Relation between abundance of nirS containing denitrifying bacteria and soil nitrogen as well as other nutrient levels

1. Introduction:

The current project to look into soil microbiota of an experimental farm forms part of a precision farming project called Tru-Nject (Tru-Nject, 2015). The project involves design of a subsurface nitrogen injector using nutrient maps to ensure efficient distribution of nitrogen (Tru-Nject, 2015). A gap in the project was the study of soil microbes and how their distribution was influenced by varying nitrogen administration. Although PCR has emerged as a method of determining soil microbe species, the amplicons are usually sequenced or qPCR is performed (Li *et al*., 2014)(Brümmer *et al*., 2003)(Janssen, 2006)(Throbäck *et al*., 2004)(Braker *et al*., 2000)(Braker *et al*., 1998). Sequencing is not usually available to all labs and is considerably expensive as is qPCR (Sue *et al*., 2014). Studies on a new method called PCR-ELISA have recently been published. It is used as a semi-quantitative method to determine presence and abundance of bacteria and viruses in a wide range of samples, usually for clinical diagnosis and for food pathogen detection (Sue *et al*., 2014). A PCR-ELISA protocol for detection of certain soil microbes was, therefore, sought. The following explains how the type of bacterium for the soil study was determined, factors influencing distribution of the bacteria and effect of agriculture on soil bacterial communities. Finally, a short explanation of how the PCR-ELISA protocol for the bacteria was designed is also mentioned.

Dominant bacterial phyla in soil are usually proteobacteria, actinobacteria and acidobacteria (Janssen *et al* 2006). Proteobacteria have an average composition of 39%, Acidobacteria 20% and actinobacteria 13%, although there is much variation in this composition depending on various biotic and abiotic factors (Janssen, 2006). Presence of a carbon source for energy is a requirement for microbial growth (Hoorman and Islam, 2010). Although fungi tend have a higher biomass than bacteria as they dominate soils, they do not thrive well in tilled soils (Hoorman and Islam, 2010). Therefore, soils frequently tilled and treated with herbicide have an abundance of bacteria; these bacteria form most of the decomposition and nutrient cycles in cultivated soils. (Moore and De Ruiter, 1991)(Lovell *et al.,* 1995)(Allison *et al.,* 2005). Undisturbed and untilled soils usually have higher microbe levels, more readily available carbon, soil organic matter and stored carbon than cultivated soils (Hoorman and Islam, 2010).

Studies on the effect of agriculture on soil microbial communities have shown the importance of environmental factors in controlling microbial community composition; the following factors can be ranked from most to the least important: soil type, time, type of farming operation (e.g., use of cover crop), management system, spatial variation of crop field (García-Orenes *et al.,* 2013). Soil organic matter content is one of the major influences on microbial community composition (García-Orenes *et al.,* 2013).

Effects of specific agricultural activities on soil microbes have also been determined. Herbicide use, for example, is known to be toxic to soil microbes and is a limiting factor in their growth (Zurnoza *et al.,*

2009). Organic matter is linked with higher soil microbial biomass (Ruppel *et al.,* 2007)(Frostegård *et al.,* 2011). In terms of soil water content, study on flooded soil showed an effect on soil microbial community composition as certain electron acceptors in flooded soils affect microbial community composition (Sylvia *et al.,* 1999). Focusing on nitrogen availability, nitrogen is known to influence microbe abundance (Vitousek & Howarth 1991). It is also known that with increase agricultural activities, the rate of nitrogen deposition and fixation has doubled (Vitousek *et al.,* 1997). Effect of nitrogen additions on soil nutrient and microbe levels is summarised in Figure 1 (Treseder, 2008). A study on nitrogen gradient fields (a grassland and an agricultural field) showed nitrogen addition lead to increased amounts of gammaproteobacteria and actinobacteria, with a decrease in acidobacteria, cyanobacteria and nitrospora (Ramirez *et al.,* 2010). In the grassland, 21 bacterial groups and 30 bacterial groups in the agricultural field were linked with changes in added nitrogen (N) (Ramirez *et al.,* 2010). There were also changes in soil pH. Although bacteria are sensitive to soil pH, this study showed that bacterial composition was more dependent on N levels and carbon availability than changes in pH or the plant community due to N additions (Ramirez *et al.,* 2010). However, for pH less than 6.5 negative correlation with bacterial diversity was observed (Ramirez *et al.,* 2010).

N additions in tropical forest soil reduce soil microbial biomass, with populations reaching initial levels in the long term (Liu *et al.,* 2013). Reduction in microbe levels was also reported in other studies (Soderstrom *et al*., 1983)(Nohrstedt *et al*., 1989). The initial decrease could be due to soil osmotic potentials reaching toxic levels from introduction of ions in fertilizers (Broadbent *et al.,* 1965). The increase in number could be due to bacterial adaptation to increased N levels (Liu *et al.,* 2013). The decrease in pH caused by N addition could lead to leeching of magnesium and calcium and mobilization of aluminium (Vitousek *et al.,* 1997). Microbes, especially denitrifying bacteria, are known to have higher abundance close to plant roots (Linne von Berge and Bothe, 1992). Excess nitrogen would mean plants would be less prone to invest in fine roots as these structures become less important for nutrient uptake (Aerts & Chapin 2000; Treseder 2004). Consequently, reduced carbon would be deposited in the soil from root turnover (Soderstrom *et al.,* 1983; Fog 1988). This was in contrast to other studies on tropical forest soil microbial biomass where microbial biomass temporarily increased (Zhang and Zak, 1998) but decreased over a long time period (Wallenstein *et al.,* 2006)(Arnebrant 1996)(Corre *et al.,* 2003)(DeForest *et al.,* 2004). Other studies on tropical forests concluded no considerable change in microbial biomass due to N addition but reduced gram negative bacterial abundance (Liu *et al.,* 2015).

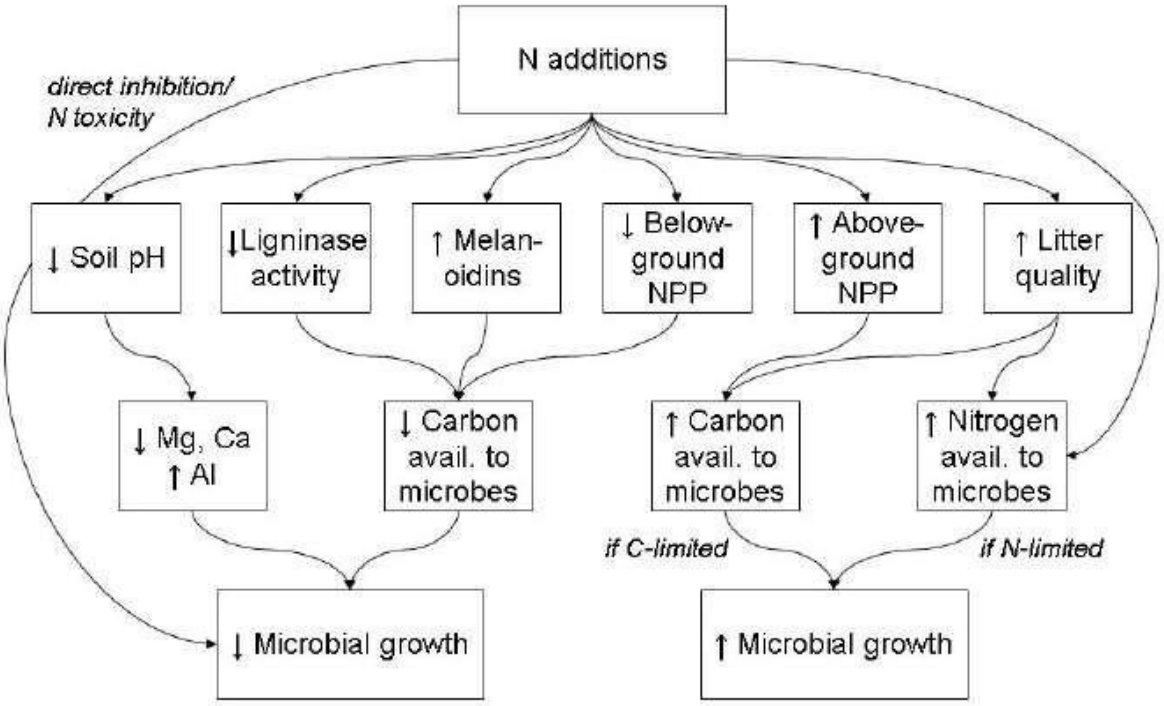

Figure 1: Effects of nitrogen additions on soil microbes and abiotic factors (Treseder, 2008)

More use of nitrogen fertilizers in agricultural fields is thought to be the main cause of increases in global N_2O levels and soils produce about 57% of global N_2O emissions (Mosier *et al.,* 1998).
N_2O is a greenhouse gas and has a global warming potential 298 times that of carbon dioxide (IPCC, 2007)(Crutzen *et al.,* 1970) (Dickinson *et al.,* 1986). It accounts for 7.9% (CO2 equivalent emission) of anthropogenic greenhouse gas emissions (Forster *et al.,* 2007). The rate of its emissions in the atmosphere is increasing at approximately 0.3% per year. In anaerobic conditions, microbial denitrification reduces nitrate (NO3-) from nitrogenous fertilizers (Dandie *et al.,* 2008). Denitrification forms part of the nitrogen cycle and reduces oxides of nitrogen and releases nitrogen gas back into the atmosphere (Fondreidst, 2015). Through the application of N fertilizer, agricultural soils often contain elevated NO3- levels and previous studies have reported high rates of denitrification and N_2O emissions in agricultural soils, especially in humid environments (Bateman & Baggs, 2005; Hofstra & Bouwman, 2005).

Incomplete denitrification releases N_2O (Knowles, 1982), as not all denitrifying bacteria possess the enzymes required for complete denitrification (Ingraham, 1981). Studies suggest that most bacteria that are selected to anaerobically reduce NO^{3-} by using it as a terminal electron acceptor only reduce NO^{3-} to N2O (Chèneby *et al.,* 1998, Gamble *et al.,* 1977, Germon 1985) This process evidently leads to loss of nitrogen from agricultural soils (Dandie *et al.,* 2011). In Canada, N2O is the main greenhouse gas from agricultural production (Helgason *et al.,* 2005).

Limited oxygen supply in agricultural soils is linked with N_2O emissions. This could be due to high water-filled pore space (WFPS) (Bateman and Baggs, 2005) or usage of oxygen due to readily usable carbon (Gillium *et al.,* 2008). An important factor controlling denitrification rates is soil water content, since soil aeration, temperature, NO_3, N and carbon availability depend on soil moisture (Mosier, 1998)(Payne and Zebarth, 1997)(Ruser *et al.*, 2006). Soil chemical and physical processes affecting denitrification rates are also known (Hofstra and Bowman, 2005).

Bacteria are known to complete the denitrification step of the nitrogen cycle. These are facultative anaerobes and in the absence of free oxygen use nitrate (NO^{3-}) and nitrite (NO^{2-}) as alternative terminal electron acceptors and reduce them to gaseous nitric oxide (NO), nitrous oxide (N_2O) and then to nitrogen gas (N_2) (Chéneby *et al.*, 1998). Besides soil denitrification, they are utilised in waste water treatments for excess nitrogen removal and degradation of organic pollutants (Fries *et al.,* 1994, Hess *et al.,* 1997, Zhou *et al.,* 1997). Denitrifiers belong to different groups of bacteria including proteobacteria as well as thermophilic and halophilic archaea (Throbäck *et al.*, 2004). The distribution of denitrifiers in a wide range of phylogenetic groups is thought to be due to lateral gene transfer (Bothe *et al*., 2000).

Studies focusing on denitrificaiton rates and N2O emissions and their association with denitrifying bacterial abundance have shown different results. Quite a few studies determined that denitrification rates and N_2O emissions do not correlate with denitrifying bacteria levels in soil (Dandie *et al.,* 2011). This was also mentioned in 2 studies in riparian and agricultural zones (Rich & Myrold,2004) (Dandie *et al.*, 2011), in denitrifier communities in cultivated and uncultivated wetlands (Ma *et al*., 2008), in marsh sediment (Cao *et al*., 2008) and potato field soil (Dandie *et al*., 2008) and in a study on diversity of denitrifying bacteria (Chèneby *et al*., 1998).

Other studies, however, have shown correlation between denitrifier abundance and N_2O emission or denitrification rates in 8 crop cultivations with differing systems of management (Morales *et al*., 2010) and in estuary sediments (Dong *et al*., 2009). As mentioned in Dandie *et al.*, (2011) determining factors relating denitrifying bacterial abundance to denitrification rates or N_2O emission rates is difficult since highly diverse bacterial species possess denitrification ability and the effect of soil properties and other factors also have to be included.

Functional genes (nirK, nirS, cnorB, qnorB, nosZ) in the denitrification pathway have been used as genetic markers to determine denitrifier abundance and diversity (as reviewed in Philippot *et al*., 2007). This is done by using conserved regions of the functional genes (Throbäck *et al*.. 2004). Ability to reduce nitrite (NO^{2-}) to nitric oxide (NO) is a distinguishing feature of denitrifiers (Zumft, 1997). This important process in denitrification is catalysed by 2 forms of nitrite reductases: me cd1 enzyme from the nirS gene and a copper containing enzyme encoded by the nirK gene (Throbäck *et al*., 2004). The nitrous oxide produced is then reduced in the final step to dinitrogen by the nitrous oxide reductase encoded by nosZ (Throbäck *et al*., 2004).

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nirK and nirS are mutually exclusive in a bacterial strain (Coyne, *et al.,* 1989). They are functionally the same (Glockner *et al.,* 1993, Zumft *et al.,* 1997). Various methods have been developed to detect these genes in soil bacteria. This involved the use of antisera against the dissimilatory nitrite reductases (dNirS (Coyne *et al.,* 1989, Southern 1975, Ward *et al.,* 1993) and dNirK (Coyne *et al.,* 1989, Michalski *et al.,* 1988). Gene probes were also used: nirK (Kloos *et al*., 1995)(Ye *et al*., 1992) and nirS (Kloos *et al*., 1995), Linne Von Berg and Bothe, 1992)(Southern, 1975)(Ward *et al*., 1993). The probes, however, had various issues. Although they were specific for the strains they were designed for, the nirK probe hybridised with other nir containing strains (Ye *et al*., 1992) and the nirS probe hybridised to a low number of bacterial strains (Southern, 1975)(Ward, 1995).

Molecular biology is now frequently used to detect nirk, nirs and nosz (Braker *et al*., 2000)(Scala and Kerkhof, 1999) with PCR being the main method of detection of denitrifying bacteria in soil and other environmental samples (Ward *et al.,* 1995, Cascioti *et al.,* 2001, Hallin *et al.,* 1999, Mergel *et al.,* 2001, Rösch *et al.,* 2002, Yan *et al.,* 2003). Studies on denitrifying bacterial communities in soils have shown the presence of nirK and nirS genotypes from uncultured denitrifying bacteria and 500 different nirK sequences have been isolated, the majority form soil samples (Molecular studies on denitrifier communities: (Bremer *et al*., 2007(Falk *et al*., 2007)(Hallin and Lindgren, 1999)(Hannig *et al.,* 2006)(Henry *et al*., 2004)(Henry *et al*., 2008)(Throbäck *et al*., 2004)(Wolsing and Priemè, 2004)(Yan *et al*., 2003) (Isolation of 500 nirk sequences (Avrahami *et al*., 2002)(Bremer *et al*., 2007) (Priemè *et al*., 2002)(Wolsing and Priemè, 2004)(Zhang *et al*., 2006). Primer design of nir and nos genes has been a focus of many studies (Ward *et al.,* 1995, Cascioti *et al.,* 2001, Hallin *et al.,* 1999, Mergel *et al.,* 2001, Rösch *et al.,* 2002, Yan *et al.,* 2003, Braker *et al.,* 2000, Braker *et al.,* 2001, Liu *et al.,* 2003, Scala *et al.,* 1999, Scala *et al.,* 1998, Scala *et al.,* 2000, Wu *et al.,* 2001). The nirk and nirs gene sequences were made using a small number of known denitrifying bacterial gene sequences (Throbäck *et al*., 2004). Since then known denitrifying bacterial sequences have increased and a reassessment of amplification efficiency of published nirk and nos primers was done (Throbäck *et al*., 2004). New primers were designed and their amplification was also assessed. A pair of new primers each for nirK and nirS was suggested to be more efficient at amplification than previously suggested primers (Throbäck *et al.,* 2004).

Factors influencing nirk and nirS levels in soil bacterial communities have been suggested. pH has been shown to be the dominant factor responsible for varying levels of nirK and nirS gene numbers in the soil (Dandie *et al*., 2011). Total copper content of soil is said to be the second most important variable responsible for nirK gene abundance (Bru *et al*., 2011)(Enwall *et al*., 2010). Spatial and temporal abundance of nirK, nirS and nosZ bacteria abundance was shown to be stable in an agricultural field with the exception of nirK, which showed temporal variation (Dandie *et al.,* 2011). The stability was despite the tillage methods and plant species differing between the different regions. A study of agricultural and

riparian lands reported a small response of the community to different usage of land (Dandie *et al.,* 2011). nirK showed greater difference in abundance than nirS between agricultural and riparian land. nirK containing bacteria seemed more sensitive to different land uses than nirs containing bacteria (Dandie *et al*., 2011). This was also the case on plant succession on a land (Smith & Ogram, 2008) and for different crop management systems (Enwall *et al*., 2010). Study on primary succession in glacial foreland showed changes in nirS gene abundance and no significant changes in nirk gene abundance (Kandeler *et al.,* 2006). In contrast study on potato crop growth showed variation in nirK levels in soil throughout the growing season (Dandie *et al*., 2008). Dandie *et al.,* (2008), however, reported that no factors seemed to affect levels of nirk, nosZ and another denitrification gene, cnorB, in a crop. However, nirK levels seemed to decrease with crop growth. Week correlation between cnorB and extractable organic carbon and temperature was, however, reported (Dandie *et al.,* 2008). The same outcome was also reported for denitrifying bacteria in a grassland (Philippot *et al*., 2009).

A major point of discussion is the link between denitrification rates and N_2O emissions with denitrifying bacteria levels. Contradictory outcomes have been reported. Some studies suggest no correlation such as 2 studies in riparian and agricultural zones (Rich & Myrold,2004) (Dandie *et al.*, 2011), in denitrifier communities in cultivated and uncultivated wetlands (Ma *et al*., 2008), in marsh sediment (Cao *et al*., 2008) and potato field soil (Dandie *et al*., 2008).
Some studies, however, have shown correlation in 8 crop cultivations with differing systems of management (Morales *et al*., 2010) and in estuary sediments (Dong *et al*., 2009). As mentioned in Dandie *et al.* (2011) determining factors relating denitrifying bacterial abundance to denitrification rates or N_2O emission rates is difficult since highly diverse bacterial species possess denitrification ability and the effect of soil properties and other factors have to be included.

The aim of this study, therefore, is to determine abundance of nirs containing bacteria in four different crops in the Tru-Nject project and its relation to nitrogen levels in the soil (abundance will also be correlated to pH and other soil nutrients). PCR-ELISA will be used as the method for detection of the bacteria as a cheaper alternative to qPCR (Sue *et al*., 2014). PCR-ELISA has been applied as a diagnostic tool for HIV, leishmania, salmonella, listeria monocytogenes and for detection of other bacteria and viruses in clinical samples (Sue *et al*., 2014). The process is illustrated in figure 2 and utilises the binding abilities of biotin and streptavidin as well as DIG and anti-DIG peroxidase conjugate antibody to detect labelled target genes (Sue *et al*., 2014). The binding produces a colour that can be quantified using a spectrophotometer (Sue *et al*., 2014). DIG labelled dUTP is added in the PCR step which incorporates in the newly amplified gene (Sue *et al*., 2014). A biotin labelled probe is designed complementary to a region of the amplified gene (Sue *et al*., 2014). The probe is hybridised with the amplified gene (Sue *et al*., 2014). The hybridised solution is added to streptavidin coated 96 well plates to allow binding of biotin on the probe to streptavidin (Sue *et al*., 2014). Anti-DIG peroxidase conjugate antibody with ABTS

substrate solution is then added which binds to DIG dUTP (Sue *et al*., 2014). The plates are then left for color development and later read on an ELISA plate reader (Sue *et al*., 2014).

In order to study the effect of nitrogen application on soil bacterial abundance it was initially thought to

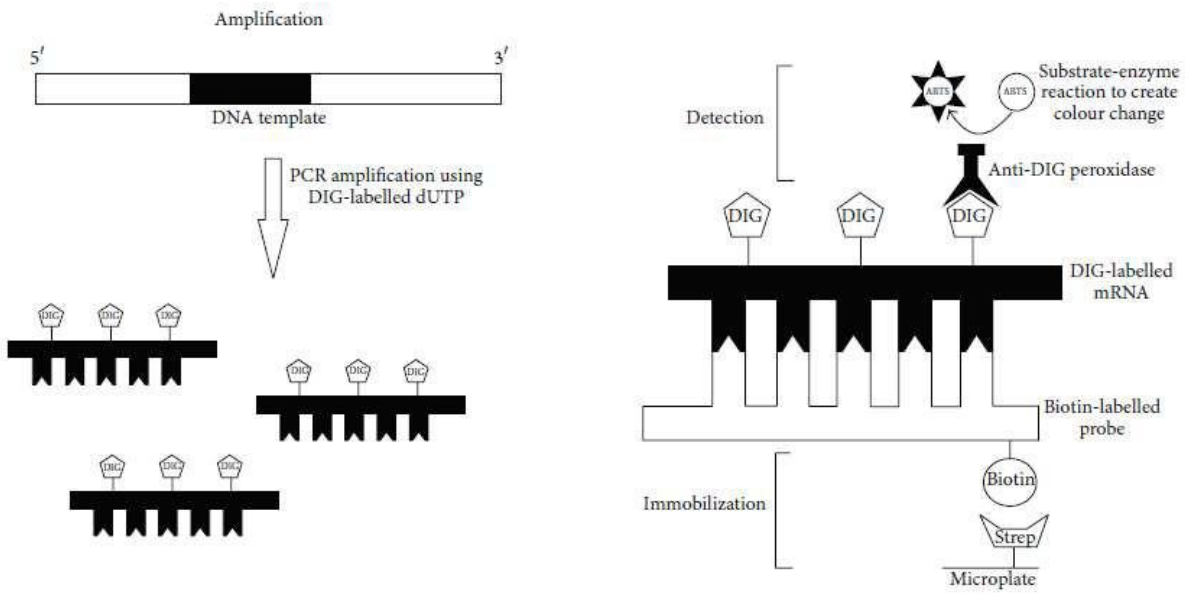

Figure 2: Illustration of PCR-ELISA: DIG labelled dUTP in the PCR step is incorporated in the amplified target gene. Hybridization of amplicon with biotin labelled probe is followed by attachment of biotin to streptavidin coated on a 96 well microplate. Anti-DIG peroxidase conjugate antibody with ABTS substrate solution is then added which produces a blue-green color change that can be detected by an ELISA plate reader (Sue *et al*., 2014).

use most dominant soil bacteria. However, PCR amplification of the dominant soil bacteria such as proteobaceria, actinobacteria and acidobacteria are based on 16S rRNA sequences (Janssen, 2006). 16S rRNA genes are ubiquitous in bacteria (Janssen, 2006). The difference in 16S rRNA sequences in different types of bacteria are used to detect different bacterial strains (Janssen, 2006). However, this method would not be suitable for PCR-ELISA as chances of non-specific amplification and hybridisation would be high in soil samples. An approach to target functional genes as molecular markers was, therefore sought. Since the study involved the effect of nitrogen application on soil microbes and because high N_2O production from agricultural soils is reported, the study was focused on determining abundance of denitrifying bacteria. Although there is contradictory evidence on the correlation between N_2O emission and denitrification rates with denitrifying bacteria levels, an understanding of the composition of soil denitrifying bacteria is necessary. There is also a need to design a widely available and cheaper method of detecting abundance of denitrifying bacteria. The nirk and nirs genes will be amplified as they have been a focus of soil molecular biology techniques with several publications on their PCR amplification.

The availability of these amplification protocols means carrying out the PCR- ELISA protocol would require minor changes to published amplification methods for the genes.

A general overview of the methods is as follows: Following soil DNA extraction, soil DNA was amplified using universal bacterial primers Eub 338 and Eub 518 (Fierer *et al.,* 2004). Then, PCR for nirK and nirS was optimised, first using the MJ Research PTC-100 thermal cycler (MJ Research Inc., St. Bruno, Quebec, Canada) and later on using BIOER's Life Touch Thermal cycler (Hangzhou Bioer Technology Co. Ltd., China), as the Life Touch thermal cycler had a higher ramp rate and PCR tubes fitted in its wells better. This was then followed by PCR of nirS using Digoxigenin-11-dUTP and then ELISA of DIG-dUTP labelled nirS amplicons to make dilution curves of absorbance vs. dilution.

Gel electrophoresis was also performed to determine relative amounts of nirS amplicons in soil samples and imageJ used to give relative intensity of PCR bands. Relative gel band intensity was then correlated to different soil nutrient levels such as total nitrogen including pH using Pearson's correlation co-efficient test.

2.**Materials and methods:**

2.1 Soil Sampling and storage:

Soil sampling was performed using a soil corer. Around 5cm of sample in the corer (with a diameter of 3 cm) at a depth of 7 – 10 cm from the soil surface was obtained for analyses. Soil samples were placed in individual plastic bags and placed in a heat insulated cool-box containing ice bags to keep samples cool.

Samples were frozen at below – 20 C within 4 hours of sampling.

2.2 Soil DNA extraction:

Soil DNA extraction was performed using the Powersoil® DNA isolation kit (MO BIO Laboratories, Carlsbad, California, USA) as per the manufacturer's instructions. Prior to soil DNA extraction, each soil sample was thawed for 45 min – 2 hours and the sample mixed and 0.25 g weighed out and used for DNA extraction.

2.3 DNA concentration measurement:

Soil DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (software version 3.7.1) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The Nanodrop was blanked using C6 solution of MO BIO's PowerSoil® DNA isolation kit. 1 ul of samples. Atleast 3 repeats of each sample were measured and the average taken.

2.4 PCR amplification of universal bacterial primers:

5 soil DNA samples from cabbage crop and one pond sediment were used. PCR amplification was performed in a 25 ul reaction using 1x Phusion U Hotstart DNA Polymerase High Fidelity buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 200 uM dNTPs (Sigma-Aldrich, St. Louis, Missouri, USA), 1 uM forward primer (Eub 338), 1 uM reverse primer (Eub 518) (Fierer *et. al.*, 2004), 0.02 U/ul of Phusion U Hotstart DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1000 ng/ul BSA (Sigma-Aldrich, St. Louis, Missouri, USA) and 3% DMSO (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Thermal cycler used was MJ Research's PTC-100 thermal cycler (MJ Research, St. Bruno, Quebec, Canada). 5ul of soil DNA was used. Primers were ordered from Sigma-Aldrich (Sigma-Aldrich, St. Louis, Missouri, USA). Refer to table 1 for primer sequences.

25 ul of PCR reaction was used as this was used in the paper by Throwback *et al*. (2004) and Braker *et al.* (1998). The polymerase was chosen owing to its HotStart function and short extension time of 15-30 sec/kp (For further details see reference: Thermo Fisher Scientific (2015). Buffer, dNTP and polymerase concentrations were as recommended by the protocol for the polymerase (protocol can be found using reference: Thermo Fisher Scientific (a) (2015). Primer concentrations were chosen based on methods in Braker *et al.*, (2000) where the concentration of primer used (1 uM) was twice that in the 1998 publication (Braker *et al.,* 1998).

Cycling conditions included an initial denaturation at 98 C for 3 min and 35 cycles of denaturation at 98 C for 40 seconds, primer annealing at 68.9 C for 1 min and extension at 72 C for 1 min, followed by a final extension at 72 C for 10 min. Primer annealing temperatures for all PCR reactions were determined using Thermo Fisher Scientific's Tm calculator webpage (for further details see reference: Thermo Fisher Scientific (b) (2015)). This polymerase was used in all PCR reactions.

PCR product size and relative amount was determined by gel electrophoresis using 2% agarose gels (Bio-Rad Laboratories, Hercules, California, USA). SYBR® Safe DNA gel stain (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for visualising gels under blue light using a blue light transilluminator (Pearl Biotech, San Francisco, Calfornia, USA).

2.5 Optimisation of PCR reaction for universal bacterial primers:

PCR optimisation was performed in terms of reagent concentrations and cycling conditions.

Use of BSA (Bovine Serum Albumin) in PCR of soil DNA had been suggested by Braker *et al.* (2000). To confirm whether this was needed, PCR was performed with and without the reagent. Reagent concentrations were the same as subsection 2.4, except no DMSO was used. Cycling conditions were also the same as in subsection 2.4, except that 40 PCR cycles were performed. 40 cycles were initially used to determine whether a PCR product would be seen at this cycle number. The initial concentration of BSA (1000 ng/ul) was based on the same concentration used in Throwback *et al.,* (2004) for nirS PCR of environmental samples.

DMSO was suggested to be used to amplify GC rich amplicons in the instructions provided for use of the Phusion U Hotstart DNA polymerase (Thermo Fisher Scientific (a), 2015). PCR reactions were also performed with and without DMSO and at reduced cycle number (35 instead of 40, which was the case with BSA). This was used to determine whether any difference to the PCR product was made. Reagent concentrations and cycling conditions were the same as in subsection 2.4 and BSA was used.

Tube wells of the MJ Research PTC-100 thermal cycler were designed for custom designed ELISA plates and not PCR tubes. As such 0.2 ml and 0.5 ml PCR tubes did not fit the thermal cycler and mineral oil was added to the wells to allow thermal contact with the tubes. To allow sufficient time for thermal transfer, time for each denaturation, primer annealing and extension step was raised to 30 seconds more than that recommended by the Phusion U Hotstart polymerase protocol (Thermo Fisher Scientific (a), 2015).

PCR products were observed using 2% agarose gels. SYBR® Safe DNA gel stain and a blue light transilluminator were used to visualise gal bands.

2.6 PCR amplification of nirK and nirS for PCR-ELISA (with DIG-labelled dUTP):

nirK and nirS genes were amplified in a total volume of 25 ul using BIOER's Life Touch Thermal Cycler (Hangzhou Bioer Technology Co. Ltd., China). 35 ng of soil DNA sample was used. Reagent composition and concentrations for both genes were the same as for amplification of universal bacterial primers in section 2.4, except the following concentrations of nucleotides were used: 200 uM of dATP, dCTP and dGTP, 190 uM dTTP and 10 uM Digoxigenin-11-dUTP (alkali stable) (All nucleotides obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, Missouri, USA)). Soil DNA from 3 different crops (cabbage, carrots and onions) was used.

For nirS, the forward primer was cd3aF (Michotey *et al.,* 2000) and the reverse R3cd (Throwback et al, 2004) and for nirK, forward primer was nirK1F and reverse nirK5R (Braker et al 1998) (Primers ordered from Eurofins Genomics, Ebersberg, Germany). Primer sequences are as shown in table 1.

Table 1. Primer sequences

Primer name	Primer length (nucleotides)	Sequence	Source
Eub 338	21	ACT CCT ACG GGA GGC AGC AG	Lane *et al.*, 1991
Eub 518	17	ATT ACC GCG GCT GCT GG	Muyzer *et al.,* 1993
cd3aF	20	GT(C/G) AAC GT(C/G) AAG GA(A/G) AC(C/G) GG	Michotey *et al.* (2000)
R3cd	19	GA(C/G) TTC GG(A/G) TG(C/G) GTC TTG A	Throwback *et al.* (2004)
nirK1F	17	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA	Braker *et al.,* 1998
nirK5R	18	GCCTCGATCAG(A/G)TT(A/G)TGG	Braker *et al.,* 1998

Cycling conditions for nirS consisted of an initial denaturation at 98 C for 3 min, followed by 30 cycles of denaturation at 98 C for 20 seconds, primer annealing at

68.9 C for 35 seconds and an extension at 72 C for 35 seconds. A final extension at 72 C for 10 min was also performed.

For the nirK gene, a denaturation step of 3 min at 98 C was followed by touchdown PCR. This consisted of 35 cycles of denaturation at 98 C for 20 sec, primer annealing for 35 sec and extension at 72 C for 35 sec. For the first 15 cycles, annealing temperature was decreased by 1 C per cycle from 75 C to 60 C. The remaining 20 cycles were performed at an annealing temperature of 60 C. Finally, an extension at 72 C for 10 min was performed. Here, the PCR cycle was kept the same as that optimised for the MJ Research thermal cycler, but times for denaturation, extension and annealing were reduced. Denaturation was changed from 40 sec to 20 sec and extension and annealing from 1 min to 35 sec. Amplification products were observed via gel electrophoresis using SYBR® Safe DNA gel and gel bands visualised using a blue light transilluminator.

2.7 Optimisation of PCR reaction for nirK and nirS gene:

Optimisation of nirS primers was performed, but initially without the use of Digoxigenin-11-dUTP. Same reagent concentrations as section 2.4 were used, except that 1 uM of nirS forward and reverse primers instead of universal bacterial primers were added. Cycling conditions were also the same as for universal bacterial primers (section 2.4) with 35 PCR cycles being performed. Primer annealing temperature was changed to 64.5 C. These reactions were performed on the MJ Research PTC-100 thermal cycler. 5 ul extracted soil DNA was used.

As both nirK and nirS forward and reverse primers are degenerate, annealing temperatures of all possible sequences of forward and reverse primers for each gene were determined using the Thermo Fisher Scientific's Tm calculator webpage (Thermo Fisher Scientific (b), 2015). The range of annealing temperatures for nirS for both forward and reverse primers was from 64.5 C -68.5 C (For forward primer cd3aF, it was 68.1 – 68.5 C and for reverse primer, R3cd it was 64.5 – 68.1C). As user instructions for the Phusion U Hotstart DNA polymerase recommend using annealing temperature of the primer with the lower Tm, 64.5 C was used for nirS.

For nirK, degenerate primers gave a very wide range of annealing temperatures (59.9 – 75.0 C)(For nirK1F it was 66.5 – 75.0 C and for nirK5R it was 59.9 – 66.2 C). Using the MJ Research PTC-100 thermal cycler, initially, 60 C annealing temperature was used, with reagent concentration and cycling conditions same as in subsection 2.4 and so no Digoxigenin-11-dUTP was used. As this showed multiple bands as shown in fig w in the results' sub-section 3.4, touchdown PCR was used. Braker *et al.,* (1998) and Braker *et al.* (2000) had also used touchdown PCR. The same reagent concentrations as for universal bacterial primers in subsection 2.4 were used, with nirK primers added instead of universal primers. Cycling conditions were the same as those used for nirK in subsection 2.6 except that time for each denaturation was 40 sec instead of 20 sec and for annealing temperature and extension it was 1

min instead of 35 sec. Initial denaturation and final extension time were kept the same. 5 ul of extracted soil DNA was used.

As access to a different thermal cycler (BIOER'S Life Touch) was obtained in which PCR tubes fitted in well and it had a much higher ramp rate than the PTC-100, the extra 30 seconds for denaturation, primer annealing and extension were not necessary. Access to a nanodrop was also obtained which showed that 2 of the 6 soil DNA samples used for nirK and nirS PCR optimization contained 35 ng in 5 ul that was used for PCR, with others containing higher amounts of DNA. 35 ng was therefore used for later PCR reactions for all soil samples as it seemed to give PCR products for the optimized reagent and cycle conditions for each gene.

With the BIOER Life Touch thermal cycler, for nirS, reagent concentrations were the same as in section 2.4. Primers used were cd3aF and R3cd (Table 1). Cycling conditions were as follows: initial denaturation at 98 C for 3 min, followed by 36 cycles of denaturation at 98 C for 10 seconds, primer annealing at 68.9 C for 30 seconds and an extension at 72 C for 30 seconds. A final extension at 72 C for 10 min was also performed.

PCR amplification for nirK using BIOER was only performed with Digoxigenin-11-dUTP, the methods for which are detailed in section 2.6.

2.8 Detection of nirS gene amplicon via ELISA

ELISA was performed using the PCR-ELISA (DIG-Detection), 5-Pack (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

The nirS probe had the same sequence as the forward primer cd3af. This ensured the probe would not bind to primers in PCR solution but to complementary amplified DNA. The probe consisted of a biotin label at the 5' to bind to streptavidin coated on ELISA strips (Probes ordered from Eurofins Genomics, Ebersberg, Germany).

The protocol involved addition of 1 ul of dilutions of a PCR product to 20 ul of denaturation solution and incubation at room temperature for 10 min. This solution was then made up to 250 ul using hybridisation solution, which consisted of either 7.5 or 15 pmol/ul of biotin labelled nirS probe in hybridisation buffer. This solution was then vortexed, 200 ul of the solution added to a well of the supplied microplate strip and placed on a vortex at its lowest speed in an incubator for 1.5 hours at 55 C. Wells were then washed 4 times with washing solution and incubated with 200 ul of Anti-DIG-POD working solution at 37 C for 30 min on a vortex in an incubator. Wells were again washed 4 times with washing solution and incubated in the dark with 200 ul of ABTS solution for 20 – 30 min at 37 C on a vortex in an incubator until color development occurred. Absorbance was read using the Opsys MR® microplate reader (Dynex Technologies Inc., Chantilly, Virginia, USA) at a wavelength of 405 nm (reference wavelength 490 nm). Software used for analyses was 'Revelation Quicklink' version 4.25 (Dynex Technologies Inc., Chantilly,

Virginia, USA). Values of PCR negative controls and reagent blanks were removed from sample readings.

First, dilutions of a PCR product (1:2, 1:3, 1:4, 1:5, 1:6 and 1:7 using PCR grade water as well as undiluted sample) were used to plot a curve of absorbance vs. dilution to determine whether a straight line curve was produced (PCR grade water description: DEPC treated water from Thermo Fisher Scientific was used (Thermo Fisher Scientific, Waltham, Massachusetts, USA)). Probe concentration was 7.5 pmol/ul. Absorbance values of blanks using only ELISA reagents were removed from sample absorbance values.

Then, ELISA was performed at twice the probe concentration (15 pmol/ul). A different PCR product was used in this case. Dilutions used were: 1:3, 1:4, 1:5 and 1:6 using PCR grade water. The experiment was then repeated at lower dilution ratios (1:2, 1:3 and 1:4 as well as undiluted PCR Product) but with the same probe concentration (15 pmol/ul) to see whether any differences to the absorbance vs. dilution curve were observed. Pooled PCR products from soil of 3 different crops (cabbages, carrots and onions was pooled) were used. For both dilution experiments using 15 pmol/ul probe, PCR negative controls with the same dilutions as samples were used. Absorbance values of negative controls were removed from values of the corresponding sample dilutions and graphs of absorbance vs. dilution factor were plotted.

2.9 Gel electrophoresis for determining relative abundance of nirS PCR amplicons:

Soil DNA samples were amplified for the nirS gene using the same reagent conditions as in section 2.4 and the same PCR cycle conditions as in section 2.6. However, 28 instead of 30 PCR cycles were performed. PCR products were run on 2% agarose gels containing SYBR® Safe DNA gel stain at a concentration of 1x. Gels were run for 10 min at 80 Volts. One PCR product was used in all gels as a standard to determine relative abundance of amplicons. This was done for a total of 24 soil samples. This included 8 samples from carrots, 5 from cabbage and 11 from leeks crop.

Image J was used for analyses of relative intensity of gel bands. This involved opening the gel image in ImageJ and changing the image to an 8-bit (producing a grey image with bands appearing white). A box was drawn around the first gel band and ctrl + 1 pressed. The box was then dragged to the neighbouring band and ctrl + 2 pressed and this was repeated for the remaining bands. Then inverted peaks/troughs representing intensity of each band were plotted by going to Analyze > Gels > Plot lanes. This produced inverted peaks for each band as shown in fig 3. A line was drawn across the base or top end of each trough and using the wand (tracing) tool, the centre or inside of the trough was clicked. A window showing the area of the trough and therefore, the intensity of the corresponding gel band appeared. These values were divided by that for the standard gel band that was used in all gel images to give relative intensities.

2.10 Soil nutrient measurement:

Values of the following soil nutrients was measured via NRM laboratories (NRM laboratories, Bracknell, Berkshire, UK):

Potassium (mg/l), phosphate (mg/l), calcium (mg/l), magnesium (mg/l) and sodium (mg/l) and percentages of total nitrogen and organic carbon content.

Soil samples taken to determine nirS gene abundance were taken either at the location where samples for nutrient analyses were taken or within 10-20 cm of that location.

2.11 Statistical analyses:

Parametric correlation analyses (using Pearson's correlation co-efficient) via Graph Pad prism 6 (GraphPad Inc., San Diego, CA, USA) was used to compare relative abundance of nirS gene in soil from three different crops (cabbage, carrot and leeks) to corresponding soil nutrient levels. *P<0.05* was considered to be significant.

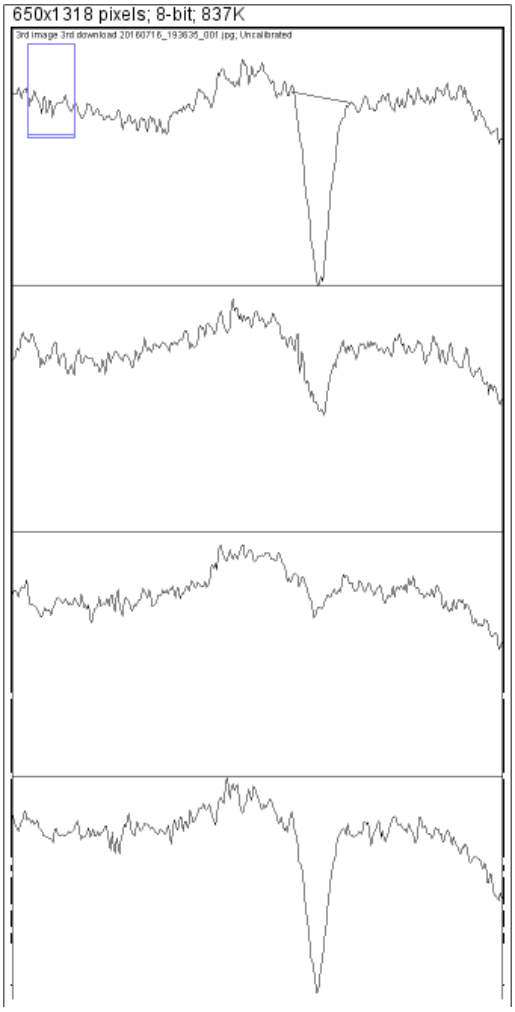

Fig. 3. Inverted peaks produced in image J showing relative gel band intensity. Here inverted peaks / troughs for 4 gel bands are shown. A line is drawn across the top end of each trough as shown on the top most trough. Using the wand (tracing) tool, the center or inside of the trough is clicked. This produces a window showing the area of the peaks and hence, the intensity of the corresponding gel band. This is the repeated for the remaining troughs.

3.**Results:**

3.1 PCR amplification of universal bacterial primers:

Amplification of universal bacterial primers (Eub 338 and Eub 518) was performed to determine whether soil DNA extracts contained bacterial DNA, whether PCR was inhibited and generally, whether the reagents and methods used in soil DNA extraction and PCR lead to the production of a PCR amplicon.

Bands of expected size (approximately 200 bp) were observed in all 5 soil DNA samples tested and in the pond sediment sample (fig 4). Pond sediment is known to contain more denitrifying bacteria and so was used as a positive control (Fries *et al.,* 1994).

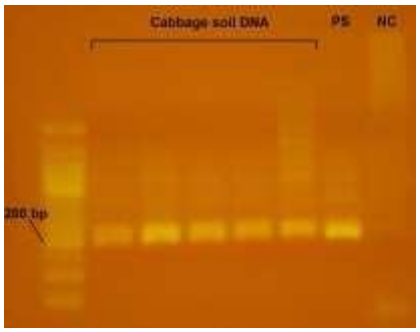

Fig 4. Gel electrophoresis following PCR of cabbage soil DNA samples and pond sediment DNA using universal bacterial primers (forward primer: Eub 338 and reverse primer: Eub 518) (Fierer *et al.,* 2004). PS indicates band from pond sediment DNA PCR and NC is the PCR negative control.

3.2 Optimisation of PCR reaction using universal bacterial primers:

Fig 5. BSA is needed for better amplification using universal bacterial primers at cycle number 40 (no DMSO used here). Bands at around 200 bp, which was expected based on the primers used, showed bands only when BSA was used and no bands when BSA was not present in the PCR reaction mixture. Different volumes of the same soil sample used also showed 5 µl gave the highest band intensity.

Results showed that BSA was required to produce a PCR product at 40 cycles (fig 5). Soil DNA volumes of 1, 5 and 7 ul were used and showed bands following PCR of all three volumes when BSA was present in the PCR reaction. The soil sample used here was from cabbage crop. The same sample was also used in the DMSO PCR reaction, results of which are explained below (sample number 8).

Incorporating DMSO along with BSA at 35 cycles (5 cycles less than when only BSA was used) showed DMSO was needed to get visible PCR gel bands at the lower cycle number (fig 6).

3.3 PCR amplification of nirK and nirS for PCR-ELISA (with Digoxigenin-11-dUTP):

For nirS PCR using forward primer cd3aF (Michotey *et al.,* 2000) and reverse primer R3cd (Throwbäck *et al*., 2004) a band size of approximately 500 bp instead of 425 bp was seen (Fig 7). In this image, a cycle number of 32 instead of 35 is shown. For nirK, primers nirK1F and nirK5R bands were also seen at 35 cycles (data not shown).

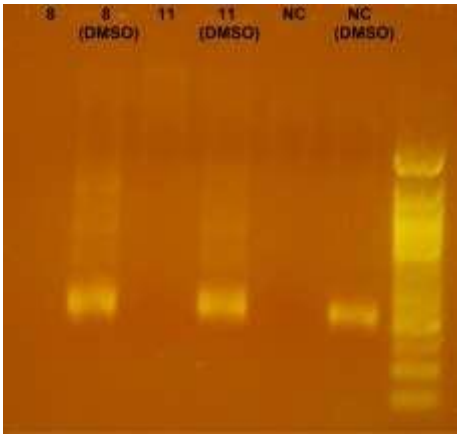

Fig 6. DMSO is needed for better amplification using universal bacterial primers (Eub 338 and Eub 518) with BSA at 35 PCR cycles. Number 8 and 11 are for different cabbage soil samples. NC means negative control. Although a band is seen for the negative control, this was most likely due to DNA contamination as no PCR band was seen in the negative control when the same PCR was repeated as shown in fig 2.

3.4 Optimisation of PCR reaction for nirK and nirS gene:

For nirS PCR using the MJ Research PTC-100 Thermal Cycler, BSA and DMSO were used as it had already been established during PCR amplification of universal bacterial primer that these were required (sub-section 2.4) (fig 8). With nirS PCR using BIOER, bands of approximate size of 450 - 500 bp were seen (fig 9).

For nirK, using PTC-100, using same reagent and cycle conditions as sub-section 2.4 for universal bacteria primers, multiple bands were seen (fig 10). Touchdown PCR was then performed and this showed single bands for all cabbage soil samples used at a band size of 450 – 500 bp (fig 11). DNA volume of 5 ul was used as this showed highest PCR band intensity when amplifying universal bacterial primers (see section 3.2).

NirK PCR using BIOER'S Life Touch thermal cycler was only performed with DIG-labelled Dutp. Although a band was obtained, a gel picture is not shown here.

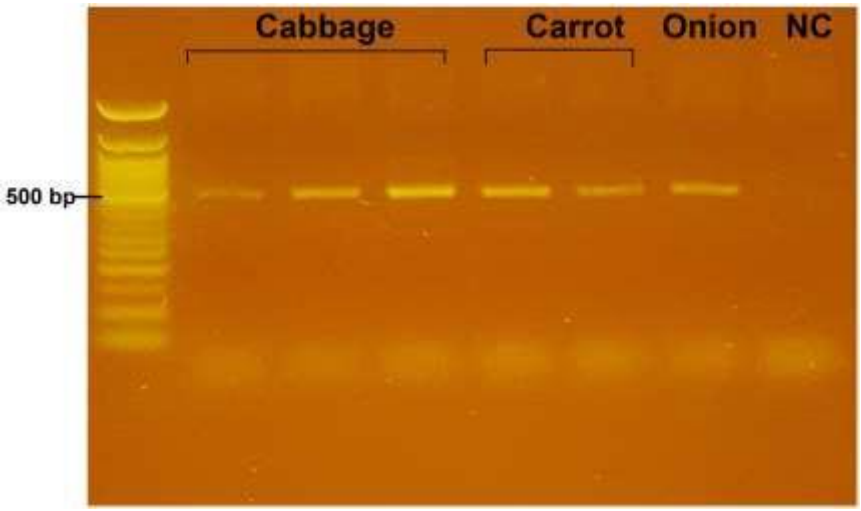

Fig 7. nirS PCR using Digoxigenin-11-dUTP. BIOER's Life Touch thermal cycler was used. A band size of approximately 500 bp was obtained.

3.5 nirS gene amplicon detection using ELISA:

The PCR product dilutions 1:2 to 1:7 using 7.5 pmol/ul probe showed a straight line distribution from dilutions 1:3 to 1:5 (fig 12), indicating possible lower and higher detection limits based on probe concentration and time and temperature of the first incubation. Increasing variation in absorbance from dilution factors 4 to 6 can be seen with errors bars for dilutions 4 – 7 showing a lot overlap with each other. Blank absorbance values shave been removed from sample absorbance.

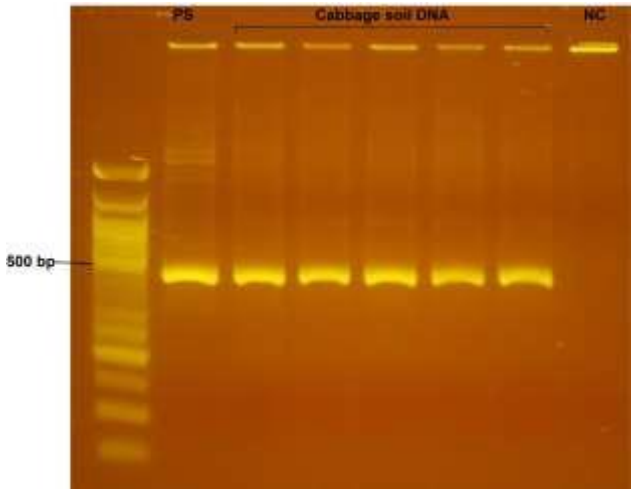

Fig 8. nirS PCR using 1000 ng/µl BSA and 3% DMSO without Digoxigenin-11-dUTP. PS indicates pond sediment samples and NC means negative control. Estimation of band size might not be accurate as ladder does not seem to have flowed down the gel properly. MJ Research's PTC-100 thermal cycler was used.

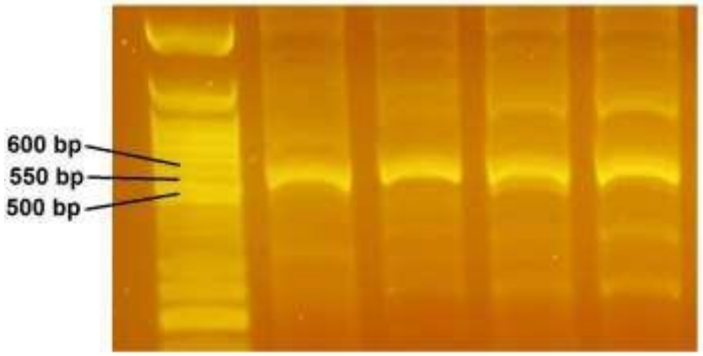

Fig 10. nirK PCR without digoxigenin-11-dUTP. Forward primer nirK1F and reverse primer nirK5R were used (Braker *et al.,* 1998). Bands are approximately of expected size at about 614 bp. Multiple bands are due to high number of degenerate base sequences in the primers. These were removed using touch-down PCR (Fig. 9).

Using twice the concentration of probe (15 pmol/ul instead of 7.5 pmol/ul) showed less variation in dilutions 3 to 6 with less overlap of error bars between the dilutions (Fig 13). Mean absorbance values and standard deviations of 1:3 dilution was 0.3665 ±0.1039, of 1:4 was 0.2115 ±0.0799, 1:5 was 0.1083 ±0.1144 and 1:6 was 0.1305 ±0.0375. However, dilution factor 6 did not follow the straight line pattern shown by 3 to 5. This was also the case in the previous experiment, but here it seemed farther away from the straight line distribution. However, it must be noted that these dilutions were made using a different PCR product to that used in the previous dilution experiment. This may have caused absorbance values of dilutions to be slightly different. Fig 14 shows the same graph without accounting for PCR negative controls. PCR negative control dilutions from 1:3 to 1:6 were used and the values removed from the corresponding sample absorbance values. Mean absorbance values and standard deviations without removing absorbance for PCR negative controls were 0.5175 ±0.1039 for dilution 1:3, 0.4565 ±0.0799 for 1:4, 0.3973 ±0.1144 for 1:5 and 0.4505 ±0.0375 for 1:6. In this case, absorbance values are closer together. Hence, error bars show more overlap. Absorbance for dilution 6 also falls further away from the straight line curve and is closer to the value for dilution 4. This graph shows absorbance values without accounting for any blanks, since reagent blanks were not used in this

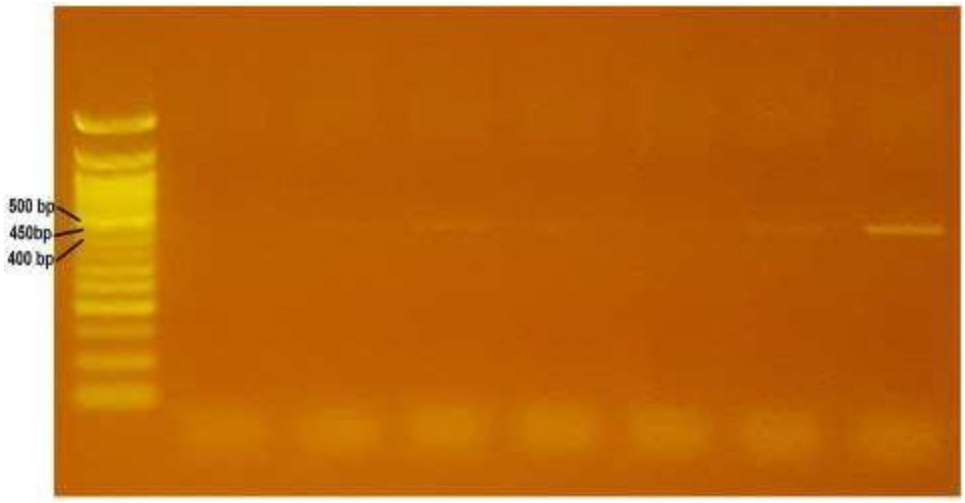

Fig 9. nirS PCR using BIOER's Life Touch Thermal cycler without Digoxigenin-11-dUTP. Only faint bands can be seen for as a low cycle number of 28 was used. Band sizes are approximately 450 – 500 bp. (Image slightly stretched to show band size more clearly). All bands are for soil DNA from carrot or leek crop.

experiment, only PCR negative controls were. Two repeats of each dilution were used, except for 1:5 which had 3 repeats.

Repeating the experiment with the same probe concentration (15 pmol/ul) but at slightly lower dilutions produced a graph as shown in fig. 15. Here dilution factor 4 falls out of the straight line distribution, with dilutions 1 to 3 falling within the straight line distribution. However, even at lower dilutions error bars show overlap.

3.6 Gel electrophoresis for determining relative abundance of PCR amplicons:

Parametric correlation analyses (using Pearson's correlation co-efficient) comparing relative intensity of PCR gel bands of 24 soil samples with soil nutrient content showed no correlation with any nutrients except calcium. Correlation analyses of calcium levels with nirS containing bacteria abundance gave a Pearson correlation co-efficient value of 0.5623 at $P < 0.05$ (Fig. 16). A two-tailed P-value was used in all Pearson correlation co-efficient analyses.

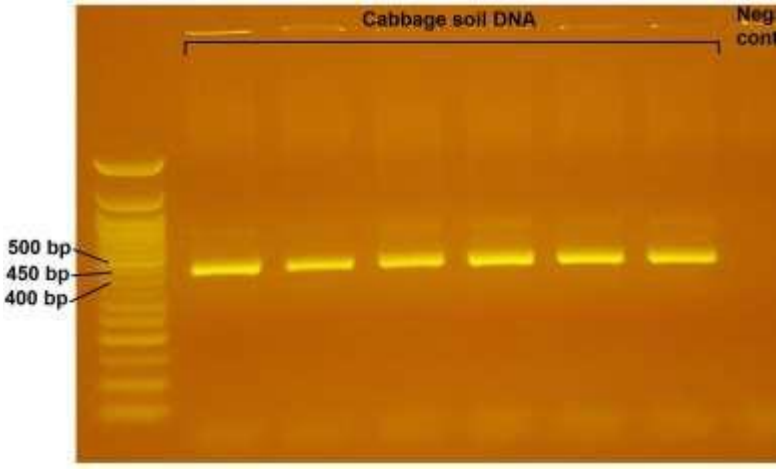

Fig 11. NirK touch-down PCR without digoxigenin-11-dUTP. Band sizes of approximately 450 – 500 bp are seen.

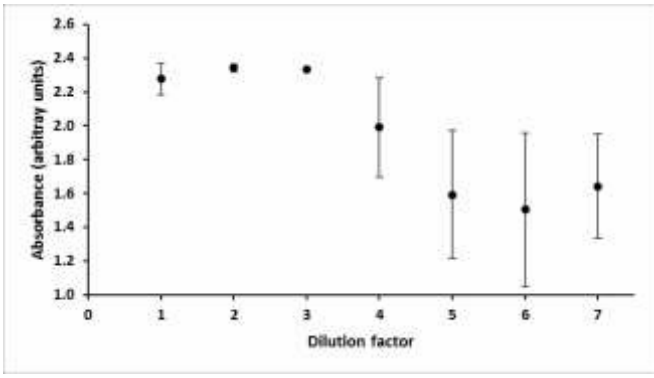

Fig. 12. ELISA absorbance values at nirS probe concentration of 7.5 pmol/ul. Dilutions factor 2 indicates 1 ul of PCR product was dissolved in 2 ul of total solution. Note that dilution factor 1 means PCR sample was undiluted. Carrot soil DNA sample was used. Note that error bars show standard deviations. Two repeat of each dilution were done. From a dilution factor of 4 to 6, a continuous increase in variation of absorbance can be seen. Although a linear graph region is seen from dilutions 3 to 5, error bars for dilutions 4 to 7 show a lot of overlap.

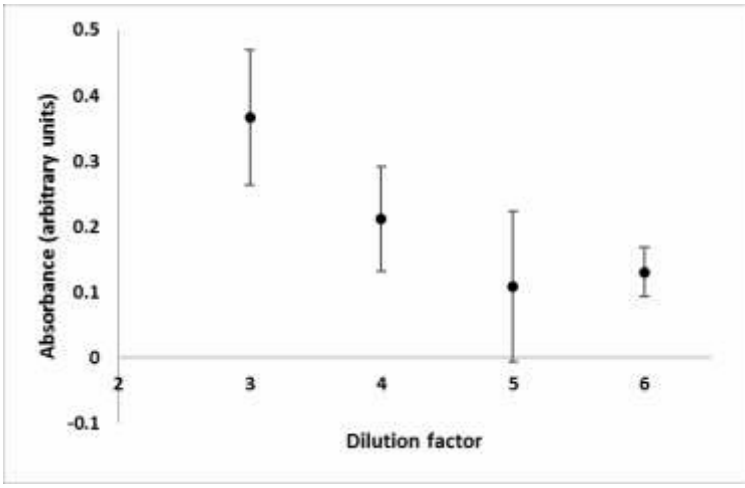

Fig. 13: ELISA absorbance values at nirS probe concentration of 15 pmol/ul.. Absorbance values of corresponding negative control dilutions have been removed from the actual absorbance values of samples. An explanation of the dilution factor is as follows: 3 signifies 1:3 dilution of PCR product in total volume of solution and so on. Dilution 1:6 could be an anomaly or indicate lower detection limit at a probe concentration of 15 pmol/ul. Error bars show the first standard deviation. Two repeats of each dilution were performed except for 1:5 where three repeats were used.

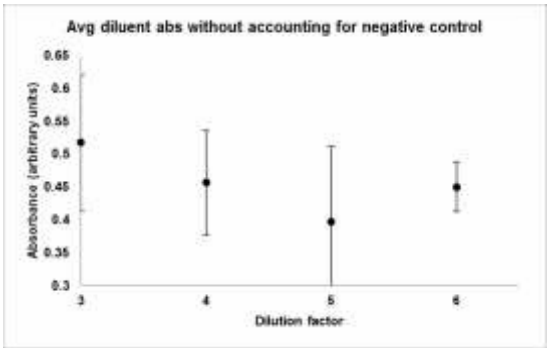

Fig. 14: ELISA absorbance values at nirS probe concentration of 15 pmol/ul without accounting for negative controls. No ELISA reagent blanks were used in this experiment. Hence blank values have not been removed from these absorbance values. Dilution factor 3 indicates 1:3 dilution of PCR product in total volume of solution using PCR grade water. Dilution factor 6 appears to be an anomaly. Error bars show standard deviation.

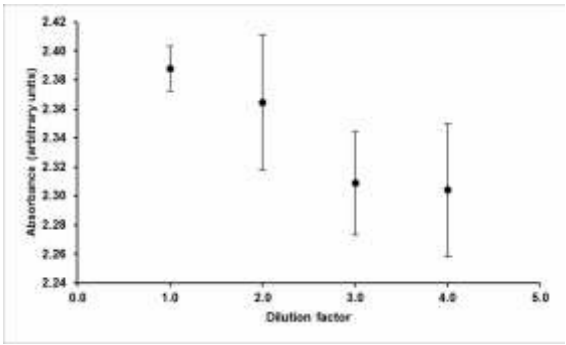

Fig. 15. ELISA absorbance values at nirS probe concentration of 15 pmol/ul but at higher dilutions than figure 9 and 10. Dilution factor 2 indicates 1 ul of PCR product was made up to 2ul using PCR grade water. Dilution factor 1 indicates the sample was undiluted. Straight line distribution is seen from dilution factor 1 to 3. However, the error bars show overlap. Dilution 4 does not follow the straight line distribution. Average of the PCR negative controls has been removed from sample absorbance values.

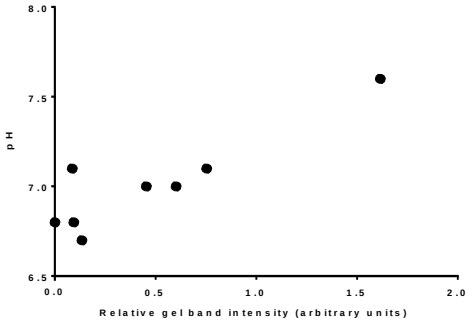

Fig. 16. Positive correlation found between pH and relative gel band intensity of nirS PCR products in carrot crop sample. Pearson correlation co-efficient value = 0.8971. P < 0.05 was considered to be significant and a two-tailed P-value was used.

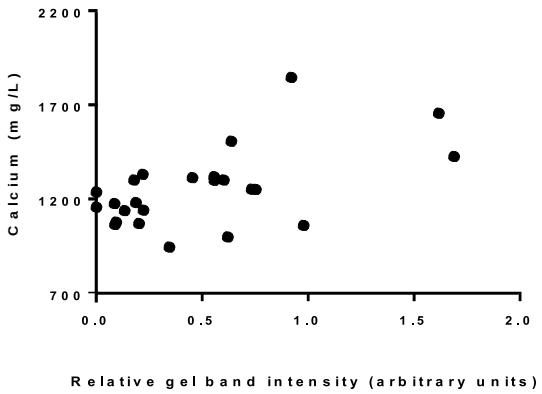

Fig 17: Positive correlation found between soil calcium levels and relative gel band intensity of nirS PCR products. Pearson correlation co-efficient value = 0.5623 (P< 0.05 was considered to be significant. A two-tailed P-value was used.

nirS gene abundance in 8 soil samples from carrots showed positive correlation with pH (Fig. 17) (Pearson correlation co-efficient value = 0.8971 at P < 0.05). No correlation was found between total nitrogen content and nirS gene abundance (Pearson correlation co-efficient value = 0.3045 at P<0.05) (fig 18). Fig 19 – Fig 25 show graphs for other nutrients (magnesium, phosphorus, sodium, potassium and organic carbon) and pH against relative intensity of gel bands for nirS PCR. As shown in these graphs, no significant correlation was found at P < 0.05 (Pearson correlation co-efficient test and two tailed P-value used).

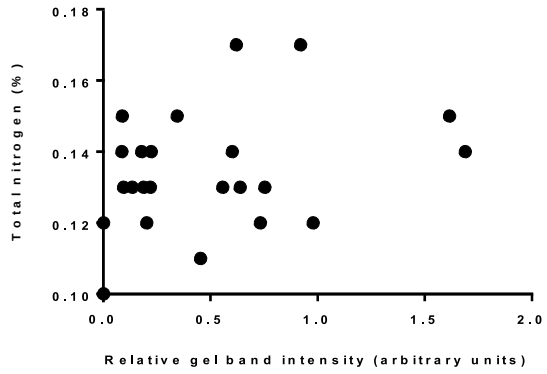

Fig 18: No significant correlation found between total nitrogen content and relative gel band intensity of nirS PCR products. Pearson correlation co-efficient value = 0.3045. P<0.05 was considered significant. Two -tailed P value used.

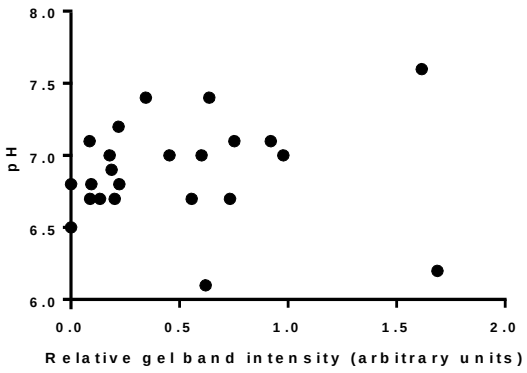

Fig. 19. No significant correlation found between pH and relative gel band intensity of nirS PCR products of all soil samples. Pearson correlation co-efficient value = 0.08033. P < 0.05 was considered to be significant and a two-tailed P-value was used.

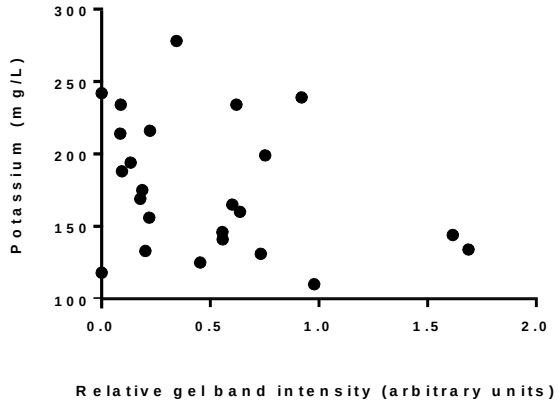

Fig. 20: No significant correlation seen between soil potassium content and relative gel band intensity of nirS PCR products. Pearson correlation co-efficient value = -0.3146 (P<0.05 was considered to be significant. Two -tailed P-value used)

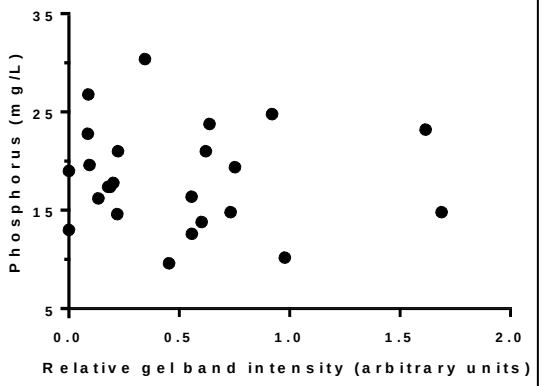

Fig. 21: No significant correlation found between soil phosphorus content and relative gel band intensity of nirS PCR products. Pearson correlation co-efficient value = -0.0630. P<0.05 was considered significant. Two -tailed P value used.

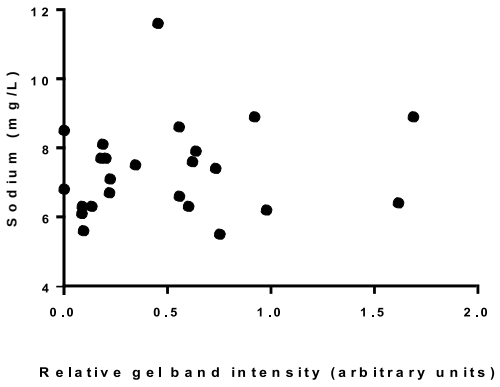

Fig. 22: No significant correlation found between soil sodium content and relative gel band intensity of nirS PCR products. Pearson correlation co-efficient value = -0.0630. P<0.05 was considered significant. Two -tailed P value used.

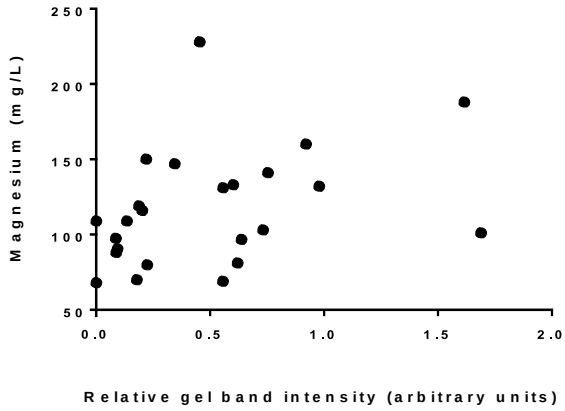

Fig. 23: No significant correlation found between soil magnesium content and relative gel band intensity of nirS PCR products. Pearson correlation co-efficient value = 0.3624. P<0.05 was considered significant. Two -tailed P value used.

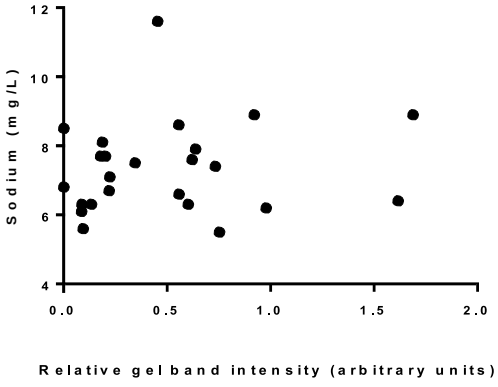

Fig. 24 : No significant correlation found between soil sodium content and relative gel band intensity of nirS PCR products. Pearson correlation co-efficient value = -0.0630. P<0.05 was considered significant. Two -tailed P value used.

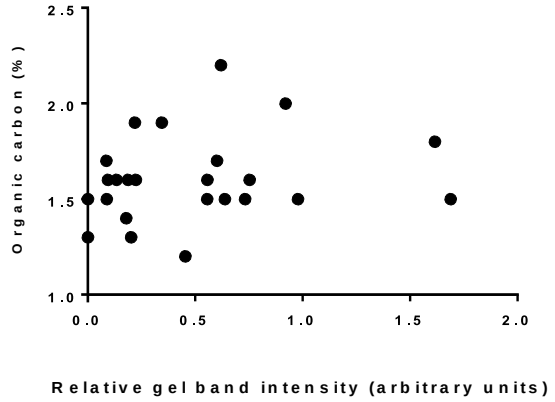

Fig. 24: No significant correlation found between organic carbon content and relative gel band intensity of nirS PCR products. Pearson correlation co-efficient value = 0.2091 (P<0.05 was considered to be significant. Two -tailed P-value used)

4. Discussion:

nirK and nirS PCR showed gel band sizes different to that mentioned in Throbäck *et al.* (2004). This could be due to a different range of nirS and nirK containing bacteria in the agricultural soil used. There is also a need for redesigning primers for nirK and nirS as gene sequences of more denitrifying bacteria species are now available to allow for better amplification of nirS and nirK containing bacteria.

Although the literature suggested PCR-ELISA to be more sensitive to gel electrophoresis (Sue *et al.,* 2004) reproducible data could not be obtained. The protocol required extensive optimisation in terms of probe concentration, dilution factor for PCR products and time and temperature of first incubation. Attempt at optimising probe concentrations and assessing its effect on absorbance of nirS PCR dilutions did not lead to repeats that accurately depicted the relative abundance of PCR products; repeats of the same PCR dilutions were widely different with many overlapping with those of other dilutions. Straight line graphs were obtained for a certain range of dilutions, but errors bars overlapped with other dilutions. This could be due to lack of a microplate thermoshaker and the use of a vortex places in an incubator to shake plates at the required temperature. The use of probes containing degenerate bases might also be the reason for this. This would lead to a range of melting temperatures for the probes making it difficult to optimise incubation temperature for the first incubation when PCR solution along with probe added to hybridisation buffer is added to streptavidin coated wells. Different sequences of the degenerate probes might bind with different affinities to their complementary PCR sequences depending on the incubation temperature. The incubation temperature of 55 C might have lead to a specific sequence within the degenerate probe solution to have higher binding affinity and therefore the ELISA might be effectively looking for a specific nirS sequence.

The finding that there was no correlation of the relative intensity of nirS gel bands with pH is contrary to the literature where most bacteria have been shown to be affected by soil pH (Griffiths *et al.,* 2011). This was despite the fact that a range of pH values were present in the soil samples (from 6.0 – 7.6). Positive correlation was seen with soil samples from carrot crop, which suggests nirS containing bacteria from these soils are perhaps more sensitive to changes in pH than those in leeks and cabbages. There was also no correlation seen with nitrogen levels, which was the primary nutrient being tested. It should be noted here that denitrification requires a range of genes such as *nosZ, narG* (Kandeler *et al.,* 2006) and

nirS forms only a part of the pathway for denitrification. It could therefore be that nitrogen levels are affecting denitrifying bacteria which contain different genes to nirS. The literature contains studies with contradictory results on whether there is a correlation between denitrification rates and denitrifying bacteria levels. This could also be the case with nitrogen and denitrifying bacteria levels. A higher sample number might have lead to different conclusions about the data. Due to time restrictions a higher sample number than 2 was not used. The positive correlation seen with calcium will need to be looked into further by performing experiments using soil samples spiked with calcium to see whether a gradual change in nirS gene abundance is seen.

As mentioned earlier, agricultural activities influence soil microbiota. Tilling, bedding, harvesting, application of pesticides and fertilisers and crop rotation mean that agricultural soil is subject to harsh physical and chemical interventions which drastically alter soil microbial composition. The Tru-Nject project aims to use sub-soil nitrogen fertiliser application based on the current nitrogen content of soil with the aim to optimise nitrogen application and reduce excessive fertiliser use. The objective to make nitrogen content in soil of a specific crop more uniform lead to the thought of whether this would cause more uniform levels of denitrifying bacteria. nirS and nirK were chosen as a class of denitrifying bacteria owing to reasons mentioned earlier. Results from the current study have, however, indicate that there might not be any correlation between nirS containing denitrifying bacteria levels and nitrogen.

To confirm whether the MO BIO PowerSoil® kit successfully extracted DNA, the DNA was run on a gel and the band sizes corresponded to greater than 10,000 base pairs (data not shown). The FastDNA® Spin Kit for soil (Qbiogene, Carlsbad, CA,USA) was used by Throwback *et al.* (2004). However, the kit required a homogeniser, the cost of which was above the budget limit at which equipment could be ordered.

Quantitative analyses of nirS containing bacteria would have been performed better via qPCR. Fluorescence curves obtained via qPCR would have allowed determination of whether amplification in any specific soil samples was being inhibited by looking at the time difference between the linear phase of different samples. The time difference would have indicated towards several underlying causes including low target DNA and possible inhibition of PCR. However, owing to budget limits this could not be done.

Freeze-thawing of soil samples was an issue and it had to be taken care of that not more than one freeze-thaw cycle was performed. However, some soil samples were frozen and thawed twice. According to MO BIO's PowerSoil® DNA isolation kit manufacturers freeze thaw cycles lead to more fragmented soil DNA. However, the manufacturers suggested that one or two freeze thaw cycles would not lead to significant changes in extracted DNA.

The next steps would be to look at correlation of nirK with pH and soil nutrient levels. As nirK and nirS are mutually exclusive in a specific bacterial strain and have the same function in terms of denitrification, it would be interesting to know whether there is any difference between correlation of nirK and nirS

containing bacteria with nutrient levels. It would also be interesting to see whether relative intensity of PCR gel bands performed using universal bacterial primers shows correlation to nutrient levels and compare it to nirK and nirS.

5. Conclusion:

Successful PCR amplification of nirK and nirS containing bacteria from crop soil part of the Tru-Nject project lead to quantification of relative abundance of nirS containing bacteria. Levels of these bacteria were correlated to soil pH, nitrogen, potassium, magnesium, calcium, phosphorus, sodium and organic carbon levels. Positive correlation of these bacteria with calcium was found using Pearson's correlation co-efficient test. Positive correlation was also found of abundance of nirS containing bacteria in carrot crop to pH. It would be helpful to look at whether nirK and amplification using the universal bacteria primers (Eub338 andEub518) shows similar results for correlation to soil nutrients.

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