Paradigm Shift: Does River Metabolism Mask the Isotopic Signal of Nitrate Sources?

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.
Abstract

Nitrate (NO₃⁻) is the most ubiquitous contaminant in surface and groundwaters in Canada. Synthetic fertilizer application and manure production in intensive agricultural areas contribute large quantities of NO₃⁻ to the landscape with a proportion seasonally lost to groundwaters and streams. Elevated concentrations of NO₃⁻ in freshwater systems can result in problems for drinking water supplies and aquatic ecosystem health. The Grand River is the largest Canadian river draining to Lake Erie and the catchment’s land-use is predominantly agricultural (~80%). It receives NO₃⁻ inputs from point (WWTPs) and non-point (agricultural manure and fertilizer) sources.

Isotopes of NO₃⁻ are commonly used in ecosystem studies to apportion sources (e.g. manure, septic systems, wastewater treatment plant effluent and synthetic fertilizers) and to determine the important NO₃⁻ transformation processes (nitrification and denitrification). For decades, several assumptions have governed these studies such as: 1) δ¹⁸O-NO₃⁻ from nitrification can be predicted using the 2:1 rule (two O in NO₃⁻ come from H₂O and one O from O₂), 2) NO₃⁻ isotopes indicate denitrification in freshwater environments when elevated in a 2:1 ratio for δ¹⁵N: δ¹⁸O, and 3) The δ¹⁵N- and δ¹⁸O of NO₃⁻ are conservative in oxic environments and thus if δ¹⁸O-NO₃⁻ is not elevated, the δ¹⁵N-NO₃⁻ can be used for source apportionment.

This research indicates that these assumptions may not always be correct. The overall objectives of this thesis are to improve the use of NO₃⁻ isotopes for source apportionment in rivers and streams and if the isotopes cannot be used to separate sources then can a mechanistic model be used to estimate rates of N transformation processes that can ultimately help to determine the fate of NO₃⁻ in rivers.

Nitrate isotope data from the Grand River shows no clear denitrification line. A seasonal trend is only observed in δ¹⁵N-NO₃⁻ (high in the summer, low in the spring and fall), not in δ¹⁸O-NO₃⁻. Incubation experiments conducted using two sites on the Grand
River with different source inputs demonstrate that the δ¹⁵N- and δ¹⁸O-NO₃⁻ are not conservative and cannot be used to indicate denitrification or to discern source inputs of NO₃⁻. The NO₃⁻ isotopes changed over time even when NO₃⁻ concentrations did not. Results from an in-river experiment were consistent with incubations and confirmed that in a highly productive river, such as the Grand River, source apportionment is difficult as internal N recycling can be rapid, and the effect on the isotopic signal of NO₃⁻ cannot be ignored.

Isotopic O-exchange between nitrite (NO₂⁻) and water (H₂O) during nitrification is a mechanism that can alter the δ¹⁸O-NO₃⁻ signal from nitrification. This study found considerable amounts of O-exchange (40-100%) occurring at both sample sites in all incubation experiments under nitrifying conditions indicating that the δ¹⁸O-NO₃⁻ is “reset” toward the δ¹⁸O-H₂O value of the water medium.

The δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ cannot be used as conservative tracers in the river. However, a mechanistic model was developed using these isotopes to explain the results from incubation experiments and include the effects of O-exchange and large kinetic ¹⁸O isotope effects on the δ¹⁸O-NO₃⁻ during nitrification. With this model, gross rates of the N cycling processes nitrification, denitrification, mineralization and uptake were obtained in order to describe the biogeochemical cycling of N in the Grand River. This model helps to determine the variability in N cycling between sites and at different times of the year.
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Chapter 1 - Introduction

1.1 Introduction

Nitrogen (N) is naturally abundant on Earth’s surface and vital for living organisms. However, few organisms (e.g. N fixing bacteria) can fix inert N\textsubscript{2} into forms of N such as ammonium (NH\textsubscript{4}\textsuperscript{+}), nitrite (NO\textsubscript{2}\textsuperscript{-}) and nitrate (NO\textsubscript{3}\textsuperscript{-}) that can be utilized by other organisms. Prior to human alteration of biogeochemical cycles, biological N\textsubscript{2} fixation (110 Tg N per year) served as the primary source of terrestrial reactive N (NO\textsubscript{3}\textsuperscript{-}, NO\textsubscript{x}, NH\textsubscript{4}\textsuperscript{+}, N\textsubscript{2}O). It was not until the intensification of human activities in the 20\textsuperscript{th} century that energy production and intensive agriculture resulted in the fixation of an additional ~150 Tg N per year by 1990 (Galloway & Cowling, 2002). The introduction of synthetic N fertilizers has supported population growth and the associated food demand; however, doubling the N loading globally has come at a cost to the environment and to human health (Galloway, 1998; Gruber & Galloway, 2008). Excess N not utilized by crops or microorganisms enters the environment in various forms. In aquatic ecosystems, it enters mainly in the highly mobile form, NO\textsubscript{3}\textsuperscript{-} (DeSimone & Howes, 1998). Anthropogenic N loading from the post-WWII industrialization of agriculture is thought to be the major cause of NO\textsubscript{3}\textsuperscript{-} pollution (Galloway, 1998; Novotny, 1999). NO\textsubscript{3}\textsuperscript{-} is now a ubiquitous contaminant in soil, surface water and groundwater globally and is a concern for drinking water quality and aquatic health especially in agriculture-dominated watersheds (Almasri, 2007; Power & Schepers, 1989).

Prolific inorganic N fertilizer and manure application in agricultural landscapes has led to large contributions of NO\textsubscript{3}\textsuperscript{-} as a non-point source contaminant into aquatic ecosystems (Charbonneau & Kondolf, 1993; Hill, 1983; Kato et al. 2009). The portion that does not run-off into receiving waters or is taken up by plant biota will leach through the soil into groundwater (Lang et al. 2013; Liu et al. 2005; Vitousek et al. 1997) or be retained as part of the residual soil nitrogen (RSN) (Janzen et al., 2003). The RSN can produce a legacy...
N effect and a time lag of many decades before it enters the groundwater system and is eventually discharged to surface water (Schlesinger, 2009; Sebilo et al. 2013; Worrall et al. 2015). Van Meter & Basu (2015) recently produced a watershed model that quantifies catchment-scale time estimates of this time lag N based on soil nutrient accumulations and groundwater travel time distributions. Although this model will be an important contribution to future watershed management, the legacy effect remains a direct risk to groundwater quality, aquatic health and human health. Also of concern is point source NO₃⁻ which enters aquatic environments via wastewater treatment plants (WWTPs) in urban centers (MacQuarrie et al. 2001).

The Ontario Safe Drinking Water Act, (O. Reg. 169/03, Sched. 2; O. Reg. 268/03, s. 1; O. Reg. 248/06, s. 2; O. Reg. 242/07, s. 1.) has set the maximum acceptable concentration of NO₃⁻ as 10 mg N/L as concentrations exceeding this limit may result in harmful health effects to humans (Knobeloch et al. 2000; Townsend et al. 2003). NO₃⁻ has been linked with methemoglobinemia, more commonly referred to as “blue-baby syndrome” in infants. This disease can result when NO₂⁻ (a precursor of NO₃⁻) oxidizes the ferrous iron in the hemoglobin of an infant’s red blood cell into the ferric form to produce methemoglobin (Fan et al. 1987; Fewtrell, 2004). Methemoglobin is unable to transport oxygen leading to a blue-grey colour in the infant’s skin and can lead to death. NO₃⁻ has also been implicated in other health outcomes such as digestive cancers, hypertension and reproductive problems (De Roos et al. 2003); however, the results have not been very conclusive (Powlson et al. 2008). Elevated NO₃⁻ can also be toxic to aquatic organisms (Camargo & Alonso, 2006) and the Canadian Council of Ministers of the Environment has set a guideline of 3.0 mg N/L for the protection of aquatic life (CCME, 2012). Source water protection and aquatic ecosystem monitoring are both needed to minimize NO₃⁻ concentrations in order to ensure a potable water supply and healthy aquatic ecosystems.
1.2 Eutrophication

Eutrophication is the greatest water quality concern for aquatic ecosystems in the world (Smith & Schindler, 2009; Smith, 2003). The results of excessive nutrient inputs of phosphorus (P) and/or N, eutrophication can lead to the growth of large, potentially toxic algae blooms in aquatic ecosystems (Schindler, 2006; Smith et al. 2006). Eutrophication has unfavorable and potentially detrimental effects that include: depletion of dissolved oxygen (DO) and the formation of harmful algal blooms (HABs). HABs may produce toxins that have adverse health effects, and also decrease aesthetic value of the water bodies and produce undesirable odours (Smith et al. 1998).

Many studies have concluded that P is the limiting nutrient in all but a few freshwater bodies and that the control of P inputs will reduce algal blooms and improve water quality (Schindler, 2012; Schindler et al. 2008; Schindler & Fee, 1974). In some cases, maintaining water quality and healthy ecosystems in coastal environments has been found to be dependent on managing nitrogen inputs (Howarth & Marino, 2006; Ryther & Dunstan, 1971); however, this issue is still up for debate and continues to be the focus of much research (Schindler et al. 2008; Smith & Schindler, 2009).

Agriculture is a major non-point source of N and P to aquatic environments and it is important that fertilizer and manure application is well understood and adequately managed to avoid eutrophication in both freshwater and marine environments. Cyanobacteria sometimes called blue-green algae are frequently found in eutrophic freshwater systems. These organisms can potentially produce the harmful toxins and some are capable of fixing N.
1.3 The Nitrogen Cycle

Inert nitrogen (N₂) in the atmosphere must be fixed to reactive N (NO₃, NH₃, organic N) before it is available to organisms. N₂ comprises 80% of Earth’s atmosphere but is only biologically available to N₂-fixing bacteria and archaea that reduce it to NH₄⁺ and incorporate it into biomass (Thamdrup, 2012). Other means of N-fixation include fixation by lightning and human activities (energy production, fertilizer production, etc.). Most prokaryotes and all eukaryotes require fixed N to live. Uptake is the incorporation (uptake) of NH₄⁺, NO₂⁻ or NO₃⁻ into organisms. NO₃⁻ and NO₂⁻ are initially reduced by nitrite and nitrate reductases into NH₄⁺ and then assimilated into organic matter. During decomposition, soil organic N is mineralized into NH₄⁺ and NH₄⁺ can be volatilized to the atmosphere or assimilated.

The transformation of NO₃⁻ in the environment is broadly governed by two biological reactions: nitrification and denitrification (Figure 1.1). Nitrification produces NO₃⁻ through the oxidation of ammonium (NH₄⁺), while denitrification removes NO₃⁻ by reduction.

![Diagram of the nitrogen cycle](image)

Figure 1.1: Nitrification and denitrification in the nitrogen cycle.
1.3.1 Nitrification

Nitrification is a two-step biologically mediated reaction where NH_4^+ is aerobically oxidized into NO_3^-.

The microorganisms responsible for the conversion of NH_4^+ into NO_3^- are chemolithoautotrophic bacteria and archaea. In the first step of NH_4^+ oxidation an intermediate hydroxylamine (NH_2OH) is produced. A small fraction of NH_2OH is converted directly to N_2O and the rest into NO_2^- (Stein & Yung, 2003). Classically, the genera responsible for this conversion are the ammonium oxidizing bacteria (AOB), *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus*. Recent research has found that in some soils (mainly acidic) and rivers ammonium oxidizing archaea (AOA) of the phylum Crenarchaeota were the dominant nitrifying organisms present (Gubry-Rangin et al. 2010; Leininger et al. 2006; Sonthiphand et al., 2013; Treusch et al. 2005). Nitrite oxidizing bacteria in the genera *Nitrobacter* or *Nitrospira* are responsible for the second oxidation step of NO_2^- into NO_3^- (Bartosch et al. 2002). Evidence from anoxic oceanic waters and deep sediments has shown NH_4^+ can be oxidized anaerobically to N_2 in the presence of nitrate and nitrite, a process termed anammox (Thamdrup, 2012). Recently, complete nitrification by a bacterium of the genus *Nitrospira* has been identified: this has been termed comammox (Daims et al., 2015).

Environmental controls on rates of nitrification include oxygen availability, pH and temperature. NH_4^+ will be rapidly nitrified into NO_3^- in soils, river sediments or biofilm with sufficient amounts of NH_3/NH_4^+, CO_2 and O_2. The optimal pH conditions for nitrifying bacteria has been found to be neutral to slightly alkaline; however, nitrification has been confirmed at pH extremes of 3 (De Boer & Kowalchuk, 2001) and 10 (Sorokin et al. 2001). Temperatures are environment specific but are generally between 25 and 30˚C (Norton, 2008). Savard et al. (2007) conducted a groundwater study in an agricultural region and found evidence of nitrification in the winter where average temperatures were as low as -7.9˚C. Depending on environmental controls and substrate availability, NO_3^- may accumulate to high concentrations in the soils or groundwater or be discharged to streams (Norton, 2008). NO_2^- has also been found to accumulate under low oxygen conditions (≤1.5
mg/L), a pH between 8-9 and temperature of approximately 30˚C (Bae et al. 2001; Ruiz et al. 2003).

1.3.2 Denitrification

Denitrification is the sequential dissimilatory reduction of NO$_3^-$ and NO$_2^-$ into inert nitrogen (N$_2$) with gaseous oxides (NO, N$_2$O) as intermediates (Figure 1.1). The microbes that mediate this process are mostly heterotrophic facultative anaerobic bacteria from the genus *Pseudomonas* but also include: *Achromobacter*, *Bacillus*, *Micrococcus* and some fungi (Knowles, 1982; Thamdrup, 2012). Under anaerobic conditions denitrifying microbes use NO$_2^-$ and NO$_3^-$ as the terminal electron acceptor in the oxidation of a variety of compounds from organic carbon and Fe(II) to sulfur (Batchelor & Lawrence, 1978; Stein et al. 2003; Straub et al. 1996). Denitrification is temperature dependent and occurs optimally at temperatures between 25 to 30˚C (Dawson & Murphy, 1972; Saad & Conrad, 1993).

The various oxidation and reduction reactions that drive the N cycle are performed by a diverse suite of microbial species and interactions (Falkowski, Fenchel, & Delong, 2008). Nitrification and denitrification are very closely linked in agricultural soils and aquatic environments. While nitrification provides a readily available source of NO$_3^-$, denitrification is responsible for the loss of fixed N to the atmosphere. Rates of N processes are important for mitigating N pollution in drinking water and aquatic ecosystem health.

1.4 Sources and Fate of Nitrogen in the Environment

There are three main sources of NO$_3^-$ to agriculture-dominated catchments; the atmosphere, nitrified inorganic fertilizer and nitrified manure/sewage. Lightning and biological fixation from bacteria and archaea are natural processes that derive reactive N from the atmosphere. Vehicle emissions and industrial sources have increased atmospheric NO$_3^-$ input globally (Elliot et al. 2007; Galloway & Cowling, 2002).
In aquatic ecosystems, N fertilizer and wastewater from farms, livestock facilities and septic systems/WWTPs are major non-point and point sources. Inorganic N fertilizer is produced using the Haber-Bosch process in which N from the air is combined with hydrogen gas to synthesize NH$_4^+$. This process was developed in 1909 and revolutionized the agricultural industry doubling fertilizer production every 8 years from 1950 to 1973 (Erisman et al. 2008; Galloway, 1998).

Organic fertilizer, such as manure and effluent discharge from septic systems/WWTPs, provides an organic source of N that can be mineralized to NH$_4^+$. Manure holdings must be emptied at least once a year and land applications in Ontario are regulated under the Nutrient Management Act and O. Reg. 267. Maximum application rate is calculated based on soil N concentrations. The time of year fertilizers are applied, crop demand, and climate conditions (dry versus wet year) determine the amount of NO$_3^-$ that enters groundwater or surface water.

1.5 Stable Isotopes of Nitrate and the Nitrogen Cycle

The stable isotopes of NO$_3^-$ can be useful for tracing the sources of NO$_3^-$ in an aquatic system and help us better understand N-cycling processes (Figure 1.2). The dual isotope approach to NO$_3^-$ studies uses the stable isotopes of both nitrogen ($^{15}$N/$^{14}$N) and oxygen ($^{18}$O/$^{16}$O) atoms in NO$_3^-$. Stable isotope ratios are typically reported as a delta (δ) with units in per mil (‰) in accordance to (Equation 1.1).

$$
\delta = \left( \frac{R_{Sample}}{R_{Standard}} - 1 \right) \times 1000
$$

Equation 1.1

Where $R_{Sample}$ is the isotopic ratio of the sample and $R_{Standard}$ is the isotopic ratio of the standard. The isotopic standard for nitrogen is N$_2$ in atmospheric air ($^{15}$N/$^{14}$N = 0.0036765) and the isotopic standard for oxygen is the Vienna Standard Mean Ocean Water (VSMOW) ($^{18}$O/$^{16}$O = 0.0020052).
Isotopes of NO$_3^-$ have slightly different chemical and physical properties due to their difference in mass. Those differences are large enough for biological and chemical processes or reactions to fractionate (Kendall 1998). Fractionation is when the relative proportions of the isotopes in a reaction change because one isotope reacts faster than another (Kendall 1998; Peterson & Fry, 1987). Typically the lighter isotope reacts faster and is incorporated into the product faster than the heavier isotope which accumulates in the residual reactants (Kendall 1998; Kendall & Aravena, 2000). These reactions are viewed as either reversible equilibrium reactions or irreversible unidirectional kinetic reactions. Fractionation factors associated with biological or chemical reactions are necessary to trace sources of NO$_3^-$. In kinetic reactions, isotope fractionation factors ($\alpha$) can be defined as:

$$\alpha_{p-s} = \frac{R_p}{R_s}$$

Equation 1.2

Where $R_p$ and $R_s$ are the ratios of the heavy isotope relative to the light isotope in the product and substrate (reactant). An isotope enrichment factor, $\varepsilon$ can be defined as:

$$\varepsilon_{p-s} = (\alpha - 1)$$

Equation 1.3

Biological reactions such as nitrification and denitrification are unidirectional and there is potential for fractionation at every step (Kendall 1998). The step in the reaction where most of the fractionation occurs is the rate-determining or slowest step. The rate-limiting step involves a large pool of substrate where the amount of material used is small in comparison to the total pool (Kendall 1998). Estimation of fractionation factors in natural systems can be very complex because the overall fractionation of the reaction is dependent on many environmental conditions (number and type of intermediate steps, species of organism, pH, etc.).
1.5.1 Nitrogen Fixation

Nitrogen fixation is defined as the process that converts inert $N_2$ from the atmosphere into reactive $N$ species ($NO_3^-$, $NH_4^+$, organic $N$). $\delta^{15}N$ of $N_2$ in the atmosphere is used as the standard and is $0\%$. For that reason, the $\delta^{15}N$ of $N$ produced by $N$-fixers like blue-green algae or bacteria associated with legumes is very low ($-3-0\%$) (Fogel & Cifuentes, 1993; Gu & Alexander, 1993) (Figure 1.2). When looking at aquatic systems, low $\delta^{15}N$ can be an indicator of $N$-fixing cyanobacteria responsible for some HABs around the world (Anderson et al., 2002; Kendall, 1998).

1.5.2 Uptake

Uptake is the incorporation of $NH_4^+$, $NO_3^-$ and $NO_2^-$ into organisms. $N$ isotopic fractionations during uptake depend on the organism involved and the environmental conditions (Kendall, 1998). Assimilation of $NH_4^+$ or $NO_3^-$ exhibits fractionations of $N$ within the range of -1.6 to $1\%$ for microorganisms in soil and a range of -2.2 to $0.5\%$ for vascular plants (Kendall, 1998) (Figure 1.2). A larger range in fractionation factors exist in aquatic environments mainly because of the potential for more isotopic effects (Kendall, 1998). In addition, it can also be the result of greater variability of $NH_4^+$ and $NO_3^-$ concentrations and lower growth rates (Fogel & Cifuentes, 1993; Kendall, 1998). Fogel & Cifuentes (1993) compiled measured values from field and laboratory studies of aquatic algae and found the range in fractionation to be $-27$ to $0\%$ for the incorporation of $NH_4^+$ and $NO_3^-$. From cultures of marine phytoplankton, Granger et al. (2010) found the O isotope effect associated with $NO_3^-$ uptake by marine phytoplankton was very similar to the $N$ isotope effects such that $N$ and $O$ isotopes of $NO_3^-$ fractionated to the same extent (1:1), with values ranging between -8\% and -5\%. 
1.5.3 Mineralization

In the context of the N cycle, mineralization is the conversion of organic N into NH$_4^+$ during decomposition of plants and animals. This process causes only a small fractionation in $\delta^{15}$N (±1‰) between soil organic matter and soil ammonium (Kendall, 1998; Kendall & Aravena, 2000) (Figure 1.2). Production of NO$_3^-$ can be an indicator of mineralization when NH$_4^+$ is subsequently nitrified.

1.5.4 Nitrification

Nitrification is the oxidation of NH$_4^+$ into NO$_3^-$ by chemolithoautotrophic bacteria and archaea (Figure 1.2). Two oxidation steps characterize this process: the oxidation of NH$_4^+$ and the oxidation of NO$_2^-$ . NH$_4^+$ is oxidized into NH$_2$OH and the source of this oxygen molecule is from O$_2$ (Equation 1.4) (Hollocher et al., 1981). An oxygen molecule from H$_2$O contributes next to the oxidation of NH$_2$OH into NO$_2^-$ (Equation 1.5) with the potential for further oxygen exchange between NO$_2^-$ and H$_2$O (Andersson & Hooper, 1983; Casciotti, 2015; Casciotti et al., 2010; Hollocher et al., 1981). Lastly, NO$_2^-$ is oxidized into NO$_3^-$ in the final step and it is a second water molecule that supplies an oxygen atom (Hollocher, 1984) (Equation 1.6).

$$NH_4^+ + O_2 \rightarrow NH_2OH$$  \hfill (Equation 1.4)

$$NH_2OH + H_2O \rightarrow NO_2^- \leftrightarrow H_2O$$  \hfill (Equation 1.5)

$$NO_2^- + H_2O \rightarrow NO_3^-$$  \hfill (Equation 1.6)

The fractionations in $\delta^{15}$N that result from this process depend on the rate-limiting step. This is most often the slow oxidation of NH$_4^+$ by Nitrosomonas in soils (Equation 1.4) because the second oxidation step (NO$_2^-$ to NO$_3^-$) has been found to be rapid in the environment.
The amount of substrate present dictates the extent of the fractionation and systems that are N-limited have low fractionations. The total isotope effect has been found to range between -12‰ and -29‰ in soils (Shearer & Kohl, 1992).

Casciotti et al. (2010) estimated the fractionation of δ¹⁸O in nitrification with cultures of marine bacteria where the combined isotope effect for O₂ and H₂O in the first oxidation step of NH₄⁺ into NO₃⁻ was found to range between -38‰ to -18‰. The isotope effect of the final oxidation step of NO₂⁻ into NO₃⁻ was found to range between -25‰ to -9‰ (Buchwald & Casciotti, 2010). Prior to these studies, nitrification fractionations of δ¹⁸O were not taken into account and values of δ¹⁸O-NO₃⁻ were calculated based on the 2:1 model (Equation 1.7) (Snider et al., 2010).

\[
\delta^{18}O\text{-NO}_{3}(\text{relVSOW}) = \left[\frac{1}{3} R_{O_2} + \frac{2}{3} R_{H_2O}\right] R_{VSOW} - 1
\]

Equation 1.7

In aquatic systems microbial nitrification is an important NO₃⁻ production process. Knowing the stable isotope fractionations has been useful for understanding the transformations of NO₃⁻ and has often been used to determine the sources of NO₃⁻ in impacted aquatic ecosystems (Anisfeld et al., 2007; Mayer et al., 2002).

1.5.5 Volatilization

In the N cycle, volatilization refers to the loss of NH₃ gas to the atmosphere. Increase in temperature and/or soil pH has been found to increase volatilization when there is adequate soil moisture (Ernst & Massey, 1960). In agricultural areas most manure N used as fertilizer is in the form of urea, which is hydrolyzed to NH₄⁺. This increases the pH and favours the loss of NH₃ gas via volatilization. The NH₃ gas produced has a δ¹⁵N lower than the remaining NH₄⁺ because the lighter isotopes react faster (Kendall, 1998). Under optimal conditions this process may also occur in streams where there are large N inputs from runoff or wastewater treatment plant effluent (Cejudo et al., 2014; Hood et al., 2013).
actual fractionation of $\delta^{15}$N for volatilization is dependent on temperature and other factors. Residual $\delta^{15}$N-$\text{NH}_4^+$ has been found to have values >20‰ after volatilization (Kendall, 1998) (Figure 1.2).

1.5.6 Denitrification

Denitrification is the reduction of NO$_3^-$ under low oxygen conditions to N$_2$ with obligate intermediates N$_2$O and NO (Figure 1.2). Redox conditions establish the availability of oxidized material that organisms use as electron acceptors in the order of O$_2$, NO$_3^-$ and SO$_4^-$ (Kendall, 1998). When conditions are favourable, denitrification will occur in soil, groundwater and streams. It is a very important process for mitigating levels of N in impacted aquatic systems. The $\delta^{15}$N of the residual nitrate increases exponentially as the reaction proceeds and NO$_3^-$ concentrations decrease. Based on experiments conducted with pure cultures of denitrifying bacteria and other soil studies, the fractionations that have been recorded for $\delta^{15}$N range from -40‰ to -5‰ (Kendall, 1998; Snider et al., 2009). As the reaction proceeds there will also be an increase in $\delta^{18}$O-NO$_3^-$. In marine environments a relationship of 1:1 for $\delta^{18}$O: $\delta^{15}$N has been observed (Granger et al., 2004; D. M. Sigman et al., 2003). Granger et al. (2008) found the combined isotope effects of N and O among four facultative anaerobic denitrifiers to range between -5‰ to -25‰. In freshwater environments, an approximate 1:2 relationship has been observed reliably in groundwater studies (Aravena et al., 1998; Cey et al., 1999; Mengis et al., 1999). Using the coupled isotope effects of both $\delta^{15}$N and $\delta^{18}$O has thus become a commonly used tool for identifying denitrification and isolating other biogeochemical processes (Kendall et al., 2007a); however there is still much to learn as recent studies have found that O-exchange between NO$_2$ and H$_2$O may indirectly affect or in-part, control the final isotopic effect on $\delta^{18}$O-NO$_3^-$ during denitrification (Knöller et al., 2011; Snider et al., 2009; Snider et al., 2013).
Figure 1.2: The main N transformation processes in soil, freshwater and groundwater and potential isotopic values and fractionations for NO$_3^-$ sources and N processes (Kendall et al., 2007b) modified from (Xu et al. 2015).

1.6 Nitrate Source Determination: Current Paradigms

Dual isotopes of NO$_3^-$($\delta^{15}$N- and $\delta^{18}$O-NO$_3^-$) have been widely used to apportion sources of NO$_3^-$ in surface water (Anisfeld et al. 2007; Lee et al. 2008; Li et al. 2010; Mayer et al. 2002) and groundwater studies (Aravena et al., 1993; Aravena & Robertson, 1998; Cey et al., 1999; Mengis et al., 1999; Wassenaar, 1995). For the past 20-25 years researchers have used the dual isotope plot (Figure 1.3) where sources of NO$_3^-$ reside within constrained boxes. Values that fall outside the boxes are then thought to result from denitrification and would plot along a 1:1 (Granger et al., 2004; Sigman et al., 2003; Sigman et al., 2005) or 1:2 (Amberger & Schmidt, 1987; Böttcher et al., 1990; Mengis et al., 1999) line for $\delta^{18}$O/$\delta^{15}$N in marine and freshwater environments, respectively.
Sources of NO$_3^-$ have wide ranges in values of $\delta^{18}$O and $\delta^{15}$N. Atmospheric NO$_3^-$ has become a more important source of acidic deposition since stricter regulations on SO$_x$ emissions were introduced (Kendall et al., 2007a). Generally, the $\delta^{15}$N-NO$_3^-$ from wet atmospheric deposition ranges between -15‰ to +15‰ (Kendall et al., 2007a). Less is known about the $\delta^{18}$O and large variability exists in literature values (Kendall, 1998; Kendall et al., 2007a). Compiled values from various studies have been found to range between +25‰ to +115‰ (Kendall, 1998; Michalski et al., 2012). However, values are generally constrained between +40‰ and +80‰ (Figure 1.3) (Kendall, 1998).

Many different N fertilizers, with widely varying compositions are used in agriculture and differentiating between them is important depending on whether they are naturally, synthetically or microbially derived (nitrification). The $\delta^{15}$N of synthetic fertilizer reflects the signal of N$_2$ in the atmosphere and usually resides around 0‰ but can be in the range of -4‰ to +4‰ (Kendall et al., 2007a). Similarly the $\delta^{18}$O reflects atmospheric O$_2$ (+23.5‰) and typically ranges between +17‰ to +25‰ (Kendall & Aravena, 2000; Kendall et al., 2007a; Wassenaar, 1995). Organic fertilizers that include plant composts and liquid and solid animal waste can have $\delta^{15}$N values that range between +2‰ to +30‰, the wide range reflecting the different origins (Kendall et al., 2007a; Wassenaar et al., 2006). Septic waste or N manure held in holding tanks can have even higher values of $\delta^{15}$N due to volatilization of NH$_4^+$ and subsequent oxidation of the residual into NO$_3^-$. The $\delta^{15}$N of microbially derived NO$_3^-$ depends on the pool of NH$_4^+$ available and which step in the process is rate limiting and therefore produces a very wide range of values from -8‰ to +30‰ (Kendall et al., 2007a).
Figure 1.3: Values typical of $\delta^{18}$O- and $\delta^{15}$N-NO$_3^-$ originating or nitrified from various N sources. Atmospheric and fertilizer sources are constrained to the boxes labeled “Atmospheric NO$_3^-$” and “NO$_3^-$ fertilizer”, respectively (Kendall & Aravena, 2000; Kendall et al., 2007b; Wassenaar, 1995). The solid lines represent denitrification and the typical slopes resulting in a marine environment (1:1) or freshwater (1:2) with initial $\delta^{15}$N = +9‰ and $\delta^{18}$O = +17‰. Values produced from nitrification of NH$_4^+$ and organic matter will reside in the box labeled “Nitrified NH$_4^+$” (Kendall et al., 2007b; Wassenaar et al., 2006). Make note that denitrification lines are for illustrative purposes (to show slopes) and are not reflective of source values.
There are several assumptions associated with the source plot (Figure 1.3):

1) $\delta^{18}$O-NO$_3^-$ from nitrification can be predicted using the 2:1 rule (two O in NO$_3^-$ come from H$_2$O and one O from O$_2$)

The first oxygen molecule in the oxidation of NH$_4^+$ comes from O$_2$ and H$_2$O contributes the second and third oxygen molecules to produce NO$_2^-$ and NO$_3^-$ (Andersson & Hooper, 1983; Hollocher et al., 1981; Hollocher, 1984). Based on the initial isotopic value of NO$_3^-$ sources, expected ranges in isotopic values of NO$_3^-$ have been determined for microbial nitrification based on their origin i.e. fertilizer or manure/septic waste. Historically, very few studies measure the actual value of $\delta^{18}$O-NO$_3^-$ and rather determine this value using this 2:1 model (Equation 1.7) (Snider et al., 2010).

2) 

a. Expected $\delta^{18}$O-NO$_3^-$/$\delta^{15}$N-NO$_3^-$ from denitrification is 1:2 for freshwater and 1:1 for marine environments.

For decades it has been known that denitrification causes a predictable increase in the $\delta^{18}$O and $\delta^{15}$N of the residual NO$_3^-$ (Amberger & Schmidt, 1987). Data collected largely from groundwater studies has consistently shown increases of $\delta^{18}$O and $\delta^{15}$N to be in an approximately 1:2 ratio (Aravena et al., 1993; Aravena & Robertson, 1998; Cey et al., 1999; Lehmann et al., 2003; Mengis et al., 1999). It has therefore been generally accepted that natural freshwater data plotting along a slope of 0.5 for $\delta^{18}$O and $\delta^{15}$N indicates denitrification. Findings from culture experiments with marine denitrifiers show a 1:1 relationship of $\delta^{18}$O and $\delta^{15}$N for denitrification (Granger et al., 2004; D. M. Sigman et al., 2003).
b.

i. $\delta^{18}$O-NO$_3^-$ elevated beyond expected ranges of sources at a particular site indicates denitrification.

ii. $\delta^{18}$O-NO$_3^-$ is conservative, and thus if $\delta^{18}$O-NO$_3^-$ is not elevated, then $\delta^{15}$N-NO$_3^-$ can be used for source apportionment

$\delta^{15}$N-NO$_3^-$ is very well understood and a greater abundance of source data exists relative to that for $\delta^{18}$O-NO$_3^-$ (Kendall et al., 2007b); however, when dealing with complex N pools and processes $\delta^{18}$O-NO$_3^-$ isotopes are more indicative of NO$_3^-$ source (Amberger & Schmidt, 1987). This is because NO$_3^-$ produced from an inorganic fertilizer should exclusively reflect $\delta^{18}$O-O$_2$ (+23.5‰) and NO$_3^-$ from nitrification should contain $\delta^{18}$O-H$_2$O (-8 to -10‰) (Amberger & Schmidt, 1987). $\delta^{18}$O-NO$_3^-$ isotopes are therefore conservative and if elevated beyond expected ranges of source values (in conjunction with $\delta^{15}$N-NO$_3^-$) will indicate denitrification. However, if not elevated then $\delta^{15}$N-NO$_3^-$ can be used independently for source apportionment.

1.7 Nitrate Source Determination: Challenging Paradigms

Recent research has found greater complexity in the N cycle than previously thought (Thamdrup, 2012) and discovery of new N processing mechanisms (Casciotti, 2015; Casciotti et al. 2010; Snider et al. 2010; Wunderlich et al. 2013), suggest that the previously listed assumptions may not always hold true. It can be shown that $\delta^{18}$O-NO$_3^-$ is not conservative and is influenced by a number of factors, including rapid abiotic O-exchange and large kinetic isotope effects, varying microbial community composition and changes to diel cycling of dissolved O$_2$ on a temporal or spatial scale.

O-exchange has been found to occur between nitrite (NO$_2^-$) and water (H$_2$O) during nitrification (Andersson et al. 1982). Depending on the relative amount and rate of O-exchange that occurs, the original $\delta^{18}$O-O$_2$ signal may be greatly reduced or eliminated. In
studies that have measured the actual value of δ\(^{18}\)O-NO\(_3^-\), values range between 0‰ and 16‰. These values are lower than those that would result if calculated using the 2:1 model (Equation 1.7) (Snider et al., 2010). The fraction of abiotic O-exchange (fabiotic) was determined for three types of soil NO\(_3^-\) by Snider et al. (2010) in a series of laboratory incubations. They found the three soil types had a different fabiotic from each other and from that which assumed a 2:1 incorporation of oxygen from H\(_2\)O and O\(_2\). In addition, the δ\(^{18}\)O fractionation factors of O\(_2\) and H\(_2\)O uptake during nitrification had never been included in the 2:1 model until Buchwald and Casciotti (2010) and Casciotti et al. (2010) estimated these factors with marine bacteria. If denitrification has occurred, the current model states that δ\(^{18}\)O-NO\(_3^-\) will be elevated beyond an expected range based on known isotope effects (Amberger & Schmidt, 1987). However, it is possible that rapid O-exchange and cycling could result in the loss of the denitrification signal all together (Wunderlich et al., 2013).

Exploring the community composition of nitrifying organisms will provide more information on their role in this complex process (Thamdrup, 2012). Ammonium oxidizing archaea (AOA) rather than ammonium oxidizing bacteria (AOB) have been found in greater abundance in soils (Gubry-Rangin et al. 2010; Leininger et al. 2006) and had the most important role in acidic soils (Zhang et al. 2012). In a freshwater study, Sonthiphand et al. (2013) explored the sediment and water column inside and outside a WWTP effluent plume in the Grand River, Ontario, and found a dominance of AOA outside the plume, AOB within and distinct in-river AOA patterns compared to those in the effluent. This study shows how multifaceted a system can be. Although there is limited literature on how much community composition influences isotopic transformations, these studies emphasize the importance of multidisciplinary research combining microbial, molecular and isotopic techniques.

Venkiteswaran et al. (2015) conducted a whole-river study on the Grand River. Nighttime δ\(^{18}\)O-O\(_2\) values were always higher than daytime values; however, nighttime values did not
always return to the atmospheric equilibrium value for δ¹⁸O-\text{O}_2 of +24.2‰. Diel O\textsubscript{2} values could change the isotopic signal of the resulting NO\textsubscript{3}⁻, however they are also unaccounted for in the 2:1 model. Current δ¹⁸O-NO\textsubscript{3}⁻ research, largely focused on soil, contains many gaps for highly impacted freshwater ecosystems. Years of data collected from the Grand River in southwestern Ontario, Canada, suggest that the δ¹⁸O-NO\textsubscript{3}⁻ signal for denitrification is lost in the river. This information in addition to the recent literature has lead to this research in which data from incubation experiments and river-chamber experiments supports literature suggesting that δ¹⁸O-NO\textsubscript{3}⁻ is not conservative and that other mechanisms play a role in the cycling of NO\textsubscript{3}⁻ and in effect, river metabolism masks NO\textsubscript{3}⁻ sources.

### 1.8 Objectives of this Research

The overall objective of this thesis is to improve the use of NO\textsubscript{3}⁻ isotopes for apportioning sources in rivers. This research will provide a greater understanding of the complexity of within-river N cycling and processing. The study site, the Grand River watershed is dominated by agriculture and NO\textsubscript{3}⁻ pollution is a widespread concern as many communities use the Grand River as their source of drinking water.

The specific objectives of Chapter 2 are to answer the question of whether or not δ¹⁸O-NO\textsubscript{3}⁻ is conservative and can be reliably used for source apportionment of NO\textsubscript{3}⁻. This question is addressed through the examination of historical data collected from the Grand River as well as data generated during three laboratory incubation experiments and two river-chamber experiments. Chapter 3 will examine the mechanisms responsible for changes to nitrate isotopes that contradict current isotope paradigms. A new mathematical model of nitrification and associated isotope fractionations is used to model observed data and more importantly, to determine rates of uptake, incorporation and release of NO\textsubscript{3}⁻. Chapter 4 will conclude the findings of this research and offer a prospective for future research and the use of the stable isotopes of NO\textsubscript{3}⁻ in source apportionment studies.
1.9 Study Site – Grand River, Ontario

The Grand River watershed located in southern Ontario is the largest (6800km²) Canadian river draining to Lake Erie (Figure 1.4). It is 7th order system at the mouth and flows north to south for ~300km with an average annual discharge of ~80m³/s (Venkiteswaran et al., 2015). The land-use is predominantly agriculture (~80%) with five large urban areas (Kitchener, Waterloo, Cambridge, Guelph and Brantford) (Rosamond, 2013). There are 30 wastewater treatment plants discharging to the river and its tributaries. These treatment plants serve >75% of population (950,000) living in the watershed (Venkiteswaran et al., 2015). The geology in the upper section of the river is largely glacial till and the lower section is clay and silt (Gao et al., 2006). High nutrient loading from agriculture and wastewater treatment plants (WWTPs) is a threat to drinking water quality making the Grand River an ideal study site for this research (GRCA, 2014).
Figure 1.4: The Grand River watershed located in southwestern Ontario, Canada is the largest Canadian river discharging to Lake Erie. Urban areas are concentrated at the center of the catchment. WWPT = wastewater treatment plant. Figure modified from Venkiteswaran et al. (2015).
Chapter 2 – Challenging Paradigms: NO₃⁻ Isotopes in the Grand River are not Conservative

2.1 Introduction

Nitrate (NO₃⁻) is one of the most pervasive contaminants in surface and groundwaters in Canada (Power & Schepers, 1989; Ritter et al., 2002) and worldwide. Since the intensification of agricultural practices during the 20th century, synthetic fertilizer application and manure production contribute large quantities of NO₃⁻ to the landscape with a proportion lost to groundwaters and streams depending on season (Galloway & Cowling, 2002). Elevated NO₃⁻ in freshwater systems can result in problems for drinking water supplies and aquatic ecosystem health. Determining the sources of NO₃⁻ to the environment is important for managing impacted systems like the Grand River in southwestern Ontario that, at times, approaches the drinking water limit of 10 mg N/L (Figure 2.1). Concentrations exceeding this limit are most harmful to infants (Fan et al., 1987).

The Grand River supplies drinking water to approximately 650,000 people across Brantford, Waterloo Region, Guelph and Six Nations Territory (GRCA, 2014) and dilutes water from a total of 30 wastewater treatment plants (WWTPs), 6 of which are above Waterloo region and 15 above Brantford. Two of these major WWTPs are located in Kitchener and Waterloo. The Kitchener WWTP underwent system upgrades in 2010 to modify the system in order to nitrify (NH₄⁺ into NO₃⁻) the wastewater before release. Prior to these modifications wastewater was released primarily as NH₄⁺. The combination of point and non-point sources of NO₃⁻ from WWTPs and agricultural runoff from intensive agricultural practices in the area pose many risks to water quality for municipalities that draw water directly from the river. In order to implement best management practices to protect the aquatic system and keep drinking water NO₃⁻ levels to a minimum, it is important for watershed managers to understand whether NO₃⁻ is coming from point or non-point sources.
Figure 2.1: Nitrate in the Grand River from 2005-2013 for three sites: West Montrose, Bridgeport and Blair that are 98km, 119km and 145km from the headwaters, respectively. Blair is downstream from the WWTP outlets of the two largest cities in the watershed, Waterloo and Kitchener. The solid black line represents the 10 mg N/L drinking water limit set by The Ontario Safe Drinking Water Act, (O. Reg. 169/03, Sched. 2; O. Reg. 268/03, s. 1; O. Reg. 248/06, s. 2; O. Reg. 242/07, s. 1.).

Dual isotopes of NO₃⁻ are commonly used in ecosystem studies to apportion sources, e.g., manure, septic systems and synthetic fertilizers (Anisfeld et al., 2007; Aravena et al., 1993; Burns & Kendall, 2002; Cey et al., 1999; Kendall, 1998; Lee et al., 2008; Li et al., 2010; Mayer et al., 2002; Mengis et al., 1999; Pardo et al., 2004; Spoelstra et al., 2001; Xu et al., 2015). For decades, several assumptions have governed these studies, including: 1) δ¹⁸O-NO₃⁻ from nitrification can be predicted using the “1:2 rule” (oxygen incorporation of one O atom from δ¹⁸O-O₂ and two O atoms from δ¹⁸O-H₂O, respectively), 2) δ¹⁸O-NO₃⁻ elevated beyond expected ranges of sources indicate denitrification, 3) both δ¹⁵N-NO₃⁻ and δ¹⁸O-NO₃⁻ are conservative, and thus if δ¹⁸O-NO₃⁻ is not elevated, then δ¹⁵N-NO₃⁻ can be used for
source apportionment, 4) manure/septic and soil sources of NO$_3^-$ are sufficiently separated in $\delta^{15}$N and 5) that biological assimilation of NO$_3^-$ imparts no isotopic fractionation. However, recent research in agricultural catchments suggests that these assumptions may not always be correct (Casciotti et al., 2010; Snider et al., 2010; Wunderlich et al., 2013).

In the past, $\delta^{18}$O-NO$_3^-$ has been used to provide information on NO$_3^-$ sources, based on the fact that industrially produced NO$_3^-$ will have a $\delta^{18}$O signal close to that of atmospheric O$_2$ while nitrified NO$_3^-$ will be much lower due to the much lower value of $\delta^{18}$O of H$_2$O. Snider et al. (2010) assembled field and incubation data of $\delta^{18}$O-NO$_3^-$ produced from nitrification in groundwater, surface water and various soil types. They found that <10% of the assembled $\delta^{18}$O-NO$_3^-$ data fit theoretical values based on the “1:2 rule” for oxygen incorporation during nitrification. Using the “1:2 rule” to calculate $\delta^{18}$O-NO$_3^-$ values is problematic, as it does not take into account potentially large isotope effects or rapid oxygen exchange between $\delta^{18}$O-H$_2$O and $\delta^{18}$O-NO$_2^-$ or changes in the $\delta^{18}$O-O$_2$ during nitrification (Snider et al., 2010; Venkiteswaran et al., 2007). It was not until Casciotti et al. (2010) estimated the combined effect for O$_2$ and H$_2$O incorporation in the initial oxidation steps of nitrification and Buchwald & Casciotti (2010) calculated the isotope effect for the incorporation of H$_2$O in the final oxidation step that kinetic isotope effects could be considered. Researchers have documented O-exchange between H$_2$O and NO$_2^-$ (Andersson & Hooper, 1983; K. Casciotti, 2015; K. L. Casciotti et al., 2010; Martin & Casciotti, 2016; Nishizawa et al., 2016) however the amount or fraction of O-exchange is not well known, especially in aquatic systems. Isotopic exchange causes $\delta^{18}$O-NO$_3^-$ values to be “reset” toward the $\delta^{18}$O-H$_2$O of the medium and this could alter the signal of nitrified sources and mask the denitrification signal.

The $\delta^{18}$O-O$_2$ should also be considered as diel changes in the isotope signal on both a temporal and spatial scale have been reported (Venkiteswaran et al., 2015, Venkiteswaran et al., 2007; Wassenaar et al., 2010). Bacterial biofilms in streams and rivers have been found to play a large role in the function and metabolism of many aquatic ecosystems (Battin et al.,
2016; Findlay, 2010) and understanding the diversity of microorganisms may also influence the nitrification signal.

High abundances of ammonia oxidizing archaea (AOA) rather than ammonia oxidizing bacteria (AOB) have been discovered in some soils (Gubry-Rangin et al., 2010; Jia & Conrad, 2009; Leininger et al., 2006), although it is unknown how the microbial community composition may influence the isotopic signal of NO₃⁻ in the river. In a microbial characterization freshwater study, Sonthiphand et al. (2013) explored the sediment and water column inside and outside a WWTP effluent plume in the Grand River, Ontario. Diverse assemblages of AOA and AOB on a spatial scale were found. AOA and AOB have potentially different ammonia-oxidation pathways (Stahl & de la Torre, 2012; Walker et al., 2010) and this could mean the end-member isotopic signal of NO₃⁻ is different between microbial groups. Research conducted by Nishizawa et al. (2016) found no apparent difference in the isotopic fractionation between AOA and AOB. However, the N and O isotope effects determined for AOA are limited compared to what has been obtained for AOB (Buchwald & Casciotti, 2010; Casciotti et al., 2010). Given that recently discovered comammox bacteria oxidize NH₄⁺ all the way through to NO₃⁻, this further complicates the situation as the final isotopic composition of NO₃⁻ could potentially be altered by these bacteria (Laura Sauder, personal communication April 2017).

Furthermore, recent research identifies the significance of including large isotope effects, rapid O-exchange, variations in microbial community composition and diel δ¹⁸O-O₂ changes when looking at N transformation processes in order to confidently use δ¹⁸O-NO₃⁻ isotopes to identify nitrified sources or denitrification.

The objectives of this chapter are to (1) determine if the (δ¹⁵N)²⁵N- and (δ¹⁸O)¹⁸O-NO₃⁻ are conservative in surface waters unless altered by denitrification, (2) if determined to be conservative, can the isotopes be used to indicate denitrification and sources of NO₃⁻ to aquatic surface water ecosystems and (3) is the interpretation of the isotopic systematics and
N cycling processes consistent for either non-point agriculture and point WWTP input. This study uses δ¹⁸O-H₂O and δ¹⁸O- and δ¹⁵N-NO₃⁻ labeled water in laboratory incubation experiments and in-river chamber experiments to observe changes to δ¹⁸O-NO₃⁻ and δ¹⁵N-NO₃⁻ isotopes with N cycling processes. The goal of the laboratory incubations was to quantify the influence of rock biofilm on NO₃⁻ isotopes and concentrations over time and to ultimately determine the fraction of O-exchange occurring between NO₂⁻ and H₂O during nitrification.

2.2 Study Sites

The Grand River watershed is the study site for this research. Land use in the Grand River watershed is largely agricultural (~80%) with five urban cities located centrally in the watershed (70% of the population) (Figure 2.2) (Rosamond, 2013; Venkiteswaran et al., 2015). The treatment plants serving the five major cities (Brantford, Cambridge, Guelph, Kitchener and Waterloo) handle ~90% of the wastewater for the entire watershed (GRCA, 2014). The sample sites for this study, West Montrose and Blair (Figure 2.2) encompass a ~100km stretch of the Grand River’s 300km length. Depending on river flow, it takes two to three days for water to travel this distance between sample sites (Mark Anderson, personal communication). Diffuse agricultural NO₃⁻ sources enter the river upstream of West Montrose and mainly point source NO₃⁻ from the Kitchener and Waterloo WWTP enters upstream of Blair. Elevated NO₃⁻ concentrations that result from increased loading tend not to decrease between these sites (Venkiteswaran et al., 2015). The surficial geology in these areas is largely glacial till and gravel (Gao et al., 2006). River flow in the Grand varies seasonally with high flow in the spring and fall and low-flow summer conditions of 5 m³/s and 11 m³/s for West Montrose and Blair, respectively (GRCA, 2014).

The sampling sites are located in the Huron Slopes climate zone that receives moisture picked up by winds over Lake Huron (Brown et al., 1980; GRCA, 2014). The moisture condenses and creates a “snow belt” with higher than average rainfall or snow
accumulation in some regions, depending on the year (Brown et al., 1980). Annual precipitation for Waterloo Region typically ranges between 900-920mm (University of Waterloo Weather Station). The Region of Waterloo has experienced both “wet” (defined as above average precipitation) and “dry” (defined as below average precipitation) years over the last decade. The amount of precipitation has a profound effect on nutrient loading to the river and nutrient dynamics such as N transformations (nitrification, denitrification, uptake, volatilization and mineralization) within the river (Cummings, 2015). This study took place in the summer (June-August) and fall months (September-November) of 2014 and 2015. In 2014, according to the University of Waterloo weather station, total precipitation was 949.6mm, ~45mm more than what was estimated for that year (904mm). The average temperature was 24.5°C during the summer (June, July and August) of 2014 and 12.7°C in the fall (September, October, November) of 2014. Total precipitation in 2015 was 814.2mm, ~100mm less than what was estimated for that year (916.3mm). The average temperature was 24.7°C in the summer and 16.4°C in the fall of that year.
Figure 2.2: West Montrose and Blair sampling sites. Modified from Venkiteswaran et al. (2015).
2.3 Methods

2.3.1 Laboratory Incubation Experiments

To conduct the laboratory incubation experiments, one biofilm-covered rock was placed in each of six 2L beakers (Figure 2.3). Beakers contained river water that was spiked with a solution of KNO₃ (USGS 32) with known isotopic values for δ¹⁸O-NO₃⁻ (+25.7‰) and δ¹⁵N-NO₃⁻ (+180‰). Prior to field sampling, an isotopic mass balance (Equation 2.1) was used to determine the amount of KNO₃⁻ solution needed to alter the NO₃⁻ isotopes in the river water to provide the necessary sensitivity to observe isotopic changes over time.

\[
\delta_f = \frac{NO_3(River) \times \delta(River) + NO_3(Standard) \times \delta(Standard)}{NO_3(River) + NO_3(Standard)}
\]

Equation 2.1

Where \(\delta_f\) is the value of either δ¹⁸O-NO₃⁻ or δ¹⁵N-NO₃⁻ after mixing, \(\delta(River)\) and \(\delta(Standard)\) represent the approximate value of either δ¹⁸O-NO₃⁻ or δ¹⁵N-NO₃⁻ at the study site (based on average NO₃⁻ isotope values for this time of year) and the known isotope values of the KNO₃⁻ standard. The concentration of the standard was made to be approximately the same as the river concentration at each site, determined by field measurements from routine field sampling and continuous data provided from a SUNA NO₃⁻ sonde located at Bridgeport, just downstream of West Montrose. \(NO_3(River)\) and \(NO_3(Standard)\) was the mass of NO₃⁻ calculated using the concentrations and volumes of water that would be used in the experiment. The target values after mixing were determined to be approximately +6‰ and +48‰ for δ¹⁸O-NO₃⁻ or δ¹⁵N-NO₃⁻, respectively.

The water was also labeled in four of the six beakers with a distinct water isotope (δ¹⁸O-H₂O) value. Incubations were run in duplicate (Beaker A and B) and designated as a “level” of δ¹⁸O-H₂O: low, medium and high. The low beakers had no spike and therefore had a δ¹⁸O-H₂O typical of the central Grand River, ranging between -9‰ and -11‰ relative to VSMOW (Venkiteswaran et al., 2015). The δ¹⁸O-H₂O spike for medium and high beakers...
was prepared by diluting a 97-atom % $^{18}$O-H$_2$O standard to a 10-atom % solution. A second isotope mass balance was conducted to determine the correct volume of 10-atom % to add to the beakers to reach target values of $+50{\%}$ and $+90{\%}$ for medium and high, respectively.

![Laboratory incubation experiment set-up](image)

Figure 2.3: Laboratory incubation experiment set-up. Six 2L beakers are shown, each containing one biofilm-covered rock and 1.4L of river water spiked with KNO$_3$ of known isotopic value for $\delta^{18}$O-NO$_3^-$ (+25.7‰) and $\delta^{15}$N-NO$_3^-$ (+180‰). Duplicate beakers were labeled according to the $\delta^{18}$O-H$_2$O value of -8‰, +50‰ and +90‰ for Low, Medium and High beakers, respectively.

Two laboratory incubations were initiated on July 7, 2015 and September 2, 2015 with water and biofilm covered rocks from West Montrose. These incubations lasted for 60 and 72 hours, respectively. A third incubation, using water from Blair began on July 29, 2015 and ran for 60 hours. Field sampling took place the morning of each incubation experiment. River temperature and dissolved oxygen (DO) concentration were measured at the site using a handheld HACH 40Qd field meter, and 10L of water was retrieved for the
incubations in plastic carboys. Additional small volume samples were collected for total nitrogen (TN), inorganic N species (NH₄⁺, NO₃⁻ and NO₂⁻) and NO₃⁻ isotopes. Samples for chemical analysis were syringe filtered (0.45µm) into 30 mL HDPE bottles in the field. Filtered samples were also collected for δ¹⁸O-H₂O in 1mL chromatography vials in the field. Six biofilm-covered rocks were collected and stored in plastic containers for transport back to the laboratory. Upon return to the laboratory, samples for chemical analysis were frozen and δ¹⁸O-H₂O samples were stored at room temperature until analysis.

Based on previous calculations, 0.5L of the enriched KNO₃ solution was added to 0.9L of river water yielding a total of 1.4L in each beaker. All three pairs of beakers rested on magnetic stir plates and contained a magnetic stir bar. The purpose of the magnetic stir plates was to simulate river flow and also keep the system well oxygenated. A rock was placed into each beaker, and was positioned against the beaker wall so that flow simulated by the stir bar would create a current above the biomass, similar to that in the river. Following NO₃⁻ isotope enrichment, river ¹⁸O-H₂O was enriched by adding 1.5mL and 2.85mL of 10-atom % ¹⁸O-H₂O into the medium and high beakers, respectively. Two lamps with 630 lumens incandescent bulbs were utilized to simulate daytime light conditions. At sundown, the beakers were wrapped with dark brown felt (“beaker cozies”) that loosely covered the tops to mimic nighttime conditions and were removed at sunrise the following morning (Figure 2.4).
Figure 2.4: Laboratory incubation experiment set-up with six 2L beakers. At sundown “beaker cozies” shown as the brown fabric were used to cover the beakers mimic nighttime conditions.

Beakers were sampled immediately after sufficient time (~2 minutes) was allowed for the sample and spike to mix completely (time 0 \([T_0]\)). Subsequent samples were taken at time \(T_{0.1}\), \(T_{0.5}\), \(T_1\), \(T_2\), \(T_4\), \(T_8\), \(T_{12}\), \(T_{24}\), \(T_{36}\), \(T_{48}\), \(T_{60}\), and \(T_{72}\) hours (West Montrose September 2015 only). Each time the beakers were sampled, temperature and DO were also measured with the same handheld HACH meter used in the field. To prevent cross-contamination between beakers, two 300mL beakers were filled with excess river water and after rinsing probes with DI were used to rinse the probe between sampling events. Beakers used for rinsing the probe were rinsed and refilled with new water after every sampling event. Temperature and DO were consistent for all experiments and over the course of each experiment, temperatures fluctuated between 22°C (night) and 24°C (daytime) and the DO between 5mg/L and 8mg/L, sufficient for nitrification. At each time point samples were
taken from each beaker using a 60mL syringe and filtered to 0.45µm. Two 30mL HDPE bottles were filled at each time point for TN, NO₃⁻, NH₄⁺, and NO₂⁻ analysis. Two 1mL chromatography vials were filled at time points T0.1 and T60 or T72 (West Montrose September 2015) for δ¹⁸O-H₂O and an additional 30mL HDPE bottle was taken for NO₃⁻ isotopes analysis at times: T0.1, T4, T12, T24, T48 and T60 or T72 (West Montrose September 2015) hours. Following sampling at each time point, 30mL samples for chemical analysis were immediately frozen and 1mL vials stored at room temperature until analysis. All samples, including those from initial field sampling were analyzed as soon as possible following the incubations.

Rock samples from the July West Montrose experiment and the July Blair experiment were scraped with a stainless steel spatula followed by a soft brush. Biofilm surface area was determined using a standard weight relationship in which the scraped area was estimated by covering it with aluminum foil that was then weighed. The samples were rinsed with nanofiltered water and poured into 90mL Starplex containers where they were frozen and subsequently then freeze-dried. Sub-samples were taken for isotopic and nutrient analyses.

2.3.2 In-River Chamber experiments

The purpose of the in-river chamber experiments was to observe how river metabolism alters NO₃⁻ isotopes and concentrations over time and to provide in-situ results under conditions comparable to those created in the laboratory incubations. In-river chamber experiments run in duplicate (Chamber A and Chamber B) were initiated at West Montrose on September 27, 2015 (CHWM1) for 24 hours. Prior to the experiment, a KNO₃ solution of known isotope values (δ¹⁸O = +25.7‰ and ¹⁵N = +180‰) was prepared (Equation 2.1) to sufficiently enrich the NO₃⁻ isotopes in the chambers. Target isotope values were calculated to be approximately +12‰ and +65‰ for δ¹⁸O and δ¹⁵N, respectively.
Recirculating chambers as described by Bott et al. (1997) were used to allow containment of the spiked sample and five biofilm-covered rocks (Figure 2.5). The circulation of the 7L sample simulated river flow conditions. Before the chambers were deployed, water samples were collected and filtered to 0.45µm for TN, NO₃⁻, NO₂⁻, NH₄⁺ and NO₃⁻ isotope (δ¹⁸O- and δ¹⁵N-NO₃⁻) and δ¹⁸O-H₂O analysis. Samples for N species and N isotopes were placed on ice in a cooler until they could be taken back to the laboratory and frozen. Temperature and DO were measured in the river with a handheld HACH meter.

Figure 2.5: In-river chamber experiment set-up. Two 7L chambers contained seven biofilm covered rocks and river water spiked with KNO₃⁻ (δ¹⁸O = +25.7‰ and ¹⁵N = +180‰). A pump circulated the water in the chambers to simulate river flow. At sampling time points, the chamber was removed from the water and opened at the top to collect samples.
Five rocks selected from the river bottom were placed in the duplicate chambers along with 2L of KNO₃ solution and approximately 5L of river water to fill the chamber. After the pumps were started and the water sufficiently mixed, the first sample (T0) was taken using a 60mL syringe. Both chambers were then sealed and submerged in the river to maintain constant temperature until the next sampling time point. Samples for TN, NO₃⁻, NO₂⁻, NH₄⁺ and NO₃⁻ isotope (¹⁸O- and ¹⁵N-NO₃⁻) were collected and filtered to 0.45µm at time points: T0, T8, T18, T22, T24 hours. Water isotopes were sampled only at time T0 and T24 hours. Samples were returned to the Environmental Geochemistry Laboratory and frozen immediately. Batteries were checked and/or changed every four hours to ensure continuous flow in the chambers. As a result, DO levels closely reflected the DO in the river, with concentrations ranging between a high of 14 mg/L during the day to a low of 5mg/L at night. This range is typical of diel O₂ fluctuations previously observed at the study location (Venkiteswaran et al., 2007).

The Environmental Geochemistry Lab at the University of Waterloo conducted routine field sampling every two weeks for seven main channel sites on the Grand River (including West Montrose and Blair) that supplemented concentration and isotopic data for this study.

2.3.3 Analytical Methods

Nitrate Concentration

A Dionex ICS-2100 ion chromatograph, with an IonPac AS18 analytical and guard column and AS40 automated sampler was used to analyze water samples for NO₃⁻ concentrations. Samples were corrected to a calibration curve, created by standards run at the same time. The minimum detection limit (MDL) for this analysis was 0.02 mg N/L with a precision of 0.07 mg N/L.
Nitrite Concentration

A Unity Scientific SmartChem 200 (Discrete Analyzer) was used to analyze NO₂⁻ concentrations in water samples based on the method outlined by the USEPA EPA 353.2 Revision 2.0 (1993). NO₂⁻ concentration is measured by diazotizing the sample with sulphanilamide followed by coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly coloured azo dye which is measured colorimetrically at 550 or 529nm. The MDL for this method is 0.01 mg NO₂⁻ N/L with a precision of 5%.

Ammonium Concentration

Total ammonia N (NH₄⁺ + NH₃) concentrations from water samples were analyzed using a Unity Scientific SmartChem 200 (Discrete Analyzer) using the method outlined by the USEPA EPA 350.1 Revision 2.0 (1993). The sample is buffered at a pH of 9.5 with a borate buffer and then distilled into a 2% solution of boric acid. Ammonia reacts with alkaline phenol and hypochlorite to form indophenol blue and the colour is intensified through the addition of sodium nitroprusside and measured colorimetrically at 630nm. The MDL using this method for this method is 0.01 mg NH₄-N/L with a precision of 5%.

Nitrate Isotopes: δ¹⁸O-NO₃⁻ and δ¹⁵N-NO₃⁻

Stable isotopes analysis of NO₃⁻ was conducted in the Environmental Isotope Laboratory (UWEIL) at the University of Waterloo, using the chemical denitrification method (conversion of NO₃⁻ to N₂O) as described by McIlvin and Altabet (2005). All samples were kept frozen prior to analysis. Analysis of δ¹⁸O-N₂O and δ¹⁵N-N₂O was conducted using a Trace Gas – Isoprime, Continuous Flow Stable Isotope Ratio Mass Spectrometer (Thermo Finnigan/Bremen-Germany). The isotopic composition of the original NO₃⁻ is ultimately determined by creating a correction equation using the NO₃⁻ standards (EGC 17, USGS 34 and USGS 35) prepared and analyzed with each sample run. Stable isotope ratios are expressed as delta (δ) and expressed in per mil (‰). The precision for this analysis is ±0.3‰ for δ¹⁵N and ±0.8‰ for δ¹⁸O.
\( \delta^{15}N \) of Rock Biofilm

All dry samples for \( \delta^{15}N \) were analyzed on a Delta Plus, Continuous Flow Stable Isotope Ratio Mass Spectrometer (Thermo Finnigan Bremen-Germany) coupled to a Carlo Erba Elemental Analyzer (CHNS-O EA1108 – Italy) in the Environmental Isotope Laboratory (UWEIL) at the University of Waterloo. Stable isotope ratios are measured relative to atmospheric air for nitrogen and Peedee Belemnite for carbon. The isotope ratios are expressed as delta values. Carlo Erba Elemental Standards B2005, B2035, and B2036 standards were used to calculate percentage compositions of nitrogen with a precision of +/- 1%.

2.4 Results

2.4.1 Temporal and Spatial Trends of NO\(_3^-\) isotopes in the Grand River

Throughout the year, samples from three sites (West Montrose, Bridgeport and Blair) in the main channel of the Grand River consistently plot in the nitrified septic/manure N box of the “Kendall plot” with values between +5‰ and +15‰ for \( \delta^{15}N \) and values between -0.1‰ and +2‰ for \( \delta^{18}O \) (Figure 2.6). Considering previous research, this plot would be enough to confirm that the main source of NO\(_3^-\) to the river is nitrified NH\(_4^+\) originating from an allochthonous septic/manure source. However, it is unlikely that the soil N signal is not observed for any of these sites, particularly in the agricultural headwaters where animal population is lower (Figure 2.7). Visually, a denitrification trend could be observed in this dual isotope plot, however, few of these data actually follow the 0.5 slope that is diagnostic of denitrification (Figure 2.8). Slopes of the best-fit linear model II regression for West Montrose, Bridgeport and Blair are all greater than 0.5 (Figure 2.8). This would typically indicate that no denitrification had occurred and therefore \( \delta^{15}N\)-NO\(_3^-\) could be used for source apportionment.
Seasonal trends in δ¹⁵N values (lowest in the spring and fall and highest in the summer) exist for these sites on the Grand River but there is no apparent seasonal trend in δ¹⁸O values (Figure 2.9). Some of the highest δ¹⁵N values (˃+10‰) are accompanied by the lowest NO₃⁻ concentrations (0-2mg N/L) suggestive of denitrification (Figure 2.10). However, the δ¹⁸O values observed at these times are among the lowest (-2‰ to +2‰). Almost all the samples at the Blair site have NO₃⁻ concentrations greater than 2 mg N/L because of the upstream nutrients inputs from two major WWTPs. These data makes source apportionment difficult using the dual isotope plot (Figure 2.6) and therefore a better explanation for these δ¹⁸O-NO₃⁻ values must be developed.

Figure 2.6: Kendall (2007b) source plot of δ¹⁵N-NO₃⁻ vs. δ¹⁸O-NO₃⁻ with data from three sites on the Grand River: West Montrose 21km and 47.7km upstream of Bridgeport and Blair, respectively. All data plot on the low end of the nitrified septic/manure N box with a range between +5‰ and +15‰ for δ¹⁵N and the majority of the δ¹⁸O between -0.1‰ and +2‰.
Figure 2.7: Density of nutrient production from all livestock types (A) and proportion of sub watershed area where there is use of agricultural fertilizer (B) (GRCA, 2013). Data from 2006 Census of Agriculture.
Figure 2.8: NO$_3^-$ isotope data from three sites on the Grand River: West Montrose 21km and 47.7km upstream of Bridgeport (A) and Blair (B). The dashed line represents the best-fit model II regression lines for these sites with slopes of 0.83, 0.69 and 1.09 and r$^2$ of 0.079, 0.41 and 0.37 for West Montrose, Bridgeport and Blair, respectively.
Figure 2.9: Seasonal variation in the $\delta^{18}O$- and $\delta^{15}N$-$\text{NO}_3^-$ for all Grand River isotope data collected at West Montrose and Bridgeport (A) and Blair (B) from 2007-2015. Ranges in concentration as mg N/L (<1, 1-2, 2-4, >5) are depicted by the shapes identified in the legend.

Figure 2.10: $\text{NO}_3^-$ vs. $\delta^{15}N$-$\text{NO}_3^-$ plots for three central Grand River sites: West Montrose and Bridgeport (A) and Blair (B). Two major WWTPs in Kitchener and Waterloo, Ontario discharge into the river between the Bridgeport and Blair sites.
2.4.2 Laboratory Incubations

In all incubations, the trends in the N concentration were different between the initial and final periods (T0-T24 hours and T24-T60).

I. West Montrose Experiment #1: July 7, 2015 (WM1)

The initial concentration of NO$_3^-$ in the river was 1.9 mg N/L (Figure 2.11). This was reflected in the initial incubation values (T0) in all but one beaker (High B) where the initial concentration was higher. NO$_3^-$ concentrations changed very little over the first 24 hours (T0-T24) in all beakers (Table 2.1). By the end of the incubation at T60, there was a net increase of NO$_3^-$ in all six beakers by 1-2 mg N/L.

NO$_2^-$ was not detected in the initial river sample and was low or undetected during the first 24 hours (Figure 2.11). The highest NO$_2^-$ concentration was 0.5 mg N/L in Low A at T60. Accumulation of NO$_2^-$ could be the result of slower rates of nitrification where NO$_2^-$ has a higher residence time. Overall, NO$_2^-$ concentrations in the Medium and High beakers remained constant and low (<0.2 mg N/l) from T24 to T60 except in Low A where concentration gradually increased over time.

NH$_4^+$ was not detected in the initial river sample and the concentration remained low or below the detection limit until T24 in all beakers (Figure 2.11). NH$_4^+$ in beaker High A changed very little from T36-T60 in the range of 0.03 to 0.06 mg N/L. The other beakers fluctuated on a 12-hour cycle as follows: NH$_4^+$ increased at T24 and T48 and decreased at T36 and T60 with the exception of Medium A that continued to increase after T24 to T36. At the final time point (T60) NH$_4^+$ was not detected in any of the six beakers.

The initial river NO$_3^-$ isotope values obtained were typical of summer isotope values in the Grand River at West Montrose (Figure 2.9). The initial river $\delta^{15}$N-NO$_3^-$ value was +12‰ and similar to the $\delta^{15}$N of biomass (+8‰ to +9‰) (Gris, 2016). The initial incubation values ranged between +73‰ to +84‰ (Figure 2.11). Final $\delta^{15}$N values ranged between +33‰ and +46‰ by the end of the experiment (T60). Values decreased by about 35‰ in all beakers.
over time (Table 2.2). The δ¹⁵N changed by 15‰ and 20‰ whether there was very little NO₃⁻ concentration change (T0-T24) or 1.1 mg N/L change (Table 2.1). Thus NO₃⁻ isotope values are changing even when NO₃⁻ concentrations are not changing. The net δ¹⁵N added to the system could not be calculated for this experiment because there was not enough change in concentration relative to the shift in isotopic values.

The separation in δ¹⁸O-NO₃⁻ between Low, Medium and High showed the incorporation of the δ¹⁸O-H₂O even when NO₃⁻ concentrations remained constant or changed very little (Figure 2.11). The δ¹⁸O-NO₃⁻ of the initial river sample was +0.6‰ and the initial experimental values of δ¹⁸O-NO₃⁻ ranged between +10‰ and +13‰ for all beakers. At T24 the δ¹⁸O-NO₃⁻ in the Low beakers had not shifted much (~1‰) from the initial values but by T60 values had decreased by 6‰ (Table 2.3). The δ¹⁸O increased in the Medium and High beakers by 8‰ and 16‰ from T0 to T24 and continued to increase throughout the experiment. The δ¹⁸O values changed/tended toward their δ¹⁸O-H₂O values regardless of any net NO₃⁻ concentration change (Table 2.3).
Figure 2.11: NO$_3^-$, NO$_2^-$ and NH$_4^+$ concentration (top) and $\delta^{18}$O- and $\delta^{15}$N-NO$_3^-$ isotope results (bottom) vs. time for the first West Montrose incubation conducted on July 7, 2015. The three pairs of beakers were labeled according to levels of $\delta^{18}$O-$\text{H}_2\text{O}$: Low (-9‰), medium (+62‰) and high (+100‰) and denoted by colours orange, purple and green in the figure, respectively. Each pair of beakers was labeled either ‘A’ or ‘B’ and are represented by circles and triangles in the figures, respectively. Initial river samples that were not spiked are marked by pink squares.
II. West Montrose Experiment #2: September 2, 2015 (WM2)

A second West Montrose incubation was conducted in September 2015 to confirm the results of the first experiment and to address changes over the growing season. The initial river NO$_3^-$ concentration was 1.3 mg N/L (Figure 2.12), and similar to the river concentrations in July 2015. Initial NO$_3^-$ concentrations in the beakers ranged between 0.9-1.0 mg NL. Similar to July WM1, NO$_3^-$ concentration showed very little change in the first 24 hours (T24) (Table 2.1). NO$_3^-$ increased between T24 and T72 hours by about 0.9 mg N/L in all beakers except for High A that only increased by 0.2 mg N/L. Similar to WM1 there was net NO$_3^-$ production over the course of the incubation. However, the change in NO$_3^-$ over the course of the experiment was smaller (~1 mg N/L) compared to the July experiment (1.3-1.7 mg N/L) (Table 2.1).

NO$_2^-$ was not detected in the initial river sample (Figure 2.12). Similar to the July incubation NO$_2^-$ concentrations were low (0.02 mg N/L) in the Low and Medium beakers and undetected in the High beakers. At T36 hours, NO$_2^-$ remained in only one beaker (Medium B) and then was undetected in all beakers until T72 when it was detected at a low concentration of 0.01 mg N/L in Medium B and High A.

Similar to the July incubations, NH$_4^+$ was not detected in the initial river sample and NH$_4^+$ was low (0.02-0.04 mg N/L) in the Low and High beakers at T0 (Figure 2.12). The exception was the Medium beakers where it was undetected. NH$_4^+$ was not detected in any beaker until T36 when a low concentration of 0.02 mg N/L was observed in Low A. Similar to July, NH$_4^+$ fluctuated on a 12-hour cycle increasing between T36 and T48 hours then decreasing between T48 and T60 hours in most beakers. The highest NH$_4^+$ concentration of 0.07 mg N/L was observed in High A at T72. The lowest net NO$_3^-$ production was also observed in this beaker. With the exception of High A, NH$_4^+$ was low (0.01-0.02 mg N/L) or undetected over the course of the incubation.
Isotopes of NO₃⁻ also responded similarly to the July incubations. The initial δ¹⁵N-NO₃⁻ river value was +12‰ and the δ¹⁵N of the rock biofilm was +8‰. The initial δ¹⁵N-NO₃⁻ values ranged between +77‰ and +86‰ and they decreased uniformly as they did in the July experiment by close to 42‰ over the course of the incubation in most of the beakers (Table 2.2). The exception was High A where there was only an 18‰ decrease in δ¹⁵N overtime. This can be attributed to low net NO₃⁻ production in this beaker. The NO₃⁻ concentration showed little change, especially in the first 24 hours, while the δ¹⁵N values decreased around 17‰ and then further decreased around 25‰ from T24 to T72 in most beakers (Table 2.2). Much like the first West Montrose experiment, the net δ¹⁵N added to the system could not be calculated for this experiment because there was not enough change in concentration relative to the shift in isotopic values.

The initial river value of δ¹⁸O-NO₃⁻ was +1.1‰ and the initial experiment value (T0) was +11.5‰ in all beakers (Figure 2.12). The δ¹⁸O-NO₃⁻ in the beakers with low δ¹⁸O-H₂O decreased by approximately 10‰ over the course of the experiment and increased in most of the Medium and High beakers by approximately 14‰ and 40‰, respectively (Table 2.3). The exception was High A that only increased by 14‰ over the course of the experiment and was the result of having the lowest net NO₃⁻ production (0.2 mg N/L) of all the beakers. Even though the final δ¹⁸O-NO₃⁻ value of High A closely resembled Medium A and B, the shift in δ¹⁸O of 14‰ was still substantial. The δ¹⁸O-NO₃ isotope shifted by ~4‰, ~8‰ and ~16‰ in the Low, Medium and High beakers, respectively in the first 24 hours despite the lowest change in NO₃⁻ concentration during this time period (0.1 mg N/L net production) (Table 2.3). The separation in δ¹⁸O-NO₃⁻ values was distinct for the three water labels (Low, Medium and High) and consistent with the results from the West Montrose July incubations. Overall, the results from this second experiment supported the findings from the July experiment.
Figure 2.12: NO₃⁻, NO₂⁻ and NH₄⁺ concentration (top) and δ¹⁸O- and δ¹⁵N-NO₃⁻ isotope results (bottom) vs. time for the second West Montrose incubation conducted on September 2, 2015. The three pairs of beakers were labeled according to levels of δ¹⁸O-H₂O: Low (-9‰), medium (+62‰) and high (+100‰) and denoted by colours orange, purple and green in the figure, respectively. Each pair of beakers was labeled either ‘A’ or ‘B’ and are represented by circles and triangles in the figures, respectively. Initial river samples that were not spiked are marked by pink squares.
An incubation was also conducted with biofilms from the Blair site to observe if changes in concentrations and isotopes downstream of the wastewater treatment plants where concentrations of all nutrients including NO$_3^-$ are typically higher were similar to the upstream site with lower nutrient concentrations.

The initial river NO$_3^-$ concentration at Blair was 3.27 mg N/L (Figure 2.13). This is a typical concentration at Blair in July (Figure 2.1) and higher than the concentrations at the West Montrose site. The initial experiment NO$_3^-$ concentration was 2.9 mg N/L in the Low beakers and ranged between 3.5-4 mg N/L in the Medium and High beakers. NO$_3^-$ concentrations showed little change in the first 24 hours similar to the West Montrose incubations and then gradually increased over time. The NO$_3^-$ concentration increased in the Low and Medium beakers by about 2.5 mg N/L and by about 2.0 mg N/L in the High beakers from T0 hours to T60 hours. Net production of NO$_3^-$ in this incubation was at least 1 mg N/L greater than the net production in both West Montrose incubations (Table 2.1). Visually and texturally the rocks at West Montrose had a finer, slimy green film whereas the biomass on the Blair rocks tended to be more rough and stringy.

The initial river NO$_2^-$ concentration was 0.40 mg N/L and the initial incubation concentrations in all six beakers were around 0.30 mg N/L (Figure 2.13), much higher than the concentrations at West Montrose. Between West Montrose and Blair there are two large WWTPs whereas upstream of West Montrose, there are mostly diffuse non-point source nutrient inputs. NO$_2^-$ concentrations decreased over time but NO$_2^-$ was present in almost all beakers from T0 to T48 (0.1-0.2 mg N/L). NO$_2^-$ was mostly absent by T60 (except in Low A and Medium A at 0.02 and 0.03 mg N/L, respectively). The highest NO$_2^-$ concentrations were consistently seen in the Medium and Low beakers.

Of the three incubations, Blair had some of the highest observed NH$_4^+$ concentrations initially and throughout the experiment. The initial river NH$_4^+$ concentration was 0.12 mg
NH₄⁺ was detected in three of the six beakers at the start of the incubation (0.1 mg N/L in Low A and Medium B and 0.04 mg N/L in Low B). The highest NH₄⁺ concentrations (>1.0 mg N/L) were observed in Low B and Medium B at T24 and T36, respectively. NH₄⁺ concentrations were undetectable in all beakers by the end of the experiment (T60). The greatest net NO₃⁻ production (1.9 mg N/L) was observed following the decrease in NH₄⁺ concentration in beaker Medium B.

The initial river δ¹⁵N-NO₃⁻ value was +12‰, similar to the West Montrose site but the δ¹⁵N of the rock biofilm was much higher, +14‰ to 17‰ (Gris, 2016). The initial δ¹⁵N in the beakers ranged between +30‰ and +40‰. Similar to the West Montrose incubations, δ¹⁵N-NO₃⁻ values uniformly decreased over time by approximately 8‰ in most beakers (Table 2.2). The exception was High B that had decreased by 14‰ at T60. Of the three incubations, Blair had the greatest net NO₃⁻ production throughout the experiment and the net δ¹⁵N approached the δ¹⁵N of rock biofilm in most beakers (Figure 2.14). Blair had the lowest change in δ¹⁵N-NO₃⁻ isotopes overtime compared to the two West Montrose experiments (Table 2.1, 2.2 and 2.3). This suggests rate of processing may have been different between the two sites but the higher δ¹⁵N of biomass influenced the amount of isotopic shift during the experiment. The initial river isotope value of δ¹⁸O-NO₃⁻ was -0.73‰ (Figure 2.13), similar to that at West Montrose in July and September. Isotope values of δ¹⁸O-NO₃⁻ at T0 of the experiment were +6‰ in Low A, +9‰ and +12‰ in Medium A and B, respectively and +7‰ in the High beakers. The δ¹⁸O-NO₃⁻ in the low beakers decreased over time by 6‰ and the values increased in the Medium beakers by 22‰. The δ¹⁸O-NO₃⁻ in the High beakers increased over time by about the same amount as the Medium beakers (25‰) and the final δ¹⁸O-NO₃⁻ values in both High beakers closely resembled those of the Medium beakers that had lower δ¹⁸O-H₂O. However, the highest NO₂⁻ concentrations were observed in Medium beakers (0.22 mg N/L). Given that O-exchange has been found to occur between NO₂⁻ and H₂O during nitrification, slower rates of nitrification could have caused NO₂⁻ to accumulate and enhanced O-exchange yielding a similar final δ¹⁸O-NO₃⁻ in the Medium and High
beakers. To account for differences in initial $\delta^{18}$O-NO$_3^-$ values, results of these incubations were recalculated to report isotopic ratios relative to the initial $\delta^{18}$O-NO$_3^-$ values (Figure 2.15). The $\delta^{18}$O-NO$_3^-$ values approached the $\delta^{18}$O-H$_2$O of each water medium (-9‰, +52‰ and +102‰) regardless of any net NO$_3^-$ concentration change (Table 2.3).
Figure 2.13: NO$_3^-$, NO$_2^-$ and NH$_4^+$ concentration (top) and δ$^{18}$O- and δ$^{15}$N-NO$_3^-$ isotope results (bottom) vs. time for the Blair incubation conducted on July 29, 2015. The three pairs of beakers were labeled according to levels of δ$^{18}$O-H$_2$O: Low (-8‰), medium (+62‰) and high (+100‰) and denoted by colours orange, purple and green in the figure, respectively. Each pair of beakers was labeled either ‘A’ or ‘B’ and are represented by circles and triangles in the figures, respectively. Initial river samples that were not spiked are marked by pink squares.
Figure 2.14: Net δ¹⁵N for the Blair (July) incubation experiment. Net ¹⁵N added to the system was calculated from an isotope mass balance similar to Equation 2.1. Triangles and circles represent duplicate beakers, A and B used in each experiment.
Figure 2.15: Isotope results for the Blair incubation conducted on July 29, 2015 shown relative to the initial δ¹⁸O-NO₃⁻ in each beaker. The three pairs of beakers were labeled according to levels of δ¹⁸O-H₂O: low (-9‰), medium (+52‰) and high (+102‰) and denoted by colours orange, purple and green in the figure, respectively. Each pair of beakers were labeled either ‘A’ or ‘B’ and are represented by circles and triangles in the figures, respectively.
2.4.3 River Chamber Experiments

Chamber Experiment – September 17, 2015

The chamber experiment conducted in the river showed different concentration changes but isotopic effects similar to the three incubation experiments. The initial NO₃⁻ concentration in the river and chambers were 0.71 mg N/L and 0.99 mg N/L, respectively (Figure 2.16). Over time the NO₃⁻ concentration decreased by over half to 0.4 mg N/L at T24 in both chambers (Table 2.1). Net NO₃⁻ loss rather than net production (incubations) suggests that the environment in the beaker and chambers was different.

NO₂⁻ was not detected in the initial river sample or in either chamber throughout the experiment, similar to most beakers in the West Montrose incubations (Figure 2.16).

The initial concentration of NH₄⁺ in the river was 0.01 mg N/L and increased to 0.03 mg N/L in both chambers at T0. NH₄⁺ was not detected until T24 in both Chamber A (0.01 mg N/L) and Chamber B (0.08 mg N/L) (Figure 2.16).

The initial river δ¹⁵N-NO₃⁻ value was 10.3‰ and the initial experiment values were +54.6‰ in Chamber A and +55.5‰ in Chamber B (Figure 2.16). The δ¹⁵N values decreased continually over the 24 hours in both chambers by about 10‰ (Table 2.2).

The initial river value of δ¹⁸O-NO₃⁻ was -1.6‰ and the initial experiment values were +14.7‰ in Chamber A and +15.3‰ in Chamber B. In 24 hours, δ¹⁸O-NO₃⁻ values decreased by 2‰ in Chamber A and increased by 2‰ in Chamber B (Table 2.3).

Though the NO₃⁻ concentrations were low and decreased over time, the isotopes changed comparably to the incubation beakers as N was processed in the chambers (Table 2.2 and Table 2.3).
Figure 2.16: NO$_3^-$, NO$_2^-$ and NH$_4^+$ concentration (top) and $\delta^{18}$O- and $\delta^{15}$N-NO$_3^-$ isotope results (bottom) vs. time for the chamber experiment conducted on September 17, 2015. The two chambers were labeled ‘A’ or ‘B’ denoted as green circles and orange triangles, respectively. Initial river samples that were not spiked are denoted by purple squares.
Table 2.1: Change in NO$_3^-$ concentration for two distinct time periods during each of three incubation experiments and one chamber experiment. Positive (+) values indicate net production of NO$_3^-$ and negative (-) values indicate net loss. Net changes were calculated as final minus initial.

<table>
<thead>
<tr>
<th></th>
<th>T0-T24 hours (mg N/L)</th>
<th>T24-T60 or T72 Hours (mg N/L)</th>
<th>Total change in NO$_3^-$ (mg N/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Montrose July 2015</td>
<td>+0.3</td>
<td>+1.1</td>
<td>+1.4</td>
</tr>
<tr>
<td>West Montrose September 2015</td>
<td>+0.1</td>
<td>+0.9</td>
<td>+1</td>
</tr>
<tr>
<td>Blair July 2015</td>
<td>+0.6</td>
<td>+1.7</td>
<td>+2.3</td>
</tr>
<tr>
<td>West Montrose Chamber September 2015</td>
<td>-0.45</td>
<td>NA</td>
<td>-0.45</td>
</tr>
</tbody>
</table>

Table 2.2: Change in δ$^{15}$N-NO$_3^-$ values (in ‰) for two distinct time periods during each of three incubation experiments and one chamber experiment. Positive (+) values indicate an increase and negative (-) values indicates a decrease in isotopic values. Net changes were calculated as final minus initial and the net δ$^{15}$N added was calculated with an isotope mass balance.

<table>
<thead>
<tr>
<th></th>
<th>T0-T24 hours (‰)</th>
<th>T24-T60 or T72 Hours (‰)</th>
<th>Total change in δ$^{15}$N (‰)</th>
<th>Net δ$^{15}$N Added (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Montrose July 2015</td>
<td>-15</td>
<td>-20</td>
<td>-35</td>
<td>NA</td>
</tr>
<tr>
<td>West Montrose September 2015</td>
<td>-17</td>
<td>-25</td>
<td>-42</td>
<td>NA</td>
</tr>
<tr>
<td>Blair July 2015</td>
<td>-3</td>
<td>-5</td>
<td>-8</td>
<td>5-17</td>
</tr>
<tr>
<td>West Montrose Chamber September 2015</td>
<td>-10</td>
<td>NA</td>
<td>-10</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 2.3: Change in $\delta^{18}$O-NO$_3^-$ values (in ‰) for two distinct time periods during each of three incubation experiments and one chamber experiment. Positive (+) values indicate an increase and negative (-) values indicates a decrease in isotopic values. Net changes were calculated as final minus initial.

<table>
<thead>
<tr>
<th>Beaker</th>
<th>T0-T24 hours</th>
<th>T24-T60 or T72 (BL1) Hours</th>
<th>Total change in $\delta^{18}$O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
</tr>
<tr>
<td>West Montrose July 2015</td>
<td>-0.8</td>
<td>+8</td>
<td>+16</td>
</tr>
<tr>
<td>West Montrose September 2015</td>
<td>-4</td>
<td>+8</td>
<td>+16</td>
</tr>
<tr>
<td>Blair July 2015</td>
<td>-2</td>
<td>+12</td>
<td>+13</td>
</tr>
<tr>
<td>West Montrose Chamber September 2015</td>
<td>-2</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
2.5 Discussion

Without a complete understanding of the δ¹⁸O values, one might disregard the role of denitrification and rapid internal N cycling in the river (Figure 2.17B). Even when NO₃⁻ concentrations are not changing in rivers, stable isotopes of NO₃⁻ can indicate the presence of NO₃⁻ cycling. No change in concentration means only balanced rates and not that δ¹⁵N- and δ¹⁸O-NO₃⁻ are conservative in the river.

2.5.1 Concentrations of N Species

Similar sized rocks were collected for the incubations but some of the variability between duplicate beakers is likely due to differences in rock surface area or biofilm biomass. Concentrations were not corrected for these two variables.

I. Role of NO₃⁻ in Rivers

No change in NO₃⁻ simply means that net rates are balanced. This was observed in most beakers during the first 24 hours of all three incubation experiments indicating balanced rates of N cycling processes (mineralization, nitrification, denitrification and uptake). Net NO₃⁻ production can occur but may be difficult to discern in rivers if there are additional sources of NO₃⁻. Net NO₃⁻ production from 24-60 or 72 hours in the beakers could be due to biomass mineralizing into NH₄⁺ and subsequently nitrifying into NO₃⁻. Rates of nitrification would have been greater than the rate of denitrification and uptake combined (Figure 2.17). Blair had higher NO₃⁻ concentrations and the greatest net NO₃⁻ production compared to West Montrose. Blair is directly downstream from the Waterloo and Kitchener WWTPs and receives point-source nutrients while West Montrose is upstream of the WWTPs and receives mostly non-point source agricultural inputs. Nutrient concentrations are therefore higher at Blair but also the concentrations of other chemicals besides nutrients are present and thus the water quality is different than West Montrose. Rate and/or amount of nitrification were highest at the Blair site compared to the upstream West Montrose site (Table 2.4). These net rates were comparable to literature rates for watersheds with mixed
land-use (Table 2.5) similar to the Grand River watershed. The lowest NO$_3^-$ concentrations were observed in the river chamber experiments at West Montrose. Lower and decreasing NO$_3^-$ concentrations suggest that rates of uptake and/or denitrification were greater than nitrification in the chambers. The two scenarios from the laboratory incubation experiments: 0-24 hours (no net change in NO$_3^-$) and 24-60/72 hours (net NO$_3^-$ production) allowed the opportunity to examine changes in δ$^{15}$N- and δ$^{18}$O-NO$_3^-$ under both conditions over time.

II. Role of NO$_2^-$ in Rivers

The low NO$_2^-$ that in most beakers in the West Montrose incubations could have resulted from fast rates of nitrification compared to mineralization such that the lifetime of NO$_2^-$ in the beakers was short. Sufficient DO, temperature and light in the beakers would have also facilitated the reaction to go to completion. High NO$_2^-$ observed in the river at the Blair site and in the Blair incubations could have been the result of higher NO$_2^-$ input from upstream WWTPs and/or slower rates of nitrification that allowed for the accumulation of NO$_2^-$.

Although NO$_2^-$ exchange with H$_2$O can occur without an accumulation of NO$_2^-$, the presence of NO$_2^-$ indicates that the residence time of NO$_2^-$ was likely longer at the Blair site compared to the West Montrose site. Where NO$_2^-$ was detected in the laboratory incubations, there was a greater opportunity for O-exchange between NO$_2^-$ and H$_2$O to occur which would cause a greater overall shift in δ$^{18}$O-NO$_3^-$ toward the δ$^{18}$O-H$_2$O of the medium.

III. Role of NH$_4^+$ in Rivers

NH$_4^+$ has a short half-life in the Grand River and is usually nitrified or taken up by biota (Cejudo, 2016) but higher concentrations may arise intermittently because of upstream nutrients or rainfall events. NH$_4^+$ was only observed at the Blair site during initial river sampling because of nutrient inputs from WWTPs. NH$_4^+$ concentrations in all three incubation experiments showed small increases and subsequent decreases that fluctuated on a 12-hour cycle and sometimes corresponded inversely with NO$_3^-$ concentrations. These
changes were likely the result of mineralization and subsequent nitrification. The δ^{15}N-NH_4^+ released from mineralization should reflect that of the rock biomass (if there is little or no fractionation) and would affect the end member value of δ^{15}N-NO_3^- after nitrification.

Denitrification of NO_3^- and uptake of NH_4^+ or NO_3^- may have also altered the concentrations of these N species; however, neither were expected to be important relative to mineralization and nitrification (Battin et al., 2016; Burgin & Hamilton, 2007; Mulholland et al., 2008). Denitrification would have been unlikely in these experiments because the beakers were well oxygenated and stirred continuously. In addition, the increase in NO_3^- concentrations over time in all incubations experiments indicates the rate of mineralization and subsequent nitrification of NH_4^+ was greater than any uptake of N species. It is difficult to discern the relative amounts of uptake/denitrification versus mineralization and subsequent nitrification in rivers with concentration data alone and with unquantified inputs of NO_3^- Isotopes are required to constrain relative rates of these processes.

### 2.5.2 Role of δ^{15}N- and ^18O-NO_3^- in Source Apportionment of a Highly Impacted River

1. **Are δ^{15}N- and ^18O-NO_3^- conservative in surface waters?**

   Results from this study indicate that the δ^{15}N- and δ^{18}O-NO_3^- are *not* conservative in the Grand River. NO_3^- isotopes changed even when NO_3^- concentrations more or less stayed the same (T0-T24 of incubations). Changes were also observed when NO_3^- concentration both increased (T24-T60 or T72 of incubations) and decreased (West Montrose chamber experiment). This contradicts the necessary assumption for source apportionment where no change in NO_3^- means conservative NO_3^- isotopes (Figure 2.17A).

   Studies have found the δ^{15}N of biomass to range between +8‰ and +9‰ at the West Montrose site and from +14‰ to +17‰ at the Blair site (Gris, 2016). The high initial δ^{15}N-NO_3^- in the incubations was expected to decrease in all experiments over time as NO_3^- was cycled because any NO_3^- produced from biomass mineralization and nitrification would have a much lower δ^{15}N than the initial incubation value. The δ^{15}N-NO_3^- values decreased
uniformly in all beakers overtime in all experiments, and net δ¹⁵N for the Blair site approached the δ¹⁵N biomass values. The δ¹⁸O-NO₃⁻ changed toward the δ¹⁸O-H₂O value of the medium in all beakers and chambers over time even when NO₃⁻ did not change. Equilibrium O-exchange between NO₂⁻ and H₂O during nitrification thus alters end member isotope values.

II. Can δ¹⁵N- and ¹⁸O-NO₃⁻ be used to indicate denitrification and sources to the Grand River?

In most cases, denitrification produces higher values of δ¹⁵N and δ¹⁸O and nitrification produces lower values of δ¹⁵N and δ¹⁸O (Figure 2.17A). However, these isotopes are not conservative and values of δ¹⁸O-NO₃⁻ are variable making it difficult to predict end member values. Therefore δ¹⁵N- and δ¹⁸O-NO₃⁻ cannot be used to indicate denitrification in the Grand River. River metabolism influences rapid isotopic changes and masks the original source and denitrification signal even where and when we know denitrification is occurring (Rosamond, 2013).

III. Is the interpretation of isotopic systematics consistent between two sample sites: West Montrose and Blair?

Differences were observed between the two study sites, adding to the complexity of riverine NO₃⁻ cycling and the use of NO₃⁻ isotopes for source apportionment. These differences were evident in the concentrations of nutrients, the net production of NO₃⁻ and the value of the δ¹⁵N of biomass (all greatest at the Blair site). These differences can be attributed to the input of the additional nutrients from the WWTPs upstream of the Blair site compared to the West Montrose site.

In addition, rates of incorporation of the three δ¹⁸O-H₂O labels into NO₃⁻ and the uptake and release of NO₃⁻ were not the same over time (between beakers and sites) and therefore, relative rates of N transformation processes should be considered as well as the mechanism(s) for the “reset” of δ¹⁸O-NO₃⁻ toward the δ¹⁸O-H₂O (Chapter 3).
Figure 2.17: Schematic representation of NO₃⁻ transformation processes and the expected trends for NO₃⁻ concentration and NO₃⁻ isotopes. The top figure (A) indicates what would be expected if the δ¹⁸O- and δ¹⁵N-NO₃⁻ were conservative in the river and B represents potential trends in values if the δ¹⁸O- and δ¹⁵N-NO₃⁻ were not conservative.
Table 2.4: Net rates of NO₃⁻ production for two distinct time periods (0-24 hours and 24-60 hours) for each incubation experiment, two at West Montrose in July and September and one at Blair and one chamber experiment. Positive (+) values indicate net release and negative (-) values indicate net consumption. Areal biomass corrections were made for the West Montrose and Blair incubations in July.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>0-24 Hours (µg N L⁻¹ h⁻¹)</th>
<th>0-24 Hours (µg N cm⁻² h⁻¹)</th>
<th>24-60 Hours (µg N L⁻¹ h⁻¹)</th>
<th>24-60 Hours (µg N cm⁻² h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Montrose July 2015</td>
<td>15.5</td>
<td>0.11</td>
<td>36.8</td>
<td>0.26</td>
</tr>
<tr>
<td>West Montrose September 2015</td>
<td>4.51</td>
<td>NA</td>
<td>21.3</td>
<td>NA</td>
</tr>
<tr>
<td>Blair 2015</td>
<td>26.0</td>
<td>0.17</td>
<td>45.2</td>
<td>0.30</td>
</tr>
<tr>
<td>West Montrose Chamber September 2015</td>
<td>-23.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 2.5: Net rates of NO₃⁻ production found in literature across different stream and watershed characteristics.

<table>
<thead>
<tr>
<th>Rate (µg N cm⁻² h⁻¹)</th>
<th>Source</th>
<th>Stream/Watershed Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.77</td>
<td>(Dodds et al., 2000)</td>
<td>Upland prairie stream</td>
</tr>
<tr>
<td>0.65</td>
<td>(Grimm et al., 1991)</td>
<td>Intermittent desert stream</td>
</tr>
<tr>
<td>0.48</td>
<td>(Starry et al., 2005)</td>
<td>Appalachian headwater stream</td>
</tr>
<tr>
<td>0.26</td>
<td>(Starry et al., 2005)</td>
<td>Appalachian headwater stream</td>
</tr>
<tr>
<td>0.45</td>
<td>(Strauss &amp; Lamberti, 2000)</td>
<td>3rd-order stream; watershed of mixed land use</td>
</tr>
</tbody>
</table>
2.6 Summary and Implications

The objectives of this chapter were to determine if the $\delta^{18}$O-NO$_3^-$ and the $\delta^{15}$N-NO$_3^-$ are conservative and, if so, can those isotopes be used as an indicator of denitrification and source in the Grand River at differences sites.

Isotope data from routinely sampled sites on the Grand River showed seasonal patterns in the NO$_3^-$ concentrations and $\delta^{15}$N-NO$_3^-$ but no clear trend in $\delta^{18}$O-NO$_3^-$ values. A “Kendall source plot” illustrated that all Grand River isotope data was in the nitrified manure source box and that denitrification does not affect the isotopes at any of these sites. It is unlikely that the seasonal shift in $\delta^{18}$N is caused by a change in N from fertilizer to manure within the same season because of the lower livestock density in the agricultural headwaters.

To examine the use of NO$_3^-$ isotopes for source apportionment and identification of denitrification in rivers, three laboratory incubation experiments and one in-river chamber experiment were conducted. The first two incubation experiments were conducted with river water and biofilm covered rocks from West Montrose in July and September. The third laboratory incubation conducted in July used river water and rocks from Blair, downstream of West Montrose and inputs to the river from the Waterloo and Kitchener WWTPs.

The highest concentrations of NO$_3^-$, NO$_2^-$ and NH$_4^+$ and the highest net NO$_3^-$ production were observed at Blair (Figure 2.13). In all incubations from both sites NO$_3^-$ concentration changed very little in the first 24 hours (T0-T24) and then steadily increased from T24-T60 or T72 (Table 2.1). The production of NO$_3^-$ was due to mineralization and subsequent nitrification, as NH$_4^+$ did not increase. The $\delta^{15}$N-NO$_3^-$ values decreased by approximately the same amount in each incubation experiment regardless of any change in N concentrations (Table 2.2). The $\delta^{18}$O-NO$_3^-$ changed during the experiment toward the $\delta^{18}$O-H$_2$O of the medium even when NO$_3^-$ concentrations did not change or changed very little (Table 2.3). All three incubations showed a separation in $\delta^{18}$O-NO$_3^-$ corresponding to
each of the distinct isotopic water labels indicating that water exchange was the involved mechanism that altered the $\delta^{18}O$-NO$_3^-$ (Low, Medium and High). This is consistent with the effect of O-exchange between NO$_2^-$ and H$_2$O which is known to be rapid.

A submerged chamber experiment that ran for 24 hours at West Montrose was conducted to compare in-situ river conditions with the laboratory beaker incubations. The NO$_3^-$ concentration decreased over time because rates of uptake and/or denitrification were greater than nitrification. The data from the chamber experiment and the laboratory incubations was comparable because the NO$_3^-$ isotope values in the chambers shifted approximately the same amount as they did within the first 24 hours in the Low beakers of the incubation experiments (Table 2.2 and Table 2.3).

The $\delta^{18}O$-NO$_3^-$ values changed relative to the $\delta^{18}O$-H$_2$O in the beakers while $\delta^{15}N$-NO$_3^-$ decreased over time and net $\delta^{15}N$ of biomass at the Blair site resembled the $\delta^{15}N$ of rock biomass. This trend was observed in every experiment and provided evidence that both the $\delta^{15}N$ and $\delta^{18}O$ of NO$_3^-$ are not conservative and therefore cannot be used as an indicator of source and denitrification in the Grand River. As N was transformed in the beakers and the chambers, NO$_3^-$ isotopes were rapidly cycled even when net NO$_3^-$ production was low (Table 2.1). This study has shown that the biofilm that covers the rocks and the river bottom has a profound effect on river metabolism and N cycling processes in the Grand River. The use of isotopes for source apportionment of NO$_3^-$ in rivers needs to be revised for highly impacted systems like the Grand River. This information is valuable to those concerned with the fate of elevated NO$_3^-$ and who have used NO$_3^-$ isotopes for source determination in the past. Managers should be cautious before isotopes are used to determine source and first understand the complexity of N cycling and NO$_3^-$ isotopes.

In order to improve the use of NO$_3^-$ isotopes as a tool for source apportionment in rivers, the possible mechanisms responsible for altering the $\delta^{18}O$-NO$_3^-$ should be examined. Abiotic O-exchange that occurs between NO$_2^-$ and H$_2$O is one such mechanism. Research
has found that the amount of abiotic O-exchange that occurs between NO$_2^-$ and H$_2$O (fabiotic) can influence the $\delta^{18}$O-NO$_3^-$ (Snider et al., 2010). The “1:2 rule” for oxygen incorporation assumes that during nitrification, the fraction of abiotic O-exchange is 0 (Snider et al., 2010). The fabiotic in the Grand River can be calculated from the $\delta^{18}$O-NO$_3^-$ results of these incubation experiments. If the fraction of abiotic exchange could be placed on a scale of 0 to 1, rates of N cycling in the Grand River could potentially be characterized. This could be a useful tool for managers in conjunction with the NO$_3^-$ isotopes to understand the fate of NO$_3^-$ in the river and improve water quality. For instance, fabiotic of 0 and no O-exchange could mean low or high rates of N transformations processes. NO$_3^-$ would be conservative in the river and persist. A fabiotic value of 1 and equilibration of H$_2$O and NO$_2^-$ would mean high rates of N processing: greater nitrification, denitrification and potentially more transient incorporation of NO$_3^-$ into biomass. A model must be created next to elucidate the rates of N transformation processes (mineralization, uptake, nitrification and denitrification) in the Grand River. Chapter 3 will use incubation data to examine the potential mechanisms responsible for altered riverine $\delta^{18}$O-NO$_3^-$ and use a mathematical model to look at the rates of N transformation processes.
Chapter 3 – Improving NO₃⁻ Isotope Tools in Productive Rivers

3.1 Introduction

In-river productivity can have a profound impact on N cycling and the resulting NO₃⁻ isotope values. Field and experimental data (Chapter 2) indicate that the δ¹⁵N- and δ¹⁸O-NO₃⁻ in the Grand River are not conservative. In-river metabolism alters the isotopic signal such that it cannot be used to indicate denitrification or sources of NO₃⁻ to an impacted river.

Nitrification is the oxidation of NH₄⁺ to NO₂⁻ and then NO₃⁻ and involves the incorporation of three oxygen molecules: one from O₂ and two from H₂O. The O₂ is the first to be incorporated to form NH₂OH, followed by one water-oxygen to form NO₂⁻ and then another water-oxygen to form NO₃⁻ (Figure 3.1). Studies have documented further O-exchange between H₂O and NO₂⁻ during NH₄⁺ oxidation (Andersson et al., 1982; Casciotti et al., 2010; DiSpirito & Hooper, 1986; Snider et al., 2010). The kinetic ¹⁸O isotope fractionations that result from the incorporation of O₂ (¹⁸εₒ₂) and H₂O (¹⁸ε_H₂O,1 and ¹⁸ε_H₂O,2) in nitrification are large and were first estimated by Casciotti et al. (2010) and Buchwald & Casciotti (2010) (Figure 3.1). The combined isotope fractionations for O₂ and H₂O incorporation (¹⁸εₒ₂ + ¹⁸ε_H₂O,1) in the initial oxidation stages was found to range between -38‰ and -18‰ for four marine ammonia-oxidizing bacteria (AOB) and the isotope effect for H₂O incorporation (¹⁸ε_H₂O,2) in the final oxidation step ranged between -25‰ and -9‰ for three marine nitrite-oxidizing bacteria. Casciotti et al. (2010) also found that the percentage of isotopic O-exchange between NO₂⁻ and H₂O during NH₄⁺ oxidation among four species of marine nitrifying bacteria varied between 1% and 25% among species. The results of this study on O-exchange between NO₂⁻ and H₂O can likely be explained by the abiotic equilibrium isotope effect (εₑq) (Casciotti et al., 2007). At equilibrium, this effect will cause the δ¹⁸O-NO₂⁻ to be ~14‰ higher than the δ¹⁸O-H₂O with which it is in equilibrium. Contrary to the
findings from Casciotti et al. (2010), other studies (Buchwald & Casciotti, 2010; DiSpirito & Hooper, 1986; Hollocher et al., 1981) found very little isotopic exchange between NO₂⁻ and H₂O (0-3%).

![Bacterial nitrification schematic modified from Snider et al. (2010).](image)

Figure 3.1: Bacterial nitrification schematic modified from Snider et al. (2010). NH₃ is oxidized to NO₃⁻ and one O from O₂ molecule (purple) and two O from H₂O molecules (green). The ¹⁸O effects for nitrification are shown as ¹⁸εO₂, ¹⁸εH₂O,1, ¹⁸εeq, and ¹⁸εH₂O,2, respectively. The δ¹⁸O value of O₂ is +24.2 assuming river water had fully equilibrated with atmospheric air and -8‰ for δ¹⁸O-H₂O a typical value at the study site.

Isotopic O-exchange between H₂O and NO₂⁻ during denitrification has also been documented (Kool et al., 2007). Denitrification is the reduction of NO₃⁻ into N₂O and N₂. If high amounts of O-exchange occur during nitrification or denitrification, the δ¹⁸O signal of NO₃⁻ will be altered and will vary unpredictably depending on the amount of exchange that occurs (Figure 3.2). As O molecules between H₂O and NO₂⁻ are exchanged and begin to equilibrate during nitrification (Figure 3.1), the final microbial end-member value of δ¹⁸O-NO₃⁻ will approach the δ¹⁸O-H₂O of the medium and may eliminate the initial δ¹⁸O-O₂ signal.
Figure 3.2: Schematic representation of the variability in $\delta^{18}$O-NO$_3^-$ with different amounts of abiotic O-exchange between NO$_2^-$ and H$_2$O during nitrification and denitrification. This schematic is assuming internal sources of NO$_3^-$ only (no additional NO$_3^-$ sources entering from the environment). Solid lines represent a hypothetical trajectory of values for $\delta^{18}$O-NO$_3^-$ as NO$_3^-$ concentration increases or decreases and no O-exchange has occurred. Dashed lines represent varying amounts of O-exchange. The grey box is a region where $\delta^{18}$O-NO$_3^-$ values are not likely to occur. The grey dashed arrow indicates NO$_3^-$ uptake, which was not expected to influence NO$_3^-$ production or NO$_3^-$ isotope values.

Prior to research on the equilibrium isotope effect and estimates of the O isotope effects for nitrification, neither large kinetic $^{18}$O fractionations nor the effect of isotopic equilibrium with water were considered in NO$_3^-$ isotope research. Instead, the $\delta^{18}$O-NO$_3^-$ values that were used were estimated from the “1:2 rule” for oxygen incorporation (Snider et al., 2010) rather than actual calculated values (Equation 3.1).

$$\delta^{18}O - NO_3^{-(relVSMOW)} = \left[\frac{1}{3} R_{O_2} + \frac{2}{3} R_{H_2O}\right] \div R_{VSMOW} - 1$$

Equation 3.1
Where \( R \) is the \(^{18}\text{O}/^{16}\text{O} \) ratio of \( \text{O}_2, \text{H}_2\text{O} \) or VSMOW (Snider et al., 2010). Fewer than 10% of the values of \( \delta^{18}\text{O}-\text{NO}_3^- \) produced from nitrification in groundwater, surface water and soils collected from field data fit theoretical values for \( \delta^{18}\text{O}-\text{NO}_3^- \) formed from nitrification using the “1:2 rule” (Snider et al., 2010). They showed that using this model to estimate \( \delta^{18}\text{O}-\text{NO}_3^- \) values is problematic and does not work in many systems.

Recent literature has enhanced our understanding of kinetic isotope effects and O-exchange during nitrification and denitrification. Snider et al. (2010) were the first to consider the large kinetic effects, the equilibrium isotope effect and the fraction of O-exchange (\( f_{\text{abiotic}} \)) in the formation of \( \delta^{18}\text{O}-\text{NO}_3^- \) during nitrification in soils (Equation 3.2). Their study highlighted the importance of oxygen isotope effects and the variability in O-exchange during nitrification to alter the final \( \delta^{18}\text{O}-\text{NO}_3^- \) value. Snider et al. (2010) conducted incubation experiments with three levels of \(^{18}\text{O} \) enriched water and three soil types. They calculated the \( f_{\text{abiotic}} \) for the different soil types and found that it differed among the soils types and differed from the \( f_{\text{abiotic}} \) that was calculated based on the 1:2 model for O-incorporation.

\[
\delta^{18}\text{O} - \text{NO}_3^- = \frac{1}{3} (2 + f_{\text{abiotic}}) \delta^{18}\text{O} - \text{H}_2\text{O} + \frac{1}{3} f_{\text{abiotic}} (2 \delta^{18}\text{O} - \delta^{18}\text{O} - 2^{18}\text{O}_2 - 2^{18}\text{H}_2\text{O}) + \delta^{18}\text{O} - \text{O}_2 + 2^{18}\text{O}_2 + 2^{18}\text{H}_2\text{O} + 2^{18}\text{H}_2\text{O})
\]

Equation 3.2

Although the \( \delta^{18}\text{O}-\text{NO}_3^- \) cannot be used to indicate that denitrification has altered the isotopic values in productive rivers like the Grand River, it may be possible that \( \text{NO}_3^- \) isotopes can be used to obtain the relative rates of \( \text{NO}_3^- \) transformation processes (nitrification and denitrification). Calculating the \( f_{\text{abiotic}} \) can provide insight into how much O-exchange has occurred relative to what is expected with the 1:2 “rule” for O incorporation (Equation 3.3 and Equation 3.4).
When no abiotic O-exchange has occurred between NO₂⁻ and H₂O such that \( f_{\text{abiotic}} = 0 \) then:

\[
\delta^{18}O - NO_2^- = \frac{2}{3} (\delta^{18}O - H_2O) + \frac{1}{3} (\delta^{18}O - O_2 + \epsilon_{O_2} + \epsilon_{H_{2O,1}} + \epsilon_{H_{2O,2}})
\]

Equation 3.3

When O-exchange has occurred and NO₂⁻ and H₂O have fully equilibrated such that \( f_{\text{abiotic}} = 1 \) then:

\[
\delta^{18}O - NO_2^- = \delta^{18}O - H_2O + \frac{2\epsilon_{O_2} + \epsilon_{H_{2O,2}}}{3}
\]

Equation 3.4

This research focuses on how abiotic O-exchange and large isotope effects can alter the isotopic values of riverine NO₃⁻. However, it is important to also understand the two other potential mechanisms that could alter the \( \delta^{18}O-\text{NO}_3^- \) and result in varying end-member NO₃⁻ isotopic values: diel changes in \( \delta^{18}O-\text{O}_2 \) and different nitrifying organisms.

Temporal and spatial changes of \( \delta^{18}O-\text{O}_2 \) have been identified in several Canadian streams and reservoirs (Venkiteswaran et al., 2015; Venkiteswaran et al., 2008; Venkiteswaran et al., 2007; Wassenaar et al., 2010). Reported diel values of \( \delta^{18}O-\text{O}_2 \) in the Grand River range between +6‰ and +29‰ with the lowest \( \delta^{18}O \) values observed in the central part of the river where \( O_2 \) saturation is the highest (Venkiteswaran et al., 2015). Dynamics of \( O_2 \) should be considered because a value greater than or less than the atmospheric \( \delta^{18}O-\text{O}_2 \) constant of +24.2‰ can drastically alter the end member nitrification values of \( \delta^{18}O-\text{NO}_3^- \) especially in a productive system.

River biofilms are composed of complex assemblages of microorganisms. Isotopic effects of nitrifying bacteria (AOB) have been extensively studied (Buchwald & Casciotti, 2010; Casciotti et al., 2010; Kool et al., 2007). However, isotopic effects of ammonia oxidizing
archaea (AOA) have not. Several studies document a substantial presence of AOA (Gubry-Rangin et al., 2010; Leininger et al., 2006; Sonthiphand et al., 2013) compared to ammonia oxidizing bacteria (AOB). This presents another mechanism with which the end member $\delta^{18}$O-NO$_3^-$ nitrification signal could be altered as AOA and AOB have potentially different ammonia-oxidation pathways (Stahl & de la Torre, 2012; Walker et al., 2010). There have been several proposed AOA oxidation pathways (Stahl & de la Torre, 2012), however most of these have recently been disproved (Kozlowski et al., 2016) and still very little is known. There have been no definitive results that suggest different isotopic fractionations between the two groups (Nishizawa et al., 2016), however this research is very limited. Comammox bacteria, which oxidize NH$_4^+$ all the way to NO$_3^-$ could also have the potential to alter the $\delta^{18}$O-NO$_3^-$ during nitrification, however the comammox pathway has yet to be studied but may resemble a combination of AOB and NOB (nitrite oxidizing bacteria) pathways (Laura Sauder, personal communication April 2017).

Biofilm covers the river bottom and submerged rocks and vegetation in the Grand River. This biofilm has been shown to influence river metabolism and rapid in-river N cycling is evident in the isotopic values (Chapter 2). Two contrasting productivity scenarios could exist with regard to O-exchange: 1) A productive river with rapidly cycling N will afford a greater opportunity for O-exchange to occur; or 2) A productive river with rapidly cycling N will have less O-exchange because the half-life of NO$_2^-$ is short. It is possible for both these scenarios to be occurring in the river but it is difficult to discern without further isotopic analysis.

The amount of O-exchange (fabiotic) can be used in conjunction with the $^{18}$O isotope effects during nitrification (Figure 3.1) in a model to obtain rates of processes. The $\delta^{15}$N-NO$_3^-$ is equally important in the model to further constrain rates of N cycling processes and further provide a look at turnover in the NO$_3^-$ pool. Based on the findings from Chapter 2, the $\delta^{15}$N should show predictable changes over time in the model and the $\delta^{18}$O should vary with respect to the $\delta^{18}$O-H$_2$O of the medium when rates (and concentrations) are held
constant. If NO$_3^-$ isotopes can be used to estimate rates of processes then the fate of NO$_3^-$ and how it is cycled in the river can be understood.

The objectives of this chapter are to: (1) Observe any large isotopic effects and calculate the amount of O-exchange between NO$_2^-$ and H$_2$O that alters the observed/measured $\delta^{18}$O values in the Grand River, (2) develop and apply a mechanistic model that can be used to explain the variation in observed $\delta^{18}$O-NO$_3^-$ values in incubation experiments and in the Grand River and (3) determine gross rates of NO$_3^-$ cycling in the Grand River and incubation experiments to understand the fate of NO$_3^-$ using the mechanistic model.

3.2 Methods

Incubations of river water from West Montrose and Blair on the Grand River (described in Chapter 2) were used to calculate the fraction of abiotic O-exchange (fabiotic) during nitrification and without denitrification at these sites. The fabiotic for each incubation (West Montrose July 2015, West Montrose September 2015 and Blair July 2015) was calculated in a similar manner to Snider et al. (2010) using the slopes from the model II regression of the $\delta^{18}$O-NO$_3^-$ values at T60 hours (T72 hours for the West Montrose September incubation) for each of the Low, Medium and High $\delta^{18}$O-H$_2$O treated beakers. The model II regressions were completed in R with the lm2 model package (Legendre, 2014). To correct for differing NO$_3^-$ production rates, final $\delta^{18}$O-NO$_3^-$ values were normalized for NO$_3^-$ production using an isotope mass balance (Equation 3.5).

$$\delta^{18}O - NO_3^- = \frac{(\delta^{18}O_{(f)} \cdot NO_3^-_{(f)}) - (\delta^{18}O_{(i)} \cdot NO_3^-_{(i)})}{NO_3^-_{(f)} - NO_3^-_{(i)}}$$

Equation 3.5

Where $\delta^{18}O - NO_3^-_{(N)}$ represents the NO$_3^-$ normalized $\delta^{18}$O value used in the model II regression to calculate the fabiotic. $\delta^{18}O_{(f)}$ and $NO_3^-_{(f)}$ are the $\delta^{18}$O-NO$_3^-$ value and the NO$_3^-$ concentration at the final time point of the incubation experiments. While $\delta^{18}O_{(i)}$ and $NO_3^-_{(i)}$
are the δ¹⁸O-NO₃⁻ value and the NO₃⁻ concentration at the start of the incubations (Snider et al., 2010).

The fabiotic expected if the δ¹⁸O-NO₃⁻ derived from nitrification were formed by only one δ¹⁸O-O₂ and two δ¹⁸O-H₂O was calculated using the 1:2 “rule” for O-incorporation (Equation 3.6). The fabiotic value formed from the 1:2 “rule” for O-incorporation during nitrification and without denitrification was used to compare the fabiotic from the incubation experiments. The \( \frac{2}{3} \) represents the two δ¹⁸O-H₂O and the \( \frac{1}{3} \) represents the one δ¹⁸O-O₂ that are incorporated during nitrification. The +24.2‰ is the atmospheric δ¹⁸O-O₂ constant for O₂ dissolved in water and is used in this calculation assuming river water had fully equilibrated with atmospheric air and no respiratory O₂ consumption took place. This value was also assumed to be constant throughout the duration of the incubation experiments:

\[
y = \frac{2}{3}x + \frac{1}{3}(24.2)
\]

Equation 3.6

3.2.1 NO₃⁻ Isotope Model

To examine the rates of N cycling processes in the incubation experiments (Chapter 2) a box model was created using Stella version 10.0.1, http://www.iseesystems.com/(Appendix A). The model had three stocks: NO₃⁻, ¹⁸O-NO₃⁻ and ¹⁵N-NO₃⁻ (Figure 3.3) and included rates as well as isotope effects for nitrification, denitrification, mineralization and uptake (Table 3.1). The model produced transient curves of NO₃⁻ concentration and NO₃⁻ isotopes that were compared with data from the incubations experiments. Data was modeled for two different time periods for each incubation experiment: Time 0-24 and Time 24-60. The model assumed that O-exchange only occurred in nitrification and the amount of O-exchange that occurred was constant. It also assumed that the δ¹⁸O-O₂ and δ¹⁸O-H₂O was constant throughout the experiment and in the river and first order rates (nitrification, denitrification and uptake) were constant over time and assumed constant production (zero order kinetics)
of NH₄⁺ over time. This model was based on a closed system meaning no new N inputs from upstream environments or the atmosphere was added. The ranges in isotope values (Table 3.1) were considered in this model and median values were used where necessary (¹⁸ε Uptake and ¹⁸ε Denitrification). The intercept value and fabiotic calculated (Figure 3.4) were used in Error! Reference source not found. to obtain the value for ¹⁸ε Nitrification in the model (Appendix A) to encompass any large variation in isotope effects (¹⁸εO₂, ¹⁸εH₂O₁ and ¹⁸εH₂O₂). Model curves were obtained through inverse modelling. Observed incubation values for NO₃⁻ concentration and NO₃⁻ isotopes as well as calculated values like the fabiotic were input and gross rates were determined based on the fit of the model output values to the observed incubation values.
Figure 3.3: Simplified schematic representation of the box model for estimating rates of N cycling processes: nitrification, denitrification, mineralization and uptake for three stocks $^{15}$N-NO$_3^-$ (A), NO$_3^-$ (B) and $^{18}$O-NO$_3^-$ (C). Arrows represent connecting links between the three stocks.
Table 3.1: Ranges of literature values for the fractionation factors used in the model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value Ranges (‰)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{15}\varepsilon$ Mineralization</td>
<td>+1</td>
<td>(Kendall, 1998)</td>
</tr>
<tr>
<td>$^{15}\varepsilon$ Nitrification</td>
<td>-29 to -12</td>
<td>(Shearer &amp; Kohl, 1988)</td>
</tr>
<tr>
<td>$^{15}\varepsilon$ Uptake</td>
<td>-8 to -5</td>
<td>(Granger et al., 2010)</td>
</tr>
<tr>
<td>$^{15}\varepsilon$ Denitrification</td>
<td>-40 to -12</td>
<td>(Kendall, 1998)</td>
</tr>
<tr>
<td>$^{\delta^{15}}N$ Biomass</td>
<td>+11</td>
<td>Measured, N=6 (Schiff, unpublished data)</td>
</tr>
<tr>
<td>$^{18}\varepsilon$ Nitrification (Equation 3.2):</td>
<td>$\delta^{18}O - NO_3^{\text{added}} = \frac{1}{3}(2 + f_{\text{ABiotic}})\delta^{18}O - H_2O + \frac{1}{3}[f_{\text{ABiotic}}(2^{\delta^{18}}eq - \delta^{18}O - O_2 - ^{18}O_{02} - ^{18}O_{H2O,1}) + \delta^{18}O - O_2 + ^{18}O_{02} + ^{18}O_{H2O,1} + ^{18}O_{H2O,2}]$</td>
<td>(Snider et al., 2010)</td>
</tr>
<tr>
<td>$^{18}\varepsilon$O$_2$ + $^{18}\varepsilon$H$_2$O,1</td>
<td>-38 to -18</td>
<td>(K. L. Casciotti et al., 2010)</td>
</tr>
<tr>
<td>$^{18}\varepsilon$H$_2$O,2</td>
<td>-25 to -9</td>
<td>(Buchwald &amp; Casciotti, 2010)</td>
</tr>
<tr>
<td>$^{\varepsilon_{eq}}$</td>
<td>+14</td>
<td>(Casciotti et al., 2007)</td>
</tr>
<tr>
<td>$^{18}\varepsilon$ Uptake</td>
<td>-8 to -5</td>
<td>(Granger et al., 2010)</td>
</tr>
<tr>
<td>$^{18}\varepsilon$ Denitrification</td>
<td>-23 to -5</td>
<td>(Granger et al., 2008)</td>
</tr>
</tbody>
</table>
3.3 Results

3.3.1 Abiotic O-Exchange and Equilibrium Isotope Effects

The δ¹⁸O-NO₃⁻ in each of the beakers from the three incubations were strongly correlated with the δ¹⁸O-H₂O values in the medium with r² values for Time 0-24 hours of 0.98 and 0.99 for West Montrose July 2015 and Blair July 2014, respectively and r² values for Time 24-60 of 0.98, 0.99 and 0.99 for West Montrose July 2015, West Montrose September 2015 and Blair July 2015, respectively (Figure 3.4). The slopes of the lines of each incubation experiment for both time periods were different than the slope calculated from the “1:2” rule of 0.67.

The $f_{\text{abiotic}}$ calculated for each incubation experiment and both time periods were all greater and statistically significant than the $f_{\text{abiotic}}$ from the “1:2” rule of 0 (Table 3.2). The $f_{\text{abiotic}}$ was always higher in the first 24 hours and corresponded to some of the greatest changes in δ¹⁸O-NO₃⁻ when NO₃⁻ changed the least (Table 2.1 and Table 2.3). The slope of 0.90 for the West Montrose July 2015 incubation during the time period of 24-60 hours was not statistically significantly different from the slope of 0.92 for the West Montrose September 2015 incubation but was different from a slope of 0.80 for the Blair 2015 incubation (Appendix A). The calculated y-intercept for each experiment ranged between 3.5‰ to 9‰ in the first 24 hours and -1.75‰ to 2.8‰ in the second time period (24-60 or 72 hours). These experimental intercepts were almost all different from the intercept of 8.06‰ calculated with the “1:2” rule.
Figure 3.4: $\delta^{18}$O-NO$_3^-$ values of NO$_3^-$ added from nitrification vs. $\delta^{18}$O-H$_2$O. Three regression lines were drawn from the results of the model II regression for two time periods: 0-24 hours (A) and 24-60 hours (B). West Montrose July 2015 is in orange and the shapes represent the actual incubation data values. The second West Montrose experiment in September 2015 is shown in purple and the Blair July 2015 experiment is in green. The black dashed line represents the fourth regression drawn from the “1:2” rule for oxygen incorporation during nitrification. The second West Montrose incubation (September) could not be represented in the first 24 hours because the change in concentration was so small that net $\delta^{18}$O of NO$_3^-$ added could not be calculated.
Table 3.2: The fraction of abiotic O-exchange between $\text{H}_2\text{O}$ and $\text{NO}_2^-$ during nitrification was calculated the same as in Snider et al. (2010) using the slopes from Figure 3.4 and Equation 3.2. Slopes, $f_{\text{abiotic}}$ and y-intercepts were found for each incubation experiment and the two different time periods.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time Period (Hours)</th>
<th>Slope</th>
<th>$f_{\text{abiotic}}$</th>
<th>y-Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>“1:2” Rule (Snider et al., 2010)</td>
<td>-</td>
<td>0.67</td>
<td>0</td>
<td>8.06</td>
</tr>
<tr>
<td>West Montrose July 2015</td>
<td>0-24</td>
<td>0.97</td>
<td>0.88</td>
<td>9.32</td>
</tr>
<tr>
<td>West Montrose July 2015</td>
<td>24-60</td>
<td>0.90</td>
<td>0.70</td>
<td>0.12</td>
</tr>
<tr>
<td>West Montrose September 2015</td>
<td>0-24</td>
<td>-</td>
<td>0.88*</td>
<td>-1.75*</td>
</tr>
<tr>
<td>West Montrose September 2015</td>
<td>24-60</td>
<td>0.92</td>
<td>0.77</td>
<td>-1.75</td>
</tr>
<tr>
<td>Blair July 2015</td>
<td>0-24</td>
<td>1.02</td>
<td>1.06</td>
<td>3.53</td>
</tr>
<tr>
<td>Blair July 2015</td>
<td>24-60</td>
<td>0.80</td>
<td>0.43</td>
<td>2.79</td>
</tr>
</tbody>
</table>

*$f_{\text{abiotic}}$/intercept was approximated because concentration change between T0-T24 was so small that net $\delta^{18}$O added could not be calculated.

3.3.2 NO$_3^-$ Isotope Best-fit Model

Small changes in model parameters were found to generate predictable and important changes in the model output (Figure 3.5). Four biogeochemical rate constants and three levels of enriched $\delta^{18}$O-$\text{H}_2\text{O}$ clearly produced different results in the model output patterns for concentrations and NO$_3^-$ isotope values of two distinct time periods (0-24 hours and 24-60 or 72 hours). The model results for all three experiments: West Montrose July 2015 (Figure 3.6), West Montrose September 2015 (Figure 3.7) and Blair July 2015 (Figure 3.8) had strong correlations with the actual incubation data for all concentrations and NO$_3^-$ isotopes (Table 3.3, Table 3.4 and Table 3.5). This model could almost entirely explain the variability in the NO$_3^-$ concentrations, $\delta^{18}$O-NO$_3^-$ and $\delta^{15}$N-NO$_3^-$ from the incubation data sets (Table 3.3, Table 3.4 and Table 3.5). The NO$_3^-$ concentrations were almost always within measurement error (<0.05 mg N/L) as were the NO$_3^-$ isotope values (<1‰).
Gross rates of uptake were held constant at 0.010 µg N L\(^{-1}\) h\(^{-1}\) for both time periods and all three experiments because uptake was not expected to change (Table 3.6). Rates of nitrification and mineralization were almost always the lowest in the first 24 hours (44-67 µg N L\(^{-1}\) h\(^{-1}\)) where \(\text{fabiotic}\) was the highest and NO\(_3^-\) concentrations changed the least (Table 2.1) during all three experiments. Higher rates of nitrification and mineralization (40 to 122 µg N L\(^{-1}\) h\(^{-1}\)) were observed during 24-60 hours for the first West Montrose (July 2015) and Blair incubations when increase in NO\(_3^-\) concentration was the greatest. The nitrification and mineralization rates for the second West Montrose experiment (September 2015) had the lowest rates of the three incubation experiments and changed little between the two time periods. This experiment overall had the smallest change in NO\(_3^-\) concentration in both 0-24 hours and 24-60 hours time periods. The greatest overall gross rates of nitrification and mineralization were observed in the Blair experiment where the greatest changes in NO\(_3^-\) concentrations and NO\(_3^-\) isotopes were observed (Table 2.1, Table 2.2 and Table 2.3).

Rates of denitrification were expected to be less than the rates of nitrification and mineralization because the experimental system was well oxygenated, stirred and the biofilm layer was thin. Gross rates were low and ranged between 0.10 to 4.0 µg N L\(^{-1}\) h\(^{-1}\) depending on the incubation beaker. There was no distinctive trend between the two time periods and were very similar across all three experiments.

Literature values reported for gross NO\(_3^-\) consumption (denitrification + uptake) ranged between 0.044 to 0.084 µg N cm\(^{-2}\) h\(^{-1}\) (Table 3.7) and average incubation rates corrected for biomass area ranged between 0.025 to 0.031 µg N cm\(^{-2}\) h\(^{-1}\) (Table 3.6). Gross rates are similar to literature values. In general, gross rates are not often reported because they more difficult to obtain than net rates so there was not much to compare in terms of catchment characteristics, etc. No literature rates were found for gross NO\(_3^-\) release (mineralization and nitrification).
Figure 3.5: Model sensitivity analysis for five model parameters: three first order rate constants ($k_{\text{Denitrification}}$, $k_{\text{Mineralization}}$ and $k_{\text{Nitification}}$), the $f_{\text{abiotic}}$ and the y-intercept of Equation 3.2. Three outcomes are shown for $\delta^{15}\text{N-NO}_3^-$, $\delta^{18}\text{O-NO}_3^-$ and NO$_3^-$ concentration.
Figure 3.6: Modeled results of the first incubation experiment at the West Montrose site conducted on July 7, 2015. The modeled output values are depicted as the solid lines and the observed incubation values are the dots. $\delta^{15}$N-NO$_3^-$ (top), $\delta^{18}$O-NO$_3^-$ (middle) and NO$_3^-$ concentration (bottom) were the parameters modeled vs. time. The model used a calculated fabiotic (Figure 3.4) of 0.88 and 0.70 (for 0-24 hours and 24-60 hours, respectively), fractionation factors for N cycling processes (Appendix A), and incubation data (Figure 2.11) to look at the rates of N cycling processes. Incubation water that was not spiked with enriched $\delta^{18}$O-H$_2$O (Low) is shown in purple. The incubation water that was spiked with enriched $\delta^{18}$O-H$_2$O was shown as orange (Medium = +50‰) and green (High = +90‰).
Figure 3.7: Modeled results of the incubation experiment conducted on September 2, 2015 at West Montrose. The model used an estimated fabiotic of 0.88 for 0-24 hours and a calculated (Figure 3.4) of 0.77 for 24-72 hours, fractionation factors for N cycling processes (Appendix A), and incubation data to look at the rates of N cycling processes (Figure 2.12). Incubation water that was not spiked with enriched δ¹⁸O-H₂O (Low) is depicted as purple lines (modeled) and dots (actual incubation values). The incubation water that was spiked with enriched δ¹⁸O-H₂O was shown as orange (Medium = +50‰) and green (High = +90‰) lines and dots. NO₃⁻ (top), δ¹⁸O-NO₃⁻(middle) and δ¹⁵N-NO₃⁻ (bottom) were the parameters modeled.
Figure 3.8: Modeled results of the Blair incubation experiment conducted on July 29, 2015. The model used calculated fabiotic values from Figure 3.4 of 1.06 and 0.43 for Time (0-24 and 24-60 hours, respectively), fractionation factors for N cycling processes (Appendix A), and incubation data to look at the rates of N cycling processes (Figure 2.13). Incubation water that was not spiked with enriched δ¹⁸O-H₂O (Low) is depicted as purple lines (modeled) and dots (actual incubation values). The incubation water that was spiked with enriched δ¹⁸O-H₂O was shown as orange (Medium = +50‰) and green (High = +90‰) lines and dots. NO₃⁻ (top), δ¹⁸O-NO₃⁻ (middle) and δ¹⁵N-NO₃⁻ (bottom) were the parameters modeled.
Table 3.3: Root mean squared error values for $\delta^{15}$N-$\text{NO}_3^-$ (‰) for each modeled incubation experiment

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-24 h</td>
<td>24-60 h</td>
<td>0-24 h</td>
</tr>
<tr>
<td>West Montrose</td>
<td>0.11</td>
<td>0.28</td>
<td>0.58</td>
</tr>
<tr>
<td>July 2015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>West Montrose</td>
<td>0.11</td>
<td>0.84</td>
<td>0.23</td>
</tr>
<tr>
<td>September 2015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blair July 2015</td>
<td>0.058</td>
<td>0.19</td>
<td>0.053</td>
</tr>
</tbody>
</table>

Table 3.4: Root mean squared error values for $\delta^{18}$O-$\text{NO}_3^-$ (‰) for each modeled incubation experiment

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-24 h</td>
<td>24-60 h</td>
<td>0-24 h</td>
</tr>
<tr>
<td>West Montrose</td>
<td>0.17</td>
<td>0.44</td>
<td>0.24</td>
</tr>
<tr>
<td>July 2015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>West Montrose</td>
<td>0.21</td>
<td>0.47</td>
<td>0.18</td>
</tr>
<tr>
<td>September 2015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blair July 2015</td>
<td>0.14</td>
<td>0.37</td>
<td>4.45</td>
</tr>
</tbody>
</table>
Table 3.5: Root mean squared error values for NO$_3^-$ (mg N/L) for each modeled incubation experiment

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-24 h</td>
<td>24-60 h</td>
<td>0-24 h</td>
</tr>
<tr>
<td>West Montrose</td>
<td>0.073</td>
<td>0.053</td>
<td>0.029</td>
</tr>
<tr>
<td>July 2015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>West Montrose</td>
<td>0.013</td>
<td>0.014</td>
<td>0.025</td>
</tr>
<tr>
<td>September 2015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blair July 2015</td>
<td>0.021</td>
<td>0.058</td>
<td>0.021</td>
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<tr>
<td></td>
<td></td>
<td></td>
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</table>
Table 3.6: Average Gross rates of N cycling processes for the West Montrose July and September 2015 incubations and the Blair July 2015 incubation. Gross rates were corrected for the West Montrose July 2015 and the Blair July 2015 incubation experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>0-24 Hours (µg N L⁻¹ h⁻¹)</th>
<th>0-24 Hours (µg N cm⁻² h⁻¹)</th>
<th>24-60 Hours (µg N L⁻¹ h⁻¹)</th>
<th>24-60 Hours (µg N cm⁻² h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>West Montrose July 2015</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineralization</td>
<td>52.7</td>
<td>0.38</td>
<td>60.3</td>
<td>0.45</td>
</tr>
<tr>
<td>Nitrification</td>
<td>44.7</td>
<td>0.30</td>
<td>84.4</td>
<td>0.65</td>
</tr>
<tr>
<td>Denitrification</td>
<td>4.07</td>
<td>0.025</td>
<td>2.73</td>
<td>0.023</td>
</tr>
<tr>
<td>Uptake</td>
<td>0.010</td>
<td>7.43x10⁻⁵</td>
<td>0.010</td>
<td>7.43x10⁻⁵</td>
</tr>
<tr>
<td><strong>West Montrose September 2015</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineralization</td>
<td>30.3</td>
<td></td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td>Nitrification</td>
<td>67.7</td>
<td></td>
<td>39.9</td>
<td></td>
</tr>
<tr>
<td>Denitrification</td>
<td>0.47</td>
<td></td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Uptake</td>
<td>0.010</td>
<td></td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td><strong>Blair July 2015</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineralization</td>
<td>46.0</td>
<td>0.29</td>
<td>71.5</td>
<td>0.51</td>
</tr>
<tr>
<td>Nitrification</td>
<td>59.0</td>
<td>0.33</td>
<td>122.3</td>
<td>0.57</td>
</tr>
<tr>
<td>Denitrification</td>
<td>0.50</td>
<td>0.031</td>
<td>0.500</td>
<td>0.031</td>
</tr>
<tr>
<td>Uptake</td>
<td>0.010</td>
<td>6.23x10⁻⁵</td>
<td>0.010</td>
<td>6.23x10⁻⁵</td>
</tr>
</tbody>
</table>

Table 3.7: Gross literature rates from different stream and watershed characteristics.

<table>
<thead>
<tr>
<th>Rate (µg N cm⁻² h⁻¹)</th>
<th>Source</th>
<th>Stream/Watershed Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.040</td>
<td>(Mulholland et al., 2008)</td>
<td>1st order stream, forested catchment</td>
</tr>
<tr>
<td>0.080</td>
<td>(Ensign &amp; Doyle, 2006)</td>
<td>Compilation of 404 studies with stream orders 1-5 and mixed catchments</td>
</tr>
</tbody>
</table>
3.4 Discussion

Productive systems allow for build-up of inorganic N and/or rapid N cycling that would allow for greater O-exchange. A mechanistic model can be used to get at the rates of NO$_3^-$ transformation processes in the Grand River.

i. Role of O-exchange between NO$_2^-$ and H$_2$O in the Grand River

The fabiotic calculated for each of the three incubation experiments provided valuable information for two Grand River sampling sites. All the slopes of the regression lines (Figure 3.4) were greater than 0.67 (“1:2” rule) and the calculated fabiotic values were greater than 0. The greatest fabiotic O-exchange was in the first 24 hours of each incubation experiment when NO$_3^-$ concentrations changed the least. This clearly indicates substantial isotopic O-exchange between NO$_2^-$ and H$_2$O that altered the δ$^{18}$O-NO$_3^-$ value produced from nitrification so that it no longer represents the “1:2” rule. This presents further evidence that the δ$^{18}$O of NO$_3^-$ is not conservative because of large amounts of biological cycling and O-exchange.

ii. Can a mechanistic model explain the variation in the observed experimental data?

The model was successful in reproducing the patterns observed in the incubation experiments (Chapter 2) and adequately described N biogeochemical cycling in the Grand River at two locations with different NO$_3^-$ source inputs.

The δ$^{15}$N-NO$_3^-$ predictably decreased by the same amount in each of the incubation experiments and thus was useful for constraining the rates of the NO$_3^-$ transformation processes.

The fabiotic was useful for best-fit modeling in each of the incubations. The greater the fabiotic, the more δ$^{18}$O-NO$_3^-$ values tended to shift toward the respective δ$^{18}$O-H$_2$O of the experimental medium, further confirming that this shift was the result of isotopic O-
exchange between NO₂⁻ and H₂O. The δ¹⁸O-O₂ signal (+24.2‰) present during the first oxidation step in nitrification is effectively eliminated in the NO₃⁻ isotope value after nitrification. This also means that if isotopic O-exchange were to occur between H₂O and denitrification intermediates (in addition to nitrification), the denitrification signal as well as any other potential NO₃⁻ source signals would be eliminated.

The y-intercept that was calculated (Figure 3.4) varied between the two time points (0-24 hours and 24-60 or 72 hours) and between each of the three experiments. The y-intercept is a function of the δ¹⁸O-O₂ as well the potentially large isotope effects associated with the incorporation of O during nitrification (Figure 3.1 and Equation 3.2). The unknown affect of dielectric shifts in δ¹⁸O-O₂ on the final NO₃⁻ isotope values as well as the variability of ¹⁸O isotopic fractionation amplifies the complexity of apportioning NO₃⁻ sources.

iii. Can isotopes of NO₃⁻ be used to estimate the gross rates of NO₃⁻ cycling in the Grand River?

Gross rates were successfully estimated for the West Montrose and Blair incubation experiments. The Blair experiment has the highest overall rates of nitrification and mineralization that likely resulted from the effects of the upstream WWTPs. The second West Montrose experiment conducted in September 2015 had the lowest overall rates of NO₃⁻ transformation processes which could have been a function of seasonality if the biofilm is more active in mid summer (July) compared to early fall (September). The model was able to capture differences between sites and thus can be used as a tool for determining the variability in the river metabolism that is caused by different source inputs.

These results and the success of the mechanistic model demonstrate that in-river metabolism has considerable effect on NO₃⁻ isotope values. Although there are still many unknowns (diel δ¹⁸O-O₂, large ¹⁸O isotopic effects and variable microbial communities) the isotopes still provide a useful tool to quantify consumption and release of NO₃⁻ in an impacted river such as the Grand River.
3.5 Conclusion

The fraction of abiotic O-exchange (fabiotic) between NO$_2^-$ and H$_2$O was successfully calculated using a model II regression for all three incubation experiments. All the regression slopes for each incubation and two different time periods (0-24 hours and 24-60 or 72 hours) were greater than the slope dictated by the “1:2 rule” for O-incorporation during nitrification. The fabiotic ranged between 0.88-1.06 for two Grand River sites in the first 24 hours and ranged between 0.43 and 0.77 after 24 hours. A large amount of O-exchange was evident at both West Montrose and Bridgeport. The largest fabiotic was in the first 24 hours of the Blair experiment (1.06). A higher fabiotic meant a greater shift in δ$^{18}$O-NO$_3^-$ toward the δ$^{18}$O-H$_2$O of the medium. O-exchange plays a substantial role in NO$_3^-$ cycling and it was important to determine the extent to which it occurs in the Grand River.

The biofilm in the Grand River had a considerable impact on the in-river metabolism and resulting isotopic values that were observed and not conservative. It is difficult however, to determine whether the O-exchange that occurred was the result of a productive river where rapid N cycling affords a greater opportunity for exchange or a productive river with slower rates where NO$_2^-$ persists and affords a greater opportunity for O-exchange.

The modeled data fit very well with the concentrations and NO$_3^-$ isotope data in all three incubations and was able to explain almost all variability. Estimates of gross rates of mineralization, nitrification, denitrification and uptake were successfully obtained. These relative rates of NO$_3^-$ transformations processes estimated from the model can be up-scaled to the river assuming that a surface area measurement of the river bottom is made. These rates in conjunction with estimations of the fraction of abiotic O-exchange provide valuable information on the fate of NO$_3^-$ in the river. Higher rates of mineralization and nitrification mean a greater internal release of NO$_3^-$ to the river whereas lower rates of mineralization and nitrification and greater rates of denitrification and/or uptake mean the river is more of an internal NO$_3^-$ sink.
Overall these results confirmed the findings from Chapter 2 that δ¹⁵N- and δ¹⁸O-NO₃⁻ are not conservative and cannot be used to indicate denitrification or NO₃⁻ sources because of in-river biological cycling. The combined fabiotic and ¹⁸O isotope effects that occurred produced results that could not be predicted by the “1:2 rule” (Equation 3.1) but were best described by Equation 3.2. Researchers that have used or continue to use the “1:2 rule” disregard large ¹⁸O isotope effects and isotopic O-exchange between NO₂⁻ and H₂O that influence riverine NO₃⁻ isotopic values.

This model will offer researchers and watershed managers (that use NO₃⁻ isotopes for source apportionment) with a new tool that provides estimates on the relative rates of N cycling processes. Having gross rates rather than only net rates is important because they are inclusive of all microbial processing rather than having to infer NO₃⁻ transformation processes by only a change in concentration. Understanding the fate of NO₃⁻ in the river using these rates of NO₃⁻ transformation processes will provide better estimates of N recycling and the contribution of microbially derived NO₃⁻ to the mixed source pool.
Chapter 4 – Conclusions and Recommendations

4.1 Conclusions

Nitrate (NO$_3^-$) is the most ubiquitous contaminant in surface and groundwaters in Canada, NO$_3^-$ is transformed in the river by two main processes: nitrification (oxidation of NH$_4^+$ to NO$_3^-$) and denitrification (reduction of NO$_3^-$ to N$_2$). The catchment of the Grand River is dominated by agricultural use (~80%). The Grand River receives NO$_3^-$ loading from point (urban WWTPs) and non-point sources (agricultural manure and fertilizer). NO$_3^-$ loading threatens drinking water quality where water is drawn directly from the river as well as overall aquatic ecosystem health.

The use of $\delta^{18}$O- and $\delta^{15}$N-NO$_3^-$ isotopes has been extensively used in source apportionment studies to differentiate between NO$_3^-$ inputs and transforming processes such as nitrification and denitrification in rivers, groundwater, soils and surface water. Sources of NO$_3^-$ in the Grand River could be estimated with isotopes if they work as conservative tracers. When looking at seasonal NO$_3^-$ isotope data from the Grand River there is no clear denitrification trend or seasonal trend but the river is highly productive. A source plot of this data suggested that all NO$_3^-$ was derived from a nitrified WWTP or manure source; however, it is unlikely that the soil-N signal is not observed, especially in agricultural headwaters where livestock populations are lower.

Three laboratory-beaker incubations with three levels of isotopically labeled river water and biofilm covered rocks demonstrated that $\delta^{15}$N- and $\delta^{18}$O-NO$_3^-$ are not conservative. Two distinct phases that occurred in all three experiments: one where NO$_3^-$ concentrations did not change over time (0-24 hours), and the other with a net increase in NO$_3^-$ concentration over time (24-60 hours). In all three experiments, the $\delta^{15}$N-NO$_3^-$ decreased consistently toward the respective $\delta^{15}$N of biomass value for each site during both time periods. The $\delta^{18}$O-NO$_3^-$ was altered relative to the $\delta^{18}$O-H$_2$O such that three distinct water labels were evident by the end of the experiment. The NO$_3^-$ isotopes were altered even when NO$_3^-$
concentrations did not change. This result proved that the δ^{18}O-NO₃⁻ values were driven by the δ^{18}O-H₂O of the medium while the δ^{15}N of biomass, as well as the δ^{15}N-NH₄⁺ being nitrified and the associated isotopic fractionations controlled the δ^{15}N-NO₃⁻. These incubation results matched the results from the in-river chamber experiment confirming the non-conservative nature of NO₃⁻ isotopes.

Incubation data can be explained with a mechanistic model that allows for isotopic O-exchange between NO₂⁻ and H₂O and a consideration of large kinetic ¹⁸O isotope effects. Recent literature has found rapid N-cycling and abiotic O-exchange between NO₂⁻ and H₂O during nitrification complicates the use of NO₃⁻ isotopes for source partitioning and the δ^{18}O-NO₃⁻ can be “reset” toward the δ^{18}O-H₂O of the medium. This was confirmed in this study as a substantial amount of isotopic O-exchange was found to have occurred between NO₂⁻ and H₂O during incubation experiments. In addition, the model permitted for an estimation of gross rates for NO₃⁻ transformation processes and was successful in determining the variability in nitrogen cycling at different parts of the Grand River and at different times of the year.

In summary, source apportionment is difficult because NO₃⁻ isotopes are not conservative and river metabolism does mask the isotopic signal of NO₃⁻. However, N cycling in a productive river, such as the Grand River is understood enough to build a mechanistic model to describe N biogeochemical cycling. This is important for those concerned with the fate of elevated NO₃⁻ in impacted rivers. Researchers should be cautious when using the dual isotopes of NO₃⁻ for source apportionment and must first understand the complexity of NO₃⁻ isotopes and transformation processes especially in productive systems.
4.2 Recommendations

The results from this thesis lead to a number of interesting questions that can be pursued through further research. A map of the microbial community composition of the rock biofilm from this study should be conducted to determine if ammonia-oxidizing archaea (AOA) and comammox in addition to ammonia-oxidizing bacteria (AOB) are present. It is possible that AOA and comammox have more of an influence on the isotopic composition of δ¹⁸O-NO₃⁻ compared to AOB (because of potentially different ammonia oxidation pathways). If all organisms are present in the biofilm this might be another potential mechanism that alters the δ¹⁸O-NO₃⁻ from nitrification.

Large diel effects of DO and δ¹⁸O-O₂ should be considered in the mechanistic model because they occur both temporally and spatially in the Grand River and would alter the NO₃⁻ isotope value that results from nitrification. In addition, the model should also include NO₂⁻ and the full denitrification reaction because it would provide a more accurate representation of N biogeochemical cycling in the river, especially if abiotic O-exchange during denitrification could be calculated.

Considering the diel measurements of δ¹⁸O-NO₃⁻ would be beneficial to decipher whether O-exchange in the river is the result of rapid biological N cycling or slower N cycling with a persistence of NO₂⁻.

It would also be advised to do another incubation experiment using Blair rocks and West Montrose water because estimated rates were the greatest at Blair, and West Montrose had the lowest NO₃⁻ concentrations. The effect of the biofilm on the amount of O-exchange would be enhanced because there would likely be a more pronounced change in the NO₃⁻ isotopes due to the faster rates and low initial concentration.
References


Cejudo, E. (2016). *Dissolved inorganic nitrogen cycling in a river receiving wastewater: the*


isotope fractionation during dissimilatory nitrate reduction by denitrifying bacteria. 


Mengis, M., Schiff, S. L., Harris, M., English, M. C., Aravena, R., Elgood, R. J., & MacLean, A.


Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. a., Schindler, D. W., …


Appendix A

Chapter 2 - Model II Regression (Figure 2.8 - 1:2 denitrification line)

Model II regression: West Montrose (WM)

\[ n = 29 \quad r = 0.2813632 \quad r\text{-square} = 0.07916524 \]

Parametric P-values: 2-tailed = 0.1392479 1-tailed = 0.06962396

Angle between the two OLS regression lines = 58.5334 degrees

Regression results

<table>
<thead>
<tr>
<th>Method</th>
<th>Intercept</th>
<th>Slope</th>
<th>Angle (degrees)</th>
<th>P-perm (1-tailed)</th>
</tr>
</thead>
<tbody>
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<td>14.92491</td>
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<tr>
<td>2</td>
<td>MA</td>
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<td>0.8259535</td>
<td>39.55512</td>
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<tr>
<td>3</td>
<td>SMA</td>
<td>-8.553758</td>
<td>0.9473341</td>
<td>43.45081</td>
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Confidence intervals

<table>
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<tr>
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<th>97.5%-Intercept</th>
<th>2.5%-Slope</th>
<th>97.5%-Slope</th>
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<tbody>
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<td>-12.588811</td>
<td>-5.7677089</td>
<td>0.65409777</td>
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Eigenvalues: 3.245237 1.80138

H statistic used for computing C.I. of MA: 0.4372429
Model II regression: Bridgeport (BR)

\( n = 16 \quad r = 0.6441039 \quad r\)-square = 0.4148699

Parametric P-values: 2-tailed = 0.007083136 1-tailed = 0.003541568

Angle between the two OLS regression lines = 23.79459 degrees

Regression results

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<td>2 MA</td>
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<td>0.6872557</td>
<td>34.49902</td>
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<td>3 SMA</td>
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Confidence intervals

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<th>2.5%-Slope</th>
<th>97.5%-Slope</th>
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<td>3 SMA</td>
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<td>0.5115350</td>
<td>1.1980531</td>
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Eigenvalues: 5.555598 1.098747

H statistic used for computing C.I. of MA: 0.1009747
Model II regression: Blair (BL)

n = 17   r = 0.5649005   r-square = 0.3191126
Parametric P-values:  2-tailed = 0.01813951    1-tailed = 0.009069754
Angle between the two OLS regression lines = 31.06915 degrees

Regression results

<table>
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<th>P-perm (1-tailed)</th>
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Confidence intervals

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<th>97.5%-Slope</th>
<th>2.5%-Slope</th>
<th>97.5%-Slope</th>
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<td></td>
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Eigenvalues: 9.224 2.562246
H statistic used for computing C.I. of MA: 0.1612954
Chapter 3 - Model II Regression (Figure 3.4-fabiotic)

West Montrose July 2015 – 24 Hours

<table>
<thead>
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<th>Method</th>
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<th>Angle (degrees)</th>
<th>P-perm (1-tailed)</th>
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<tbody>
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Confidence intervals

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<th>97.5%-Slope</th>
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<td>-123.2528</td>
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Eigenvalues: 5990.091 27.17346

H statistic used for computing C.I. of MA: 0.7390816

West Montrose July 2015 – 24-60 Hours

<table>
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<tr>
<th>Method</th>
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<th>Slope</th>
<th>Angle (degrees)</th>
<th>P-perm (1-tailed)</th>
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<tbody>
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Confidence intervals

<table>
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<tr>
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<th>2.5%-Slope</th>
<th>97.5%-Slope</th>
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</thead>
<tbody>
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Eigenvalues: 5027.265 16.53433

H statistic used for computing C.I. of MA: 0.5345005
West Montrose September 2015 – 24-60 Hours

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<th>P-perm (1-tailed)</th>
</tr>
</thead>
<tbody>
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Confidence intervals

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<th>Slope 2.5%</th>
<th>Slope 97.5%</th>
<th>P-value</th>
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Eigenvalues: 4072.794 0.3964017

H statistic used for computing C.I. of MA: 0.01571663
### Blair July 2015 – 24 Hours

<table>
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<th>Intercept</th>
<th>Slope</th>
<th>Angle (degrees)</th>
<th>P_perm (1-tailed)</th>
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<tbody>
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<td>1.019336</td>
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**Confidence intervals**

<table>
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<th>97.5% - Intercept</th>
<th>2.5% - Slope</th>
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**Eigenvalues:** 5041.694 1.869427

H statistic used for computing C.I. of MA: 0.05990813

### Blair July 2015 – 24-60 Hours

<table>
<thead>
<tr>
<th>Method</th>
<th>Intercept</th>
<th>Slope</th>
<th>Angle (degrees)</th>
<th>P_perm (1-tailed)</th>
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<tbody>
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**Confidence intervals**

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**Eigenvalues:** 3740.484 41.9357

H statistic used for computing C.I. of MA: 1.851317
Equation 3.2: \[ \delta^{15}O - NO_3^- = \frac{2}{3} \left( 2 + f_{\text{ABBIOTIC}} \right) \delta^{18}O - H_2O + \frac{2}{3} f_{\text{ABBIOTIC}} \left( 2^{18}H_2O - \delta^{18}O - 0_2 - ^{18}O_2 - ^{18}H_2O_2 \right) + \delta^{18}O - 0_2 + ^{18}O_2 + ^{18}H_2O_2 \]
MODEL EQUATIONS:

Model parameters that are adjusted for each incubation are indicated as $a-f$ and values for these parameters are in Table A1

$^{15}\text{N-NH}_4(t) = ^{15}\text{N-NH}_4(t \text{- dt)} + (^{15}\text{N}\_\text{Rate\_Mineralization} - ^{15}\text{N}\_\text{Rate\_Nitrification}) \times \text{dt}$

**INITIAL** $^{15}\text{N-NH}_4 = a \times (b/1000+1) \times 0.0036764$

**INFLOWS:**

$^{15}\text{N}\_\text{Rate\_Mineralization} =$

$(\text{Rate\_Mineralization}) \times (^{15}\alpha\_\text{Mineralization}) \times (\text{Ratio\_^{15}\text{N\_Biomass}})$

**OUTFLOWS:**

$^{15}\text{N}\_\text{Rate\_Nitrification} = (\text{Ratio\_^{15}\text{N-NH}_4}) \times (^{15}\alpha\_\text{Nitrification}) \times (\text{Rate\_Nitrification})$

$^{15}\text{N-NO}_3(t) = ^{15}\text{N-NO}_3^- (t \text{- dt)} + (^{15}\text{N}\_\text{Rate\_Nitrification} - ^{15}\text{N}\_\text{Rate\_Uptake} - ^{15}\text{N}\_\text{Rate\_Denitrification}) \times \text{dt}$

**INITIAL** $^{15}\text{N-NO}_3^- = d \times ((e/1000+1) \times 0.0036764)$

**INFLOWS:**

$^{15}\text{N}\_\text{Rate\_Nitrification} = (\text{Ratio\_^{15}\text{N-NH}_4}) \times (^{15}\alpha\_\text{Nitrification}) \times (\text{Rate\_Nitrification})$

**OUTFLOWS:**

$^{15}\text{N}\_\text{Rate\_Uptake} = (^{15}\alpha\_\text{Uptake}) \times (\text{Rate\_Uptake}) \times (\text{Ratio\_^{15}\text{N-NO}_3})$

$^{15}\text{N}\_\text{Rate\_Denitrification} = (\text{Rate\_Denitrification}) \times (^{15}\alpha\_\text{Denitrification}) \times (\text{Ratio\_^{15}\text{N-NO}_3})$

$^{18}\text{O-NO}_3(t) = ^{18}\text{O-NO}_3^- (t \text{- dt)} + (^{18}\text{O}\_\text{Rate\_Nitrification} - ^{18}\text{O}\_\text{Rate\_Uptake} - ^{18}\text{O}\_\text{Rate\_Denitrification}) \times \text{dt}$

**INITIAL** $^{18}\text{O-NO}_3^- = d \times ((f/1000+1) \times 0.0020052)$

**INFLOWS:**
18O_Rate_Nitrification = (Rate_Nitrification) x ((Equation 3.2/1000+1) x Ratio_VSMOW)

OUTFLOWS:

18O_Rate_Uptake = (Rate_Uptake) x (18α_Uptake) x (Ratio_18O-NO3)

18O_Rate_Denitrification = (Rate_Denitrification) x (18α_Denitrification) x (Ratio_18O-NO3)

NH4(t) = NH4(t - dt) + (Rate_Mineralization - Rate_Nitrification) x dt

INITIAL NH4 = a

INFlOWS:

Rate_Mineralization = k_Mineralization

OUTFLOWS:

Rate_Nitrification = k_Nitrification x NH4

NO3(t) = NO3(t - dt) + (Rate_Nitrification - Rate_Denitrification - Rate_Uptake) x dt

INITIAL NO3 = d

INFlOWS:

Rate_Nitrification = k_Nitrification x NH4

OUTFLOWS:

Rate_Denitrification = k_Denitrification x NO3

Rate_Uptake = k_Uptake x NO3

15α_Denitrification = -24/1000+1

15α_Nitrification = -15/1000+1

15α_Uptake = -8/1000+1

16α_Denitrification = -12/1000+1

18α_Uptake = -8/1000+1
$^{15}\alpha_{\text{Mineralization}} = 1$

$\delta^{15}\text{N}_{\text{Biomass}} = c$

$\delta^{15}\text{N}_{\text{NH}_4} = (\text{Ratio}_{^{15}\text{N}-\text{NH}_4}/\text{Ratio}_{\text{AIR}} - 1) \times 1000$

$\delta^{15}\text{N}_{\text{NO}_3} = (\text{Ratio}_{^{15}\text{N}-\text{NO}_3}/\text{Ratio}_{\text{AIR}} - 1) \times 1000$

$\delta^{18}\text{O}_{\text{NO}_3} = (\text{Ratio}_{^{18}\text{O}-\text{NO}_3}/\text{Ratio}_{\text{VSMOW}} - 1) \times 1000$

$\text{Ratio}_{^{15}\text{N}_{\text{Biomass}}} = (c/1000+1) \times \text{Ratio}_{\text{AIR}}$

$\text{Ratio}_{^{15}\text{N}-\text{NH}_4} = ^{15}\text{N}_{\text{NH}_4}/\text{NH}_4$

$\text{Ratio}_{^{15}\text{N}-\text{NO}_3} = ^{15}\text{N}_{\text{NO}_3}/\text{NO}_3$

$\text{Ratio}_{\text{AIR}} = 0.0036764$

$\text{Ratio}_{\text{H}_2\text{O}} = (\delta^{18}\text{O}_{\text{H}_2\text{O}}/1000+1) \times (\text{Ratio}_{\text{VSMOW}})$

$\text{Ratio}_{^{18}\text{O}-\text{NO}_3} = ^{18}\text{O}_{\text{NO}_3}/\text{NO}_3$

$\text{Ratio}_{\text{O}_2} = (\delta^{18}\text{O}_{\text{O}_2}/1000+1) \times (\text{Ratio}_{\text{VSMOW}})$

$\text{Ratio}_{\text{VSMOW}} = 0.0020052$

Equation 3.2 ($^{18}\alpha_{\text{Nitrification}}$) = $\frac{1}{2}(2 + f_{\text{abiotic}})\delta^{18}\theta - H_2\theta + y - \text{int}$

$\delta^{18}\text{O}_{\text{O}_2} = 24.2$
Table A1: Values used in the model for each of the three incubation experiments West Montrose (WM) July 2015, WM September and Blair (BL) July, 2015.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$\delta^{18}$O-H$_2$O (‰)</th>
<th>f*abiotic</th>
<th>y-int</th>
<th>NH$_4^+$ (mgN/L)</th>
<th>$^{15}$N-NH$_4$ (‰)</th>
<th>$^{15}$N of Biomass (‰)</th>
<th>NO$_3^-$ (mgN/L)</th>
<th>$^{15}$N-NO$_3^-$ (‰)</th>
<th>$^{18}$O-NO$_3^-$ (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M = Measured E= Estimated</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>E</td>
<td>E</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
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<tr>
<td>WM July Low 0-24h</td>
<td>-9.0</td>
<td>0.88</td>
<td>9.32</td>
<td>0.030</td>
<td>+12</td>
<td>+8.5</td>
<td>2.00</td>
<td>+76.0</td>
<td>+11.2</td>
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<tr>
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<td>0.12</td>
<td>0.030</td>
<td>+12</td>
<td>+8.5</td>
<td>2.16</td>
<td>+57.0</td>
<td>+10.5</td>
</tr>
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<td>0.88</td>
<td>9.32</td>
<td>0.030</td>
<td>+12</td>
<td>+8.5</td>
<td>2.00</td>
<td>+78.5</td>
<td>+10.5</td>
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<tr>
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<td>0.70</td>
<td>0.12</td>
<td>0.030</td>
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<td>+8.5</td>
<td>2.34</td>
<td>+65.4</td>
<td>+18.3</td>
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<td>0.88</td>
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<td>0.030</td>
<td>+12</td>
<td>+8.5</td>
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<td>0.030</td>
<td>+12</td>
<td>+8.5</td>
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<tr>
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<td>0.030</td>
<td>+12</td>
<td>+8.5</td>
<td>0.95</td>
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<td>+8.5</td>
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<td>+7.24</td>
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<td>+18</td>
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<td>+40.1</td>
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<tr>
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<td>0.030</td>
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<td>+18</td>
<td>3.80</td>
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<tr>
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<td>1.06</td>
<td>3.53</td>
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<td>+18</td>
<td>3.30</td>
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<tr>
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<td>+18</td>
<td>3.50</td>
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<td>+18.9</td>
</tr>
</tbody>
</table>

*Experiment values used in the model equations