MEASURING DENITRIFICATION IN THE SUBSURFACE ENVIRONMENT OF MANAWATU RIVER CATCHMENT

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Abstract

Denitrification is an important nitrate (NO_3^-) attenuation process in soil-water systems. A sound understanding of this process will aid in the management and mitigation of the impacts of NO_3^- on groundwater and surface water quality. Denitrification in surface soils has been widely studied, but there are relatively few studies of its occurrence and distribution in the subsurface environment, particularly in the Manawatu River catchment, New Zealand. Challenges around the measurement of denitrification in the subsurface environment is one of the reasons that there has been limited research in this important area. Acetylene inhibition (AI) is a commonly employed method to measure denitrification in soil-water systems. However, subsurface denitrification studies using the AI method vary in methodological details, and this variation has implications for the reliability and comparability of results.

An experimental site was established at Massey University's No. 1 Dairy Farm to determine appropriate procedures for measuring denitrifying enzyme activity (DEA) in the subsoil, and to examine the ability of the push-pull test to quantify denitrification in shallow groundwater in the Manawatu River catchment. Two laboratory incubation techniques, flask and vacuum bag incubation, were evaluated for their ability to measure DEA in subsoil samples (depth: 0-200 cm bgl). A single-well, push-pull test was conducted on a piezometer (depth: 6.5 m bgl) to measure denitrification rate in the shallow groundwater. The test involved re-injection of extracted groundwater with added; nitrate (KNO_3), conservative tracer (bromide) and acetylene, and was followed by sampling at specific time intervals till about four hours after completing the solution re-injection. Preliminary results suggest that the DEA measurements for subsoils may require more nitrate input than what is commonly added in incubations with surface soils. Improvements in the DEA estimates by the vacuum bag technique were achieved with the use of larger amounts of soil and larger gas sample volumes. Initial results from the push-pull experiment indicate the occurrence of denitrification in shallow groundwater at the test site. This is shown by a higher nitrate-nitrogen loss than can be attributed to dilution and dispersion, coupled with the increasing N_2O concentration, and existence of favourable environmental conditions in the saturated zone.

While it is apparent that DEA assays for subsurface soils require standardisation, this study demonstrates the usefulness of the AI method as a simple and economical method to measure denitrification in the subsurface environment. The methods developed here are being used to measure denitrification in the vadose and saturated zones at other selected sites in Manawatu River catchment. These measurements are being conducted as part of a wider research programme by Massey University Institute of Agriculture and Environment and Horizons Regional Council to investigate the transport and fate of nitrogen from farms to the waterways in the catchment.

1.   Introduction

To meet the growing demand for food and fibre, expansion and intensification of primary production need to be managed properly to minimize its effects on water quality and freshwater ecosystems. Farm nutrients, such as nitrogen and phosphorus from inorganic and organic fertilisers, once released from soils, take different flow pathways (via surface runoff, subsurface drainage, and subsurface flow) to reach and contaminate surface and groundwater bodies. An increase in nitrate levels in water bodies may have undesirable environmental consequences such as eutrophication, algal blooms, and fish poisoning (Di & Cameron, 2002; Puckett et al., 1999).

The amount of nitrate leached to groundwater and subsequently transported to surface waters such as streams, rivers or lakes depends on a number of physical, chemical and biological factors that influence nitrate flow pathways and attenuation processes (Haag & Kaupenjohann, 2001; Mastrocicco et al., 2011; Paramasivam et al., 2002). These nitrate attenuation processes include plant uptake, assimilation into microbial biomass, dissimilatory nitrate reduction to ammonium, and denitrification (Martin et al., 1999; Porubsky et al., 2011; Puckett & Brian Hughes, 2005; Puckett et al., 2008; Rivett et al., 2008). In particular, denitrification – a microbial-mediated transformation of nitrate (NO_3^-) to harmless dinitrogen (N_2) gas – becomes more prominent compared to other processes in the subsurface environment (Rivett et al., 2008). A sound understanding of this process will, therefore, aid in the management and mitigation of the impacts of NO_3^- on groundwater and surface water quality.

While denitrification in surface soils 'root zone' has been widely studied (e.g. Saggar et al., 2013; Xu et al., 2013), there are relatively few studies of its occurrence and distribution in the subsurface environment (both the deeper vadose zone and shallow groundwater) (e.g. Jahangir et al., 2012; Jahangir 2013; Kamewada, 2007; Korom, 2012). This is particularly the case in the Manawatu River catchment, New Zealand. Denitrification investigations in the Manawatu catchment have been limited to surface soils and concentrated in the western part of the Manawatu gorge (e.g., Jha et al., 2011; Luo et al., 1998; Luo et al., 1999; Ruz-Jerez et al., 1994). To our knowledge, there have been no measurements on denitrification in the deeper vadose and saturated zones in the Manawatu River catchment. Denitrification studies in the deeper vadose and saturated zones, however, are being conducted elsewhere in New Zealand, e.g. in the Waikato region (Barkle et al., 2007; Clague et al., 2013; Stenger et al., 2008) and in the Canterbury region (Peterson et al., 2013; Thomas et al., 2012).

Challenges around the measurement of denitrification in the subsurface environment is one of the reasons that there has been limited research in this important area. One of the most common methods used to quantify denitrification is the acetylene inhibition (AI) method (Yoshinari et al., 1977). This method has been widely used in the determination of the denitrifying enzyme activity (DEA), which gives a snapshot of the potential of the soil to denitrify at the time of sampling. Given that DEA is affected by a number of measurement factors including the amount of soil and substrate (nitrate and organic carbon) as well as by incubation and sampling procedures that could influence the composition in gas samples, there is a need to standardise the AI method. Luo et al. (1996) conducted a study to standardise DEA measurements for New Zealand pasture soils, but it was carried out with samples from the surface soil layer (0-10 cm). Subsurface denitrification studies using the AI method, however, vary in methodological details (e.g. Hayakawa et al., 2012; Jarvis & Hatch,

1994; Oehler et al., 2007) and this variation may have implications for the reliability and comparability of results.

This study aims to evaluate existing procedures and standardise the AI method for measuring denitrification in the subsurface environment. The study has two main objectives as follows: a) to determine the appropriate procedures for measuring denitrifying enzyme activity (DEA) in the subsoil, and b) to examine the applicability of a single-well push-pull method to quantify denitrification in shallow groundwater in the Manawatu catchment. The first objective was further divided into two specific objectives; (i) to determine the optimum nitrate concentration for DEA in composite subsoil samples; and (ii) to determine the appropriate incubation medium such as vacuum pouch or Erlenmeyer flasks to measure DEA. In this paper, we present and discuss the preliminary results achieved so far.

## 2.   Material and Methods

2.1   Study site

An experimental study site has been established at Massey University No. 1 Dairy Farm in Palmerston North, New Zealand where soil samples were collected from 0 to 200 cm below ground level (bgl), for further testing and analysis in the laboratory. The soil samples collected were classified as Rangitikei silt loam, with sand being found at a depth of 200 cm. A total of six piezometers were installed in different locations in the farm at a depth ranging from 3.30 m to 8.70 m bgl. The piezometers were made up of polyvinyl chloride pipes of 28 mm internal diameter, with perforations (5 mm diameter) at the bottom 0.50 m of the piezometer wrapped with 250 μm mesh nylon screen. With natural backfill comprising as primary filter pack around the screened portion of the piezometer, sand (<0.6mm) was used as the secondary filter pack, and bentonite as the annular seal.

2.2   Denitrifying enzyme activity (DEA) measurement

DEA was investigated with soils taken from 0 to 200 cm bgl and grouped according to depth as follows: 0-30, 30-60, 60-100, and 100-200 cm. Soil samples were collected from three coring locations with at least 5 kg of soil from each depth. Composite samples from each depth were sieved (2 mm) and stored at 4°C until denitrification analysis. The denitrification assay involved anaerobic incubation of soil samples in a DEA solution containing sources of nitrate-nitrogen (KNO_3) and carbon (glucose), and 10 ppm chloramphenicol to inhibit microbial growth and de novo synthesis of new enzymes. To ensure non-limiting amount of electron donor, 300 μg glucose-C g^{-1} dry soil was included in the DEA solution. In determining the optimum nitrate concentration in the DEA assays, i.e. the concentration needed to obtain the highest DEA per unit amount of soil, the following concentrations were evaluated based on several studies (Barkle et al., 2007; Singh et al., 1989; Luo et al, 1996; Murray et al., 2004; Paramasivam et al., 1999; Yeomans et al., 1992): 25, 50, 75, 100, and 125 μg nitrate-N g^{-1} dry soil.

Two incubation methods were used: Erlenmeyer flask and vacuum pouch (Fig. 1). While the use of flasks is very common, gas-tight plastic bags or vacuum pouches have also been used in incubations for denitrification studies (Bishop et al., 2014; Klemedtsson et al., 1987; Twining et al., 2007).

For DEA measurement with Erlenmeyer flasks, fresh soil samples in five replicates (10 g dry equivalent, sieved <2 mm) were placed with 10 mL of DEA solution into 125 mL flasks fitted with suba-seals and incubated in the dark at 20°C on a rotary shaker. Anaerobic conditions were created by removing air from the flask and flushing with N_2 three times. Purified acetylene was added (until it comprised 10% of headspace volume) to prevent the conversion of N_2O to N_2. Gas samples (5 mL) were collected at 0, 2, 4, and 6 hrs from the start of incubation.
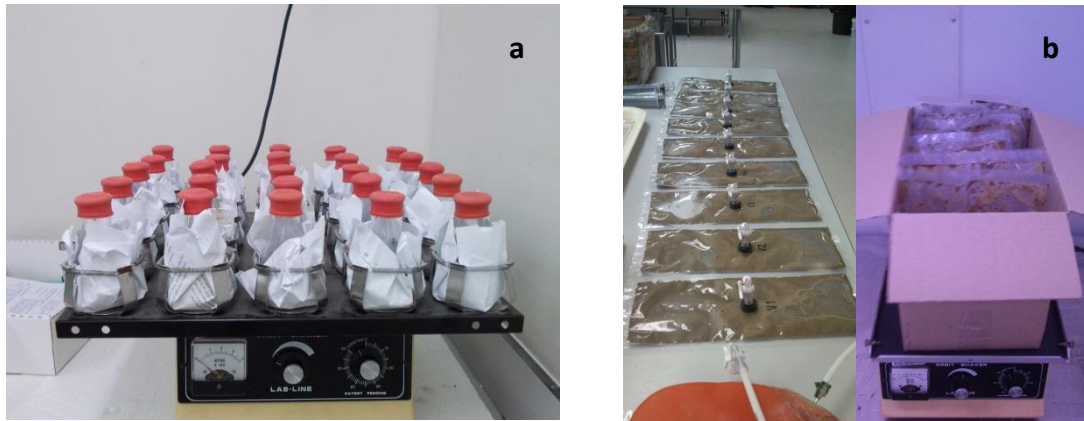
Figure 1 Incubation methods used in this study to measure DEA:
a) Erlenmeyer flask, b) vacuum pouches

For DEA measurement with vacuum pouches (100x285mm, gauge 70 μm), fresh soil samples in three replicates (40 g dry equivalent, sieved <2 mm) were placed with 40 mL of DEA solution into the pouches fitted with a luer-lock valve and rubber fitting sealed with F2 contact adhesive. Vacuum pouches were sealed and air was removed by syringe and flushed with N_2 once. After removing the N_2 gas used to flush the sample, 180 mL of N_2 and 20 mL of acetylene were added to comprise the headspace and the samples were incubated in the dark at 20 °C on a rotary shaker. Gas samples (25 mL) were collected at 0, 3, and 6 hrs from the start of incubation.

2.3 *Single-well push-pull test*

A number of studies have used the push-pull test to measure denitrification in shallow groundwater (e.g., Addy et al., 2002; Istok, 2013; Istok et al., 1997; Jahangir et al., 2013; Sanchez-Perez et al., 2003; Tesoriero et al., 2000; Well et al., 2003). The push-pull test allows straightforward and less time consuming measurement of denitrification rate at any desired depth at any location depending on the well design. In this study, a single-well, push-pull test was conducted on a piezometer (depth: 6.5 m bgl) to measure denitrification rate in the shallow groundwater at the study site. This test involved extraction of groundwater, preparation and injection of test solution, and retrieval of injected solution at specific time intervals till 3.5 hours after completing the solution re-injection. The test solution was prepared by adding bromide (KBr), nitrate (KNO_3) and acetylene into the extracted groundwater in a 20-L flexible container, with the following resulting target concentrations: 10 mg L^{-1} Br^-, 10 mg L^{-1} NO_3-N, and 50 ml L^{-1} acetylene. A total of 40 L of test solution was used in this study. Groundwater samples collected in triplicates (approximately 60 mL each) for chemical analysis were filtered (0.45 μm) on site and frozen until analysed for nitrate-N, ammonium-N, dissolved organic carbon (DOC), and bromide.

Groundwater samples (120 mL) for N_2O analysis were collected by syringe through a t-type luer-lock mini valve (Addy et al., 2002) to avoid sample contact with the atmosphere and transferred into evacuated vacuum pouches. To create a headspace, 50 mL of N_2 was added to each sample and samples were stored at 4°C until further analysis within 24 hours. Extraction of N_2O from the headspace was based on similar studies (Addy et al., 2002; Hill et al., 2000; Lemon & Lemon, 1981; Rudd et al., 1974; Sanchez-Perez et al., 2003; Well et al., 2003) with samples brought to a constant temperature (20°C), shaken on a rotary shaker for 1.5 hours, and 25 mL gas sample taken from headspace in each sample and placed in 12 mL evacuated glass vials for N_2O analysis.

2.4 *Laboratory analysis of samples and DEA estimation*

Nitrate-N and bromide in groundwater samples were analysed by ion chromatography (Lachat Instruments IC5000), ammonium-N by continuous flow analysis (Technicon® AutoAnalyzer II), and DOC by potassium dichromate wet oxidation and titration (method 5220B, APHA-AWWA-WEF [2005], but with some adjustments: using 10 mL sample, 20 mL H_2SO_4 with 5 g Ag_2SO_4 L^{-1}, and 10 mL 0.025N $K_2Cr_2O_7$ in digestion, and 0.01N ferrous ammonium sulphate for titration). Gas samples were analysed for N_2O with a Shimadzu Gas Chromatograph (GC) 17 A (Japan) which has a ^{63}Ni-electron capture detector. Concentrations of N_2O were plotted against time and DEA was determined from the resulting slope divided by the mass of dry soil.

3. Preliminary Results and Discussion

3.1 *Optimum nitrate concentration*

Table 1 summarizes the DEA values measured using the flask incubation method to determine the optimum nitrate concentration for incubations. The results show an apparent trend of increasing DEA values with increasing nitrate concentrations up 125 µg NO_3-N g^{-1} dry soil in the surface (Table 1). However, below 30 cm no significant increase in DEA was observed with increasing concentrations of nitrate above 25 µg NO_3-N g^{-1} dry soil. These results seem to support the study of Luo et al. (1996) which showed 50 µg NO_3-N g^{-1} dry soil as giving the optimum DEA value. However, the results also do not rule out other concentrations (75 and 100) to give optimum DEA values. This is further supported by the DEA values measured using the vacuum pouch technique (see next section 3.2), where the DEA values seem to peak at 75 µg NO_3^--N g^{-1} dry soil, especially for subsoil samples (Fig. 2b and 3b).

**Table 1** DEA (µg N_2O-N kg^{-1} dry soil d^{-1}, mean ± stdev) in soil samples from Massey No. 1 Dairy Farm

Depth, cm	Treatments (µg NO_3-N g^{-1} dry soil)				
	25	50	75	100	125
0-30	435 ± 126^b	725 ± 209^{ab}	882 ± 382^a	901 ± 302^a	410 ± 107^b
30-60	3.89 ± 0.68^a	5.30 ± 1.92^a	5.24 ± 0.80^a	5.10 ± 1.66^a	4.92 ± 0.98^a
60-100	4.06 ± 1.11^a	4.09 ± 0.59^a	3.92 ± 1.84^a	4.12 ± 0.48^a	3.66 ± 0.38^a
100-200	5.47 ± 1.55^{ab}	6.39 ± 1.61^a	3.92 ± 0.91^b	5.24 ± 0.70^{ab}	5.56 ± 1.20^{ab}

Note: No. of replicates: 5. Different superscripts denote significant statistical differences at p<0.05 within each soil depth category.

It is also apparent that DEA is much higher in surface soils (depth: 0-30 cm) than in subsoil (30-200 cm) (Table 1). No significant differences were found in DEA values among different nitrate concentrations for subsoil samples (30-200 cm). This could be attributed to the very low N_2O concentrations measured in incubations with subsoil samples which are within the detection error of the GC: GC readings of 500 ppb standards vary by up to 62 ppb (413 ppb to 475 ppb), whereas N_2O concentrations in gas samples in vials were in the range of 20-87 ppb only, with most gas samples having N_2O concentrations below 50 ppb. This, therefore, entails that DEA values in subsoil measured with flasks are not very reliable. This issue of low N_2O concentrations in subsoil sample incubations was further investigated with the use of larger soil amounts and larger gas sample volumes collected from incubations by using the vacuum pouch technique.

3.2   Comparison of DEA measured with different incubation methods

For surface soil samples (0-30 cm), DEA values obtained from both incubation techniques are comparable (Figure 2) indicating the applicability of the vacuum pouch technique for measuring DEA. However, this comparable result is not observed in the subsurface samples (100-200 cm) where the DEA values obtained with the flasks were significantly lower as compared to the vacuum pouch incubations (Figure 3). The discrepancies could be attributed to the very low N_2O concentrations measured in the flasks. It is also apparent that the variations in the DEA values were smaller in the vacuum pouch incubations than in the flask incubations (Figure 2).
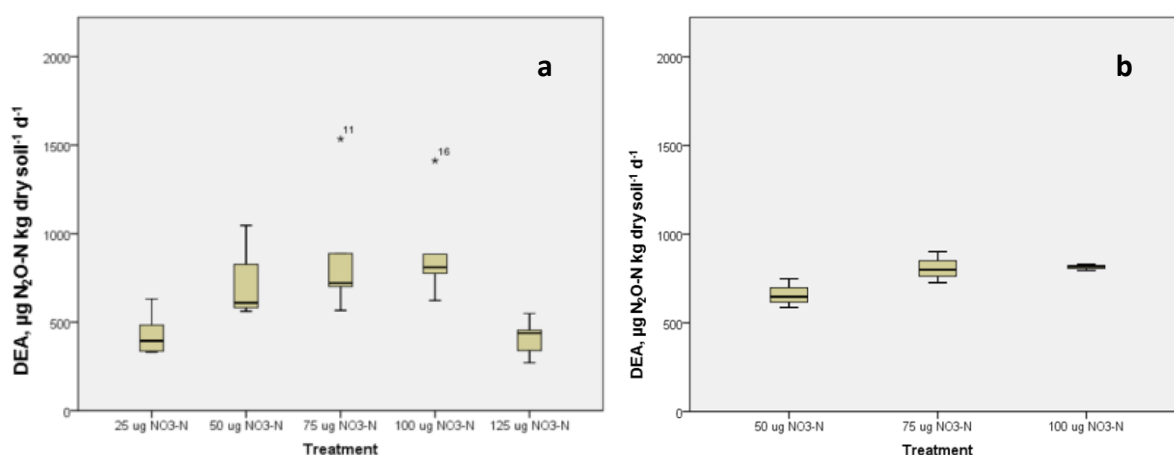
Figure 2 DEA in surface soil samples (0-30 cm) measured by incubations using:
a) Erlenmeyer flask, b) vacuum pouches

These results suggest that both incubation methods are applicable for surface soils (Figure 2). However, vacuum pouches appear to be more reliable for subsoil samples (Figure 3). With vacuum pouches, larger amount of soil can be used and larger gas sample volumes can be taken from incubations to obtain N_2O concentrations much higher than the detection limit of the gas chromatograph. Larger gas sample volumes also give a better representation of the headspace composition resulting to lesser variations in the DEA values. On the other hand, there are some constraints in using larger amount of soil and gas samples in flasks, such as flask volume limitation and negative pressure when extracting larger gas sample.
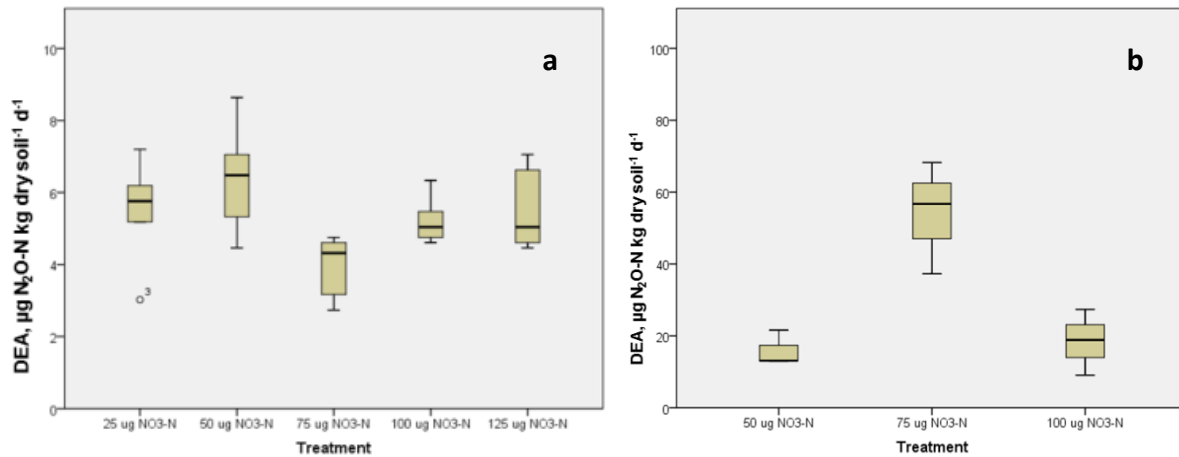
Figure 3 DEA in surface soil samples (100-200 cm) measured by incubations using:
a) Erlenmeyer flask, b) vacuum pouches

The DEA values obtained in this study are generally comparable to the values obtained in other areas in the country using the AI technique. The DEA values for the top layer (0-30 cm bgl) are much lower than the values (18,960-23,760 μg N_2O-N kg^{-1} dry soil d^{-1}) obtained from surface soil samples (0-10 cm bgl) at Massey No. 1 Dairy Farm (Deslippe et al., 2014). This is expected due to the higher denitrifier population (Paramasivam et al., 1999; Yeomans et al., 1992) and/or available electron donor such as organic carbon (Cannavo et al., 2004) in the surface soil. On the other hand, the values were in the range of the DEA values (300-5,000 μg N_2O-N kg^{-1} soil d^{-1}) measured from subsurface soils (10-40 cm bgl) in other areas in Manawatu (Luo et al., 1998). The DEA values obtained with the vacuum pouches in this study for the subsurface layer (100-200 cm bgl) are within the range of the DEA values (5-100 μg N_2O-N kg^{-1} dry soil d^{-1}) found in subsoil (120-235 cm bgl) in Canterbury (Peterson et al., 2013).

### 3.3   *Preliminary results of the push-pull test*

Figure 4 shows the changes in the concentrations of nitrate-N and bromide during the single push-pull test conducted at the study site. A similar decreasing trend can be observed in nitrate-N and bromide concentrations with nitrate-N decreasing more than bromide, as shown by the decreasing nitrate-N/bromide ratio. The decreasing ratio indicates that the overall nitrate reduction could not be fully attributed to dispersion and advection (Sanchez-Perez et al., 2003). The additional reduction in nitrate (apart from dispersion and advection) is shown as the plot of dilution-corrected nitrate-N concentration, which is the product of the dilution factor and observed nitrate-N concentration at time, t. The dilution factor was computed as the ratio of the bromide concentration of the test solution to the bromide concentration at time, t (Istok, 2013; Sanchez-Perez et al., 2003; Tesoriero et al. 2000; Trudell et al., 1986).

Given that dissimilatory nitrate reduction to ammonium (DNRA) is not apparent due to very low or negligible NH_4^+-N concentrations (0-0.077 mg L^{-1}) (Tesoriero et al., 2000), this additional reduction in nitrate-N could be attributed to denitrification at the study site. This potential occurrence of denitrification is further supported by an increase in N_2O concentrations measured in the groundwater samples during the test (Figure 5). The high N_2O concentration at time t = 0 could be due to the fact that the extracted sample at time t = 0 was less diluted with groundwater compared to samples taken at a later time. The first sample was

retrieved immediately after the completion of injection, in which no chaser was used to flush the test solution from the piezometer casing into the aquifer.
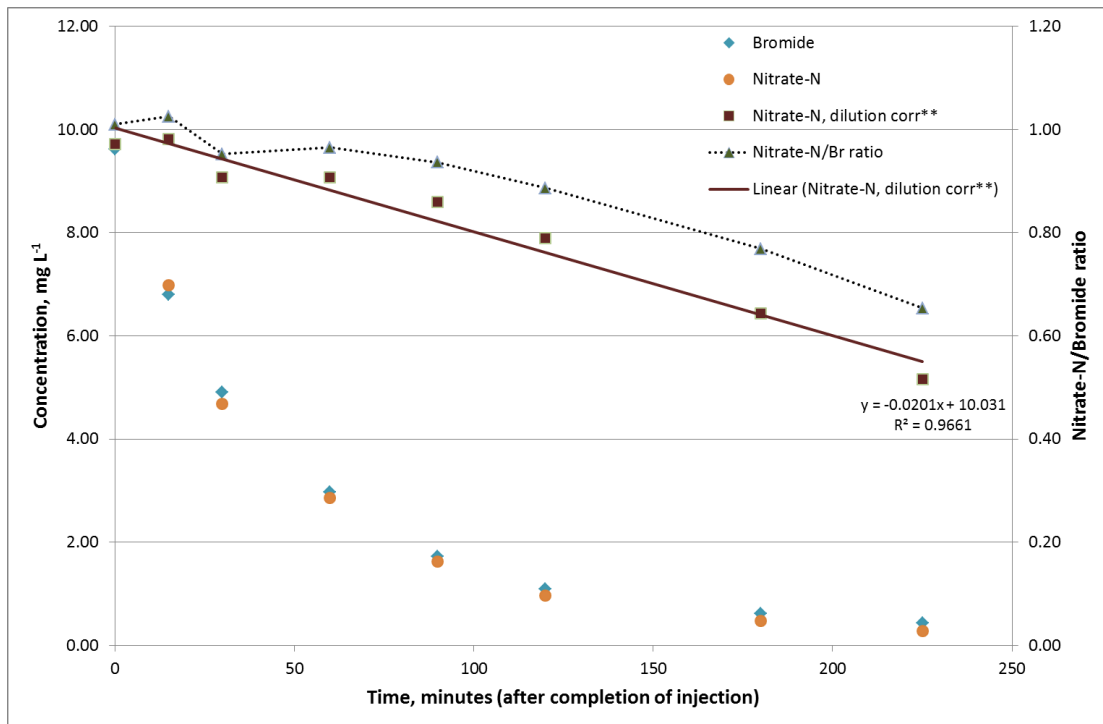
Figure 4 Changes in NO_3^--N and Br^- concentrations during the push-pull test conducted at the study site in Massey No. 1 Dairy Farm. No. of replicates per sampling time: 3. Background concentrations: NO_3-N: 0.049 mg L^{-1} and bromide: 0.099 mg L^{-1}.
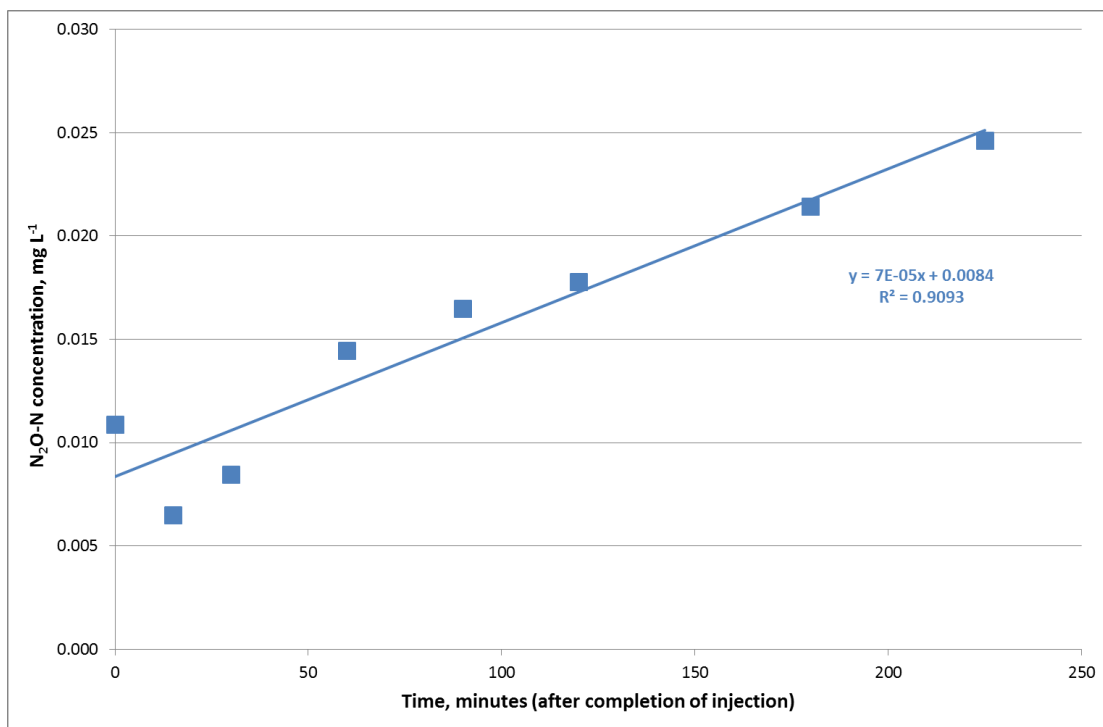
Figure 5 Changes in nitrous oxide concentration during the push-pull test conducted at the study site in Massey No. 1 Dairy Farm. No. of replicates per sampling time: 2. Values are not corrected for dilution.

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In this preliminary push-pull test, the denitrification rate (zero-order kinetics) was estimated at 1.20 mg N L^{-1} h^{-1} based on the slope of the regression line of dilution-corrected nitrate concentrations (Figure 4). This rate appears higher than those reported in the literature (0.01 – 1.12 mg N L^{-1} h^{-1}) obtained by push-pull tests (Addy et al., 2002; Istok et al., 1997; Sanchez-Perez et al., 2003; Starr & Gillham, 1993; Toda et al., 2002; Trudell et al., 1986; Well et al., 2003). The first-order rate computed according to Haggerty et al. (1998) was 2.79 d^{-1}, which is much higher than the rates (0.118-0.367 d^{-1}) measured in push-pull tests in the Waikato region (Hadfield & Gibbs, 2007 in Burbery et al., 2013). The higher rate obtained in this study could be due to the favourable environmental conditions, such as the availability of electron donor (organic carbon) and anaerobic condition (dissolved oxygen concentrations observed during the test ranged from 1.05 to 0.36 mg L^{-1}).

Although the results (Figure 4 and 5) indicate the applicability of the push-pull test for measuring denitrification, further tests would be needed to confirm the occurrence of high denitrification at the study site.

4.   Concluding Remarks

This study demonstrates the usefulness of the acetylene inhibition (AI) incubations and single-well push-pull method as simple and economical methods to measure denitrification in the subsurface environment. The preliminary results suggest that the DEA measurements for subsoils may require more nitrate input (75 µg NO_3^--N g^{-1} dry soil) than what is commonly added in incubations with surface soils (50 µg NO_3^--N g^{-1} dry soil) (Luo et al., 1996; Drury et al., 2008). It was also observed that N_2O concentrations measured using flasks for incubation were very low and within the detection error of the GC, particularly for the subsoil samples. This could be overcome by the vacuum pouch technique using larger amounts of soil and larger gas sample volumes to measure DEA values for subsoils. The preliminary results from the single-well push-pull test also indicate the usefulness of the method and the occurrence of denitrification in shallow groundwater at the study site. This is shown by a higher nitrate-nitrogen loss than can be attributed to dilution (dispersion and advection), coupled with the increasing N_2O concentration, and existence of favourable environmental conditions in the saturated zone.

However, further tests with the vacuum pouch technique would be needed to confirm the optimum nitrate concentration for different soil types and depths, and additional push-pull tests are required to refine the method and confirm the high denitrification rate at the study site. The methods developed in this study are to be used to measure denitrification in the subsurface environment at selected sites in the Manawatu River catchment.

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