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ABSTRACT

Excess nitrate (NO$_3^-$) in terrestrial and aquatic ecosystems has resulted in numerous water quality problems throughout the U.S., which emphasizes the need to develop effective management plans to deal with NO$_3^-$ pollution, especially in mixed land-use watersheds. Stable NO$_3^-$ isotopes ($\delta^{15}N$-NO$_3^-$ and $\delta^{18}O$-NO$_3^-$) have been promoted as a useful tool for studying NO$_3^-$ sources and cycling, but the application of these tracers in mixed land-use watersheds remains limited. To address this need, five studies were undertaken to improve the ability to trace NO$_3^-$ sources and movements in Spring Creek, a small (201 km$^2$) mixed land-use karst watershed located in the Ridge and Valley Province of central Pennsylvania. The first study (Chapter 2) was intended to improve our understanding of NO$_3^-$ stable isotope variations in precipitation, which is essential for tracing sources of NO$_3^-$ in streams during storm runoff periods. Results of the study showed that information about oxidant levels in the atmosphere and knowledge of storm tracks can be very useful for explaining seasonal and within-storm variations of NO$_3^-$ stable isotopes in precipitation. The second study (Chapter 3) assessed NO$_3^-$ isotope transformations, especially denitrification, in losing and gaining stream reaches along the main-stem of Spring Creek within the karst valley to test the assumption that NO$_3^-$ isotopes in stream water conserve their original source signatures. Results of this study showed that NO$_3^-$ stable isotopes were relatively unaffected by in-stream processes in losing reaches, and only changed in the gaining reaches when new and isotopically distinct NO$_3^-$ sources were introduced into the stream. The third study (Chapter 4) was undertaken to better understand NO$_3^-$ sources and flow pathways during storm events in a mixed land-use watershed. The study illustrated for the first time how stable isotopes in NO$_3^-$ and H$_2$O could be combined to show major differences in NO$_3^-$ delivery mechanisms between forested uplands and karst valleys, and confirmed the dominance of overland flow pathways in urbanizing basins. The fourth study (Chapter 5) tested whether microbial source tracking techniques with $E.~coli$ bacteria could be used to develop indicators of human and animal pollution in stream water to address difficulties in distinguishing sewage (human) and manure (animal) sources of NO$_3^-$ using stable
isotopes. Applications of a phenotypic (serotyping) and a molecular (ERIC-PCR) source tracking technique indicated that animal sources of *E. coli* were predominant throughout the Spring Creek watershed and that human *E. coli* sources were much less important. The results suggested that differentiation of human and animal pollution sources may be difficult in watersheds that are primarily serviced by sewage treatment (e.g. Spring Creek), and that future studies of this sort should consider identifying watersheds where human and animal pollution sources are more widely distributed. The final study (Chapter 6) was designed to determine how well land-use and NO₃⁻ pollutant sources could be discriminated using NO₃⁻ stable isotopes, inorganic chemistry, and bacterial indicator variables in a mixed land-use setting. The results of this study showed that NO₃⁻ isotopes were sufficient for capturing basic differences in principal NO₃⁻ sources from precipitation, forested land-uses, and sewage; however, inorganic chemical tracers enabled discrimination among land-uses and geology that could not necessarily be captured using NO₃⁻ isotopes alone. Future studies applying combined tracer approaches in mixed land-use watersheds should therefore consider the value of using NO₃⁻ isotopes alone to discriminate basic NO₃⁻ sources as well as the added benefit of using inorganic chemistry to discriminate on other watershed characteristics such as land-use and geology. Overall, the five studies in this dissertation demonstrated valuable new techniques and highlighted new directions of research that will help to further our understanding of NO₃⁻ sources, transformations, and flow pathways in mixed land-use watersheds.
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Chapter 1

INTRODUCTION

NITRATE POLLUTION PROBLEM

Human use of nitrogen (N) in agriculture and the burning of fossil fuels have essentially doubled the amount of reactive N in the terrestrial environment since pre-industrial times (Galloway et al., 1995; Vitousek et al., 1997). Much of this N can be converted to soluble nitrate (NO$_3^-$), which is the most mobile form of N, and consequently is most easily leached to groundwater and surface water. In the United States, excess NO$_3^-$ in terrestrial and aquatic ecosystems has resulted in numerous problems including the alteration of nutrient cycles in eastern U.S. forests (e.g. Likens et al., 1996), water quality impairments in acid-sensitive headwater streams (e.g. Kahl et al., 2004), and eutrophication of streams, rivers, and estuaries, namely the Chesapeake Bay (e.g. NRC, 2000; Boesch et al., 2001).

Large-scale water quality problems such as atmospheric deposition and eutrophication in the Chesapeake Bay emphasize the need to develop effective management plans to deal with NO$_3^-$ pollution. In response to this need, a great deal of research has been conducted to study N in terrestrial and aquatic environments including the development of watershed-scale N budgets (e.g. Howarth et al., 1996; Boyer et al., 2002), watershed and in-stream modeling of N fate and transport (e.g. Alexander et al., 2000), and studies linking land-use patterns to NO$_3^-$ concentrations (e.g. Miller et al., 1997; Jordan et al., 1997; Herlihy et al., 1998; Williams et al., 2005). While many of these techniques have yielded valuable information on N movement and patterns in the environment, they have not provided explicit information on sources and fate of mobile N sources in streams, specifically NO$_3^-$.

An efficient way to minimize NO$_3^-$ pollution is to identify the actual sources and better understand the processes that mobilize and transport NO$_3^-$ from the terrestrial environment to aquatic systems. This is a difficult task for several reasons, which include
the fact that NO$_3^-$ can originate from multiple sources (e.g. atmosphere, fertilizer, sewage, etc.), point sources and non-point sources of NO$_3^-$ tend to overlap and mix within a given basin, and simultaneous biogeochemical cycles assimilate and transform NO$_3^-$ in the aquatic environment (Kendall, 1998). Stable isotopes in NO$_3^-$ (e.g. $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$) provide a unique opportunity to study NO$_3^-$ pollution because the ratios of these isotopes change predictably throughout the nitrogen cycle and allow researchers to draw conclusions regarding the fate and transport of NO$_3^-$.

**NITRATE STABLE ISOTOPES IN WATERSHED RESEARCH**

**Brief Background on Nitrogen and Oxygen Isotopes**

The ability to isotopically identify NO$_3^-$ pollution sources relies on the use of nitrogen (N) and oxygen (O) isotopes, which makeup the NO$_3^-$ molecule. Nitrogen has two stable isotopes ($^{14}$N and $^{15}$N). The relative abundance of $^{14}$N is 99.63% and the relative abundance of $^{15}$N is 0.37% (Kendall, 1998). Nitrogen has a broad range of oxidation states, ranging from +5 (NO$_3^-$) to -3 (NH$_4^+$) (Kendall, 1998). As a result, natural isotopic compositions of N are extremely variable in the environment, which represents an important consideration when interpreting $\delta^{15}$N-NO$_3^-$ signatures in NO$_3^-$ source tracking applications. In contrast to N, O has three stable isotopes ($^{16}$O, $^{17}$O, and $^{18}$O). The relative abundances of all three in the environment, as reported by Kendall (1998), are 99.63% for $^{16}$O, 0.0375% for $^{17}$O, and 0.1995% for $^{18}$O. The isotope of O used most frequently in NO$_3^-$ pollution studies is $\delta^{18}$O-NO$_3^-$ (Kendall, 1998), although recent attention also has been given to $\delta^{17}$O-NO$_3^-$ as a useful tracer of NO$_3^-$ sources (e.g. Michalski et al., 2004).

The measurement of stable isotopes in the environment is based on comparing stable isotope ratios in natural samples (e.g. from terrestrial material, water, etc.) to stable isotope ratios in standards of known isotopic composition. For example, the stable isotopic composition of N and O isotopes in NO$_3^-$ would be determined as follows:

$$\delta^{15}\text{N-NO}_3^- \text{ or } \delta^{18}\text{O-NO}_3^- = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \cdot 1000$$
where R is the ratio of the heavy to light isotope (e.g. $^{15}\text{N}/^{14}\text{N}$ or $^{18}\text{O}/^{16}\text{O}$) in the sample ($R_{\text{sample}}$) and in the standard ($R_{\text{standard}}$), and $\delta$ (delta) is interpreted as parts per thousand or permil (%). The international reference standard for $\delta^{15}\text{N}-\text{NO}_3^-$ is atmospheric N$_2$ gas, and the international reference standard for $\delta^{18}\text{O}-\text{NO}_3^-$ is Vienna Standard Mean Ocean Water (VSMOW).

The partitioning of isotopes by way of physical, chemical, and biological transformations is known as fractionation (Coplen, 1993). This is what permits NO$_3^-$ isotopes to be utilized as tracers of NO$_3^-$ pollution sources in the environment. Two types of fractionation are defined in the literature (see Kendall, 1998): (1) equilibrium (reversible reactions), and (2) kinetic (irreversible reactions). While both of these types of reactions can result in significant isotope fractionation, many of the most important fractionating processes are kinetic reactions that occur due to the cycling of N in the environment (see Figure 1.1). Kinetic fractionation processes that affect NO$_3^-$ stable isotopes include N fixation, assimilation by organisms (e.g. plants, aquatic algae), mineralization (conversion of organic-N to NH$_4^+$), nitrification (conversion of NH$_4^+$ to NO$_3^-$), volatilization (loss of NH$_3$ gas to the atmosphere), and denitrification (conversion of NO$_3^-$ to N$_2$O and N$_2$ gas) (Kendall, 1998). Different NO$_3^-$ sources undergo different isotopic fractionation processes, which essentially has permitted investigators to delineate unique source regions using $\delta^{15}\text{N}-\text{NO}_3^-$ and $\delta^{18}\text{O}-\text{NO}_3^-$ isotopes. Ultimately, four major sources of NO$_3^-$ have been determined using $\delta^{15}\text{N}-\text{NO}_3^-$ and $\delta^{18}\text{O}-\text{NO}_3^-$ (see Kendall et al., 1998; Rock and Mayer, 2004): (1) NO$_3^-$ in precipitation, (2) NO$_3^-$ from microbial nitrification in soils, (3) N in synthetic fertilizers, and (4) NO$_3^-$ derived from sewage or manure sources (see Figure 1.2 for sources). The ability to separate these sources using $\delta^{15}\text{N}-\text{NO}_3^-$ and $\delta^{18}\text{O}-\text{NO}_3^-$ is what generated the interest in using NO$_3^-$ isotopes to identify NO$_3^-$ pollution sources in watershed research.

**Applications of Nitrate Isotopes in Watershed Research**

One of the first studies to trace sources of NO$_3^-$ in surface water was performed in the Sangamon River basin in Illinois (Kohl et al., 1971). The authors used $\delta^{15}\text{N}-\text{NO}_3^-$ in surface water as well as $^{15}\text{N}$ in soils and fertilizers to estimate the percentage contribution
of fertilizer NO$_3^-$ to Lake Decatur, an impoundment on the Sangamon River. Based on two-component separation models, the authors concluded that 55% of the NO$_3^-$ in Lake Decatur was derived from fertilizer applications and the remainder was from virgin soils. The results of the study by Kohl et al. (1971) were strongly criticized by Hauck et al. (1972) in part for failing to consider the effects of biogeochemical N transformations on $\delta^{15}$N-NO$_3^-$ during transport from the river to the lake. As a result of this criticism, studies using $\delta^{15}$N-NO$_3^-$ in surface water were limited for a period of time, especially in agricultural watersheds.

New motivation for surface water study was generated when $\delta^{18}$O-NO$_3^-$ techniques were developed (see Amberger and Schmidt, 1987) and refined (see Silva et al., 2000). The majority of $\delta^{18}$O-NO$_3^-$ applications initially focused on quantifying sources of atmospheric deposition in acid-sensitive forested watersheds in North America and Europe. Durka et al. (1994) used $\delta^{18}$O-NO$_3^-$ to estimate the percentage contribution of atmospheric NO$_3^-$ and microbially-derived NO$_3^-$ to spring water in northeast Germany. The authors used $\delta^{18}$O-NO$_3^-$ instead of $\delta^{15}$N-NO$_3^-$ because it exhibited a larger isotopic difference between precipitation and soil NO$_3^-$ sources (see Figure 1.2). Using two-component mixing models, the authors were able to show that 30% to 46% of the total NO$_3^-$ in spring water was derived from atmospheric sources. Following this landmark study, future work confirmed the importance of microbially-derived NO$_3^-$ as the dominant NO$_3^-$ source in small forested watersheds (e.g. Spoelstra et al., 2001; Williard et al., 2001; Burns and Kendall, 2002; Campbell et al., 2002; Pardo et al., 2004; Ohte et al., 2004; Piatek et al., 2005).

Recently, studies using NO$_3^-$ stable isotopes to trace surface water NO$_3^-$ sources in developed watersheds have regained importance. While Kohl et al. (1971) attempted to quantify NO$_3^-$ sources in an agricultural watershed using mixing model approaches, more recent studies in developed watersheds have backed away from the idea of quantifying NO$_3^-$ source contributions and have focused more on NO$_3^-$ source identification. For example, recent studies in agricultural watersheds have used $\delta^{15}$N-NO$_3^-$ to investigate the importance of animal waste (e.g. Karr et al., 2003) and fertilizer NO$_3^-$ (e.g. Kellman, 2005) sources in stream water. Studies in urban watersheds have suggested that enriched $\delta^{18}$O-NO$_3^-$ at higher flow rates may be a useful indicator of urban runoff during storm
events (e.g. Ging et al., 1996; Silva et al., 2002). Mixed land-use watershed studies primarily have focused on identifying the importance of broad NO$_3^-$ source categories in stream water (e.g. precipitation, soil-derived NO$_3^-$, manure/sewage, and fertilizers) during predominately low flow conditions (e.g. Mayer et al., 2002; Chang et al., 2002; Rock and Mayer, 2004; Segal-Rozenhaimer, 2004; Panno et al., 2006).

OPPORTUNITIES TO IMPROVE NITRATE SOURCE TRACKING

In reviewing the literature, several key opportunities were identified to improve NO$_3^-$ source tracking in mixed land-use watersheds. Firstly, most surface water studies using NO$_3^-$ isotopes have worked exclusively in one type of land-use setting (e.g. forest, urban, or agriculture). Of the studies that have been conducted in mixed land-use basins, the majority have been conducted at the scale of large river basins (> 10,000 km$^2$) (e.g. Chang et al., 2002; Rock and Mayer, 2004; Segal-Rozenhaimer, 2004, Panno et al., 2006). Delineating subwatersheds with relatively homogenous land-uses (e.g. forest, agriculture, urban) often is not possible in large river basins, and thus meaningful comparisons of NO$_3^-$ sources and cycling among different land-use types can be difficult to make (see Chang et al., 2002). The premise of this research is that studying a smaller mixed land-use watershed may result in a better understanding of land-use influences on NO$_3^-$ sources and cycling.

Spring Creek, a 371 km$^2$ mixed land-use watershed in central Pennsylvania, was selected as an ideal watershed in which to test ideas about improving NO$_3^-$ source tracking using NO$_3^-$ stable isotopes. Spring Creek is a tributary to the Susquehanna River, and ultimately the Chesapeake Bay. The watershed is situated within the Appalachian Section of the Ridge and Valley Physiographic Province, which is characterized by forested sandstone ridges (elevation ~ 550 - 600 m) and wide karst valleys with a mixture of forest, agricultural, and urban land-uses. The mixture of land-uses and geologic settings allowed us to delineate subwatersheds with uniquely different land-uses such as forest, agriculture, urban, and mixtures thereof. Furthermore, the forested watershed was underlain by sandstone, and the remaining watersheds were
underlain by carbonate bedrock. The differences in land-use and geologies among the selected watersheds allowed us to test ideas about how land-use and geology affected NO$_3^-$ sources and flow paths within the Spring Creek watershed. With this basic experimental design in mind, we identified five key areas where more study was needed to help improve NO$_3^-$ source tracking in small mixed land-use watersheds. These areas are outlined in more detail and correspond to individual chapters in this dissertation.

**NO$_3^-$ Isotope Variations in Precipitation**

The ability to trace sources of NO$_3^-$ relies on the assumption that source signatures are relatively fixed and do not change much over time. Precipitation is an important potential source of NO$_3^-$ in mixed land-use watersheds, and temporal variations of NO$_3^-$ stable isotopes in precipitation (e.g. seasonal, within individual storm events) may overlap NO$_3^-$ isotope signatures from other important NO$_3^-$ sources (e.g. fertilizer; see Figure 1.2) and adversely affect the ability to clearly identify precipitation NO$_3^-$ in stream water. Several studies have documented seasonal variations of δ$^{15}$N-NO$_3^-$ (e.g. Freyer, 1978; Heaton, 1987; Freyer et al., 1991; Russell et al., 1998; Campbell et al., 2002; Hastings et al., 2003) and δ$^{18}$O-NO$_3^-$ (Durka et al., 1994; Williard et al., 2001; Campbell et al., 2002; Hastings et al., 2003; Pardo et al., 2004). Only a few studies have considered the effects of within-storm variations on NO$_3^-$ stable isotopes, and these studies have focused solely on δ$^{15}$N-NO$_3^-$ (e.g. Moore, 1973; Heaton and Collett, 1985; Heaton, 1987). Of the studies that have been conducted, the primary explanations for NO$_3^-$ isotope variations have centered on oxidant concentrations in the atmosphere (e.g. Freyer et al., 1993; Hastings et al., 2003). Other possible explanations of within-storm isotope variations may be related to storm origin and prevailing climatic conditions. Therefore, in order to use isotopes to trace NO$_3^-$ sources, it was important initially to study seasonal and within-storm variations of NO$_3^-$ stable isotopes in precipitation and attempt to explain these variations in terms of oxidant chemistry, storm tracks, and atmospheric conditions.
**NO₃⁻ Isotope Transformations in Streams**

NO₃⁻ isotope transformations in streams due to biogeochemical fractionations (e.g. denitrification) also may affect the ability to trace sources of NO₃⁻ in streams. The failure to document NO₃⁻ isotope transformations was one of the main reasons that Hauck et al. (1972) strongly criticized the study by Kohl et al. (1971) for the Sangamon River. In-stream processes such as denitrification (e.g. Kellman and Hillaire-Marcel, 1998; Battaglin et al., 2002; Panno et al., 2006), nitrification (Kendall, 1998), assimilation by plants and phytoplankton (e.g. Fogel and Cifuentes, 1993; Granger et al., 2004), biological and food-web transformations (e.g. Fry, 2006), and simple dilution or mixing (Kendall, 1998) all can affect δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ in stream water. On the main stem of Spring Creek, determining the importance of NO₃⁻ isotope transformations may further be complicated by the presence of gaining and losing stream sections due to the occurrence of karst geologic features. In order to compare stream stations spatially separated it was important to understand the importance of in-stream NO₃⁻ isotope transformations in gaining and losing stream reaches and determine whether different in-stream processes such as denitrification were important in Spring Creek.

**NO₃⁻ Sources and Flow Paths during Storms**

In the context of NO₃⁻ source tracing in mixed land-use watersheds, few studies have explicitly considered the effects of storm flows on NO₃⁻ sources, as most have been conducted during largely non-storm periods (e.g. Chang et al., 2002; Rock and Mayer, 2004; Segal-Rozenhaimer, 2004, Panno et al., 2006). Studying storms is important because the majority of NO₃⁻ export occurs at high flows, and proper management of NO₃⁻ pollution requires a thorough understanding of NO₃⁻ sources and hydrologic flow paths (Kendall, 1998) in watersheds with different land-use and geology.

Monitoring changes in NO₃⁻ stable isotopes prior to and during storm events in streams may provide useful information regarding NO₃⁻ sources and flow paths. Changes in δ¹⁵N-NO₃⁻ from baseflow to peakflow may help to identify shifts in the relative importance of NO₃⁻ from inorganic versus organically-derived sources during storm events. Changes in δ¹⁸O-NO₃⁻ from baseflow to peakflow may provide useful information on the significance of precipitation NO₃⁻ sources during runoff periods.
\(\delta^{18}O-\text{NO}_3^-\) also may represent a useful indicator of hydrologic flow pathways for dissolved \(\text{NO}_3^-\). The possibility of combining \(^{18}O\) isotopes in water (\(\delta^{18}O-\text{H}_2\text{O}\)), which yield specific information on water flow paths, with \(\text{NO}_3^-\) stable isotopes with information about \(\text{NO}_3^-\) sources has not been thoroughly explored. Therefore, a study was initiated to test ideas regarding potential differences in \(\text{NO}_3^-\) sources and flow paths using \(\text{NO}_3^-\) isotopes and \(\delta^{18}O-\text{H}_2\text{O}\) in watersheds with different land-use and geology at times of both baseflow and peakflow during storm events.

**Differentiating Manure and Sewage \(\text{NO}_3^-\) Sources**

One of the significant challenges in identifying \(\text{NO}_3^-\) sources in mixed land-use watersheds with large proportions of agricultural and urban land-uses is separating manure and sewage sources of \(\text{NO}_3^-\). These sources have overlapping \(\text{NO}_3^-\) isotope signatures (see Figure 1.2; Kendall, 1998), which makes them largely indistinguishable from each other in stream water by isotopes alone. As a result of this isotopic overlap problem, new tracers are needed in addition to \(\text{NO}_3^-\) stable isotopes to help resolve the relative importance of manure and sewage sources of \(\text{NO}_3^-\) in stream water. One possible solution to this problem is to test whether microbial source tracking techniques with *E. coli* bacteria are capable of identifying the relative importance of human and animal *E. coli* in streams.

A variety of microbial source tracking techniques have been reviewed (Sinton et al., 1998; Simpson et al., 2002; Scott et al., 2002; Meays et al., 2004; EPA, 2005; Seurinck et al., 2005; Stoeckel, 2005) and compared in inter-laboratory comparisons (Griffith et al., 2003, Stewart et al., 2003; Stoeckel et al., 2004; Moore et al., 2006). Two techniques that may be useful and complementary include enterobacterial repetitive intergenic consensus PCR (ERIC-PCR; see Versalovic et al., 1991; Meacham et al., 2003), a molecular technique, and serotyping (e.g. Ørskov et al., 1977; Ørskov and Ørskov, 1984), a phenotypic technique. Parveen et al. (2001) suggested that combining serotyping with a molecular technique such as PCR may be a powerful method for identifying the importance of human and animal sources of *E. coli* in stream water. The ability to identify the relative importance of human and animal *E. coli* in Spring Creek was attempted in order to improve differentiation of sewage and manure sources of \(\text{NO}_3^-\).
Differentiating NO$_3^-$ in Mixed Land-Use Watersheds

Statistical methods that link land-use, water quality, and NO$_3^-$ sources provide important information that can promote successful management of NO$_3^-$ pollution. Recent studies in mixed land-use watersheds have shown that NO$_3^-$ in forested land-uses can be differentiated from NO$_3^-$ in more urbanized and agricultural land-uses using $\delta^{15}$N-NO$_3^-$ (e.g. Harrington et al., 1998; Mayer et al., 2002; Rock and Mayer, 2004; Voss et al., 2006). Other studies have been less successful in linking NO$_3^-$ isotopes and land-use (e.g. Chang et al., 2002; Douglas et al., 2002). One of the reasons for mixed success was likely due to scale (e.g. Chang et al., 2002), as many of the studies were conducted in large river basins with complex land-use patterns. Another confounding factor could potentially be the seasonal variations in NO$_3^-$ stable isotopes, which might affect land-use differentiation at different times of the year. Finally, the inability to differentiate NO$_3^-$ among developed watersheds in agriculture and urban land-uses also has hampered efforts to identify NO$_3^-$ sources from different land-uses. One possible solution to these problems would be to test whether additional chemical and microbial tracers improve the ability to identify NO$_3^-$ from different land-uses. Spruill et al. (2002) combined $\delta^{15}$N-NO$_3^-$ with sixteen inorganic chemicals and ionic ratios of those chemicals using multivariate statistics to provide additional statistical power in differentiating NO$_3^-$ sources between manure, sewage, and septic systems in groundwater. A study was thus needed to test the relative importance of NO$_3^-$ stable isotopes, inorganic chemicals, and microbial tracers as discriminators of NO$_3^-$ from different land-uses using multivariate statistics.

DISSERTATION OBJECTIVES

The overall objective of this dissertation was to improve the ability to trace NO$_3^-$ sources and movements in a mixed land-use watershed. Six storm events were sampled during 2005 using a nested and spatially-distributed sampling design (5 sites). The sites represented a downstream progression of forested uplands underlain by resistant sandstone to karst lowlands with agricultural, urban, and mixed land-uses. Specific sub-objectives of the dissertation were to:
1. Document and provide explanations for variations of isotopes in precipitation (Chapter 2).
2. Evaluate and quantify the importance of NO₃⁻ isotope transformations (e.g. denitrification) in gaining and losing stream sections during a summer low flow period (Chapter 3).
3. Identify NO₃⁻ sources and flow paths in watersheds with different land-uses and karst versus sandstone bedrock geology at times of baseflow and peakflow during events (Chapter 4).
4. Develop indicators of human and animal E. coli sources that could potentially improve the ability to discriminate between sewage and manure NO₃⁻ sources (Chapter 5).
5. Test NO₃⁻ source discrimination among watersheds with different land-uses using a combination of NO₃⁻ stable isotopes, inorganic chemical tracers, and microbial indicators (Chapter 6).
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Figure 1.1: Processes in the nitrogen cycle that affect NO$_3^-$ stable isotopes (adapted from Nadelhoffer and Fry, 1994; Kendall, 1998).
Figure 1.2: $\delta^{15}N$-$\text{NO}_3^-$ and $\delta^{18}O$-$\text{NO}_3^-$ can be used to help discriminate among different sources of NO$_3^-$ in streams (modified from Kendall, 1998).
Chapter 2

USE OF ATMOSPHERIC OXIDANTS AND STORM TRACKS TO EXPLAIN VARIATIONS OF STABLE ISOTOPES IN NITRATE AND WATER IN PRECIPITATION IN CENTRAL PENNSYLVANIA

ABSTRACT

Stable isotopes of NO$_3^-$ ($\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$) and H$_2$O ($\delta^{18}$O-H$_2$O) were monitored sequentially in precipitation at a central Pennsylvania site during six storm events in 2005 to determine whether information on atmospheric oxidants (e.g. O$_3$, NO$_2$, and NO$_x$), and storm-tracks (using the NOAA HYSPLIT model) were capable of explaining observed seasonal and within-storm isotopic variations. Results showed that $\delta^{15}$N-NO$_3^-$, $\delta^{18}$O-NO$_3^-$, and $\delta^{18}$O-H$_2$O in precipitation varied significantly during individual storm events. Seasonally, $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in precipitation followed a pattern of depletion during the summer months and enrichment during the winter months. In contrast, the highest values of $\delta^{18}$O-H$_2$O in precipitation occurred during the summertime and most depleted values occurred during the winter months. Atmospheric oxidants were useful for explaining seasonal and within-storm variations of $\delta^{15}$N-NO$_3^-$ for all six storm events as evidenced by negative relationships with NO$_2$:NO$_x$ ratios and ozone (O$_3$). In comparison, $\delta^{18}$O-NO$_3^-$ was positively related to O$_3$ in three dormant season storms, which suggested that the O$_3$ oxidation pathway was important for producing the high $\delta^{18}$O-NO$_3^-$ observed in wintertime precipitation. Storm track information was especially useful for describing differences in $\delta^{15}$N-NO$_3^-$. Cool-sector storms originating from the E/NE produced low $\delta^{15}$N-NO$_3^-$ characteristic of automobile emissions, whereas warm-sector storms with tracks from the SW/S/SE produced high $\delta^{15}$N-NO$_3^-$ characteristic of coal-fired emissions. Lightning also may have been an important source of atmospheric NO$_3^-$ during two warm-sector thunderstorms. With regard to $\delta^{18}$O-H$_2$O, an increasing relationship with air temperature was observed, which indicated that greater isotopic fractionation occurred at lower temperatures of condensation. This observation helped to
explain why four warm-sector storms produced significantly more enriched $\delta^{18}O$-H$_2$O than two cool-sector storms. Overall, this study showed that (1) information about oxidant levels can be useful to predict seasonal and within-storm variations of NO$_3^-$ stable isotopes in precipitation, and (2) knowledge of storm tracks (warm-sector versus cool-sector) may be important for determining sources of NO$_3^-$ in wet deposition and formation processes that affect H$_2$O.
INTRODUCTION

Stable isotopes of nitrogen (\(^{15}\text{N}\)) and oxygen (\(^{18}\text{O}\)) are increasingly being used to understand the sources and cycling of nitrate (\(\text{NO}_3^-\)) in precipitation and its fate as wet deposition in terrestrial and aquatic ecosystems. Much of this interest is driven by the fact that \(\text{NO}_3^-\) is a major component of acidic deposition, which has altered nutrient cycles in eastern U.S. forests (e.g. Likens et al., 1996) and impaired water quality in acid-sensitive headwater streams (e.g. Kahl et al., 2004). Additional motivation for isotope studies of \(\text{NO}_3^-\) in precipitation has been generated because wet deposited nitrogen (N), of which \(\text{NO}_3^-\) is a significant component, may account for as much as 23-32% of the total N load delivered to estuarine systems such as the Chesapeake Bay (Castro et al., 2001; Sheeder et al., 2002; Castro et al., 2003).

The majority of the research on \(^{15}\text{N}\) and \(^{18}\text{O}\) isotopes in precipitation \(\text{NO}_3^-\) (referred to as \(\delta^{15}\text{N-NO}_3^-\) and \(\delta^{18}\text{O-NO}_3^-\) from this point forward) has focused on two major themes: (1) assessing the significance of atmospheric processes and pollutant sources that may affect \(\delta^{15}\text{N-NO}_3^-\) and \(\delta^{18}\text{O-NO}_3^-\) signatures (e.g. Hastings et al., 2003), and (2) using \(\delta^{18}\text{O-NO}_3^-\) as a tracer to quantify the contribution of atmospheric wet \(\text{NO}_3^-\) deposition in nitrogen-limited forest ecosystems (e.g. Williard et al, 2001) and to evaluate the importance of atmospheric \(\text{NO}_3^-\) sources during high stream flow periods in mixed land-use (e.g. Chang et al., 2002) and urbanized watersheds (e.g. Ging et al., 1996). It is in the context of each of these types of studies that temporal variations (e.g. within-storm, seasonal, etc.) of \(\delta^{15}\text{N-NO}_3^-\) and \(\delta^{18}\text{O-NO}_3^-\) in precipitation must be considered. In terms of studying the sources and cycling of \(\text{NO}_3^-\) in precipitation, temporal variations of \(\delta^{15}\text{N-NO}_3^-\) and \(\delta^{18}\text{O-NO}_3^-\) have typically been very useful as they have permitted the investigator to consider whether other atmospheric variables such as oxidant chemistry or temperature may help explain the observed isotopic variations. In contrast, temporal variations in \(\delta^{15}\text{N-NO}_3^-\) and \(\delta^{18}\text{O-NO}_3^-\) have been often viewed as problematic for those interested in using a particular isotopic signal to quantify or identify atmospheric \(\text{NO}_3^-\) sources in stream systems, especially during storm events. Clearly, one must identify and characterize the different types of variation that might be expected when using \(\delta^{15}\text{N-NO}_3^-\)
and Δ^{18}O-NO₃⁻ stable isotopes to study NO₃⁻ sources and cycling in precipitation and its fate on the landscape.

Of the types of variation anticipated for Δ^{15}N-NO₃⁻ and Δ^{18}O-NO₃⁻ stable isotopes in precipitation, seasonal variation has received by far the most attention in the literature. Many studies have documented seasonal variations in Δ^{15}N-NO₃⁻ (Freyer, 1978; Heaton, 1987; Freyer et al., 1991; Russell et al., 1998; Yeatman et al., 2001; Campbell et al., 2002; Hastings et al., 2003; Cooney, 2005) and Δ^{18}O-NO₃⁻ (Durka et al., 1994; Williard et al., 2001; Campbell et al., 2002; Hastings et al., 2003; Pardo et al., 2004; Cooney, 2005) in atmospheric and precipitation NO₃⁻ at various sites around the world. In general, these studies have shown that both Δ^{15}N-NO₃⁻ and Δ^{18}O-NO₃⁻ are highest during the winter months and lowest during the summer months. An exception for Δ^{15}N-NO₃⁻ was found at a site in Bermuda, where the highest Δ^{15}N-NO₃⁻ values occurred during the warm season (April – September) and the lowest Δ^{15}N-NO₃⁻ values occurred during the cool season (October – March) (Hastings et al., 2003). Recent explanations for the observed seasonal variations in Δ^{15}N-NO₃⁻ and Δ^{18}O-NO₃⁻ have centered on seasonal shifts in prevailing oxidant chemistry in the atmosphere (e.g. OH versus O₃ concentrations) and the associated effects on different NO₃⁻ precursor concentrations (e.g. NO, NO₂ and NOₓ) (see Freyer et al., 1993; Hastings et al., 2003).

In contrast to seasonal variations of Δ^{15}N-NO₃⁻ and Δ^{18}O-NO₃⁻ in NO₃⁻, much less is known about how these isotopes vary during storm events. Early work by Moore (1973), Heaton and Collett (1985), and Heaton (1987) identified within-storm variations of Δ^{15}N-NO₃⁻. Based on limited samples, the authors generally concluded that the within-storm variations were not consistent on a storm-to-storm basis. Heaton (1987) suggested that variations of Δ^{15}N-NO₃⁻ during storms in Pretoria, South Africa may have been due to selective washout of N bearing compounds. Other mechanisms may also have been important, but these have yet to be thoroughly explored. With regard to Δ^{18}O-NO₃⁻, no published studies were found that have documented within-storm variations. Some evidence for the possibility of Δ^{18}O-NO₃⁻ variations during storms stems from the results of a study conducted in central Pennsylvania, where ^{18}O in water (Δ^{18}O-H₂O from this point forward) was shown to vary greatly during the course of 10 different storm events.
sampled in 1989 and 1990 (Pionke and DeWalle, 1992). $\delta^{18}$O-H$_2$O is potentially related to $\delta^{18}$O-NO$_3^-$ because $\delta^{18}$O-H$_2$O has been shown to influence the $^{18}$O of hydroxyl radicals (OH) in the atmosphere (Dubey et al., 1997). OH is an important oxidant involved in atmospheric NO$_3^-$ formation (Calvert et al., 1985) and contributes oxygen (O) atoms to newly formed NO$_3^-$ molecules. As a result of this known relationship, we anticipate that $\delta^{18}$O-NO$_3^-$ also would vary during storm events, although the exact mechanisms responsible (e.g. importance of OH versus O$_3$) for the variations would require further exploration.

While seasonal variation and within-storm variation are clearly important for $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$, storm origin and atmospheric conditions may also result in observable effects on these isotopes as suggested by several recent studies. Russell et al. (1998) collected bulk precipitation samples from 60 storm events at a site near Lewes, DE at the mouth of the Delaware Bay from 1993 through 1994 and analyzed them for $\delta^{15}$N-NO$_3^-$. Back trajectory analysis using the NOAA HYbrid Single-Particle Lagrangian Integrated Trajectory (HYSPLIT) model (see Draxler and Hess, 2004) showed that transport patterns originating from the northwest and west of Lewes, DE produced the highest fluxes of NO$_3^-$ and had $\delta^{15}$N-NO$_3^-$ signatures indicative of fossil fuel combustion. Hastings et al. (2003) collected precipitation samples on an event basis on the island of Bermuda from 2000 through 2001 and analyzed the samples for $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$. Results using back trajectory analysis showed that air masses with origins from the southeast and southwest produced higher $\delta^{15}$N-NO$_3^-$ and lower $\delta^{18}$O-NO$_3^-$ isotope ratios than air masses originating from the north and northwest. Further, the two general groups of transport patterns to Bermuda were well correlated with air mass conditions, with warm season (April - September) air masses exhibiting southeast and southwest transport patterns and cool season (October – March) air masses exhibiting north and northwest transport patterns. More recently, Cooney (2005) analyzed $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in precipitation for 46 storm event samples of bulk precipitation collected from 2002 through 2004 at a site in Frederick, MD. Unlike the previous studies, no relationship between storm origin and NO$_3^-$ stable isotopes could be identified using back trajectory analysis. Clearly, other factors may be important in addition to storm origin to explain the variability of $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in precipitation samples.
Additional insight may be gained from studies combining back trajectory analysis and atmospheric air mass characterization to help explain observed variations of stable isotopes in water for different types of storm tracks. Studies in central New York State conducted by Lawrence et al. (1982) and Gedzelman and Lawrence (1982) using deuterium (δD) and Burnett et al. (2004) using δ^{18}O-H_2O showed that these isotope ratios were generally lowest in the cool-sector of storms and highest in the warm-sector of storms. The positioning of frontal boundaries and low pressure systems relative to the sites in central New York primarily determined the temperature of the air mass and the resultant isotope ratios for δD and δ^{18}O-H_2O. It appears that characterizing storm track patterns and air mass conditions together may help further explain storm-to-storm variations in δ^{15}N-NO_3^- and δ^{18}O-NO_3^- in precipitation.

The overall goal of this study was to characterize the stable isotopic (δ^{15}N-NO_3^- and δ^{18}O-NO_3^-, δ^{18}O-H_2O) and inorganic chemical composition of precipitation for six different storm events at a site located within the Spring Creek watershed in central Pennsylvania. Specific sub-objectives of the study were to:

1. Assess the significance of inter- and intra-storm variations of stable isotopes.
2. Utilize available information on atmospheric oxidant (e.g. O_3) and NO_3^- precursor concentrations (e.g. NO_x and NO_2) as well as local climate information (e.g. hourly precipitation amounts and temperature) to help explain observed variations of stable isotopes in precipitation.
3. Use surface weather maps and back trajectory analysis to test the hypothesis that storm origin and atmospheric air mass conditions result in observable differences in the stable isotopic composition of precipitation.
MATERIALS AND METHODS

Study Site Location and Description

Precipitation samples were collected at a single station located within the upper portion of the Spring Creek watershed in central Pennsylvania (Figure 2.1). Spring Creek is a 371 km² mixed land-use watershed, which is situated within the Nittany Valley in the Valley and Ridge physiographic region. Central Pennsylvania has a humid continental type climate, with average annual precipitation approximately 97 cm based upon 80 years of data (1926-2005) measured at State College, PA (PA State Climatologist, 2006). During the period of study (2005), the total observed precipitation for the year was 104 cm, which was slightly above average. The precipitation sampling site was established adjacent to the Land and Water Research Building in University Park, PA (40.81°N, -77.85°W). All precipitation samples were collected on a storm event basis.

Precipitation Collection

Six storm events were sampled using a passive precipitation sampler designed and constructed to collect sufficient volumes of rainwater for NO₃⁻ stable isotope and inorganic chemical analysis. The sampler was approximately 5.7 m² in area and was equipped with a polyethylene plastic tarp, which funneled water to a series of three 20-L polypropylene carboys (Figure 2.2). The bottles were arranged such that they filled sequentially during storms according to a slightly modified design from that of Kennedy et al. (1979). This enabled within-storm variations of stable isotopes and chemistry to be evaluated for each storm event.

A total of 8 20-L carboys were available for precipitation sampling during storms. Approximately 0.36 cm (0.13 in) of rain produced enough water to fill one 20-L carboy. As a result, storms that produced approximately 2.88 cm (1.13 in) of precipitation could be adequately sampled using 8 carboys. Unfortunately, most sequentially collected precipitation samples had to be integrated to some degree for two main reasons: (1) storms that produced more than 2.88 cm of precipitation resulted in more water than could be sampled using 8 carboys, and (2) high-intensity rainfall during thunderstorms often resulted in short-circuiting of the funnel and tube connections.
A strict procedure was developed for washing the carboys and the plastic tarp. The carboys were washed, rinsed with 10% hydrochloric acid (HCl), and triply rinsed with deionized water before each storm event. The plastic tarp was changed between events and was washed three times with deionized water just prior to the onset of precipitation to assure that only wet deposition from rainfall was collected in the sample bottles. Wash-off from the tarp was collected in a bottle after a three-week long dry period in November 2005 to provide some isotope information on dry NO$_3^-$ deposition.

**Water Chemistry and Stable Isotope Analyses**

Precipitation samples were analyzed for inorganic chemistry at the Water Quality Laboratory in the Penn State Institutes of the Environment (PSIE). All water samples were filtered through 0.45 µm filters. Water samples were analyzed for pH (electrometric), specific conductance (electrometric), SO$_4^{2-}$ and Cl$^-$ (ion chromatography), NO$_3$-N (cadmium reduction), Ca$^{2+}$ and Na$^+$ (atomic absorption direct aspiration), and SiO$_2$ (ammonium molybdate). All analyses were conducted using standard methods (APHA, 2005). See Appendix A.1 for all raw chemistry data.

Stable isotopes in NO$_3^-$ ($^{15}$N and $^{18}$O) were analyzed at the University of Waterloo Environmental Isotope Laboratory (EIL). $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ were analyzed according to the methods outlined by Chang et al. (1999) and Silva et al. (2000). Each water sample was processed through a series of filters (see Figure 2.3) designed to facilitate the collection of NO$_3^-$ for stable isotope analysis. The first filter in the series was a Pall© 0.45 µm groundwater filter used to remove all particulate matter from the water sample. Next, a column packed with BIO-RAD© AG 50W-X8 cation exchange resin (100-200 mesh) was used to remove dissolved organic carbon (DOC), which was shown to cause problems with $\delta^{18}$O-NO$_3^-$ (Chang et al., 1999; Silva et al., 2000). Finally, a column packed with BIO-RAD© AG 2-X8 anion exchange resin (100-200 mesh) was used to collect dissolved NO$_3^-$ and other anions in solution. This procedure was performed at the Penn State Water Quality Laboratory. Samples were usually filtered within 48 hours of collection, although occasionally samples had to be stored for longer than 48 hours. Spoelstra et al. (2004) showed that samples of precipitation NO$_3^-$ could be stored for up to two weeks with minimal effects of biological isotope fractionation.
During the course of this study, all precipitation samples were filtered onto anion exchange resins within one week of collection. Anion exchange resins loaded with NO$_3^-$ were then stored in a 4°C cooler until they could be shipped to the EIL at Waterloo for further processing and analysis. The results for δ$^{15}$N-NO$_3^-$ and δ$^{18}$O-NO$_3^-$ are reported in delta notation (δ$^{15}$N and δ$^{18}$O in permil units) versus their respective international reference standards. The international reference standard for δ$^{15}$N-NO$_3^-$ is atmospheric N$_2$ gas, while the international reference standard for δ$^{18}$O-NO$_3^-$ is Standard Mean Ocean Water (SMOW). The analytical error for both isotopes was approximately ±0.2‰ based on duplicate samples (26 duplicates for δ$^{15}$N-NO$_3^-$ and 14 duplicates for δ$^{18}$O-NO$_3^-$).

Stable isotopes of δ$^{18}$O-H$_2$O also were analyzed at the University of Waterloo EIL. Water samples for δ$^{18}$O-H$_2$O were collected and stored in airtight bottles prior to shipment to the EIL at Waterloo. The analysis for δ$^{18}$O-H$_2$O was conducted using the CO$_2$ equilibration method outlined by Epstein and Mayeda (1953). All results are reported in delta notation (δ$^{18}$O in permil units) relative to Standard Mean Ocean Water (SMOW). The analytical error for δ$^{18}$O-H$_2$O isotopes was approximately ±0.1‰ based on 20 duplicate samples. Appendix A.1 shows raw stable isotope data for precipitation.

**Storm Characterization and Back Trajectories**

All sampled storms were characterized using available meteorological data to assess the potential influence of air mass and storm tracks on NO$_3^-$ stable isotopes and precipitation chemistry. Weather surface maps for the continental U.S. were obtained from the National Weather Service (NWS) Hydrometeorological Prediction Center (HPC) map archive (NWS, 2006) for each of the six storm events sampled during 2005. These maps were used to characterize the general synoptic conditions occurring during the course of each event. The location of the cold frontal boundary was used to define whether State College remained in the cold sector or the warm sector of the storm (e.g. Lawrence et al., 1982). If the storm track occurred along a cold front that had not yet passed through State College, the storm was classified as a warm-sector storm. On the other hand, if a storm moved along a cold frontal boundary that had already passed through State College, then the storm was classified as a cool-sector storm.
In addition to using surface weather maps, back trajectories were computed with Version 4 of the HYSPLIT model (see Draxler and Hess, 2004 for more details; NOAA, 2006) using data from the Eta Data Assimilation System (EDAS - 40 km resolution). Back trajectories were calculated at 500 meters above ground level (agl), which is within the atmospheric boundary layer. Back trajectories were traced for 48 hours just prior to the end of each storm.

**Climate and Atmospheric Chemistry Data**

Climate and atmospheric chemistry data were collected for the duration of each storm event to help explain variations in the stable isotopic and chemical composition of precipitation during events. Total precipitation amounts were monitored using a simple rain gauge attached to the precipitation sampler. Hourly precipitation and temperature data were obtained from a NWS weather station located at the nearby University Park (UP) Airport. Hourly nitrogen oxide (NO₂ and NOₓ) data were obtained from the Air Quality Learning and Demonstration Center located on the Penn State University campus (PA DEP, 2006), and hourly O₃ data were obtained from the Clean Air Status and Trends Network (CASTNET) Leading Ridge monitoring site located about 17 km south of State College. All hourly data were aggregated to obtain a representative observation of climate and atmospheric chemistry conditions for each precipitation chemistry sample taken during a storm event. See Appendix A.1 for climate and atmospheric data.

**RESULTS AND DISCUSSION**

**Storm Descriptions and Characterization**

A total of six storms were sampled during the 2005 calendar year. Table 2.1 presents information on the start and end times for each storm as well as precipitation type, amount, average intensity, and number of samples collected during the event. The storms were characterized according to storm track (see Figure 2.4a-f) and classified as cool-sector or warm-sector storms (Table 2.1). Appendix A.2 summarizes detailed information on each storm event that was sampled.
Inter-Storm and Seasonal Variations

$\delta^{18}O-H_2O$

$\delta^{18}O-H_2O$ varied significantly on a storm-to-storm basis during the 2005 sampling period (Table 2.2, Figure 2.5a). Using Figure 2.5a as a guide, one can see that $\delta^{18}O-H_2O$ was very negative during the storms on March 23 and March 28-29 (-13.2‰ and -9.16‰, respectively), increased substantially on July 5 and August 31 (-5.3‰ and -4.2‰, respectively), and then decreased slightly for the storms sampled on October 7 and November 16 (-7.4‰ and -7.1‰, respectively). Thus, despite only using data from six storm events, a distinct seasonal pattern is apparent in the dataset.

Seasonal variations of $\delta^{18}O-H_2O$ have been well documented in the literature, and we expected to see this pattern in our data. Gat (1996, and references therein) have shown that $\delta^{18}O-H_2O$ varies seasonally at many sites around the world, with high $\delta^{18}O-H_2O$ during the summer months and low $\delta^{18}O-H_2O$ during the winter. The magnitude of variation observed for this central Pennsylvania site using mean values (-4.2‰ to -13.2‰) is well within the ranges of variation reported by past investigators working in the same region (DeWalle et al., 1997; O’Driscoll et al., 2005).

$\delta^{15}N-NO_3^-$

$\delta^{15}N-NO_3^-$ varied on a storm-to-storm basis during the 2005 study period (Table 2.2, Figure 2.5b). According to Figure 2.5b, $\delta^{15}N-NO_3^-$ began to decline from the March 23 storm (-0.1‰) to its lowest value, which was recorded during the July 5 storm (-2.9‰). $\delta^{15}N-NO_3^-$ was much higher on August 31 (+0.6‰) and then reached its peak on October 7 (+2.5‰). It remained elevated on the November 16 storm (1.6‰). Like $\delta^{18}O-H_2O$, a distinct seasonal pattern in $\delta^{15}N-NO_3^-$ is apparent in the dataset.

Seasonal patterns of $\delta^{15}N-NO_3^-$ have been demonstrated by many investigators. Studies in South Africa (Heaton, 1987), Germany (Freyer, 1991), England (Yeatman et al., 2001), Delaware, USA (Russell et al., 1998), and Maryland, USA (Cooney, 2005) all have shown that $\delta^{15}N-NO_3^-$ in precipitation is lowest during the summer and generally peaks during the autumn and winter months. In general, the same pattern of variation for
\(\delta^{15}\text{N-NO}_3^-\) in precipitation was observed here in central Pennsylvania using data from the six storm events sampled in 2005. Further, the range of variability in this study using mean values (-2.91‰ in mid summer to +2.5‰ in early fall) compared favorably to the range of variability observed in the Maryland study (-3‰ in summer to +2.5‰ in fall) (Cooney, 2005). One notable exception to the seasonal pattern of \(\delta^{15}\text{N-NO}_3^-\) was observed in Bermuda precipitation, where Hastings et al. (2004) reported the highest \(\delta^{15}\text{N-NO}_3^-\) in the spring and the lowest \(\delta^{15}\text{N-NO}_3^-\) in the late autumn during the course of a one-year study. While several investigators have used oxidant chemistry and variations in NO\(_2\) and NO\(_x\) to explain seasonal differences in the \(\delta^{15}\text{N-NO}_3^-\) in precipitation (e.g. Freyer et al., 1993), Hastings et al. (2004) postulated that changes in air mass and pollutant sources were more likely responsible for the seasonal differences in \(\delta^{15}\text{N-NO}_3^-\) in precipitation on Bermuda.

\(\delta^{18}\text{O-NO}_3^-\)

\(\delta^{18}\text{O-NO}_3^-\) exhibited storm-to-storm variation that was generally similar to the pattern of variation observed for \(\delta^{15}\text{N-NO}_3^-\) (Table 2.2, Figure 2.5c). \(\delta^{18}\text{O-NO}_3^-\) increased from the storm on March 23 (37.6‰) to the storm on March 28-29 (51.6‰), and then decreased substantially to its lowest value during the thunderstorms on July 5 (19.7‰). After July 5, \(\delta^{18}\text{O-NO}_3^-\) in precipitation steadily increased through the storms on August 31 (26.9‰) and October 7 (40.6‰), reaching its peak value on November 16 (51.9‰). A similar seasonal trend to \(\delta^{15}\text{N-NO}_3^-\) was noted. Organizing the storm data according to dormant season (March 23, March 28-29, November 16) and growing season (July 5, August 31, October 7) also showed differences in \(\delta^{18}\text{O-NO}_3^-\), with dormant season \(\delta^{18}\text{O-NO}_3^-\) in precipitation being significantly higher than growing season \(\delta^{18}\text{O-NO}_3^-\) \((p = 0.013)\).

The seasonality of \(\delta^{18}\text{O-NO}_3^-\) in precipitation has been widely reported by many researchers in different locations around the world. Studies in Germany (Durka et al., 1994), Pennsylvania and West Virginia (Williard et al., 2001), Colorado (Campbell et al., 2002), Bermuda (Hastings et al, 2003), New Hampshire (Pardo et al., 2004), and Maryland (Cooney, 2005) all have illustrated seasonal changes in \(\delta^{18}\text{O-NO}_3^-\) where the
lowest values occurred during the summer months and the highest values occurred during the winter months. This basic pattern of variability also was observed during the 2005 study period in central Pennsylvania using data from six storm events. The magnitude of variation for $\delta^{18}O$-NO$_3^-$ in precipitation in central Pennsylvania was $+19.7\%$ to $+51.9\%$o using mean values for each storm, which encompasses a small portion of the total range of values for $\delta^{18}O$-NO$_3^-$ in precipitation reported by Kendall (1998) for sites in North America and Germany ($+14\%$ to $+71\%$). If the full range of values from this study are considered ($+11.9\%$ to $+69\%$), then a larger portion of the range reported by Kendall (1998) is accounted for, and the value of $+11.9\%$ for $\delta^{18}O$-NO$_3^-$ likely represents one of the lower values among previously reported observations for sites in North America.

**Intra-Storm Variations**

$\delta^{18}O$-H$_2$O

$\delta^{18}O$-H$_2$O stable isotopes varied during the course of all six storm events sampled during 2005 (Table 2.2), and the magnitude of these variations was different for each event. For all six storm events, the mean change in rainwater $\delta^{18}O$-H$_2$O was $1.9\%$, with the maximum change occurring during the October 7 storm event ($4.1\%$) where five samples were taken throughout the event. The storm on March 28-29, where eight samples were taken, also showed a substantial change in $\delta^{18}O$-H$_2$O during the event ($3.27\%$). The observed changes in $\delta^{18}O$-H$_2$O during storms within the Spring Creek watershed were comparable to results obtained by Pionke and DeWalle (1992). The authors sampled ten storm events in central Pennsylvania during 1989 and 1990 and found that intra-storm variability of $\delta^{18}O$-H$_2$O ranged from $3.4$ to $16.0\%$, with the largest variability occurring during the longer and largest storms. The storms on March 28-29 and October 7, which had the largest observed changes in $\delta^{18}O$-H$_2$O, also were the two largest and longest storms sampled during the study period (Table 2.1).

Patterns of $\delta^{18}O$-H$_2$O variation during storms also were characterized for all six storm events. In general, $\delta^{18}O$-H$_2$O tended to become progressively lighter during the course of all six storms sampled (Table 2.2). Figure 2.6a shows the progression of $\delta^{18}O$-
H₂O for the March 28-29 storm and the October 7 storm. Both storms show a typical pattern of depletion of the heavy isotopes in H₂O as the storm progressed. The pattern of δ¹⁸O-H₂O stable isotopes becoming progressively lighter during the course of storm events has been observed by other investigators in the past. Gat (1996, and references therein) suggested that the beginning part of storm events were often more enriched in the heavy isotopes due to the effects of partial evaporation on falling rain drops through the air column. Further study by McDonnell (1990) and Pionke and DeWalle (1992) suggested that changes in the stable isotopic composition of H₂O during storms were primarily due to differences in moisture source, rainout history, and rainfall intensity. These and other possible controls on δ¹⁸O-H₂O variations in precipitation are explored and discussed in a later section.

δ¹⁵N-NO₃⁻

Like δ¹⁸O-H₂O stable isotopes, δ¹⁵N-NO₃⁻ also varied during the course of individual storm events sampled within the Spring Creek watershed (Table 2.2). The magnitude of the intra-storm variations in δ¹⁵N-NO₃⁻ ranged from about 1‰ to greater than 8‰ for all six storms (mean = 4.8‰). The maximum change in δ¹⁵N-NO₃⁻ was 8.8‰ and it occurred during the March 28-29 storm event.

Few studies have documented the types of changes in δ¹⁵N-NO₃⁻ that might occur during storms. Moore (1973) showed isotopic changes of about 1‰ for δ¹⁵N-NO₃⁻ sampled from a storm event near Boulder, CO. He tentatively noted that the δ¹⁵N-NO₃⁻ increased as concentrations of NO₃⁻ increased during the storm. Later, Heaton and Collett (1985) and Heaton (1987) sampled a series of storms near Pretoria, South Africa and documented fairly large changes in δ¹⁵N-NO₃⁻ (~ 2-9‰) during different events. Unlike Moore (1973), Heaton (1987) did not observe any distinct relationships between δ¹⁵N-NO₃⁻ and NO₃⁻ concentrations during events. Relationships between δ¹⁵N-NO₃⁻ and NO₃⁻ concentrations were observed during the course of individual storm events in the current study, although these relationships were quite weak and differed in terms of magnitude and direction for different storm events.
Patterns of $\delta^{15}$N-NO$_3^-$ variation during storms also were assessed for each event. For the two storms sampled during 2005 with 5 or more precipitation samples, the patterns of $\delta^{15}$N-NO$_3^-$ variation were very different (Figure 2.6b). For the storm on March 28-29, $\delta^{15}$N-NO$_3^-$ increased slightly at the beginning of the storm (+0.7‰), dropped during the mid-point of the event (-3.3‰), and then increased considerably (+8.3‰) before dropping substantially again (-8.8‰) toward the end of the storm. On October 7, $\delta^{15}$N-NO$_3^-$ increased gradually throughout the event (+1.7‰), fell moderately (-3.5‰) and then increased again (+2.2‰) at the end of the storm. These results are in contrast to Heaton (1987), who, with the exception of one event, reported that $\delta^{15}$N-NO$_3^-$ generally followed an increasing pattern during storms. Both Moore (1973) and Heaton (1987) suggested that atmospheric oxidation reactions may have caused the observed $\delta^{15}$N-NO$_3^-$ variations in precipitation, but did not have the data available to test those hypotheses. The potential effects of climate and atmospheric chemistry on $\delta^{15}$N-NO$_3^-$ variations are explored later in this paper.

$\delta^{18}$O-NO$_3^-$

During the course of this study, large variations in $\delta^{18}$O-NO$_3^-$ ranging from 6.7 to 35.1‰ were observed during individual storm events (mean = 21.5‰) (Table 2.2). The maximum change in $\delta^{18}$O-NO$_3^-$ occurred during the March 28-29 storm event, which was the longest duration event sampled during 2005. Interestingly enough, the magnitude of observed changes in $\delta^{18}$O-NO$_3^-$ was not related to the size or duration of the storm event, with the second largest change (30.0‰) occurring during the shortest duration event (August 31).

Patterns of $\delta^{18}$O-NO$_3^-$ changes during storms appeared to be more systematic than patterns of $\delta^{15}$N-NO$_3^-$ (Table 2.2). For the three storms that were sampled where greater than 3 samples were collected for $\delta^{18}$O-NO$_3^-$ (March 28-29, October 7, and November 16), $\delta^{18}$O-NO$_3^-$ was typically enriched at the beginning of the event, decreased as the storm progressed, and then increased to a value similar to the starting isotopic composition. Figure 2.6c shows the $\delta^{18}$O-NO$_3^-$ trend for the storms sampled on March 28-29 and October 7. A plot of NO$_3^-$ concentration variations is also showed for the
same two storm events (Figure 2.6d). From the two plots, it appears that $\delta^{18}$O-NO$_3^-$ and NO$_3^-$ concentrations vary together during storms. Pearson correlation analysis showed correlation coefficients between $\delta^{18}$O-NO$_3^-$ and NO$_3^-$ concentrations were 0.55 ($p < 0.001$) and 0.39 ($p < 0.001$) for the March 28-29 and October 7 storms, respectively. The reason for these correlations is not known, but like $\delta^{15}$N-NO$_3^-$, the atmospheric processes responsible for NO$_3^-$ formation may be an important driver.

To our knowledge, no published studies have documented variations of $\delta^{18}$O-NO$_3^-$ during storm events. The observed variations of $\delta^{18}$O-NO$_3^-$ are primarily significant in the context of using $\delta^{18}$O-NO$_3^-$ as a tracer of NO$_3^-$ in atmospheric wet deposition. Many past studies have utilized $\delta^{18}$O-NO$_3^-$ stable isotopes to estimate the percent contribution of wet NO$_3^-$ deposition to total stream NO$_3^-$ in forested watersheds using simple two-component mixing models (e.g. Williard et al., 2001; Campbell et al., 2002; Pardo et al., 2004). These studies often have used bulk precipitation samples to characterize the $\delta^{18}$O-NO$_3^-$ signature of wet NO$_3^-$ deposition and generally have concluded that wet NO$_3^-$ deposition accounts for anywhere from 30-50% of the total amount of NO$_3^-$ exported from forested watersheds, with NO$_3^-$ from soil nitrification making up the remaining 50-70%. Since the degree to which $\delta^{18}$O-NO$_3^-$ varies in rainfall is not usually known for a given event, the range of possible solutions using two-component mixing models is rarely if ever reported. As a result of within-storm variations of precipitation $\delta^{18}$O-NO$_3^-$, care should be taken when interpreting the reported contributions of wet NO$_3^-$ deposition to total stream NO$_3^-$.

Possible Explanations for Observed $\delta^{18}$O-H$_2$O Variations

Temperature is an important controlling variable on the resultant $\delta^{18}$O-H$_2$O isotopic composition of precipitation. Rain is formed as water vapor cools and condenses within a cloud, and this formation process usually occurs under isotopic equilibrium conditions (Dansgaard, 1964). According to Rayleigh fractionation theory, heavy isotopes ($\delta^{18}$O-H$_2$O) are preferred during the condensation process, and the remaining cloud vapor becomes progressively depleted in $\delta^{18}$O-H$_2$O (Rozanski et al., 1992). The amount of fractionation is dependent on the temperature of formation at the base of the
Therefore, many investigators have used air temperature as a surrogate for cloud-base temperatures and reported significant long-term (> 20 years) relationships between $\delta^{18}$O-H$_2$O and air temperature (°C) (e.g. Dansgaard, 1964 and references therein; Rozanski et al., 1992). The slopes of these long-term relationships ($\delta^{18}$O-H$_2$O‰ per °C) have been used to indicate the temperature at which water was condensed within the cloud, and thus have shown potential as indicators of past climatic conditions.

Long-term relationships between $\delta^{18}$O-H$_2$O and temperature have clearly been demonstrated for many sites around the world. For the central Pennsylvania study, only one year of data was available, and much of the data was collected during the course of individual storm events. Despite the shortness of the dataset, a significant ($p < 0.001$, $R^2 = 0.56$) positive relationship was found between $\delta^{18}$O-H$_2$O and air temperature (°C) (Figure 2.7a). The slope of this relationship was 0.28‰ per °C, which is much less than the reported slopes for many of the long-term relationships between $\delta^{18}$O-H$_2$O and air temperature (0.52‰ to 0.72‰ per °C) (Rozanski et al., 1992). However, it does compare quite well to relationships that have been developed on a short-term or seasonal basis (0.30‰ to 0.54‰ per °C) (Rozanski et al., 1992). Thus, it appears that for this site in central Pennsylvania, air temperature is an important controlling variable on the observed variations in $\delta^{18}$O-H$_2$O, even for data that was largely collected during the course of individual storm events. It appears to imply that air temperature recorded at ground level is a useful indicator for changes that may be occurring in the cloud during storm events, although this would have to be verified using additional within-storm data.

In addition to air temperature, precipitation amount has been shown to affect the observed $\delta^{18}$O-H$_2$O isotopic composition (e.g. Dansgaard, 1964). In general, the amount effect has been demonstrated by comparing $\delta^{18}$O-H$_2$O data from small and large rainstorms. Water collected during smaller rainstorms is typically more enriched in the heavy isotopes than the water collected from larger rainstorms (Ingraham, 1998), which is primarily due to the different effects of evaporation on isotope fractionation. As a result, a negative relationship between $\delta^{18}$O-H$_2$O and precipitation amount (mm) has been demonstrated, with slopes ranging from -1.6‰ to -2.2‰ per 100 mm of rainfall (Dansgaard, 1964).
Data on the cumulative amount of precipitation (mm) were available during this study to test whether there was an observable amount effect on **δ**<sup>18</sup>O-H<sub>2</sub>O isotopes in precipitation. For the two longest duration storms sampled during the 2005 study period (March 28-29 and October 7), strong negative relationships between **δ**<sup>18</sup>O-H<sub>2</sub>O and precipitation amount were observed (Figure 2.7b). The relationship for the March 28-29 storm had a slope of -4.9‰ per 100 mm of precipitation (p < 0.001, R<sup>2</sup> = 0.97), whereas the relationship for the October 7 storm had a slope of -5.2‰ per 100 mm of precipitation (p < 0.001, R<sup>2</sup> = 0.58). Thus, a clear effect of the amount of precipitation on **δ**<sup>18</sup>O-H<sub>2</sub>O could be demonstrated during the course of individual storm events, and these slopes were comparable to those reported by Dansgaard (1964) for longer-term studies.

The track that an individual storm takes as well as the characteristics of its air mass has been shown to affect the isotopic composition of precipitation (Lawrence et al., 1982; Gedzelman and Lawrence, 1982; Burnett et al., 2004). In general, these studies showed that precipitation originating in the cool-sector of storms was much lighter in **δ**D and **δ**<sup>18</sup>O-H<sub>2</sub>O stable isotopes than precipitation originating in the warm-sector for sites located in central New York. The observed differences in the isotopic composition of precipitation primarily were attributed to the proximity and location of the frontal boundaries that produced the precipitation. Lawrence et al. (1982) explained that precipitation often condenses in the air mass above the frontal boundary, which results in a progressive depletion of the heavy isotopes in H<sub>2</sub>O (**δ**D and **δ**<sup>18</sup>O-H<sub>2</sub>O). The altitude of the frontal boundary also is important, because the precipitation is further depleted in heavy isotopes as the condensation process occurs at higher and higher altitudes. The farther a frontal boundary is located from the sampling location, the higher the altitude is at which the frontal boundary can be found. For the studies in central New York, cool-sector events were typically characterized by storms and frontal boundaries that remained well to the south of the sampling location, with the frontal boundary at considerable altitude. These events mostly occurred during the winter months and produced precipitation that was depleted in the heavier isotopes.

Using data collected from the six storms during 2005 in central Pennsylvania, a similar difference in **δ**<sup>18</sup>O-H<sub>2</sub>O was found when cool-sector and warm-sector events were compared. The two storms sampled during March had storm tracks well to the south of
State College, keeping the region within the cool-sector of the storm during the entire event (Figure 2.4a-b). In comparison, the four successive storm events in 2005 all had storm tracks where the rain fell entirely within the warm-sector of the storm, just ahead of the advancing frontal boundary (Figure 2.4c-f). When the mean δ¹⁸O-H₂O values were compared for warm- and cool-sector events, we found that precipitation in the cool-sector had significantly (p < 0.001) more negative values of δ¹⁸O-H₂O (-10.3‰) than precipitation in the warm-sector (-6.4‰). This confirms the results reported by Lawrence et al. (1982) and implies that storm track and air mass characteristics can be used to explain the observed variability of δ¹⁸O-H₂O in precipitation at this central Pennsylvania site.

Possible Explanations for Observed δ¹⁵N-NO₃⁻ Variations

Several possible controls on the variation of δ¹⁵N-NO₃⁻ were explicitly considered in the context of this study, including (1) effects of atmospheric oxidation chemistry on the formation of NO₃⁻, (2) possible changes in the sources of atmospheric NO₃⁻, and (3) storm track and air mass characteristics. The potential importance of lightning also was explored, as lightning can be an important source of NO₃⁻ in precipitation (e.g. Hastings et al., 2003).

Some evidence has been presented to suggest that atmospheric oxidation chemistry may explain the seasonal variations of δ¹⁵N in NO₂ gas (Freyer et al., 1993), and thus potentially the δ¹⁵N of the resultant HNO₃ (Hastings et al., 2004). In a study performed in Jülich, Germany, Freyer et al. (1993) showed that the cyclic reactions responsible for converting between NO and NO₂ affected the δ¹⁵N signature of NO₂ gas. The seasonality of the δ¹⁵N in NO₂ was attributed to changes in the ratio of NO₂ to NOₓ (NO₂:NOₓ), where NOₓ is defined as the sum of NO and NO₂. Freyer et al. (1993) argued that the large equilibrium isotope effect between NO and NO₂ (α = 1.028 at 25°C) becomes more pronounced as the ratio of NO₂:NOₓ approaches 1 during the winter months. This in turn would be expected to produce higher values of δ¹⁵N in NO₂. During the summer months, when more complete oxidation of NO to NO₂ occurs due to higher concentrations of O₃, the ratio of NO₂:NOₓ declines, decreasing the influence of
the isotopic exchange reaction between NO₂ and NOₓ and producing lower values of δ¹⁵N in NO₂ (Freyer et al., 1993).

Hourly data on atmospheric concentrations of NO₂ and NOₓ were available from the Air Quality Learning and Demonstration Center at Penn State, and were used to determine whether the ratio of NO₂:NOₓ could explain the observed inter- and intra-storm variations of δ¹⁵N-NO₃⁻ in precipitation in central Pennsylvania. Figure 2.8a shows a significant (p = 0.002, R² = 0.47) negative relationship between δ¹⁵N-NO₃⁻ and NO₂:NOₓ. The slope of this relationship (-21) compared favorably to the slopes reported by Freyer et al. (1993) (-16 to -22) for the reported relationships between δ¹⁵N in NO₂ and NO₂:NOₓ. Further support for the effects of oxidation reactions on δ¹⁵N-NO₃⁻ can be seen in Figure 2.9, which shows a significant (p < 0.001, R² = 0.54) negative relationship between δ¹⁵N-NO₃⁻ and atmospheric O₃. Freyer et al. (1993) observed that δ¹⁵N-NO₃⁻ in precipitation was often highest when O₃ concentrations were lowest during the winter months, and the relationship in Figure 2.8b confirms this observation. The relationships in Figures 2.8 and 2.9 suggest that changes in the atmospheric oxidation chemistry, including effects on the ratio of NO₂ to NOₓ and atmospheric concentrations of O₃, help explain observed changes in the δ¹⁵N-NO₃⁻ in precipitation both during and in between storm events in central Pennsylvania.

Changes in anthropogenic and natural sources of atmospheric NO₃⁻ also have been suggested as possible drivers for variations of δ¹⁵N-NO₃⁻ in precipitation. Heaton (1990) reported that the δ¹⁵N of NOₓ resulting from vehicle exhaust emissions ranged from -13‰ to -2‰, whereas the δ¹⁵N of NOₓ from coal combustion ranged from +6‰ to +9‰. Natural NOₓ produced by lightning is very important during the summer months in the northeast U.S. (see Levy II et al., 1996; Price et al., 1997), and a study by Hoering (1957) showed that the δ¹⁵N of NOₓ from electrical discharges such as lightning was found to be between -0.5‰ and +1.4‰. For this particular study, it is possible that mixtures of NO₃⁻ from coal emissions and coal-fired power plant emissions affected δ¹⁵N-NO₃⁻ in rainfall during all six storms, with lightning representing a potentially important third source affecting δ¹⁵N-NO₃⁻ during the two thunderstorms on July 5 and
August 31. The relative importance of these sources is explored further in the context of storm origin and air mass characteristics.

Back trajectory analysis (e.g. Russell et al., 1998; Hastings et al., 2003) and air mass characterization (e.g. Lawrence et al., 1982) were used to assess whether there were any effects of air mass source on $\delta^{15}$N-NO$_3^-$ that might indicate a change in the source of NO$_3^-$ in precipitation. In essence, the two storms in March were characterized by east / northeast (E/NE) transport patterns with precipitation occurring in the cool-sector of the storm (Figure 2.4a-b). In contrast, the four storms that followed were characterized by southwest / south / southeast SW/S/SE transport patterns and produced precipitation in the warm-sector of the storm (Figure 2/4c-f). Table 2.3 shows that $\delta^{15}$N-NO$_3^-$ was significantly lower ($p = 0.051$) for storms with E/NE transport and cool-sector precipitation (-1.2‰) as compared to storms with SW/S/SE transport and warm-sector precipitation (+1.4‰). Thus, storms occurring with transport patterns from the E/NE exhibited negative $\delta^{15}$N-NO$_3^-$ signatures that may have been influenced by NO$_x$ derived from automobile emissions. Storms with SW/S/SE transport patterns over the Ohio River Valley exhibited higher $\delta^{15}$N-NO$_3^-$ signatures, which indicated a stronger influence of NO$_3^-$ from coal-fired power plant emissions and/or lightning (July 5 and August 31 thunderstorms). These conclusions should be interpreted with care, as they are based upon the limited available information on $\delta^{15}$N signatures in atmospheric NO$_3^-$ sources reported in the literature.

**Possible Explanations for Observed $\delta^{18}$O-NO$_3^-$ Variations**

Variations of $\delta^{18}$O-NO$_3^-$, especially those observed with changes in seasons, primarily have been explained in the context of different atmospheric oxidation pathways. Simplified atmospheric reactions responsible for producing HNO$_3$ from NO$_x$ in the atmosphere are summarized below (e.g. Calvert et al., 1985; Hastings et al., 2004).
**Summertime Reactions**

\[
\text{NO}_2 + h\nu \rightarrow \text{NO} + \text{O} \\
\text{NO} + \text{O}_3 \rightarrow \text{NO}_2 + \text{O}_2 \\
\text{NO}_2 + \text{OH} + \text{M} \rightarrow \text{HNO}_3 + \text{M}
\]

**Wintertime Reactions**

\[
\text{NO}_2 + \text{O}_3 \rightarrow \text{NO}_3 + \text{O}_2 \\
\text{NO}_2 + \text{NO}_3 + \text{M} \rightarrow \text{N}_2\text{O}_5 + \text{M} \\
\text{N}_2\text{O}_5 + \text{H}_2\text{O} \rightarrow 2\text{HNO}_3
\]

Reactions during the summertime are dominated by the oxidation of NO\textsubscript{x} via OH. During the wintertime, when OH concentrations are typically lower than in summer, the oxidation of NO\textsubscript{x} is dominated by O\textsubscript{3}. The $\delta^{18}$O composition of OH is expected to reflect the isotopic composition of H\textsubscript{2}O vapor (Dubey et al., 1997), which is typically negative. O\textsubscript{3} on the other hand has $^{18}$O values in the range of $+90\%o$ to $+122\%o$ (Krankowsky et al., 1995; Johnston and Thiemens, 1997). As a result, oxidation of NO\textsubscript{2} via the OH pathway produces the lower $\delta^{18}$O-NO\textsubscript{3} observed during the summer months. In contrast, reactions between NO\textsubscript{x} and O\textsubscript{3} during the wintertime produce the higher values of $\delta^{18}$O-NO\textsubscript{3}. This process helps to explain the seasonal (storm-to-storm) variation of $\delta^{18}$O-NO\textsubscript{3} observed in precipitation during the 2005 study period in central Pennsylvania (Figure 2.6c).

Further evidence was sought to verify the importance of these different reaction pathways by using available information on atmospheric O\textsubscript{3} concentrations and the $\delta^{18}$O isotopic content of rainwater. $\delta^{18}$O-NO\textsubscript{3} data for all six storms were classified according to the time of year that the precipitation occurred. Wintertime (dormant season) storms included the events on March 23, March 28-29, and November 16. Summertime (growing season) storms included the events on July 5, August 31, and October 7. A significant ($p = 0.03$, $R^2 = 0.36$) and positive relationship was found between $\delta^{18}$O-NO\textsubscript{3} and O\textsubscript{3} concentrations (ppb) for the three storms that occurred during the wintertime months, when oxidation via the O3 pathway would be expected to dominate (Figure 2.9a). Thus, as O\textsubscript{3} becomes progressively more available to oxidize NO\textsubscript{x} to HNO\textsubscript{3} in the troposphere, $\delta^{18}$O-NO\textsubscript{3} in precipitation increases. In contrast, during the summertime a non-significant negative relationship ($p = 0.15$, $R^2 = 0.31$) was found between $\delta^{18}$O-NO\textsubscript{3} and $\delta^{18}$O-H\textsubscript{2}O in precipitation (Figure 2.9b). While the relationship was not statistically
significant at the $\alpha = 0.05$ level, it does suggest that the $\delta^{18}\text{O-H}_2\text{O}$ may help to explain the effects of OH oxidation on $\delta^{18}\text{O-NO}_3^{-}$.

The influence of storm track and air mass characteristics also were investigated as possible controls on the $\delta^{18}\text{O-NO}_3^{-}$ in precipitation. While $\delta^{18}\text{O-NO}_3^{-}$ in precipitation was higher for storms that exhibited east / northeast (E/NE) transport patterns (cool-sector) than for storms that exhibited southwest / south / southeast SW/S/SE transport patterns (warm-sector), the difference was not statistically significant ($p = 0.12$). Thus, unlike $\delta^{15}\text{N-NO}_3^{-}$, storm track and air mass characteristics did not help to explain observed differences in $\delta^{18}\text{O-NO}_3^{-}$.

**CONCLUSIONS**

Significant inter- and intra-storm variations of $\delta^{18}\text{O-H}_2\text{O}$, $\delta^{15}\text{N-NO}_3^{-}$, $\delta^{18}\text{O-NO}_3^{-}$ in precipitation were observed during six storms sampled during 2005 in central Pennsylvania. Seasonal variations of $\delta^{18}\text{O-H}_2\text{O}$ were similar to those found in past studies in central Pennsylvania, with the highest values during the summertime and most depleted values during the winter. Increases in $\delta^{18}\text{O-H}_2\text{O}$ with surface temperature data, which served as a useful analog for cloud condensation temperatures, suggested that greater fractionation and more depleted $\delta^{18}\text{O-H}_2\text{O}$ coincided with cooler temperatures. Further, progressive depletion of $\delta^{18}\text{O-H}_2\text{O}$ isotopes occurred during two long-duration storm events, and this clearly was related to cumulative precipitation amounts. Storm tracks also were important for explaining variations in $\delta^{18}\text{O-H}_2\text{O}$, with two cool-sector storms producing significantly more depleted $\delta^{18}\text{O-H}_2\text{O}$ than four warm-sector storms.

With regard to NO$_3^{-}$ stable isotopes, both $\delta^{15}\text{N-NO}_3^{-}$ and $\delta^{18}\text{O-NO}_3^{-}$ were most depleted during the summer months and were most enriched during winter. Patterns of $\delta^{15}\text{N-NO}_3^{-}$ variation during two long-duration storms were very different, whereas $\delta^{18}\text{O-NO}_3^{-}$ variations showed a common tendency to be enriched at the beginning and ending of storms, with the most depleted signatures occurring during the middle of the event. $\delta^{15}\text{N-NO}_3^{-}$ decreased with increasing ratios of NO$_2$:NO$_x$ and O$_3$ in the atmosphere, which
suggested that atmospheric oxidation chemistry was an important driver of seasonal and within-storm variations of these isotopes. $\delta^{18}$O-NO$_3^-$ variations during three dormant season storm events were positively related to O$_3$, which implied that the O$_3$ oxidation pathway was important for producing the high $\delta^{18}$O-NO$_3^-$ observed in wintertime precipitation. Cool-sector storms with tracks from the E/NE produced low $\delta^{15}$N-NO$_3^-$ characteristic of automobile emissions, whereas warm-sector storms with tracks from the SW/S/SE produced high $\delta^{15}$N-NO$_3^-$ characteristic of coal-fired emissions. No relationship between $\delta^{18}$O-NO$_3^-$ and storm track was observed in the data.

Overall, the results of this study demonstrated that: (1) information about oxidant levels can be useful to predict seasonal and within-storm variations of NO$_3^-$ stable isotopes, and (2) knowledge of storm tracks (warm-sector versus cool-sector) may be important for determining sources of NO$_3^-$ in wet deposition and formation processes that affect H$_2$O.
REFERENCES


Environmental Protection Agency (EPA). 2006. Clean Air Status and Trends Network (CASTNET). [http://www.epa.gov/castnet/](http://www.epa.gov/castnet/). This website provides data and information on dry deposition of sulfur and nitrogen compounds for more than 70 sites throughout the United States.


Figure 2.1: Map showing general location where precipitation samples were collected within the Spring Creek watershed. Dark black outline delineates upper portion of the Spring Creek watershed, which served as the watershed boundary for the precipitation study.
Figure 2.2: Precipitation collector used to collect sequential precipitation samples for water chemistry and stable isotope analyses.
Figure 2.3: Picture showing how water samples were processed to collect NO$_3^-$ for stable isotope analysis. Water samples were successively filtered through: (1) a 0.45 µm groundwater filter, (2) a column packed with BIO-RAD© AG 50W-X8 cation exchange resin (100-200 mesh), and (3) a column packed with BIO-RAD© AG 2-X8 anion exchange resin (100-200 mesh).
Figure 2.4 (a-b): Weather surface maps (left) and 48-hour back trajectories (right) at 500 meters above ground level for storms sampled on (a) March 23, 2005 and (b) March 28-29, 2005. Star indicates the collection site in State College, PA. Heavy black arrow indicates forward movement of storm system. Shaded region indicates the location of the warm-sector of storms that were sampled.
Figure 2.4 (c-d): Weather surface maps (left) and 48-hour back trajectories (right) at 500 meters above ground level for storms sampled on (a) July 5, 2005 and (b) August 31, 2005. Star indicates the collection site in State College, PA. Heavy black arrow indicates forward movement of storm system. Shaded region indicates the location of the warm-sector of storms that were sampled.
Figure 2.4 (e-f): Weather surface maps (left) and 48-hour back trajectories (right) at 500 meters above ground level for storms sampled on (a) October 7, 2005 and (b) November 16, 2005. Star indicates the collection site in State College, PA. Heavy black arrow indicates forward movement of storm system. Shaded region indicates the location of the warm-sector of storms that were sampled.
Figure 2.5: Box plots of (a) $\delta^{18}$O-H$_2$O, (b) $\delta^{15}$N-NO$_3^-$, (c) $\delta^{18}$O-NO$_3^-$, (d) NO$_3^-$, (e) SO$_4^{2-}$, and (f) Cl$^-$ for six storm events sampled in 2005. Isotopic ratios (plots a-c) are reported in delta notation and the units are in permil (‰). Inorganic chemicals (plots d-f) are reported in mg L$^{-1}$. For the plot of $^{18}$O-H$_2$O (plot a), n=2 samples for the storm sampled on 7-5.
Figure 2.6: Plots showing within-storm variation in (a) $\delta^{18}O\text{-H}_2\text{O}$ (‰), (b) $\delta^{15}N\text{-NO}_3$ (‰), (c) $\delta^{18}O\text{-NO}_3$ (‰), and (d) NO$_3$ (mg L$^{-1}$) for two storm events. Left-hand plot (1) shows a storm event that was sampled on March 28-29, 2005. Right-hand plot (2) shows a storm event that was sampled on October 7, 2005.
Figure 2.7: Variations in $\delta^{18}O$-H$_2$O (‰) with (a) temperature (°C) for storms 2 (3-28-05) and 5 (10-7-05) and (b) cumulative precipitation amount (mm) for all six storms.
Figure 2.8: Variations in δ¹⁵N-NO₃⁻ (%o) with (a) NO₂:NOₓ and (b) ozone (ppb) for all six storms sampled during 2005 in central PA.
Figure 2.9: Variations in δ¹⁸O-NO₃⁻ (‰) with (a) ozone (ppb) for precipitation occurring during the dormant season (n = 3 storms) and (b) δ¹⁸O-H₂O (‰) for precipitation occurring during the growing season (n = 3 storms). Samples were collected in central PA.
Table 2.1: Information on start and end times, precipitation type, precipitation amount, and storm-type characterization for the six storms sampled during 2005 in central PA.

<table>
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<tr>
<th>Storm Number</th>
<th>Start Time (EST)</th>
<th>End Time (EST)</th>
<th>Type of Precipitation</th>
<th>Amount (cm)</th>
<th>Mean Intensity (cm hr(^{-1}))</th>
<th>Number of Samples</th>
<th>Air Mass Type</th>
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<td>0.30</td>
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Table 2.2: Precipitation chemistry and stable isotopic composition for all precipitation samples collected during the 2005 study. Bolded values show the mean value for each storm event. The row in italics represents the results from a melted snow sample.

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<th>Storm</th>
<th>pH</th>
<th>EC (µS cm⁻¹)</th>
<th>NO₃⁻</th>
<th>SO₄²⁻</th>
<th>Na⁺</th>
<th>Cl⁻</th>
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<th>δ¹⁵N-NO₃⁻</th>
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Table 2.3: Comparison of precipitation amount, inorganic chemistry, and stable isotope characteristics of cool-sector and warm-sector storm events.

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<td>Mean NO$_3^-$ concentration (mg L$^{-1}$)</td>
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<td>Mean NO$_3^-$ deposition (kg ha$^{-1}$)</td>
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<td>$\delta^{15}$N-NO$_3^-$ (‰)**</td>
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<td>$\delta^{18}$O-NO$_3^-$ (‰)</td>
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<td>$\delta^{18}$O-H$_2$O (‰)*</td>
<td>-10.27</td>
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*Results of a one-way ANOVA were significant at $\alpha = 0.05$

**Results of a one-way ANOVA were significant at $\alpha = 0.1$
Chapter 3

NITRATE ISOTOPE TRANSFORMATIONS IN GAINING AND LOSING STREAM REACHES WITHIN A MIXED LAND-USE KARST WATERSHED IN CENTRAL PENNSYLVANIA

ABSTRACT

The effect of in-channel processes on NO₃⁻ isotope signatures was studied in a mixed land-use karst basin to isolate effects of natural processes such as denitrification as well as point-source spring and sewage inputs. The study was conducted in Spring Creek, a 201 km² mixed land-use watershed with karst geology located in central Pennsylvania. A Lagrangian sampling design was used to identify changes in NO₃⁻ stable isotopes and inorganic chemistry in four distinct stream reaches during a low streamflow period in early summer 2005. Two reaches were gaining reaches (Reaches 1 and 4), with Reach 1 being impacted by a large spring input and Reach 4 affected by an upstream sewage treatment plant discharge. NO₃⁻ transformations such as denitrification were identified using plots of NO₃⁻ stable isotopes against NO₃⁻ concentrations for each reach. Overall changes in δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ were minimal (± 0.6‰) in the losing reaches (Reaches 2 and 3), and suggested that NO₃⁻ transformation processes (e.g. denitrification) were efficient and resulted in minimal isotopic fractionation. In contrast, gaining reaches (Reaches 1 and 4) exhibited significant changes in NO₃⁻ stable isotopes due to inputs from a large spring and a sewage treatment plant discharge, respectively. In the gaining reaches, results showed that undetected spring inputs in karst terrain can alter NO₃⁻ isotope signatures. Furthermore, plumes of sewage effluent can have a gradually expanding zone of influence on NO₃⁻ isotopes in the downstream direction. Overall, this study showed that NO₃⁻ stable isotope levels were relatively unaffected by NO₃⁻ transformation processes, and that unknown or undetected inputs of NO₃⁻ within streams can have major impacts on inferred in-stream processes.
INTRODUCTION

A great deal of research has been devoted to identifying the principal natural and anthropogenic sources of dissolved nitrate (NO$_3^-$) in streams using stable isotopes of nitrogen ($^{15}$N) and oxygen ($^{18}$O). Studies have been conducted in a variety of different settings, including forested (e.g. Williard et al., 2001), agricultural (e.g. Karr et al., 2003), urban (e.g. Ging et al., 1996), and mixed land-use (e.g. Chang et al., 2002) watersheds. Stable isotopes ($\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$) have been used to trace NO$_3^-$ in streams because they attain unique signatures for different NO$_3^-$ sources (see Kendall, 1998; Figure 3.1), which can provide critically important information to support water quality management programs.

One of the major assumptions in stream studies using natural abundance levels of $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ is that the isotopes conserve their pollutant source signatures during in-stream transport. However, both point source and diffuse source additions of new NO$_3^-$ with differing isotope signatures and in-stream NO$_3^-$ transformation processes represent ways that $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ signatures can be altered. In-stream processes such as denitrification (Kellman and Hillaire-Marcel, 1998), nitrification (Kendall, 1998), assimilation by plants and phytoplankton (e.g. Fogel and Cifuentes, 1993; Granger et al., 2004), biological and food-web transformations (e.g. Fry, 2006), and simple dilution or mixing (Kendall, 1998) all can affect $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$. As a result, quantifying the changes in NO$_3^-$ stable isotopes and determining whether these changes are due to the influx of new NO$_3^-$ sources or in-stream processing should be an important objective in studies of watershed NO$_3^-$ pollution.

One simple way to illustrate potentially important changes in NO$_3^-$ isotopes is to develop relationships between NO$_3^-$ stable isotopes and NO$_3^-$ concentrations. Past studies of NO$_3^-$ isotope transformations in groundwater (e.g. Mariotti et al., 1988; Fustec et al., 1991; Koba et al., 1997) and stream water (e.g. Kellman and Hillaire-Marcel, 1998; Sebilo et al., 2003) have used negative relationships between NO$_3^-$ stable isotopes (e.g. $^{15}$N-NO$_3^-$) and the natural logarithm of NO$_3^-$ concentrations to indicate the importance of denitrification. This simple approach may be useful for identifying important NO$_3^-$
isotope transformation trends in streams in addition to denitrification; however, several factors exist that must be addressed in order to successfully interpret these trends.

Mixed land-use watersheds provide challenging environments in which to use δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ isotopes to distinguish between the importance of in-stream NO₃⁻ processing and external NO₃⁻ sources. Mixed land-use watersheds typically have a mixture of land-uses (e.g. forest, agriculture, and urban) and NO₃⁻ sources such as treated sewage effluent, fertilizer inputs from residential and agricultural lands, and stormwater runoff. NO₃⁻ concentrations are typically elevated in developed watersheds (e.g. Walsh et al., 2005), which enhances the possibility that in-stream NO₃⁻ processes (e.g. assimilation and denitrification) will be important.

Karst geology represents an added level of complexity because streams in these settings can gain and lose significant volumes of water in discrete stream reaches (e.g. O’Driscoll and DeWalle, 2006). Streams that lose water to groundwater may not exhibit significant changes in NO₃⁻ stable isotopes. In contrast, gains of NO₃⁻ from point-sources, springs, or upwelling groundwater may significantly alter δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ signatures and confound the use of these isotopes as tracers. In karst regions, it is important to gain a better understanding of NO₃⁻ isotope transformations in losing and gaining streams.

The overall goal of this study was to assess NO₃⁻ isotope transformations in four distinct stream reaches within a 13.5 km section of Spring Creek, a rapidly urbanizing karst watershed located in central Pennsylvania. We hypothesized that NO₃⁻ stable isotopes in stream reaches with minimal gains of new water would be more representative of in-stream NO₃⁻ processing whereas NO₃⁻ stable isotopes in reaches that were impacted by point source gains from springs or sewage effluent would be more indicative of NO₃⁻ sources. The specific objectives of the study were to:

1. Compare the magnitude and pattern of NO₃⁻ isotope transformations in losing stream reaches to the magnitude and pattern of NO₃⁻ isotope transformations in reaches that gained water from groundwater and a sewage effluent discharge.
2. Evaluate the effectiveness of using natural abundance levels of $\delta^{15}\text{N-NO}_3^-$ and $\delta^{18}\text{O-NO}_3^-$ to infer in-stream processing of NO$_3^-$ in stream water in urbanizing karst watersheds.

MATERIALS AND METHODS

Study Site Description

The study was performed within the upper portion of the Spring Creek watershed, located in central Pennsylvania (Figure 3.2a). The upper portion of the Spring Creek watershed is 201 km$^2$ in area and includes the borough of State College and the campus of Penn State University (Figure 3.2a). While land-use in the upper basin is still predominately agriculture (44%) and forest (34%), urban land-uses (22%) such as residential and commercial development are increasing in importance as population increases around the borough of State College.

In terms of hydrology, Spring Creek is situated within the karst-underdrained Nittany Valley in the Valley and Ridge physiographic region (O’Driscoll and DeWalle, 2006). Due to the karst nature of the valley, Spring Creek can be classified as a perched or losing stream in many sections (O’Driscoll and DeWalle, 2006), with major groundwater gains often occurring at distinct spring inputs (Fulton et al., 2005). The gradient of Spring Creek is low (slope ~ 0.5%), and the stream channel sediments can be characterized as predominately cobble and gravel with silt-sand mixtures in the slower pools (Wohl and Carline, 1996). In the upper portion of the watershed stream channel widths range from 3-6 m in the headwaters to 9-15 m towards the defined outlet at Rock Road. During periods of low streamflow, average stream depths in the thalweg range from 0.2-0.5 m deep.

Selection of Sampling Period

In-stream NO$_3^-$ transformation processes are often considered most efficient during the summer and fall months when streamflows are low (shallow depths) and stream temperatures are elevated (e.g. Hill, 1988). The dates chosen to conduct this study
were June 21 and June 23, 2005, which followed a period of extremely low rainfall and thus were characterized by very low streamflow rates. Total rainfall for April, May, and June 2005 at State College was only 13.8 cm, which represented the driest April – June period since records began in 1926 (PA State Climatologist, 2006). In response to the lack of precipitation, streamflow in May and June averaged only 1.15 m$^3$ s$^{-1}$, which was much below the average for those two months during the period of record at the USGS stream gauge in Houserville (1985 – 2005; mean = 2.0 m$^3$ s$^{-1}$). Figure 3.3 shows a picture of the low streamflow conditions observed in Spring Creek during the sampling period, which is located amid the study section. Figure 3.4 shows precipitation (observed at State College) and streamflow rates (observed at Houserville) for June 2005, just prior to the onset of sampling. A period of gradual streamflow recession, beginning on June 12, led up to the sampling on June 21 and 23.

**Study Design and Sample Collection**

A Lagrangian sampling design was used to assess NO$_3^-$ isotope transformations in Spring Creek. Lagrangian sampling (e.g. Moody, 1993; Battaglin et al., 2001) using measured stream velocity was used to sample a parcel of water repeatedly as it flowed downstream to identify changes in chemistry and hydrology that may be indicative of a given process. Four distinct stream reaches of varying lengths were sampled along the main stem of Spring Creek (Figure 3.2a, Table 3.1). More detailed descriptions of each stream reach are given in Appendix A.3.

Travel-time estimation was conducted during June 13-17, 2005, a period during which streamflow rates in Spring Creek remained quite stable (Figure 3.4). Stream velocities were estimated for each reach using a Marsh-McBirney current meter and Manning’s equation (see Chow et al., 1988). Travel-times were then used to estimate the times when a water sample would be collected at each sampling site within a given reach (Table 3.1).

Stream sampling was conducted during the daytime hours on June 21 and June 23, 2005. Both of these days were sunny and clear, with maximum air temperatures reaching 30°C (85°F). Reaches 1 and 4 were sampled on June 21, and Reaches 2 and 3 were sampled on June 23. Water samples were collected at all sites for stable isotopes in
NO₃⁻ and inorganic chemistry. The times when water samples were collected are shown in Table 3.1. Samples for NO₃⁻ stable isotope analysis were collected in 5-L polypropylene carboys. Each carboy was rinsed with 10% hydrochloric acid (HCl), and rinsed three times with deionized water prior to sample collection. Samples for inorganic water chemistry were collected in 1-L high-density polyethylene bottles that had been washed in phosphate-free soap, rinsed and soaked in a 20% HCl bath, and then rinsed with deionized water.

Finally, stream discharge was computed from measurements at the top and bottom of each reach using the wading-discharge method with a Marsh-McBirney current meter. Discharge measurements were taken the same day as the water samples. Streamflow in Reaches 1 and 4 increased by 75% and 5%, respectively (see Table 3.2). In contrast, streamflow in Reaches 2 and 3 decreased by 16% and 15%, respectively. Assuming that errors in current meter measurement (see Fulford, 2001) were relatively consistent, Reaches 1 and 4 were therefore classified as gaining stream reaches, and Reaches 2 and 3 were classified as losing stream reaches. These classifications formed the basis of comparison for in-stream NO₃⁻ isotope transformations.

Water Chemistry and Stable Isotope Analyses

Stream water samples were analyzed for inorganic chemistry at the Water Quality Laboratory in the Penn State Institutes of the Environment (PSIE). All water samples were filtered through 0.45 µm filters. Samples were analyzed for pH (electrometric), specific conductance (electrometric), SO₄²⁻ and Cl⁻ (ion chromatography), NO₃⁻ (cadmium reduction), NH₄⁻ (phenate), and Ca²⁺ and Na⁺ (atomic absorption direct aspiration). All analyses were conducted using standard methods (APHA, 2005). Stream temperature measurements also were taken during field visits using a portable temperature probe.

Stable isotopes in NO₃⁻ (¹⁵N and ¹⁸O) were analyzed at the University of Waterloo Environmental Isotope Laboratory (EIL). δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ were analyzed according to the methods outlined by Chang et al. (1999) and Silva et al. (2000). Anion exchange resins were used to collect dissolved NO₃⁻ in stream water at the Water Quality Laboratory located within the Penn State Institutes of the Environment (see Chapter 2 for
a more complete description of stable isotope methodology). Anion exchange resins loaded with NO$_3^-$ were stored in a 4°C cooler until they could be shipped to the EIL at Waterloo for further processing and analysis. The results for δ$^{15}$N-NO$_3^-$ and δ$^{18}$O-NO$_3^-$ are reported in delta notation (δ$^{15}$N and δ$^{18}$O in permil units, ‰) versus their respective international reference standards. The international reference standard for δ$^{15}$N-NO$_3^-$ is atmospheric N$_2$ gas, while the international reference standard for δ$^{18}$O-NO$_3^-$ is Standard Mean Ocean Water (SMOW). The analytical error for both isotopes was approximately ±0.2‰ based on duplicate samples (26 duplicates for δ$^{15}$N-NO$_3^-$ and 14 duplicates for δ$^{18}$O-NO$_3^-$). See Appendix A.4 for all chemistry and stable isotope data.

Assessing In-Stream NO$_3^-$ Isotope Transformations

Graphs of δ$^{15}$N-NO$_3^-$ and δ$^{18}$O-NO$_3^-$ versus the natural logarithm of NO$_3^-$ (Ln NO$_3^-$) were created to assess the potential for denitrification in each stream reach. The slopes of significant negative relationships between both isotopes in NO$_3^-$ and Ln NO$_3^-$ yielded estimates of the isotopic enrichment factor (ε) for denitrification (e.g. Mariotti et al., 1988, Koba et al., 1997). The isotope enrichment factors for δ$^{15}$N-NO$_3^-$ and δ$^{18}$O-NO$_3^-$ could be compared to verify whether a denitrification signal was present. Recent studies have shown that ε$^{15}$N: ε$^{18}$O is about 2:1 (e.g. Cey et al., 1999; Lehmann et al., 2003). This corresponds to a slope of approximately 0.51 when δ$^{18}$O-NO$_3^-$ is plotted against δ$^{15}$N-NO$_3^-$ (Chen and MacQuarrie, 2005).

RESULTS AND DISCUSSION

Losing Stream Reaches (Reaches 2 and 3)

NO$_3^-$ Cycling

NO$_3^-$ cycling in Reaches 2 and 3 was very interesting because each reach showed a general pattern of decreasing NO$_3^-$ concentrations (Figure 3.5). NO$_3^-$ loads decreased concomitantly with streamflow in Reaches 2 (-20%) and 3 (-23%) (Table 3.2). Part of
these losses was due to reductions in streamflow (Table 3.2); however, NO$_3^-$ concentrations also decreased by 5% and 9% in Reaches 2 and 3, respectively (Table 3.2). The fact that both reaches lost streamflow indicated that dilution by new water was probably not an important factor affecting NO$_3^-$ concentrations and loads. As a result the observed changes in NO$_3^-$ were most likely due to in-stream processing.

**NO$_3^-$ Isotope Transformations**

Stable isotope data provided the most useful means for assessing the primary mechanisms responsible for NO$_3^-$ losses in Reaches 2 and 3. Figure 3.5 shows that as NO$_3^-$ concentrations decreased throughout both reaches (-2.7 mg L$^{-1}$ from Site 2.1 to Site 3.3), $\delta^{15}$N-NO$_3^-$ stable isotope values generally increased (+1.2‰ from Site 2.1 to Site 3.3). A plot of $\delta^{15}$N-NO$_3^-$ versus Ln NO$_3^-$ (Figure 3.7) clearly showed this inverse relationship (slope = -5.25, $R^2 = 0.52$, $p = 0.07$) for the data collected from Reaches 2 and 3. The slope of this plot also allowed for the estimation of the isotope enrichment factor ($\varepsilon^{15}$N) for denitrification (e.g. Mariotti et al., 1988), which was determined to be -5.25‰. This value was in the range of previously reported estimates of $\varepsilon^{15}$N from several denitrification studies in groundwater (Mariotti et al., 1988; Fustec et al., 1991; Koba et al., 1997) and surface water (Kellman and Hillaire-Marcel, 1998; Sebilo et al., 2003).

The value of $\varepsilon^{15}$N obtained for Spring Creek was on the lower end of values reported by other studies, especially those conducted in groundwater (Mariotti et al., 1988 and references therein; Böttcher et al., 1990). This low value can be explained in the context of denitrification efficiency. Hill (1988) suggested that in-stream denitrification was most efficient at low stream flow rates when stream temperatures were highest, and Mariotti et al. (1988) showed that the lowest values of $\varepsilon^{15}$N typically were observed at high rates (efficiencies) of denitrification. As a result, the low value of $\varepsilon^{15}$N in Spring Creek seems to indicate that denitrification is occurring, that it is efficient, and that it results in limited fractionation of $\delta^{15}$N-NO$_3^-$. Unfortunately, the use of $\delta^{15}$N-NO$_3^-$ alone did not indicate that denitrification was solely responsible for the losses of stream water NO$_3^-$ in Reaches 2 and 3. Denitrification is known to fractionate $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ (Kendall, 1998) at a ratio of $\varepsilon^{15}$N:$\varepsilon^{18}$O...
Therefore, plots of δ¹⁸O-NO₃⁻ versus Ln NO₃⁻ also were investigated to verify the interpretation that was made using δ¹⁵N-NO₃⁻. Interestingly enough, a non-significant relationship between δ¹⁸O-NO₃⁻ and Ln NO₃⁻ (slope = -0.39, R² = 0.01, p = 0.87) was found (Figure 3.8). Further, a plot of δ¹⁸O-NO₃⁻ versus δ¹⁵N-NO₃⁻ yielded a non-significant (p = 0.24), but slightly increasing relationship with a slope of only 0.39. One would anticipate a significant slope of 0.51 (Chen and MacQuarrie, 2005) for the δ¹⁸O-NO₃⁻ versus δ¹⁵N-NO₃⁻ relationship and ε¹⁵N:ε¹⁸O to generally be in the range of 2:1 if denitrification were the sole process responsible for NO₃⁻ losses in Reaches 2 and 3. Taken together, Figures 3.7 and 3.8 suggest that denitrification may likely have occurred in Reaches 2 and 3, but some other process(es) clearly impacted the interpretation of the δ¹⁸O-NO₃⁻ results.

Assimilative uptake of inorganic nitrogen by algae, phytoplankton, and macrophytes is one possible mechanism that may have been occurring concurrently with denitrification. During the assimilation of inorganic nitrogen (NO₃⁻ or NH₄⁺), organisms discriminate in favor of the lighter isotopes, thereby leaving the residual inorganic nitrogen enriched in the heavier isotopes (Kendall, 1998). Early work on δ¹⁵N showed that observed fractionations due to assimilation ranged from almost 0‰ in plants (Mariotti et al., 1980) to anywhere from 0‰ to -27‰ in aquatic algal assemblages (Fogel and Cifuentes, 1993). Recently, a study by Granger et al. (2004) suggested that ε¹⁵N:ε¹⁸O ≈ 1:1 during the assimilative uptake of NO₃⁻ by phytoplankton, with the primary fractionating mechanism being the reduction of NO₃⁻ to NH₄⁺ by the NO₃⁻ reductase enzyme. Since ε¹⁵N:ε¹⁸O was significantly greater than 1:1 in Reaches 2 and 3 (~ 13:1), it is unlikely that assimilation alone, or in combination with denitrification, could adequately explain the observed δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ patterns shown in Figures 3.7 – 3.8. Nonetheless, it is clear that more information is still needed regarding the range of fractionations in δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ that may be expected during assimilation of inorganic nitrogen by aquatic organisms. We cannot rule out the possibility the assimilation was occurring in Spring Creek, especially since this stream contains a healthy summertime population of aquatic plants and algae and sampling was conducted in full sunlight.
Coupled nitrification and denitrification represented another possible mechanism to explain different changes in $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ during in-stream NO$_3^-$ transformation. Denitrification is known to affect $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ similarly, and if it is the dominant NO$_3^-$ transformation process, it can be detected using a known increasing relationship between $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ (e.g. Chen and MacQuarrie, 2005) or by demonstrating concomitant negative relationships between NO$_3^-$ stable isotopes and NO$_3^-$ concentrations. It is widely assumed that NO$_3^-$ produced during in-stream nitrification (in the water column or within the stream sediments) represents a significant source of NO$_3^-$ for denitrification (Seitzinger, 1988). The production of NO$_3^-$ via nitrification ($\text{NH}_4^+ \rightarrow \text{NO}_3^-$) results in depletion of $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in the residual NO$_3^-$, but unlike denitrification, nitrification causes much larger isotopic fractionations in $\delta^{15}$N-NO$_3^-$ than in $\delta^{18}$O-NO$_3^-$ (Lehmann et al., 2004). In fact, little if any fractionation of $\delta^{18}$O-NO$_3^-$ is expected in response to nitrification (Casciotti et al., 2002). Thus, if these two processes were occurring concurrently, one might expect a larger overall isotopic effect for $\delta^{18}$O-NO$_3^-$ following denitrification due to a dampening effect on $\delta^{15}$N-NO$_3^-$ caused by the efflux of depleted (lower) $\delta^{15}$N-NO$_3^-$ from nitrification. The overall effects on the residual NO$_3^-$ from denitrification are likely to depend on the beginning isotopic compositions of the source materials in nitrification ($\delta^{15}$N in NH$_4^+$ and $\delta^{18}$O in H$_2$O), which further complicates the issue. Overall, the data in Figures 3.7 and 3.8 are not suggestive of coupled nitrification and denitrification causing the differences in $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in Reaches 2 and 3 because the largest isotope effects were observed for $\delta^{15}$N-NO$_3^-$, not $\delta^{18}$O-NO$_3^-$.

More detailed work using NH$_4^+$ concentrations, $\delta^{18}$O in H$_2$O, and $\delta^{15}$N-NH$_4^+$ would be needed to further constrain the effects of nitrification and denitrification on $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in these reaches.

One final possibility to explain the different behavior of $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in Reaches 2 and 3 is mixing of isotopically distinct groundwater from within the channel or from small seeps or springs. A few samples taken from local springs indicated that groundwater had $\delta^{15}$N-NO$_3^-$ compositions similar to stream water ($\sim +5\%$), but had $\delta^{18}$O-NO$_3^-$ compositions ($\sim +2\%$) that were slightly less than stream water ($\sim +3.5\%$).
Therefore, localized inputs of groundwater or spring water may have diluted the $\delta^{18}$O-$\text{NO}_3^-$ signal while minimally affecting the $\delta^{15}$N-$\text{NO}_3^-$ signal in the reach. Denitrification could have produced the observed increase in $\delta^{15}$N-$\text{NO}_3^-$ while the expected concomitant $\delta^{18}$O-$\text{NO}_3^-$ increase may have been slightly diluted and confounded due to mixing with groundwater. This hypothesis would require further testing using detailed information on surface water and groundwater exchanges within the stream channel, especially since Reaches 2 and 3 were classified as reaches that lost streamflow overall.

**Gaining Stream Reaches (Reaches 1 and 4)**

*NO$_3^-$ Cycling and Isotope Transformations in Reach 1 – Effect of Spring Inputs*

In-stream NO$_3^-$ processing was more difficult to infer in Reach 1 using $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ as compared to Reaches 2 and 3. This was primarily due to the discharge of a large spring in-between Sites 1.3 and 1.4, which caused streamflow discharge to increase by 75% in this reach and temperature to decrease from 16°C at Site 1.3 to 13°C at Site 1.4 (Figure 3.5). The discharge of this spring substantially increased conductivity and concentrations of NO$_3^-$, Cl$^-$, and SO$_4^{2-}$ from Sites 1.3 to 1.4 (Figure 3.5). In contrast, $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ decreased by -0.6‰ and -1.5‰, respectively. As a result, it was difficult to infer in-stream NO$_3^-$ processing in the entire reach using chemical and isotopic data from Site 1.4.

Ignoring Site 1.4 and the effects of the spring input, very minor changes in stream chemistry occurred in Reach 1 (Figure 3.5). From Site 1.1 to Site 1.3, overall changes in NO$_3^-$, SO$_4^{2-}$, and Cl$^-$ were less than 0.3 mg L$^{-1}$, and changes in conductivity were less than 30 µS cm$^{-1}$. With regard to $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ stable isotope data, only $\delta^{18}$O-NO$_3^-$ showed any significant changes in the reach. $\delta^{18}$O-NO$_3^-$ increased from +5.1‰ at Site 1.1 to +7.8‰ at Site 1.2 with virtually no changes in $\delta^{15}$N-NO$_3^-$ (Figure 3.5), a slight decrease in NO$_3^-$ concentration (-0.2 mg L$^{-1}$), and an increase in NH$_4^+$ concentration from non-detectable levels to about 0.02 mg L$^{-1}$. Therefore it is possible that a coupling of nitrification and denitrification occurred in-between these two sites producing a greater isotopic effect for $\delta^{18}$O-NO$_3^-$ as opposed to $\delta^{15}$N-NO$_3^-$ (e.g. Lehmann et al., 2004).
Downstream from Site 1.2, \( \delta^{18}O-\text{NO}_3^- \) decreased from 7.8‰ to 4.3‰ at Site 1.3 accompanied by no change in \( \delta^{15}N-\text{NO}_3^- \). The only possible explanation for a decrease in \( \delta^{18}O-\text{NO}_3^- \) with no accompanying change in \( \delta^{15}N-\text{NO}_3^- \) would have been dilution from a low \( \delta^{18}O-\text{NO}_3^- \) source entering upstream of Site 1.3. A small spring discharge from the Blue Spring in Boalsburg does enter upstream from Site 1.3. Unfortunately no data on \( \delta^{18}O-\text{NO}_3^- \) were available for this spring so it is not possible to determine whether the decrease in \( \delta^{18}O-\text{NO}_3^- \) was in fact due to dilution. Finally, reach-scale processes such as assimilation or denitrification were not evident using plots of \( \delta^{15}N-\text{NO}_3^- \) and \( \delta^{18}O-\text{NO}_3^- \) versus \( \text{Ln NO}_3^- \) (Figure 3.9).

**NO\textsubscript{3}\textsuperscript{-} Cycling and Isotope Transformations in Reach 4 – Effects of Sewage Effluent**

Like Reach 1, the ability to make inferences about NO\textsubscript{3} \textsuperscript{-} transformation processes using \( \delta^{15}N-\text{NO}_3^- \) and \( \delta^{18}O-\text{NO}_3^- \) stable isotopes was significantly confounded by inputs from a point source discharge. In this case, the discharge originated from the University Area Joint Authority (UAJA) sewage treatment plant located just upstream of Site 4.1 (see Figure 3.2). The reach began just below the point source input and a plume of wastewater along one bank was gradually mixed into the bulk stream water with distance downstream. During the sampling period (6/21-6/23), UAJA discharged about 0.2 m\textsuperscript{3} s\textsuperscript{-1} (~ 4.5 MGD) of treated effluent into Spring Creek. This increased the flow in Spring Creek from about 0.9 m\textsuperscript{3} s\textsuperscript{-1} (Site 3.3) to 1.1 m\textsuperscript{3} s\textsuperscript{-1} (Site 4.1), or by about 25%. The effluent discharge from UAJA was characterized by high electrical conductivity (980 μS cm\textsuperscript{-1}) and had elevated concentrations of NO\textsubscript{3} \textsuperscript{-} (52 mg L\textsuperscript{-1}), Cl\textsuperscript{-} (193 mg L\textsuperscript{-1}), and SO\textsubscript{4}\textsuperscript{2-} (82 mg L\textsuperscript{-1}) relative to stream water just above the discharge at Site 3.3 (see Table 3.2 and Figure 3.5 for stream water concentrations). Further, NO\textsubscript{3} \textsuperscript{-} in the sewage effluent was greatly enriched in \( \delta^{15}N-\text{NO}_3^- \) (+19.2‰) relative to \( \delta^{15}N-\text{NO}_3^- \) in stream water (+5.9‰) due to the effects of NH\textsubscript{3} volatilization and denitrification during the sewage treatment process. \( \delta^{18}O-\text{NO}_3^- \) in the effluent (+4.3‰) only was slightly enriched relative to the \( \delta^{18}O-\text{NO}_3^- \) observed in stream water above the discharge point (+3.3‰).

The effects of the UAJA wastewater treatment plant were clearly observable within Reach 4. Essentially, stream water conductivity and concentrations of NO\textsubscript{3} \textsuperscript{-}, Cl\textsuperscript{-},
and SO₄²⁻ all increased substantially from Sites 4.1 to 4.2 in response to the sewage effluent discharge. Further δ¹⁵N-NO₃⁻ also increased from +5.8‰ to +9.1‰ due to the mixing of high δ¹⁵N-NO₃⁻ water from the effluent. These increases were mostly likely due to the gradual mixing of sewage effluent with stream water in Reach 4. This interpretation can be confirmed by using a simple mixing equation for a side channel discharge (taken from Fischer et al., 1979; page 114), which is used by UAJA at its discharge location in Spring Creek. Based on width, depth, slope, and velocity information, the equation indicated that it would take about 2 – 3 km of straight stream channel to completely mix (concentrations within 5% of mean value across the width of the stream) the sewage effluent with stream water in Spring Creek. Spring Creek is not a straight channel, and therefore the length required for complete mixing would be much less than 3 km. In essence the effects of the sewage effluent discharge still would be expected to be observed for some distance downstream. Using the stream chemistry results shown in Figure 3.5, it appeared that most chemical concentrations and stable isotope values changed little downstream of Site 4.2. Thus, based upon samples taken from the mid-point of the stream channel, the sewage effluent appeared to be mostly mixed with stream water after this point. Since the sewage effluent was high in NO₃⁻ and had measurable NH₄⁺ concentrations, we expected that significant in-stream processing of NO₃⁻ was occurring. The most important impact of the sewage effluent was to diminish the effectiveness of δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ techniques to detect whether NO₃⁻ was being affected by in-stream processes (e.g. Figure 3.9). Future sampling efforts should incorporate integrated sampling across the stream to account for potentially important effects of side channel discharges of sewage effluent.

**Implications for Assessing In-Stream NO₃⁻ Transformation Processes**

This study has several implications for using the natural abundances of δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ to study in-stream transformations of NO₃⁻ in mixed land-use karst watersheds. Most importantly, reach selection is very important. Reaches should be selected where inputs of new water or new (different) NO₃⁻ sources are minimal, as was the case in Reaches 2 and 3. This allows the investigator to strictly study changes in stream chemistry and δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ and attribute those changes to in-stream
processing of NO₃⁻. Point source discharges from a spring (Reach 1) or a sewage treatment plant (Reach 4) can adversely affect the ability to use δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ at natural abundance levels to study in-stream NO₃⁻ transformations, especially when the inputs occur within the mid-point of a study reach. Depending on the size and flow of the stream, the effects of mixing in sewage effluent may last for considerable distances downstream. It was clear from this study that the length of Reach 4 was far too short to account for the effects of the sewage treatment plant on stream chemistry and stable isotopes. Thus, in order to study NO₃⁻ transformation processes reaches with sewage effluent discharges, the length of the study reach should be sized accordingly to account for these effects. Finally, gains and losses of stream water, which are especially important in karst watersheds, should be accounted for at all sampling locations.

**CONCLUSIONS**

Comparisons of losing and gaining stream reaches represented a useful technique for determining the significance of NO₃⁻ isotope transformations in Spring Creek during a summer low flow period. In losing stream reaches (Reaches 2 and 3), NO₃⁻ stable isotopes were most suggestive of in-stream denitrification. Overall changes in δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ were minimal (± 0.6‰) in the losing reaches, and suggested that NO₃⁻ transformation processes were efficient and resulted in minimal isotopic fractionation during extreme low flows and relatively high temperatures. In contrast, gaining reaches (Reaches 1 and 4) exhibited significant changes in NO₃⁻ stable isotopes due to inputs from a large spring and a sewage treatment plant discharge, respectively, and these changes confounded the ability to utilize isotopes to study in-stream transformations of NO₃⁻. The results of this study showed that NO₃⁻ stable isotopes were relatively unaffected by in-stream processes, and only changed when new and isotopically distinct NO₃⁻ sources were introduced into the stream.
REFERENCES


Figure 3.1: $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in NO$_3^-$ can be used to discriminate among different sources of NO$_3^-$ in streams (modified from Kendall, 1998). Diagonal arrow shows the results of denitrification, which can alter the initial $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ in NO$_3^-$ source signatures. The slope of this line has been estimated to be approximately 0.51 (see Chen and MacQuarrie, 2005).
Figure 3.2: Maps showing the four stream reaches within the Spring Creek watershed that were monitored for denitrification. In the upper map (a), the dark black outline delineates upper portion of the Spring Creek watershed, which served as the watershed boundary for the denitrification study. The lower map (b) shows a close-up of the individual stream reaches that were monitored.
Figure 3.3: Picture of Spring Creek upstream from Puddintown Road Bridge showing low and clear conditions that were present during the sampling period.
Figure 3.4: Stream hydrograph for Spring Creek at Houserville, PA (USGS Gage 01546400 – Site #3.2 on Figure 3.2) and precipitation amounts for State College, PA for the month of June 2005. Precipitation amounts are shown as white bars. Streamflow is shown as the blue line.
Figure 3.5: Downstream changes in streamflow (m³ s⁻¹), NO₃⁻ (mg L⁻¹), Cl⁻ (mg L⁻¹), SO₄²⁻ (mg L⁻¹), electrical conductivity (µS cm⁻¹), stream temperature (°C), δ¹⁵N-NO₃ (‰), and δ¹⁸O-NO₃ (‰) for all four monitored reaches in Spring Creek.
Figure 3.7: $\delta^{15}$N-NO$_3^-$ (‰) versus the natural logarithm of NO$_3^-$ (Ln NO$_3^-$) for losing reaches 2 and 3. The slope of the relationship between $\delta^{15}$N-NO$_3^-$ and Ln NO$_3^-$ is -5.25, which provides an estimate of $\varepsilon$, the isotope enrichment factor for denitrification effects on $\delta^{15}$N-NO$_3^-$ (after Mariotti et al., 1988). The relationship is significant at $\alpha = 0.10$. Site location numbers are indicated adjacent to the points in the plot (see Figure 3.2 and Table 3.1).
Figure 3.8: $\delta^{18}$O-NO$_3^-$ (‰) versus the natural logarithm of NO$_3^-$ (Ln NO$_3^-$) for losing reaches 2 and 3. The slope of the relationship between $\delta^{18}$O-NO$_3^-$ and Ln NO$_3^-$ is -0.39, which provides an estimate of $\varepsilon$, the isotope enrichment factor for denitrification effects on $\delta^{18}$O-NO$_3^-$ (after Mariotti et al., 1988). Unlike Figure 3.7 ($\delta^{15}$N-NO$_3^-$ vs. Ln NO$_3^-$), this relationship is not significant (p = 0.87). Site location numbers are indicated adjacent to the points in the plot (see Figure 3.2 and Table 3.1).
Figure 3.9: Plots of $\delta^{15}$N-NO$_3^-$ (‰) and $\delta^{18}$O-NO$_3^-$ (‰) versus the natural logarithm of the NO$_3^-$ (Ln NO$_3^-$) for gaining reaches 1 and 4. Site location numbers are indicated adjacent to the points in the plot (see Figure 3.2 and Table 3.1).
Table 3.1: Site description, site number, date and time of sampling, and information on distances and travel times for all four reaches sampled during the June denitrification study. Conditions during both days of sampling were clear and sunny with air temperatures near 30°C (85°F).

<table>
<thead>
<tr>
<th>Site Name</th>
<th>Site No.*</th>
<th>Date</th>
<th>Time</th>
<th>Distance from Previous Site (km)</th>
<th>Estimated Velocity (m s⁻¹)</th>
<th>Estimated Travel Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring behind car wash in Boalsburg</td>
<td>1.1</td>
<td>06/21/2005</td>
<td>9:50 AM</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Boalsburg Military Museum</td>
<td>1.2</td>
<td>06/21/2005</td>
<td>11:48 AM</td>
<td>0.9</td>
<td>0.12</td>
<td>2.0</td>
</tr>
<tr>
<td>Mary Elizabeth Street Bridge</td>
<td>1.3</td>
<td>06/21/2005</td>
<td>1:08 AM</td>
<td>0.6</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Upstream of Cedar Run</td>
<td>1.4</td>
<td>06/21/2005</td>
<td>3:52 PM</td>
<td>1.2</td>
<td></td>
<td>2.7</td>
</tr>
<tr>
<td>Downstream of Cedar Run</td>
<td>2.1</td>
<td>06/23/2005</td>
<td>7:05 AM</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Upstream of Lemont</td>
<td>2.2</td>
<td>06/23/2005</td>
<td>9:21 AM</td>
<td>1.3</td>
<td>0.15</td>
<td>2.4</td>
</tr>
<tr>
<td>Spring Creek Park</td>
<td>2.3</td>
<td>06/23/2005</td>
<td>3:01 PM</td>
<td>3.1</td>
<td></td>
<td>5.7</td>
</tr>
<tr>
<td>Upstream of Slab Cabin Run</td>
<td>2.4</td>
<td>06/23/2005</td>
<td>4:46 PM</td>
<td>0.9</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
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<td>06/23/2005</td>
<td>8:00 AM</td>
<td>--</td>
<td>0.18</td>
<td>--</td>
</tr>
<tr>
<td>Houserville Bridge</td>
<td>3.2</td>
<td>06/23/2005</td>
<td>11:01 AM</td>
<td>2.0</td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>Upstream of UAJA Effluent</td>
<td>3.3</td>
<td>06/23/2005</td>
<td>1:00 PM</td>
<td>1.4</td>
<td></td>
<td>2.1</td>
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<tr>
<td>UAJA Sewage Effluent</td>
<td>--</td>
<td>06/21/2005</td>
<td>1:55 PM</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<tr>
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<td>06/21/2005</td>
<td>2:00 PM</td>
<td>--</td>
<td>0.15</td>
<td>2.3</td>
</tr>
<tr>
<td>Along Rock Road</td>
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<td>1.0</td>
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<tr>
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<td>06/21/2005</td>
<td>5:09 PM</td>
<td>0.6</td>
<td></td>
<td>--</td>
</tr>
</tbody>
</table>

*Site numbers include identifying information on the reach and the site (reach.site). Site numbers correspond to Figure 3.1.
Table 3.2: Changes in NO$_3^-$ concentration (mg L$^{-1}$), flow (m$^3$ s$^{-1}$), NO$_3^-$ loads (kg km$^{-1}$ day$^{-1}$), $\delta^{15}$N-NO$_3^-$ (‰), and $\delta^{18}$O-NO$_3^-$ (‰) for the top and bottom sites in each of the 4 Reaches sampled during the denitrification study.

<table>
<thead>
<tr>
<th>Reach</th>
<th>Site</th>
<th>NO$_3^-$ (mg L$^{-1}$)</th>
<th>Flow (m$^3$ s$^{-1}$)</th>
<th>NO$_3^-$ loads (kg km$^{-1}$ day$^{-1}$)</th>
<th>$\delta^{15}$N-NO$_3^-$ (‰)</th>
<th>$\delta^{18}$O-NO$_3^-$ (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reach 1 (Gaining Reach)</td>
<td>1.1 - Top</td>
<td>2.8</td>
<td>0.06</td>
<td>5.18</td>
<td>5.3</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>1.4 – Bottom</td>
<td>13.4</td>
<td>0.22</td>
<td>96.49</td>
<td>4.6</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Change*</td>
<td>+10.6</td>
<td>+0.17</td>
<td>+91.3</td>
<td>-0.8</td>
<td>-2.3</td>
</tr>
<tr>
<td></td>
<td>% Change</td>
<td>+78.9%</td>
<td>+74.6%</td>
<td>+94.6%</td>
<td>-14.3%</td>
<td>-44.4%</td>
</tr>
<tr>
<td>Reach 2 (Losing Reach)</td>
<td>2.1 – Top</td>
<td>16.5</td>
<td>0.61</td>
<td>163.60</td>
<td>4.7</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>2.4 – Bottom</td>
<td>15.7</td>
<td>0.51</td>
<td>130.79</td>
<td>5.2</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Change*</td>
<td>-0.8</td>
<td>-0.10</td>
<td>-32.8</td>
<td>+0.5</td>
<td>-0.5</td>
</tr>
<tr>
<td></td>
<td>% Change</td>
<td>-5.1%</td>
<td>-15.8%</td>
<td>-20.1%</td>
<td>+9.4%</td>
<td>-14.8%</td>
</tr>
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<td>Reach 3 (Losing Reach)</td>
<td>3.1 – Top</td>
<td>15.2</td>
<td>1.01</td>
<td>389.63</td>
<td>5.3</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>3.3 – Bottom</td>
<td>13.8</td>
<td>0.86</td>
<td>300.79</td>
<td>5.9</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Change*</td>
<td>-1.4</td>
<td>-0.15</td>
<td>-88.8</td>
<td>+0.6</td>
<td>+0.1</td>
</tr>
<tr>
<td></td>
<td>% Change</td>
<td>-9.4%</td>
<td>-14.8%</td>
<td>-22.8%</td>
<td>+9.8%</td>
<td>+3.4%</td>
</tr>
<tr>
<td>Reach 4 (Gaining Reach)</td>
<td>4.1 – Top</td>
<td>15.7</td>
<td>1.13</td>
<td>452.05</td>
<td>5.8</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>4.3 – Bottom</td>
<td>19.9</td>
<td>1.20</td>
<td>604.46</td>
<td>9.5</td>
<td>3.6</td>
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<tr>
<td></td>
<td>Change*</td>
<td>+4.2</td>
<td>+0.06</td>
<td>+152.4</td>
<td>+3.7</td>
<td>+0.2</td>
</tr>
<tr>
<td></td>
<td>% Change</td>
<td>+21.2%</td>
<td>+5.1%</td>
<td>+25.2%</td>
<td>+38.7%</td>
<td>+4.4%</td>
</tr>
</tbody>
</table>

*Change represents the absolute change in the stream reach (value at bottom of reach – value at top of reach)
Chapter 4

NITRATE SOURCE AND FLOW PATH IDENTIFICATION USING STABLE ISOTOPES IN A MIXED LAND-USE KARST BASIN

ABSTRACT

Potential sources and flow paths of stream water NO$_3^-$ were assessed using stable isotopes in NO$_3^-$ ($\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$) and H$_2$O ($\delta^{18}$O-H$_2$O) in Spring Creek, a 201 km$^2$ mixed land-use watershed located in central Pennsylvania. Six storm events were sampled during 2005 using a nested and spatially-distributed sampling design (5 sites). The sites represented a downstream progression of forested uplands underlain by resistant sandstone to karst lowlands with agricultural, urban, and mixed land-uses. Stream samples were collected during antecedent baseflow conditions and at or near peakflow for each event. Contributions of event NO$_3^-$ and event water during each storm were computed using two-component mixing models. Comparisons among sites of the fractions of event NO$_3^-$ and event water at peakflow showed three distinct types of flowpath and NO$_3^-$ source variations: urban, forested uplands, and karst lowlands. At peakflow for the urbanized site, high proportions of event water at peakflow were matched closely by high fractions of event NO$_3^-$ (mean ratio of event NO$_3^-$ to event water was 1.03) showing the dominant influence of overland flow pathways and precipitation as the NO$_3^-$ source. The forested upland site with steeper terrain, shallow soils, and sandstone bedrock showed an intermediate response with about 35% of peakflow and about 22% of event NO$_3^-$ due to precipitation (mean ratio of event NO$_3^-$ to event water was 0.65). This suggested that both channel precipitation and soil interflow flow path processes were important and that NO$_3^-$ was a mixture of soil and precipitation sources. In the karst lowlands, very low fractions of event water and even lower fractions of event NO$_3^-$ at peakflow (mean ratios of event NO$_3^-$ to event water ranged from 0.11 to 0.25) showed the dominance of piston-flow flow generation processes and flushing of stored NO$_3^-$.

Overall, this study illustrated for the first time how NO$_3^-$ isotopes and $\delta^{18}$O-H$_2$O could be combined to show major differences in water and NO$_3^-$ delivery mechanisms.
between forested uplands and karst valleys, and confirm the dominance of overland flow pathways in urbanizing basins.
INTRODUCTION

Excess nitrogen (N) in streams and rivers has resulted in large-scale eutrophication of estuarine systems in coastal regions of the United States, namely the Chesapeake Bay (NRC, 2000). On an annual basis, much of this N is exported as soluble NO₃⁻ during high stream flow periods in response to storms. While storm events are an important driver of NO₃⁻ export on all watersheds, other factors that affect NO₃⁻ such as land-use conditions (e.g. Jordan et al., 1997) and geologic differences (e.g. Miller et al., 1997) exert unique influences on the mobilization and subsequent hydrologic transport of NO₃⁻ to streams during events. In order to minimize NO₃⁻ pollution in watersheds with mixed land-use and geology, methods are needed that can help identify NO₃⁻ sources and flow pathways during storm events.

One valuable method for identifying NO₃⁻ sources in mixed land-use watersheds is the use of NO₃⁻ isotopes (δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻) (see Figure 4.1; Kendall, 1998). Many recent studies in mixed land-use basins have used NO₃⁻ isotopes to identify NO₃⁻ sources (e.g. Chang et al., 2002; Rock and Mayer, 2004; Segal-Rozenhaimer, 2004; Panno et al., 2006). Unfortunately, few studies in mixed land-use basins have explicitly considered the effects of storm events on NO₃⁻ isotopes and sources. One reason for this is that many mixed land-use studies have been conducted in large river basins (> 10,000 km² in area) with complex mixtures of land-use and geology.

In order to compare storm responses among watersheds with different characteristics, it would be useful to work at the scale of a small mixed land-use watershed where individual watersheds with relatively homogenous land-uses (e.g. forest, agriculture, urban) and geology (e.g. karst versus sandstone) could be delineated and simultaneously monitored during storms. A study in two heavily urbanized watersheds in Texas conducted by Ging et al. (1996) monitored δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ in baseflow and stormflow. The study showed that δ¹⁵N-NO₃⁻ was a useful tracer of organically-derived NO₃⁻ from sewage at baseflow, whereas increases in δ¹⁸O-NO₃⁻ during storms were useful for inferring the importance of precipitation NO₃⁻ in direct runoff from impervious surfaces at stormflow. Therefore, changes in NO₃⁻ isotopes from baseflow to stormflow in the Ging et al. (1996) study yielded direct information on NO₃⁻ sources and suggested possible shifts in flow pathways during storm events in urban basins. A
similar monitoring strategy in a mixed land-use basin may represent a practical way for generating new information on how NO₃⁻ sources and flow paths change during storms on watersheds with distinctly different land-use and geology.

While NO₃⁻ stable isotopes clearly yield information on NO₃⁻ sources, they only provide supplementary information on possible flow pathways. A more direct way to determine hydrologic flow pathways is to monitor stable isotopes in water (e.g. δ¹⁸O-H₂O), which are more directly related to water sources. There has been widespread application of water isotopes to separate “event” water and “pre-event” water flow paths during storm events using two-component mixing models (e.g. Sklash, 1990; Pionke et al., 1993; DeWalle and Pionke, 1994). Extending this idea to NO₃⁻ isotopes would permit the estimation of “event” and “pre-event” NO₃⁻ sources during storms, which would represent a new application of two-component separation models. Complementary estimates of “event” water and “event” NO₃⁻ for the same storm events would allow us to more clearly determine the differences in flow pathways for water and NO₃⁻ among watersheds with different land-use and geology.

The overall goal of this study was to combine stable isotopes in NO₃⁻ with stable isotopes in H₂O to infer predominant sources and flow paths of NO₃⁻ during storms in nested and spatially-distributed subwatersheds with different land-uses and geology (sandstone uplands and karst valleys) within the upper Spring Creek watershed in central Pennsylvania. The specific objectives of the study were to:

1. Quantify changes in NO₃⁻ stable isotopes from baseflow to peakflow and evaluate whether these changes reflect changes in NO₃⁻ sources during storm events in watersheds with different land-uses.
2. Use two-component hydrograph separation techniques to determine whether fractions of new NO₃⁻ accompany fractions of new water, and assess whether these methods may be useful for comparing predominant flow pathways and NO₃⁻ delivery mechanisms among watersheds with different land-uses and bedrock geology.
MATERIALS AND METHODS

Spring Creek Watershed

Spring Creek is a 371 km² watershed located in central Pennsylvania, and is a tributary to the Susquehanna River, which eventually flows into the Chesapeake Bay estuary (Figure 4.2). The climate in central Pennsylvania is a humid continental type climate, with average annual precipitation of approximately 97 cm based upon 80 years of data (1926-2005) measured at State College, PA (PA State Climatologist, 2006). Average annual stream runoff is approximately 38 cm based on 65 years of data measured at the USGS stream gauge on Spring Creek at Axemann (02050204).

The Spring Creek watershed is situated within the Appalachian Section of the Ridge and Valley Physiographic Province, which is characterized by sandstone and shale ridges (elevation ~ 550 - 600 m) and wide valleys underlain by carbonate geology (elevation ~ 300 - 400 m). The complex geology within the Spring Creek watershed is an important factor that affects the local hydrology. Streams in the valley are fed by a combination of mountain runoff from the forested ridges and large groundwater spring inputs (Fulton et al., 2005; O’Driscoll and DeWalle, 2006). Due to the highly soluble limestone and dolomite bedrock, significant subsurface drainage pathways exist. In parts of the watershed, perched and losing streams are common for large parts of the year (O’Driscoll and DeWalle, 2006).

Spring Creek is a mixed land-use watershed, with forests covering the ridges and a mixture of agriculture, residential, and industrial land-uses in the valleys (Figure 4.2). The watershed is undergoing rapid urbanization, with agricultural land-uses being replaced by urban and suburban land-uses. As a result of the continued development, a variety of significant point and non-point sources of NO₃⁻ pollution are active within the Spring Creek watershed, including one sewage treatment plant (University Area Joint Authority) and runoff from agriculture and urban lands during baseflow and stormflow conditions. Several miles of Spring Creek are listed on the Federal 303(d) list for impairments due to nutrient pollution from point sources, urban runoff, and crop-related agriculture (PA DEP, 2004). Recent monitoring by the ClearWater Conservancy has
shown that the main stem of Spring Creek typically has NO$_3^-$ concentrations ranging from 10 to 20 mg L$^{-1}$ (2.3 to 4.5 mg L$^{-1}$ as NO$_3$-N) (Sengle, 2002).

**Watershed Sampling Design**

The study was designed to take advantage of differences in land-use, geology, and predominant NO$_3^-$ sources that existed within the upper Spring Creek watershed, which is 201 km$^2$ in area. Five watershed sampling sites were selected within the upper Spring Creek watershed (Figure 4.2, Table 4.1), which included three tributary streams with uniquely different land-uses (forest, agricultural, and urban) as well as two downstream mixed land-use sites located on the main-stem of Spring Creek. This design allowed us to compare tracer signatures and NO$_3^-$ sources among watersheds with different land-uses and bedrock geology. Study sites were established on the following watersheds: (1) Galbraith Gap Run, a 13 km$^2$ forested watershed; (2) Cedar Run, a 45 km$^2$ agricultural watershed; (3) Thompson Run, an 11 km$^2$ urban / developed watershed; (4) Spring Creek at Houserville, a 150 km$^2$ mixed land-use watershed; and (5) Spring Creek at Rock Road, a 201 km$^2$ mixed land-use watershed (see Figure 4.2). The forested watershed was underlain by sandstone and shale bedrock, while the remaining four watersheds were predominately carbonate geology (Table 4.1). Treated sewage effluent was collected at the University Area Joint Authority (UAJA) outfall along Spring Creek located about 1.4 km downstream of the Houserville sampling site (Figure 4.2). This allowed us to assess the impacts of sewage discharges on NO$_3^-$ concentrations and stable isotopes in Spring Creek. Finally, two groundwater springs (Figure 4.2, Table 4.1) were sampled once in summer and once again in late fall to characterize the chemistry and stable isotopic composition of shallow groundwater. Appendix A.5 shows pictures of the sampling sites during characteristic storm events and provides more details on specific sampling site locations.
Stream Sampling and Precipitation Monitoring

All stream sites and the sewage treatment plant effluent were sampled during six storm events in 2005. For each storm event, streams were sampled once during antecedent baseflow and again at or near peakflow. The sewage treatment plant effluent only was sampled once per storm during baseflow conditions. Baseflow sampling was used to reflect local groundwater inputs for each stream site. See Appendix A.6 for more details.

Precipitation during each event was sampled using a sequential passive precipitation sampler designed and constructed to collect sufficient volumes of rainwater for NO$_3^-$ stable isotope and inorganic chemical analysis. The sampler was approximately 5.7 m$^2$ in area and was equipped with a polyethylene plastic tarp, which funneled water to a series of three 20-L polypropylene carboys. A new tarp was used for each event that was sampled, and the tarp was washed three times with deionized water just prior to the onset of precipitation (see Chapter 2 for more information on precipitation sampling). Approximately 0.36 cm (0.13 in) of rainfall filled an individual carboy. The bottles were arranged such that they filled sequentially during storms according to a slightly modified design from that of Kennedy et al. (1979). This enabled within-storm variations of stable isotopes and chemistry to be evaluated for each storm event. Sub-samples were collected from individual carboys for inorganic chemistry and $\delta^{18}$O-H$_2$O analysis. Finally, wash-off from the tarp was collected in a carboy after a three-week long dry period in November 2005 to provide some information on the isotopic composition of dry NO$_3^-$ deposition. See Appendix A.6 for more details.

Streamflow Data

Streamflow rates were recorded for all baseflow and peakflow samples. Continuous streamflow data were available at Spring Creek Houserville, Cedar Run, and Thompson Run. The data at the Houserville site were available from the United States Geological Survey (USGS). Data from Cedar Run and Thompson Run were collected by the ClearWater Conservancy, a local watershed advocacy group active within the Spring Creek watershed. Streamflow rates at Galbraith Gap Run were estimated using a simple stage-discharge relationship developed for the site during the study period in 2005.
Streamflow rates at Rock Road were estimated using data from the upstream gauge (3.6 km upstream) at Houserville (USGS 01546400), a downstream gauge (9.6 km downstream) at Axemann (USGS 01546500), and observed flow rates from the UAJA sewage treatment plant.

**Water Chemistry and Stable Isotope Analyses**

All stream water and precipitation samples were analyzed for inorganic chemistry at the Water Quality Laboratory in the Penn State Institutes of Energy and the Environment (PSIEE). Water samples were filtered through 0.45 µm filters. Samples were analyzed for pH (electrometric), specific conductance (electrometric), SO$_4^{2-}$ and Cl$^-$ (ion chromatography), NO$_3$-N (cadmium reduction), NH$_4$-N (phenate), and Ca$^{2+}$ and Na$^+$ (atomic absorption direct aspiration). All analyses were conducted using standard methods (APHA, 2005). Stream temperature measurements also were taken during field visits using a portable temperature probe.

Stable isotopes in NO$_3^-$ ($^{15}$N and $^{18}$O) were analyzed at the University of Waterloo Environmental Isotope Laboratory (EIL). $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ were analyzed according to the methods outlined by Chang et al. (1999) and Silva et al. (2000). Anion exchange resins were used to collect dissolved NO$_3^-$ in stream water at the Water Quality Laboratory located within the Penn State Institutes of the Environment (see Chapter 2 for a more complete description of stable isotope methodology). Anion exchange resins loaded with NO$_3^-$ were stored at 4°C until they could be shipped to the EIL at Waterloo for further processing and analysis. The results for $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ are reported in delta notation ($\delta^{15}$N and $\delta^{18}$O in permil units, ‰) versus their respective international reference standards. The international reference standard for $\delta^{15}$N-NO$_3^-$ is atmospheric N$_2$ gas, while the international reference standard for $\delta^{18}$O-NO$_3^-$ is Standard Mean Ocean Water (SMOW). The analytical error for both isotopes was approximately ±0.2‰ based on duplicate samples (26 duplicates for $\delta^{15}$N-NO$_3^-$ and 14 duplicates for $\delta^{18}$O-NO$_3^-$).

$\delta^{18}$O-H$_2$O also was analyzed at the University of Waterloo EIL. Water samples for $\delta^{18}$O-H$_2$O were collected and stored in airtight 20-mL HDPE scintillation bottles prior to shipment to the EIL at Waterloo. The analysis for $\delta^{18}$O-H$_2$O was conducted using the
CO2 equilibration method outlined by Epstein and Mayeda (1953). All results are reported in delta notation ($\delta^{18}O$ in permil units, ‰) relative to Standard Mean Ocean Water (SMOW). The analytical error for $\delta^{18}O$-H$_2$O isotopes was approximately $\pm 0.1\%$ based on 20 duplicate samples. Appendix A.7 displays raw chemistry and isotope data for all stream sites.

**Data Analysis**

One of the major objectives of this study was to evaluate whether new sources of water were accompanied by new sources of NO$_3^-$ during storms events. A unique method for possibly answering this question was developed using two-component hydrograph separation models. The two-component model (1) has been used by many investigators to separate peakflow into “pre-event” (old) and “event” (new) water sources using $\delta^{18}O$-H$_2$O isotopes and dissolved SiO$_2$ as tracers (e.g. Pionke et al., 1993; DeWalle and Pionke, 1994).

\[
\frac{Q_{EW}}{Q_{TW}} = \frac{\delta_{TW} - \delta_{PW}}{\delta_{EW} - \delta_{PW}}
\]  

(1)

Where:  
$Q_{TW}$ = Streamflow rate ($m^3$ s$^{-1}$) at peakflow  
$\delta_{TW}$ = Isotope (or chemical concentration) at peakflow  
$Q_{PW}$ = Streamflow rate ($m^3$ s$^{-1}$) at baseflow  
$\delta_{PW}$ = Isotope (or chemical concentration) of pre-event water (assigned to baseflow)  
$Q_{EW}$ = Streamflow rate ($m^3$ s$^{-1}$) of event water  
$\delta_{EW}$ = Isotope (or chemical concentration) of event water (assigned to incoming precipitation)

On four basins, $\delta^{18}O$-H$_2$O was used to separate pre-event and event water fractions because it directly traced sources of water (Wels et al., 1991). On the urban basin, dissolved SiO$_2$ was used instead of $\delta^{18}O$-H$_2$O because it varied less than $\delta^{18}O$-H$_2$O in event water (see Chapter 2 for a discussion of $\delta^{18}O$-H$_2$O variations in precipitation), which minimized the effects of poor timing between individual precipitation samples and the occurrence of peakflows.
The new approach applied in this study was to use NO$_3^-$ stable isotopes to partition peakflow NO$_3^-$ into its “pre-event” and “event” source fractions (2). $\delta^{18}$O-NO$_3^-$ was selected as the ideal tracer to perform these calculations because it exhibited better separations between precipitation (event NO$_3^-$) and terrestrial NO$_3^-$ sources.

\[
\frac{M_{EN}}{M_{TN}} = \frac{\delta_{TN} - \delta_{PN}}{\delta_{EN} - \delta_{PN}}
\]

Where:
- $M_{TN}$ = Mass (mg) of total NO$_3^-$
- $\delta_{TN}$ = Isotope value of total NO$_3^-$ at peakflow
- $\delta_{PN}$ = Isotope value of pre-event NO$_3^-$ at baseflow
- $M_{EN}$ = Mass (mg) of event NO$_3^-$
- $\delta_{EN}$ = Isotope value of event NO$_3^-$ (assigned to incoming precipitation)

If event water were accompanied by event NO$_3^-$ sources, then one would expect these two calculations to be similar. If the fraction of event NO$_3^-$ differed from the fraction of event water, then other factors (e.g. sources, biochemical transformations) may be affecting the NO$_3^-$ stable isotopes during storm events.

In order to use two-component hydrograph separations to calculate fractions of event water and event NO$_3^-$, it is important to state the major assumptions involved (summarized from Sklash, 1990; Buttle, 1994).

1. $\delta_{EW}$ (or $\delta_{EN}$) does not vary while being routed through the watershed
2. $\delta_{PW}$ (or $\delta_{PN}$) remains constant during the storm event
3. $\delta_{PW}$ (or $\delta_{PN}$) is significantly different from ($\delta_{NW}$ or $\delta_{NN}$)

With regard to the first assumption, $\delta_{EW}$ (or $\delta_{EN}$) was assumed to represent incoming precipitation. During the course of all six storm events, significant variations of $\delta^{18}$O-H$_2$O and NO$_3^-$ stable isotopes in precipitation were observed (see Chapter 2). In order to assign an individual isotopic composition for event water and/or event NO$_3^-$ ($\delta_{EW}$ or $\delta_{EN}$), a precipitation-weighted mean was calculated for each storm using the incremental mean method proposed by McDonnell (1990). With regard to the second assumption, recent research has shown that the $\delta^{18}$O-H$_2$O isotopes in pre-event water ($\delta_{PW}$) can vary
significantly during storm events (see Gremillion and Wanielista, 2000a). In addition to $\delta^{18}$O-H$_2$O variations, NO$_3^-$ stable isotopes also may vary in baseflow during storm events. While potential variations of $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in baseflow ($\delta_{PN}$) were not known during individual storm events monitored in 2005, a previous study suggested that fractionation of NO$_3^-$ isotopes during typical baseflow conditions in Spring Creek was minimal (see Chapter 3). Overall, a study by Gremillion and Wanielista (2000b) showed that the significance of isotope variations in baseflow during storms did not affect the results of steady-state two-component hydrograph separations in a Florida river. Therefore, we chose to use the steady-state form of the two-component separation model to calculate fractions of event water (1) and event NO$_3^-$ (2) in the Spring Creek watershed.

In addition to estimating event water and event NO$_3^-$ fractions, we also were interested in estimating the isotopic signature of the “stormflow” NO$_3^-$ that mixed with NO$_3^-$ at baseflow to generate the observed NO$_3^-$ isotopic signature at peakflow. The isotopic signatures of “stormflow” NO$_3^-$ were calculated using the event water fractions estimated from (1) and the observed NO$_3^-$ isotopic compositions at baseflow and peakflow.

\[
\delta_{SF} = \frac{\delta_{TN} - \delta_{PN}}{f_{EW}} + \delta_{PN} \tag{3}
\]

Where:
- $\delta_{SF}$ = NO$_3^-$ isotope signature in stormflow NO$_3^-$
- $f_{EW}$ = fraction of event water, $\left(\frac{Q_E}{Q_T}\right)$ using $\delta^{18}$O-H$_2$O
- $\delta_{TN}$ = NO$_3^-$ isotope signature of total NO$_3^-$ at peakflow
- $\delta_{PN}$ = NO$_3^-$ isotope signature of pre-event NO$_3^-$ at baseflow

Stormflow NO$_3^-$ signatures for $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ were estimated independently using (3). Occasionally a situation occurred where $(\delta_{TN} - \delta_{PN})$ was large and $f_{EW}$ was very small (< 0.05), which produced anomalously large estimates (positive or negative) of $\delta_{SF}$ for stormflow NO$_3^-$, which were not used in the results.
RESULTS AND DISCUSSION

Urban Watershed

Changes in NO$_3^-$ Sources

Baseflow to peakflow changes in $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ were significant on the urban watershed (Figure 4.2) during the 2005 study period. During baseflow conditions, the urban watershed had enriched $\delta^{15}$N-NO$_3^-$ (see Figure 4.3) resembling NO$_3^-$ from sewage and manure sources. During five storm events, $\delta^{15}$N-NO$_3^-$ decreased from baseflow to peakflow (mean decrease = -5.2‰), with the November 16 storm being the only exception (slight increase of +1‰) (Figure 4.4). Increases in $\delta^{18}$O-NO$_3^-$ were consistent and substantial during all six storms (mean increase = +27.1‰) (Figure 4.4). Changes in NO$_3^-$ isotopes on the urban watershed were not significantly affected by storm size, duration, intensity, or antecedent moisture conditions.

Using all data collected from baseflow and peakflow, a significant negative correlation was observed between $\delta^{18}$O-NO$_3^-$ and $\delta^{15}$N-NO$_3^-$ ($R^2 = 0.63$, $p = 0.002$). This inverse relationship strongly suggested that sewage and manure NO$_3^-$ sources with low $\delta^{18}$O-NO$_3^-$ and high $\delta^{15}$N-NO$_3^-$ in baseflow were overwhelmed by atmospheric (precipitation or wash-off of dry deposition) NO$_3^-$ sources with high $\delta^{18}$O-NO$_3^-$ and low $\delta^{15}$N-NO$_3^-$ in peakflow. Further evidence for the importance of atmospheric NO$_3^-$ in peakflow was shown by the fact that estimated stormflow NO$_3^-$ isotope signatures were clearly in the range of NO$_3^-$ isotope signatures in wet and dry deposition (see Figure 4.5).

Storms sampled on March 23, July 5, and August 31 all had peakflow samples with higher $\delta^{18}$O-NO$_3^-$ than that observed in precipitation samples taken on the same date. One possible explanation for this may have been inadequate temporal sampling of precipitation during some storms, especially the intense thunderstorms on July 5 and August 31 that yielded only one bulk precipitation sample each. By collecting only one integrated precipitation sample during these events, a higher $\delta^{18}$O-NO$_3^-$ sample may simply have been missed.
Another possible explanation may have been significant wash-off of dry NO$_3^-$ deposition that accumulated on impervious surfaces prior to storm events. Dry NO$_3^-$ deposition was measured once during November 2005, which had enriched $\delta^{18}$O-NO$_3^-$ and much higher $\delta^{15}$N-NO$_3^-$ than that of incoming precipitation (Figure 4.3). If dry NO$_3^-$ deposition had higher $\delta^{18}$O-NO$_3^-$ than precipitation in other pre-storm periods, then this might explain why $\delta^{18}$O-NO$_3^-$ in peakflow samples was greater than precipitation for the storms on March 23, July 5, and November 16. Clearly, additional samples of dry NO$_3^-$ deposition would be needed to test this idea. Further characterization of NO$_3^-$ isotopes in dry deposition should be performed in future studies because wash-off of dry deposited NO$_3^-$ is likely to be a significant component of the overall NO$_3^-$ load during peakflow conditions on urban watersheds.

Changes in NO$_3^-$ Flow Paths

Overland flow was by far the most dominant flow pathway during storm events in the urban watershed. While this observation on urbanized watersheds was not entirely new (see review by Paul and Meyer, 2001), it was interesting to note that isotopic techniques helped to identify and quantify the effects. Initial evidence for the importance of overland flow in the urban watershed was shown by the fact that peakflow NO$_3^-$ isotope signatures closely resembled NO$_3^-$ from the atmosphere (Figure 4.3), which was observed in a few past studies on urbanized basins during high flow periods (e.g. Ging et al., 1996; Silva et al., 2002). Hydrograph separations provided further evidence for the importance of overland flow. Using dissolved SiO$_2$, the percentage of event water added during peakflow conditions ranged from 79% to 94% (Figure 4.6). The mean event water contribution on the urbanized watershed (87%) was significantly greater than the mean event water contributions on the four lesser developed watersheds ($p < 0.001$). Finally, event NO$_3^-$ fractions (0.80) and event water fractions (0.77) were quite similar, which suggested that event NO$_3^-$ from precipitation was efficiently delivered in event water during storms (see Figure 4.7).

While mean fractions of event NO$_3^-$ compared fairly well with mean event water fractions (Figure 4.7), more detailed analysis showed significant variations in the results. Correlations between fractions of event NO$_3^-$ and fractions of event water among all six
storm events were not very strong (see Figure 4.8), and this was likely due to significant variations of NO$_3^-$ isotopes in precipitation during individual storm events (see Chapter 2), and the inability to adequately match peakflow samples with samples of the precipitation bursts that caused the peak. Furthermore, variable contributions from wash-off of dry-deposition and organically-derived NO$_3^-$ on impervious surfaces may also have affected the isotopic results. Better sampling resolution would likely have improved the regression fit (Figure 4.8) and shown that event NO$_3^-$ did accompany event water on an event-basis within the urban watershed.

The overall results on the urban watershed showed land-use clearly dominated the hydrologic response to all six storm events, despite the karst geologic nature of the watershed (see Table 4.1). The urban stormwater problem could clearly be identified using NO$_3^-$ isotopes and hydrograph separation techniques. This suggested that simple isotopic measurements such as the ones conducted in this study could be especially valuable in evaluating the effectiveness of stormwater management alternatives in reducing overland flow from precipitation in urbanizing watersheds.

**Forested Watershed**

*Changes in NO$_3^-$ Sources*

The forested watershed (Figure 4.2) showed interesting changes in NO$_3^-$ stable isotopes during the six monitored storm events in 2005 (Figures 4.3 and 4.9). Changes in $\delta^{18}$O-NO$_3^-$ from baseflow to peakflow were consistently upward (mean increase of +13.7‰ for five storms), with the only exception being a slight decrease (-2.3‰) for the storm that occurred on October 7 (Figure 4.9). In addition, mean $\delta^{18}$O-NO$_3^-$ during peakflow (+15.9‰) was significantly higher than mean $\delta^{18}$O-NO$_3^-$ in baseflow (+4.9‰) for all six storms ($p = 0.002$). This indicated that NO$_3^-$ from the atmosphere, which typically had enriched values of $\delta^{18}$O-NO$_3^-$ (+19.7‰ to +79.0‰ during 2005, see Chapter 2 for discussion of seasonal effects), was important during peakflow conditions on the forested watershed.

In contrast, changes in $\delta^{15}$N-NO$_3^-$ during storms were more variable than changes in $\delta^{18}$O-NO$_3^-$. Increases in $\delta^{15}$N-NO$_3^-$ were observed on March 29 (+1.6‰), October 7
(±0.1‰), and November 16 (±1.1‰). Decreases in δ¹⁵N-NO₃⁻ occurred on March 23 (-2.1‰), July 5 (-0.2‰), and August 31 (-1.0‰). Overall, the changes in δ¹⁵N-NO₃⁻ were subtle, and only two storms showed changes that were significantly larger than ±1‰, which is within the error in isotope analyses. The 2.1‰ decrease in δ¹⁵N-NO₃⁻ on March 23 (+2.6‰ at baseflow and +0.5‰ at peak flow) may have been due to runoff from melting snow, which had a δ¹⁵N-NO₃⁻ signature of -0.6‰ (Figure 4.3). The storm on August 31 produced a δ¹⁵N-NO₃⁻ decrease of 1.0‰, which also was in the direction of precipitation (Figure 4.9). This storm produced intense precipitation (~ 4.8 cm hr⁻¹) (Table 4.2), and likely resulted in some saturated overland flow. Channel precipitation also may have been important due to intense precipitation amounts in a short period of time. Increases in δ¹⁵N-NO₃⁻ that occurred on March 29 and November 16 were both in the direction of nitrogen from soils (Figures 4.3 and 4.9).

Changes in δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ from baseflow to peakflow during storm events indicated that a mixture of atmospheric NO₃⁻ and nitrogen from soils were the two most important NO₃⁻ sources added during storm events (Figure 4.4). If an δ¹⁸O-NO₃⁻ signature of +8‰ was assumed for microbially-produced NO₃⁻ (taken from Williard et al., 2001; average of soil samples from 27 forested watersheds in Pennsylvania), then the percent contribution of atmospheric NO₃⁻ for the forested watershed for all six storm events in 2005 could be estimated using the following two-component mixing equation (e.g. Williard et al., 2001):

\[
\% \text{ atmospheric } NO_3^- = \frac{(18O - NO_3^- \text{ peakflow}) - (18O - NO_3^- \text{ soil})}{(18O - NO_3^- \text{ atmosphere}) - (18O - NO_3^- \text{ soil})}
\]

The results of these calculations suggested that atmospheric NO₃⁻ accounted for 22% to 36% of the stream water NO₃⁻ during peakflow conditions in the forested watershed. These results mostly agreed with recent evidence using δ¹⁸O-NO₃⁻ in forested watershed studies, which generally showed that atmospheric (wet + dry deposition) NO₃⁻ accounted for less than 30% of the total NO₃⁻ in stream water during storm events (e.g. Williard et al., 2001) and snowmelt runoff episodes (e.g. Burns and Kendall, 2002; Campbell et al.,
2002; Piatek et al., 2005). More recent work using $\delta^{17}$O-NO$_3^-$ in a semi-arid watershed showed that atmospheric NO$_3^-$ may account for up to 40% of total NO$_3^-$ exported during storms (Michalski et al., 2004).

Based on the simple two-component models and the plots of NO$_3^-$ stable isotopes in baseflow and peakflow (Figure 4.9), it is plausible to suggest that the majority of NO$_3^-$ being exported during storm events in the forested watershed was from recent microbial soil nitrification (64% to 78% of the NO$_3^-$). The observed NO$_3^-$ isotope signatures in the peakflow mixture and the estimated NO$_3^-$ isotope signatures for stormflow NO$_3^-$ added during storms generally support this conclusion (Table 4.5). Under ideal conditions (e.g. when soil moisture is low), microbial nitrification can produce significant pools of NO$_3^-$ in the mineral soils, which then can be flushed into the stream during storm events (e.g. Creed et al., 1996) due to the expansion of variable source areas (e.g. Creed and Band, 1998). This flushing mechanism appeared to be important on the forested watershed because NO$_3^-$ concentrations consistently increased from baseflow to peakflow for all six storm events in 2005 (mean increase = +0.9 mg L$^{-1}$). Further support for this interpretation is provided by the fact that concentrations of SO$_4^{2-}$, Cl$^-$, and Ca$^{2+}$ also increased during all six storms, indicating sources of water at peakflow that were recently in contact with mineral soils.

Higher pulses of NO$_3^-$ during storms were strongly related to drier 30-day antecedent precipitation amounts (see Figure 4.10). The largest increases in NO$_3^-$ concentrations occurred during the July 5 (+0.7 mg L$^{-1}$; 30-day antecedent precipitation = 4.4 cm), August 31 (+1.3 mg L$^{-1}$; 30-day antecedent precipitation = 4.6 cm) and October 7 (+2.1 mg L$^{-1}$; 30-day antecedent precipitation = 2.3 cm) storm events, and each of these events was preceded by 30 days of relatively low precipitation (see Figure 4.10). As a result, this may have resulted in extended periods of microbial soil nitrification (e.g. Creed et al., 1996), and therefore higher flushes of soil-derived NO$_3^-$ into the stream. The driest 30-day period occurred previous to the storm on October 7, which also coincided with the largest increase in NO$_3^-$ from baseflow to peakflow for any of the six storms. If nitrification was enhanced during this dry period, then it might explain the anomalously high $\delta^{18}$O-NO$_3^-$ value that was recorded during baseflow sampling on October 6 ($\delta^{18}$O-
NO₃⁻ = +13.6‰). This value occurred at the upper range of δ¹⁸O-NO₃⁻ values expected for nitrification in soils (+2‰ to +14‰) reported by Mayer et al. (2001).

An alternate explanation for the high pulses of NO₃⁻ that were associated with dry antecedent conditions is wash-off of dry deposition from vegetated surfaces. Studies of nitrogen deposition in forested watersheds have estimated that dry deposition is a significant pathway of nitrogen input, comprising 46% (Lovett and Lindberg, 1993) to 80% (Lindberg et al., 1986) of total nitrogen deposition. Large amounts of dry deposition could build up during long dry periods, which would be available for wash-off during storm events. As a result, it is possible that the high pulses of NO₃⁻ observed following the dry periods on July 5, August 31, and October 7 were partly due to wash-off of dry deposition.

**Changes in NO₃⁻ Flow Paths**

Sources of NO₃⁻ and event water during storms in the forested, sandstone bedrock watershed varied for different types of events. Hydrograph separations using δ¹⁸O-H₂O showed a large variation in responses on the forested watershed, with event water contributions ranging from 1% (March 23) to 49% (March 29) (Figure 4.6). A more detailed analysis was conducted by plotting event NO₃⁻ fractions against event water fractions using δ¹⁸O-NO₃⁻ (Figure 4.8), which showed an interesting pattern that appeared to indicate an effect of storm size on flow path responses. Storms that produced small amounts of event water (fₑ₃ < 0.2) yielded the greatest fraction of event NO₃⁻, while larger storms that produced more event water yielded substantially less event NO₃⁻ during peakflow conditions (Figure 4.8). On average, about 66% of event NO₃⁻ accompanied event water (precipitation) during storm events (see Figure 4.7) suggesting that the delivery of event NO₃⁻ was fairly efficient. The remaining 34% of event NO₃⁻ may have originated from soils or other terrestrial sources.

The overall contrast between small storms and large storms on the forested, sandstone bedrock watershed was interesting in terms of flow paths and NO₃⁻ delivery mechanisms. During larger storm events, changes in δ¹⁸O-NO₃⁻ were likely muted by fluxes of NO₃⁻ from the soil with lower δ¹⁸O-NO₃⁻ signatures (e.g. soil NO₃⁻). Studies in forested watersheds have shown that soil water with a different δ¹⁸O-H₂O signature than
baseflow can be rapidly mobilized (through macropores, etc.) and can generate substantial volumes of streamflow during large storm events (e.g. Swistock et al., 1989). If this type of flow path was active during the three largest storm events (March 29, August 31, and October 7) then high event water contributions and flushing of soil-derived NO$_3^-$ with low $\delta^{18}$O-NO$_3^-$ could represent a plausible explanation for the pattern shown in Figure 4.8.

During small storm events, changes in $\delta^{18}$O-NO$_3^-$ were large suggesting that event NO$_3^-$ sources were more important than pre-event NO$_3^-$ sources from soils. Two non-competing hypotheses to explain this pattern include the influences of channel precipitation and wash-off of dry-deposited NO$_3^-$ in throughfall, both of which have enriched $\delta^{18}$O-NO$_3^-$ relative to terrestrial NO$_3^-$ sources. A study conducted in a forested Appalachian watershed in central Pennsylvania showed that percentages of channel precipitation in stormflow were highest during small events, with overall contributions approaching 7% (Crayosky et al., 1999). Thus, significant contributions of channel precipitation could in part explain why fractions of event NO$_3^-$ were greatest during small storms where hydrograph separations indicated less than 20% event water was generated. It also could be argued that wash-off of dry-deposited NO$_3^-$ from vegetated surfaces was important during small storms. As mentioned previously, studies of nitrogen deposition in forested watersheds have estimated that dry deposition is a significant pathway of nitrogen input (Lindberg et al., 1986; Lovett and Lindberg, 1993). Based upon the potential isotopic difference between NO$_3^-$ in wet and dry deposition observed in this study (see Figure 4.3), future research should explicitly address the relative importance of event NO$_3^-$ from dry deposition and wet deposition in forested watersheds using measurements of NO$_3^-$ stable isotopes.

**Karst Watersheds**

**Changes in NO$_3^-$ Sources on the Agricultural Watershed**

Changes in $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ from baseflow to peakflow were notably smaller and more variable during storms on the agricultural watershed (Figure 4.2) as compared to the urban and forested watershed responses. Changes in $\delta^{15}$N-NO$_3^-$ during
storms were typically less than ±1‰, with the exceptions being the storms on March 23 (+1.1‰) and March 29 (-1.4‰) (Figure 4.11). Changes in \( \delta^{18}O-\text{NO}_3^- \) also were small and usually within ±1.5‰. The only exception was the storm on March 29, which resulted in a significant decrease in \( \delta^{18}O-\text{NO}_3^- \) (-3.2‰) (Figure 4.11). No relationship existed between storm characteristics and changes in NO\(_3^-\) isotopes from baseflow to peakflow.

Overall, the peakflow NO\(_3^-\) isotope signatures added during storms were very similar to the NO\(_3^-\) stable isotope signatures observed at baseflow. This indicated that NO\(_3^-\) sources probably did not change much during storms on the agricultural watershed. The combined baseflow to peakflow changes in NO\(_3^-\) isotopes along with the estimated NO\(_3^-\) isotope signatures of newly added NO\(_3^-\) in stormflow (Figure 4.5) indicated that a fairly constant mixture of NO\(_3^-\) from organically-derived (manure or septic system effluent) and soil-derived sources was present prior to and during storm events.

**Changes in NO\(_3^-\) Sources on the Mixed Land-Use Watershed at Houserville**

The mixed land-use site at Houserville (Figure 4.2) was an interesting site because it basically integrated the upstream effects of forested, agricultural, and urban land-uses during storm events. Observed changes in \( \delta^{15}N-\text{NO}_3^- \) from baseflow to peakflow were typically small (< ±0.5‰) (Figure 4.12). The one exception occurred on March 23, when \( \delta^{15}N-\text{NO}_3^- \) decreased by 2.7‰ (Figure 4.12). This was likely due to runoff from melting snow, which had a \( \delta^{15}N-\text{NO}_3^- \) signature of -0.6‰ (Figure 4.3). In contrast to \( \delta^{15}N-\text{NO}_3^- \), changes in \( \delta^{18}O-\text{NO}_3^- \) were slightly more significant during storms, and these changes were typically positive (+1.3‰ to +4.2‰) (Figure 4.7). The largest increase of +4.2‰ occurred on August 31, which was a high intensity thunderstorm (4.8 cm/hr), and therefore this change likely represented the influence of NO\(_3^-\) from precipitation. Overall, small changes in NO\(_3^-\) isotopes from baseflow to peakflow and estimated stormflow NO\(_3^-\) signatures (Table 4.5) indicated that NO\(_3^-\) sources at the mixed land-use site in Houserville primarily were of soil origin (Figure 4.3), and these sources did not change much during storm events.
Changes in NO₃⁻ Sources on the Mixed Land-Use Watershed at Rock Road

The mixed land-use site at Rock Road (Figure 4.2) generally behaved similarly to the upstream site at Houserville during storms, although an effect of the UAJA sewage treatment could clearly be identified (Figure 4.3). δ¹⁸O-NO₃⁻ typically increased from baseflow to peakflow (+0.1‰ to +2.7‰), although the changes were of a lesser magnitude than what was observed at the upstream site in Houserville (Figure 4.13). Furthermore, the estimated new δ¹⁸O-NO₃⁻ signature (Figure 4.5) was similar to that of baseflow (see Figure 4.3). The major difference between the site at Rock Road and the upstream site in Houserville was the influence of the UAJA sewage treatment on δ¹⁵N-NO₃⁻ signatures during storms. Baseflow δ¹⁵N-NO₃⁻ signatures were significantly higher at Rock Road than at Houserville (Figure 4.3), and this was due to the enriched δ¹⁵N-NO₃⁻ constantly discharged in the sewage effluent. As a result, δ¹⁵N-NO₃⁻ decreased significantly during all six storms at the Rock Road site (-1.0‰ to -5.8‰) (Figure 4.13) due to dilution by upstream waters and newly added NO₃⁻ in stormflow (see Figure 4.5) with low δ¹⁵N-NO₃⁻ signatures mostly in the range of soil-derived NO₃⁻.

Changes in NO₃⁻ Flow Paths on the Karst Watersheds

Hydrograph separations were used to provide initial information regarding flow path dynamics on the three karst watersheds. The most intense storm on August 31 (see Table 4.2) produced the highest event water fractions observed on the agricultural (fₑW = 0.18) and mixed land-use watersheds at Houserville (fₑW = 0.53) and Rock Road (fₑW = 0.49) (Figure 4.6). The remaining five storms produced more varied event water responses among the three karst basins (Figure 4.6). For all six storms, mean event water fractions on the mixed land-use watersheds at Houserville (mean = 0.33) and Rock Road (mean = 0.24) were much higher (p = 0.07) than the agricultural watershed (mean = 0.08) during storms. This was expected because the two mixed land-use watersheds were more urbanized (~ 23% developed) and had higher densities of impervious surfaces (e.g. roads, parking lots, etc.) than the agricultural watershed (see Table 4.1). As a result, some of the event water observed in the mixed land-use watersheds was likely direct runoff from these surfaces.
Despite the range of event water responses, a fairly uniform response was noted when event NO$_3^-$ fractions were plotted against event water fractions for all three karst watersheds (Figure 4.8). Fractions of event NO$_3^-$ calculated using $\delta^{18}$O-NO$_3^-$ increased with increasing fractions of event water during storms (Figure 4.8) (slope = 0.22, $R^2 = 0.51$, $p = 0.001$) (also see Figure 4.7). The results suggested that the majority of NO$_3^-$ delivered during storms was from older stored sources and did not accompany event water.

The combined evidence from hydrograph separations and NO$_3^-$ stable isotopes suggested that most of the NO$_3^-$ during storms on the three karst watersheds originated from deep groundwater flow paths. On the agricultural watershed, where urbanization effects were least evident, the predominant flow path was best explained as a piston-flow type mechanism (e.g. Hewlett and Hibbert, 1967; reviewed by Buttle, 1994; Bonell, 1998), where older groundwater with similar $\delta^{18}$O-H$_2$O and NO$_3^-$ stable isotope signatures was likely displaced by incoming precipitation and released to the channel as streamflow. This conceptual model would explain the small fractions of event water, the small changes in NO$_3^-$ isotopes, and the large fraction of pre-event NO$_3^-$ that accompanied groundwater sources during storm events. Flow paths in the two more developed mixed land-use, karst watersheds were more challenging to interpret because event water fractions were much greater during storms than in the agricultural watershed, but very little event NO$_3^-$ accompanied event water (similar overall response to agricultural watershed).

If event water was not contributing event NO$_3^-$ to the mixed land-use sites during storms, then what other flow pathways could have been active? A significant portion of the carbonate valley aquifers in the Spring Creek watershed are of the conduit flow aquifer type (see Fulton et al., 2005), which are characterized by flows through large pipes of considerable diameter (see Shuster and White, 1971; White, 1988). White and Reich (1970) suggested that conduit-flow aquifers can temporarily store large volumes of runoff and stormwater, and then release it slowly through springs over the next few days following a storm event. Thus it is possible that during a storm event, overland flow from impervious surfaces does not flow directly to the stream, but instead is transported into the conduit flow system within the epikarst (upper karst aquifer). As a result, recent
water from a previous storm event may be displaced into the stream via a piston-flow type mechanism. While this water may still have a different $\delta^{18}$O-H$_2$O signature than the older baseflow water, the event NO$_3^-$ isotope signature (see Figure 4.5) may be stored long enough for it to undergo biochemical fractionation and/or mixing with other NO$_3^-$ sources such that it remains mostly similar to baseflow. This conceptual model may help to explain how larger fractions of event water could still result in small changes in NO$_3^-$ isotopes and large fractions of event NO$_3^-$ accompanying pre-event water sources during storm events.

**Implications of Flow Paths in Karst Watersheds for NO$_3^-$ Pollution Management**

Based upon analysis of hydrograph separations and NO$_3^-$ stable isotopes in karst watersheds, groundwater appeared to be significantly influencing NO$_3^-$ dynamics during baseflow and peakflow conditions in the Spring Creek watershed. This has several implications for NO$_3^-$ pollution management. First, data on springs collected during this study (Figure 4.3) as well as other local springs (ClearWater Conservancy, unpublished data) has shown that NO$_3^-$ concentrations range from 10 to 20 mg L$^{-1}$, with average concentrations of 20 mg L$^{-1}$ more common in springs that drain agricultural regions. This indicates that there is a potentially large reservoir of NO$_3^-$ in the karst aquifers supplying water to Spring Creek and several of its karst tributaries during baseflow and peakflow conditions. Therefore, future considerations for mitigating NO$_3^-$ pollution in Spring Creek will need to consider methods that prevent NO$_3^-$ leaching to groundwater such as limiting fertilizer and manure applications during wet periods and protecting areas that may enhance denitrification such as wetlands and forested riparian corridors. A second implication for NO$_3^-$ pollution management is related to the apparent age of the local groundwater, or the time elapsed between recharge and discharge (e.g. Focazio et al., 1997). Recent studies using data collected from the Benner Spring (just downstream of the site at Rock Road) have estimated the apparent age of discharging groundwater to be on the order of 6 to 7 years using ratios of tritium to helium $\left(\frac{^3H}{^3He}\right)$ (Lindsey et al., 2003). In general, the combination of groundwater age with active deep groundwater
flow paths during storms suggests that NO$_3^-$ mitigation policies implemented in the near future may not show immediate short-term results in the Spring Creek watershed.

CONCLUSIONS

NO$_3^-$ stable isotopes were useful for showing how NO$_3^-$ sources changed from baseflow to peakflow during storm events in the Spring Creek watershed. Large changes in $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ on the urban watershed during storm events clearly showed that atmospheric NO$_3^-$ sources (wet + dry deposition) were predominant during peakflow conditions, and that no other significant urban NO$_3^-$ sources beyond those from the atmosphere were detectable. Further, mean fractions of event NO$_3^-$ and event water were equally high (~80%), which suggested an active overland flow pathway due to direct runoff from impervious surfaces. On the forested, sandstone watershed, atmospheric NO$_3^-$ also was significant, and accounted for 22% to 36% of the NO$_3^-$ at peakflow. Channel precipitation and/or wash-off of dry-deposited NO$_3^-$ from vegetated surfaces was a potentially important NO$_3^-$ source during small storms, while shallow subsurface flow paths were more important NO$_3^-$ delivery mechanisms during larger storms following longer periods of dry conditions.

A much different response to storms was observed on the agricultural and mixed land-use karst basins. Smaller changes in NO$_3^-$ isotopes occurred during storm events, which suggested that NO$_3^-$ sources from those at baseflow did not change much on these three karst basins. Essentially, a mixture of organically-derived and soil-derived NO$_3^-$ sources were present prior to and during storms at these sites. In addition to small changes in isotopes during storm events, comparisons of event NO$_3^-$ and event water fractions on the three karst basins suggested that the majority of NO$_3^-$ was pre-event NO$_3^-$ that largely accompanied pre-event water during storm events. As a result, deeper groundwater flow paths were likely responsible for most of the NO$_3^-$ delivered during storms on the karst basins. Overall, this study illustrated for the first time how NO$_3^-$ isotopes and $\delta^{18}$O-H$_2$O could be combined to show major differences in water and NO$_3^-$.
delivery mechanisms between forested uplands and karst valleys, and confirm the dominance of overland flow pathways in urbanizing basins.
REFERENCES


Figure 4.1: \( \delta^{15}N \) and \( \delta^{18}O \) in NO\(_3^-\) can be used to discriminate among different sources of NO\(_3^-\) in streams (modified from Kendall, 1998).
Figure 4.2: Map showing sampling sites and subwatershed boundaries within the study portion of the Spring Creek watershed.
Figure 4.3: $\delta^{18}$O-NO$_3^-$ versus $\delta^{15}$N-NO$_3^-$ for all Spring Creek subwatersheds sampled at baseflow (circles) and peakflow (open squares) during the 2005 study period (n = 6 storms). Boxes outlined on the plot indicate approximate boundaries for the isotopic composition of different sources of NO$_3^-$ according to Kendall (1998).
Figure 4.4: Urban watershed baseflow to peakflow changes in δ18O-NO₃⁻ versus δ15N-NO₃⁻ for all six storm events sampled during 2005. Numbers are used to label each storm event (see Table 4.2).
Figure 4.5: Stormflow mean new NO$_3^-$ isotope signatures for the forested watershed (n = 3), agricultural watershed (n = 4), urban watershed (n = 6), mixed land-use watershed at Houserville (n = 6), and mixed land-use watershed at Rock Road (n = 3). Boxes outlined on the plot indicate approximate boundaries for the isotopic composition of different sources of NO$_3^-$ according to Kendall (1998).
Figure 4.6: Fraction of event water for the six storms sampled during 2005. SiO$_2$ was used to calculate event water fractions on the urban watershed, whereas $\delta^{18}$O-H$_2$O was used to calculate event water fractions on the remaining watersheds. The total amount of precipitation and the duration of the storm are shown below the date of each storm.
Figure 4.7: Mean fractions of event NO$_3^-$ predicted using $\delta^{18}$O-NO$_3^-$ and mean fractions of event water predicted using $\delta^{18}$O-H$_2$O and SiO$_2$ (urban watershed only) for each sub-watershed in Spring Creek during 2005. Ratios of event NO$_3^-$ to event water are shown for each watershed. The number (n) of storms used to calculate the mean fractions for each watershed differed because some calculations yielded unreasonable results (see pages 8-9 in the methods for more details).
Figure 4.8: Comparison of the fraction of event NO$_3^-$ predicted using $\delta^{18}$O-NO$_3^-$ versus the fraction of event water predicted using $\delta^{18}$O-H$_2$O and SiO$_2$ (urban watershed only) for all six storms sampled in the Spring Creek watershed during 2005. Green points show data for the forested basin, red points show data for the urban basin, and white points are used to label three karst watersheds (agricultural, mixed land-use at Houserville, and mixed land-use at Rock Road).
Figure 4.9: Forested watershed baseflow to peakflow changes in $\delta^{18}$O-NO$_3^-$ versus $\delta^{15}$N-NO$_3^-$ for all six storm events sampled during 2005. Numbers are used to label each storm event (see Table 4.2).
Figure 4.10: Increase in NO$_3^-$ concentration (mg L$^{-1}$) during six storms in 2005 on the forested watershed plotted against 30-day antecedent precipitation amounts (cm).

\[ \Delta \text{NO}_3^- = -0.84 \times \ln(30\text{-Day Precip}) + 2.5 \]

\[ R^2 = 76.5, \ p = 0.023 \]
Figure 4.11: Agricultural karst watershed baseflow to peakflow changes in $\delta^{18}$O-NO$_3^-$ versus $\delta^{15}$N-NO$_3^-$ for all six storm events sampled during 2005. Numbers are used to label each storm event (see Table 4.2).
Figure 4.12: Mixed land-use watershed at Houserville baseflow to peakflow changes in $\delta^{18}$O-NO$_3^-$ versus $\delta^{15}$N-NO$_3^-$ for all six storm events sampled during 2005. Numbers are used to label each storm event (see Table 4.2).
Figure 4.13: Mixed land-use watershed at Rock Road baseflow to peakflow changes in $\delta^{18}$O-NO$_3^-$ versus $\delta^{15}$N-NO$_3^-$ for all six storm events sampled during 2005. Numbers are used to label each storm event (see Table 4.2).
Table 4.1: Information on sampling sites, predominant land-use classification, watershed area (km$^2$), land-use distribution based on data from Landsat Thematic Mapper, and percent geology.

<table>
<thead>
<tr>
<th>Site Name (Abbreviation)</th>
<th>Site Description</th>
<th>Watershed Area (km$^2$)</th>
<th>Land-Use (% Area)</th>
<th>Geology (% Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galbraith Gap Run (GGR)</td>
<td>Forested</td>
<td>13</td>
<td>100 0 0</td>
<td>6 81 13</td>
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<tr>
<td>Cedar Run (CRL)</td>
<td>Agricultural</td>
<td>45</td>
<td>20 74 1</td>
<td>88 5 7</td>
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<tr>
<td>Thompson Run (THR)</td>
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<td>11</td>
<td>13 19 66</td>
<td>100 0 0</td>
</tr>
<tr>
<td>Spring Creek – Houserville (SPH)</td>
<td>Mixed Land-Use</td>
<td>150</td>
<td>36 37 23</td>
<td>76 16 8</td>
</tr>
<tr>
<td>University Area Joint Authority</td>
<td>Sewage Treatment</td>
<td>--</td>
<td>-- -- --</td>
<td>-- -- --</td>
</tr>
<tr>
<td>(UAJA)</td>
<td>Plant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring Creek Rock Road (SPR)</td>
<td>Mixed Land-Use</td>
<td>201</td>
<td>31 44 22</td>
<td>81 13 6</td>
</tr>
<tr>
<td>Thompson Spring (TS)</td>
<td>Groundwater Spring</td>
<td>--</td>
<td>-- -- --</td>
<td>-- -- --</td>
</tr>
<tr>
<td>Bathgate Spring (BS)</td>
<td>Groundwater Spring</td>
<td>--</td>
<td>-- -- --</td>
<td>-- -- --</td>
</tr>
</tbody>
</table>

For. = Forested  
Ag. = Agricultural  
Dev. = Developed  
Carb. = Carbonate  
Sand. = Sandstone  

Table 4.2: Information on start and end times, antecedent precipitation (past month), observed precipitation type, precipitation amount, and storm characterization for the six storms sampled during 2005.

<table>
<thead>
<tr>
<th>Storm Number</th>
<th>Start Time (EST)</th>
<th>End Time (EST)</th>
<th>Antecedent Precipitation – Past Month (cm)</th>
<th>Precipitation Type</th>
<th>Amount (cm)</th>
<th>Mean Intensity (cm hr$^{-1}$)</th>
<th>Number of Samples</th>
</tr>
</thead>
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<td>1</td>
<td>03-23-05 5:00 AM</td>
<td>03-23-05 3:00 PM</td>
<td>11.0</td>
<td>Rain</td>
<td>1.27</td>
<td>0.18</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>03-27-05 7:00 PM</td>
<td>03-29-05 3:00 AM</td>
<td>12.7</td>
<td>Rain / Snow</td>
<td>4.57</td>
<td>0.30</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>07-05-05 7:00 PM</td>
<td>07-05-05 8:00 PM</td>
<td>4.4</td>
<td>Rain</td>
<td>1.07</td>
<td>1.07</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
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<td>08-31-05 4:00 AM</td>
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</tr>
<tr>
<td>5</td>
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<td>10-07-05 7:00 PM</td>
<td>2.3</td>
<td>Rain</td>
<td>7.37</td>
<td>0.79</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>11-16-05 7:00 AM</td>
<td>11-16-05 12:00 PM</td>
<td>8.9</td>
<td>Rain</td>
<td>1.22</td>
<td>0.27</td>
<td>3</td>
</tr>
</tbody>
</table>
Chapter 5

DEVELOPMENT OF INDICATORS OF HUMAN VERSUS ANIMAL
ESCHERICHIA COLI SOURCES USING ERIC-PCR AND
SEROTYPING FOR SOURCE TRACKING IN A MIXED LAND-USE
WATERSHED

ABSTRACT

In order to better differentiate human from animal sources of NO₃⁻ in stream water, concentration dynamics and potential sources of *Escherichia coli* were investigated in upper Spring Creek, a 201 km² mixed land-use watershed located in central Pennsylvania. Five sites representing a downstream progression of forested, agricultural, urban, and mixed land-uses were monitored at baseflow and peakflow during six storm events in 2005. *E. coli* was measured using grab sampling of stream water, and concentrations were determined using a membrane filtration technique. Sources of randomly selected *E. coli* isolates from stream water samples were characterized by serotyping and analyzed using ERIC-PCR. A local host library was developed for ERIC-PCR profiles using 156 *E. coli* isolates collected from humans and animals (cattle, pigs, horses, pigs, sheep, deer, geese, ducks, and dogs) from the Spring Creek watershed. Statistical analysis of the library showed that average correct classification rates for the two source groups were 78.3% using Jackknife analysis and 81.4% using discriminant analysis. Matching unknown isolates from streams to known bacterial sources was based upon a large serotyping database (23,519 human and animal isolates) and the local watershed library used for ERIC-PCR. A unique aspect of this study was that stream water isolate matches were expressed using indicator variables that showed the weighted percentage match for a given stream isolate to humans and animals. Indicator variables were calculated by coding humans = 0 and animals = 1. Results of the study showed that *E. coli* concentrations were typically elevated at all watershed sites, and that the highest concentrations were observed at peakflow conditions during all six storm events. Adjusting for the effects of rainfall intensity, peakflow concentrations of *E. coli* were highest on the agricultural and urban watersheds and lowest on the forested watershed.
Microbial source tracking using indicator variables developed from ERIC-PCR and serotyping showed that animal sources of *E. coli* dominated human isolates (roughly 70% vs. 30%) during both baseflow and peakflow conditions. Furthermore, ERIC-PCR and serotyping provided remarkably consistent results. Library size and representation of wildlife sources represented potential study limitations, and these aspects should be addressed in future studies. Overall, the application of serotyping and ERIC-PCR showed that animal sources of *E. coli* were predominant in the Spring Creek watershed, and these techniques provided confirmatory evidence regarding the effectiveness of wastewater treatment processes in removing potential human sources of contamination.
INTRODUCTION

Fecal pollution in natural waters is responsible for the impairment of a significant portion of surface waters throughout the United States (EPA, 2005). These impairments are typically identified using microbial indicators such as *Escherichia coli* (EPA, 2000). *E. coli* is a complicated microbial pollutant, and its delivery to surface water can depend on time of the year, flow rate (e.g. storms versus dry periods), and land-use (Triaster and Anisfeld, 2006). As a result, monitoring of *E. coli* is critical to gaining an initial understanding of fecal contamination in a given water body prior to undertaking more sophisticated methods used to identify fecal pollutant sources. The ability to identify these sources is important from a public health perspective (drinking water, contact recreation) as well for helping to manage and reduce point and nonpoint sources of pollution.

Recently, there has been a great deal of interest in testing a variety of methods, collectively known as microbial source tracking (MST), to identify sources of fecal indicator bacteria (e.g. *E. coli*) in natural waters. While MST has shown some promise in recent literature reviews (Sinton et al., 1998; Simpson et al., 2002; Scott et al., 2002; Meays et al., 2004; EPA, 2005; Seurinck et al., 2005; Stoeckel, 2005; Stoeckel and Harwood, 2007), there are still many potential limitations that have been documented in method inter-comparison studies (see Griffith et al., 2003, Stewart et al., 2003; Stoeckel et al., 2004; Moore et al., 2006). These limitations may be the reason that only a few studies have attempted to evaluate MST in watershed settings (see for example Whitlock et al., 2002; Hyer et al., 2004; Somarelli et al., 2007).

A variety of methods exist for identifying sources of fecal pollution in water. Most methods focus on the use of *E. coli*, although several other indicator organisms have gained popularity in recent years (e.g. *Bacteroides fragilis*, *Enterococcus spp.*, and *C. perfringens*) (see Scott et al., 2002). The methods that are used to differentiate sources of *E. coli* can generally be broken down into two broad categories: (1) those that use molecular (genetic) characteristics, and (2) those that assess physiological (phenotypic) characteristics. Examples of molecular techniques include pulsed-field gel electrophoresis (PFGE), repetitive element polymerase chain reaction (PCR), amplified
fragment length polymorphism (AFLP), and ribotyping. Examples of phenotypic techniques include serotyping, antibiotic resistance analysis (ARA), and carbon source utilization (CSU).

Of the available molecular methods, repetitive element PCR-based techniques have widely been used due to their ease of application, cost-efficiency, and high discriminatory power (see EPA, 2005; Stoeckel, 2005). Repetitive element PCR uses primers corresponding to repetitive DNA elements to generate highly specific genomic fingerprints (Scott et al., 2002), which can be used to differentiate among different sources of *E. coli* bacteria. Three primers have been used in past studies including repetitive extragenic palindromic sequence PCR (REP-PCR) (e.g. Carson et al., 2003), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) (e.g. McLellan et al., 2003), and PCR with extragenic repeating elements (BOX-PCR) (e.g. Dombek et al., 2000). REP-PCR and BOX-PCR have been the most widely applied PCR methods to differentiate *E. coli* sources, and relatively few studies have documented the utility of using ERIC-PCR. One recent study by McLellan et al. (2003) showed that ERIC-PCR was useful for separating *E. coli* from animal and human isolates collected from the Milwaukee River basin in Wisconsin. In contrast, a more recent study by Leung et al. (2004) suggested that ERIC-PCR was not successful in discriminating human and animal sources collected in Ontario, Canada due to limited DNA fragment production during PCR analysis. Clearly, more research is needed to determine whether ERIC-PCR primers can distinguish human and animal sources of *E. coli*.

In addition to repetitive element PCR, phenotypic techniques such as serotyping have been suggested to be a potentially useful tool in conjunction with molecular techniques to improve the ability to differentiate human and animal sources of *E. coli* (see Parveen et al., 2001). Early serotyping research by Bettelheim et al. (1974) and Bettelheim et al. (1976) implied that the distribution of *E. coli* serotypes might be different for humans and animals, although significant sharing of serotypes between humans and animals still was observed (Bettelheim et al., 1976). More recently, Parveen et al. (2001) analyzed 100 isolates from animals (n=50) and humans (n=50) and found that serotypes in humans were rarely found in animals, which suggested a possible application in MST. The possibility of shared serotypes between humans and animals
precludes serotyping from being used as a stand-alone method, but it may be useful in combination with ERIC-PCR techniques to better evaluate the importance of human and animal sources of E. coli.

The desired outcome of most MST methods such as ERIC-PCR and serotyping is the specific identification and quantitative differentiation of E. coli sources (e.g. human, cow, pig, deer, etc.). The ability to make specific identifications is often limited by the size and discriminatory power of the E. coli source library (e.g. Johnson et al., 2004) as well as the algorithm used to identify the sources. Examples of typical algorithms used for classification of E. coli isolates in MST studies include discriminant analysis (based on band-matching) and nearest-neighbor rules (similarity-based) (EPA, 2005). Often times investigators are solely interested in identifying the predominant source of E. coli as opposed to quantitatively differentiating between large numbers of diverse sources. One solution to this problem is creating broad categories of sources (e.g. human versus animal) (see Johnson et al., 2004) to improve classification success.

Herein, we report the results of a one-year survey assessing E. coli dynamics and sources within the upper Spring Creek watershed during baseflow and peakflow conditions. Four overall objectives were outlined for this study:

1. Assess E. coli concentration dynamics in the Spring Creek watershed and test for potential differences due to storms, seasons, and land-use.
2. Develop and test the ability of a library of E. coli strains to differentiate between known sources of human and animal E. coli.
3. Implement library-based source tracking to help identify the importance of human and animal E. coli in the upper Spring Creek watershed using discriminant analysis of ERIC-PCR fingerprint data.
4. Compare indicator variables of animal and human E. coli sources developed from analysis by ERIC-PCR and characteristic serotypes.
MATERIALS AND METHODS

Spring Creek Watershed

Spring Creek is a 371 km² watershed located in central Pennsylvania, and is a tributary to the Susquehanna River, which eventually flows into the Chesapeake Bay estuary (Figure 5.1). Spring Creek is a mixed land-use watershed, with forests covering the ridges and a mixture of agriculture, residential, and industrial land-uses in the valleys. The watershed is undergoing rapid urbanization, with agricultural land-uses being replaced by urban and suburban land-uses. As a result of the continued development, a variety of significant point and non-point sources of *E. coli* are present within the Spring Creek watershed, including one sewage treatment plant (University Area Joint Authority) and runoff from agriculture and urban lands during baseflow and stormflow conditions. Examples of potentially important animal sources of *E. coli* bacteria include domestic livestock (e.g. cattle, horses, pigs, sheep), wildlife (e.g. deer, geese, ducks), and pets (e.g. dogs). Human sources of bacteria would likely originate from septic systems and from the treated sewage effluent.

Watershed Sampling Design

The study was designed to take advantage of differences in land-use that existed within the upper Spring Creek watershed. Five watershed sampling sites were selected within the upper Spring Creek watershed (Figure 5.1, Table 5.1), which included three tributary streams with uniquely different land-uses (forest, agricultural, and urban) as well as two downstream mixed land-use sites located on the main-stem of Spring Creek. This design allowed us to compare potentially important *E. coli* sources among watersheds with different land-uses. Study sites were established on Galbraith Gap Run (13 km² forested watershed), Cedar Run (45 km² agricultural watershed), Thompson Run (11 km² urban / developed watershed), Spring Creek at Houserville (150 km² mixed land-use watershed), and Spring Creek at Rock Road (201 km² mixed land-use watershed).
Water Sampling Procedures

All stream sites were sampled during six storm events in 2005 (see Table 5.2 for summary of storm characteristics). For each storm event, streams were sampled once during antecedent baseflow and again at or near peakflow. The University Area Joint Authority (UAJA) sewage treatment plant effluent was sampled only during baseflow conditions.

The collection of stream water samples for bacterial analysis closely adhered to the guidelines set forth by Myers and Wilde (2003). Stream water samples were taken near the channel thalweg, and sewage effluent samples were filled at the outfall. All water samples were collected in 1 L polypropylene sample bottles that were sterilized in an autoclave at 121°C prior to usage. Sample bottles used for sewage effluent sampling were buffered with approximately 0.5 mL of a sterilized 10% solution of sodium thiosulfate (Na₂S₂O₃). Sodium thiosulfate was used to prevent losses of *E. coli* due to the effects of residual chlorine. Laboratory gloves always were worn during water sample collection and handling. All water samples were stored on ice and transported back to the Gastroenteric Disease Center (GDC) at Penn State University for further analysis. Every attempt was made to analyze samples within the six-hour holding time established by the U.S. Environmental Protection Agency (EPA, 2000; APHA, 2005). When samples could not be analyzed within 6 hours, analysis always was performed within 24 hours of sample collection (see Pope et al., 2003 for a discussion of holding time effects on *E. coli* concentrations).

Enumeration and Isolation of *E. coli* in Water Samples

The membrane filtration technique was used to enumerate and isolate *E. coli* from stream water and sewage effluent samples (see Dufour et al., 1981; EPA, 2000; EPA, 2002, Myers and Wilde, 2003). Each sample was serially diluted using sterile phosphate buffer solution (HACH®) according to guidelines recommended by Myers and Wilde (2003). A range of serial dilutions (3 to 5) was used for each water sample based upon anticipated *E. coli* concentrations. Each dilution was filtered through a pre-sterilized Millipore® filtration apparatus onto a sterile 0.45 µm filter paper, which then was placed onto DIFCO® plate-modified mTEC agar (see EPA, 2002; Myers and Wilde, 2003). The
mTEC agar plates were incubated at 35°C for two hours and 44.5°C for 22 hours. Redish or purplish colonies were considered to be *E. coli* (see Ciebin et al., 1995; Guadet et al., 1996; and EPA, 2002 for reviews and comparisons of mTEC agar to other selective media), and these colonies were counted and enumerated according to protocols outlined by Myers and Wilde (2003). It is important to note that periodic testing of field blanks, filter blanks, and procedure blanks (see Myers and Wilde, 2003) showed no apparent contamination bias during the sampling and analysis process.

Once the *E. coli* colonies had been enumerated for all water samples, one mTEC agar plate per sample was selected for isolation of *E. coli* colonies that would be tested using serotyping and ERIC PCR methods. Ten *E. coli* isolates were randomly selected from each plate. These isolates then were tested using a Real-Time PCR technique to determine whether they were *E. coli*. Positive *E. coli* isolates were stored on tryptic soy agar slants and eventually were frozen down for storage at -80°C.

**Watershed *E. coli* Library Development**

A library of *E. coli* isolates was developed for the Spring Creek watershed in order to compare molecular profiles from known animal sources to unknown sources obtained from stream water samples. Fecal samples were collected from a variety of animals living within the Spring Creek watershed during the summer of 2005 including beef cattle, dairy cows, horses, sheep, pigs, chickens, deer, ducks, geese, dogs, and humans. Table 5.3 provides more information on the total number of animals sampled, the number of isolates collected per animal, and the sources used to obtain animal fecal samples.

In general, sterile swabs (BD CultureSwab® with Amies Agar) were used to collect fresh fecal material from each animal (10 swabs per animal). Human fecal samples were obtained from 24-hour integrated samples of settled solids provided by the UAJA sewage treatment plant. Fecal sample collection from cattle (beef and dairy), horses, pigs, sheep, chickens, and deer was conducted by swabbing fresh feces from individual animal pens, and this process was administered by trained animal handlers at Penn State. Fecal samples from dogs were obtained by using rectal swabs from healthy animals. Goose and duck feces were collected by swabbing fresh feces at a local pond.
Swabs containing fresh fecal material were transported on ice back to the Penn State GDC immediately following collection (within 6 hours). The swabs were placed directly into vials of EC Medium (Difco®) broth and incubated at 44.5°C for 24 hours. After 24 hours, samples from each vial were streaked for isolation onto agar plates with EC Medium and incubated overnight at 35°C. Three isolates were randomly selected from each streaked plate, and these isolates were tested using a Real-Time PCR technique to determine whether they were *E. coli*. Positive *E. coli* isolates were stored on tryptic soy agar slants and eventually were frozen down for storage at -80°C.

**Serotyping**

Serotyping was used to determine which O serogroup best characterized different *E. coli* bacteria that were isolated from water samples. A total of 271 *E. coli* isolates from stream water samples (at least 2 isolates per water sample) were randomly selected for serotyping at the Penn State GDC. Serotyping was performed on the bacterial isolates via agglutination reaction with antisera raised against 179 different *E. coli* serogroups. Laboratory methods closely followed those outlined by Ørskov et al. (1977) and Ørskov and Ørskov (1984).

O-serotyping involves the addition of antibodies generated against 179 O antigens in a suspension. Agglutination of the suspension reflects that the reaction is positive for a particular O serogroup (summarized from Stoeckel, 2005). O antigens are part of the lipopolysaccharide present on the outer membrane of gram negative bacteria (e.g. *E. coli*) (DebRoy et al., 2004), and a series of antibodies have been defined against these antigens (Stoeckel, 2005). The World Health Organization recognizes 179 different O serogroups (O1 to O30, O32 to O46, O48 to O71, O73 to O92, O95 to O121, O123 to O175, X6, X9, X10, X13, X18, X19, X21, X23, X25, X28, X38, and X43) (Ørskov et al., 1977).

**ERIC-PCR**

Enterobacterial repetitive intergenic consensus (ERIC) PCR (see Versalovic et al., 1991) was used to develop molecular profiles of *E. coli* isolates from stream water and animals in the Spring Creek watershed. Sixty-four *E. coli* isolates from stream water were typed using ERIC-PCR (~ one isolate per site at baseflow and peakflow). Further,
156 *E. coli* isolates from animals (see Table 5.3 for summary) were analyzed using ERIC-PCR. These animal ERIC profiles were used in the final Spring Creek watershed library.

ERIC-PCR was performed according to a modified protocol that was outlined by Meacham et al. (2003). Briefly, *E. coli* isolates were plated the night prior to running ERIC-PCR in order to obtain fresh growth. DNA from fresh *E. coli* cultures was isolated by resuspending a loopful of culture in 150 μL of sterile H₂O. The mixture was vortexed until homogenous (~1 minute), and then was placed into a Techne heat block at 100°C for 10 minutes. After heating, the mixture was centrifuged at 13,000 rpm for 2 minutes to isolate DNA in the supernatant. Reaction contents for each PCR (11 μL total reaction volume) consisted of 3 μL of template DNA, 0.5 μM of primers (Integrated DNA Technologies, Inc., Coralville, IA), 0.18 mM concentration of each of the four deoxyribonucleotide triphosphates (dNTPs), 4 mM MgCl₂, 0.4 units (U) of *Taq* DNA polymerase (PGC Scientific, Gaithersburg, MD), 50 mM Tris (pH 8.3), 250 μg mL⁻¹ BSA, 2% sucrose, and 0.1 mM cresol red. The PCR was performed in a RapidCycler (Idaho Technologies Inc., Salt Lake City, UT) by using a Rapid-Cycle DNA amplification method and consisted of 30 cycles of template denaturation at 94°C for 10 seconds, annealing at 52°C for 10 seconds, and extension at 72°C for 2 minutes. Amplified ERIC-PCR products were resolved by electrophoresis on 2% agarose gels stained with ethidium bromide, and were normalized using a 100-bp ladder in the first and last lanes of a 14-lane gel. Gels were run at 100 volts for 4 hours. After electrophoresis was completed, gels were scanned using a Kodak EDAS system and the images were saved as tagged image files (TIF) (see Figure 5.2 for example).

**ERIC-PCR Fingerprint Processing**

All gel images were analyzed using GelCompar II Software (version 3.5; Applied Maths, Belgium). Each gel was normalized using the 100-bp reference ladder in lanes 1 and 14. This procedure allowed us to compare fingerprint patterns from multiple gels. All bands were aligned and quantified automatically using an algorithm available in the GelCompar II Software (Applied Maths, 2003). Fully processed fingerprint images were added to a database and compared using several statistical analysis techniques.
Statistical Analysis of Library *E. coli* Isolates

Cluster analysis was used to estimate similarity coefficients for all animal and stream water *E. coli* isolates in the database using GelCompar II Software. Similarity coefficients were generated using the Pearson product-moment correlation coefficient, which calculated similarities based on densitometric curves (see Figure 5.3) as opposed to common and different bands (e.g. algorithms of Jaccard, Dice, etc.). Curve-based coefficients such as the Pearson coefficient and the Cosine coefficient are often preferred because they do not rely on manual assignment of bands, and therefore produce less biased analysis of DNA fingerprints (see Albert et al., 2003). Furthermore curve-based coefficients have been recommended over band-based coefficients because they often result in higher rates of correct classification within *E. coli* reference libraries analyzed using PCR techniques (e.g. Hassan et al., 2005; Goldberg et al., 2006).

The ability of the Spring Creek watershed library to correctly differentiate human and animal *E. coli* isolates was assessed using Jackknife analysis and discriminant analysis. Jackknife analysis was performed using similarity coefficients generated in GelCompar II. Briefly, all *E. coli* isolates in the Spring Creek watershed library (see Table 5.3) were identified as human or animal in origin. Each isolate then was removed one by one from the library database and classified against the existing entries based on maximum similarity. The percentage of cases correctly classified into a particular source group (e.g. human or animal) represented the significance of that group and how well one could expect to classify unknown isolates from stream water (or another source). Discriminant analysis also was performed on the Spring Creek watershed library as an additional test of differentiation between human and animal *E. coli* sources. Discriminant analysis was conducted using quantitative band-matching data that was exported from GelCompar II into SPSS statistical analysis software (version 15.0; SPSS, 2007).
Indicator Variables to Identify Human and Animal *E. coli* Isolates in Stream Water

A sample-weighted indicator variable was calculated for each unknown stream water isolate using information from serotyping and ERIC-PCR data. The purpose was to develop an indicator variable for each stream water isolate that quantified its relatedness to human and animal *E. coli* sources. Variables developed using serotyping and ERIC-PCR data could then be compared to assess whether the methods provided complementary information regarding the relatedness of a stream water isolate to human or animal *E. coli*.

Serotyping indicator variables were developed using an extensive database of previously serotyped human and animal *E. coli* isolates available at the Penn State GDC. The database used for this analysis contained 23,519 *E. coli* isolates taken mostly from chickens, cows (dairy and beef), deer, dogs, ducks, geese, humans, pigs, and turkeys. Other animals included in the database were bear, beavers, chipmunks, horses, raccoons, sheep, and squirrels (grey and red), although these were not well represented for most serotypes. Essentially, a database query was performed for each O serogroup identified in a stream water sample that also was analyzed using ERIC-PCR. The percentage of humans and animals with that particular O serogroup was estimated, and a weighted indicator variable was calculated by coding humans = 0 and animals = 1.

\[
\text{Weighted Indicator Variable} = (\text{Animals})(1) + (\text{Humans})(0) \quad (1)
\]

Thus, if O21 were identified in a stream water sample, and a database query indicated that O21 was found 75% of the time in animals and 25% of the time in humans, the coefficient would be calculated as follows:

\[
\text{Weighted Indicator Variable} = (0.75)(1) + (0.25)(0) = 0.75
\]

The coefficient basically identifies that this particular isolate is more similar to an animal than to a human because it occurred in animals 75% of the time. It does not indicate that the isolate is purely of animal origin. The number of isolates varied for different serogroups in the database, and this certainly may impact the interpretation of human or
animal sources for different serogroups. This represents a potential limitation of using a serotyping database to identify *E. coli* from human or animal isolates.

Indicator variables also were developed using ERIC-PCR fingerprint data. For each stream water isolate, the top 20 human and animal matches based on percent similarity were analyzed. The percentage of humans and animals within the top 20 matches was estimated, and a weighted indicator variable was calculated according to (1) by coding humans = 0 and animals = 1. The interpretation was similar to that described for serotyping indicator variable, although the rate of matching to human or animal sources was based on a fixed number of samples (n = 20). Using this approach, indicator variables from ERIC-PCR data could qualitatively be compared to those generated using serotyping information.

**RESULTS AND DISCUSSION**

*E. coli* Concentration Dynamics in the Upper Spring Creek Watershed

A clear effect of storm events could be detected by comparing baseflow and peakflow *E. coli* concentrations on all five watersheds. For the most part, *E. coli* concentrations increased substantially from baseflow to peakflow for all storm events on all five watersheds (see Figure 5.4). Interestingly enough, decreases in *E. coli* concentrations were observed from baseflow to peakflow on the forested watershed and the urban watershed during the first storm event on March 23, 2005, and these represented the only two exceptions (Figure 5.4). It is unclear what may have caused these decreases, as testing of field blanks, filter blanks, and procedure blanks (see Myers and Wilde, 2003) indicated no clear contamination problems during the March 21-23 sampling period. Overall, increased concentrations from baseflow to peakflow were likely due to wash off of *E. coli* in overland flow (e.g. Olyphant et al., 2003; Traister and Anisfeld, 2006) and resuspension of *E. coli* residing in stream channel sediments (e.g. Jamieson et al., 2005).

In addition to determining the general pattern of increasing *E. coli* concentrations in streams during storm events, we tested whether specific storm characteristics (e.g.
intensity, duration, and amount; see Table 5.2) influenced the observed variation in *E. coli* concentration responses to storms among sites (see Figure 5.4). Overall, simple linear regression models showed that *E. coli* concentrations increased significantly with precipitation intensity on the urban watershed ($R^2 = 0.99, p < 0.001$), mixed land-use watershed at Houserville ($R^2 = 0.92, p = 0.002$), and mixed land-use watershed at Rock Road ($R^2 = 0.94, p = 0.001$). The peakflow *E. coli* concentrations on the agricultural watershed were positively related to amount and intensity, and negatively related to duration ($R^2 = 0.99, p = 0.012$). Changes in *E. coli* concentrations on the forested watershed could not adequately be explained using any of the observed storm characteristics. In general, it was not surprising to find that higher intensity storms produced higher peakflow concentrations of *E. coli* because these types of storms would produce more overland flow and in-channel flow, which could therefore wash off greater numbers of *E. coli*. It should be noted that the high $R^2$ values in the regression models were likely inflated somewhat due to the strong influence of the most intense storm sampled on August 31 (see Table 5.2). This storm produced 4.8 cm of precipitation in one hour, and was responsible for the highest observed *E. coli* concentrations on each of the five watersheds during 2005 (Figure 5.4). Therefore, the influence of rainfall intensity on *E. coli* concentrations should be interpreted with caution as a wide range of rainfall intensities could not be sampled using only six storm events.

*E. coli* concentrations in the upper Spring Creek watershed also were evaluated for differences between growing (May – October) and dormant (November – April) seasons. During baseflow conditions, no distinct seasonal pattern in *E. coli* concentrations was evident among the five watersheds sampled in 2005. The agricultural watershed and the mixed land-use watershed at Houserville had higher *E. coli* concentrations during the growing season (Table 5.5), whereas the forested watershed, urban watershed, and mixed land-use watershed at Rock Road all had higher *E. coli* concentrations during the dormant season. At peakflow, all five watersheds had substantially higher *E. coli* concentrations during the growing season as opposed to the dormant season (Table 5.5). Using two-sample T-tests, no significant seasonal difference was detected for any watershed during baseflow or peakflow conditions. Overall, growing season *E. coli* concentrations (mean of baseflow and peakflow) were higher than
dormant season *E. coli* concentrations (table 5.5), and this seasonal pattern has been observed in past studies (e.g. Traister and Anisfeld, 2006). Higher *E. coli* concentrations during the growing season typically have been attributed to increased activity of animals and the application of manures for crops.

Basic differences in *E. coli* concentrations due to land-use were observed between the 5 watersheds at baseflow and peakflow, although these differences were not significant using a simple one-way analysis of variance. Adjusting for the effects of rainfall intensity during peakflow conditions showed that *E. coli* concentrations were significantly different among all five watersheds during these high flow rates (see Table 5.6). In general, the agricultural watershed had the highest mean *E. coli* concentrations at baseflow and peakflow, followed by the urban watershed, and the two mixed land-use watersheds at Houserville and Rock Road (Table 5.6). The forested watershed had the lowest mean *E. coli* concentrations at baseflow and peakflow (Table 5.6). The differences were likely due to the fact that the more developed watersheds had a greater proportion of potential *E. coli* sources available for wash off (e.g. manure, domestic livestock, pets, septic, wildlife) as compared to the forested watershed, where mostly wildlife sources would have been anticipated. This pattern agreed with the study conducted by Traister and Anisfeld (2006), which showed that developed watersheds typically had higher *E. coli* concentrations than undeveloped watersheds (e.g. those in forests).

In the discussion of baseflow and peakflow *E. coli* concentration variations in Spring Creek, it should briefly be noted that many instances were observed where *E. coli* concentrations exceeded EPA water quality standards for contact recreation (see Appendix A.8 for *E. coli* concentration data). The EPA maintains a two-tiered system of water quality criteria for *E. coli*: (1) an instantaneous standard of 235 colony forming units (cfu) per 100 mL, and (2) a geometric mean of 126 cfu per 100 mL based on two or more samples taken during baseflow conditions (EPA, 1986). If either criterion is exceeded, then the site is in violation of EPA water quality standards. Of the six baseflow samples that were collected on each of the five watersheds in Spring Creek, the instantaneous standard was violated once on the forested watershed (17%), twice on the urban and two mixed land-use watersheds (33%), and three times on the agricultural
watershed (50%). The geometric mean of all six baseflow samples showed violations \((E. coli > 126 \text{ cfu per } 100 \text{ mL})\) on the agricultural (258 cfu per 100 mL) and urban (256 cfu per 100 mL) watersheds. Instantaneous violation rates increased substantially during peakflow conditions when \(E. coli\) concentrations were much higher (Figure 5.4), with the forested watershed recording four violations (67%), the agricultural and two mixed land-use watersheds recording five violations (83%), and the urban watershed violating the instantaneous standard during all six storms (100%). Consistently elevated \(E. coli\) concentrations, especially during peakflow conditions, suggested that significant sources of \(E. coli\) were present within the Spring Creek watershed, and presented a strong argument for the testing of microbial source tracking techniques to help resolve whether \(E. coli\) was predominantly originating from human or animal sources.

Testing Library Differentiation of Human and Animal \(E. coli\)

A library of 156 \(E. coli\) isolates from humans \((n = 38)\) and animals \((n = 118)\) was assessed using Jackknife analysis and discriminant analysis to test the ability of the library to correctly differentiate between human and animal bacteria sources. Jackknife analysis showed that human \(E. coli\) isolates were correctly classified 65.8% of the time and animal \(E. coli\) isolates were correctly classified 90.7% of the time (Table 5.7) using maximum similarity values based on the Pearson product-moment correlation coefficient. Human isolates were misidentified as animal isolates 34.2% of the time, and animal isolates were misidentified as human isolates 9.3% of the time (Table 5.7). Similarly, discriminant analysis on quantitative band-matching data showed that 76.3% of the human \(E. coli\) isolates and 86.4% of the animal \(E. coli\) isolates could correctly be identified in the library database (see Figure 5.5, Table 5.8). Misidentification rates were 23.7% for humans and 13.6% for animals (Table 5.8). Overall, average rates of correct classification (ARCC) were 78.3% using Jackknife analysis and 81.4% using discriminant analysis. The average rates of correct classification reported herein compared favorably with those reported in previous studies where human and animal \(E. coli\) sources were separated (e.g. Parveen et al., 1999; Carson et al., 2003), and suggested that the isolate library was capable of successfully identifying the importance of human and animal \(E. coli\) sources in the upper Spring Creek watershed.
While ARCCs using Jackknife analysis and discriminant analysis were fairly high, it should be pointed out that library size was an important issue when implementing library-based source tracking at the scale of a 201 km$^2$ watershed such as Spring Creek. Most libraries used in recent bacteria source tracking studies have been quite small, ranging from 35 to 500 isolates (Johnson et al., 2004; EPA, 2005). The library used in this study (156 isolates) clearly fits into this category. Small libraries are typically used when budgets or time considerations do not allow the collection and analysis of larger $E. \text{coli}$ populations. Unfortunately, smaller libraries limit the ability to identify $E. \text{coli}$ from specific animal sources such as cows, geese, deer, or humans because the genetic diversity of $E. \text{coli}$ bacteria in the environment is very large (McLellan, 2004; Lasalde et al., 2005), and is due in part to factors such as feeding and dietary differences among animals (e.g. Hartel et al., 2003), temporal and geographic differences between animal species (Gordon, 2001; Hartel et al., 2002; Scott et al., 2003; Jenkins et al., 2003) and the number of strains present within a particular animal (e.g. McLellan et al., 2003). Further, significant genetic variation of $E. \text{coli}$ also has been demonstrated in secondary habitats such as septic tanks (Gordon et al., 2002) and in streams during baseflow and peakflow conditions (Hartel et al., 2004). As a result of these factors, it has been estimated that libraries of 10,000 or more isolates may be needed to adequately characterize the genetic variation of $E. \text{coli}$ in its primary and secondary habitats (see Johnson et al., 2004).

To circumvent some of the drawbacks of a small host library, we chose only to identify the relative importance of human and animal $E. \text{coli}$ in stream water, as opposed to attempting detailed animal identifications using maximum similarity coefficients. This decision was based in part on the fact that organizing $E. \text{coli}$ into broader source groupings (e.g. human and animal) has been shown to increase the likelihood of successful discrimination using PCR-based approaches (e.g. Carson et al., 2003; Johnson et al., 2004). Furthermore, recent inter-laboratory comparison studies have suggested that library-based methods (e.g. PCR) performed better when only identification of the dominant source of $E. \text{coli}$ (e.g. human or animal) was attempted (see Griffith et al., 2003).
Identifying the Importance of Human and Animal *E. coli* in Stream Water

**Discriminant Analysis**

Discriminant analysis was performed on quantitative band-matching data from 156 human and animal *E. coli* isolates and 63 stream water isolates from the five watersheds in upper Spring Creek. A plot of the first two discriminant functions, which explained 55.5% of the variation in quantitative band-matching data among library and stream water isolates, showed that the majority of *E. coli* in stream water was more similar to animal sources of *E. coli* than to human sources (Figure 5.6). The centroids for all five watersheds showed stronger affiliations with animal sources of *E. coli* as opposed to human sources (Figure 5.6). The centroids for the two mixed land-use watersheds at Houserville and Rock Road, while still strongly within the animal cluster, showed the closest affiliation with human *E. coli* sources among all five watersheds based on the discriminant analysis (Figure 5.6). While a small group of *E. coli* isolates from both these sites was observed to cluster near the centroid for the human *E. coli* isolates, a significant portion of the animal isolates also were in close proximity, making the interpretation of human or animal source difficult using this plot alone. A second point to make was that isolates from the forested (1 isolate), agricultural (2 isolates), and urban (1 isolate) watersheds plotted some distance from the main grouping of stream water isolates (Figure 5.6). These isolates clearly were not similar to any animal or human isolates in the watershed library, which suggested that they could not easily be identified and points to the need for a larger, more representative library.

**Comparison of Serotyping and ERIC-PCR Indicator Variables**

Indicator variables calculated using serotyping data and similarity data from ERIC-PCR fingerprints provided further information regarding the importance of human and animal *E. coli* sources in the upper Spring Creek watershed. Like the discriminant analysis (Figure 5.6), serotyping and ERIC-PCR indicator variables revealed that animal sources were likely to be more important than human *E. coli* sources during baseflow (Figure 5.7) and peakflow (Figure 5.8) conditions. Essentially, the figures suggested that serotypes and ERIC-PCR profiles from animal isolates were more likely than human
isolates to match *E. coli* isolates collected from stream water. The mean rate of animal matches was greater than 70% for baseflow and peakflow isolates collected at all sites. While the indicator variables at peakflow were somewhat higher than during baseflow (see Figures 5.7 and 5.8), they were not statistically different from one another. Furthermore, differences in indicator variables due to watershed land-use also were not observed. Overall, these observations are in general agreement with early work by Glantz (1973), who showed that the majority of 3,200 serogroups isolated from Spring Creek and its tributaries were animal in origin. The most unique result was that serotyping and ERIC-PCR indicator variables showed remarkable agreement for all sites (Figures 5.7 and 5.8). This finding supports the recommendations of Parveen et al. (2001), who suggested that combining serotyping with a molecular approach (e.g. ERIC-PCR in this case) would represent a useful approach for identifying sources of fecal pollution in water. In the case of the Spring Creek study, it also points to the possibility that one method (the simplest or most cost effective) may have been sufficient for tracking sources of *E. coli* in streams.

**Overall Interpretations**

Discriminant analysis on quantitative band-matching data and the comparison of ERIC-PCR and serotyping indicator variables suggested that animals were the predominant source of *E. coli* throughout the upper Spring Creek watershed for all flow conditions. This did not mean that human sources of *E. coli* were unimportant. The canonical discriminant plot (Figure 5.6) suggested that some isolates, especially those from the two mixed land-use sites, potentially matched human isolates in the library. The two indicator variables (Figures 5.7 and 5.8) suggested that for a given stream water isolate, up to 30% of the potential matching *E. coli* isolates could have been from humans. Therefore, based on these results, it is possible that humans could represent an important background source of *E. coli* at all sites during baseflow and peakflow conditions. As of the year 2000, the majority (94%) of the households in the upper Spring Creek watershed were hooked up to public sewer, while only a small portion (7%) used on-lot septic systems for waste treatment (U.S. Census Bureau, 2000). UAJA, which is the largest wastewater treatment plant in the Centre Region, effectively treated
the sewage effluent such that concentrations of *E. coli* remained well below water quality standards (< 80 cfu per 100 mL) during the study period (see Figure 5.4). Therefore, the finding that human isolates were of lower importance was not surprising in the context of this study.

It should be mentioned that the results of this study were mostly in line with two other recent studies that tested microbial source tracking in watershed settings. Whitlock et al. (2002) applied antibiotic resistance analysis in a mostly urban watershed in Florida to identify sources of *E. coli* from wild animals, humans, and dogs. Unlike the current study in Spring Creek, where animals were important at all flow rates, results from the Florida study by Whitlock et al. (2002) showed that humans were an important background source of *E. coli* during low flows. At high flow rates, wild animal sources became the most predominant *E. coli* source. In a more recent study by Somarelli et al. (2007), rep-PCR with BOX-A1R primers was used to identify wildlife (geese and deer) as the most predominant source of *E. coli* in a mixed land-use watershed located in the Finger Lakes region of New York. Humans were the least important of the sources tested (geese, deer, cows, and humans).

**Potential Limitations of Study**

Several potential study limitations warrant further discussion, and these are reviewed briefly. One of the potential limitations in the current study was the small size of the host library of *E. coli* isolates. This issue was discussed in more detail in a previous section. A second potential limitation was related to the composition of the host library and the serotyping database. The host library was unintentionally biased toward animal sources of *E. coli*, with animal sources accounting for 118 isolates (76%) and human sources accounting for 38 isolates (24%). The serotyping database (n = 23,519 isolates) also was heavily biased toward animal *E. coli* sources, with animals accounting for 81% of the database composition, and humans accounting for 19%. Of the animal sources, 75% of the serotyped isolates were domestic livestock animals (e.g. cattle, horses, pigs, sheep, etc.) and only 6% were from wildlife (mostly deer and geese). As a result, the heavy emphasis of animal sources may have influenced the overall importance of animal sources of *E. coli* in Spring Creek. Future work in this watershed would
definitely require obtaining better representation of human *E. coli* sources in both databases. Further, the serotyping database would require additional serotype information from wildlife sources.

**CONCLUSIONS**

*E. coli* monitoring in the upper Spring Creek watershed showed that all watersheds had measurable concentrations during baseflow and peakflow conditions. All sites recorded frequent (> 50%) violations of the EPA instantaneous water quality standard (235 cfu per 100 mL) during six storms sampled at peakflow conditions. Overall, the largest variations in *E. coli* concentrations were observed during storm events, with *E. coli* concentrations during peakflow greatly exceeding those observed during baseflow conditions. The highest peaks in *E. coli* appeared to coincide with the most intense storm events. Adjusting for the effects of rainfall intensity, peakflow concentrations of *E. coli* were highest on the agricultural and urban watersheds and lowest on the forested watershed.

Microbial source tracking showed that using ERIC-PCR and serotyping provided consistent results regarding potential sources of *E. coli* in the upper Spring Creek watershed. Indicator variables comprised of the weighted mean percentage of matches to human (indicator = 0) and animal (indicator = 1) sources using ERIC-PCR and serotyping were used to make comparisons among sites. Both methods suggested that animal sources of *E. coli* were more likely than human isolates to match a stream isolate (roughly 70% vs. 30%). The small host library used for ERIC-PCR (156 isolates) combined with heavy biases toward animals in the serotyping and library databases represented potential limitations of the study. Overall, the application of serotyping and ERIC-PCR showed that animal sources of *E. coli* were predominant in the Spring Creek watershed, and these techniques provided confirmatory evidence regarding the effectiveness of wastewater treatment processes in removing potential human sources of contamination.
REFERENCES


\url{http://www.census.gov/census2000/states/pa.html}. 

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Figure 5.1: Map showing sampling sites and subwatershed boundaries within the study portion of the Spring Creek watershed.
Figure 5.2: Representative ERIC2 profiles of *E. coli* isolates collected from stream water during the 2005 Spring Creek study. Lanes 1 and 14: 100 bp marker (New England Biolabs cat# N3231S); 2: forested watershed (baseflow), 3: agricultural watershed (baseflow), 4: urban watershed (baseflow), 5: mixed land-use watershed at Houserville (baseflow), 6: mixed land-use watershed at Rock Road (baseflow), 7: UAJA sewage effluent, 8: groundwater spring, 9: groundwater spring, 10: forested watershed (peakflow), 11: agricultural watershed (peakflow), 12: urban watershed (peakflow), mixed land-use watershed at Houserville (peakflow).
Figure 5.3: Calculation of the Pearson product-moment correlation between two animal *E. coli* isolates. Red (1) and blue (2) lines depict the densitometric curves for each isolate. The banding patterns developed from the ERIC-PCR gels are shown below.
Figure 5.4: $\log_{10} E. coli$ concentrations (cfu per 100 mL) measured at baseflow (light blue bars) and peakflow (dark blue bars) for the five watersheds in Spring Creek and the UAJA sewage treatment plant (baseflow only) during 2005.
Figure 5.5: Two dimensional plot using discriminant analysis of quantitative band-matching data obtained from ERIC-PCR. The graph was generated by plotting the first discriminant function (accounting for 35.1% of the variance) against the second discriminant function (accounting for 20.4% of the variance). Results are shown for *E. coli* collected from known sources such as animals and humans.
Figure 5.6: Two dimensional plot using discriminant analysis of quantitative band-matching data obtained from ERIC-PCR. The graph was generated by plotting the first discriminant function (accounting for 35.1% of the variance) against the second discriminant function (accounting for 20.4% of the variance). Results are shown for E. coli collected from known sources such as animals (white outlined region) and humans (black outlined region) as well as unknown stream water sources from the forested, agricultural, urban, and mixed land-use watersheds at baseflow and peakflow.
Figure 5.7: Baseflow comparison of the mean rate at which animal isolates matched individual stream water isolates using serotyping (white bars) and ERIC-PCR (shaded bars). Error bars indicate ± 1 standard deviation from the mean. Each bar represents the mean of six isolates, one from each storm event, that were tested using both ERIC-PCR and serotyping methods.
Figure 5.8: Peakflow comparison of the mean rate at which animal isolates matched individual stream water isolates using serotyping (white bars) and ERIC-PCR (shaded bars). Error bars indicate ± 1 standard deviation from the mean. Each bar represents the mean of six isolates, one from each storm event, that were tested using both ERIC-PCR and serotyping methods.
Table 5.1: Information on sampling sites, predominant land-use classification, watershed area (km²), and land-use distribution based on data from Landsat Thematic Mapper.

<table>
<thead>
<tr>
<th>Site Name (Abbreviation)</th>
<th>Site Description</th>
<th>Watershed Area (km²)</th>
<th>Land-Use / Land-Cover (% Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galbraith Gap Run (GGR)</td>
<td>Forested Watershed</td>
<td>13</td>
<td>Forest 100 Agriculture 0 Developed 0</td>
</tr>
<tr>
<td>Cedar Run (CRL)</td>
<td>Agricultural Watershed</td>
<td>45</td>
<td>Forest 20 Agriculture 74 Developed 1</td>
</tr>
<tr>
<td>Thompson Run (THR)</td>
<td>Urban Watershed</td>
<td>11</td>
<td>Forest 13 Agriculture 19 Developed 66</td>
</tr>
<tr>
<td>Spring Creek – Houserville (SPH)</td>
<td>Mixed Land-Use Watershed</td>
<td>150</td>
<td>Forest 36 Agriculture 37 Developed 23</td>
</tr>
<tr>
<td>University Area Joint Authority (UAJA)</td>
<td>Sewage Treatment Plant</td>
<td>--</td>
<td>-- Developed 23</td>
</tr>
<tr>
<td>Spring Creek Rock Road (SPR)</td>
<td>Mixed Land-Use Watershed</td>
<td>201</td>
<td>Forest 31 Agriculture 44 Developed 22</td>
</tr>
</tbody>
</table>

Table 5.2: Information on start and end times, antecedent precipitation (past month), observed precipitation type, precipitation amount, and storm characterization for the six storms sampled during 2005.

<table>
<thead>
<tr>
<th>Storm Number</th>
<th>Start Time (EST)</th>
<th>End Time (EST)</th>
<th>Antecedent Precipitation – Past Month (cm)</th>
<th>Precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Type of Precipitation</td>
</tr>
<tr>
<td>1</td>
<td>03-23-05 5:00 AM</td>
<td>03-23-05 3:00 PM</td>
<td>11.0</td>
<td>Rain</td>
</tr>
<tr>
<td>2</td>
<td>03-27-05 7:00 PM</td>
<td>03-29-05 3:00 AM</td>
<td>12.7</td>
<td>Rain / Snow</td>
</tr>
<tr>
<td>3</td>
<td>07-05-05 7:00 PM</td>
<td>07-05-05 8:00 PM</td>
<td>4.4</td>
<td>Rain</td>
</tr>
<tr>
<td>4</td>
<td>08-31-05 3:00 AM</td>
<td>08-31-05 4:00 AM</td>
<td>4.6</td>
<td>Rain</td>
</tr>
<tr>
<td>5</td>
<td>10-07-05 6:00 AM</td>
<td>10-07-05 7:00 PM</td>
<td>2.3</td>
<td>Rain</td>
</tr>
<tr>
<td>6</td>
<td>11-16-05 7:00 AM</td>
<td>11-16-05 12:00 PM</td>
<td>8.9</td>
<td>Rain</td>
</tr>
</tbody>
</table>
Table 5.3: Information on numbers of animals sampled, *E. coli* isolates per animal, and total *E. coli* isolates collected during the library development phase of the project. Sources of animal samples also are given.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sample Date</th>
<th>Number of Animals</th>
<th>Isolates per Animal</th>
<th>Total Isolates Collected</th>
<th>Source of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef Cow</td>
<td>07/29/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>PSU Beef Center</td>
</tr>
<tr>
<td>Dairy Cow</td>
<td>07/29/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>PSU Dairy Barns</td>
</tr>
<tr>
<td>Horse</td>
<td>07/29/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>PSU Equine Facilities</td>
</tr>
<tr>
<td>Sheep</td>
<td>08/03/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>PSU Sheep Center</td>
</tr>
<tr>
<td>Pig</td>
<td>08/03/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>PSU Swine Center</td>
</tr>
<tr>
<td>Chicken</td>
<td>08/15/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>PSU Poultry Education and Research Center</td>
</tr>
<tr>
<td>Deer</td>
<td>07/29/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>PSU Deer Research Center</td>
</tr>
<tr>
<td>Duck</td>
<td>06/27/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>The “Duck” Pond (local pond)</td>
</tr>
<tr>
<td>Goose</td>
<td>06/27/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>The “Duck” Pond (local pond)</td>
</tr>
<tr>
<td>Dog</td>
<td>08/08/2005</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>Metzger Animal Hospital (Veterinary Clinic)</td>
</tr>
<tr>
<td>Human</td>
<td>08/15/2005</td>
<td>--</td>
<td>--</td>
<td>50</td>
<td>UAJA Settled Solids</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td></td>
<td>350</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.4: Number of human and animal *E. coli* isolates analyzed using ERIC-PCR and included in the final watershed library.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Source of Isolates</th>
<th>(number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef Cow</td>
<td>Penn State Beef Center</td>
<td>(17)</td>
</tr>
<tr>
<td>Dairy Cow</td>
<td>Penn State Dairy Barns</td>
<td>(12)</td>
</tr>
<tr>
<td></td>
<td>GDC Collection</td>
<td>(8)</td>
</tr>
<tr>
<td>Horse</td>
<td>Penn State Equine Facilities</td>
<td>(5)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Penn State Sheep Center</td>
<td>(1)</td>
</tr>
<tr>
<td>Pig</td>
<td>Penn State Swine Center</td>
<td>(6)</td>
</tr>
<tr>
<td>Deer</td>
<td>Penn State Deer Research Center</td>
<td>(8)</td>
</tr>
<tr>
<td></td>
<td>GDC Collection</td>
<td>(11)</td>
</tr>
<tr>
<td>Duck</td>
<td>Local ponds</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>GDC Collection</td>
<td>(9)</td>
</tr>
<tr>
<td>Goose</td>
<td>Local ponds</td>
<td>(8)</td>
</tr>
<tr>
<td></td>
<td>GDC Collection</td>
<td>(12)</td>
</tr>
<tr>
<td>Dog</td>
<td>Local veterinary clinic</td>
<td>(8)</td>
</tr>
<tr>
<td></td>
<td>GDC Collection</td>
<td>(12)</td>
</tr>
<tr>
<td>Human</td>
<td>UAJA Settled Solids</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td>GDC Collection</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>UAJA Treated Effluent – Water</td>
<td>(6)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>156</strong></td>
</tr>
</tbody>
</table>
Table 5.5: Mean *E. coli* concentrations (cfu per 100 mL) for growing season (May – October) and dormant season (November – April) periods.* Results are summarized separately for baseflow, peakflow and all flow conditions.

<table>
<thead>
<tr>
<th>Watershed</th>
<th>Baseflow</th>
<th>Peakflow</th>
<th>All Flows</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growing (n = 3)</td>
<td>Dormant (n = 3)</td>
<td>Growning (n = 3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forested</td>
<td>80</td>
<td>246</td>
<td>5,250</td>
</tr>
<tr>
<td>Agricultural</td>
<td>1,167</td>
<td>115</td>
<td>54,567</td>
</tr>
<tr>
<td>Urban</td>
<td>293</td>
<td>449</td>
<td>25,600</td>
</tr>
<tr>
<td>Mixed Land-Use Houserville</td>
<td>499</td>
<td>38</td>
<td>10,415</td>
</tr>
<tr>
<td>Mixed Land-Use Rock Road</td>
<td>31</td>
<td>391</td>
<td>10,473</td>
</tr>
</tbody>
</table>

*Seasonal differences were not detected at the α = 0.05 level for baseflow and peakflow samples using two-sample T-tests.

*High concentrations of *E. coli* at peakflow during the growing season were largely driven by the intense thunderstorm on August 31, which produced concentrations >> 10,000 cfu per 100 mL at all stream sites.

Table 5.6: Mean *E. coli* concentrations (cfu per 100 mL) for baseflow and peakflow conditions.*

<table>
<thead>
<tr>
<th>Watershed</th>
<th>Baseflow</th>
<th>Peakflow</th>
<th>All Flows</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (n = 6)</td>
<td>(Std. Dev.)</td>
<td>Mean (n = 6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forested</td>
<td>163</td>
<td>(243)</td>
<td>2,723</td>
</tr>
<tr>
<td>Agricultural</td>
<td>641</td>
<td>(976)</td>
<td>27,840</td>
</tr>
<tr>
<td>Urban</td>
<td>371</td>
<td>(352)</td>
<td>13,058</td>
</tr>
<tr>
<td>Mixed Land-Use Houserville</td>
<td>269</td>
<td>(378)</td>
<td>5,618</td>
</tr>
<tr>
<td>Mixed Land-Use Rock Road</td>
<td>211</td>
<td>(257)</td>
<td>5,645</td>
</tr>
</tbody>
</table>

*Land-use differences were not detected at the α = 0.05 level for baseflow and peakflow samples using one-way Analysis of Variance.
Table 5.7: Percentage of *E. coli* isolates in the Spring Creek watershed library (n = 156) correctly assigned to human and animal source groups based on Jackknife Analysis of Pearson product-moment similarity coefficients. The average rate of correct classification (ARCC) was 78.3%.

<table>
<thead>
<tr>
<th></th>
<th>Humans</th>
<th>Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td>65.8</td>
<td>9.3</td>
</tr>
<tr>
<td>Animals</td>
<td>34.2</td>
<td>90.7</td>
</tr>
</tbody>
</table>

Table 5.8: Percentage of *E. coli* isolates in the Spring Creek watershed library (n = 156) correctly assigned to human and animal source groups based on discriminant analysis of quantitative band-matching data. Only the first discriminant function was used because it accounted for 100% of the observed variation. The average rate of correct classification (ARCC) was 81.4%.

<table>
<thead>
<tr>
<th></th>
<th>Humans</th>
<th>Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td>76.3</td>
<td>23.7</td>
</tr>
<tr>
<td>Animals</td>
<td>13.6</td>
<td>86.4</td>
</tr>
</tbody>
</table>

Wilks’ Lambda: 0.647  
$\chi^2$: 60.263  
Degrees of Freedom: 31.000  
Significance (p): 0.001
Chapter 6

COMPARING DISCRIMINATION OF LAND-USE AND STREAM POLLUTION USING NITRATE ISOTOPES, INORGANIC CHEMISTRY, AND BACTERIAL INDICATORS

ABSTRACT

Discrimination of land-use and NO$_3^-$ pollution sources was evaluated and compared in upper Spring Creek, a 201 km$^2$ mixed land-use watershed located in central Pennsylvania. Five sites representing a downstream progression of forested uplands underlain by resistant sandstone to karst lowlands with agricultural, urban, and mixed land-uses were monitored at baseflow and peakflow during six storm events in 2005. Water samples were analyzed for NO$_3^-$ stable isotopes ($\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$), inorganic chemistry (pH, electrical conductivity, NO$_3^-$, SO$_4^{2-}$, Cl$^-$, Na$^+$, Ca$^{2+}$, SiO$_2$), and E. coli bacteria. Discriminant analysis using NO$_3^-$ stable isotopes showed that watersheds with different land-uses could correctly be discriminated 70% of the time at baseflow and 72% of the time at peakflow. $\delta^{15}$N-NO$_3^-$ and NO$_3^-$ were important discriminatory variables at baseflow and peakflow, respectively, and both variables essentially separated forested land-use and sewage effluent from all other sources. The most problematic discrimination occurred among the agricultural, urban, and mixed land-use watersheds, and mixing and seasonal trend analyses confirmed that these sites could not be distinguished isotopically. The use of inorganic chemical tracers showed a marked improvement in correct discrimination among watersheds with different land-uses and pollutant sources. The average correct classification rates among land-use and pollutant sources at baseflow and peakflow improved to 97.5% and 93.2%. During baseflow and peakflow conditions, Na$^+$ was a useful tracer for separating sewage as well as urban, agricultural, and mixed land-uses. Ca$^{2+}$ and conductivity also were important for capturing geologic differences between sandstone and karst basins at baseflow. During peakflow conditions, pH and Ca$^{2+}$ accounted for differences in dilution among sites during storms due to the influences of overland flow. The full discriminant model using
NO$_3^-$ isotopes, inorganic chemistry, and microbial indicators showed that Na$^+$ and Ca$^{2+}$ remained very important discriminating variables, while NO$_3^-$ isotopes diminished in relative importance. Microbial indicators designed to detect differences in human and animal sources of pollution were not significant discriminators of land-use at baseflow or peakflow, and this mostly was due to a lack of significant human *E. coli* sources within the watershed. Overall correct classifications in the full model were not much different than using inorganic chemistry alone. Based upon the results of this study, NO$_3^-$ stable isotopes were sufficient for capturing basic differences in principal NO$_3^-$ sources from forested land-uses and sewage, although isotopic overlap among developed land-uses presented problems. The addition of inorganic chemical tracers clearly enabled discrimination among land-uses and geology that could not necessarily be captured using NO$_3^-$ isotopes alone, but results using inorganic chemistry are not generalizable and are specific to the Spring Creek watershed.
INTRODUCTION

NO$_3^-$ pollution of streams and rivers represents a significant water quality challenge facing watershed managers throughout the United States, especially within the Chesapeake Bay estuary (NRC, 2000). In order to effectively manage NO$_3^-$, it is important to identify key NO$_3^-$ sources within the watershed. Land-use is an important driver of water quality, and many past studies have linked water quality concentrations (e.g. nitrogen or NO$_3^-$) to land-use (e.g. Miller et al., 1997; Jordan et al., 1997; Herlihy et al., 1998; Williams et al., 2005). Unfortunately, linking water quality to land-use does not typically yield critical information on NO$_3^-$ sources. In order to mitigate NO$_3^-$ pollution, it is advantageous to develop methods that link land-use, water quality, and information on NO$_3^-$ sources.

Stable isotopes in NO$_3^-$ ($\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$) offer a way to track principal NO$_3^-$ sources in mixed land-use watersheds (see Figure 6.1; Kendall, 1998 and references therein). Ultimately, four major sources of NO$_3^-$ can be determined using $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ (see Kendall et al., 1998; Rock and Mayer, 2004): (1) NO$_3^-$ in precipitation, (2) NO$_3^-$ from microbial nitrification in soils, (3) synthetic N fertilizers, and (4) NO$_3^-$ derived from sewage or manure sources (see Figure 6.1). The linkage between NO$_3^-$ isotopes and NO$_3^-$ sources suggests there may be opportunities to differentiate NO$_3^-$ from different types of land-use.

A number of studies have been conducted looking into whether NO$_3^-$ stable isotopes may be useful discriminators of NO$_3^-$ from different land-uses. Several studies have been able to differentiate NO$_3^-$ in forested land-uses from NO$_3^-$ in more urbanized and agricultural land-uses using $\delta^{15}$N-NO$_3^-$ (e.g. Harrington et al., 1998; Mayer et al., 2002; Rock and Mayer, 2004; Voss et al., 2006). While these studies reported some success in using NO$_3^-$ isotopes to detect land-use NO$_3^-$ source signals, other studies have reported much less success (e.g. Chang et al., 2002; Douglas et al., 2002).

One confounding factor affecting the results of previous studies was scale (e.g. Chang et al., 2002). Most studies attempting to relate NO$_3^-$ isotopes to land-use were conducted in large river basins ($> 10,000$ km$^2$ in area) with a complex mixture of land-uses. In order to better determine the ability of NO$_3^-$ isotopes to resolve NO$_3^-$ from
different land-uses, it would be useful to work at the scale of a small mixed land-use watershed, where individual watersheds with relatively homogenous land-uses (e.g. forest, agriculture, urban) could be delineated.

Mixing of NO$_3^-$ from broad land-use categories and seasonal NO$_3^-$ isotope variations represent additional confounding factors that must be considered. In terms of mixing, past land-use studies using NO$_3^-$ isotopes essentially have differentiated two main sources: (1) low NO$_3^-$ concentrations with low $\delta^{15}$N-NO$_3^-$ from forest land (e.g. NO$_3^-$ from soil nitrification), and (2) high NO$_3^-$ concentrations with high $\delta^{15}$N-NO$_3^-$ from agricultural and urban land (e.g. a mixture of manure and sewage sources) (e.g. Harrington et al., 1998; Mayer et al., 2002; Voss et al., 2006). Simple two-component mixing models (e.g. Burg and Heaton, 1998) may be useful to demonstrate whether NO$_3^-$ isotopes indicate binary mixing among land-use, geology, and pollutant sources. Furthermore, seasonal variations of NO$_3^-$ stable isotopes also must be characterized because it is helpful to know whether NO$_3^-$ stable isotopes can distinguish different land-uses, geologic settings, and pollutant sources on a year-round basis, or whether there are certain periods when some (or all) sources are indistinguishable using NO$_3^-$ stable isotopes.

One possible improvement over the use of isotopes alone is to combine additional tracers with NO$_3^-$ stable isotopes. Ions such as Na$^+$ and Cl$^-$ may be useful for identifying the importance of sewage, septic, or other anthropogenic NO$_3^-$ sources that are commonly found in agricultural and urban watersheds. Furthermore, microbial indicators of human and animal pollution developed in a previous study (see Chapter 5) may be useful for differentiating between sewage and manure sources of NO$_3^-$, which tend to have overlapping $\delta^{15}$N-NO$_3^-$ source signatures (see Figure 6.1; Kendall et al., 1998). Finally, geologic differences are important, and Ca$^{2+}$ and SiO$_2$ tracers may help identify potentially higher NO$_3^-$ concentrations in karst versus sandstone settings (see Boyer and Pasquarell, 1995; Boyer and Pasquarell, 1996; Miller et al., 1997).

A good example of combining NO$_3^-$ isotopes with additional tracers is a recent study by Spruill et al. (2002). The authors combined $\delta^{15}$N-NO$_3^-$ with sixteen inorganic chemicals and ionic ratios of those chemicals in a multivariate statistical model to provide additional statistical power in differentiating NO$_3^-$ sources between manure,
sewage, and septic systems in groundwater. While this type of approach appeared to be valuable in a groundwater setting, there was an underlying assumption that the inorganic chemical tracers combined with NO$_3^-$ stable isotopes related directly to NO$_3^-$ sources. Similar studies in surface water within mixed land-use watersheds should address: (1) the degree of improvement attained by adding other tracers, and (2) whether the added tracers relate in any way to NO$_3^-$ sources or instead capture other watershed characteristics such as land-use and geology.

Herein we report the results of a one-year survey in Spring Creek, a mixed land-use watershed in central Pennsylvania, to assess NO$_3^-$ isotope discrimination among watersheds with different land-uses, geologies, and pollutant sources of NO$_3^-$, and the level of improvement achieved by adding inorganic chemical and bacterial indicators. Two main objectives were outlined for this study:

1. Assess and compare the effectiveness of NO$_3^-$ stable isotopes versus inorganic chemistry in stream water to discriminate among different land-uses and NO$_3^-$ from sewage and precipitation by accounting for potential effects of mixing and seasonality on isotope tracers.
2. Test whether the combination of inorganic chemistry and bacterial indicators with NO$_3^-$ stable isotopes improves discrimination among different land-uses and NO$_3^-$ from sewage and precipitation over either set of tracers alone.

MATERIALS AND METHODS

Spring Creek Watershed

Spring Creek is a 371 km$^2$ watershed located in central Pennsylvania, and is a tributary to the Susquehanna River, which eventually flows into the Chesapeake Bay estuary (Figure 6.2). The Spring Creek watershed is situated within the Appalachian Section of the Ridge and Valley Physiographic Province. Several sandstone ridges (elevation ~ 550 - 600 m) are present within the watershed, and karst geologic features are predominant in the valleys (elevation ~ 300 - 400 m), which include sinkholes,
groundwater conduits, and large springs (see Fulton et al., 2005). The karst nature of the watershed results in complicated hydrology, with gaining and losing stream sections a common occurrence (Fulton et al., 2005; O’Driscoll and DeWalle, 2006). The climate in central Pennsylvania is a humid continental type climate, with average annual precipitation of approximately 97 cm based upon 80 years of data (1926-2005) measured at State College, PA (PA State Climatologist, 2006). Average annual stream runoff is approximately 38 cm based on 65 years of data measured at the USGS stream gauge on Spring Creek at Axemann (02050204).

**Watershed Sampling Design**

The study was designed to take advantage of differences in land-use, geology, and predominant NO$_3^-$ sources that existed within the upper Spring Creek watershed, which is 201 km$^2$ in area. Five watershed sampling sites were selected within the upper Spring Creek watershed (Figure 6.2, Table 6.1), which included three tributary streams with uniquely different land-uses (forest, agricultural, and urban) as well as two downstream mixed land-use sites located on the main-stem of Spring Creek. This design allowed us to compare tracer signatures and NO$_3^-$ sources among watersheds with different land-uses and bedrock geology. Study sites were established on the following watersheds: (1) Galbraith Gap Run, a 13 km$^2$ forested watershed; (2) Cedar Run, a 45 km$^2$ agricultural watershed; (3) Thompson Run, an 11 km$^2$ urban / developed watershed; (4) Spring Creek at Houserville, a 150 km$^2$ mixed land-use watershed; and (5) Spring Creek at Rock Road, a 201 km$^2$ mixed land-use watershed. The forested watershed was underlain by sandstone and shale bedrock, while the remaining four watersheds were predominately carbonate geology (Table 6.1). Treated sewage effluent was discharged and samples were collected at the University Area Joint Authority (UAJA) outfall along Spring Creek located about 1.4 km downstream of the Houserville sampling site (Figure 6.2). This allowed us to assess the impacts of sewage discharges on NO$_3^-$ concentrations and stable isotopes in Spring Creek. Finally, two groundwater springs (Figure 6.2, Table 6.1) were sampled once in summer and once again in late fall to characterize the chemistry and stable isotopic composition of local groundwater. Appendix A.6 provides more details on specific sampling site locations.
Stream Sampling and Precipitation Monitoring

All stream sites and the sewage treatment plant effluent were sampled during six storm events in 2005. For each storm event, streams were sampled once during antecedent baseflow and again at or near peakflow. The sewage treatment plant effluent only was sampled once per storm during baseflow conditions. Baseflow sampling was used to reflect local groundwater inputs for each stream site. Storm sampling was spread out through the year in order to assess seasonality of tracer data.

Precipitation during each event was sampled using a sequential passive precipitation sampler designed and constructed to collect sufficient volumes of rainwater for NO$_3^-$ stable isotope and inorganic chemical analysis. The sampler was approximately 5.7 m$^2$ in area and was equipped with a polyethylene plastic tarp, which funneled water to a series of three 20-L polypropylene carboys. A new tarp was used for each event that was sampled, and the tarp was washed three times with deionized water just prior to the onset of precipitation (see Chapter 2 for more information on precipitation sampling). Approximately 0.36 cm (0.13 in) of rainfall filled an individual carboy. The bottles were arranged such that they filled sequentially during storms according to a slightly modified design from that of Kennedy et al. (1979). Sub-samples were collected from individual carboys for analysis of inorganic chemistry and isotopes in water.

Water Chemistry and Stable Isotope Analyses

All stream water and precipitation samples were analyzed for inorganic chemistry at the Water Quality Laboratory in the Penn State Institutes of Energy and the Environment (PSIEE). Water samples were filtered through 0.45 µm filters. Samples were analyzed for pH (electrometric), specific conductance (electrometric), SO$_4^{2-}$ and Cl$^-$ (ion chromatography), NO$_3$-N (cadmium reduction), NH$_4$-N (phenate), and Ca$^{2+}$ and Na$^+$ (atomic absorption direct aspiration). All analyses were conducted using standard methods (APHA, 2005). Stream temperature measurements also were taken during field visits using a portable temperature probe.

Stable isotopes in NO$_3^-$ ($^{15}$N and $^{18}$O) were analyzed at the University of Waterloo Environmental Isotope Laboratory (EIL). $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ were analyzed according to the methods outlined by Chang et al. (1999) and Silva et al. (2000).
exchange resins were used to collect dissolved NO$_3^-$ in stream water at the Water Quality Laboratory located within the Penn State Institutes of the Environment (see Chapter 2 for a more complete description of stable isotope methodology). Anion exchange resins loaded with NO$_3^-$ were stored in a 4°C cooler until they could be shipped to the EIL at Waterloo for further processing and analysis. The results for δ$^{15}$N-NO$_3^-$ and δ$^{18}$O-NO$_3^-$ are reported in delta notation (δ$^{15}$N and δ$^{18}$O in permil units, ‰) versus their respective international reference standards. The international reference standard for δ$^{15}$N-NO$_3^-$ is atmospheric N$_2$ gas, while the international reference standard for δ$^{18}$O-NO$_3^-$ is Standard Mean Ocean Water (SMOW). The analytical error for both isotopes was approximately ±0.2‰ based on duplicate samples (26 duplicates for δ$^{15}$N-NO$_3^-$ and 14 duplicates for δ$^{18}$O-NO$_3^-$).

Stable isotopes of δ$^{18}$O-H$_2$O also were analyzed at the University of Waterloo EIL. Water samples for δ$^{18}$O-H$_2$O were collected and stored in airtight 20-mL HDPE scintillation bottles prior to shipment to the EIL at Waterloo. The analysis for δ$^{18}$O-H$_2$O was conducted using the CO$_2$ equilibration method outlined by Epstein and Mayeda (1953). All results are reported in delta notation (δ$^{18}$O in permil units, ‰) relative to Standard Mean Ocean Water (SMOW). The analytical error for δ$^{18}$O-H$_2$O isotopes was approximately ±0.1‰ based on 20 duplicate samples.

**E. coli Collection, Enumeration, and Development of Indicator Variables**

*E. coli* was analyzed in stream water and sewage effluent samples during the 2005 study period. The collection of samples for bacterial analysis closely adhered to the guidelines set forth by Myers and Wilde (2003). All water samples were collected in 1 L polypropylene sample bottles that were sterilized in an autoclave at 121°C prior to usage. Upon completion of field sampling, water samples were stored on ice and transported back to the Gastroenteric Disease Center (GDC) at Penn State University for further analysis. More details on field sampling are provided in Chapter 5. *E. coli* in stream water and sewage effluent samples were enumerated and isolated using the membrane filtration technique with modified mTEC agar (see Dufour et al., 1981; EPA, 2000; EPA, 2002, Myers and Wilde, 2003). *E. coli* colonies (at least 1 per
site per storm) were isolated and later analyzed using serotyping (phenotypic technique) and ERIC-PCR (molecular technique) (see Chapter 5 for more details on methodology). Stream water isolates analyzed using ERIC-PCR were compared to a library of 150 ERIC profiles obtained from beef cattle, dairy cows, horses, sheep, pigs, chickens, deer, ducks, geese, dogs, and humans using GelCompar II software (version 3.5; Applied Maths, Belgium) (see Chapter 5 for more details on library development and analysis of fingerprint profiles). Water isolates analyzed using serotyping were compared to an existing GDC database of 23,519 *E. coli* serotypes taken mostly from chickens, cows (dairy and beef), deer, dogs, ducks, geese, humans, pigs, and turkeys (domestic) (see Chapter 5). *E. coli* indicator variables comprised of the weighted mean percentage of best matches to human (indicator = 0) and animal (indicator = 1) sources were calculated using ERIC-PCR and serotyping data for each stream site during baseflow and peakflow conditions. These variables were used as indicators of human and animal sources of *E. coli* (see Chapter 5). In this study, they were tested to see whether further discrimination between human (sewage and septic) and animal (manure) sources of NO$_3^-$ could be achieved in watersheds with different land-uses.

**Data Analysis – Mixing**

Binary mixing models were used to provide insight into potentially important NO$_3^-$ isotope relationships with land-use and NO$_3^-$ pollutant sources from sewage and precipitation in the Spring Creek watershed. Simple binary mixing curves (after Burg and Heaton, 1998) were developed using the following series of equations:

1. $C_M = C_H f + C_L (1 - f)$  
2. $C_M \delta_M = C_H \delta_H f + C_L \delta_L (1 - f)$  
3. $\delta_M = \frac{C_H \delta_H f + C_L \delta_L (1 - f)}{C_M}$

where:  

- $C_M$ = Concentration of mixed waters  
- $\delta_M$ = Isotopic signature of mixed waters  
- $C_H$ = Concentration of high NO$_3^-$ water  
- $\delta_H$ = Isotopic signature of high NO$_3^-$ water
\[ C_L = \text{Concentration of low NO}_3^- \text{ water} \]
\[ \delta_L = \text{Isotopic signature of low NO}_3^- \text{ water} \]
\[ f = \text{fraction of high NO}_3^- \text{ water in mixture} \]

Hypothetical high and low end-members for NO\(_3^-\) and NO\(_3^-\) stable isotopes were selected using observed data from actual NO\(_3^-\) sources (e.g. stream water, precipitation, sewage effluent) and the curves were visually fit to the observed data collected from Spring Creek and its tributaries. Logarithmic regression models also were used to generate fitting parameters for the data.

**Statistical Methods – Discriminant Analysis**

Discriminant analysis is a multivariate statistical method that generates linear equations (analogous to regression) to describe graphically (in three or fewer dimensions) the differential features of observations from several known groups (see Johnson and Wichern, 2002). Therefore, discriminant analysis was selected as an appropriate statistical tool to differentiate among watersheds with different land-use and NO\(_3^-\) from sewage and precipitation in the Spring Creek watershed. For the purposes of this study, stepwise discriminant analysis was first used to compare NO\(_3^-\) stable isotopes (\(\delta^{15}N\)-NO\(_3^-\), \(\delta^{18}O\)-NO\(_3^-\)), stable isotopes in water (\(\delta^{18}O\)-H\(_2\)O), and NO\(_3^-\) concentrations versus the use of inorganic chemistry as discriminators of land-use and the two NO\(_3^-\) pollutant sources. Discriminant analysis then was used to ascertain the relative importance of NO\(_3^-\) isotopes, inorganic chemical tracers (pH, electrical conductivity, SO\(_4^{2-}\), Cl\(^-\), Na\(^+\), Ca\(^{2+}\), SiO\(_2\)), oxygen isotopes in water (\(\delta^{18}O\)-H\(_2\)O), and microbial indicators (E. coli concentrations, serotyping and ERIC-PCR indices – see Chapter 5).

SPSS statistical analysis software (version 15.0; SPSS, 2007) was used to perform stepwise discriminant analysis of the Spring Creek water quality dataset. Each variable was entered into the model if the significance of its F-value was less than 0.05. Variables in the model were removed when the F-value significance was greater than 0.10. Stepwise discriminant models were run on baseflow and peakflow datasets separately. Results were graphed using two-dimensional canonical discriminant function plots.
RESULTS AND DISCUSSION

NO$_3^-$ Isotope Discrimination of Watersheds and Sources

**Baseflow Conditions**

Stepwise discriminant analysis was performed on baseflow samples from the five watersheds in Spring Creek as well as on sewage effluent from UAJA and two local groundwater springs. Significant discriminatory variables in the final analysis were NO$_3^-$ concentrations and $\delta^{15}$N-NO$_3^-$. The overall correct classification rate for all sources in the model was 70% (see Figure 6.3, Table 6.3).

The first discriminant function accounted for the majority of the variation (98.3%) in $\delta^{15}$N-NO$_3^-$ and NO$_3^-$ concentrations among sources, and therefore was the most important discriminator. $\delta^{15}$N-NO$_3^-$ showed the strongest correlation with the first function (+0.632), followed by NO$_3^-$ concentrations (+0.564) (Table 6.3). As a result, discrimination among sources was mainly due to differences in $\delta^{15}$N-NO$_3^-$, with NO$_3^-$ concentration differences being slightly less important. Forested land-use (centroid 1 on Figure 6.3) was efficiently separated from all other sources because it had the lowest mean $\delta^{15}$N-NO$_3^-$ (+1.4‰) and NO$_3^-$ concentrations (0.7 mg L$^{-1}$) during baseflow conditions (see Table 6.4). On the other hand, sewage effluent (centroid 6 on Figure 6.3) plotted farthest to the right because it had the highest mean $\delta^{15}$N-NO$_3^-$ (+14.8‰) and NO$_3^-$ concentrations (40.9 mg L$^{-1}$) among all sources (see Table 6.4).

While the forested watershed and sewage effluent were efficiently discriminated using $\delta^{15}$N-NO$_3^-$ and NO$_3^-$ concentrations, discrimination of the remaining sites was more challenging. The agricultural watershed, urban watershed, mixed land-use watershed at Houserville, and groundwater springs had similar $\delta^{15}$N-NO$_3^-$ (range: +5.1‰ to +6.9‰) and NO$_3^-$ concentrations (range: 15.4 mg L$^{-1}$ to 20.9 mg L$^{-1}$) (see Table 6.4), and as a result the centroids plotted fairly close together in the two-dimensional discriminant plot (see Figure 6.5). The mixed land-use watershed at Rock Road was a slight exception because $\delta^{15}$N-NO$_3^-$ (+10.9‰) and NO$_3^-$ concentrations (24.1 mg L$^{-1}$) were slightly higher.
due to upstream discharges of high NO$_3^-$ and enriched $\delta^{15}$N-NO$_3^-$ from the UAJA sewage treatment plant.

**Peakflow Conditions**

Stepwise discriminant analysis was performed on peakflow samples from the five watersheds in Spring Creek as well as on samples collected from precipitation and sewage effluent during storm events. Significant discriminatory variables used in the final analysis were NO$_3^-$ concentrations, $\delta^{18}$O-NO$_3^-$, and $\delta^{15}$N-NO$_3^-$. The overall correct classification rate for all sources in the model was 72% (see Figure 6.4, Table 6.5).

The first discriminant function accounted for the majority of the variation (91.4%) in $\delta^{15}$N-NO$_3^-$, $\delta^{18}$O-NO$_3^-$, and NO$_3^-$ concentrations among sources, and therefore provided the most discriminatory power among sources at peakflow. NO$_3^-$ concentrations showed the strongest overall correlation with the first function ($+0.775$) (Table 6.5). $\delta^{18}$O-NO$_3^-$ was negatively correlated with the first function ($-0.318$), while $\delta^{15}$N-NO$_3^-$ showed a small positive correlation ($+0.422$) (Table 6.7). As a result, discrimination among sources was mainly due to differences in NO$_3^-$ concentrations, with $\delta^{18}$O-NO$_3^-$ and $\delta^{15}$N-NO$_3^-$ differences being slightly less important. The forested watershed (centroid 1 on Figure 6.4) was efficiently separated from all other sources because it had low mean NO$_3^-$ concentrations ($1.6$ mg L$^{-1}$) and relatively high $\delta^{18}$O-NO$_3^-$ ($+15.9\%$) during peakflow conditions (Table 6.6). The sewage effluent (centroid 6 on Figure 6.4) also was efficiently discriminated because it had the highest mean NO$_3^-$ concentrations ($40.9$ mg L$^{-1}$) and the lowest mean $\delta^{18}$O-NO$_3^-$ ($+2.2\%$) among all sources (Table 6.6). Finally, precipitation (centroid 7 on Figure 6.4) was discriminated from all other sources on the basis of low mean NO$_3^-$ concentrations ($0.8$ mg L$^{-1}$) and enriched mean $\delta^{18}$O-NO$_3^-$ ($+43.8\%$) (Table 6.6).

**Problems with Discrimination Using NO$_3^-$ Stable Isotopes**

Discriminant analysis using NO$_3^-$ isotopes was most successful in discerning forested land-use from more developed land-uses at baseflow and peakflow in Spring Creek. Binary mixing curves were used to illustrate this point further using equation (3) (after Burg and Heaton, 1998) for all watersheds and NO$_3^-$ sources. A decreasing
curvilinear relationship between $\delta^{18}$O-NO$_3^-$ and NO$_3^-$ concentrations (Figure 6.5a) and an increasing curvilinear relationship between $\delta^{15}$N-NO$_3^-$ and NO$_3^-$ concentrations (Figure 6.5b) indicated that Spring Creek was mostly a conservative mixture of low NO$_3^-$ concentration waters (0.3 mg L$^{-1}$) having low $\delta^{15}$N-NO$_3^-$ (-5.6‰) and high $\delta^{18}$O-NO$_3^-$ (+67.7‰) (e.g. precipitation, runoff from forested lands) mixed with high NO$_3^-$ concentration waters (11 mg L$^{-1}$) having high $\delta^{15}$N-NO$_3^-$ (+9.9‰) and low $\delta^{18}$O-NO$_3^-$ (-0.2‰) (e.g. manure runoff, sewage and septic wastes, etc.). The high NO$_3^-$ concentration waters included the urban, agricultural, and mixed land-use watershed sites, which were mostly indistinguishable using NO$_3^-$ stable isotopes.

Seasonality of NO$_3^-$ stable isotopes was evaluated to determine whether isotopic overlap was a problem throughout the year. $\delta^{15}$N-NO$_3^-$ patterns mostly were similar on the agricultural watershed, the urban watershed, and the mixed land-use watershed in Houserville (Figure 6.6a). The mixed land-use watershed at Rock Road had much higher $\delta^{15}$N-NO$_3^-$ than the other four sites due to the influence of the sewage treatment plant (see Figure 6.6a; Table 6.7). In contrast, $\delta^{15}$N-NO$_3^-$ on the forested watershed remained distinct from the other four watersheds for all of 2005 due to the influence of low $\delta^{15}$N-NO$_3^-$ natural background soil nitrogen sources (see Figure 6.6a; Table 6.7). Significant overlap was noted for $\delta^{18}$O-NO$_3^-$ on all five watersheds throughout the year, especially during the summer and fall months (see Figure 6.6b). On March 26, a 4.2‰ difference in $\delta^{18}$O-NO$_3^-$ was observed between the two mixed land-use sites (+4.1‰ to +8.2‰), with the remaining sites falling within this range. From August through November, most watershed sites showed significant overlap of $\delta^{18}$O-NO$_3^-$ signatures and essentially were isotopically indistinguishable (see Table 6.8).

In essence, the main limitation was that NO$_3^-$ stable isotopes were not capable of discriminating NO$_3^-$ from urban, agricultural, and mixed land-uses. This observation is in agreement with many past studies that have attempted to discriminate NO$_3^-$ according to land-use using only NO$_3^-$ stable isotopes (Harrington et al., 1998; Mayer et al., 2002; Douglas et al., 2002; Rock and Mayer, 2004; Voss et al., 2006; Cole et al., 2006). A variety of NO$_3^-$ sources exist within these land-uses, including manure from animals, septic wastes, and sewage effluent discharges. These organically-derived NO$_3^-$ sources
are isotopically indistinguishable, especially using $\delta^{15}$N-NO$_3^-$ (see Kendall, 1998). Isotopic overlap among organically-derived NO$_3^-$ sources has received substantial attention in the literature (see Kendall 1998 and references therein; Bedard-Haughn, 2003), and remains a major drawback to using NO$_3^-$ stable isotopes to differentiate among developed land-uses in mixed land-use watersheds.

**Chemical Discrimination of Watersheds and Sources**

**Baseflow Conditions**

Stepwise discriminant analysis was performed on baseflow samples from the five watersheds in Spring Creek as well as on sewage effluent from UAJA and two local groundwater springs. Significant variables used in the final discriminant analysis included pH, conductivity, NO$_3^-$, SO$_4^{2-}$, Cl$^-$, Na$^+$, Ca$^{2+}$, and SiO$_2$. The overall rate of correct classification for the model was 97.5% (see Figure 6.7, Table 6.3).

The first discriminant function explained 48.4% of the variation in inorganic chemistry among sites and pollutant sources during baseflow conditions. Na$^+$ (+0.703) mostly strongly correlated with the first discriminant function (Table 6.3), while the remaining variables in the model were less important discriminators of land-use and pollutant sources. Sewage effluent (centroid 6 on Figure 6.7) was efficiently separated from all other sources because it had the highest mean Na$^+$ concentrations (100.0 mg L$^{-1}$) during the study period (see Table 6.4). The mixed land-use watershed at Rock Road (centroid 5 on Figure 6.7) could be distinguished from the other three developed watersheds because it was significantly influenced by discharges from the UAJA sewage treatment plant. The agricultural (centroid 2), urban (centroid 3), and mixed land-use watershed at Houserville (centroid 4) also could be discriminated using Na$^+$ concentrations (Figure 6.7, Table 6.4). The urban watershed had the highest mean Na$^+$ concentrations in baseflow (27.3 mg L$^{-1}$), followed by the mixed land-use site in Houserville (14.0 mg L$^{-1}$) and the agricultural watershed (4.7 mg L$^{-1}$).

The second discriminant function explained 42.1% of the variation in inorganic chemistry at baseflow. Ca$^{2+}$ was the discriminatory variable that showed the strongest positive correlation with the second function (+0.661), followed closely by conductivity.
The forested watershed was easily distinguished from all other watersheds and pollutant sources because it had the lowest mean conductivity (37 µS cm\(^{-1}\)) and Ca\(^{2+}\) (2.9 mg L\(^{-1}\)) during the study period (Table 6.4; Figure 6.7). The remaining sites were not well discriminated using Ca\(^{2+}\) and conductivity because the mean concentrations among sites were quite similar (see Table 6.4).

The results of discriminant analysis suggested that Na\(^{+}\) was an important discriminatory variable during baseflow conditions in the Spring Creek watershed (see Table 6.4). While NO\(_3^-\) stable isotopes were unable to differentiate NO\(_3^-\) from the agricultural watershed, urban watershed, and mixed land-use watershed in Houserville (upstream from UAJA sewage plant), the use of Na\(^{+}\) clearly enabled a separation to be made among these three sites. Na\(^{+}\) is an important component of human and animal waste as well as in road salt, and these pollutants are likely transported to streams in runoff. It was shown in Chapter 4 that new water sources due to runoff were most important in the urban watershed. The mixed land-use watershed site in Houserville experienced moderate amounts of surface runoff during storms, whereas the agricultural watershed mostly was dominated by older water sources from groundwater. As a result, the difference in Na\(^{+}\) concentrations among the three watersheds may have been due to differences in the importance of runoff mechanisms transporting animal waste and road salt pollutant sources to stream water.

Ca\(^{2+}\) concentrations and conductivity also were important for separating differences in watershed characteristics within Spring Creek. The importance of Ca\(^{2+}\) and conductivity was likely due to geologic differences between the forested watershed, which was underlain by resistant sandstone and shale formations (Table 6.1), and the remaining four watersheds that were underlain by carbonate bedrock (Table 6.1). Past studies have shown that carbonate watersheds have much higher NO\(_3^-\) concentrations than sandstone watersheds (Gerhart, 1986; Boyer and Pasquarell, 1995; Boyer and Pasquarell, 1996; Miller et al., 1997), and this has been attributed to the preference of calcareous soils for agriculture and the propensity for karst landforms to rapidly transmit soluble NO\(_3^-\) to groundwater. As a result, Ca\(^{2+}\) was useful for discriminating high NO\(_3^-\) waters in the carbonate watersheds from low NO\(_3^-\) waters in the forested, sandstone watershed.
**Peakflow Conditions**

Stepwise discriminant analysis also was performed on peakflow samples from the five watersheds in Spring Creek as well as on samples collected from precipitation during storm events. Significant variables in the final discriminant analysis included pH, NO$_3^-$, SO$_4^{2-}$, Cl$^-$, Na$^+$, Ca$^{2+}$, and SiO$_2$. The overall correct classification rate for the final model was 93.2% (see Figure 6.8, Table 6.5).

The first discriminant function explained 66.8% of the variation in inorganic chemistry at peakflow (Table 6.5). Na$^+$ was most strongly correlated with the first discriminant function (+0.671) followed by SO$_4^{2-}$ (+0.598) and Cl$^-$ (+0.536) (Table 6.5). Similar to baseflow conditions, this function mostly discriminated sewage effluent (centroid 6 on Figure 6.8) from all other land-uses and pollutant sources at peakflow because sewage effluent had the highest mean concentrations of Na$^+$ (100.0 mg L$^{-1}$), SO$_4^{2-}$ (63.3 mg L$^{-1}$), and Cl$^-$ (148.8 mg L$^{-1}$). The remaining watersheds and pollutant sources were not easily discriminated using the first discriminant function (see Figure 6.8).

The second discriminant function explained 23.0% of the variation in inorganic chemistry at peakflow, and appeared to discriminate among the land-use and precipitation sources more efficiently than the first discriminant function. The second function was most strongly correlated with pH (+0.729) and Ca$^{2+}$ (+0.574) (Table 6.5). The agricultural watershed had the highest mean Ca$^{2+}$ concentrations (59.5 mg L$^{-1}$) of all sites (Table 6.5), and therefore plotted the highest on the Y-axis of Figure 6.8. In contrast, precipitation plotted the lowest on the Y-axis of Figure 6.8 because it had the lowest mean Ca$^{2+}$ (0.3 mg L$^{-1}$) and pH (4.9) of all the sources (Table 6.5). The forested and urban watersheds were discriminated from the two mixed land-use watersheds based mostly upon differences in Ca$^{2+}$ concentrations (see Figure 6.8, Table 6.5).

The results of discriminant analysis on peakflow samples showed that most all sites could be differentiated using selected inorganic chemicals. For the most part, differences among sites were captured using Ca$^{2+}$ concentrations. Essentially, Ca$^{2+}$ was diluted less during storms in the agricultural watershed due to minimal influences of overland flow (see Chapter 4). In contrast, the remaining sites exhibited different effects of dilution with the forest and urban sites showing more significant effects than the two
mixed land-use sites. Therefore, the addition of inorganic chemical parameters, especially Ca$^{2+}$ concentrations, was useful from the standpoint of characterizing differences in storm response in the Spring Creek watershed.

**Isotopic, Chemical, and Bacterial Discrimination of Watersheds and Sources**

In the previous sections, NO$_3^-$ stable isotopes correctly discriminated approximately 70% of the land-use and NO$_3^-$ pollution sources, whereas inorganic chemistry performed much better, discriminating better than 90% of the sources. One final discriminant analysis was used to test the relative importance of NO$_3^-$ stable isotopes and inorganic chemical tracers in a combined model. In addition, microbial tracers were added to this model to test whether they could further discriminate among human (e.g. septic and sewage) and animal (e.g. manure) pollution sources in these watersheds.

**Baseflow Conditions**

Stepwise discriminant analysis on baseflow samples using NO$_3^-$ stable isotopes, inorganic chemical tracers, and bacterial indicators produced interesting results that helped determine the relative importance of these variables as discriminators of land-use, geology, and pollutant sources. Significant variables used in the final discriminant analysis included conductivity, NO$_3^-$, SO$_4^{2-}$, Na$^+$, Ca$^{2+}$, SiO$_2$, and $\delta^{15}$N-NO$_3^-$. The overall rate of correct classification for the model was 97.5% (see Figure 6.9, Table 6.3), which was the same rate reported for the model using only inorganic chemical tracers.

Discriminant analysis using the combined tracer dataset produced similar overall results to the previous analysis using inorganic chemical tracers alone. The first discriminant function described 48.2% of the variation, and it was most highly correlated with Na$^+$ (+0.838) (Table 6.3). The second discriminant function accounted for 44.5% of the variation, and it correlated most strongly with Ca$^{2+}$ (+0.732) (Table 6.3). As a result, Na$^+$ still was the most important variable for discriminating the effects of sewage, manure, and road salt influences (see Figure 6.9), while Ca$^{2+}$ captured the geologic differences between the forested, sandstone watersheds and the remaining karst sites (see Figure 6.9). Despite the fact that $\delta^{15}$N-NO$_3^-$ was included in the final model (see Table
6.3), isotopic tracers did not play a large role in discriminating among the watersheds and pollutant sources.

**Peakflow Conditions**

Discriminant analysis using the combined tracer dataset at peakflow also showed the importance of inorganic chemical tracers as discriminators of land-use and pollutant sources. Significant variables in the final discriminant analysis included pH, NO$_3^-$, SO$_4^{2-}$, Cl$^-$, Ca$^{2+}$, SiO$_2$, $\delta^{18}$O-NO$_3^-$, and $\delta^{15}$N-NO$_3^-$. The overall rate of correct classification for the model was 96.4% (see Figure 6.10, Table 6.5), which represented a slight (3.2%) improvement in discrimination using only inorganic chemical tracers. The first discriminant function described 64.0% of the variation, and it was most highly correlated with SO$_4^{2-}$ (+0.595) and Cl$^-$ (+0.528) (Table 6.5). The second discriminant function accounted for 25.0% of the variation, and it correlated most strongly with pH (+0.642) and Ca$^{2+}$ (+0.468) (Table 6.5). In the case of peakflow samples, the SO$_4^{2-}$ and Cl$^-$ clearly discriminated sewage effluent from all other sources (see Table 6.6, Figure 6.10), whereas pH and Ca$^{2+}$ captured differences in dilution due to low pH and Ca$^{2+}$ precipitation inputs (see Table 6.6) on watersheds during storms (see Figure 6.10). Like in baseflow, NO$_3^-$ stable isotopes were included in the final model, but neither $\delta^{18}$O-NO$_3^-$ nor $\delta^{15}$N-NO$_3^-$ played an important role in discriminating sites based on land-use or pollutant sources (see Table 6.5).

**Variables That Were Not Significant Discriminators of NO$_3^-$ Sources**

One of the hypotheses in this study was that microbial indicator variables developed using ERIC-PCR and serotyping (see Chapter 5) would significantly improve the ability to differentiate NO$_3^-$ between human (sewage and septic) and animal (manure) sources in mixed land-use watersheds. We expected these variables would be important in discriminating agricultural NO$_3^-$ sources (mostly manure) from more urbanized sources of NO$_3^-$, especially downstream of the UAJA sewage treatment plant. Stepwise discriminant analysis indicated that *E. coli* concentrations and microbial indicator variables were not useful discriminators of NO$_3^-$ sources among watersheds with different land-uses in the Spring Creek watershed. One reason for this was due to the overall
similarity of indicator variables among all five watershed sampled in Spring Creek during baseflow and peakflow (see Tables 6.7 and 6.10; Chapter 5), which suggested that stream water isolates were more likely to be similar to animal sources (~70%) as opposed to human sources (~30%). The predominance of animal source matching to E. coli from stream water samples was attributed to the fact that the majority (94%) of the households in the upper Spring Creek watershed were hooked up to public sewer, while only a small portion (7%) used on-lot septic systems for waste treatment (U.S. Census Bureau, 2000). Additionally, UAJA effectively treated the sewage effluent such that concentrations of E. coli remained well below water quality standards, which further decreased the probability of detecting human sources of E. coli in Spring Creek. The minimization of human E. coli influences in Spring Creek represented the most plausible explanation for why microbial indicator variables were not useful discriminators of NO$_3^-$ sources. Future testing of this idea should focus on rural watersheds with a much greater percentage of people on septic systems. This would be expected to contribute human sources of NO$_3^-$ and E. coli, which is an important prerequisite to detecting important differences between human and animal sources of NO$_3^-$ in mixed land-use watersheds.

**Overall Importance of NO$_3^-$ Isotopes versus Inorganic Chemical Tracers**

One of the goals of this study was to document the relative importance of NO$_3^-$ stable isotopes and inorganic chemistry as discriminators of land-use and pollutant sources in mixed land-use watersheds. Initial discriminant analysis using NO$_3^-$ stable isotopes showed that land-uses and pollutant sources could successfully be discriminated 70.0% and 76.8% of the time at baseflow and peakflow, respectively. The use of inorganic chemical tracers showed more improved discrimination among sites and sources, with correct classification rates of 97.5% and 93.2% at baseflow and peakflow, respectively. When isotopic and chemical tracers were combined in a full model, NO$_3^-$ stable isotopes were considered significant variables, but their overall importance as discriminators of land-use and pollutant sources diminished (see Tables 6.3 and 6.5). For the most part, Na$^+$ and Ca$^{2+}$ concentrations were more important discriminators of watersheds with different land-uses and geology than NO$_3^-$ stable isotopes in the combined model. Furthermore, there was little difference in correct classification
between the model with only inorganic chemistry and the full model using NO$_3^-$ stable isotopes and inorganic chemistry at baseflow (see Table 6.3) and peakflow (see Table 6.5).

Despite the fact that inorganic chemistry provided better discriminatory power over NO$_3^-$ stable isotopes, there are several important factors that should be outlined. NO$_3^-$ stable isotopes provide explicit information on NO$_3^-$ sources (see Kendall, 1998), while inorganic chemical tracers capture only some aspects of NO$_3^-$ sources (e.g. manure, sewage, septic), but also capture differences in geology, soils, and vegetation that may or may not directly be related to NO$_3^-$ sources. If the goal of the study is to discriminate land-use only, then sampling inorganic tracers may be sufficient. However, if the goal is to make inferences regarding NO$_3^-$ sources from different land-uses in mixed land-use watersheds, then NO$_3^-$ isotopes would be an important addition. Overall, the combined information from the two types of tracers in this study allowed inferences to be made regarding differences in NO$_3^-$ sources, land-use, and geology that were not possible using either type of tracer alone.

**CONCLUSIONS**

The results of this study showed that the decision to use isotopes, chemistry, and microbial indicators to differentiate NO$_3^-$ from different land-uses and pollutant sources should be based on specific watershed conditions and the desired outcomes of the study. If the goal is to make inferences regarding principal NO$_3^-$ sources, then NO$_3^-$ stable isotopes may be sufficient. NO$_3^-$ stable isotopes captured basic differences in principal NO$_3^-$ sources from forested land-uses and sewage, although isotopic overlap among developed land-uses presented some problems. On the other hand, if the goal is to account for potentially important differences in land-use and geology that cannot necessarily be captured using NO$_3^-$ isotopes alone, then inorganic chemical tracers may represent a powerful addition. In the case of Spring Creek, inorganic chemical tracers clearly enabled discrimination between sandstone and carbonate geology, but the results may not be generalizable to watersheds in other physiographic regions. Finally, the lack of significant sources of human *E. coli* in the Spring Creek watershed limited the
effectiveness of microbial indicator variables to detect human versus animal sources of NO$_3^-$ pollution. Future studies looking to test the utility of these indicator variables should identify watersheds where significant sources of human and animal bacteria exist.
REFERENCES


Douglas, T.A., C.P. Chamberlain, and J.D. Blum. 2002. Land use and geologic controls on the major elemental and isotopic ($\delta^{15}$N and $^{87}$Sr/$^{86}$Sr) geochemistry of the Connecticut River watershed, USA. *Chemical Geology*. 189: 19-34.


Figure 6.1: $\delta^{15}$N and $\delta^{18}$O in NO$_3^-$ can be used to discriminate among different sources of NO$_3^-$ in streams (modified from Kendall, 1998).
Figure 6.2: Map showing sampling sites and subwatershed boundaries within the study portion of the Spring Creek watershed.
Figure 6.3: Baseflow plot showing results of forward stepwise discriminant analysis of stable isotopes and NO$_3^-$ concentrations in the Spring Creek watershed. The graph was generated by plotting the first discriminant function (accounting for 98.3% of the variance) against the second discriminant function (accounting for 1.7% of the variance). Significant variables included in the analysis were NO$_3^-$ (mg L$^{-1}$) and $\delta^{15}$N-NO$_3^-$ (‰). Table 6.5 reports correlations between the significant discriminatory variables and the two discriminant functions. Relevant statistics also are summarized in Table 6.5.
Figure 6.4: Peakflow plot showing results of forward stepwise discriminant analysis of stable isotopes and NO$_3^-$ concentrations in the Spring Creek watershed. The graph was generated by plotting the first discriminant function (accounting for 91.5% of the variance) against the second discriminant function (accounting for 7.9% of the variance). Significant variables included in the analysis were NO$_3^-$ (mg L$^{-1}$), $\delta^{15}$N-NO$_3^-$ (‰), and $\delta^{18}$O-NO$_3^-$ (‰). Table 6.7 reports correlations between the significant discriminatory variables and the two discriminant functions. Relevant statistics also are summarized in Table 6.7.
Figure 6.5: Seasonal changes in baseflow levels of $\delta^{15}$N-NO$_3^-$ (a) and $\delta^{18}$O-NO$_3^-$ (b) during the 2005 sampling period for all five Spring Creek subwatersheds the sewage treatment plant, and mean precipitation signatures for each event (see Table 6.2 for the number of precipitation samples).
Figure 6.6: (a) Plot of $\delta^{18}$O-NO$_3^-$ (%) versus NO$_3^-$ (mg L$^{-1}$) and (b) $\delta^{15}$N-NO$_3^-$ (%) versus NO$_3^-$ (mg L$^{-1}$) for all sites within the Spring Creek watershed. The solid black line shows a best-fit log-linear regression model to the data (relevant statistics summarized in each plot). The dashed black line represents a hypothetical mixing curve between two sources (see Burg and Heaton, 1998): (1) a source mixture with high NO$_3^-$ concentration ($C_H$) and associated $\delta^{15}$N-NO$_3^-$ isotopic signature ($\delta_H$), and (2) a source mixture with low NO$_3^-$ concentration ($C_L$) and associated $\delta^{15}$N-NO$_3^-$ isotopic signature ($\delta_L$). End-member concentrations and isotopic signatures used to create the mixing curves are summarized in each plot.
Figure 6.7: Baseflow plot showing results of forward stepwise discriminant analysis of inorganic chemical tracers in the Spring Creek watershed. The graph was generated by plotting the first discriminant function (accounting for 48.4% of the variance) against the second discriminant function (accounting for 42.1% of the variance). Significant variables included in the analysis were pH, conductivity (µS cm⁻¹), NO₃⁻ (mg L⁻¹), SO₄²⁻ (mg L⁻¹), Cl⁻ (mg L⁻¹), Na⁺ (mg L⁻¹), Ca²⁺ (mg L⁻¹), and SiO₂ (mg L⁻¹). Table 6.5 reports correlations between the significant discriminatory variables and the two discriminant functions. Relevant statistics also are summarized in Table 6.3.
Figure 6.8: Peakflow plot showing results of forward stepwise discriminant analysis of inorganic chemical tracers in the Spring Creek watershed. The graph was generated by plotting the first discriminant function (accounting for 66.8% of the variance) against the second discriminant function (accounting for 23.0% of the variance). Significant variables included in the analysis were pH, NO₃⁻ (mg L⁻¹), SO₄²⁻ (mg L⁻¹), Cl⁻ (mg L⁻¹), Na⁺ (mg L⁻¹), Ca²⁺ (mg L⁻¹), and SiO₂ (mg L⁻¹). Table 6.7 reports correlations between the significant discriminatory variables and the two discriminant functions. Relevant statistics also are summarized in Table 6.5.
Figure 6.9: Baseflow plot showing results of forward stepwise discriminant analysis of inorganic chemistry, stable isotopes, and microbial indicators in the Spring Creek watershed. The graph was generated by plotting the first discriminant function (accounting for 48.2% of the variance) against the second discriminant function (accounting for 44.5% of the variance). Significant variables included in the analysis were conductivity (µS cm⁻¹), NO₃⁻ (mg L⁻¹), SO₄²⁻ (mg L⁻¹), Na⁺ (mg L⁻¹), Ca²⁺ (mg L⁻¹), SiO₂ (mg L⁻¹), and δ¹⁵N-NO₃ (‰). Table 6.5 reports correlations between the significant discriminatory variables and the two discriminant functions. Relevant statistics also are summarized in Table 6.3.
Figure 6.10: Peakflow plot showing results of forward stepwise discriminant analysis of inorganic chemistry, stable isotopes, and microbial indicators in the Spring Creek watershed. The graph was generated by plotting the first discriminant function (accounting for 64.0% of the variance) against the second discriminant function (accounting for 25.0% of the variance). Significant variables included in the analysis were pH, Cl\(^{-}\) (mg L\(^{-1}\)), NO\(_3\)\(^{-}\) (mg L\(^{-1}\)), SO\(_4\)\(^{2-}\) (mg L\(^{-1}\)), Ca\(^{2+}\) (mg L\(^{-1}\)), SiO\(_2\) (mg L\(^{-1}\)), $\delta^{15}$N-NO\(_3\)\(^{-}\) (‰), and $\delta^{18}$O-NO\(_3\)\(^{-}\) (‰). Table 6.7 reports correlations between the significant discriminatory variables and the two discriminant functions. Relevant statistics also are summarized in Table 6.5.
Table 6.1: Information on sampling sites, predominant land-use classification, watershed area (km²), land-use distribution based on data from Landsat Thematic Mapper, and percent geology.

<table>
<thead>
<tr>
<th>Site Name (Abbreviation)</th>
<th>Site Description</th>
<th>Watershed Area (km²)</th>
<th>Land-Use (% Area)</th>
<th>Geology (% Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galbraith Gap Run (GGR)</td>
<td>Forested</td>
<td>13</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Cedar Run (CRL)</td>
<td>Agricultural</td>
<td>45</td>
<td>20</td>
<td>74</td>
</tr>
<tr>
<td>Thompson Run (THR)</td>
<td>Urban</td>
<td>11</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>Spring Creek – Houserville (SPH)</td>
<td>Mixed Land-Use</td>
<td>150</td>
<td>36</td>
<td>37</td>
</tr>
<tr>
<td>University Area Joint Authority (UAJA)</td>
<td>Sewage Treatment Plant</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Spring Creek Rock Road (SPR)</td>
<td>Mixed Land-Use</td>
<td>201</td>
<td>31</td>
<td>44</td>
</tr>
<tr>
<td>Thompson Spring (TS)</td>
<td>Groundwater Spring</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Bathgate Spring (BS)</td>
<td>Groundwater Spring</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

For. = Forested
Ag. = Agricultural
Dev. = Developed
Carb. = Carbonate
Sand. = Sandstone

Table 6.2: Information on start and end times, antecedent precipitation (past month), observed precipitation type, precipitation amount, and storm characterization for the six storms sampled during 2005.

<table>
<thead>
<tr>
<th>Storm Number</th>
<th>Start Time (EST)</th>
<th>End Time (EST)</th>
<th>Antecedent Precipitation – Past Month (cm)</th>
<th>Precipitation</th>
<th>Type of Precipitation</th>
<th>Amount (cm)</th>
<th>Mean Intensity (cm hr⁻¹)</th>
<th>Number of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>03-23-05 5:00 AM</td>
<td>03-23-05 3:00 PM</td>
<td>11.0</td>
<td>Rain</td>
<td>1.27</td>
<td>0.18</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>03-27-05 7:00 PM</td>
<td>03-29-05 3:00 AM</td>
<td>12.7</td>
<td>Rain / Snow</td>
<td>4.57</td>
<td>0.30</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>07-05-05 7:00 PM</td>
<td>07-05-05 8:00 PM</td>
<td>4.4</td>
<td>Rain</td>
<td>1.07</td>
<td>1.07</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>08-31-05 3:00 AM</td>
<td>08-31-05 4:00 AM</td>
<td>4.6</td>
<td>Rain</td>
<td>4.83</td>
<td>4.83</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10-07-05 6:00 AM</td>
<td>10-07-05 7:00 PM</td>
<td>2.3</td>
<td>Rain</td>
<td>7.37</td>
<td>0.79</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>11-16-05 7:00 AM</td>
<td>11-16-05 12:00 PM</td>
<td>8.9</td>
<td>Rain</td>
<td>1.22</td>
<td>0.27</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.3: Baseflow pooled within-groups correlations between significant discriminating variables and standardized canonical discriminant functions for Spring Creek. The percentage of the total explained variation is listed below each function. Bolded values indicate the highest correlation with each function.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Discriment Analysis NO$_3$ and Isotopes Only</th>
<th>Discriment Analysis with Chemistry Only</th>
<th>Discriment Analysis Isotopes with Chemistry and E. coli Indicators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Function 1 (98.3%) Function 2 (1.7%)</td>
<td>Function 1 (48.4%) Function 2 (42.1%)</td>
<td>Function 1 (48.2%) Function 2 (44.5%)</td>
</tr>
<tr>
<td>NO$_3$</td>
<td>+0.564 +0.825</td>
<td>+0.240 +0.447</td>
<td>+0.420 +0.233</td>
</tr>
<tr>
<td>$\delta^{15}$N-NO$_3$</td>
<td>+0.632 -0.775</td>
<td>-- --</td>
<td>+0.399 +0.141</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>-0.199 +0.060</td>
<td>-- --</td>
</tr>
<tr>
<td>Conductivity</td>
<td>-- --</td>
<td>+0.195 +0.608</td>
<td>+0.465 +0.394</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td></td>
<td>+0.411 +0.387</td>
<td>-- --</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>-- --</td>
<td>+0.703 +0.501</td>
<td>+0.838 +0.025</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>-- --</td>
<td>+0.364 +0.420</td>
<td>+0.510 +0.142</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>-- --</td>
<td>-0.344 +0.661</td>
<td>+0.039 +0.732</td>
</tr>
<tr>
<td>SiO$_2$</td>
<td>-- --</td>
<td>+0.112 +0.136</td>
<td>+0.165 +0.056</td>
</tr>
</tbody>
</table>

Wilks' Lambda: 0.038 0.000 0.000
$\chi^2$: 112.516 308.892 299.995
Degrees of Freedom: 12.000 48.000 42.000
Significance (p): <0.001 <0.001 <0.001
ARCC: 70.0% 97.5% 97.5%
Table 6.4: Baseflow mean (n=6) chemical concentrations and isotope signatures for significant variables included in the discriminant analysis in the Spring Creek watershed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Forest</th>
<th>Agriculture</th>
<th>Urban</th>
<th>Mixed Houserville</th>
<th>UAJA Effluent</th>
<th>Mixed Rock Road</th>
<th>Ground Water¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>--</td>
<td>7.5</td>
<td>8.2</td>
<td>8.2</td>
<td>8.3</td>
<td>7.3</td>
<td>8.1</td>
<td>7.5</td>
</tr>
<tr>
<td>Conductivity</td>
<td>µS cm⁻¹</td>
<td>36.5</td>
<td>487.7</td>
<td>637.2</td>
<td>531.6</td>
<td>911.3</td>
<td>641.9</td>
<td>694.5</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>mg L⁻¹</td>
<td>0.7</td>
<td>20.9</td>
<td>19.3</td>
<td>15.4</td>
<td>40.9</td>
<td>24.1</td>
<td>19.8</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>mg L⁻¹</td>
<td>5.8</td>
<td>20.7</td>
<td>22.5</td>
<td>22.9</td>
<td>63.3</td>
<td>37.0</td>
<td>27.2</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>mg L⁻¹</td>
<td>0.8</td>
<td>13.4</td>
<td>72.1</td>
<td>37.5</td>
<td>148.8</td>
<td>70.5</td>
<td>66.1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>mg L⁻¹</td>
<td>2.9</td>
<td>64.3</td>
<td>58.5</td>
<td>58.2</td>
<td>43.3</td>
<td>55.0</td>
<td>60.5</td>
</tr>
<tr>
<td>Na⁺</td>
<td>mg L⁻¹</td>
<td>0.7</td>
<td>4.7</td>
<td>27.3</td>
<td>14.0</td>
<td>100.0</td>
<td>35.4</td>
<td>25.1</td>
</tr>
<tr>
<td>SiO₂</td>
<td>mg L⁻¹</td>
<td>5.3</td>
<td>5.7</td>
<td>7.2</td>
<td>5.7</td>
<td>7.4</td>
<td>5.9</td>
<td>6.9</td>
</tr>
<tr>
<td>δ¹⁸O-H₂O</td>
<td>‰</td>
<td>5.3</td>
<td>5.7</td>
<td>7.2</td>
<td>5.7</td>
<td>7.4</td>
<td>5.9</td>
<td>6.9</td>
</tr>
<tr>
<td>δ¹⁵N-NO₃⁻</td>
<td>‰</td>
<td>1.4</td>
<td>+5.1</td>
<td>+6.9</td>
<td>+5.8</td>
<td>+14.8</td>
<td>+10.9</td>
<td>+5.8</td>
</tr>
<tr>
<td>δ¹⁸O-NO₃⁻</td>
<td>‰</td>
<td>4.9</td>
<td>+3.7</td>
<td>+3.7</td>
<td>+3.5</td>
<td>+2.2</td>
<td>+4.7</td>
<td>+2.4</td>
</tr>
<tr>
<td>E. coli</td>
<td>cfu per 100 mL</td>
<td>163</td>
<td>641</td>
<td>371</td>
<td>269</td>
<td>22</td>
<td>211</td>
<td>458</td>
</tr>
<tr>
<td>Serotyping Index²</td>
<td>unitless</td>
<td>0.79</td>
<td>0.76</td>
<td>0.81</td>
<td>0.77</td>
<td>--</td>
<td>0.81</td>
<td>0.72</td>
</tr>
<tr>
<td>ERIC PCR Index²</td>
<td>unitless</td>
<td>0.76</td>
<td>0.81</td>
<td>0.82</td>
<td>0.85</td>
<td>--</td>
<td>0.82</td>
<td>0.70</td>
</tr>
</tbody>
</table>

¹n=4 for groundwater spring samples
²See methods for interpretation of the Serotyping and ERIC-PCR Indices
Table 6.5: Peakflow pooled within-groups correlations between significant discriminating variables and standardized canonical discriminant functions for Spring Creek. The percentage of the total explained variation is listed below each function. Bolded values indicate the highest correlation(s) with each function.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Discriminant Analysis NO₃⁻ and Isotopes Only</th>
<th>Discriminant Analysis with Chemistry Only</th>
<th>Discriminant Analysis Isotopes with Chemistry and <em>E. coli</em> Indicators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Function 1 91.5%</td>
<td>Function 2 7.9%</td>
<td>Function 1 66.8%</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>+0.775</td>
<td>+0.172</td>
<td>+0.490</td>
</tr>
<tr>
<td>δ¹⁸O-NO₃⁻</td>
<td>-0.318</td>
<td>+0.927</td>
<td>--</td>
</tr>
<tr>
<td>δ¹⁵N-NO₃⁻</td>
<td>+0.422</td>
<td>-0.081</td>
<td>--</td>
</tr>
<tr>
<td>pH</td>
<td>+0.157</td>
<td>+0.729</td>
<td>+0.175</td>
</tr>
<tr>
<td>Na⁺</td>
<td>+0.671</td>
<td>-0.010</td>
<td>--</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>+0.598</td>
<td>+0.223</td>
<td>+0.595</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>+0.536</td>
<td>+0.053</td>
<td>+0.528</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>+0.191</td>
<td>+0.574</td>
<td>+0.207</td>
</tr>
<tr>
<td>SiO₂</td>
<td>+0.319</td>
<td>-0.002</td>
<td>+0.327</td>
</tr>
</tbody>
</table>

Wilks’ Lambda: 0.013 0.000 0.000
χ²: 215.790 494.310 482.222
Degrees of Freedom: 18.000 42.000 48.000
Significance (p): <0.001 <0.001 <0.001
ARCC: 76.8% 93.2% 96.4%
Table 6.6: Peakflow mean (n=6) chemical concentrations and isotope signatures for significant variables included in the discriminant analysis of peakflow samples in the Spring Creek watershed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Forest</th>
<th>Agriculture</th>
<th>Urban</th>
<th>Mixed</th>
<th>Houserville</th>
<th>UAJA</th>
<th>Mixed</th>
<th>Rock Road</th>
<th>Precip.¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>--</td>
<td>7.6</td>
<td>8.0</td>
<td>8.2</td>
<td>7.9</td>
<td>7.3</td>
<td>7.9</td>
<td>7.9</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>Conductivity</td>
<td>µS cm⁻¹</td>
<td>51.7</td>
<td>449.7</td>
<td>139.6</td>
<td>362.7</td>
<td>911.3</td>
<td>419.8</td>
<td>11.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>mg L⁻¹</td>
<td>1.6</td>
<td>17.3</td>
<td>2.9</td>
<td>9.2</td>
<td>40.9</td>
<td>12.6</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>mg L⁻¹</td>
<td>7.7</td>
<td>15.7</td>
<td>8.0</td>
<td>17.2</td>
<td>63.3</td>
<td>20.8</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl⁻</td>
<td>mg L⁻¹</td>
<td>1.3</td>
<td>12.9</td>
<td>18.3</td>
<td>28.9</td>
<td>148.8</td>
<td>37.3</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>mg L⁻¹</td>
<td>5.4</td>
<td>59.5</td>
<td>12.7</td>
<td>37.3</td>
<td>43.3</td>
<td>39.6</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>mg L⁻¹</td>
<td>1.0</td>
<td>4.8</td>
<td>10.6</td>
<td>13.7</td>
<td>100.0</td>
<td>18.8</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SiO₂</td>
<td>mg L⁻¹</td>
<td>4.2</td>
<td>5.3</td>
<td>1.0</td>
<td>4.1</td>
<td>7.4</td>
<td>3.9</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ¹⁸O-H₂O</td>
<td>‰</td>
<td>-8.7</td>
<td>-8.7</td>
<td>-8.5</td>
<td>-8.4</td>
<td>-9.1</td>
<td>-8.6</td>
<td>-8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ¹⁵N-NO₃⁻</td>
<td>‰</td>
<td>+1.3</td>
<td>+5.2</td>
<td>+2.8</td>
<td>+5.4</td>
<td>+14.8</td>
<td>+7.8</td>
<td>+0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ¹⁸O-NO₃⁻</td>
<td>‰</td>
<td>+15.9</td>
<td>+2.9</td>
<td>+30.8</td>
<td>+5.4</td>
<td>+2.2</td>
<td>+5.4</td>
<td>+43.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>cfu per 100 mL</td>
<td>2,723</td>
<td>27,840</td>
<td>13,058</td>
<td>5,618</td>
<td>22</td>
<td>5,645</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotyping Index²</td>
<td>unitless</td>
<td>0.87</td>
<td>0.84</td>
<td>0.87</td>
<td>0.75</td>
<td>--</td>
<td>0.86</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERIC PCR Index²</td>
<td>unitless</td>
<td>0.83</td>
<td>0.84</td>
<td>0.82</td>
<td>0.91</td>
<td>--</td>
<td>0.85</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹n=23 for precipitation samples
²See methods for interpretation of the Serotyping and ERIC-PCR Indices
Table 6.7: Baseflow T-tests for significant differences in $\delta^{15}$N-NO$_3^-$ among the sites sampled in the Spring Creek watershed during six times in 2005. $\delta^{15}$N-NO$_3^-$ signatures (‰) are given adjacent to (or below) the site name. P-values are listed for a two-sided T-test between each of the sites. Bolded values indicate statistical significance at $\alpha = 0.05$. N = 6 samples for all sites except springs, where only 4 samples were collected.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Means</th>
<th>Forested (GGR)</th>
<th>Agricultural (CRL)</th>
<th>Urban (THR)</th>
<th>Springs (Groundwater)</th>
<th>Mixed LU (SPH)</th>
<th>Sewage (UAJA)</th>
<th>Mixed LU (SPR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forested (GGR)</td>
<td>+1.4‰</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agricultural (CRL)</td>
<td>+5.1‰</td>
<td>p &lt; 0.001</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban (THR)</td>
<td>+6.9‰</td>
<td>p = 0.001</td>
<td>p = 0.106</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Springs$^1$ (Groundwater)</td>
<td>+5.5‰</td>
<td>p &lt; 0.001</td>
<td>p = 0.557</td>
<td>p = 0.186</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed LU (SPH)</td>
<td>+5.8‰</td>
<td>p &lt; 0.001</td>
<td>p = 0.331</td>
<td>p = 0.302</td>
<td>p = 0.655</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sewage (UAJA)</td>
<td>+14.8‰</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.001</td>
<td>p = 0.001</td>
<td>p = 0.001</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Mixed LU (SPR)</td>
<td>+10.9‰</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.006</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.035</td>
<td>--</td>
</tr>
</tbody>
</table>

$^1$n=4 for groundwater spring samples
Table 6.8: Baseflow T-tests for significant differences in $\delta^{18}$O-NO$_3^-$ among the sites sampled in the Spring Creek watershed during six times in 2005. $\delta^{18}$O-NO$_3^-$ signatures (‰) are given adjacent to (or below) the site name. P-values are listed for a two-sided T-test between each of the sites. Bolded values indicate statistical significance at $\alpha = 0.05$. N = 6 samples for all sites except springs, where only 4 samples were collected.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Means</th>
<th>Forested (GGR)</th>
<th>Agricultural (CRL)</th>
<th>Urban (THR)</th>
<th>Springs (Groundwater)</th>
<th>Mixed LU (SPH)</th>
<th>Sewage (UAJA)</th>
<th>Mixed LU (SPR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forested (GGR)</td>
<td>+4.9‰</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agricultural (CRL)</td>
<td>+3.7‰</td>
<td>p = 0.580</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban (THR)</td>
<td>+3.7‰</td>
<td>p = 0.583</td>
<td>p = 0.988</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Springs$^1$ (Groundwater)</td>
<td>+2.4‰</td>
<td>p = 0.246</td>
<td>p = 0.154</td>
<td>p = 0.133</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed LU (SPH)</td>
<td>+3.5‰</td>
<td>p = 0.521</td>
<td>p = 0.860</td>
<td>p = 0.843</td>
<td>p = 0.181</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sewage (UAJA)</td>
<td>+2.2‰</td>
<td>p = 0.226</td>
<td>p = 0.162</td>
<td>p = 0.146</td>
<td>p = 0.779</td>
<td>p = 0.196</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed LU (SPR)</td>
<td>+4.7‰</td>
<td>p = 0.945</td>
<td>p = 0.404</td>
<td>p = 0.401</td>
<td>p = 0.056</td>
<td>p = 0.315</td>
<td>p = 0.050</td>
<td>--</td>
</tr>
</tbody>
</table>

$^1$n=4 for groundwater spring samples
Chapter 7

SYNTHESIS

Overview

Minimizing the impacts of excess NO$_3^-$ on aquatic ecosystems, especially within the Chesapeake Bay watershed, requires the development of tools to identify NO$_3^-$ sources. NO$_3^-$ stable isotopes represent a valuable tool for studying NO$_3^-$ pollution because the isotope ratios relate directly to NO$_3^-$ sources and allow investigators to study the fate and transport of NO$_3^-$ in the environment. Unfortunately, efforts to use NO$_3^-$ stable isotopes in mixed land-use watersheds characteristic of those found within the Chesapeake Bay watershed have been difficult due to variations of NO$_3^-$ isotopes in time and space, biogeochemical transformations of NO$_3^-$ isotope source signatures in stream water, and the presence of complicated mixtures of NO$_3^-$ sources from developed land-uses. The five studies in this dissertation addressed opportunities to improve NO$_3^-$ source tracking using stable isotopes in the Spring Creek watershed, a mixed land-use basin located in central Pennsylvania and a tributary to the Chesapeake Bay estuary. Major conclusions from the five studies are summarized, and direct implications for NO$_3^-$ pollution management in the Spring Creek watershed are discussed.

Summary of Major Conclusions

The first study (Chapter 2) was conducted to improve source tracking of water and NO$_3^-$ in precipitation by characterizing variations of isotopes in precipitation, which has not been studied extensively. The study showed that seasonal and within-storm variations of $\delta^{15}$N-NO$_3^-$, $\delta^{18}$O-NO$_3^-$, and $\delta^{18}$O-H$_2$O were significant at a site in central Pennsylvania during 2005. Results indicated that variations of $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in precipitation could be explained using knowledge of oxidant chemistry in the atmosphere. Ozone (O$_3$) oxidation is important during the winter, and this factor was likely responsible for enriched wintertime values of $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ in precipitation. Lower values of $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ during the summer months were
related to ratios of NO$_2$:NO$_x$ and OH oxidation, respectively. Storm tracks were important for explaining variations in NO$_3^-$ isotopes, with cool-sector storms from the E/NE producing low $\delta^{15}$N-NO$_3^-$ and warm-sector storms from the SW/S/SE producing high $\delta^{15}$N-NO$_3^-$. $\delta^{18}$O-H$_2$O isotopes in precipitation also showed significant variations during seasons and storm events. More depleted $\delta^{18}$O-H$_2$O was observed during the winter months and during cool-sector storm events, and this was most likely due to increased fractionation of $\delta^{18}$O-H$_2$O isotopes at cooler temperatures. Progressive depletion of $\delta^{18}$O-H$_2$O isotopes that occurred during storm events was mostly related to cumulative precipitation amounts. The results of this study showed that information about oxidant levels can be useful to predict seasonal and within-storm variations of NO$_3^-$ stable isotopes in precipitation, and that knowledge of storm tracks (warm-sector versus cool-sector) may be important for determining sources of NO$_3^-$ in wet deposition and formation processes that affect H$_2$O.

The assumption that NO$_3^-$ isotopes conserved their original source signatures was tested in the second study (Chapter 3) by assessing NO$_3^-$ isotope transformations, especially denitrification, in losing and gaining stream reaches along the main-stem of Spring Creek within the karst valley. Results showed that minimal NO$_3^-$ isotope transformations recurred in two losing reaches due to efficient denitrification during extreme low flows and elevated stream temperatures. In contrast, two gaining reaches that were affected by inputs from springs and a sewage treatment plant showed larger changes in NO$_3^-$ isotopes, which potentially confounded the ability to utilize isotopes to study in-stream NO$_3^-$ transformations. In general, changes in NO$_3^-$ isotopes appeared to be most affected by new NO$_3^-$ inputs as opposed to biogeochemical fractionations and therefore had minimal impacts on NO$_3^-$ source tracking efforts.

The third study (Chapter 4) was undertaken to better understand NO$_3^-$ sources and flow pathways during storm events in a mixed land-use watershed. Comparisons among sites of the fractions of event NO$_3^-$ and event water at peakflow showed three distinct types of flowpath and NO$_3^-$ source variations: urban, forested uplands, and karst lowlands. Large changes in $\delta^{18}$O-NO$_3^-$ on the urban watershed accompanied high fractions of event NO$_3^-$ and event water and pointed to the importance of overland flow from urban stormwater runoff. On the forested, sandstone watershed, small storms
resulted in significant contributions of NO$_3^-$ from channel precipitation, whereas larger storms following longer dry periods resulted in flushing of recently nitrified NO$_3^-$ via shallow soil subsurface flow pathways. On the karst basins, small changes in NO$_3^-$ isotopes during storms suggested that a mixture of organically-derived and soil-derived NO$_3^-$ was present prior to and during storms at these sites. Furthermore, analysis of event NO$_3^-$ and event water fractions on karst basins showed that old NO$_3^-$ largely accompanied old water from deeper groundwater flow paths during storm events. Overall, this study illustrated for the first time how NO$_3^-$ isotopes and $\delta^{18}O$-$H_2O$ could be combined to show major differences in water and NO$_3^-$ delivery mechanisms between forested uplands and karst valleys, and confirm the dominance of overland flow pathways in urbanizing basins.

Isotopic overlap among organically-derived NO$_3^-$ sources (e.g. sewage and manure) has been problematic in many past NO$_3^-$ source tracking studies, and this challenge was addressed in the fourth study (Chapter 5) by testing whether *E. coli* bacteria could be used to develop indicators of human and animal pollution in stream water within the Spring Creek watershed. Weighted mean indicator variables were developed to handle frequency data generated from ERIC-PCR and serotyping that quantified the strength of matching between unknown stream water isolates and known *E. coli* sources from humans and animals. The results revealed that animal sources of *E. coli* were more likely than human isolates to match stream isolates (roughly 70% vs. 30%) during baseflow and peakflow conditions. Overall, the application of serotyping and ERIC-PCR showed that animal sources of *E. coli* were predominant in the Spring Creek watershed, and these techniques provided confirmatory evidence regarding the effectiveness of wastewater treatment processes in removing potential human sources of contamination.

The final study (Chapter 6) was designed to determine how well land-use and NO$_3^-$ pollutant sources could be discriminated using NO$_3^-$ stable isotopes, inorganic chemistry, and bacterial indicator variables in a mixed land-use setting. Discriminant analysis using NO$_3^-$ stable isotopes alone showed that forested land-use and precipitation could easily be separated from sewage effluent using $\delta^{15}N$-NO$_3^-$ and NO$_3^-$ concentrations; however, discriminating among the urban, agricultural, and mixed land-use watersheds in karst using isotopes alone was more problematic and therefore resulting in overall correct
classifications of only 70% during baseflow and peakflow conditions. Inorganic chemical tracers performed much better than NO$_3^-$ stable isotopes, with overall correct classification rates greater than 90%. Na$^+$ captured the influences of sewage, manure, and road salt pollution and Ca$^{2+}$ accounted for geologic differences between karst and sandstone watersheds. A full discriminant model using NO$_3^-$ isotopes, inorganic chemicals, and bacterial indicators demonstrated that inorganic tracers such as Na$^+$ and Ca$^{2+}$ remained important discriminators of land-use, pollution, and geologic influences while NO$_3^-$ isotopes were relatively less important. Bacterial indicators were not included in the model at all, and this was likely due to the limited influences of human *E. coli* sources within the Spring Creek watershed. Based upon the results of this study, NO$_3^-$ stable isotopes were sufficient for capturing basic differences in principal NO$_3^-$ sources from precipitation, forested land-uses, and sewage, although isotopic overlap among developed land-uses still presented problems. The addition of inorganic chemical tracers clearly enabled discrimination among land-uses and geology that could not necessarily be captured using NO$_3^-$ isotopes alone.

**Implications of Results and Suggestions for Future Research**

The results of this dissertation research have several important implications for the identification of NO$_3^-$ sources and management of NO$_3^-$ pollution within Spring Creek and similar mixed land-use watersheds in the central Appalachian Mountain region of Pennsylvania.

- During storm events the urban watershed generated large volumes of stormwater runoff, which was dominated by NO$_3^-$ from precipitation and wash-off of dry deposition as indicated using $\delta^{18}$O-NO$_3^-$ isotopes. This phenomenon was observed in a watershed where urban land-uses accounted for about 66% of the total land-use. Therefore, $\delta^{18}$O-NO$_3^-$ may represent a useful tracer for identifying stormwater problems in urbanizing basins and also could help to evaluate the effectiveness of best management practices. Future work also should focus on better characterization of the NO$_3^-$ isotope signature of dry deposition.
- NO$_3^-$ flow pathways in agricultural and mixed land-use karst watersheds were significantly dominated by groundwater during baseflow and peakflow conditions, which suggested that a large reservoir of groundwater NO$_3^-$ from human activities was active prior to and during storm events. Furthermore, previous work suggested that local groundwater residence times were on the order of 6 to 7 years. Therefore,
future management of NO$_3^-$ pollution in Spring Creek and similar mixed land-use karst watersheds should consider methods that prevent NO$_3^-$ leaching to groundwater. The results of these measures may not be realized in the short-term.

- Sewage treatment increased stream water NO$_3^-$ concentrations downstream of the UAJA effluent discharge and the impacts could easily be detected during baseflow conditions using $\delta^{15}N$-NO$_3^-$. This suggested that $\delta^{15}N$-NO$_3^-$ could be used to test whether enhanced sewage treatment processes reduced the influence of NO$_3^-$ from sewage discharges.

- *E. coli* was not a significant indicator of human and animal sources of pollution in the Spring Creek watershed, and this was mostly due to the limited presence of septic systems and efficient treatment of *E. coli* in sewage at the UAJA treatment facility. Bacterial indicator variables still may be useful in future studies of NO$_3^-$ pollution in mixed land-use watersheds, and future studies should consider testing these indicators in settings where a more significant contribution of human *E. coli* could be expected such as from failing septic systems or poor sewage treatment. Furthermore, library size and representativeness should be enhanced to improve the development of these indicator variables.

- In the Ridge and Valley physiographic region, differences in geology and land-use within Spring Creek were partially captured using NO$_3^-$ stable isotopes in stream water. Improved discrimination was provided by Na$^+$, which captured the influences of sewage, manure, and road salt pollution and Ca$^{2+}$, which accounted for geologic differences between karst and sandstone watersheds. Combining these tracers in similar mixed land-use watersheds within the central Appalachian Mountain region of Pennsylvania may be useful in future studies of land-use and geologic impacts on NO$_3^-$ pollution.

Overall, NO$_3^-$ stable isotopes were useful for tracing sources, transformations, and flow pathways of NO$_3^-$ during baseflow and peakflow conditions, and the addition of water isotopes and inorganic chemical tracers showed promise as useful tools for further constraining important influences of mixed geology and land-use on NO$_3^-$ sources in central Pennsylvania watersheds.
APPENDICES

FIELD, SITE, AND HYDROLOGICAL DATA
Appendix A.1: Meteorological, chemical, and isotopic data collected for precipitation study.

<table>
<thead>
<tr>
<th>Date (2005)</th>
<th>Precip (mm)</th>
<th>Air Temp (°C)</th>
<th>Cond (µS cm⁻¹)</th>
<th>pH</th>
<th>NO₃⁻</th>
<th>SO₄²⁻</th>
<th>Cl⁻</th>
<th>Na⁺</th>
<th>Ca²⁺</th>
<th>δ¹⁸O-NO₃⁻</th>
<th>δ¹⁵N-NO₃⁻</th>
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</table>

Storm Number 1: March 23, 2005

This storm developed along a frontal boundary in the panhandle of Oklahoma on Monday, March 21. It developed rapidly and moved into the Tennessee Valley on Tuesday March 22 (Figure 2.4a). Precipitation began falling as rain in State College around 5:00 AM on Wednesday March 23 in response to the approaching low-pressure system. At around 1:00 PM on March 23, the precipitation changed to a mixture of snow and rain and continued at varying rates throughout the afternoon hours. A total of 1.27 cm of precipitation was recorded when the precipitation ended around 3:00 PM. Two rainfall samples and one melted snow sample were collected during the storm (Table 2.1). The storm system took a path to the south of State College, and thus the region remained entrenched in the cool-sector of the storm for the entire duration of the event.

Storm Number 2: March 27-29, 2005

The storm that occurred on March 27-29 in State College, PA followed directly on the heels of the storm that occurred on March 23. This particular storm was a much larger storm than its predecessor. It had its origins in the Pacific Ocean on Thursday March 24, crossed through California and entered into the Colorado Rocky Mountain Range on Friday March 25. By Saturday March 26, the low-pressure system dropped into Texas and approached the Gulf Coast where it began to intensify rapidly along a stationary frontal boundary. On Sunday March 27, the storm was tracking north-northeast through the Tennessee Valley and approached the panhandle of Virginia by 7:00 AM on Monday March 28 (Figure 2.4b). Precipitation began falling as rain in State College around 7:00 PM on March 27 well in advance of the storm and continued at varying intensities through 2:00 AM on March 28. During the early morning hours on March 28, the parent low-pressure system transferred its energy to a coastal low-pressure system, which underwent further cyclogenesis off the coast of Delaware. Rain began falling again in State College at around 8:00 AM on March 28 and continued through 3:00 AM on March 29. At this point, the low-pressure system was located off the coast of New England with the flow of air and moisture into State College out of the north and
east. A total of 4.57 cm of rain was recorded from this storm, and eight precipitation samples were collected (Table 2.1). Like the previous storm on March 23, the track of this storm center was well to the south of State College, which as a result remained in the cool-sector of the storm for duration of the event.

**Storm Number 3: July 5, 2005**

The storm event on July 5 occurred in response to an advancing cold-front that originated in the northern Great Plains on July 3 and by July 5 was positioned in the lower Great Lakes (Figure 2.4c). Strong thunderstorms, with individual cells moving northeast along a line that extended from West Virginia into central New York, moved through State College around 7:00 PM on July 5. Intense heavy rain fell in State College for about an hour, which resulted in 1.07 cm of rainfall. Despite the large amount of rainfall, the intense rainfall rates caused short-circuiting of the funnel and tube connections in the precipitation sampler, and only two rainfall samples were collected (Table 2.1). The precipitation formed ahead of the frontal boundary due to the forcing of colder air over the warm air mass that was in place. Thus, the entire rain event occurred while State College was within the warm-sector of the storm.

**Storm Number 4: August 31, 2005**

On Monday August 29, Hurricane Katrina made landfall on the Louisiana-Mississippi coastline. The storm became a tropical depression on August 30 and moved due north into Tennessee and Kentucky. By August 31, the low-pressure system was centered in northwest Pennsylvania near Erie, PA with a cold front trailing the southern end of the storm (Figure 2.4d). A line of very intense thunderstorms formed along this frontal boundary during the early morning hours on August 31 and moved rapidly northeastward through State College, PA. These storms lasted only about 45 minutes, but produced 4.83 cm of rainfall in that short time period. Like the previous storm, rainfall rates were extremely intense and caused problems with the precipitation sampler. Only one integrated rain sample was recovered from this event (Table 2.1). Overall, the precipitation fell in the warm-sector of the storm, which was ahead of the approaching cold front from the west.
**Storm Number 5: October 7, 2005**

Tropical Storm Tammy made landfall along the Atlantic Coast near Jacksonville, Florida on Wednesday October 5. The storm weakened to a tropical depression and then moved into southern Georgia on October 6. The moisture from Tammy was funneled northward along a slow moving frontal boundary that was aligned north to south from eastern Ohio to the panhandle of Florida (Figure 2.4e). Rain began falling around 6:00 AM on Friday October 7 in response to the approaching front and moisture from Tammy. Rain continued throughout the daytime hours and ended around 7:00 PM. Rainfall totaled about 7.67 cm, and fell in the warm-sector of the storm while the front was positioned to the west of State College. The moderate intensity of the storm permitted the collection of five rainfall samples from this event (Table 2.1).

**Storm Number 6: November 16, 2005**

The final storm that was sampled during 2005 occurred in response to a cold front that approached State College from the west. The front trailed a low-pressure system that moved northward through the Great Lakes on Wednesday November 16 (Figure 2.4f). Rain fell ahead of the frontal boundary and lasted from 7:00 AM through 12:00 PM. Rainfall occurred in the warm-sector of the storm ahead of the cold front. The total rainfall was 1.22 cm, and three rainfall samples were collected (Table 2.1).
Appendix A.3: Detailed descriptions of stream reaches used in Chapter 3 (NO$_3^-$ Isotope Transformations).

**Reach 1**
Reach 1 was about 2.7 km in length and began at a major spring located approximately 1 km upstream of the Boalsburg Military Museum (Site 1.1, Figure 3.2b). The reach contained 4 sampling sites and was terminated just upstream of the confluence with Cedar Run in Oak Hall, PA (Site 1.4, Figure 3.2b). Specific site locations are detailed below:

- Site 1.1 was located approximately 25 m downstream of the confluence between a small spring and the main stem of Spring Creek. The spring emerges just behind the car wash located along South Atherton Street (Business Route 322) in Boalsburg, and then flows underneath the roadway into Spring Creek.
- Site 1.2 was located about 50 m downstream of the stone arch bridge over Spring Creek in the Boalsburg Military Museum.
- Site 1.3 was located about 50 m upstream of the Mary Elizabeth Street bridge in Boalsburg.
- Site 1.4 was located just upstream of the Linden Hall Road bridge over Spring Creek in Oak Hall, PA. A stream gauging station developed by the ClearWater Conservancy is located at the sampling site.

**Reach 2**
Reach 2 began downstream of the confluence with Cedar Run (Site 2.1, Figure 3.2b) and was about 5.3 km in length. It had 4 sampling sites and extended to a point just upstream of the confluence with Slab Cabin Run (Site 2.4, Figure 3.2b). Specific site locations are detailed below:

- Site 2.1 was located just downstream of the Boalsburg Road bridge over Spring Creek and was adjacent to the Oak Hall Quarry property.
- Site 2.2 was located along Boalsburg Road approximately 0.6 miles upstream of the intersection with East Branch Road. A large gravel pulloff is located on the right-hand side of the road. From the pulloff, a narrow walking trail can be taken to Spring Creek where the stream samples were collected.
- Site 2.3 was located just upstream of the picnic area in Spring Creek Park. The park was accessed from the Houserville Road entrance in Houserville, PA.
- Site 2.4 was located on Spring Creek just upstream of the confluence with Slab Cabin Run. The site was accessed using a walking path that begins adjacent to the bridge over Slab Cabin Run along Puddintown Road.
Reach 3

Reach 3 began just below the confluence with Slab Cabin Run (Site 3.1, Figure 3.2b) and extended 3.4 km to a site located just upstream of the University Area Joint Authority (UAJA) Wastewater Treatment Plant effluent discharge (Site 3.3, Figure 3.2b). Specific site locations are detailed below:

- Site 3.1 was located on Spring Creek just downstream of the confluence with Slab Cabin Run. The site was accessed using a walking path that begins adjacent to the bridge over Slab Cabin Run along Puddintown Road.
- Site 3.2 was located just downstream of the Trout Road bridge over Spring Creek. The USGS Stream Gauging Station in Houserville (01546400) is located at this bridge.
- Site 3.3 was located just upstream of the UAJA effluent discharge into Spring Creek.

Reach 4

Reach 4 began just downstream of UAJA (Site 4.1, Figure 3.2b), and it was 2.1 km in length, ending at the green bridge at Rock Road (Site 4.3, Figure 3.2b). Reaches 3 and 4 had only 3 sampling sites each. Specific site locations are detailed below:

- Site 4.1 was located just downstream of the UAJA effluent discharge into Spring Creek.
- Site 4.2 was located approximately 0.9 miles downstream of the UAJA effluent discharge point. The site was accessed from a gravel parking area along Rock Road, which was located about 0.4 miles (650 m) downstream of the I-99 overpass.
- Site 4.3 was located about 20 m upstream of the blue steel bridge over Spring Creek, which permits access to the Rockview Penitentiary property. The blue bridge is located about 0.5 miles downstream of Site 4.2 in a large gravel parking area that can be accessed from Rock Road just before it makes a hard bend to the left. A small run-of-the-river dam is located just downstream of the bridge.
Appendix A.4: Streamflow, physical, chemical, and isotopic data used in Chapter 3 (NO₃ Isotope Transformations).

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<th>Cond. (µS cm⁻¹)</th>
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<th>Concentration (mg L⁻¹)</th>
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Appendix A.5: Location information and photographs of sites sampled in the Spring Creek watershed during 2005.

Galbraith Gap Run (Forested Watershed)
Galbraith Gap Run was sampled adjacent to the Rothrock State Forest parking area off of Bear Meadows Road, which is about 0.5 miles up from the Tussey Mountain Ski Area. The access point for the parking area is located just before the first bridge over Galbraith Gap Run on Bear Meadows Road.

Cedar Run (Agricultural Watershed)
Cedar Run was sampled at the ClearWater Conservancy monitoring location, which is about 200 meters upstream of the confluence with Spring Creek in Oak Hall, PA. The site is located on private property and access is restricted to those working in cooperation with ClearWater Conservancy.

Thompson Run (Urban Watershed)
Thompson Run was sampled about 100 meters upstream of where it flows into the Duck Pond. The Duck Pond can be accessed from Service Road, which is located directly across the street from where Porter Road intersects East College Avenue.

Spring Creek Houserville (Mixed Land-Use Watershed)
Spring Creek at Houserville was located just downstream of the Trout Road bridge over Spring Creek. The USGS Stream Gauging Station in Houserville (01546400) is located at this bridge.

University Area Joint Authority (Sewage Effluent)
UAJA sewage effluent was sampled directly from the outfall discharge.

Spring Creek Rock Road (Mixed Land-Use Watershed)
Spring Creek Rock Road was sampled just upstream of the blue steel bridge along Rock Road, which provides access into Rockview Penitentiary property. The site was located about 20 m upstream of the bridge. The blue bridge is located about 0.5 miles downstream of Site 4.2 in a large gravel parking area that can be accessed from Rock Road just before it makes a hard bend to the left towards the airport. A small run-of-the-river dam is located just downstream of the bridge.
Baseflow and stormflow photos from: (a) the forested watershed (Galbraith Gap Run), (b) the agricultural watershed (Cedar Run), (c) the urban watershed (Thompson Run), (d) the mixed land-use watershed (Spring Creek at Houserville), and (e) the mixed land-use watershed (Spring Creek at Rock Road).
University Area Joint Authority outfall on Spring Creek (1.4 km downstream of Spring Creek at Houserville; 2.2 km upstream of Spring Creek at Rock Road). Mean daily discharge of sewage effluent is approximately 5 MGD, or 0.22 m$^3$ s$^{-1}$, which increases flow in Spring Creek by approximately 25% during baseflow conditions.
Examples of stormwater response on the urban watershed (Thompson Run) following an intense rainfall event (storm occurred on 11-16-06 – not during study period). Flows increased by almost two orders of magnitude and resulted in extreme flooding in and around State College borough. (a) taken just downstream of the Duck Pond (established sampling location was upstream of the Duck Pond). (b) taken 0.2 km downstream of Duck Pond along East College Avenue. The stream rose by more than 10 feet, and flooded the parking lot of a car dealership.
Appendix A.6: Information on sampling procedures and representative storm hydrograph for all six storms sampled in 2005.

The timing of peakflow sampling was determined using information from real-time streamflow data and staff gauges. Real-time streamflow data from the USGS gauge at Houserville (01546400) were used to guide peakflow sampling on the two mainstem Spring Creek sites, while periodic staff gauge monitoring was used to aid peakflow sampling on Galbraith Gap Run and Cedar Run. Peakflows on the urban watershed (Thompson Run) often were very large, and precluded the installation and utility of a staff gauge at that site. Therefore, peakflow samples at Thompson Run were taken shortly after the occurrence of peak intensity rainfall during the course of a storm event.

Stream water samples were collected for stable isotopes (NO$_3^-$ and H$_2$O) and inorganic chemistry. Most stream samples for NO$_3^-$ stable isotope analysis were collected in 5-L polypropylene carboys, however 20-L carboys were used to accommodate low stream water NO$_3^-$ concentrations in the forested watershed (Galbraith Gap Run). Each carboy was rinsed with 10% hydrochloric acid (HCl), and was then rinsed three times with deionized water prior to sample collection. Samples for inorganic water chemistry were collected in 1-L high-density polyethylene (HDPE) bottles that had been washed in phosphate-free soap, rinsed and soaked in a 20% HCl bath, and then rinsed with deionized water.

Streamflow rates were recorded for all baseflow and peakflow samples. Continuous streamflow data were available for three of the five stream sampling sites used in this study. The USGS operates a real-time stream gauge at the Houserville sampling site (01546400), and 15-minute streamflow data were available at this site. Streamflow rates at Cedar Run and Thompson Run were monitored continuously by the ClearWater Conservancy, a local watershed advocacy group active within the Spring Creek watershed. These data were collected on a 20-minute basis. For Galbraith Gap Run, streamflow was calculated using a simple staff-discharge relationship developed for the site during the study period in 2005. Baseflow rates for Spring Creek at Rock Road were estimated by summing flows at the UAJA sewage treatment plant with flows at the upstream site at Houserville. During baseflow, the only major water input between Houserville and Rock Road was the sewage effluent discharge. Peakflow rates for Rock Road were estimated using 15-minute streamflow data from the upstream site at Houserville (3.6 km upstream from Rock Road) and a downstream USGS gauge at Axemann (9.6 km downstream from Rock Road).
Streamflow hydrograph for the 2005 study period recorded at the USGS gauge in Houserville, Pennsylvania (01546400). Streamflow data represent 15-minute averages, and are reported in m³ s⁻¹. The red circles indicate dates when peakflow samples were collected.
Appendix A.7a: Relevant physical, chemical, and stable isotope data for baseflow, peakflow, and precipitation samples collected during the period March 21-23, 2005. Information on a snow sample (SNOW) also is provided.

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Appendix A.7b: Relevant physical, chemical, and stable isotope data for baseflow, peakflow, and precipitation samples collected during the period March 26-29, 2005.

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Appendix A.7c: Relevant physical, chemical, and stable isotope data for baseflow, peakflow, and precipitation samples collected on July 5, 2005.

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Appendix A.7d: Relevant physical, chemical, and stable isotope data for baseflow, peakflow, and precipitation samples collected during the period August 29-31, 2005.

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Appendix A.7e: Relevant physical, chemical, and stable isotope data for baseflow, peakflow, and precipitation samples collected during the period October 6-7, 2005.

<table>
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<th>Conditions</th>
<th>Site</th>
<th>Date / Time</th>
<th>Sample Conditions</th>
<th>Selected Stream Chemistry (mg L⁻¹)</th>
<th>Stable Isotopes (%)</th>
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<tr>
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<td>Flow (m³ s⁻¹)</td>
<td>Cond (µS cm⁻¹)</td>
<td>pH</td>
<td>Temp (°C)</td>
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Appendix A.7f: Relevant physical, chemical, and stable isotope data for baseflow, peakflow, and precipitation samples collected during the period November 14-16, 2005. Information on a dry deposition (DRY DEP) sample also is provided.

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<th>Sample Conditions</th>
<th>Selected Stream Chemistry (mg L⁻¹)</th>
<th>Stable Isotopes (%)</th>
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</thead>
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<td>NO₃⁻ 0.7 SO₄²⁻ 5.8 Cl⁻ 1.0 Na⁺ 1.0 Ca²⁺ 3.2 SiO₂ 5.3</td>
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<tr>
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<td>NO₃⁻ 19.6 SO₄²⁻ 18.1 Cl⁻ 13.7 Na⁺ 4.8 Ca²⁺ 64.0 SiO₂ 5.9</td>
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<td>Flow (m³ s⁻¹) 0.23 Cond (µS cm⁻¹) 638 pH 8.3 Temp (°C) 15.3</td>
<td>NO₃⁻ 18.5 SO₄²⁻ 19.5 Cl⁻ 53.3 Na⁺ 19.8 Ca²⁺ 59.0 SiO₂ 7.0</td>
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<tr>
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<td>SPH</td>
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<td>NO₃⁻ 21.2 SO₄²⁻ 20.2 Cl⁻ 46.0 Na⁺ 17.2 Ca²⁺ 61.0 SiO₂ 7.0</td>
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<td>NO₃⁻ 1.1 SO₄²⁻ 7.9 Cl⁻ 1.7 Na⁺ 1.4 Ca²⁺ 5.1 SiO₂ 4.8</td>
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Appendix A.8: *E. coli* counts (cfu per 100 mL) used in Chapter 5.

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<th>Storm 3 7/5 to 7/5</th>
<th>Storm 4 8/29 to 8/31</th>
<th>Storm 5 10/6 to 10/7</th>
<th>Storm 6 11/14 to 11/16</th>
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Appendix A.9: ERIC-PCR gel images used in Chapter 5 – see attached Powerpoint file named “Appendix_A9_(ERIC-PCR_Images.ppt”.

Appendix A.10: Serotyping data and calculations used in Chapter 5 – see attached Excel file named “Appendix_A10_(Serotyping_Data)”.

Vita
Anthony Robert Buda

EDUCATION

Ph.D., Forest Resources (Forest Hydrology) 2007
School of Forest Resources
Pennsylvania State University
Dissertation: Tracing stream nitrate in a central Pennsylvania mixed land-use basin using stable isotopes, bacteria, and inorganic chemicals

M.S., Forest Resources with Option in Watershed Stewardship 2000
School of Forest Resources
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Thesis: The potential effects of changes in precipitation and temperature on acidic wet deposition in central Pennsylvania

B.S., Environmental Science, cum laude with Departmental Honors 1998
Department of Geological and Environmental Science
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Thesis: A longitudinal and temporal characterization of Penns Creek: physical and chemical parameters and potential effects of a limestone quarry

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Postdoctoral Research Associate (Research Hydrologist) Start: May 2007
USDA Agricultural Research Service (ARS)
Pasture Systems and Watershed Management Research Unit

Graduate Research Assistant January 2003 – May 2007
School of Forest Resources, Penn State University

Environmental Scientist June 2001 – December 2002
Interstate Commission on the Potomac River Basin (ICPRB)

SELECTED PUBLICATIONS


