American Journal of Agricultural and Biological Sciences, 2012, 7 (3), 357-369
ISSN: 1557-4989

doi:10.3844/ajabssp.2012.357.369 Published Online 7 (3) 2012 (http://www.thescipub.com/ajabs.toc)

Enumeration of Microbes and Gas Production During Denitrification and Nitrogen Fixation Processes in Soil

Ghaly A.E. and K.N. MacDonald

Department of Process Engineering and Applied Science, Dalhousie University, Halifax, Canada
Email: Abdel.Ghaly@dal.ca

Received 2012-08-17, Revised 2012-09-11; Accepted 2012-09-11

ABSTRACT

Dry plant material contains 2-4% nitrogen, making it an essential nutrient for all plants. The nitrogen cycle regulates the pathways which transform nitrogen from a relatively inert dinitrogen gas to forms of organic nitrogen such as proteins and nucleic acids. Denitrification and nitrogen fixation are the two most important processes that remove and add nitrogen to the soil, respectively. The aim of the study was to gain information on the denitrification and nitrogen fixing activities in soil and sediment employing the acetylene technique and assuring the gas chromatography analysis by total plate count and most probably number. The results indicated that acetylene (0.1 atm) inhibited N_2O reduction and caused stoichiometric accumulation of N_2O during the conversion of NO_3^- to N_2. N_2O was an obligatory intermediate in the sequence of steps between N_2O^- and N_2. The appearance of CO_2 and accumulation of N_2O would be suitable criteria for the presence of denitrifiers in appropriately enriched media and the acetylene reduction test is a suitable assay for nitrogen fixing activity. There was an obligatory requirement for organic carbon as a carbon and energy source for denitrification and nitrogen fixation to take place. The results showed that acetylglucosamine can be used as a carbon and energy source for denitrification but not as a nitrogen source (C:N ratio of 5:1). NH_4^+ has no effect on denitrification activity but it inhibited the nitrogenase activity. The presence of air in the gas phase affects both the denitrification and nitrogen fixing activity while adding H_2O encouraged anaerobic conditions.

Keywords: Denitrification, Nitrogen fixation, Soil, Bacteria, Enumeration, NO_3^-, NO_2^-, N_2O, N_2, C_2H_2, C_2H_4; CO_2, Acetylglucosamine, Carbon, Energy

1. INTRODUCTION

Generally, natural agricultural soils are made of five major components as shown in **Fig. 1**: (a) organic matter (3-6%), which is made of the remains of plants and animals and the products of their decomposition, (b) minerals (over 50%), which are the products of rock breakage by physical and chemical processes, (c) air and water, which take up to 25-30% of the total volume of the soil depending on the soil type and moisture content and (d) the living organism (less than 1%) which are the primary decomposers of dead organic matter and are responsible for the cycles of elements such as nitrogen, carbon, sulfur and phosphorus (Ghaly *et al*., 1999). A gram of soil may contain up to 10^{12} bacteria, 10^6 algae, 10^8 protozoa and 10 km of fungal hyphae (Trevors, 2010).

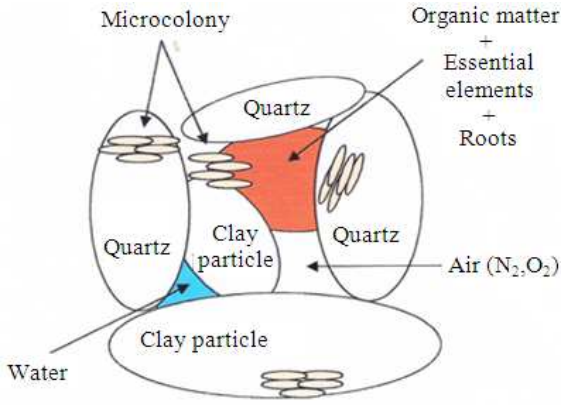


Fig. 1. Soil aggregates in the root zone

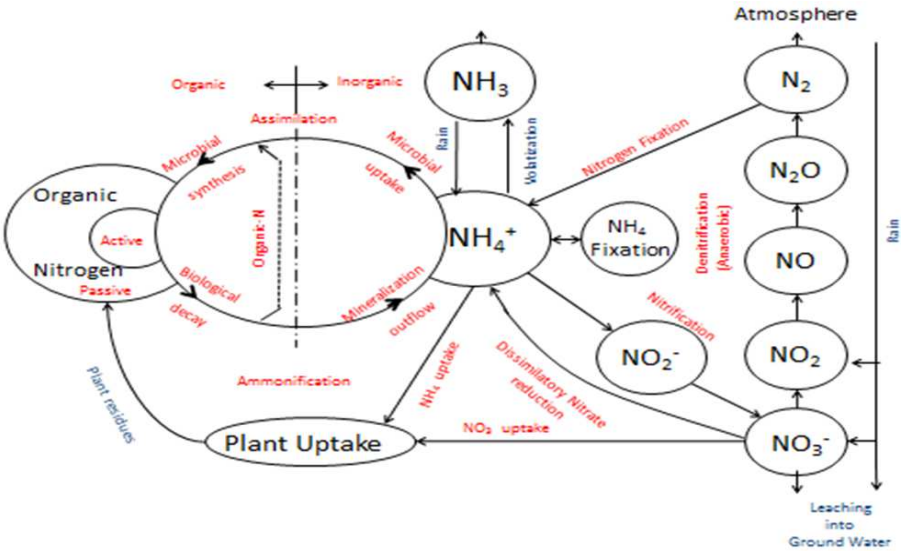


Fig. 2. The nitrogen cycle

Because of its very conspicuous importance to crop nutrition, the nitrogen cycle (**Fig. 2**) has attracted considerable attention from soil microbiologists, plant nutritionists and soil fertility specialists. Dry plant material contains 2-4% nitrogen. Nitrogen is a constituent of many organic compounds including amino acids, proteins, nucleic acids and enzymes. Through a sequence of microbial reactions, organic nitrogen is transformed into ammonium and then to nitrate (Cranfield *et al*., 2010). The plants absorb nitrogen from soil in the form of NO_3^- or NH_4^+. The uptake of NH_4^+ is depressed under acidic conditions while the uptake of NO_3^- is depressed under alkaline conditions. High concentrations of nitrogen can result in toxicity to plants as they become dark green in color with abundant foliage but with restricted root growth. Nitrogen deficiency also results in restricted growth and the plants will turn yellow from lack of chlorophyll especially in older leaves (Ross *et al*., 2011; Tremblay *et al*., 2012).

The overall transformations in which microorganisms are involved in the nitrogen cycle range from nitrogen gas to protein and other complex organic nitrogenous compounds with a tremendously large array of substrates between these extremes. A great many intricate enzymatic reactions are involved in bringing about these changes (Cranfield *et al*., 2010; Robertson and Kuenen, 1990). However, the two processes that remove and add nitrogen from the soil are denitrification and nitrogen fixation, respectively. The aim of this study was to examine soil and sediment for both denitrification and nitrogen fixation bacteria using gas chromatography and acetylene reduction techniques. The Most Probable Number (MPN) and Total Plate Count (TPC) were employed to assure the results of gas chromatography and C_2H_2 tests.

2. DENITRIFICATION

Certain microorganisms are capable of transforming nitrate and nitrite to nitrogen oxides and nitrogen gas by a process called denitrification, or dissimilatory nitrate reduction, which leads to a net loss of nitrogen from the soil. Denitrification is herein defined as the biochemical reduction of NO_3-N and NO_2-N to gaseous nitrogen in the form of Nitric Oxide (NO) and Nitrous Oxide (N_2O) and molecular Nitrogen (N_2) Eq. 1 (Cranfield *et al*., 2010; Torrento *et al*., 2010):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

Denitrification takes place in anoxic soils where the O_2 is absent or the diffusion of O_2 to the center of soil aggregate is slow allowing for anoxic micro sites (Burgin *et al*., 2010). Denitrification is a major process in the nitrogen cycle that returns N to the atmosphere thereby completing the biogeochemical cycle. Loss of nitrogen via denitrification is typically 2 kg/ha/yr for unfertilized soil (Hofstra and Bouwman, 2005).

In denitrification, nitrate serves as terminal electron acceptor for the oxidation of substrate. Many facultative anaerobic bacteria, predominantly of the genera *Pseudomonas*, *Achromobacter*, *Alkaligenes* and *Paracoccus* (**Table 1**) are capable of this reaction (Kim *et al*., 2008; Torrento *et al*., 2010). The reaction appears

to be coupled with specific enzymes and cofactors. The electron transport system is identical under aerobic and anaerobic conditions (Lin *et al*., 2009). The aerobic and anaerobic systems for the oxidation of glucose by these facultative bacteria are as follows Eq. 2 and 3:

Aerobic system:

$$C_6H_{12}O_6 + O_2 \xrightarrow{\text{Facultative bacteria}} 6CO_2 + 6H_2O \quad (2)$$

Anaerobic system:

$$5C_6H_{12}O_6 + HNO_3 \xrightarrow{\text{Facultative bacteria}} 30CO_2 + 4HNO_3 + 12\,N_2 \quad (3)$$

Assuming availability of substrate (NO_3^- or NO_2^-) and reasonable conditions of temperature (10-30°C) and pH(7.0-8.5), the denitrification reaction is strongly influenced by the partial pressure of O_2 as well as the availability of an energy source (Calderer *et al*., 2010). Factors that indirectly affect O_2 availability such as water logging have a significant effect on the denitrification process (Burgin *et al*., 2010).

It is generally assumed that during denitrification, bacteria oxidize organic substances completely to carbon dioxide concomitant with the reduction of nitrate or nitrite to nitric oxide, nitrous oxide and nitrogen. The ratio of carbon dioxide to nitrogen in denitrifying cultures corresponds to the theoretical ratio to be expected for complete oxidation of the organic substrate (Bothe *et al*., 2007; Warneke *et al*., 2011). In their carbon nitrogen balance experiments, Calderer *et al*. (2010) and Senbayram *et al*. (2012) clearly established that the organic substrate (carbon and energy source) is completely oxidized to carbon dioxide during denitrification, except for a small part that is converted to bacterial cells. Their results also indicated that the quantity of substrate assimilated to bacterial cells under anaerobic conditions was close to that observed under aerobic conditions.

Patriquin and Knowles (1974) suggested that disappearance of accumulated nitrite and presence of N_2O in nitrite containing systems were reliable criteria for the presence of denitrifiers. Other researchers used gas chromatography to identify and quantitate gasses arising from denitrification including nitric oxide, nitrous oxide and molecular dinitrogen (Yoshinari and Knowles, 1976; Hara *et al*., 2009; Jia and Conrad, 2009; Warneke *et al*., 2011; Senbayram *et al*., 2012). However, Warneke *et al*. (2011) reported that certain denitrifying bacteria (including *Agrobacterium tumerfaciens)* lack nitrous oxide reductase and cannot complete the denitrification process to produce N_2, generating NO as the end product of denitrification.

Table 1. Denitrifying bacteria of the genus *Pseudomonias*, *Paracoccus*, *Alcaligenes* and *Achromobacter*

Genus	Species	Reference
Pseudomonias	*putida*	Dandie *et al*. (2007)
	stutzeri	Ward (1995)
	aureofaciens	Ward (1995)
	denitrificans	Ward (1995)
	atlantica	Ward (1995)
	fluorescens	Ward (1995)
	aeruginosa	Ward (1995)
	savastanoi	Dandie *et al*. (2007)
	brassicacearum	Dandie *et al*. (2007)
	chlororaphis	Dandie *et al*. (2007)
	frederiksbergensis	Dandie *et al*. (2007)
	grimontii	Dandie *et al*. (2007)
	kilonesis	Dandie *et al*. (2007)
	lini	Dandie *et al*. (2007)
	mandelii	Dandie *et al*. (2007)
	migulae	Dandie *et al*. (2007)
Paracoccus	*denitrificans*	Ward (1995)
	halodinitrificans	Ward (1995)
Alcaligenes	*faecalis*	Ward (1995)
	eutrophus	Hallin and Lindgren (1999)
	denitrificans	Hallin and Lindgren (1999)
Achromobacter	*piechaudii*	Dandie *et al*. (2007)
	cycloclastes	Hallin and Lindgren (1999)

Nakajima *et al*. (2005) reported that the lack of significant gas production in inverted vials in the presence of denitrifier might result from: (a) toxicity or accumulated nitrite (b) inappropriate organic matter-nitrate ratio in the medium (c) formation of N_2O, which is highly soluble, but not N_2 and (d) nitrate-nitrite suppression of gas formation by denitrifiers, with concomitant use of energy substrates by competing organisms in the mixed culture systems.

Studies on inhibition of Nitrous Oxide (N_2O) reductase by azide (N_3^-), Cyanide (CN^-) and Dinitrophnol (DNP) in *Pseudomonas denitrificans* showed that N_2O was an obligatory intermediate in the reduction of NO_2^- to N_2 (Warneke *et al*., 2011). Yoshinari and Knowles (1976) reported that C_2H_2 inhibited reduction of N_2O by three denitrifying bacteria, *Pseudomonas perfectomarinus*, *Pseudomonas aeruginosa* and *Micrococcus denitrificans*. They also reported that during reduction of NO_3^- or NO_2^-, C_2H_4 caused accumulation of N_2O with a stoichiometry which suggested that N_2O is an obligatory intermediate in the reduction of NO_2^- to N_2 with all three species of denitrifiers.

Senbayram *et al*. (2012) reported that the ratio of N_2O to N_2 produced by a denitrifying soil is related to the initial NO_3^- content available to the micro flora. High NO_3^- contents lead to greater N_2O production which has a greater greenhouse effect and contributes to ozone depletion.

3. NITROGEN FIXATION

If molecular nitrogen was completely inert biologically, the activities of the denitrifying bacteria would very rapidly deplete the biosphere of all nitrogen available for growth and life would cease on earth. Although atmospheric nitrogen is not a suitable nutrient for most organisms, it can be used by a few specialized types as a source of nitrogen for growth. It is these nitrogen fixing organisms that compensate for the losses of combined nitrogen due to denitrification and maintain a more or less constant amount of nitrogen in the biosphere through the process of nitrogen fixation (Cranfield *et al*., 2010; Jia and Conrad, 2009). Nitrogen fixation is herein defined as a biological process by which nitrogen gas (N_2) in the soil is converted into ammonium (NH_4) by either symbiotic or asymbiotic associations Eq. 4:

$$N_2 + 4H_2O \xrightarrow{\text{Nitrogenase}} 2NH_4 + 2O_2 \quad (4)$$

Nitrogen fixation is one of the most metabolically intensive processes regulated by the available cellular energy (Reed *et al*., 2011). In free nitrogen fixation energy needs must be met through either photosynthesis or the consumption of carbon based energy sources (organic matter). In the symbiotic association, the ectotrophic mycorrhizal fungi (which possess the enzyme nitrogenase) forms a sheath around the active fine roots of plants and provide them with NH_4 while obtaining their carbohydrate (energy requirements) from the plant (**Fig. 3**). Because of the large surface area, fungi also obtain other soil minerals from the soil and transfer them to the plant roots. In the asymbiotic association, bacteria and blue green algae possess the enzyme nitrogenase and can fix N_2 into NH_4. However, this process takes place in soils that have high organic matter content which provide a ready source of energy to these microorganisms.

The nitrogen fixation process is controlled by N:P ratio as phosphorus activates the gene required for the synthesis of the enzyme nitrogenase. Molybdenum (Mo) and iron (Fe) are also required as they serve as structural compounds for nitrogenase. Investigations with cell-free preparation have implicated three similar two-component proteins (an Fe-Mo protein, a V-Fe protein and an Fe-Fe protein), each of these protein pairs has been observed to produce an active N complex (enzyme nitrogenase) upon combination. Proteins from different genera appear to be very closely related, indicating broad generally applicable N fixation schemes (Jetten, 2008; Santos *et al*., 2012; Gaby and Buckley, 2011). Reed *et al*. (2011) indicated that organisms are not limited to expressing a single form of nitrogenase enzyme and may carry the genes necessary for encoding all three types. Nitrogenase is remarkably nonspecific, possessing the capability to reduce such compounds as nitrous oxide and acetylene (Keeney, 1973). The reduction of acetylene to ethylene has become the basis for a widely applied assay for nitrogen fixation Eq. 5 (Hara *et al*., 2009):

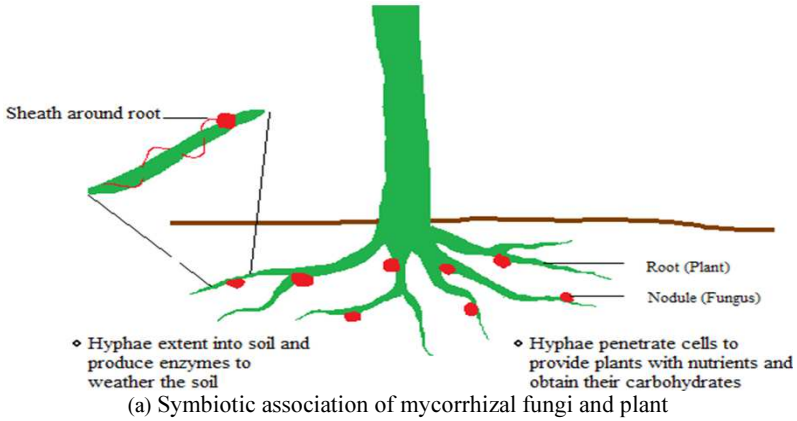
$$C_2H_2 + 2e^- + 2H^+ \xrightarrow{\text{Nitrogenase}} C_2H_4 \quad (5)$$

Bertics *et al*. (2010) stated that nitrogenase can reduce a number of substrates with a facility that is apparently dependent upon the number of electrons required for reduction. Acetylene (C_2H_2) is an alternative substrate for nitrogenase and will compete effectively with N_2 at the active site of the enzyme to be reduced to ethylene C_2H_4. The reduction of acetylene to ethylene requires two electrons whereas the reduction of N_2 to NH_3 requires six electrons so that nitrogenase activity as measured by acetylene reduction is theoretically three times as high as that rated for N_2. Bertics *et al*. (2010) stated that in practice the equivalent ratio may be as high as 4 due to the inhibition of H_2 production which normally occurs with the reduction of N_2, thereby increasing the rate of reaction Eq. 6:

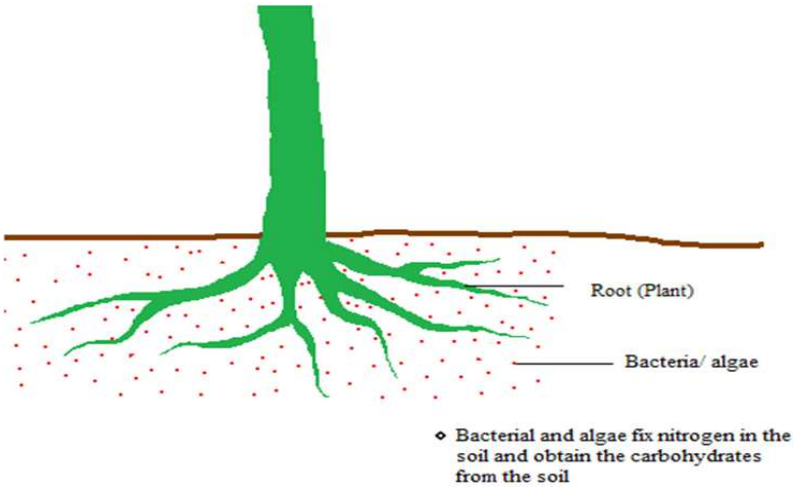
$$N_2 + 6H^+ + 6e^- \xrightarrow{\text{Nitrogenase}} 2NH_3 \quad (6)$$

Burns and Hardy (1975) reported that nitrous oxide and C_2H_2 are substrates for and competitive inhibitors of nitrogenase. Dalton and Whittenburry (1976) reported that the addition of 0.5 mL of acetylene immediately inhibited gas metabolism and growth of nitrogen fixing methanol oxidizing *Methylococcus capsulatus*. However oxygen concentration, CO_2 production and growth were restored when methanol was added to the culture. When ethylene was added to a similar culture the rate of uptake and production of the other gases decreased to 45% of their normal values, indicating that metabolism was not completely halted.

Factors affecting nitrogen fixation include: (a) availability of energy source, (b) moisture content and (c) presence of NO_3 and NH_4 in soil. Vitousek and Hobbie (2000) reported that 65% of the variation in nitrogen fixation rates in tropical forest litters was accounted for in the variation in carbon availability. Accessible carbon substrate was observed as the most commonly limiting factor in litter decomposition. Low lignin litters (10-15% lignin) fixed 0.9-1.3 mg nitrogen/g initial mass while high lignin litters (25-30% lignin) fixed 0.03-0.06 mg nitrogen/g initial mass. In a similarly designed experiment, Perez *et al*. (2010) found that the greatest factor affecting the rate of nitrogen fixation was the carbon to nitrogen ratio of the energy source provided.



(a) Symbiotic association of mycorrhizal fungi and plant



(b) Asymtiotic assiciation of free bacteria/algae and plant

Fig. 3. Symbiotic and asymbiotic nitrogen fixation.

They observed a 3.4 fold increase in nitrogen fixation when C:N ratio of the litter used was changed from 50.6 to 78.9%. It was hypothesised that the increased nitrogen fixation rates were due to an increased competitive advantage for nitrogen fixing bacteria in a low nitrogen environment.

Larrainzar *et al*. (2009) investigated the effect of mild drought conditions on the root nodule nitrogen fixation of the legume *Medicago truncatula.* Their results indicated that soil moisture was an important factor in the nitrogen fixation rate in soil. During drought conditions, decreases in nitrogen fixation occurred although increases were observed in soil carbon sources (including various sugars). Similarly, Zhao *et al*. (2010) reported a significant decrease in nitrogenase activity of biocrust when moisture content was reduced to below 20% field holding capacity. However, there was no significant variation in the nitrogenase activity reported when the moisture content varied from 40-100% field holding capacity.

Ohyama *et al*. (2008) reported the inhibition of root nodule growth within hours of the addition of nitrate or ammonia nitrogen. Changes in the level of nitrogen fixation were observed 24 hours after nitrate or ammonia addition. Salvagiotti *et al*. (2008) reviewed the literature on nitrogen fixation and found a negative exponential decay in the nitrogen fixation rate when the amount of nitrogen added as either ammonium or nitrate fertilizer increased.

4. MATERIALS AND METHODS

4.1. Soil and Sediment Collection

A Nova Scotia soil was obtained from a commercial farm in Turo Nova Scotia, 100 km from Halifax. The top vegetation/trash cover of the soil was scrapped away and the top 30 cm of the soil was removed with a shovel. The soil was placed in a heavy duty polyethylene bag and

transported from the collection site to the Waste Management Laboratory, Dalhousie University, Halifax, Nova Scotia. The soil was washed with water 3 times to minimize the level of nutrients in it. The soil characteristics are presented in **Table 2**. The sediment was obtained from a natural wetland in the Waverly Game Sanctuary, approximately 25 km from Halifax, Nova Scotia. The sediment was collected in a plastic container and transported to the Waste Management Laboratory of Dalhousie University. The sediment characteristics are shown in **Table 2.**

4.2. Experimental Design

The denitrification and nitrogen fixation in soil and sediment were evaluated. The effects of gas phase composition (air or N_2), addition of glucose (0 and 0.5%), addition of C_2H_2 (0 and 5 mL), addition of water (0, 0.5, 1.0, 4.0 and 4.5 mL) and addition of a nitrogen source (0, NH_4^+, NH_4+NO_3, acetylglucosamine, acetylglucosamine + NO_3^-) were studied. The experiments were divided into seven groups with 4 flasks in each group (**Table 3**). Three replicated were carried out for each treatment. This resulted in 84 flasks.

4.3. Experimental Procedure

Ten grams of soil (or sediment) were placed into each of the 50 mL Erlenmeyer flasks. After closing with an appropriate size of serum stopper, some flasks were evacuated through a needle and back filled with N_2 to one atmosphere. When required, 5.0 mL of gas phase was replaced with the same volume of acetylene (C_2H_2) to give a final concentration of 0.1 atmospheres. Also, when desired 0.5 mL of glucose and/ or 0.5 NH_4+, NO_3^- and /or glucosamine was added to give a final concentration of 0.5% w/w glucose and 100 ppm w/w/ NO_3^- -N. Water was added as desired. All flasks were incubated at room temperature for 7 days. **Table 3** showed the treatment combinations and the analysis carried out on the samples.

4.4. Gas Analyses

The CO_2, N_2O, C_2H_2 and C_2H_4 measurements were carried out using a gas chromatograph (Hewlett Packard model HP 5980A, Palo Alto, California, U.S.A), with helium as a carrier gas (at a flow rate of 30 mL min^{-1}) and a Poropak Q stainless steel column (152.4 x 3.2 mm O.D.). The column was set up in a bypass arrangement with a Molecular Sieve 5A 60/80 stainless steel column (152.4 x 3.2 mm O.D.). 1 mL of the gas samples was injected into the column and a switch valve was adjusted to store the nitrogen until the elution of the other compounds (carbon dioxide, nitrate, acetylene and ethylene). The injector and column temperatures were set to 150 and 45°C, respectively. Detection was performed with a flame ionization detector as well as a thermal conductivity detector set to 250°C.

4.5. Bacterial Count Analysis

Bacterial count of *Azotobacter, Clostridium* and qualitative enrichments of denitrifiers were performed. Plate counts were performed by creating tenfold dilutions of the soil in sterile water. To create the first dilution (10^{-1}) 1 g of soil was added to a test tube with 9 mL of water and shaken to produce an even mixture. Further dilutions were prepared by pipetting 1 mL of the previous dilution into 9 mL of water until dilutions were made for 10^{-1} to 10^{-7}. Agar plates of Azotobacter agar (manitol), clostridium agar and fluorescein denitrification agar were prepared for the bacteria counts by dissolving the ingredients listed in **Table 4** in 1 L of distilled water and autoclaving. Spread plates were prepared by pipetting 0.1 mL of the respective dilution onto a labelled (specimen and dilution) agar plate and spreading with a sterile glass spreader. All samples were plated in triplicate. Agar plates were sealed, inverted and incubated for 48 hours at 30°C. Plates that grew between 3 and 300 colonies were counted. The cell count was obtained by multiplying the plate count by the respective dilution, to obtain a value in cells/g soil.

5. RESULTS

Table 5 shows the data obtained from the gas chromatographic analysis and bacterial count from the experiments in which denitrification and nitrogen fixation were studied using the treatment combinations described in **Table 3**.

5.1 Group 1 (Soil with N_2).

5.1.1. Denitrification

There was no NO_3^- added to the soil samples. Therefore, the denitrification process did not proceed. The presence of 0.5 µ moles of N_2O in flask 4 may be from the initial gas phase or due to traces of NO_3^- or NO_2^- in the soil sample which were converted to N_2O and the later accumulated due to the inhibitory effect of C_2H_2 on the conversion of N_2O to N_2. The numbers of denitrifiers obtained by the most probably numbers (MPN) methods were smaller than that of other flasks (other groups where denitrification proceeded) which supported the results obtained by gas chromatography analysis.

5.1.2. Nitrogen Fixation

There was no nitrogen-fixing activity in flask 1 and 2 due to the lack of organic carbon which required as a carbon and energy source. The 1.4 n moles C_2H_4 detected in flask 2 may be due to organic carbon traces in the soil sample.

Table 2. Soil and sediment characteristics

Parameter	Soil	Sediment
Soil type	Stewiack	Queen
Texture	Medium	Moderately fine
Drainage	Well to moderately drained	Moderately drained
Permeability	Slow (0.36×10^{-2})	Slow (25×10^{-2})
Field bulk density	1.4 kg/m^3	1.3 kg/m^3
pH	6.5	5.9
Particle size	21 clay, 20 silt, 59 sand	25 clay, 23 silt, 54 sand
Classification	Sandy loam	Sandy clay loam

Table 3. Experimental parameters

Group	Sample	Flask	Gas Phase	0.5 ml Glucose (0.5% w/w)	0.5 ml N (100µg/g)	5ml C_2H_2 (0.1 atm)	H_2O (ml)
1	Soil	1	N_2	-	-	-	1.0
		2	N_2	-	-	+	1.0
		3	N_2	+	-	-	0.5
		4	N_2	+	-	+	0.5
2	Soil	1	N_2	+	-	+	0.5
		2	N_2	+	NH_4^+	+	-
		3	N_2	+	NO_3^-	+	-
		4	N_2	+	NH_4^+ NO_3^-	+	-
3	Soil	1	N_2	-	NO_3^-	-	0.5
		2	N_2	-	NO_3^-	+	0.5
		3	N_2	+	NO_3^-	-	-
		4	N_2	+	NO_3^-	+	-
4	Soil	1	Air	+	-	+	0.5
		2	Air	+	NO_3^-	+	-
		3	Air	+	-	+	4.5
		4	Air	+	NO_3^-	+	4.0
5	Soil	1	N_2	+	AG	+	-
		2	N_2	-	AG	+	0.5
		3	N_2	+	-	+	0.5
		4	N_2	-	AG + NO_3^-	+	-
6	Sediment	1	N_2	-	-	+	1.0
		2	N_2	+	-	+	0.5
		3	N_2	+	NH_4^+	+	-
		4	N_2	+	NO_3^-	+	-
7	Sediment	1	N_2	+	-	+	0.5
		2	N_2	+	NO_3^-	+	-
		3	N_2	+	AG	+	-
		4	N_2	+	AG + NO_3^-	+	-

AG- Acetylglucosamine

Table 4. Plate count medium composition

Azotobacter Agar		Clostridium Agar		Fluorescence Denitrification Agar	
Ingredient	g	Ingredient	g	Ingredient	g
Mannitol	20.00	Yeast Extract	3.00	Agar	15.00
Agar	15.00	Meat Extract	10.00	Proteose peptone No. 3	10.00
Soil Extract	5.00	Meat Peptone	5.00	KNO_3	2.00
K_2HPO_4	1.00	Starch	1.00	K_2HPO_4	1.50
$MgSO_4$	0.20	D(+) Glucose	5.00	$MgSO_2 \cdot 7H_2O$	1.50
NaCl	0.20	NaCl	5.00	$NaNO_2$	0.50
$FeSO_4$	Trace	CH_3COONa	3.00		
		L-cysteine Hydrochloride	0.50		
		Agar	12.50		

Table 5. Gas chromatographic analysis and bacterial count

Group	Sample	Flask	Gas Phase	GC Analysis				Bacterial Count/ g		
				N_2O (µm/g)	CO_2 (µm/g)	C_2H_4 (µm/g)	C_2H_2 (µm/g)	Az.	Cl.	De.
1	Soil	1	N_2			0.0	0	0	240	3.2×10^3
		2	N_2			1.4	0			
		3	N_2			0.0	0			
		4	N_2	0.5	20	1000.0	0			
2	Soil	1	N_2			1400.0	11			
		2	N_2			300.0	15			
		3	N_2	3.0	17	580.0	14	0	330	1.6×10^0
		4	N_2	2.0	29					
3	Soil	1	N_2	0.3	1					
		2	N_2			1.0	6			
		3	N_2	0.0	23					
		4	N_2	2.0	9	370.0	6	260	3500	2.4×10^6
4	Soil	1	Air			167.0	14	1000	0	17
		2	Air	21.0	39					
		3	Air			575.0	11			
		4	Air	23.0	38	375.0	8			
5	Soil	1	N_2			1400.0	9			
		2	N_2	1.0	7	26.0	8	280	220	2.1×10^4
		3	N_2			2500.0	9			
		4	N_2	4.0	7					
6	Sediment	1	N_2			2.0	14			
		2	N_2			37.0	14			
		3	N_2	0.2	11	3.0	15	0	350	1.1×10^3
		4	N_2	0.3	5	1.0	14			
7	Sediment	1	N_2			86.0	15			
		2	N_2	2.0	5					
		3	N_2	0.1	5	2.0	10	0	240	45
		4	N_2	0.6	4					

Az= Azotobacter Agar; Cl= Clostridium Agar; De= Denitrification Agar

In flasks 3 and 4, which received glucose, there was considerable amount of C_2H_4 in flask 4 (C_2H_2 added) which suggested that glucose was utilized by nitrogen fixers as a carbon and energy source, while there was no significant C_2H_4 production in flask 3 (no C_2H_2 added). The total plate count *(Azotobacter)* and MPN (*Clostridium*) results in flask 1 showed the absence of nitrogen fixation activity which agreed with the results obtained by gas chromatographic analysis.

5.2. Group 2 (Soil with N_2 & N-Fertilizers)

5.2.1. Denitrification

The presence of CO_2 and accumulation of N_2O due to the inhibition of the last step (conversion of N_2O to N_2O by C_2H_2 indicated the proceeding of denitrification process. Since there was no significant differences between flask 3 (no NH_4^+ added) and flask 4 (NH_4^+ added), this would indicate that, NH_4^+ has no effect on denitrification activity.

5.2.2. Nitrogen Fixation

The amounts of C_2H_4 produced in flask 2 (NH_4^+ added) and flask 3 (NO_3 added) were 5 and 3 times less than that of flask 1, respectively. The results of flask 2 supported the fact that NH_4^+ assimilated via glucosamine dehydrogenase and the nitrogen fixing organisms use NH_4^+-N for growth as they do not produce the enzymes required for nitrogen fixation. The accumulation of NH_4^+ switches off *nif* genes (Leigh and Dodsworth, 2007). In flask 3 the accumulation of N_2O from denitrification activity (due to the inhibitory effect of C_2H_2 on the conversion of N_2O to N_2O) inhibited, to a certain extent, the nitrogenase activity (Newton and Dilworth, 2011). The total plate count and MPN results of flask 3 assured this fact as compared to flask 1 group 1.

5.3. Group 3 (Soil with N_2 and NO_3^-)

5.3.1. Denitrification

In flask 1, the denitrification did not take place due to the lack of organic carbon. The traces of N_2O and CO_2 may be from the initial gas phase. The large amount of CO_2 and the absence of N_2O are the result of the denitrification and the complete conversion of N_2O to N_2. The accumulation of N_2O in flask 4 indicated that, C_2H_2 has an inhibitory effect on the conversion of N_2O to N_2 by denitrifiers. This agreed with the results

obtained from flasks 3 and 4 in group 2. The MPN count assured the activity of denitrification and agreed with the results of the gas chromatograph analysis.

5.3.2. Nitrogen Fixation

There was no significant production of C_2H_4 in flask 2, as compared to flask 4, due to the lack of organic carbon source which agrees with the results of flask 2 group 1. The higher bacterial count in flask 4 showed that the nitrogen fixation process proceeded adequately.

5.4. Group 4 (Soil with Air & NO_3^-)

5.4.1. Denitrification

The only difference between flasks 2 and 4 was the received amount of H_2O. The 4.5 mL H_2O in flask 4 encouraged the anaerobic condition in the soil more than that in flask 2 (0.5 mL H_2O), since the gas phase was air in both, which gave rise to denitrification earlier in flask 4 and resulted in more N_2O accumulation. Flask 2 and 4 seem to have much higher CO_2 production and N_2O accumulation than the similar flasks receiving the same treatment (flask 4 group 2, flask 4 group 3, flask 4 group 6, flask 3 group 7). The only difference was the amount of water added. However, flask 2 group 4 has no H_2O and gained approximately the same amount of CO_2 and N_2O. The reason for that is not understood. The MPN showed low numbers of denitrifiers in flask 1 because denitrification did not proceed due to the lack of NO_3^- or NO_2^-.

5.4.2. Nitrogen Fixation

Comparing flasks 1 and 3 (both contained air as the gas phase), the 4.5 mL H_2O in flask 4 encouraged anaerobic conditions in the soil more than that in flask 2 (0.5 mL H_2O) which favored the nitrogenase activity. The amount of C_2H_4 produced in flask 1 was due to the fact that the soil sample may contain aerobic organisms which consumed the available O_2 and brought about an anaerobic condition favoring the activity of nitrogenase. Flasks 3 and 4 received the same amount of H_2O. The lower C_2H_4 production in flask 4 was due to the effects of NO_3^- and NO_2^- on nitrogenase activity (Newton and Dilworth, 2011) which agreed with the results of flask 3 group 2. N_2O showed more effect on nitrogenase activity in flask 1 (0.5 mL H_2O) than flask 4 (4.0 mL H_2O) due to the fact that N_2O is soluble in water. The amount of C_2H_4 produced in flask 1 is much smaller than those of flask 4 group 1 and flask 1 group 2. The reason for that may be due to the effect of oxygen in the gas phase of the first on the nitrogenase activity causing some delay until the aerobes consumed the O_2 from the gas phase. Bacterial count of flask 4 showed that the predominant nitrogen fixer was the *Azotobacter* bacteria which can grow in the presence of O_2.

5.5. Group 5 (Soil with N_2 & Acetylglucosamine)

5.5.1. Denitrification

The results indicated that acetylglucosamine cannot be used as an alternative N source for denitrification but can be used as organic carbon and energy sources. This finding is reasonable since it has a C:N ratio of approximately 5:1. The MPN count indicated the absence of denitrification in flask 3 due to the lack of inorganic nitrogen sources (NO_3^- or NO_2^-).

5.5.2. Nitrogen Fixation

By comparing the results of flask 2 to those of flasks 1 and 3, it can be concluded that acetylglucosamine was not utilized as a carbon and energy source by nitrogen fixing bacteria. Flask 3 (which has the same treatment as flasks 4 group 1, flask 1 group 2, flask 1 group 4, flack 2 group 6 and flask 1 group 7) seems to have much more production of C_2H_4. That could be the result of experimental differences. Bacterial count of flask 2 was consistent with the results of gas chromatography analysis.

5.6. Group 6 (Sediment with N_2 & N-Fertilizers)

5.6.1. Denitrification

The results indicated that NH_4^+ cannot be used as alternative N source for denitrification. The MPN indicated that the initial inoculum of denitrifiers in soil samples was higher than that of sediment samples.

5.6.2. Nitrogen Fixation

There was no significant C_2H_4 produced in flasks 1, 2 and 4. In flask 1, due to the tack of an organic carbon source which agreed with the results of flask 1 and 2 group 1 and flask 2 group 3. In flask 3, there was an effect of NH_4^+ on nitrogenase which again agrees with the results of flask 2 group 2. In flask 4, the accumulation of N_2O affected the nitrogenase activity which is in agreement with the results of flask 3, group 2 and flask 4 group 3. Flask 2, which has the same treatment as flask 4 groups 1 and flask 1 group 2, gained much smaller amount of C_2H_4 production. The only difference between the first flask and the others is that the first has sediment sample which suggested that sediment may have less nitrogen fixing organisms than the soil. The data from the bacterial count supports this conclusion.

5.7. Group 7 (Sediment with N_2 and N-Fertilizers)

5.7.1. Denitrification

The results of flask 2 are in agreement with the results of flask 3 group 2, flask 4, group 3 and flask 4, group 6 which has similar treatments. The results of flask

3 indicated that aceteleglucosamine cannot be used as alternative N source for denitrification which is agreement with the results of flask 2, group 5. Flask 4 was expected to yield at least the same amount of N_2O as flask 4, group 5 which has no glucose (or flasks 1 and 2, group 5 and flask 3, group 7). The reason for the low production of N_2O is unknown.

5.7.2. Nitrogen Fixation

The results indicated that acetylglucosamine is not a recommended substrate for nitrogen fixation. The reasons for that are because either it is not easily utilizable substrate by nitrogen fixers or it may be broken down by some microorganisms to NH_4^+ which inhibits nitrogenase activity. Again the results indicated that the sediment sample had less initial nitrogen fixing organisms that the soil and bacterial count supported this fact too.

6. DISCUSSION

6.1. Denitrification

Acetylene (0.1 atm) caused complete inhibition of the reduction of N_2O to N_2 by denitrifiers. During the denitrification, bacteria oxidize organic substances completely to carbon dioxide concomitant with reduction of nitrate or nitrite to nitrous oxide and nitrogen. The ration of carbon dioxide to nitrogen in denitrifying culture corresponded to the theoretical ratio to be expected for complete oxidation of the organic substrate (Koeve and Kahler, 2010). The results of our experiment support this fact. Since CO_2 was produced, approximately at the same concentration in the absence and presence of C_2H_2 and N_2O only accumulated in the presence of C_2H_2 we can draw the conclusion that acetylene did not inhibit the reduction of NO_3^- to NO_2^- and then to N_2O by the organisms. It is, therefore, interesting that C_2H_2 should be an inhibitor of N_2O reductase. The reported effects of N_3^- and CN^- on denitrification are explained by the effects on specific cytochrome systems (Shoun and Tanimoto, 1991; Yang *et al*., 2010).

The fact that C_2H_2 specifically inhibits N_2O reduction and causes stoichiometric accumulation of N_2O during reduction of NO_3^- and NO_2^- suggests that N_2O is an obligatory intermediate in the sequence of steps between NO_3^- and N_2 (**Eq. 1**). This agrees with the findings of Yoshinari and Knowles (1976) and Cabrera *et al*. (2011).

Denitrification proceeded most efficiently as indicated by the appearance of CO_2 in systems with organic carbon which contained detectable quantities of N_2O in presence of C_2H_2. These observations suggest that the appearance of CO_2 and accumulation of N_2O would be suitable criteria for the presence of denitrifiers in appropriately enriched media. The most probably number (MPN) count supported this suggestion.

Since the results indicated that obligatory requirement for organic carbon for denitrification to take place, they also suggested the possibility of using acetylglucosamine as a carbon and energy source, not as nitrogen source. This is due to the fact that acetylglucosamine has a C:N ration of approximately 5:1 and some microorganisms may break it down to NH_4^+ (Scheepers and Raun, 2008) which is not the suitable nitrogen substrate for denitrifiers. It is also noted that NH_4^+ has no effect on denitrification activity. This was similar to the results reported by Park *et al*. (2010); Veillette *et al*. (2011) and Qiu *et al*. (2012).

6.2. Nitrogen Fixation

The acetylene reduction test has been used as an assay for nitrogen fixation activity. The results reported here support the validity of the C_2H_2 - C_2H_4 assay as sensitive analysis for nitrogen –fixing activity. In the assay, acetylene was reduced to ethylene by nitrogenase in which it acts as an alternative substrate to N_2 for the enzyme. Reduction of C_2H_2 to C_2H_4 (**Eq. 5**), like reduction of N_2 to $2NH_3$ (**Eq. 4**), requires and enzyme extracts containing nitrogenase and an energy source. The reduction product (ethylene) was easily measured by gas chromatography. C_2H_2 is the preferred assay substrate, since more products are formed because of its requirement for 2 electrons versus 6 electrons for N_2 (Hardy and Hevelka, 1975; Bertics *et al*., 2010).

Since the results indicated that obligatory requirement for organic carbon substrate for nitrogen fixation process to proceed, acetylglucosamine was not the suitable substrate for nitrogen fixation process, either because it was not easily utilizable substrate or some microorganisms broke it down to NH_4^+ which inhibits the nitrogenase activity (Park *et al*., 2010).

The presence of air in the gas phase supresses the nitrogenase activity while addition of H_2O encourages the anaerobic condition. NH_4^+ inhibited the nitrogenase activity.

7. CONCLUSION

The aim of the study was to gain information on the denitrification and nitrogen fixing activities in soil and sediment employing the acetylene technique and assuring the gas chromatography analysis by total plate count and most probably number. The results indicated that acetylene (0.1 atm) inhibited N_2O reduction and caused stoichiometric accumulation of N_2O during the

conversion of NO_3^- to N_2. N_2O was an obligatory intermediate in the sequence of steps between N_2O^- and N_2. The appearance of CO_2 and accumulation of N_2O would be suitable criteria for the presence of denitrifiers in appropriately enriched media and the acetylene reduction test is a suitable assay for nitrogen fixing activity. There was an obligatory requirement for organic carbon as a carbon and energy source for denitrification and nitrogen fixation to take place. The results showed that acetylglucosamine can be used as a carbon and energy source for denitrification but not as a nitrogen source (C:N ratio of 5). NH_4^+ has no effect on denitrification activity but it inhibited the nitrogenase activity. The presence of air in the gas phase affects both the denitrification and nitrogen fixing activity while adding H_2O encouraged anaerobic conditions.

8. ACKNOWLEGDEMENT

This research was funded by the Natural Science and Engineering Research Council (NSERC) of Canada.

REFERENCES

Bertics, V.J., J.A. Sohm, T. Treude, C.E.T. Chow and D.G. Capone *et al*., 2010. Burrowing deeper into benthic nitrogen cycling: the impact of bioturbation on nitrogen fixation coupled to sulfate reduction. Marine Ecol. Progress Ser., 409: 1-15. DOI: 10.3354/meps08639.

Bothe, H., S.J. Ferguson and W.E. Newton. 2007. Biology of the Nitrogen Cycle. 1st Edn., Elsevier, Amsterdam, ISBN-10: 0444528571, pp: 452.

Burgin, A.J., P.M. Groffman and D.N. Lewis, 2010. Factors regulating denitrification in a riparian wetland. Soil Sci. Soc. Am. J., 74: 1826-1833.

Burns, R.C. and R.F. Hardy, 1975. Nitrogen Fixation in Bacteria and Higher Plants. 1st Edn., Springer, Berlin, ISBN-10: 038707192X, pp: 189.

Cabrera, J.J., C. Sanchez, A.J. Gates, E.J. Bedmar and S. Mesa *et al*., 2011. The nitric oxide response in plant-associated endosymbiotic bacteria. Biol. Soc. Trans., 39: 1880-1998. DOI: 10.1042/BST20110732.

Calderer, M., O. Gibert, V. Marti, M. Rovira and J.D. Pablo *et al*., 2010. Denitrification in presence of acetate and glucose for bioremediation of nitrate-contaminated groundwater. Environ. Technol., 31: 799-814. DOI: 10.1080/09593331003667741.

Cranfield, D.E., A.N. Glazer and P.G. Falkowski, 2010. The evolution and future of earth's nitrogen cycle. Science, 330: 192-196. DOI: 10.1126/science.1186120.

Dalton, H. and R. Whittenbury, 1976. The acetylene reduction technique as an assay for nitrogenase activity in the methane oxidizing bacterium *Methylococcus capsulatus* strain bath. Arch. Microbiol., 109: 147-151. DOI: 10.1007/BF00425127.

Dandie, C.E., D.L. Burton, B.J. Zebarth, T.J. Trevors and C. Goyer, 2007. Analysis of denitrification genes and comparison of *nosZ*, *cnorB* and 16S rDNA from culturable denitrifying bacteria in potato cropping systems. Syst. Applied Microbiol., 30: 128-138. DOI: 10.1016/j.syapm.2006.05.002.

Gaby, J.C. and D.H. Buckley, 2011. A global census of nitrogenase diversity. Environ. Microbiol., 13: 1790-1799. PMID: 21559425.

Ghaly, R.A., J.B. Pyke, A.E. Ghaly and V.I. Urgusal, 1999. Physical and thermochemical properties of uncontaminated and diesel-contaminated peat. Energy Sour., 21: 433-451. DOI: 10.1080/00908319950014740.

Hallin, S. and P.E. Lindgren, 1999. PCR detection of genes encoding nitrite reductase in denitrifying bacteria. Applied Environ. Microbiol., 65: 1652-1658.

Hara, S., Y. Hashidoko, R.V. Desyatkin, R. Hatano and S. Tahara, 2009. High rate of N_2 fixation by east siberian cryophilic soil bacteria as determined by measuring acetylene reduction in nitrogen-poor medium solidified with gellan gum. Applied Environ. Microbiol., 75: 2811-2819. DOI: 10.1128/AEM.02660-08.

Hardy, R.W. and U.D. Havelka, 1975. Nitrogen fixation research: A key to world food? Science, 188: 633-642. DOI: 10.1126/science.188.4188.633.

Hofstra, N. and A.F. Bouwman, 2005. Denitrification in agricultural soils: Summarizing published data and estimating global annual rates. Nutr. Cycl. Agroecosyst., 72: 267-278. DOI: 10.1007/s10705-005-3109-y.

Jetten, M.S., 2008. The microbial nitrogen cycle. Environ. Microbiol., 10: 2903-2909. PMID: 18973618.

Jia, Z. and R. Conrad, 2009. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environ. Microbiol., 11: 1658-1671. PMID: 19236445.

Keeney, D.R., 1973. The nitrogen cycle in sediment-water systems. J. Environ. Q., 2: 15-29.

Kim, M., S.Y. Jeong, S.J. Yoon, S.J. Cho and Y.H. Kim *et al*., 2008. Aerobic denitrification of *Pseudomonas putida* AD-21 at different C/N ratios. J. Biosci. Bioeng., 106: 489-502. DOI: 10.1263/jbb.106.498.

Koeve, W. and P. Kahler, 2010. Heterotrophic denitrification vs. autotrophic anammox-quantifying collateral effects on the oceanic carbon cycle. Biogeosciences, 7: 2327-2337. DOI: 10.5194/bg-7-2327-2010.

Larrainzar, E., S. Wienkoop, C. Scherling, S. Kempa and R. Ladera *et al*., 2009. Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in *Medicago truncatula* under drought and recovery. Mol. Plant-Microbe Interact., 22: 1565-1576. DOI: 10.1094/MPMI-22-12-1565.

Leigh, J.A. and J.A. Dodsworth, 2007. Nitrogen regulation in bacteria and archaea. Annual Rev. Microbiol., 61: 349-377. DOI: 10.1146/annurev.micro.61.080706.093409.

Lin, Y.M., J.H. Tay, Y. Liu and Y.T. Hung, 2009. Biological nitrification and denitrification processes. Biolog. Treatment Processes, Handbook Environ. Eng., 8: 539-599. DOI: 10.1007/978-1-60327-156-1_13.

Nakajima, Y., S. Ishizuka, H. Tsuruta, A. Iswandi and D. Murdiyarso. 2005. Microbial processes responsible for nitrous oxide production from acid soils in different land-use patterns in Pasirmayang, central Sumatra, Indonesia. Nutr. Cycl. Agroecosyst., 71: 33-42. DOI: 10.1007/s10705-004-1196-9.

Newton, W.E. and M.J. Dilworth, 2011. Assays of nitrogenase reaction products. Methods Mol. Biol., 766: 105-127. PMID: 21833864.

Ohyama, T., A. Yamazaki, N. Yamashita, T. Kimura and S. Ito *et al*., 2008. Mechanism of quick and reversible inhibition of soybean nodule growth and nitrogen fixation activity by nitrate and its metabolites. Curr. Plant Sci. Biotechnol. Agric., 42: 89-90. DOI: 10.1007/978-1-4020-8252-8_29.

Park, S., W. Bae and B.E. Rittmann, 2010. Operational boundaries for nitrite accumulation in nitrification based on minimum/maximum substrate concentrations that include effects of oxygen limitation, pH and free ammonia and free nitrous acid inhibition. Environ. Sci. Technol., 44: 335-342. DOI: 10.1021/es9024244.

Patriquin, D.G. and R. Knowles, 1974. Denitrifying bacteria in some shallow-water marine sediments: Enumeration and gas production. Can. J. Microbiol., 20: 1037-1041. DOI: 10.1139/m74-160.

Perez, C.A., M.R. Carmona and J.J. Armesto, 2010. Non-symbiotic nitrogen fixation during leaf litter decomposition in an old-growth temperate rain forest of Chiloe Island, southern Chile: Effects of single versus mixed species litter. Austral Ecol., 35: 148-156. DOI: 10.1111/j.1442-9993.2009.02020.x.

Qiu, X., T. Wang, X. Zhong, G. Du and J. Chen, 2012. Screening and characterization of an aerobic nitrifying-denitrifying bacterium from activated sludge. Biotechnol. Bioprocess Eng., 17: 353-360. DOI: 10.1007/s12257-011-0467-y.

Reed, S.C., C.C. Cleveland and A.R. Townsend, 2011. Functional ecology of free-living nitrogen fixation: A contemporary perspective. Annual Rev. Ecol. Evolut. Syst., 42: 489-512. DOI: 10.1146/annurev-ecolsys-102710-145034.

Robertson, L.A. and J.G. Kuenen, 1990. Combined heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha* and other bacteria. Antonie Van Leeuwenhoek, 57: 139-152. DOI: 10.1007/BF00403948.

Ross, K.A., J.G. Ehrenfeld and M.V. Patel, 2011. The effects of nitrogen addition on the growth of two exotic and two native forest understory plants. Biol. Ivasions, 13: 2203-2216. DOI: 10.1007/s10530-011-0034-7.

Salvagiotti, F., K.G. Cassman, J.E. Specht, D.T. Walters and A. Weiss *et al*., 2008. Nitrogen uptake, fixation and response to fertilizer N in Soybeans: A review. Field Crops Res., 108: 1-13. DOI: 10.1016/j.fcr.2008.03.001.

Santos, P.C.D., Z. Fang, S.W. Mason, J.C. Setubal and R. Dixon, 2012. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics, 13: 162-162. DOI: 10.1186/1471-2164-13-162.

Scheepers, J.S. and W.R. Raun, 2008. Nitrogen in Agricultural Systems. 1st Edn., ASA, Madison, Madison, ISBN-10: 0891181644, pp: 965.

Senbayram, M., R. Chen, A. Budai, L. Bakken and K. Ditter, 2012. N2O emission and the $N_2O/(N_2O+N_2)$ product ratio of denitrification as controlled by available carbon substrates and nitrate concentrations. Agric., Ecosyst. Environ., 147: 4-12. DOI: 10.1016/j.agee.2011.06.022.

Shoun, H. and T. Tanimoto, 1991. Denitrification by the fungus F*usarium oxysporum* and involvement of cytochrome P-450 in the respiratory nitrite reduction. J. Biol. Chem., 266: 11078-11082.

Torrento, C., J. Cama, J. Urmeneta, N. Otero and A. Soler, 2010. Denitrification of groundwater with pyrite and *Thiobacillus denitrificans*. Chem. Geol., 278: 80-91. DOI: 10.1016/j.chemgeo.2010.09.003

Tremblay, N., Z. Wang and Z.G. Cerovic, 2012. Sensing crop nitrogen status with fluorescence indicators. A review. Agron. Sustain. Dev., 32: 451-464. DOI: 10.1007/s13593-011-0041-1.

Trevors, J.T., 2010. One gram of soil: A microbial biochemical gene library. Antonie Van Leeuwenhoek, 97: 99-106. PMID: 19921459.

Veillette, M., P. Veins, A.A. Ramirez, R. Brzezinski and M. Heitz, 2011. Effect of ammonium concentration on microbial population and performance of a biofilter treating air polluted with methane. Chem. Eng. J., 171: 1114-1123. DOI: 10.1016/j.cej.2011.05.008.

Vitousek, P.M. and S. Hobbie, 2000. Heterotrophic nitrogen fixation in decomposing litter: Patterns and regulation. Ecology, 81: 2366-2376. DOI: 10.1890/0012-9658(2000)081[2366:HNFIDL]2.0.CO;2.

Ward, B.B., 1995. Diversity of culturable denitrifying bacteria: Limits of rDNA RFLP analysis and probes for the functional gene, nitrite reductase. Arch. Microbiol., 163: 167-175.

Warneke, S., L.A. Schipper, M.G. Matiasek, K.M. Scow and S. Cameron *et al*., 2011. Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds. Water Res., 45: 5463-5475. DOI: 10.1016/j.watres.2011.08.007.

Yang, Y., A. Dey and R.A. Decreau, 2010. Spectroscopic characterization and competitive inhibition studies of azide binding to a functional NOR model. Eur. J. Inorganic Chem., 2010: 4870-4874. DOI: 10.1002/ejic.201000566.

Yoshinari, T. and R. Knowles, 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. 69: 705-710. DOI: 10.1016/0006-291X(76)90932-3.

Zhao, Y., M. Xu and J. Belnap, 2010. Potential nitrogen fixation activity of different aged biological soil crusts from rehabilitated grasslands of the hilly Loess Plateau, China. J. Arid Environ., 74: 1186-1191. DOI: 10.1016/j.jaridenv.2010.04.006.