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Anaerobic Ammonium Oxidation in Waste Water - An Isotope Hydrological Perspective

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1. Introduction

Excess nitrogen components must be removed from wastewater to protect the quality of the water bodies that it will be eventually discharged to. A conventional wastewater treatment system for nitrogen removal is often involved with two processes, nitrification and denitrification. Nitrification is mostly achieved by complete oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) by the appropriate aerobic bacteria and then oxidation of the nitrite to nitrate ion (NO_3^-) by another variety of aerobic bacteria. Subsequently, the formed nitrate will be reduced to dinitrogen gas under anoxic conditions at the expense of organic carbon and released into the atmosphere as a harmless product (van Dongen et al., 2001). The introduction of oxygen into wastewater for nitrification requires a large amount of energy. Furthermore, the carbon source is often limited in wastewater, so purchasing of carbon source (typically methanol) is necessary too. A newly discovered anaerobic ammonium oxidation (anammox) may circumvent the limitations and open up a new possibility for nitrogen removal from wastewater. The alternative approach is a microbiological involved activity which requires less energy and enables more efficiency on N removal.

2. The history and physiology of anammox

The discovery of anammox activity and anammox bacteria is quite recent. Even though Richards (1965) has noticed NH_4^+ deficits in anoxic marine basins, and proposed that the missing NH_4^+ was anaerobically oxidized to N_2 by some unknown microbe using nitrate as an oxidant, which was coined one of two “lithotrophs missing in nature” by Broda (1977). Because there was no known biological pathway for this transformation, biological anaerobic ammonium oxidation received little further attention (Arrigo, 2005). It was not until mid-1990s, work with bioreactors designed to remove NH_4^+ from wastewater provided direct evidence for anaerobic ammonium oxidation, and the process was termed “anammox” by Mulder and his colleagues (1995). A series of ^{15}N -labelling experiments were carried out to study the metabolic mechanism and intermediates of anammox reaction (van de Graaf et al., 1995; 1997). It is a chemolithotrophic process in which 1 mol of NH_4^+ is oxidized by 1 mol of NO_2^- to produce N_2 gas in the absence of oxygen (Strous et al., 1999).



The pathway of N_2 formation clearly distinguishes anammox from denitrification which combines N from two NO_3^- molecules to form N_2 and presents as an elegant shortcut in the natural nitrogen cycles (Fig 1.) Physical purification of the anammox microbes from the multispecies biofilms yielded a 99.6% pure culture that was capable of carrying out PCR amplification of the DNA. The microbes responsible for anammox process were identified as members of the bacterial order Planctomycetales (Strous et al., 1999). The first genome sequence of a representative anammox bacterium was published in 2006 (Strous et al., 2006). To date, five anammox genera have been described, *Candidatus Brocadia*, *Candidatus Kuenenia*, *Candidatus Scalindua*, *Candidatus Anammoxoglobus* and *Candidatus Jettenia*.

A range of studies have been conducted for the detection of anammox bacteria and activities in variable environments from natural to man-made ecosystems (Risgaard-Petersen et al., 2003; Schmid et al., 2005). Anammox activity was found in marine environments, such as the Black Sea, the coast of Namibia, Chile, Peru and some freshwater and estuarine systems like, Lake Tanganyika and mangroves (Kuypers et al. 2003; 2005; Risgaard-Petersen et al., 2004; Meyer et al., 2005; Thamdrup et al., 2006; Schubert et al., 2006; Hamersley et al., 2009). In addition to widespread distribution, the activity of anammox bacteria in the environments also be substantial. The maximum reported contribution of anammox is 67-79%, occurring in sediments at a depth of 700m of the Norwegian Trench (Engström et al., 2005). Considerable supporting evidences have confirmed that anammox has global importance (Kuene, 2008).

Owing to the availability of laboratory enrichment cultures, the physiology of anammox bacteria has been relatively well characterized (Jetten et al, 2005). Anammox is characterized by slow growth and its cell doubles only once per 11 days under optimum conditions and 2-3 weeks on average (Strous et al., 2006). The low growth rate of anammox bacteria is not caused by inefficient energy conservation but by a low substrate-conversion rate. Furthermore, anammox bacteria are obligate anaerobes and their metabolism is reversibly inhibited when oxygen concentration is above 2 μM and nitrite is higher than 10 mM (Strous et al., 1997a). The temperature range suitable for anammox bacteria has been reported between -2°C (sea ice, Rysgaard & Glud, 2004) and 43°C (Strous et al., 1999). A recent study has observed anammox activity at temperature from 60°C to 85°C at hydrothermal vents located along Mid-Atlantic Ridge (Byrne et al., 2008). At optimal condition, anammox biomass could be enriched from activated sludge within hundred days. Enriched anammox bacteria in active sludge or biofilm present as brownish or red granule (Fig 2.). Under the microscope, the bacteria are observed as small coccoid cells with diameter of approximately 800 nm. They all possess one anammoxosome, a membrane bound compartment inside the cytoplasm which is the locus of anammox catabolism. Further, the intracytoplasmic is surrounded by unique lipids, called ladderanes (Sinninghe Damsté et al., 2004). Due to their unique characteristics, ladderane lipids have also been used as a biomarker for the presence of anammox bacteria (Kuypers et al., 2003). Besides, an interesting special feature is the turnover of hydrazine (normally used as a high-energy rocket fuel and poisonous to most living organisms) as an intermediate.

In addition, anammox bacteria have been found to be metabolically flexible, exhibiting alternative metabolic pathways. For instance, anammox can subsequently reduce NO_3^- to NO_2^- to NH_4^+ , followed by the conversion of NH_4^+ and NO_2^- to N_2 through anammox pathway, allowing anammox bacteria to overcome NH_4^+ limitation. Anammox bacteria are also a potential source of N_2O production by nitric oxide detoxification (Kartal et al., 2007). Apart from NO_2^- and NO_3^- , anammox bacteria also employ Fe^{3+} , manganese oxides as electron acceptors (Strous et al., 2006), which further expended the metabolic diversity of the anammox bacteria.

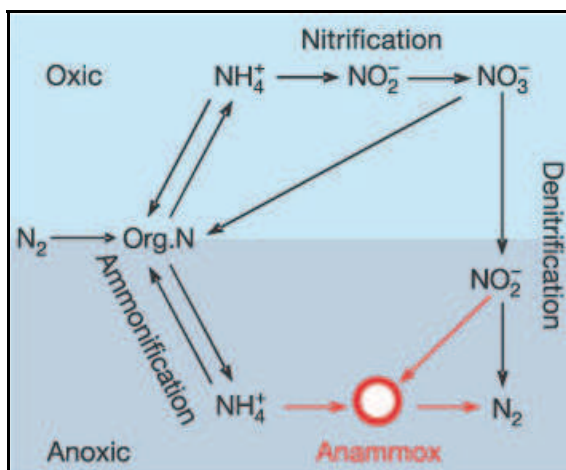


Fig. 1. Anammox in the context of nitrogen cycle (Modified from Kuyper, et al., 2003).

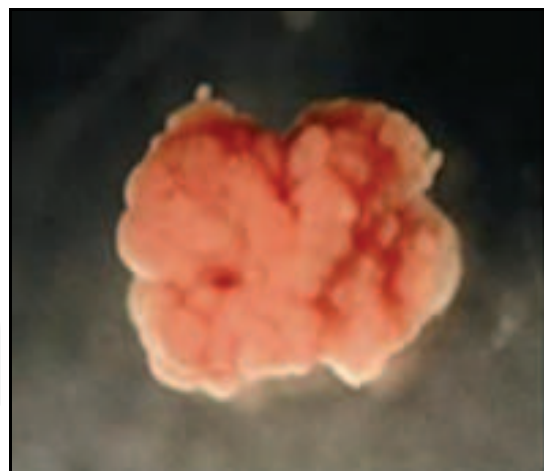


Fig. 2. Typical anammox granular sludge (Photo modified from Van Loosdrecht, 2006).

3. The application of anammox in waste water

Since anammox was discovered in a denitrifying fluidized bed reactor for wastewater treatment, it was realized that having a great potential for the removal of undesired NH_4^+ from wastewater from the beginning. The introduction of anammox process to N-removal would lead to a 90% reduction in operation costs because by using anammox process, nitrification process normally employed in wastewater treatment can be stopped at the nitrite level which can save aeration and carbon sources. For this reason, Mulder and colleagues patented the process immediately, even without direct proof and understanding of its biological nature (Mulder, 1992). In recent years, many research efforts dedicated to the application aspects of anammox reaction. The feasibility of the anammox process for the removal of NH_4^+ from sludge digester effluents was evaluated. Experiments with a laboratory-scale (2L) fluidized bed reactor showed that the anammox process was capable to remove NH_4^+ and NO_2^- (externally added) efficiently from the sludge digester effluent. And anammox biomass could be enriched from activated sludge within 100 days (Strous et al., 1997 b; Jetten et al., 1997). The possible reactors are sequencing batch reactors (SBR), moving bed reactor, blanket reactor or gas-lift-loop reactor. In these studies, NO_2^- was supplied from a concentrated stock solution. However, for application in real wastewater practice, a suitable system for biological NO_2^- has to be developed. One such system is the combination of the anammox process and SHARON (Sustainable high rate ammonium removal over nitrite) process. The principle of the combined process is that the NH_4^+ in the sludge digester effluent is oxidized in the SHARON reactor to NO_2^- for only 50% in the reaction I. The mixture of NO_2^- and NH_4^+ is ideally suited as influent for the anammox process in reaction II. With this system sludge digester effluent can be treated independently. In the study, the SHARON process was operated stably for more than 2 years. During the test period the overall NH_4^+ removal efficiency was 83% (Van Dongen et al., 2001). In the earlier design, reactions I and II were carried out in consecutive reactors, but these were later combined in a single oxygen-limited reactor where nitrite-producing bacteria and anammox bacteria coexist. However, anammox bacteria grow slowly and because of the low specific conversion rates of one reactor process, the bottleneck in this combination has been insufficient biomass retention (Kartal et al., 2010). A granular-sludge reactor is developed to achieve a high volumetric conversion rate due to a large surface area for mass transfer (Kartal

et al., 2010). The selective production of granules has been successfully applied on nitrifying/anammox sludge in a sludge blanket reactor, which substantially improved the energy management of wastewater facilities. Granular-sludge system not only overcome the limit of conversion rate, but also offers the possibility for application of anammox for wastewater treatment at low temperature and concentrations. The upper limits of nitrogen loading to anammox process were explored in gas lift reactors. The results showed that anammox bacteria were able to remove 8.9 kg N m⁻³ reactor day⁻¹ (Jetten et al., 2004). Due to extensive explorations of anammox process and combinations with other processes in the practices of application, there are numerous developed systems from SHARON-anammox, OLAND (Oxygen-limited autotrophic nitrification-denitrification, Kuai & Verstraete, 1998) to CANON (Completely autotrophic nitrogen removal over nitrite, Third et al., 2001) and DEAMOX (Denitrifying ammonium oxidation, Kalyuzhnyi et al., 2006). Van der Star et al., (2007) have made an overview and suggested that a uniform naming of these process as shown in table 1.

Process name proposed by van der Star et al., (2007)	Source of nitrite	Alternative process name	Reference
Two reactor Nitritation-anammox process	Nitritation of NH ₄ ⁺	SHARON ^{a,b} - anammox	Van Dongen et al., 2001
		Two stage OLAND	Wyffels et al., 2004
One- reactor Nitritation-anammox	Nitritation of NH ₄ ⁺	OLAND ^c	Kuai and Verstraete, 1998
		CANON ^d	Third et al., 2001
		Aerobic/anoxic deammonification	Hippen et al., 2001
		SNAP ^e DEMON ^f DIB ^{f,g}	Lieu et al., 2005 Wett, 2006 Ladiges et al., 2006
One reactor denitrification- anammox process	NO ₃ ⁻ of denitrification	Anammox ^h DEAMOX ⁱ	Mulder et al., 1995 Kalyuzhnyi et al., 2006

^a Sustainable high rate ammonium removal over nitrate; the name only refers to nitritation when nitrite oxidation is avoided by choice of residence time and operation at elevated temperature.

^b Sometimes the nitrification-denitrification over nitrite is addressed by this term.

^c Oxygen-limited autotrophic nitrification denitrification.

^d Completely autotrophic nitrogen removal over nitrite.

^e Single-stage nitrogen removal using the Anammox and partial nitritation.

^f Name refers to the deammonification process in an SBR under pH-control.

^g Deammonification in Interval-aerated Biofilm systems.

^h System where Anammox was found originally. The whole process was originally designated as Anammox.

ⁱ Denitrifying ammonium oxidation: this name only refers to denitrification with sulphide as electron donor.

Table 1. Process names for nitrogen removal systems involving the anammox process (modified from van der Star et al., 2007).

To date, there are several full-scale installations of anammox applications in the wastewater treatment plants. The first full scale reactor was built in Netherlands in 2002. The prototype has been set up as part of a municipal wastewater treatment plant in Rotterdam and is performing well. The internal circulation type reactor used in Rotterdam is especially suited for use of granular sludge. As of 2006, three full scale processes intended for the application of anammox have been built in Europe. In addition, anammox bacteria have been found that can be enriched from various types of wastewater sludge, indicating that anammox bacteria are indigenous in many treatment plants throughout the world (Op den Camp et al., 2006). Therefore, the ubiquitous characteristic of anammox bacteria makes no real limit to its application at normal wastewater treatment plants.

4. Tracing anammox in contaminated ground water- a case study

Groundwater contamination by NH_4^+ typically occurs because of surface activities such as composting, landfilling (Erksine, 2000), disposal of animal wastes and animal carcasses (Ritter & Chirnside, 1995; Umezawa et al., 2008), fertilizer storage (Barcelona & Naymik, 1984), and septic system effluent (Aravena & Robertson, 1998). NH_4^+ contaminated groundwater is a likely site for anammox activity. NH_4^+ enters the groundwater system and competes for exchange sites on soil particle surfaces; then nitrifying organisms in the oxic zone oxidize NH_4^+ to NO_2^- and then to NO_3^- . Movement of the groundwater through the soil matrix carries the products of partial nitrification (NH_4^+ and $\text{NO}_2^-/\text{NO}_3^-$) as the plume spreads due to the effects of retardation by aquifer material (Erksine, 2000). It is expected that contaminated groundwater environments will favor the anammox reaction when both NO_2^- and NH_4^+ are present in areas of low oxygen. In landfills, NH_4^+ is rarely detected over a few hundred meters away from the source, suggesting that attenuation of NH_4^+ is occurring along the flowpath (Erksine, 2000), and this is likely to be the case regardless of the source of NH_4^+ . We think that groundwater provides anammox organisms with an ideal environment for growth. Isotope evidence for anammox in groundwater has been shown by Clark and colleagues (Clark et al., 2008), but the presence and activity of anammox organisms has yet to be confirmed. In the case study, a series of geochemical, isotopic, labelling experiments and microbiological techniques including FISH, PCR, are used to assess whether anammox organisms are present and active in NH_4^+ -contaminated groundwater sites.

4.1 Isotopic evidence of anammox

Tracing the fate of NH_4^+ and NO_3^- in ground water is greatly aided by measurement of ^{15}N and ^{18}O , which can be used to characterize sources of these compounds and the reaction pathways they may have followed (Delwiche & Steyn, 1970; Hübner, 1986; Kendall, 1998). The reactions of nitrogen species in the environment are associated with characteristic fractionations that provide additional insights to subsurface processes and fate. Transformation of NO_3^- to N_2 by denitrifying bacteria is accompanied by a ^{15}N fractionation on the order of $\epsilon^{15}\text{N}_{\text{N}_2-\text{NO}_3} = -15\text{‰}$ to -20‰ (Wada et al., 1975; Böttcher et al. 1990). Böttcher et al. (1990) also showed that ^{18}O is also enriched in the residual NO_3^- product, with $\epsilon^{18}\text{O}_{\text{N}_2-\text{NO}_3} = -8\text{‰}$. Accordingly, stable isotopes provide important constraints on plausible reaction pathways for nitrogen species in the subsurface. Within the context of tracing anammox in ground water through the use of stable isotopes, a detailed investigation was undertaken at the site of a municipal water supply aquifer contaminated by the activities of

a chemical plant and fertilizer blending operation (Fig 3.). Wastewater contribution comes from the chemical company and fertilizer blending company with ammonium approaching 840 ppm N and nitrate up to 350 ppm N.

4.1.1 Field and analytical work

A program of field sampling and analytical work was carried out in 2003 and again in 2004, involving sampling ground water from 62 piezometers and extraction wells both on two companies sites. Total NH_4^+ concentrations were analyzed on unfiltered samples by distillation and titration with sulphuric acid. NO_3^- and NO_2^- concentrations were measured by liquid chromatography. The 2004 series of samples were analyzed for isotopes of NH_4^+ (^{15}N) and NO_3^- (^{15}N and ^{18}O) in the G.G. Hatch Isotope Laboratory in University of Ottawa. $^{15}\text{N}\text{-NH}_4^+$ was measured by a diffusion procedure. The sample was first distilled at high pH into a sulphuric acid solution, and concentrated to ammonium sulphate salt by evaporation. The salt precipitation was analyzed as N_2 gas by continuous flow isotope ratio mass spectrometry using a Finigan MAT Delta Plus directly interfaced with a Carlo Erba elemental analyzer (EA). Isotopes in NO_3^- were analyzed by quantitative conversion of NO_3^- to N_2O gas according to the bacterial denitrifier method (Sigman et al. 2001; Casciotti et al. 2002). The bacterial N_2O was analyzed for both ^{15}N and ^{18}O by injection through a gas bench interfaced with a Finnigan MAT Delta Advantage continuous flow mass spectrometer. The

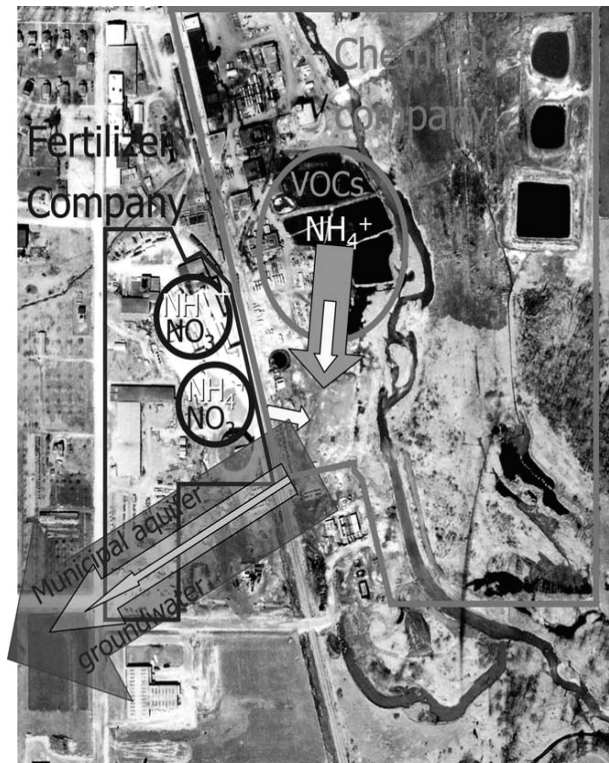


Fig. 3. Air photo of study area showing the direction ground water flow from the waste water ponds from chemical company and fertilizer company to the confined municipal aquifer.

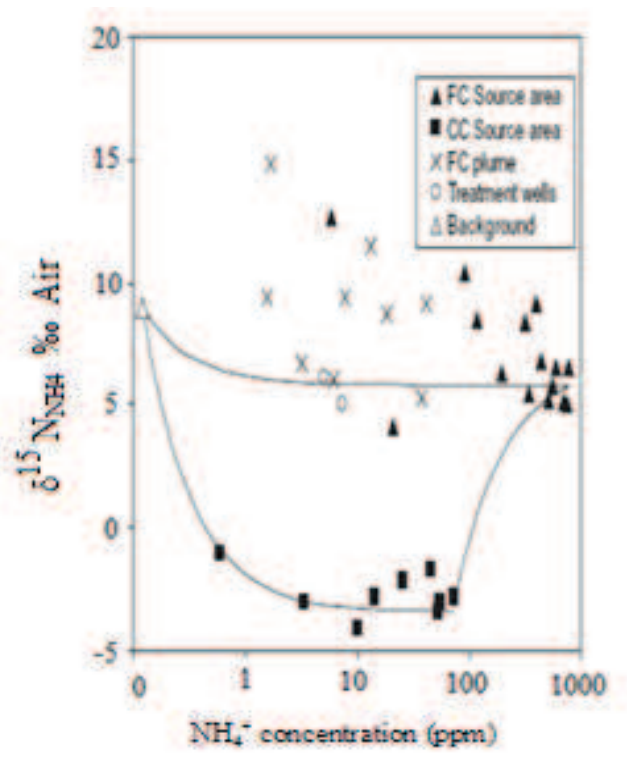


Fig. 4. $\delta^{15}\text{N}_{\text{NH}_4}$ vs. total NH_4^+ for waste of water source area and treatment well ground water. Conservative mixing envelope shown with black line.

N₂ gas concentrations were measured in ground water sampled in septum vials by purging with Helium (He) and direct injection into a Finnigan MAT Delta Advantage mass spectrometer.

4.1.2 Isotope results and discussion

The regional background geochemistry of the confined municipal upper aquifer was measured in two wells. The concentrations of NH₄⁺ and NO₃⁻ of background water is lower than 1 ppm N, and the redox conditions are considered to be moderately reducing, with dissolved oxygen less than 1 mg L⁻¹ and Eh of 137mV. Ground water at fertilizer company source area was dominated by NH₄-NO₃ with an average NH₄⁺ 632 ppm N and 250ppm NO₃⁻ ppm N. The NH₄⁺ concentration at chemical company source area was lower with an average value of 40.6ppm N and this water has no detectable NO₃⁻. However within municipal upper aquifer, concentration of NH₄⁺ was highly diluted with a maximum of 7.3 ppm N in the water treatment wells. The comparison between the really measured NH₄⁺ and the predicted concentration of NH₄⁺ by a conservative mixing model indicated that a significant loss of NH₄⁺ in ground water aquifer. The missing NH₄⁺ was calculated to be 30.7 and 21.2 ppm N in treatment well 1 and treatment well 2, respectively. In the same way, NO₃⁻ was found a loss of 8.0 and 3.2 ppm N from the two wells. These values are minimum estimate because NO₃⁻ is not retarded by sorption in the aquifer like NH₄⁺ (For more information, please refer to the publication (Clark et al., 2008)).

The missing of NH₄⁺ was believed as a reactive loss involved with anammox reaction which is based on isotopic evolution of associated nitrogen species. In conservative mixing, δ¹⁵N will reflect the concentration-weighted contribution of NH₄⁺ from each primary source. In the present case, if no reaction, δ¹⁵N of nitrogen species would be weighted results of wastewater from chemical company, fertilizer company with dilution water from background water. Fractionation of ¹⁵N during cation exchange is considered to be minor to negligible (Kendall 1998), and so retardation is not expected to affect the δ¹⁵N of NH₄⁺ in the municipal aquifer. By contrast, reactive loss of NH₄⁺ by oxidation, whether through nitrification or anammox, will impart a clear enrichment trend independent of any mixing relationships. A plot of δ¹⁵N_{NH₄} against NH₄⁺ concentration shows a strong contrast between the two waste water source areas and background NH₄⁺ in the municipal aquifer (Fig 4.). The values for δ¹⁵N_{NH₄} for the high NH₄⁺ concentration sites near the former fertilizer company water storage pond average 5.8‰, while those for the chemical company average -2.7‰, providing an 8.5‰ contrast between the two. The conservative mixing envelope, calculated from binary mixing between each of the three endmembers, is shown in figure 4. Nonconservative behaviour of samples from the fertilizer company source area is observed by their trend toward δ¹⁵N-enriched values at lower NH₄⁺ concentrations. Similarly, samples from the chemical company plume show nonconservative enrichment in ¹⁵N. This is consistent with the conservative mixing calculations, showing reactive loss of NH₄⁺ along the flowpath. Because cation exchange has been shown to be essentially nonfractionating (Ceazan et al., 1989; Kendall, 1998; Buss et al., 2004), this reactive loss must be through oxidation.

The usual pathway for NH₄⁺ oxidation is nitrification by O₂. This is an energetically favourable reaction in oxic water. It follows a two-step reaction through nitrite by a mixture of aerobic bacteria, including *Nitrosomonas*, *Nitrobacter nitrosospira*, and *Nitrobacter pseudomonas*. However, according to our measurement, redox conditions are unfavourable for aerobic bacteria, and so NH₄⁺ loss by nitrification is unlikely in these ground water. Further evidence against nitrification is found by the positive correlation between NO₃⁻ and

NH_4^+ in this water. NH_4^+ loss by oxidation to NO_3^- would show an inverse correlation and NO_3^- would remain the dominant species in the municipal aquifer. A third line of evidence against NH_4^+ nitrification is found in the comparison of $\delta^{15}\text{N}$ values in NH_4^+ coexisting with NO_3^- in individual water samples. Essentially all samples, NO_3^- were enriched in ^{15}N over coexisting NH_4^+ . These rules out NH_4^+ nitrification as a source for NO_3^- , which would produce NO_3^- with lower $\delta^{15}\text{N}$ than the NH_4^+ precursor (Kendall, 1998). Furthermore, the $\delta^{15}\text{N}_{\text{NH}_4}$ enrichment trends with decreasing NH_4^+ concentration against the possibility of NH_4^+ nitrification (Fig 5.). The positive correlation for NH_4^+ and NO_3^- , the enrichment in $^{15}\text{N}_{\text{NH}_4}$, and the greater enrichment for ^{15}N in NO_3^- over NH_4^+ suggest that the loss of NH_4^+ is due to anaerobic oxidation by anammox bacteria. Two Rayleigh distillation trend lines trace the enrichment in $\delta^{15}\text{N}_{\text{NH}_4}$ in the residual NH_4^+ from different initial concentrations. The enrichment factor $\epsilon^{15}\text{N}_{\text{NH}_4-\text{N}_2} = 4\text{‰}$ used for these trend lines provided the best fit for the range of source area data points and thus provides a first-order estimate of ^{15}N fractionation during anammox reaction. Additional evidence for anammox reaction in the NH_4^+ - NO_3^- ground water at fertilizer company source area is found in the overpressing of N_2 gas in these samples. Normalization of measured N_2 concentrations to atmospherically derived Argon gas (Fig 6.) showed that overpressing in N_2 in excess of three times of atmospheric saturation. The $\delta^{18}\text{O}$ composition of NO_3^- further supported reactive loss of NO_3^- , where enrichment of $\delta^{18}\text{O}$ and $\delta^{15}\text{N}$ was seen for most samples (Data not shown).

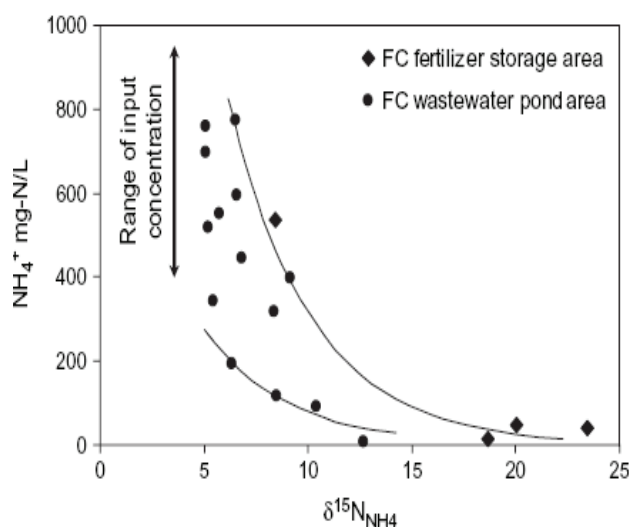


Fig 5. Evolution of $\delta^{15}\text{N}_{\text{NH}_4}$ during anammox reaction for the high NH_4^+ - NO_3^- fertilizer company ground water. Trend lines calculated from a Rayleigh distillation with a reaction enrichment factor of 4‰.

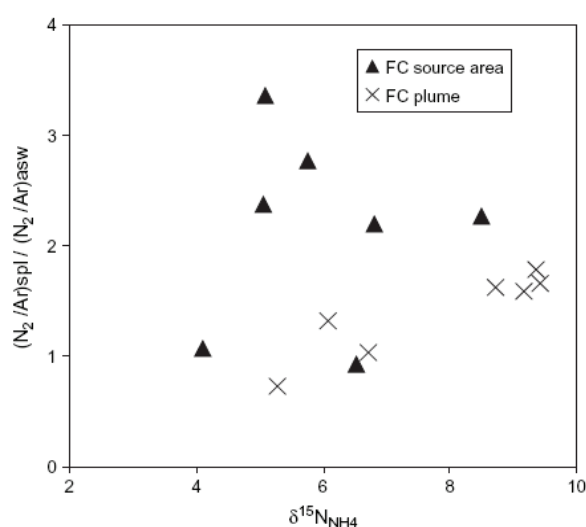


Fig. 6. Excess N_2 in fertilizer company ground water from reactive loss of NO_3^- and/ or NH_4^+ , normalized to dissolved argon gas.

4.1.3 Summary

Anaerobic oxidation of the ammonium by anammox bacteria is concluded as the reason of the strong attenuation of NH_4^+ and NO_3^- observed between the source areas and the municipal ground water treatment wells. Several lines of evidence suggest the conclusion:

1. $\delta^{15}\text{N}$ measurements of NH_4^+ show progressive enrichment with decreasing concentration, demonstrating reactive loss by ammonium oxidation. Volatilization

- along the flowpath is unlikely because it requires unsaturated conditions and because of the neutral pH of the water (negligible un-ionized NH_3).
2. NO_3^- concentrations decline along the flowpath and into the municipal aquifer. This precludes nitrification for the observed loss of NH_4^+ for which an increase in NO_3^- concentrations should be observed. The measured redox conditions are too low to support aerobic nitrification of NH_4^+ .
 3. $\delta^{15}\text{N}_{\text{NO}_3}$ is consistently 5‰ to 10‰ enriched over that of $\delta^{15}\text{N}_{\text{NH}_4}$ for water carrying both species, demonstrating that NH_4^+ loss is not by nitrification. Oxidation of NH_4^+ to NO_3^- would produce NO_3^- with depleted $\delta^{15}\text{N}$ values.
 4. Strong correlations between $\delta^{15}\text{N}_{\text{NH}_4}$ and $\delta^{15}\text{N}_{\text{NO}_3}$ demonstrate reactive loss of both species, consistent with anammox reaction. Enrichment of $\delta^{15}\text{N}_{\text{NO}_3}$ correlates with enrichments in $\delta^{15}\text{N}_{\text{NH}_4}$, further supporting reactive loss of NO_3^- .
 5. N_2 overpressuring above atmospheric equilibrium is observed to increase with increasing $\delta^{15}\text{N}_{\text{NH}_4}$ values along the flowpath from the FC source area. Increased N_2 in conjunction with enrichment in $\delta^{15}\text{N}_{\text{NH}_4}$ can occur only through anaerobic oxidation of NH_4^+ to N_2 by the anammox reaction.

4.2 Tracer experiments

Tracer experiments with ^{15}N -labeled nitrogen species are commonly used for elucidating nitrogen fate in both sediments and groundwater environments. Consumption of $^{15}\text{NH}_4^+$ and concomitant production of ^{15}N -labeled N_2 provided the first clear experimental evidence for anammox activity in a fluidized bed reactor (van de Graaf et al., 1995). So far, few labelling experiments have provided evidence of anammox in anoxic basin and in the suboxic zone of sea and lakes (Dalsgaard et al., 2003; Kuypers et al., 2003; Schubert et al., 2006; Hamersley et al., 2009), but there is no analogue application in groundwater systems yet. ^{15}N -labelling also provides a very sensitive technique for the determination of anammox rates. And a simultaneous determination of anammox and denitrification, gives insights to the relative importance of the two N removal pathways (Thamdrup & Dalsgaard, 2002; Risgaard-Petersen et al., 2003). In addition, potential isotopic fractionation associated with anammox bacteria activity also indicates the presence of anammox reaction. From the simultaneous attenuation of NH_4^+ and NO_3^- , and a progress enrichment of $\delta^{15}\text{N}\text{-NH}_4^+$ and $\delta^{15}\text{N}\text{-NO}_3^-$, Clark et al., (2008) suggested that anammox may play a role in ground water. As a follow-up study, a series of ^{15}N labelling incubation experiments have been established to investigate anammox activity and reaction rates at several ground water sites.

4.2.1 ^{15}N labelling experiments

For ^{15}N -labelling experiments, the method was slightly modified from the previous publication (Dalsgaard et al., 2003). Ground water or sediment and groundwater in an industrial contaminated site Elmira and a turkey manure polluted site Zorra were collected directly to 12-mL exetainers (Labco, UK). In terms of the mixture of sediment and ground water incubation, around 4.5mL sediment and 7.5mL of groundwater were collected. In order to minimize oxygenation, exetainer was submerged into a big container completely filled with ground water and neither headspace nor bubbles in the vial. From each site, triplicates were sampled for ^{15}N labelling experiments. ^{15}N labelling experiments were conducted immediately after return to the laboratory (less than 2 hours). In brief, 3mL of water was withdrawn by a syringe to make a headspace for helium (He) flushing. Each

exetainer was flushed with He for at least 15min to remove background N₂ and dissolved O₂ and N₂. ¹⁵N enriched compounds were added with syringe to a final concentration of 100μmol in 10ml of sample as ¹⁵NH₄Cl and Na¹⁵NO₃ (all >99% ¹⁵N, Sigma-Aldrich). Even though the final concentration of enriched ¹⁵N was variable in previous studies, ranging from 40 μmol to 10mmol L⁻¹ (Dalsgaard et al, 2003; Thamdrup et al., 2006), the present addition was in higher range because that the concentration of ¹⁴N species in study samples were very high and sometime can reach to 20mmol L⁻¹. An additional trial was carried out without any tracer addition as control to confirm that the whole incubation system functions well. ¹⁵N-labelling experiments were incubated in a dark incubation chamber at 15°C, which is very close to the in situ temperature. ¹⁴N¹⁵N:¹⁴N¹⁴N and ¹⁵N¹⁵N:¹⁴N¹⁴N were determined by gas chromatography-isotope ratio mass spectrometry and expressed as δ¹⁴N¹⁵N values ($\delta^{14}\text{N}^{15}\text{N} = \left[\frac{(^{14}\text{N}^{15}\text{N}:^{14}\text{N}^{14}\text{N})_{\text{sample}}}{(^{14}\text{N}^{15}\text{N}:^{14}\text{N}^{14}\text{N})_{\text{standard}}} - 1 \right] \times 1000$; air was used as the standard)

(GG Hatch isotope laboratory, University of Ottawa). In terms of anammox contribution to total N₂ production, assuming that the ¹⁵NH₄⁺ pool turns over at the same rate as the ambient ¹⁴NH₄⁺ pool, the total anammox N₂ production can be calculated from the production of ²⁹N₂ and the proportionate ¹⁵N labelling in the whole NH₄⁺ pool (Thamdrup & Dalsgaard, 2002; Thamdrup et al., 2006). The rates of anammox were extrapolated from linear regression of ¹⁴N¹⁵N as a function of time in the incubation with ¹⁵NH₄⁺ and the rates of denitrification were determined from the slope of linear regression of ¹⁵N¹⁵N over time in the incubation with ¹⁵NO₃⁻.

4.2.2 Results and discussion

At both of sampling sites except a pristine background well (Pu86 having not been impacted by NH₄⁺ from the compost plume), the formation of ¹⁴N¹⁵N was observed in the incubation trials with ¹⁵NH₄⁺ (Fig 7 a and c). However, the formation of ¹⁴N¹⁵N was very slow, and the concentration was lower than the detection limit after 72 hours incubation and the enrichment signal δ¹⁵N/¹⁴N was only 22.1 ± 4.2‰. The incubation experiments were extended to 3 months. The highest δ¹⁵N/¹⁴N increased to 14,278.03‰ at the end of incubation. At Elmira site, ¹⁴N¹⁵N accumulated linearly and stably with time without a lag phase, which indicates that anammox was the active process and no intermediates were involved in the reaction (Galán et al., 2009). Furthermore, the production of only ¹⁴N¹⁵N rather ¹⁵N¹⁵N was a clear evidence for the stoichiometry of N₂ production through anammox (van de Graaf et al., 1995; Jetten et al., 2001). At Zorra site, the formation of ¹⁴N¹⁵N reached the maximum at 1500hours incubation and started to decline. This is maybe due to the lack of another N donor NO₃⁻ which concentration was low at Zorra site. In control incubations without added tracer there was no production of ¹⁵N-enriched N₂, indicating the eligibility of the incubation system. At Elmira sites, the average ¹⁴N¹⁵N formation rate was 0.014±0.003μmol L⁻¹ h⁻¹, and the rate at Zorra site was 0.02±0.0021 μmol L⁻¹ h⁻¹. The rate of ¹⁴N¹⁵N production essentially corresponded to the anammox rate (van de Graaf et al., 1995; Thamdrup & Dalsgaard 2002; Dalsgaard et al., 2003). So, according to the equation from Thamdrup & Dalsgaard (2002), the calculated anammox reaction was 0.04±0.008 μmol L⁻¹ h⁻¹ at Elmira and 0.021±0.0022 μmol L⁻¹ h⁻¹ at Zorra. Compared to Dalsgaard et al., (2003) reported reaction rates 42 to 61mmol N m⁻² d⁻¹ in anoxic water column of Golfo Dulce, the reaction rate in ground water was much lower. However, many lower rates have been found in the oxygen-deficient water such as in eastern South Pacific (≤0.7nmol L⁻¹ h⁻¹;

Thamdrup et al., 2006) and in the Black Sea ($\sim 0.007 \mu\text{mol d}^{-1}$; Kuypers et al., 2003). Our results were very close the reported reaction rates in freshwater lakes, ranging from 6 to 504 $\text{nmol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ (Hamersley et al., 2009).

The pronounced accumulation of $^{15}\text{N}^{15}\text{N}$ in the incubation of $^{15}\text{NO}_3^-$ indicated that active and strong denitrification process (Fig 7b and d). The production of $^{15}\text{N}^{15}\text{N}$ was the major product at Zorra sites with an order magnitude higher than the mass of $^{14}\text{N}^{15}\text{N}$. In the incubation of $^{15}\text{NH}_4^+$, using the calculated anammox produced N_2 as a numerator and the total produced N_2 ($^{14}\text{N}^{14}\text{N} + ^{14}\text{N}^{15}\text{N} + \text{insignificant } ^{15}\text{N}^{15}\text{N}$) as a denominator, at Elmira sites 32.7% of N_2 gas was attributed to anammox; 21.4% for Zorra sites. $^{15}\text{NO}_3^-$ tracer labelling experiment showed that anammox accounted for 44.79% of N_2 production at Elmira sites and 29.03% at Zorra sites. The two techniques demonstrated a fair agreement at both of study sites. To date, the reported relative contribution of anammox to N_2 production was variable with a wild range from below detection to 67% (Thamdrup & Dalsgaard 2002; Dalsgaard et al., 2005). The contribution of anammox activity to N cycle was fairly corresponding to the percentage of anammox bacteria biomass (bacteria biomass data will be shown following). In conclusion, ^{15}N labelling experiments directly and clearly proved that the presence and activity of anammox in ground water.

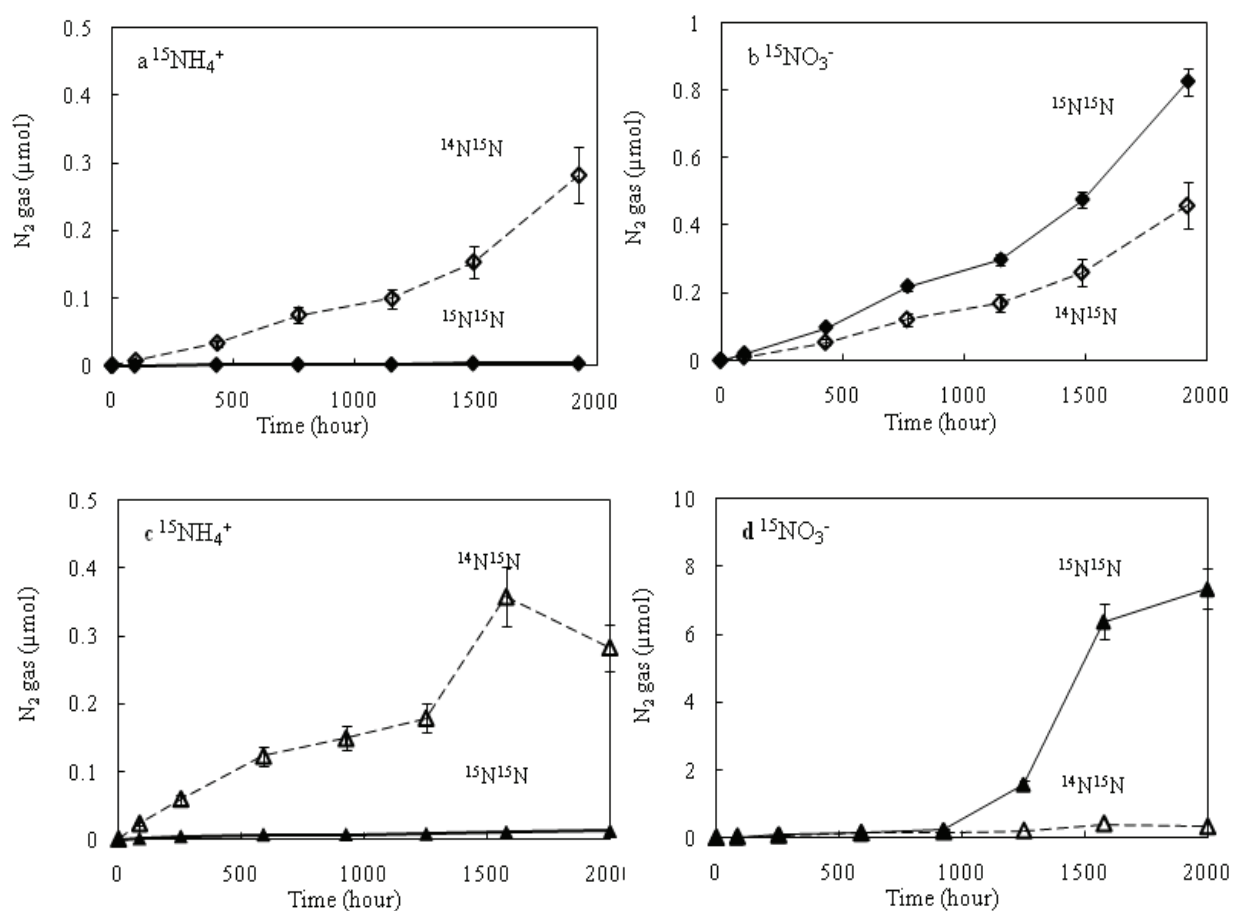


Fig. 7. Formation of $^{14}\text{N}^{15}\text{N}$ (open square) and $^{15}\text{N}^{15}\text{N}$ (solid square) in 3mL of headspace of incubation vials with samples from Elmira site(a and b) and Zorra site(c and d) after addition of $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$.

4.3 Microbiological analyses

Molecular methods have been extensively utilized to identify the presence of anammox bacteria in environmental and wastewater samples. Fluorescence in situ hybridization (FISH) targeting the 16S rRNA gene has been used extensively, and described in detail by Schmid et al. (2005). Anammox bacteria have also been identified using PCR, using a variety of primers, often based on FISH probes, targeting the group as a whole or specific members (Schmid et al. 2005; Penton & Tiedje, 2006). Quantitative PCR (q-PCR) has been used for direct quantification of all known anammox-like bacteria in water columns (Hamersley et al. 2009), in wastewater enrichment cultures (Tsushima et al., 2007) and in terrestrial ecosystems (Humbert et al., 2010).

4.3.1 Microbiological methods

For the present study, between 240 mL and 1 L of groundwater was collected and filtered via piezometer for DNA extraction; filtrate was collected on a 0.22µm filter surface (Millipore). Filters were stored at -70°C until DNA extraction. Nucleic acids were extracted from the filter surface using a phenol chloroform extraction technique, described previously by Neufeld et al., (2007). General bacterial 16S rRNA gene primers for denaturing gradient gel electrophoresis (DGGE; GC-341f and 518r; Muyzer et al., 1993) and anammox-specific 16S rRNA gene primers (An7f and An1388r; Penton et al., 2006) were used for PCR along with a series of reaction conditions (Moore et al, submitted). PCR products were cloned using a TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. DNA sequencing was performed at the Biochemistry DNA sequencing facility at the University of Washington (ABI 3700 sequencer), at The Center for Applied Genomics in Toronto (ABI 3730XL sequencer), and at the sequencing facility at the University of Waterloo (Applied Biosystems 3130xl Genetic Analyzer). DNA chromatograms were manually edited for base mis-calls and were visually inspected and trimmed to ensure only quality reads were included. Redundant sequences were removed using Jalview. Alignment and building phylogenetic trees were done with MEGA4.0 (Tamura et al., 2007). Sequences were aligned with known anammox reference sequences obtained from Genbank (DQ459989, AM285341, AF375994, DQ317601, DQ301513, AF375995, AF254882, AY257181, and AY254883) and a Planctomycete outgroup (EU703486). Phylogenetic trees were built using the neighbor joining method and the maximum composite likelihood model. Total bacterial community pie charts were constructed using phylum assignments provided by the Ribosomal Database Project and NCBI Blast. Anammox specific qPCR used An7f and An1388r (Penton et al., 2006) and general bacterial qPCR used 341f and 518r (Muyzer et al., 1993).

Fluorescently labelled oligonucleotide probes: EUB 338 (specific for all bacteria cells), Amx368 (specific for all anammox species) and Kst- 0157-a-A-18 (specific for an anammox species "*Kuenenia stuttgartiensis*") all labelled with different fluorescent color were used to ground water and sediment samples in order to determine the abundance of the specific anammox bacteria cells in samples. Several protocols have been used and a suitable protocol for this type of environmental samples was modified. In order to give a quantitative point view of total cell versus anammox, cell counting was established. Total cell counting was carried by DAPI (4',6-diamidino-2-phenylindole) staining, which is a special fluorescent stain that binds strongly to the DNA's of only all bacterial cells (Tekin, in preparation).

4.3.2 Results and discussion

Planctomycete abundance in the total bacterial community increased with depth at Zorra according to clone library data, and planctomycetes reached 5.2 and 20.8% of the total

bacterial community at depths greater than 5 m below ground surface. Large Illumina libraries (~100 000) sequences indicated that anammox organisms made up ~10% of the bacterial community at Zorra. Quantitative PCR using anammox specific primers (An7f An1388r; Penton et al. 2006) confirmed that the abundance of anammox organisms increased with the observed increase in planctomycete abundance at Zorra site. The number of anammox 16S rRNA gene copies at Elmira was lower on average than that of Zorra. A pristine background well (having not been impacted by NH_4^+ from the compost plume) showed two orders of magnitude fewer anammox gene copies per nanogram of genomic DNA than at impacted area. Clone libraries targeting the 16S rRNA genes of anammox bacteria were used to examine the communities of anammox performing organisms at field sites. All Anammox organisms were present at the two contaminated groundwater sites however the community compositions differ (Fig 8). At Zorra site, *Can. Brocadia* dominated anammox community, where the vast majority of anammox sequence also grouped with known *Can. Brocadia* reference sequence, and a few clones grouped with known *Can. Scalindua*. FISH images also showed the presence of anammox bacteria in both of two ground water sites (Data not shown).

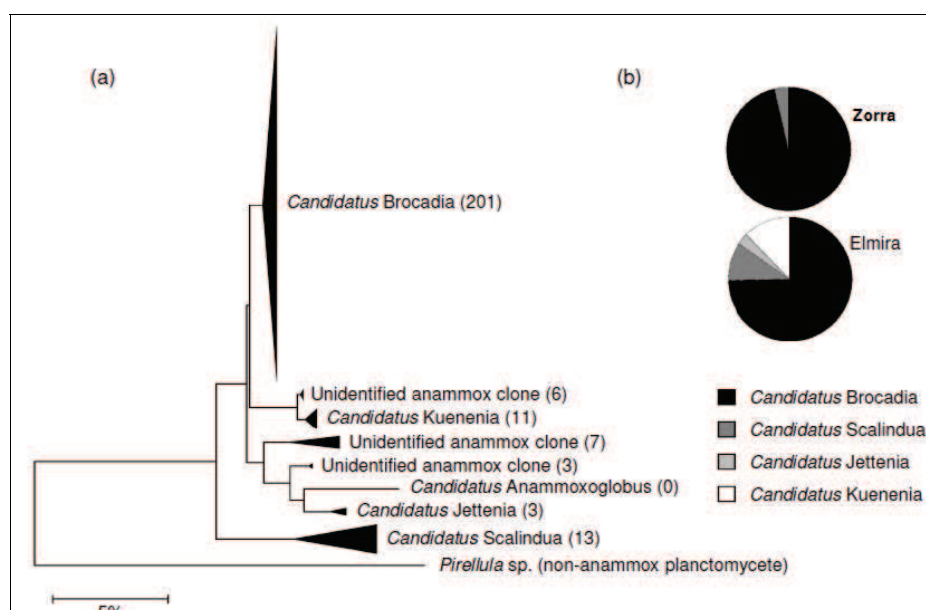


Fig. 8. (a) Phylogenetic tree of environmental anammox sequences aligned with known anammox reference sequences. Numbers in brackets represent the number of clones identifying with each cluster. (b) Distribution of anammox related 16S rRNA gene sequences found at each field site, by genus. (Modified from Moore et al., in preparation).

Anammox organisms are very hard to culture due to extremely slow growth rates, so there is a high reliance on molecular techniques for finding and identifying these organisms in mixed communities. PCR of environmental DNA extracts with general bacterial primers to generate clone libraries has been shown to underestimate the proportion of anammox organisms in the environment due to mismatches with “universal” primers (Jetten et al., 2009; Penton et al., 2006; Schmid et al., 2007). Anammox organism abundance may be greater than estimated by molecular methods due to known mismatches of anammox organisms with several “anammox,” “planctomycete” or “universal bacterial” primer sets. Anammox organisms have at least 10 mismatches with 27f and 2 mismatches with 1492r,

primers used to create general bacterial 16S rRNA gene libraries for Zorra where the abundance of planctomycetes was estimated to be between 5.2 and 20.8% of the total bacterial population at 7.5 m. In summary, the results of microbiological investigation provided further evidence for anammox presence in ground water and additional insight of anammox bacteria community in ground water environments.

5. Anammox and denitrification in waste water

From a geochemical perspective, anammox and denitrification have the same implication, i.e., they both lead to a loss of fixed nitrogen, albeit with a somewhat different stoichiometry. The biogeochemical relationship between anammox bacteria and denitrifiers appears quite complex. They always coexist in the same environment where they can be competitor to each other and also can play as a booster too.

In some environments with low NH_4^+ , anammox depends on ammonification, which may connect with denitrifiers' function on N-containing organics. In addition, the electron acceptor of anammox NO_2^- also highly relies on the production of denitrification. Therefore, the combination of anammox and denitrification is introduced in most of application in waste water treatment as above stated. Under the assumption that NO_2^- consumption by anammox can be described by Michaelis-Menten kinetics (Dalsgaard et al., 2003), the apparent half-saturation concentrations, K_m for NO_2^- during anammox in natural environments has been constrained to $<3 \mu\text{M}$ (Trimmer et al., 2003). Since maximum NO_2^- concentrations in natural environments are only few μmol per liter, tighter competition for NO_2^- may affect the balance between anammox and denitrification (Kuyper et al., 2006). The competition ability relies on the availability of organic matter and the physiology of bacteria. Anammox bacteria is regarded as autotrophic, so the activity of anammox bacteria may not be directly associated with organic matter. In contrast, organic matter provides both of energy and substrates to denitrification which sometime limits denitrification activity, especially in waste water treatment (Ruscalleda et al., 2008), but denitrifiers grow faster than anammox bacteria which make the organisms easily outgrown in the competition. Similarly, NH_4^+ sometime derives from ammonification as mentioned above which more complicate the relationship of the two processes.

With more studies, more and more scientists argue that it is possible that anammox account for a substantial 30-50% of N_2 production in the ocean or oxygen minimum zone. Theoretically, 29% of N_2 production during the complete mineralization of Redfieldian organic matter through denitrification and anammox, is produced through anammox (Dalsgaard et al., 2003; Devol, 2003). Kuyper et al., (2006) supposed the number can exceed 48%. However, Gruber (2008) think this conclusion can not be easily extrapolated, since the dependence of anammox on denitrification, but he also pointed out that there is ample room for surprises since how little we know about the process and the associated organisms.

6. Conclusions and outlook

Over 40 years have passed since the anaerobic oxidation of ammonium with nitrite reduction was first proposed. However, our understanding of anammox is till far from complete. Anammox research is still in a very early state. All over the world, research groups are working on diverse aspects of the molecular biology, biochemistry, ultrastructure, physiology and metabolism and ecology of anammox process. As well as

assessing the impact of the activity on the environment and their application in waste water treatment. A lot of interesting facts have been revealed and certainly more will come in future. Identifying the genomes of anammox bacteria will help to cultivate these bacteria in pure cultures what wasn't achieved until now. Pure cultures could optimize the application of anammox in wastewater treatment plants and facilitate the research on the anammox bacteria. Several important questions remain to be answered are: how important the anammox process is in freshwater ecosystems, especially contaminated aquifer? How do anammox organisms interact with other nitrogen involved bacteria? From an isotope hydrological perspective, the relevant fractionation factors have yet to be established. Also, the limited applications on waste water treatment indicate that a further understanding of anammox is needed.

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