Evidence for Participation of Anammox in Nitrogen Attenuation Observed in Groundwater Impacted by a Manure Lagoon

by

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Author's Declaration

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

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Abstract

Decades of agricultural use of fertilizer and manure has resulted in nitrogen being the most common groundwater contaminant. Of the known processes for nitrogen attenuation, both denitrification and anammox produce a complete transformation of nitrogen species to dinitrogen gas (N_2); however, denitrification is typically also associated with the release of N_2O and CO_2, both greenhouse gases. Anaerobic ammonium oxidation (Anammox), which has been recently discovered to be more prevalent in groundwater environments than previously thought, simultaneously removes NH_4^+ and nitrate (NO_3^-), does not require dissolved organic carbon (DOC), and does not produce greenhouse gas by-products. This study evaluates the natural occurrence of anammox in a manure lagoon plume, as well as the feasibility of enhancing anammox activity by mixing NH_4^+ rich groundwaters and NO_3^- rich groundwaters together. Fifteen experiments were undertaken with NH_4^+-N concentrations ranging between 5-100 mg/L, and a NO_3^--N ranging from 5-88 mg/L. These experiments suggest a nitrogen removal rate (based on NH_4^+ removal in anaerobic conditions) from anammox generally in the range of 0.1-0.2 mg/L/day. Based on an absence of dissolved oxygen (DO), and concomitant loss of NO_3^--N with associated ^{15}N-NO_3^- enrichment (2.1-8.7‰) in 11 experiments, it is considered unlikely that nitrification was the cause of the NH_4^+ loss observed in these experiments. Concurrent ^{15}N-NH_4^+ enrichment of 4.1-11.5‰ was observed in these 11 experiments. Real-time quantitative polymerase chain reaction (qPCR) DNA analyses were used to show the presence of anammox bacteria and to demonstrate temporal population increases during the experiments (up to 16.3% anammox in total bacteria population) in the three experiments analyzed. Although anammox-related N removal rates were modest in these trials, such rates could be significant with respect to the multi-year residence times associated with most groundwater flow systems.

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1.0 Introduction

1.1 Nitrogen and Manure Application

Between 1981 and 2006, manure production in Canada has increased 16%, from 156,265,000 to 180,960,000 tonnes (Hofmann 2006). Nitrogen constitutes a substantial portion of this manure, especially manure produced by poultry farming. Between 1980 and 2010, poultry production in Ontario has more than doubled from 106,000 to 216,000 birds (Statistics Canada 2007; Statistics Canada 2011), and a recent study by the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) showed that poultry manure contains the highest percentage of nitrogen (solid manure average = 2.45%, n = 809), making it a rich fertilizer (Brown 2008). With such an increase in manure loading, combined with the introduction of synthetic fertilizers, contamination of groundwater has in turn increased, to the point where nitrogen (as NO_3^-) is considered the most common groundwater contaminant (Freeze and Cherry 1979).

The problem of increased manure production is exacerbated by its greater association with point sources, as more intensive farming has evolved in Canada. In 2000, 25% of Ontario farms accounted for 75% of all farming revenue in the province (Miller 2000). Of these large-scale farms, hog farms in particular produce significant amounts of manure. According to the Environmental Commissioner of Ontario, Ontario had over 3.4 million hogs in the province, with 400,000 in Huron County alone (Miller 2000). The hogs in Ontario produce as much manure (sewage) as the entire human population in the province (Miller 2000).

The overall increase in nitrogen-based agricultural contamination is a concern because of its potential effects on health and on the environment, including its contribution to eutrophication

and to health concerns like methemoglobinemia and the carcinogenic effects of nitrosamines (Appendix A).

A 106 well Ontario groundwater survey conducted in 1986 showed that 15.5% of wells exceeded the water quality guideline for nitrate of 10 mg N/L (Frank et al. 1991). 36% of the wells had concentrations greater than 1 mg N/L. In 1991, a more extensive survey was completed in Huron County, Ontario (Fleming 1992). In this survey, 45 of 301 wells (or 15%) exceeded 10 mg N/L, while the average concentration was still substantial at 3.5 mg N/L. In 1992, an Ontario-wide survey of 1237 wells was undertaken, which again found 15% of surveyed wells exceeding 10 mg N/L (Agriculture Canada 1993; Goss et al. 1998). Another 11% had NO_2^- concentrations between 5-10%. Further to this study, 141 multi-level wells were installed in fields adjacent to the supply wells sampled. 21% of these multi-level wells exceeded 10 mg/L NO_3^--N (Agriculture Canada 1993; Goss et al. 1998).

These surveys suggest that NO_3^- contamination is prevalent in Ontario. Considering the health and ecological risks associated with nitrogen, there is growing interest in remediation methods.

1.2 Mechanisms of Nitrogen Attenuation

The nitrogen cycle (Figure 1) is complex, and under varying circumstances, nitrogen species can be highly reactive or conservative, mobile or retarded. These traits can make remediation of nitrogen difficult, as explained below. Between 60-70% of nitrogen excreted in poultry manure is in the form of organic nitrogen (uric acid and urea, Nahm 2003), which under low pH, moist, and warm conditions can mineralize to NH_3 and NH_4^+ (Eq. 1.1):

$$\text{Organic N} \rightarrow NH_3 \leftrightarrow NH_4^+ \qquad \text{(Eq. 1.1)}$$

Ammonium, though generally of lesser focus in many groundwater studies, is of substantial importance to this study, as it is the main parameter that distinguishes anaerobic ammonium oxidation (anammox) from the many other nitrogen transformation processes.

1.2.1 Ammonium Attenuation

In 1977, it was postulated that the existence of a chemolithotrophic bacteria able to oxidize NH_4^+ was possible, due to the thermodynamically favourable reaction (Broda 1977). This theory indicated that NH_4^+ could be oxidized under anaerobic conditions. Prior to this theory, it was generally accepted that aerobic nitrifiers were the only bacteria able to oxidize NH_4^+. Evidence of anaerobic oxidation of NH_4^+ (anammox reaction) was discovered 18 years later (Eq. 1.2, Mulder et al. 1995):

$$3NO_3^- + 5NH_4^+ \rightarrow 4N_2 + 9H_2O + 2H^+ \qquad \text{(Eq. 1.2)}$$

Prior to this discovery, the attenuation of NH_4^+ was thought to be dominated by the processes of nitrification, sorption, volatilization and assimilation. Assimilation of NH_4^+ converts NH_4^+ to essential cellular nitrogenous compounds such as glutamine ($C_5H_{10}N_2O_3$) and glutamate ($C_5H_9NO_4$), under the enzymatic activity of glutamate dehydrogenase (Nagatini et al. 1971). However, it is generally assumed that assimilatory metabolism consumes a small amount of nitrogen relative to dissimilatory reactions (Madigan et al. 2003).

Deprotonation (or dissociation) of ammonium (NH_4^+) to ammonia (NH_3) is a pH dependent process (Eq. 1.3, Lide and Haynes 2010):

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad \text{(Eq. 1.3)}$$

where pKa = 9.25 at 25°C. As pH decreases, NH_4^+ becomes the dominant ion. During volatilization, there is a strong isotopic enrichment effect; as $NH_{3\,(aq)}$ converts to $NH_{3\,(g)}$ the residual NH_3 becomes enriched in ^{15}N. The isotopic enrichment (ε) from volatilization and dissociation can be as high as 34‰ at 25 °C (Kirschenbaum et al. 1947; Urey 1947).

As ammonium is a cation, it is also susceptible to cation exchange, as well as other means of sorption (adsorption, absorption, surface complexation, and surface precipitation) (Buss et al. 2004).

The strength on which a cation will sorb to a negatively charged surface is determined by the exchange coefficient, which considers both the nature of the soil surface and the cation (Buss et al. 2004; Appelo & Postma 1993). One exchange order that has been proposed (Buss et al. 2004; Schwartz & Zhang 2003) for typical cations found in groundwater is: $Al^{3+} > Ca^{2+} > Mg^{2+} > NH_4^+ > K^+ > H^+ > Na^+$. The effects of cation exchange are defined by the retardation factor (R, Eq. 1.4):

$$R = \frac{v_{water}}{v_{solute}} \qquad \text{(Eq. 1.4)}$$

where v is the linear groundwater velocity. R is also a function of grain size (Eq. 1.5, 1.6; Böhlke et al. 2006):

$$R = 1 + K_d \qquad \text{(Eq. 1.5)}$$

$$\text{where } K_d = K'_d \cdot \rho_{solid} \cdot (1-n)/n, \qquad \text{(Eq. 1.6)}$$

where K'_d is the sorption coefficient (g_{H2O}/g_{solid}), ρ_{solid} is the grain density (g/cm^3), and n is porosity.

Retardation factors for NH_4^+ in sands range from 1 to 6.4 (Dance and Reardon 1983; Ceazan et al. 1989; Thornton et al. 2000; Böhlke et al. 2006).

Further, the capacity for an aquifer to exchange ions is known as the Cation Exchange Capacity (CEC), which is proportional to the amount of clay (high surface area) and organic carbon (Eq. 1.7, Appello and Postma 2009):

$$\text{CEC (meq/kg)} = 7 \cdot (\%\ \text{Clay}) + 35 \cdot (\%\ \text{Organic Carbon}) \quad \text{(Eq. 1.7)}$$

Sorption of NH_4^+ will be accompanied by the release of the desorbed ion, often Ca^{2+}, Mg^{2+}, and Na^+ (Ceazan et al. 1989).

Though sorption is a fully reversible process, isotopic fractionation can be observed as $^{15}N\text{-}NH_4^+$ will be preferentially sorbed to exchange sites. Early studies suggest that depletion of residual $^{15}N\text{-}NH_4^+$ can range from 1-11‰ in clay rich environments (Delwiche and Steyn 1970; Karamanos and Rennie 1978), but more recent studies have suggested minimal fractionation in coarser grained sediment (Böhlke et al. 2006; Sills 2006).

In the presence of oxygen, NH_4^+ can also be oxidized to NO_3^- by the following microbially mediated reaction (Eq. 1.8):

$$NH_4^+ + 2O_2 \rightarrow H_2O + 2H^+ + NO_3^- \quad \text{(Eq. 1.8)}$$

This reaction can be further described as two partial oxidation reactions based on the genera of bacteria responsible (Eq. 1.9, 1.10, 1.11, Kendall 1998):

$$\text{(Nitrosomonas)}\ NH_4^+ + H_2O \rightarrow NH_2OH + 2[H] + H^+ \quad \text{(Eq. 1.9)}$$

$$NH_2OH + O_2 \rightarrow NO_2^- + [H] + H^+ \quad \text{(Eq. 1.10)}$$

$$\text{(Nitrobacter)}\ NO_2^- + H_2O \rightarrow NO_3^- + 2[H] \quad \text{(Eq. 1.11)}$$

Other intermediate products of nitrification include NO and NO_2^- (Casciotti et al. 2003). The presence of any of the intermediate products, and an increase in dissolved NO_3^- in an aerobic environment is reasonable evidence of nitrification.

As nitrifiers will preferentially consume $^{14}N\text{-}NO_3^-$, isotope fractionation is another method of identifying nitrification. Residual $^{15}N\text{-}NH_4^+$ has been observed to enrich between 12-29‰ (Shearer and Kohl 1986; Kendall 1998), which implies an equal depletion in newly formed NO_3^-.

1.2.2 Nitrate Attenuation (Including Anammox)

Until recently, the two important microbiological reactions for N-attenuation were considered to be nitrification and denitrification (reduction to N_2). Under anaerobic conditions, nitrate is the next most energetically preferred compound (next to O_2) for use in oxidation reactions. During denitrification in the groundwater zone, there are two dominant electron donors, organic carbon and pyrite (Payne 1976; Zumft 1997; Bottcher et al. 1990; Aravena and Robertson 1998; Appelo and Postma 2009):

$$4NO_3^- + 5CH_2O + 4H^+ \rightarrow 2N_2 + 5CO_2 + H_2O \qquad \text{(Eq. 1.12)}$$

$$14NO_3^- + 5FeS_2 + 4H^+ \rightarrow 7N_2 + 10SO4^{2-} + 5Fe^{2+} + H_2O \qquad \text{(Eq. 1.13)}$$

Each of these processes goes through a NO_x pathway, where the nitrogen compound is increasingly reduced until the reaction completes at dinitrogen gas (Eq. 1.14):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad \text{(Eq. 1.14)}$$

Isotope fractionation during denitrification is believed to be a rate dependent process (Kendall and Aravena 2000), with reported fractionation factors ranging from

-45 to -5‰, however most studies indicate enrichment greater than -15‰ (Blackmer and Bremner 1977; Kendall and Aravena 2000; Casciotti et al. 2002; Menyailo and Hungate 2006).

Nitrate assimilation is possible for some bacteria, but in order for assimilation to proceed, NO_3^- must be converted to NH_4^+, which can then be converted to $C_5H_{10}N_2O_3$ or $C_5H_9NO_4$ (Nagatini et al. 1971; Marzluf 1993; Lin and Stewart 1997). However, the nitrate assimilation pathway is not observed in all bacteria, and has only recently been observed in bacteria (Lin and Stewart 1997). Further, it is generally assumed that assimilatory metabolism consumes a small amount of nitrogen relative to dissimilatory reactions (Madigan et al. 2003).

A less studied NO_3^- transformation mechanism is dissimilatory nitrate reduction to ammonium (DNRA). There are two known pathways for this reduction, DNRA coupled with sulfur oxidation, and fermentation (Megonigal et al. 2004; Burgin and Hamilton 2007; Tobias and Neubauer 2009). Both reactions occur under anaerobic conditions and can be favoured in highly reducing, carbon rich environments. The overall fermentation reaction is:

$$NO_3^- + 4H_2 + 2H^+ \rightarrow NH_4^+ + 3H_2O \qquad \text{(Eq. 1.15)}$$

Although little is known about fractionation under DNRA (University of Waterloo 2006), it is speculated that the $^{15}N\text{-}NH_4^+$ product will be depleted with respect to the parent NO_3^- (McCready et al. 1983; Ostrom et al. 2002).

Lastly, there has been more recent attention to NO_3^- attenuation via the anammox process (Eq. 1.2). Anammox bacteria belong to the order Planctomycetes (Strous et al. 1999b), with 5 potential genera currently identified (*Candidatus* Brocadia, *Candidatus* Scalindua, *Candidatus* Kuenenia, *Candidatus* Jettenia, and *Candidatus* Anammoxoglobus, Moore et al. 2011). Anammox bacteria may be responsible for up to 50% of the world's nitrogen loss from marine

waters (Dalsgaard et al. 2005) but the estimates are highly variable. Moore et al. (2011) identified *Candidatus* Brocadia and *Candidatus* Scalindua (estimates of anammox N_2 production of 18.0 +/- 6.5%) in groundwater at 3 sites in Canada, including a poultry manure composting site in Southern Ontario (Zorra), which is the site of principle interest in this study.

Anammox activity is associated with simultaneous consumption of NO_3^- and NH_4^+, as well as concurrent enrichment of residual $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$ (Clark et al. 2008). Few isotope studies have focused on anammox, but a fractionation factor of ~ 4‰ has been estimated for $^{15}N\text{-}NH_4^+$ (Clark et al. 2008; Robertson et al. 2011; Lazenby 2011). The bacteria species responsible for the anammox reaction are autotrophs, using the acetyl CoA pathway to fix CO_2 (Jetten et al. 2009), and the anammox pathway is believed to be inhibited by pyruvate, ethanol, glucose, and high concentrations of NO_2^- (Strous et al. 2009a). High concentrations of dissolved organic carbon (DOC) are believed to result in denitrification dominating over anammox (Chamchoi et al. 2008).

Anammox is believed to occur strictly under anaerobic conditions. Some studies suggest that reversible oxygen inhibition will occur above 13 μM dissolved oxygen (DO) (Jensen et al. 2008, Kuypers et al. 2005). However, many waste water treatment plants actually inject air into their anammox reactors, to increase concentrations of nitrite via nitritation (Furukawa et al. 2006, Pynaert et al. 2004). It has been suggested that anammox activity can occur within small anaerobic microzones within an otherwise aerobic environment, relying on other bacteria to locally consume oxygen and produce substrate for anammox activity (Woebken et. al. 2007).

1.3 Site Description

The field site that is the focus of this study is a poultry manure composting facility located in Zorra township, in southwestern Ontario. The site consists of multiple manure composting piles (windrows) which are watered as part of the composting process (Figure 2). The runoff from this composting process collects in a ~ 20 x 50 m lagoon, which varies in depth seasonally between 1 and 2 m (Lazenby 2011).

The geology in the area consists of a Pleistocene glacial spillway aquifer (Robertson and Schiff 2008), with a range of sediments present from silt and clay to coarse gravel (Robertson and Schiff 2008; Lazenby 2011). Lazenby (2011) installed a network of 40 multilevel monitoring wells (Figure 3), and delineated the groundwater plume originating from the lagoon. The centreline geology consists of mostly of sand and gravel, but somewhat finer sediments occur at the distal end of the section (Figure 4). Between 2007-2010, the plume core zone had NH_4^+-N values ranging from 32 ± 3 mg/L near the lagoon to 2 ± 3 mg/L at the distal end of the monitoring network, 101 m downgradient from the lagoon (Lazenby 2011). Nitrate-N was generally below detection (< 0.01 mg/L) in the plume core zone, while regional background groundwater had NO_3^-N averaging 10 ± 1 mg/L, as a result of other agricultural activities occurring in the area (Lazenby 2011). Nitrate-N concentrations ranged from 19-40 mg/L in the shallow water table zone overlying the lagoon plume (Lazenby 2011).

1.4 Research Objectives

Two previous studies at the Zorra site suggest possible anammox activity. Lazenby (2011) estimated a TN degradation rate of 0.4 mg/L/day in the groundwater plume which was attributed to both denitrification and anammox. He inferred that anammox was likely present, based on ^{15}N enrichment and decreases in both NO_3^- and NH_4^+ particularly along the plume

edges, but a highly fluctuating lagoon $\delta^{15}N\text{-}NH_4^+$ signature (28-72‰, Lazenby 2011) made anammox specific enrichment difficult to quantify.

In a separate study at the Zorra site, Moore et al. (2011) used a DNA-based microbiological approach (qPCR) to quantify groundwater-based anammox communities, which identified up to 5% anammox bacteria populations in groundwater from wells PU103 and PU106.

The objective of this study is to gain a better understanding of anammox activity at the Zorra site, including providing initial estimates of rates of anammox activity in water containing both NO_3^- and NH_4^+. The longer term goal of this research program is to assess the possibility of mixing high NO_3^- and NH_4^+ groundwaters together to enhance nitrogen attenuation by the anammox pathway. The methodology used here includes a variety of microcosm experiments undertaken to observe potential anammox activity using various substrate mixtures (sediment and groundwater) from the Zorra site. Rates of anammox activity are estimated in an attempt to determine if this process might represent a potentially important new process for natural attenuation of nitrogen loading from point-source agricultural operations.

1.5 Experimental Approach

The experimental approach began with a set of batch tests and field reactors trials to obtain a general understanding of potential anammox activity and how to apply this to a larger field scale experiment. The plan for the installed field mesocosms was to convert them from an initial static condition, to dynamic-flow conditions, by initiating timed pump-dosing from adjacent monitoring wells. However, upon observing the relatively low reaction rates in these initial tests, it was determined that observing informative field trends would be difficult under dynamic flow conditions due to the many variables associated with a field scale groundwater

plume (heterogeneous media, potential diffusion of oxygen at saturated-unsaturated interface, seasonal variability of the plume N parameters, seasonal temperature variability, etc). Thus, the field barrels were maintained in their initial static condition (no further pumping) because of the likely seasonal variability of piezometer chemistry and because of the possibility of O_2 contamination during pumping.

The next best option was then considered to be a dynamic flow column test in the laboratory. However, due to the importance of excluding oxygen in the experiments, and the inherent difficulties with oxygen contamination, given the column apparatus available, it was subsequently decided to focus on microcosm batch tests that could potentially provide the most reliable, oxygen free, experimental environments.

As the first set of batch tests did show evidence of O_2 incursion, along with, again, relatively low reaction rates, several successive sacrificial microcosm experiments were performed, seeking to achieve improved reaction rates while increasing O_2 exclusion.

The approach used for evaluating the possibility of anammox activity was primarily based on mass balance and isotopic evidence, similar to previous researchers (Mulder et al. 1995, Clark et al. 2008, Robertson et al. 2011). These indicators include the concomitant consumption of NO_3^- and NH_4^+, along with the progressive enrichment of the residual $\delta^{15}N$-NO_3^- and $\delta^{15}N$-NH_4^+ under anaerobic conditions (Figure 5). However, it is important to note that in laboratory studies, a lack of observed dissolved oxygen (DO) does not prove an absence of atmospheric leakage, as oxygen consumption reactions (such as nitrification) can rapidly consume small amounts of O_2 leakage. This led to the implementation of a DO control in Experiment 3, and the use of an anaerobic chamber and gas tight sacrificial bottles in Experiment 4.

Further to the previous studies, Rayleigh curves were used to distinguish between NH_4^+ undergoing nitrification and anammox activity. Additionally, a modified tagged $^{15}N\text{-}NH_4^+$ experiment was also attempted, similar to previous studies (Thamdrup and Dalsgaard 2002; Moore et al. 2011) in collaboration with the University of Ottawa. This experiment had the potential to quantify the relative magnitude of denitrification versus anammox reactions, which could not be readily done using the other lines of evidence. However, the tagged experiment encountered a number of problems including:

- F_{NH4} (Fraction of NH_4^+ as $^{15}N\text{-}NH_4^+$) was too low (0.04-0.05) to consider $^{15}N\text{-}NO_3^-$ contribution to N_2 as being negligible, which violates the terms of the basic IPT equation (Appendix F).

- Analysis of samples employed insufficient QA/QC procedures, including removing varying amounts of sample, injecting varying amounts of He (both unrecorded), and manually shaking bottles for 1 minute to obtain headspace 'equilibrium' conditions; in addition, pressures and temperatures were not recorded, and bottles were not weighed. Sample volume injected into the mass spectrometer ranged drastically from 0.95 to 90 uL, and the machine was calibrated using a 1-point (lab air) calibration.

- Analytical error was estimated based on the standard deviation of the standard used for analysis (lab air). The analytical error for $^{29}N_2$ was equal to or greater than the measured $^{29}N_2$ in some samples. Accounting for this error using the updated equation (Appendix F) results in a range of anammox produced N_2 between 0 and greater than 100%, making these analyses of questionable reliability.

These concerns made the results of this experiment unreliable. Consequently, the results are excluded from the discussion here, although the data and experimental procedures undertaken are preserved in Appendix F.

It is important to note that denitrification is expected to occur concomitantly with anammox at the Zorra site because DOC values are quite high in the groundwater. Although this complicates quantifying the anammox contribution, overall, this would be considered a favourable circumstance because additional natural attenuation of TIN occurs. Denitrification becomes a concern only when it goes to completion, as it then removes the available substrate (NO_3^-) required for anammox to proceed. Generally, it is beneficial for both denitrification and anammox to occur together, as both can play an important role in the natural attenuation of anthropogenic contamination.

Evidence that is indicative of anammox activity includes the concomitant consumption of NO_3^- and NH_4^+ at a 3:5 molar ratio (Eq. 1.4, Mulder et al. 1995) under anaerobic conditions. If NO_3^- is removed in a higher amount, then denitrification could be the cause of additional removal. Another possibility is that NH_4^+ concentrations are being buffered, either by ammonification or by desorption from the aquifer solids, both of which would give an impression of excess NO_3^- removal. If NO_3^- is removed at a lower ratio (i.e. more NH_4^+ removed than anticipated), nitrification, biomass assimilation or adsorption could be the cause. If an increase in Ca^{2+}, Mg^{2+} or Na^+ was observed along with NH_4^+ loss, sorption would be the expected pathway. A loss of NO_3^- combined with increasing NH_4^+ concentrations could suggest either DNRA or that a combination of denitrification and mineralization is occurring.

The isotopic evidence expected for anammox activity includes the concurrent enrichment of both residual $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$ (Clark et al. 2008). A fractionation factor of ~ 4‰ has

been estimated previously for $^{15}N\text{-}NH_4^+$ (Clark et al. 2008; Robertson et al. 2011; Lazenby 2011), and though a fractionation factor has yet to be determined for $^{15}N\text{-}NO_3^-$ during anammox, enrichment is expected. If substantially higher fractionation factors are observed (e.g. 12-29‰) for $^{15}N\text{-}NH_4^+$, this could provide evidence that nitrification is occurring (Shearer and Kohl 1986; Kendall 1998) . If depletion of residual $^{15}N\text{-}NH_4^+$ is observed along with a concentration decrease, sorption could be the cause, although literature currently suggests that NH_4^+ fractionation during adsorption in sands is weak (Bohlke et al., 2006) . Assimilation is not expected to have a significant effect on the isotopic signature (Aravena and Mayer, 2010)

Denitrification enrichment is a rate dependent process, and fractionation factors for $^{15}N\text{-}NO_3^-$ have been reported between -45 and -5‰, however most studies indicate values greater than -15‰ (Blackmer and Bremner 1977; Kendall and Aravena 2000; Casciotti et al. 2002; Menyailo and Hungate 2006). As the $^{15}N\text{-}NO_3^-$ isotope effect has not been studied in detail for anammox, differentiation from denitrification is difficult. As a consequence, an observation of NH_4^+ consumption in an anaerobic environment, combined with $^{15}N\text{-}NH_4^+$ enrichment at the expected fractionation factor, has become the principle line of evidence in support of anammox in this study. However without proof of an oxygen-free environment, the possibility of coupled nitrification-denitrification remains, which could potentially produce a similar net loss of NH_4^+ and NO_3^- together, combined with enrichment of both $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$.

2.0 Methods

2.1 Cations

Major cation (Al^{3+}, Ca^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Na^{+}) concentrations were determined by inductively coupled plasma-atomic emission spectroscopy using a Horiba Jobin Yvon Ultima 2 ICP (Horiba Jobin Yvon) with a detection limit of 0.005-0.01 mg/L at the labs of the Groundwater Quality and Assessment Section, National Water Research Institute, Environment Canada, Burlington ON. Sample concentrations were calibrated against multi-ion standards that were included within each run of samples. When necessary, samples were diluted with Milli-Q water to bring their concentration within the working range of the standards (Spoelstra 2010).

NH_4^+ concentrations were determined colorimetrically using a Beckman D600 UV-Vis Spectrophotometer (Beckman Coulter Canada, Mississauga, ON) (650-660 nm) after reaction with Indophenol Blue indicator solution at the Environmental Geochemistry Laboratory (EGL), University of Waterloo. Samples were routinely diluted on a 1:100 or 1:20 basis as determined by the colour of sample (indicative of organic and NH_4^+ concentration) to prevent interference with colorimetry and to keep sample concentrations within the range of standards. The analytical precision for dissolved NH_4^+ concentration using this method (with Zorra samples) is approximately ± 4%.

2.2 Anions

NO_3^-N analyses was performed using two methods, both at the EGL at the University of Waterloo. The first method employed ion chromatography using a Dionex ICS-90 (Dionex, Sunnyvale, CA) which provided a detection limit of 0.5 mg/L. The second method used a Westco SmartChem 200 Analyzer (Westco, Brookfield, CT). The Westco unit used a nitrite-

nitrate colourimetric method based on USEPA Method 353.2, Revision 2.0 (1993) and Standard Methods Method 4500 NO_3^-, with a detection limit of 0.05 mg/L. Samples were auto diluted to ½, ¼, or 1/6 the original concentration (method range 0.05-20.00 mg N/L). The analytical precision for dissolved NO_3^- concentration using this method (with Zorra samples) is approximately ± 0.3%.

NO_2^-N concentrations were determined colorimetrically, using two methods at the EGL at the University of Waterloo. The first method used a Beckman D600 UV-Vis Spectrophotometer (Beckman Coulter Canada, Mississauga, ON). The method selectively quantifies nitrite ions in solution by first reacting with sulfanilamide to form a diazonium salt. When this salt couples with the N-(1-naphthyl) ethylenediamine hydrochloride dye a reddish purple colour is generated and a spectrophotometer can then be used to determine how much nitrite is in the sample. Concentration is determined by absorbance of the solution at 545nm wavelength. The second method used a Westco SmartChem 200 Analyzer (Westco, Brookfield, CT). The Westco determines nitrite concentrations by diazotizing with sulphanilamide followed by coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly coloured azo dye, which is measured colorimetrically at 550 or 520 nm. The analytical precision for dissolved NO_2^- concentration using this method (with Zorra samples) is approximately ± 3%.

Other anions (Cl^-, SO_4^{2-}, Br^-, PO_4^{3-}, F^-), were also analyzed by ion chromatography in the EGL at the University of Waterloo, with a detection limit of < 0.5 mg/L. DOC was measured using a Dohrman DC-190 total carbon analyzer (Dohrmann, Santa Clara, CA) also at the EGL. For anion analyses, dilutions were undertaken for highly coloured and concentrated samples. Standards were included in all analyses. The analytical precision for DOC concentration using this method (with Zorra samples) is approximately ± 2%.

2.3 N_2O

N_2O analyses were performed at the EGL using a headspace equilibrium technique and a gas chromatograph. Thuss (2008) describes this technique as follows: headspace is created to produce positive pressure inside bottles by injecting 10mL of He into the samples while removing 5mL of samples, then sample bottles were shaken for about 90 minutes until dissolved N_2O reaches equilibrium with headspace. The N_2O concentrations were then determined with an Electron Capture Detector (ECD) on a Varian CP 3800 greenhouse gas analyzer (Varian Canada, Inc.). Dissolved N_2O concentrations were then calculated according to Henry's Law which provided a detection limit of 0.2 μg/L. The analytical precision for dissolved N_2O concentration using this method is approximately ± 5%.

2.4 Total Nitrogen, TKN, DON

TN was determined at the EGL using a Dohrmann Apollo Carbon Anlyzer with TN Module (Dohrman, Santa Clara, CA). DON was calculated as the difference between TN and (NH_4^+-N + NO_3^-N + NO_2^-N). TKN analysis at the Soil and Nutrient Laboratory follows a modified Kjeldahl digestion (Thomas et al., 1967), with nitrogen concentrations measured using a Technicon Auto Analyzer. DON was calculated from TKN as the difference between TKN and NH_4^+-N. TKN was analyzed at Agriculture and Food Laboratory at the University of Guelph. The analytical precision for dissolved TN concentration using this method (with Zorra samples) is approximately ± 1%; however, this error does not account for uncertainty in percent recovery of nitrogen.

2.5 $\delta^{15}N$ —NO_3^-

NO_3^-N isotopic composition was also analyzed at the EGL. The method of preparation follows a modified version of chemical denitrification method (McIlvin and Altabet 2005). This method is acceptable for samples with relatively low concentrations of NO_2^-N (<10%). For preparation, samples filtered to 0.45 µm were frozen in glass vials and freeze dried prior to reconstitution with 2 mL of 0.75M NaCl. 0.1 mL of Cd is then added, after which vials are shaken for 24 hours to reduce NO_3^-N to NO_2^-N. Samples are then filtered (to remove Cd) and injected into he filled headspace vial where conversion to N_2O is accomplished by a 2M NaN_3 and 20% glacial acetic acid buffer solution for 30 minutes. The reaction is quenched by the addition of 1 mL of 6M NaOH. The samples are then over pressurized with 10 mL of He, and shaken for 1 hour. N_2O samples are analyzed for $\delta^{15}N$ and $\delta^{18}O$ by injection of ~ 6 nmol of N_2O into a GV Trace Gas pre-concentrator system, attached to a GV Isoprime mass spectrometer at the University of Waterloo Isotope Laboratory (UWEILAB). Raw molecular ratios (mass 44, 45 and 46) from the mass spectrometer are converted to isotopic ratios ($^{15}N/^{14}N$ and $^{18}O/^{16}O$) using the data correction method described by Kaiser et al. (2003). N_2O isotopic data is then corrected to yield $\delta^{15}N$ and $\delta^{18}O$ values using internal standards prepared and run as samples. Isotopic results are expressed in delta (δ) notation in per mil units, relative to the reference standard of atmospheric N_2 for $\delta^{15}N$ and $\delta^{18}O$. The analytical precision for both $\delta^{15}N$ and $\delta^{18}O$ is approximately ± 1‰.

2.6 $\delta^{15}N$-NH_4^+

Preparation for determination of $\delta^{15}N$-NH_4^+ was performed at the Environmental Isotope Laboratory based on the methods in Spoelstra et al. (2006). This is a modified version of the

standard ammonium diffusion technique for $\delta^{15}N$ determination (Brooks et al., 1989; Sørensen and Jensen 1991; Holmes et al. 1998; Sebilo et al. 2004). Unfiltered, preserved samples with concentrations as low as 0.6 mg/L NH_4^+-N are prepared, in duplicate, with a solution of 4M potassium chloride (KCl) so that the total volume in a 50 mL Wheaton serum bottle is 20 mL and the mass of NH_4^+-N contained in the bottle is at least 20 µg. Separately, diffusion 'traps' are made by sealing an acidified (10 uL 0.2M H_2SO_4) quartz filter disk (Whatman 4.7cm QMA filters, baked) in a section of polytetrafluoroethylene (PTFE) tape ('T-Tape'), which allows gas, but not water diffusion across the membrane. After the addition of a magnetic stir bar (Fisherbrand, 1''), each solution containing the nitrogen and KCl mixture is made basic (as indicated by the addition of a phenolphthalein indicator) by the addition of 0.2M sodium hydroxide (NaOH) and buffered to a pH of ~ 9.3 by the addition of 2 mL of a sodium tetraborate ($Na_2B_4O_7$) solution, at which time the PTFE traps are added to the solution and bottles are capped with 20 mm butyl blue septum stoppers (Belco Glass Co.). Bottles are left to stir (using a magnetic stir plate) for a minimum of 10 days, during which time the dissolved NH_4^+ progressively volatilizes to gaseous NH_3 in the bottle headspace and then precipitates on the acidified filter disk as ammonium sulphate ($(NH_4)_2SO4$). At the conclusion of the diffusion period, PTFE traps are removed and the filter disks placed in vials (Fisherbrand 1 dr.) that are frozen, then freeze dried. The disks are then combusted and the vapour produced is analyzed on a Carlo Erba Instruments NA 1500 Series 2 Nitrogen/Carbon/Sulphur analyzer coupled with a Finnigan Mat Delta Plus at the EIL. Isotopic results are expressed in the standard δ units (per mil difference) relative to the reference standard of atmospheric N_2 for $\delta^{15}N$. The analytical precision for $\delta^{15}N$-NH_4^+ is approximately ± 0.3‰.

2.7 EC, DO, Eh, pH

Field and laboratory measurements were performed routinely for electrical conductivity (EC), dissolved oxygen (DO), reduction potential (Eh) and pH of groundwater. A Barnant 20 brand digital pH meter (Barnant, Barrington, IL) was used to determine *in situ* pH and Eh of groundwater after being calibrated to buffers of pH 4, 7 and 10 and checking against a Zobell's solution. DO (mg/L), EC (uS/cm) and temperature (^{o}C) were measured using an HQ20d Dissolved Oxygen meter (Hach Company, Loveland, CO) which also gave corroborating measurements of pH using interchangeable probes on the same meter. Occasionally a Winkler Mini Winkler titration (modified from Stainton et al. 1974) was performed in the lab to corroborate values obtained with the DO probe. This method uses manganese (II) chloride and sodium hydroxide, which oxidize to form manganese (II) hydroxide, which fixes all available oxygen in the sample. Sulphuric acid (H_2SO_4) acidifies the sample and sodium iodide (NaI) is oxidized to iodine (oxygen in the sample oxidized Mn^{2+} to Mn^{4+}, Mn^{4+} oxidised I to I_2). Titrating the iodine with sodium thiosulphate ($Na_2S_2O_3$) and a starch indicator gives a value that is directly proportional to the concentration of dissolved oxygen in the sample. 30mL of sample was taken in a Trutest Precision (Geo. S. Trudell Co., London ON) glass syringe, stoppered with a Plasticoid Sleeve rubber stopper (Fischer Scientific, Nepean ON) and immediately preserved with $Mn^{2+}Cl$ and NaOH to preserve the sample, which was titrated within minutes of being taken.

For all field and laboratory sampling, DO and pH were determined immediately after sampling. For the sacrificial DO bottle, the probes were placed directly in the sacrificial bottles, as soon as the stopper was removed (DO probe, then pH, as only one could fit at a time). Reduction potential, conductivity, and temperature were measured immediately thereafter. For

the sacrificial serum bottles, each sample was decanted into a 200 mL beaker, where all probes could be placed simultaneously. For the field reactors, a 30mL syringe (without plunger) was attached to each of the sampling port tubes. The DO probe was immediately placed in the syringe. Approximately 15 mL of sample was pumped into the syringe, after which the DO probe was activated. Continual additions of fresh water (to a total of approximately 35 mL or more) were added to the syringe until the DO stabilized. Due to the limiting amount of reactor water available, the addition of fresh water during DO stabilization stopped if the DO value decreased below 1 mg/L (therefore <1 mg/L is considered the detection limit for this method). This preserved the reactor volume, but still demonstrated low DO. Measurement of pH, EC, temperature, and occasionally Eh then followed. All groundwater field parameters were measured using a flow through cell, pumped for 3-5 minutes to allow for stabilization of each parameter.

2.8 qPCR

A DNA-based microbiological approach was used to estimate the population of anammox bacteria relative to all bacteria present. Quantitative real-time PCR (qPCR) was performed using a C1000 thermal cycler with a CFX96 real-time system (Bio-Rad). PCR was performed using the following reaction components in 10-µl volumes: 5 µl of SYBR-green Supermix (Bio-Rad), 0.05 µl equivalent of each forward and reverse primer (100 mM), 0.5 µl of bovine serum albumin (10 mg mL^{-1}; Kreader 1996), and 0.5 µl of extracted and quantified environmental nucleic acids (0.5 to 5 ng μl^{-1}) or DNA standards. Anammox-specific qPCR was conducted similarly to a previously published protocol (Ward et al. 2009) using primers Amx368f (Schmid et al., 2003) and Amx820r (Schmid et al. 2000). General bacterial qPCR used primers 341f and 518r (Muyzer et al. 1993). The qPCR thermal program for Amx368f and

Amx820r involved an initial denaturation of 3 minutes at 95°C followed by 40 cycles of 45 seconds at 95°C, 1 min at 62°C and 1 min at 72°C. Melt curve analysis used a gradient of 62°C to 95°C with 0.5°C temperature increments. For qPCR with 341f and 518r, the reaction involved an initial denaturation step of 3 min at 95°C, followed by 40 cycles of 45 seconds at 95°C, 1 min at 55°C, and 1 min at 72°C. The melt curve analysis involved an increase of temperature from 55°C to 95°C in 0.5°C increments. The efficiency of general bacterial and anammox qPCRs were 88.4% and 92.4%, with R^2 values of 0.99 and 0.964, respectively. All qPCR products were run on a 1% agarose gel along with a 1 kb Plus DNA ladder (Invitrogen) to confirm the size and quality of PCR products.

PCR products from groundwater samples were used to generate standard curves for qPCR. PCR products were purified using a MinElute kit (Qiagen) and quantified with a spectrophotometer (Nanodrop ND-1000). Products were diluted to 10 ng μl^{-1} and eight serial 10-fold dilutions were performed using sterile distilled and deionized water. All qPCR amplifications were conducted in duplicate. The analyses were performed by Tara Moore of the Environmental Microbiology Laboratory at the University of Waterloo.

3.0 Results

3.1 Experiment 1 – Tedlar Bag Microcosms

3.1.1 Introduction

Two initial batch test trials were performed to obtain a preliminary understanding of the potential role of anammox at the Zorra site. Experiment 1 was completed using approximately 100 g of dry core mixed in a Tedlar bag with groundwater from the site. Two types of groundwater mixtures were used, a single source water, and a dual source mixture, to emulate potential anammox 'engineered' designs for future studies. As NO_3^- is typically found in aerobic zones, and NH_4^+ only persists in anaerobic environments, natural mixtures of these two ions will likely only occur near aerobic-anaerobic groundwater interfaces. However, engineered systems could enhance this mixing. Future remediation projects may require engineered mixing of NO_3^- and NH_4^+ rich waters, therefore a study showing successful nitrogen removal from a mixed water source was warranted. This experiment consisted of two trials as follows:

- Trial 1, Dual Source Groundwater: PU125-5.1m (6 mg/L NO_3^-N) and PU96-2.6m (14 mg/L NH_4^+-N, mixed with 95 g sediment from core PU103)
- Trial 2, Single Source Groundwater: PU117-2.2m (29 mg/L NO_3^-N and 25 mg/L NH_4^+-N, mixed with 91 g sediment from core PU103)

3.1.2 Methodology

Trial 1 used dual sourced groundwater mixed with sediment core from the Zorra site, while Trial 2 used single source groundwater mixed with the same sediment. Concentrations of NH_4^+ and NO_3^-were expected to be high at the single well location (PU117) based on previous

monitoring. The first trial used a 5L polyvinyl fluoride (PVF) Tedlar bag, with a 3 mm spigot. These bags are relatively airtight, but the small spigot only allowed for fines smaller than 3 mm diameter to be placed in the bag. 95 g (dry weight) of sediment was used in Trial 1 and 91 g in Trial 2, after which the bags were filled with groundwater to capacity (5 litres). The sediment used in both Tedlar bags was air dried core samples collected during the drilling of well PU103 (Lazenby 2011), using a Geoprobe direct push drill rig with a Macro-Core MC5 Soil Sampling System. The cores were stored at the University of Waterloo for approximately 12 months before use. The groundwater chosen for the single source required high concentrations of both NH_4^+ and NO_3^-, while the dual sourced groundwater required one water with high NH_4^+, and a second with high NO_3. Groundwater with high DOC was avoided, as previous studies have suggested high DOC groundwater will favour denitrification over anammox (Chamchoi et al. 2008). Groundwater was collected on September 25, 2009, using a peristaltic pump, and retained in two 20L plastic Reliance carboy vessels, filled with virtually zero headspace. The water was taken back to the lab and immediately pumped into the Tedlar bags. During loading, the water was pumped from the middle of the 20L vessel. The mixed water (PU125-5.1m and PU96-2.6m) was filled (50:50) in the field to reduce potential aeration in the lab. The groundwater for the dual source trial was expected to be high in NO_3^- (>10 mg N/L, PU125) and NH_4^+ (>20 mg N/L, PU96), based on previous monitoring (Lazenby 2011).

The advantage of the Tedlar bag was that a water sample could be taken from the bag by simply squeezing the bag, and extracting the sample from the spigot using a 60 cc syringe with a 3 way valve. The bags were manually shaken 1 hour prior to sampling. Samples were either analyzed immediately or were frozen. The experiment ran from September 2009 to January 2010 (122 days). Nitrate samples were filtered using 0.45µm syringe filters (PALL acrodisk

PSF or Whatman). Samples for NH_4^+ analysis were preserved immediately after collection by acidification to pH 2-4 using sulphuric acid (H_2SO_4). Both samples were retained in 30 mL Nalgene bottles.

3.1.3 Results and Discussion

Trial 1 (mixed groundwater) consumed 7.4 mg/L of NH_4^+-N, 3.1 mg/L of NO_3^-N, produced 2.7 mg/L of NO_2^-N (Figure 6a), and had overall TN loss of 9.4 mg/L (based on TN analysis), over the 125 day experiment period (Figure 6c). This implied a TN consumption at a rate of 0.1 mg/L/day. Ammonium ($\delta^{15}N$-NH_4^+) increased over the course of the experiment by 7.4‰ (25.6‰ increasing to 33.0‰), while $\delta^{15}N$-NO_3^- increased by 8.4‰ (33.4‰ increasing to 41.8‰) (Figure 6b, Table B1). The precision of $\delta^{15}N$-NH_4^+ and $\delta^{15}N$-NO_3^- analysis is 0.3% and 1%, respectively, suggesting that increases >1‰ can not be explained by analytical error.

Somewhat greater loss of NH_4^+ was observed than can be attributed to anammox alone (based on concomitant NO_3^- loss). 7.4 mg/L of NH_4^+-N was consumed during the experiment, and based on the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995), 4.5 mg/L of NO_3^-N loss was expected. This experiment consumed only 3.1 mg/L of NO_3^-N, which was somewhat less than the expected amount (30% less), but was within the general range of consumption expected for anammox activity.

A possible cause for observed NH_4^+ loss is that oxygen contamination nitrified some of the NH_4^+ to NO_3^- or NO_2^-, which was then subsequently consumed by denitrification. If this was the case, a lag or even depletion of ^{15}N-NO_3^- should be observed as the experiment progressed (Figure 6b). This was not the case. Though there is ^{15}N-NO_3^- depletion observed between time

zero and 21 days (Figure 6b), overall $\delta^{15}N\text{-}NO_3^-$ increase (8.4‰) is greater than the increase observed with $\delta^{15}N\text{-}NH_4^+$ (7.4‰).

Figure 7 shows the expected isotopic evolution for $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ based on representative literature enrichment factors for both nitrification and anammox, and using the Rayleigh model (Eq. 3.1, Kendall and McDonnell 1998):

$$R = R_o f^{(\alpha-1)} \qquad \text{(Eq. 3.1)}$$

where R is the $^{15}N/^{14}N$ ratio for a given fraction (*f*) of reactant remaining, R_o is the initial ratio of the reactant, and α is the fractionation factor. The fractionation factors used in Figures 7 and 8 are literature values as follows:

- Nitrification (α = 0.988 to 0.971); enrichment range for nitrification expected to be between -12 to -29‰ (University of Waterloo, 2006)
- Anammox (α = 0.996 for $^{15}N\text{-}NH_4^+$, 0.993 for $^{15}N\text{-}NO_3^-$); $\delta^{15}N\text{-}NH_4^+$ enrichment based on previous studies (4‰, Clark et al. 2008; Robertson et al. 2010; Lazenby 2011), $\delta^{15}N\text{-}NO_3^-$ estimated to be similar to $\delta^{15}N\text{-}NH_4^+$ enrichment.

The relationship between α and ε is as follows (Eq. 3.2):

$$\varepsilon_{p\text{-}r} = (\alpha-1) \times 1000 \qquad (‰) \qquad \text{(Eq. 3.2)}$$

Note that the term 'enrichment' describes the isotopic evolution of a chemical (ie. $^{15}N\text{-}NH_4^+$), which can be variable over time. The enrichment factor (ε) is a constant value describing the isotopic fractionation between a product and reactant at a given point in time.

Figure 8 compares the predicted curves for both anammox and nitrification reactions to the experimental data. The progressive increase of both $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ over the course of the experiment matches very well with the predicted anammox curves, but is inconsistent with a nitrification reaction because more depleted $^{15}N\text{-}NO_3^-$ would be expected (Figure 8a). This assumes that nitrification goes to completion, and does not end with NO_2^- as the final product. However, it should be noted that denitrification would also result in an observed increase of $\delta^{15}N\text{-}NO_3^-$.

Additionally, there is evidence suggesting that denitrification is not the main source of enrichment. First, NO_3^- concentrations were observed to be lower than expected for anammox; if denitrification was occurring at a greater rate than anammox, NO_3^- consumption would presumably be greater than that of NH_4^+. Second, though denitrification can have enrichment in-situ values between 13-19‰, research suggests that enrichment factors of 20-40‰ are more common, assuming denitrification doesn't go to completion, and substrate supply to denitrifiers isn't diffusion limited (Blackmer and Bremner 1977; Kendall and Aravena 2000; Casciotti et al. 2002; Menyailo and Hungate 2006). Finally, previous studies suggest that anammox will dominate over denitrification in environments with high NH_4^+ concentrations (Hamersley et al. 2009, Kupyers et al. 2005), as was the case in this experiment. This does not imply that denitrification is not occurring, but based on the mass balance approach used above (which assumes NH_4^+-N is consumed by anammox), most of the nitrogen consumption is accounted for with anammox. It is possible that some NH_4^+ loss could be accounted for via coupled nitrification-denitrification at the start of the experiment (when O_2 was likely introduced during experiment setup), as well as NH_4^+ assimilation into biomass.

The single groundwater batch test (Figure 9) had promising early results. Nitrogen consumption was higher than the Trial 1 mix for the first 69 days when NH_4^+ and NO_3^- were being consumed at a rate of approximately 0.80 mg N/L/day, which was ~ eight times greater than for Trial 1. The higher rate may be explained by the substrate concentrations, as PU117-2.2m was initially richer in both NO_3^-N (29 mg/L) and NH_4^+-N (25 mg /L) compared to Trial 1 (6 mg/L NO_3^-N, 15 mg/L NH_4^+-N). However, after day 69, NO_3^- dramatically increased in the Trial 2 microcosm over the duration of the experiment (Figure 9). A possible explanation for this is a nitrification effect due to oxygen contamination that may have occurred as a result of repetitive sampling.

3.1.4 Experiment 1 – Conclusions

The first preliminary experiment was a success in that it showed consumption of both NH_4^+-N and NO_3^-N, although intermittently, and showed isotopic enrichment that could be suggestive of anammox. The major conclusions are:

- Concomitant NH_4^+ and NO_3^- consumption, along with δ^{15}N-NH_4^+ and δ^{15}N-NO_3^- increases were observed after mixing two N-rich groundwater sources and incubating with sediment under anaerobic conditions, suggesting that the anammox might be responsible for some N attenuation.

- The Rayleigh models predict enrichment of ^{15}N-NH_4^+ and depletion of ^{15}N-NO_3^- for a nitrification effect. The observed enrichment of both parameters suggests that nitrification is not the dominant process, but rather this behaviour supports the possibility of anammox.

- Oxygen contamination appears to have resulted in an abrupt production of NO_3^- after day 69 in Trial 2. Future experiments should thus ensure completely anaerobic conditions to promote anammox attenuation.

- A conservative anammox reaction rate (based on NH_4^+ loss) for Trial 1 (mixed groundwater) was 0.1 mg N/L/day

- Before suspected oxygen contamination, Trial 2 (single source microcosm) attenuated N at a rate of 0.80 mg N/L/day, eight times faster than the dual source microcosm. It is possible that the higher rate in the single source microcosm was due to a pre-existing and more active anammox community, or was due to the higher initial substrate concentrations (NH_4^+-N and NO_3^-N of 25 and 29 mg/L, respectively, while NH_4^+-N and NO_3^-N for the mixed groundwater were only 6 and 14 mg/L, respectively).

- This experiment demonstrated the need for a durable experiment, one that can maintain integrity throughout the experiment period. Going forward, the need to ensure anaerobic conditions must be addressed.

3.2 Experiment 2 – Kimex Flask Microcosms

3.2.1 Introduction

Due to the limiting nature of the Tedlar bags (experiment 1) to contain sediment, a parallel study was initiated using a different incubation vessel. The second experiment was completed using approximately 500 g of dry core in a 2L Kimex Heavy Walled Filter Flask. The large (50 mm) flask opening allowed 500% more sediment to be used compared to the Tedlar bags, but it might have carried a higher risk of oxygen contamination. Whereas the Tedlar bag had an air-tight valve, the flasks used a taped and vacuum greased stopper with two glass tubes for sampling. As with Experiment 1, a minimum number of parameters were initially analyzed (NO_3^-, NH_4^+, NO_2^- and TN). The same two types of groundwater were used in the study, a single point source water, and a dual source mixture, to emulate potential 'anammox engineered' designs for future studies. The groundwater used for this experiment was taken from:

- Trial 1, Dual Source Groundwater: PU125-5.1m (6 mg/L NO_3^-N) and PU96-2.6m (14 mg/L NH_4^+-N, mixed with 500 g sediment from core PU103)
- Trial 2, Single Source Groundwater: PU117-2.2m (29 mg/L NO_3^-N and 25 mg/L NH_4^+-N, mixed with 500 g sediment from core PU103)

3.2.2 Methodology

Experiment 2 used a 2L Kimax heavy walled glass filter flask, with a 50 mm opening. This method was used as an alternative to the Tedlar bags, to further minimize the possibility of atmospheric O_2 diffusion into the microcosms. Two hollow glass rod tubes were installed into a rubber stopper, which sealed the jar. The first glass rod was used to remove sample, while the second rod was attached to an aluminum balloon, filled with helium. This design allowed for

helium to replace any sample removed (Figure B1). Another advantage of the glass jar design was to allow the use of all sediment sizes in the mixture. However, it was found that during the test, the microcosms were likely affected by oxygen contamination due to the difficulty in effectively sealing the rubber stopper and glass rods. Sample was extracted from the flask by applying pressure to the helium balloon, which would push sample into a syringe that was affixed to the second glass rod. Both rods were sealed immediately following sampling. The flasks were shaken 1 hour prior to sampling. The experiment ran from September 2009 to January 2010, concurrently with Experiment 1.

Experiment 2 was initiated at the same time as Experiment 1, and used the same groundwater and sediment samples, as well as the same sample collection procedures (see section 2.1.1). Approximately 500 g of dry sediment (<50 mm diameter) was used in each flask. No mechanical sorting of the sediment was done in this case, other than the exclusion of pebbles that were too large to fit through the 50 mm flask opening.

3.2.3 Results and Discussion

Trial 1 consumed NH_4^+ and NO_3^- at a combined rate of 0.26 mg N/L/day during the first 56 days of the study (Figure 10a). Similar to Trial 2 in Experiment 1, NO_3^- concentrations then rose rapidly after day 56, again as a possible result of oxygen contamination that occurred during repetitive sampling.

Trial 2 (single source) consumed NH_4^+ and NO_3^- at a combined rate of 0.5 mg N/L/day during the first 35 days (Figure 10b), and then again rapid increase of NO_3^- concentrations occurred after day 35 suggesting a possible oxygen contamination.

Both trials show an overall loss of TIN ($NH_4^+ + NO_3^- + NO_2^-$) over the 125 experiment period (Figure 10). Trial 1 and Trial 2 lost 8 and 14 mg/L TIN, respectively.

3.2.4 Experiment 2 – Conclusions

Though Experiment 2 showed promising nitrogen removal which could be indicative of anammox, possible oxygen contamination of both trials within the first 56 days of the 125 day experiment make the case for anammox less clear. However, some conclusions can still be made:

- Oxygen is a major concern when trying to support an anammox reaction at the microcosm scale, which may also prove to be a substantial *in situ* issue. If all NH_4^+ is nitrified to NO_3^-, anammox reactions can not occur. Though anammox bacteria have been shown to produce both NH_4^+ and NO_2^- from NO_3^- (Kartel et al. 2007), it is unclear if reaction rates in that scenario are lower than in a NH_4^+ rich environment. Anammox has been shown to convert NO_3^- to NH_4^+, even in the presence of external NH_4^+.

- Assuming NH_4^+ and NO_3^- loss during the initial 56 days of the mixed well flask was a result of anammox, it would appear that the amount of sediment affects reaction rates. The flask experiment (~ 500 g sediment) reaction rate was 0.3 mg N/L/day, while the Tedlar experiment (~ 100 g sediment) rate was only 0.1 mg N/L/day. Less sediment may result in lower sediment-based biomass being present, resulting in reduced reaction rates. It is also possible that a slow O_2 leak was present throughout the experiment, resulting in the initially high reaction rates observed (nitrification + denitrification) before a larger leak resulted in complete oxygen contamination.

3.3 Experiment 3 – Sacrificial DO Bottles

3.3.1 Introduction

Due to the probable oxygen contamination as a result of repetitive sampling of the Experiment 1 and 2 microcosms, a different approach was taken to reduce the possibility of oxygen ingress. The single microcosm method used in the first two experiments was replaced with a sacrificial bottle design. This sacrificial bottle experiment used six identical water and sediment mixtures per trial, each contained in a 250 mL DO bottle. One bottle per trial was sampled for each sampling event, leaving the remaining bottles undisturbed. An additional upgrade for Experiment 3 was the use of freshly drilled core material obtained from the suspected anammox zone at the Zorra site (Moore et al. 2011). This experiment consisted of four trials as follows:

- Trial 1, Single Source Groundwater: PU115-2.2m (43 mg/L NO_3^--N and 5 mg/L NH_4^+-N, mixed with 50 g of fresh core collected at 3-6m depth beside PU103)
- Trial 2, Dual Source Groundwater: PU80-1.7m (7 mg/L NO_3^--N) and PU122-2.2m (43 mg/L NH_4^+-N), mixed with 50 g of fresh core collected at 3-6m depth beside PU103)
- Trial 3, DO Control: He sparged DI water, mixed with 100 g of boiled silica sand. These bottles were used to observe any potential oxygen ingress over time.
- Trial 4, Dual Source Groundwater: PU80-1.7m (7 mg/L NO_3^--N) and PU125-2.7m (6 mg/L NO_3^--N, 9 mg/L NH_4^+-N), mixed with 50 g of fresh core collected at 3-6m depth beside PU103).

3.3.2 Methodology

Two lab microcosm experiments were completed (Experiments 3 and 4) in which sets of sacrificial bottles were used to avoid the problem of possible atmospheric O_2 introduction associated with repeatedly sampling the same microcosm container. In Experiment 3, each trial consists of 6 identical mixtures contained in 6 separate 250 mL DO bottles. One bottle was sampled per sampling event. A second advantage to the sacrificial bottle experiment is the abundance of sample water (250 mL per bottle). The main drawback of the sacrificial bottle approach is the difficulty in obtaining 6 identical microcosms, considering soil heterogeneities, etc.

Experiment 3 consisted of 4 trials comprised of various mixes of Zorra water, combined this time with fresh core obtained from the suspected anammox zone at the Zorra site identified by Moore (2011). The core was retrieved on June 29, 2010, beside well PU103, between 3-6 m depth, using the Geoprobe drill rig with the Macro-Core MC5 Soil Sampling System. The cores were placed in a 19L pail, which was filled with groundwater from PU125-2.7 to preserve the anaerobic condition of the sample. This experiment employed the same groundwater collection procedure as the batch tests, using 20L jugs in the field that were then decanted into each DO bottle in the lab. The four trials consisted of differing sediment-water mixtures, each with 6 sacrificial bottles (250 mL Wheaton dissolved oxygen bottles) filled with 50 g (wet wt) of fresh core (except PU-C series, which used boiled silica sand) and groundwater from the field site. Each bottle was sealed with a glass stopper, with DI water placed on top of the stopper to further ensure it was sealed, then Parafilm wax wrapped around the stopper and water. The bottles were placed in a shaker, in the dark, and were shaken for 10 minutes per day. One bottle from each mix was sampled (and sacrificed) approximately once per month between July 6, 2010 and

January 14, 2011. Analyses included DO (both by Hach probe and meter, and occasional Winkler titration for verification), pH, Eh, electrical conductivity, temperature, NO_3^- and NH_4^+ concentrations and isotopes, as well as NO_2^-, TN, DON, DOC, Cl^-, SO_4^{2-}, Br^-, N_2O, CO_2, and CH_4 (Appendix D for table). Details on sampling and analytical procedures can be found in section 2.2.

3.3.3 Results and Discussion

All 4.5 mg/L of available NH_4^+-N were consumed in Trial 1 (single groundwater), and between 3.6 and 11.0 mg/L of NO_3^--N (pronounced difference in concentrations between days 140 and 190, Figure 11a). 1.9 mg/L of NO_2^--N was produced but consumed during the experiment (Table D1), and N_2O was produced (Figure 13a), suggesting an overall TIN loss of between 8.1 and 15.5 mg/L, over the 192 day experiment period (Figure 11a). This implied a TIN consumption at a rate of up to 0.1 mg/L/day. Ammonium ($\delta^{15}N$-NH_4^+) change over the course of the experiment could not be determined due to the rapid consumption of NH_4^+ and lack of sample volume at those concentrations. $\delta^{15}N$-NO_3^- increased by 2.1‰ (32.2 to 34.4‰) (Figure 12a, Table D1).

Somewhat greater loss of NO_3^- was observed than can be attributed to anammox alone (based on concomitant NH_4^+ loss). 4.5 mg/L of NH_4^+-N was consumed during the experiment, and based on the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995), 2.7 mg/L of NO_3^--N loss was expected. In this experiment, between 3.6 and 11 mg/L of NO_3^--N was consumed, which was somewhat more than the expected amount.

One possible scenario to account for the greater loss of NO_3^- could be anammox activity. The formation of NH_4^+ from NO_3^- in anammox bacteria may be facilitated by short chain organic

acids (such as formic acid, acetic acid, and propionic acid) which reduce NO_3^- (Kartal et al. 2007). The method in which the NO_3^- is reduced to NH_4^+ is proposed to operate similarly to dissimilatory nitrate reduction to ammonium (DNRA) (Galán et al. 2009), with some of the NO_2^- intermediate retained for reaction with the NH_4^+ (Jensen et al. 2008).

Figure 14 compares the experimental data to the expected isotopic evolution for $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ based on literature enrichment factors for both nitrification and anammox, using the Rayleigh model (Kendall and McDonnell 1998, Section 3.1.2 for details). Though low concentrations of NH_4^+ prevented a sufficient $\delta^{15}N\text{-}NH_4^+$ data comparison, the $\delta^{15}N\text{-}NO_3^-$ data shows progressive enrichment. However, as the initial isotope value for $\delta^{15}N\text{-}NO_3^-$ was lower than the initial $\delta^{15}N\text{-}NH_4^+$ value, both nitrification and anammox would exhibit progressive enrichment of $\delta^{15}N\text{-}NO_3^-$ (Figure 14).

Though it is impossible to rule out denitrification as playing a role in the consumption of NO_3^- in experiment 1, there is evidence to support anammox activity is also occurring. Though DO was present during experimental setup (3.43 mg/L, Table D1), it remained below detection (<0.3 mg/L) for the remainder of the experiment. Ammonium concentrations increased between the first two sampling events (potentially the result of NH_4^+-N in sediment), which does not allow for quantification of NH_4^+-N loss via nitrification during the initial stage of the experiment. The consumption of NH_4^+ under observed anaerobic conditions (DO <0.3 mg/L, and DO control supports anaerobic conditions maintained, Table D1) after experimental setup suggests consumption of nitrogen by bacteria other than denitrifiers. Dissolved oxygen concentrations suggest that nitrification likely is not the main contributor to NH_4^+ loss. The presence of CH_4 in samples (326-523 nmol/L, Table D1) also suggest reducing conditions throughout the experiment. Dissolved organic carbon (DOC) remained relatively stable between

60-70 mg/L (Table D1) over the duration of the experiment, suggesting a heterotrophic reaction such as denitrification may not be a main contributor to nitrogen consumption. However, only 11.8 mg/L DOC is required to consume all 11.0 mg/L of NO_3^--N (Appendix D). Based on the high concentrations of DOC and dilutions required for analysis (10-20x), the precision of DOC analysis could potentially mask consumption. 11.8 mg/L CO_2-C would be produced if denitrification consumed all available nitrate, however CO_2-C concentrations remain fairly stable at 25 mg/L throughout the experiment (Table D1).

Pyrite oxidation is another typical electron donor that can accompany denitrification (Aravena and Robertson 1998; Bottcher et al. 1990; Postma et al. 1991), however as the expected SO_4^{2-} increase was not observed over the course of the experiment (29-31 mg/L), pyrite oxidation seems unlikely (Table D1). Other forms of denitrification with reduced metal pairing (Mn, etc) are possible, but were not assessed.

Nitrous oxide (N_2O), an intermediate product of denitrification and an alternate product of nitrification, appears to suggest denitrification occurred in the trial. Concentrations of N_2O-N ranged from 5 to 4200 nmol/L, which is substantial (atmospheric saturation of N_2O is approximately 9 nmol/L). Observed anaerobic conditions suggest that nitrification is an unlikely source of N_2O. As neither the nitric nor nitrous oxide reductase have been identified within the anammox bacterium, it has been suggested that N_2O does not play a role in the anammox process (Strous et al. 2006). Though concentrations of N_2O were high from the outset of the experiment, and sporadic throughout, N_2O production (via dentrification) during the experiment appears to be likely (Figure 13a). An undiscovered N_2O producing anammox enzyme is also a possibility.

Trial 2 (dual groundwater) differed from Trial 1 in groundwater sources (single vs. dual source), but also in limiting substrate conditions (NH_4^+ limited in Trial 1, NO_3^- limited in Trial 2, Table D1). Concurrent loss of NO_3^- and NH_4^+, combined with progressive enrichment of both $\delta^{15}N$-NO_3^- and $\delta^{15}N$-NH_4^+ were observed as the test proceeded. All 7.4 mg/L of available NO_3^--N was consumed over the 192 day experiment, while 13.7 mg/L of NH_4^+-N was consumed (Figure 11b). No significant concentrations of NO_2^--N or N_2O-N were produced suggesting TIN loss of 21.1 mg/L, or a consumption rate of 0.1 mg/L/day. Ammonium ($\delta^{15}N$-NH_4^+) increased over the course of the experiment by 11.5‰ (22.6 to 34.1‰), while $\delta^{15}N$-NO_3^- increased by an unexpectedly high amount between the two points analyzed (34‰: 20.0 to 54.0‰) (Figure 12b, Table D1). Though the results were reproducible, it is unlikely that the enrichment observed is related to anammox. Due to low NO_3^- concentrations, isotope values during the later stages of the experiment could not be analyzed.

Similar to Experiment 1, Trial 1 (also NO_3^- limiting), a slightly greater loss of NH_4^+ was observed than can be attributed to anammox alone (based on concomitant NO_3^- loss). 13.7 mg/L of NH_4^+-N was consumed during the experiment; however, DO was observed to be high during experiment setup (3.41 mg/L, Table D1). The NH_4^+-N concentration for the first bottle (30.17 mg/L) appears to be an outlier, as the following bottles have much higher concentrations (Table D1). To account for potential nitrification, a conservative approach of assuming all NH_4^+-N consumed between experiment setup and day 30 was consumed by nitrification was used. This suggests an NH_4^+-N loss of 4.8 mg/L via nitrification, suggesting a loss of 8.9 mg/L NH_4^+-N under anaerobic conditions. Based on the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995), a loss of 5.3 mg/L NO_3^--N was expected. This experiment consumed

7.4 mg/L of NO_3^--N, which was more than the expected amount (30% more), suggesting that an additional NO_3^--N pathway was likely active.

In further support of anammox activity, dissolved oxygen (DO) was also measured during the experiment (Figure 13b). Though DO was slightly increased during the experimental setup (3.41 mg/L, Figure 13b), concentrations remained <0.3 mg/L in each sacrificial bottle. This, combined with the DO control (Trial 3) indicating the bottles remained air-tight, suggests that loss of NH_4^+ was not by nitrification. The presence of CH_4 in samples (109-277 nmol/L, Table D1) also suggest reducing conditions throughout the experiment.

DOC, CO_2, and N_2O concentrations can be used to evaluate potential denitrification activity (Table D1). DOC concentrations decrease enough to consume all available NO_3^-, but the precision of DOC analysis, combined with highly diluted samples (10x), could be enough to produce the appearance of consumption. 7 mg/L of CO_2 is produced, which is within the range expected if denitrification was consuming the net NO_3^--N loss observed (Appendix D). N_2O is generally low, though an increase is observed towards the end of the experiment (26-73 nanomoles/L, Figure 13b).

Though there is some evidence that could support denitrification consumption of NO_3^-, it does not resolve the fate of NH_4^+. If all consumed NH_4^+ was being oxidized to NO_3^- (via nitrification or other currently unknown method) and subsequently denitrified, the resulting DOC consumption and CO_2 production would be about three times the amount observed.

Due to the unexpectedly high increase between the two δ^{15}N-NO_3^- isotopic values measured, and a lack of additional isotope date (a result of low NH_4^+ concentrations), the δ^{15}N-NO_3^- data was not sufficient to provide evidence for anammox activity.

Figure 15 compares the experimental data to the expected isotopic evolution for both $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ based on literature enrichment factors for both nitrification and anammox, using the Rayleigh model (Kendall and McDonnell 1998). The progressive increase of $\delta^{15}N\text{-}NH_4^+$ over the course of the experiment is generally consistent with both predicted anammox and nitrification curves. There is insufficient $\delta^{15}N\text{-}NO_3^-$ data to evaluate.

The increase of $\delta^{15}N\text{-}NH_4^+$ is substantially greater (11.5‰ @ $f_{NH4} = 0.68$) than that observed in Experiment 1, Trial 1 (7.4‰ @ $f_{NH4} = 0.37$). As such, fractionation factors used in Experiment 1 will underestimate observed increases in this trial. An ε of 20‰ is required to match the enrichment evolution observed, which is 14‰ higher than Experiment 1, and 16‰ higher than current literature values describing anammox enrichment (4‰, Clark et al. 2008; Robertson et al. 2010; Lazenby 2011).

One possible explanation for $\delta^{15}N\text{-}NH_4^+$ increases above what was expected could be the result of ammonification. Though ammonification does not have a large enrichment effect it could have a large impact on the presumed amount of NH_4^+ remaining in the system. For instance, if a measured NH_4^+-N concentration decreases from 10 to 5 mg/L, but ammonification produced 5 mg/L of new NH_4^+-N, only 50% of actual NH_4^+ consumption would be accounted for. This would result in a presumed fraction of remaining NH_4^+ (f_{NH4}) of 0.5, when the actual fraction should be 0. Attempts at quantifying TN (and subsequently organic nitrogen) were made, but organic nitrogen recovery was unreliable (75%-80% recovery of organic standards).

Trial 3 (He sparged DI with boiled silica sand medium) was designed to observe potential oxygen ingress into the sacrificial bottles over time. The silica sand was boiled to prevent microbial consumption of DO. DO concentrations remained stable (within error) throughout the 192 day experiment (Figure 13c), suggesting that oxygen ingress is likely not occurring. This

further indicates that nitrification should not be a major source of NH_4^+ consumption. Trial 3 also served as a sample blank during analysis (Table D1), as it was absent of all measured contaminants.

Trial 4 (dual groundwater) was quite similar to Trial 2, except the NH_4^+ source (PU125) also had its own NO_3^- pool, which could result in an already active anammox community. As both water sources for Trial 4 contained NO_3^-, NH_4^+ was the limiting substrate (13.2 mg/L NO_3^--N, 8.9 mg/L NH_4^+-N). 7.3 mg/L of NH_4^+-N was consumed, along with 5.5 mg/L of NO_3^--N (Figure 11d). Approximately 1 mg/L of NO_2^--N was produced, but apparently consumed before the end of the experiment, suggesting a TIN loss 12.8 mg/L, or 0.1 mg/L/day. Ammonium (δ^{15}N-NH_4^+) increased over the course of the experiment by 7‰ (37.8 to 44.9‰), while δ^{15}N-NO_3^- increased by 3.9‰ (21.4 to 25.3‰) (Figure 12d, Table D1).

Somewhat greater loss of NO_3^- was observed than can be attributed to anammox alone (based on concomitant NH_4^+ loss). 7.3 mg/L of NH_4^+-N was consumed during the experiment, and based on the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995), 4.4 mg/L of NO_3^--N loss was expected. This experiment consumed 5.5 mg/L of NO_3^--N, which was somewhat more than the expected amount (25% more), but generally was within the range of consumption expected for anammox activity.

To determine if loss of NH_4^+ might be the result of nitrification, DO was measured during the experiment. Though DO was present during experimental setup (4.36 mg/L, Table D1), it remained below detection (<0.3 mg/L) for the remainder of the experiment. Ammonium concentrations increased between the first two sampling events (potentially the result of NH_4^+-N in sediment), which does not allow for quantification of NH_4^+-N loss via nitrification during the initial stage of the experiment. This suggests that the net loss of NH_4^+ was not by nitrification.

The presence of CH_4 in samples (52-146 nmol/L, Table D1) also suggest reducing conditions throughout the experiment.

DOC, CO_2, and N_2O concentrations can be used to evaluate potential denitrification activity. DOC concentrations generally increase over the course of the experiment (29 to 44 mg/L, Table D1) which is likely an artefact of analysis accuracy combined with highly diluted samples (10x). Similarly CO_2 (26 to 31 mg/L) generally remains at the same or lower concentrations to the first sampling event (Table D1). N_2O decreases over the course of the experiment from 2175 nmol/L (prior to incubation) to 11 nmol/L at the conclusion of the experiment (Table D1). Only the consumption of N_2O seems to indicate that denitrification is a contributor to nitrogen loss, but due to the range in values, denitrification can not be ruled out as playing a larger role.

Due to the initial isotope value for $\delta^{15}N\text{-}NO_3^-$ being lower than the initial $\delta^{15}N\text{-}NH_4^+$ value, both nitrification and anammox could exhibit progressive enrichment of $^{15}N\text{-}NO_3^-$ (Figure 16). Though increases are observed in both $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$, isotope data alone does not support anammox over nitrification. However, the DO control (Trial 3) demonstrated an air-tight seal over the course of the experiment (and Trial 4 consistently measured DO <0.3 mg/L), which suggests that nitrification is unlikely.

3.3.4 Experiment 3 – Conclusions

Experiment 3 was a success in that it provided the same evidence of potential anammox activity as Experiment 1, but due to unexpectedly low $\delta^{15}N\text{-}NO_3^-$ values, Rayleigh modeling was not as effective as Experiment 1 in discounting nitrification activity. Consumption of both

NH_4^+-N and NO_3^--N was observed in all trials, and both substrates showed isotopic enrichment that could be suggestive of anammox. The major conclusions include:

- Concomitant NH_4^+ and NO_3^- consumption, along with $\delta^{15}N$-NH_4^+ and $\delta^{15}N$-NO_3^- enrichment was observed in multiple mixes of single and dual N-rich groundwater sources, suggesting that the anammox reaction might be active.

- When initial $\delta^{15}N$-NO_3^- isotope values are greater than initial $\delta^{15}N$-NH_4^+ values (Experiment 1, Trial 1), Rayleigh models can predict enrichment of $\delta^{15}N$-NH_4^+ and depletion of $\delta^{15}N$-NO_3^- for a nitrification effect. However, $\delta^{15}N$-NO_3^- enrichment is expected for both anammox and nitrification when initial $\delta^{15}N$-NH_4^+ values are greater than $\delta^{15}N$-NO_3^-. This case makes it very important to confirm anaerobic conditions for differentiation between anammox and nitrification.

- Oxygen contamination was not observed in any of the trials (after initial setup). However, further steps can be taken to ensure nitrification is not a major source of NH_4^+ consumption. These steps could include the use of a gas tight sacrificial bottle and an anaerobic chamber to store them.

- Denitrification is likely a source of NO_3^- consumption in the experiments. It is possible that some NH_4^+ loss could be accounted for via coupled nitrification-denitrification at the start of the experiment (when O_2 was likely introduced during experiment setup), as well as NH_4^+ assimilation into biomass. Going forward, quantifying the relative magnitudes of denitrification and anammox, as well as potential microbial NH_4^+ assimilation need to be addressed.

- Experiments 1-3 have exhibited a higher presumed anammox rate of 0.1 mg N/L/day when NH_4^+ is abundant (Experiment 1, Trial 1; Experiment 4, Trial 2), and a lower rate of 0.06 to 0.07 mg N/L/day when NH_4^+ is limiting (Experiment 4, Trials 1 and 4).

- Given the current analytical uncertainty, it is highly unlikely to completely rule out denitrification as a source of nitrogen removal. Further analyses (such as enriched isotope experiments and DNA testing) would help to further differentiate between denitrification and anammox.

- Substrate concentrations should be increased in order to allow easier late-time isotopic analyses.

3.4 Experiment 4 – Sacrificial Serum Bottles

3.4.1 Introduction

Although Experiment 3 was successful in providing evidence supporting anammox activity, further refinements were made in a second sacrificial bottle experiment. This sacrificial bottle experiment used ten 160 mL Wheaton glass serum bottles containing identical water and sediment mixtures per trial. One bottle per trial was sampled for each sampling event, leaving the remaining bottles undisturbed. As with Experiment 3, freshly drilled core obtained from the suspected anammox zone at the Zorra site (Moore et al. 2011) was used. To increase substrate concentrations, a concentrated NH_4NO_3 salt solution was injected into each bottle. Quantitative real-time PCR (qPCR) analyses were also completed to obtain a temporal evolution of anammox community growth. Trials 1 through 5 were set up and stored in an anaerobic chamber, further reducing the possibility of oxygen contamination. This experiment consisted of five trials as follows:

- Trial 1, Single Source Groundwater: PU121-3.0m (80 mg/L NO_3^--N and 75 mg/L NH_4^+-N with injected NH_4NO_3, mixed with 100 g of fresh core collected at 3-6m depth beside PU103).
- Trial 2, a Sediment Biomass Control: PU121-3.0m (85 mg/L NO_3^--N and 84 mg/L NH_4^+-N with injected NH_4NO_3, mixed with 100 g of boiled silica sand).
- Trial 3, Dual Source Groundwater: PU115-3.0m (80 mg/L NO_3^--N with injected NH_4NO_3) and PU84-3.1m (70 mg/L NO_3^--N with injected NH_4NO_3), mixed with 100 g of fresh core collected at 3-6m depth beside PU103).

- Trial 4, Single Source Groundwater: PU86-3.1m (background well, 75 mg/L NO_3^--N and 96 mg/L NH_4^+-N with injected NH_4NO_3, mixed with 100 g of fresh core collected at 3-6m depth beside PU103).

- Trial 5, Single Source Groundwater: PU115-2.2m (centreline well, 75 mg/L NO_3^--N and 100 mg/L NH_4^+-N with injected NH_4NO_3, mixed with 100 g of fresh core collected at 3-6m depth beside PU103).

3.4.2 Methodology

In Experiment 3 it was found that in many of the microcosms, the substrate (either NO_3^- or NH_4^+) was entirely consumed after a short period of time, due to unexpectedly low initial concentrations. To rectify this, Experiment 4 was initiated in which the same sediment groundwater mixtures were spiked with an additional NH_4NO_3 salt solution. After the mixes had been added to the bottles, and the bottles were sealed, a concentrated NH_4NO_3 solution (57 mg in 10 mL helium sparged DI water) was injected into each bottle. To further ensure that oxygen ingress (and therefore nitrification) was not significant, this experiment took place in an anaerobic chamber.

For this experiment, groundwater samples from the Zorra site were collected in 1L Bernardin glass mason jars with snap lids, filled with zero headspace and immediately placed in the anaerobic chamber upon return to the lab. Mixing of the well water was done in the anaerobic chamber. Five trial mixtures, each with 10 160 mL Wheaton glass serum bottles were undertaken. Four of the 5 trial mixtures consisted of 100 g of fresh sediment core from the field site, mixed with groundwater from various plume zones. The core samples were collected from the probable anammox zone (borehole PU103, Moore et al. 2011) using the University of

Waterloo Geoprobe drill rig. Both the core samples and the groundwater samples were collected on October 12, 2010 and all 80 bottles were loaded by October 14, 2010. Both core and groundwater samples were transported directly to the University of Waterloo where they were placed in an anaerobic chamber prior to loading. The fifth trial mixture was a sediment biomass control containing boiled silica sand mixed with the same groundwater as one of the other trials. The bottles were sealed with a 20mm Bellco butyl rubber stopper and crimped with an aluminum crimp cap. The bottles were stored in a anaerobic chamber (covered to prevent light exposure), and were shaken by hand multiple times per week.

3.4.3 Results and Discussion

Trial 1 (single groundwater) consumed 25.4 mg/L of available NH_4^+-N, and 27.2 mg/L of NO_3^--N (Figure 17a). 0.8 mg/L of NO_2^--N was produced but consumed during the experiment, suggesting an overall TIN loss of 52.6 mg/L, over the 247 day experiment period. This implied a TIN consumption at a rate of 0.2 mg/L/day. Ammonium ($\delta^{15}N$-NH_4^+) increased over the course of the experiment by 5.0‰ (-3.4 to 1.9‰), while $\delta^{15}N$-NO_3^- increased by 8.4‰ (-1.3 to 7.1‰) (Figure 18a, Table E1).

Somewhat greater loss of NO_3^- was observed than can be attributed to anammox alone (based on concomitant NH_4^+ loss). 25.4 mg/L of NH_4^+-N was consumed during the experiment, and based on the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995), 15.2 mg/L of NO_3^--N loss was expected. This experiment consumed 27.2 mg/L of NO_3^--N, which was more than the expected amount (75% more), suggesting that another mechanism of nitrogen removal was also possibly occurring.

To determine if loss of NH_4^+ was the result of nitrification, DO was measured during the experiment. DO remained <0.3 mg/L in each sacrificial bottle, suggesting that loss of NH_4^+ was not by nitrification. The presence of CH_4 in samples (48-210 nmol/L, Table E1) also suggests reducing conditions throughout the experiment.

DOC, CO_2, and N_2O concentrations can be used to evaluate potential denitrification activity. Based on the additional 12 mg/L NO_3^--N consumed (not accounted for by anammox), DOC and concentrations would be expected to decrease by approximately 13 mg/L (Appendix E), however DOC data generally increases over the course of the experiment, from 53-66 mg/L (Table E1) which is likely an artefact of analysis uncertainty combined with highly diluted samples (10x). It is also possible that high rates of ammonification are resulting in the observed increase in DOC. Similarly CO_2 would be expected to increase by 13 mg/L, which is not observed in the data (22-71 mg/L, after an time zero value of 91 mg/L, Table E1). N_2O decreases over the course of the experiment. None of these parameters provide supporting evidence that denitrification is a major contributor to nitrogen loss.

Results from qPCR analysis show an increase in anammox population over time (Figure 20). The population only grows from 0 (day 0) to 0.12% of bacteria population, but growth increases by an order of magnitude with each sample.

Figure 21 compares the predicted curves for both anammox and nitrification reactions to the experimental data. The progressive increase of both $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ over the course of the experiment matches reasonably well with the predicted anammox curves, but is inconsistent with a nitrification reaction because more depleted $\delta^{15}N\text{-}NO_3^-$ would be expected (Figure 21a). Though $\delta^{15}N\text{-}NH_4^+$ enriched only 5‰ over the course of the experiment, an ε of 11‰ was required to match the enrichment evolution observed. This could be the result

of mineralization skewing the f_{NH4} (see Section 3.3.2, Trial 2). However, it should be noted that denitrification would also result in an observed increase of $\delta^{15}N\text{-}NO_3^-$.

Trial 2 (sediment biomass control) used the same groundwater as Trial 1, but replaced the field site sediment of Trial 1 with boiled silica sand. Trial 2 consumed 12.7 mg/L of available NH_4^+-N, and 16.9 mg/L of NO_3^--N (Figure 17b). NO_2^--N was just above detection, suggesting an overall TIN loss of 29.6 mg/L, over the 247 day experiment period. This implied a TIN consumption at a rate of 0.1 mg/L/day, or ½ of observed loss in Trial 1. Ammonium ($\delta^{15}N\text{-}NH_4^+$) did not increase over the course of the experiment, while $\delta^{15}N\text{-}NO_3^-$ increased by 4.1‰ (-1.4 to 2.7‰) (Figure 18b, Table E1).

As 12.7 mg/L of NH_4^+-N was consumed, the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995) would suggest that 7.6 mg/L of NO_3^--N would be required, which is only 45% of the actual NO_3^--N loss observed. As NH_4^+ was still available, it is unlikely that the extra NO_3^- was reduced by anammox, which suggest that another nitrogen consuming mechanism also occurred.

Nitrification does not appear to be responsible for the removal of NH_4^+, as DO concentrations remained below 0.3 mg/L throughout the experiment (Table E1). Also, the presence of CH_4 in samples (33-136 nmol/L, Table E1) suggests reducing conditions throughout the experiment.

DOC and CO_2 values were inconsistent during Trial 2, and do not show a trend of consumption or production. However, N_2O was found to be very high throughout the experiment (22641 to 48452 nmol/L, 250x higher than all other trials in Experiment 4, Figure

19b). This suggests that denitrification may have been a significant component of nitrogen loss for this Trial.

The results of the qPCR analysis do not suggest anammox growth over time (Figure 22). Though the populations are significantly higher than in Trial 1 (Figure 19), they appear to be decreasing (6.6% on day 123, 2.8% on day 247).

Figure 23 compares the predicted curves for both anammox and nitrification reactions to the experimental data. The progressive increase of $\delta^{15}N\text{-}NO_3^-$ over the course of the experiment matches reasonably well with the predicted anammox curves, but is inconsistent with a nitrification reaction because more depleted $\delta^{15}N\text{-}NO_3^-$ would be expected (Figure 23a). This model suggests that a significant increase was not expected for $\delta^{15}N\text{-}NH_4^+$ with the remaining fraction of NH_4^+ observed. If mineralization is not occurring (mineralization would skew f_{NH4}, see Section 3.3.2), the $\delta^{15}N\text{-}NH_4^+$ evolution could be indicative of anammox. It should also be noted that denitrification would also result in an observed increase of $^{15}N\text{-}NO_3^-$.

Trial 3 (dual groundwater) was the only trial in Experiment 4 to use two sources of groundwater. Trial 3 consumed 17.3 mg/L of available NH_4^+-N, and 25.7 mg/L of NO_3^--N (Figure 17c). NO_2^--N was just above detection, suggesting an overall TIN loss of 43.0 mg/L, over the 247 day experiment period. This implied a TIN consumption at a rate of 0.2 mg/L/day. Ammonium ($\delta^{15}N\text{-}NH_4^+$) increased by 4.1‰ (-2.1 to 2.0‰) over the course of the experiment, while $\delta^{15}N\text{-}NO_3^-$ increased by 7.0‰ (-1.4 to 5.6‰) (Figure 18c, Table E1).

Based on the the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995), 10.4 mg/L of NO_3^--N would be required to consume the observed NH_4^+ loss. Almost 2.5

times the expected amount of NO_3^- was consumed, suggesting another nitrogen removal process was also occurring.

As with all Trials in Experiment 4, DO concentrations remained below 0.3 mg/L throughout the experiment (Table E1), suggesting nitrification was likely not a major contributor to NH_4^+ removal. The presence of CH_4 in samples (370-1935 nmol/L, Table E1) also suggests reducing conditions throughout the experiment.

Neither DOC-C (21 to 94 mg/L) nor CO_2-C (14 to 132 mg/L) are indicative of denitrification, as data for each parameter lacks a consistent accumulation or loss trend (Table E1). N_2O-N accumulation was observed at days 88 (306 nmoles/L) and 123 (189 nmoles/L), but was otherwise low (Table E1).

Results from qPCR analysis show an increase in anammox population over time (Figure 24). The population grows from 10.2 (day 123) to 16.3% (day 247) of bacteria population, indicating a substantial growth in anammox bacteria. This is considered strong evidence for nitrogen consumption by anammox.

Figure 25 compares the predicted curves for both anammox and nitrification reactions to the experimental data. The progressive increase in $\delta^{15}N\text{-}NO_3^-$ over the course of the experiment matches reasonably well with the predicted anammox curve, but is inconsistent with a nitrification reaction because more depleted $\delta^{15}N\text{-}NO_3^-$ would be expected (Figure 25a). The $\delta^{15}N\text{-}NH_4^+$ data did not match the predicted anammox curve entirely, due to the light isotope value determined at the beginning of the experiment (-2.1‰), otherwise the evolution fits the anammox curve well (Figure 25b). However, it should be noted that denitrification would also result in an observed increase of $\delta^{15}N\text{-}NO_3^-$.

Trial 4 (single groundwater) consumed 30.3 mg/L of NH_4^+-N, however no significant loss of NO_3^--N was observed (Figure 17d). NO_2^--N was just above detection, suggesting an overall TIN loss of 30.3 mg/L, over the 247 day experiment period. This implied a TIN consumption at a rate of 0.1 mg/L/day. Ammonium ($\delta^{15}N$-NH_4^+) increased by 4.3‰ (-4.8 to -0.5‰) over the course of the experiment, while $\delta^{15}N$-NO_3^- increased by 8.7‰ (-7.0. to 1.7‰) (Figure 18d, Table E1).

Based on the the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995), 18.2 mg/L of NO_3^--N would be required to consume the observed NH_4^+ loss. This is the only experiment in this study where observed NO_3^- loss was not sufficient to account for observed NH_4^+ consumption by anammox.

Aside from the first sampling event (DO = 0.9 mg/L), DO concentrations remained below 0.3 mg/L throughout the experiment (Table E1), suggesting nitrification was likely not a major contributor to NH_4^+ removal. The presence of CH_4 in samples (65-546 nmol/L, Table E1) also suggests reducing conditions throughout the experiment.

Neither DOC nor CO_2 were strongly indicative of denitrification. The DOC range could potentially suggest denitrification, but CO_2 concentrations were low and steadily declining. N_2O concentrations remained relatively stable for the first 123 days of the experiment, after which a decline of approximately 86% was observed (Table E1).

Results from qPCR analysis show an increase in anammox population over time (Figure 26). The population grows steadily from 0.1.(day 32) to 4.2% (day 247) of bacteria population, indicating a reasonable growth in anammox bacteria. This is considered strong evidence for nitrogen consumption by anammox.

Figure 27 compares the predicted curves for both anammox and nitrification reactions to the experimental data. The progressive increase of $\delta^{15}N\text{-}NO_3^-$ over the course of the experiment matches reasonably well with the predicted anammox curve, but is inconsistent with a nitrification reaction because more depleted $\delta^{15}N\text{-}NO_3^-$ would be expected (Figure 27a). The $\delta^{15}N\text{-}NH_4^+$ data did not match the predicted curve very well, as most of the data points plot above the predicted curve. This could be accounted for by mineralization. A change in ε would not provide a significantly better match of the data.

Trial 5 (single groundwater) consumed 36.0 mg/L of NH_4^+-N and between 7.5 and 20.5 mg/L of NO_3^--N (large concentration difference between first two samples, Figure 17e). NO_2^--N was just above detection, suggesting an overall TIN loss of between 43.5 and 56.5 mg/L, over the 247 day experiment period. This implied a maximum TIN consumption at a rate of 0.2 mg/L/day. Ammonium ($\delta^{15}N\text{-}NH_4^+$) increased by 6.0‰ (-3.0 to 3.0‰) over the course of the experiment, while $\delta^{15}N\text{-}NO_3^-$ increased by 6.2‰ (0.4 to 6.6‰) (Figure 18e, Table E1).

Based on the the anammox NO_3^-:NH_4^+ consumption ratio of 3:5 (Eq. 1.4, Mulder et al. 1995), 21.6 mg/L of NO_3^--N would be required to consume the observed NH_4^+ loss, which was slightly more than the observed amount (5% more), but was still generally within the range of consumption expected for anammox activity.

DO concentrations remained below 0.3 mg/L throughout the experiment (Table E1), and were on average the lowest of all measured microcosm experiments. This suggests nitrification was likely not a major contributor to NH_4^+ removal. The presence of CH_4 in samples (315-1341 nmol/L, Table E1) also suggests reducing conditions throughout the experiment.

Neither of DOC or CO_2 was strongly indicative of denitrification. The DOC range was relatively stable around 45 mg/L (Table E1), although the range in values could potentially suggest denitrification. CO_2 and N_2O concentrations generally decreased over the 247 day experiment (Table E1).

Results from qPCR analysis were inconclusive (Figure 28), as two of the three filters were destroyed during analysis.

Figure 29 compares the predicted curves for both anammox and nitrification reactions to the experimental data. The progressive increase of $\delta^{15}N\text{-}NO_3^-$ over the course of the experiment matches reasonably well with the predicted anammox curve, but is inconsistent with a nitrification reaction because more depleted $\delta^{15}N\text{-}NO_3^-$ would be expected (Figure 29a). Though $\delta^{15}N\text{-}NH_4^+$ enriched only 6‰ over the course of the experiment, an ε of 20‰ was required to match the enrichment evolution observed. This could be the result of mineralization skewing the f_{NH4} (see Section 3.3.2, Trial 2). It should also be noted that denitrification would also result in an observed increase of $\delta^{15}N\text{-}NO_3^-$.

3.4.4 Experiment 4 – Conclusions

Though Experiment 4 exhibited some of the strongest evidence in support of nitrogen consumption by anammox bacteria, it also produced a series of unexpected results. The major conclusions include:

- Each trial (with the exception of Trial 4) showed concomitant loss of NH_4^+ and NO_3^-. Higher nitrogen removal rates were generally observed (none lower than 0.1 mg/L/day), which could be a result of higher substrate concentrations or the use of additional amounts of fresh sediment (50g in Experiment 3 vs 100g in Experiment 4).

- The sediment biomass control experiment (Trial 2) showed less increase in $\delta^{15}N\text{-}NH_4^+$ (~0.6‰ vs 5.0‰ in Trial 1), half the increase of $\delta^{15}N\text{-}NO_3^-$ (4.1‰ vs 8.2‰ in Trial 1), produced 250 times more N_2O than Trial 1, yet consumed nitrogen at half the rate of Trial 1 (0.2 mg/L/day). However, the control contained a much higher percentage of anammox bacteria (2.8-6.6% compared to 0.01-0.1% in Trial 1).

- qPCR analysis confirmed the presence of anammox in all trials. Trials 1, 3, and 4 all observed increasing anammox populations over time, suggesting nitrogen removal by anammox is likely occurring. Trial 2 (sediment biomass control) observed a large initial anammox population, but one that declined over time. Trial 5 was inconclusive due to filter destruction during analysis.

- Dissolved Oxygen (DO) showed that each trial remained anaerobic throughout the experiment. This suggests that nitrification is likely not a main contributor to NH_4^+ loss. Further, DO concentrations measured in the trials of Experiment 4 (in anaerobic chamber) were similar to those observed in Experiment 3, suggesting that Experiment 3 microcosms likely remained anaerobic over the course of the experiment. Also, the presence of CH_4 in all trials suggests highly reducing conditions, further indicating that O_2 is likely not abundant.

3.5 Experiment 5 – Field Reactors

3.5.1 Introduction

With the ultimate goal of this research being to provide an anammox-based solution for passive nitrogen remediation in farm-field environments, a larger scale mesocosm experiment was undertaken to observe potential anammox activity under field conditions. Two field reactors were installed at the Zorra Site in October 2009 (Figure 1). Each reactor consisted of a 170 litre barrel with a 2.54 cm diameter PVC multidepth piezometer installed, with screens at depths 8, 25, 43, 61 and 79 cm (Appendix F). The first reactor, referred to as the "Inoculated Reactor", contained a 10% mixture of core from the plume zone that was potentially anammox active. This core was mixed with dry sand from an on-site pit. The reactor was installed adjacent to PU125 (Figure 2). The control (control for initial sediment biomass) reactor contained only dry sand from the sand pit, and was installed beside PU103 (Figure 2). The reactors underwent two separate experiment phases between October 2009 and August 2011. During Phase I, the reactors consumed all available NO_3^- after 300 days, and then for Phase II they were replenished with substrate (NH_4NO_3 salt solution) in September, 2010. The results of each phase of the experiment are described below.

3.5.2 Methodology

The over-arching goal of this research is to provide an anammox-based solution for *in-situ* nitrogen remediation in farm-field environments. With this in mind, two large scale field reactors were installed at the Zorra Site in October 2009. Each reactor consisted of a 170 litre barrel with a 2.54 cm diameter PVC pipe, multidepth piezometer installed through the barrel lid. The piezometer was wrapped with five mini piezometers, with screens at depths 8, 25, 43, 61 and

79 cm below the barrel top (Figure F1). The first reactor, referred to as the "Inoculated Reactor", contained a 10% mixture of core from the plume zone that was potentially anammox active. This core was mixed with dry sand from an on-site pit. The sediment biomass control reactor contained only dry sand from the sand pit.

Fresh core for the inoculated reactor was taken directly beside well PU125, a 3-6 m depth, a zone which was previously determined to have a higher than average population of anammox bacteria (Moore et al. 2011). Drilling was completed using the Geoprobe drill rig with Macro-Core MC5 Soil Sampling System on October 19, 2009. The bottom and top 10 cm of barrel space were filled with pea gravel. The barrels were installed with tops approximately 30 cm bgs; the inoculated barrel was installed adjacent to well PU125, and the control beside well PU103 (Figure 3).

The reactors underwent two separate experimental phases between October 2009 and August 2011. Initially, both reactors were filled with groundwater from well PU115-2.2m using a peristaltic pump over a 4 hour period. The reactors then remained static, with no new groundwater introduced over the next 11 months. Once per month, each reactor port (8, 25, 43, 61 and 79 cm depth) was sampled for DO, pH, and temperature in the field, and samples were retained for NO_2^-, NO_3^-, NH_4^+, TN, and isotopic analyses. Chapter 2 describes the laboratory analytical procedures. To reduce exposure to oxygen, helium sparged DI water was used to replace the sample water extracted from the reactors. DI water was added through a vent at the top of the reactor, so that dilution at the bottom of the barrel was minimized. To minimize dilution, only 30-60mL of water (in two bottles) was taken from each port during each sampling event, and was replaced with an equivalent volume of He sparged DI water. Nitrate and NH_4^+ samples were filtered using 0.45µm syringe filters (PALL acrodisk PSF or Whatman) and stored

in Nalgene bottles. Samples analyzed for NH_4^+ were preserved in the field to pH 2-4 using sulphuric acid (H_2SO_4). Samples were stored in an ice filled cooler during transport to the University of Waterloo, where they were either immediately analyzed or frozen.

The second experimental phase involved recharging the reactors with additional NH_4^+ and NO_3^- substrate after the NO_3^- in the initial experiment was found to be fully consumed. On August 19, 2010, 4 Litres of 3500 mg N/L ammonium nitrate was added to the sediment biomass control reactor, and 7 Litres was added to the inoculated reactor (the extra 3 L was an attempt to fill the reactor to the top after a leak was detected). Sodium bromide was also added to each reactor, to act as a conservative tracer. To ensure uniform mixing of the added substrate, the water in each reactor was then circulated from top to bottom for 7 days using a peristaltic pump pumping at a rate of ~ 1L/min. Conductivity was measured during the first sampling event (September 30, 2010) which indicated that the injected solutions had mixed well through both barrels.

3.5.3 Results and Discussion

The top three reactor ports were not included in the analysis due to high DO concentrations (>1 mg/L in control ports 8cm and 25cm), and the generally observed increasing NH_4^+-N trend in the upper three ports (Figures G2 and G3). This trend could potentially be explained by ammonification, but is not consistent with nitrogen removal trends for this study. The bottom two ports in each reactor are of the most interest, as they are the most likely to remain anoxic, reducing the likelihood of nitrification. Inoculated (Figure 30) and control (Figure 31) both exhibit concurrent loss of NH_4^+ and NO_3^- bottom two ports (61 and 79 cm depth).

The inoculated reactor consumed TIN at a rate of 0.2 mg/L/day (76.7 to 23.1 mg/L) at 61 cm, and 0.1 mg/L/day (68.7 to 46.1 mg/L) at 79 cm depth. The sediment biomass control reactor consumed TIN at a rate of 0.1 mg/L/day at both 61cm (93.7 to 39.3 mg/L) and 79 cm (80.5 to 39.7 mg/L) depths (Table F1). Though the total nitrogen loss was similar, consumption of NH_4^+ was not. The inoculated barrel consumed NH_4^+ at a rate of 0.06 mg/L/day (42.9 to 22.6 mg/L, Figure 30a) at 61 cm depth, and 0.02 mg/L/day (53.5 to 46.1 mg/L, Figure 30b) at 79 cm depth. The control consumed NH_4^+ at a rate of 0.03 mg/L/day at both 61 cm (53.2 to 39.3 mg/L, Figure 31a) and 79 cm (54.5 to 39.6 mg/L, Figure 31b) depths. That the reaction rate of the inoculated reactor (61 cm) is two times that of the control suggests that the inoculated sand may have played a role in the removal of NH_4^+. Note that the samples taken on November 13, 2009 were taken with sample vials that tended to rupture when frozen, and as such, the data on that date was not reliable.

The isotopic data show minor increases of $\delta^{15}N\text{-}NH_4^+$ in the lower ports of the inoculated barrel (Figure 32). Though the endpoints are the same, the 61cm port was enriched by 2.3‰ (24.0 to 26.6‰) between day 163 and day 299. Depth 79cm was enriched by 2.2‰ (23.9 to 26.1‰) between days 0 and 248, but had a final increase of only 0.8‰ (24.7‰). The control barrel did not show enrichment at the 61cm port over the experimental period (27.6‰), while the 79cm port became depleted by 2‰ (28.7 to 25.5‰) over the course of the trial. Due to the low increase observed for $\delta^{15}N\text{-}NH_4^+$, $\delta^{15}N\text{-}NO_3^-$ samples were not analyzed.

Dissolved Oxygen (DO) was found to be generally higher in the sediment biomass control reactor. In ports 61cm and 79cm, DO averaged 1.5 and 0.9 mg/L between April and August 2009, respectively. In comparison, the same depths for the inoculated reactor had average DO concentrations of 0.8 and 0.7 mg/L over the same time period (Table F1).

Concentrations below 1 mg/L are generally considered anaerobic conditions, and as such, nitrification may not have played a major role in NH_4^+ consumption observed in the inoculated reactor.

Results of qPCR analysis show inoculated anammox groundwater communities at 0.13% at depth 61 cm, and 0.22% at depth 79 cm (Figure 33). In the control reactor, anammox communities were 3-13 times less prevalent at 0.037% at depth 61 cm, and 0.016% at 79 cm depth. Though a higher percentage of anammox bacteria were observed in the inoculated barrel, the values suggest only a minor amount of anammox activity.

On August 19, 2010, the reactors were recharged with a concentrated NH_4NO_3 salt solution, after which they were pumped for 7 days in order to effectively circulate the substrate through the reactors. In the inoculated barrel, NH_4^+ was consumed at a rate of 0.1 mg/L/day (135.0 to 94.9 mg/L) at the 61 cm port, and 0.05 mg/L/day (129.1 to 118.9 mg/L) at the 79 cm port (Table F1). Both of these rates underestimate the reaction rate, as NH_4^+ increased substantially during the last sampling event (Figure 34). NO_3^- increased at a rate of 0.2 mg/L/day at both the 61 cm (211.5 to 273.2 mg/L, Figure 34a) and 79 cm (175.3 to 231.5 mg/L, Figure 34b) ports.

The control reactor produced different results (Figure 35). Ammonium was consumed at a rate of 0.2 mg/L/day (102.2 to 67.24 mg/L, Figure 35a) at the 61 cm port and 0.04 mg/L/day (85.0 to 77.7 mg/L, Figure 35b). Nitrate was produced at a rate of 0.01 mg/L/day (82.3 to 81.8 mg/L, Figure 35a) at the 61 cm port, and consumed at a rate of 0.01 mg/L/day (104.7 to 77.3 mg/L, Figure 35b) at the 79 cm port.

A combination of NO_3^- production, NH_4^+ consumption, and higher DO concentrations (Table F1) suggests that both inoculated and control reactors underwent nitrification, possibly due to oxygen contamination while circulating the salt solution over seven days. Though the bottom port of the control reactor appears to have consumed both NO_3^- and NH_4^+, the overall apparent oxygen contamination makes the ability to observe anammox activity difficult.

3.5.4 Experiment 5 – Conclusions

The field reactors did not give as strong of evidence for anammox activity as the previous experiments; however, evidence that could be indicative of anammox was observed. The major conclusions made were:

- The inoculated reactor consumed NH_4^+ at a rate (0.15 mg/L/day) 3 times greater than that of the sediment biomass control (0.051 mg/L/day). As DO was relatively low, or absent, and NO_3^- was consumed simultaneously, it would appear that anammox may have consumed the NH_4^+, and inoculated sediment increased the anammox reaction rate.

- Observed isotope enrichment was significantly less in the reactors than in previous laboratory experiments. One possible explanation for this could be mineralization of urea. If substantial mineralization was occurring, it would buffer any enrichment expected from NH_4^+ consumption. Also, the relatively small percentage consumption of NH_4^+ in these trials (26 to 27% in control, 14 to 47% in inoculated) made observation of isotopic trends more difficult.

- Anammox communities were shown to be present with qPCR, but those communities were much smaller than those observed in the laboratory microcosms.

- Future field mesocosms should focus on temperature and DO control to maximize anammox growth. As the reactors were installed close to the ground surface, reactor temperature appeared to be controlled by atmospheric temperature (Table F1), which is less reflective of groundwater conditions. Controlling DO concentrations will reduce the chances of nitrification and inactivation of anammox bacteria.

3.6 Ongoing Site Characterization

3.6.1 Introduction

In a previous study, Lazenby (2011) detailed the characterization of the groundwater plume impacted by the Zorra manure lagoon during 2007-2010. Because of the observed seasonal transience of both NH_4^+ and NO_3^- concentrations within the plume, ongoing sampling of the plume was continued as part of this study. Local groundwater sampling occurred prior to the commencement of each laboratory experiment and also two major sampling events were also completed along the centreline of the plume on September 23, 2010 and October 26, 2010. The results from these sampling events are discussed below.

3.6.2 Methodology

Lazenby (2011) completed a detailed characterization of the groundwater plume impacted by the Zorra manure lagoon was undertaken during the period 2007-2010. Because of the considerable seasonal transience of NH_4^+ and NO_3^- concentrations observed within the plume, ongoing sampling of the plume was continued as part of this study.

Local groundwater sampling occurred prior to the commencement of each laboratory experiment and also two major sampling events were completed along the centreline of the plume in September and October 2010. Field samples were collected from multi-level

piezometers using peristaltic pumps. Before sampling, each well was purged of at least three well volumes (pumped for 3-5 minutes).

The first detailed sampling episode (September 23, 2010) targeted high NO_3^- and NH_4^+ areas for potential use in the Experiment 4 trials. Large volumes of groundwater (500-1000 mL) were collected in Nalgene bottles for NO_3^- and NH_4^+ concentrations. Samples for NH_4^+ analysis were preserved in field with H_2SO_4 to pH 2-4, whereas samples for NO_3^- analysis were filtered (0.45 μm) but were retained unpreserved. All samples were stored on ice during transport to the University of Waterloo, where they were either immediately analyzed or frozen.

The second detailed sampling event (October 26, 2010) was completed when it was discovered that a large portion of the plume contained substantially lower NH_4^+ and NO_3^- concentrations than was expected, suggesting possible anammox activity. Sampling included field measurement of pH, reduction potential (Eh,) DO, electrical conductivity (EC), temperature, water levels and sampling for multiple parameters as follows: three 30 mL Nalgene bottles were field filtered (0.45 μm) and used for 1) NO_3^- (untreated) 2) cations (preserved to pH 2 using HNO_3) and 3) NH_4^+ (preserved to pH 2-4 using H_2SO_4). Samples were also collected for NO_3^- and NH_4^+ isotope analyses at this time, in 125-1000 mL Nalgene bottles, using the same filtering and preservation methods as concentration bottles. Samples for dissolved N_2O and N_2 analyses were also collected using 160 mL Wheaton serum bottles. These bottles were completely filled, with no headspace and were sealed with a serum stopper (Vaccutainer brand) containing an inserted hypodermic needle to allow overflow to escape. These bottles were then sealed with electrical tape and preserved with 0.4 mL of mercuric chloride ($HgCl_2$) injected using the hypodermic needle. A total of 60 monitoring points were sampled during this event.

3.6.3 Results and Discussion

On September 23, 2010, 16 discrete points were sampled along the centre line plume (Lazenby 2011, Figure 36) to determine areas of interest (high NO_3^-, NH_4^+) for use in Experiment 4. A large section of the plume core was observed to have a lower concentration of NH_4^+-N than expected (<4 mg/L, Figure 36a). Nitrate (as N) was also low in these wells (~ 0.1 mg/L, Figure 37a), but this was consistent with previous sampling.

As a result of this finding, a more detailed sampling (60 points) was undertaken on October 26, 2010. The area of low NH_4^+ in September appeared to have expanded by the October sampling event, as concentrations at PU124 also decreased sharply (Figure 36b). Also, the region of low NH_4^+-N was observed to have decreased concentrations in October (most <1 mg/L, Figure 36b). In the October event NO_3^--N concentrations in the core zone increased slightly (Figure 37b). DO concentrations remained <0.3 mg/L within this area (Table G1), suggesting that nitrification was not the dominant NH_4^+ removal mechanism.

To determine if the observed nitrogen loss along the flowpath was the result of transient source conditions, or a possible change in plume location, NH_4^+ concentrations were compared to the conservative element Na^+. Sodium was used previously as the preferred "conservative" tracer at the Zorra site (Lazenby 2011). Figure 38 illustrates the results of a Na: NH_4^+ ratio for the two sampling events. The large observed increase in the Na: NH_4^+ ratio suggests that NH_4^+ was being attenuated relative to Na^+. Sodium increased in concentration at some wells compared to previous years (Table G1), strongly suggesting that the plume core position had not shifted, and that loss of NH_4^+ is likely the result of degradation processes. Concurrent loss of NH_4^+ and NO_3^- in this region suggests anammox may have played a role in nitrogen removal.

Sorption is likely not a major factor in the large difference in NH_4^+ concentrations observed between PU124 and PU125. If cation exchange were occurring, it would likely play a role in the finer grained regions along the cross section (Figure 3). Though the geology between PU124 and PU125 is a sandy material, the grain size tends to fine east of PU125 (Lazenby 2011), where sorption would be more likely. As NH_4^+ were observed to be much higher east of PU125, higher sorption rates in the coarser grained region between PU124 and PU125 are unlikely.

Further, previous work at the site has determined a groundwater velocity of ~ 400 m/year (Lazenby 2011). As the lagoon has been in operation since 2006, approximately 16-20 pore volumes have passed through the 100 m plume, which would suggest that most available sorption sites along the flowpath have likely been filled.

The average NH_4^+-N loss between PU92 and PU125 was 4.1 mg/L between the two sampling events. Assuming this was the result of anammox activity, 2.5 mg/L of NO_3^--N would be required to consume the observed NH_4^+ loss (Eq. 1.4, Mulder et al. 1995). This would suggest an anammox consumption rate of 0.2 mg/L/day, which is consistent with previous experiments.

Overall, similar trends were noted during the two sampling events that were described in the previous study (Lazenby 2011). The NH_4^+, NO_3^-, and Na^+ concentrations along the centre line were similar to those previously reported, though a few exceptions were noted for NH_4^+. High concentrations (exceeding 50 mg/L) at the proximal end of the plume, which were noted previously (Lazenby 2011) were not observed during this study (Figure 36). Also, although low concentrations of NH_4^+ were previously observed in a few small zones along the plume, they were not noted to the extent and low concentration observed during this study.

As with the previous study, an estimate on overall dilution and natural attenuation was performed for this study. Table 2 summarizes the reduction of total nitrogen and NH_4^+ from this study and the previous study (Lazenby 2011). Decay rates noted in this study are half an order to an order of magnitude lower than previously noted, which may be a result of noted reduction in lagoon volume over the two years of this study.

3.6.4 Site Characterization – Conclusions

Two sampling events were undertaken along the previously delineated plume core in the fall of 2010 to further the ongoing site characterization as well as determine preferred sampling locations for groundwater used in Experiment 4. Based on this study, the following conclusions were made:

- In September 2010, a large segment of the plume core was depleted in NH_4^+ and NO_3^- between wells PU92 and PU125. In October 2010, this area expanded and also moved upgradient to well PU124. The concurrent loss of both nitrogen species, as was observed in this zone, is typical of anammox activity.

- Using the conservative tracer Na^+, it was determined that the plume core had not moved, suggesting that the observed NH_4^+ and NO_3^- loss was not due to a physical alteration of the flowpath. As the lagoon has been in place for over 5 years, and an estimated 16 to 20 pore volumes had passed through the length of the plume, sorption was suggested to be an unlikely cause of NH_4^+ loss. This suggests that the observed nitrogen loss is likely the result of bacterial degradation.

- NH_4^+-N concentrations were observed to reach values approaching the detection limit (0.01 mg/L) in October, while NO_3^- concentrations increased slightly compared to the September values. This is consistent with a potential anammox reaction, which would consume less NO_3^- once the NH_4^+ substrate was consumed.

- The plume core zone was observed to be highly anaerobic (DO <0.5 mg/L), suggesting that nitrification was not a dominant process.

- The cumulative observations above suggest that NH_4^+ and NO_3^- were concurrently remediated by biological means in an anaerobic environment, which is strong evidence for the possibility of anammox activity.

4.0 Conclusions & Summary

A summary of results for Experiments 1-5 and the site characterization monitoring are found in Table 1. Each experiment (with the exception of Experiment 4, Trial 4) exhibited concurrent loss of NH_4^+ and NO_3^-. Most experiments had an observed NH_4^+ and NO_3^- isotopic enrichment, under anoxic conditions. Rayleigh curves were produced for multiple experiments, showing a good fit between observed data and predicted anammox isotope evolution, although the inferred fractionation factors were higher in some cases than values reported in previous groundwater studies . Anammox bacteria communities were confirmed for each sample analyzed, and many displayed growth of anammox population over time. These findings are summarized below.

4.1 Ammonium Attenuation

Each experiment in this study exhibited NH_4^+ attenuation. In experiments where oxygen contamination was suspected (Experiment 1, Trial 2; Experiment 2, Trials 1 & 2, Experiment 5), there was characteristic NH_4^+ consumption with simultaneous NO_3^- production. This behaviour was distinctly different from all the other experiments, which exhibited either concurrent NH_4^+ and NO_3^- loss, or NH_4^+ loss with no observed change to NO_3^- concentrations (Experiment 4, Trial 4).

DO was monitored over the course of Experiments 3-5, as well as during the site characterization. In Experiments 4 and 5, DO was observed to be consistently below 0.3 mg/L, strongly suggesting anerobic conditions were maintained throughout the experiments. The bottom two ports in Experiment 5 (field barrels) were generally found to have DO concentrations < 1 mg/L prior to the addition of the NH_4NO_3 salt solution, during Phase II of Experiment 5.

This required pumping to circulate the water, whereafter a general increase in DO was observed (to 0.5-1.5 mg/L), which lead to simultaneous NH_4^+ loss and NO_3^- production. This suggested that nitrification occurred during the second phase of the experiment as a result of O_2 contamination that occurred during the circulation procedure.

Sorption is considered an unlikely contributor to the observed NH_4^+ loss at the Zorra site. The site geology is coarse grained sand and gravel, and while sorption is known to retard NH_4^+ migration in similar sand and gravel aquifers (e.g. Ceazan et al. 1989), the combined age of the source (>5 years) and the high groundwater velocity (~ 400m/year, Lazenby 2011) suggests that available exchange sites within the monitored plume zone, which is only ~ 100 m in length, have likely been filled for years.

Additionally, in each experiment where $\delta^{15}N\text{-}NO_3^-$ data is available, isotopic enrichment, rather than depletion was observed, which supports the likelihood of anammox activity over nitrification activity. However, note that in experiments where nitrification was suspected (Experiments 1-2, Phase II of Field Barrel study), limited $\delta\ ^{15}N\text{-}NO_3^-$ data is available.

4.2 Anammox

In experiments not contaminated by oxygen, NH_4^+ was attenuated under anaerobic conditions, and nitrification, volatilization and sorption were considered less likely to be major contributors to the observed NH_4^+ loss. Rather, there are multiple examples of data indicating conditions consistent with anammox attenuation. Concurrent loss of NH_4^+ and NO_3^- (except Experiment 4, Trial 4), along with increases of both $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}NO_3^-$ (except in controls, as expected) was observed in each experiment. Inferred enrichment factors ranged from -6 to -20‰ for $^{15}N\text{-}NH_4^+$ and from -12 to -30‰ for $^{15}N\text{-}NO_3^-$ which is somewhat higher

but still generally consistent with values reported for anammox activity (-4 to -8‰, for $^{15}N\text{-}NH_4^+$, Clark et al. 2008; Robertson et al., 2011). The precision of $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$ analysis is estimated at 0.3% and 1%, respectively, which suggests that analytical error was not a significant contributor to the distinct enrichment observed.

Quantitative PCR (qPCR) analysis confirmed the presence of anammox DNA in each experiment analyzed (Experiments 4 and 5). A temporal analysis of the anammox community was performed for Experiment 4 (via qPCR), which revealed that the percentage of anammox bacteria increased in all trials over time (except for sediment biomass control). In experiment 4 (Trial 3) 16% of the bacteria population was found to be anammox at the end of the trial.

Using previously suggested fractionation factors (Lazenby 2011; Robertson et al. 2011; Clark et al. 2008), Rayleigh models were designed to predict the isotope evolution of $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}NO_3^-$ under anammox activity. Of the 9 modeled runs, all but one (Experiment 4, Trial 4) provided a good fit consistent with anammox activity. Five of the 9 model fits used an ε value of 6 ‰, which is similar to previous estimations of NH_4^+-N isotopic fractionation during anammox. The remaining three model runs required larger ε values of -11 to -20‰ to fit the experimental data. A possible explanation for this difference could be ammonification or desorption effects buffering the residual NH_4^+ concentration, leading to relatively low apparent f_{NH4} values where these effects were stongest . Enrichment of $^{15}N\text{-}NO_3^-$ was not analyzed in as much detail in this study as such enrichment could also be indicative of denitrification.

Rates for anammox attenuation were estimated based on the assumption that all NH_4^+ was consumed by anammox bacteria (Table 1). Rates typically ranged from 0.1-0.2 mg N/L/day, though Experiments 1 and 2 exhibited rates as high as 0.5 mg N/L/day before suspected oxygen contamination occurred. Substantial differences in NH_4^+ consumption was observed between

field site sediment and control sediment experiments in Experiment 4, Trials 1 and 2, however it was unclear whether an increase in sediment volumes used (Experiment 3 vs. Experiment 4) resulted in increased rates of anammox activity.

Differentiating quantitatively between anammox and denitrification activity proved to be difficult. Half of the experiments exhibited NO_3^- loss greater than that expected based on anammox alone (based on NH_4^+ loss, Table 1), which was assumed to be the result of denitrification. Increased concentrations of N_2O and CO_2 that were observed, along with generally high concentrations of DOC suggests that denitrification likely contributed to nitrogen loss observed in these experiments. The isotopically tagged ^{15}N experiments (Appendix F) suggested that less than 5% of N_2 was formed by anammox, however these results are questionable because of experimental and analytical problems encountered (Section 1.5). Previous studies at the site have suggested that anammox contributes 18 +/- 6.5% of N_2 gas production (Moore et al. 2011), which appears more consistent with the evidence found in this study; particularly, the observed net NH_4^+-N loss under anaerobic conditions.

Nitrite production was noted in multiple experiments, though it does not strongly support one reaction over the multiple reactions involving NO_2^-; as denitrification, nitrification, and anammox can all potentially result in increasing NO_2^--N. Though the reduction of NO_3^- to NO_2^- would theoretically occur in the anammoxosome (Jetten et al. 2009), it is possible that anammox activity resembles the 'hole in the pipe' model of nitrification and denitrification, where intermediate products of the reaction are measureable outside of the cell (Firestone and Davidson 1989).

Another possible complication associated with the microcosm experiments could be the effect of NH_4^+ desorption from the solids. Ammonium is normally strongly adsorbed in typical

aquifer sands, for example in the Otis aquifer sands Ceazan et al. (1989) report Kd vales of 0.59-0.87 for NH_4^+. Although NH_4^+ Kd values in the Zorra sand were not measured directly in this study, if a typical sand value of 0.7 is considered, in the Zorra aquifer (per unit volume), the mass of NH_4^+ adsorbed on the solids would be 3.5 times higher than the mass in solution (using the standard retardation equation, and assuming porosity of 0.35). If then, anammox activity is specifically consuming NH_4^+ from the aqueous phase, lowering concentrations would then initiate desorption from the solid phase. The desorbed material would then tend to mask the amount of NH_4^+ that has been consumed and would generate an additional pool of aqueous NH_4^+ that would dilute the isotopic signature of the residual NH_4^+. This phenomenon would likely have the effect of causing underestimation of the anammox reaction rate and would also cause underestimation of the isotopic fractionation factor. Thus, NH_4^+ desorption effect would tend to mask anammox activity inferred from the mass balance and isotopic approaches utilized in these experiments.

Another possible process that could underestimate the role of anammox is solute diffusion. Mariotti et al. (1988) describe denitrification going to completion within the matrix of an aquifer, where dissolved NO_3^- enters via diffusion. No isotope effect is associated with this case, as all NO_3^- is consumed. A similar situation could potentially occur in the bottom sediment layer of the sacrificial bottles if the sediment wasn't shaken regularly and a combination of anammox and denitrification consumed all of the nitrogen in the sediment. This would result in masking of enrichment from both processes.

Finally, as anammox bacteria are known for their slow growth, with a doubling time between 1.8 (Isaka et al, 2005; Dalsgaard et al, 2005) and 21 (Jetten et al, 2005) days, experimental duration needs to considered to allow for adequate biota acclimation (lag) time.

Previous anammox studies include a wide variety of experimental periods ranging from; 9 hours (van de Graff et al. 1995), 60 hours (Thamdrup et al. 2006), 160 days (Jetten et al. 1997), to as high as 800 days (Mulder et al. 1995). This study had experiment lengths between 122-299 days, which is longer than most of the previous studies. Further, an increase in nitrogen consumption was generally not observed towards the end of these tests, thus experimental duration appears to have been adequate.

4.3 Denitrification

Based on multiple lines of evidence (N_2O and CO_2 production, DOC consumption, NO_3^--N consumption, $\delta^{15}N$-NO_3^- enrichment) denitrification is likely a source of NO_3^- consumption in this study. Though an attempt was made at quantifying the relative magnitudes of anammox and denitrification (IPT study), a reliable conclusion was not obtained. In most situations, denitrification is expected to occur concomitantly with anammox. Overall, this is generally considered to be a favourable circumstance as it contributes to additional N attenuation. Denitrification only becomes of concern if it goes to completion, which then removes the NO_3^- required for anammox activity.

4.4 Future Work

A combination of mass balance, microbiological and isotopic evidence developed in this study has strongly suggested that anammox could be a significant contributor to nitrogen attenuation in agricultural settings. A recommendation would be to develop a more robust procedure for preparation of $\delta^{15}N$-NO_3^- samples in high DOC environments such as the Zorra site. Inconsistency in reproducibility of Zorra samples suggests that a procedure including dialysis of samples with high organic content should be implemented. A better method for determination of organic nitrogen, and a better understanding of the potential for mineralization

of this N in groundwater environments will provide a more reliable rate of nitrogen attenuation. Two separate analyses of organic N (TN and TKN) were undertaken by different laboratories (University of Waterloo, University of Guelph), and comparative results suggest incomplete recovery of organic nitrogen (Table C1, Table D1). Organic nitrogen could be an important contributor to inorganic concentrations in agricultural settings, and as such, a greater understanding of potential transformations is needed.

Another future goal should be to more accurately quantify the relative amounts of anammox and denitrification N loss. A more rigorous IPT study, corroborated with more precise DOC, N_2O and CO_2 analyses, would assist in this goal. A more appropriate IPT study would overwhelm the experiment with $^{15}N\text{-}NH_4^+$ (generally 90 atom percent or higher), which was not the case in the current set of experiments. This would then permit the assumption that any $^{29}N_2$ gas produced in the experiment is the result of the combining of $^{14}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ (Thamdrup and Dalsgaard, 2006).

To obtain a more robust estimate on NH_4^+ consumption via anammox, quantification of microbial NH_4^+ assimilation should be addressed. Assimilation has the potential to play a significant role in NH_4^+ removal in situations where overall nitrogen removal is low.

Microbial nitrogen assimilation may be an important component of nitrogen loss in experiments where low nitrogen rates exist, such as in this study. Though it is generally assumed that assimilative metabolism consumes less nitrogen than dissimilative reactions, further work can be done to validate this, such as estimating microbial assimilation (Spoelstra, 2011):

$$\text{Net Microbial N Assimilation (mass)} = \Delta\text{Biomass (mass)} \times \%\text{N in biomass} \qquad \text{Eq. 4.1}$$

An important next step in this process is the implementation of a larger scale field study; either another reactor, or potentially tiling high NO_3^- water into an NH_4^+ plume. If a reactor is employed, a much higher percentage of fresh core should be used (10% core was used in this study), due to the slow doubling time of anammox bacteria.

Anammox has been used effectively in many waste water treatment plant reactors around the world, with N nitrogen removal rates that are up to four orders of magnitude greater than those observed in this study (Table 3). Though higher temperatures used in these studies (35°C) may influence reaction rates, and other N loss processes (denitrification and volatilization) likely also occur, the reactors suggest that much higher anammox reaction rates are potentially achievable. Waste water treatment plants generally have higher substrate concentrations, which would lead to higher removal rates if the reaction kinetics are first order, however the 15 experiments conducted in this study did not show dramatically different anammox reaction rates at different substrate concentrations (Figure 39). Finally, Experiments 1-4 contained a maximum of only 40% porous media, thus in field settings with 100% porous media and with greater biomass acclimation periods, higher reaction rates could be expected.

Although the current study has provided compelling evidence for the occurrence of anammox, through an observation of net NH_4^+ consumption under apparently anoxic conditions (particularly experiment 4 in the anaerobic chamber), combined with isotopic enrichment of the residual NH_4^+ in the presence of anammox bacteria, the current tests can not rule out that other processes may have also contributed to the observed NH_4^+ consumption. In future phases of this research, additional microcosm tests should be undertaken using the procedures of Experiment 4 (in an anaerobic chamber) but targeting specific processes that may have also influenced NH_4^+ consumption. With the successful completion of these trials it might then be possible to more

firmly assign the NH_4^+ consumption rates observed in this study specifically to anammox. Suggested targeted trials include:

Trial 1, O_2 diffusion; reactive mixtures affected only oxygen influx

Mix 1; 20 mg/L $FeCl_2$ in DI water

Mix 2; 20 mg/L NH_4Cl in DI water

Trial 2, Sorption/desorption

Mix 1; 20 mg/L NH_4-N in DI water

Mix 2; Mix 1 + silica sand

Mix 3; Mix 1 + Borden sand

Mix 4; Mix 1 + washed Zorra sand

Mix 5; Mix 1 + fresh Zorra sand

Trial 3, Biological assimilation

Mixes and procedures to be determined in conjunction with biology department (e.g. measure increase in solids dry weight over time).

Trial 4, Substrate Concentration effect

Mix 1; 20 mg/L NH_4-N in DI water + Zorra sediment (no NO_3)

Mix 2; 20 mg/L NH-N in DI water + Zorra sediment + 50 mg/L NO_3^-N

Mix 3; 20 mg/L NH_4-N in DI water + Zorra sediment + 20 mg/L NO_3^-N

Mix 4; 20 mg/L NH_4-N in DI water + Zorra sediment + 5 mg/L NO_3^--N

Trial 5, Heterogeneity Uncertainty

Do Trial 4 in triplicate, then error bars due to media heterogeneity can be assigned.

These experiments could be the focus of a new M.Sc. level project or specific trials could be undertaken as B.Sc. level research projects.

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Table 1. Summary of results for Experiments 1-5; full-test consumption of NH_4^+ and NO_3^-, Total N and anammox reaction rates, δ $\delta^{15}N$-NH_4^+ and $\delta^{15}N$-NO_3^- increase, model results (ε, α_{NH4} α_{NO3}) , and percent anammox based on qPCR results.

Experiment	Trial	Full Test Consumption (mg/L as N)		Total N Removal (Observed)	N Removal (Anammox)[1]	Enrichment (‰)		Isotopic Fractionation Parameters				qPCR
		NH_4^+	NO_3^-	(mg N/L/day)	(mg N/L/day)	$\delta^{15}N$ - NH_4^+	$\delta^{15}N$ - NO_3^-	ε[3] NH_4^+ (‰)	ε[3] NO_3^- (‰)	α[4] NH_4^+	α[4] NO_3^-	% Anammox
1 (5L Tedlar Bag)	1	7.4	3.1	0.1	0.1	7.4	8.4	-6.0	-15.0	0.994	0.985	n/a
	2[2]	21.4	20.3	0.6*	0.5	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2 (2L Kimex Flask)	1[2]	12.0	2.8	0.2*	0.3	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	2[2]	11.0	6.3	0.4*	0.5	n/a	n/a	n/a	n/a	n/a	n/a	n/a
3 (Sacrificial DO Bottles)	1	4.5	3.6-11.0	0.1	<0.1	n/a	2.1	-6.0	-15.0	0.994	0.985	n/a
	2	13.7	7.4	0.1	0.1	11.5	34.0	-15.0	-12.0	0.985	0.988	n/a
	3	Dissolved Oxygen Control - No substrate										
	4	7.3	5.5	0.1	0.1	7.0	3.9	-6.0	-15.0	0.994	0.985	n/a
4 (Sacrificial Serum Bottles)	1	25.4	27.2	0.2	0.2	5.0	8.4	-11.0	-15.0	0.989	0.985	0.1
	2	12.7	16.9	0.1	0.1	0.0	4.1	-6.0	-15.0	0.994	0.985	6.6
	3	17.3	25.7	0.2	0.1	4.1	7.0	-6.0	-15.0	0.944	0.985	16.3
	4	30.3	0.0	0.1	0.2	4.3	8.7	-6.0	-15.0	0.944	0.985	4.2
	5	36.0	7.5-20.5	0.2	0.2	6.0	6.2	-20.0	-30.0	0.980	0.970	n/a
5 (Field Reactors)	Inoculated	37.7	144.6	0.6	0.2	2.2	n/a	n/a	n/a	n/a	n/a	0.2
	Control	31.9	139.4	0.5	0.2	-2.0	n/a	n/a	n/a	n/a	n/a	<0.1

Notes: 1. Anammox consumption values based on assumption that all NH_4^+ is consumed by anammox, NO_3^- consumed at 3:5 NO_3^-:NH_4^+ ratio (Mulder et al. 1995).
2. Data based on results prior to oxygen contamination.
3. $\varepsilon_{p-r} = (\alpha-1) \times 1000‰$
4. α =Rproduct/Rreactant

Table 2. Percent reduction of NH_4^+-N and total nitrogen (TN), based on mean Na^+, NH_4^+-N, DON and total nitrogen (TN) concentrations measured at distal transect E-E' (88-101 m from the lagoon) compared to the mean lagoon values (n = 5-15). Distal values include plume core wells (Na^+ > 30 mg/L) at nests PU81, 84, 96 and 121 where available (n = 8-16). Nitrogen values for July to August 2009 from Lazenby (2011). The piezometers used in calculations include: July 15, 2009 (PU81, 84, 96, 121; n = 16), August 20, 2009 (PU96, 121; n = 8), November 13, 2009 (PU96, 121; n = 8), September 25, 2010 (PU84, 96, 121; n = 9), October 26, 2010 (PU84, 96, 121; n = 10).

	Measured (mg/L)				**% Reduction**					
	Na^+	**NH_4^+-N**	**DON**	**TN**	**NH_4^+-N**			**TN**		
Lagoon (C_o)	220	121	218	361	**Total**	**Dilution**	**Decay**	**Total**	**Dilution**	**Decay**
Distal Transect E-E'										
July 15, 2009	33	4.3	-	-	96	85	11	-	-	-
Aug. 20, 2009	36	8.2	10	23	93	84	9	94	84	10
Nov. 13, 2009	41	14	10	25	88	81	7	93	81	12
Sept. 25, 2010	29	15	5.4	27	88	87	1	92	87	5
Oct. 26, 2010	25	9	8.3	26	93	89	4	93	89	4
Distal Average	**33**	**10**	**8.4**	**25**	**92**	**85**	**6**	**93**	**85**	**8**

Table 3. Summary of waste water treatment plant reactor nitrogen removal rates where the anammox reaction is utilized, compared to Experiment 4, Trial 3 of this study.

Reactor	N Removal (mg/L-day)	Temp (^oC)	pH	Notes	Reference
SHARON	850	30-40	7-8	No sludge retention prevents nitrite oxidation. Denitrification controls pH. Costs approx. $1.7 per kg N-$NH_4^+$ removed.	Hellinga et al. 1998
CANON	315	30	7.8	Completely Autotrophic Process (no denitrification). Anammox/partial nitrification process. Under oxygen limiting conditions, 15% of NH4 converted to NO3.	Sliekers et al. 2002
OLAND	1807	30 +/- 2	6.5-8	Nitritation (partial nitrification of NH4 to NO2) is used to provide nitrite for the anammox reaction. Low DO (0.31 +/- 0.18 mg/L).	Pyraert et al. 2004
SNAP	289-384	35	7.5-7.7	Single Stage Reactor. DO constant at 2-3 mg/L.	Furukawa et al. 2006
SNAD	483	35	8-8.2	Single Stage Reactor. DO kept between 0.4-0.6 mg/L	Chen et al. 2009
Experiment 4, Trial 3	0.2	22	6.7-7.2	Sacrificial Bottle with 40% media. Low DO (0.14 +/- 0.09 mg/L)	This Study

Figures

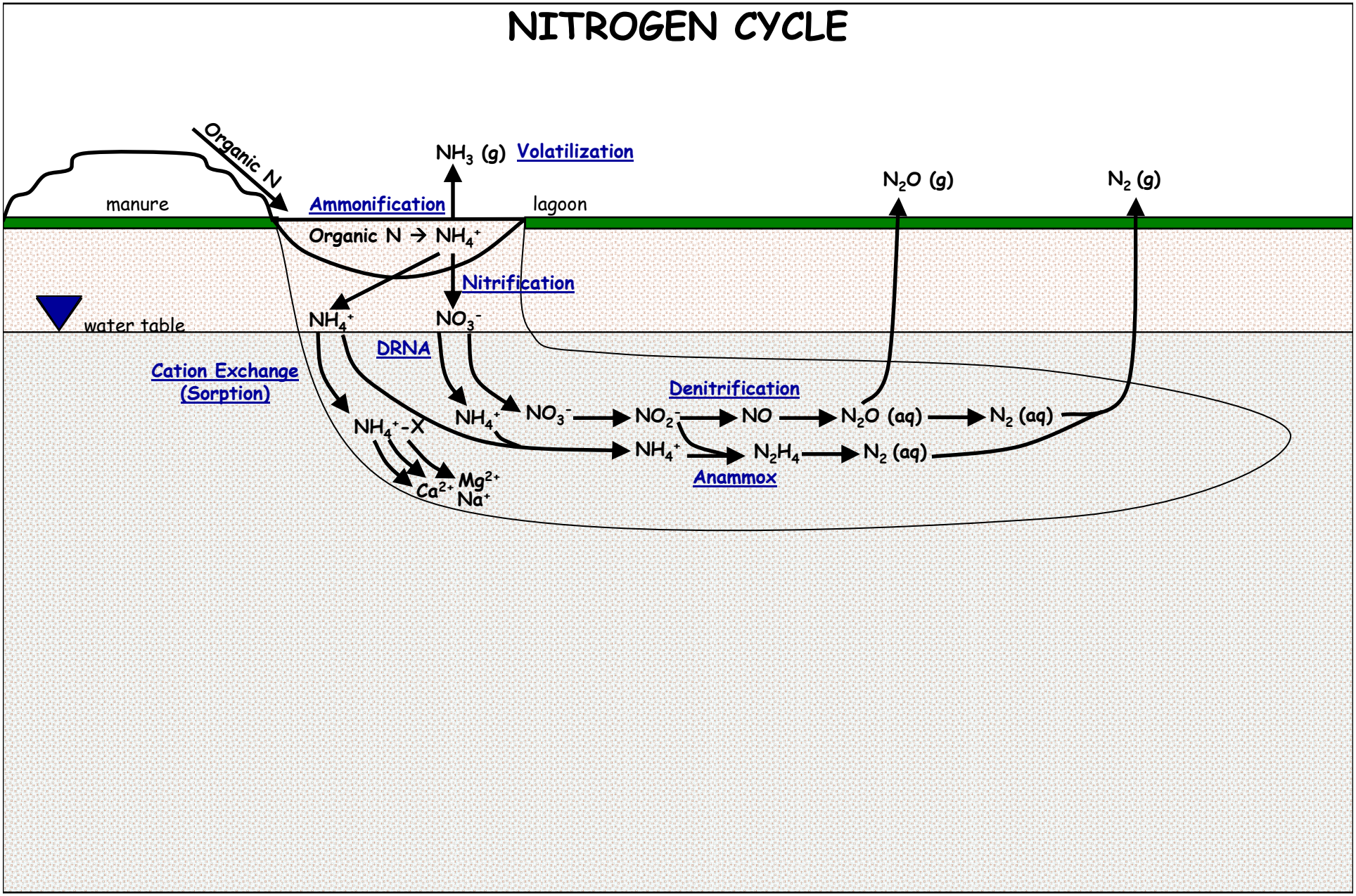

Figure 1. Biogeochemical and physical processes affecting speciation of nitrogen at a manure lagoon. Each of the highlighted are processes detailed in the study, including ammonification, volatilization, nitrification, sorption, dissimilatory reduction of nitrate to ammonium (DRNA), denitrification, and anammox.

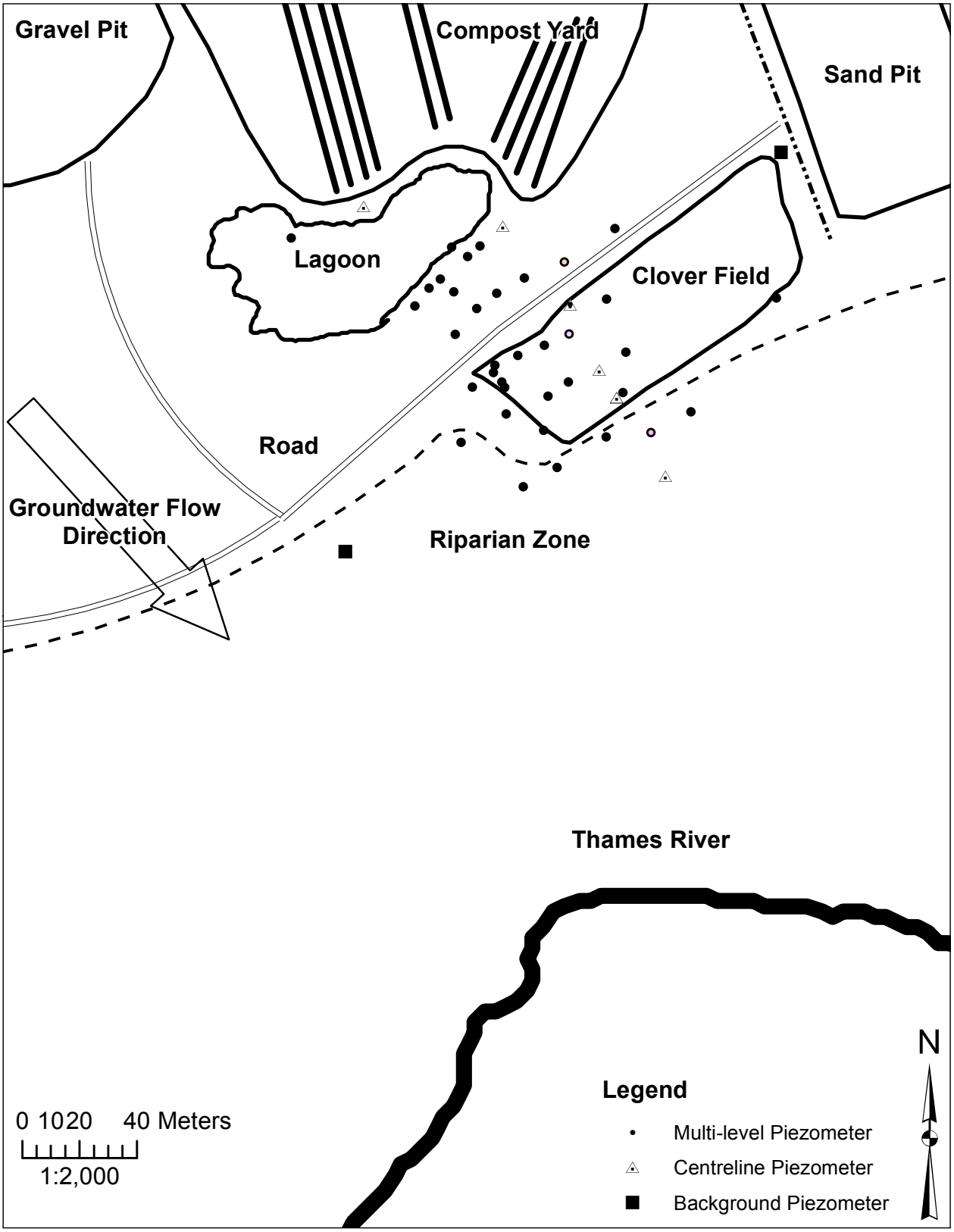

Figure 2: Zorra site monitoring network and local features. The site consists of a manure composting operation and runoff lagoon. The multi-level piezometer monitoring network was installed during a prior study (Lazenby 2011). Adapted from Lazenby (2011).

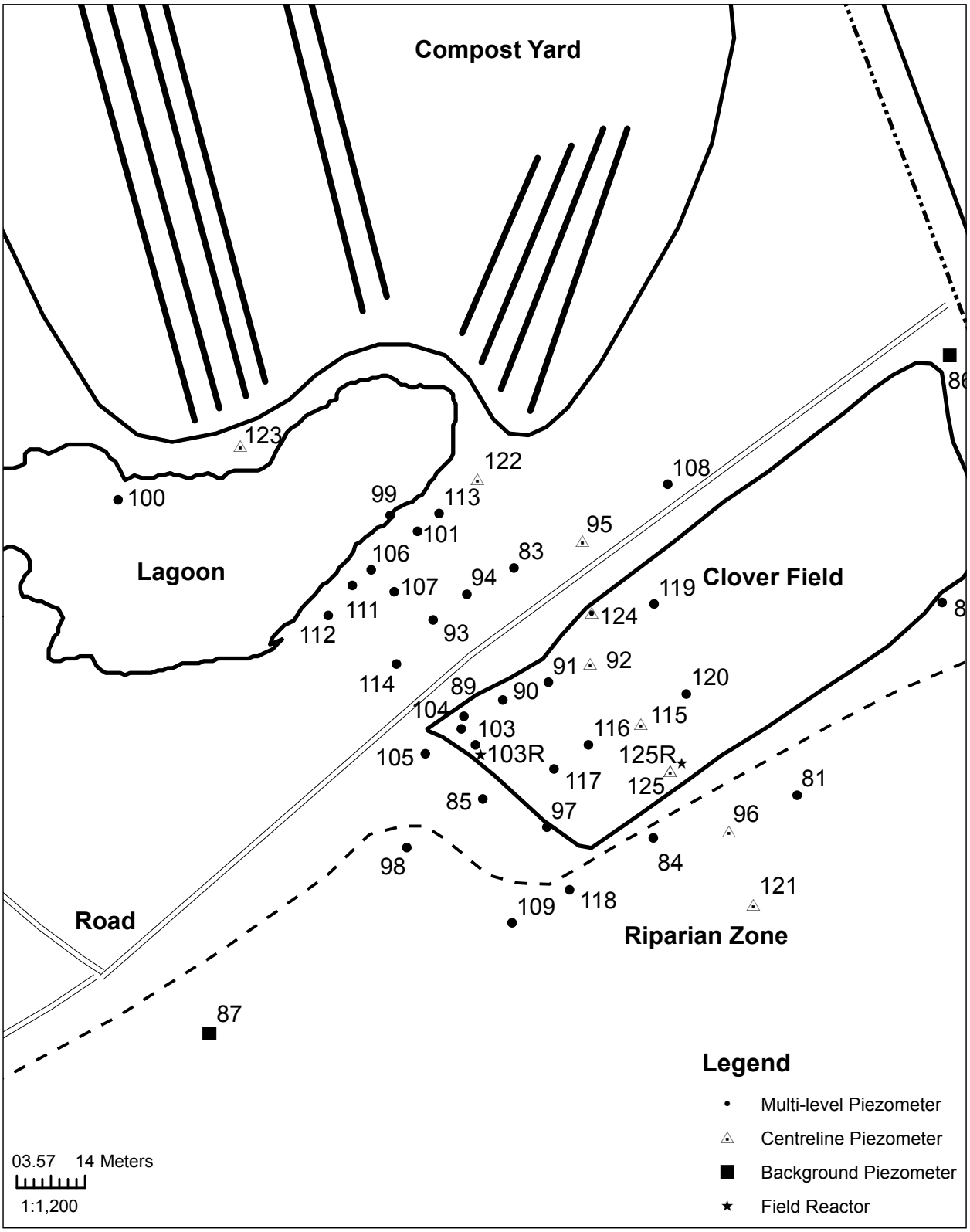

Figure 3: Zorra monitoring well network and locations of field reactos 103 and 125. Adapted from Lazenby (2011).

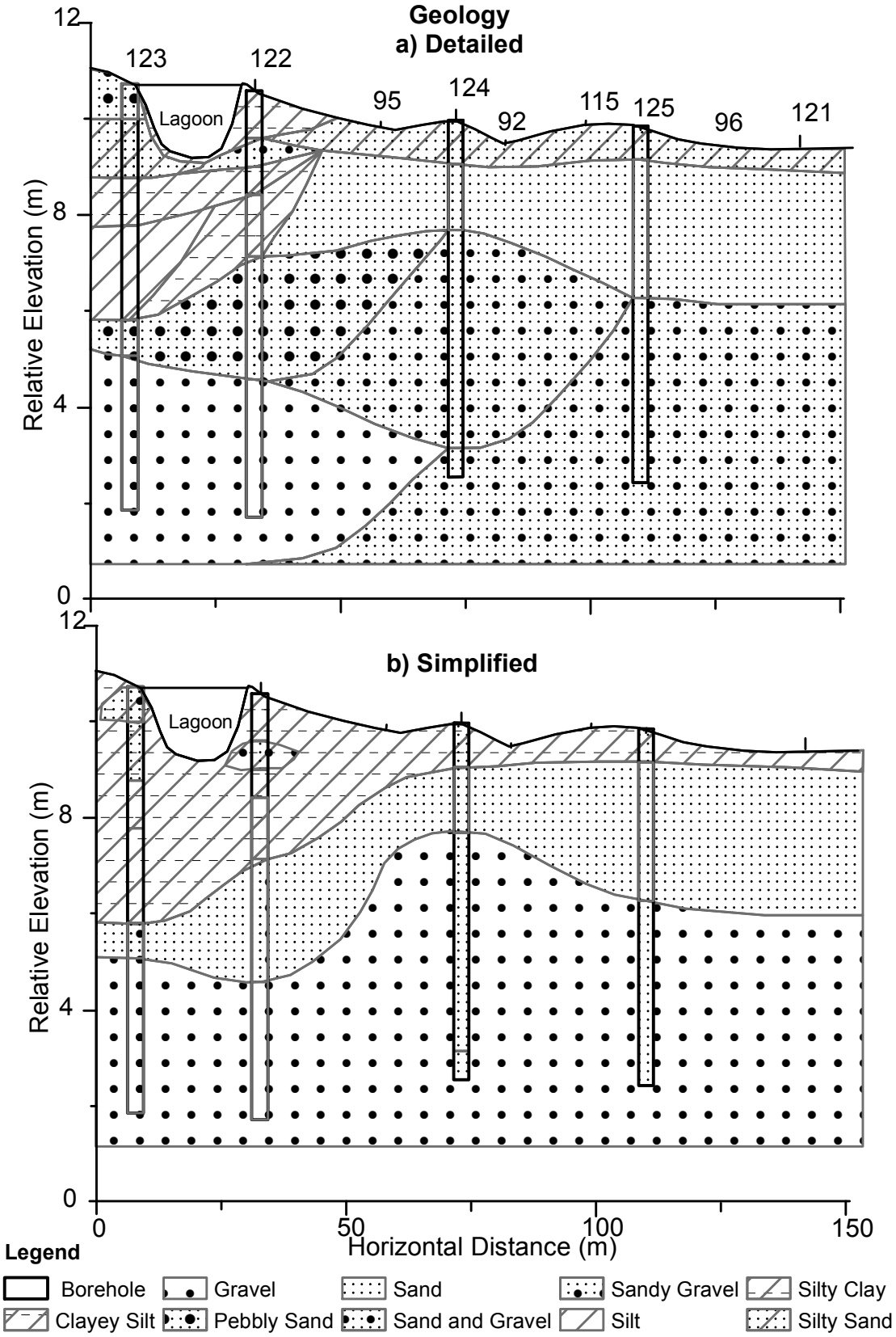

Figure 4: Geology along the centre line: **a)** detailed **b)** simplified. Note the cross section has a vertical exaggeration of approx. 10.

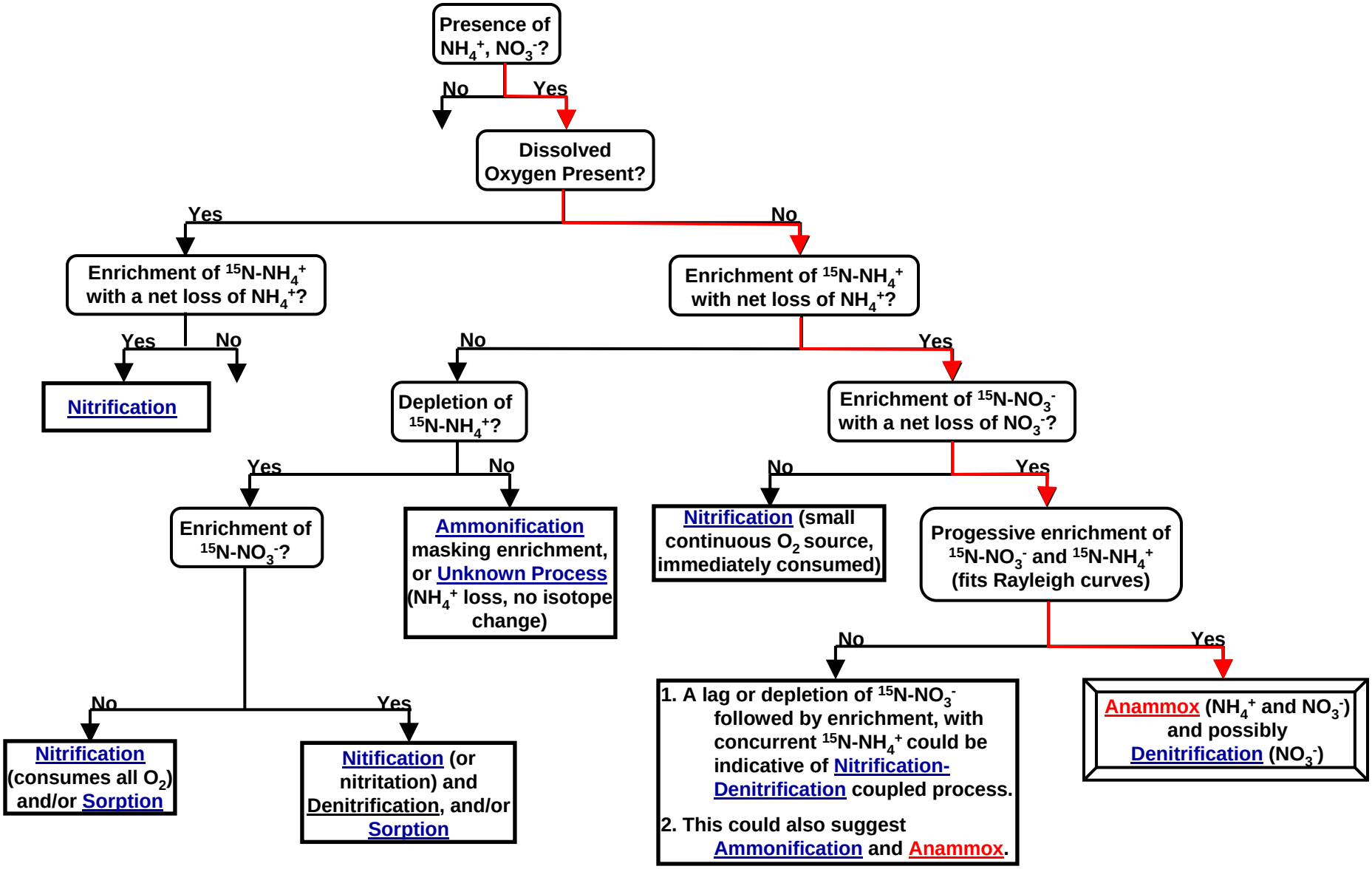

Figure 5. Flow chart illustrating the potential pathways of transformation for NH_4^+ and NO_3^-, based on current understanding of the nitrogen cycle. Note that initial ^{15}N-NH_4^+ pool is assumed to have an isotope signature equal to or less than the ^{15}N-NO_3^- pool.

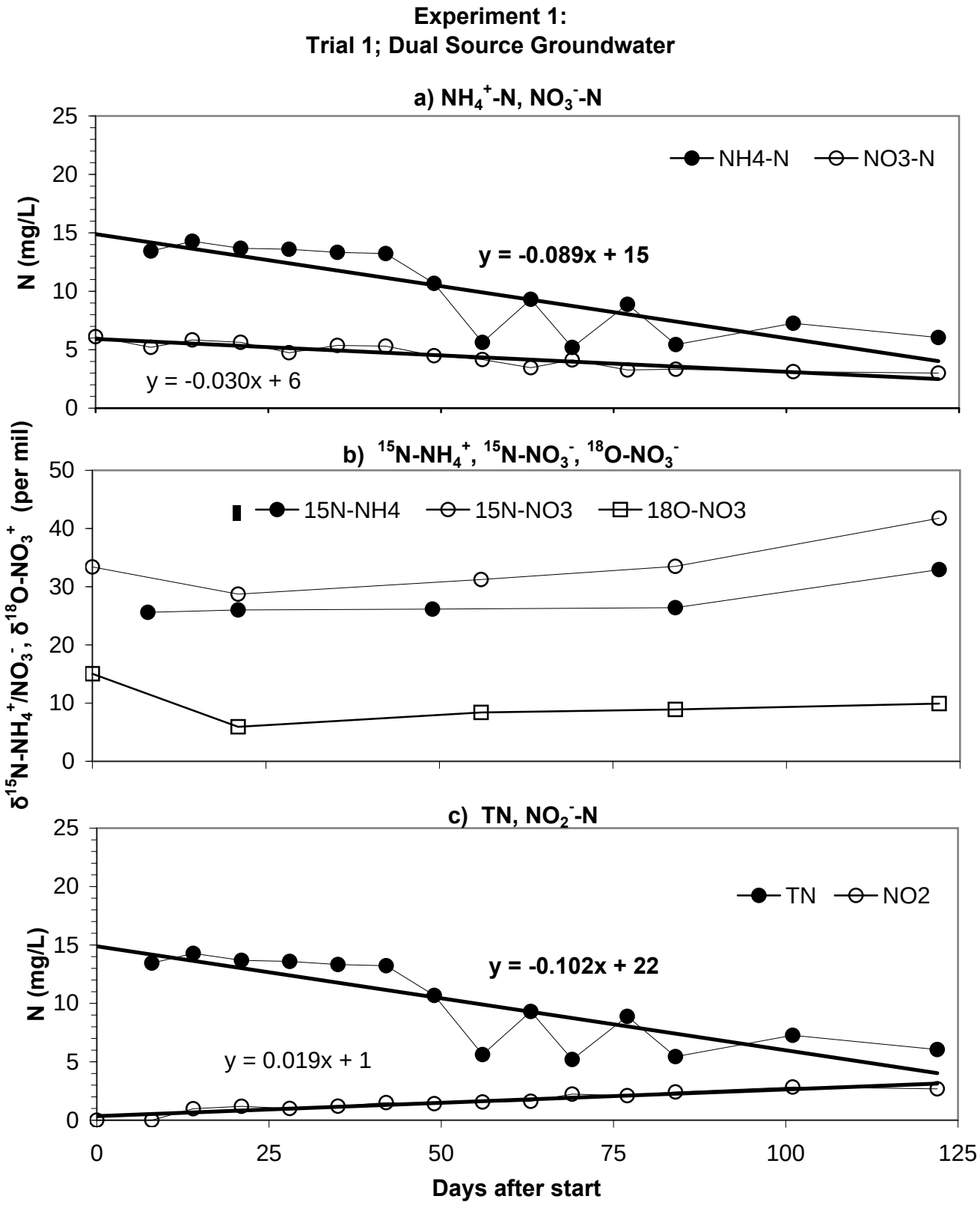

Figure 6. Experiment 1, Trial 1; dual source groundwater (PU125-5.1m and PU96-2.6m) mixed with 100g sediment (core PU103) in laboratory microcosm (5L Tedlar bag); nitrogen trends: **a)** NH_4^+-N and NO_3^--N, **b)** NH_4^+ and NO_3^- isotopic trends and **c)** total nitrogen (TN) and NO_2^--N trends; Sept. 25, 2009 to Jan. 25, 2010.

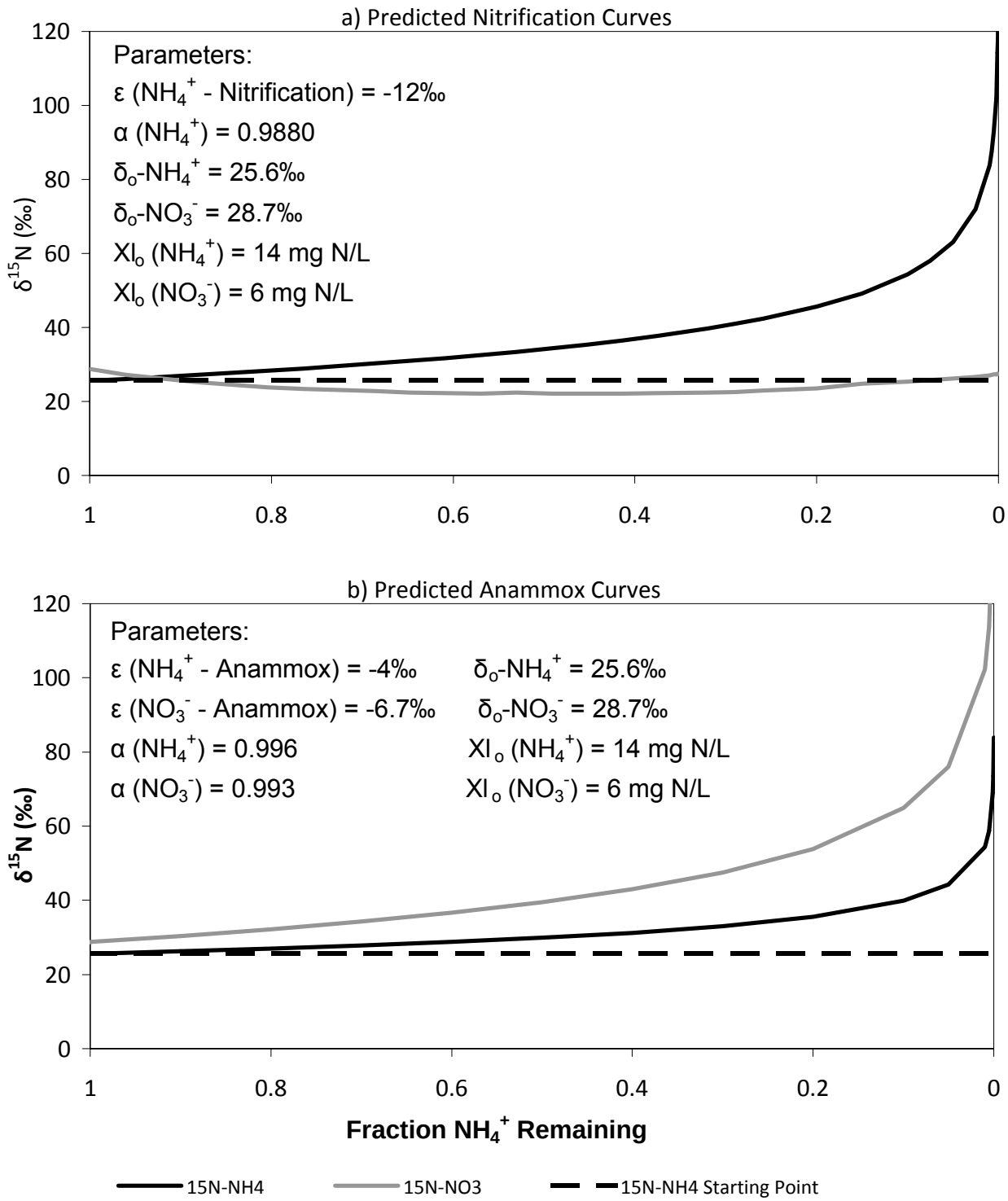

Figure 7. Theoretical Rayleigh curves illustrating $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}NO_3^-$ isotope evolution during: **a)** nitrification of NH4+ (to completion), **b)** anammox (to completion). These curves were generated based on a closed Rayleigh model, using literature enrichment factors (except for $\delta^{15}N\text{-}NO_3^-$ anammox, which was estimated). See Appendix B for details.

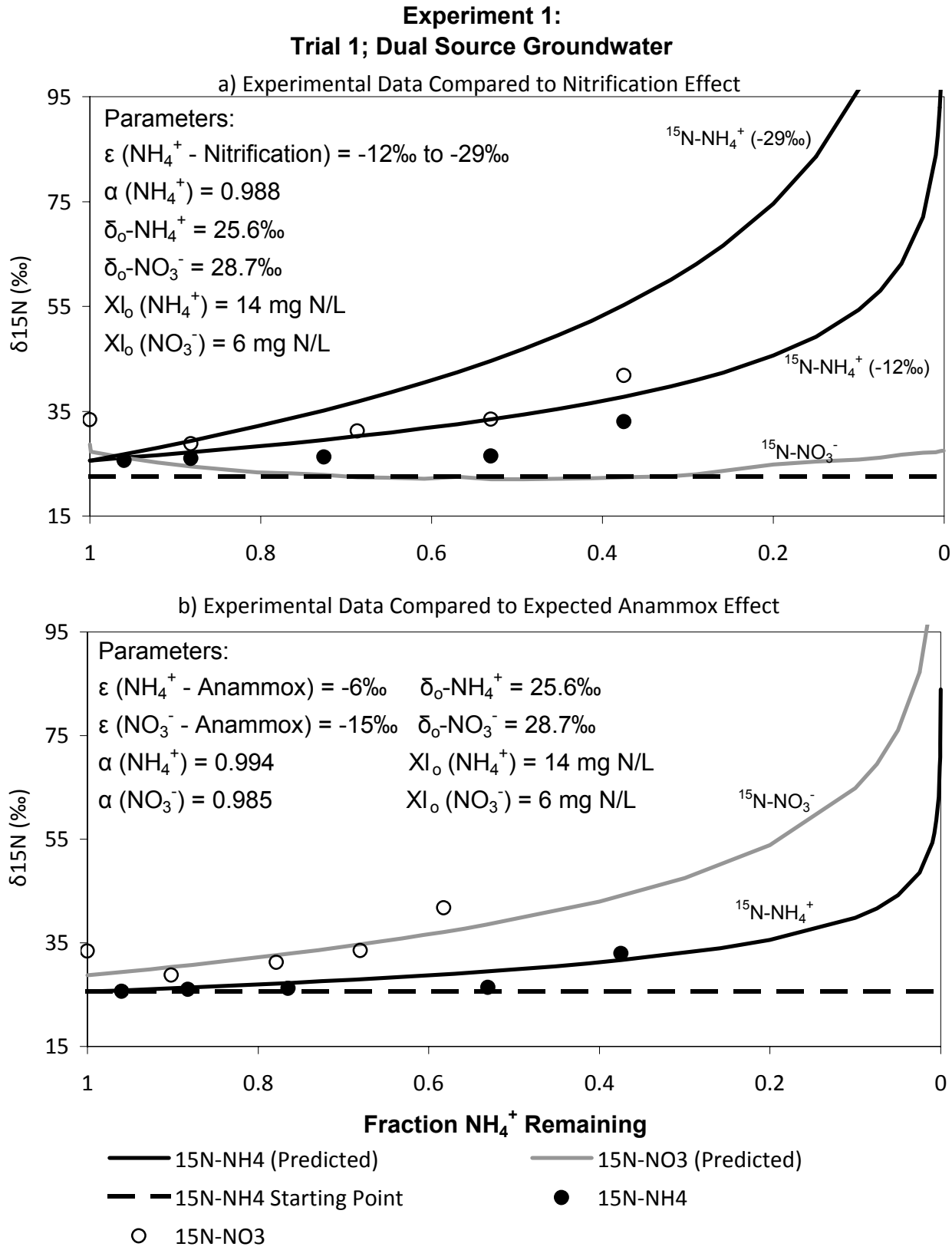

Figure 8. Experiment 1, Trial 1; N isotopic trends compared to isotope evolution expected from: **a)** NH4 nitrification and **b)** anammox.

Experiment 1:
Trial 2; Single Source Groundwater

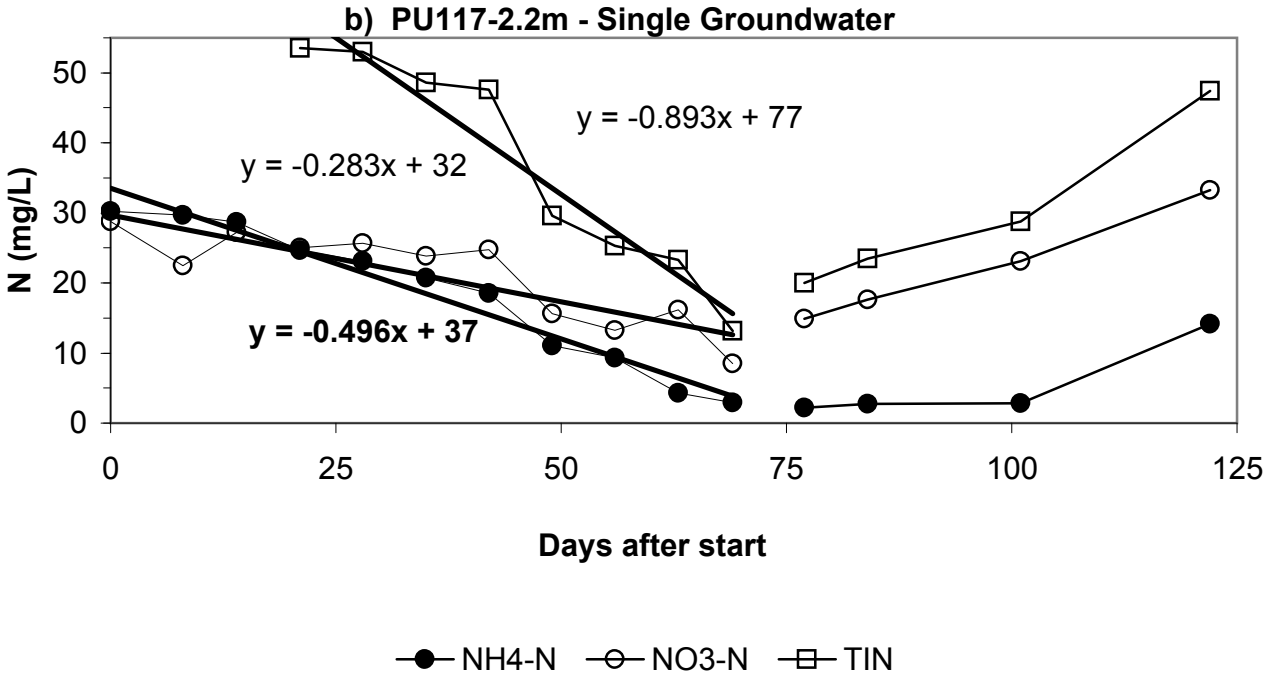

Figure 9. Experiment 1, Trial 2; Single sourced groundwater (PU117-2.2m) mixed with 100 g sediment (core PU103) in laboratory microcosm (5L Tedlar Bag); nitrogen trends, Sept. 25, 2009 to Jan. 25, 2010.

Experiment 2:
2L Glass Flasks

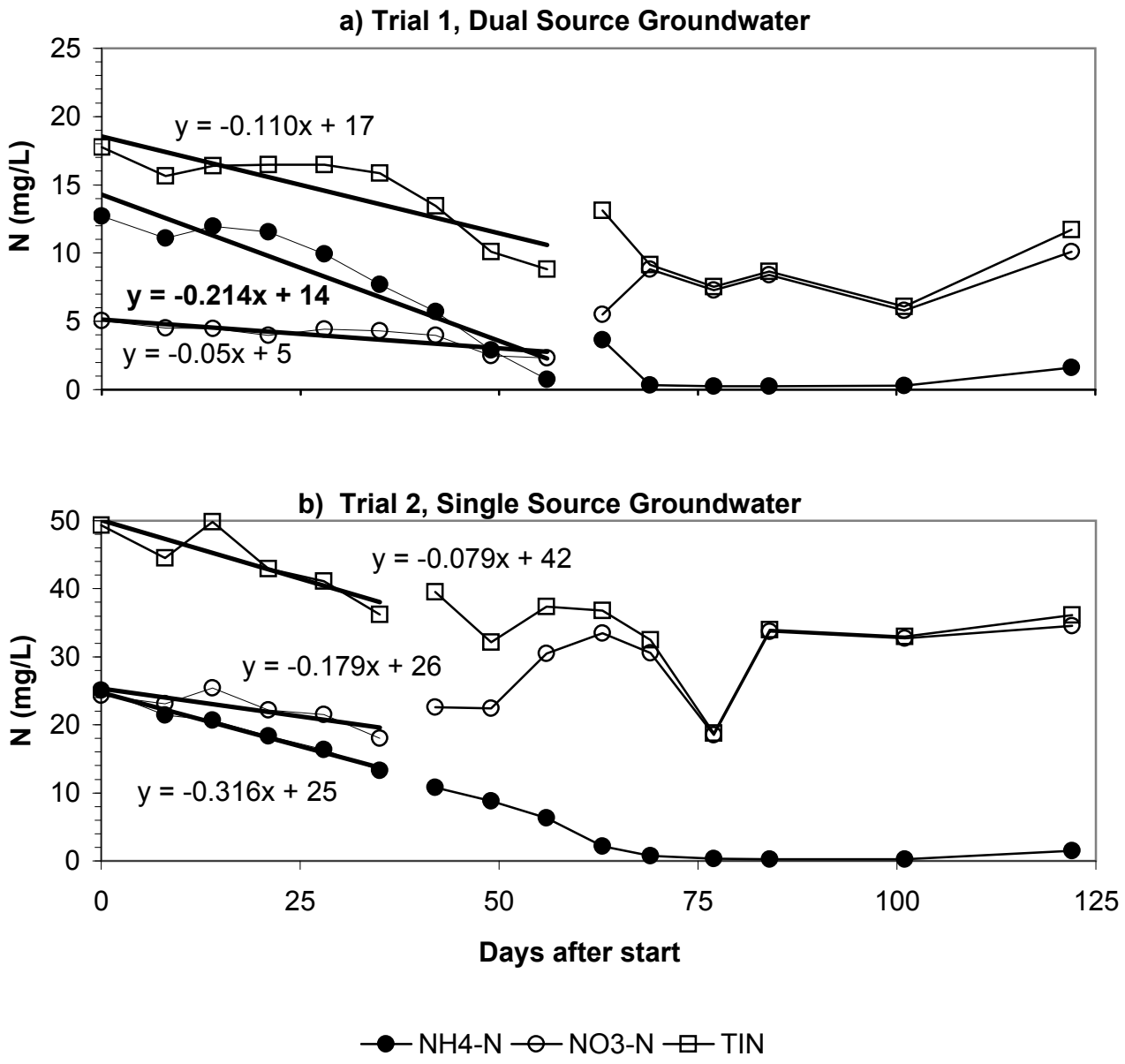

Figure 10. Experiment 2; Putnam groundwater mixed with ~ 500 g sediment in laboratory microcosms (2L glass flasks): **a)** dual source groundwater (PU125-5.1m and PU96-2.6m) and **b)** single source groundwater (PU117-2.2m), Sept. 25, 2009 to Jan. 25, 2010.

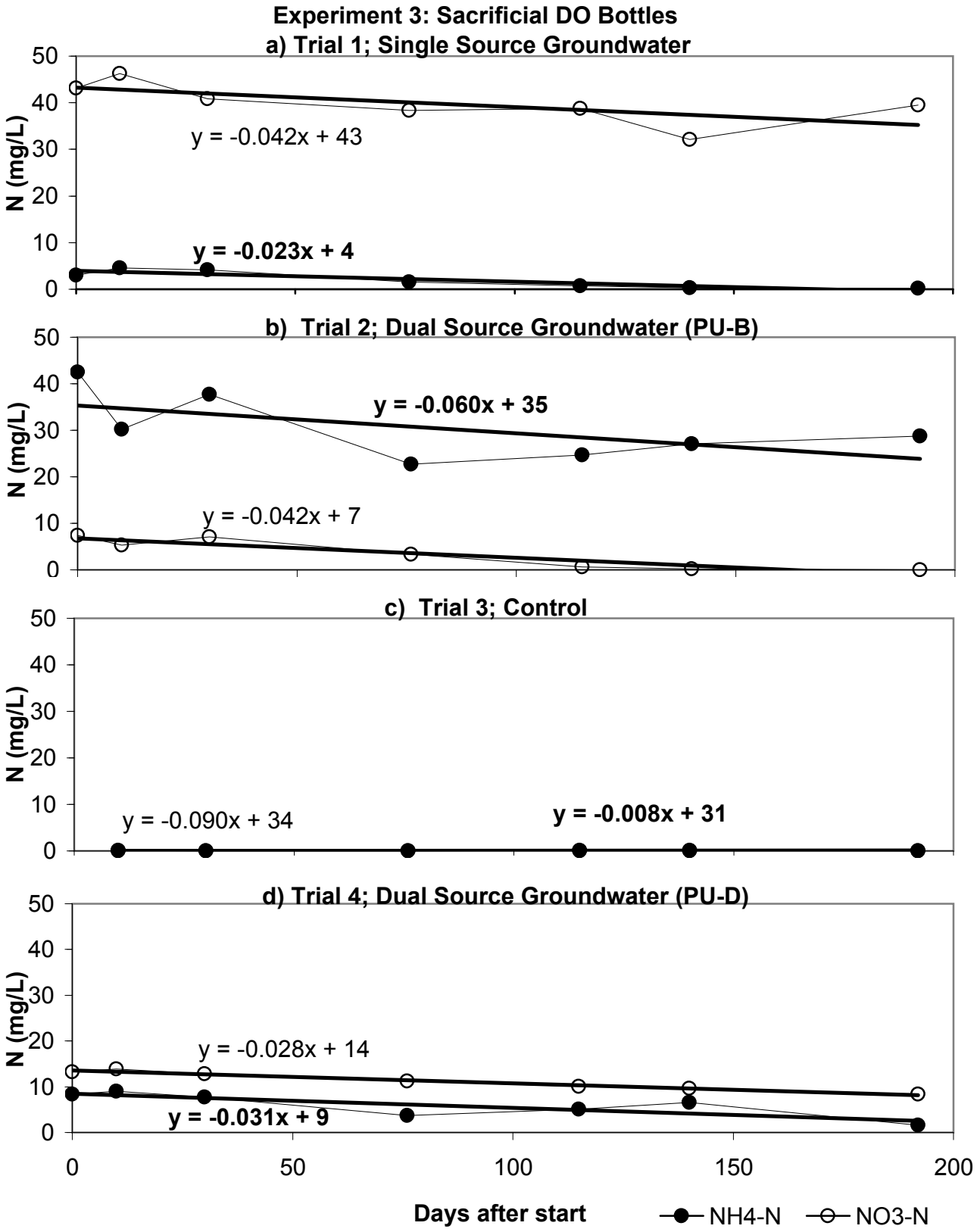

Figure 11. Experiment 3; Putnam groundwater mixed with ~ 50 g sediment in sacrificia bottle microcosms (250 mL DO bottles): **a)** single source groundwater (PU115-2.2m), **b)** dual source groundwater (PU122-2.2m and PU80-1.7m), c) DO control and d) dua source groundwater (PU125-2.7m and PU80-1.7m), Sept. 25, 2009 to Jan. 25, 2010.

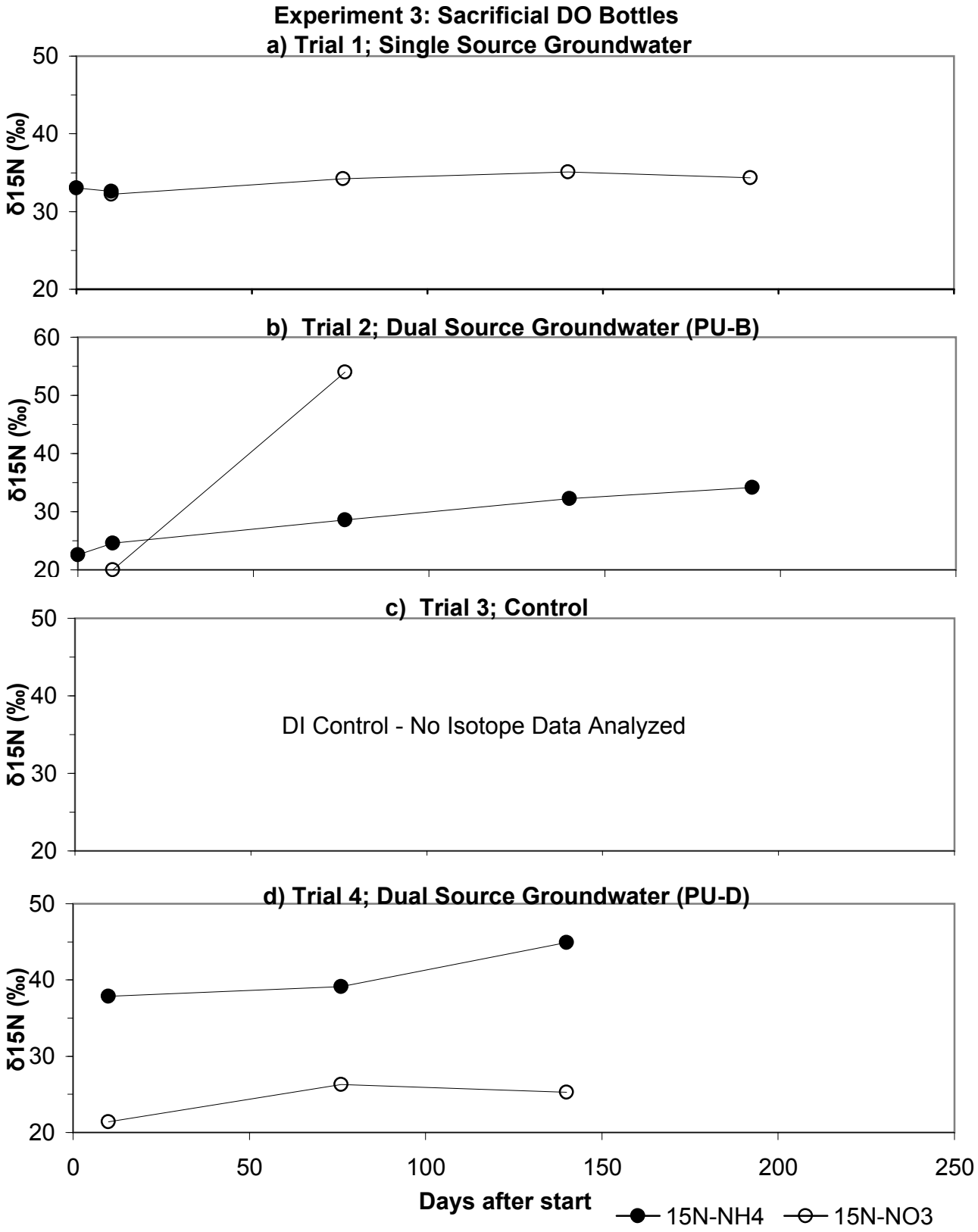

Figure 12. Experiment 3; Zorra groundwater mixed with ~ 50 g sediment in sacrificia bottle microcosms (250 mL DO bottles): Isotope trends for **a)** single source groundwater (PU115-2.2m), **b)** dual source groundwater (PU122-2.2m and PU80-1.7m), **c)** DO control, **d)** dual source groundwater (PU125-2.7m and PU80-1.7m), Sept. 25, 2009 to Jan. 25, 2010.

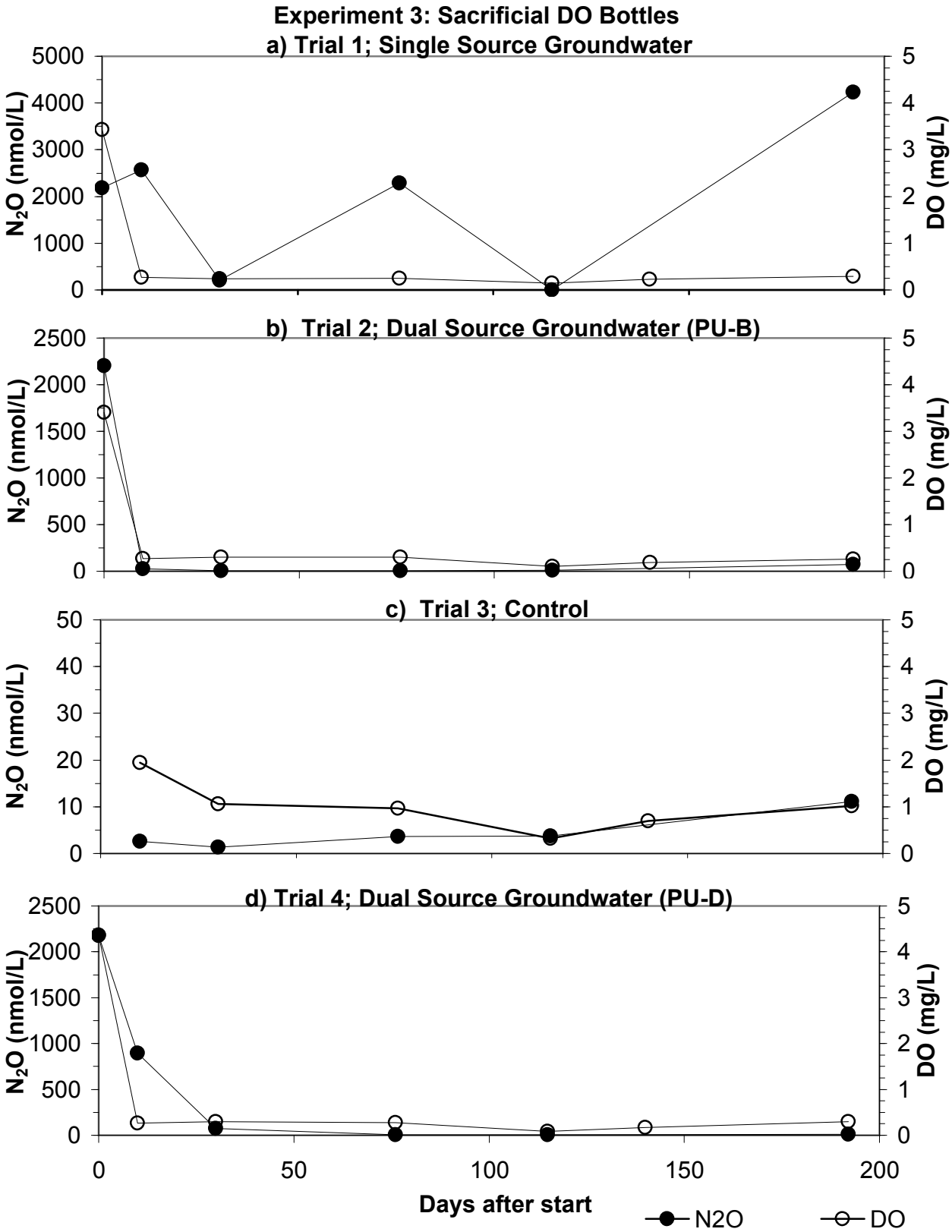

Figure 13. Experiment 3; Putnam groundwater mixed with ~ 50 g sediment in sacrificial bott microcosms (250 mL DO bottles): N_2O and DO trends for **a)** single source groundwater (PU115-2.2m), **b)** dual source groundwater (PU122-2.2m and PU80-1.7m), **c)** DO control anc **d)** dual source groundwater (PU125-2.7m and PU80-1.7m), Sept. 25, 2009 to Jan. 25, 2010.

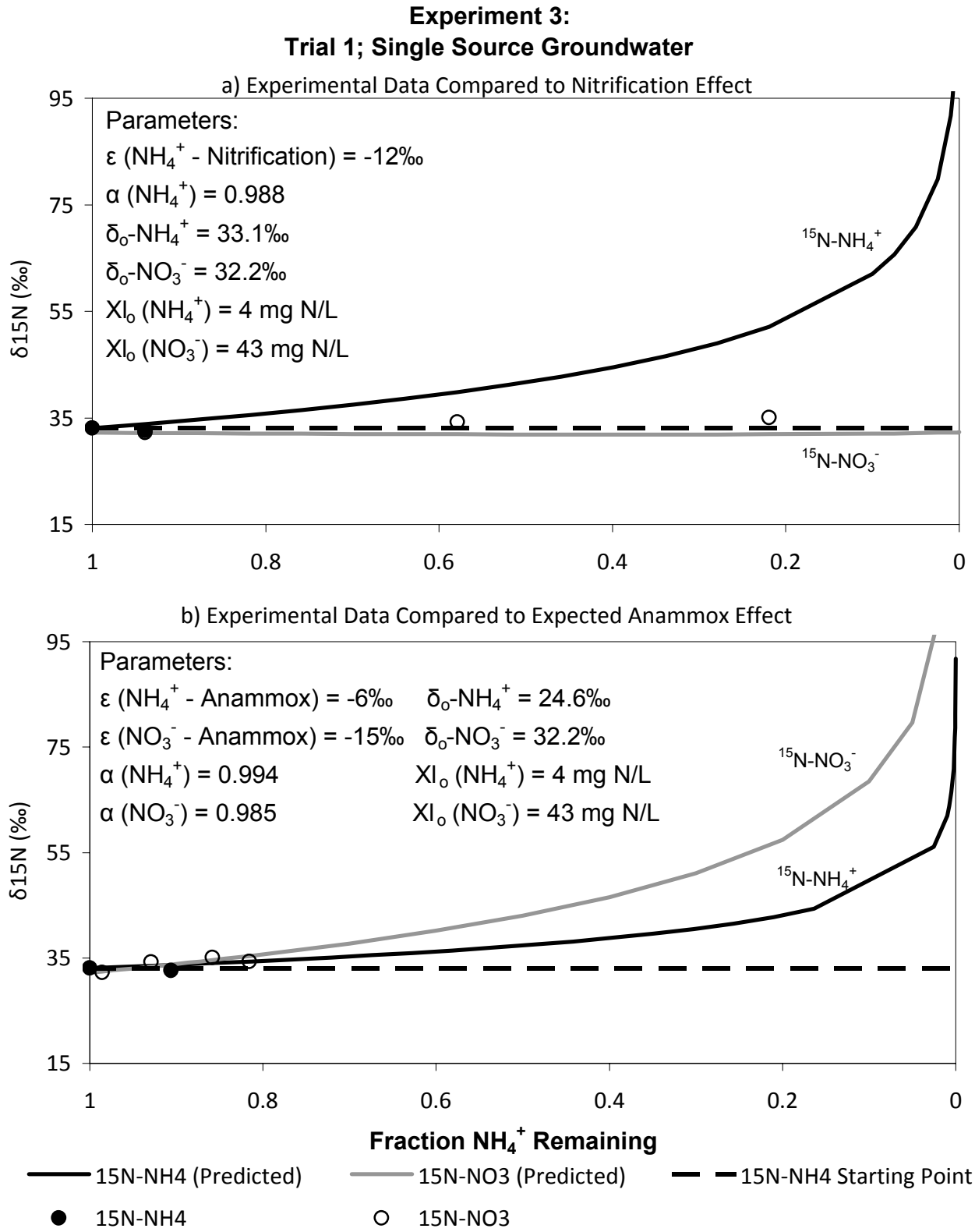

Figure 14. Experiment 3, Trial 1; N isotopic trends compared to isotope evolution expected from **a)** NH_4^+ nitrification and **b)** anammox for a single source groundwater (PU115-2.2m).

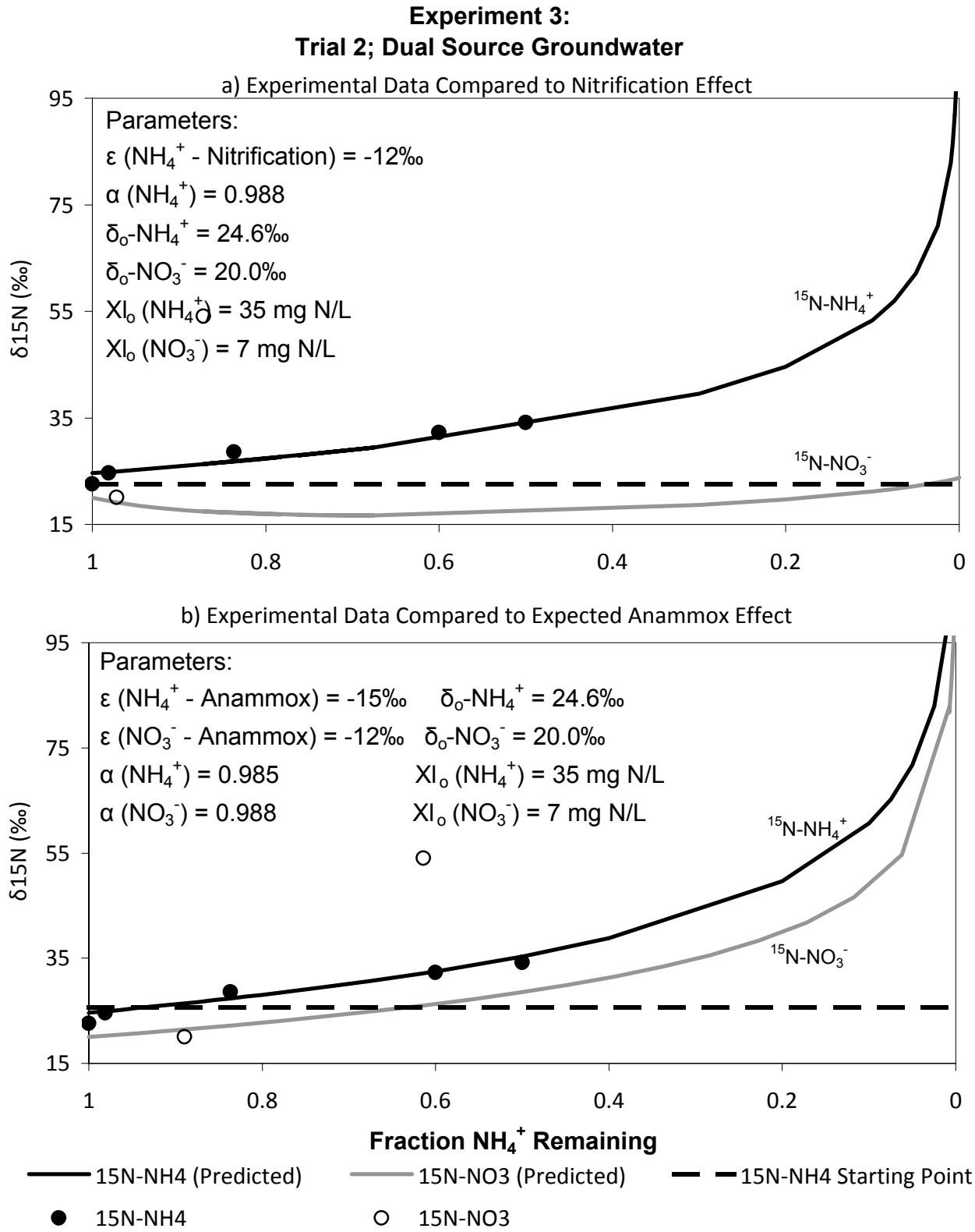

Figure 15. Experiment 3, Trial 2; N isotopic trends compared to isotope evolution expected from **a)** NH_4^+ nitrification and **b)** anammox for a dual source groundwater (PU122-2.2m and PU80-1.7m).

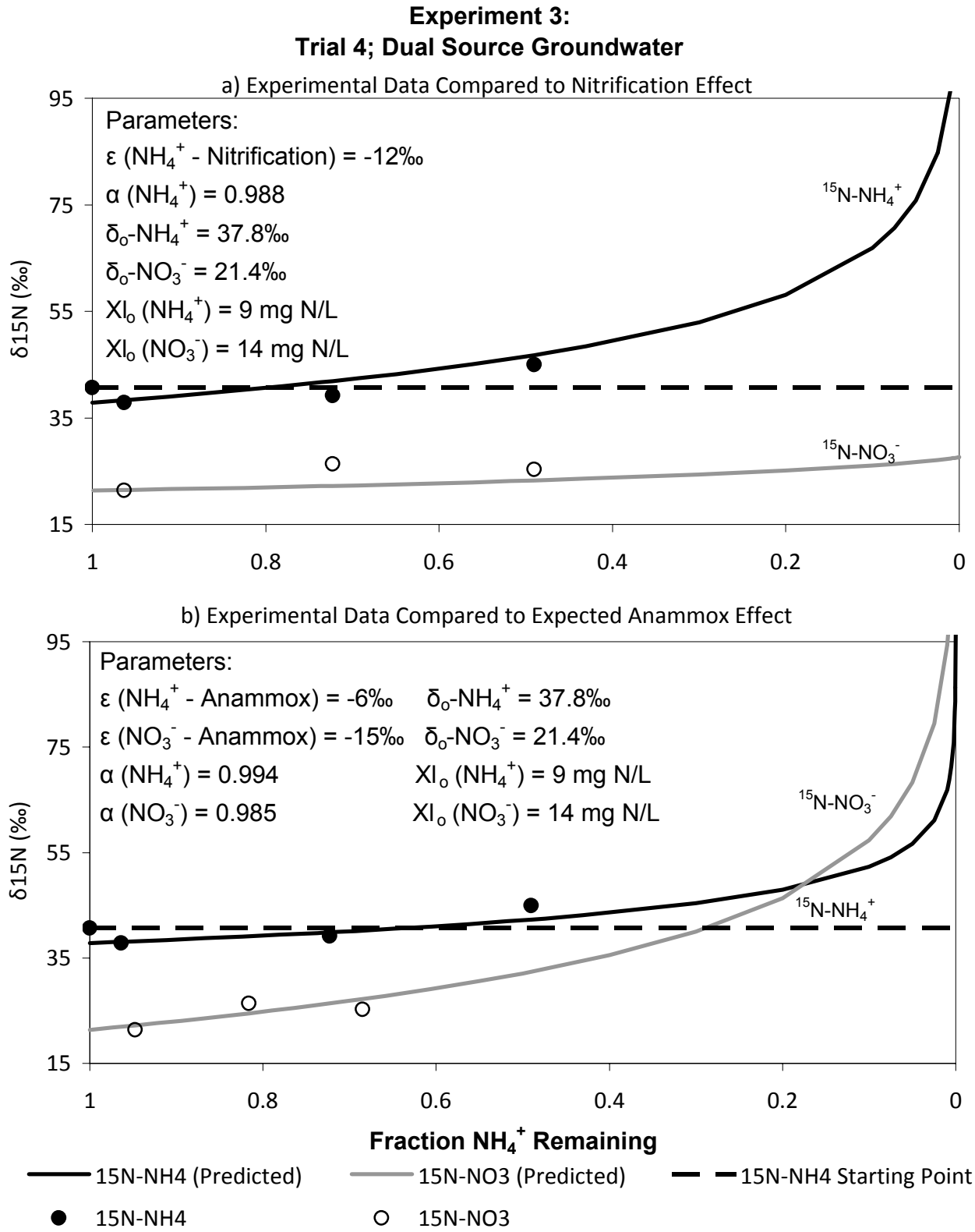

Figure 16. Experiment 3, Trial 4; N isotope trends compared to isotope evolution expected from **a)** NH4+ nitrification and **b)** anammox for a dual source groundwater (PU125-2.7m and PU80-1.7).

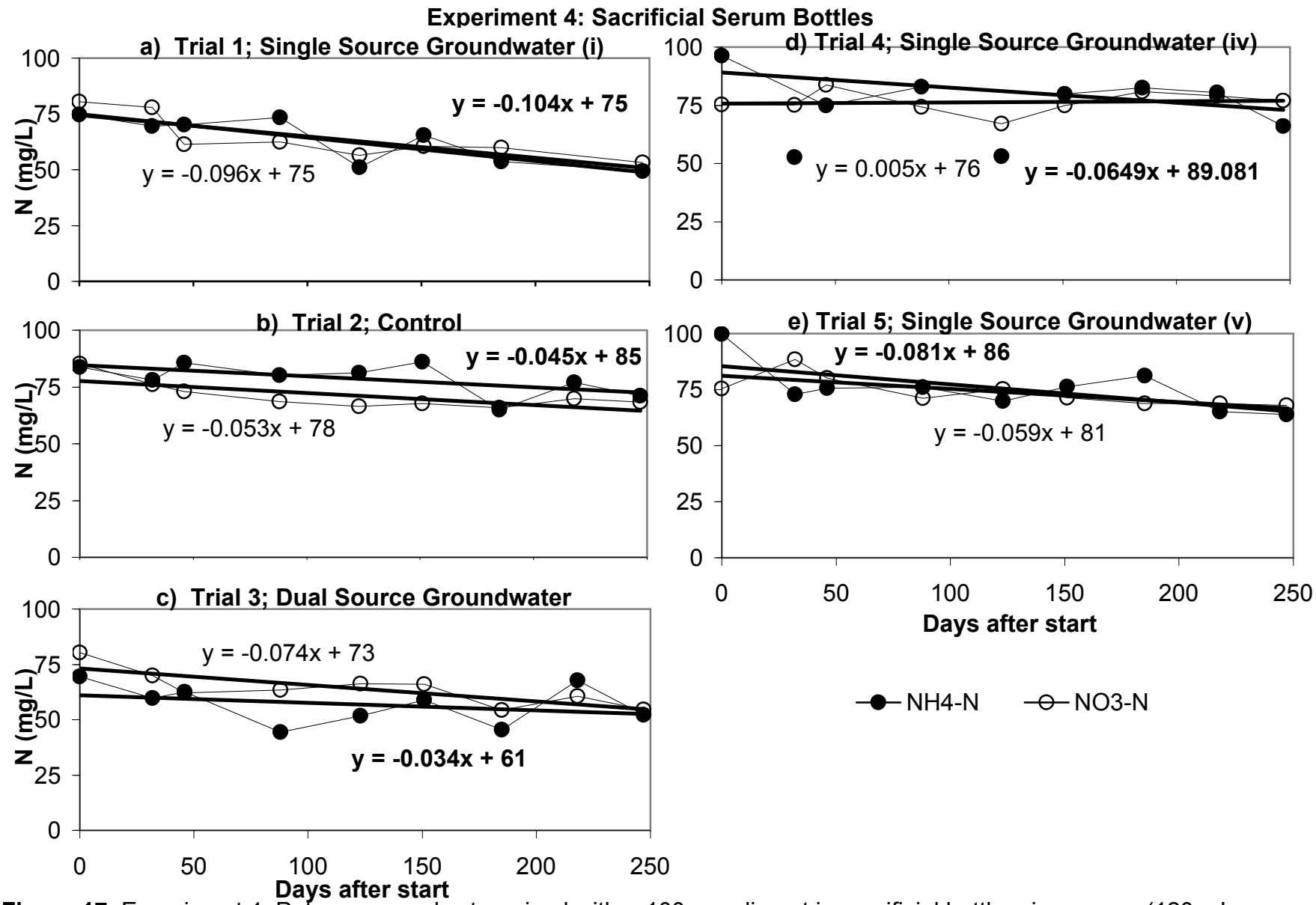

Figure 17. Experiment 4; Putnam groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (120 mL Serum bottles): **a)** single source groundwater (PU121-3.0m), **b)** control (PU121-3.0m), **c)** dual source groundwater (PU115-3.0m and PU84-3.1m), **d)** single source groundwater (PU86-3.1m) and **e)** single source groundwater (PU115-2.2m Each bottle set was injected with NH4NO3 for increased substrate concentrations, Oct. 25, 2010 to Jun. 24, 2010.

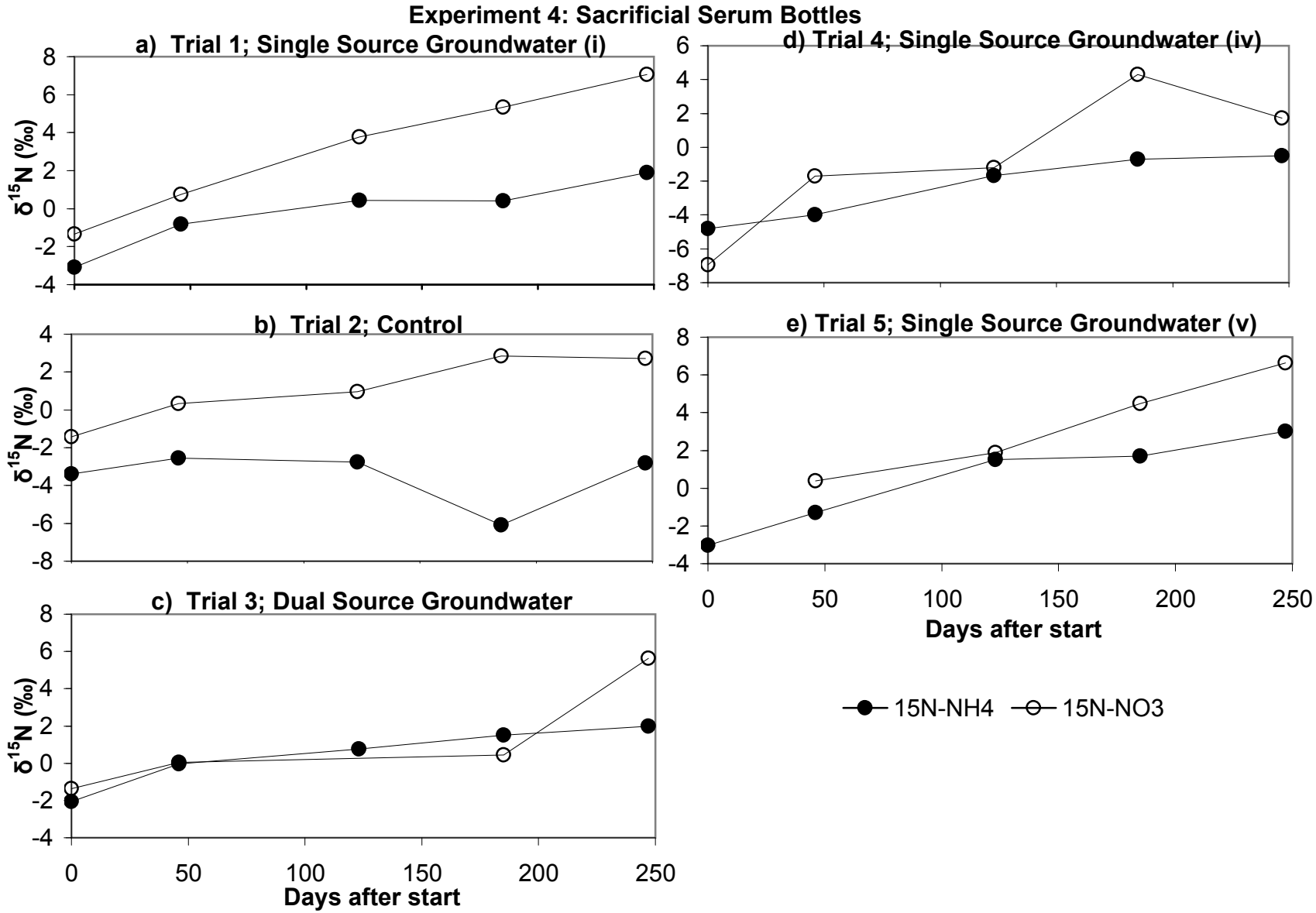

Figure 18. Experiment 4; isotopic evolution trends in sacrificial bottles: **a)** Trial 1 (121-3.0m), **b)** Trial 2 (Control, 121-3.0m **c)** Trial 3 (PU115-3.0m and PU84-3.1m), **d)** Trial 4 (PU86-3.1m) and **e)** Trial 5 (PU115-2.2m), Oct. 25, 2010 to Jun. 24, 2010

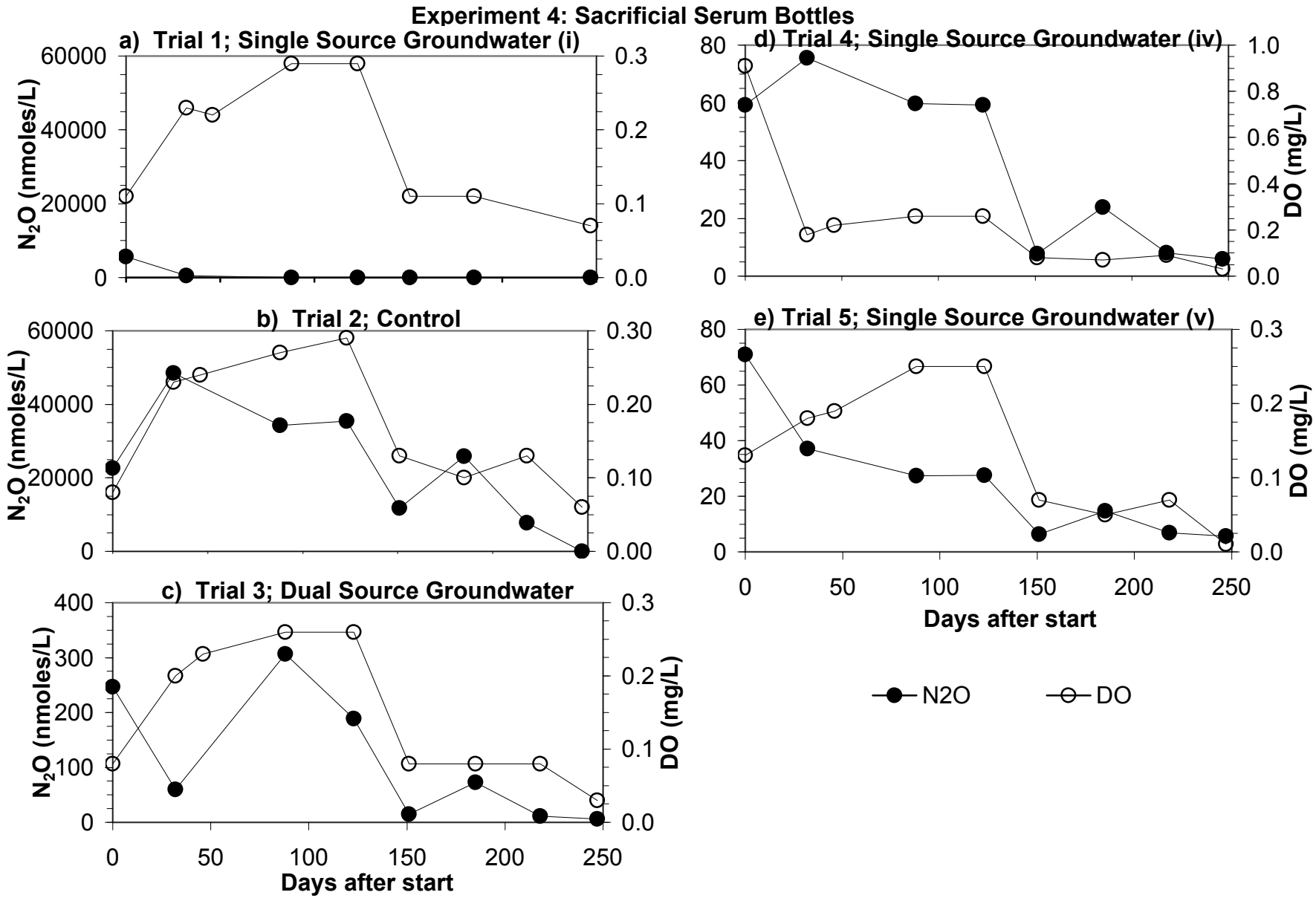

Figure 19. Experiment 4; N_2O and DO evolution trends in sacrificial bottles: **a)** Trial 1 (121-3.0m), **b)** Trial 2 (Control, 121-3.0m), **c)** Trial 3 (PU115-3.0m and PU84-3.1m), **d)** Trial 4 (PU86-3.1m) and **e)** Trial 5 (PU115-2.2m), Oct. 25, 2010 to Jun. 24, 2010

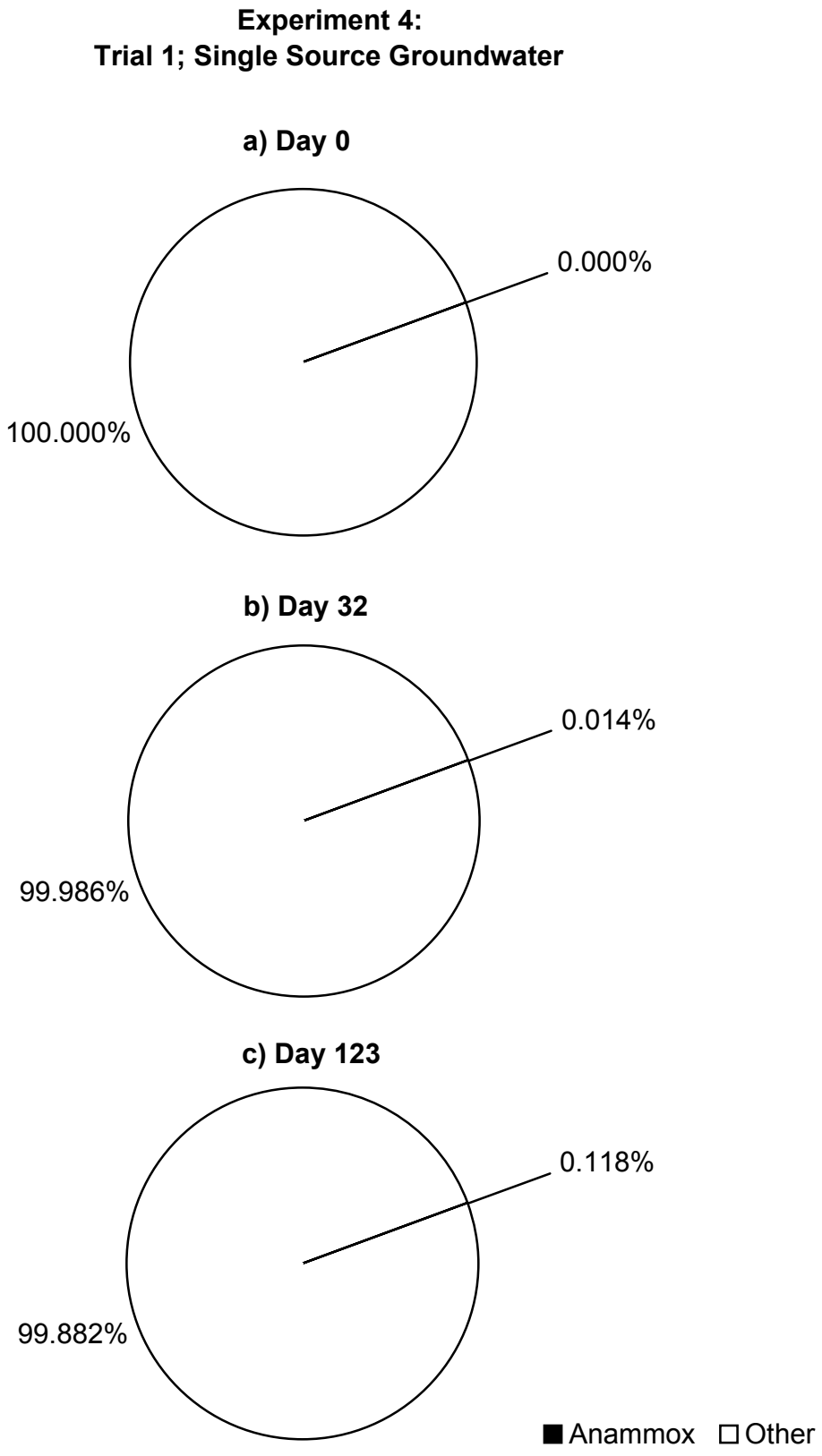

Figure 20. Experiment 4, Trial 1; single source groundwater (PU121-3.0m) anammox bacterial community evolution via qPCR analysis at **a)** 0 days **b)** 32 days and **c)** 123 days. Data analysis by Tara Moore of the University of Waterloo Biology Department

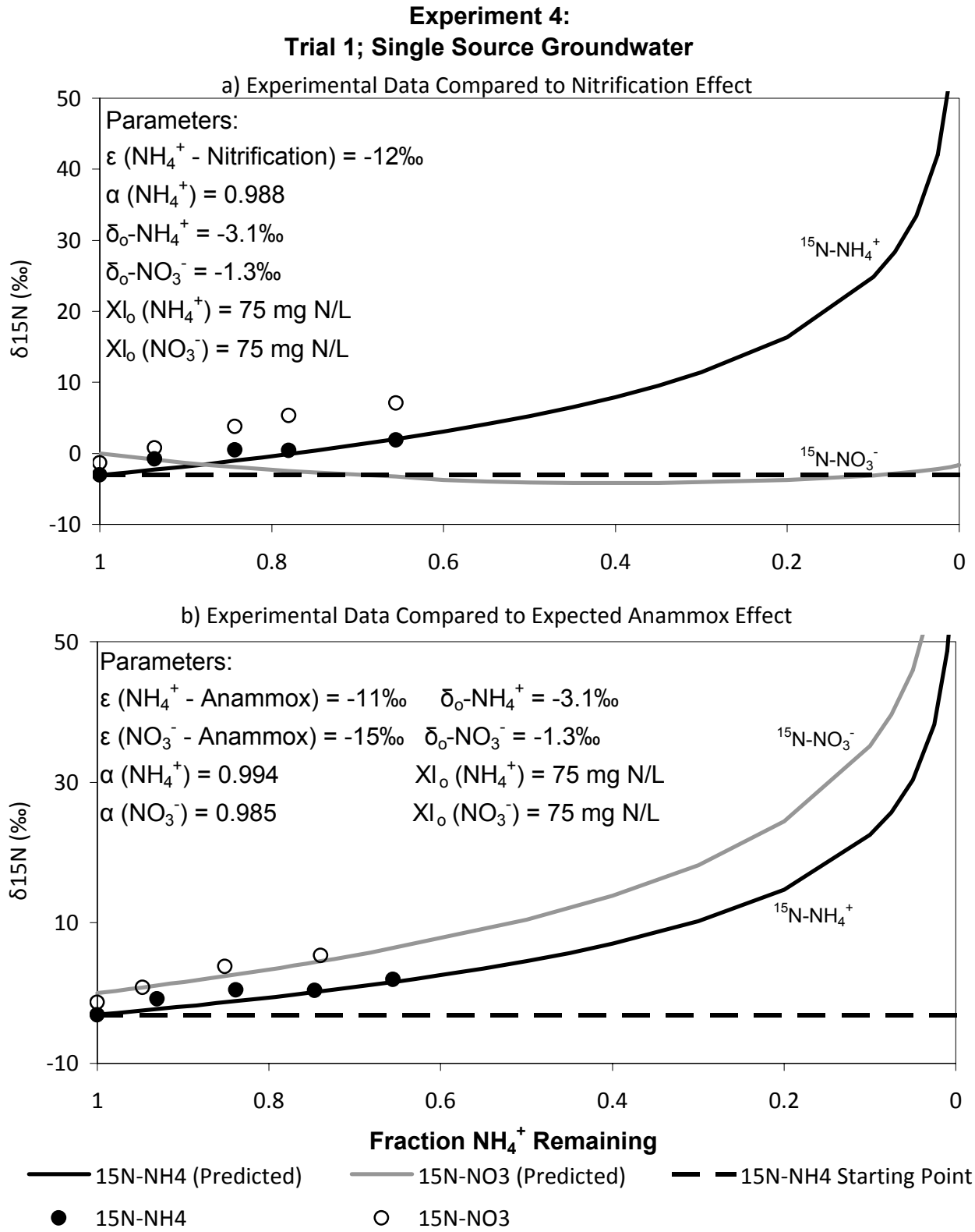

Figure 21. Experiment 4, Trial 1; N isotopic trends compared to isotope evolution expected from **a)** NH_4^+ nitrification and **b)** anammox for a single source groundwater (PU121-3.0m spiked with NH_4NO_3.)

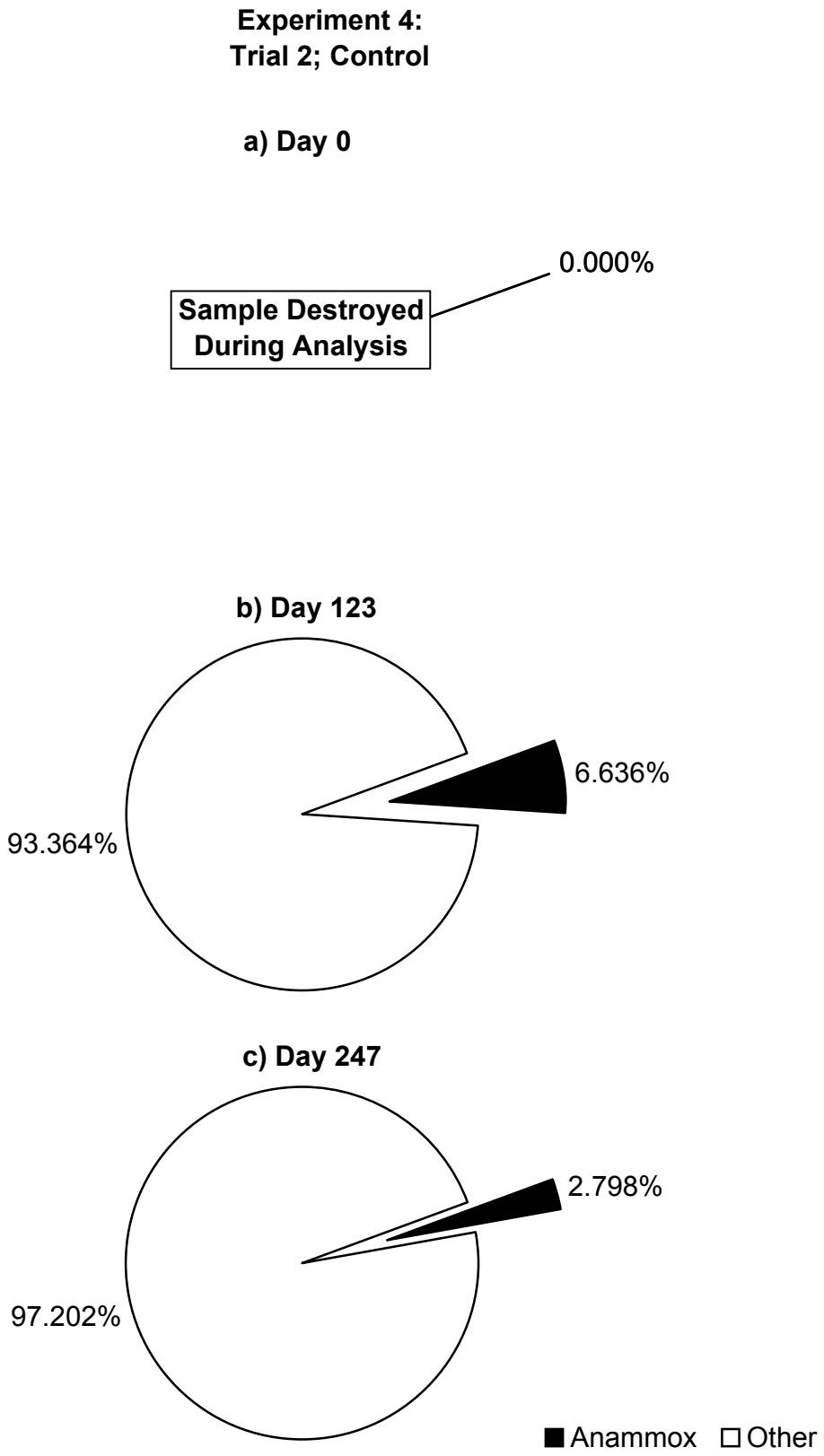

Figure 22. Experiment 4, Trial 2; control (single source groundwater, PU121-3.0m, in silica sand) anammox bacterial community evolution via qPCR analysis at **a)** 0 days, **b)** 123 days and **c)** 247 days. Data analysis by Tara Moore of the University of Waterloo Biology Department.

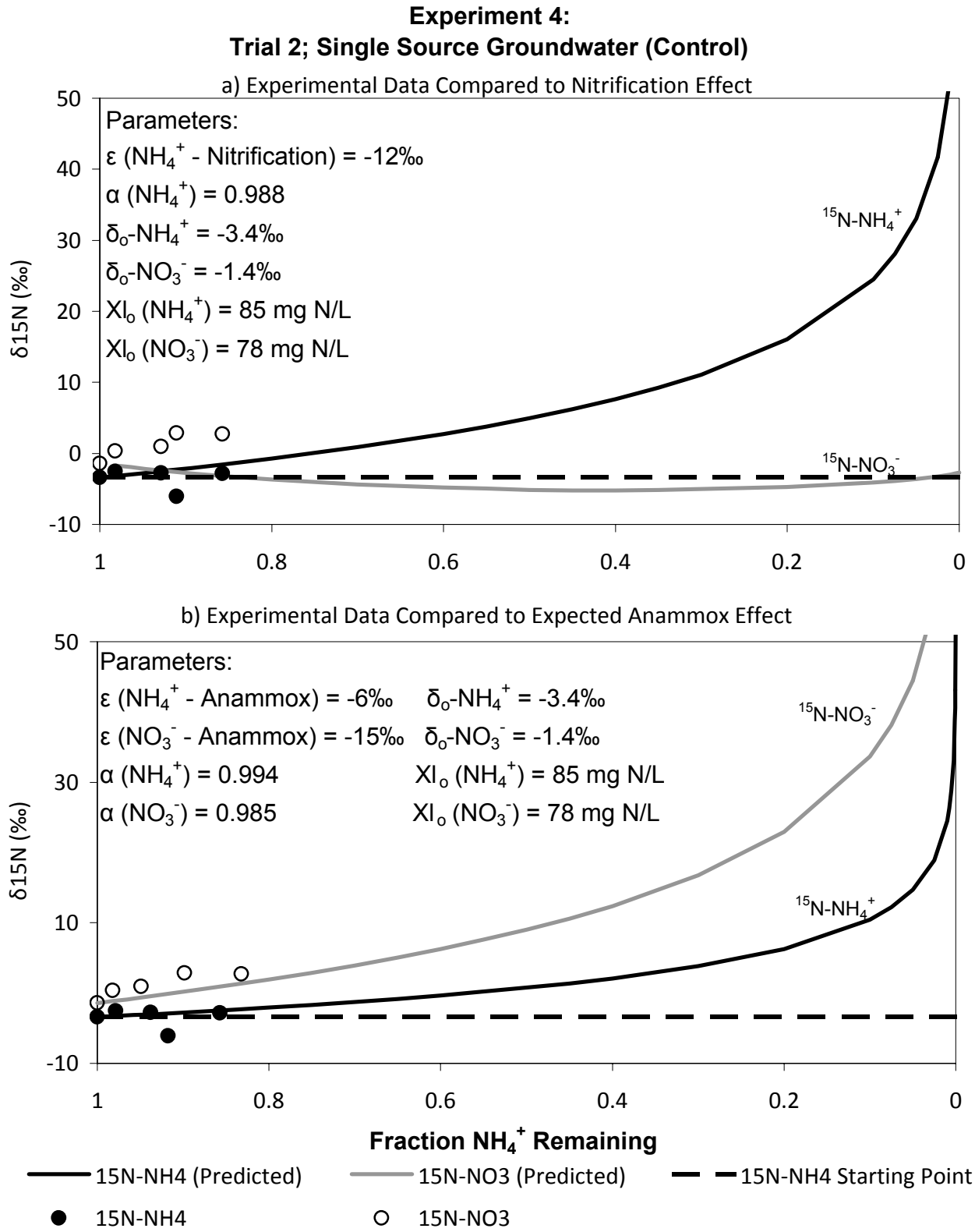

Figure 23. Experiment 4, Trial 2 (Control); N isotopic trends compared to isotope evolution expected from **a)** NH_4^+ nitrification and **b)** anammox for a single source groundwater (PU121-3.0m Control, spiked with NH_4NO_3, with silica sand instead of sediment).

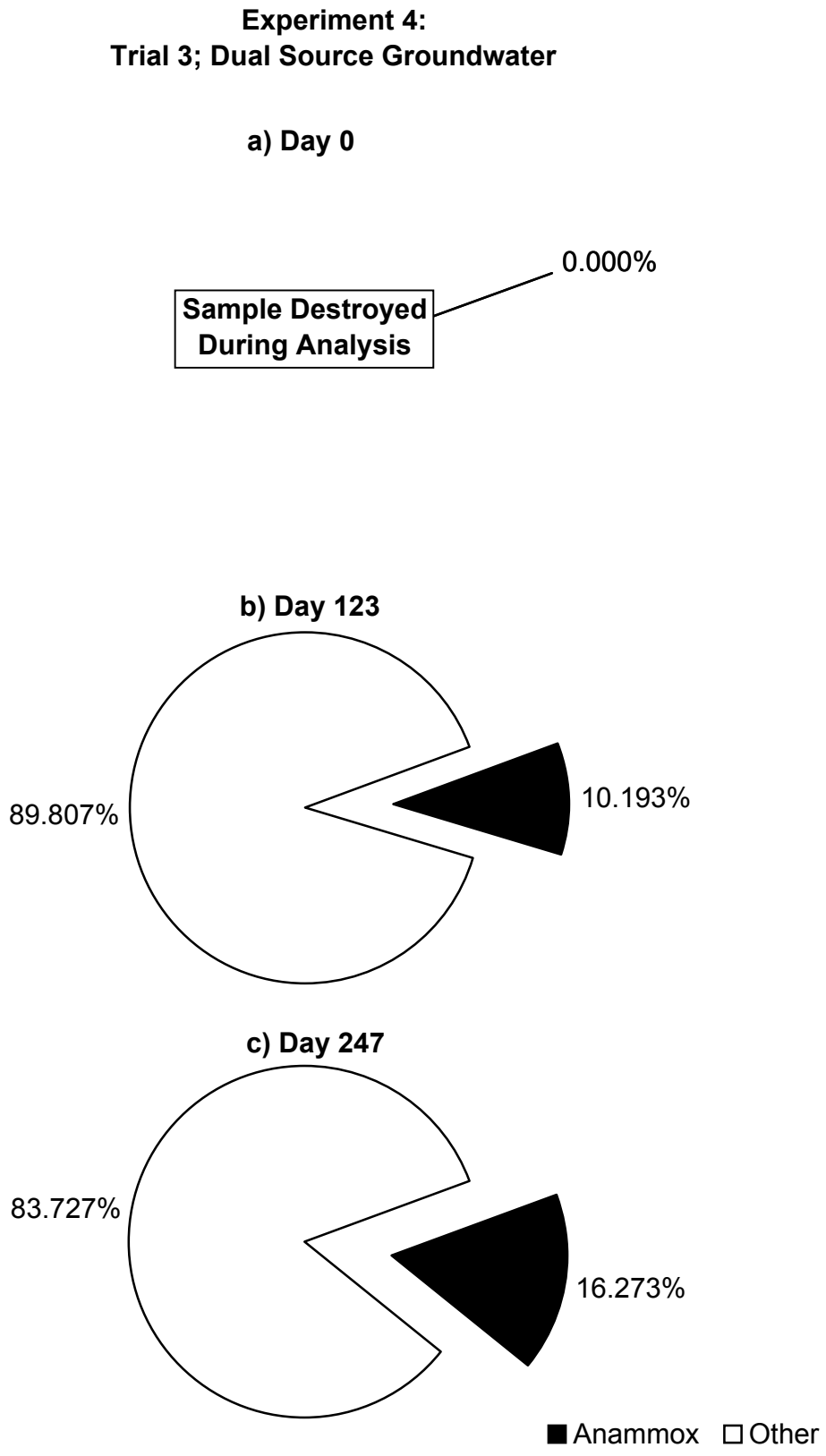

Figure 24. Experiment 4, Trial 3; dual source groundwater (PU115-3.0m and PU84-3.1m) anammox bacterial community evolution via qPCR analysis at**a)** 0 days, **b)** 123 days and **c)** 247 days. Data analysis by Tara Moore of the University of Waterloo Biology Department

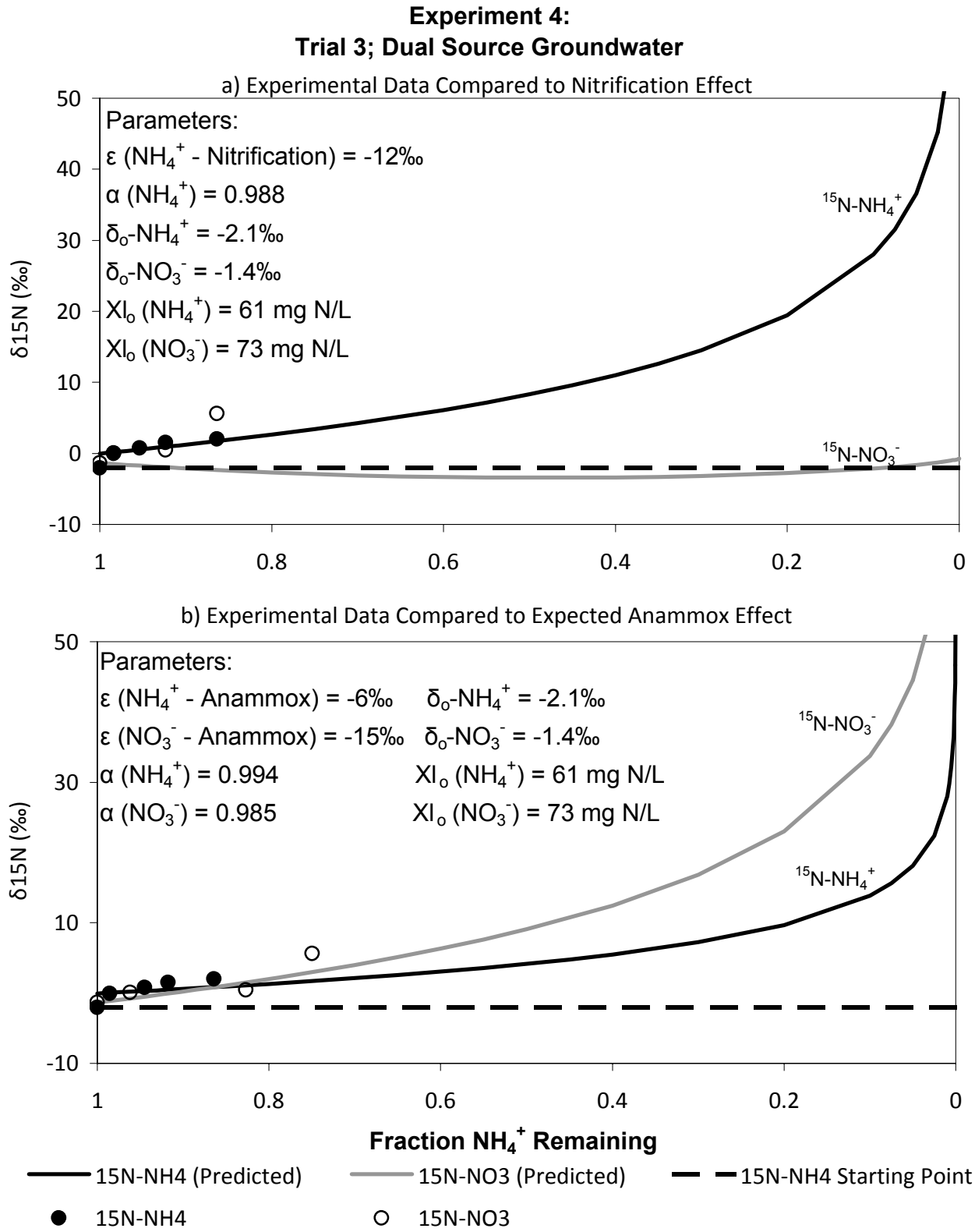

Figure 25. Experiment 4, Trial 3; N isotopic trends compared to isotope evolution expected from **a)** NH_4^+ nitrification and **b)** anammox for a dual source groundwater (PU115-3.0m and PU121-3.0m, spiked with NH_4NO_3).

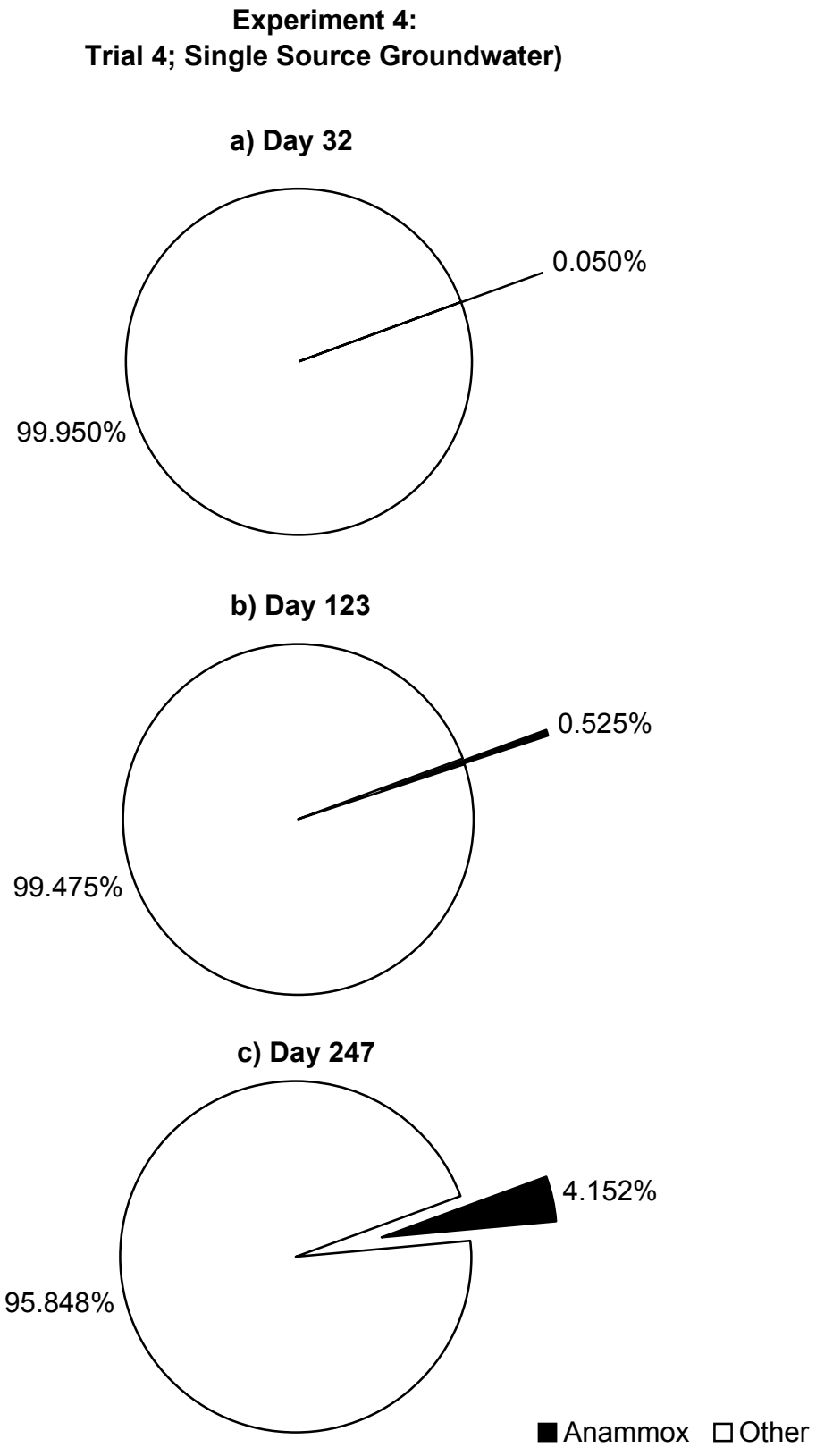

Figure 26. Experiment 4, Trial 4; single source groundwater (PU86-3.1m) anammox bacterial community evolution via qPCR analysis at **a)** 32 days **b)** 123 days and **c)** 247 days. Data analysis by Tara Moore of the University of Waterloo Biology Department

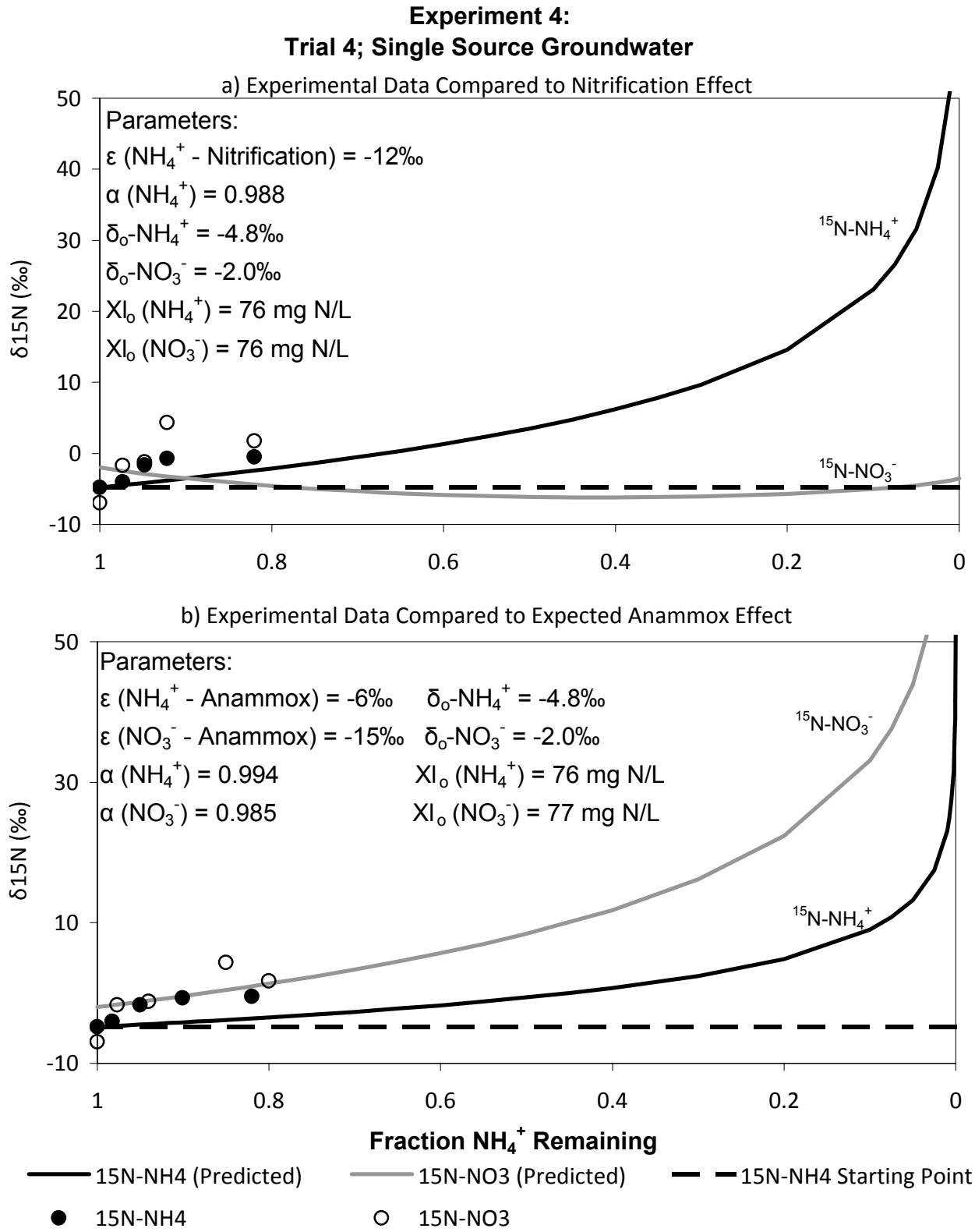

Figure 27. Experiment 4, Trial 4; N isotopic trends compared to isotope evolution expected from **a)** NH_4^+ nitrification and **b)** anammox for a single source groundwater (PU86-3.1m, spiked with NH_4NO_3).

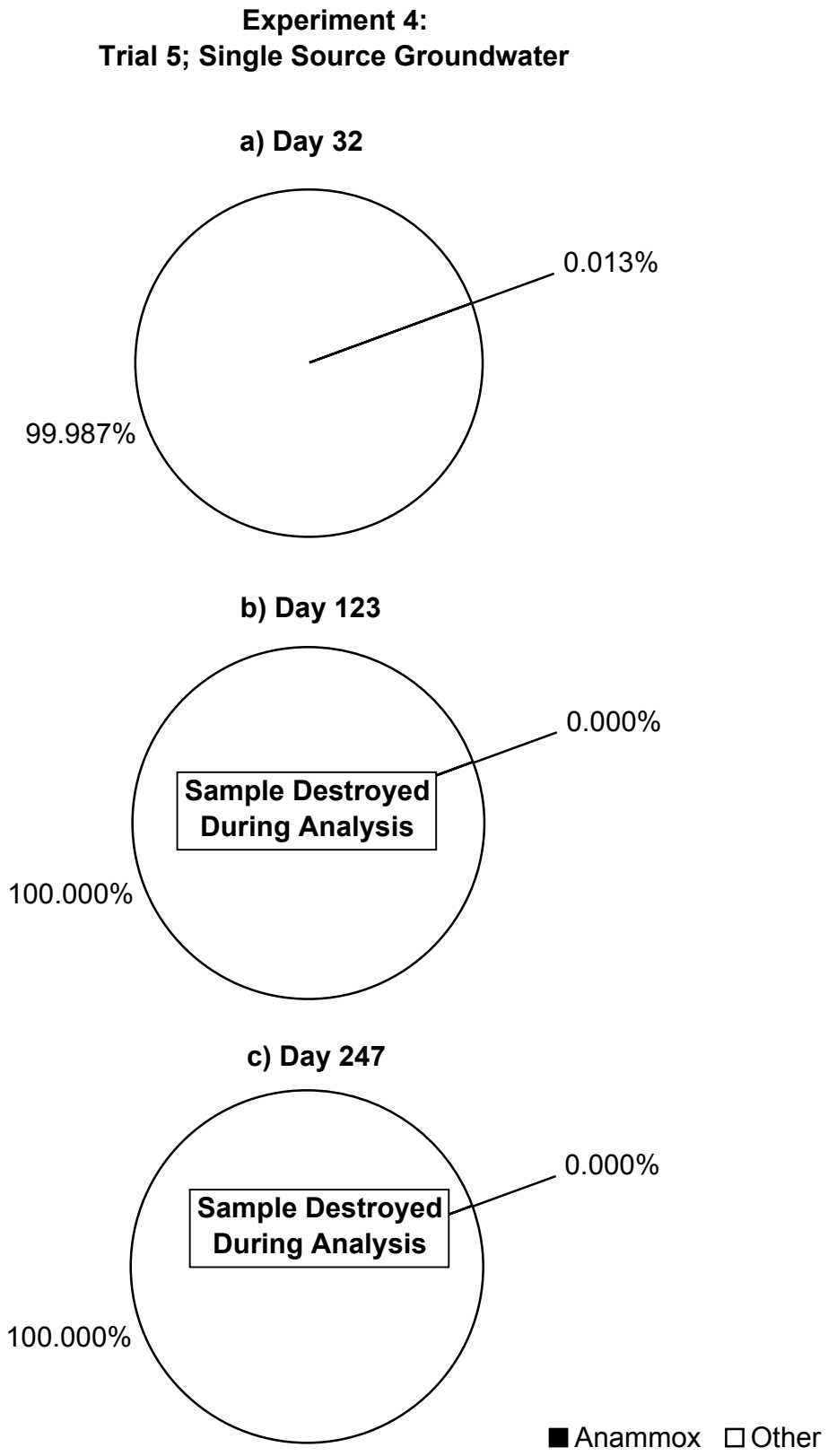

Figure 28. Experiment 4, Trial 1; single source groundwater (PU121-3.0m) anammox bacterial community evolution via qPCR analysis at **a)** 0 days **b)** 32 days and **c)** 123 days. Data analysis by Tara Moore of the University of Waterloo Biology Department

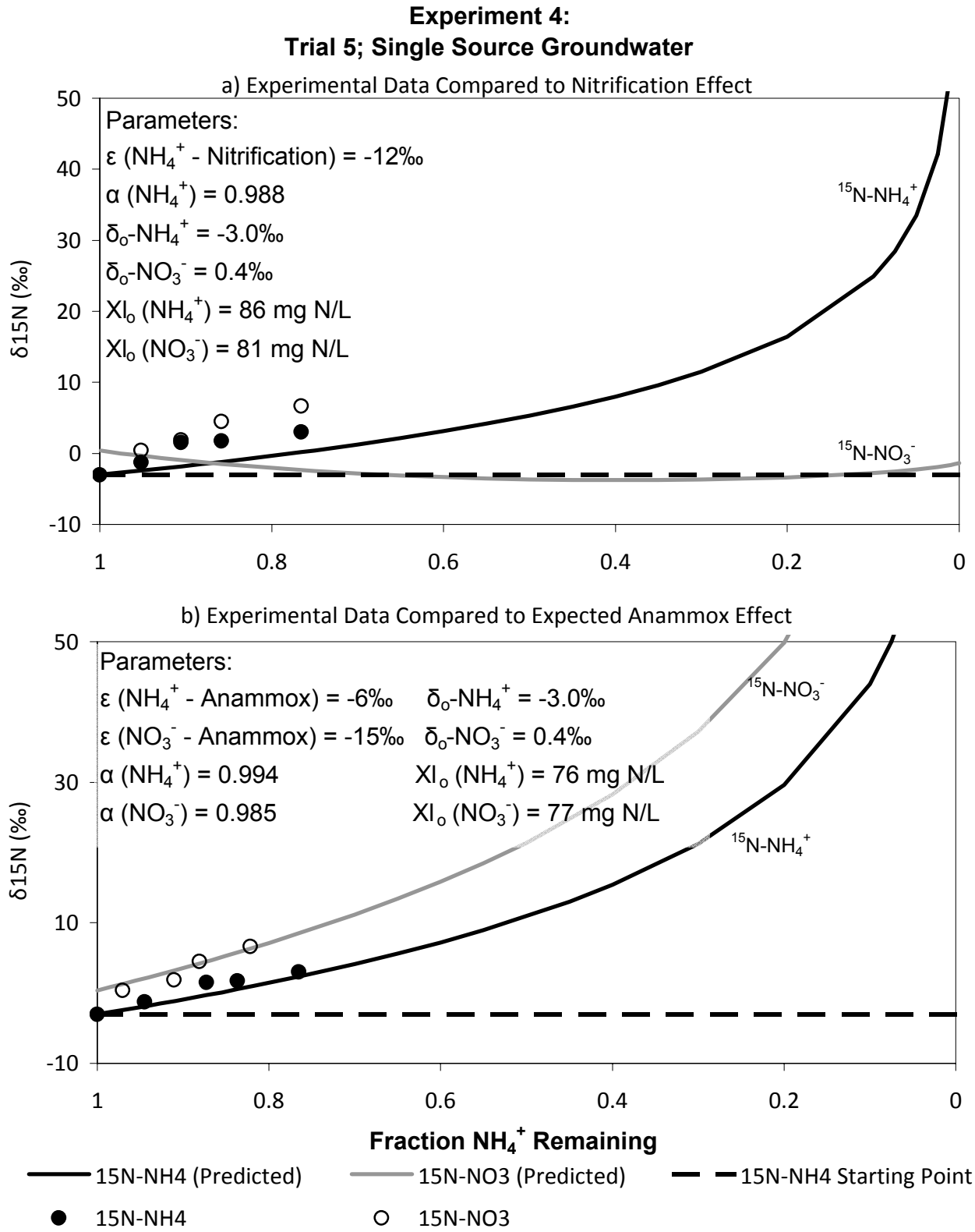

Figure 29. Experiment 4, Trial 5; N isotopic trends compared to isotope evolution expected from **a)** NH_4^+ nitrification and **b)** anammox for a single source groundwater (PU115-2.2m, spiked with NH_4NO_3).

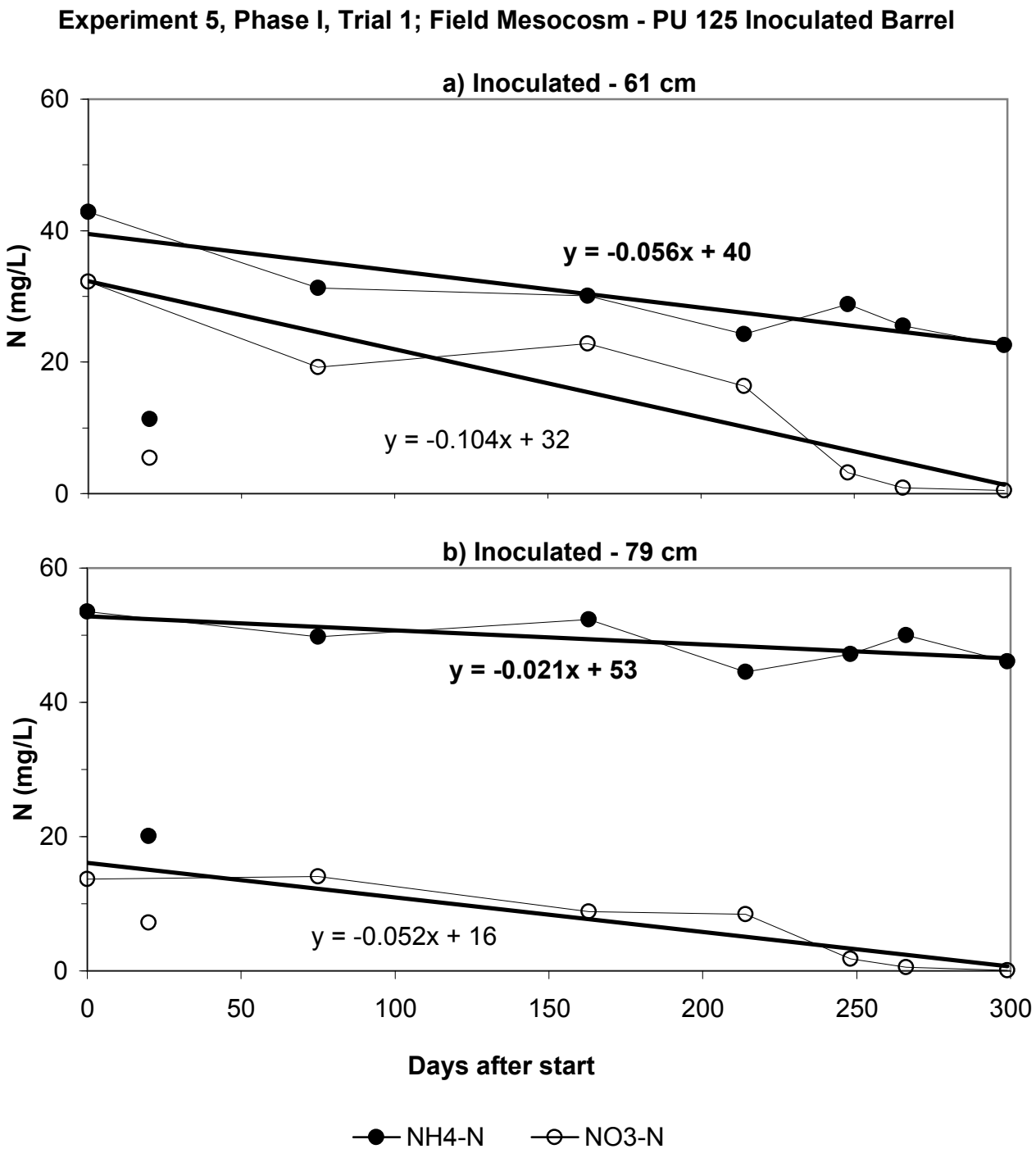

Figure 30. Experiment 5 (Field Barrels), Phase I, Trial 1 (Inoculated) Putnam (PU115-2.2m) groundwater mixed with ~ 10% core drilled from suspected anammox zones, sampled at bottom two ports: a) 61 cm and b) 79 cm, Sept. 25, 2009 to Aug. 19, 2010.

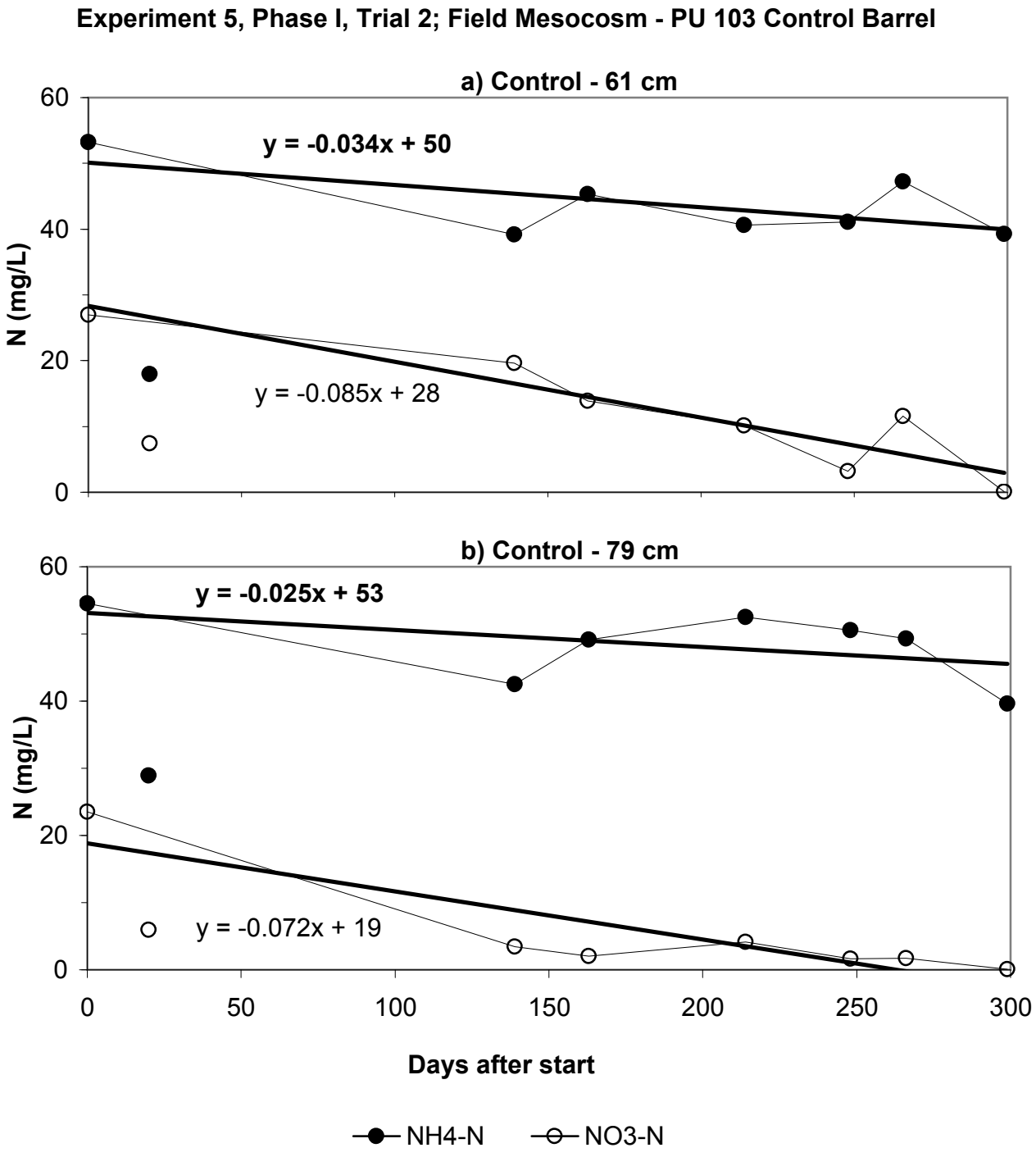

Figure 31. Experiment 5 (Field Barrels), Phase I, Trial 2 (Control) Putnam (PU115-2.2m) groundwater mixed with sand from local pit, sampled at bottom two ports: a) 61 cm anc b) 79 cm, Sept. 25, 2009 to Aug. 19, 2010.

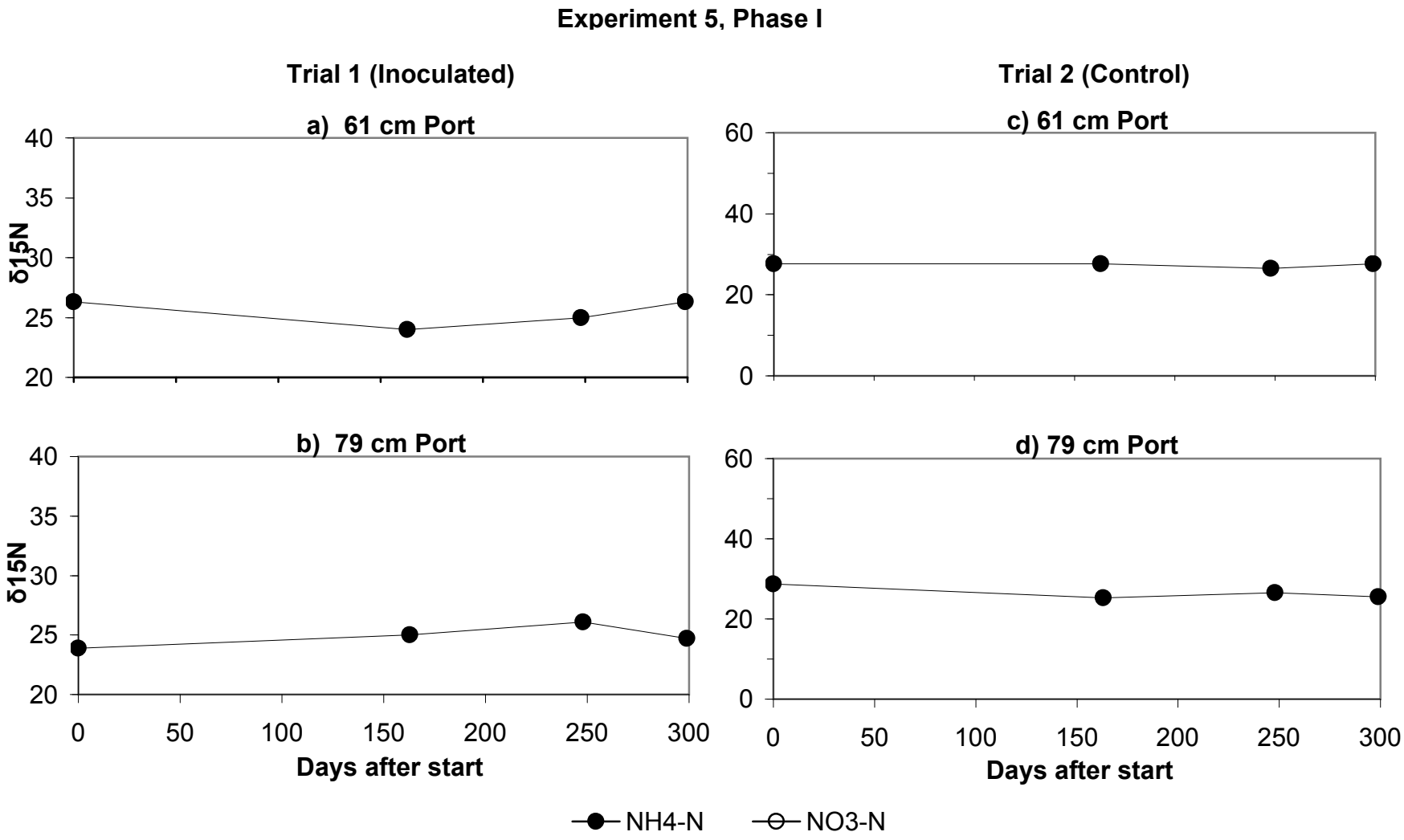

Figure 32. Experiment 5; isotopic evolution trends in field mesocosms: **a)** Innoculated Reactor (61 cm), **b)** Innoculated Reactor (79 cm), **c)** Control Reactor (61cm), and **d)** Control Reactor (79cm), Sept. 25, 2009 to Aug. 19, 2010

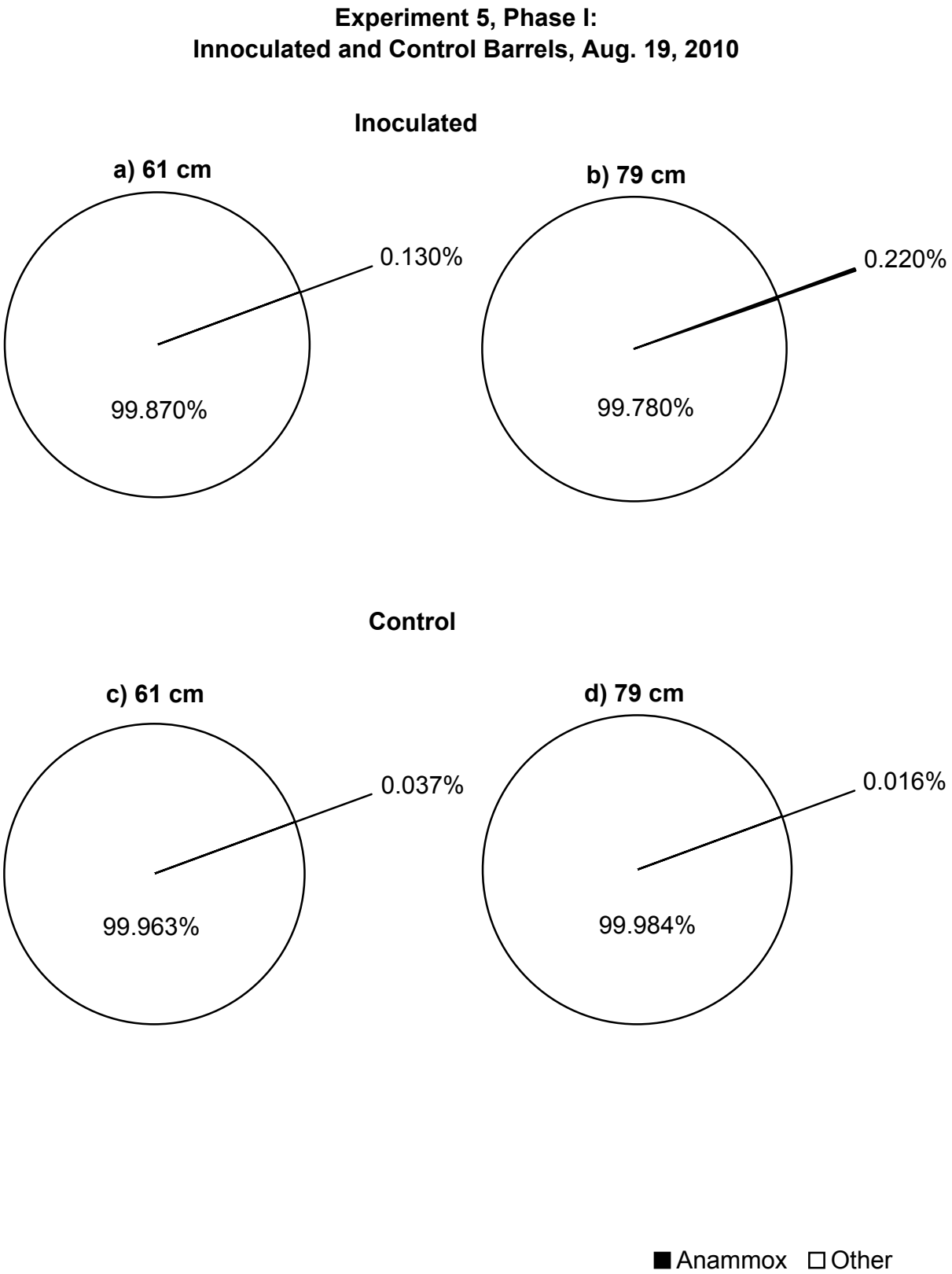

Figure 33. Experiment 5 (Field Reactors); single source groundwater (PU115-2.2m) anammox bacterial community evolution via qPCR analysis on Aug. 19, 2010 at: **a)** Inoculated - 61 cm **b)** Inoculated - 79 cm **c)** Control - 61 cm and **d)** Control - 79 cm. Data analysis by Tara Moore of the University of Waterloo Biology Department

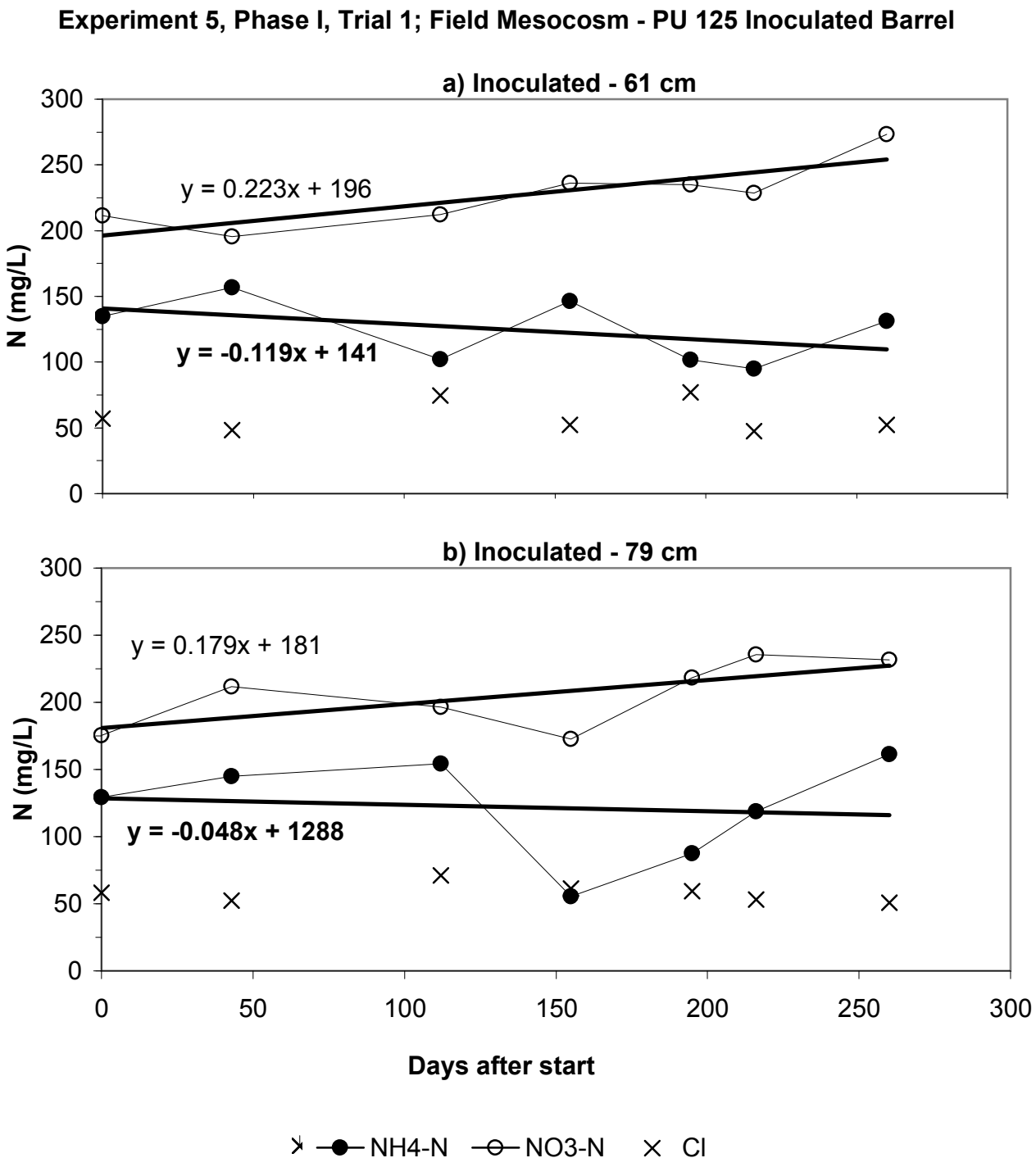

Figure 34. Experiment 5 (Field Barrels), Phase II, Trial 1 (Inoculated) Putnam (PU115-2.2m) groundwater mixed with ~ 10% core drilled from suspected anammox zones, sampled at bottom two ports: a) 61 cm and b) 79 cm, Sept. 30, 2010 to Jun. 17, 2011.

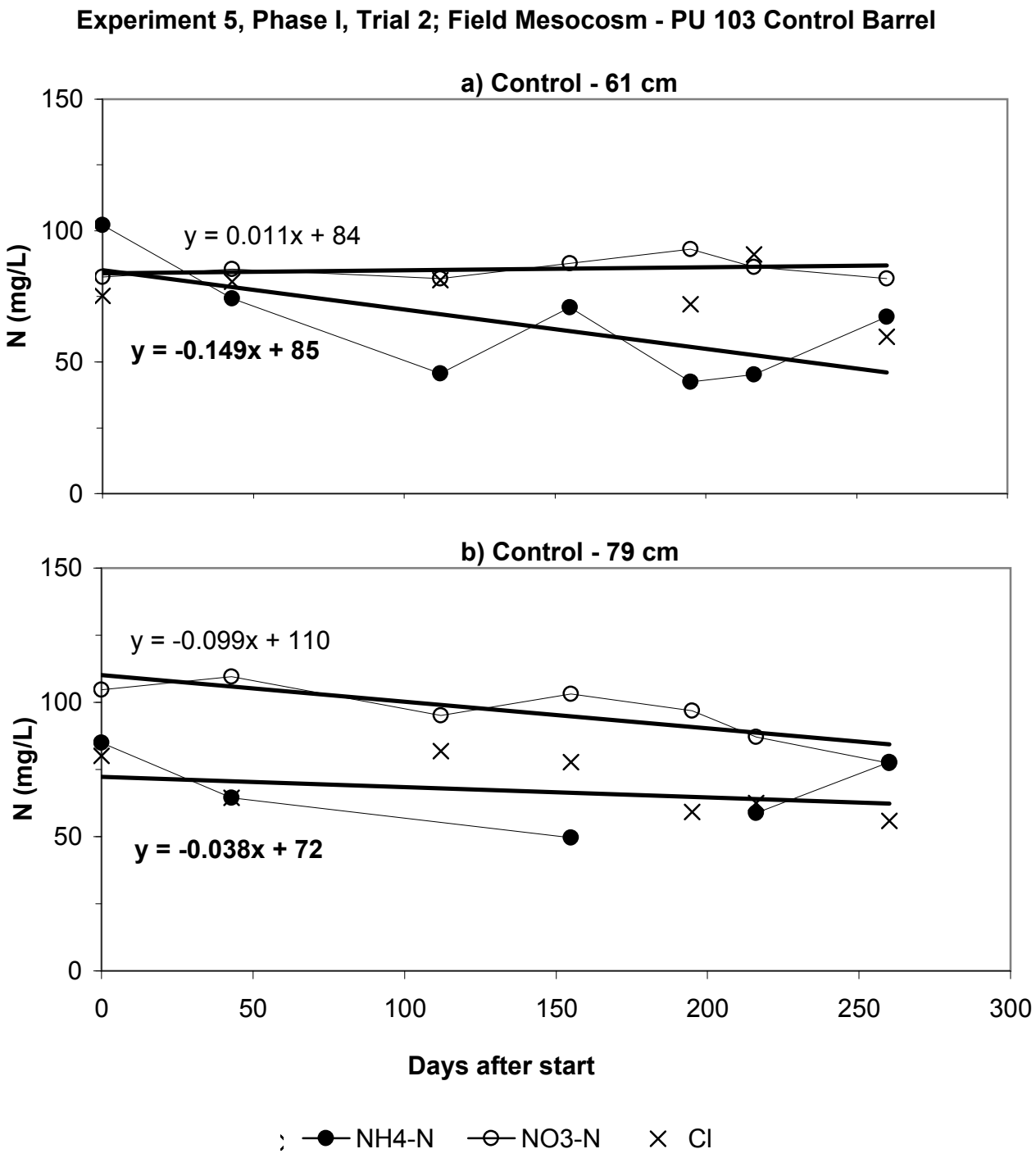

Figure 35. Experiment 5 (Field Barrels), Phase II, Trial 2 (Control) Putnam (PU115-2.2m) groundwater mixed with sand from local pit, sampled at bottom two ports: a) 61 cm anc b) 79 cm, Sept. 30, 2010 to Jun. 17, 2011.

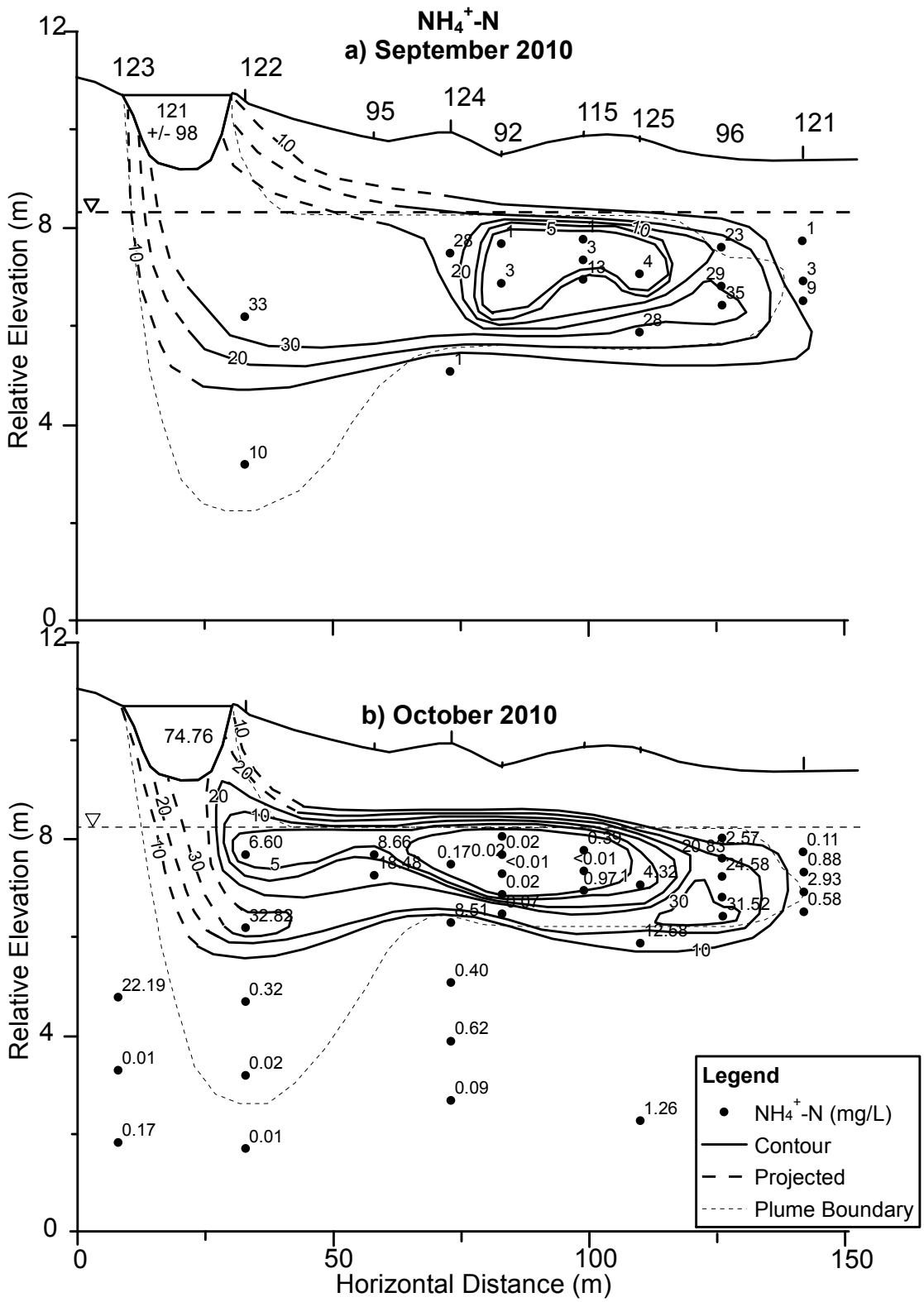

Figure 36. Zorra site; NH_4^+ distribution along the plume centre line: **a)** September 2010 and **b)** October 2010 (vertical exaggeration approx. 10.) Sept. 2010 pond value is mean and standard deviation from Aug. 28 2008 to Nov. 13 2009 (n=9) by Lazenby (2011). Plume boundary based on Na^+=30 mg/L, as described in Lazenby (2011).

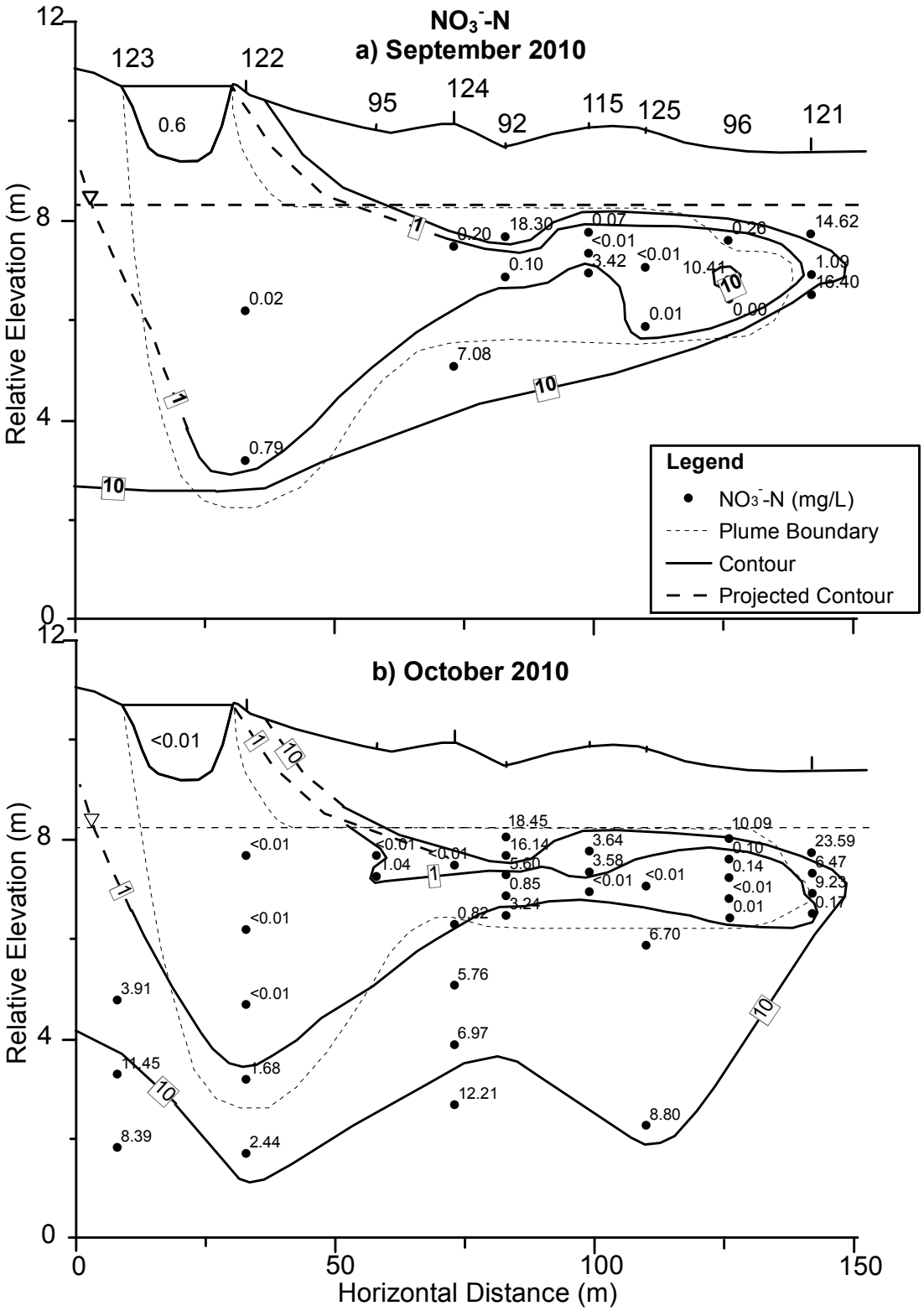

Figure 37. Zorra site; NO_3^- distribution along the centre line: **a)** September 2010 and **b)** October 2010 (vertical exaggeration of approx. 10). Sept. 2010 pond value is the mean and standard deviation measured during Aug. 28 2008 to Nov. 13 2009 (n=9) by Lazenby (2011). Plume boundary based on Na^+=30 mg/L, described in Lazenby (2011)

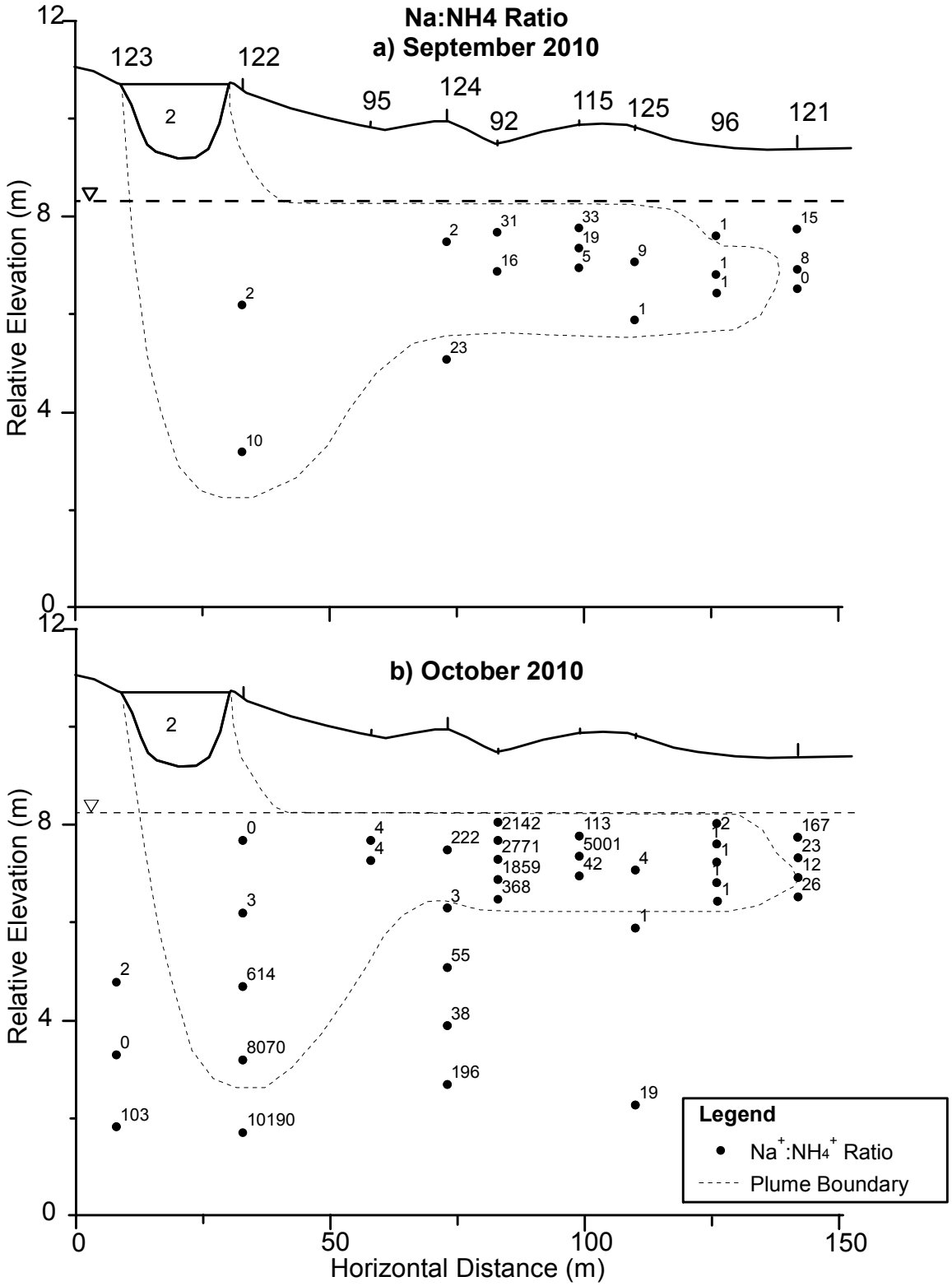

Figure 38. Zorra site; Na^+:NH_4^+ distribution along the centre line: **a)** September 2010 and **b)** October 2010 (vertical exaggeration of approx. 10). Sept. 2010 pond value is the mean measured during Aug. 28 2008 to Nov. 13, 2009 (n=9) by Lazenby (2011). Plume boundary based on Na^+=30 mg/L, as described in Lazenby (2011).

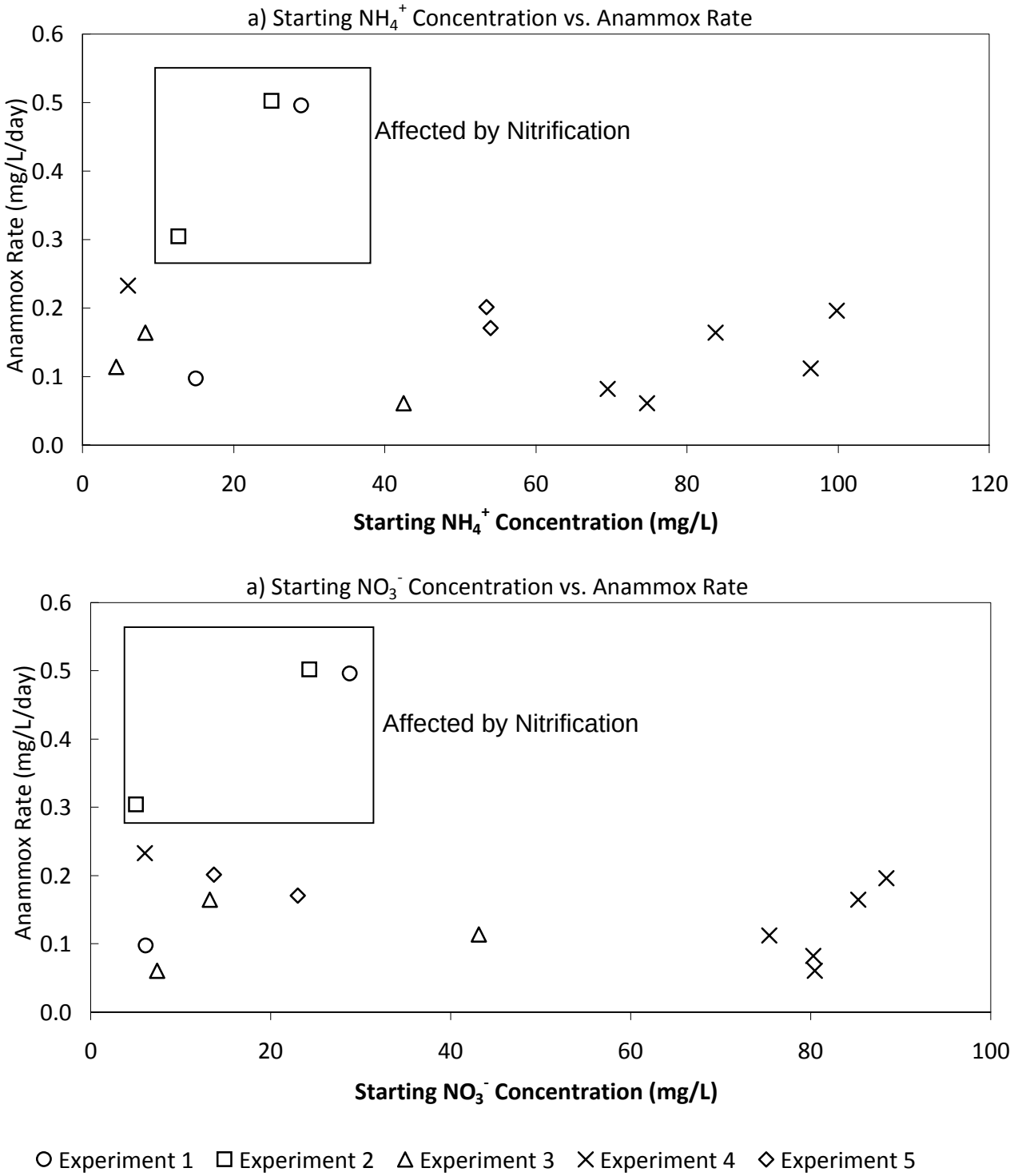

Figure 39. Anammox rates vs. starting concentrations of **a)** NH_4^+ and **b)** NO_3^-. Anammox rates based on assumption that all NH_4^+ loss is consumed by anammox bacteria. Note the three experiments with >0.3 mg/L/day anammox rate experienced nitrification, likely resulting in an overestimation of the anammox rate.

Appendix A:
Nitrogen Contamination – Health and Environmental Concerns

Health & Environmental Concerns: Agricultural Nitrogen Groundwater Contamination

A.1 Nitrogen Species as Carcinogens

Nitrate consumption is also starting to be linked to increases in cancer rates (Ward et al., 2010). Though N-nitroso compounds have long been suspected of a role in cancer rates (WHO, 1978), increasing studies, though debatable, are suggesting evidence supports an increase in some forms of cancer (bladder, ovarian, thyroid, etc) with increasing levels of drinking water NO_3^- (Weyer et al, 2001, Ward et al, 2010, others).

Though it was discovered as early as 1956 that nitrosamines were carcinogenic (Williams 1988), most N-nitroso compounds are now considered as "potent animal carcinogens" (Ward et al., 2011). N-nitroso compounds are formed during the process known as nitrosation, where organic compounds (amines and amides) are converted into nitroso products (Mirvish 1995), typically by transforming NO_2^-:

$$NO_2^- + 2H^+ \rightarrow NO^+ + H_2O \qquad \text{(Eq. 1.1)}$$

$$R_2NH + NO^+ \rightarrow R_2N\text{-}NO + H^+ \qquad \text{(Eq. 1.2)}$$

Nitrosation has been of interest to organic chemists since 1846, when Piria performed the nitrosation process on sliphatic primary amines (Williams 1988). Nitrosation occurs under acidic conditions, such as those located in the stomach, where NO_3^- is readily oxidized to NO_2^- (Banbury Report, 1982) and reactions such as Equation 1.1 and 1.2 proceed.

In 1970, Sugimura et al. showed that a N-nitroso compound (N-methyl-N^1-nitro-N-nitrosoguanidine) in the water supply caused cancer in the stomach, duodenum, jejunum, liver, and mesentery of rats (Sugimura et al. 1970). Recent studies (Correa 1992, Ward et al. 2011) suggest that a high NO_3^- and NO_2^- intake, combined with a low vitamin C (a nitrosation inhibitor) consumption, increases the chances of stomach cancer.

Though human tolerance of N-nitroso compounds in water is currently unknown, N-nitroso intake has been examined in another field. In the known cancer-causing tobacco industry, tobacco specific nitrosomines are ingested at rates up to 48 µg/person/day in cigarette smokers, and up to 223 µg/person/day in tobacco chewers (Mirvish 1995).

A.2 Methemoglobinemia

Nitrate concentrations greater than 10 mg N/L can cause methemoglobinemia (Blue Baby Syndrome), where the human body reduces NO_2^- in the body (Skipton, 1995). Nitrite then oxidizes the ferrous iron (Fe^{2+}) molecule in □aemoglobin, resulting in ferric iron (Fe^{3+}). At this point □aemoglobin becomes a new compound, methemoglobin (Lee and Ferguson, 2009). As methemoglobin is unable to carry oxygen through the body, the bloodstream becomes oxygen deficient, resulting in the blue discolouration of skin. Adults have two built in defense mechanisms for this in gastric acid and enzymes (Skipton, 1995). Gastric acid production lowers the body's pH, which decreases the ability of NO_3^- oxidation to NO_2^-. If methemoglobin is still produced, adult bodies contain two enzymes (Diaphorase I and Diaphorase II) that will reduce methemoglobin back to □aemoglobin (Lee and Ferguson, 2009). Infants under 6 months of age are prone

to methemoglobinemia as both of these mechanisms aren't fully developed at their stage of life.

A.3 Depleted Oxygen in Rivers and Lakes

Eutrophication is defined as the natural or artificial addition of nutrients (such as NO_3^- or PO_4^{3-}) to bodies of water, and the effect these nutrients have on the water and its ecosystem (National Academy of Sciences, 1969). The effects are algae, cyanobacteria, and macrophytes (Madigan et al. 2003). When they die, their high biochemical oxygen demand (BOD) results in large scale oxygen depletion in the water (Madigan et al. 2003). If the nutrient source is substantial enough, enough plant and algal matter will make the water body uninhabitable for aquatic life.

Eutrophication occurs in both rivers and lakes, however, lakes are more susceptible to oxygen depletion. Due to the dynamic nature of rivers, natural aeration and mixing allows rivers to handle BODs that a lake couldn't (National Academy of Sciences, 1969).

Typically, the source of nutrient loading of water bodies comes from runoff, or overloaded treatment plants. However, a source coming under greater scrutiny in the Grand River watershed is agricultural contamination via groundwater plume discharge or drainage tile.

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Appendix B:
Experiment 1 – 5L Tedlar Microcosms

Geochemistry and Isotope Data
Rayleigh Model Data

Table B1: Geochemistry and isotope data for Experiment 1: microcosms of sediment-groundwater mixtures in 5L Tedlar bags. Trial 1 (PU12596-B) contains 100 g sediment from core PU103 with starting concentrations of NO_3^--N and NH_4^--N of 15 and 21 mg/L, respectively.. Trial 2 (PU117 2.2-B) contains 100 g sediment from core PU103, with starting concentrations of NO3-N and NH4-N of 29 and 30 mg/L, respectively.

Experiment	Date	Days	NO_2^--N	NO_3^--N	NH_4^--N	TIN	TN	DOC	^{15}N-NO_3^-	^{18}O-NO_3^-	^{15}N-NH_4^+
			mg N/L						‰		
Experiment 1, Trial 1	25-Sep-09	0	-	6.12	15*	21.12*	40.86	44.61	33.40	15.04	
	3-Oct-09	8	-	5.21	13.45	18.66	44.44	44.80			25.60
	9-Oct-09	14	0.98	5.85	14.28	21.11	51.78	45.35			
	16-Oct-09	21	1.19	5.63	13.68	20.51	44.21	41.62	28.73	5.93	26.00
	23-Oct-09	28	1.01	4.74	13.60	19.34	36.89	52.25			
	30-Oct-09	35	1.20	5.36	13.33	19.89	48.94	45.44			
	6-Nov-09	42	1.51	5.31	13.23	20.04	39.01	49.99			
	13-Nov-09	49	1.41	4.48	10.68	16.57	38.39	39.73			26.15
	20-Nov-09	56	1.56	4.16	5.62	11.34		23.63	31.23	8.40	
	27-Nov-09	63	1.62	3.46	9.31	14.39	22.05				
	3-Dec-09	69	2.24	4.13	5.19	11.56	27.26	34.81			
	11-Dec-09	77	2.10	3.27	8.88	14.25					
	18-Dec-09	84	2.42	3.33	5.44	11.19			33.50	8.91	26.40
	4-Jan-10	101	2.84	3.13	7.26	13.23					
	25-Jan-10	122	2.69	3.00	6.04	11.73	23.56	36.05	41.77	9.92	32.95
Experiment 1, Trial 2	25-Sep-09	0	-	28.80	30.23	59.03					
	3-Oct-09	8	-	22.49	29.69	52.18					
	9-Oct-09	14	0.00	27.25	28.68	55.93					
	16-Oct-09	21	3.85	25.05	24.65	53.54					
	23-Oct-09	28	4.23	25.71	23.08	53.02					
	30-Oct-09	35	4.04	23.86	20.73	48.63					
	6-Nov-09	42	4.32	24.75	18.56	47.64					
	13-Nov-09	49	2.92	15.63	11.02	29.57					
	20-Nov-09	56	2.69	13.27	9.31	25.27					
	27-Nov-09	63	2.81	16.16	4.29	23.26					
	3-Dec-09	69	1.71	8.50	2.93	13.14					
	11-Dec-09	77	2.95	14.87	2.21	20.03					
	18-Dec-09	84	3.14	17.59	2.77	23.50					
	4-Jan-10	101	2.86	23.07	2.82	28.75					
	25-Jan-10	122	-	33.26	14.16	47.42					

Table B2. Rayleigh model data for Experiment 1, Trial 1 (PU12596B), model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 1 (PU12596)

Theoretical (Ideal Rayleigh) - Nitrification Model									
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative		
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - no prior pool	δ (‰) with prior NO_3^- pool)
20.00	0.00	1.46	0.00375	20.94			0		
19.98	0.02	1.46	0.00375	20.95	0.02	8.69	0.02	8.69	28.66
18.00	2.00	1.32	0.00376	22.23	1.98	9.32	2.00	9.31	23.64
14.40	5.60	1.05	0.00377	24.97	3.60	11.26	5.60	10.57	19.67
10.08	9.92	0.74	0.00378	29.37	4.32	14.71	9.92	12.37	18.29
6.05	13.95	0.44	0.00381	35.69	4.03	19.87	13.95	14.54	18.62
3.02	16.98	0.22	0.00384	44.35	3.02	27.04	16.98	16.77	19.75
1.21	18.79	0.09	0.00388	55.89	1.81	36.65	18.79	18.69	21.00
0.36	19.64	0.03	0.00394	71.26	0.85	49.31	19.64	20.01	21.95
0.07	19.93	0.01	0.00402	92.15	0.29	66.03	19.93	20.68	22.45
0.01	19.99	0.00	0.00413	122.75	0.07	88.75	19.99	20.90	22.62
0.00	20.00	0.00	0.00435	181.98	0.01	121.92	20.00	20.94	22.65

Table B2 cont'd. Rayleigh model data for Experiment 1, Trial 1 (PU12596B), model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 1 (PU12596)

Experimental Data - Nitrification (PU125-5.1m and PU96-2.6m, Tedlar Bag)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
15.00	0.00	1.00	0.00377	25.60			0.00			33.4	
14.99	0.02	1.00	0.00377	25.61	0.02	13.30	0.02	13.30	28.69		
14.23	0.77	0.96	0.00377	26.10	0.76	16.39	0.77	16.33	27.23		25.6
13.81	1.19	0.92	0.00377	26.61	0.41	9.05	1.19	13.79	26.13		
13.23	1.77	0.88	0.00378	27.15	0.59	14.56	1.77	14.04	25.21	28.73	26.0
12.64	2.36	0.84	0.00378	27.71	0.59	15.10	2.36	14.30	24.47		
12.06	2.94	0.80	0.00378	28.29	0.59	15.66	2.94	14.57	23.87		
11.47	3.53	0.76	0.00378	28.91	0.59	16.25	3.53	14.85	23.38		
10.64	4.36	0.73	0.00379	29.55	0.83	20.64	4.36	15.96	23.15		26.2
10.02	4.98	0.69	0.00379	30.24	0.62	18.57	4.98	16.28	22.89	31.23	
9.72	5.28	0.65	0.00379	30.96	0.30	6.86	5.28	15.75	22.44		
9.13	5.87	0.61	0.00379	31.73	0.59	18.96	5.87	16.07	22.27		
8.54	6.46	0.57	0.00380	32.55	0.59	19.75	6.46	16.40	22.14		
7.52	7.48	0.53	0.00380	33.43	1.02	26.06	7.48	17.72	22.45	33.5	26.4
7.37	7.63	0.49	0.00380	34.38	0.15	-12.91	7.63	17.11	22.05		
6.79	8.21	0.45	0.00381	35.41	0.59	22.47	8.21	17.50	22.06		
6.20	8.80	0.41	0.00381	36.53	0.59	23.53	8.80	17.90	22.12		
5.62	9.38	0.37	0.00382	37.76	0.59	24.69	9.38	18.32	22.22	41.77	33.0
4.80	10.20	0.32	0.00382	39.72	0.82	26.25	10.20	18.96	22.43		
4.35	10.65	0.29	0.00383	40.95	0.45	27.84	10.65	19.33	22.58		
3.88	11.13	0.26	0.00383	42.39	1.74	29.16	11.13	20.02	22.94		
3.00	12.00	0.20	0.00384	45.60	2.62	31.40	12.00	21.17	23.59		
2.25	12.75	0.15	0.00386	49.22	3.95	34.75	12.75	23.12	24.84		
1.50	13.50	0.10	0.00388	54.33	0.75	38.98	13.50	24.00	25.39		
1.13	13.88	0.08	0.00389	57.98	0.38	43.40	13.88	24.53	25.74		
0.75	14.25	0.05	0.00391	63.14	0.38	47.66	14.25	25.14	26.15		
0.38	14.63	0.03	0.00394	72.02	0.38	54.26	14.63	25.88	26.67		
0.15	14.85	0.01	0.00398	83.87	0.23	64.12	14.85	26.46	27.09		
0.11	14.89	0.01	0.00400	87.62	0.04	72.63	14.89	26.58	27.17		
0.08	14.93	0.01	0.00402	92.93	0.04	77.01	14.93	26.70	27.26		
0.04	14.96	0.00	0.00405	102.05	0.04	83.80	14.96	26.85	27.36		
0.02	14.99	0.00	0.00410	114.24	0.02	93.93	14.99	26.95	27.44		
0.01	14.99	0.00	0.00411	118.09	0.00	102.68	14.99	26.97	27.45		
0.01	14.99	0.00	0.00413	123.54	0.00	107.18	14.99	26.99	27.46		
0.00	15.00	0.0001	0.00421	145.45	0.01	118.07	15.00	27.02	27.49		

Table B2 cont'd. Rayleigh model data for Experiment 1, Trial 1 (PU12596B), model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 1 (PU12596)

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00377	25.60	0.00378	28.73
5.99	5.99	1.00	1.00	0.00377	25.61	0.00378	28.75
5.40	5.40	0.90	0.90	0.00377	26.25	0.00379	30.36
4.80	4.80	0.80	0.80	0.00378	26.97	0.00379	32.18
4.20	4.20	0.70	0.70	0.00378	27.80	0.00380	34.25
3.60	3.60	0.60	0.60	0.00378	28.75	0.00381	36.64
3.00	3.00	0.50	0.50	0.00379	29.87	0.00382	39.48
2.40	2.40	0.40	0.40	0.00379	31.25	0.00383	42.97
1.80	1.80	0.30	0.30	0.00380	33.04	0.00385	47.48
1.20	1.20	0.20	0.20	0.00381	35.55	0.00387	53.87
0.60	0.60	0.10	0.10	0.00382	39.87	0.00392	64.88
0.30	0.30	0.05	0.05	0.00384	44.20	0.00396	76.01
0.06	0.06	0.01	0.01	0.00388	54.33	0.00405	102.30
0.03	0.03	0.01	0.01	0.00389	58.73	0.00409	113.82
0.01	0.01	0.00	0.00	0.00393	69.00	0.00420	141.04
0.00	0.00	0.00	0.00	0.00395	73.46	0.00424	152.97
0.00	0.00	0.00	0.00	0.00398	83.87	0.00434	181.14

Table B2 cont'd. Rayleigh model data for Experiment 1, Trial 1 (PU12596B), model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 1 (PU12596)

Experimental Data Anammox (PU125-5.1m and PU96-2.6m, Tedlar Bag)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment - PU12596 Bag	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Measured δ15N-NO3	Measured δ15N-NH4
15.00	5.63	1.00	1.00	0.00377	25.60	0.00378	28.73	33.40	
14.99	5.49	1.00	0.98	0.00377	25.61	0.00378	29.11		
14.23	5.35	0.96	0.95	0.00377	25.85	0.00378	29.51		25.60
13.81	5.21	0.92	0.93	0.00377	26.11	0.00379	29.91		
13.23	5.08	0.88	0.90	0.00377	26.37	0.00379	30.33	28.73	26.00
12.64	4.94	0.84	0.88	0.00377	26.65	0.00379	30.76		
12.06	4.80	0.80	0.85	0.00378	26.94	0.00379	31.20		
10.64	4.66	0.76	0.83	0.00378	27.25	0.00379	31.65		26.15
10.89	4.52	0.73	0.80	0.00378	27.57	0.00379	32.12		
10.02	4.38	0.69	0.78	0.00378	27.92	0.00380	32.60	31.23	
9.72	4.25	0.65	0.75	0.00378	28.28	0.00380	33.09		
9.13	4.11	0.61	0.73	0.00378	28.66	0.00380	33.61		
8.54	3.97	0.57	0.70	0.00378	29.07	0.00380	34.14		
7.52	3.83	0.53	0.68	0.00378	29.51	0.00380	34.69	33.50	26.40
7.37	3.69	0.49	0.66	0.00379	29.98	0.00381	35.26		
6.79	3.55	0.45	0.63	0.00379	30.49	0.00381	35.85		
6.20	3.42	0.41	0.61	0.00379	31.05	0.00381	36.47		
4.14	3.28	0.37	0.58	0.00379	31.66	0.00381	37.12	41.77	32.95
3.88	3.14	0.26	0.56	0.00380	33.96	0.00382	37.79		
3.00	3.00	0.20	0.53	0.00381	35.55	0.00382	38.49		
1.50	2.25	0.10	0.40	0.00382	39.87	0.00383	42.97		
1.13	1.69	0.08	0.30	0.00383	41.66	0.00385	47.48		
0.75	1.13	0.05	0.20	0.00384	44.20	0.00387	53.87		
0.38	0.56	0.03	0.10	0.00386	48.55	0.00392	64.88		
0.15	0.42	0.01	0.08	0.00388	54.33	0.00393	69.49		
0.11	0.28	0.01	0.05	0.00388	56.15	0.00396	76.01		
0.08	0.14	0.01	0.03	0.00389	58.73	0.00400	87.26		
0.04	0.06	0.00	0.01	0.00391	63.14	0.00405	102.30		
0.02	0.04	0.00	0.01	0.00393	69.00	0.00407	107.07		
0.01	0.03	0.00	0.01	0.00394	70.85	0.00409	113.82		
0.01	0.01	0.00	0.00	0.00395	73.46	0.00414	125.47		
0.00	0.01	0.0001	0.001	0.00398	83.87	0.00420	141.04		

Table B2 cont'd. Rayleigh model data for Experiment 1, Trial 1 (PU12596B), model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 1 (PU12596)

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.60	‰
δ_o-NO_3^-	28.73	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	13.68	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	5.63	mg/L

Nitrification: Actual Data - Input

Experiment:	PU12596B	
Initial NH_4^+-N (mg/L)	15.00	mg N/L
Final NH_4^+-N (mg/L)	3.88	mg N/L
f NH_4^+	0.26	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	25.60	‰
Initial NO3--N (mg/L)	5.63	mg N/L
Final NO3--N (mg/L)	3.00	mg N/L
f NO_3^-	0.53	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	28.73	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-6.0000	‰
α (NH4)	0.9940	
(α-1) NH4	-0.0060	
ε (NO3- - Nitrification)	-15.0000	‰
α (NO3-)	0.9850	
(α-1) NO3-	-0.0150	

Table B2 cont'd. Rayleigh model data for Experiment 1, Trial 1 (PU12596B), model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 1 (PU12596)

Equations:

$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{react}$	Fractionation (ii)
$\delta = [R_{sample}/R_{star}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio

where:

$\varepsilon p\text{-}r$	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N = 0.0036765$
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

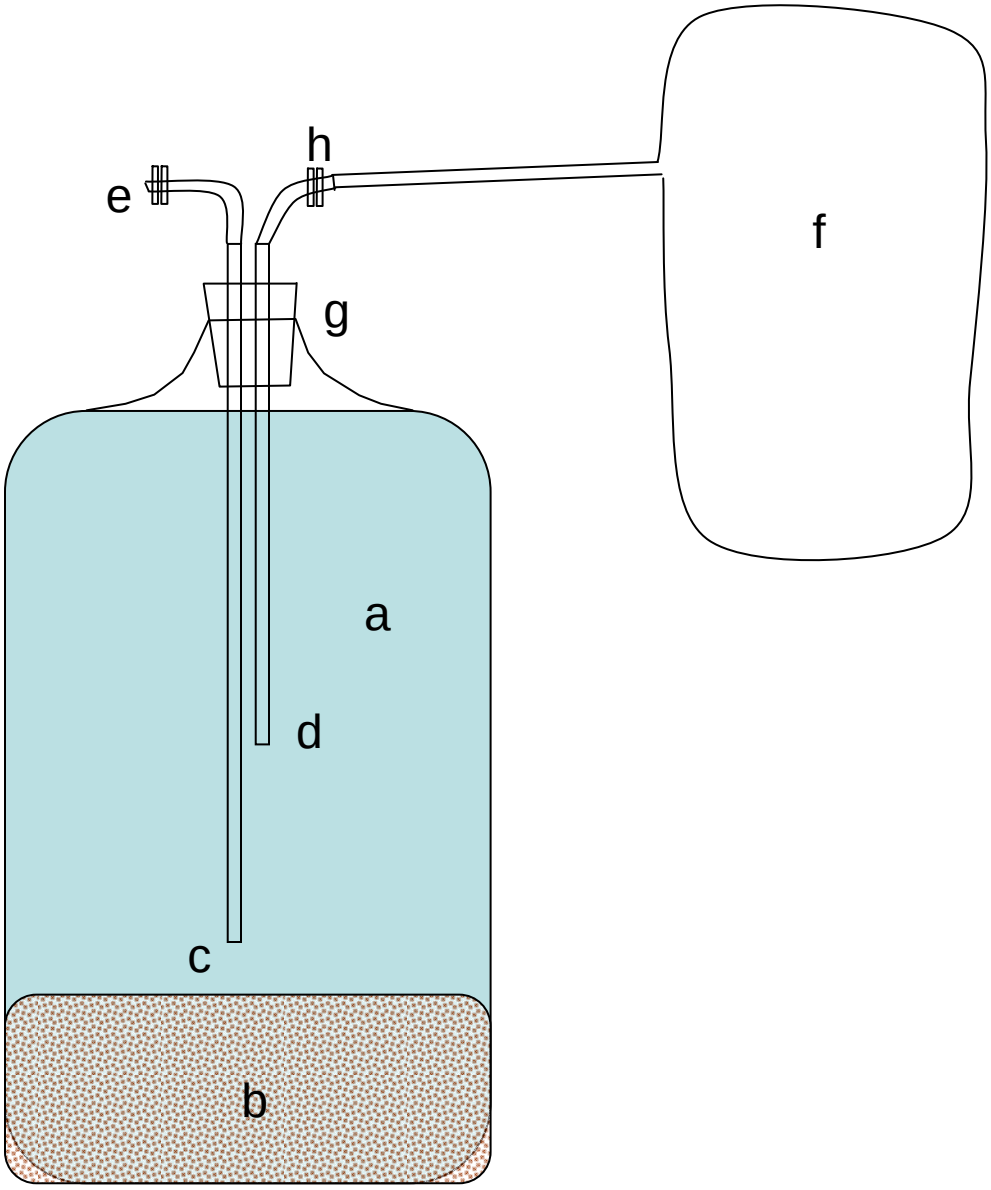
Appendix C:
Experiment 2 – 2L Kimex Microcosms

Geochemistry and Isotope Data
Microcosm Design

Table C1 : Geochemistry and isotope data for Experiment 2: microcosms of sediment-groundwater mixtures in 2L Kimex glass jars. Trial 1 (PU12596-B) contains 100 g sediment from core PU103 with starting concentrations of NO_3^--N and NH_4^--N of 5 and 13 mg/L, respectively. Trial 2 (PU117 2.2-B) contains 100 g sediment from core PU103, with starting concentrations of NO3-N and NH4-N of 29 and 25 mg/L, respectively.

Experiment	Date	Days	NO_2^--N	NO_3^--N	NH_4^--N	TIN	TN	DOC	^{15}N-NO_3^-	^{18}O-NO_3^-	^{15}N-NH_4^+
			mg N/L						‰		
Experiment 2, Trial 1	25-Sep-09	0	-	5.06	12.71	17.77					
	3-Oct-09	8	-	4.53	11.10	15.63					
	9-Oct-09	14	0.00	4.46	11.95	16.41					
	16-Oct-09	21	0.95	3.96	11.55	16.45					
	23-Oct-09	28	2.10	4.43	9.95	16.47					
	30-Oct-09	35	3.87	4.29	7.69	15.84					
	6-Nov-09	42	3.78	3.99	5.69	13.47					
	13-Nov-09	49	4.70	2.50	2.91	10.11					
	20-Nov-09	56	5.80	2.31	0.73	8.84					
	27-Nov-09	63	4.00	5.50	3.62	13.12					
	3-Dec-09	69	0.00	8.80	0.35	9.15					
	11-Dec-09	77	0.00	7.30	0.25	7.55					
	18-Dec-09	84	0.00	8.40	0.25	8.65					
	4-Jan-10	101	0.00	5.80	0.29	6.09					
	25-Jan-10	122	0.00	10.09	1.63	11.72					
Experiment 2, Trial 2	25-Sep-09	0	-	24.30	25.04	49.34					
	3-Oct-09	8	-	23.10	21.45	44.55					
	9-Oct-09	14	3.66	25.45	20.69	49.80					
	16-Oct-09	21	2.37	22.17	18.37	42.92					
	23-Oct-09	28	3.20	21.53	16.37	41.11					
	30-Oct-09	35	4.90	18.01	13.32	36.23					
	6-Nov-09	42	6.11	22.63	10.79	39.53					
	13-Nov-09	49	1.00	22.40	8.77	32.17					
	20-Nov-09	56	0.50	30.50	6.34	37.34					
	27-Nov-09	63	1.18	33.50	2.13	36.81					
	3-Dec-09	69	1.20	30.60	0.71	32.51					
	11-Dec-09	77	0.00	18.50	0.30	18.80					
	18-Dec-09	84	0.00	33.70	0.26	33.96					
	4-Jan-10	101	0.00	32.70	0.25	32.95					
	25-Jan-10	122	-	34.56	1.54	36.10					

2L Kimex Flask Microcosm Design



Legend

a) Sample Water	e) Sample port
b) Dry core from PU103 (<50 mm)	f) Helium Balloon
c) Sample intake (glass rod)	g) Taped, greased stopper
d) Helium port	h) Two hose clamps

Figure C1: Design for 2L Kimex glass flask (Experiment 2). Two of these vessels were constructed, for Trial 1 (PU12596 – mixed groundwater) and Trial 2 (PU117 2.2m – single groundwater). The experiment ran from 25-Sept-09 to25-Jan-10 .

Appendix D:
Experiment 3 – Sacrificial DO Bottles

Geochemistry and Isotope Data
Theoretical Denitrification Calculations
Rayleigh Model Data

Table D1. Analytical results for Experiment 3; Zorra groundwater mixed with ~ 50 g sediment in sacrificial bottle microcosms (250 mL DO bottles): Trial 1, single source groundwater (PU115-2.2m); Trial 2, dual source groundwater (PU122-2.2m and PU80-1.7m); Trial 3, DO control (He sparged DI in bioled silica sand); and Trial 4, dual source groundwater (PU125-2.7m and PU80-1.7m), Sept. 25, 2009 to Jan. 25, 2010.

Trial	Date	Day	Analytical (mg/L)										Isotopes (‰)		
			NO_3^--N	NO_2^--N	NH_4^+-N	TN	TKN	DON	DOC	Cl^-	$SO4^{2-}$	Br^-	^{15}N-NH_4^+	^{15}N-NO_3^-	^{18}O-NO_3^-
Trial 1 (pre incubation)	6-Jul-10	0	43.10	0.01	3.04	73			412	76			33.06		
	16-Jul-10	10	46.28	1.78	4.54	62	16	12	70	76			32.57	32.217	9.150
	5-Aug-10	30	40.87	1.88	4.21	62			59	75					
	20-Sep-10	76	38.40	0.02	1.59				60	76	29	0.40	NH_4^+ too low	34.20	9.49
	29-Oct-10	115	38.80	0.03	0.75	70	15	14	58	80	31	0.48			
	23-Nov-10	140	32.10	0.54	0.34				61	59	24	n.a	NH_4^+ too low	35.08	11.11
	14-Jan-11	192	39.50	2.13	0.17	45	13	13	69	76	31	0.01	NH_4^+ too low	34.35	10.21
Trial 2 (pre incubation)	6-Jul-10	0	7.40	0.05	42.48	114			730	135			22.61		
	16-Jul-10	10	5.35	0.15	30.17	65	54	24	115	125			24.62	20.02	3.10
	5-Aug-10	30	7.08	0.04	37.66	47			81	132					
	20-Sep-10	76	3.33	0.07	22.73		45	23	74	107	16	1.84	28.55	54.04	29.41
	29-Oct-10	115	0.63	0.08	24.67	53			91	119	18	1.00			
	23-Nov-10	140	0.21	0.05	27.10	47			87	135	20	0.72	32.24	low concentration	
	14-Jan-11	192	0.05	0.04	28.79	47	45	16	93	112	17	0.71	34.14		
Trial 3 (Control)	16-Jul-10	10	0.00	0.00	0.07	-2			2	-1					
	5-Aug-10	30	-0.01	-0.01	0.02	-2			-2	2					
	20-Sep-10	76	n.a.	0.00	0.03				2	1	1	n.a.			
	29-Oct-10	115	0.08	0.00	-0.03				2	2	0	n.a.			
	23-Nov-10	140	0.14	0.14	0.00	1			2	1	0	0.00			
	14-Jan-11	192	0.03	0.00	0.00				1	2	0	n.a			
Trial 4 (pre incubation)	6-Jul-10	0	13.24	0.16	8.32	44			109	41			40.63		
	16-Jul-10	10	13.88	0.64	8.97	25	11	2	29	39			37.84	21.378	5.929
	5-Aug-10	30	12.78	0.78	7.74	26			29	42					
	20-Sep-10	76	11.22	0.00	3.76		9	5	47	34	15	0.03	39.13	26.318	6.974
	29-Oct-10	115	10.05	0.02	5.03	29			20	33	13	0.26			
	23-Nov-10	140	9.61	0.01	6.52				27	37	13	0.20	44.93	25.264	7.063
	14-Jan-11	192	8.42	0.00	1.64		10	8	44	33	13	n.a.	NH_4^+ too low		

Table D1 cont'd. Analytical results for Experiment 3; Zorra groundwater mixed with ~ 50 g sediment in sacrificial bottle microcosms (250 mL DO bottles): Trial 1, single source groundwater (PU115-2.2m); Trial 2, dual source groundwater (PU122-2.2m and PU80-1.7m); Trial 3, DO control (He sparged DI in bioled silica sand); and Trial 4, dual source groundwater (PU125-2.7m and PU80-1.7m), Sept. 25, 2009 to Jan. 25, 2010.

Trial	Date	Day	Gases (nmol/L, unless specified)			Field Parameters				
			N_2O	CO_2 (mg/L)	CH_4	DO (mg/L)	Conductivity (µS/cm)	Eh (mV)	pH	Temperature (°C)
Trial 1 (pre incubation)	6-Jul-10	0	2181	7	1227	3.43	1202	242	7.22	10.7
	16-Jul-10	10	2567	24	378	0.27	1832	110	7.28	19.7
	5-Aug-10	30	206	15	354	0.24	1682	172	7.25	19.2
	20-Sep-10	76	2292	17	326	0.25	1604	229	7.27	20.1
	29-Oct-10	115	5	25	326	0.15	1690	105	6.65	23.1
	23-Nov-10	140				0.23	1587	81	7.04	22.0
	14-Jan-11	192	4234	27	523	0.29	1586	142	6.92	20.7
Trial 2 (pre incubation)	6-Jul-10	0	2204	7	21823	3.41	1946	235	6.94	8.8
	16-Jul-10	10	26	38	109	0.27	1953	172	7.07	19.9
	5-Aug-10	30	4	28	112	0.30	1818	200	7.03	19.0
	20-Sep-10	76	6	28	193	0.30	1736	230	7.06	19.9
	29-Oct-10	115	10	45	193	0.10	1814	15	6.70	23.5
	23-Nov-10	140				0.19	1760	-54	6.59	21.3
	14-Jan-11	192	73	51	277	0.26	1714	66	6.87	20.7
Trial 3 (Control)	16-Jul-10	10	3	-1	5	1.94	20	138	8.36	19.8
	5-Aug-10	30	1	1	7	1.06	10	161	8.13	19.0
	20-Sep-10	76	4	1	70	0.97	10	173	8.35	19.9
	29-Oct-10	115	4	2	70	0.32	28	-61	6.72	23.4
	23-Nov-10	140		1		0.70	11	-95	6.83	20.9
	14-Jan-11	192	11		-21	1.02	1225	72	7.16	20.6
Trial 4 (pre incubation)	6-Jul-10	0	2175	26	6061	4.36	1202	273	6.78	8.4
	16-Jul-10	10	897	25	52	0.26	1206	182	7.24	19.9
	5-Aug-10	30	75	15	52	0.30	1117	216	7.18	18.9
	20-Sep-10	76	7	23	146	0.28	1087	223	7.23	19.9
	29-Oct-10	115	5	31	146	0.09	1150	15	6.98	23.4
	23-Nov-10	140				0.17	1066	-33	6.55	22.0
	14-Jan-11	192	11	26	84	0.30	1067	40	6.97	20.8

Appendix D – Theoretical Denitrification Calculation for Trial 1:

11 mg/L of NO_3^--N was consumed. How much DOC would be consumed, and how much CO_2 would be produced, if the nitrate was completely consumed by denitrification?

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

DOC:

11 mg/L NO_3^--N x 1 g/1000 mg x 1 mole/ 14.00674 g x 0.250 L/bottle
= 0.000196 moles N/bottle

4moles N: 5 moles DOC :. DOC = 0.000196 x 5/4 = 0.000245 moles DOC

0.000245 moles/bottle DOC x 12.0107 g/mole x 1 bottle/0.250 L x 1000 mg/g
= 11.791 mg/L DOC-C consumed

CO_2:

As both DOC and CO_2 are measured as C (and both have 5 moles in rxn), both calculations are identical, so 11.791 mg/L CO_2-C will be produced.

Appendix D – Theoretical Denitrification Calculation for Trial 2:

7.4 mg/L of NO_3^--N was consumed. How much DOC would be consumed, and how much CO_2 would be produced, if the nitrate was completely consumed by denitrification?

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

DOC:

7.4 mg/L NO_3^--N x 1 g/1000 mg x 1 mole/ 14.00674 g x 0.250 L/bottle
= 0.000132 moles N/bottle

4moles N: 5 moles DOC :. DOC = 0.000132 x 5/4 = 0.000165 moles DOC

0.000165 moles/bottle DOC x 12.0107 g/mole x 1 bottle/0.250 L x 1000 mg/g
= 7.927 mg/L DOC-C consumed

CO_2:

As both DOC and CO_2 are measured as C (and both have 5 moles in rxn), both calculations are identical, so 7.927 mg/L CO_2-C will be produced.

Appendix D – Theoretical Denitrification Calculation for Trial 4:

5.5 mg/L of NO_3^--N was consumed. How much DOC would be consumed, and how much CO_2 would be produced, if the nitrate was completely consumed by denitrification?

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

DOC:

5.5 mg/L NO_3^--N x 1 g/1000 mg x 1 mole/ 14.00674 g x 0.250 L/bottle
= 0.0000982 moles N/bottle

4moles N: 5 moles DOC :. DOC = 0.0000982 x 5/4 = 0.000123 moles DOC

0.000123 moles/bottle DOC x 12.0107 g/mole x 1 bottle/0.250 L x 1000 mg/g
= 5.895 mg/L DOC-C consumed

CO_2:

As both DOC and CO_2 are measured as C (and both have 5 moles in rxn), both calculations are identical, so 5.895 mg/L CO_2-C will be produced.

Table D2. Rayleigh model data for Experiment 3, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU115-2.2m)

Experimental Data - Nitrification: Experiment 3 (PU115-2.2m)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
3.94	0.00	1.00	0.00380	33.06			0.00				33.063
3.94	0.00	1.00	0.00380	33.08	0.00	20.67	0.00	20.67	32.22		
3.71	0.23	0.94	0.00380	33.84	0.23	20.46	0.23	20.47	32.16	32.22	32.568
3.47	0.48	0.88	0.00380	34.66	0.47	21.45	0.48	21.44	32.10		
3.23	0.71	0.82	0.00381	35.54	0.24	22.68	0.71	21.85	32.05		
2.99	0.95	0.76	0.00381	36.49	0.24	23.58	0.95	22.28	32.01		
2.76	1.19	0.70	0.00381	37.51	0.24	24.55	1.19	22.73	31.97		
2.52	1.42	0.64	0.00382	38.63	0.24	25.61	1.42	23.21	31.93		
2.19	1.75	0.58	0.00382	39.86	0.32	30.32	1.75	24.53	31.92	34.2	
2.05	1.90	0.52	0.00383	41.23	0.47	27.40	1.90	24.26	31.89		
1.81	2.13	0.46	0.00383	42.76	0.24	29.47	2.13	24.83	31.87		
1.57	2.37	0.40	0.00384	44.52	0.24	31.09	2.37	25.46	31.87		
1.34	2.61	0.34	0.00385	46.56	0.24	32.96	2.61	26.14	31.87		
1.10	2.84	0.28	0.00386	49.01	0.47	34.07	2.84	26.89	31.89		
0.72	3.22	0.22	0.00387	52.06	0.61	40.09	3.22	28.80	31.98	35.08	
0.39	3.55	0.10	0.00390	62.01	0.71	41.75	3.55	29.85	32.04		
0.30	3.65	0.08	0.00392	65.68	0.10	50.99	3.65	30.42	32.08		
0.20	3.75	0.05	0.00394	70.88	0.10	55.28	3.75	31.07	32.13		
0.10	3.84	0.03	0.00397	79.82	0.30	56.07	3.84	31.86	32.19		
0.04	3.90	0.01	0.00401	91.76	0.06	71.86	3.90	32.47	32.24		
0.03	3.91	0.01	0.00403	95.53	0.01	80.43	3.91	32.59	32.25		
0.02	3.92	0.01	0.00405	100.88	0.02	82.64	3.92	32.72	32.26		
0.01	3.93	0.00	0.00408	110.07	0.01	91.68	3.93	32.87	32.27		
0.00	3.94	0.00	0.00413	122.35	0.01	101.89	3.94	32.97	32.28		
0.00	3.94	0.00	0.00414	126.23	0.00	110.70	3.94	32.99	32.28		
0.00	3.94	0.00	0.00416	131.72	0.00	115.24	3.94	33.01	32.29		
0.00	3.94	0.00	0.00420	141.17	0.00	122.27	3.94	33.04	32.29		
0.00	3.94	0.00	0.00424	153.79	0.00	132.76	3.94	33.05	32.29		
0.00	3.94	0.00	0.00426	157.78	0.00	141.82	3.94	33.05	32.29		
0.00	3.94	0.00	0.00428	163.43	0.00	146.49	3.94	33.06	32.29		
0.00	3.94	0.00	0.00431	173.14	0.00	153.71	3.94	33.06	32.29		
0.00	3.94	0.00	0.00436	186.12	0.00	164.50	3.94	33.06	32.29		
0.00	3.94	7.5E-06	0.00438	190.22	0.00	173.81	3.94	33.06	32.29		

Table D2 cont'd. Rayleigh model data for Experiment 3, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU115-2.2m)

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00380	33.06	0.00379	32.22
5.99	5.99	1.00	1.00	0.00380	33.07	0.00380	32.24
5.40	5.40	0.90	0.90	0.00380	33.72	0.00380	33.85
4.80	4.80	0.80	0.80	0.00380	34.45	0.00381	35.68
4.20	4.20	0.70	0.70	0.00381	35.28	0.00382	37.76
3.60	3.60	0.60	0.60	0.00381	36.23	0.00382	40.16
3.00	3.00	0.50	0.50	0.00381	37.37	0.00383	43.01
2.40	2.40	0.40	0.40	0.00382	38.76	0.00385	46.51
1.80	1.80	0.30	0.30	0.00383	40.55	0.00386	51.03
1.20	1.20	0.20	0.20	0.00383	43.09	0.00389	57.44
0.60	0.60	0.10	0.10	0.00385	47.43	0.00393	68.49
0.30	0.30	0.05	0.05	0.00387	51.80	0.00397	79.66
0.06	0.06	0.01	0.01	0.00390	62.01	0.00407	106.04
0.03	0.03	0.01	0.01	0.00392	66.43	0.00411	117.60
0.01	0.01	0.00	0.00	0.00396	76.78	0.00421	144.91
0.00	0.00	0.00	0.00	0.00398	81.27	0.00425	156.88
0.00	0.00	0.00	0.00	0.00401	91.76	0.00436	185.15

Table D2 cont'd. Rayleigh model data for Experiment 3, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU115-2.2m)

Experimental Data Anammox: Experiment 3 (PU115-2.2m)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Measured δ15N-NO3	Measured δ15N-NH4
3.94	*43.20*	1.00	1.00	0.00380	33.06	0.00379	32.22		33.06
3.94	*42.59*	1.00	0.99	0.00380	33.07	0.00380	32.44	32.22	
3.76	41.98	0.95	0.97	0.00380	33.36	0.00380	32.67		
3.71	41.37	0.91	0.96	0.00380	33.67	0.00380	32.89		32.57
3.57	41.37	0.91	0.96	0.00380	33.67	0.00380	32.89		
3.39	40.75	0.86	0.94	0.00380	34.00	0.00380	33.12		
3.21	*40.14*	0.81	0.93	0.00380	34.34	0.00380	33.36	34.20	
3.02	39.53	0.77	0.91	0.00380	34.71	0.00380	33.60		
2.84	38.91	0.72	0.90	0.00381	35.10	0.00380	33.84		
2.66	38.30	0.67	0.89	0.00381	35.51	0.00380	34.09		
2.47	37.69	0.63	0.87	0.00381	35.95	0.00380	34.34		
2.29	*37.07*	0.58	0.86	0.00381	36.43	0.00380	34.59	35.08	
2.19	36.46	0.53	0.84	0.00381	36.95	0.00380	34.85		
2.11	36.46	0.53	0.84	0.00381	36.95	0.00380	34.85		
1.93	*35.24*	0.49	0.82	0.00381	37.51	0.00381	35.38	34.35	
1.74	30.24	0.44	0.70	0.00382	38.14	0.00382	37.76		
1.56	25.92	0.40	0.60	0.00382	38.83	0.00382	40.16		
1.38	21.60	0.35	0.50	0.00382	39.60	0.00383	43.01		
1.19	17.28	0.30	0.40	0.00383	40.49	0.00385	46.51		
1.01	12.96	0.26	0.30	0.00383	41.53	0.00386	51.03		
0.83	8.64	0.21	0.20	0.00383	42.78	0.00389	57.44		
0.72	4.32	0.16	0.10	0.00384	44.35	0.00393	68.49		
0.64	2.16	0.16	0.05	0.00384	44.35	0.00397	79.66		
0.10	0.43	0.03	0.01	0.00388	56.09	0.00407	106.04		
0.04	0.32	0.01	0.01	0.00390	62.01	0.00408	110.83		
0.03	0.22	0.01	0.01	0.00391	63.84	0.00411	117.60		
0.02	0.11	0.01	0.00	0.00392	66.43	0.00415	129.28		
0.01	0.04	0.00	0.00	0.00394	70.88	0.00421	144.91		
0.00	0.03	0.00	0.00	0.00396	76.78	0.00423	149.86		
0.00	0.02	0.00	0.00	0.00397	78.64	0.00425	156.88		
0.00	0.01	0.00	0.00	0.00398	81.27	0.00430	168.97		
0.00	0.00	0.0001	0.0001	0.00401	91.76	0.00436	185.15		

Table D2 cont'd. Rayleigh model data for Experiment 3, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU115-2.2m)

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.00	‰
δ_o-NO_3^-	40.00	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	20.00	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	20.00	mg/L

Nitrification: Actual Data - Input

Experiment:	PU-A	
Initial NH_4^+-N (mg/L)	3.94	mg N/L
Final NH_4^+-N (mg/L)	0.10	mg N/L
f NH_4^+	0.03	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	33.06	‰
Initial NO3--N (mg/L)	**43.20**	mg N/L
Final NO3--N (mg/L)	35.24	mg N/L
f NO_3^-	0.82	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	**32.22**	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-6.00	‰
α (NH4)	0.9940	
(α-1) NH4	-0.0060	
ε (NO3- - Nitrification)	-15.00	‰
α (NO3-)	0.9850	
(α-1) NO3-	-0.0150	

Table D2 cont'd. Rayleigh model data for Experiment 3, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU115-2.2m)

Equations:	
$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{reacta}$	Fractionation (ii)
$\delta = [R_{sample}/R_{stan}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio
where:	
εp-r	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N$ = 0.0036765
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

Table D3. Rayleigh model data for Experiment 3, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (Experiment 3 (PU122-2.2m and PU80-1.7m))

Experimental Data - Nitrification: Experiment 3 (PU122-2.2m and PU80-1.7m)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
35.31	0.00	1.00	0.00377	24.62			0.00				22.6
35.28	0.04	1.00	0.00377	24.63	0.04	12.33	0.04	12.33	19.98		
34.32	0.99	0.97	0.00377	24.97	0.96	12.50	0.99	12.50	19.13	20.02	24.6
33.37	1.95	0.94	0.00377	25.32	0.96	12.84	1.95	12.67	18.49		
32.41	2.90	0.92	0.00377	25.67	0.96	13.19	2.90	12.84	18.00		
31.46	3.86	0.89	0.00377	26.04	0.96	13.55	3.86	13.01	17.62		
30.50	4.81	0.86	0.00377	26.42	0.96	13.92	4.81	13.19	17.33		
29.55	5.77	0.84	0.00378	26.81	0.96	14.30	5.77	13.38	17.11		
28.59	6.72	0.81	0.00378	27.22	0.96	14.69	6.72	13.56	16.95		
27.64	7.68	0.78	0.00378	27.64	0.96	15.10	7.68	13.75	16.83		
30.78	4.53	0.87	0.00377	26.31	-3.14	14.64	4.53	13.14	17.41	54.04	28.6
26.68	8.63	0.76	0.00378	28.07	0.96	15.52	8.63	13.95	16.75		
23.85	11.46	0.68	0.00378	29.46	2.83	16.41	11.46	14.56	16.70		
26.96	8.35	0.76	0.00378	27.94	-3.11	16.34	8.35	13.89	16.77		32.2
23.85	11.46	0.68	0.00378	29.46	3.11	16.34	11.46	14.56	16.70		34.1
10.59	24.72	0.30	0.00382	39.53	13.26	21.41	24.72	18.23	18.64		
7.06	28.25	0.20	0.00384	44.60	3.53	29.39	28.25	19.62	19.71		
3.53	31.78	0.10	0.00387	53.33	3.53	35.88	31.78	21.43	21.16		
2.65	32.67	0.08	0.00389	56.97	0.88	42.40	32.67	22.00	21.63		
1.77	33.55	0.05	0.00390	62.12	0.88	46.66	33.55	22.65	22.17		
0.88	34.43	0.03	0.00394	71.00	0.88	53.25	34.43	23.43	22.83		
0.35	34.96	0.01	0.00398	82.84	0.53	63.10	34.96	24.03	23.33		
0.26	35.05	0.01	0.00399	86.58	0.09	71.60	35.05	24.15	23.43		
0.18	35.14	0.01	0.00401	91.88	0.09	75.98	35.14	24.28	23.54		
0.09	35.23	0.00	0.00405	101.00	0.09	82.76	35.23	24.43	23.66		
0.04	35.28	0.00	0.00409	113.17	0.05	92.89	35.28	24.53	23.75		
0.03	35.29	0.00	0.00411	117.02	0.01	101.62	35.29	24.55	23.77		
0.02	35.30	0.00	0.00413	122.47	0.01	106.13	35.30	24.57	23.78		
0.00	35.31	0.0001	0.00421	144.36	0.01	117.00	35.31	24.61	23.81		

Table D3 cont'd. Rayleigh model data for Experiment 3, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (Experiment 3 (PU122-2.2m and PU80-1.7m))

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00377	24.62	0.00375	20.02
5.99	5.99	1.00	1.00	0.00377	24.64	0.00375	20.03
5.40	5.40	0.90	0.90	0.00377	26.24	0.00375	21.31
4.80	4.80	0.80	0.80	0.00378	28.06	0.00376	22.75
4.20	4.20	0.70	0.70	0.00379	30.12	0.00377	24.40
3.60	3.60	0.60	0.60	0.00380	32.50	0.00377	26.29
3.00	3.00	0.50	0.50	0.00381	35.33	0.00378	28.54
2.40	2.40	0.40	0.40	0.00382	38.80	0.00379	31.30
1.80	1.80	0.30	0.30	0.00384	43.29	0.00380	34.86
1.20	1.20	0.20	0.20	0.00386	49.66	0.00382	39.91
0.60	0.60	0.10	0.10	0.00390	60.63	0.00386	48.60
0.30	0.30	0.05	0.05	0.00394	71.71	0.00389	57.36
0.06	0.06	0.01	0.01	0.00404	97.90	0.00396	77.97
0.03	0.03	0.01	0.01	0.00408	109.37	0.00400	86.98
0.01	0.01	0.00	0.00	0.00418	136.48	0.00407	108.18
0.00	0.00	0.00	0.00	0.00422	148.36	0.00411	117.43
0.00	0.00	0.00	0.00	0.00433	176.42	0.00419	139.22

Table D3 cont'd. Rayleigh model data for Experiment 3, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (Experiment 3 (PU122-2.2m and PU80-1.7m))

Experimental Data Anammox (PU122-2.2m and PU80-1.7m, Experiment 3, Trial 2)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment - PU12596 Bag	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Measured δ15N-NO3	Measured δ15N-NH4
35.31	7.40	1.00	1.00	0.00377	24.62	0.00375	20.02		22.61
35.28	6.99	1.00	0.94	0.00377	24.64	0.00375	20.72		
34.64	6.58	0.98	0.89	0.00377	24.92	0.00376	21.45	**20.02**	24.62
34.01	6.18	0.96	0.83	0.00377	25.20	0.00376	22.24		
33.37	5.77	0.94	0.78	0.00377	25.49	0.00376	23.08		
32.73	5.36	0.93	0.72	0.00377	25.79	0.00376	23.98		
32.09	4.95	0.91	0.67	0.00377	26.09	0.00377	24.95		
31.46	4.54	0.89	0.61	0.00377	26.40	0.00377	26.01	54.04	
30.82	4.13	0.87	0.56	0.00377	26.71	0.00378	27.17		
30.18	3.73	0.85	0.50	0.00378	27.04	0.00378	28.46		
30.78	3.32	0.84	0.45	0.00378	27.36	0.00379	29.89		28.55
28.91	2.91	0.82	0.39	0.00378	27.70	0.00379	31.52		
28.27	1.00	0.80	0.34	0.00378	28.04	0.00380	33.39		
27.64	0.74	0.78	0.28	0.00378	28.39	0.00381	35.60		
27.00	0.50	0.76	0.23	0.00378	28.75	0.00382	38.31		
26.36	0.25	0.75	0.17	0.00378	29.12	0.00383	41.77		
25.73	0.09	0.73	0.12	0.00378	29.50	0.00385	46.61		
25.09	0.08	0.71	0.06	0.00379	29.89	0.00388	54.64		
23.85	0.05	0.68	0.01	0.00379	30.67	0.00398	83.06		
21.19	0.06	0.60	0.01	0.00380	32.50	0.00398	81.70		32.24
17.66	0.04	0.50	0.01	0.00381	35.33	0.00400	86.98		34.14
14.13	0.02	0.40	0.00	0.00382	38.80	0.00403	96.06		
7.06	0.01	0.20	0.00	0.00386	49.66	0.00407	108.18		
3.53	0.01	0.10	0.00	0.00390	60.63	0.00409	112.01		
2.65	0.00	0.08	0.00	0.00392	65.21	0.00411	117.43		
1.77	0.00	0.05	0.0001	0.00394	71.71	0.00419	139.22		
0.88	0.00	0.03	0.00	0.00398	82.91	0.00420	143.16		
0.35	0.00	0.01	0.00	0.00404	97.90	0.00422	148.74		
0.26	0.00	0.01	0.00	0.00405	102.65	0.00426	158.33		
0.18	0.00	0.01	0.00	0.00408	109.37	0.00431	171.14		
0.09	0.00	0.00	0.00	0.00412	120.97	0.00432	175.19		
0.04	0.00	0.00	0.00	0.00418	136.48	0.00434	180.92		
0.03	0.00	0.00	0.00	0.00420	141.40	0.00438	190.79		
0.02	0.00	0.00	0.00	0.00422	148.36	0.00443	203.95		
0.00	0.00	0.0001	0.00000075	0.00433	176.42	0.00444	208.11		

Table D3 cont'd. Rayleigh model data for Experiment 3, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (Experiment 3 (PU122-2.2m and PU80-1.7m))

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.00	‰
δ_o-NO_3^-	40.00	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	20.00	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	20.00	mg/L

Nitrification: Actual Data - Input

Experiment:	PU-B	
Initial NH_4^+-N (mg/L)	35.31	mg N/L
Final NH_4^+-N (mg/L)	23.85	mg N/L
f NH_4^+	0.68	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	24.62	‰
Initial NO3--N (mg/L)	**7.40**	mg N/L
Final NO3--N (mg/L)	0.05	mg N/L
f NO_3^-	0.01	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	**20.02**	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-15.00	‰
α (NH4)	0.9850	
(α-1) NH4	-0.0150	
ε (NO3- - Nitrification)	-12.00	‰
α (NO3-)	0.9880	
(α-1) NO3-	-0.0120	

Table D3 cont'd. Rayleigh model data for Experiment 3, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (Experiment 3 (PU122-2.2m and PU80-1.7m))

Equations:

$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{reacta}$	Fractionation (ii)
$\delta = [R_{sample}/R_{stan}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio

where:

εp-r	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N$ = 0.0036765
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

Table D4. Rayleigh model data for Experiment 3, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU125-2.7m and PU80-1.7m)

Experimental Data - Nitrification: Experiment 3 (PU125-2.7m and PU80-1.7m)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
8.48	0.00	1.00	0.00382	37.84			0.00				40.6
8.47	0.01	1.00	0.00382	37.86	0.01	25.40	0.01	25.40	21.38		
8.17	0.31	0.96	0.00382	38.31	0.30	25.62	0.31	25.62	21.47	21.38	37.8
8.10	0.38	0.96	0.00382	38.41	0.37	25.67	0.38	25.67	21.49		
7.73	0.75	0.91	0.00382	39.00	0.37	26.24	0.75	25.95	21.61		
7.36	1.12	0.87	0.00382	39.61	0.37	26.83	1.12	26.24	21.74		
6.99	1.49	0.82	0.00382	40.26	0.37	27.45	1.49	26.54	21.88		
6.62	1.86	0.78	0.00383	40.94	0.37	28.11	1.86	26.85	22.03		
6.25	2.23	0.74	0.00383	41.66	0.37	28.80	2.23	27.18	22.18		
6.13	2.35	0.72	0.00383	41.90	0.12	29.28	2.35	27.28	22.23	26.32	39.1
5.88	2.61	0.69	0.00383	42.42	0.37	29.53	2.61	27.51	22.35		
5.50	2.98	0.65	0.00384	43.24	0.37	30.31	2.98	27.86	22.52		
5.13	3.35	0.61	0.00384	44.12	0.37	31.15	3.35	28.23	22.71		
4.76	3.72	0.56	0.00384	45.06	0.74	31.60	3.72	28.61	22.91		
4.39	4.09	0.52	0.00385	46.07	0.37	33.01	4.09	29.01	23.12		
4.16	4.32	0.49	0.00385	46.75	0.23	33.85	4.32	29.27	23.25	25.26	44.9
4.02	4.46	0.47	0.00385	47.18	0.37	34.06	4.46	29.43	23.34		
3.65	4.83	0.43	0.00385	48.40	0.37	35.21	4.83	29.87	23.57		
2.55	5.93	0.30	0.00387	52.95	1.84	36.57	5.93	31.35	24.37		
1.70	6.79	0.20	0.00389	58.08	0.85	42.73	6.79	32.78	25.12		
0.85	7.63	0.10	0.00392	66.92	0.85	49.24	7.63	34.61	26.07		
0.64	7.85	0.08	0.00394	70.61	0.21	55.85	7.85	35.19	26.36		
0.42	8.06	0.05	0.00396	75.83	0.21	60.17	8.06	35.84	26.69		
0.21	8.27	0.03	0.00399	84.82	0.21	66.85	8.27	36.64	27.08		
0.08	8.40	0.01	0.00403	96.81	0.13	76.82	8.40	37.25	27.36		
0.06	8.42	0.01	0.00405	100.60	0.02	85.43	8.42	37.37	27.41		
0.04	8.44	0.01	0.00407	105.97	0.02	89.87	8.44	37.50	27.47		
0.02	8.46	0.00	0.00410	115.21	0.02	96.73	8.46	37.65	27.54		
0.01	8.47	0.00	0.00415	127.54	0.01	106.99	8.47	37.75	27.59		
0.01	8.48	0.00	0.00416	131.44	0.00	115.84	8.48	37.77	27.59		
0.00	8.48	0.00	0.00418	136.96	0.00	120.40	8.48	37.79	27.60		
0.00	8.48	0.0001	0.00426	159.13	0.00	131.41	8.48	37.83	27.62		

Table D4 cont'd. Rayleigh model data for Experiment 3, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU125-2.7m and PU80-1.7m)

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00382	37.84	0.00376	21.38
5.99	5.99	1.00	1.00	0.00382	37.85	0.00376	21.40
5.40	5.40	0.90	0.90	0.00382	38.50	0.00376	23.00
4.80	4.80	0.80	0.80	0.00382	39.23	0.00377	24.80
4.20	4.20	0.70	0.70	0.00382	40.07	0.00378	26.86
3.60	3.60	0.60	0.60	0.00383	41.03	0.00378	29.24
3.00	3.00	0.50	0.50	0.00383	42.17	0.00379	32.05
2.40	2.40	0.40	0.40	0.00384	43.56	0.00381	35.52
1.80	1.80	0.30	0.30	0.00384	45.37	0.00382	39.99
1.20	1.20	0.20	0.20	0.00385	47.91	0.00385	46.34
0.60	0.60	0.10	0.10	0.00387	52.28	0.00389	57.27
0.30	0.30	0.05	0.05	0.00388	56.67	0.00393	68.32
0.06	0.06	0.01	0.01	0.00392	66.92	0.00402	94.43
0.03	0.03	0.01	0.01	0.00394	71.37	0.00407	105.87
0.01	0.01	0.00	0.00	0.00398	81.76	0.00417	132.89
0.00	0.00	0.00	0.00	0.00399	86.27	0.00421	144.73
0.00	0.00	0.00	0.00	0.00403	96.81	0.00431	172.70

Table D4 cont'd. Rayleigh model data for Experiment 3, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU125-2.7m and PU80-1.7m)

Experimental Data Anammox (PU125-2.7m and PU80-1.7m)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment - PU12596 Bag	
X_I (NH_4^+)	X_I (NO_3^+)	$f NH_4^+$	$f NO_3^-$	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Data From Experiment	Measured δ15N-NH4
8.48	13.89	1.00	1.00	0.00382	37.84	0.00376	21.38		40.63
8.47	13.52	1.00	0.97	0.00382	37.85	0.00376	21.79		
8.17	13.16	0.96	0.95	0.00382	38.07	0.00376	22.21	21.38	37.84
8.10	12.79	0.96	0.92	0.00382	38.13	0.00376	22.64		
7.73	12.43	0.91	0.89	0.00382	38.42	0.00376	23.08		
7.36	12.06	0.87	0.87	0.00382	38.73	0.00376	23.54		
6.99	11.70	0.82	0.84	0.00382	39.05	0.00376	24.01		
6.62	11.33	0.78	0.82	0.00382	39.39	0.00377	24.50	26.32	
6.25	10.97	0.74	0.79	0.00382	39.75	0.00377	25.00		
6.13	10.60	0.72	0.76	0.00382	39.86	0.00377	25.52		39.13
5.88	10.24	0.69	0.74	0.00382	40.13	0.00377	26.06		
5.50	9.88	0.65	0.71	0.00383	40.54	0.00377	26.61		
5.13	9.51	0.61	0.68	0.00383	40.97	0.00378	27.19	25.26	
4.76	9.15	0.56	0.66	0.00383	41.44	0.00378	27.80		
4.39	8.42	0.52	0.61	0.00383	41.95	0.00378	29.08		
4.16	7.64	0.49	0.55	0.00383	42.30	0.00379	30.58		44.93
4.02	6.94	0.47	0.50	0.00383	42.50	0.00379	32.05		
3.65	5.55	0.43	0.40	0.00383	43.11	0.00381	35.52		
2.55	4.17	0.30	0.30	0.00384	45.37	0.00382	39.99		
1.70	2.78	0.20	0.20	0.00385	47.91	0.00385	46.34		
0.85	1.39	0.10	0.10	0.00387	52.28	0.00389	57.27		
0.64	1.04	0.08	0.08	0.00388	54.10	0.00390	61.85		
0.42	0.69	0.05	0.05	0.00388	56.67	0.00393	68.32		
0.21	0.35	0.03	0.03	0.00390	61.07	0.00397	79.49		
0.08	0.14	0.01	0.01	0.00392	66.92	0.00402	94.43		
0.06	0.10	0.01	0.01	0.00393	68.76	0.00404	99.16		
0.04	0.07	0.01	0.01	0.00394	71.37	0.00407	105.87		
0.02	0.03	0.00	0.00	0.00396	75.83	0.00411	117.42		
0.01	0.01	0.00	0.00	0.00398	81.76	0.00417	132.89		
0.01	0.01	0.00	0.00	0.00398	83.63	0.00418	137.79		
0.00	0.01	0.00	0.00	0.00399	86.27	0.00421	144.73		
0.00	0.00	0.0001	0.0001	0.00403	96.81	0.00431	172.70		

Table D4 cont'd. Rayleigh model data for Experiment 3, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU125-2.7m and PU80-1.7m)

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.00	‰
δ_o-NO_3^-	40.00	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	20.00	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	20.00	mg/L

Nitrification: Actual Data - Input

Experiment:	PU-D	
Initial NH_4^+-N (mg/L)	8.48	mg N/L
Final NH_4^+-N (mg/L)	2.55	mg N/L
f NH_4^+	0.30	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	37.84	‰
Initial NO3--N (mg/L)	**13.89**	mg N/L
Final NO3--N (mg/L)	8.42	mg N/L
f NO_3^-	0.61	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	**21.38**	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-6.00	‰
α (NH4)	0.994	
(α-1) NH4	-0.006	
ε (NO3- - Nitrification)	-15.00	‰
α (NO3-)	0.985	
(α-1) NO3-	-0.015	

Table D4 cont'd. Rayleigh model data for Experiment 3, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 3 (PU125-2.7m and PU80-1.7m)

Equations:

$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{reacta}$	Fractionation (ii)
$\delta = [R_{sample}/R_{stan}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio

where:

εp-r	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N$ = 0.0036765
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

Appendix E:
Experiment 4 – Sacrificial Serum Bottles

Geochemistry and Isotope Data
Theoretical Denitrification Calculations
Rayleigh Model Data

Table E1. Analytical results for Experiment 4; Zorra groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 1, single source groundwater (PU121-3.0m); Trial 2, Control (PU121-3.0m); Trial 3, dual source groundwater (PU115-3.0m and PU84-3.1m); Trial 4, single source groundwater (PU86-3.1m); and Trial 5, single source groundwater (PU115-2.2m). Each bottle set was injected with NH4NO3 for increased substrate concentrations, Oct. 25, 2010 to Jun. 24, 2010.

Trial	Date	Days	Analytical (mg/L)											Isotopes (‰)		
			NO_3^--N	NO_2^--N	NH_4^+-N	TN	DON	TKN	DON	DOC	Cl^-	$SO4^{2-}$	Br^-	^{15}N-NH_4^+	^{15}N-NO_3^-	^{18}O-NO_3^-
Trial 1	25-Oct-10	0	80.44	2.32	74.75	149		85.4	10.6	53	60	22	n.a.	-3.08	-1.34	23.11
	26-Nov-10	32	77.93	0.07	69.60	149				25	61	22	n.a.			
	10-Dec-10	46	61.34	0.00	70.10					48	59	20	0.35	-0.82	0.76	25.07
	21-Jan-11	88	62.40	0.04	73.32	128				28	73	29	1.47			
	25-Feb-11	123	56.50	0.01	51.16			56.8	5.6	27	76	23	n.a	0.44	3.78	26.29
	25-Mar-11	151	60.50	0.03	65.41					35	67	23				
	28-Apr-11	185	59.88	0.00	53.73	110				28	56	21		0.40	5.33	26.71
	31-May-11	218	**Bottle Destroyed on 11-Jan-11 while manua**													
	29-Jun-11	247	53.27	0.01	49.31			55.5	6.2	66	51	19	0.26	1.90	7.06	28.84
Trial 2	25-Oct-10	0	85.27	0.02	83.80			71.6	-12.2	53	66	23	n.a.	-3.39	-1.42	22.18
	26-Nov-10	32	76.16	0.36	77.97	164				41	68	23	n.a.			
	10-Dec-10	46	72.90	0.79	85.70	153				59	63	23	0.62	-2.56	0.34	22.68
	21-Jan-11	88	68.60	2.42	80.20	159				34	75	28	1.40			
	25-Feb-11	123	66.40	0.27	81.25					81	80	25	n.a	-2.77	0.97	23.22
	25-Mar-11	151	67.70	0.02	86.01	154				31	67	24				
	28-Apr-11	185	65.72	0.00	65.02	143				50	57	21		-6.10	2.83	25.22
	31-May-11	218	69.81	0.00	76.94					42	59	21	0.28			
	29-Jun-11	247	68.37	-	71.07			59.2	-11.9	99	56	21	0.85	-2.80	2.73	24.51
Trial 3	25-Oct-10	0	80.27	0.01	69.51	144				49	92	33	n.a.	-2.06	-1.36	22.86
	26-Nov-10	32	69.90	0.02	59.68	122				33	95	30	n.a.			
	10-Dec-10	46	62.10	0.00	62.50					49	86	27	1.31	-0.03	0.05	23.61
	21-Jan-11	88	63.50	0.02	44.41	135				38	102	33	0.03			
	25-Feb-11	123	66.30	<0.001	51.64	105				54	106	35	1.56	0.77		
	25-Mar-11	151	66.00	0.01	58.84	224				41	99	35				
	28-Apr-11	185	54.34	<0.001	45.52					21	93	29	0.75	*1.50*	0.45	22.50
	31-May-11	218	60.58	0.01	67.72	100				46	95	28	0.63			
	29-Jun-11	247	54.53	0.00	52.22					94	77	27	0.66	*2.00*	5.62	27.54

Table E1 cont'd. Analytical results for Experiment 4; Zorra groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 1, single source groundwater (PU121-3.0m); Trial 2, Control (PU121-3.0m); Trial 3, dual source groundwater (PU115-3.0m and PU84-3.1m); Trial 4, single source groundwater (PU86-3.1m); and Trial 5, single source groundwater (PU115-2.2m). Each bottle set was injected with NH4NO3 for increased substrate concentrations, Oct. 25, 2010 to Jun. 24, 2010.

Trial	Date	Days	Analytical (mg/L)											Isotopes (‰)		
			NO_3^--N	NO_2^--N	NH_4^+-N	TN	DON	TKN	DON	DOC	Cl^-	$SO4^{2-}$	Br^-	^{15}N-NH_4^+	^{15}N-NO_3^-	^{18}O-NO_3^-
Trial 4	13-Oct-10	0	75.40	0.50	96.38			63.8	-32.6	20	15	6	0.55	-4.80	-6.95	15.82
	26-Nov-10	32	75.17	0.13	52.63	129				6	17	3	0.29			
	10-Dec-10	46	83.74	0.00	74.80	77				6	18	3	0.09	-4.00	-1.69	21.47
	21-Jan-11	88	74.30	0.00	82.85	133				3	34	10	0.85			
	25-Feb-11	123	67.00	0.00	53.10	101		56.9	3.7	15	103	9		-1.66	-1.20	19.59
	25-Mar-11	151	74.80	0.02	79.79	155				5	16	8				
	28-Apr-11	185	80.95	0.02	82.59					92	20	3	0.21	-0.70	4.31	27.56
	31-May-11	218	79.00	0.01	80.43	121				11	23	3	1.18			
	29-Jun-11	247	76.98	-0.01	66.04			47.2	-18.9	22	15	3	0.11	*-0.50*	1.73	23.76
Trial 5	13-Oct-10	0	75.40	-	99.81			96.3	-3.5	56	87	22	0.97	-3.02		
	26-Nov-10	32	88.40	0.04	72.84	147				42	94	27	1.24			
	10-Dec-10	46	80.07	0.01	75.70	143				52	89	23	n.a	-1.30	0.39	24.53
	21-Jan-11	88	71.10	0.05	75.99	91				44	99	30	1.68			
	25-Feb-11	123	75.30	0.02	69.90			69.8	-0.1	61	95	28	0.91	1.51	1.87	22.42
	25-Mar-11	151	71.40	0.04	76.31	152				44	88	27				
	28-Apr-11	185	68.85	0.02	81.12					43	74	24		1.70	4.47	27.61
	31-May-11	218	68.70	0.02	65.26	116				54	81	24	0.17			
	29-Jun-11	247	67.90	-	63.79			65.5	1.7	98	76	24	0.72	3.00	6.63	29.65

Table E1 cont'd. Analytical results for Experiment 4; Zorra groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 1, single source groundwater (PU121-3.0m); Trial 2, Control (PU121-3.0m); Trial 3, dual source groundwater (PU115-3.0m and PU84-3.1m); Trial 4, single source groundwater (PU86-3.1m); and Trial 5, single source groundwater (PU115-2.2m). Each bottle set was injected with NH4NO3 for increased substrate concentrations, Oct. 25, 2010 to Jun. 24, 2010.

Trial	Date	Days	Gases (nmol/L, unless specified)			Field Parameters				
			N_2O	CO_2 (mg/L)	CH_4	DO (mg/L)	Conductivity (µS/cm)	Eh (mV)	pH	Temperature (°C)
Trial 1	25-Oct-10	0	5725	91	48	0.11	1849	132	6.94	22.4
	26-Nov-10	32	459	22	198	0.23	1824	92	7.08	21.4
	10-Dec-10	46				0.22	1797	55	6.91	21.6
	21-Jan-11	88	37	23	163	0.29	1804	130	6.84	22.0
	25-Feb-11	123	10	31	203	0.29	1801	157	7.17	21.5
	25-Mar-11	151	9	14	169	0.11	1725	188	6.86	21.5
	28-Apr-11	185	9	68	210	0.11	1735	186	7.08	21.2
	31-May-11	218	**Bottle Destroyed on 11-Jan-11 while manually shaking**							
	29-Jun-11	247	7	71	314	0.07	1753	195	7.02	22.1
Trial 2	25-Oct-10	0	22641	92	33	0.08	1996	75	6.84	22.5
	26-Nov-10	32	48452	22	68	0.23	1879	98	6.75	21.1
	10-Dec-10	46				0.24	1908	26	6.94	21.3
	21-Jan-11	88	34266	25	89	0.27	1915	91	6.81	22.0
	25-Feb-11	123	35400	42	136	0.29	1937	163	7.16	21.5
	25-Mar-11	151	11707	16	123	0.13	1865	167	6.81	21.6
	28-Apr-11	185	25835	86	107	0.10	1884	169	7.21	21.2
	31-May-11	218	7790	93	11	0.13	1975	170	7.12	20.1
	29-Jun-11	247	7	77	73	0.06	1868	89	7.02	22.1
Trial 3	25-Oct-10	0	247	132	370	0.08	2030	97	6.83	22.5
	26-Nov-10	32	60	31	1171	0.20	1915	87	6.69	21.3
	10-Dec-10	46				0.23	1929	10	6.93	21.6
	21-Jan-11	88	306	30	841	0.26	1907	66	6.88	22.1
	25-Feb-11	123	189	36	993	0.26	1942	148	7.20	21.4
	25-Mar-11	151	15	14	793	0.08	1905	132	6.78	21.6
	28-Apr-11	185	73	75	1935	0.08	1882	144	7.07	21.4
	31-May-11	218	11	99	924	0.08	1977	13*	7.07	20.5
	29-Jun-11	247	6	68	900	0.03	1908	46	6.99	22.3

Table E1 cont'd. Analytical results for Experiment 4; Zorra groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 1, single source groundwater (PU121-3.0m); Trial 2, Control (PU121-3.0m); Trial 3, dual source groundwater (PU115-3.0m and PU84-3.1m); Trial 4, single source groundwater (PU86-3.1m); and Trial 5, single source groundwater (PU115-2.2m). Each bottle set was injected with NH4NO3 for increased substrate concentrations, Oct. 25, 2010 to Jun. 24, 2010.

Trial	Date	Days	Gases (nmol/L, unless specified)			Field Parameters				
			N_2O	CO_2 (mg/L)	CH_4	DO (mg/L)	Conductivity (µS/cm)	Eh (mV)	pH	Temperature (°C)
Trial 4	13-Oct-10	0	59	39	95	0.91	1313	40	7.45	24.6
	26-Nov-10	32	76	32	65	0.18	1261	69	6.96	21.2
	10-Dec-10	46				0.22	1266	7	7.23	21.8
	21-Jan-11	88	60	15	167	0.26	1253	22	7.12	22.1
	25-Feb-11	123	59	17	160	0.26	1676	167	7.50	21.4
	25-Mar-11	151	8	4	168	0.08	1232	92	7.10	21.7
	28-Apr-11	185	24	52	546	0.07	1234	108	7.43	21.5
	31-May-11	218	8	44	222	0.09	1260	13*	7.39	20.4
	29-Jun-11	247	6	48	396	0.03	1227	0	7.31	22.5
Trial 5	13-Oct-10	0	71	43	1341	0.13	1991	53	7.27	24.7
	26-Nov-10	32	37	14	700	0.18	1885	72	6.86	21.3
	10-Dec-10	46				0.19	1887	5	7.08	21.8
	21-Jan-11	88	27	21	322	0.25	1866	10	6.96	22.2
	25-Feb-11	123	28	22	315	0.25	1879	165	7.32	21.5
	25-Mar-11	151	6	8	601	0.07	1822	93	6.90	21.7
	28-Apr-11	185	15	70	1204	0.05	1835	99	7.18	21.6
	31-May-11	218	7	109	762	0.07	1861	13*	7.14	20.6
	29-Jun-11	247	6	56	518	0.01	1801	12	7.08	22.5

Appendix D – Theoretical Denitrification Calculation for Trial 1:

12 mg/L of NO_3^--N was consumed. How much DOC would be consumed, and how much CO_2 would be produced, if the nitrate was completely consumed by denitrification?

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

DOC:

12 mg/L NO_3^--N x 1 g/1000 mg x 1 mole/ 14.00674 g x 0.160 L/bottle
= 0.000137 moles N/bottle

4moles N: 5 moles DOC :. DOC = 0.000137 x 5/4 = 0.000171 moles DOC

0.000171 moles/bottle DOC x 12.0107 g/mole x 1 bottle/0.160 L x 1000 mg/g
= 12.862 mg/L DOC-C consumed

CO_2:

As both DOC and CO_2 are measured as C (and both have 5 moles in rxn), both calculations are identical, so 12.862 mg/L CO_2-C will be produced.

Table E2. Rayleigh model data for Experiment 4, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m)

Experimental Data - Nitrification: Experiment 4 (PU121-3.0m)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
74.79	0.00	1.00	0.00367	-3.08			0.00			-1.34	-3.1
74.72	0.07	1.00	0.00367	-3.07	0.07	-15.04	0.07	-15.04	-0.02		
72.37	2.42	0.97	0.00367	-2.69	2.34	-14.84	2.42	-14.85	-0.47		
69.99	4.80	0.94	0.00367	-2.29	2.38	-14.25	4.80	-14.55	-0.88	0.76	-0.8
67.69	7.10	0.91	0.00367	-1.89	2.30	-14.28	7.10	-14.46	-1.26		
65.35	9.44	0.87	0.00367	-1.46	2.34	-13.66	9.44	-14.26	-1.60		
63.01	11.78	0.84	0.00367	-1.03	2.34	-13.23	11.78	-14.06	-1.92	3.78	0.4
60.66	14.13	0.81	0.00367	-0.57	2.34	-12.79	14.13	-13.85	-2.21		
58.32	16.47	0.78	0.00368	-0.10	2.34	-12.33	16.47	-13.63	-2.47	5.33	0.4
55.98	18.81	0.75	0.00368	0.39	2.34	-11.86	18.81	-13.41	-2.70		
49.03	25.76	0.66	0.00368	1.98	6.95	-10.85	25.76	-12.72	-3.27	7.06	1.9
44.87	29.92	0.60	0.00369	3.05	4.16	-9.51	22.97	-15.99	-3.77		
41.13	33.66	0.55	0.00369	4.10	3.74	-8.48	26.71	-14.94	-3.94		
37.40	37.40	0.50	0.00370	5.25	3.74	-7.39	30.45	-14.01	-4.06		
33.66	41.13	0.45	0.00370	6.52	3.74	-6.20	34.19	-13.16	-4.14		
29.92	44.87	0.40	0.00371	7.94	3.74	-4.87	37.92	-12.34	-4.16		
26.18	48.61	0.35	0.00371	9.56	3.74	-3.37	41.66	-11.53	-4.14		
22.44	52.35	0.30	0.00372	11.43	3.74	-1.66	45.40	-10.72	-4.06		
14.96	59.83	0.20	0.00374	16.36	7.48	1.56	52.88	-8.98	-3.73		
7.48	67.31	0.10	0.00377	24.85	7.48	7.87	60.36	-6.90	-3.09		
5.61	69.18	0.08	0.00378	28.39	1.87	14.22	62.23	-6.26	-2.85		
3.74	71.05	0.05	0.00380	33.41	1.87	18.36	64.10	-5.54	-2.56		
1.87	72.92	0.03	0.00383	42.04	1.87	24.78	65.97	-4.68	-2.20		
0.75	74.04	0.01	0.00387	53.56	1.12	34.36	67.09	-4.03	-1.91		
0.56	74.23	0.01	0.00389	57.21	0.19	42.63	67.28	-3.90	-1.85		
0.37	74.42	0.01	0.00391	62.36	0.19	46.89	67.47	-3.76	-1.79		
0.19	74.60	0.00	0.00394	71.24	0.19	53.49	67.65	-3.60	-1.71		
0.07	74.72	0.00	0.00398	83.08	0.11	63.34	67.77	-3.49	-1.66		
0.06	74.73	0.00	0.00400	86.82	0.02	71.84	67.78	-3.47	-1.65		
0.04	74.75	0.00	0.00402	92.13	0.02	76.22	67.80	-3.45	-1.64		
0.01	74.78	0.0001	0.00409	113.42	0.03	86.80	67.83	-3.41	-1.62		

Table E2 cont'd. Rayleigh model data for Experiment 4, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m)

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00367	-3.08	0.00368	0.00
5.99	5.99	1.00	1.00	0.00367	-3.07	0.00368	0.02
5.40	5.40	0.90	0.90	0.00367	-1.92	0.00368	1.58
4.80	4.80	0.80	0.80	0.00367	-0.63	0.00369	3.35
4.20	4.20	0.70	0.70	0.00368	0.84	0.00370	5.36
3.60	3.60	0.60	0.60	0.00369	2.54	0.00370	7.69
3.00	3.00	0.50	0.50	0.00369	4.55	0.00371	10.45
2.40	2.40	0.40	0.40	0.00370	7.02	0.00373	13.84
1.80	1.80	0.30	0.30	0.00371	10.21	0.00374	18.22
1.20	1.20	0.20	0.20	0.00373	14.73	0.00377	24.44
0.60	0.60	0.10	0.10	0.00376	22.49	0.00381	35.14
0.30	0.30	0.05	0.05	0.00379	30.32	0.00385	45.96
0.06	0.06	0.01	0.01	0.00386	48.72	0.00394	71.52
0.03	0.03	0.01	0.01	0.00389	56.75	0.00398	82.72
0.01	0.01	0.00	0.00	0.00395	75.62	0.00408	109.17
0.00	0.00	0.00	0.00	0.00398	83.86	0.00412	120.77
0.00	0.00	0.00	0.00	0.00406	103.22	0.00422	148.15

Table E2 cont'd. Rayleigh model data for Experiment 4, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m)

Experimental Data Anammox: Experiment 4 (PU121-3.0m)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment - PU12596 Bag	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Measured δ15N-NO3	Measured δ15N-NH4
74.79	**74.51**	1.00	1.00	0.00367	-3.08	0.00368	0.00	**-1.34**	-3.08
74.72	72.93	1.00	0.98	0.00367	-3.07	0.00368	0.32		
73.00	71.74	0.98	0.96	0.00367	-2.81	0.00368	0.57		
71.28	**70.09**	0.95	0.95	0.00367	-2.55	0.00368	0.82	0.76	
69.99	69.37	0.93	0.93	0.00367	-2.29	0.00368	1.07		-0.82
67.85	68.18	0.91	0.92	0.00367	-2.01	0.00368	1.33		
66.13	66.99	0.88	0.90	0.00367	-1.73	0.00368	1.60		
64.41	65.81	0.86	0.88	0.00367	-1.44	0.00368	1.87		
61.96	64.62	0.84	0.87	0.00367	-1.14	0.00368	2.14		0.44
60.98	**62.69**	0.82	0.85	0.00367	-0.84	0.00369	2.42	3.78	
59.26	62.24	0.79	0.84	0.00367	-0.52	0.00369	2.70		
57.54	61.06	0.77	0.82	0.00368	-0.20	0.00369	2.99		
55.49	59.87	0.75	0.80	0.00368	0.13	0.00369	3.29		0.40
54.11	58.68	0.72	0.79	0.00368	0.48	0.00369	3.59		
49.02	57.50	0.66	0.77	0.00368	1.56	0.00369	3.90		1.90
48.61	56.31	0.65	0.76	0.00368	1.66	0.00369	4.21		
47.12	**56.73**	0.63	0.74	0.00368	2.00	0.00369	4.53	5.33	
44.87	53.94	0.60	0.72	0.00369	2.54	0.00369	4.86		
41.13	52.75	0.55	0.71	0.00369	3.50	0.00370	5.19		
37.40	**50.77**	0.50	0.68	0.00369	4.55	0.00370	5.77	7.06	
33.66	37.26	0.45	0.50	0.00370	5.72	0.00371	10.45		
29.92	29.80	0.40	0.40	0.00370	7.02	0.00373	13.84		
49.03	22.35	0.30	0.30	0.00371	10.21	0.00374	18.22		
14.96	14.90	0.20	0.20	0.00373	14.73	0.00377	24.44		
7.48	7.45	0.10	0.10	0.00376	22.49	0.00381	35.14		
5.61	5.59	0.08	0.08	0.00377	25.73	0.00382	39.62		
3.74	3.73	0.05	0.05	0.00379	30.32	0.00385	45.96		
1.87	1.86	0.03	0.03	0.00382	38.20	0.00389	56.89		
0.75	0.75	0.01	0.01	0.00386	48.72	0.00394	71.52		
0.56	0.56	0.01	0.01	0.00387	52.05	0.00396	76.15		
0.37	0.37	0.01	0.01	0.00389	56.75	0.00398	82.72		
0.19	0.19	0.00	0.00	0.00391	64.84	0.00402	94.03		
0.07	0.07	0.00	0.00	0.00395	75.62	0.00408	109.17		
0.06	0.06	0.00	0.00	0.00397	79.03	0.00410	113.97		
0.04	0.04	0.00	0.00	0.00398	83.86	0.00412	120.77		
0.01	0.01	0.0001	0.0001	0.00406	103.22	0.00422	148.15		

Table E2 cont'd. Rayleigh model data for Experiment 4, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m)

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.00	‰
δ_o-NO_3^-	40.00	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	20.00	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	20.00	mg/L

Nitrification: Actual Data - Input

Experiment:	i	
Initial NH_4^+-N (mg/L)	74.79	mg N/L
Final NH_4^+-N (mg/L)	49.03	mg N/L
f NH_4^+	0.66	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	-3.08	‰
Initial NO3--N (mg/L)	**74.51**	mg N/L
Final NO3--N (mg/L)	50.77	mg N/L
f NO_3^-	0.68	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	**0.00**	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-11.00	‰
α (NH4)	0.989	
(α-1) NH4	-0.011	
ε (NO3- - Nitrification)	-15.00	‰
α (NO3-)	0.985	
(α-1) NO3-	-0.015	

Table E2 cont'd. Rayleigh model data for Experiment 4, Trial 1; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m)

Equations:

$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{react}$	Fractionation (ii)
$\delta = [R_{sample}/R_{sta}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio

where:

εp-r	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N = 0.0036765$
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

Table E3. Rayleigh model data for Experiment 4, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m - Control)

Experimental Data - Nitrification: Experiment 4 (PU121-3.0m - Control)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
84.58	0.00	1.00	0.00366	-3.39			0.00			-1.42	-3.4
82.32	2.26	0.98	0.00366	-3.18	2.26	-11.24156	2.26	-11.24156208	-1.697812112	0.34	-2.6
81.56	3.02	0.96	0.00367	-2.96	0.76	-26.71	3.02	-15.14	-1.93		
80.05	4.53	0.95	0.00367	-2.73	1.51	-14.81	4.53	-15.03	-2.17		
78.56	6.01	0.93	0.00367	-2.50	1.49	-14.79	6.01	-14.97	-2.39	0.97	-2.8
75.53	9.05	0.91	0.00367	-2.27	3.03	-8.29	9.05	-12.73	-2.60	2.83	-6.1
75.52	9.06	0.89	0.00367	-2.04	4.53	-14.36	9.06	-14.69	-2.81		
72.50	12.08	0.86	0.00367	-1.55	6.06	-13.96	12.08	-14.46	-3.18	2.73	-2.8
67.66	16.92	0.80	0.00367	-0.72	7.86	-13.37	16.92	-14.08	-3.69		
59.21	25.37	0.70	0.00368	0.88	8.46	-11.94	25.37	-13.37	-4.36		
54.98	29.60	0.65	0.00368	1.77	4.23	-10.69	29.60	-12.98	-4.61		
50.75	33.83	0.60	0.00369	2.74	4.23	-9.78	33.83	-12.58	-4.81		
46.52	38.06	0.55	0.00369	3.78	4.23	-8.79	38.06	-12.16	-4.95		
42.29	42.29	0.50	0.00369	4.93	8.46	-8.24	46.52	-11.45	-5.18		
38.06	46.52	0.45	0.00370	6.20	4.23	-6.51	50.75	-11.04	-5.22		
33.83	50.75	0.40	0.00370	7.63	4.23	-5.18	54.98	-10.59	-5.22		
29.60	54.98	0.35	0.00371	9.24	4.23	-3.68	59.21	-10.09	-5.17		
25.37	59.21	0.30	0.00372	11.11	12.69	-3.61	71.89	-8.95	-5.04		
16.92	67.66	0.20	0.00374	16.04	8.46	1.25	80.35	-7.88	-4.70		
8.46	76.12	0.10	0.00377	24.53	8.46	7.56	88.81	-6.41	-4.08		
6.34	78.24	0.08	0.00378	28.07	2.11	13.90	90.92	-5.93	-3.86		
4.23	80.35	0.05	0.00380	33.09	2.11	18.04	93.04	-5.39	-3.58		
2.11	82.46	0.03	0.00383	41.72	2.11	24.46	95.15	-4.73	-3.24		
0.85	83.73	0.01	0.00387	53.23	1.27	34.04	96.42	-4.22	-2.97		
0.63	83.94	0.01	0.00389	56.88	0.21	42.31	96.63	-4.11	-2.91		
0.42	84.16	0.01	0.00390	62.03	0.21	46.57	96.84	-4.00	-2.85		
0.21	84.37	0.00	0.00394	70.90	0.21	53.16	97.05	-3.88	-2.79		
0.08	84.49	0.00	0.00398	82.74	0.13	63.01	97.18	-3.79	-2.74		
0.06	84.52	0.00	0.00399	86.49	0.02	71.51	97.20	-3.77	-2.73		
0.04	84.54	0.00	0.00401	91.79	0.02	75.89	97.22	-3.76	-2.72		
0.01	84.57	0.0001	0.00409	113.08	0.03	86.46	97.26	-3.73	-2.70		

Table E3 cont'd. Rayleigh model data for Experiment 4, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m - Control)

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00366	-3.39	0.00367	-1.42
5.99	5.99	1.00	1.00	0.00366	-3.39	0.00367	-1.41
5.40	5.40	0.90	0.90	0.00367	-2.76	0.00368	0.16
4.80	4.80	0.80	0.80	0.00367	-2.06	0.00368	1.93
4.20	4.20	0.70	0.70	0.00367	-1.26	0.00369	3.94
3.60	3.60	0.60	0.60	0.00368	-0.33	0.00370	6.26
3.00	3.00	0.50	0.50	0.00368	0.76	0.00371	9.02
2.40	2.40	0.40	0.40	0.00368	2.10	0.00372	12.40
1.80	1.80	0.30	0.30	0.00369	3.83	0.00374	16.78
1.20	1.20	0.20	0.20	0.00370	6.28	0.00376	22.98
0.60	0.60	0.10	0.10	0.00372	10.47	0.00380	33.67
0.30	0.30	0.05	0.05	0.00373	14.68	0.00384	44.48
0.06	0.06	0.01	0.01	0.00377	24.53	0.00393	70.00
0.03	0.03	0.01	0.01	0.00378	28.80	0.00397	81.18
0.01	0.01	0.00	0.00	0.00382	38.78	0.00407	107.60
0.00	0.00	0.00	0.00	0.00384	43.11	0.00411	119.18
0.00	0.00	0.00	0.00	0.00387	53.23	0.00422	146.52

Table E3 cont'd. Rayleigh model data for Experiment 4, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m - Control)

Experimental Data Anammox: Experiment 4 (PU121-3.0m - Control)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment - PU12596 Bag	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Measured δ15N-NO3	Measured δ15N-NH4
84.58	**77.60**	1.00	1.00	0.00366	-3.39	0.00367	-1.42	-1.42	-3.39
84.49	77.53	1.00	1.00	0.00366	-3.39	0.00367	-1.41		
82.33	**75.18**	0.98	0.98	0.00366	-3.26	0.00367	-1.15	0.34	-2.56
81.04	74.92	0.96	0.97	0.00366	-3.14	0.00367	-0.89		
78.56	**71.11**	0.94	0.95	0.00367	-3.01	0.00367	-0.63	0.97	-2.77
75.53	72.31	0.92	0.93	0.00367	-2.88	0.00368	-0.36		-6.10
75.87	71.01	0.90	0.91	0.00367	-2.74	0.00368	-0.09		
72.50	**67.84**	**0.86**	0.90	0.00367	-2.47	0.00368	0.19	2.83	-2.80
70.79	68.40	0.84	0.88	0.00367	-2.33	0.00368	0.47		
63.43	**64.56**	0.80	**0.83**	0.00367	-2.06	0.00368	1.34	2.73	
63.43	62.08	0.75	0.80	0.00367	-1.67	0.00368	1.93		
61.32	58.30	0.73	0.75	0.00367	-1.47	0.00369	2.87		
59.21	54.32	0.70	0.70	0.00367	-1.26	0.00369	3.94		
54.98	50.44	0.65	0.65	0.00367	-0.81	0.00370	5.05		
50.75	46.56	0.60	0.60	0.00368	-0.33	0.00370	6.26		
46.52	42.68	0.55	0.55	0.00368	0.19	0.00370	7.58		
42.29	38.80	0.50	0.50	0.00368	0.76	0.00371	9.02		
38.06	34.92	0.45	0.45	0.00368	1.40	0.00372	10.61		
33.83	31.04	0.40	0.40	0.00368	2.10	0.00372	12.40		
72.50	23.28	0.30	0.30	0.00369	3.83	0.00374	16.78		
16.92	15.52	0.20	0.20	0.00370	6.28	0.00376	22.98		
8.46	7.76	0.10	0.10	0.00372	10.47	0.00380	33.67		
6.34	5.82	0.08	0.08	0.00372	12.22	0.00382	38.14		
4.23	3.88	0.05	0.05	0.00373	14.68	0.00384	44.48		
2.11	1.94	0.03	0.03	0.00375	18.91	0.00388	55.39		
0.85	0.78	0.01	0.01	0.00377	24.53	0.00393	70.00		
0.63	0.58	0.01	0.01	0.00377	26.30	0.00395	74.63		
0.42	0.39	0.01	0.01	0.00378	28.80	0.00397	81.18		
0.21	0.19	0.00	0.00	0.00380	33.09	0.00402	92.48		
0.08	0.08	0.00	0.00	0.00382	38.78	0.00407	107.60		
0.06	0.06	0.00	0.00	0.00383	40.58	0.00409	112.39		
0.04	0.04	0.00	0.00	0.00384	43.11	0.00411	119.18		
0.01	0.01	0.0001	0.0001	0.00387	53.23	0.00422	146.52		

Table E3 cont'd. Rayleigh model data for Experiment 4, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m - Control)

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.00	‰
δ_o-NO_3^-	40.00	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	20.00	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	20.00	mg/L

Nitrification: Actual Data - Input

Experiment:	i	
Initial NH_4^+-N (mg/L)	84.58	mg N/L
Final NH_4^+-N (mg/L)	72.50	mg N/L
f NH_4^+	0.86	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	-3.39	‰
Initial NO3--N (mg/L)	**77.60**	mg N/L
Final NO3--N (mg/L)	64.56	mg N/L
f NO_3^-	0.83	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	**-1.42**	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-6.00	‰
α (NH4)	0.994	
(α-1) NH4	-0.006	
ε (NO3- - Nitrification)	-15.00	‰
α (NO3-)	0.985	
(α-1) NO3-	-0.015	

Table E3 cont'd. Rayleigh model data for Experiment 4, Trial 2; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU121-3.0m - Control)

Equations:	
$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{reacta}$	Fractionation (ii)
$\delta = [R_{sample}/R_{star}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio
where:	
εp-r	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N = 0.0036765$
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

Table E4. Rayleigh model data for Experiment 4, Trial 3; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU115-3.0m & PU121-3.0m)

Experimental Data - Nitrification: Experiment 4 (PU115-3.0m & PU121-3.0m)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
60.96	0.00	1.00	0.00368	-0.03			0.00			-1.36	-2.1
60.90	0.06	1.00	0.00368	-0.02	0.06	-12.02	0.06	-12.02	-1.37		
59.42	1.55	0.98	0.00368	0.17	1.48	-7.35	1.55	-7.53	-1.49	0.05	0.0
59.06	1.91	0.97	0.00368	0.35	1.84	-11.84	1.91	-11.84	-1.63		
56.83	4.13	0.95	0.00368	0.54	2.59	-8.07	4.13	-7.87	-1.71		0.8
57.21	3.75	0.94	0.00368	0.73	1.84	-11.47	3.75	-11.66	-1.86		
54.75	6.22	0.92	0.00368	0.93	2.08	-9.63	6.22	-8.46	-1.92	0.45	1.5
55.37	5.59	0.91	0.00368	1.13	1.84	-11.08	5.59	-11.47	-2.08		
52.66	8.30	**0.86**	0.00368	1.73	2.08	-19.31	8.30	-11.18	-2.36	5.62	2.0
48.77	12.19	0.80	0.00369	2.65	6.60	-10.15	12.19	-10.75	-2.70		
45.72	15.24	0.75	0.00369	3.43	3.05	-9.00	15.24	-10.40	-2.92		
42.67	18.29	0.70	0.00369	4.26	3.05	-8.21	18.29	-10.04	-3.10		
39.62	21.34	0.65	0.00370	5.15	3.05	-7.36	21.34	-9.65	-3.23		
36.58	24.38	0.60	0.00370	6.12	3.05	-6.44	24.38	-9.25	-3.33		
33.53	27.43	0.55	0.00370	7.17	3.05	-5.44	27.43	-8.83	-3.40		
30.48	30.48	0.50	0.00371	8.32	6.10	-4.90	30.48	-8.38	-3.43		
27.43	33.53	0.45	0.00371	9.60	3.05	-3.16	33.53	-7.91	-3.42		
24.38	36.58	0.40	0.00372	11.03	3.05	-1.83	36.58	-7.40	-3.37		
21.34	39.62	0.35	0.00372	12.65	3.05	-0.32	39.62	-6.86	-3.29		
18.29	42.67	0.30	0.00373	14.52	9.14	-0.25	42.67	-6.27	-3.17		
12.19	48.77	0.20	0.00375	19.47	6.10	4.63	48.77	-4.91	-2.78		
6.10	54.86	0.10	0.00378	27.99	6.10	10.96	54.86	-3.14	-2.12		
4.57	56.39	0.08	0.00379	31.54	1.52	17.32	56.39	-2.59	-1.90		
3.05	57.91	0.05	0.00381	36.57	1.52	21.48	57.91	-1.96	-1.62		
1.52	59.44	0.03	0.00384	45.23	1.52	27.91	59.44	-1.19	-1.28		
0.61	60.35	0.01	0.00389	56.79	0.91	37.53	60.35	-0.60	-1.02		
0.46	60.50	0.01	0.00390	60.44	0.15	45.82	60.50	-0.49	-0.96		
0.30	60.66	0.01	0.00392	65.61	0.15	50.10	60.66	-0.36	-0.91		
0.15	60.81	0.00	0.00395	74.51	0.15	56.71	60.81	-0.22	-0.84		
0.06	60.90	0.00	0.00399	86.39	0.09	66.59	60.90	-0.12	-0.80		
0.05	60.92	0.00	0.00401	90.15	0.02	75.12	60.92	-0.10	-0.79		
0.03	60.93	0.00	0.00403	95.47	0.02	79.52	60.93	-0.08	-0.78		
0.01	60.95	0.0001	0.00411	116.83	0.02	90.13	60.95	-0.04	-0.76		

Table E4 cont'd. Rayleigh model data for Experiment 4, Trial 3; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU115-3.0m & PU121-3.0m)

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00368	-0.03	0.00367	-1.36
5.99	5.99	1.00	1.00	0.00368	-0.02	0.00367	-1.35
5.40	5.40	0.90	0.90	0.00368	0.60	0.00368	0.22
4.80	4.80	0.80	0.80	0.00368	1.31	0.00368	1.99
4.20	4.20	0.70	0.70	0.00368	2.11	0.00369	4.00
3.60	3.60	0.60	0.60	0.00369	3.04	0.00370	6.32
3.00	3.00	0.50	0.50	0.00369	4.14	0.00371	9.08
2.40	2.40	0.40	0.40	0.00370	5.48	0.00372	12.46
1.80	1.80	0.30	0.30	0.00370	7.22	0.00374	16.84
1.20	1.20	0.20	0.20	0.00371	9.67	0.00376	23.04
0.60	0.60	0.10	0.10	0.00373	13.88	0.00380	33.73
0.30	0.30	0.05	0.05	0.00374	18.11	0.00384	44.54
0.06	0.06	0.01	0.01	0.00378	27.99	0.00393	70.06
0.03	0.03	0.01	0.01	0.00380	32.27	0.00398	81.25
0.01	0.01	0.00	0.00	0.00383	42.29	0.00407	107.67
0.00	0.00	0.00	0.00	0.00385	46.63	0.00411	119.24
0.00	0.00	0.00	0.00	0.00389	56.79	0.00422	146.59

Table E4 cont'd. Rayleigh model data for Experiment 4, Trial 3; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU115-3.0m & PU121-3.0m)

Experimental Data Anammox: Experiment 4 (PU115-3.0m & PU121-3.0m)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Measured δ15N-NO3	Measured δ15N-NH4
60.96	**73.15**	1.00	1.00	0.00368	-0.03	0.00367	-1.36	**-1.36**	-2.06
60.90	71.74	1.00	0.98	0.00368	-0.02	0.00367	-1.07		
59.42	**69.74**	0.99	0.96	0.00368	0.06	0.00367	-0.77	0.05	-0.03
59.24	68.92	0.97	0.94	0.00368	0.14	0.00367	-0.47		
58.41	67.51	0.96	0.92	0.00368	0.23	0.00368	-0.16		
56.83	**64.03**	0.94	0.90	0.00368	0.31	0.00368	0.16		0.77
56.75	64.70	0.93	0.88	0.00368	0.40	0.00368	0.48		
54.75	63.29	0.92	0.87	0.00368	0.49	0.00368	0.81		1.50
55.09	61.88	0.90	0.85	0.00368	0.58	0.00368	1.15		
52.66	**59.44**	**0.86**	0.83	0.00368	0.85	0.00368	1.49	0.45	2.00
51.82	59.07	0.85	0.81	0.00368	0.95	0.00368	1.85		
48.77	57.66	0.80	0.79	0.00368	1.31	0.00368	2.21		
45.72	**54.84**	0.75	**0.75**	0.00368	1.70	0.00369	2.96	5.62	
42.67	51.20	0.70	0.70	0.00368	2.11	0.00369	4.00		
39.62	47.54	0.65	0.65	0.00369	2.56	0.00370	5.11		
36.58	43.89	0.60	0.60	0.00369	3.04	0.00370	6.32		
33.53	40.23	0.55	0.55	0.00369	3.56	0.00370	7.64		
27.43	36.57	0.45	0.50	0.00369	4.77	0.00371	9.08		
24.38	29.26	0.40	0.40	0.00370	5.48	0.00372	12.46		
52.66	21.94	0.30	0.30	0.00370	7.22	0.00374	16.84		
12.19	14.63	0.20	0.20	0.00371	9.67	0.00376	23.04		
6.10	7.31	0.10	0.10	0.00373	13.88	0.00380	33.73		
4.57	5.49	0.08	0.08	0.00373	15.63	0.00382	38.20		
3.05	3.66	0.05	0.05	0.00374	18.11	0.00384	44.54		
1.52	1.83	0.03	0.03	0.00376	22.35	0.00388	55.46		
0.61	0.73	0.01	0.01	0.00378	27.99	0.00393	70.06		
0.46	0.55	0.01	0.01	0.00379	29.76	0.00395	74.69		
0.30	0.37	0.01	0.01	0.00380	32.27	0.00398	81.25		
0.15	0.18	0.00	0.00	0.00381	36.57	0.00402	92.55		
0.06	0.07	0.00	0.00	0.00383	42.29	0.00407	107.67		
0.05	0.05	0.00	0.00	0.00384	44.09	0.00409	112.46		
0.03	0.04	0.00	0.00	0.00385	46.63	0.00411	119.24		
0.01	0.01	0.0001	0.0001	0.00389	56.79	0.00422	146.59		

Table E4 cont'd. Rayleigh model data for Experiment 4, Trial 3; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU115-3.0m & PU121-3.0m)

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.00	‰
δ_o-NO_3^-	40.00	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	20.00	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	20.00	mg/L

Nitrification: Actual Data - Input

Experiment:	iii	
Initial NH_4^+-N (mg/L)	60.96	mg N/L
Final NH_4^+-N (mg/L)	52.66	mg N/L
f NH_4^+	0.86	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	-0.03	‰
Initial NO3--N (mg/L)	**73.15**	mg N/L
Final NO3--N (mg/L)	54.84	mg N/L
f NO_3^-	0.75	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	**-1.36**	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-6.00	‰
α (NH4)	0.994	
(α-1) NH4	-0.006	
ε (NO3- - Nitrification)	-15.00	‰
α (NO3-)	0.985	
(α-1) NO3-	-0.015	

Table E4 cont'd. Rayleigh model data for Experiment 4, Trial 3; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU115-3.0m & PU121-3.0m)

Equations:	
$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{reacta}$	Fractionation (ii)
$\delta = [R_{sample}/R_{star}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio
where:	
εp-r	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N$ = 0.0036765
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

Table E5. Rayleigh model data for Experiment 4, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Experimental Data - Nitrification: Experiment 4 (PU86-3.1m)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
89.08	0.00	1.00	0.00366	-4.80			0.00			-6.95	-4.8
88.99	0.09	1.00	0.00366	-4.79	0.09	-16.74	0.09	-16.74	-2.02		
86.10	2.99	0.97	0.00366	-4.48	2.90	-14.04	2.99	-14.12	-2.46	-1.69	-4.0
81.10	7.98	0.95	0.00366	-4.16	5.00	-9.67	7.98	-11.33	-2.89	-1.2	-1.7
77.07	12.01	0.92	0.00366	-3.83	4.02	-10.45	12.01	-11.04	-3.24	4.31	-0.7
79.83	9.25	0.90	0.00366	-3.49	9.16	-16.10	9.25	-16.11	-3.53		
73.05	16.03	**0.82**	0.00367	-2.43	13.04	-15.95	16.03	-15.61	-4.38	1.73	-0.5
71.26	17.82	0.80	0.00367	-2.13	8.57	-14.79	17.82	-15.47	-4.57		
66.81	22.27	0.75	0.00367	-1.36	4.45	-13.73	22.27	-15.12	-4.98		
64.58	24.50	0.73	0.00367	-0.95	2.23	-13.14	24.50	-14.94	-5.16		
62.36	26.72	0.70	0.00367	-0.53	2.23	-12.73	26.72	-14.76	-5.33		
60.13	28.95	0.68	0.00368	-0.10	2.23	-12.31	28.95	-14.57	-5.48		
57.90	31.18	0.65	0.00368	0.36	2.23	-11.87	31.18	-14.38	-5.61		
53.45	35.63	0.60	0.00368	1.32	4.45	-11.18	35.63	-13.98	-5.83		
48.99	40.09	0.55	0.00369	2.37	4.45	-10.19	40.09	-13.56	-6.00		
44.54	44.54	0.50	0.00369	3.51	8.91	-9.65	44.54	-13.11	-6.11		
40.09	48.99	0.45	0.00369	4.78	4.45	-7.91	48.99	-12.64	-6.18		
35.63	53.45	0.40	0.00370	6.20	4.45	-6.59	53.45	-12.14	-6.19		
31.18	57.90	0.35	0.00371	7.82	4.45	-5.09	57.90	-11.59	-6.16		
26.72	62.36	0.30	0.00371	9.68	13.36	-5.02	62.36	-11.01	-6.07		
17.82	71.26	0.20	0.00373	14.61	8.91	-0.17	71.26	-9.65	-5.71		
8.91	80.17	0.10	0.00376	23.08	8.91	6.13	80.17	-7.90	-5.03		
6.68	82.40	0.08	0.00377	26.62	2.23	12.47	82.40	-7.35	-4.79		
4.45	84.63	0.05	0.00379	31.63	2.23	16.61	84.63	-6.72	-4.49		
2.23	86.85	0.03	0.00382	40.24	2.23	23.01	86.85	-5.95	-4.11		
0.89	88.19	0.01	0.00387	51.74	1.34	32.58	88.19	-5.37	-3.81		
0.67	88.41	0.01	0.00388	55.38	0.22	40.83	88.41	-5.25	-3.75		
0.45	88.64	0.01	0.00390	60.53	0.22	45.09	88.64	-5.13	-3.69		
0.22	88.86	0.00	0.00393	69.39	0.22	51.67	88.86	-4.99	-3.61		
0.09	88.99	0.00	0.00398	81.21	0.13	61.51	88.99	-4.89	-3.56		
0.07	89.01	0.00	0.00399	84.95	0.02	69.99	89.01	-4.87	-3.55		
0.04	89.04	0.00	0.00401	90.24	0.02	74.37	89.04	-4.85	-3.54		
0.01	89.07	0.0001	0.00409	111.50	0.04	84.93	89.07	-4.81	-3.52		

Table E5 cont'd. Rayleigh model data for Experiment 4, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00366	-4.80	0.00367	-2.00
5.99	5.99	1.00	1.00	0.00366	-4.79	0.00367	-1.99
5.40	5.40	0.90	0.90	0.00366	-4.17	0.00367	-0.42
4.80	4.80	0.80	0.80	0.00366	-3.47	0.00368	1.35
4.20	4.20	0.70	0.70	0.00367	-2.67	0.00369	3.35
3.60	3.60	0.60	0.60	0.00367	-1.75	0.00370	5.68
3.00	3.00	0.50	0.50	0.00367	-0.65	0.00371	8.43
2.40	2.40	0.40	0.40	0.00368	0.69	0.00372	11.81
1.80	1.80	0.30	0.30	0.00369	2.42	0.00374	16.19
1.20	1.20	0.20	0.20	0.00369	4.86	0.00376	22.39
0.60	0.60	0.10	0.10	0.00371	9.04	0.00380	33.07
0.30	0.30	0.05	0.05	0.00373	13.25	0.00384	43.87
0.06	0.06	0.01	0.01	0.00376	23.08	0.00393	69.38
0.03	0.03	0.01	0.01	0.00378	27.35	0.00397	80.55
0.01	0.01	0.00	0.00	0.00381	37.31	0.00407	106.96
0.00	0.00	0.00	0.00	0.00383	41.64	0.00411	118.53
0.00	0.00	0.00	0.00	0.00387	51.74	0.00421	145.86

Table E5 cont'd. Rayleigh model data for Experiment 4, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Experimental Data Anammox: Experiment 4 (PU86-3.1m)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Measured δ15N-NO3	Measured δ15N-NH4
89.08	**75.76**	1.00	1.00	0.00366	-4.80	0.00367	-2.00	-6.95	-4.80
88.99	74.89	1.00	0.99	0.00366	-4.79	0.00367	-1.83		
86.10	74.01	0.98	0.98	0.00366	-4.70	0.00367	-1.65	-1.69	-4.00
86.08	72.73	0.97	0.96	0.00366	-4.60	0.00367	-1.39		
81.10	71.21	0.95	0.94	0.00366	-4.49	0.00367	-1.07	-1.20	-1.66
83.16	69.70	0.93	0.92	0.00366	-4.39	0.00367	-0.75		
81.71	66.29	0.92	0.88	0.00366	-4.28	0.00368	0.00		
77.07	64.40	0.90	0.85	0.00366	-4.18	0.00368	0.44	4.31	-0.70
78.79	62.50	0.88	0.83	0.00366	-4.07	0.00368	0.88		
77.33	60.61	0.87	0.80	0.00366	-3.96	0.00368	1.35		
73.05	60.61	**0.82**	0.80	0.00366	-3.61	0.00368	1.35	1.73	-0.50
71.26	56.82	0.80	0.75	0.00366	-3.47	0.00369	2.32		
62.36	53.03	0.70	0.70	0.00367	-2.67	0.00369	3.35		
57.90	49.24	0.65	0.65	0.00367	-2.22	0.00369	4.47		
53.45	45.46	0.60	0.60	0.00367	-1.75	0.00370	5.68		
48.99	41.67	0.55	0.55	0.00367	-1.22	0.00370	6.99		
40.09	37.88	0.45	0.50	0.00368	-0.02	0.00371	8.43		
35.63	30.30	0.40	0.40	0.00368	0.69	0.00372	11.81		
73.05	22.73	0.30	0.30	0.00369	2.42	0.00374	16.19		
17.82	15.15	0.20	0.20	0.00369	4.86	0.00376	22.39		
8.91	7.58	0.10	0.10	0.00371	9.04	0.00380	33.07		
6.68	5.68	0.08	0.08	0.00372	10.79	0.00381	37.54		
4.45	3.79	0.05	0.05	0.00373	13.25	0.00384	43.87		
2.23	1.89	0.03	0.03	0.00374	17.47	0.00388	54.78		
0.89	0.76	0.01	0.01	0.00376	23.08	0.00393	69.38		
0.67	0.57	0.01	0.01	0.00377	24.85	0.00395	74.00		
0.45	0.38	0.01	0.01	0.00378	27.35	0.00397	80.55		
0.22	0.19	0.00	0.00	0.00379	31.63	0.00401	91.85		
0.09	0.08	0.00	0.00	0.00381	37.31	0.00407	106.96		
0.07	0.06	0.00	0.00	0.00382	39.11	0.00409	111.74		
0.04	0.04	0.00	0.00	0.00383	41.64	0.00411	118.53		
0.01	0.01	0.0001	0.0001	0.00387	51.74	0.00421	145.86		

Table E5 cont'd. Rayleigh model data for Experiment 4, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.00	‰
δ_o-NO_3^-	40.00	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	20.00	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	20.00	mg/L

Nitrification: Actual Data - Input

Experiment:	iv	
Initial NH_4^+-N (mg/L)	89.08	mg N/L
Final NH_4^+-N (mg/L)	73.05	mg N/L
f NH_4^+	0.82	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	-4.80	‰
Initial NO3--N (mg/L)	**75.76**	mg N/L
Final NO3--N (mg/L)	77.00	mg N/L
f NO_3^-	1.02	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	**-2.00**	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-6.00	‰
α (NH4)	0.994	
(α-1) NH4	-0.006	
ε (NO3- - Nitrification)	-15.00	‰
α (NO3-)	0.985	
(α-1) NO3-	-0.015	

Table E5 cont'd. Rayleigh model data for Experiment 4, Trial 4; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Equations:	
$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{reacta}$	Fractionation (ii)
$\delta = [R_{sample}/R_{star}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio
where:	
εp-r	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N$ = 0.0036765
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

Table E6. Rayleigh model data for Experiment 4, Trial 5; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Experimental Data - Nitrification: Experiment 4 (PU86-3.1m)											
Nitrogen from NH_4^+ Pool (Assuming Nitrification)			NH_4^+ - Rayleigh Curve		NO_3^- - Newly formed		NO_3^- - Cumulative			Data From Experiment	
X_I (NH_4^+)	X_I (NO_3^+)	f NH_4^+	R (sample - NH_4^+)	δ (sample - NH_4^+)	mass (mg N/L)	δ (‰)	mass (mg N/L)	δ (‰) - From Nitrification	δ (‰) Total, with NO_3^- pool	Measured δ15N-NO3	Measured δ15N-NH4
85.46	0.00	1.00	0.00367	-3.02			0.00				-3.0
85.38	0.09	1.00	0.00367	-3.01	0.09	-14.98	0.09	-14.98	0.37		
83.37	2.09	0.98	0.00367	-2.72	2.01	-14.83	2.09	-14.84	0.01		
81.73	3.74	0.95	0.00367	-2.43	1.64	-17.21	3.74	-15.88	-0.33	0.39	-1.3
79.36	6.10	0.93	0.00367	-2.13	4.01	-14.40	6.10	-14.55	-0.65		
75.48	9.99	0.91	0.00367	-1.83	6.25	-9.74	9.99	-12.04	-0.97	1.87	1.5
75.35	10.11	0.88	0.00367	-1.51	4.01	-13.80	10.11	-14.25	-1.23		
70.44	15.02	0.86	0.00367	-1.19	5.03	-10.75	15.02	-11.61	-1.48	4.47	1.7
71.34	14.12	0.83	0.00367	-0.86	4.01	-13.17	14.12	-13.95	-1.73		
65.41	20.06	**0.77**	0.00368	0.18	5.03	-19.04	20.06	-13.47	-2.35	6.63	3.0
64.10	21.37	0.75	0.00368	0.43	7.24	-12.22	21.37	-13.36	-2.47		
59.82	25.64	0.70	0.00368	1.26	4.27	-11.17	25.64	-13.00	-2.82		
55.55	29.91	0.65	0.00368	2.15	4.27	-10.32	29.91	-12.62	-3.11		
51.28	34.19	0.60	0.00369	3.11	4.27	-9.41	34.19	-12.22	-3.34		
47.01	38.46	0.55	0.00369	4.16	4.27	-8.42	38.46	-11.79	-3.52		
42.73	42.73	0.50	0.00370	5.31	8.55	-7.88	42.73	-11.35	-3.66		
38.46	47.01	0.45	0.00370	6.58	4.27	-6.14	47.01	-10.87	-3.74		
34.19	51.28	0.40	0.00371	8.00	4.27	-4.81	51.28	-10.37	-3.77		
29.91	55.55	0.35	0.00371	9.62	4.27	-3.31	55.55	-9.83	-3.76		
25.64	59.82	0.30	0.00372	11.49	12.82	-3.24	59.82	-9.24	-3.69		
17.09	68.37	0.20	0.00374	16.42	8.55	1.62	68.37	-7.88	-3.39		
8.55	76.92	0.10	0.00377	24.91	8.55	7.93	76.92	-6.12	-2.78		
6.41	79.05	0.08	0.00378	28.46	2.14	14.28	79.05	-5.57	-2.55		
4.27	81.19	0.05	0.00380	33.47	2.14	18.42	81.19	-4.94	-2.27		
2.14	83.33	0.03	0.00383	42.10	2.14	24.84	83.33	-4.18	-1.92		
0.85	84.61	0.01	0.00387	53.63	1.28	34.42	84.61	-3.59	-1.64		
0.64	84.82	0.01	0.00389	57.27	0.21	42.70	84.82	-3.48	-1.58		
0.43	85.04	0.01	0.00391	62.43	0.21	46.96	85.04	-3.35	-1.52		
0.21	85.25	0.00	0.00394	71.30	0.21	53.55	85.25	-3.21	-1.45		
0.09	85.38	0.00	0.00398	83.14	0.13	63.40	85.38	-3.11	-1.40		
0.06	85.40	0.00	0.00400	86.89	0.02	71.91	85.40	-3.09	-1.39		
0.04	85.42	0.00	0.00402	92.19	0.02	76.29	85.42	-3.07	-1.38		
0.01	85.46	0.0001	0.00409	113.49	0.03	86.87	85.46	-3.03	-1.36		

Table E6 cont'd. Rayleigh model data for Experiment 4, Trial 5; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Theoretical (Ideal Rayleigh) - Anammox Model							
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)
6.00	6.00	1.00	1.00	0.00367	-3.02	0.00368	0.39
5.99	5.99	1.00	1.00	0.00367	-3.00	0.00368	0.42
5.40	5.40	0.90	0.90	0.00367	-0.92	0.00369	3.56
4.80	4.80	0.80	0.80	0.00368	1.44	0.00370	7.11
4.20	4.20	0.70	0.70	0.00369	4.12	0.00372	11.15
3.60	3.60	0.60	0.60	0.00370	7.22	0.00373	15.84
3.00	3.00	0.50	0.50	0.00372	10.90	0.00376	21.41
2.40	2.40	0.40	0.40	0.00373	15.42	0.00378	28.27
1.80	1.80	0.30	0.30	0.00375	21.28	0.00381	37.18
1.20	1.20	0.20	0.20	0.00379	29.59	0.00386	49.88
0.60	0.60	0.10	0.10	0.00384	43.97	0.00394	71.94
0.30	0.30	0.05	0.05	0.00389	58.54	0.00402	94.46
0.06	0.06	0.01	0.01	0.00402	93.17	0.00422	148.60
0.03	0.03	0.01	0.01	0.00408	108.43	0.00431	172.74
0.01	0.01	0.00	0.00	0.00421	144.69	0.00452	230.75
0.00	0.00	0.00	0.00	0.00427	160.67	0.00462	256.61
0.00	0.00	0.00	0.00	0.00441	198.63	0.00485	318.77

Table E6 cont'd. Rayleigh model data for Experiment 4, Trial 5; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Experimental Data Anammox: Experiment 4 (PU86-3.1m)									
Nitrogen Loss (Assuming Anammox)				NH_4^+ - Rayleigh Curve		NO_3^- - Rayleigh Curve		Data From Experiment	
X_l (NH_4^+)	X_l (NO_3^+)	f NH_4^+	f NO_3^-	R (sample - NH_4^+)	δ (sample - NH_4^+)	R (sample - NO_3^-)	δ (sample - NO_3^-)	Measured δ15N-NO3	Measured δ15N-NH4
85.46	**81.24**	1.00	1.00	0.00367	-3.02	0.00368	0.39		-3.02
85.38	80.03	1.00	0.99	0.00367	-3.00	0.00368	0.84		
83.84	**78.54**	0.98	0.97	0.00367	-2.64	0.00368	1.30	0.39	
82.29	77.61	0.96	0.96	0.00367	-2.27	0.00368	1.76		
81.73	76.41	0.94	0.94	0.00367	-1.89	0.00368	2.23		-1.30
79.21	75.20	0.93	0.93	0.00367	-1.50	0.00369	2.71		
77.66	**74.02**	0.91	0.91	0.00367	-1.11	0.00369	3.20	1.87	
76.12	72.78	0.89	0.90	0.00367	-0.71	0.00369	3.69		
75.48	**70.38**	0.87	0.88	0.00368	-0.30	0.00369	4.20	4.47	1.51
73.04	70.36	0.85	0.87	0.00368	0.12	0.00369	4.71		
70.44	66.74	0.84	0.85	0.00368	0.55	0.00370	5.23		1.70
69.95	**66.74**	0.82	**0.82**	0.00368	0.98	0.00370	6.31	6.63	
65.41	64.99	**0.77**	0.80	0.00369	2.33	0.00370	7.11		3.00
59.82	56.87	0.70	0.70	0.00369	4.12	0.00372	11.15		
55.55	52.80	0.65	0.65	0.00370	5.61	0.00373	13.40		
51.28	48.74	0.60	0.60	0.00370	7.22	0.00373	15.84		
47.01	44.68	0.55	0.55	0.00371	8.97	0.00374	18.49		
38.46	40.62	0.45	0.50	0.00372	13.03	0.00376	21.41		
34.19	32.50	0.40	0.40	0.00373	15.42	0.00378	28.27		
65.41	24.37	0.30	0.30	0.00375	21.28	0.00381	37.18		
17.09	16.25	0.20	0.20	0.00379	29.59	0.00386	49.88		
8.55	8.12	0.10	0.10	0.00384	43.97	0.00394	71.94		
6.41	6.09	0.08	0.08	0.00386	49.99	0.00398	81.23		
4.27	4.06	0.05	0.05	0.00389	58.54	0.00402	94.46		
2.14	2.03	0.03	0.03	0.00395	73.32	0.00411	117.46		
0.85	0.81	0.01	0.01	0.00402	93.17	0.00422	148.60		
0.64	0.61	0.01	0.01	0.00404	99.47	0.00426	158.56		
0.43	0.41	0.01	0.01	0.00408	108.43	0.00431	172.74		
0.21	0.20	0.00	0.00	0.00413	123.90	0.00440	197.38		
0.09	0.08	0.00	0.00	0.00421	144.69	0.00452	230.75		
0.06	0.06	0.00	0.00	0.00423	151.29	0.00456	241.42		
0.04	0.04	0.00	0.00	0.00427	160.67	0.00462	256.61		
0.01	0.01	0.0001	0.0001	0.00441	198.63	0.00485	318.77		

Table E6 cont'd. Rayleigh model data for Experiment 4, Trial 5; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Parameters:

Parameters	Value	Unit
ε (NH_4^+ - Nitrification)	-12.00	‰
α (NH_4^+)	0.99	
(α-1) NH_4^+	-0.01	
δ_o-NH_4^+	25.00	‰
δ_o-NO_3^-	40.00	‰
$R_{standard}$ ($^{15}N/^{14}N$)	0.00	
R_o (sample - NH_4^+)	0.00	
R_o (sample - NO_3^-)	0.00	
X_I^o (NH_4^+)	20.00	mg/L
X_I^o (NO_3^-)	0.00	mg/L
Original NO_3^- Conc.	20.00	mg/L

Nitrification: Actual Data - Input

Experiment:	v	
Initial NH_4^+-N (mg/L)	85.46	mg N/L
Final NH_4^+-N (mg/L)	65.41	mg N/L
f NH_4^+	0.77	
R_o (sample - NH4)	0.00	
Initial 15N-NH4+	-3.02	‰
Initial NO3--N (mg/L)	**81.24**	mg N/L
Final NO3--N (mg/L)	66.74	mg N/L
f NO_3^-	0.82	
R_o (sample - NO3-)	0.00	
Initial 15N-NO3-	**0.39**	‰

Anammox Parameters:

ε (NH4 - Nitrification)	-20.00	‰
α (NH4)	0.980	
(α-1) NH4	-0.020	
ε (NO3- - Nitrification)	-30.00	‰
α (NO3-)	0.970	
(α-1) NO3-	-0.030	

Table E6 cont'd. Rayleigh model data for Experiment 4, Trial 5; model data for predicted anammox and nitrification isotope evolution curves.

Rayleigh Model and Experimental Data - Experiment 4 (PU86-3.1m)

Equations:

$R = R_o f^{(\alpha-1)}$	Rayleigh Equation
$\varepsilon_{p-r} = (\alpha-1) \times 10$	Enrichment
$\alpha=(\varepsilon_{p-r}/1000‰$	Fractionation (i)
$\alpha=R_{product}/R_{reacta}$	Fractionation (ii)
$\delta = [R_{sample}/R_{star}$	Isotopic value (del)
$R_{sample} = [\delta/100$	Isotopic Ratio

where:

$\varepsilon p\text{-}r$	Enrichment between product (NH_4^+) and reactant (NO_3^-)
δ	isotope value for a given fraction of substrate remaining
δ_o	initial isotopic value
f	X_l/X_l^o, where X_l is the given concentration of the light isotope (ie. Your NH4-N value at a given time), X_l^o is the initial concentration.
α	=isotopic fractionation factor, which can be calculated by:
R	Ratio ($^{15}N/^{14}N$) of product ($NH4^+$) at a given fraction of converted substrate
R_o	Original ratio ($^{15}N/^{14}N$) of product ($NH4^+$), before any substrate is consumed
R_{sample}	same as R, but in this case, the equation with R_{sample} was used to calculate R_o
$R_{standard}$	Ratio Standard - Atmospheric ratio of $^{15}N/^{14}N = 0.0036765$
Source	http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchch2.html

Appendix F:
Experiment 4 – Sacrificial Serum Bottles:
Labeled ^{15}N-NH_4^+ Experiment

Appendix F: Tagged $^{15}N\text{-}NH_4^+$ Isotope Pairing Technique Experiment

Introduction

To augment the microcosm experiments, particularly those from Experiment 4, a modified version of the isotope pairing technique (Thamdrup and Dalsgaard, 2002) was initiated where labeled, enriched $^{15}N\text{-}NH_4^+$ (annomium sulphate, $(NH_4)_2SO_4$) was applied to duplicate sacrificial bottles sets from Experiment 4 (Trials 6, 7, and 8 from the labeled experiment represent Trials 4, 5, and 1 from Experiment 4, respectively). A number of problems arose throughout the process, which resulted in the removal of the experiment from the thesis. The experiment is instead presented in the appendix. The problems encountered include:

- Out of concern for analyzing samples with extremely high $^{15}N\text{-}NH_4^+$ signatures, a conservative amount of label was used, resulting in F_{NH4} values of 0.05 for Trials 6 and 7, and 0.04 for Trial 8 (F_{NH4} represents the fraction of $^{15}N\text{-}NH_4^+$ in the NH_4^+ pool). This suggests that the naturally occurring $^{15}N\text{-}NO_3^-$ in the sample could not be considered negligible, which violated the terms of the basic anammox equation (Thamdrup et al. 2006):

$$N_{2\text{-anammox}} = {}^{14}N^{15}N_{NH4+} \times F^{-1}_{ammonium}, \qquad \text{(Eq. 3.3)}$$

where $F^{-1}_{ammonium}$ is the fraction of $^{14}N\text{-}NH_4$ in the sample. A revised equation (Spoelstra 2011) was implemented instead (Appendix F).

- Analysis of $^{28,29,30}N_2$ and associated isotope signatures were completed at the University of Ottawa, where highly variable procedures were implemented. These procedures included manual shaking of samples for 1 minute or less for headspace equilibration, varying amounts of sample removed and He injected for headspace, pressure and temperatures not recorded, and bottles were not weighed. The analytical procedure included volumes injected into the mass spec ranging from 0.95 to 90 uL, and the mass spec was calibrated using a 1-point (lab air) calibration.

- Analytical error was estimated based on the standard deviation of the standard, which in this case was lab air. The analytical error for $^{29}N_2$ was equal to or greater than the actual measured $^{29}N_2$ in some samples. Accounting for this error in the equation for N_2 production by anammox (Appendix F) resulted in a range from 0 to greater than 100%, making it impossible to define an accurate value for anammox activity.

Method

Labelled, enriched $^{15}N\text{-}NH_4^+$ (annomium sulphate, $(NH_4)_2SO_4$) was used in three additional trials to Experiment 4. This was based on the premise that under anaerobic conditions, $^{29}N\text{-}N_2$ gas ($^{14}N\text{-}NO_3^-$ + $^{15}N\text{-}NH_4^+$) would only be produced via anammox (Risgaard-Petersen et al. 2003). By adding a substrate with a much higher $^{15}N/^{14}N$ ratio (0.111, final mixture 0.00627, Appendix F) than naturally found (0.00367), it can be assumed that virtually all of the ^{15}N in $^{29}N_2$ produced originated from the labelled

$^{15}N\text{-}NH_4^+$. If nitrification is not involved, anammox can be inferred as the source of $^{29}N_2$. A 2L solution of labelled $^{15}N\text{-}NH_4^+$ was prepared, where 14 mg of 10 atom percent $(NH_4)_2SO_4$ was added to 0.5 L of DI water, and 571 mg of unlabelled NH_4NO_3 was added to 0.5 L of DI water, both of which were then mixed with 1L of sample water (see Appendix F for mixing calculations). The solution was then sparged with He for 30 minutes to remove dissolved O_2 and N_2. These experiments were undertaken at the same time as the Moore et al. (2011) incubations, and differ in multiple aspects, including the use of freshly cored sediment, large scale microcosms (vs Exetainers), and each bottle is sampled only once, as apposed to sampling one Exetainer multiple times. The sacrificial bottle approach may be the most significant difference, as multiple sampling events using the same septa could result in oxygen contamination, which could lead to nitrification of labelled $^{15}N\text{-}NH_4^+$, resulting in $^{29}N_2$ (from denitrification) being incorrectly interpreted as anammox activity.

Three sets of sacrificial bottle trials were undertaken, designated Trials 6-8. The design for the trials was the same for the all trials (Section 2.1.4), with the exception that Trials 6-8 contained labeled $^{15}N\text{-}NH_4^+$. Also, due to their isotopically enhanced nature, Trials 6-8 were placed in a covered shaker (not an anaerobic chamber) in another lab, where they were shaken for 10 minutes per day.

Analytical Methods

N_2 gas in the headspace of each serum bottle was sampled at the G.G. Hatch Stable Isotope Lab at the University of Ottawa using a Thermo-Finnigan DeltaPlus XP Isotope-Ratio Mass Spectrometer (IRMS). Initially, a headspace of approximately 5mL was created by injecting 5mL of helium, and removing approximately the same amount of sample. Each bottle was shaken manually for 1 minute prior to sampling in order to equilibrate the dissolved N_2 gas with the headspace. Gas samples were obtained using a helium-flushed 0.1 mL gas tight syringe and injected into the GC. The injection volume and split ratio were recorded in addition to the results. Standard air samples were run after every tenth sample.

A refined methodology was implemented for the final three sampling events. After injecting a 7mL headspace (and removing 6.5-7.0 mL sample), the bottles were shaken for 2 hours to equilibrate dissolved N_2 gas with the headspace. In addition to standard air samples, two calibration standards were introduced: injection of lab air at multiple volumes, and injection of a range of N_2 concentrations at the same volume.

$^{14}N^{15}N$:$^{14}N^{14}N$ and $^{15}N^{15}N$:$^{14}N^{14}N$ ratios were determined by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) and expressed as $\delta^{15}N/^{14}N$ values (GG Hatch isotope laboratory, University of Ottawa):

$$\delta^{15}N/^{14}N = [(^{15}N:^{14}N)\text{sample}/(^{15}N:^{14}N)\text{standard} - 1] \times 1000‰,$$

where $(^{15}N:^{14}N)$standard was lab air.

Results

Three of the 8 trials were also injected with Labelled $^{15}N\text{-}NH_4^+$ (annomium sulphate, $(NH_4)_2SO_4$) and analyzed for $^{29}N_2$ production. These experiments differ from Moore et al. (2011) as they use freshly cored sediment, large scale microcosms (vs Exetainers), and each bottle is sampled only once, as opposed to sampling one Exetainer multiple times (which increases the risk of oxygen contamination). The labeled portion of the experiment consisted of three trials as follows:

- Trial 6, Same design as Trial 4, but also injected with $(NH_4)_2SO_4$. Used to analyze for $^{28,29,30}N_2$ and $\delta^{15}N$.
- Trial 7, Same design as Trial 5, but also injected with $(NH_4)_2SO_4$. Used to analyze for $^{28,29,30}N_2$ and $\delta^{15}N$.
- Trial 8, Same design as Trial 1, but also injected with $(NH_4)_2SO_4$. Used to analyze for $^{28,29,30}N_2$ and $\delta^{15}N$.

Trial 6

A labelled $^{15}N\text{-}NH_4^+$ experiment was also undertaken using the same water and sediment as Trial 4 (Trial 8, Figure E1). The data was analyzed using a 1-point calibration procedure, which resulted in unreliable absolute concentrations, but trends that could be analyzed. $^{29}N_2$ concentrations increased over the duration of the experiment, increasing by over an order of magnitude. The concentration range is consistent with a similar labelled $^{15}N\text{-}NH_4^+$ experiment (Thamdrup et al. 2006). Like the concentrations, $\delta^{15}N$ was not calibrated as standards were not run until day 196, but

trends can be noted. $\delta^{15}N$ steadily increases from 11‰ (day 17) to 140‰ (day 172), and from 1312‰ (day 196) to 1350‰ (258) with the corrected data. Anammox contribution to N_2 production was calculated based on a modified version of Thamdrup and Dalsgaard (2002) and Spoelstra (2011), which suggests that only ~3% of N_2 produced is from anammox activity (Table E8). However, the analytical error for $^{29}N_2$ production (based on standard deviation of lab air, >100% error in some samples) alone suggests that N_2 production from anammox could range from 0 to greater than 100%. This, combined with concerns already mentioned, made the reliability of this data low.

Trial 7

A labelled $^{15}N\text{-}NH_4^+$ experiment was also undertaken using the same water and sediment as Trial 5 (Trial 7, Figure E1). The data was analyzed using a 1-point calibration procedure, which resulted in unreliable absolute concentrations, but trends that could be analyzed. $^{29}N_2$ concentrations increased over the duration of the experiment, increasing by over an order of magnitude. Like the concentrations, $\delta^{15}N$ was not calibrated as standards were not run until day 196, but trends can be noted. Interestingly, $\delta^{15}N$ was substantially enriched at the beginning of the experiment (131‰, day 17) and remained relatively constant throughout the first 172 days (125‰, day 172) and between 1295‰ (day 196) and 1248‰ (258) with the corrected data. Anammox contribution to N_2 production was calculated based on a modified version of Thamdrup and Dalsgaard (2002) and Spoelstra (2011), which suggests that only ~4% of N_2 produced is from anammox activity (Appendix F). As mentioned for Trial 6, accounting for analytical error in $^{29}N_2$ alone will give an anammox produced N_2 range from 0 to

greater than 100%. This, combined with concerns already mentioned, made the reliability of this data low.

Trial 8

As with Trials 1 and 4, a labelled $^{15}N\text{-}NH_4^+$ experiment was also undertaken using the same water and sediment as Trial 1 (Trial 8, Figure E1). As with Trial 4, the data was analyzed using a 1-point calibration procedure, which resulted in unreliable absolute concentrations, but trends that could be analyzed. $^{29}N_2$ concentrations increased over the duration of the experiment, increasing by over an order of magnitude. The concentration range is consistent with a similar labelled $^{15}N\text{-}NH_4^+$ experiment (Thamdrup et al. 2006). Like the concentrations, $\delta^{15}N$ was not calibrated as standards were not run until day 196, but trends can be noted. $\delta^{15}N$ progressively enriched from the beginning of the experiment (3‰, day 17) to day 172 (172‰), with the calibrated data enriching from 1335‰ (day 196) to 1366‰ (day 258) . Anammox contribution to N_2 production was calculated based on a modified version of Thamdrup and Dalsgaard (2002) and Spoelstra (2011), which suggests that only ~2% of N_2 produced is from anammox activity (Appendix F). As with the previous trials, accounting for analytical error in $^{29}N_2$ will give an Anammox produced N_2 range from 0 to greater than 100%, which, combined with previous concerns, makes the reliability of the data low.

Conclusions

$^{15}N\text{-}NH_4^+$ experiments showed an increasing trend in concentrations of $^{29}N_2$ over time; however, according to a modified version of the Thamdrup and Dalsgaard (2002) and Spoelstra (2011) equations for calculating anammox, less than 5% of all N_2 produced can be attributed to anammox. Due to the fact that accounting for analytical error in $^{29}N_2$ analysis gives a range of 0 to greater than 100% for anammox contribution to N_2, the data reliability was considered low, and not reported in the main thesis. Future experiments using the IPT method should focus on data precision, to ensure a reasonable approximation of anammox contribution to dinitrogen production.

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Thamdrup, B., T. Dalsgaard, M.M. Jensen, O. Ulloa, L. Farias and R. Escribano. 2006. Anaerobic ammonium oxidation in the oxygen-deficient waters off northern Chile, Limno. Oceanogr., 51(5):2145-2156.

Table F1. Analytical results for Experiment 4; Putnam groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 6, single source groundwater (PU121-3.0m); Trial 7, single source groundwater (PU86-3.1m); and Trial 8, single source groundwater (PU115-2.2m), NH4NO3 and 15N-NH4+ enriched (NH4)2SO4 added; raw data from Clark lab (Ottawa ON).

Sample ID	Sample Date	Lab ID	Volume ul	Split ratio	Amplitude	Area $^{28}N_2$	Area $^{29}N_2$	Area $^{30}N_2$	δ $^{15}N/^{14}N$	δ $^{30}N/^{28}N$
AIR STANDARDS										
Standard air	October	N-6910	100	10		37.909	0.284	0.017	0.462	125.752
Standard air	October	N-6910	100	10		37.015	0.277	0.017	0.420	143.030
Standard air	October	N-6910	100	10		38.957	0.291	0.018	0.453	145.845
Standard air	October	N-6910	100	10		37.909	0.283	0.019	0.483	173.996
Standard air	October	N-6910	100	10		36.240	0.271	0.019	0.517	197.004
Standard air	October	N-6910	100	10		36.534	0.273	0.020	0.585	204.116
Standard air	October	N-6910	100	10		36.053	0.262	0.020	0.599	216.843
Standard air	October	N-6910	100	10		34.963	0.261	0.020	0.604	210.211
Standard air	October	N-6910	100	10		34.849	0.260	0.020	0.583	220.708
Standard air	October	N-6910	100	10		32.170	0.240	0.019	0.541	309.008
Standard air	October	N-6910	100	10		31.267	0.233	0.019	0.470	295.682
Standard Deviation	**October**					**2.376**	**0.018**	**0.001**	**0.066**	**58.383**
Standard air	November	N-6910	100	10		47.487	0.357	0.027	0.147	334.920
Standard air	November	N-6910	100	10		47.348	0.355	0.027	0.146	389.668
Standard air	November	N-6910	100	10		47.867	0.359	0.027	0.145	363.210
Standard air	November	N-6910	100	10		48.786	0.366	0.028	0.220	354.875
Standard air	November	N-6910	100	10		46.831	0.352	0.028	0.118	340.875
Standard air	November	N-6910	100	10		48.105	0.361	0.027	0.169	340.790
Standard air	November	N-6910	100	10		48.274	0.362	0.027	0.170	342.920
Standard air	November	N-6910	100	10		47.780	0.359	0.028	0.207	311.052
Standard air	November	N-6910	100	10		47.215	0.354	0.028	0.219	330.602
Standard air	November	N-6910	100	10		47.231	0.354	0.028	0.192	314.516
Standard air	November	N-6910	100	10		48.332	0.363	0.029	0.176	308.633
Standard air	November	N-6911	100	10		46.713	0.351	0.029	0.200	325.886
Standard Deviation	**November**					**0.636**	**0.005**	**0.001**	**0.033**	**23.318**

Table F1 cont'd. Analytical results for Experiment 4; Putnam groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 6, single source groundwater (PU121-3.0m); Trial 7, single source groundwater (PU86-3.1); and Trial 8, single source groundwater (PU115-2.2m), NH4NO3 and 15N-NH4+ enriched (NH4)2SO4 added; data from Clark lab (Ottawa ON).

Sample ID	Sample Date	Lab ID	Volume ul	Split ratio	Amplitude	Area $^{28}N_2$	Area $^{29}N_2$	Area $^{30}N_2$	δ $^{15}N/^{14}N$	δ $^{30}N/^{28}N$
Standard air	December	N-6910	100	10		50.213	0.376	0.032	0.217	186.203
Standard air	December	N-6910	100	10		51.797	0.388	0.034	0.358	178.435
Standard air	December	N-6910	100	10		49.018	0.367	0.034	0.237	176.672
Standard air	December	N-6910	100	10		49.186	0.369	0.034	0.233	172.311
Standard air	December	N-6910	100	10		50.094	0.376	0.035	0.259	181.603
Standard air	December	N-6910	100	10		48.701	0.365	0.035	0.282	178.249
Standard air	December	N-6910	100	10		50.268	0.377	0.037	0.285	181.702
Standard air	December	N-6910	100	10		51.264	0.384	0.037	0.236	176.711
Standard air	December	N-6910	100	10		50.331	0.377	0.037	0.265	174.996
Standard air	December	N-6910	100	10		49.682	0.372	0.036	0.272	169.849
Standard air	December	N-6911	100	10		51.018	0.382	0.038	0.273	172.146
Standard air	December	N-6912	100	10		48.957	0.369	0.036	0.217	170.507
Standard air	December	N-6913	100	10		50.110	0.376	0.038	0.261	175.182
Standard air	December	N-6914	100	10		49.583	0.372	0.037	0.272	173.341
Standard air	December	N-6915	100	10		49.782	0.373	0.032	0.234	153.156
Standard air	December	N-6916	100	10		49.678	0.372	0.036	0.268	163.393
Standard Deviation	**December**					**0.739**	**0.005**	**0.002**	**0.021**	**7.981**

Table F1 cont'd. Analytical results for Experiment 4; Putnam groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 6, single source groundwater (PU121-3.0m); Trial 7, single source groundwater (PU86-3.1); and Trial 8, single source groundwater (PU115-2.2m), NH4NO3 and 15N-NH4+ enriched (NH4)2SO4 added; data from Clark lab (Ottawa ON).

Sample ID	Sample Date	Lab ID	Volume ul	Split ratio	Amplitude	Area $^{28}N_2$	Area $^{29}N_2$	Area $^{30}N_2$	δ $^{15}N/^{14}N$	δ $^{30}N/^{28}N$
Standard air	Jan/Feb	N-6910	100	10	10675	46.117	0.346	0.031	0.285	313.400
Standard air	Jan/Feb	N-6910	100	10	11032	47.515	0.356	0.033	0.155	298.600
Standard air	Jan/Feb	N-6910	100	10	11360	46.867	0.352	0.034	0.167	297.500
Standard air	Jan/Feb	N-6910	100	10	10673	46.405	0.348	0.034	0.107	307.560
Standard air	Jan/Feb	N-6910	100	10	11357	47.970	0.360	0.030	0.054	274.293
Standard air	Jan/Feb	N-6910	100	10	10244	44.809	0.336	0.030	-0.051	284.401
Standard air	Jan/Feb	N-6910	100	10	10911	46.043	0.345	0.030	-0.021	277.188
Standard air	Jan/Feb	N-6910	100	10	10285	46.712	0.350	0.032	0.016	273.590
Standard air	Jan/Feb	N-6910	100	10	11327	46.892	0.352	0.033	0.087	274.724
Standard air	Jan/Feb	N-6910	100	10	10878	47.656	0.357	0.035	0.057	277.302
Standard air	Jan/Feb	N-6911	100	10	10540	46.454	0.348	0.034	0.008	264.428
Standard air	Jan/Feb	N-6912	100	10	10521	43.965	0.330	0.033	0.052	283.026
Standard air	Jan/Feb	N-6913	100	10	10463	45.585	0.342	0.035	0.020	293.272
Standard air	Jan/Feb	N-6914	100	10	10257	45.277	0.339	0.035	0.042	288.592
Standard air	Jan/Feb	N-6915	100	10	10555	45.517	0.341	0.035	-0.013	290.127
Standard air	Jan/Feb	N-6916	100	10	10575	46.659	0.350	0.036	0.025	285.864
Standard air	Jan/Feb	N-6917	100	10	10505	45.698	0.342	0.036	0.055	296.246
Standard air	Jan/Feb	N-6918	100	10	11092	45.366	0.340	0.035	0.055	296.655
Standard air	Jan/Feb	N-6919	100	10	10485	46.167	0.346	0.034	0.012	395.853
Standard Deviation	**Jan/Feb**					**1.003**	**0.008**	**0.002**	**0.078**	**27.795**

Table F1 cont'd. Analytical results for Experiment 4; Putnam groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 6, single source groundwater (PU121-3.0m); Trial 7, single source groundwater (PU86-3.1); and Trial 8, single source groundwater (PU115-2.2m), NH4NO3 and 15N-NH4+ enriched (NH4)2SO4 added; data from Clark lab (Ottawa ON).

Sample ID	Sample Date	Lab ID	Volume ul	Split ratio	Amplitude	Area $^{28}N_2$	Area $^{29}N_2$	Area $^{30}N_2$	δ $^{15}N/^{14}N$	δ $^{30}N/^{28}N$
Standard air	Mar/Apr	N-6910	100	10	10804	46.455	0.348	0.033	0.186	156.055
Standard air	Mar/Apr	N-6910	100	10	11187	47.252	0.354	0.034	0.197	141.644
Standard air	Mar/Apr	N-6910	100	10	11338	47.075	0.353	0.033	0.135	126.540
Standard air	Mar/Apr	N-6910	100	10	11451	48.430	0.363	0.034	0.178	116.735
Standard air	Mar/Apr	N-6910	100	10	11050	47.719	0.358	0.034	0.184	117.697
Standard air	Mar/Apr	N-6910	100	10	11412	47.977	0.360	0.035	0.485	119.125
Standard air	Mar/Apr	N-6910	100	10	11188	47.562	0.357	0.034	0.138	109.246
Standard air	Mar/Apr	N-6910	100	10	11017	47.204	0.354	0.034	0.197	109.677
Standard air	Mar/Apr	N-6910	100	10	11212	47.054	0.353	0.034	0.179	110.748
Standard air	Mar/Apr	N-6910	100	10	11237	47.765	0.358	0.034	0.286	111.593
Standard air	Mar/Apr	N-6910	100	10	11026	47.477	0.356	0.033	0.156	105.354
Standard air	Mar/Apr	N-6910	100	10	10619	46.877	0.351	0.033	0.143	111.595
Standard air	Mar/Apr	N-6910	100	10	11392	48.854	0.366	0.031	0.254	92.239
Standard air	Mar/Apr	N-6910	100	10	11736	48.206	0.361	0.039	0.174	81.024
Standard air	Mar/Apr	N-6910	100	10	11008	47.471	0.356	0.228	0.130	72.556
Standard air	Mar/Apr	N-6910	100	10	10382	47.685	0.357	0.357	0.142	127.105
Standard Deviation	**Mar/Apr**					**0.607**	**0.005**	**0.091**	**0.088**	**20.513**

Table F1 cont'd. Analytical results for Experiment 4; Putnam groundwater mixed with ~ 100 g sediment in sacrificial bottle microcosms (160 mL serum bottles): Trial 6, single source groundwater (PU121-3.0m); Trial 7, single source groundwater (PU86-3.1); and Trial 8, single source groundwater (PU115-2.2m), NH4NO3 and 15N-NH4+ enriched (NH4)2SO4 added; data from Clark lab (Ottawa ON).

Sample ID	Sample Date	Lab ID	Volume ul	Split ratio	Amplitude	Area $^{28}N_2$	Area $^{29}N_2$	Area $^{30}N_2$	δ $^{15}N/^{14}N$	δ $^{30}N/^{28}N$
SAMPLES										
Vi-time1-Pu86	October	N-8724	100	10		3.506	0.026	0.002	2.503	61.452
Vii-time1-Pu115	October	N-8725	100	10		2.635	0.020	0.001	10.784	-425.415
Viii-time1-Pu121	October	N-8726	100	10		2.550	0.022	0.001	130.942	-424.946
Vi-time 2-Pu86	November	N-8798	100	10		4.854	0.037	0.001	29.250	-349.401
Vii-time2-Pu115	November	N-8799	100	10		7.225	0.058	0.002	74.532	-324.754
Viii-time2-Pu121	November	N-8800	100	10		7.366	0.062	0.002	124.737	-300.383
Vi-time1-Pu86-sec	December	N-8798	100	10		10.885	0.087	0.004	67.229	-316.487
Vii-time1-Pu115-sec	December	N-8799	100	10		9.367	0.077	0.003	99.693	-354.652
Viii-time1-Pu121-sec	December	N-8800	100	10		10.539	0.089	0.004	130.273	-336.269
Vi-time3-Pu86	Jan/Feb	N-9020	100	10		1.175	0.009	0.000	71.730	-386.379
Repeat vi-time 3-pu86	Jan/Feb	N-9020	1000	10		11.408	0.094	0.006	95.645	-276.200
Vii-time3-Pu115	Jan/Feb	N-9021	100	10		1.606	0.013	0.000	92.148	-520.016
Repeat-vii-time 3-Pu115	Jan/Feb	N-9021	100	10		1.636	0.013	0.000	92.481	-513.219
Viii-time3-Pu121	Jan/Feb	N-9022	100	10		0.742	0.006	0.000	116.347	-614.085
repeat Viii-time 3-Pu121	Jan/Feb	N-9022	1000	10		9.625	0.082	0.004	129.807	-382.500
Vi-time4-Pu86	Jan/Feb	N-9023	100	10		0.657	0.005	0.000	113.834	-614.215
Repeat- Vi-time4-Pu86	Jan/Feb	N-9023	1000	10		7.719	0.065	0.003	128.240	-249.926
Vii-time4-Pu115	Jan/Feb	N-9024	1000	10		11.534	0.098	0.004	128.261	-246.034
Viii-time4-Pu121	Jan/Feb	N-9025	100	10		1.593	0.014	0.000	145.926	-554.866
Vi-time 5Waterloo	Mar/Apr	N-9091	1000	10		13.548	0.116	0.006	146.488	-254.506
Vii-time 5-Waterloo	Mar/Apr	N-9092	1000	10		24.408	0.207	0.011	133.899	-235.471
Viii-time 5-Waterloo	Mar/Apr	N-9093	1000	10		26.425	0.222	0.012	119.265	-230.146
Vi-time 7Waterloo	Mar/Apr	N-9094	1000	10		31.810	0.300	0.014	172.406	-223.563
Vii-time 6-Waterloo	Mar/Apr	N-9095	1000	10		29.796	0.255	0.014	139.523	-225.123
Viii-time 7-Waterloo	Mar/Apr	N-9096	1000	10		26.648	0.225	0.012	125.282	-240.482

Appendix F – Experiment 3: Sacrificial Serum Bottles
<u>Calculations for Ottawa 15N-NH4 Experiments (Calculate moles/injection)</u>

How many moles of N_2 are in 1L of lab air?

$$PV = nRT$$

$$n = \frac{PV}{RT}$$

where: P = 1 atm (lab conditions, approx)
T = 296.15 K (23°C, approx lab conditions)
R = 0.08205746 atm·L·K^{-1}mol^{-1} (gas constant)
V = 1 L of air x 78.084% (% of N_2 in air)
= 0.78084 L

$$n = \frac{(1atm)(0.78084L)}{(0.082057\text{atm}\cdot\text{L}\cdot\text{K-1mol-1})(296.15\text{K})}$$

$$n = 0.0321\text{moles}$$

For our experiments, we're interested in the amount of nitrogen (moles) in a 9.09µL injection [Why 9.09µL? The split on the GC is 10:1, meaning 10 of 11µL injected goes to waste → 100µL are injected per sample, so 100µL x 1/11 = 9.09µL analyzed]

V = 9.09µL x 78.084% = 7.097µL = 7.097E-06 L

$$n = \frac{(1atm)(7.097\text{E-06L})}{(0.08205746\text{atm}\cdot\text{L}\cdot\text{K-1mol-1})(296.15\text{K})}$$

$$n = 2.9204\text{E-7 moles}$$

$$\text{n} = 0.29204\mu\text{mol of N2/injection of lab air}$$

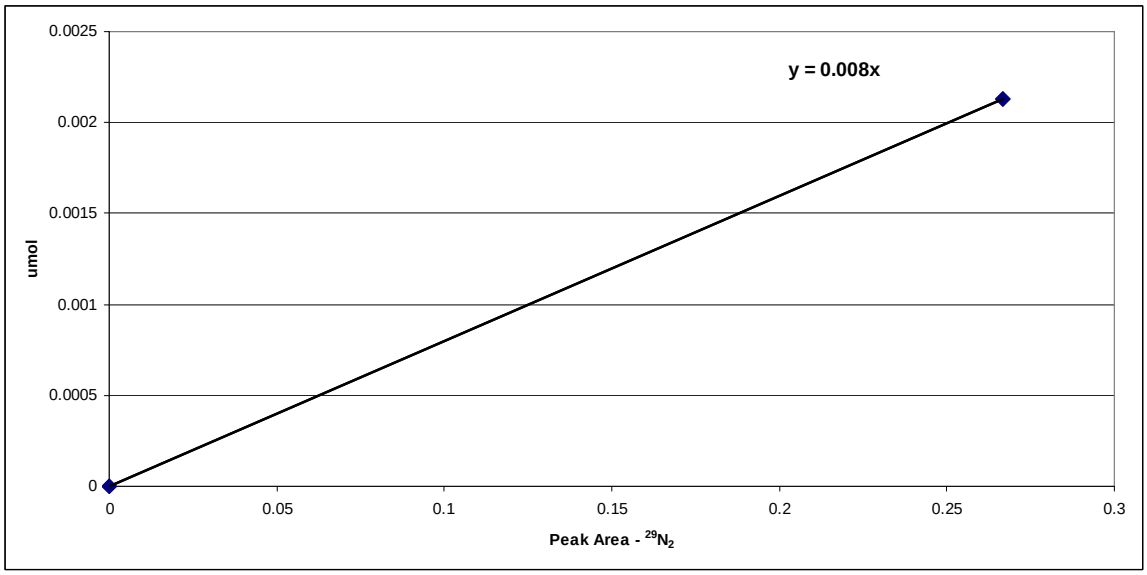
Now, to factor in isotopic abundance:

Isotope Pair	Percent Abundance	x N_2 in lab air injection (µmol)	µmol of isotope pair per injection
$^{28}N_2$	0.99268	0.29204	0.28990
$^{29}N_2$	0.007299		0.00213
$^{30}N_2$	1.34E-05		3.913E-06

For a one-point calibration curve, each of these isotope pairs will be plotted against their average peak area for each particular run. A trendline will be made (forced through 0), and we will use the trendline equation (y = mx + 0) to determine the factor (m) we will apply to the sample values.

Example: $29N_2$ (data from October 2010 injections):

Average peak area for $29N_2$ lab air = 0.266714
µmol $29N_2$ per lab air injection = 0.00213 µmol (theorized value from table above):



Therefore, we will apply a factor of 0.008 to our sample peak areas for this run, which gives us our sample values in umol:

Sample	Peak Area – $^{29}N_2$	Slope of trendline (y = **m**x + 0)	µmol Sample
vi – PU86	0.02622	0.008	0.00020976
vii – PU115	0.01986		0.00015888
viii – PU121	0.02151		0.00017208

Now we have the amount of $^{29}N_2$ in the sample per 9.09µL injection, which can be converted to the total amount in the bottle by other means.

Note: N2 concentrations determined from the headspace measurement require full equilibration between dissolved and gas phases (not done in previous data) and correction for volume injected (not done in previous data, john showed this effect).

Table F2. Corrected headspace and dissolved concentrations for N_2 data.

Sample	Date	Day	Corrected (Headspace)			Corrected (Dissolved)		
			$^{28}N_2$ (mg/L)	$^{29}N_2$ (mg/L)	$^{30}N_2$ (mg/L)	$^{28}N_2$ (umol/L)	$^{29}N_2$ (umol/L)	$^{30}N_2$ (umol/L)
Trial 6 (PU86-3.1m)	31-Oct-10	17	87.51703552	5.876090784	2.65527E-05	0.444101364	0.029815408	1.34728E-07
	26-Nov-10	43	91.23307746	6.299653392	1.23219E-05	0.451933635	0.031203306	6.10324E-08
	21-Dec-10	68	194.5444853	13.90302241	0.000827203	1.033880245	0.073871127	4.39512E-06
	21-Jan-11	99	22.81061129	1.646654545	9.18811E-06	0.111532642	0.008051158	4.49243E-08
	21-Jan-11	99	22.14687103	16.26715727	0.000137754	0.108276255	0.079529813	6.73463E-07
	22-Feb-11	131	12.74870505	0.952304328	1.63183E-06	0.06600897	0.004930684	8.44903E-09
	22-Feb-11	131	14.98453322	11.33803146	4.34551E-05	0.078428713	0.059342772	2.2744E-07
	15-Mar-11	152	254.6616395	19.56455856	9.09142E-05	1.271898424	0.097690389	4.53946E-07
	4-Apr-11	172	59.7928537	50.3400798	5.17328E-05	0.284530595	0.23954631	2.46161E-07
	28-Apr-11	196	5432.821085	41.7242785	0.010747207	14.59488662	0.111484991	2.87148E-05
	31-May-11	229	4421.941859	35.99917994	0.006322492	21.36813988	0.173195757	3.04171E-05
	29-Jun-11	258	5172.334788	38.90669072	0.008418492	13.89147045	0.103956258	2.24928E-05
Trial 7 (PU115-2.2m)	31-Oct-10	17	65.76195234	4.450768992	8.95283E-06	0.331931452	0.022463682	4.5186E-08
	26-Nov-10	43	135.8131501	9.789002496	2.81269E-05	0.684406191	0.049323356	1.4172E-07
	21-Dec-10	68	167.4050193	12.32701654	0.000579489	0.88506432	0.065161492	3.06318E-06
	21-Jan-11	99	31.17579631	2.282195671	1.30252E-05	0.152424111	0.011157745	6.36808E-08
	21-Jan-11	99	31.75042958	2.325616441	1.34336E-05	0.155236219	0.011370217	6.56784E-08
	22-Feb-11	131	22.39147844	16.94278445	9.62834E-05	0.115954118	0.087737587	4.98591E-07
	15-Mar-11	152	45.87875676	34.85987496	3.03075E-05	0.228090806	0.173307343	1.5067E-07
	15-Mar-11	152	56.00657573	42.7764618	4.56006E-05	0.266512183	0.203552837	2.16982E-07
	4-Apr-11	172	5159.717328	40.31548461	0.002918336	24.67892681	0.191841745	1.38864E-05
	28-Apr-11	196	5479.732997	42.92325202	0.009762692	14.72160652	0.11468872	2.60843E-05
	31-May-11	229	5526.084865	43.75054374	0.009519234	14.84682535	0.116899298	2.54338E-05
	29-Jun-11	258	6913.840688	55.00291023	0.015016862	18.60123948	0.14696672	4.01226E-05
Trial 8 (PU121-3.0m)	31-Oct-10	17	63.65782945	4.820545872	8.17188E-06	0.319033618	0.024157635	4.09523E-08
	26-Nov-10	43	138.4633379	10.44787766	3.00604E-05	0.708217085	0.053431823	1.53731E-07
	21-Dec-10	68	188.358796	14.25590721	0.000747596	0.994698738	0.075269374	3.94715E-06
	21-Jan-11	99	14.41242367	1.07874561	2.12439E-06	0.074804648	0.005598922	1.1026E-08
	21-Jan-11	99	18.68509383	14.15517102	6.622E-05	0.096803149	0.073334314	3.43064E-07
	22-Feb-11	131	30.92730625	2.375984534	1.12637E-05	0.159911513	0.012284801	5.82379E-08
	15-Mar-11	152	49.6708618	37.26342468	3.54948E-05	0.236361638	0.177317938	1.68896E-07
	4-Apr-11	172	50.09041065	37.76766588	3.57345E-05	0.238358185	0.179717459	1.70036E-07
	28-Apr-11	196	4815.553884	37.25810213	0.007602796	12.92861828	0.099551167	2.03134E-05
	31-May-11	229	5396.517064	42.2038679	0.009130907	14.49682929	0.112766482	2.43963E-05
	29-Jun-11	258	6824.727821	53.68403936	0.014714113	18.35983962	0.143442534	3.93137E-05

Notes: Data highlighted in red were poor results from initial analysis in Ottawa, and do not reflect the rest of the data. In each case, the data was rerun with more accurate results.

Appendix F: Experiment 4 -Labelled NH_4^+ (^{15}N tagged) Experiment – N_2 Contribution Calculations

N_{2TOTAL} = total N_2 produced in incubation
A_{TOTAL} = total N_2 produced by anammox
D_{TOTAL} = total N_2 produced by denitrification

Assume: $N_{2TOTAL} = A_{TOTAL} + D_{TOTAL}$ (1)

$^{28}N_{2TOTAL}$ = Total $^{28}N_2$ produced in incubation
^{28}A = Total $^{28}N_2$ produced by anammox
^{28}D = Total $^{28}N_2$ produced by denitrification

Assume: $^{28}N_{2TOTAL} = {}^{28}A + {}^{28}D$ (2)
The same statements apply for $^{29}N_2$ and $^{30}N_2$:
Assume: $^{29}N_{2TOTAL} = {}^{29}A + {}^{29}D$ (3)
$^{30}N_{2TOTAL} = {}^{30}A + {}^{30}D$ (4)

F_{NH4} = Fraction of ^{15}N- NH_4^+ in NH_4^+ pool
F_{NO3} = Fraction of ^{15}N- NO_3^- in NO_3^- pool = natural abundance of ^{15}N

and ^{28}A = unlabelled NH_4^+ + unlabelled NO_3^-:
$= A_{TOTAL}(1- F_{NH4})(1-F_{NO3})$ (5)

^{29}A = unlabelled NH_4^+ + labelled NO_3^- and
labelled NH_4^+ + unlabelled NO_3^-
$= A_{TOTAL} \times (1- F_{NH4})(F_{NO3})] + [A_{TOTAL} \times (F_{NH4})(1-F_{NO3})]$
$= A_{TOTAL} [(1- F_{NH4})(F_{NO3}) + (F_{NH4})(1-F_{NO3})]$
$= A_{TOTAL} [(F_{NO3})- (F_{NH4}F_{NO3}) + (F_{NH4})- (F_{NH4}F_{NO3})]$
$= A_{TOTAL} [(F_{NO3})- 2(F_{NH4}F_{NO3}) + (F_{NH4})]$ (6)

^{30}A = labelled NH_4^+ + labelled NO_3^-
$= A_{TOTAL} \times (F_{NH4})(F_{NO3})$ (7)

^{28}D = 2 unlabelled NO_3^-
$= D_{TOTAL} \times (1-F_{NO3})^2$ (8)

^{29}D = unlabelled NO_3^- + labelled NO_3^-
$= D_{TOTAL} \times (1-F_{NO3})(F_{NO3}) \times 2$ (9)

^{30}D = 2 labelled NO_3^-
$= D_{TOTAL} \times (F_{NO3})^2$ (10)

a) Sub (5) and (8) into (2)
b) Sub (6) and (9) into (3) (to check if you get the same A_{TOTAL})

a) $^{28}N_{2TOTAL} = {}^{28}A + {}^{28}D$

$^{28}N_{2TOTAL} = A_{TOTAL}(1- F_{NH4})(F_{NO3}) + D_{TOTAL} \times (1-F_{NO3})^2$

Rearrange (1), replace D_{TOTAL}:

$^{28}N_{2TOTAL} = A_{TOTAL}(1- F_{NH4})(F_{NO3}) + (N_{2TOTAL} - A_{TOTAL}) \times (1-F_{NO3})^2$

$^{28}N_{2TOTAL} = A_{TOTAL}(1- F_{NH4})(F_{NO3}) + N_{2TOTAL}(1-F_{NO3})^2 - A_{TOTAL}(1-F_{NO3})^2$

$A_{TOTAL}(1-F_{NO3})^2 - A_{TOTAL}(1- F_{NH4})(F_{NO3}) = N_{2TOTAL}(1-F_{NO3})^2 - {}^{28}N_{2TOTAL}$

$A_{TOTAL}[(1-F_{NO3})^2 - (1- F_{NH4})(F_{NO3})] = N_{2TOTAL}(1-F_{NO3})^2 - {}^{28}N_{2TOTAL}$

$$A_{TOTAL} = \frac{N_{2TOTAL}(1-F_{NO3})^2 - {}^{28}N_{2TOTAL}}{[(1-F_{NO3})^2 - (1- F_{NH4})(F_{NO3})]} \qquad (11)$$

To solve for D_{TOTAL} instead of A_{TOTAL}, Rearrange (1), replace A_{TOTAL}:

$^{28}N_{2TOTAL} = (N_{2TOTAL} - D_{TOTAL})(1- F_{NH4})(F_{NO3}) + (D_{TOTAL}) \times (1-F_{NO3})^2$

$^{28}N_{2TOTAL} = N_{2TOTAL}(1- F_{NH4})(F_{NO3}) - D_{TOTAL}(1- F_{NH4})(F_{NO3}) + D_{TOTAL}(1-F_{NO3})^2$

$D_{TOTAL}(1- F_{NH4})(F_{NO3}) - D_{TOTAL}(1-F_{NO3})^2 = N_{2TOTAL}(1- F_{NH4})(F_{NO3}) - {}^{28}N_{2TOTAL}$

$D_{TOTAL}[(1- F_{NH4})(F_{NO3}) - (1-F_{NO3})^2] = N_{2TOTAL}(1- F_{NH4})(F_{NO3}) - {}^{28}N_{2TOTAL}$

$$D_{TOTAL} = \frac{N_{2TOTAL}(1- F_{NH4})(F_{NO3}) - {}^{28}N_{2TOTAL}}{[(1- F_{NH4})(F_{NO3}) - (1-F_{NO3})^2]} \qquad (12)$$

b) $^{29}N_{2TOTAL} = {}^{29}A + {}^{29}D$

$^{29}N_{2TOTAL} = A_{TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] + [D_{TOTAL} \times (1-F_{NO3})(F_{NO3}) \times 2]$

Rearrange (1), replace D_{TOTAL}:

$^{29}N_{2TOTAL} = A_{TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] + [(N_{2TOTAL} - A_{TOTAL}) \times (1-F_{NO3})(F_{NO3}) \times 2]$

$^{29}N_{2TOTAL} = A_{TOTAL}(F_{NO3}) - 2A_{TOTAL}(F_{NH4}F_{NO3}) + A_{TOTAL}(F_{NH4}) + 2N_{2TOTAL}(1-F_{NO3})(F_{NO3}) - 2A_{TOTAL}(1-F_{NO3})(F_{NO3})$

$A_{TOTAL}(F_{NO3}) - 2A_{TOTAL}(F_{NH4}F_{NO3}) + A_{TOTAL}(F_{NH4}) - 2A_{TOTAL}(1-F_{NO3})(F_{NO3}) = {}^{29}N_{2TOTAL} - 2N_{2TOTAL}(1-F_{NO3})(F_{NO3})$

$A_{TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4}) - 2(1-F_{NO3})(F_{NO3})] = {}^{29}N_{2TOTAL} - 2N_{2TOTAL}(1-F_{NO3})(F_{NO3})$

$$A_{TOTAL} = \frac{{}^{29}N_{2TOTAL} - 2N_{2TOTAL}(1-F_{NO3})(F_{NO3})}{[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4}) - 2(1-F_{NO3})(F_{NO3})]} \qquad (13)$$

To solve for D_{TOTAL} instead of A_{TOTAL}, Rearrange (1), replace A_{TOTAL}:

$^{29}N_{2TOTAL} = (N_{2TOTAL} - D_{TOTAL})[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] + [D_{TOTAL} \times (1-F_{NO3})(F_{NO3}) \times 2]$

$^{29}N_{2TOTAL} = N_{2TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] - D_{TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] + 2D_{TOTAL}(1-F_{NO3})(F_{NO3})$

$D_{TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] - 2D_{TOTAL}(1-F_{NO3})(F_{NO3}) = N_{2TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] - {}^{29}N_{2TOTAL}$

$D_{TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4}) - 2(1-F_{NO3})(F_{NO3})] = N_{2TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] - {}^{29}N_{2TOTAL}$

$$D_{TOTAL} = \frac{N_{2TOTAL}[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4})] - {}^{29}N_{2TOTAL}}{[(F_{NO3}) - 2(F_{NH4}F_{NO3}) + (F_{NH4}) - 2(1-F_{NO3})(F_{NO3})]} \qquad (14)$$

Table D3. Anammox N_2 contribution table, modified and expanded on from Thamdrup and Dalsgaard (2006) to account for natural abundance levels of $^{15}N\text{-}NO_3^-$ Spoelstra (2011).

Sample	Date	Day	Corrected (Dissolved, umol/L)				Calculation (modified from Thamdrup and Dalsgaard 2006, Spoelstra 2011)												
			$^{28}N_2$	$^{29}N_2$	$^{30}N_2$	N_2 Total	A_{tot} (^{28}N equation)	A_{tot} (^{29}N equation)	Anammox %	^{28}A	^{29}A	^{30}A	$^{28,29,30}A$ Sum	D_{tot} (^{28}N equation)	D_{tot} (^{29}N equation)	^{28}D	^{29}D	^{30}D	$^{29}D/N2_{tot}$ (Nitrification Indicator)
Trial 6	31-Oct-10	17	0.444	0.003	4.74E-06	0.447	0.00033	0.00035	0.001	0.00034	0.00002	0.00000	0.0004	0.4471	0.4470	0.4438	0.0033	5.99E-06	0.0073
(PU86-3.1m)	26-Nov-10	43	0.452	0.003	2.06E-06	0.455	0.00226	0.00235	0.005	0.00222	0.00013	0.00000	0.0024	0.4531	0.4530	0.4497	0.0033	6.07E-06	0.0073
	21-Dec-10	68	1.034	0.008	6.96E-05	1.042	0.01224	0.01111	0.011	0.01050	0.00061	0.00000	0.0111	1.0298	1.0310	1.0234	0.0075	1.38E-05	0.0072
	21-Jan-11	99	0.112	0.001	6.07E-06	0.112	0.00148	0.00139	0.012	0.00131	0.00008	0.00000	0.0014	0.1109	0.1110	0.1102	0.0008	1.49E-06	0.0072
	21-Jan-11	99	0.108	0.001	9.37E-06	0.109	0.00182	0.00166	0.015	0.00157	0.00009	0.00000	0.0017	0.1073	0.1075	0.1067	0.0008	1.44E-06	0.0072
	22-Feb-11	131	0.066	0.001	2.04E-06	0.067	0.00122	0.00120	0.018	0.00114	0.00007	0.00000	0.0012	0.0653	0.0653	0.0649	0.0005	8.75E-07	0.0072
	22-Feb-11	131	0.078	0.001	4.68E-06	0.079	0.00168	0.00161	0.020	0.00152	0.00009	0.00000	0.0016	0.0774	0.0775	0.0769	0.0006	1.04E-06	0.0071
	15-Mar-11	152	1.272	0.011	5.49E-06	1.283	0.02904	0.02939	0.023	0.02778	0.00161	0.00001	0.0294	1.2536	1.2533	1.2441	0.0091	1.68E-05	0.0071
	4-Apr-11	172	0.285	0.003	1.27E-06	0.287	0.01132	0.01142	0.040	0.01079	0.00062	0.00000	0.0114	0.2758	0.2757	0.2737	0.0020	3.69E-06	0.0070
	28-Apr-11	196	14.595	0.117	1.55E-04	14.712	0.20460	0.20624	0.014	0.19493	0.01127	0.00004	0.2062	14.5075	14.5059	14.3999	0.1058	1.94E-04	0.0072
	31-May-11	229	21.368	0.182	2.02E-04	21.550	0.51784	0.52158	0.024	0.49299	0.02849	0.00010	0.5216	21.0324	21.0286	20.8750	0.1534	2.82E-04	0.0071
	29-Jun-11	258	13.891	0.109	1.28E-04	14.001	0.14703	0.14883	0.011	0.14067	0.00813	0.00003	0.1488	13.8537	13.8519	13.7507	0.1010	1.86E-04	0.0072
Trial 7	31-Oct-10	17	0.332	0.002	2.12E-06	0.334	0.00063	0.00068	0.002	0.00065	0.00004	0.00000	0.0007	0.3338	0.3337	0.3313	0.0024	4.47E-06	0.0073
(PU115-2.2m)	26-Nov-10	43	0.684	0.005	3.22E-06	0.690	0.00822	0.00838	0.012	0.00792	0.00046	0.00000	0.0084	0.6816	0.6815	0.6765	0.0050	9.13E-06	0.0072
	21-Dec-10	68	0.885	0.007	5.64E-05	0.892	0.01491	0.01402	0.016	0.01326	0.00076	0.00000	0.0140	0.8774	0.8783	0.8718	0.0064	1.18E-05	0.0072
	21-Jan-11	99	0.152	0.001	6.29E-06	0.154	0.00235	0.00227	0.015	0.00214	0.00012	0.00000	0.0023	0.1513	0.1514	0.1503	0.0011	2.03E-06	0.0072
	21-Jan-11	99	0.155	0.001	6.37E-06	0.156	0.00241	0.00232	0.015	0.00220	0.00013	0.00000	0.0023	0.1541	0.1542	0.1530	0.0011	2.07E-06	0.0072
	22-Feb-11	131	0.116	0.001	6.86E-06	0.117	0.00249	0.00239	0.020	0.00226	0.00013	0.00000	0.0024	0.1144	0.1145	0.1137	0.0008	1.53E-06	0.0071
	15-Mar-11	152	0.228	0.002	1.01E-06	0.230	0.00480	0.00486	0.021	0.00460	0.00026	0.00000	0.0049	0.2252	0.2251	0.2235	0.0016	3.02E-06	0.0071
	15-Mar-11	152	0.267	0.002	1.19E-06	0.269	0.00585	0.00593	0.022	0.00560	0.00032	0.00000	0.0059	0.2629	0.2628	0.2609	0.0019	3.52E-06	0.0071
	4-Apr-11	172	24.679	0.201	7.92E-05	24.880	0.41751	0.42445	0.017	0.40130	0.02307	0.00008	0.4244	24.4629	24.4560	24.2773	0.1784	3.28E-04	0.0072
	28-Apr-11	196	14.722	0.120	1.40E-04	14.842	0.25653	0.25872	0.017	0.24461	0.01406	0.00005	0.2587	14.5856	14.5834	14.4769	0.1064	1.95E-04	0.0072
	31-May-11	229	14.847	0.123	1.35E-04	14.970	0.28584	0.28828	0.019	0.27255	0.01567	0.00005	0.2883	14.6839	14.6814	14.5742	0.1071	1.97E-04	0.0072
	29-Jun-11	258	18.601	0.154	1.71E-04	18.756	0.36932	0.37239	0.020	0.35208	0.02024	0.00007	0.3724	18.3864	18.3833	18.2490	0.1341	2.46E-04	0.0071
Trial 8	31-Oct-10	17	0.319	0.003	1.98E-06	0.322	0.00895	0.00905	0.028	0.00867	0.00038	0.00000	0.0090	0.3127	0.3126	0.3104	0.0023	4.19E-06	0.0071
(PU121-3.0m)	26-Nov-10	43	0.708	0.006	3.42E-06	0.714	0.01921	0.01946	0.027	0.01865	0.00081	0.00000	0.0195	0.6949	0.6946	0.6896	0.0051	9.31E-06	0.0071
	21-Dec-10	68	0.995	0.008	6.46E-05	1.003	0.02941	0.02803	0.028	0.02686	0.00117	0.00000	0.0280	0.9736	0.9750	0.9679	0.0071	1.31E-05	0.0071
	21-Jan-11	99	0.075	0.001	2.36E-06	0.075	0.00195	0.00191	0.025	0.00183	0.00008	0.00000	0.0019	0.0735	0.0735	0.0730	0.0005	9.85E-07	0.0071
	21-Jan-11	99	0.097	0.001	5.66E-06	0.098	0.00287	0.00275	0.028	0.00264	0.00011	0.00000	0.0028	0.0947	0.0949	0.0942	0.0007	1.27E-06	0.0071
	22-Feb-11	131	0.160	0.001	5.80E-06	0.161	0.00518	0.00509	0.032	0.00488	0.00021	0.00000	0.0051	0.1561	0.1562	0.1550	0.0011	2.09E-06	0.0071
	15-Mar-11	152	0.236	0.002	1.05E-06	0.238	0.00609	0.00617	0.026	0.00592	0.00026	0.00000	0.0062	0.2322	0.2321	0.2304	0.0017	3.11E-06	0.0071
	4-Apr-11	172	0.238	0.002	1.05E-06	0.240	0.00643	0.00651	0.027	0.00624	0.00027	0.00000	0.0065	0.2339	0.2338	0.2321	0.0017	3.13E-06	0.0071
	28-Apr-11	196	12.929	0.105	1.24E-04	13.033	0.27299	0.27546	0.021	0.26394	0.01148	0.00004	0.2755	12.7603	12.7578	12.6646	0.0930	1.71E-04	0.0071
	31-May-11	229	14.497	0.118	1.33E-04	14.615	0.34035	0.34343	0.023	0.32906	0.01432	0.00005	0.3434	14.2750	14.2719	14.1677	0.1041	1.91E-04	0.0071
	29-Jun-11	258	18.360	0.151	1.69E-04	18.511	0.45000	0.45394	0.025	0.43495	0.01892	0.00006	0.4539	18.0606	18.0567	17.9248	0.1317	2.42E-04	0.0071

Fractions of ^{15}N in NH_4^+ and NO_3^-

F_{NH4} (Trial 6)	0.05133807
F_{NH4} (Trial 7)	0.051070541
F_{NH4} (Trial 8)	0.038308479
F_{NO3}	0.00366

Theoretical fractional abundances of N2 isotope masses

^{28}A	0.9451898
^{29}A	0.0546223
^{30}A	0.0001879
Total	1

^{28}D	0.993
^{29}D	0.007
^{30}D	0.000
Total	1.000

Notes: Data highlighted in red were poor results from initial analysis in Ottawa, and do not reflect the rest of the data. In each case, the data was rerun with more accurate results.

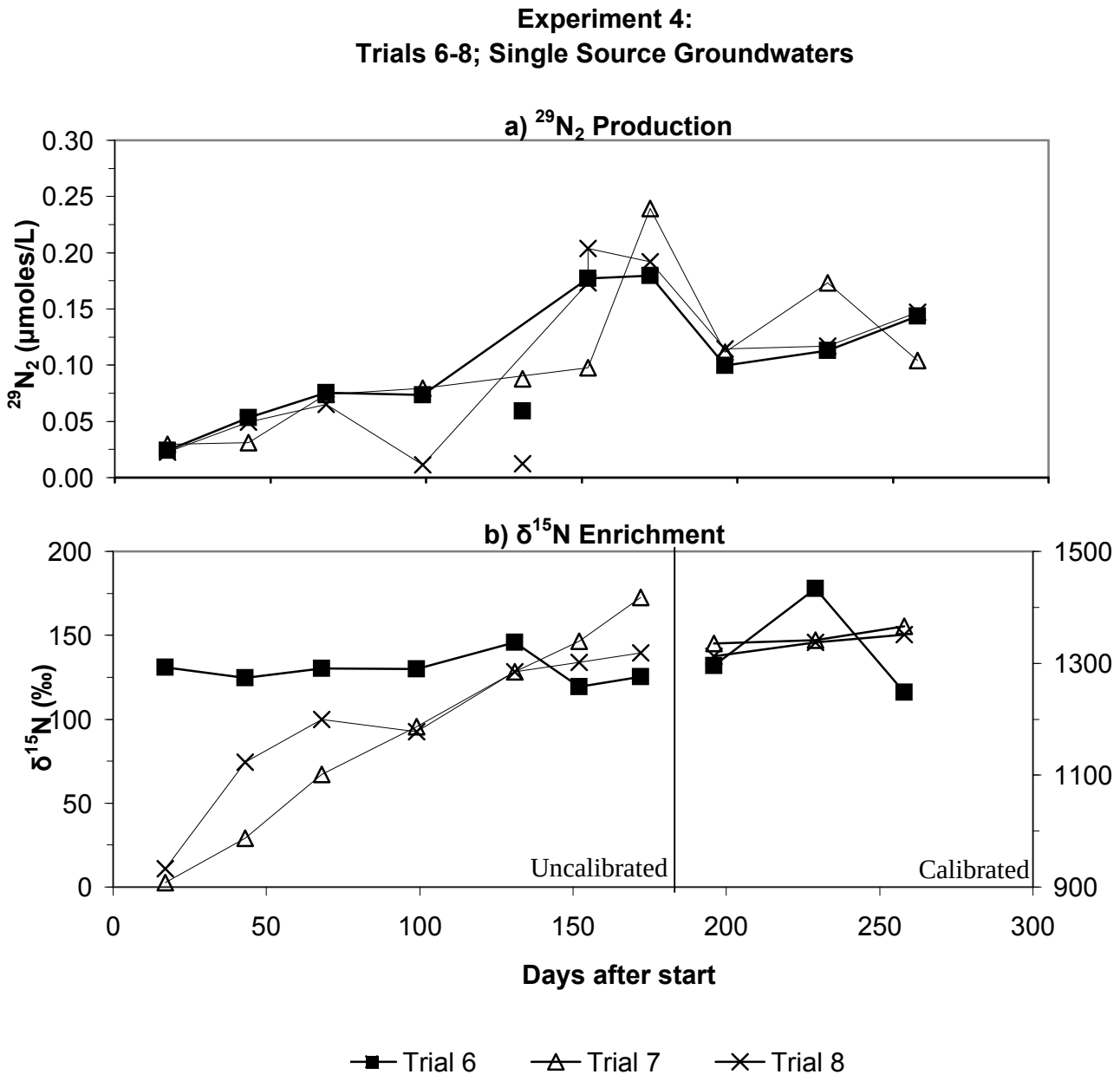


Figure F1. Experiment 4, Trial 6 (PU86-3.1m), Trial 7 (PU115-2.2m) and Trial 8 (PU121-3.0m single source groundwater, with NH_4NO_3 and $^{15}N\text{-}NH_4^+$ enriched $(NH_4)_2SO_4$ added; evolution of **a)** $^{29}N_2$ production and **b)** $\delta^{15}N$ Enrichment, Oct. 25, 2010 to Jun. 24, 2011. Note that during the first 172 days of the experiment, δ15N data was uncorrected, after which a dual correction (volume and concentration) was applied (Appendix E). Concentration data was calculated with a 1-point calibration (Appendix E).

Appendix G:
Experiment 5 – Field Mesocosms, Zorra Site

Geochemistry and Isotope Data
Reactor Design
Reactor Figures (all ports)

Table G1. Analytical results for Experiment 5; Zorra Reactors (Control: 100% sand from pit; Inoculated: 10% core drilled from PU103 3-6m depth), port depths (both reactors): 8 cm, 25 cm, 43 cm, 61 cm, 79 cm, both reactors filled with groundwater from PU115-2.2m on Oct. 24, 2009, experiment ran until Aug. 19, 2010.

Reactor	Date	Day	Analytical (mg/L)							Isotopes (‰)		Field Parameters		
			NO_2^--N	NO_3^--N	NH_4^+-N	TIN	TN	Cl^-	DOC	^{15}N-NH_4^+	^{15}N-NO_3^-	DO (mg/L)	Conductivity (µS/cm)	Temp (°C)
Control: 8 cm	24-Oct-09	0	1.97	31.42	0.73	34.11	43.20	71.20	39.84			6.91	1491	10.2
	13-Nov-09	20	0.39	3.95	1.80	6.14	11.83	47.96	28.75			3.81	1341	11.4
	12-Mar-10	139	0.52	2.19	5.73	8.45	11.21	17.63	6.72			3.50	524	7.7
	5-Apr-10	163	0.14	3.09	4.94	8.17	7.66	25.83	17.32			3.30	890	14.2
	26-May-10	214	0.03	0.19	8.58	8.79	11.33	53.85	23.39			3.80	1050	26.5
	29-Jun-10	248	0.00	0.00	7.74	7.74	9.84	64.87	28.28			4.30	1120	7.0
	17-Jul-10	266	0.41	5.99	4.00	10.39	9.24	63.54	10.79			2.90	1187	25.0
	19-Aug-10	299	-0.01	0.06		0.05		52.56	30.55			1.33	1196	19.0
Control: 25 cm	24-Oct-09	0	3.00	31.33	5.64	39.98	46.69	69.72	45.51			7.32	1619	10.5
	13-Nov-09	20	0.77	1.78	0.55	3.09	11.32	65.04	50.24			1.41	1359	9.7
	12-Mar-10	139	2.57	20.35	12.34	35.25		72.68	24.32			5.89	1460	6.5
	5-Apr-10	163	2.42	27.96	13.25	43.64	40.11	74.24	37.68			1.98	1409	14.1
	26-May-10	214	0.05	8.22	11.66	19.93	23.66	67.51	38.76			1.80	1301	24.9
	29-Jun-10	248	0.26	5.31	14.68	20.24	24.24	71.12	39.78			2.44	1415	7.0
	17-Jul-10	266	0.00	1.47	9.10	10.57	13.29	68.57	35.36			2.04	1333	25.0
	19-Aug-10	299	-0.01	0.00	7.09	7.08		53.11	31.30			2.39	1233	19.0
Control: 43 cm	24-Oct-09	0	2.49	26.62	31.23	60.34	74.38	76.29	134.29			9.06	1844	11.3
	13-Nov-09	20	1.40	10.68	18.56	30.64	20.02	21.25	24.58			1.05	1726	11.0
	12-Mar-10	139	2.51	27.75	28.28	58.53	60.30	74.07	103.22			1.24	1579	7.2
	5-Apr-10	163	1.79	22.61	28.11	52.51	52.20	71.36	40.39			0.91	1612	14.2
	26-May-10	214	0.13	22.97	32.65	55.75	53.53	76.61	48.14			0.78	1772	23.7
	29-Jun-10	248	0.02	9.22	34.10	43.34	44.81	79.32	45.95			0.85	1747	7.0
	17-Jul-10	266	0.00	6.16	24.74	30.90	39.51	81.94	49.26			0.60	1754	25.0
	19-Aug-10	299	-0.01	0.27	27.42	27.68		69.80	32.80			0.69	1621	19.0

Table G1. Analytical results for Experiment 5; Zorra Reactors (Control: 100% sand from pit; Inoculated: 10% core drilled from PU103 3-6m depth), port depths (both reactors): 8 cm, 25 cm, 43 cm, 61 cm, 79 cm, both reactors filled with groundwater from PU115-2.2m on Oct. 24, 2009, experiment ran until Aug. 19, 2010.

Reactor	Date	Day	Analytical (mg/L)							Isotopes (‰)		Field Parameters		
			NO_2^--N	NO_3^--N	NH_4^+-N	TIN	TN	Cl^-	DOC	^{15}N-NH_4^+	^{15}N-NO_3^-	DO (mg/L)	Conductivity (µS/cm)	Temp (°C)
Control: 8 cm	24-Oct-09	0	1.97	31.42	0.73	34.11	43.20	71.20	39.84			6.91	1491	10.2
	13-Nov-09	20	0.39	3.95	1.80	6.14	11.83	47.96	28.75			3.81	1341	11.4
	12-Mar-10	139	0.52	2.19	5.73	8.45	11.21	17.63	6.72			3.50	524	7.7
	5-Apr-10	163	0.14	3.09	4.94	8.17	7.66	25.83	17.32			3.30	890	14.2
	26-May-10	214	0.03	0.19	8.58	8.79	11.33	53.85	23.39			3.80	1050	26.5
	29-Jun-10	248	0.00	0.00	7.74	7.74	9.84	64.87	28.28			4.30	1120	7.0
	17-Jul-10	266	0.41	5.99	4.00	10.39	9.24	63.54	10.79			2.90	1187	25.0
	19-Aug-10	299	-0.01	0.06		0.05		52.56	30.55			1.33	1196	19.0
Control: 25 cm	24-Oct-09	0	3.00	31.33	5.64	39.98	46.69	69.72	45.51			7.32	1619	10.5
	13-Nov-09	20	0.77	1.78	0.55	3.09	11.32	65.04	50.24			1.41	1359	9.7
	12-Mar-10	139	2.57	20.35	12.34	35.25		72.68	24.32			5.89	1460	6.5
	5-Apr-10	163	2.42	27.96	13.25	43.64	40.11	74.24	37.68			1.98	1409	14.1
	26-May-10	214	0.05	8.22	11.66	19.93	23.66	67.51	38.76			1.80	1301	24.9
	29-Jun-10	248	0.26	5.31	14.68	20.24	24.24	71.12	39.78			2.44	1415	7.0
	17-Jul-10	266	0.00	1.47	9.10	10.57	13.29	68.57	35.36			2.04	1333	25.0
	19-Aug-10	299	-0.01	0.00	7.09	7.08		53.11	31.30			2.39	1233	19.0
Control: 43 cm	24-Oct-09	0	2.49	26.62	31.23	60.34	74.38	76.29	134.29			9.06	1844	11.3
	13-Nov-09	20	1.40	10.68	18.56	30.64	20.02	21.25	24.58			1.05	1726	11.0
	12-Mar-10	139	2.51	27.75	28.28	58.53	60.30	74.07	103.22			1.24	1579	7.2
	5-Apr-10	163	1.79	22.61	28.11	52.51	52.20	71.36	40.39			0.91	1612	14.2
	26-May-10	214	0.13	22.97	32.65	55.75	53.53	76.61	48.14			0.78	1772	23.7
	29-Jun-10	248	0.02	9.22	34.10	43.34	44.81	79.32	45.95			0.85	1747	7.0
	17-Jul-10	266	0.00	6.16	24.74	30.90	39.51	81.94	49.26			0.60	1754	25.0
	19-Aug-10	299	-0.01	0.27	27.42	27.68		69.80	32.80			0.69	1621	19.0

Table G1 cont'd. Analytical results for Experiment 5; Zorra Reactors (Control: 100% sand from pit; Inoculated: 10% core drilled from PU103 3-6m depth port depths (both reactors): 8 cm, 25 cm, 43 cm, 61 cm, 79 cm, both reactors filled with groundwater from PU115-2.2m on Oct. 24, 2009, experiment ran until Aug. 19, 2010.

Reactor	Date	Day	Analytical (mg/L)							Isotopes (‰)		Field Parameters		
			NO_2^--N	NO_3^--N	NH_4^+-N	TIN	TN	Cl^-	DOC	^{15}N-NH_4^+	^{15}N-NO_3^-	DO (mg/L)	Conductivity (µS/cm)	Temp (°C)
Control: 61 cm	24-Oct-09	0	2.58	26.96	53.19	82.73	93.74	81.37	105.01	27.6		9.82	1967	11.7
	13-Nov-09	20	1.20	7.42	17.91	26.54	23.73	36.13	26.69			0.85	1929	11.8
	12-Mar-10	139	1.61	19.60	39.17	60.38	49.37	59.86	39.48			2.29	1306	6.4
	5-Apr-10	163	2.15	13.90	45.32	61.37	59.71	68.33	50.95	27.6		1.04	1865	14.3
	26-May-10	214	0.33	10.17	40.58	51.08	58.15	79.48	71.44			1.07	1834	21.1
	29-Jun-10	248	0.03	3.17	41.08	44.28	53.39	80.47	66.91	26.5		1.84	1782	7.0
	17-Jul-10	266	0.00	11.58	47.23	58.81	48.39	83.50	62.35			0.90	1801	25.0
	19-Aug-10	299	0.01	0.05	39.25	39.31		81.20	69.61	27.6		0.82	1769	19.0
Control: 79 cm	24-Oct-09	0	2.48	23.54	54.52	80.53	86.15	78.83	99.99	28.7		9.02	1977	11.1
	13-Nov-09	20	1.51	5.97	28.94	36.43		49.46	44.29			1.06	1875	11.7
	12-Mar-10	139	0.98	3.44	42.54	46.95	39.33	79.74	18.34			2.29	1720	7.5
	5-Apr-10	163	0.57	2.01	49.11	51.68	57.25	73.17	81.70	25.2		0.90	1822	14.3
	26-May-10	214	1.25	4.15	52.49	57.89	60.55	86.04	83.61			0.94	1862	21.5
	29-Jun-10	248	0.42	1.66	50.54	52.63	53.49	90.78	73.03	26.6		0.89	1859	7.0
	17-Jul-10	266	0.00	1.72	49.32	51.04	50.08	93.48	72.57			0.78	1789	25.0
	19-Aug-10	299	0.01	0.04	39.63	39.68		97.57	59.54	25.5		0.85	1757	19.0
Control: Total	24-Oct-09	0	12.51	139.86	145.31	272.66	344.16		424.65					
	13-Nov-09	20	5.28	29.80	67.76	102.84	66.90		174.55					
	12-Mar-10	139	8.19	73.32	128.06	209.56	160.21		192.09					
	5-Apr-10	163	7.07	69.57	140.73	217.37	344.16		424.65					
	26-May-10	214	1.78	45.70	145.96	193.44	66.90		174.55					
	29-Jun-10	248	0.73	19.36	148.14	168.23	160.21		192.09					
	17-Jul-10	266	0.40	26.93	134.39	161.72	216.94		228.03					
	19-Aug-10	299	0.02	0.43	113.38	113.79	207.22		265.34					

Table G1 cont'd. Analytical results for Experiment 5; Zorra Reactors (Control: 100% sand from pit; Inoculated: 10% core drilled from PU103 3-6m depth port depths (both reactors): 8 cm, 25 cm, 43 cm, 61 cm, 79 cm, both reactors filled with groundwater from PU115-2.2m on Oct. 24, 2009, experiment ran until Aug. 19, 2010.

Reactor	Date	Day	Analytical (mg/L)							Isotopes (‰)		Field Parameters		
			NO_2^--N	NO_3^--N	NH_4^+-N	TIN	TN	Cl^-	DOC	^{15}N-NH_4^+	^{15}N-NO_3^-	DO (mg/L)	Conductivity (µS/cm)	Temp (°C)
Inoculated: 8 cm	24-Oct-09	0	1.37	27.50	3.55	32.42	36.33	68.74	41.10			4.43	1306	10.0
	13-Nov-09	20	0.59	2.69	1.32	4.60	2.69	15.15	17.02			4.92	1426	11.0
	7-Jan-10	75	FROZEN											
	5-Apr-10	163		0.36	1.19	1.55	2.97	51.11	18.74			2.45	845	16.4
	26-May-10	214	No sample - dry											
	29-Jun-10	248												
	17-Jul-10	266												
	19-Aug-10	299												
Inoculated: 25 cm	24-Oct-09	0	0.76	34.58	4.77	40.11	46.48	76.08	105.12			6.30	1510	9.6
	13-Nov-09	20	Sample Destroye		0.48			54.01	32.27			1.21	1451	11.1
	7-Jan-10	75	0.76	17.03	0.60	18.39	28.11	98.69	26.08			<1	573	0.0
	5-Apr-10	163	0.86	11.69	1.41	13.97	19.40	82.28	25.71			0.57	1175	14.8
	26-May-10	214	0.24	4.50	1.93	6.67	8.91	79.77	119.63			0.73	1198	22.8
	29-Jun-10	248	0.00	0.00	1.27	1.27	2.81	74.16	27.27			0.95	1183	7.0
	17-Jul-10	266	0.00	0.03	1.68	1.71	5.89	77.43	25.60					24.5
	19-Aug-10	299	-0.01	0.06	0.53			55.83	16.15			0.72	1084	18.5
Inoculated: 43 cm	24-Oct-09	0	1.15	37.42	16.03	54.60	61.12	76.08	43.73			8.29	1695	9.9
	13-Nov-09	20	1.34	18.97	3.14	23.44	30.91	54.01	30.16			1.11	1656	10.4
	7-Jan-10	75	0.95	31.99	9.39	42.33	47.53	98.69	43.86			<1	2090	0.0
	5-Apr-10	163		20.35	14.60	34.95	40.17	80.89	4.28			0.93	1558	15.2
	26-May-10	214	0.21	13.55	17.34	31.10	31.75	79.77	43.23			0.75	1539	21.5
	29-Jun-10	248	0.01	0.95	18.46	19.42	20.90	74.16	39.02			0.88	1503	7.0
	17-Jul-10	266	0.00	0.03	23.24	23.27	26.20	76.68	48.15			0.70	1498	24.5
	19-Aug-10	299	-0.01	0.22	13.81	14.02			22.92			0.77	1368	18.5

Table G1 cont'd. Analytical results for Experiment 5; Zorra Reactors (Control: 100% sand from pit; Inoculated: 10% core drilled from PU103 3-6m depth port depths (both reactors): 8 cm, 25 cm, 43 cm, 61 cm, 79 cm, both reactors filled with groundwater from PU115-2.2m on Oct. 24, 2009, experiment ran until Aug. 19, 2010.

Reactor	Date	Day	Analytical (mg/L)							Isotopes (‰)		Field Parameters		
			NO_2^--N	NO_3^--N	NH_4^+-N	TIN	TN	Cl^-	DOC	^{15}N-NH_4^+	^{15}N-NO_3^-	DO (mg/L)	Conductivity (µS/cm)	Temp (°C)
Inoculated: 61 cm	24-Oct-09	0	1.60	32.27	42.86	76.72		78.99	74.37	26.3		7.49	1960	9.6
	13-Nov-09	20	0.86	5.40	11.35	17.62		16.39	25.05			1.28	1827	10.7
	7-Jan-10	75	1.11	19.19	31.24	51.53		54.67	33.23			1.52	1650	0.4
	5-Apr-10	163		22.84	30.10	52.93		87.15	31.96	24		0.77	1730	14.0
	26-May-10	214	0.78	16.35	24.22	41.36		84.23	37.71			0.59	1704	21.1
	29-Jun-10	248	0.01	3.16	28.84	32.00	34.38	81.31	28.24	25		1.00	1690	7.0
	17-Jul-10	266	0.00	0.88	25.55	26.43		136.10	43.90			0.67	1679	24.5
	19-Aug-10	299	0.01	0.52	22.62	23.14		64.96	34.34	26.3		0.50	1602	18.5
Inoculated: 79 cm	24-Oct-09	0	1.48	13.68	53.49	68.65	53.34	39.66	58.27	23.9		5.63	2007	9.2
	13-Nov-09	20	1.69	7.17	20.07	28.93	38.90	57.92	54.88			1.12	1888	10.3
	7-Jan-10	75	1.57	14.07	49.77	65.41	3.85	79.28	60.18			<1	2000	1.3
	5-Apr-10	163	0.87	8.81	52.33	62.01	60.73	71.25	59.07	25		0.50	1800	12.7
	26-May-10	214	1.09	8.47	44.50	54.06	57.78	76.17	61.28			0.53	1754	19.2
	29-Jun-10	248	0.02	1.81	47.18	49.02	52.58	77.30	59.24	26.1		1.05	1816	7.0
	17-Jul-10	266	0.01	0.58	49.97	50.56	50.89	78.12	60.59			0.52	1801	24.5
	19-Aug-10	299	0.01	0.04	46.10	46.14		67.60	46.34	24.7		0.60	1708	18.5
Inoculated: Total	24-Oct-09	0	6.37	145.44	120.70	272.51	197.26		322.58					
	13-Nov-09	20	4.48	34.23	36.36	75.07	72.50		159.38					
	7-Jan-10	75	6.02	90.28	115.86	212.16	79.50		163.36					
	5-Apr-10	163	1.74	64.05	99.64	165.43	123.27		139.76					
	26-May-10	214	2.32	42.87	87.99	133.18	98.44		261.85					
	29-Jun-10	248	0.04	5.92	54.58	60.54	110.68		153.78					
	17-Jul-10	266	0.01	1.53	100.44	101.97	82.99		178.24					
	19-Aug-10	299	0.02	0.84	83.05	83.91	0.00		0.00					

Table G2. Analytical results for Experiment 5; Zorra Reactors (Control: 100% sand from pit; Inoculated: 10% core drilled from PU103 3-6m depth), port depths (both reactors): 8 cm, 25 cm, 43 cm, 61 cm, 79 cm, Control and Inoculated reactors filled with 4 and 7L of 3500 mg N/L NH4NO3, respectively, Sept. 30, 2010 to Jun. 17, 2011.

Reactor	Date	Day	Analytical (mg/L)					Field Parameters				
			NO_3^--N	NH_4^+-N	Cl^-	$SO4^{2-}$	Br^-	DO (mg/L)	Conductivity (µS/cm)	Eh (mV)	pH	Temperature (°C)
Control: 8 cm	30-Sep-10	0	89.08	95.60	68.51	29.30	14.04	2.00	2280			14.0
	12-Nov-10	43	85.23	59.71	60.04	29.00	16.38	2.54	2300			2.1
	20-Jan-11	112	95.03	75.09	85.60	37.09		2.82	2250			0.5
	4-Mar-11	155	100.76	68.81	83.51	39.08		4.36	2240		7.23	2.4
	13-Apr-11	195	97.55	43.49	70.32	31.77	15.28	0.98	2170			11.2
	4-May-11	216	96.20	27.97	60.41	36.61		1.55	1970	147	6.74	10.9
	17-Jun-11	260	105.48	47.91	67.23	31.19	14.82	0.79	2075	57	6.85	26.0
Control: 25 cm	30-Sep-10	0	127.34	96.77	67.87	28.69	21.65	1.97	2480			14.0
	12-Nov-10	43	74.60	55.72	71.09	30.10	20.41	1.64	2390			2.1
	20-Jan-11	112	45.88	75.49	52.57	21.49		1.43	2260			0.5
	4-Mar-11	155	95.40	55.87	70.93	32.37		1.60	2300		7.27	2.0
	13-Apr-11	195	91.52	54.18	65.06	34.41	15.87	0.75	2190			11.3
	4-May-11	216	87.97	55.14	62.61	35.28		0.80	2230	157	6.97	12.2
	17-Jun-11	260	105.20	52.69	57.22	29.79	14.59	0.53	2073	90	6.87	26.4
Control: 43 cm	30-Sep-10	0	77.17	101.89	70.89	26.86	14.88	2.25	2140			14.0
	12-Nov-10	43	93.41	77.98	81.58	34.46	19.82	2.13	2230			2.2
	20-Jan-11	112	86.87	92.07	81.74	35.34		1.46	2270			0.0
	4-Mar-11	155	84.87	280.32	83.71	31.37		>1	2340		7.52	3.0
	13-Apr-11	195	63.97	64.17	71.73	33.35	16.26	1.10	2310			11.2
	4-May-11	216	82.52	52.02	62.18	33.24		0.70	2260	158	7.13	12.3
	17-Jun-11	260	88.36	85.47	65.45	32.59	16.12	0.80	2230	90	7.20	24.4
Control: 61 cm	30-Sep-10	0	82.31	102.20	75.16	32.75	17.49	1.30	2250			14.5
	12-Nov-10	43	85.47	74.28	80.59	34.73	19.00	1.10	2540			2.2
	20-Jan-11	112	81.87	45.61	81.20	30.73		0.86	2650			0.5
	4-Mar-11	155	87.53	70.80	199.74	35.12		>1	2700		7.52	4.1
	13-Apr-11	195	92.97	42.49	71.98	30.15	18.73	0.82	2550			11.4
	4-May-11	216	86.09	45.23	90.87	31.59		0.79	2220	161	7.09	12.0
	17-Jun-11	260	81.84	67.24	59.73	28.94	16.85	0.67	2700	104	7.20	22.4

Table G2 cont'd. Analytical results for Experiment 5; Zorra Reactors (Control: 100% sand from pit; Inoculated: 10% core drilled from PU103 3-6m depth), port depths (both reactors): 8 cm, 25 cm, 43 cm, 61 cm, 79 cm, Control and Inoculated reactors filled with 4 and 7L of 3500 mg N/L NH4NO3, respectively, Sept. 30, 2010 to Jun. 17, 2011.

Reactor	Date	Day	Analytical (mg/L)					Field Parameters				
			NO_3^--N	NH_4^+-N	Cl^-	$SO4^{2-}$	Br^-	DO (mg/L)	Conductivity (µS/cm)	Eh (mV)	pH	Temperature (°C)
Control: 79 cm	30-Sep-10	0	104.65	84.99	80.16	36.05	22.49	1.50	2340			15.0
	12-Nov-10	43	109.61	64.37	64.36	64.36	23.79	1.12	2240			2.2
	20-Jan-11	112	95.02	175.96	81.74	81.74		0.96	2200			0.5
	4-Mar-11	155	103.15	49.66	77.81	31.52	20.12	>1	2400		7.54	2.6
	13-Apr-11	195	96.81		59.26	29.87	18.34	0.64	2250			11.4
	4-May-11	216	87.04	58.70	62.49	29.69	18.36	0.70	2230	162	7.17	10.9
	17-Jun-11	260	77.25	77.72	55.91	31.33	17.55	0.53	2103	122	7.24	24.9
Control: Total	30-Sep-10	0	480.55	481.45	362.59	153.64	90.54					
	12-Nov-10	43	452.16	367.95	366.14	192.95	97.06					
	20-Jan-11	112	394.87	448.85	357.29	198.30	16.38					
	4-Mar-11	155	465.99	531.73	517.79	167.47	20.12					
	13-Apr-11	195	446.02	229.66	351.56	149.77	86.30					
	4-May-11	216	441.17	254.58	348.47	161.58	33.64					
	17-Jun-11	260	448.85	311.09	298.71	159.25	65.11					
Inoculated: 8 cm	30-Sep-10	0	226.71	163.34	53.06	27.29	16.30	2.25	3140			15.0
	12-Nov-10	43	244.77	213.41	69.30	32.54	18.97	1.98	3120			2.1
	20-Jan-11	112	No Sample - Dry									
	4-Mar-11	155										
	13-Apr-11	195										
	4-May-11	216										
	17-Jun-11	260										
Inoculated: 25 cm	30-Sep-10	0	237.90	159.42	58.71	34.39	25.66					
	12-Nov-10	43	132.28	77.05	55.52	26.56	17.02	1.90	3140			14.5
	20-Jan-11	112	253.46	61.97	53.34	30.11	18.52	1.22	3240			2.1
	4-Mar-11	155	223.37	187.33	74.37	37.62		>1	3350		7.13	1.9
	13-Apr-11	195	191.21	64.47	39.05	25.09	14.60	0.93	3400			11.6
	4-May-11	216	290.91	66.07	76.50	44.57		0.99	3430	137	6.64	12.9
	17-Jun-11	260	204.44	48.79	35.86	29.53	13.85	0.94	3370	158	6.69	24.9

Table G2 cont'd. Analytical results for Experiment 5; Zorra Reactors (Control: 100% sand from pit; Inoculated: 10% core drilled from PU103 3-6m depth), port depths (both reactors): 8 cm, 25 cm, 43 cm, 61 cm, 79 cm, Control and Inoculated reactors filled with 4 and 7L of 3500 mg N/L NH4NO3, respectively, Sept. 30, 2010 to Jun. 17, 2011.

Reactor	Date	Day	Analytical (mg/L)					Field Parameters				
			NO_3^--N	NH_4^+-N	Cl^-	$SO4^{2-}$	Br^-	DO (mg/L)	Conductivity (µS/cm)	Eh (mV)	pH	Temperature (°C)
Inoculated: 43 cm	30-Sep-10	0	208.18	130.53	59.06	28.47	17.83	2.38	2920			14.5
	12-Nov-10	43	209.80	40.02	52.01	25.60	17.07	1.78	3200			2.3
	20-Jan-11	112	255.89	1.27	59.15	24.23		1.13	3350			0.5
	4-Mar-11	155	234.94	64.07	72.75	37.89		>1	3510		7.40	1.7
	13-Apr-11	195	184.90	79.64	45.67	30.89	16.23	1.15	3600			11.7
	4-May-11	216	262.13	79.71	78.05	40.90		1.02	3.31	162	6.86	12.2
	17-Jun-11	260	269.30	91.12	49.58	36.06	17.04	0.67	3.41	146	6.93	22.2
Inoculated: 61 cm	30-Sep-10	0	211.49	135.02	57.21	28.11	13.79	2.00	3000			14.5
	12-Nov-10	43	195.46	156.89	48.26	24.91	18.97	1.16	3300			2.4
	20-Jan-11	112	212.36	102.22	74.61	31.84		0.89	3480			0.5
	4-Mar-11	155	236.33	146.33	52.45	29.30		>1	3460		7.56	1.9
	13-Apr-11	195	235.11	101.71	76.95	39.11	18.64	0.92	3350			11.7
	4-May-11	216	228.42	94.90	47.31	29.63		0.82	3300	169	6.98	14.2
	17-Jun-11	260	273.15	131.35	52.38	35.49	18.33	0.83	3400	144	7.05	24.6
Inoculated: 79 cm	30-Sep-10	0	175.32	129.15	58.02	30.17	12.91	2.25	2800			14.5
	12-Nov-10	43	211.71	145.05	52.41	27.98	18.89	1.43	3150			2.5
	20-Jan-11	112	196.55	154.33	71.26	31.70		1.09	3240			0.5
	4-Mar-11	155	172.54	55.60	61.31	29.09		>1	3480		7.61	1.6
	13-Apr-11	195	218.52	87.41	59.41	29.97	17.93	0.90	3270			11.7
	4-May-11	216	235.73	118.88	52.93	32.89		0.85	3180	163	7.07	16.3
	17-Jun-11	260	231.53	161.49	50.73	32.85	17.89	0.66	3310	143	7.23	24.5
Inoculated: Total	30-Sep-10	0	1041.08	684.64	292.05	149.63	88.01					
	12-Nov-10	43	959.05	459.05	260.22	130.64	89.03					
	20-Jan-11	112	1174.15	321.05	317.49	142.10	18.52					
	4-Mar-11	155	1102.12	517.40	333.62	171.80	0.00					
	13-Apr-11	195	1014.62	412.87	266.74	155.95	83.62					
	4-May-11	216	1279.32	439.26	332.84	188.89	0.00					
	17-Jun-11	260	1247.74	523.87	238.13	170.00	84.15					

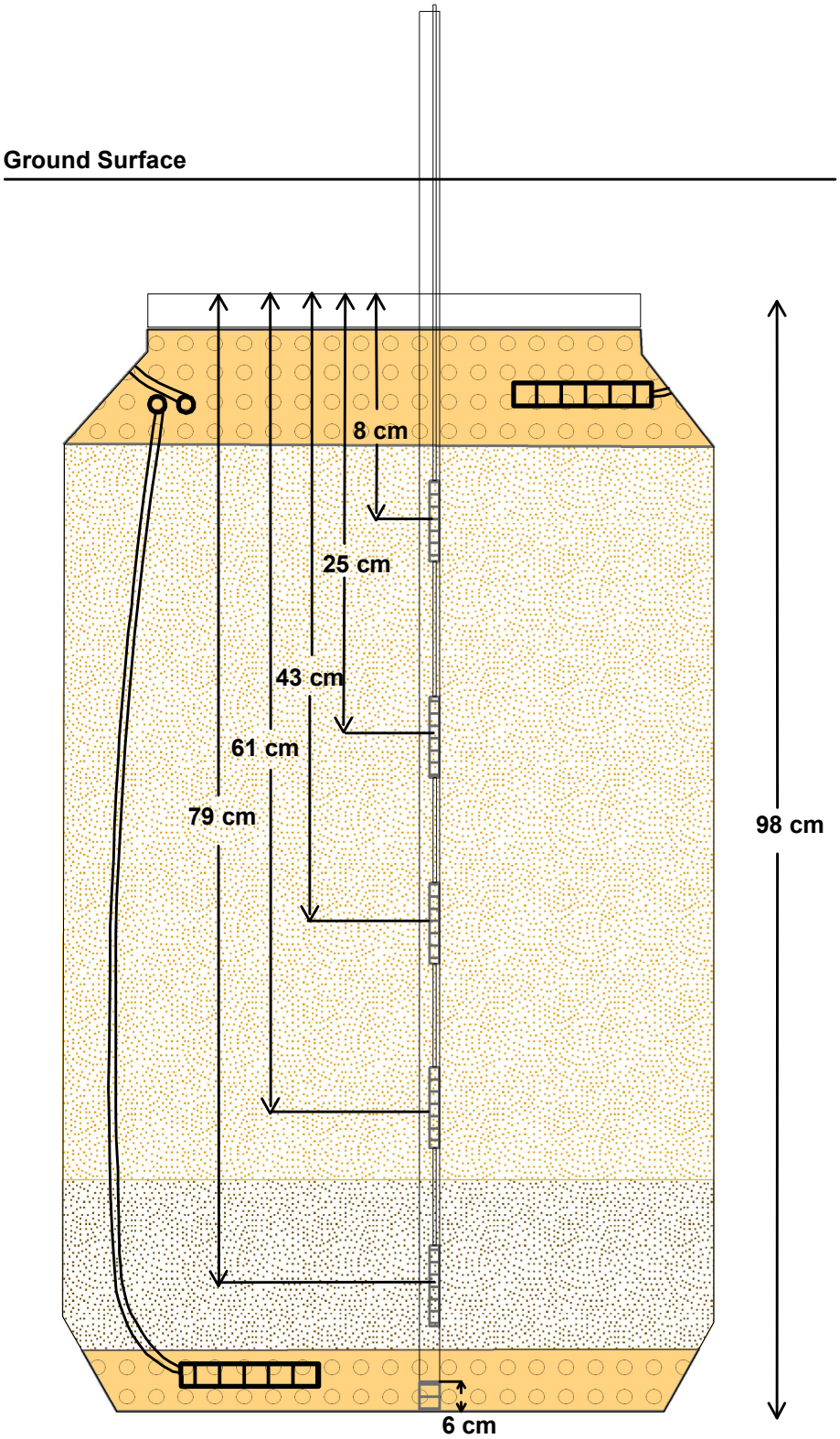


Figure G1. Construction design for Inoculated Reactor (PU125). Bottom portion contained fresh core:pit sand ratio of 1:2, the remainder was filled at a ratio of 1:10 (10% core). The Control Reactor is identically built, with the exception that it contains 100% pit sand.

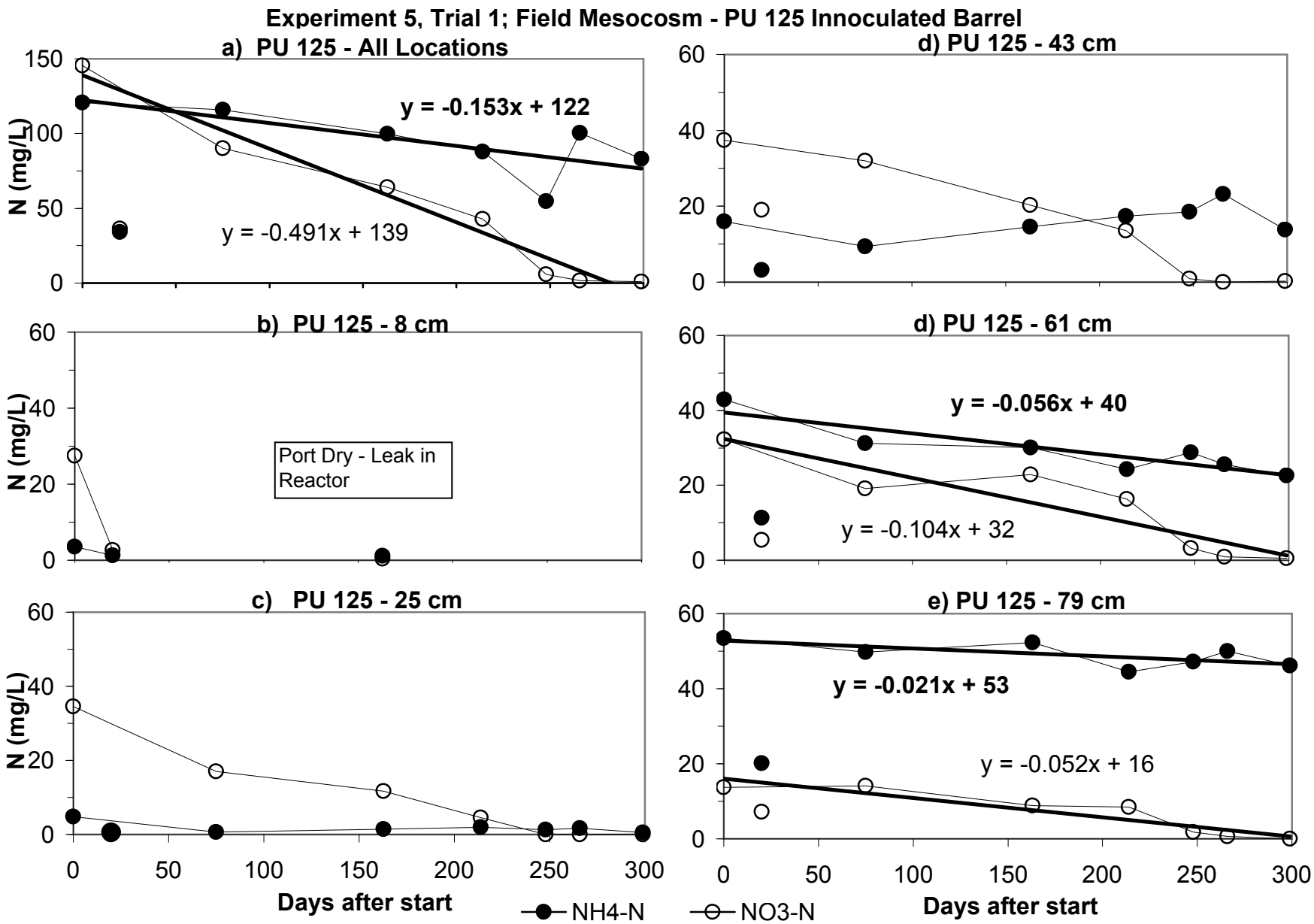


Figure G2. Experiment 5, Trial 1; Putnam (PU115-2.2m) groundwater mixed with ~ 10% core drilled from suspected anammox zones, sampled at five ports: **a)** All locations (sum of all ports) **b)** 8 cm **c)** 25 cm **d)** 43 cm and **e)** 79 cm, Sept. 25, 2009 to Aug. 19, 2010.

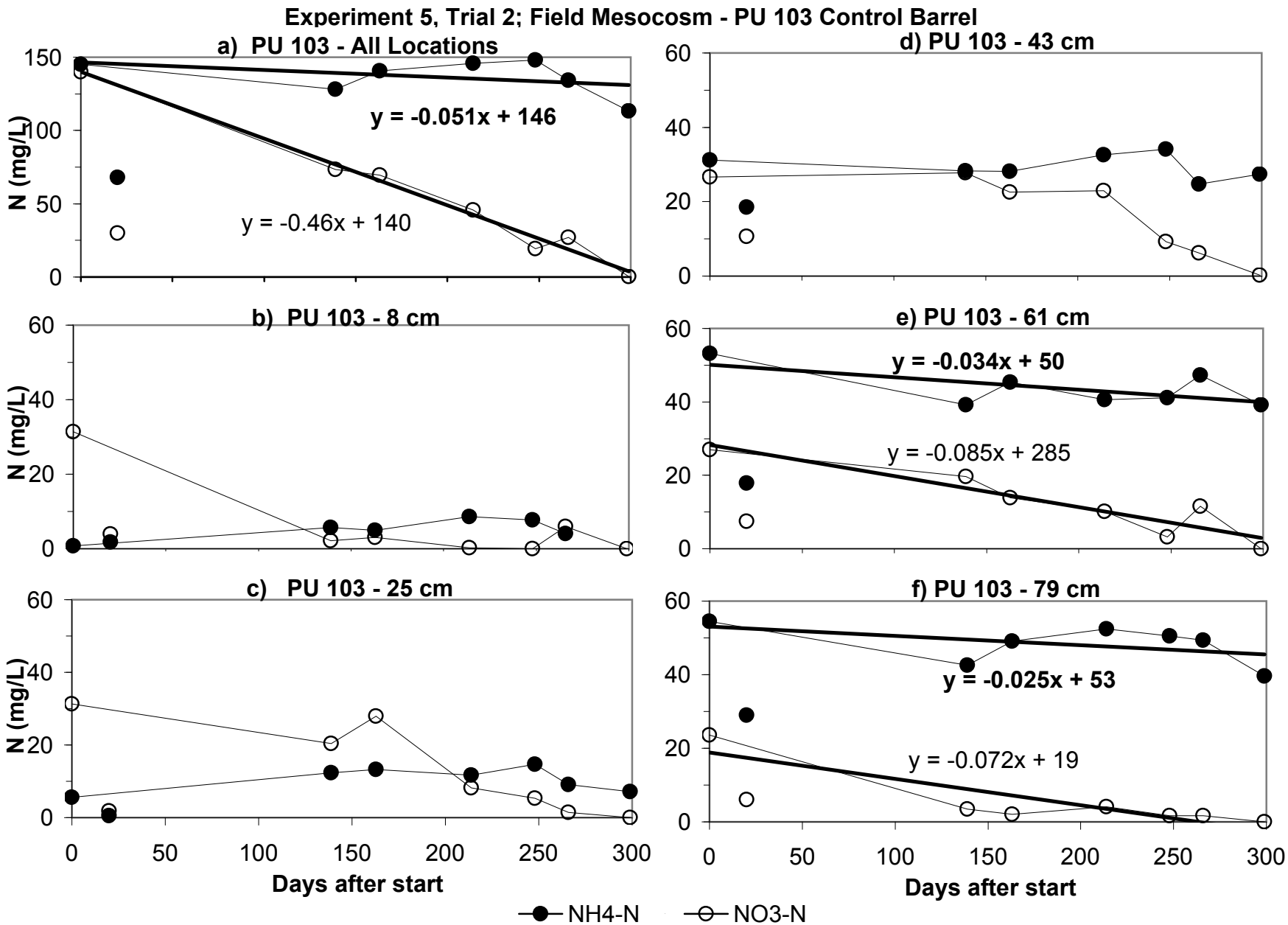


Figure G3. Experiment 5, Trial 2; Putnam (PU115-2.2m) groundwater mixed with sand from local pit. Sampled at five ports **a)** All locations (sum of all ports) **b)** 8 cm **c)** 25 cm **d)** 43 cm and **e)** 79 cm, Sept. 25, 2009 to Aug. 19, 2010.

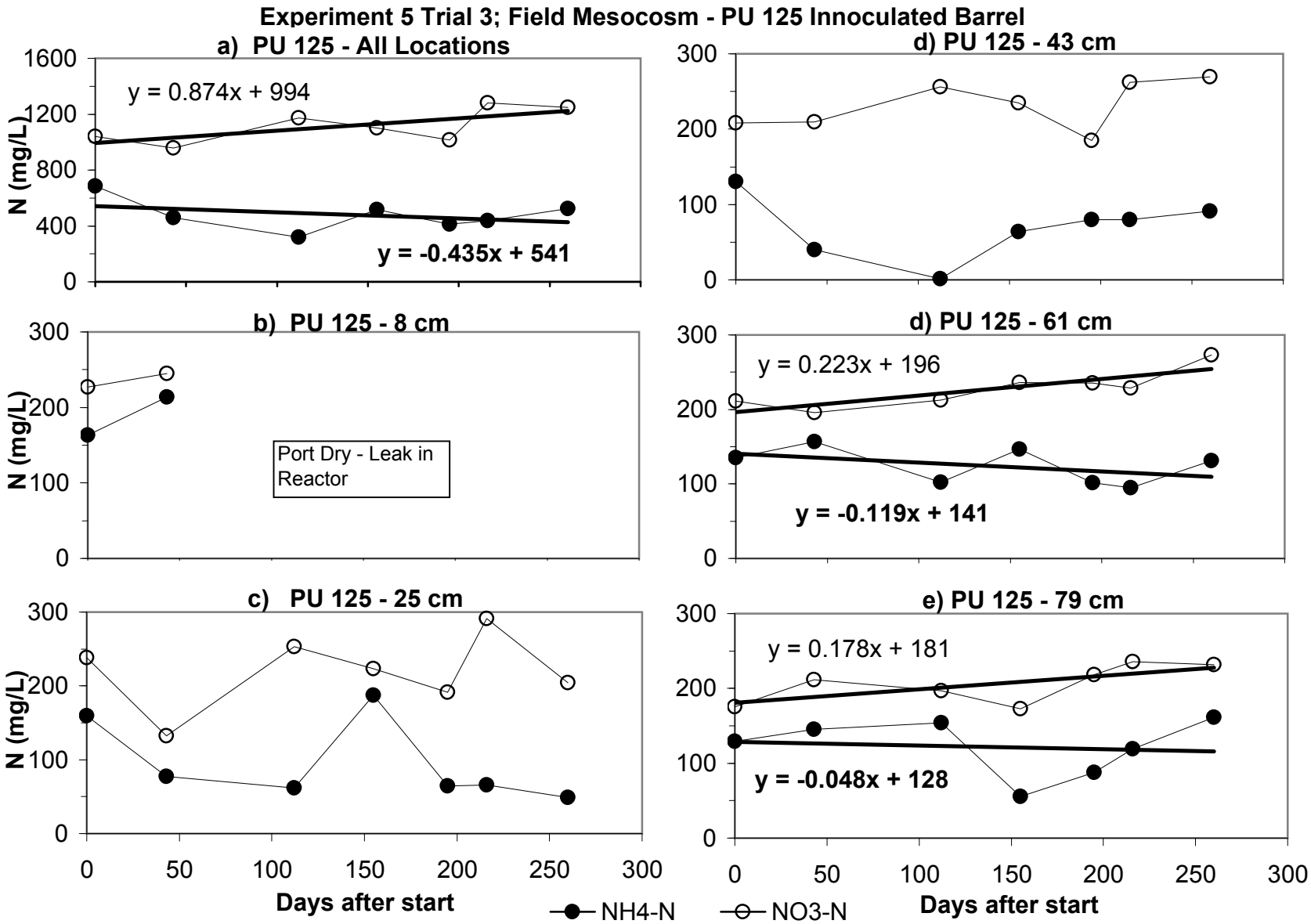


Figure G4. Experiment 5, Trial 3; Putnam (PU115-2.2m) groundwater mixed with ~ 10% core drilled from suspected anammox zones, with 7L of 3500 mg/L NH_4NO_3-N added, sampled at five ports: **a)** All locations (sum of all ports) **b)** 8 cm **c)** 25 cm **d)** 43 cm and **e)** 79 cm, Sept. 30, 2010 to Jun. 17, 2011.

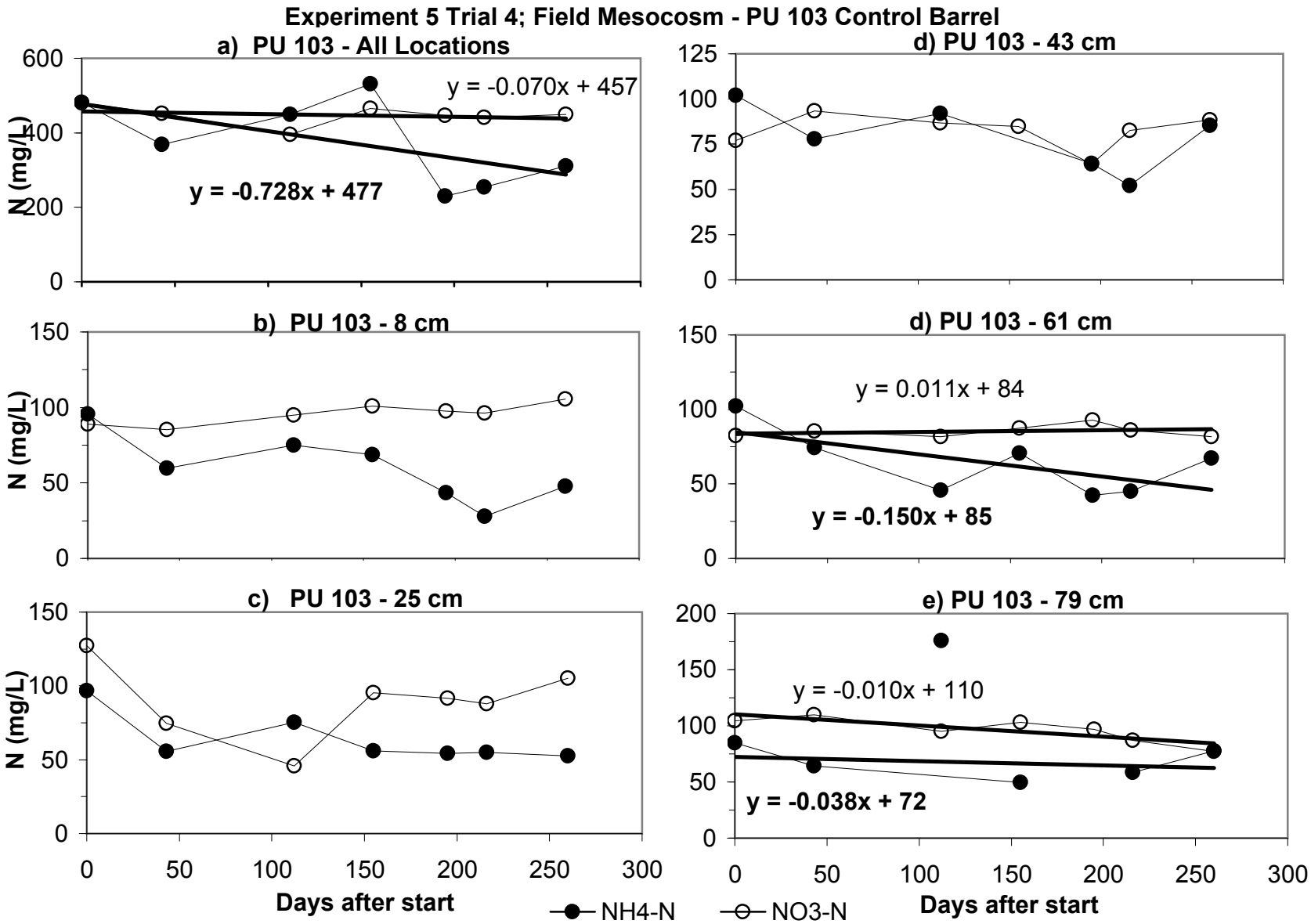


Figure G5. Experiment 5, Trial 4; Putnam (PU115-2.2m) groundwater mixed with sand from the local pit, with 4L of 3500 mg/L NH_4NO_3-N added, sampled at five ports: **a)** All locations (sum of all ports) **b)** 8 cm, **c)** 25 cm **d)** 43 cm and **e)** 79 cm, Sept. 30, 2010 to Jun. 17, 2011.

Appendix H:
Ongoing Groundwater Plume Monitoring, Zorra Site

Geochemistry and Isotope Data

Table H1. Geochemistry of Monitoring network, September 2010 and October 2010.

Well	Date	Analytical (mg/L)														
		NO_3^-	NO_2^-	NH_4^+	TN	DOC	$SO4^{2-}$	Cl^-	Br^-	PO_4^{3-}	Al^{3+}	Ca^{2+}	Fe^{2+}	K^+	Mg^{2+}	Na^+
PU103-4.1	26-Oct-10	2.53		3.67	6		11.00	58.44								
PU103-5.1	26-Oct-10	2.14		1.18	6		10.46	58.27								
PU103-7.5	26-Oct-10	0.50		2.47	7		9.69	52.23								
PU103-9.0	26-Oct-10	0.11		7.58	11		10.50	60.81								
PU115-2.2	26-Oct-10	3.64	0.63	0.39	36		34.45	83.50			<0.05	87.03	1.12	232.80	29.69	43.92
PU115-2.6	26-Oct-10	3.58	0.84	<0.01	32		45.88	132.43			<0.05	113.80	0.61	245.50	27.05	50.01
PU115-3.0	26-Oct-10	<0.01		0.97	21		37.22	121.80			0.018j	96.84	1.23	264.90	21.43	41.13
PU121-1.8	26-Oct-10	23.59	0.02	0.11	28		10.56	31.77			<0.05	148.50	<0.02	35.56	25.42	18.35
PU121-2.2	26-Oct-10	6.47		0.88	14		20.29	54.30			<0.05	86.56	<0.02	101.70	23.15	20.28
PU121-2.6	26-Oct-10	9.23		2.93	20		21.76	58.35			<0.05	137.40	<0.02	121.70	32.18	34.93
PU121-3.0	26-Oct-10	0.17		0.58	16		23.26	57.52			0.007j	42.06	<0.02	44.81	8.81	15.17
PU122-3.0	26-Oct-10	<0.01		6.60	31		10.57	168.85								
PU122-4.5	26-Oct-10	<0.01		32.82	45		89.11	379.66			<0.05	199.10	39.93	121.50	76.71	112.30
PU122-6.0	26-Oct-10	<0.01		0.32	157		54.94	428.93			<0.05	280.10	20.31	581.20	76.62	196.60
PU122-7.5	26-Oct-10	1.68		0.02	187		41.76	311.72			0.15	154.70	14.38	573.40	64.18	161.40
PU122-9.0	26-Oct-10	2.44		0.01	107		30.45	214.81			<0.05	149.50	7.49	338.40	52.03	101.90
PU123-6.0	26-Oct-10	3.91	0.03	22.19	37		31.33	103.00			<0.05	140.10	2.70	186.40	34.91	42.79
PU123-7.5	26-Oct-10	11.45		0.01	12		11.05	30.54								
PU123-9.0	26-Oct-10	8.39	0.01	0.17	9		9.18	34.16			<0.05	449.70	<0.02	12.17	28.53	17.57
PU124-2.7	26-Oct-10	<0.01		0.17	26		15.16	130.98			<0.05	116.60	12.37	206.90	34.39	37.67
PU124-3.9	26-Oct-10	0.82		8.51	13		13.79	86.50			<0.05	112.50	0.59	141.50	30.44	27.26
PU124-5.1	26-Oct-10	5.76		0.40	9		13.14	58.06			<0.05	147.10	0.03	37.98	31.66	21.93
PU124-6.3	26-Oct-10	6.97		0.62	16		13.03	55.39			<0.05	136.20	<0.02	51.98	32.94	23.81
PU124-7.5	26-Oct-10	12.21	0.01	0.09	17		13.49	39.31			<0.05	138.50	<0.02	24.01	30.57	17.63
PU125-2.7	26-Oct-10	0.00		4.32	22		31.15	86.62			0.14	59.14	0.30	100.63	16.54	17.64
PU125-3.9	26-Oct-10	6.70		12.58	24		15.33	45.64			<0.05	71.15	0.008j	135.90	20.92	18.34
PU125-7.5	26-Oct-10	8.80		1.26	14		12.95	46.47			<0.05	151.10	<0.02	43.72	34.21	24.49
PU84-1.5	26-Oct-10	35.81		0.03	38		12.79	38.67								
PU84-1.9	26-Oct-10	21.98	1.17	0.09	33		26.97	71.04								
PU84-2.2	26-Oct-10	7.80	1.80	0.10	21		28.13	86.63								

Table H1 cont'd. Geochemistry of Monitoring network, September 2010 and October 2010.

Well	Date	Analytical (mg/L)														
		NO_3^-	NO_2^-	NH_4^+	TN	DOC	$SO4^{2-}$	Cl^-	Br^-	PO_4^{3-}	Al^{3+}	Ca^{2+}	Fe^{2+}	K^+	Mg^{2+}	Na^+
PU84-2.7	26-Oct-10	4.87	1.34	0.08	19		25.46	80.82								
PU86-2.7	26-Oct-10	7.44		0.01	8		1.22	5.39			<0.05	107.40	<0.02	0.55	16.73	0.048j
PU86-3.1	26-Oct-10	5.02		0.03	5		1.07	11.23			0.034j	111.20	<0.02	0.72	17.39	0.51
PU87-1.1	26-Oct-10	0.03		4.27	4		10.27	119.07			<0.05	87.03	2.17	23.89	50.39	15.25
PU87-1.5	26-Oct-10	0.76		6.98	8		7.47	105.04			<0.05	191.40	<0.02	28.35	49.36	19.63
PU87-1.9	26-Oct-10	0.95		9.54	8		7.00	58.11								
PU87-1.9	26-Oct-10	1.00		9.54	8		6.78	54.77								
PU87-2.7	26-Oct-10	0.13		5.03	8		10.66	59.34			<0.05	109.40	0.26	70.89	23.42	28.64
PU92-1.4	26-Oct-10	18.45		0.02	45		37.15	91.43			<0.05	123.80	0.42	221.60	22.15	42.83
PU92-1.8	26-Oct-10	16.14	0.37	0.02	41		41.05	100.92			<0.05	120.80	0.45	242.90	22.66	45.61
PU92-2.2	26-Oct-10	5.60	0.55	<0.01	31		58.35	140.75			0.12	72.29	1.00	163.90	14.83	27.71
PU92-2.6	26-Oct-10	0.85	0.03	0.02	13		39.42	94.90			<0.05	103.60	0.17	249.30	22.25	37.17
PU92-3.0	26-Oct-10	3.24		0.07			26.98	71.41			<0.05	107.90	0.10	147.90	22.08	25.79
PU95-2.2	26-Oct-10	0.00		8.66	21		14.95	95.45			<0.05	65.33	3.03	158.60	52.93	34.35
PU95-2.6	26-Oct-10	1.04		18.48	41		44.78	155.73			<0.05	160.70	9.73	340.10	42.65	64.68
PU96-1.4	26-Oct-10	10.09		2.57	243		n.a.	36.50			<0.05	47.87	<0.02	14.95	8.66	5.63
PU96-1.8	26-Oct-10	0.10		20.83	17		21.31	66.14			0.029j	71.76	0.011j	176.20	17.31	23.63
PU96-2.2	26-Oct-10	0.14		24.58	33		25.26	79.54			<0.05	66.62	<0.02	175.60	20.27	24.73
PU96-2.6	26-Oct-10	<0.01		26.74	32		22.25	67.92			0.023j	68.94	0.12	189.40	19.31	28.71
PU96-3.0	26-Oct-10	0.01		31.52	39		18.79	59.94			0.01	67.33	0.26	210.80	15.61	36.79
PU97-1.4	26-Oct-10	0.01		0.40			16.93	62.47								
PU97-1.8	26-Oct-10	0.27		0.45	8		17.52	64.59								
PU97-2.2	26-Oct-10	<0.01		0.96	10		22.52	77.57								
PU97-2.5	26-Oct-10	<0.01		0.19	5		15.82	64.50								
PU97-3.0	26-Oct-10	0.00		1.01	5		13.47	54.34								