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By Lindsey R. Crawley

Entitled NITROGEN AND OXYGEN ISOTOPE-RATIO ANALYSIS OF NITRATE BY THE DENITRIFIER METHOD USING CONTINUOUS FLOW ISOTOPE-RATIO MASS SPECTROMETRY

For the degree of Master of Science

Is approved by the final examining committee:

Greg Michalski
Chair

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NITROGEN AND OXYGEN ISOTOPE-RATIO ANALYSIS OF NITRATE BY THE DENITRIFIER METHOD USING CONTINUOUS FLOW ISOTOPE-RATIO MASS SPECTROMETRY

A Thesis

Submitted to the Faculty

of

Purdue University

by

Lindsey R. Crawley

In Partial Fulfillment of the

Requirements for the Degree

of

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West Lafayette, Indiana

To my parents & my husband

for giving me all of the love and support one could ever need.

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ABBREVIATIONS

CF-IRMS = Continuous Flow Isotope-ratio Mass Spectrometer

Delta, $\delta = [(R_x-R_{ref})/R_{ref}]*1000‰$

EA = Elemental Analyzer

GC = Gas Chromatograph

IRMS= Isotope-ratio Mass Spectrometer

LN_2= Liquid Nitrogen

MS = Mass Spectrometer

Per mil, ‰ = $1/1000^{th}$

RT= Room Temperature

SOP = Standard Operating Procedure

TSA = Tryptic Soy Agar

TSB = Tryptic Soy Broth

VSMOW = Vienna Standard Mean Ocean Water

ABSTRACT

Crawley, Lindsey R., M.S., Purdue University, December 2010. Nitrogen and Oxygen Isotope-ratio Analysis of Nitrate by the Denitrifier Method Using Continuous Flow Isotope-ratio Mass Spectrometry. Major Professor: Greg Michalski.

The total isotopic composition of nitrate is used for identifying the origin and fate of nitrate in atmospheric, terrestrial and aquatic systems. The analysis of $\delta^{18}O$ and $\delta^{15}N$ values each give important and unique information about the sources and sinks of nitrate in these systems. This denitrifier method is based on the isotope ratio analysis of nitrous oxide generated through reduction of nitrate by cultured denitrifying bacteria. Here, preliminary data is presented on $\delta^{15}N$ and $\delta^{18}O$ generated by the bacterial production of N_2O from NO_3^-.

During the process of denitrification, nitrates are converted to nitrogen gas via a series of intermediate gaseous nitrogen oxide compounds. Denitrification occurs due to the presence of heterotrophic bacteria or autotrophic denitrifier pathways in select bacteria. Thus, we have chosen a distinct bacterium for the investigation of nitrate reduction for this study. *Pseudomonas aureofaciens* contains the copper-containing nitrite reductase necessary for the reduction of nitrate. Our efforts focus on the conversion of NO_3 to N_2O using techniques adapted from Cascotti[22]. In this study, a customized headspace extraction system was built in order to analyze $\delta^{18}O$ and $\delta^{15}N$ of N_2O from NO_3^-. Successful purification of *Pseudomonas aureofaciens*, conversion of NO_3^- to N_2O, and the standardization of nitrate was the result of many experiments. Tests of the impact on isotopic composition by pre-concentration methods have been performed including freeze-drying/evaporation and ion chromatography. The denitrifier

method has been implemented in the Michalski Stable Isotope Lab in order to simultaneously detect the stable isotope composition of oxygen and nitrogen in nitrate samples.

Chapter 1: INTRODUCTION

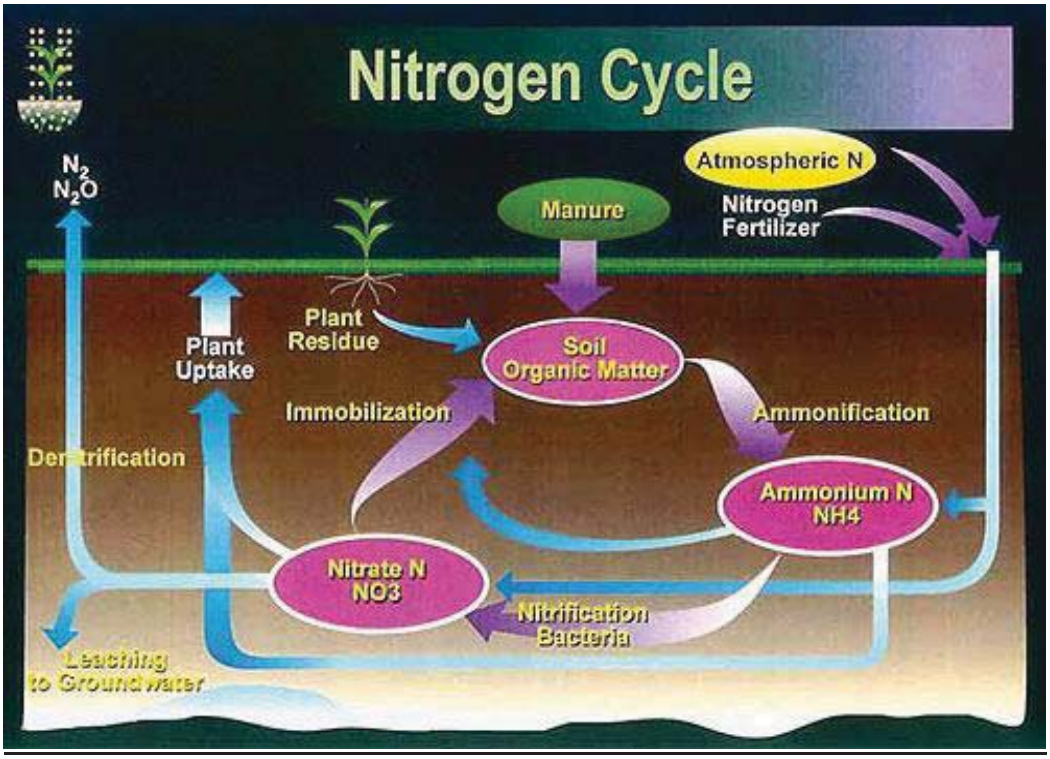


Figure 1.1: Nitrogen Cycle

1.1 Nitrate and the Nitrogen Cycle

Nitrogen (N) is a key element in biological systems as amino acids are the building blocks of life. The most abundant source of nitrogen available to life on Earth is atmospheric N_2, which makes up approximately 78% of the atmosphere. Nitrogen gas in its molecular form (N_2), has a strong triple bond (ΔH= 226 kcal mol^{-1}) rendering N_2 inert in most biological systems[1]. Therefore, unique chemical or biological mechanisms have evolved to convert N_2 into more functional forms

of N, such as nitrates, nitrites, and ammonium, in order for biological systems to maintain functionality. These trace nitrogen compounds, in various reduction or oxidation states, exist throughout the environment and cycle through the biosphere via five main processes[2]: nitrogen fixation, nitrogen uptake, ammonification, nitrification, and denitrification (Figure 1.1). These processes are used to explain how atmospheric nitrogen gas can be exploited for use by organisms and for the recycling of N in the biosphere.

1.2 Stable Isotopes of Nitrogen and Oxygen

Stable isotopes can be used as an indicator of environmental change in natural systems including the nitrogen cycle. Isotopes are defined as atoms of the same element with differing numbers of neutrons[3] and noted as

$${}^{A}_{Z}X \quad \text{(i.e. } {}^{14}_{7}N\text{, or nitrogen-14)}$$

where X stands for the chemical element or elemental symbol, A is the atomic mass number (the total number of protons and neutrons in the nucleus), and Z is the atomic number (number of protons in the nucleus). It is common in biogeochemistry to omit the proton subscript for simplicity. Stable isotopes do not radioactively decay; however, their abundances in nature do vary due to small differences in the thermodynamic properties of isotopically substituted molecules. Oxygen, hydrogen, carbon, nitrogen, and sulfur are the main elements in biogeochemical systems and variations in their isotopic abundances in different compounds can be used to trace processes such as metabolism, phase changes, and reaction rates in the C, N, S and water cycles.

1.2.1 Isotopes in Nitrate

In this study, the nitrogen and oxygen isotopes, ^{14}N, ^{15}N, ^{16}O, and ^{18}O, in nitrate are of interest. Their respective isotopic abundances are: 0.996337,

0.003663, 0.9976206, and 0.0020004 relative to N_2-Air and Standard Mean Ocean Water (SMOW)[4]. All isotopic measurements are made in comparison to these reference abundances. Isotopic enrichments or depletions are then calculated relative to the accepted standard and reported as "delta" in units of parts per thousand, or per mil (‰):

$$\delta\ (‰) = (R_{sample}/R_{standard} - 1) * 1000$$

The amount of rare isotope is usually expressed as a ratio, R, of the rare isotope relative to the abundant isotope, such as $^{15}N/^{14}N$. Delta (δ) notation is a way of making comparisons between the isotopic ratios of two materials. δ values can be used to compare 1) high vs. low values, 2) more/less positive vs. more/less negative (e.g. -10‰ is more positive than -20‰), and 3) heavier vs. lighter (the "heavy" material is the one with the higher δ value)[1]. These small changes in isotopic abundance are important ways of tracing changes in biogeochemical systems such as the nitrogen cycle.

1.2.2 Tracing Sources and Cycling of N and O Using Stable Isotopes

Isotopologues of nitrate can be valuable in tracing sources and cycling of nitrate within the nitrogen cycle. Isotopologues are molecules that have the same chemical formula but different masses and this difference in mass results in isotopologues having different thermodynamic properties. Changes in reaction rates are dependent upon the differences in vibrational energies of the isotopologues and leads to isotopic fractionation. Isotopic fractionation is the partial separation of a light isotope from a heavy isotope during a chemical reaction or physical processes. Isotope fractionations can occur during equilibrium reactions or unidirectional kinetic reactions. Equilibrium fractionations occur when there is an exchange of two isotopes of the same element between molecules in a chemical equilibrium:

$$A^aX + B^bX \leftrightarrow A^bX + B^aX$$

In this reaction, isotopic fractionation of compound *A* relative to compound *B* can be expressed as a fractionation factor, which is equivalent to the equilibrium constant:

$$\alpha_{A\text{-}B} = R_A/R_B$$

where A and B are different phases such as $H_2O_{(g)}$, $H_2O_{(liq)}$. For example, the exchange of ^{18}O between nitrate and water can be written as

$$OONO + H_2{}^{18}O \leftrightarrow OON^{18}O + H_2O$$

$$\alpha_{NO3\text{-}H2O} = (OON^{18}O/OONO) / (H_2{}^{18}O/H_2O).$$

During equilibrium reactions, the species with the higher oxidation state tends to become enriched (accumulates the heavier isotope) while the species of lower oxidation state is depleted[1]. δ values can be related to fractionation factors using the following equation[5]:

$$\alpha_{A\text{-}B} = (1000 + \delta_A)/(1000 + \delta_B)$$

The fractionation factor is also related to the isotopic enrichment factor, ε:

$$\varepsilon_{A-B} = (\alpha_{A-B} - 1)*1000,$$

For small values of ε ($\varepsilon_{A-B} \approx \delta_A - \delta_B$) is simplified as

$$\varepsilon_{A-B} \approx \delta_A - \delta_B \approx 1000 \ln \alpha_{A\text{-}B}.$$

Kinetic isotope effects (KIE) also lead to fractionation due to the mass difference of isotopologues in unidirectional kinetic reactions. Here, light isotopes typically react faster than heavy isotopes because the bonds of lighter isotopes are broken more easily than equivalent bonds of heavier isotopes[1]. This leads to the heavier isotopes becoming enriched in the reactants, while the products are depleted (lower δ value). The kinetic fractionation factor is expressed as:

$$\alpha_{P\text{-}S} = R_P / R_S,$$

where P≡product and S≡substrate (reactant), and the isotopic enrichment factor, ε, is:

$$\varepsilon_{P\text{-}S} = (\alpha_{P\text{-}S} - 1) * 1000,$$

If fractionations are small and the reactant concentration is large,

$$\varepsilon_{P\text{-}S} = \delta_P - \delta_S.$$

In both equilibrium and kinetic processes, the Rayleigh equation is used to calculate the change of isotopic composition of the residual substrate as a function of the initial isotopic composition of the substrate (δ_0), the remaining fraction of the substrate (f), and the enrichment factor $\varepsilon_{P\text{-}S} <> 0$:

$$\delta \approx \delta_0 + \varepsilon_{P\text{-}S} \ln f.$$

1.2.3 $\delta^{15}N$ and $\delta^{18}O$ in the Nitrogen Cycle

Nitrogen containing compounds cycle through the biosphere via five main processes known as the nitrogen cycle: nitrogen fixation, nitrogen uptake, ammonification, nitrification, and denitrification[6] and stable isotope variations can help understand the cycling process. Nitrogen fixation is a process in which un-reactive N_2 is converted into a nonzero oxidation state, most often as ammonium[2]. Fixation can occur via industrial fixation, lightning, or biological fixation, all of which require intense amounts of energy. Depending on the nitrogen fixation process, the fractionation factor can be greater or less than 1 (α=0.998-1.002[7]). Artificial fertilizer production is the largest source of anthropogenic fixed nitrogen on Earth. In industrial nitrogen fixation, the Haber-Bosch process uses an enriched iron or ruthenium catalyst to form ammonia from atmospheric N_2 and hydrogen at high temperature and pressure[8]: $N_2 + 3H_2 \rightarrow 2NH_3$. Nitrogen is fixed by industry for use as fertilizer and as feedstock for nitric acid production through O_2 oxidation of NH_3 (Ostwald Process). Industrial nitrogen fixation provides about 80 Tg N per year to the nitrogen cycle[9]. The $\delta^{15}N$ values in ammonium from fertilizer are significantly different when compared to $\delta^{15}N$ in nitrate from fertilizer (Figure 1.2). The $\delta^{18}O$ of synthetic nitrate is ~ 20‰ and reflects a mixture of air O_2 ($\delta^{18}O$ ~ +23‰[10]) and water ($\delta^{18}O$ ~ -5‰[10]) that is used in its production. This $\delta^{18}O$ value is significantly different than microbial nitrate (see below) and this difference can be used to trace the amount of synthetic fertilizer in ecosystems perturbed by humans.

Nitrogen fixation can also occur as a result of lightning strikes. In this form of nitrogen fixation, electrical energy breaks the N≡N, allowing N_2 to combine with atmospheric O_2, forming nitrogen oxides[11]: $N_2 + O_2 + \text{energy} \rightarrow 2NO_x$. NOx is ultimately oxidized to nitric acid (HNO_3), which is highly soluble, and falls to the ground when it dissolves into rainwater. Studies of acid rain are also of great interest to the stable isotope community; ^{15}N isotopic studies have successfully distinguished sources of NO_x emitted from coal combustion (+6 to +9‰) than from automobiles (-13 to -2‰) at a study site in South Africa[12]. Recently, Elliott et

al.[13] have also suggested that nitrate $\delta^{15}N$ values can be used to distinguish NOx sources. The $\delta^{18}O$ values in atmospherically fixed nitrate are highly elevated spanning 60-90‰ and these high values have been used to trace the deposition of atmospheric nitrate to terrestrial ecosystems.

Table 1.1: Global production and distribution rates of reactive nitrogen

	Nr (Tg N/yr)[9]
Natural Nr creation	
Terrestrial BNF	100
Marine BNF	15
Total Lightning	5
Anthropogenic Nr Creation	
Haber-Bosch	80
BNF, cultivation	40
Fossil-fuel combustion	24
Total Terrestrial	249
Total Global	264
Atmospheric emission	
NO_x, fossil-fuel combustion	24
NO_x, other	24
Terrestrial NH_3	62
Marine NH_3	13
Total Emissions	123
Atmospheric Deposition	
Terrestrial NO_y	30
Marine NO_y	14
Terrestrial NH_x	40
Marine NH_x	16
Organic N	
Total Deposition	100
Denitrification	
Continental N_2O	11.7
Marine N_2O	4
Continental N_2	13-233
Marine N_2	110
Total Denitrification	249

Biological nitrogen fixation converts atmospheric nitrogen to ammonia (NH_3) using microbes that contain the nitrogenase enzyme[8]: $N_2 + 6\ H^+ + 6\ e^- \rightarrow 2\ NH_3$. The nitrogenase enzyme often produces organic materials with $\delta^{15}N$ values slightly less than 0‰[14]. Lower isotopic values in organic matter, when compared to higher values produced by other mechanisms, is often cited as evidence of N_2 fixation[1]. Common isotopic methods for quantifying biological fixation include the ^{15}N tracer dilution method[15,16] and the ^{15}N natural abundance method [17].

The process of ammonium conversion into organic nitrogen is known as nitrogen uptake, or ammonia assimilation. In biological reactions such as assimilation, organisms tend to favor lighter isotopes over heavier isotopes during incorporation of ammonium into their biomass[181]. This may result in leaving behind an enriched ^{15}N in the ammonium pool while producing depleted amino acids. Fractionation factors of assimilation are in the range of α=1.000-1.020[7]. Changes in $\delta^{15}N$ might be used to infer the degree of assimilation in certain ecosystems.

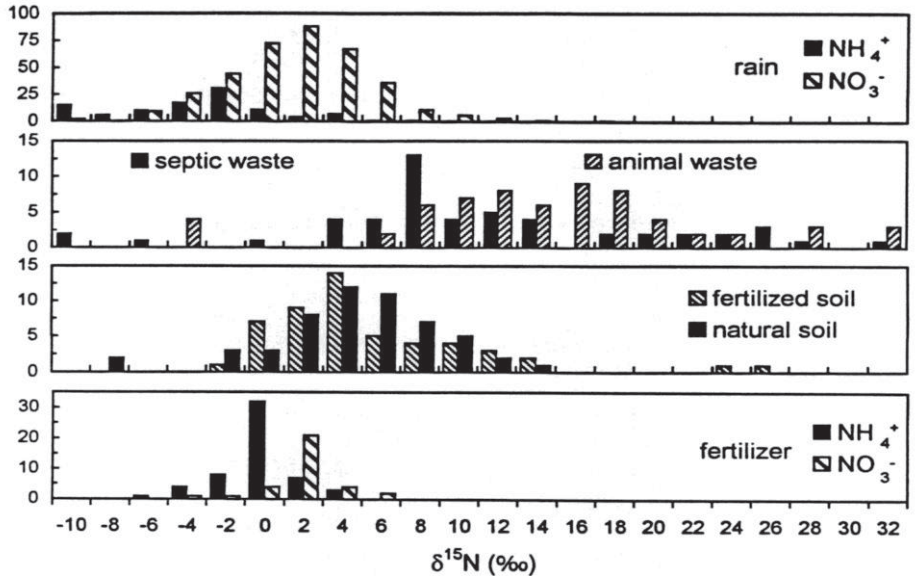


Figure 1.2: Ranges of $\delta^{15}N$ values of some major sources of N[1]

Ammonification, also known as nitrogen mineralization, is a microbial process that converts organic N into ammonium. When an organism expires, heterotrophic microbes consume and decompose the organic matter to acquire energy; residual nitrogen compounds are excreted or left behind[18]. During ammonification, N isotope fractionation is very small ($\alpha \approx 1.000$); the isotopic value of product N in NH_4^+ differs very little, if at all, from the initial $\delta^{15}N$ of organic N[7]. Soil ammonium $\delta^{15}N$ is typically found to be within a few permil of the organic N composition in the soil[1].

Nitrification is a multi-step oxidation process involving autotrophic organisms for the purpose of gaining metabolic energy. In this aerobic process, bacteria consume ammonium via oxidation in order to gain energy. In nitrification, one particular group of bacteria (e.g. *Nitrosomonas*), oxidize ammonium to nitrite:

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$$

followed by the oxidation of nitrite to nitrate by a second set of bacteria (e.g. *Nitrobactor*):

$$2NO_2^- + O_2 \rightarrow 2NO_3^-$$

The resulting nitrate product can be found in soil profiles and surface and groundwater [17] and is found to have a fairly large ^{15}N fractionation factor of 1.015-1.035[7]. This is attributed to bacterial oxidation in the first step of the process, leaving NO_2^- depleted in ^{15}N relative to NH_4^+. Oxygen incorporated into the product nitrate is believed to come from air O_2 and water in a 1/3 to 2/3 proportion. Air O_2 has a $\delta^{18}O$ value of +23‰[10] and is essentially constant in time and space in the troposphere. On the other hand, the $\delta^{18}O$ of water in soil is a function of precipitation $\delta^{18}O$ values and enrichment from evapotranspiration, which is variable. Precipitation $\delta^{18}O$ values vary as a function of water vapor source and temperature (location, season, and altitude) and typically spans -20 to +5‰[19]. Enrichment of residual soil water by evaporation is a function of

temperature and the degree of evaporation (Rayleigh process). Therefore, the $\delta^{18}O$ variations resulting from nitrification can range from roughly -10 to +10‰ depending on location and temperature. These values are very different when compared to synthetic and atmospheric nitrates and they could be used to trace nitrate sources in soil and water systems.

1.2.4 Denitrification in the Nitrogen Cycle

Denitrification is critical for understanding life processes on Earth; it is the sole process occurring in the nitrogen cycle that is a net return of N_2 to the atmosphere[8]. Denitrification is the biological reduction of nitrate (NO_3^-) to nitrogen gas (N_2) via heterotrophic bacteria under anaerobic conditions[18]:

$$5CH_2O + 4H^+ + 4NO_3^- \rightarrow 2N_2 + 5CO_2 + 7H_2O$$

Heterotrophic bacteria are those that are unable to synthesize their own organic compounds[20]. Denitrification takes place when oxygen levels are depleted and nitrate serves as an alternative to oxygen as the final electron acceptor in the respiration process. The reduction of nitrate, thus completes the nitrogen cycle by returning fixed nitrogen to the atmosphere as N_2, NO, and N_2O. Isotopic fractionation of nitrogen and oxygen isotopes occurs during bacterial denitrification. Denitrification depletes N_2O in comparison to the reactant NO_3^- (α=1.000-1.033[7]). In systems where denitrification occurs over time, the residual nitrate becomes enriched and follows Rayleigh distillation type behavior (Figure 1.3). In these cases, changes in the $\delta^{15}N$ (or $\delta^{18}O$) may be a way of assessing the degree of denitrification occurring in closed systems such as septic tanks, groundwater, and ocean sediments.

The combination of $\delta^{18}O$ and $\delta^{15}N$ values in nitrate, sometimes called the dual isotope approach, has been used to help distinguish between sources and processes in the nitrate cycle. For example, synthetic nitrate fertilizer and

microbial soil nitrate have distinct $\delta^{15}N$ values (see Figure 1.2), relative to manure nitrate, but synthetic nitrate $\delta^{18}O$ values are unique compared to soil and manure nitrate. Therefore, the combination of $\delta^{18}O$ and $\delta^{15}N$ can be used to determine the sources of nitrate contributed to groundwater or surface waters. Normal ranges of $\delta^{15}N$ and $\delta^{18}O$ are shown in Figure 1.3. Distinguishing atmospheric nitrate sources presents the opposite scenario; $\delta^{18}O$ can be used to evaluate possible nitrate sources when $\delta^{15}N$ values cannot.

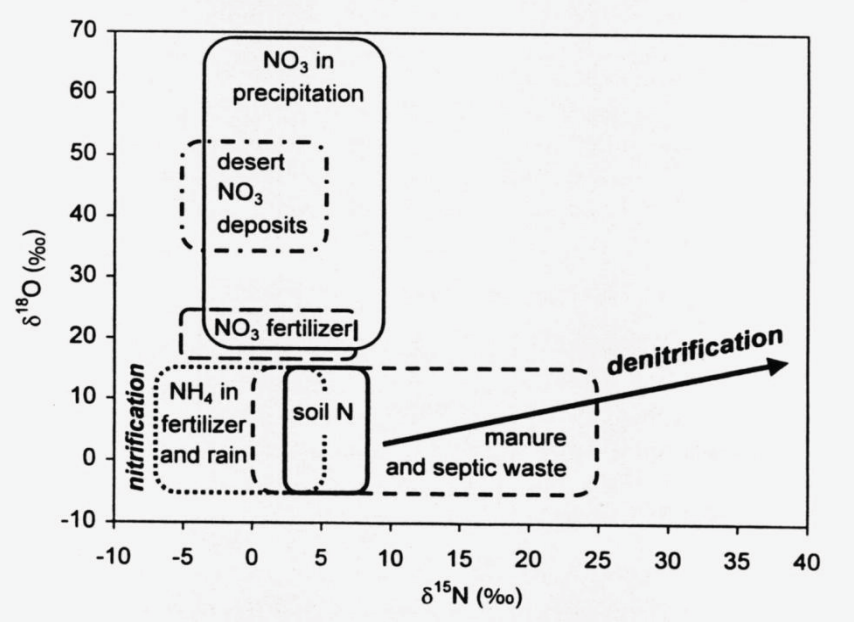


Figure 1.3: Typical ranges of $\delta^{18}O$ and $\delta^{15}N$ of nitrate from various sources[1].

The denitrifying processes can complicate identification of nitrate sources using the dual isotope approach. During denitrification, the $\delta^{15}N$ and $\delta^{18}O$ values in the residual nitrate exponentially increase (Figure 1.4, $\beta \approx \alpha$). A trend of simultaneous changes in $\delta^{15}N$ and $\delta^{18}O$ may confuse source identification, but is usually a sign that the denitrification process is active. Kendall et al.[1], described three benefits of measuring both ^{15}N and ^{18}O isotopes in nitrate: 1) oxygen

isotopic separation of some sources is greater than when compared to the nitrogen isotopes, allowing better source resolution by having two tracers, (2) some nitrate sources that are presently indistinguishable with $\delta^{15}N$ alone (e.g., fertilizer vs. soil nitrate, or atmospheric vs. soil nitrate) may be identified only when the $\delta^{18}O$ of nitrate is analyzed, and (3) oxygen isotopic compositions of nitrate vary systematically with nitrogen isotopic compositions during denitrification[1].

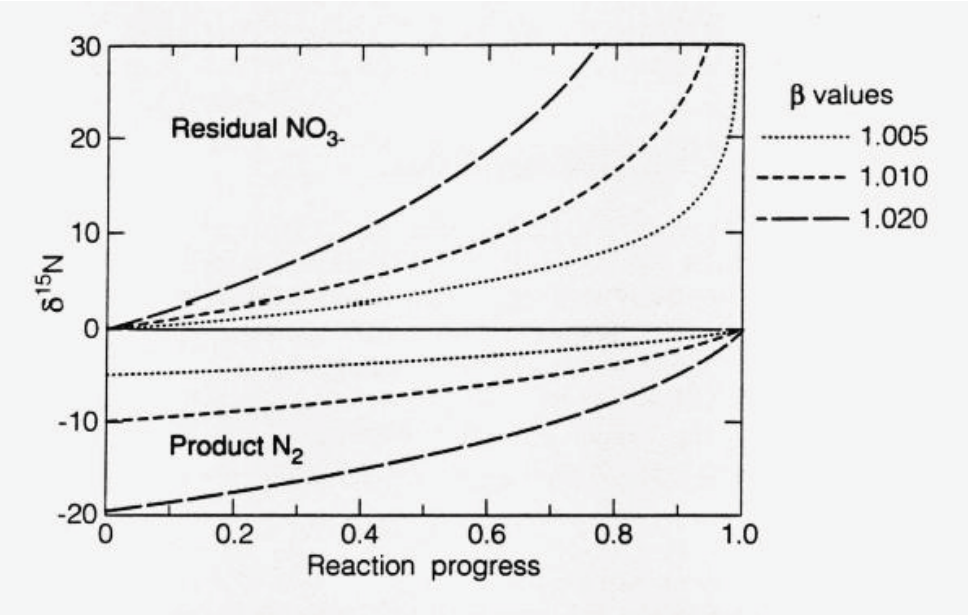


Figure 1.4: $\delta^{15}N$ values of residual nitrate and product nitrogen as a function of reaction time from denitrification[1].

CHAPTER 2: NITROGEN AND OXYGEN ISOTOPE-RATIO ANALYSIS OF NITRATE BY THE DENITRIFIER METHOD USING CONTINOUS FLOW ISOTOPE-RATIO MASS SPECTROMETRY

2.1 Experimental Section

Nitrogen and oxygen isotopes in nitrate can be determined utilizing the denitrifier method[21,22]. Denitrification is the process of converting NO_3^- or NO_2^- into reduced nitrogen gases (N_2O or N_2). Under certain conditions or specific bacteria, N_2O can act as the terminal electron acceptor in the biologic reduction of nitrate. If the nitrogen and oxygen isotopes in nitrate are directly incorporated into the N_2O product then N_2O from denitrification can be the analyte for determining isotope abundances in NO_3^- [21,22]. The determination of $\delta^{15}N$ and $\delta^{18}O$ values in NO_3^- via N_2O begins with denitrification of the sample, followed by headspace extraction and purification of the product N_2O and finally, isotopic analysis of the N_2O using an isotope-ratio mass spectrometer. Sigman et al.[21] discussed the following advantages of using bacterial derived N_2O for nitrate isotope analysis relative to other methods: a 100-fold reduction in the sample size requirement, proportional blank size for samples with lower nitrate concentrations, a reduction in the time requirement of analysis, and reproducibility of samples with 1μM nitrate or greater.

The method discussed here is a technique where the isotopic analysis of nitrate is carried out utilizing denitrification. Denitrification is a microbial process that reduces nitrate to nitrite, nitric oxide, nitrous oxide, and nitrogen gas:

$$2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_{2\,(g)}$$

Specific nitrase reductase enzymes contained within the denitrifying bacteria each facilitate a step in the reduction of nitrate to nitrogen gas. The denitrifier method takes advantage of the fact that some bacterial halt the reduction after the nitrous oxide production step[21,22], which allows incorporation of both the nitrogen and oxygen isotopes into the product N_2O. If complete denitrification were to occur the oxygen would be lost (converted to water) and isotope analysis of the N_2 gas would be challenging because the large background signal of N_2 originating from air N_2. Therefore, it is imperative that the denitrifying bacteria selected for this study lack the active N_2O-reductase enzyme (if present, this reductase would further reduce N_2O to N_2 [23] and isotopic analysis of $\delta^{18}O$ in N_2O would no longer be possible (see section 2.1.1)). It is also important that only the oxygen from NO_3^- be incorporated into N_2O, because ^{18}O exchange between NO_2^- (or NO) and H_2O during the reduction may erase the original nitrate $\delta^{18}O$ value.

2.1.1 Denitrifier Strain

One bacterium that meets the above criteria is *Pseudomonas Aureofaciens (P. Aureofaciens)*. This denitrifying strain is an aerobic bacterium, golden-yellow in color, and was isolated from Maas River clay[24]. Glockner et al. showed that the Cu-containing nitrite reductase in *P. Aureofaciens* incorporates relatively little oxygen from water during converting NO into N_2O. During the denitrification process ($2NO_3^- \rightarrow N_2O$) only one of six oxygen atoms in NO_3^- is incorporated into N_2O. This loss of oxygen is likely to be accompanied by a kinetic isotope effect, common for biologic oxidation/reduction reactions, and alter the $\delta^{18}O$ of the product N_2O relative to the NO_3^- substrate. In order for the $\delta^{18}O$ of nitrate to be determined through the isotopic analysis of N_2O, this biologic isotopic fractionation must be consistent. Fortunately, prior research has demonstrated that N atoms are conserved during the reduction, no external N source is available for isotopic exchange and the produce N_2O can be used to

determine the $\delta^{15}N$ in nitrate[22]. Therefore, N_2O produced by P. Aureofaciens reduction of nitrate allows for the determination of the nitrogen isotopic composition of nitrate (^{14}N and ^{15}N) as well as oxygen isotopic composition (^{16}O and ^{18}O).

Preparation of *P. Aureofaciens* used in the NO_3^- isotopic analysis begins with culturing pure strains of the bacteria, preparing a growth media, inoculation of a growth media with the purified strains, and then harvesting the bacteria that are then used to reduce nitrate to nitrous oxide. Single colonies of bacteria act as a clone of the original source, yet are free of impurities, and the appearance of single colonies helps to visually distinguish pure bacteria from possible contaminants. Purifying the bacteria begins with obtaining freeze-dried *P. Aureofaciens* from ATCC biological resource center (http://www.atcc.org) and making cultures. The bacteria are cultivated on tryptic soy agar plates amended with potassium nitrate at room temperature (details in Appendix D). Using a flamed inoculating loop, an agar plate (Plate #1) is streaked from the frozen pure *P. Aureofaciens* stock and is allowed to grow for 2-3 days until the streaks appear with a yellow-orangish color. A second agar plate (Plate #2) is then streaked using single colonies on Plate #1, and after two or more days, Plate #3 is streaked using single colonies from Plate #2. The purpose of streaking from the freezer source then using three sequential plates is for the stepwise purification of bacterial colonies, which results in single colony growth. After three consecutive plates, single colonies are selected from plate #3 for inoculating the growth media, which is used to produce a large amount of bacteria.

Tryptic soy broth (TSB) serves as a quality medium for bacteria growth, but this particular denitrifying bacterium requires supplementary nutrients prior to inoculation. Three additional ingredients are added to modify the store-bought supply of TSB for optimum growth conditions of *P. Aureofaciens*: potassium nitrate (10 mM), ammonium sulfate (3.8 mM), and potassium phosphate (36 mM). Nitrogen compounds within the stock TSB, as well as the addition of $(NH_4)_2SO_4$ to the culture medium provides the nitrogen necessary for

assimilation, which limits any nitrate from the sample from being incorporated into bacterial biomass[21]. Although Greenberg et al. [23] and Christensen et al. [25] noted that a similar bacteria*, Pseudomonas Chloroaphis,* matured without the addition of ammonium, like Sigman[21], the NH_4^+ addition is purely precautionary (*P. Aureofaciens* was recently reclassified as a strain of *P. Chloroaphis*[26]). The nutrient media is distributed into four 250 mL polycarbonate centrifuge bottles (VWR), then capped and autoclaved. After the solution cools to room temperature, the media is inoculated from an individual colony picked from Plate #3, re-capped and allowed to develop for 5-9 days. This incubation time allows for the complete consumption of the O_2 in the headspace making the solution anaerobic, after which the reduction of NO_3^- can take place. At any time past the 5 day incubation point, the solution is tested for incomplete conversion of nitrate with an Aqua Chek nitrate/nitrite test strip (Hach). A positive result (pink) indicates the presence of nitrate and nitrite and the bottle should be allowed to react longer, up until the 9 day mark. A negative result (off-white) indicates complete conversion of nitrate and the bacteria may then be harvested and used in nitrate isotope analysis.

2.1.2 Conversion of Sample to Nitrous Oxide

Complete conversion of any nitrate sample (or standard) to nitrous oxide requires concentration and purification of the bulk bacteria solution, reacting sample nitrate with the bacteria, extracting N_2O from the vial headspace, and analysis on an isotope-ratio mass spectrometer. The bacteria are harvested by centrifugation of the inoculated bottle(s) that have tested negative for nitrate/nitrite. Two bottles are centrifuged (Damon-IEC, model IEC CU-5000) for 10 minutes at 3000rpm. After the first round of centrifugation, the solutions are separately decanted, leaving behind two bacterial pellets. The first bacterial pellet is re-suspended with 50mL of a nitrate-free growth medium (rinse) solution and shaken. The rinse solution consists of a nitrate-free growth medium with the

addition of one drop of an anti-foaming agent (J.T. Baker Antifoam-B Silicone Emulsion Agent; see Appendix D for reagents) added just before the solution is used to rinse bacteria pellets during pelletization. The re-suspended pellet is then poured into the bottle containing the second bacteria pellet. These combined pellets are capped, shaken, centrifuged, and decanted a second time. The rinse solution dilutes any NO_3^- that may still be present in the growth medium as well as any N_2O that may be dissolved in the original growth medium. The combined solution is rinsed with a second 50mL aliquot of the rinse solution. After a third and final centrifuging of the solution, the bacterial pellet is re-suspended in 125 mL of rinse solution, leading to a 4-fold concentration of bacteria. Helium gas is purged through the bacteria solution for 2 hours using a gas bubbler; the helium serves to degas any remaining nitrous oxide that was produced by NO_3^- reduction in the original growth medium. Flushing with helium at this step also helps to reduce the signal of the blank. The solution is transferred to a sterile 125mL glass bottle and capped with a septum seal. The headspace is purged with helium gas and the solution is allowed to sit overnight. Promoting an anaerobic environment ensures that the bacteria are no longer able to reproduce, yet they will still be able to complete the NO_3^- conversion on the following day.

On the following day, the bacteria solution is aliquoted, re-flushed, inoculated with a nitrate-containing solution (either reference nitrates or samples), and incubated to convert nitrate to N_2O. 1 mL of the bacteria media is pipeted into individual 12mL sample vials (LabCo, Ltd.), sealed with septum caps, and flushed on a gas rack with helium at a rate of 40mL/min for 5 minutes (16 vial volumes). Flushing with an inert gas dispels any air within the vial and removes any N_2O that may have been adsorbed onto the bacteria during the overnight incubation. The purging gas enters through a disposable needle and exits through a second disposable needle that are inserted above the liquid level in the vial and discarded after use to avoid cross contamination. At this point, the vials contain only the denitrifying bacteria and a headspace of helium. Nitrate samples (or reference nitrates) are then added to the helium-flushed bacteria

vials where NO_3^- is converted to N_2O. The samples containing nitrate for analysis should be concentrated to the optimal 100 nmol NO_3^- per mL before they are inoculated into the bacterial solution. 100 nmol NO_3^- is optimal, because the 50nmol of N_2O produced (stoichiometry of the bacterial denitrification) is the amount of gas needed to keep the analytical blank below 1% (see section 2.2.4). Using a disposable syringe and needle, 1mL of each nitrate solution is added to an individual vial and incubated. (see section 2.2.6). After 60 minutes, 0.5 mL of 1% NaOH solution is added to each vial to scavenge any CO_2 and lyses the bacteria, which stops the bacterial reduction and releases any cell bound nitrous oxide into the solution. The sample vials are then ready to be loaded into the headspace extraction system for IRMS analysis.

2.1.3 Extraction and Isotopic Analysis of Nitrous Oxide

N_2O produced from NO_3^- denitrification in the sample vial is extracted from the headspace, cryogenically collected, and purified, before entering the isotope ratio mass spectrometer. The customized headspace extraction set-up has an autosampler (Gilson 221 XL Liquid Handler), six independent purification traps, a gas chromatography system and two cryogenic loops. The system is fully automated using a combination of Isodat and Peak Simple software (see Appendix D). After the N_2O is extracted from headspace vials, it is injected into the IRMS via a custom made open split interface.

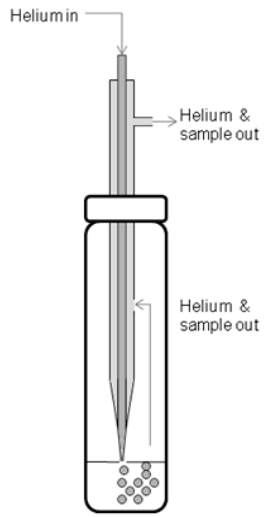


Figure 2.1: Autosampler needle extracting gas from sample vial.

Each sample vial undergoes the same headspace extraction procedure (Figure 2.2). The vial is purged with a helium carrier gas (99.99% pure, Airgas) at 30 mL/min for 12 minutes, which flushes the 12 mL glass vials roughly 30 times. Flushing is carried out using a double needle (Figure 2.1) (SerCon). The inside needle supplies the helium carrier gas down through the sample vial into the liquid causing vigorous bubbling. A hole in the outside needle permits the sample gas to exit to the purification and collection traps. It is important that the liquid volume must be <4 mL or less in the 12 mL vial or else the liquid may flow up and out through the exit hole of the outer needle, risking contamination of the extraction system.

The sample N_2O gas is sent through a series of five purification traps. The first trap is a dry 12mL headspace vial (LabCo) paired with a second autosampler needle (SerCon) and is positioned so that if any liquid leaves the sampling vial it will be isolated and not contaminate the subsequent traps The second trap is a Nafion drier (PermaPure, Inc., MD-050-72S-1) removes moisture from the sample gas stream by allowing water molecules to migrate through the Nafion membrane into a helium counter current that carries the moisture away. The next

purification step uses 7mL of solid Carbosorb (SerCon, granular 6-12 mesh) to remove CO_2 from the gas stream. The fourth trap in the series is filled with 7mL of solid magnesium perchlorate (CosTech Analytical Technologies, Inc.) that absorbs trace water from the helium purge gas that may have bypassed the Nafion drier. Both the Carbosorb and magnesium perchlorate solids are separately enclosed in 4.3”x0.32” glass tubing. The fifth trap in the series is a Supelco Type F hydrocarbon purge trap (12” in length and 0.125” in diameter) that filters out volatile organic compounds (VOCs). VOCs are produced during the incubation step and if not removed, the hydrocarbons can interfere with the mass 45 signal by producing hydrogen atoms (1 amu) that can recombine with mass 44 N_2O in the IRMS ion source, producing a signal at mass 45.

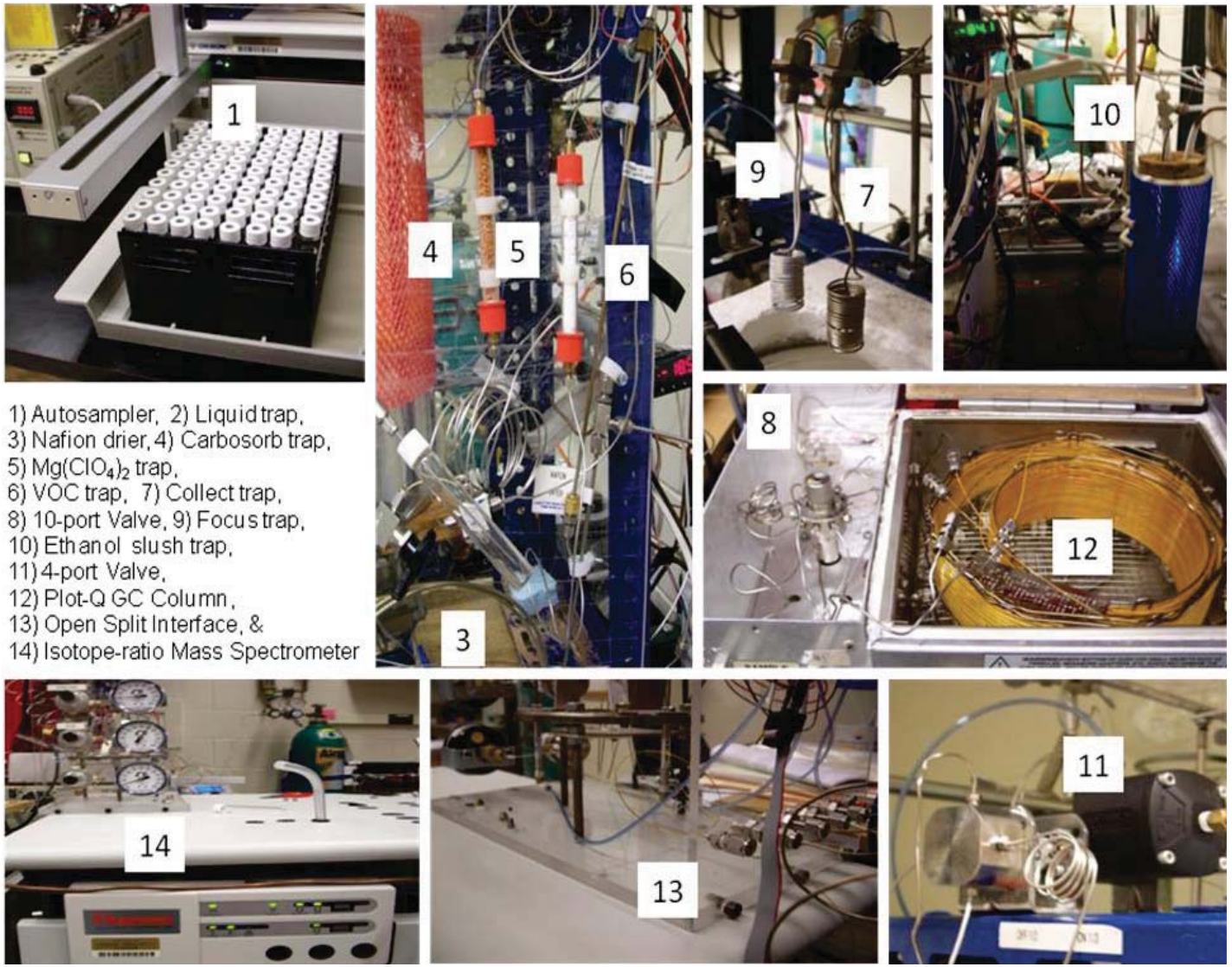


Figure 2.2: IRMS online extraction system

After passing through the five purification traps, the sample gas is preconcentrated in two cryogenic loops. The first cryogenic trap is immersed in liquid nitrogen (-196°C) and collects headspace N_2O for 10.5 minutes while the previous sample is being processed by the GC (see below). The N_2O collected in the first trap is the transferred to a second loop (focus loop) by lifting the trap out of the liquid nitrogen using a pneumatic lifter. In between the first cryogenic trap and the focus trap is an ethanol slush trap (-80°C) used as a final filter to reduce water and VOCs in the sample gas, before the N_2O is collected in the focus loop that is immersed in liquid nitrogen. After complete transfer of the N_2O, the focus loop is thawed and the helium flow carries the N_2O through a HP-Plot-Q column (Agilent) where it is separated from any other gases such as CO_2. Finally, the sample gas is injected into a custom open-split interface and analyzed in the IRMS.

During the on-line extraction of N_2O from sample vials, there are two configurations of sample collection: Inject or Load. While in Inject mode (Figure 2.3) , the 10-port valve is aligned so that focus trap raises up from the liquid N_2, sending the sample collected from the previous sample through the ethanol slush trap and into the Plot-Q column using 10-port valves where it reaches the IRMS. Simultaneously, a new isotopic analysis begins (T= 0 min) and the autosampler needle is lowered into the next vial, flushing the sample gas from the vial using a helium carrier gas. The sample gas is transported from the vial, through the Nafion drier, Carbosorb trap, and $Mg(ClO_4)_2$ trap, and finally into the collect trap submerged in liquid N_2. At approximately 3.5 minutes into collection, the focus trap is lowered into liquid N_2 and allowed to cool.

After 5 minutes of sample collection from one vial, the 10-port valve rotates to Load mode (Figure 2.4). At the 10 minute mark, the collect trap is raised above the liquid N_2 level and allowed to warm to room temperature, transferring the collected gas to the focus trap. At 12 minutes, the autosampler needle is moved to a vial containing only helium; this ensures that gases from one vial do not

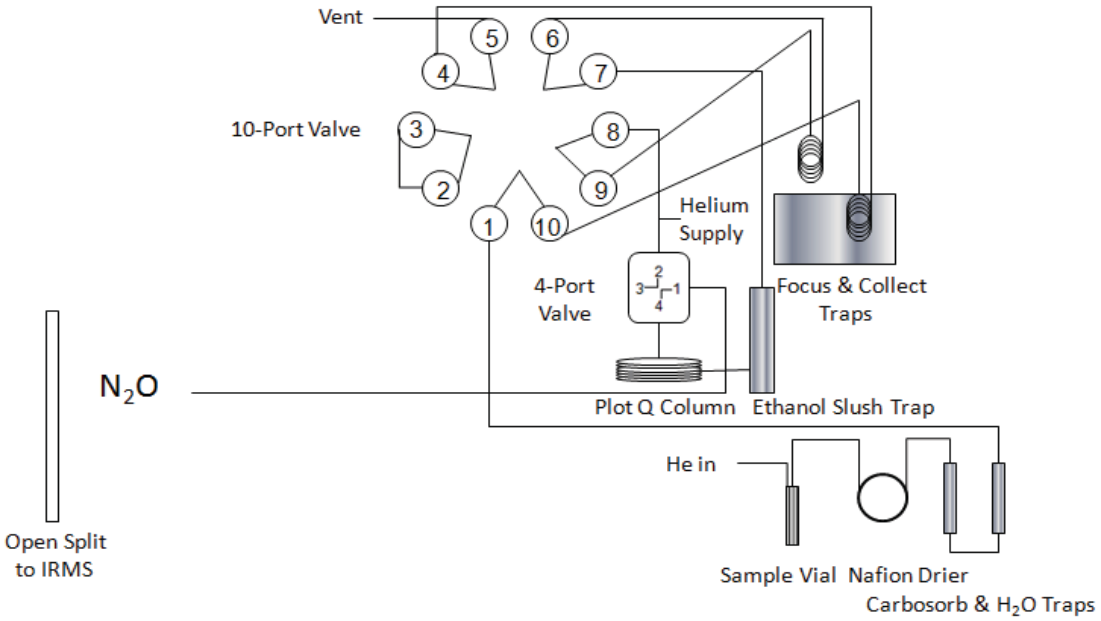


Figure 2.3: Inject: 2-3 configuration

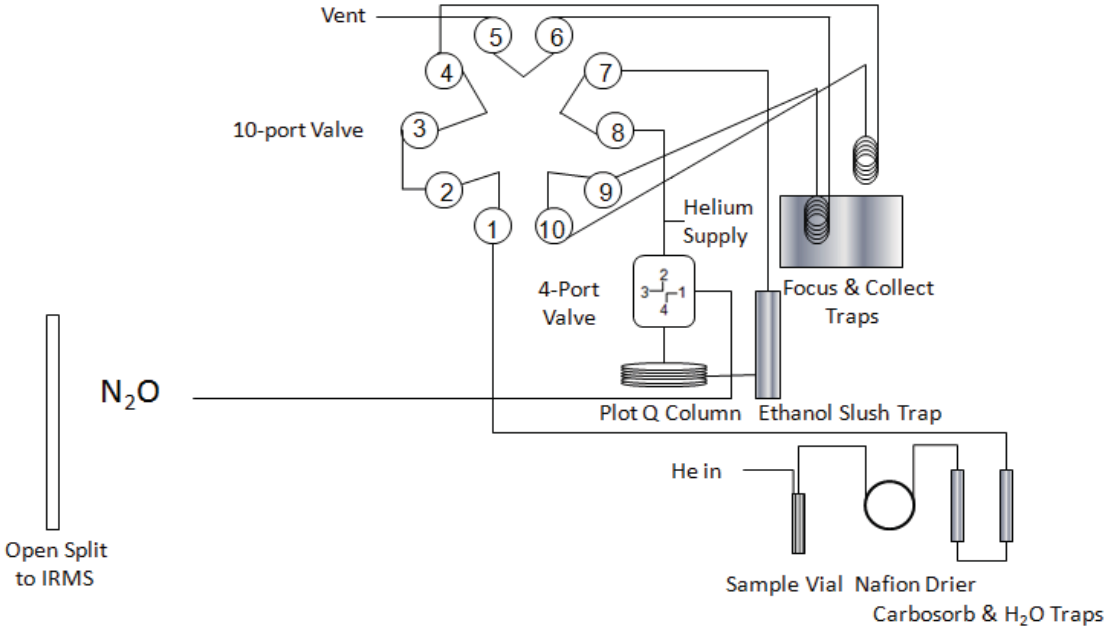


Figure 2.4: Load: 1-2 configuration

come into contact with gases in the next vial. Just before the 15 minute sampling time expires, the 10-port valve rotates to Inject mode where the previous focused gas is analyzed as mentioned above.

The Delta-V Plus isotope-ratio mass spectrometer (Thermo Electron Corporation) is ideal for nitrogen and oxygen isotope analysis of nitrous oxide because it is equipped with an 11-cup collector configuration which allows for N and O isotopes to be measured without peak jumping (magnet adjustment). The electron impact ion source is controlled with a computer and supplementary software and is self-aligning and is able to provide high sensitivity and linearity. This IRMS has the sensitivity to detect one ion per 1100 molecules in Continuous Flow mode and the flexibility to cover a mass range up to m/z 96 (FisherSci). The automated N_2O system is capable of running approximately 75 samples during an overnight run and up to 96 samples in a 24-hour time period.

In order to determine the ratio of N and O isotopes in N_2O, masses 44, 45, and 46 amu are monitored. The most common isotopologue of N_2O has a mass of 44 amu: $^{14}N^{14}N^{16}O$; the abundance of the minor isotopologues of N_2O have masses of 45 and 46 amu (available in Table 2.1) and were calculated based on the natural abundance of N and O isotopes relative to N_2-Air and SMOW, respectively. The likelihood of isotopologues $^{14}N^{15}N^{17}O$, $^{15}N^{14}N^{17}O$, and $^{15}N^{15}N^{16}O$ influencing the signal is very low and is ignored. This can be justified by determining the change permil values as affected by a change in isotopic abundance. For the mass 46 signal, a 10‰ change in the ^{15}N and ^{17}N in an isotopologue ($^{14}N^{15}N^{17}O$, $^{15}N^{14}N^{17}O$, or $^{15}N^{15}N^{16}O$) only changes ^{18}O signal in $^{14}N^{14}N^{18}O$ by less than 0.1‰. Therefore, isotopologues $^{14}N^{14}N^{16}O$, $^{14}N^{15}N^{16}O$, $^{15}N^{14}N^{16}O$, $^{14}N^{14}N^{17}O$ and $^{14}N^{14}N^{18}O$ are of importance in this study.

Since the vapor pressure of N_2O and CO_2 are comparable and have the identical molecular masses of 44, 45, and 46, a Plot-Q GC column is used to separate N_2O and CO_2 peaks. Some small CO_2 peaks may appear on the spectrographs, but they are easy to differentiate from N_2O peaks based on peak size.

Table 2.1: Abundances of N_2O isotopes and isotopologues

Isotope	*Mole Fraction*[27]	*Mass*	*Isotopologue*	*Mole Fraction*
^{14}N	0.9963	44	$^{14}N^{14}N^{16}O$	0.9902
^{15}N	0.0037	45	$^{14}N^{15}N^{16}O$	0.0037
^{16}O	0.9976		$^{15}N^{14}N^{16}O$	0.0037
^{18}O	0.0020		$^{14}N^{14}N^{17}O$	0.0004
		46	$^{14}N^{14}N^{18}O$	0.0020
			$^{14}N^{15}N^{17}O$	1.47E-06
			$^{15}N^{14}N^{17}O$	1.47E-06
			$^{15}N^{15}N^{16}O$	1.37E-05

2.1.4 Isotopic References/Standardization

Isotopic standards are used as a way to relate a measured isotopic value to a known or accepted value. An isotopic standard should be some homogeneous material with an isotopic ratio similar to that of the sample for which it will be compared to and be available in large quantities. Standards are used as a way to relate isotopic values, rather than determination of an absolute value, because absolute isotopic determination can be very difficult as well as vary among laboratories. Delta notation (as mentioned in Chapter 1) expresses isotopic abundances (or ratios) of a sample relative to an accepted isotopic ratio of a standard:

$$\delta = [(R_{smpl} - R_{std})/R_{std}]*1000‰.$$

These isotopic abundances are not absolute measurements, but rather precise determinations of the differences in isotopic ratios.

It is standard practice to use calibrated reference materials to ensure the accurate and precise analysis of stable isotope abundances. Although each N_2O analysis is referenced against a nitrous oxide gas cylinder within the mass

spectrometer, the N_2O gas is not the absolute reference. Nitrate standards are preferred over a nitrous oxide reference gas. Within each batch of samples, nitrate standards are prepared in the same fashion as nitrate samples and then analyzed in the mass spectrometer, following the "identical treatment" principle. In doing so, it is possible to obtain accurate and precise isotopic measurements and it also serves as one way to recognize or identify any possible changes in the system or analysis that can occur on a day-to-day basis.

There are a number of nitrate reference materials that have a range of accepted $\delta^{15}N$ and $\delta^{18}O$ values relative to air N_2 and VSMOW respectively. Internationally recognized nitrate standards include USGS32 potassium nitrate and USGS35 sodium nitrate. USGS35 has a $\delta^{15}N$ value of +2.7‰ relative to N_2-Air[28] and a $\delta^{18}O$ value of +57.5‰ relative to VSMOW[28] and USGS32 has a $\delta^{15}N$ value of +180.0‰ relative to N_2-Air[28] and a $\delta^{18}O$ value of +25.7‰ relative to VSMOW[28]. These international standards are available at a very limited supply therefore secondary standards were calibrated relative to the international reference materials and in turn relative to air N_2 and VSMOW.

Two secondary nitrate reference salts were prepared and calibrated and then three subsequent mixtures of these references salts resulted in a set of four secondary "working standards". Secondary standards used in this study are Hoffman nitrate fertilizer (20Hoff) and North Carolina State University (NCSU) Potassium Nitrate (William Showers, personal communication). Hoffman brand nitrate is a sodium nitrate mined from ore deposits in the Atacama Desert in Northern Chile and exported as fertilizer. The Hoffman secondary nitrate standard was calibrated relative SMOW using USGS35 as the reference by converting the nitrates to O_2 using the silver decomposition method[33]. Converting the delta value of a sample (s) measured relative to a working standard (ws) into a delta relative to the absolute standard (as) uses the following equation:

$$\delta_{s\text{-}as}\ (‰) = \delta_{s\text{-}ws} + \delta_{ws\text{-}as} + 1/1000(\delta_{s\text{-}ws} \cdot \delta_{ws\text{-}as})$$

During standardization of oxygen isotopes, the “sample” is the secondary standard Hoffman, the working standard is USGS35, and absolute standard is VSMOW yielding the conversion identity of:

$\delta^{18}O_{20Hoff\text{-}VSMOW} = \delta^{18}O_{20Hoff\text{-}USGS35} + \delta^{18}O_{USGS35\text{-}VSMOW} + (\delta^{18}O_{20Hoff\text{-}USGS35} + \delta^{18}O_{USGS35\text{-}VSMOW} / 1000)$.

A set of ten analyses of Hoffman relative to USGS35 yielded a $\delta^{18}O$= -3.2‰ which gives a Hoffman values relative to SMOW $\delta^{18}O$= +54.4‰. The $\delta^{18}O$ of NCSU was determined by thermal conversion elemental analysis at North Carolina State University and was found to be -23.6 +/- 0.2‰ (William Showers, personal communication). The $\delta^{15}N$ of Hoffman and NCSU was determined by converting it to N_2 using elemental analysis and comparing it to USGS32, USGS35 using the following equation:

$\delta^{15}N_{20Hoff\text{-}N2} = \delta^{15}N_{20Hoff\text{-}USGS35} + \delta^{15}N_{USGS35\text{-}N2} + (\delta^{15}N_{20Hoff\text{-}USGS35} + \delta^{15}N_{USGS35\text{-}N2}/ 1000)$

The $\delta^{15}N$ of Hoffman and NCSU is found to be +3.2‰ and -2.2‰, respectively, relative to air N_2 .

Mass balance equations were used to make additional secondary standards with isotopic compositions that are similar to the composition of samples being studied. This technique is used frequently in order to preserve expensive international standards available in limited quantities by mixing with other readily available standards. The desired isotopic value of a mixed standard from the masses and isotopic values of the individual standards:

$$\delta^{15}N_{Std\ Mix} = \delta^{15}N_{Std\ A} * x + \delta^{15}N_{Std\ B} * [1-x],$$

where x, mole fraction of standard= $mass_{StdA}$/ ($mass_{StdA}$ + $mass_{StdB}$), assuming both standards have the same molecular formula.

A working standard nicknamed NC32 is a combination of USGS32 and NCSU nitrate standards. Since $\delta^{18}O_{20Hoff\text{-}USGS35}$, $\delta^{15}N_{20Hoff\text{-}USGS35}$, and $\delta^{15}N_{NC32\text{-}VSMOW}$ were measured during elemental analysis $\delta^{15}N$ of NC32 can be calculated. The $\delta^{15}N$ isotopic value of working standard NC32 was calculated to be $\delta^{15}N$ = +15.3‰ using $\delta^{15}N_{NCSU}$= -2.2‰, both relative to N_2-Air, and x= $mass_{NCSU}$/ ($mass_{NCSU}$ + $mass_{USGS32}$)=0.90388.

Working standards are prepared from the aforementioned standards to provide a range of $\delta^{15}N$ and $\delta^{18}O$ values. $\delta^{18}O$ and $\delta^{15}N$ values of the working standards “10Hoff” and “1Hoff” are also calculated based on mass balance mixtures of working standards 20Hoff and NC32 (by mole fraction). The working standards 20Hoff, 10Hoff, 1Hoff, and NC32, all used on a regular basis, have a set of known values based on the mass balance calculation (Table 2.2). These standards span a range of values that is known to shift on a daily basis. For $\delta^{18}O$ the range is roughly 60 permil and the $\delta^{15}N$ range is about 15 permil and the day to day shift is +/- 1.0‰, which is tolerable. For example, the spread between 20Hoffman and NC32 might be $\delta^{18}O$= -28 to +40‰ one day then $\delta^{18}O$= -30 to + 39‰ on the following day’s analysis, but the span of values remains unchanged (~67-69‰ difference).

A calibration curve is plotted graphing the actual known isotopic values versus the measured isotopic values of the standards to correct for this shift in isotopic values. Using the equation of the line, the measured sample values, not standards, are then corrected to represent true $\delta^{15}N$ and $\delta^{18}O$ values. Table 2.2 represents of typical measurements of $\delta^{15}N$ and $\delta^{18}O$ using calibrated standards.

Table 2.2: N and O isotopic ratios (‰) for references and standards used

	$\delta^{18}O$ (VSMOW)	$\delta^{15}N$ (N_2-Air)
International References		
USGS32	25.7[28]	180[28]
USGS35	57.5[28]	2.7[28]
Secondary Standards		
20Hoff (also a working standard)	54.3	3.2
NCSU	-23.5	-2.2
Working Standards		
NC32 (93% NCSU + 7% USGS32)	-18.8	15.3
10Hoff (50% 20Hoff + 50% NC32)	17.8	9.2
1Hoff (5% 20Hoff + 95% NC32)	-15.2	14.7

Table 2.3: Example of typical isotopic values (‰) for calibrated working standards

	$^{18}O_{measured}$	$^{18}O_{actual}$	$^{15}N_{measured}$	$^{15}N_{actual}$
20Hoff	41.8	54.1	-1.5	0.5
10Hoff	10.7	17.6	4.6	7.9
1Hoff	-19.9	-15.2	11.9	14.6
NC32	-25.1	-18.8	12.6	15.3

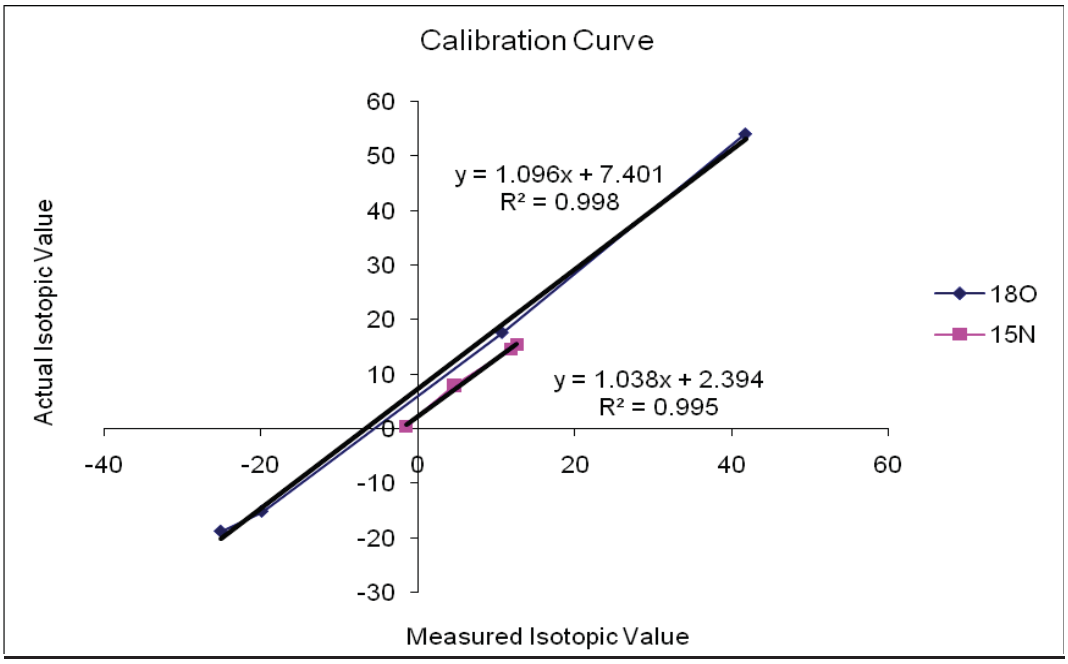


Figure 2.5: Example of a typical calibration curve

2.2 Results and Discussion

In order to understand the denitrification method's capability of determining precise and accurate isotope ratios of oxygen and nitrogen in nitrate, a series of control and test experiments were carried out. The precision and accuracy capabilities of the isotope-ratio mass spectrometer were first tested. Then, various tests were performed in order to reduce the size of the blanks. The impact of bacterial growth conditions and preparation on isotope precision and accuracy were also examined. Additionally, standardization tests were performed, followed by sample preparation and other miscellaneous improvements.

2.2.1 Precision Limits of the Mass Spectrometer

In order to test instrument's accuracy and precision of the IRMS (i.e. without sample manipulation or conversion) an "on-off" test was performed. During the "on-off" test, the reference N_2O gas is measure relative to itself (i.e. a zero test) by lowering and raising the N_2O reference capillary into the open split (Figure 2.6).

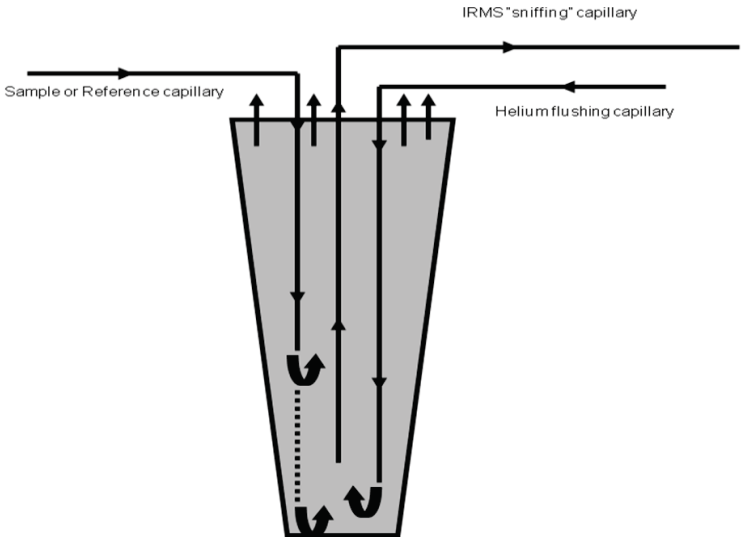


Figure 2.6: Open split tube

When the reference gas capillary is lowered into the open split, the resulting IRMS signal (in mV) is a function of the reference gas pressure, the ion source settings, and proper alignment of the electromagnet cups (Figure 2.7). The software will only be able to analyze N_2O isotope ratios precisely and accurately if the IRMS is "tuned". Improper tuning of the mass spectrometer will lead to useless data. The ion source parameters must be able to attain a focused, intense and stable ion beam. The software interface allows the user to fine tune the accelerating voltage, magnet, electron trap, and box current. The maximum number of ions produced is attained by adjusting the ion current, electron energy, high voltage (ion acceleration), box, and trap currents. Focusing the ion beam is achieved by balancing the X-Focus, X-Deflection, Y-Deflection, SE-Suppressing,

and Focus Quad. Each of these parameters can be manually optimized or autofocused using the software. It is important that peak centering and autofocusing of these parameters be completed daily to ensure optimal signal-to-noise conditions in the IRMS. Once tuned, lowering the N_2O reference gas capillary into the open split generates an ion signal is plotted versus time, a square shaped peak is produced (Figure 2.9).

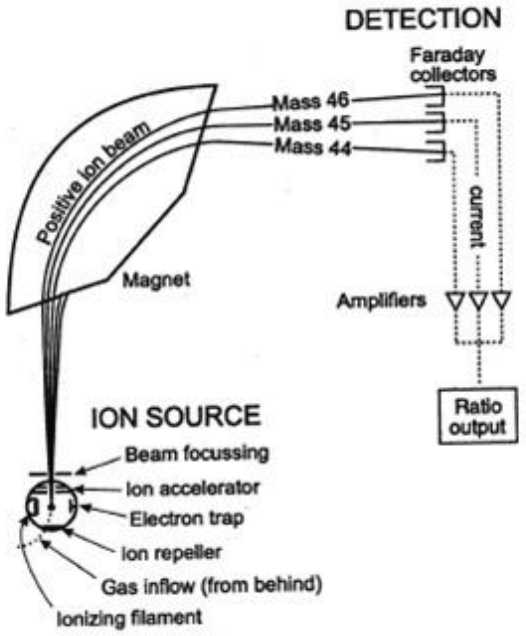
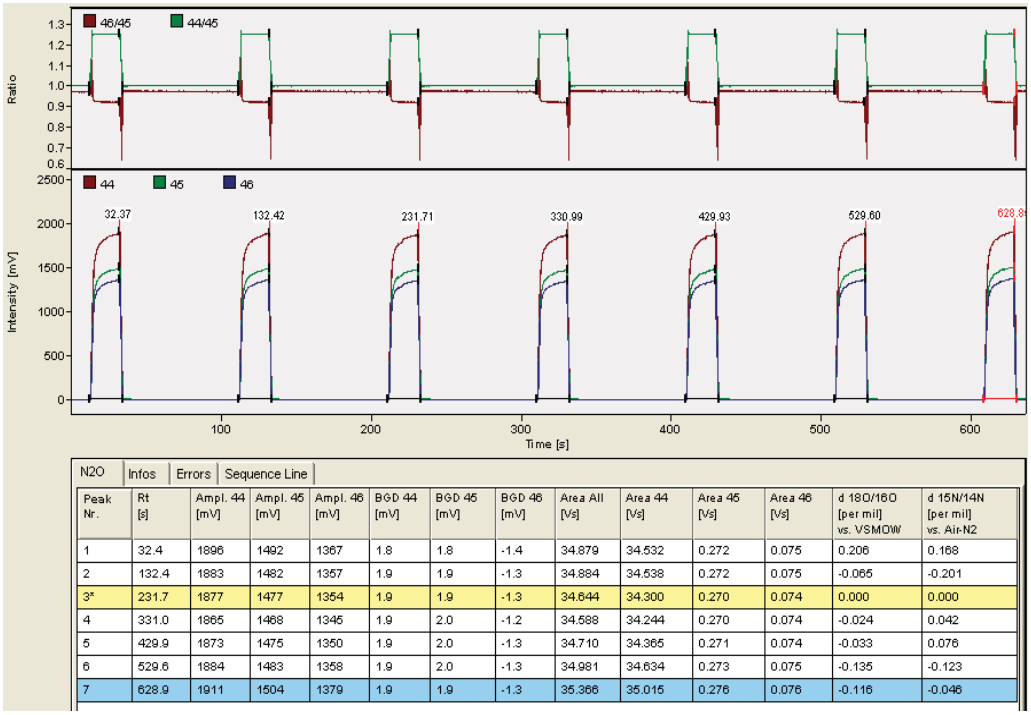


Figure 2.7: Schematic of an IRMS with triple collector cup system

The signal generated by gases entering the IRMS from the open split is a function of the gas pressures and dilutions. The N_2O reference gas (Air Products and Chemicals, Inc.) is supplied by a 196 cubic foot tank with an outflow of 200psi. The gas is connected to the open split interface, where the reference gas is delivered at a pressure of ~2psi through a silica capillary line into an open

split tube. A helium purge gas (37psi) is also directing the flow into the split tube where there is a silica capillary “sniffing” tube (100 μm I.D.). The sniffing line sends gas to the ion source through a pressure differential (~ 2 bar versus 10^{-6} mbar inside the IRMS). The purge pressure of the helium must be higher than atmospheric pressure to keep atmospheric air from mixing in the tube. The sample open split tube also has a capillary delivering a helium purge gas, a second sniffing capillary (100 μm I.D.) and a GC column capillary that brings the headspace extraction to the MS. The sample capillary is controlled by a pneumatic piston plunger, and is lowered below the helium and sniffing capillaries just before the sample leaves the GC column.



N2O | Infos | Errors | Sequence Line

Peak Nr.	Rt [s]	Ampl. 44 [mV]	Ampl. 45 [mV]	Ampl. 46 [mV]	BGD 44 [mV]	BGD 45 [mV]	BGD 46 [mV]	Area All [Vs]	Area 44 [Vs]	Area 45 [Vs]	Area 46 [Vs]	d 18O/16O [per mil] vs. VSMOW	d 15N/14N [per mil] vs. Air-N2
1	32.4	1896	1492	1367	1.8	1.8	-1.4	34.879	34.532	0.272	0.075	0.206	0.168
2	132.4	1883	1482	1357	1.9	1.9	-1.3	34.884	34.538	0.272	0.075	-0.065	-0.201
3*	231.7	1877	1477	1354	1.9	1.9	-1.3	34.644	34.300	0.270	0.074	0.000	0.000
4	331.0	1865	1468	1345	1.9	2.0	-1.2	34.588	34.244	0.270	0.074	-0.024	0.042
5	429.9	1873	1475	1350	1.9	2.0	-1.3	34.710	34.365	0.271	0.074	-0.033	0.076
6	529.6	1884	1483	1358	1.9	2.0	-1.3	34.981	34.634	0.273	0.075	-0.135	-0.123
7	628.9	1911	1504	1379	1.9	1.9	-1.3	35.366	35.015	0.276	0.076	-0.116	-0.046

Figure 2.8: N_2O reference gas “on-off” test

The purpose of the on-off test was to determine the best precision that can be expected from any conflo analysis. An arbitrary N_2O pulse is selected as the

reference peak (Second pulse, Figure 2.9) and the ion current of the other N_2O pulses are measured relative to this reference pulse. The software calculates the δ value of the each N_2O pulse relative to the reference pulse integrating the areas under 44, 45, and 46 amu voltage versus time (V/s):

$$\delta_{smpl\text{-}ref} = [(45/44_{smpl} - 45/44_{ref})/(45/44_{ref})]*1000$$
$$\delta_{smpl\text{-}ref} = [(46/44_{smpl} - 46/44_{ref})/(46/44_{ref})]*1000$$

Since the reference N_2O gas has already been calculated relative to SMOW using:

$$\delta_{ref\text{-}SMOW} = [(45/44_{ref} - 45/44_{SMOW})/(45/44_{SMOW})]*1000$$
$$\delta_{ref\text{-}SMOW} = [(46/44_{ref} - 46/44_{SMOW})/(46/44_{SMOW})]*1000$$

then,

$$\delta_{smpl\text{-}SMOW} = \delta_{smpl\text{-}ref} + \delta_{ref\text{-}SMOW} + 1/1000*\delta_{ref\text{-}SMOW} + \delta_{smpl\text{-}ref}.$$

On-off tests resulted in a peak area of 34.9 +/- 0.3 mV which is a deviation of less than 0.75% for each pulse. The $\delta^{18}O$ values of the test pulse relative to the reference pulse was -0.024 +/- 0.1 indicating both good accuracy (0 is the expected value) and good precision. Similarly, the $\delta^{15}N$ values of the pulses were -0.012 +/- 0.1‰ relative to the reference pulse value showing oxygen and nitrogen isotopes can be measure with equal accuracy and a precision of +/- 0.1‰ by the IRMS.

2.2.2 Introducing a Secondary Source of N_2O for Analysis: Headspace Extraction

The precision of the on-line extraction system coupled to the IRMS was tested by analysis of vials containing N_2O. The 12mL glass vials capped with

rubber septa (LabCo) were filled with N_2O on a custom gas flushing rack using a tank of 100ppm (+/- 2%) N_2O in helium (Airgas). This is roughly equal to the amount of N_2O produced from100nmol nitrate solutions. Vials were flushed for 5 minutes at a flow rate of 40mL/min, which purges the headspace approximately 16 times (refer to Appendix D for full details). The N_2O is extracted from the vial headspace, passing through all purification traps, before being concentrated in the focus loop as discussed in section 2.2.3. The N_2O then passes through the GC column and into a second open split capillary (0.68 mm diameter) while the sample gas enters the IRMS via a second sniffing capillary (0.50mm diameter). The position of the GC capillary is controlled by a pneumatic piston plunger, and is lowered below the helium and sniffing capillaries just before the sample leaves the GC column and raised thereafter. The N_2O sample peak eluting from the GC produces a Gaussian shape peak (Figure 2.9), but it can still be integrated and compared against the areas of square N_2O reference peak.

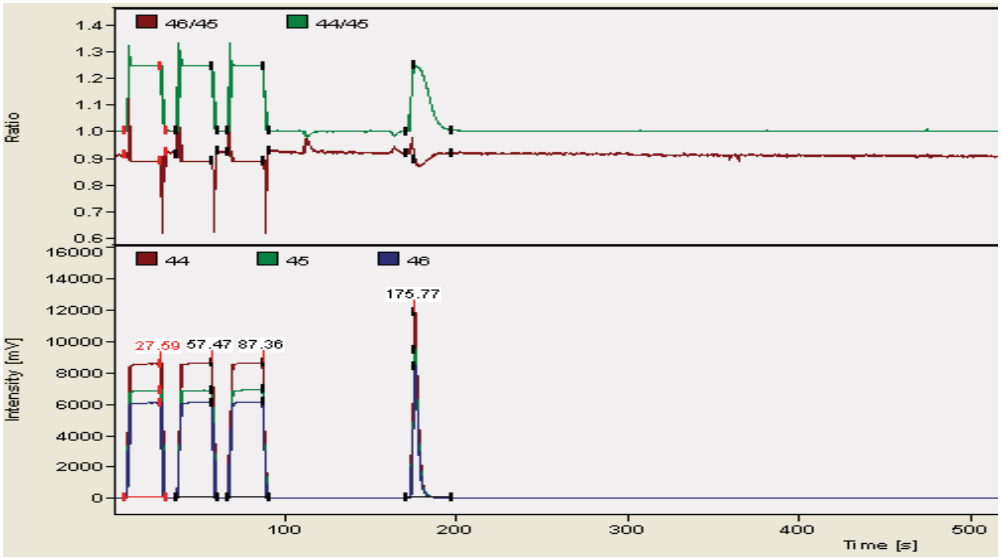


Figure 2.9: 100ppm N_2O peak preceded by reference peaks

The width and elution time of this peak is a function of the flow rate out of the GC column, which is a result of the GC helium pressure. If the flow from the helium carrier gas is increased, the sample peaks will elute sooner, but decreases the separation of gases that can cause interferences with the N_2O analysis such as CO_2, which is an isobar of N_2O (i.e. mass 44, 45, 46). The ideal helium pressure to minimize retention time (~3 min) but still achieve good separation of CO_2 is 12-15 psi. The results of one particular analysis of 100ppm N_2O flushed vials (n=52) gives a peak area of 25.5 +/- 0.6 mV, which is a deviations of less than 2.5% of each pulse. The standard deviation of $\delta^{18}O$ values of the N_2O pulse relative to the reference pulse was +/- 0.6. Likewise, $\delta^{15}N$ standard deviation was +/- 0.3. These precisions have been achieved on a regular basis and reflect the best precision possible for sample extraction and analysis.

2.2.3 Blank Signal

The investigation of the blank (nitrous oxide signal that originates from sources other than denitrification of the sample nitrate), is important because if the blank size in a given batch of samples is not small and reproducible it will affect the isotopes measured from N_2O. The N_2O blank was thoroughly investigated under many different scenarios: helium blanks, water blanks, reagent blanks, prepared bacterial solution blanks, and method blanks. All of these are considered to be ‘blanks’, as they do not contain any supplementary NO_3^- which could be converted to N_2O.

The first blank tests were on the helium purged vials and water. Since a large amount of helium gas is used as the online extraction carrier gas, trace amounts of N_2O in the helium could contribute to the overall blank. Using the headspace extraction protocol, extraction of helium purged vials did not produce any observable N_2O signal, showing the high purity helium purge gas is free of detectable N_2O. If the initial atmospheric air in vials was not flushed out and replaced with helium, the N_2O found as a trace gas in the atmosphere

(315ppb[15]), would be an additional blank in the headspace extraction. Based on the 12ml vial volume and 315 ppb mixing ratio, the blank from air would be 0.155 nmol. The absence of any detectable N_2O in the helium vials demonstrates that N_2O from air has been largely eliminated during the helium flushing procedure. When nitrate is added to the bacteria during normal sample analysis, it is dissolved in 1-2 ml of water. This water is saturated with N_2O according to Henry's Law[29],

$$[N_2O_{(aq)}] = k_{H\text{-}N2O} * p_{N2O}$$

where $k_{H\text{-}N2O}$ is the Henry's Law constant of N_2O and p_{N2O} is the partial pressure of N_2O in air, which yields a saturation concentration of N_2O in water of 7.8 nmol/L. Therefore, if 1mL of water is added to a flushed vial, only 7.8 picomoles of N_2O have been introduced, which is negligible. This solution blank was tested using vials containing only water and only broth (un-inoculated TSB). 1mL of solution was added to multiple vials that were capped and flushed with helium to dispel any air in the vial. Headspace extraction and IRMS analysis of both tests showed no detectable N_2O from either the water only or the broth only blank vials.

2.2.4 Blank from Bacterial Growth and Preparation

The blank size as a result of the prepared *P. Aureofaciens* growth solution (method blank) was also investigated. In this situation, the bacteria were grown in a media solution containing a large amount of nitrate. This nitrate should be completely reduced to N_2O during the growth period; failure to remove unreduced nitrate by rinsing or N_2O that is saturated in the solution could be a significant blank. After the bacteria were grown in large batches, vials were prepared by adding 1mL of water, flushing on the gas rack, and then injecting 1mL of the prepared *P. Aureofaciens* solution through the septa. The mixture

was allowed to sit for 1 hr, after which the bacteria were lysed by adding with NaOH. This procedure simulated the complete sample handling protocol (with no added NO_3^-). A peak area of roughly 0.8 V/s was observed when the pelletized bacteria solution was reacted with water, but without any purging of helium (Figure 2.10). Time trials of 1,2, and 3 hours bubbling with helium in a gas bubbler before inoculation showed that 2 hours of helium purging sufficiently reduces the method blank (Figure 2.10). These results prompted further testing of peak area reduction in greater detail.

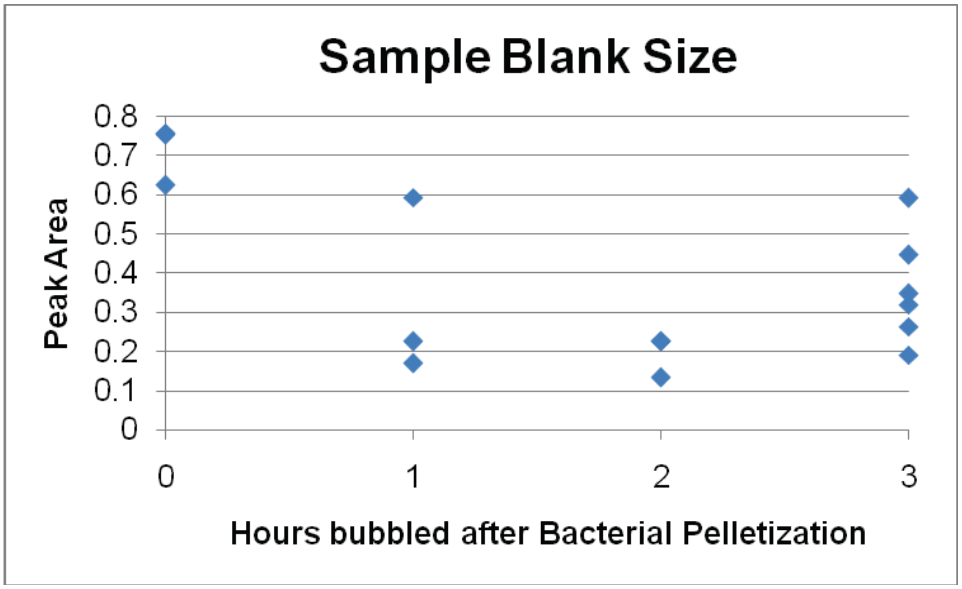


Figure 2.10: Blank size of *P. Aureofaciens* and water versus helium purging time

Using the bacterial growth solution, without pelletizing the bacteria or any further preparation step, resulted in an massive blank signal of 15.7 (Scenario 1, Figure 2.11). The second scenario (Figure 2.11) involved rinsing the bacterial solution with a nitrate-free medium. Peak areas were reduced to 1.2 +/- 0.1. Additional tests revealed that purging the growth solution with helium for 2 hrs immediately following centrifugation, but before allowing the bacterial solution to react with any other reagent, resulted in peak area of 0.1 +/- 0.04 (Scenario 3,

Figure 2.11), a factor of 10 less in both the average signal and standard deviation when compared to a solution that was not purged (Table A.3). Table A.4 shows the results of the peak area of bacteria and water blanks (1mL each of bacteria and water only in a vial, no NO_3^-), averaging 1.3 +/- 1.5 (Scenario 4, Figure 2.11) when the solution was stored overnight in the original polycarbonate bottle under air headspace. Once the prepared bacterial solution has been bubbled, it is important to store the solution under helium until the next day. Later tests showed that by replacing the air headspace with helium reduces the blank by a factor of 10 to 0.2 +/- 0.08. (Scenario 5, Figure 2.11). A final blank test revealed that by rinsing the bacterial solution, purging for 2 hr., storing under helium headspace overnight, and repeating the 2 hr purge the following day eliminated the blank signal altogether (Scenario 7, Figure 2.11) when compared to the peak area of 0.4 +/- 0.05 when rinsing, purging, sitting overnight under helium headspace, and inoculating without a second purge (Scenario 6, Figure 2.11). This means that not only does the helium serve to make the headspace anaerobic, but it also helps to reduce the signal of the blanks. Since the bottles used for growth have a wide plastic cap, the use of the 125mL glass bottle with a septum seal cap made helium flushing much easier and less uncertain that air would be able to mix. The glass bottles are also sterilized in an autoclave before use to avoid contaminants. A prepared bacteria solution sitting overnight under helium headspace must be used the following day; it cannot sit for 2 nights and then be used on the third day.

Tests of the method blank peak size based on the number of purging hours shows that a 2hr purge of helium, using a 250mL gas washing bottle with a fritted disk (Ace Glass, Inc.), is sufficient and greatly reduces the size of the blank (Tables A.3 and A.4). Tests were then conducted to see if the blank could be reduced by reversing the procedure (Scenario 7, Figure 2.11); by first adding bacteria to the vials, conducting a second helium flush of individual vials and then adding water. This resulted in elimination of blank peak areas.

The second flushing the bacteria removes any leftover nitrous oxide that may have been produced overnight as well as any excess nitrate in the growth solution. This blank has been associated with the growth media (or bacteria within) as it can vary between different batches of media, but has not shown to vary significantly within samples prepared by a single batch. Therefore, a blank is measured and can be applied as a constant correction to all of the samples within a run. The sample blank size (Peak Area= <0.3 V/s) is and should be a very low percentage of the sample. A small blank size has been found to be reproducible on a daily basis due to the bacterial solution purging with helium gas multiple times throughout the preparation process. The peak area of the method blank has been shown to be reproducible amongst a batch of samples; therefore, an offset correction can be made for the isotopic fractionation[22].

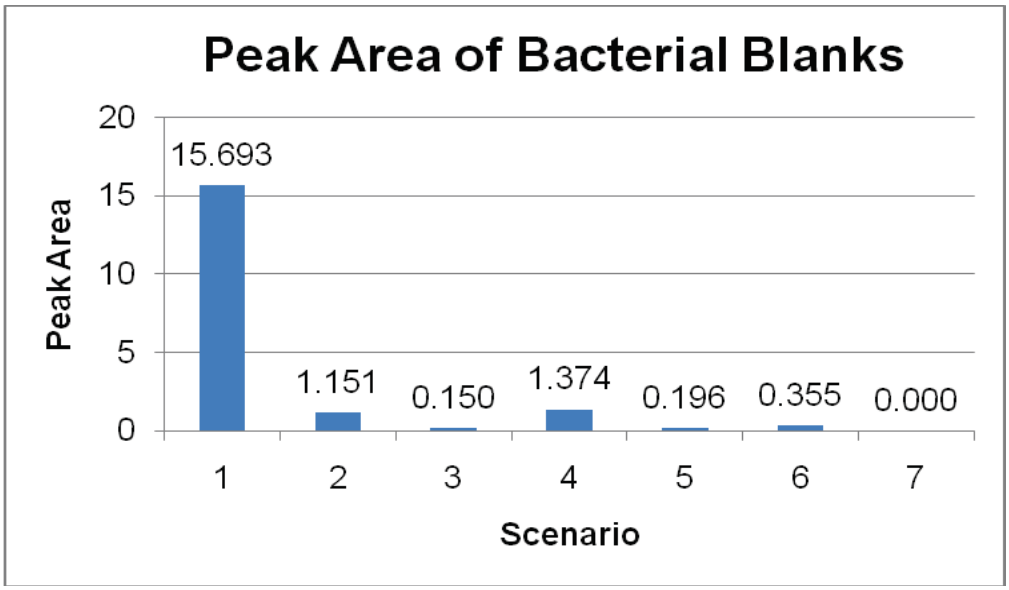


Figure 2.11: Peak areas of bacterial blanks from different scenarios:
1) No rinse, no purge, 2) Rinse, no purge,
3) Rinse, then 2 hr purge, 4) Rinse, sit overnight in air headspace,
5) Rinse, sit overnight in helium headspace,
6) Rinse, 2 hr purge, sit overnight in helium headspace, and
7) Rinse, 2 hr purge, sit overnight in helium headspace, 2 hr purge.

2.2.5 Sample Conversion

Biologic reduction is known to cause isotope fractionation therefore, complete conversion of nitrate into N_2O by the denitrifying bacteria is important for the isotopic analysis. Data showing N_2O peak area versus time suggests that the conversion of NO_3^- to N_2O via bacterial denitrification nears completion after 40 min. of reaction (Figure 2.12). The data show that a 60 min incubation period is sufficient to convert the NO_3^- to N_2O, but nitrate solutions with a concentration greater than 100nmol NO_3^- per milliliter require a longer incubated period as well (Figure 2.13). The $\delta^{18}O$ and $\delta^{15}N$ values of the product N_2O are fairly constant after 30 min. of denitrification. However, at reaction times less than 30 minutes, the δ values are consistently lower as a function of peak area

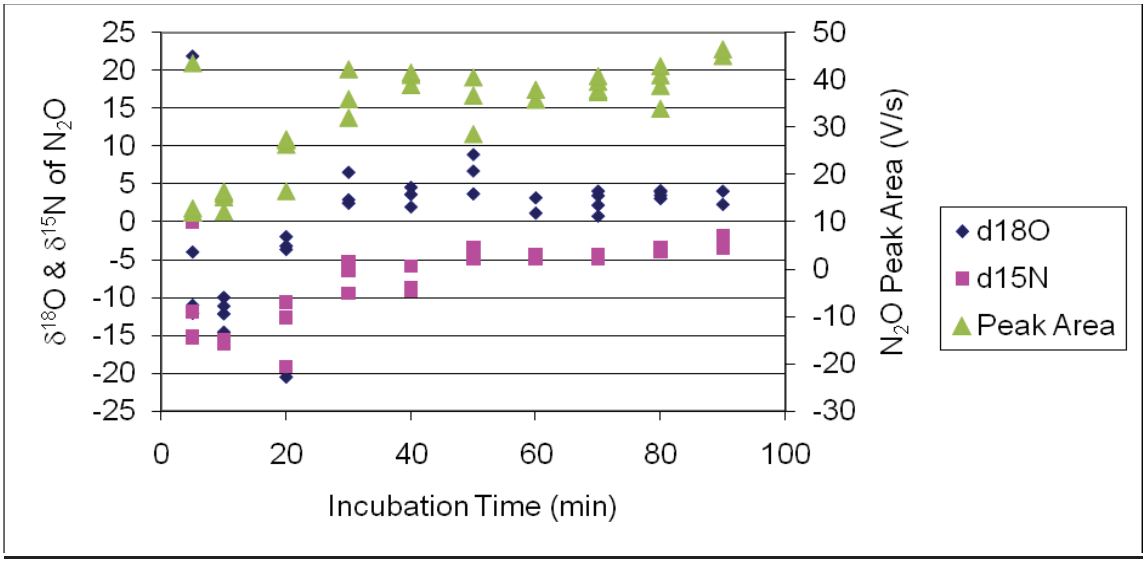


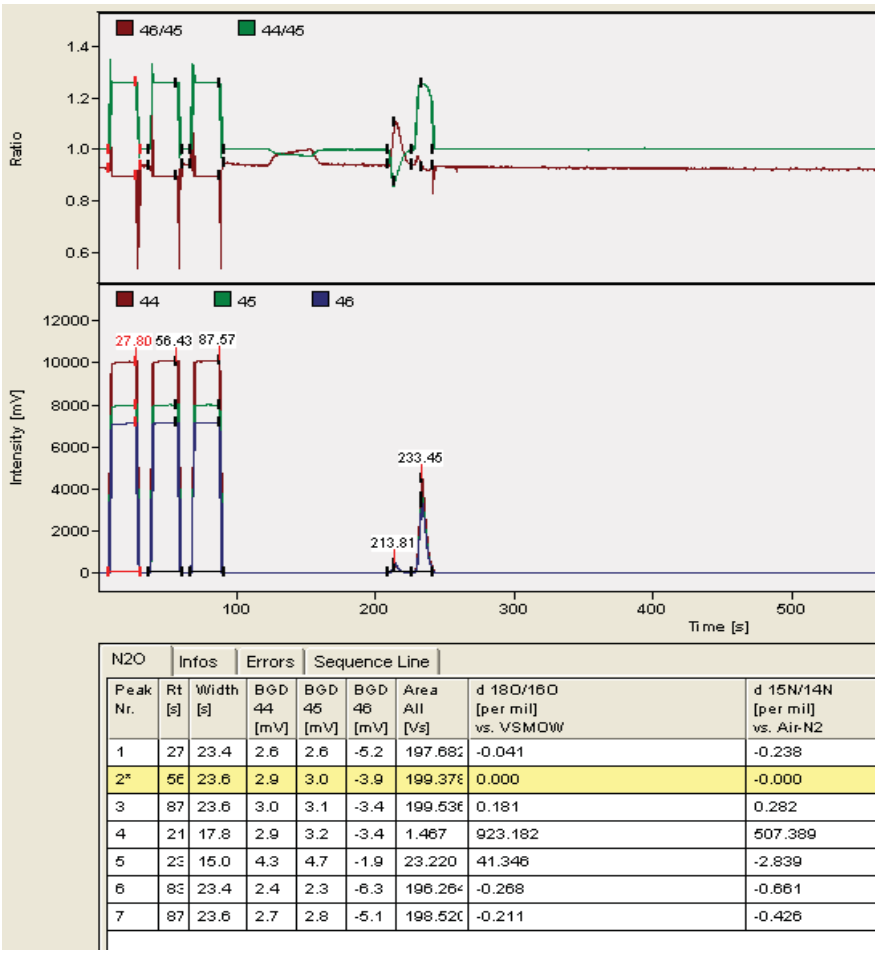
Figure 2.12: Rayleigh plot based on inoculation time of N_2O by *P. Aureofaciens*

(yield) and can be as low as -14‰ for $\delta^{15}N$ and -19‰ for $\delta^{18}O$ relative to the steady state value. As the bacterial reaction progresses, the isotopes follow a Rayleigh distillation behavior. For nitrogen, the N atoms are conserved in the

$2NO_3^- \rightarrow N_2O$ reaction, so the Rayleigh fractionation will be a function of the reaction progress. A Rayleigh plot, shown below, was plotted to show the effect of isotopic fractionation on $\delta^{18}O$ and $\delta^{15}N$ values. This figure shows that isotopic fractionation between NO_3^- and product N_2O is somewhat large. At 100% conversion, the $\delta^{15}N$ value in N_2O will be the same as the $\delta^{15}N$ in the starting NO_3^-. For oxygen, kinetics will control the isotopic effects because there is a loss of five of the six nitrate's oxygen atoms during the conversion to N_2O. This kinetic isotope effect (KIE) causes the N_2O to be enriched in ^{18}O compared to initial nitrate, even at 100% conversion. However, the data show that when >90% yields are achieved the $\delta^{18}O$ value is consistent and the actual $\delta^{18}O$ value of the nitrate can be determined using reference materials because the KIE will be the same. The $\delta^{18}O$ and $\delta^{15}N$ values are fairly constant after 30 min, while the range of δ values is ~14 for $\delta^{15}N$ and ~19 for $\delta^{18}O$ over the same time period. If the bacterial reaction doesn't go to completion, the Rayleigh process is shut down and this will not be true.

2.2.6 Results of Standard Analyses

The resulting precision and accuracy of the complete method was assessed using a large number of analyses of our nitrate standards (Table A.5). A typical voltage to time plot of one nitrate analysis is shown in Figure 2.13. The main visual difference between the 100ppm N_2O or blank analysis is the presence of CO_2 (an isobar of N_2O) that appears as a small peak just before the N_2O peak. The software "interprets" this peak as N_2O and the high natural abundance of ^{13}C relative to ^{15}N results in high $\delta^{15}N$ and $\delta^{18}O$ values (100's of ‰) for this "N_2O", which is actually CO_2. This highlights why the Plot-Q column needs to effectively separate CO_2 and N_2O; even a 1% contribution from CO_2 could change the N_2O values by 10‰ (using isotope mass balance). Over the course of one batch of standards, N_2O peaks consistently appear on the chromatogram at the 3 minute mark (+/- 3 sec.) when the helium flow is 15psi.



N2O | Infos | Errors | Sequence Line

Peak Nr.	Rt [s]	Width [s]	BGD 44 [mV]	BGD 45 [mV]	BGD 46 [mV]	Area All [Vs]	d 18O/16O [per mil] vs. VSMOW	d 15N/14N [per mil] vs. Air-N2
1	27	23.4	2.6	2.6	-5.2	197.68	-0.041	-0.238
2*	56	23.6	2.9	3.0	-3.9	199.37	0.000	-0.000
3	87	23.6	3.0	3.1	-3.4	199.53	0.181	0.282
4	21	17.8	2.9	3.2	-3.4	1.467	923.182	507.389
5	23	15.0	4.3	4.7	-1.9	23.220	41.346	-2.839
6	83	23.4	2.4	2.3	-6.3	196.26	-0.268	-0.661
7	87	23.6	2.7	2.8	-5.1	198.52	-0.211	-0.426

Figure 2.13: Example chromatograph of 20Hoff nitrate standard

The denitrifier method results in reproducible peak areas and delta values for a given reference nitrate showing that the method gives good precision. For a given sample size of 50nmol N_2O (from 100nmol NO_3^-), the peak area averages 20-30 V/s or 1.5-2.5 V/s per mole of N_2O. The 1Hoff nitrate standard has been found to have isotopic values of $\delta^{18}O$= -26.9 +/- 0.7 and $\delta^{15}N$= -4.3 +/- 0.1. An increase in the peak area does change the isotopic values. When nitrate standards had a concentration greater than 100nmol NO_3^-, there is linearity from 0-125 V/s and again from 125-200 V/s, although the linearity for $\delta^{18}O$ is not the same as $\delta^{15}N$ in either case (Figure 2.14). Changes in the peak area on $\delta^{18}O$ and

$\delta^{15}N$ can be corrected for using the equation of the line when δ is plotted versus peak area, using the line from the appropriate set of peak area linearity.

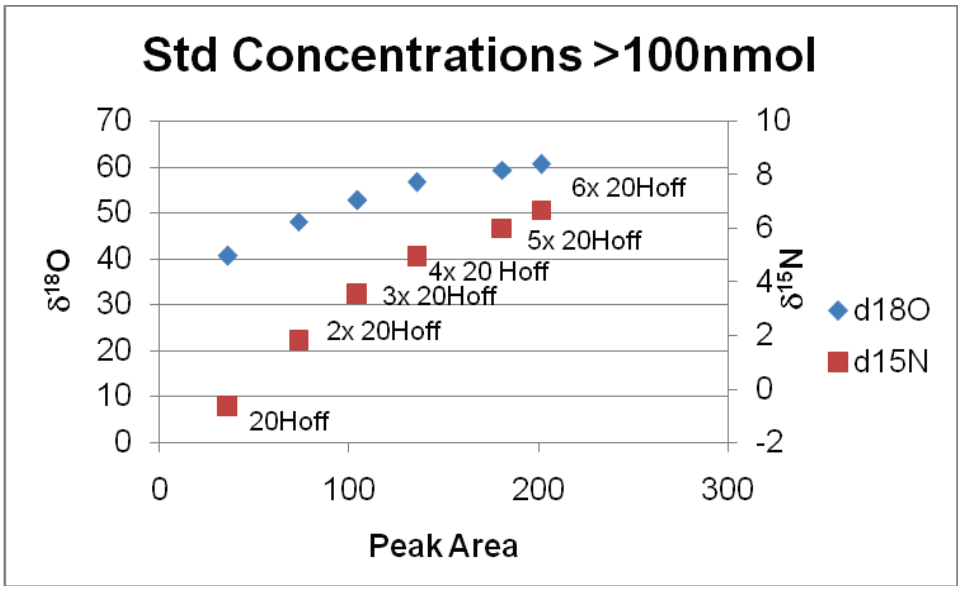


Figure 2.14: Change in $\delta^{18}O$ when nitrate concentration increases

A large number of analyses of the four working reference nitrates were conducted as a measure of the accuracy of the method. IRMS analyses resulted in the isotopic values in the following table, relative to VSMOW and N_2-Air, respectively:

Table 2.3: Example of typical isotopic values (‰) for calibrated standards

	$^{18}O_{measured}$	$^{18}O_{actual}$	$^{15}N_{measured}$	$^{15}N_{actual}$
20Hoff	41.8	54.1	-1.5	0.5
10Hoff	10.7	17.6	4.6	7.9
1Hoff	-19.9	-15.2	11.9	14.6
NC32	-25.1	-18.8	12.6	15.3

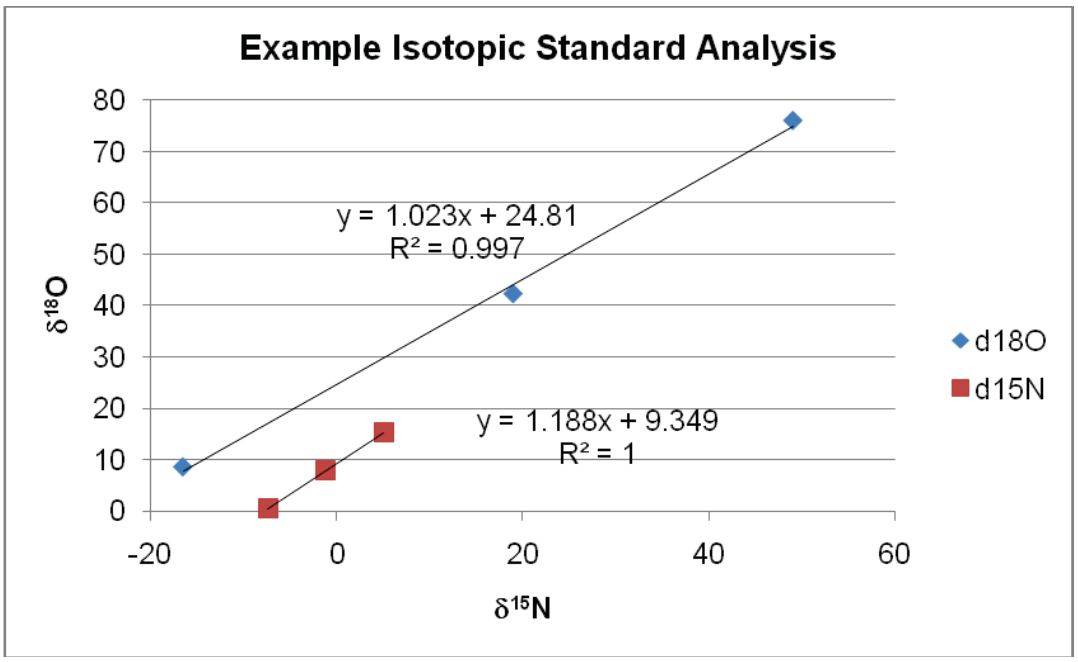


Figure 2.15: $\delta^{18}O$ and $\delta^{15}N$ of example standard analysis

The measured $\delta^{18}O$ and $\delta^{15}N$ are the isotopic values measured by the IRMS relative to the tank N_2O reference gas. The expected values have been determined based on reference calculations (section 2.1.4). The difference between the measured and actual (calculated) $\delta^{15}N$ isotopic values can be attributed to the difference in the N_2O reference tank, whereas the same differences in $\delta^{18}O$ are caused by fractionation during nitrate reduction as well as the tank. A calibration curve is constructed using the accepted isotopic values (based on calculation or prior calibration measurements) and the measured isotopic values of the standards. The equation of the line can be used to transform the measured $\delta^{15}N$ and $\delta^{18}O$ values into the correct values relative to N_2-Air and SMOW.

2.2.7 Background Signal

A large number of analyses led to the conclusion that in order to achieve reliable results, it is very important that the background levels of masses 44, 45, and 46 be low (<10 mV) and stable (Figure 2.16). Figure 2.17 shows the poor precision of isotopic values when the background is unstable. In an attempt to control the stability of background levels, a number of treatments were tested to observe how background levels affected the precision of isotopic values: sample capillary in the open split, GC column heating, and using a coldtrap. Initially, the sample capillary coming from the GC column into the open split was always left below the sniffer capillary and the IRMS was exposed to all gases eluting from the column over time. The procedure was adjusted so that the sample capillary is lowered into the open split just before the sample N_2O gas leaves the GC column and is again raised just after the entire N_2O peak has eluted. The hypothesis was that compounds such as water and VOCs might build up in the Plot-Q column and then bleed out over time causing isobaric inferences. Initially, the GC column was baked out daily at 280°C to purge any gases that might slowly elute and contribute to background isobaric interferences (e.g H_2O). Monitoring the background over a 48 hour period showed the background signal peaks and valleys every 12 analyses (3 hours), supporting the hypothesis that some compound with a long retention time was eluting and introducing a prolonged isobaric interference (Figure 2.18). To help limit the build up of these contaminating gases, a -80°C ethanol slush coldtrap was added to the extraction lines following the GC to trap any remaining water or low vapor pressure VOCs that may have made its way through the extraction system. Figure 2.19 shows that although the coldtrap reduced the background signal, there is still a jump in the values every 10 analyses or so. Reproducible low and stable background levels were achieved (Figure 2.20) by altering the procedure so that the after N_2O has eluted, the Plot-Q column is baked at 380°C for 10 minutes, followed by a 3 minute cool down that brings the column back to ambient temperatures before the next analysis. Having a low stable background at 44, 45, and 46 amu

improved standard deviations of nitrate standard analyses considerably from +/- 5‰ before these new procedures to an acceptable +/- 0.5‰ in most runs after the new treatments were implemented.

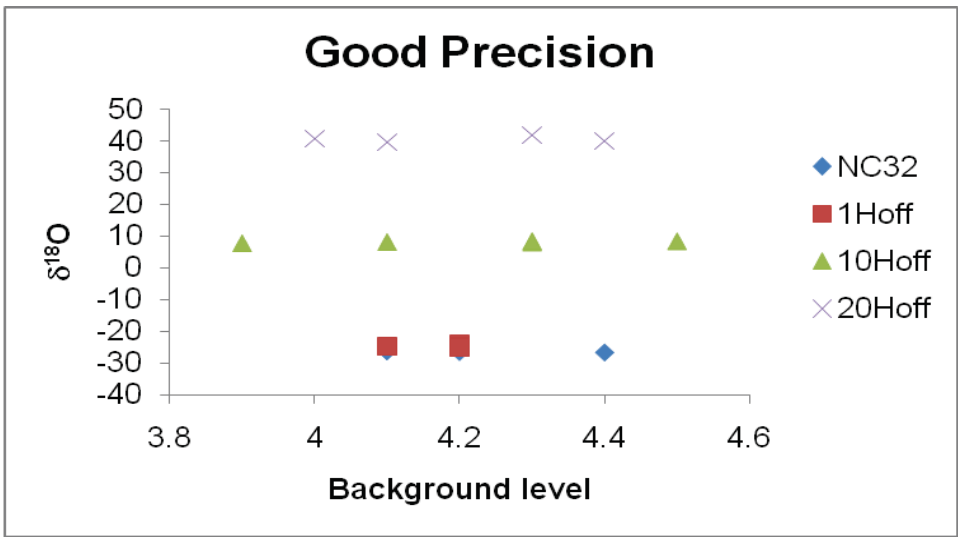


Figure 2.16: Stable backgrounds give isotopic values with low standard deviations

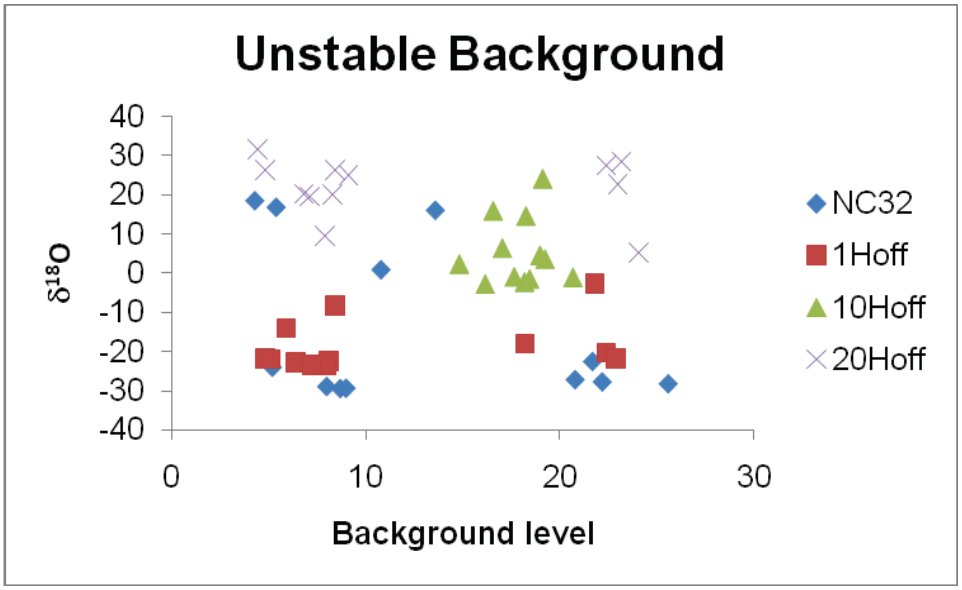


Figure 2.17: Unstable backgrounds produce unreliable isotopic values

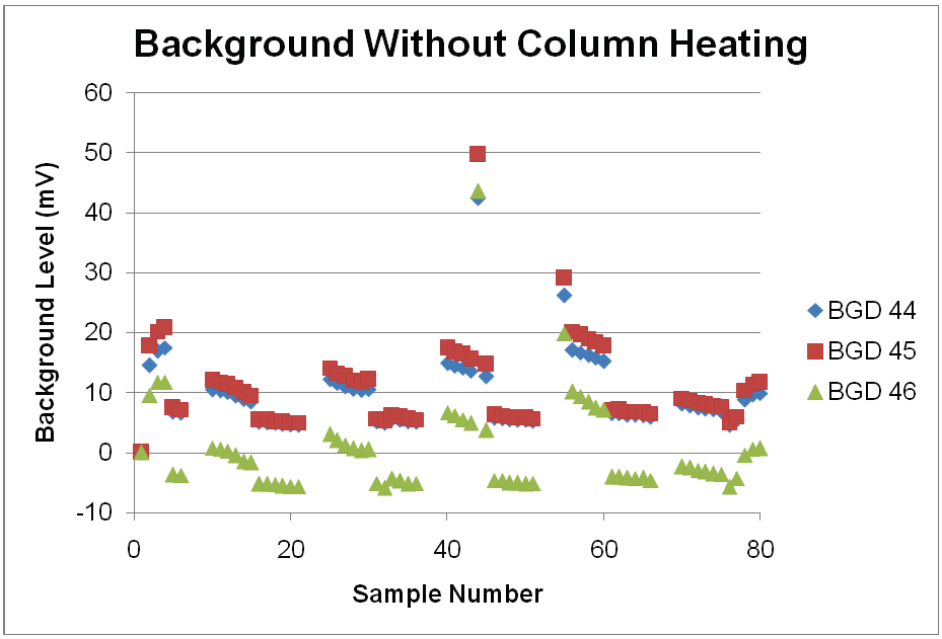


Figure 2.18: High, unstable background levels when the GC column is baked prior to each batch of sample analyses.

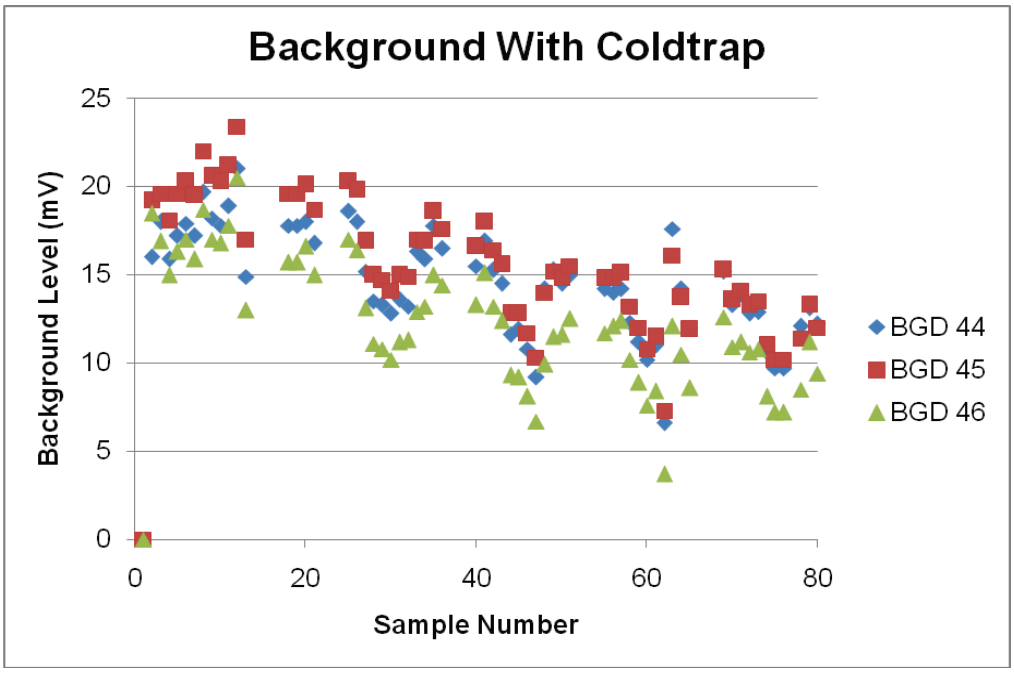


Figure 2.19: Reduced background levels with the addition of a -80°C Coldtrap.

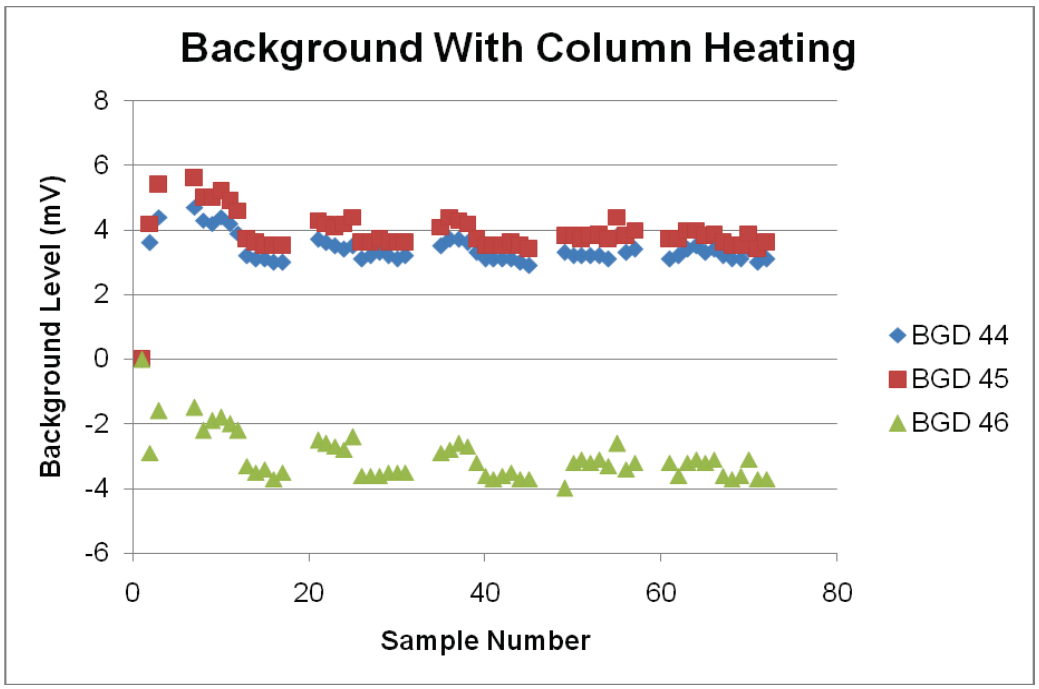


Figure 2.20: Low, stable backgrounds as a result of backing out the GC column during each sample vial analysis and using the -80°C cold trap.

2.2.8 Impact of pH and Pre-Concentration on $\delta^{15}N$ and $\delta^{18}O$

The ideal nitrate sample size and concentration for this method is based on the blank size and limitations of the extraction system. Ideally, a blank signal with a peak area of 1% or less of the sample area will give good precision and accuracy. Since the procedural blank results in a signal area of 0.3 V/s or less (see section 2.2.4), a sample peak area over 30 V/s is desirable. Analysis of the 100 ppm N_2O tank (50 nmol per 12ml vial) yields peak areas of ~35 V/s. Nitrate samples/references should then be measured out at 100nmol of NO_3^-, which would result in 50 nmol N_2O to achieve good precision/accuracy. In terms of liquid samples, injecting 1 ml of sample solution is easy to handle, so to achieve the 100 nmol requirement, the nitrate concentration of the sample needs to be 6.3mg/L. Samples with higher concentrations can be diluted or a smaller volume injected. If the nitrate concentration is less than 6.3 mg/L, the method is limited by the volume that the 12ml vial can hold. A maximum of 3 mL of sample

solution can be added before the total volume (sample plus the volume of the bacteria reagents) begins to rise to a level that the helium purge may force liquid out the exit hole of the double needle. Therefore, the low limit is ~2 mg/L of nitrate; this ensures sufficient nitrate for analysis but also allows for efficient headspace extraction. Many environmental samples often have nitrate concentrations below this level. For example, nitrate is 35 μg/L in Lake Tahoe's water column[30]. Soil extracts and aerosol filters may also be in the ug/L level. In these instances, the sample must be pre-concentrated.

Little research has been published on the impact of pre-concentration methods on isotopic composition of nitrate. Three pre-concentration techniques were tested: freeze-drying, evaporation, and ion chromatography. In each test, standards were tested for their isotopic composition after pre-concentration. Approximately 1mL of a 100ppm liquid nitrate standard was added to a 15mL plastic centrifuge tube (FisherSci) and amended with a 14mL acidic (100, 10, or 1μM H_2SO_4) or basic (0.1M Na_2CO_3) solution to a specific pH to simulate samples that are not neutral, such as rainwaters. After preparation, both sets of samples were evaporated in an oven at 80°C for up to two days or freeze-dried on a centrifuge for ~6-8 hrs. When the pH of a nitrate solution is of a value other than 7, peak areas and $\delta^{15}N$ and $\delta^{18}O$ values are negatively affected. At pH <7, the peak area decreases dramatically when the acidity increases (Figure 2.21). As shown in Figures 2.22 and 2.23, the isotopic values from acidic nitrate samples are very unreliable.

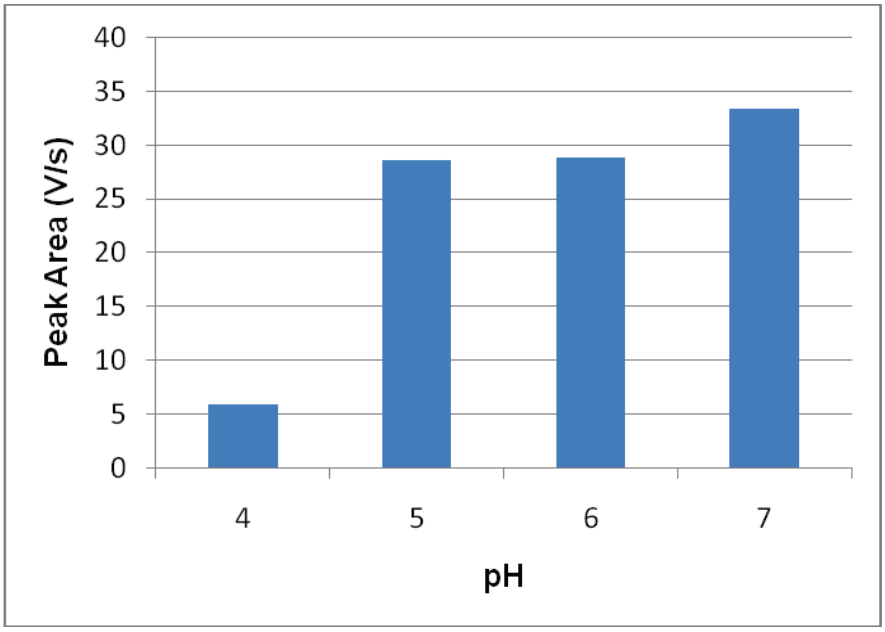


Figure 2.21: Peak area versus pH

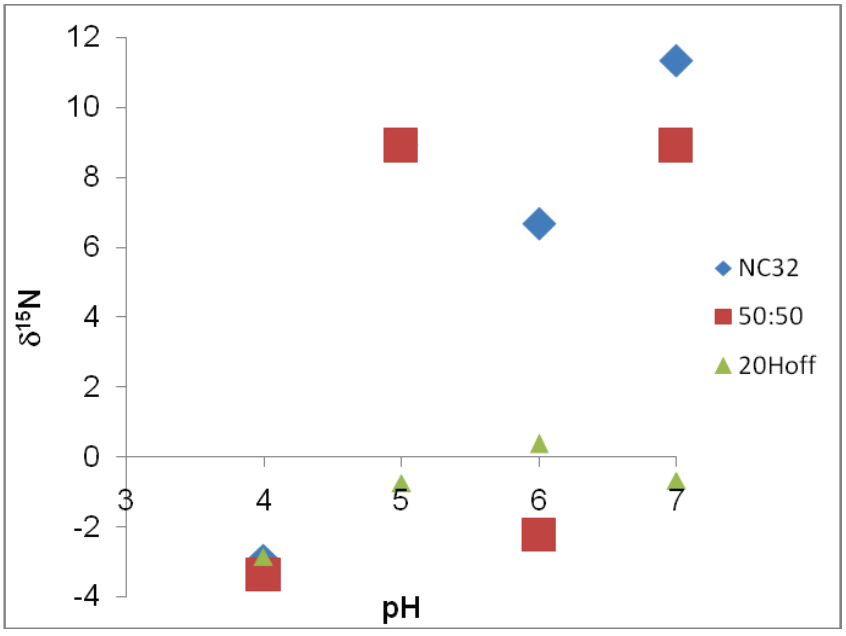


Figure 2.22: $\delta^{15}N$ of N_2O versus pH of nitrate solutions evaporated to dryness.

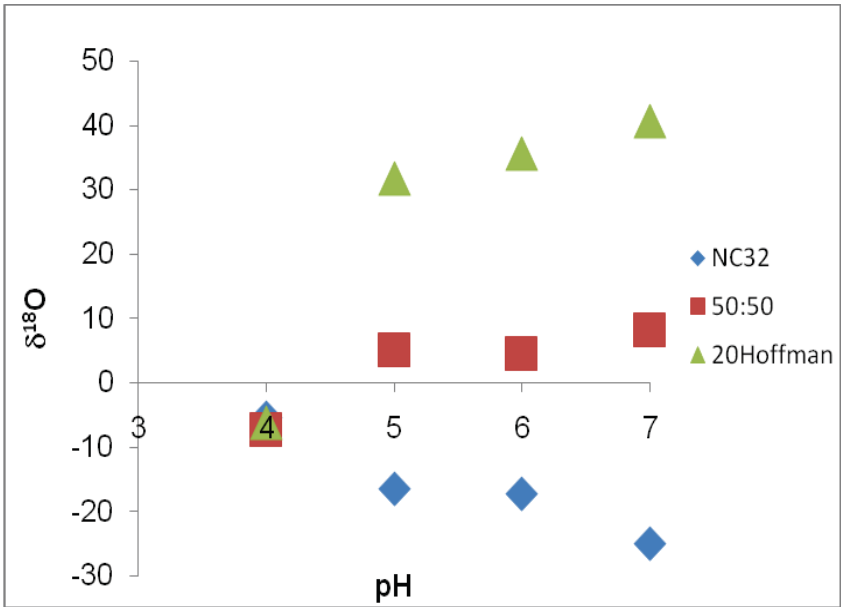


Figure 2.23: $\delta^{18}O$ N_2O versus pH of nitrate solutions evaporated to dryness.

Although it is easier to let nitrate samples evaporate to dryness during pre-concentration, samples must be evaporated only until the volume is just less than or equal to 2mL. (Evaporation to dryness greatly increases the risk of vaporizing off NO_3^- as HNO_3, which in turn has an isotope effect.) This also results in the production of nitric acid and subsequent volatilization or exchange with water at low pH levels:

$$H^+ + NO_3^- \leftrightarrow HNO_{3(aq)} \leftrightarrow HNO_{3(g)}$$

$$k_{eq} = [H^+][NO_3^-]/[HNO_3]$$

When NO_3^- is lost, the change in isotopes left in solution is exhibited in the decreasing yields (Figure 2.21). At low pH, isotope fractionation appears to be happening via volatilization because both $\delta^{15}N$ and $\delta^{18}O$ are impacted while exchange with H_2O would only impact $\delta^{18}O$ (Figures 2.22 and 2.23). What seems to be happening is that the NO_3^- is volatilizing off as HNO_3.

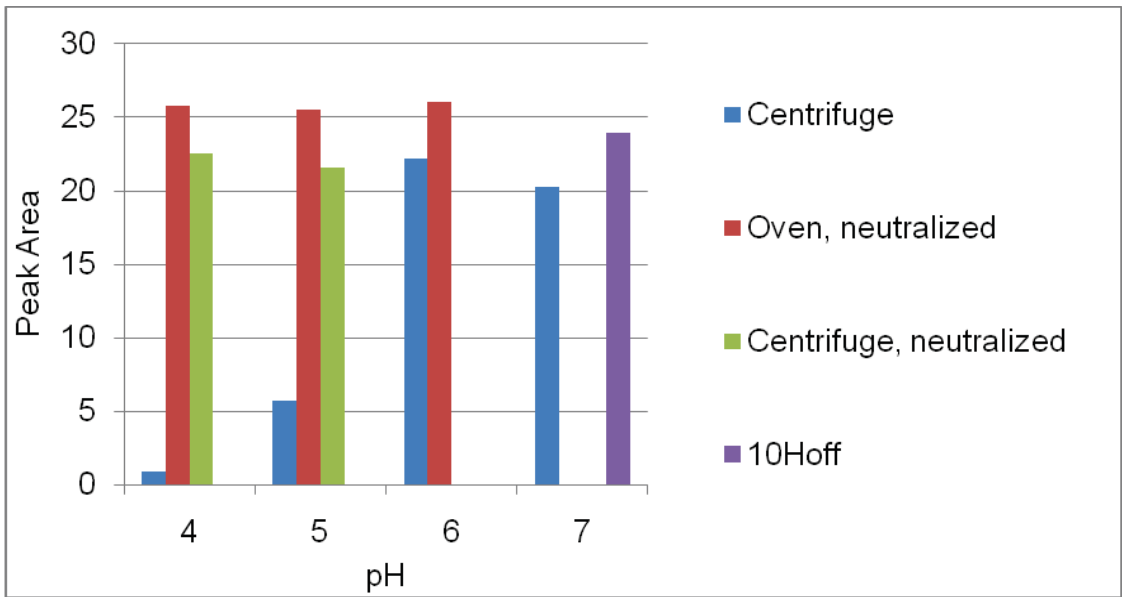


Figure 2.24: Peak area after pre-concentration of nitrate standards

A second set of identical solutions were prepared to a specific pH, as above, but were then buffered. Acidic 10Hoff nitrate standards were neutralized using a 0.35M $HNaCO_3/Na_2CO_3$ solution before pre-concentration took place. 1mL of 10Hoff nitrate standard was neutralized with the 14mL buffer and then one set of standards was evaporated in an 80°C oven while a second set was evaporated on a centrifuge attached to a freeze-drier. The original untreated 10Hoff standard gives a peak area of ~24 V/s (Figure 2.24) similar to both the neutralized oven and centrifuge freeze-dried samples that produced a peak area of ~25 V/s. This is in contrast to the un-buffered acidic samples (Figure 2.24) where the peak areas diminished as the pH became more acidic. The conclusion is that neutralizing acidic solutions before the denitrification of the sample nitrate is critical for accurate isotopic analysis of nitrate when the sample solutions are even slightly acidic.

Ion chromatography was also tested as a means of separating NO_3^- from other anions in solution that might interfere with the isotopic analysis. A control solution was made containing 100nmol of Cl^-, SO_4^{2-}, NO_2^-, and 20Hoff NO_3^-. Vials

were aliquoted with 0.75mL of the anion solution into a 15mL centrifuge tube using a pipet and diluted with 14.25 mL of water. The “separation method” uses the ion chromatography as a preparative device (as opposed to analytical device) and the entire 15mL is pumped onto the column. Once loaded onto the column, the anions are eluted using a $Na_2CO_3/NaHCO_3$ eluent as the mobile phase. Initial separations did not completely separate NO_2^- from NO_3^- (Figure 2.25a). The pump speed and mobile phase solution strengths were modified and NO_3^- can successfully be separated from Cl^- and SO_4^{2-} in the same samples (Figure 2.25b). Volumes of the eluted nitrate peaks are ~ 2-3 ml and still have trace bicarbonate and therefore can be further freeze dried without risk of isotopic alteration. The denitrifier method was used to determine the isotopic values of the separated 20Hoff NO_3^- solutions and standard deviations were low and less than 0.7‰.

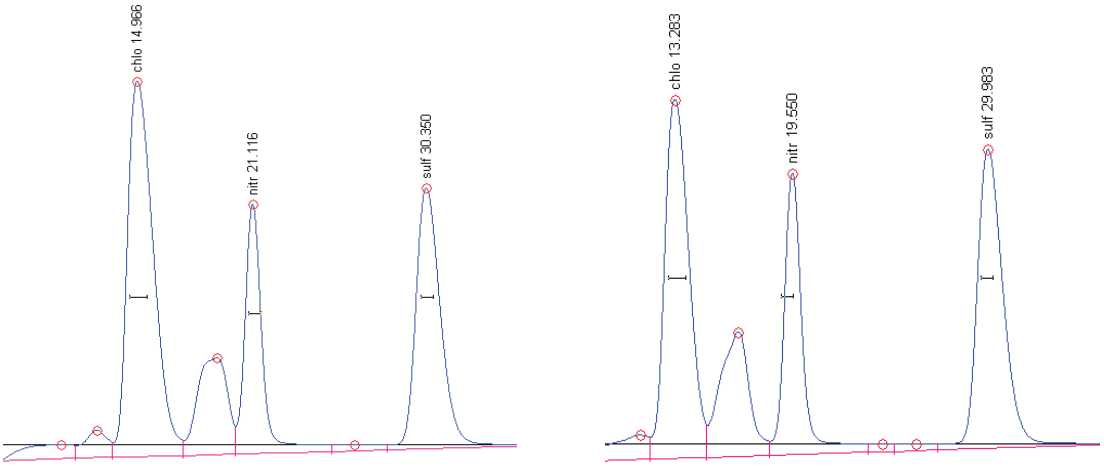


Figure 2.25: Ion chromatography used to separate NO_3^- from other anions: a) initially, NO_2^- and NO_3^- were not fully separated (left), but b) NO_3^- successfully separated from NO_2^- and other ions.

2.2.9 $\delta^{15}N$ and $\delta^{18}O$ Analyses of Precipitation Samples

The denitrifier method, as described in this thesis, was used to collect data on the $\delta^{18}O$ and $\delta^{15}N$ of nitrate extracted from rainwater at collected from two regions. The first region was precipitation samples from Hong Kong, China provided by the Global Network of Isotopes in Precipitation (GNIP) as part of the International Atomic Energy Agency (IAEA)/World Meteorological Organization (WMO) Isotopes-in-Precipitation Network project. The second set was rainwater collected weekly over three years at three sites in Indiana and Illinois (details can be found in Masters Thesis of David F. Mase). Nitrate in monthly rainwater in Hong Kong had a range of $\delta^{18}O$= +26.7 to +66.5‰ and $\delta^{15}N$= -19.2 to +1.9‰ (Figures 2.26 and 2.27). The Midwestern sites had a range of $\delta^{18}O$= +63.6 to +87.0‰ and $\delta^{15}N$= -16.3 to +20‰. Both sites exhibit a range of $\delta^{18}O$ that has been published in recent studies and some seasonal variations are observed[35,36,37]. The $\delta^{15}N$ range is typically within the values observed in atmospheric nitrate, but there are clear instances of $\delta^{15}N$ above 10‰ and below -10‰ which is atypical of atmospheric nitrate $\delta^{15}N$ values published in the existing literature[36,38,39,40]. A full description and interpretation of the meaning of the $\delta^{18}O$ and $\delta^{15}N$ values in these precipitation nitrates is beyond the scope of this thesis, which is based on developing the analytical method, but will be discussed in forthcoming publications by David Mase, Greg Michalski, and Lindsey Crawley.

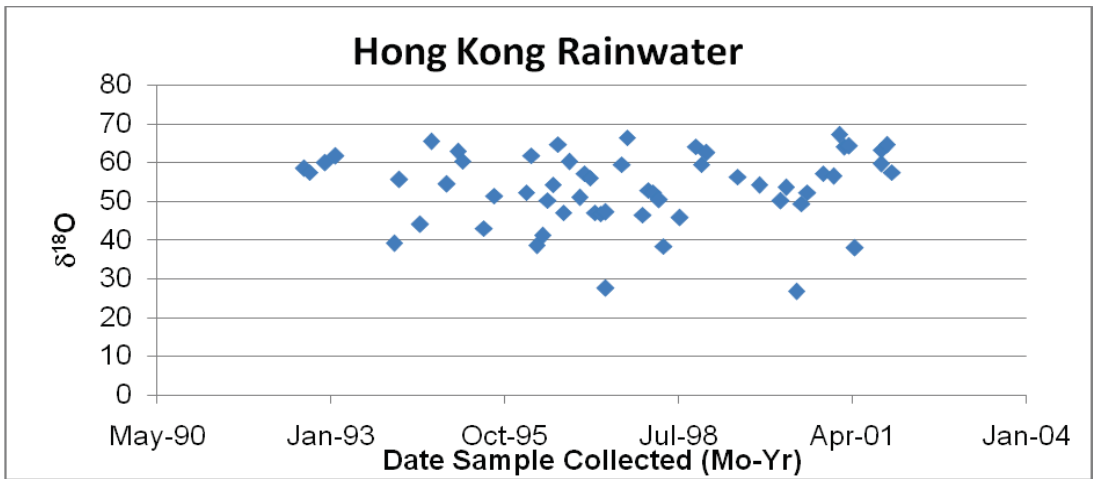


Figure 2.26: $\delta^{18}O$ of Nitrate in Hong Kong Rainwater

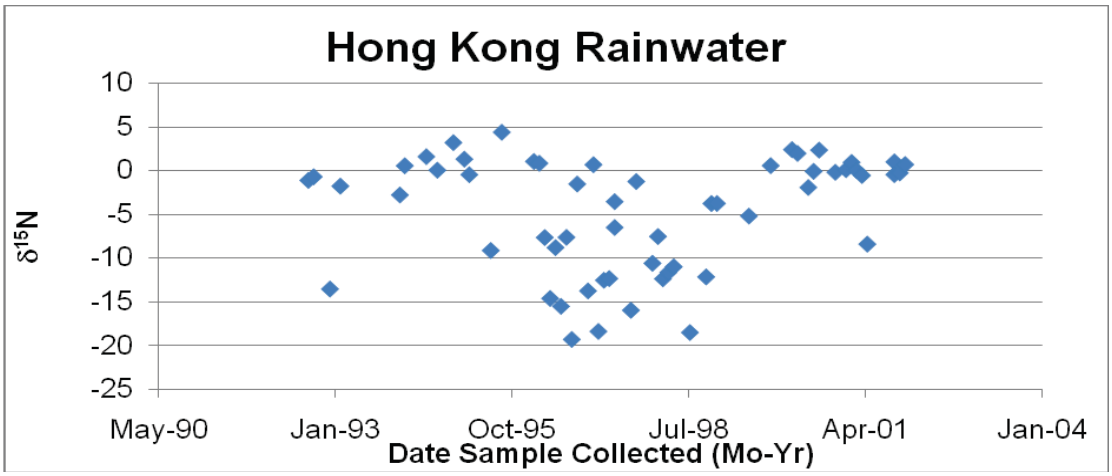


Figure 2.27: $\delta^{15}N$ of Nitrate in Hong Kong Rainwater

2.3 Conclusion

The denitrifier method has shown to be a fully compatible method for the simultaneous measurements of $^{18}O/^{16}O$ and $^{15}N/^{14}N$ of nitrate. Pre-concentration of nitrate samples using evaporation methods has been proven to reflect the original isotopic signature of samples provided that the solution is neutralized prior to denitrification. We are proud to report that the denitrifier method has been

successfully implemented into the Michalski Stable Isotope Lab as a fully operational method for the nitrogen and oxygen isotopic analysis of nitrate. The denitrifier method has been used to determine the nitrogen and oxygen composition of rainwater sample from the Midwestern United States and Hong Kong, China. Continued work for the triple oxygen isotope composition of nitrate using thermal decomposition by fellow labmates is expected to ensue in the near future.

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Appendix A: Tables of Related Data

Table A.1: 100ppm N_2O standard deviation <1

Identifier	*Peak Area (mV/s)*
100ppm N_2O	26.129
100ppm N_2O	24.992
100ppm N_2O	26.292
100ppm N_2O	25.998
100ppm N_2O	25.715
100ppm N_2O	25.738
100ppm N_2O	25.834
100ppm N_2O	26.081
100ppm N_2O	26.276
100ppm N_2O	25.864
100ppm N_2O	26.288
100ppm N_2O	25.935
100ppm N_2O	26.219
100ppm N_2O	26.323
100ppm N_2O	26.077
100ppm N_2O	26.280
100ppm N_2O	26.035
100ppm N_2O	25.752
100ppm N_2O	25.444
100ppm N_2O	23.945
100ppm N_2O	24.547
100ppm N_2O	25.323
100ppm N_2O	25.538
100ppm N_2O	25.471
100ppm N_2O	25.510
100ppm N_2O	25.521
100ppm N_2O	25.546
100ppm N_2O	25.566
100ppm N_2O	25.556
100ppm N_2O	25.447
100ppm N_2O	23.584
100ppm N_2O	24.625
100ppm N_2O	24.978
100ppm N_2O	25.360
100ppm N_2O	25.418
100ppm N_2O	25.868
100ppm N_2O	25.496
100ppm N_2O	25.316

100ppm N_2O	25.537	
100ppm N_2O	25.552	
100ppm N_2O	25.389	
100ppm N_2O	25.582	
100ppm N_2O	24.274	
100ppm N_2O	24.561	
100ppm N_2O	24.949	
100ppm N_2O	25.025	
100ppm N_2O	25.831	
100ppm N_2O	24.297	
100ppm N_2O	25.563	
100ppm N_2O	25.235	
100ppm N_2O	25.089	
100ppm N_2O	24.711	
	25.5	*Avg*
	0.6	*Std Dev*

Table A.2: Water blanks before helium purging

Identifier	*Peak Area (mV/s)*	
PAur & Water	1.356	
PAur & Water	1.069	
PAur & Water	1.177	
PAur & Water	1.162	
PAur & Water	1.022	
PAur & Water	1.045	
PAur & Water	1.246	
PAur & Water	1.213	
PAur & Water	1.065	
	1.2	*Avg*
	0.1	*Std Dev*

Table A.3: Water blanks after helium purging

Identifier	*Peak Area (mV/s)*	
PAur & Water with Purging	0.277	
PAur & Water with Purging	0.211	
PAur & Water with Purging	0.168	
PAur & Water with Purging	0.135	
PAur & Water with Purging	0.115	
PAur & Water with Purging	0.107	
PAur & Water with Purging	0.269	
PAur & Water with Purging	0.186	
PAur & Water with Purging	0.161	
PAur & Water with Purging	0.153	
PAur & Water with Purging	0.144	
PAur & Water with Purging	0.139	
PAur & Water with Purging	0.117	
PAur & Water with Purging	0.121	
PAur & Water with Purging	0.119	
PAur & Water with Purging	0.128	
PAur & Water with Purging	0.127	
PAur & Water with Purging	0.126	
PAur & Water with Purging	0.136	
PAur & Water with Purging	0.159	
PAur & Water with Purging	0.135	
PAur & Water with Purging	0.134	
PAur & Water with Purging	0.120	
PAur & Water with Purging	0.124	
	0.150	*Avg*
	0.044	*Std Dev*

Table A.4: Peak areas and standard deviations before and after sitting overnight under helium headspace.

Identifier 1	*Area All*
Bac in plastic, air headspace	5.528
Bac in plastic, air headspace	1.146
Bac in plastic, air headspace	1.079
Bac in plastic, air headspace	0.717
Bac in plastic, air headspace	0.740
Bac in plastic, air headspace	1.067
Bac in plastic, air headspace	0.875
Bac in plastic, air headspace	0.761
Bac in plastic, air headspace	0.480
Bac in plastic, air headspace	0.878
Bac in plastic, air headspace	1.172
Bac in plastic, air headspace	0.684

Bac in plastic, air headspace	6.844	
Bac in plastic, air headspace	1.152	
Bac in plastic, air headspace	0.711	
Bac in plastic, air headspace	0.992	
Bac in plastic, air headspace	0.857	
Bac in plastic, air headspace	1.228	
Bac in plastic, air headspace	0.910	
Bac in plastic, air headspace	0.965	
Bac in plastic, air headspace	1.221	
Bac in plastic, air headspace	0.969	
Bac in plastic, air headspace	1.087	
Bac in plastic, air headspace	0.929	
	1.374	*Avg*
	1.507	*Std Dev*

Bac in autoclaved btl, flushed	0.249	
Bac in autoclaved btl, flushed	0.141	
Bac in autoclaved btl, flushed	0.122	
Bac in autoclaved btl, flushed	0.140	
Bac in autoclaved btl, flushed	0.205	
Bac in autoclaved btl, flushed	0.206	
Bac in autoclaved btl, flushed	0.149	
Bac in autoclaved btl, flushed	0.195	
Bac in autoclaved btl, flushed	0.160	
Bac in autoclaved btl, flushed	0.131	
Bac in autoclaved btl, flushed	0.137	
Bac in autoclaved btl, flushed	0.487	
Bac in autoclaved btl, flushed	0.206	
Bac in autoclaved btl, flushed	0.200	
Bac in autoclaved btl, flushed	0.282	
Bac in autoclaved btl, flushed	0.211	
Bac in autoclaved btl, flushed	0.171	
Bac in autoclaved btl, flushed	0.154	
Bac in autoclaved btl, flushed	0.149	
Bac in autoclaved btl, flushed	0.160	
Bac in autoclaved btl, flushed	0.230	
Bac in autoclaved btl, flushed	0.155	
Bac in autoclaved btl, flushed	0.275	
	0.196	*Avg*
	0.078	*Std Dev*

Table A.5: Example nitrate standard analysis results

Identifier	*Peak Area*	*BGD 44*	*BGD 45*	*BGD 46*	*$\delta^{18}O$*	*$\delta^{15}N$*	
10Hoff	21.214	2.20	2.20	-8.90	18.278	-2.020	
10Hoff	22.363	2.10	2.10	-8.70	18.860	-1.287	
10Hoff	21.208	2.20	2.20	-8.50	19.726	-0.536	
	21.595				*18.955*	*-1.281*	*Avg*
	0.665				*0.729*	*0.742*	*Std Dev*
20Hoff	27.580	2.20	2.10	-8.70	48.488	-6.875	
20Hoff	27.495	2.10	2.00	-8.50	49.917	-7.184	
20Hoff	28.562	2.10	2.00	-8.70	48.708	-8.309	
	27.879				*49.038*	*-7.456*	*Avg*
	0.593				*0.769*	*0.755*	*Std Dev*
NC32	20.867	2.20	2.20	-9.00	-16.487	5.148	
NC32	18.531	2.20	2.10	-8.50	-16.664	5.416	
NC32	19.740	2.10	2.00	-8.80	-16.536	4.563	
	19.713				*-16.562*	*5.042*	*Avg*
	1.168				*0.091*	*0.436*	*Std Dev*

Table A.6: Example of good standard run for isotopic value correction

Identifier	*Peak Area*	*BGD 44*	*BGD 45*	*BGD 46*	*$\delta^{18}O$*	*$\delta^{15}N$*	
NC32	20.867	2.2	2.2	-9.0	-16.487	5.148	
NC32	18.531	2.2	2.1	-8.5	-16.664	5.416	
NC32	19.740	2.1	2.0	-8.8	-16.536	4.563	
	19.713				*-16.562*	*5.042*	*Avg*
	1.168				*0.091*	*0.436*	*Std Dev*
10Hoff	21.214	2.2	2.2	-8.9	18.278	-2.020	
10Hoff	22.363	2.1	2.1	-8.7	18.860	-1.287	
10Hoff	21.208	2.2	2.2	-8.5	19.726	-0.536	
	21.595				*18.955*	*-1.281*	*Avg*
	0.665				*0.729*	*0.742*	*Std Dev*
20Hoff	27.580	2.2	2.1	-8.7	48.488	-6.875	
20Hoff	27.495	2.1	2.0	-8.5	49.917	-7.184	
20Hoff	28.562	2.1	2.0	-8.7	48.708	-8.309	
	27.879				*49.038*	*-7.456*	*Avg*
	0.593				*0.769*	*0.755*	*Std Dev*

Table A.7: Successful IC separation of NO_3^-

Identifier	*Peak Area*	$\delta^{18}O$	$\delta^{15}N$	
NO_3^- separation by IC	29.916	50.041	-3.069	
NO_3^- separation by IC	27.068	50.639	-2.098	
NO_3^- separation by IC	25.231	50.366	-2.518	
NO_3^- separation by IC	23.548	49.629	-2.931	
NO_3^- separation by IC	21.056	49.925	-3.036	
NO_3^- separation by IC	21.182	50.680	-3.009	
NO_3^- separation by IC	18.841	49.036	-3.999	
NO_3^- separation by IC	18.695	49.604	-3.437	
NO_3^- separation by IC	20.377	48.260	-4.866	
NO_3^- separation by IC	24.726	50.023	-3.188	
NO_3^- separation by IC	26.825	50.502	-2.513	
NO_3^- separation by IC	29.453	49.779	-3.886	
NO_3^- separation by IC	30.467	50.269	-2.930	
NO_3^- separation by IC	30.809	49.705	-3.464	
NO_3^- separation by IC	28.025	50.134	-2.987	
	25.081	*49.906*	*-3.195*	*Avg*
	4.274	*0.633*	*0.678*	*Std Dev*

Table A.8: Hong Kong rainwater $\delta^{15}N$ and $\delta^{18}O$ analysis

Date (MM-YY)	*Sample ID*	$\delta^{18}O$(‰)	$\delta^{15}N$ (‰)
Sep-92	Hong Kong 9209 930223	58.467	-1.161
Oct-92	Hong Kong 9210 931666	57.504	-0.752
Jan-93	Hong Kong 9301 932208	59.977	-13.568
Mar-93	Hong Kong 9303 932209	61.700	-1.821
Feb-94	Hong Kong 9402 942535	39.185	-2.828
Mar-94	Hong Kong 9403 942536	55.638	0.502
Jul-94	Hong Kong 9407 950211	44.130	1.543
Sep-94	Hong Kong 9409 950209	65.374	-0.004
Dec-94	Hong Kong 9412 950861	54.468	3.149
Feb-95	Hong Kong 9502 951707	62.782	1.259
Mar-95	Hong Kong 9503 951708	60.365	-0.516

Jul-95	Hong Kong 9507 960380	43.051	-9.167
Sep-95	Hong Kong 9509 960379	51.391	4.327
Mar-96	Hong Kong 9603 962593	52.318	0.995
Apr-96	Hong Kong 9604 962741	61.760	0.789
May-96	Hong Kong 9605 962742	38.538	-7.687
Jun-96	Hong Kong 9606 962743	41.098	-14.639
Jul-96	Hong Kong 9607 970429	50.240	-8.840
Aug-96	Hong Kong 9608 970430	54.306	-15.544
Sep-96	Hong Kong 9609 970431	64.698	-7.663
Oct-96	Hong Kong 9610 970868	47.130	-19.324
Nov-96	Hong Kong 9611 970867	60.257	-1.582
Jan-97	Hong Kong 9701 971403	50.961	-13.778
Feb-97	Hong Kong 9702 971404	57.177	0.632
Mar-97	Hong Kong 9703 971405	56.052	-18.404
Apr-97	Hong Kong 9704 971374	47.056	-12.563
May-97	Hong Kong 9705 971375	46.850	-12.368
Jun-97	Hong Kong 9706 971376	47.160	-6.539
Jun-97	Hong Kong 9706 980088	27.547	-3.590
Sep-97	Hong Kong 9709 980089	59.496	-15.993
Oct-97	Hong Kong 9710 986739	66.484	-1.298
Jan-98	Hong Kong 9801 981531	46.552	-10.628
Feb-98	Hong Kong 9802 981532	52.819	-7.577
Mar-98	Hong Kong 9803 981533	52.240	-12.402
Apr-98	Hong Kong 9804 981886	50.412	-11.665
May-98	Hong Kong 9805 981887	38.465	-11.030
Aug-98	Hong Kong 9808 990006	45.762	-18.534
Nov-98	Hong Kong 9811 990611	64.129	-12.181
Dec-98	Hong Kong 9812 990612	59.476	-3.805
Jan-99	Hong Kong 9901 991532	62.605	-3.802
Jul-99	Hong Kong 9907 700077	56.323	-5.242
Nov-99	Hong Kong 9911 700406	54.077	0.518
Mar-00	Hong Kong 0003 701365	50.096	2.349
Apr-00	Hong Kong 0004 702028	53.653	1.915
Jun-00	Hong Kong 0006 702030	26.715	-1.972
Jul-00	Hong Kong 0007 710011	49.426	-0.137
Aug-00	Hong Kong 0008 710012	52.274	2.291
Nov-00	Hong Kong 0011 710231	57.203	-0.235
Jan-10	Hong Kong 0101 710884	56.463	0.062
Feb-10	Hong Kong 0102 710885	67.177	0.889
Mar-10	Hong Kong 0103 710886	63.946	-0.020
Apr-10	Hong Kong 0104 711797	64.184	-0.603
May-10	Hong Kong 0005 702029	38.063	-8.444
Oct-10	Hong Kong 0010 710230	59.642	0.947

Oct-10	Hong Kong 0110 721434	63.222	-0.501
Nov-10	Hong Kong 0111 721418	64.579	-0.313
Dec-10	Hong Kong 0112 721419	57.539	0.645

Appendix B: Studies of *Bacillus Halodenitrificans as a Potential Substitute for Pseudomonas Aureofaciens Using the Denitrifier Method*

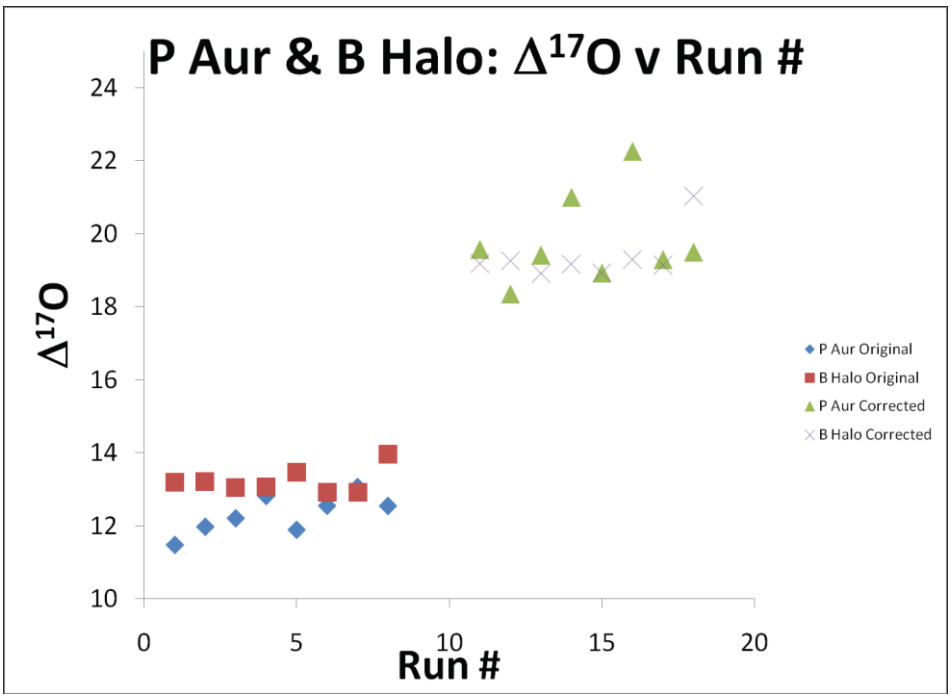


Figure B.1: *Bacillus Halodenitrificans* as a potential substitute in the denitrifier method.

Additional investigations also focused on *Bacillus halodenitrificans* as a substitute for *Pseudomonas Aureofaciens* using the denitrifier method. This bacteria also contains the copper-containing nitrite reductase (which minimizes the exchange) necessary for the catalyzation of nitrate, but still lacks the nitrous oxide reductase, therefore conversion halts at N_2O. *B. halodenitrificans* has the advantage of being an anaerobic halotolerant (salt-tolerant) denitrifier. Many of our samples have a high saline content; also pre-concentration techniques using anion resin require elution using high ionic strength solutions. Further, high saline growth solutions limit contamination from other bacteria or organisms. Some data suggested that *B. halodenitrificans* worked but there were often inconsistent results (N_2O was not always produced). Initial results suggested that *B.*

halodenitrificans has the potential to replace *P. Aureofaciens* as the denitrifying bacteria in the denitrifier method. This halotolerant bacterium is more precise in $\Delta^{17}O$ measurements and appears to be less prone to O_2 exchange when compared to *P. Aureofaciens.*

Appendix C: Studies of $\Delta^{17}O$ of O_2 Generated from Gold Disproportionation of N_2O from *P. Aureofaciens*.

For a short time, research efforts also focused on the conversion of N_2O over a gold tube into both O_2 and N_2 using techniques adapted from Cascotti[22] Kaiser[34], and Michalski[33]. Currently, there is no published method that allows for the simultaneous determination of $\delta^{18}O$, $\delta^{15}N$, and $\Delta^{17}O$ of nitrate. Some preliminary data on $\delta^{15}N$, $\delta^{18}O$, $\Delta^{17}O$ values of N_2 and O_2 generated by the disproportionation of bacterial produced N_2O by *P. Aureofaciens* has been achieved (Figure 2.29). The yellow boxes in Figure 2.29 are the $\Delta^{17}O$ values ($\Delta^{17}O \approx 0.52 * \delta^{18}O$) of working standards based on their thermal decomposition values. High $\Delta^{17}O$ values are underestimated by 20-30%, partially due to exchange blank and linearity. Detection limits are roughly 2‰. Unfortunately, collapse of *P. Aureofaciens* around January 2010 limited further exploration of the denitrifier method coupled to gold disproportionation and research was refocused on the optimal growth of *P. Aureofaciens*.

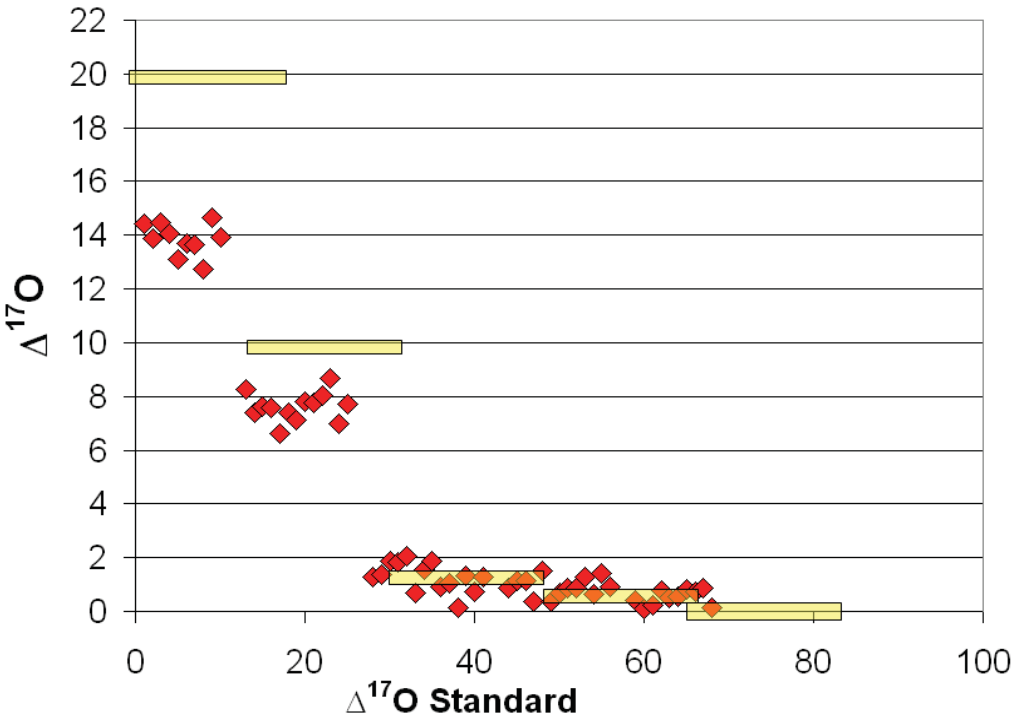


Figure C.1: $\Delta^{17}O$ values of N_2 and O_2 generated by the disproportionation of bacterial produced N_2O.

Appendix D:

Nitrogen and Oxygen Isotope-ratio Analysis of Nitrate by the Denitrifier Method Using Continuous Flow Isotope-ratio Mass Spectrometry

Standard Operating Procedure (SOP)

Lindsey Crawley

09/01/2010

Step-by-Step Preparation for Isotopic Analyses of Nitrate by Bacterial Denitrification

Preparation of Tryptic Soy Agar Plates

15% Glycerol Solution for Freezer Storage of Bacteria:

1. Sterilize glycerol:
    a. Transfer 100mL glycerol to serum bottle and seal serum bottle with stopper and aluminum crimp seal cap.
    b. Attach a vent needle to stopper.
    c. Autoclave for 1 hr. @ 17psi. (Keep this bottle as the supply of sterilized glycerol.)
    d. Warm bottle of glycerol to a less viscous liquid via hot plate for easy transfer to syringe or pipet. Add ventilation needle to cap during warming.
2. Add 1mL glycerol and 6mL TSB to a 12 mL screw cap vial via syringe or pipet.
3. Store until needed for bacterial inoculation from store-bought supply.

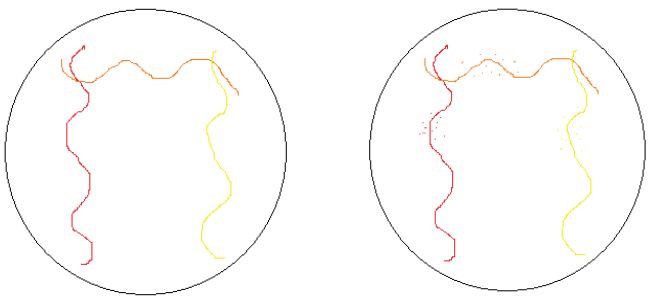
Making the plates:

1. Mix together in an Erlenmeyer flask:
    a. 10g tryptic soy agar
    b. 0.25g KNO_3
    c. 250mL E-pure or Milli-Q water
2. Dissolve solids via stir plate & stirring bar. Warm the solution if necessary.
3. Cover flask with a glass petri dish and autoclave @ 17psi for 60 min.
4. Uncover the autoclave after it has returned to RT. Remove flask only if you can handle it at a comfortable temperature.
5. Sterilize sheets of Al foil by wiping with ethanol. Place sheets above and below plates & lids to avoid contamination and dust.

6. Pour agar into sterile petri dishes just so that the bottom is covered but no more than half full, flaming the lip of the flask every 3-4 plates (250mL should make ~10 100x15 plates), leave the lids off but cover dishes and lids sheets of ethanol-wiped aluminum foil to avoid dust. Allow to solidify overnight.
7. Place covered petri dishes on top of one another into a Ziploc freezer bag. Place one uncovered dish filled with Drierite in bottom of bag.
    a. Label the bag with the date that plates were poured.

Preparation of Bacterial Growth Solution from Colonies

1. Streaking the bacteria:
    a. Locate the freezer stock of *P. aureofaciens* or desired bacteria.
    b. Flame the inoculating loop until the coil turns orange to kill any bacteria. Touch the hot loop to an un-streaked portion of the plate to cool the loop.
    c. Dip the sterilized loop into the frozen stock of bacteria and gently streak the bacteria across the width of the top 1/3 of the plate in a back-and-forth S-shape motion.
    d. Repeat step b.
    e. Touch the loop to the medium just above the end of the last streak and repeat S-shape motion in a perpendicular direction from the first streak.
    f. Repeat steps b & e for a third streak.



g. To make new plates from existing plates, pick a single colony and proceed to streaking new plates using the flame and streak steps above.

h. Plate numbering:

i. #1: Plate streaked from freezer stock

ii. #2: Plate streaked from Plate #1

iii. #3: Plate streaked from Plate #2

i. Plate identification:

i. Include: Strain (*P. Aureofaciens* would be appropriate here)

ii. Plate #

iii. Date

j. Plates typically take 2-3 days to grow and should be left out at RT.

k. After plates are spent, secure the lid in place with a strip of Parafilm and place plates in the biohazard bag for disposal. Contact REM for pick up when the box becomes full.

2. Inoculation of Bacteria:

a. Choose a colony of bacteria from a fully grown plate #2 or #3 using a sterilized inoculating loop then dip and swirl into one 250mL plastic bottle containing TSB.

b. 5-9 days growth time is optimal. The color will appear to be a dark yellow-orange.

c. Test for incomplete conversion of nitrate by dipping one test strip of Aqua Chek nitrate/nitrite test strips into each bottle to be used. A negative result (pink) indicates proper conversion of nitrate and the bottle may then be pelletized. A positive result (off-white) indicates the presence of nitrate and nitrate and may be re-capped and allowed to incubate longer, up until the 9 day mark.

d. Repeat for the desired number of bottles you wish to inoculate (most likely 2/day). A cloudy solution that appears after 2-3 days indicates growth of bacteria compared to TSB that has not been inoculated. The solution often has more of a nutty-like smell when grown properly versus a rancid, sour smell when contaminated. TSB will more of a yellow-orange color than a brown color also indicates possible contamination.

Preparation of Tryptic Soy Broth (TSB)

Making the medium:

1. Mix together in an Erlenmeyer flask:
    a. 30g tryptic soy broth
    b. 1.0g KNO_3
    c. 0.5g $(NH_4)_2SO_4$
    d. 4.9g potassium phosphate
    e. 1L E-pure or Milli-Q water
2. Mix on stir plate with a stir bar at 600rpm. Warm if necessary.
3. Distribute medium equally into four clean 300mL plastic screw-cap bottles.
4. Autoclave for 60 min. at 17 psi.

Preparation of Nitrate Standards

1. For solid nitrate standards:
    a. Using a known nitrate standard and a second source of nitrate, weigh out the appropriate ratios of each separately.
    b. Homogenize the solids by grinding together with a mortar & pestle.
    c. Retain in a small container for later use.
2. For liquid nitrate standards:

d. Measure out 840μg (0.84mg) of a 100nmol NO_3^- standard on a microbalance from the solid stock container prepared above.
e. Deliver to a 100mL volumetric flask, add water to the mark, cover with Parafilm, and invert solution many times to mix & dissolve.
f. Aliquot into single-use (daily) containers and label appropriately. 15mL centrifuge tubes work well for this.

Harvesting Tryptic Soy Broth for Analysis

1. Concentrating Culture by Centrifugation:
    a. Centrifuge the fully grown inoculated solutions from two plastic screw-cap bottles for 7 min. at 3000rpm, forming a pellet of bacteria on the bottom of the bottle.
    b. Decant off supernatant liquid from each of the two bottles into a waste jar to be autoclaved and disposed.
    c. Add 1 drop of Antifoam B to a bottle of TSB Rinse (prepared without KNO_3).
    d. Add 50mL TSB Rinse to first centrifuged bottle, cap, and shake vigorously until pellet has redissolved.
    e. Transfer resuspension liquid from the first shaken bottle into the second bottle (which has also been decanted) and re-dissolve bacterial growth again by shaking.
    g. To the first bottle, add 50mL water to balance out the centrifuge.
    h. Centrifuge remaining bottle for 7 min. at 3000rpm.
    i. Decant off supernatant into waste jar.
    j. Repeat steps d, e & g for a second rinse.
    k. Refill to bottle with remaining TSB Rinse and shake to re-suspend solution. Add more TSB Rinse from a second bottle if a larger volume is desired.

l. Purge with helium in glass bubbler for 2 hrs.

m. Pour remaining solution into a sterile125mL glass bottle and cap with septum. Flush for 5 min. with helium. Let set overnight.

2. Addition of Nitrate Standards to Bacteria:

   a. Add 1 mL prepared bacteria solution to the desired number of 12 mL vials (via pipet) then cap with septum screw-caps.

   b. Flush vials for 5min. with helium gas at ~40mL/min.

   c. Aliquot 1mL of nitrate standards into bacteria vials for analysis (via 3mL disposable syringe and needle).

   d. Let bacteria and nitrate react for 60 minutes. More concentrated standards will need to react longer (i.e. 2 hours).

   e. Lyse the bacteria by aliquoting 0.5 mL 1% NaOH to each vial via disposable syringe (without removing the cap).

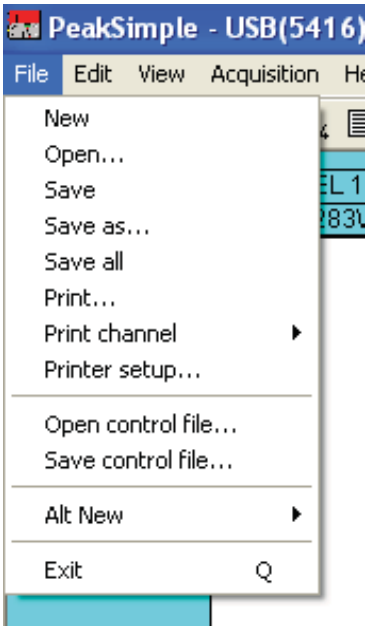
Step-by-Step Preparation for Isotopic Analyses of Nitrate via Headspace Extraction

Peak Simple 388-32bit Program **Instructions**

Open *Peak Simple* Program from the Desktop.

Click *OK* if the intro. window pops up.

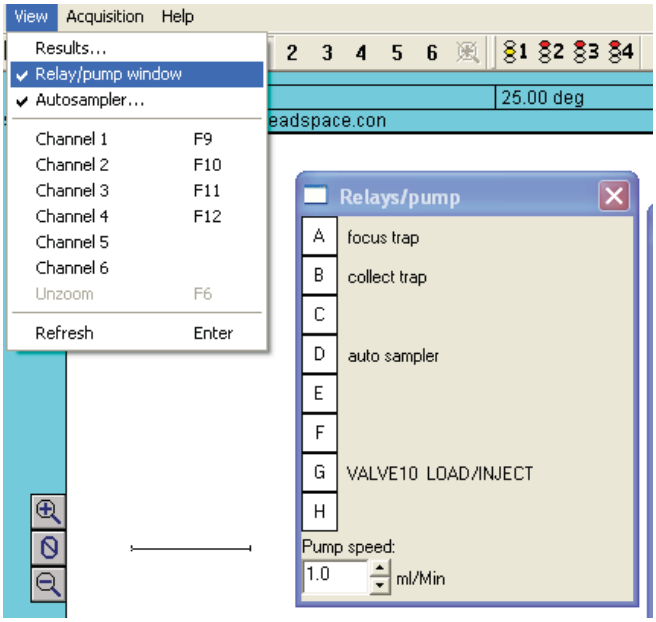
Click *File, Open Ctrl File,* then select appropriate file (usually headspace with heat.con), *Open*.



Click *File, Save all.*

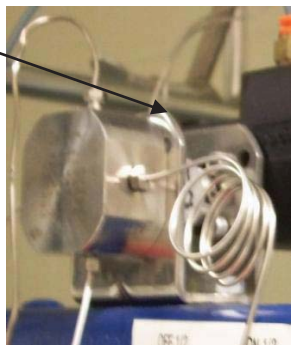
Click *Edit*, *Channels*, Channel 1: *Events*, to ensure the appropriate event has loaded (usually headspace N2O.evt). Click *OK* to exit.

Click Channel 1: *Details*, confirming the appropriate end time. Click *OK* to exit.



Click *View*, *Relays/PumpWindow*. The focus and collect traps should be raised so that both A & B boxes are white.

Click box *C* as necessary to connect the 4-port valve to the appropriate ports: 1-2 & 3-4 or 1-4 & 2-3. This is written on the side of the valve. (For headspace N2O, box C is set to black at start.)

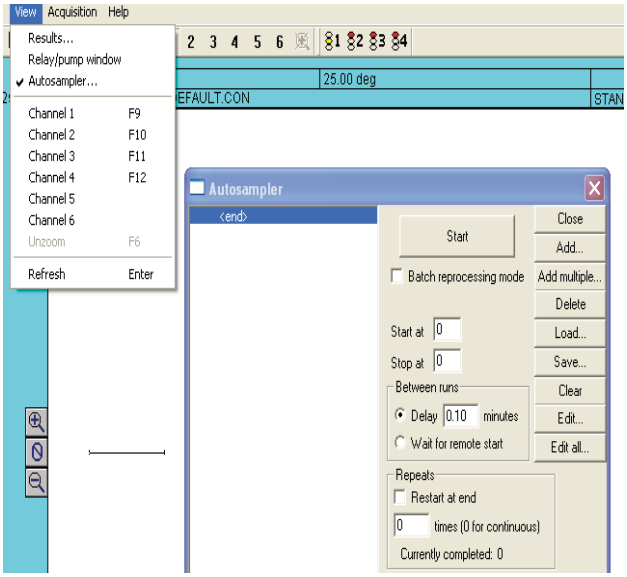


Click box *G* to black.

***Peak Simple* Instructions cont.**

Click *View, Autosampler, Add,* select the appropriate control file (usually headspace with heat.con). Verify proper files are loaded. Click *OK*.

The Batch reprocessing mode box should be left un-checked. Select *Wait for remote start* and *Restart at end* box. Click *Start*.



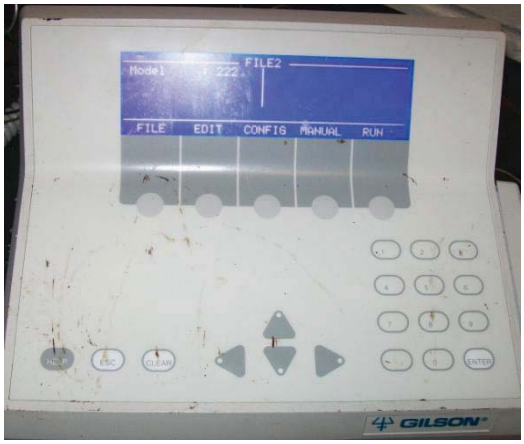
Summary of Event file: C:\Peak388-32bit\headspace N2O.evt:

Time(min)	*Event*	*Detail*
0.050	B ON (collect trap)	Collect trap lowers into liquid N_2
0.100	D ON (autosampler)	Autosampler needle moves, inserted in next vial
0.130	D OFF (autosampler)	Stops autosampler needle movement, remains in vial
3.400	A ON (focus trap)	Focus trap lowers into liquid N_2, cools, collects sample gas
5.300	G OFF (Valve10 load(w))	Connects valves 2-3, 4-5, 6-7, 8-9, 1-10
10.500	B OFF (collect trap)	Collect traps raises, warms, sends collected gas to focus trap
12.000	D ON (autosampler)	Autosampler needle moves to vial in rack position #1
12.300	D ON (autosampler)	Stops autosampler needle movement, remains in vial
14.800	G ON (Valve10 inject(b))	Connects valves 1-2, 3-4, 5-6, 7-8, 9-10
14.900	A OFF (focus trap)	Focus trap raises, warms, sends gases to GC, Coldtrap, IRMS

Instructions for Gilson Autosampler 222XL Liquid Handler

Beginning from the top left of the Autosampler rack (with the handle facing you), place two 100ppm N_2O flushed vials in positions 1 & 2 (position 2 is below position 1, not to the right of 1).

Place vials to be analyzed from positions 3 & on. Place two 100ppm N_2O flushed vials in the following two slots after all others have been positioned.



To set the Autosampler:
Using File 2, press *Run*.
First vial? 1 Press *enter* if starting with vial in position #1 on rack. Otherwise, type in starting position.
Nb vials? 132 – Leave this as is – Press *enter*.

Press *stop* at any time to cancel automation.

Instructions for Physical Set-up of IRMS

Plug in the liquid N_2 supply dewar white cord to outlet via timer.
Set timer.

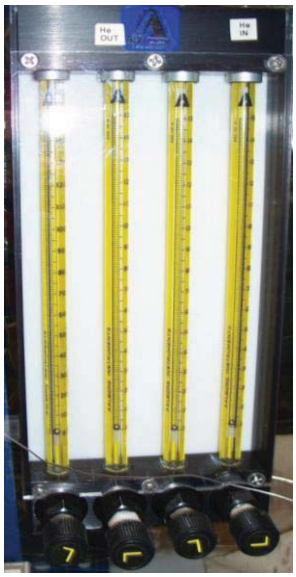
For instructions, see manual provided with timer.

Instructions for Physical Set-up of IRMS cont.

Disconnect autosampler needle, rinse with water daily (scrub with toothbrush if needed).

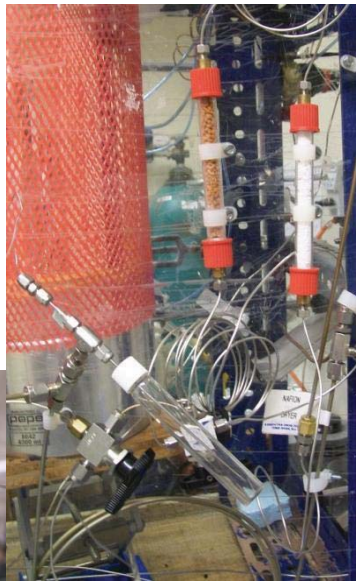
Wipe the holder with ethanol & WD-40 weekly.

Reconnect needle with reverse connections and purge with He at 100mL/min for 3-5 min. Connect properly & adjust to appropriate height, use Autosampler commands to insert into a vial, wait for He flow to equilibrate.



Set the flow of He gas in to 30mL/min.

Absorb excess H_2O from empty dewar (daily, if possible, but at least once a week).

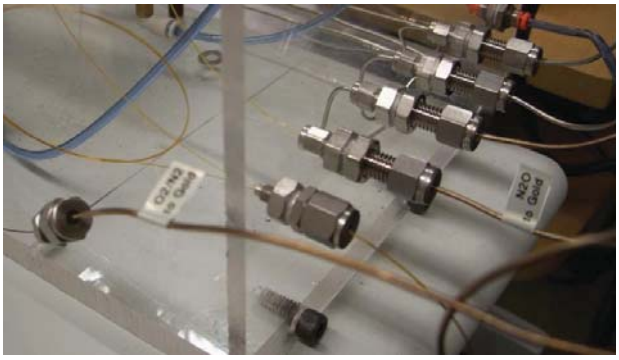


Replace water drip vial if wet (daily). Disconnect Carbosorb & $Mg(ClO_4)_2$ traps (weekly), remove wet solids, and replace with dry as needed.

Adjust valve as necessary to direct flow to proper place (normally turned to headspace).

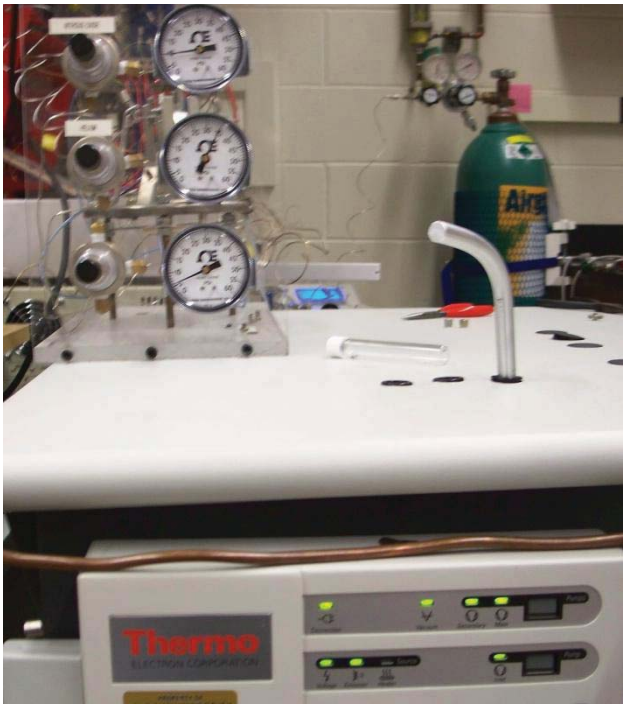
Instructions for Physical Set-up of IRMS cont.

Connect proper capillary (N_2O or N_2/O_2) to Open Split.



Set system pressure to P=12-15psi (N_2O) or
P=24 (N_2/O_2).

Rotate the conflo inlet L-valve counterclockwise to allow sample to flow from Open Split to IRMS. (Vacuum should read 10^{-6} not 10^{-8})



Fill main supply liquid N_2 dewar as needed (M-Th 9a, F 9a & 2p).
Connect liquid N_2 union to tank, open blue valve one full turn.
Use on/off switches to control flow to cryofocus traps (dewar) and -80°C coldtrap.

Isodat Acquisition **Instructions**

Open *Isodat Acquisition* Program from the Desktop.

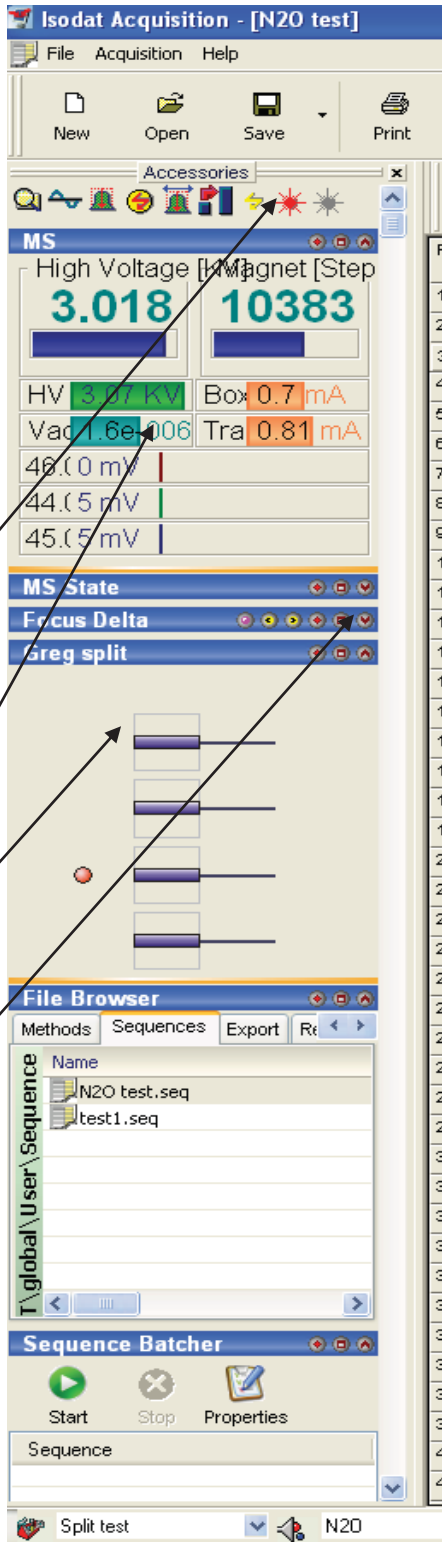
At the very bottom left-hand corner, select desired interface and gas configurations from the drop-down windows (commonly *Split Test2* and N_2O).

Check source (Voltage & Emission lights on front of MS should be green). If the values for Box and Trap read zero, the source is not on. Click the red laser under to enable the ion source.

Open the L-valve located on the top of the MS. (You should see the vacuum pressure lower from 10^{-8} to 10^{-6}.)

In the Greg Split window, lower the N_2O reference gas by clicking the top purple bar so that it disappears.

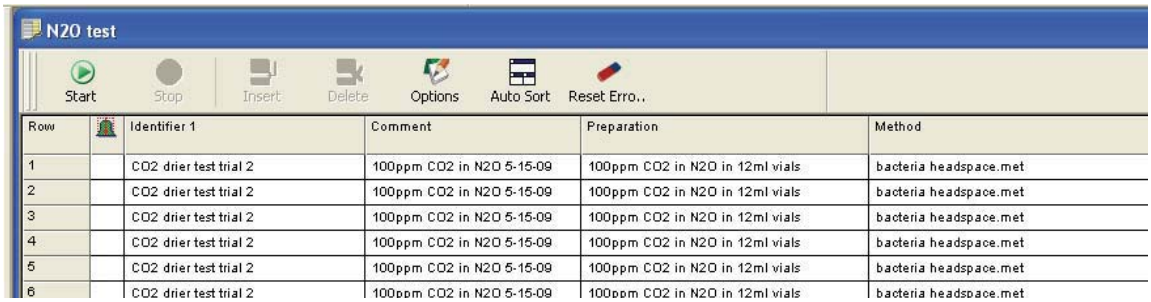
Open the Focus Delta window by clicking the down arrow. Right-click in the Focus Delta window, select *Autofocus* to tune the beam, click *OK*, and wait to finish. Right-click in the same window again, and select, *Pass to Gas configuration*. (This should be done daily.)



***Isodat Acquisition* Instructions cont.**

Under the File Browser mini-window, click *Method* tab, and choose the desired method (N2O bacteria.met). Next, click *Sequence* tab, and choose the desired sequence (headspace bacteria.seq).

Begin by inserting or deleting the appropriate number of rows based on the number of samples to be run. To add more, click *Insert* and add the desired number to be added into the count box and click *OK*. If there are excess, highlight the desired number of rows and click *Delete.*



Starting with Row 1, type the appropriate name and any other designating information under the Identifier 1, Comment, Preparation, and Method columns. It is only necessary to type in this information once all samples are the same. When finished, click once on the *text* for the Identifier 1 box in Row 2 to highlight. Next, right-click the *Identifier 1* tan box and select *Fill Grid with Data.* (This automatically fills in the desired information). If more sample identifiers are needed, manually name each box under the column separately or select multiple, separate boxes. If you wish to name a block of rows with the same name, highlight the desired rows, right-click, select *Fill Grid with Data.* Repeat as necessary. Check that all samples in racks match up with the row number on screen. If correct, click and highlight Row 1, click *Insert, Top of Selection,* type in 1, *OK.* Type *VOID* into Identifier 1 column. Click once on the comment box to highlight entire column and right-click to select *Fill Grid with Data.* Repeat for Preparation and Method columns.

Click once in the tan Row box in the top left corner to highlight all samples. Next, click the green *Start* arrow when ready to run samples. (See other instructions before clicking *Start*.)

Isodat Workspace **Data Access Instructions**

Open *Isodat Workspace* to see individual scans of each sample when they are finished. As with *Acquisition*, the appropriate interfaces (i.e. *Split Test* and N_2O) must be selected.

Under the File Browser window, click the *Results* tab. You may need to double-click the return arrow (↵) and scroll down to select the folder that you entered into File Name. Double-click the file name and select the data you wish to view.

When the entire run is complete, click on the Results tab in the File Browser to analyze data using Excel. Double-click the folder name of the chosen file and the right-click on the Excel folder and select *Launch Explorer.* Select the name of the chosen Excel file you wish to view.

Data Processing Instructions in *Excel*

With the spreadsheet open, highlight all columns starting with Column C and continuing to the right and down, selecting all data from this point. Locate the yellow exclamation point, click *Convert to Number.*

Select All, click *Data*, *Sort, Rt*, and delete the first reference peaks, 1, 2, and 3, based on their respective retention times and the last two reference peaks as well, usually peaks 6-7, but choose this based on retention times as well (Rt~800-900s).

Select All, click *Data*, *Sort, d18O/16O*, and delete O_2 peaks that can by identified by unusually large (3 digit) values.

Select All, click *Data*, *Sort, Identifier* and *Row*. Then, arrange data as you wish.