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Abundance of Functional Groups of Nitrogen Transforming Microorganisms Potentially Involved in N2O Emissions from a Subtropical Forested Watershed in China

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Acknowledgements:

This thesis was conducted at the department of chemistry, biotechnology, and food science, at the university life sciences with Professor Åsa Frostegård and Dr. Liu Binbin as my supervisors.

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Date received

ABSTRACT:

In this study, we investigated N-cycling processes and N_2O emissions along a hillslope (HS) and a hydrologically connected groundwater discharge zone (GDZ) in a subtropical forest ecosystem in southwest China, Tieshanping (TSP). The two landscape elements HS and GDZ differ fundamentally in eco-hydrological conditions, soil texture, organic carbon supply, pH (dominated by acid soils pH 4.0 - 4.5) and NO_3^- concentration. The area has received high levels of long-distance transported nitrogenous compounds for several decades. Earlier studies by our research group showed high N_2O emissions from the investigated plots, the highest emissions were measured from the hilslope (HS), while the groundwater discharge zone (GDZ) had lower emissions, possibly due to complete denitrification taking place in this zone where anoxic conditions prevailed over longer periods than in the HS. Accumulation of nitrite was also shown, and it was speculated that nitrite oxidation was retarded compared to ammonia oxidation.

For this study Soil samples were collected from Tieshanping (TSP). DNA was extracted from the soil samples. Cloning was done to make plasmids, which were used as standards for the primers for each corresponding gene. Quantitative PCR was used to quantify the genes; by quantifying the genes, abundance of functional members at different sampling sites was revealed. In addition to this a microcosm experiment was performed, to analyze the denitrification activity from both HS and the GDZ soil samples.

In the present study, I quantified functional genes that are involved in the nitrogen cycle, including genes coding for ammoniam oxidation (*amo*A of ammonia oxidizing bacteria; AOB and archaea, AOA); nitrite oxidation (*nxr*B),and denitrification (*nir*K, *nir*S, *nosZ*). 16S rRNA abundance was assessed as a general marker for bacterial abundance. In addition, sulphate reducing bacteria (*dsrA*) were quantified. Aim of this study was to see if there is correlation between abundance of N-cycle genes and N-transformation rates. It was hypothesized that nitrous oxide reductase (N_2OR) was present at HS but was not expressed due to oxic anoxic transitions while the other enzymes (NIR, NOR) are not influenced. Nitrite oxidation was retarded in HS samples, for this reason it is assumed that *amoA* (ammonia oxidizers) abundance

will be higher than *nxrB* (nitrite oxidizers) abundance in samples where nitrite accumulates. Archaeal ammonia oxidisers will dominate (higher abundance) while there will be lower amounts of bacterial ammonia oxidisers due to low pH in this soil. GDZ has low organic material, and may not provide enough electrons for denitrification. It is hypothesized that the reductive force can be provided by the sulphate reducers.

All genes showed highest abundance per gram soil in the heavily disturbed GDZ (formerly cultivated terraces), despite lower soil organic carbon content (1-4% w/w as opposed to 10-20% w/w in HS topsoil). Archaeal ammonia oxidizers (AOA) were more abundant than bacterial ammonia oxidizers (AOB) which could be due to the low pH of these soils The results of the microcosm experiment (semi-automated robotic incubation system) were in accordance with the denitrification results observed from the molecular studies i.e. GDZ has high denitrification activity than HS (normalized to Carbon content).

The reason for high abundance of genes in GDZ could be due to the presence stable anoxic conditions. N_2OR is expressed under the stable anoxic conditions leading to lower N_2O emission. Additional factors causing lower N_2O emissions from the GDZ may be the higher soil pH (4.5 at GDZ versus 4.0 at the HS). We can conclude that the GDZ is the sink, where the microbial communities are more abundant.

The work in this thesis was conducted in the Environmental Microbiology group of the Department of Chemistry, Biotechnology, and Food Science (IKBM) of the Norwegian university of Life Sciences (NMBU) in Ås, Norway.

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Abbreviations:

AOA – ammonia oxidizing archaea

AOB-ammonia oxidizing bacteria

Anammox-Anaerobic ammonium Oxidation

DNA-Deoxyribonucleic acid

DNRA-Dissimilatory nitrate reduction to ammonia

HS- hill slope

IPCC- intergovernmental panel on climate change

GDZ- ground water discharge zone

GHG- green house gas

N_2 - nitrogen

N_2O - nitrous oxide

N_2OR - nitrous oxide reductase enzyme

NAR - nitrate reductase

NH_4^+ - ammonium

NIR –nitrite reductase

NO – nitric oxide

NO_2^- - nitrite

NO_3^- - nitrate

NOR – nitric oxide reductase enzyme

O_2-Oxygen

PCR-polymerase chain reaction

qPCR-quantitative real-time PCR

RNA-ribonucleic acid

rRNA-ribosomal RNA

SRB- sulphate reducing bacteria

List of figures and tables:

1. .INTRODUCTION:

1.1. Nitrogen cycle and N_2O emission:

The earth atmosphere contains layers of gases including 78% nitrogen, 21% oxygen and 1% of other trace amount of gases which protects all form of life on earth. The numerous trace amounts of gases in the atmosphere also include the green house gases (GHG), CO_2, CH_4, N_2O, CH_3Cl, which are added by both natural sources and human activities (Kasting and Siefert 2002). The natural sources include the wetlands, termites and the oceans. Microbial processes (nitrification and denitrification) produce N_2O which is considered to be an important GHG. Of the total global N_2O emissions, it is estimated that 62% are from natural and agricultural soils (6 and 4.2 Tg N yr-1, respectively; (Thomson, Giannopoulos et al. 2012) and denitrification is traditionally considered as the main source of these emissions (Ostrom et al 2010). Human activities like industries, agricultural, transportation are responsible for all increase in GHG emission (IPCC 2007).

Human activities influence the biogeochemical cycles, possibly the most seriously affected is the nitrogen cycle. Nitrogen is an important component of all living organisms and it composes nearly 6.25% of their dry weight. It is an important component of amino acids and nucleic acids and is essential for all biochemical process in organisms. Nitrogen is also known to be one of the growth limiting factor, even if water and appropriate climate condition are available to support life (Martinez-Espinosa, Cole et al. 2011). Nitrogen in the atmosphere (78% N_2) is relatively stable; and in the atmosphere most organisms cannot use N_2 directly because the triple bond between the two nitrogen atoms make the dinitrogen (N_2) molecule inert, it must be fixed by microorganisms before it can be taken up by other organisms (Francis, Beman et al. 2007).

Many biochemical pathways are involved in the different red-ox transformations of the nitrogen cycle, and several of these are unique to prokaryotic organisms. In addition to nitrogen compounds being transformed through biochemical reactions exerted by living organisms, also purely chemical, redox reaction take place. The biologically mediated transformations are shown in Fig. 1 below.

N_2 is released into the atmosphere by anammox and denitrifying bacteria and the nitrogen is returned back to the cycle by nitrogen fixing micro organisms. Through these processes, the N_2 level in the atmosphere is kept constant (ca 78%). Important steps in nitrogen cycle include nitrogen fixation, nitrification, denitrification, anaerobic ammonia oxidation (Anammox) dissimilatory nitrate reduction to ammonium (DNRA) and nitrate assimilation. In the following sections the individual processes that comprise the nitrogen cycle are discussed in more detail.

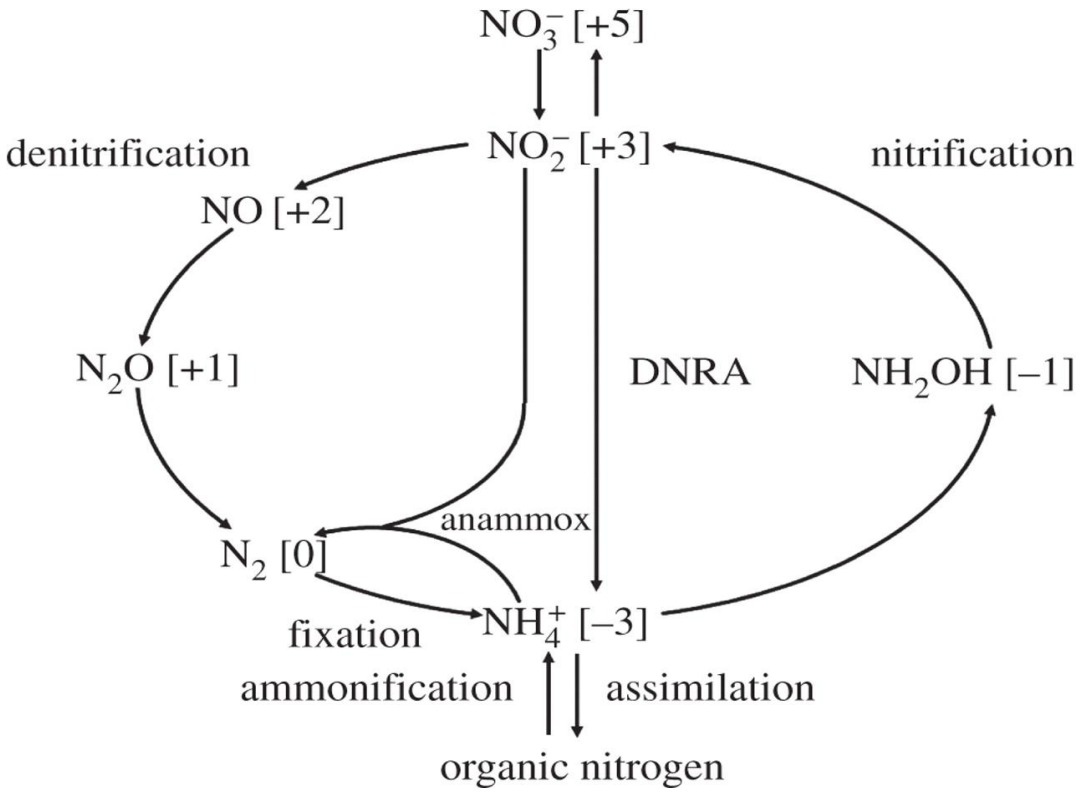


Figure 1.1 The nitrogen cycle, In the nitrogen cycle the microbial processes cycle nitrogen through the biologically available (NH_4^+, NO_2^- and NO_3^-) and unavailable forms (N_2). The oxidation state of each process is shown in parentheses (Thomson, Giannopoulos et al. 2012).

1.2. Nitrogen fixation:

Nitrogen is one of the most abundant elements in the atmosphere but is present as inert diatomic nitrogen gas (N_2), which cannot be assimilated by any organisms and unless it is at first converted into reduced reactive nitrogen. There are only a few groups of prokaryote organisms

which are capable of fixing atmospheric N_2, thus reducing it to biologically available ammonia (Galloway, Dentener et al. 2004). As mentioned in section (1.1) nitrogen fixation is the only biological process for returning back the nitrogen to the biosphere which is lost by the process of denitrification and anammox. Example of nitrogen fixing bacteria include aquatic organisms, such as cyanobacteria, free-living soil bacteria, such as *Azotobacter*, bacteria that form associative relationships with plants, such as *Azospirillum*, and most importantly, bacteria, such as *Rhizobium* and *Bradyrhizobium*, that form symbioses with legumes and other plants (Postgate 1982). Nitrogen fixing microorganisms use the enzyme nitrogenase to catalyze the reduction of dinitrogen (Karl, Letelier et al. 1997).

1.3. Nitrification:

Nitrification is a two step process, in the first step oxidation of ammonia (NH_3) into nitrite (NO_2^-) takes place. This step is carried out by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA). This step is also known as the rate limiting step of nitrification. In a second step nitrite (NO_2^-) is oxidized to nitrate (NO_3^-) which is carried out by nitrite oxidizing bacteria (NOB). The first step oxidation of ammonia to nitrite, is again a two step process in which the ammonia is first converted to hydroxylamine (NH_2OH) by the ammonia oxidizing enzyme ammonia mono-oxygenase (AMO) and in second step the hydroxylamine (NH_2OH) is then converted to nitrite (NO_2^-) by the hydroxylamine oxidoreductase (Francis, O'Mullan et al. 2003) (De Boer and Kowalchuk 2001). Less is known about NO_2^- oxidation as compared to NH_3 oxidation; in this step the key enzyme is the nitrite Oxidoreductase (NXR) which converts NO_2^- to NO_3^-. NO_2^- oxidation step is fast and prevents the accumulation of toxic NO_2^- (Zhu, Mulder et al. 2013). This process of nitrification occurs in many environments and is carried out by Chemolithoautotrophic microbes one such example is of waste water treatment, where the removal of ammonia is essential (Leininger, Urich et al. 2006).

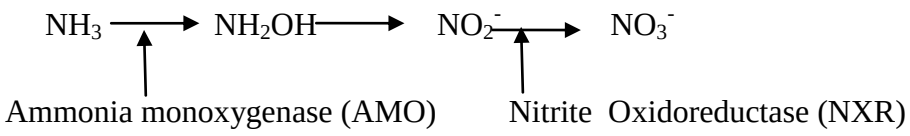


Figure 1.2 Shows the process of nitrification with intermediate steps

Nitrification in soil is important because it provides the plants with nitrate, but it can also lead to groundwater pollution due to leaching (Leininger, Urich et al. 2006). There are many factors which control the rate of nitrification. One of the major factors is the presence of oxygen since nitrification is an aerobic process. In soil the rate of oxygen diffusion depends on the pore size and moisture present in the respective soil (Schurgers, Dörsch et al. 2006). Another nitrification rate limiting factor is the availability of ammonia NH_3 rather than ammonium NH_4^+ (De Boer and Kowalchuk 2001). Yet another important controller of nitrification is pH. Nitrification is absent in highly acidic soils (although recently some nitrification activity has been observed in acidic soils) because low pH results in formation of NH_4^+ leading to unavailability of NH_3. The optimal pH range for nitrification is 4.3 (at this low pH it is the archaeal nitrification that is important) to 7.5 (Yao, Gao et al. 2011).

The ammonia oxidizers contain a membrane bound enzyme ammonia mono-oxygenase AMO, which catalyzes the oxidation of ammonia. The gene *amoA* encodes the subunit A of AMO enzyme and has been widely used as the molecular marker for studying AOB and AOA (Francis, O'Mullan et al. 2003).

1.3.1. Ammonia oxidizing bacteria (AOB) and Ammonia oxidizing archaea (AOA):

Chemolithoautotrophic ammonia-oxidizing bacteria (AOB) are important drivers of the nitrogen cycle; they aerobically oxidize ammonia to nitrate or nitrite. AOB is considered important as AOB are responsible for carrying out the first step of ammonia oxidation. Compared to other bacteria AOB are less diverse, show slow growth rate and are sensitive to acid (Jiang and Bakken 1999). Ammonia oxidizing archaea (AOA) are found to be more abundant in a wide range of soil as compared to AOB (Di, Cameron et al. 2010). Both AOA and AOB can contribute to loss of fertilizers, by converting the fertilizers like urea and ammonia to nitrite which can easily get washed away leading to pollution of water or can act as electron acceptor in denitrification (Purkhold, Pommerening-Roser et al. 2000)

1.4. Denitrification:

Denitrification is a dissimilatory process in which nitrate (NO_3^-) or nitrite (NO_2^-) is reduced to nitrogen gas (N_2) through intermediate steps catalyzed by four different reductase enzymes

which are encoded by a respective gene. Denitrification is of special importance in the nitrogen cycle as it causes the loss of nitrogen from soil; moreover the intermediate products gaseous nitrogen oxides like nitrous oxide (N_2O) and nitric oxide (NO) are produced which act as GHGs. The process is carried out by facultative anaerobic heterotrophic bacteria. Another importance of denitrification is that under oxygen limiting conditions the microbes switch from aerobic respiration to anaerobic respiration and start respiring nitrate. These gaseous oxides are the major product of denitrification and can act as electron acceptors in the absence of oxygen leading to anaerobic respiration (Knowles 1982). Denitrification takes place through the following intermediates:

$$NO_3^- \xrightarrow{Nar} NO_2^- \xrightarrow{Nir} NO \xrightarrow{Nor} N_2O \xrightarrow{Nos} N_2$$

Figure 1.3. Shows the process of dentrification with intermediate products and the four reductase which are essential for denitrification

The release intermediate product of nitrification and denitrification (N_2O and NO) to the atmosphere can be explained by ″holes in the pipe model″. This model explains that N_2O and NO are leaked to atmosphere due to overload of enzymatic capacity involved in the process of nitrification and denitrification.

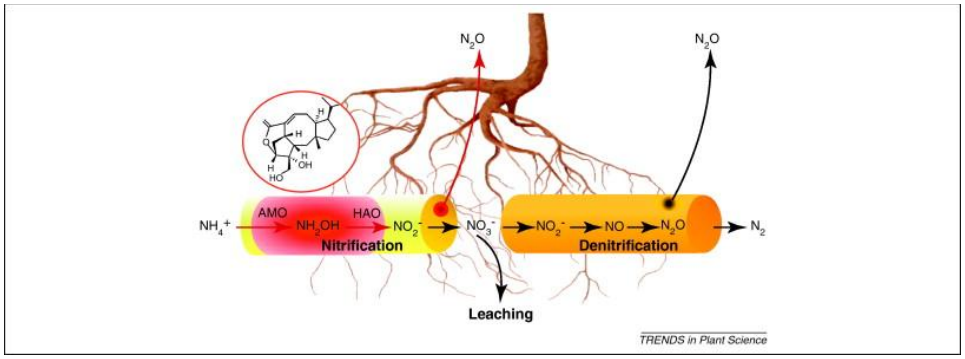


Figure 1.4 Holes in the pipe model, showing the process of nitrification and denitrification where N_2O and NO are leaked to the atmosphere, due to excess of reactive nitrogen present (Philippot and Hallin)

Denitrification enzymes:

There are four reductases (NAR, NIR, NOR, N_2OR) which are involved in denitrification. If all of the four reductase are regulated and expressed, then there will be less toxic denitrification intermediate oxides (NO_2^-, NO, and N_2O) (Zhu, Mulder et al. 2013).The position (membrane bound or in the periplasm) of denitrification enzymes in denitrifiers are shown in figure 1.4. and are further explained in the following sections .

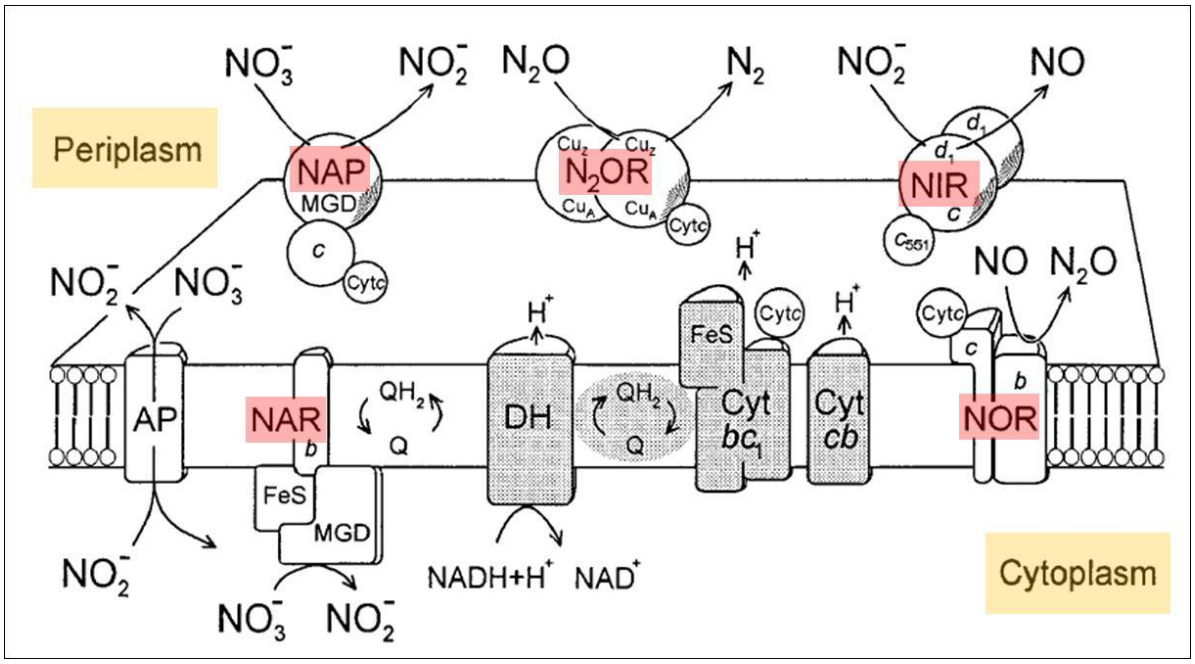


Figure 1.5 The position of denitrification enzymes in denitrifiers. Denitrification enzymes are highlighted in red. NAR and NOR are membrane-bound, whereas NAP, NIR and N2OR are in the periplasm. NAP: periplasmic nitrate reductase; NAR: membrane-bound nitrate reductase; NIR: nitrite reductase; NOR: nitric oxide reductase; N2OR: nitrous oxide reductase

1.4.1. Nitrate reductase (NAR)

Nitrate reductase is the first enzyme in the process of denitrification and catalyzes the reduction of nitrate (NO_3^-) into nitrite (NO_2^-) (Jacques, Burlat et al.). The activity of the nitrate reductases is rate limiting step in the process of denitrification.

There are three forms of NAR, one assimilatory (Nas) and two dissimilatory forms, the membrane-bound respiratory form (Nar) which is expressed under anoxic or microoxic conditions; and second the periplasmic form (Nap) which can be expressed in aerobic conditions i.e. it is oxygen tolerant. NARs ability to be expressed under both aerobic and anaerobic conditions makes it of special interest (Bell, Richardson et al. 1990). NAR is a mononuclear

molybdenum enzyme; it is a member of the dimethylsulfoxide (DMSO) reductase family. In prokaryotic NARs molybdenum (Mo) is bound to a bis-molybdopterin guanine dinucleotide (MGD). The NARs are heterotrimeric and are composed of a Mo-MGD cofactor, an iron-sulfur (FeS) center, and either a FAD group in the case of Nas; a cytochrome b prosthetic group in the case of Nap; a cytochrome c group in case of Nar. A variety of electron donors are used by NARs including ferrodoxin, flavodoxin, and NADH for the Nas enzyme, quinones for the Nap enzymes, and quinols for the Nar enzymes (Carlisle, Yarnes et al. 2014).

1.4.2. Nitrite reductase (NIR)

Nitrite reductase (nir) catalyzes the reduction of nitrite (NO_2^-) into nitric oxide (NO). This step is of special importance as it differentiates the denitrifiers from the nitrate respiring bacteria also both substrate the (NO_2^-) and the product (NO) are toxic, moreover the first gaseous product of denitrification is produced in this step (Zumft 1997).

Nitrite reductase exists in two different structural types, the first type contains copper and is encoded by the gene *nirK* while the second type contains hemeC and is encoded by the gene *nirS*. Interestingly no functional difference has been found between the two structurally different nitrite reductases but both of them have never been found in the same organism (Prieme, Braker et al. 2002)

1.4.3. Nitric oxide reductase (NOR)

Nitric oxide reductase belongs to family oxidoreductase, and catalyzes the reduction of nitric oxide (NO) to nitrous oxide (N_2O). There are two types of NOR; qNor and cNor. It is known to be the key enzyme involved in the production of nitrous oxide N_2O, the GHG (Hino, Matsumoto et al. 2010). Bacteria use NOR enzyme to reduce NO which is toxic to them at high concentrations, NOR is not active in the presence of oxygen (Zumft 1997).

NOR is a membrane bound enzyme which has a c-type cytochrome centre, and catalytic site with a high spin b-type haem and an adjacent nonhaem iron atom (Ferguson 1998).

1.4.4. Nitrous oxide reductase (N_2OR)

Nitrous oxide reductase belongs to the family oxidoredeuctase and catalyzes the reduction of nitrous oxide (N_2O) to nitrogen (N_2), the final step in the process of denitrification. This enzyme is of much interest because this is the only so far known biological mechanism that converts N_2O to inert N_2. It is a periplasmic enxyme and is considered to be more sensitive to O_2 than the other denitrification enzymes. It is a homodimeric enzyme with one domain containing copper and protein $Cu_A$and the second domain consists of 7-bladed propeller of β-sheets, this makes up the catalytic site Cuz (Brown, Tegoni et al. 2000).

1.5. Dissimilatory nitrate reduction to ammonia (DNRA):

In the dissimilatory nitrate reduction to ammonia **(**DNRA) pathway NO_3^- is reduced to ammonium (NH_4^+) under anoxic or microoxic conditions, at NO_2^- reduction stage NO and N_2O are emitted as byproduct (Kelso, Smith et al. 1997) (Mania, Heylen et al. 2014). The mechanism of DNRA is still not fully understood.

Thus, DNRA and denitrification take place under the same environmental conditions (low oxygen pressure and presence of organic carbon and NO_3^- or NO_2^-), so both processes are in competition for available NO_3^-. DNRA, which retains nitrogen in soils as (NH_4^+) is considered to be the dominant process when the NO_3^- is limited and organic carbon is present in excess, while denitrification is dominant when NO_3^- is in excess and organic carbon is limited (Kelso, Smith et al. 1997). Both of these processes occur at the same time in anoxic environments, so it is difficult to distinguish their contribution to N_2O emission.

The difference between DNRA and denitrification is that in DNRA, NO_2^- is reduced to NH_4^+ in just one reaction, in which six electrons are transferred. The reaction is catalyzed by a key enzyme respiratory cytochrome *c* NO_2^- reductase known as NrfA (Mania, Heylen et al. 2014).

1.6. Anammox

Anaerobic Ammonium Oxidation (Anammox) is an important part of the nitrogen cycle in which nitrite and ammonia are converted into dinitrogen (N_2) gas. Denitrification and anammox are the two important processes by which the fixed dinitrogen gas is returned to the atmosphere (Kartal, Maalcke et al. 2011).

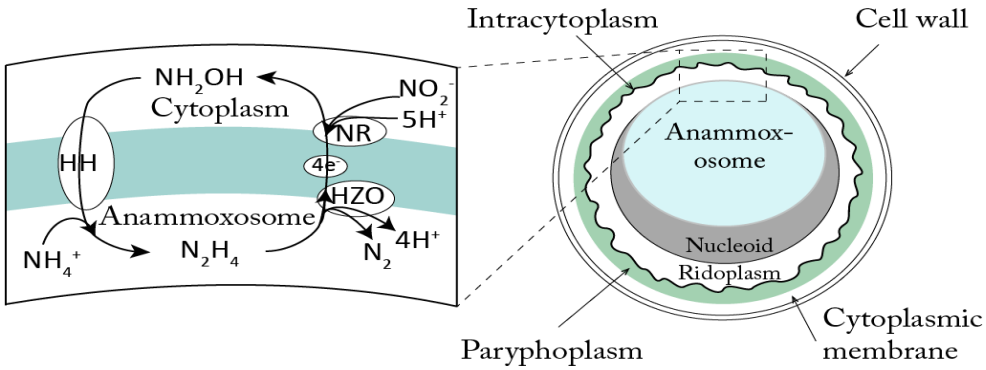


Figure 1.6 Morphology of the anammox cell and proposed model for the anammox process (Ryabenko 2013)

Anammox bacteria depend on other bacteria which provide ammonia and nitrite. It forms synergism with ammonia oxidizing bacteria (AOB) produce nitrite. As mentioned in section 1.2 nitrification is a two step process. In the first step ammonia is oxidized to nitrite so the AOB bacteria provide the anammox with substrate nitrite and in turn anammox removes the toxic product nitrite for AOB (Ding, Zheng et al. 2013). Ammonia and nitrite are limited in natural ecosystems, to get the nitrite substrate anammox and denitrifying bacteria compete with each other. Anammox are chemoautotrophs, one of the factor on which their survival rate depends is chemical oxygen demand (COD) concentrations in wastewater which is used to indirectly measure the organic carbon. When COD is low while nitrite is high than activity of anammox is not affected as anammox are strict anaerobes, as COD starts increasing than the denitrifiers will overtake and the nitrite will be used by them.

1.7. Sulphate reducing bacteria (SRB) :

Sulphate reducing bacteria (SRB) obtain energy by oxidizing organic compounds or molecular hydrogen while reducing sulphate (SO_4^{2-}) to sulphide (S^{2-}) or hydrogen sulphide (H_2S). SRB can support metal precipitation as sulphide and increase the alkalinity(Zhang and Wang 2014) The formation of sulphide causes corrosion and odour problems (van den Brand, Roest et al. 2014).

Sulphate reducing bacteria (SRB) were of special interest for our study. There is a possibility that by quantifying this group, we could understand where the reductive power in the groundwater discharge zone at TSP comes from to drive the observed strong NO_3^- sink. At TSP there is a strong observed sink for sulfate, so we speculated whether sulfide could play a role as reducing agent, somehow releasing electrons when ground water table moves up and down.

1.8. N_2O as green house gas:

Nitrous oxide (N_2O), commonly known as the laughing gas is very stable and inert green house gas in the troposphere. N_2O is known to be one of the three most important green house gases, as described in (IPCC, 2007). When this gas reaches the stratosphere it is broken down to NO (Conrad 1996). N_2O is known to be the most destructive source of stratospheric ozone depletion (Ravishankara, Daniel et al. 2009). The atmospheric N_2O concentration has increased about 20% over the past century and it is increasing by 0.25% each year (Martinez-Espinosa, Cole et al. 2011). N_2O study is of special interest, even though the concentration of N_2O (0.3 ppmv) is less as compared to CO_2 (387 ppmv). This is because it has global warming potential 300 times higher than that of carbaon dioxide and has a residence time of 120-150yrs in the atmosphere (Fields 2004) (Ravishankara, Daniel et al. 2009). Among the green house gases the contribution of N_2O to global warming accounts for 10%, of which more than two thirds comes from microbial activity taking place in soil (Richardson, Felgate et al. 2009).

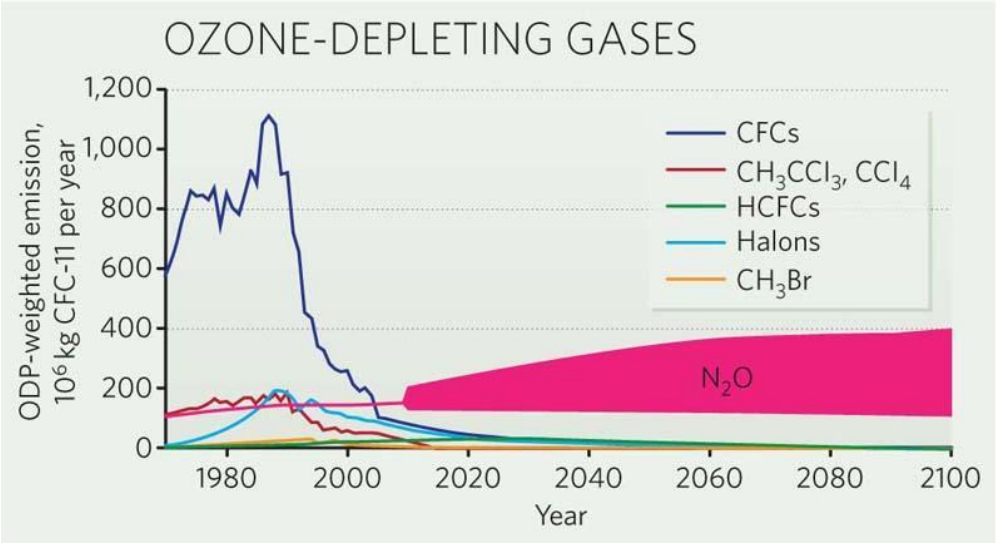
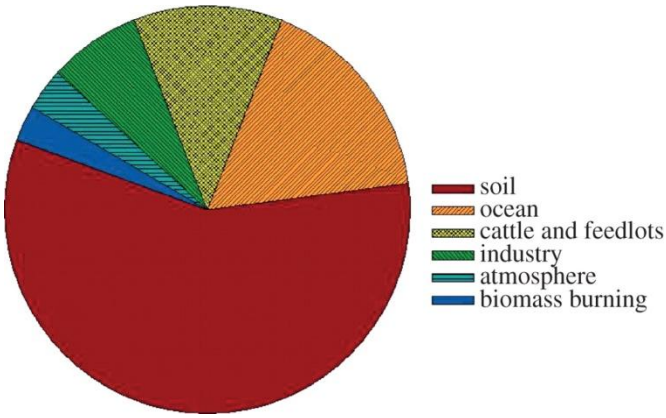


Figure 1.7 Comparison of the ozone-depleting potential of different gases (Richardson, Felgate et al. 2009)

The N_2O has become abundant in the atmosphere due to anthropogenic activities, rapid changes in agricultural practices, combustion of fossil fuels, and the human impact on the nitrogen cycle (Gruber and Galloway 2008). To save the ozone layer, it is important to control future emission

by anthropogenic activities of different ozone depleting gases. Especially N_2O, because of its destructive potential (300 times more as compared to CO_2) and its stable nature (120-150yrs) (Ravishankara, Daniel et al. 2009).



1.8 Proportions of total global nitrous oxide emitted by various sources and human activities. Adapted from data in the Contribution of Working Group III to the fourth assessment report of the intergovernmental panel on climate change, 2007. Eds B. Metz, O. R. Davidson, P .R. Bosch, R. Dave and L. A. Meyer. Cambridge, UK; New York, NY: Cambridge University Press.

1.9. Factors affecting N_2O emission:

It is possible to mitigate N_2O emission factors. The emission of N_2O from soil to the atmosphere by microbes is directly or indirectly affected by the factors which influence the rate of nitrification or denitrification.

Two of the important factors which control the emission of N_2O are the availability oxygen and water. In soil availability of oxygen to microbes depend on the structure of soil and on its pore size. If water is present in the soil it leads to water filled pores, leading to anoxic conditions because oxygen can't diffuse from environment to soil and denitrification becomes dominant process. While if less water is present and soil is relatively dry, then it becomes oxic and nitrification dominates there (Schurgers, Dörsch et al. 2006). Another master factor controlling the emission of N_2O is the pH; the pH influences the rate of nitrification and denitrification in different ways. The rate of nitrification is found to be optimum at pH 6.5-8, some activity of nitrification has been observed in acidic soils as well. And denitrification is often found to be most favorable at neutral pH, and the rate increases with decrease in pH. (ŠImek and Cooper 2002). one more factor controlling the N_2O emission from soil is the use of nitrogen fertilizers,

which provide NH_4^+ and N to microbes leading to increase in rates of nitrification and denitrifcations which result in high N_2O emissions (Beauchamp 1997).

In addition to availability of nitrogen the emission of N_2O is also influenced by the presence of carbon. In heterotrophic nitrification and denitrification, organic carbon C acts as the electron donor (Huang, Zou et al. 2004). In our study we considered alternative source for electron donation which will decrease the importance of C. We study the quantification of sulphate reducing bacteria, as their presence could indicate production of reduced forms of sulphur which may act as electron donors.

1.10. Site description and previous studies :

The catchment tieshanping (TSP), located about 25 km northeast of chonqing city, SW China (29°38'N 104°41'E) is of special interest as it is under increasing pressure of high nitrogen (N) deposition in recent decades The catchment is surrounded by lots of agricultural fields, therefore receiving N deposition from there. TSP site receives about 5 g N m^{-2} yr^{-1} through atmogenic deposition ((2/3 as ammonium), most of which is removed before discharge).

The tieshanping (TSP) catchment is 16.2 ha headwater catchment, and for the current study 4.6 ha sub-catchment was selected. This catchment consists of two landscape elements: one hillslope (HS) and one groundwater discharge zone (GDZ). In each of these landscape element, we set up a transect, T_0 ,T_1, T_3, T_5 donate the four spots top to the bottom of the HS and B_2, B_3, B_5, B_6 are four spots from the inlet to the outlet of the GDZ as shown in figure 1.6, from these spots the soil samples were taken (Zhu, Mulder et al. 2013). Both landscapes, HS and GDZ differ from each other in eco hydrological condition e.g. soil texture, moisture, organic carbon supply, pH and NO_3^- concentration. The GDZ is covered with shrubs and grasses while trees taller than 2 m are absent. GDZ had a short history of vegetable production in 1960 but abandoned shortly after, Zhu and colleagues (2013).The mean annual temperature of TSP is 18.2 ˚C, higher rain fall about 75% occurs in summer (average rainfall 2001-2003).

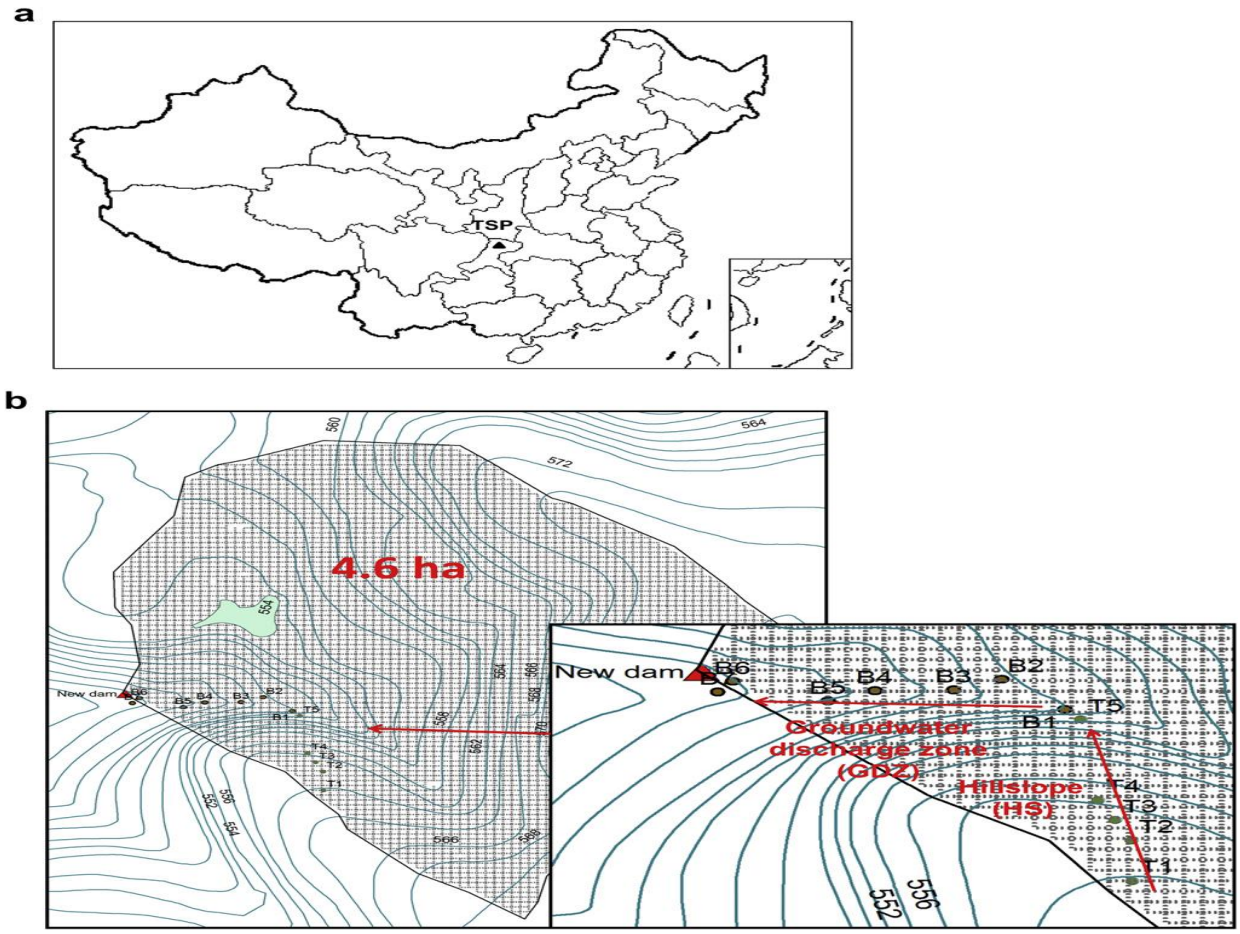


Figure 1.9 Location of Tieshanping (TSP) and sampling points in TSP. location of TSP sub-catchment in china shown in panel (a), and panel (b) shows the plots selected to collect the samples in TSP catchment, transect T (T1-T5) is the hillslope (HS) and transect B (B1-B6) is the groundwater discharge zone (GDZ) (Zhu, Mulder et al. 2013).

TSP is considered to be a hotspot for N_2O emission. Earlier field work has been done on TSP catchment to study emission of N_2O. In previous studies anoxic incubations were made to study the process of denitrification. In situ $^{15}NO_3^-$ labeling experiment was conducted to compare the process of nitrification and denitrification. My goal was to focus on the molecular part and find correlation between abundance of N-cycle genes and N-transformation rates.

Aim of this study:

As mentioned in earlier sections N_2O is green house gas and its emission is a threat to our environment, and is known to be most effective in destroying the stratospheric ozone layer. Soils samples for my study were collected from subtropical forest ecosystem in southwest China, Tieshanping (TSP) which is known to be a hotspot for N_2O emission. The current study continues from a study conducted previously by our group member Jing Zhu. In the previous study mainly field work and robot measurements plus the gas kinetics experiment were done.

This study aimed to answer the following hypthesis:

i. To see if there is correlation between abundance of N-cycle genes and N-transformation rates.
ii. To study complete denitrification genes (gene *nosZ*, gene *nirS*, and gene *nirK*) abundance and to compare the denitrification genes abundance in HS and GDZ.
iii. It is hypothesized that nitrous oxide reductase (N_2OR) was present at HS but was not expressed due to oxic anoxic transitions while the other enzymes (NIR NOR) are not influenced.
iv. It is assumed that ratio of *nos*/*nir* would be higher in GDZ.
v. Nitrite oxidation was retarded in HS samples, for this reason it is assumed that *amoA* (ammonia oxidizers) abundance will be higher than *nxrB* (nitrite oxidizers) abundance in samples where nitrite accumulates.
vi. Archaeal ammonia oxidisers will dominate (higher abundance) while there will be lower amounts of bacterial ammonia oxidisers due to low pH in this soil.
vii. Low organic material is present at the GDZ, and may not provide enough electrons for denitrification. It is hypothesized that the reductive force can be provided by the sulphate reducers.

The work was done in the following six steps:

i. Extraction and purification of DNA
ii. Optimizing the primers
iii. Making of the plasmids
iv. Quantifying the genes
v. Measuring dry weight, carbon content, nitrate and nitrite concentrations.
vi. Measuring gas kinetics of denitrification using the robotized incubation system.

2. MATERIAL AND METHODS:

INSTRUMENTS	SUPPLIERS
2720 Thermal Cycler (PCR machine)	Applied Biosystems, CA, USA
Delta 320 pH meter	Mettler Toledo AG, Greifensee, Switzerland
Drying oven	Termaks AS, Bergen, Norway
Gel Doc XR system (with Quantity One 1-D Analysis Software, ver. 4.6.7)	Bio-Rad Laboratories, CA, USA
Gel Doc XR system (with Quantity One 1-D Analysis Software, ver. 4.6.7)	Bio-Rad Laboratories, CA, USA
Electrophresis electricty supplier	Bio-Rad Laboratories, CA, USA
MiniSpin microcentrifuge	Eppendorf AG, Hamburg, Germany
NanoDrop Spectrophotometer ND-1000	Nanodrop Technologies, Thermo Fisher Scientific, MA, USA
Plate Spin II centrifuge (cooling centrifuges)	kubota
SpeedVac Concentrator (vacuum centrifuge)	speedvac Savant Instruments Inc., NY, USA
Qubit Fluorometer	Invitrogen, Life Technologies, CA, USA
StepOnePlus Real-Time PCR System (with StepOne Software v2.0) QPCR	Applied Biosystems, Life Technologies, CA,USA
Bead beating machine	AB applied biosystems

Incubator with and without shaker	provocell
Water bath	thermo scientific

LABORATORY EQUIPMENTS	SUPPLIERS
Pipette tips	Thermo Scientific
Eppendorf tubes	Axygen
Glass beads	Sigma
Syringes 10ml-50ml	Plasti Pak
Various glass equipments	Labsystems
Petri dish	
Spatula	
Beakers	
Measuring cylinde	
Funnels	

KITS	SUPPLIERS
DNA purification kit	zymo research
Gel extraction kit	Promega
Plasmid extraction kit	Qiagen
TA cloning kit	Invitrogen

CHEMICALS	SUPPLIERS
10 mg/mL ethidium bromide	VWR International, PA, USA
96% ethanol	Kemetyl Norge AS, Vestby, Norway
Acetic acid	Merck KgaA, Darmstadt, Germany
Agar	merck
Amphicillin	sigma
Calcium chloride, dihydrate (CaCl2 · 2H2O)	Merck KgaA, Darmstadt, Germany
Chloroform	Merck KgaA, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA	Sigma, Sigma-Aldrich, MO, USA
Gel red	Sigma
Isoamyl alcohol	Merck KgaA, Darmstadt, Germany
Potassium nitrate (KNO3)	Merck KgaA, Darmstadt, Germany
Sodium chloride (NaCl)	VWR International, PA, USA
Sodium hydroxide (NaOH)	Merck KgaA, Darmstadt, Germany
Sodium iodide (NaI)	J.T.Baker, Avantor, PA, USA
Sodium nitrite (NaNO2)	Merck KgaA, Darmstadt, Germany
Trizma base	Sigma, Sigma-Aldrich, MO, USA
xgal	

2.1. PRIMERS:

The primers were amplified by running PCR at different temperatures. The following primers were used in this study.

Table 2.1showing the primers used in this study

Gene	primer	Primer sequence	Reference
16S	**27F**	**AGAGTTTGATCMTGGCTCAG**	**(Weisburg, Barns et al. 1991),and (Muyzer, de Waal et al. 1993)**
	518R	**ATTACCGCGGCTGCTGG**	
***nos*Z**	**nosZ F**	**CGY TGT TCM TCG ACA GCC AG**	**(Henry, Bru et al. 2006)**
	nosZ1622R	**CGSACCTTSTTGCCSTYGCG**	**(Henry, Bru et al. 2006)**
***nir*S**	**cd3aF**	**GTSAACGTSAAGGARACSGG**	**(Throback, Enwall et al. 2004)**
	R3cd	**GASTTCGGRTGSGTCTTGA**	
***nir*K**	**nirK1F**	**GGMATGGTKCCSTGGCA**	**(Braker, Fesefeldt et al. 1998)**
	nirK5R	**GCCTCGATCAGRTTRTGG**	
***amo*A for Bacteria**	**amoA-1F**	**GGGGTTTCTACTGGTGGT**	**(Rotthauwe, Witzel et al. 1997)**
	amoA-2R	**CCCCTCKGSAAAGCCTTCTTC [K= G or T; S = G or C]**	
***dsr* (dissimilatory sulfite reductase)**	**DSR1F1^{+}**	**ACSCACTGGAAGCACGGCGG**	**Kondo 2004**
	DSR1-R	**GTGGMRCCGTGCAKRTTGG**	
***amo*A for Archea**	**cren amo_F (I)a**	**ATGGTCTGGCTAAGACGMTGTA**	**Hallam et al. 2006**
	Arch-amoAR	**GCGGCCATCCATCTGTATGT**	**Francis et al. 2005**
Nspira (16S Gene)	**518F**	**CCAGCAGCCGCGGTAAT**	**(Webster, Embley et al. 2005)**
	Nspira-705r	**GGCCTTCYTCCCGAT**	
Nspira (nitrite oxidoreductase gene)	**nxrB169F**	**TAC ATG TGG TGGAAC A**	**Pester et al. 2010**
	nxrB638R	**CGG TTC TGG TCRATC A**	

BUFFERS:

TAE, 50X 242g tris base 57.1ml acetic acid 100ml 0.5MEDTA pH8 final volume

AGAR AND MEDIA:

- S.O. C MEDIUM
  2g Bacto ™ tryptone
  0.5g Bacto™ yeast extract
  0.075g Nacl (1M)
  0.019g Kcl (1M)
  0.247 $MgSO_4$
  60 ml dH_2O
  Sterilized in an autoclave machine for 15mins at 115°C . 2ml glucose (1M after autoclaving), Add sterilized dH_2O to 100ml.

- LB medium and LB agar plates:
  10% tryptone- 10g
  0.5% yeast extract – 5g
  1.0% Nacl – 10g
  pH 7.0
  dissolve everything in 950ml water and adjust the pH to 7. Autoclave on liquid cycle for 20mins for LB medium and for LB agar plates add 15g/L agar before autoclaving.

3. METHODS:

3.1. Soil sampling:

Soil samples were collected on sixth July 2013 by Peter Dörsch from a subtropical forest ecosystem in southwest China, Tieshanping (TSP). The upper 15 cm were sampled. Soil was collected from eight different spots, four from the hillslope (HS) labeled as T0, T1, T3 and T5 and four from the ground water discharge zone (GDZ) labeled as B2, B3, B5, and B6. The soil was transported from China to Norway in cylinders, which were kept cold during the transport. In the lab soils were sieved (4 mm mesh size) and stored in closed plastic bags at 4 °C until use. Dry weight and carbon content of the samples were measured. The pH of soil from HS was and 4.5 in GDZ.

3.2. Extraction of DNA from soil microbes:

DNA can be extracted from soil bacteria either by direct soil extraction in which cells are lysed within the soil or by separating the cells from the soil before lysis (so-called "indirect extraction"). The advantage of the the direct extraction method is that the DNA is, at least theoretically obtained from the entire microbial community; the disadvantage is that the DNA is not only extracted from living microbes but also from dead microbes. Furthermore the DNA obtained will contain more impurities like humic acids, compared to when DNA is obtained from cells previously separated from the soil. Although the "indirect extraction" method provides purer DNA; it has a large disadvantage in that extracted DNA represents only 20% of the microbial community. The common important step in both methods is the lysis (Frostegard, Courtois et al. 1999). In the present thesis work I used the first method of direct DNA extraction, after extraction the impure DNA was purified. DNA was extracted and purified from the provided soil samples by following the procedure:

Materials:

- 0.25g small glass beads, 0.25g middle glass beads and 1 big glass bead in a 2ml tube.
- Phenol-chloroform-isoamyl alcohol (25:24:1) PH 8
- Extraction buffer CTAB
- Chloroform isoamyl alcohol
- PEG 30%

- -18°C 70% alcohol
- Dnase frees water

Procedure:

i. 0.25g of soil was transferred to 2ml tubes containing the beads
ii. 0.25ml of phenol and 0.25ml of chloroform isoamyl alcohol was added to the tubes followed by o.5ml of CTAB buffer.
iii. Samples were lysed at speed 6.0 in BIO101 fast prep FP120 for 2X45 (with 60 sec on ice in between)
iv. Samples were centrifuged at 16 000g (14.600 rpm) for 5 min at 4 °C
v. 400 µl of the supernatant (aqueous phase) was carefully transferred to new tubes on ice
vi. To remove the phenol, 400 µl (equal volume) of chloroform-isoamyl alcohol (24:1) was added and the samples were centrifuged at 16000g (14.600 rpm) for 5mins at 4 °C
vii. The aqueous phase was carefully transferred to new tubes on ice (avoiding the interphase).
viii. 800 µl of 30% PEG solution was added to the aqueous phase in order to ppt. the nucleic acid. The tubes were placed on ice for 2hours.
ix. The tubes were centrifuged for 20mins at 16000g (14.600PM) at 4 °C
x. The supernatant was discarded and the pellets were washed with 0.5ml of 70% ice cold (-18 °C) ethanol.
xi. The tubes were centrifuged for 5mins, 16000g (14,600rpm) at 4 °C.
xii. The supernatant was discarded (this step was done carefully to avoid losing the pellets, as they were lose) and the tubes were dried in the vacuum drier.
xiii. The pellets were re suspended in 100 µl dH_2O.
xiv. The concentration of DNA was measured by nanodrop method.
xv. The DNA was stored at -20 °C.

DNA purification kit:

Following two DNA purification kits from two different suppliers were tried

- Promega
- Zymo research

The DNA purification kit from zymo research gave the best results.

Procedure:

a) In a 1.5ml micro centrifuge tube 2Volumes of ChiP DNA Binding Buffer to each volume of DNA sample was added
b) The mixture was transferred to a provided Zymo-Spin^{TM} IC-XL column in a collection tube.
c) The Zymo-spin with collection tube was centrifuged and the flow through was discarded.
d) 200 µl of wash buffer was added to the column and was centrifuged for 1 minute. The step was repeated.
e) 50µl of water was added directly to the column matrix and left on bench for 1 minute, and then the column was centrifuged in clean 1.5ml micro centrifuge tube for 30 seconds to elude the DNA. The DNA was stored at -20 °C.

The optimizing annealing temperature of the primers was found by using the extracted DNA and by running PCR reactions.

3.3. PLASMID PREPARATION:

3.3.1. Polymerase chain reaction:

PCR is a technique that exponentially amplifies targeted sequences of DNA in *vitro* through using the enzymatic replications. It is the most commonly used technique due to its speed (fast), simplicity, specificity and sensitivity. There are three major steps in a PCR, (i) Denaturation (94°C) in which the double stranded dsDNA melts resulting in single stranded ssDNA. (ii) Annealing (~54°C) in which the primers anneasl to the complimentary template strand. And (iii) The extension (~72°C) in which the polymerase initiate the replication of DNA fragmented located between the primers by adding deoxyribonucleoside complementary to the corresponding template base in a 5’ to 3’ direction. These three steps complete one PCR cycle and in most cases

the cycles are repeated until sufficient amount of DNA concentration is reached (35-40cycles). In this study we used the PCR for amplifying the primers to find the annealing temperature and amplified the DNA to make the plasmids for cloning purposes.

Procedure:

- The PCR was performed using the protocol provided by the supplier, in a 25 µl tube containing the following reagents:
- Master mix for Omega:

Reagent	volume
DNA template	2 µl
5X PCR buffer	5 µl
dNTPs	2µl
Primer forward reverse	1µl
Primerreverse	1µl
Taq polymerase	0.2 µl
$Mgcl_2$	2 µl
Sterile water	11.8 µl
Total volume	25 µl

- The reagents were mixed and the reaction mixture was placed in a thermal cycler. The typical setting for the cycler were as follow:

PCR settings:

Temperature	steps	time	cycles

95°C	Intial denaturation	5 mins	35 cycles
94°C	Denaturation	30 sec	
55*°C	Annealing	40sec	
72°C	Extension	40sec	
72°C	Final extension	40sec	
4°C	Storage	∞	

***this temperature of annealing was varied and adjusted according to the primers used.**

3.3.2. Agarose gel electrophoresis:

Agarose gel electrophoresis is a technique in which DNA fragments are separated according to its size in an electric field. The phosphate backbone of the DNA or RNA is negatively charged so when placed in an electric field the DNA will migrate towards positively charged anode. The separated DNA fragment can be visualized under the UV light either by staining with appropriate dye or by adding a dye into the gel while making it (Lee, Costumbrado et al. 2012).

Preparation of 1% agarose gel:

Materials:

Ultra pure ™ agarose

1 x TAE buffer

Gel red 0.4 µl

Procedure:

- 1% agarose solution was prepared by dissolving 0.4 g of agarose in 40 ml of TAE buffer. This was then heated in the microwave to dissolve the agarose in TAE buffer.
- Prior to casting 0.4 µl of gel red was added to the agarose solution and mixed well. The solution was then poured into a gel rack where combs were inserted to make the wells.
- The gel was left for 30 minutes to solidify. The combs were removed and the gel was placed in electrophoresis chamber which was filled with 1 X TAE buffer
- 6 µl of the ladder and 5 µl of the PCR product was added into the wells. This was run for 40 minutes at 80 volts. The band was cut under the UV light in Gel doc machine.

3.3.3. Purification of DNA

Gel extraction kit was used to extract the DNA by following procedure:

- The gel slice with the band cut was weighed and equal volume of binding buffer (XP2) was added. This mixture was incubated at 60° C until the gel completely melted.
- The Hibind DNA mini column was placed in a collection tube. The DNA/agarose solution from first step is added to the HiBind DNA mini column and centrifuged at 10,000x g for 1 min at room temperature. The flow through liquid was discarded and the HiBind DNA mini column was placed back into the same collection tube.
- 300 µlof binding buffer XP2 was added into the HiBind DNA mini column, and centrifuged at 13,000X g for 1 minute at room temperature. The flow through liquid was discarded and the HiBind DNA mini column was placed back into the same collection tube
- 700 µl of SPW wash buffer was added (diluted with absolute ethanol) to the HiBind DNA mini column and centrifuged at 13,000 X g for 1 minute at room temperature, this was done in order to wash the HiBind mini column. The flow through liquid was discarded and the HiBind DNA mini column was placed back into the same collection tube

- In order to remove the ethanol centrifuged the empty HiBind DNA mini column for 2 minutes at maximal speed ≥13,000 x g to dry the column matrix.
- The HiBind DNA mini column was placed into a clean 1.5ml microcentrifuge tube. 50 µl of elution buffer was added to the matrix column and incubated at room temperature for 2 minutes, this was centrifuged for 1 minute at maximal speed ≥13, 000 x g to elute the DNA.

3.3.4. DNA Ligation:

An important step in making of recombinant plasmid is to connect the insert DNA to the vector. This is done by the formation of a phosphodiester bond between 5' phosphate and 3' hydroxyl termini in double stranded DNA. This process is called ligation and is carried out by T4 DNA ligase enzyme. This enzyme repairs the "nicks" in the DNA at the expense of ATP which is usually provided in the buffer.

- 10 µl of ligation mix was set up as follow:

Fresh PCR product	Xµl
10X Ligation Buffer	1µl
PCR®2.1 vector (25ng/µl)	2µl
T4 DNA ligase (4.0 Weiss units)	1µl
Sterile water	to make up the total volume of 9µl
Final volume	10µl

- The formula below was used to estimate the amount of PCR product needed to ligate with 50 ng of PCR ®2.1 vector:

$$X \text{ ng PCR product} = \frac{(Y \text{ bp PCR product})(50\text{ng PCR}^{®}2.1 \text{ vector})}{(\text{size in bp of the PCR }^{®}2.1 \text{ vector:}\sim 3900)}$$

Where ,

X ng = the amount of PCR product and

Y bp = base pairs to be ligated for a 1:1 (vector:insert) molar ratio.

- The ligation mix was incubated at 14 °C for overnight.

3.3.5. Transforming competent cells:

The following protocol was followed to transform one shot® competent cells:

- The vials containing the ligation reactions were centrifuged and placed on ice. The competent cells were thaw on ice, 50 µl vial of frozen one shot® competent cells were used for each transformation.
- 2µl of each ligation reaction was directly transferred by a pipette into the vials of competent cells; this was mixed gently by pipette tip.
- The vials were incubated on ice for 30 minutes and the remaining ligation mix was stored at -20 °C.
- The cells were heat shocked at 42 °C without shacking, and immediately were transferred back to ice.
- 250µl of room temperature S.O.C medium was added to each vial.
- The vials were kept on a horizontal shaker at 37°C for one hour at 225rpm.
- The vials were spread on LB agar plates containing X-gal and 50 µg / ml of ampicillin. Three different volumes of each vial were used 10µl, 50µl and 240µl on three different agar plates. The plates were incubated at 37°C overnight, and kept at +4 for 2-3 hours to allow proper color development.

Analyzing Transformants:

- Ten white colonies were picked and transferred to 5 ml of LB broth containing 50 µg / ml ampicillin, this colonies were grown overnight on a shaker incubater at 37° C for plasmid isolation and restriction analysis.
- Plasmid was isolated by following the provided procedure

3.3.6. Real time-PCR (qPCR):

Real time PCR as indicated by name is based on polymerase chain reaction where the DNA is amplified exponentially. The benefit of real time PCR over standard PCR is that it can detect DNA concentration after each cycle by using either fluorescent dyes or fluorescently tagged oligonucleotide probes. In our study we used the fluorescent dye syber green, which emints light signals when it bind to the dsDNA. The fluorophore, fluorescent agents is added to the reaction mix, when it interacts with the PCR product fluorescent signals are emitted. Stronger signals are detected when there is more PCR product. Based on this signals amplification curve is generated. In this study we used qPCR to quantify the functional genes.

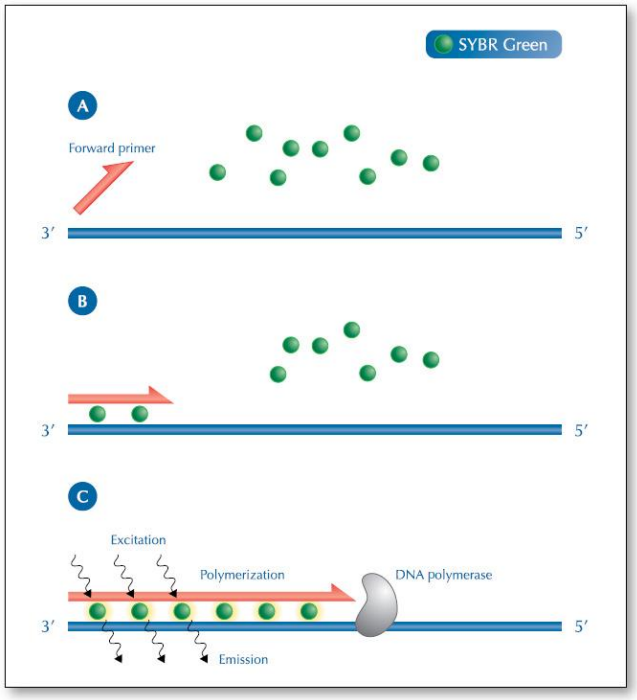


Figure 2.1 the basics of quantitave PCR (qPCR) by fluorescent dye syber green. The syber green molecules are free in the reaction mix, DNA denatures, primers aneal and syber green binds to dsDNA and emits light signals when intercalates between DNA base pairs.

Procedure:

- The qPCR was performed by following the protocol provided by the supplier. A typical reaction setup is shown in the table below:

reagents	volume	Conc.
Master mix	10 µl	
Primer (forward)	1 µl	0.4µM *1
Primer (reverse)	1 µl	0.4µM *1
ROX	0.4 µl	1 X

H_2O	5.6 µl	
Final concentration	18 µl	

- The reagents were mixed, and placed in real-time pcr system step one plus ™ to get the standard, melting and amplification curves.

3.4. Robot measurements (semi-automated robotic incubation system) and the gas kinetics experiment:

A robotized incubation system is used for phenotypic characterization of bacteria. We used this system for denitrifying bacteria. In this system gaseous metabolites and end products are measured at the end of transitions from oxic to anoxic conditions.

This system consists of an automated sampler connected to a peristaltic pump for measurement of gas products by microbes. In addition, awater bath with temperature 0-40˚C. This incubation system at a time can accommodate fifteen 120 ml sealed serum bottles. This system consists of a program called python, which controls the incubation system as the GC, integrating the NO peaks and also organizes the data (Molstad, Dorsch et al. 2007).

Measurement of nitrite NO_2^- and nitrate NO_3^- concentrations:

For this purpose 0.5g of soil was weighed from each sampling point, 700µl of water was added, vortexed and this mixture was centrifuged. The supernatant was transferred into new eppendorf tubes. For nitrite measurement the reducing agent NaI (10mg NaI /ml acetic acid) was used and for nitrate measurement the reducing agent VCL_3 (.8g of VCL_3 in 100ml of 1 M HCL) was used.

Measurement of gas flux by using the robot (semi-automated robotic incubation system):

The experiment was designed to test the denitrification activity of the TSP soil from both HS and the GDZ, a pre experiment was run on the robot to test the respiration rates and parallel flasks with volume 40ml were incubated for nitrite measurement. We used 10 g

fresh weight soil in 120ml vials to measure the gas kinetics over a period of 48h, 90h, 170h and 210 h. Parallel sets of 12 ml vials with 3.3 g wet soil were prepared to measure the nitrite concentration at different time points 48h, 90h, 170h and 210 h. Moisture contents of all the vials were adujed to the same level by adding 1.5 ml of water in the 120 ml vials and 0.5 ml of water in 12 ml vials. Soil nitrate contents were adjusted to the same level by using 1 ml of 0.1 M KNO_3 in 120ml vials and 0.3 ml in 12 ml vials. All incubations were done at temperature 20 ˚C.

4. Results:

The main purpose of my thesis was to quantify the microbial community involved in emission of N_2O in the atmosphere from a subtropical forest ecosystem in southwest China, Tieshanping (TSP). The abundance of the nitrifiers, Ammonia oxidizing bacteria (AOB) and Ammonia oxidizing archaea (AOA), for both the *amoA* gene was quantified and compared. The denitrifiers were quantified along the hillslope and in the ground water discharge zone. Denitrification was also studied by using gas kinetics measurements on a robotized incubation system.

I had 24 real samples from eight different sampling points from TSP, China. Nine different primers were selected based on existing literature as mentioned in section 2.1. I made a plasmid for each of the primer and used these plasmids as my standards in qPCR. The following genes were quantified by qPCR. For total microbial estimate gene *16S*, for denitrification the genes *nosZ, nirS, nirK*, for nitrification gene *amoA* (both from bacteria and archea), for nitrite oxidation gene *nxrB* and for sulphate reducing bacteria gene *dsrA* were quantified.

4.1. DNA quality and quantity:

Soils are difficult to extract the DNA due to the presence of inhibitors. Effort was put in DNA extraction and purification (by trying different extraction and purification methods), then the quality and quantity of DNA was measured by using the Nanodrop and Qubit method. DNA quality and quantity varied between the different samples. DNA with 260/280 ratio 1.7 or higher was preferred and samples with a ratio lower than1.7 were used only for a few cases (appendix table 1-3).

4.2. Amplification of the primers and making of plasmids:

Changes in PCR conditions were made like annealing temperature and template concentration for different primers. After PCR, the primers were observed on the agarose gel for a specific

band for each primer (see index). Plasmids were made for each gene; the band was purified and again the agarose gel was run to check the specific band. Ligation was done and the ligation mix was spread on the agar plates and incubated at 37 °C as described in section (3.3.4). Successful ligation of the PCR product into the plasmid resulted in white colonies, which comprised approx.10%-15% of the total number of colonies. Blue colonies, which theoretically should not contain any insert, were discarded. For each insert, 10 white colonies were picked randomly and further incubated. The plasmids were extracted and analyzed on agarose gel. If a band of the expected size was present, the extracted plasmid was sent for sequencing. A plasmid containing the correct sequence was used to construct the standard curve.

4.3. Abundance of the 16S rRNA gene:

To estimate the size of the total prokaryote community in the soil samples, the 16S rRNA gene was quantified using qPCR. As seen from Fig. 4.1 the abundance of this gene per g dry soil increased along the HS with gene copies around $1.5*10^8$ in T0 T1 and T3, abundance of gene per g dry soil was highest in GDZ with $1.0*10^9$ in T5 as well as in all GDZ samples. The efficiency of the qPCR was 91.1% and the R^2 was 0.994

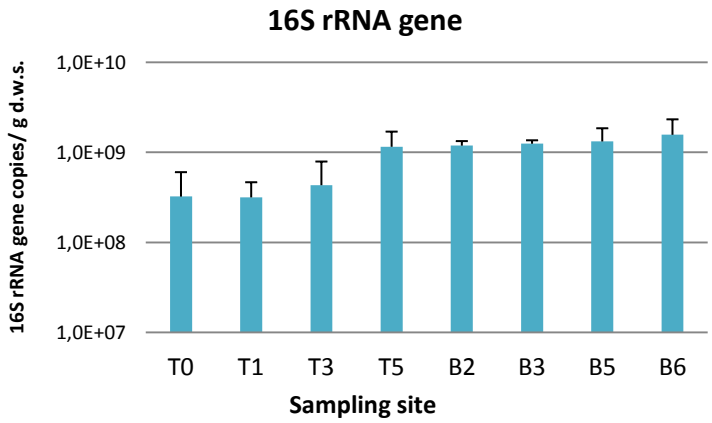


Figure4.1 Number of prokaryote organisms estimated by by qPCR of the 16S rRNA gene T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ.

4.4. Quantification of nitrifying organisms:

4.4.1. AOB and AOA :

Oxidation of ammonia can be carried out by two different groups of prokaryotes; ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA), The gene *amoA*, encoding the enzyme ammonia monooxygenase was quantified for both groups of organisms. It was found that *amoA* genes from AOA were more abundant with gene copies around $5.0*10^7$ in GDZ as compared to AOB with gene copies around 400 in GDZ (Figure 4.2a and b). Three replicate samples, taken from different squares, were analysed for each of the sampling points in the HS and GDZ. In addition the repeatability and reliability of the QPCR was investigated by analyzing the technical replicates (taken from the same soil sample) for the *amoA* gene in Archaea, which showed that the real time PCR technique was repeatable and reliable. The efficiency of the QPCR was 87.907% and R^2 0.998 for bacteria, the efficiency of the QPCR was 82.3% and R^2 0.992 for archaea

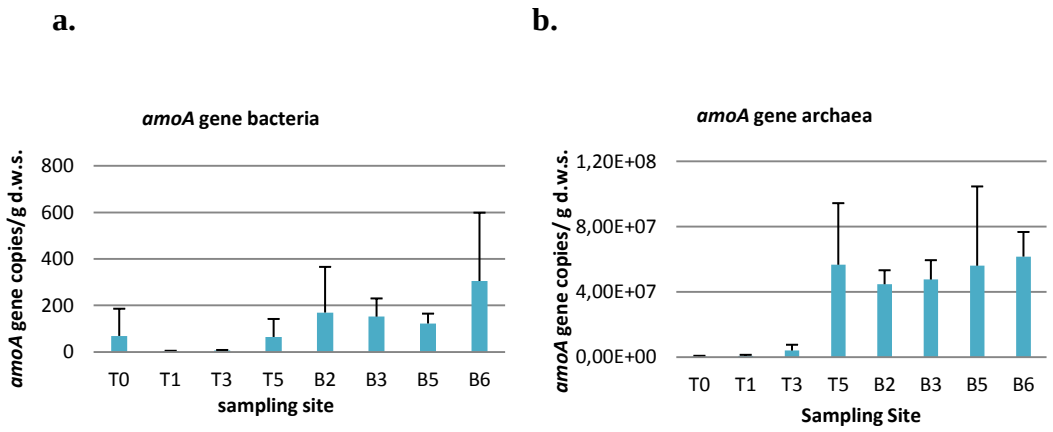


Figure4. 2 Quantification of *amoA* gene for bacteria (graph a) and archaea (graph b) from hill slope (HS) and ground water discharge zone (GDZ) samples. There is an increase in nitrification gene copy number *amoA* gene for bacteria and archaea from hillslope to groundwater discharge zone. *amoA* gene for archaea (AOA) are more abundant as compared to *amoA* gene for bacteria (AOB). T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

4.4.2. Nitrite Oxidation:

The abundance of the gene *nxrB*, which encodes the enzyme nitrite oxidoreductase responsible for the oxidation of nitrite to nitrate in nitrite oxidizing bacteria, was studied. The n*xrB* gene was quantified and its abundance was compared in HS and GDZ. The abundance of *nxrB* gene increased from HS (at sampling point T_0 the gene copies around 5 $1.0*10^5$) towards the GDZ (at sampling point B6 the gene copies observed were around $1.0*10^7$) as shown in Fig.4.3.

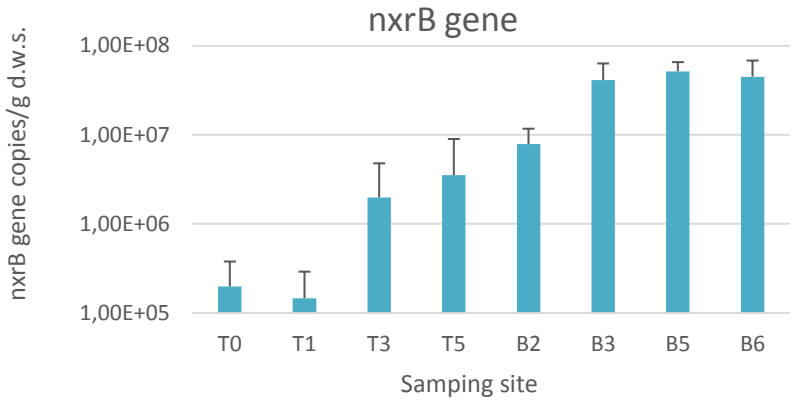


Figure 4.3 The number of *nxrB* gene copies from Hill Slope (HS) and Groundwater Discharge Zone (GDZ) samples. There is an increase in *nxrB* gene copy number from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

The ratio between gene *amoA* and gene *nxrB* for the different samples showed that gene *amoA* (archaea ammonia oxidizers) is highr as compared to *nxrB* (nitrite oxidizers) at HS as compared to GDZ as shown in graph 4.3.1.

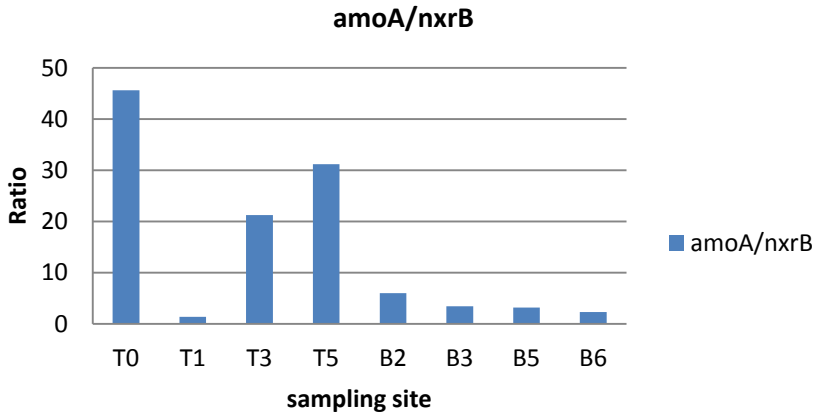


Figure 4.3.1 Ratio between gene *amoA* and gene *nxrB*, different samples showed that gene *amoA* (archaea ammonia oxidizers) is highr as compared to *nxrB* (nitrite oxidizers) at HS as compared to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ.

Ratio of functional genes *amoA* (bacteria and archaea)and *nxrB* to 16S rRNA was analyzed. This was done to see the abundance of functional community to the total microbial community. The results showed that the abundance of functional genes were higher in ground discharge zone (GDZ) as compared to the hillslope (HS) (appendix).

4.5. Denitrification:

The abundance of the genes *nosZ*, *nirS* and *nirK* genes involved in denitrification, increased from the HS towards the GDZ. This trend was similar for the three genes, but the gene copy numbers were much lower for the gene *nirK* with gene copies around $5.00*10^5$ in HS and $5.00*10^6$ in GDZ compared to gene *nosZ* with gene copies around $5.0*10^6$ in HS, $2.05*10^8$ in GDZ and *nirS* with gene copies around $1.0*10^6$ in HS, $1.0*10^8$ in GDZ. Among all the genes of denitrification, the gene *nosZ*, encoding the N_2OR, is of special interest since this enzyme is the only known enzyme that reduces N_2O. *nosZ* the efficiency of the QPCR was 97.45% and R^2 0.998, *nirK* the efficiency of the QPCR was 80.01% and R^2 0.999. *nirS* the efficiency of the QPCR was 81.1% and R^2 0.995

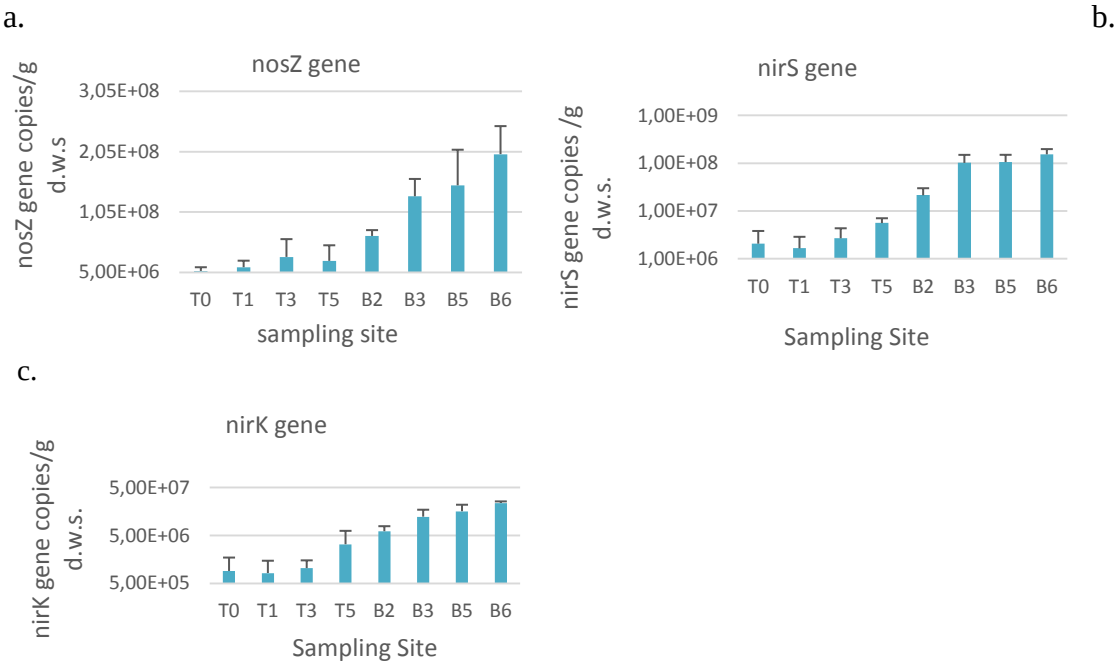


Figure 4.4 The number of denitrification gene copies from Hill Slope (HS) and Groundwater Discharge Zone (GDZ) samples. There is an increase in denitrification gene copy number (part a *nosz*, part b *nirs*, part c *nirk*) from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

Ratio of denitrification genes (*nosz*, *nirs*, *nirk*) to 16S rRNA was analyzed. This was done to see the abundance of functional community to the total microbial community. The results showed that the abundance of denitrification genes were higher in ground discharge zone (GDZ) as compared to the hillslope (HS) (result shown in appendix).

4.5.1. Ratio of *nos* and *nir*:

The ratio of *nos/nir* was declining from HS towards the GDZ. As it has been seen in graph 4a that the gene *nos*Z is higher in GDZ as compared to the HS, but the *nos/nir* ratio indicates

opposite case. Higher ratio of *nos/nir* at HS indicate that, at HS more bacteria harbor gene *nosZ* as compared to gene *nirK*.

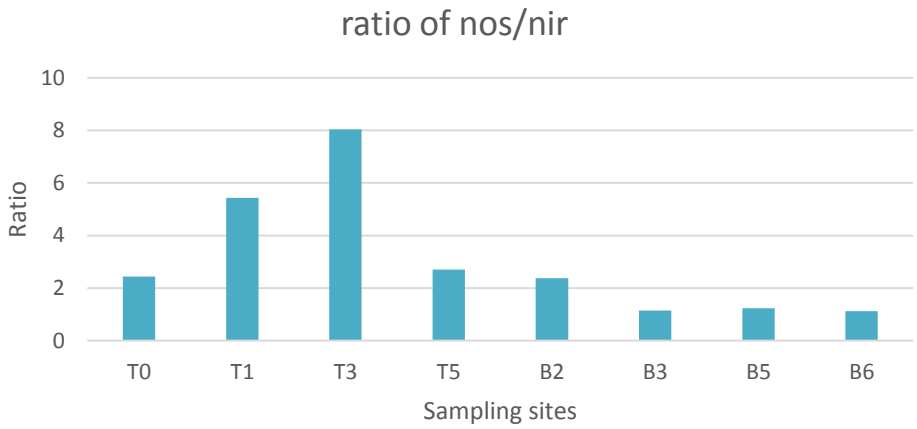


Figure4. 5 Ratio of *nosZ*/(*nirK*+ *nirS*). Graph showing the ratio of *nosZ*/(*nirK*+*nirS*) ratio, from this ratio we can predict high copies of gene *nosZ* at hillslope (HS) as compared to ground discharge zone (GDZ). T0 T1 T3 and T5 symbolize the different sampling point at HS while B2, B3, B5 and B6 symbolize the different sampling point at GDZ.

4.6. Sulphate reduction:

The reason to quantify gene *dsrA* was to understand where the reductive power in the groundwater discharge zone at TSP comes from to drive the observed strong NO_3^- sink. Sulphate reducing bacteria are increasing along the hillslope to the ground water discharge zone. High numbers of sulphate reducing bacteria are found in the GDZ (at sampling point B6 the gene copies observed were around $1.0*10^8$) as seen in graph 6. The efficiency of the qPCR was 104.40% and R^2 was0.98.

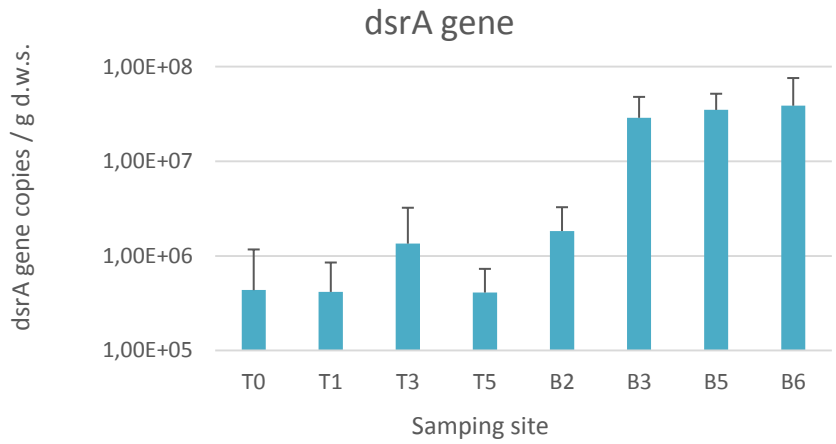


Figure4. 6 The number of gene *dsrA* copies from Hill Slope (HS) and Groundwater Discharge Zone (GDZ) samples. There is an increase in gene *dsrA* copy number from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

Ratio of genes *dsrA* to 16S rRNA was analyzed. This was done to see the abundance of sulphate reducing bacteria (SRB) to the total microbial community. The results showed that the abundance of SRB genes were higher in ground discharge zone (GDZ) as compared to the hillslope (HS) (result shown in appendix).

4.7. Carbon and nitrogen content measurement:

Carbon and nitrogen content measurement showed the same fluctuating trend. Although concentrations of carbon was higher as compared to nitrogen. Higher concentrations were observed in HS as compared to GDZ. Graph 7 shows the varying concentration of carbon and nitrogen in all 8 sampling points (3 replicates from each sampling point in total 24 samples)

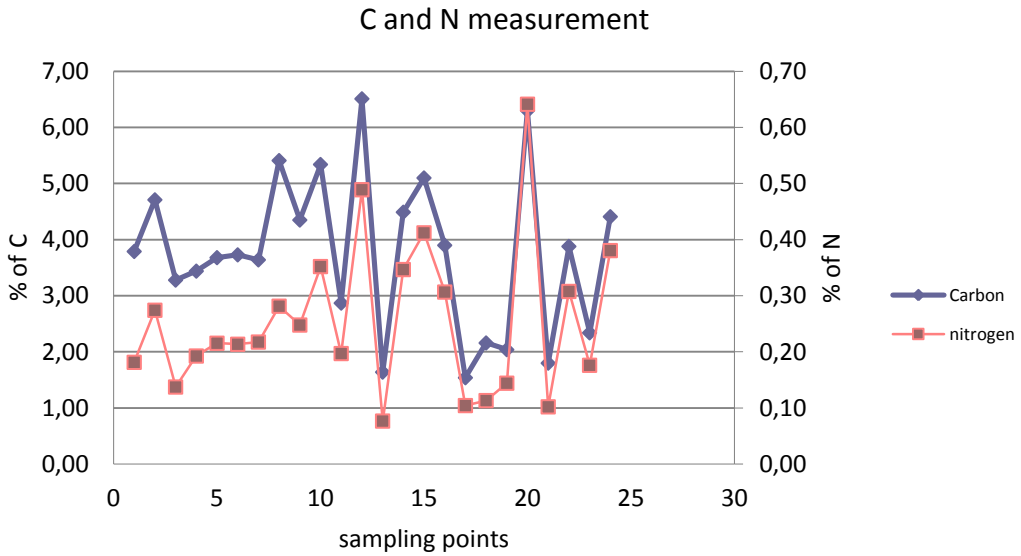


Figure 4.7 Carbon and nitrogen content measurement at the sampling site, the figure shows the varying concentration of carbon and nitrogen in all 24 samples. Carbon concentration is higher as compared to nitrogen at first sampling points in hillslope (HS) but the concentration of carbon and nitrogen is not much different in ground discharge zone (GDZ)

Higher carbon to nitrogen ratio indicates lower nitrogen as compared to carbon, figure 12 shows that C/N is high in GDZ as compared to HS.

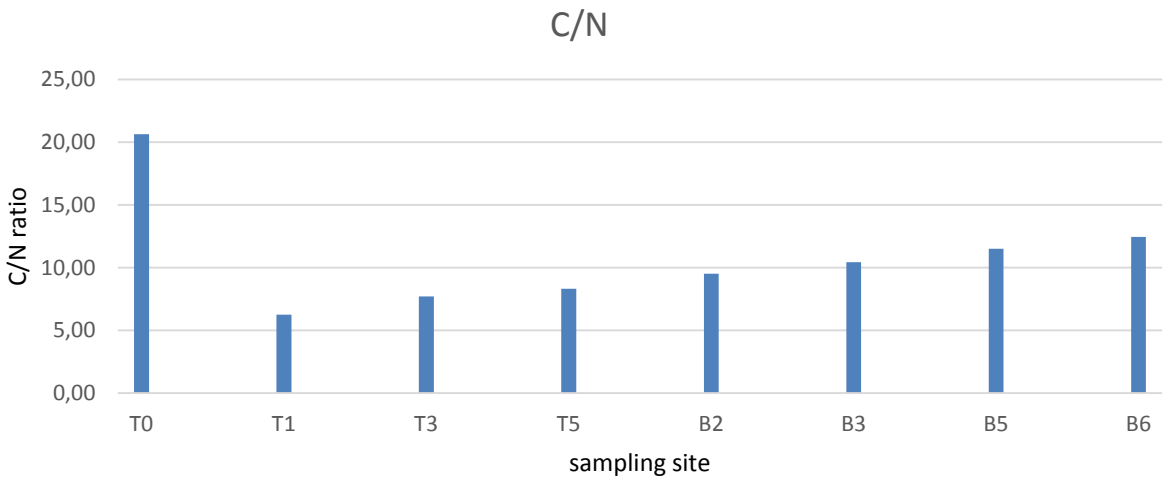


Figure4. 8 carbon to nitrogen ratio, increase in carbon to nitrogen ratio was observed from hillslope (HS) to the ground discharge zone (GDZ) exception seen at first sampling point in HS where the ratio is observed to be very high. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

4.8. Nitrate (NO^-_3) measurements:

Nitrate is produced by nitrification in a two step reaction, ammonium oxidation to nitrite by ammonia oxidizing bacteria and archaea, followed by oxidation of nitrite to nitarte. Nitrate is consumed anaerobically during denitrification and dissimilatory nitrate reduction to ammonium (DNRA). Nitrate concentrations were measured at the start of the incubations of the flasks. No significance difference in nitrate measurement was seen between the HS and GDZ (on average 70mgN/L both in HS and GDZ); high concentration of nitrate was seen at Riparian zone (T5 90mgN/L and B2 100mgN/L) points between the HS and GDZ as shown in graph 9.

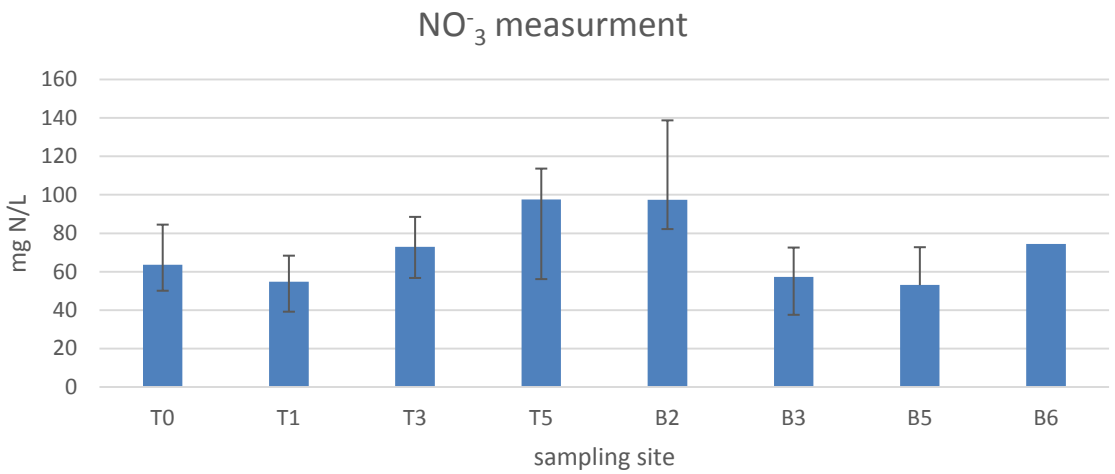


Figure4. 9 nitrate measurement, no significance difference in nitrate measurement was observed between the hillslope (HS) and ground discharge zone (GDZ), high concentration of nitrate was seen at Riparian zone (T5 and B2) points between the HS and GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

Nitrite (N_2O^-) measurement:

Nitrite measurements were done in parallel with the robotized incubation system. Samples from eight sampling points were measured (three replicates of each in total 24 samples). Measurements were taken after 48h, 90h, 170h and 210 h, with time there was seen a decrease in nitrite concentrations (figure 14).). The conditions maintained in the small flasks used for nitrite measurement were one third of the big flasks used in the robotized incubation system (section 3.4). Overall the nitrite concentrations were very low, and there was no significance difference between HS and GDZ, there was observed a slightly higher nitrite concentration in GDZ as compared to the HS, this could be because nitrate reduction rate is higher in GDZ as compared to HS. Higher nitrite at GDZ is also in accordance with the dentrification results (higher denitrification at GDZ) from both molecular and functional part.

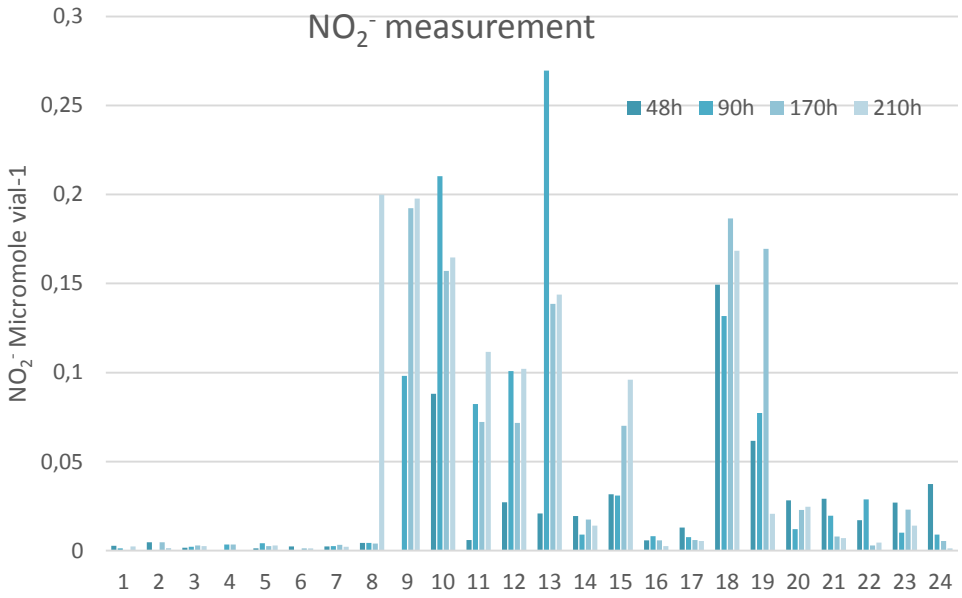


Figure 4.10 Nitrite (NO_2^-) measurement, nitrite measurements decreased with time**.** (along the x-axis the sampling point are in replicates as follow T0=1,2,3 T1=4,5,6 T3=7,8,9 T5= 10, 11, 12 B2=13,14,15 B3=16,17,18 B5=19,20,21 B6=22,23,24) T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

4.9. Robot measurements and the gas kinetics experiment:

The rate of denitrification was studied in robotized incubation system; the flasks were made anaerobic by removing the oxygen and helium washed to maintain the pressure (section 3.4). Higher rate of denitrification was observed in the GDZ (at sampling point B5 0.4 micromol N h-1-g-1 carbon was observed) as compared to the HS (at sampling point T0 0.15 micromol N h-1-g-1 carbon was observed), this result is in accordance with the denitrification results observed from the molecular studies.

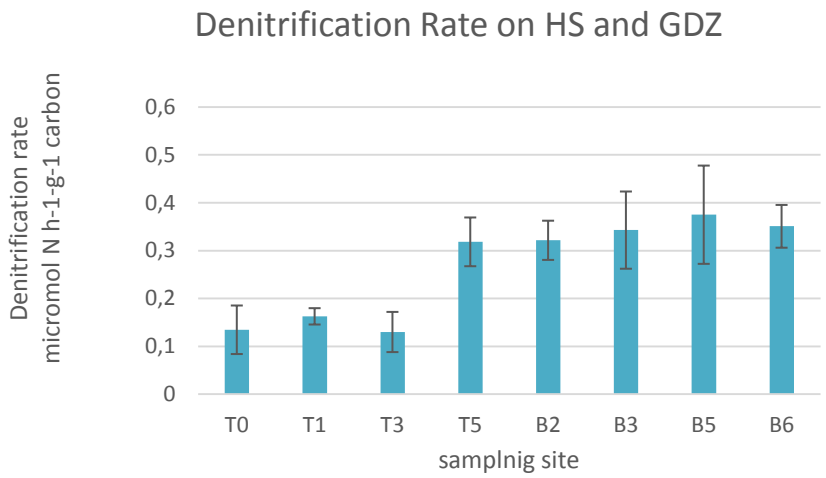
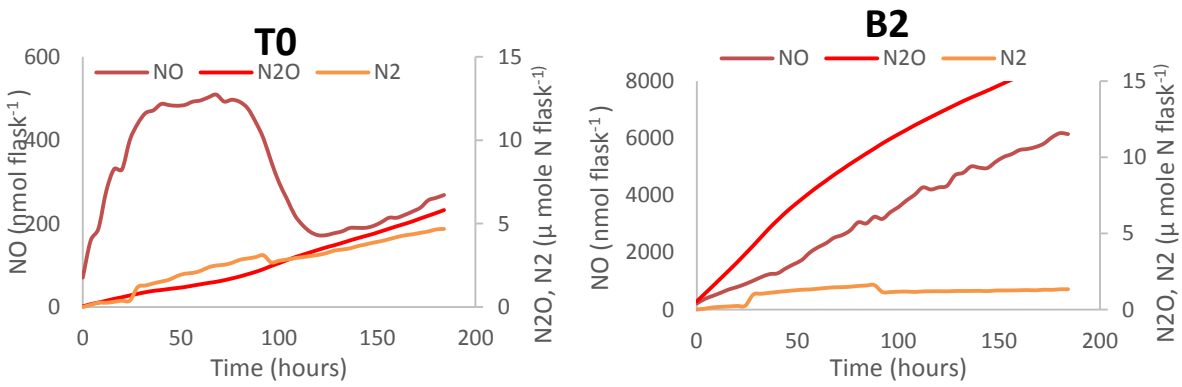


Figure4. 11 Rate of denitrification measured using an automated incubation system T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ.

Gas kinetics Graphs:

In the previous kinetics studies slurries were used while in the current study we used soil. Gas kinetics Graphs for NO, N_2O and N_2 during incubation of soil under anoxic conditions showed as expected that N_2O and NO reduction was less on the hillslope (HS) as compared to ground discharge zone (GDZ). Emission of N_2 was observed to be higher in GDZ as compared to HS as seen in figure 4.12.



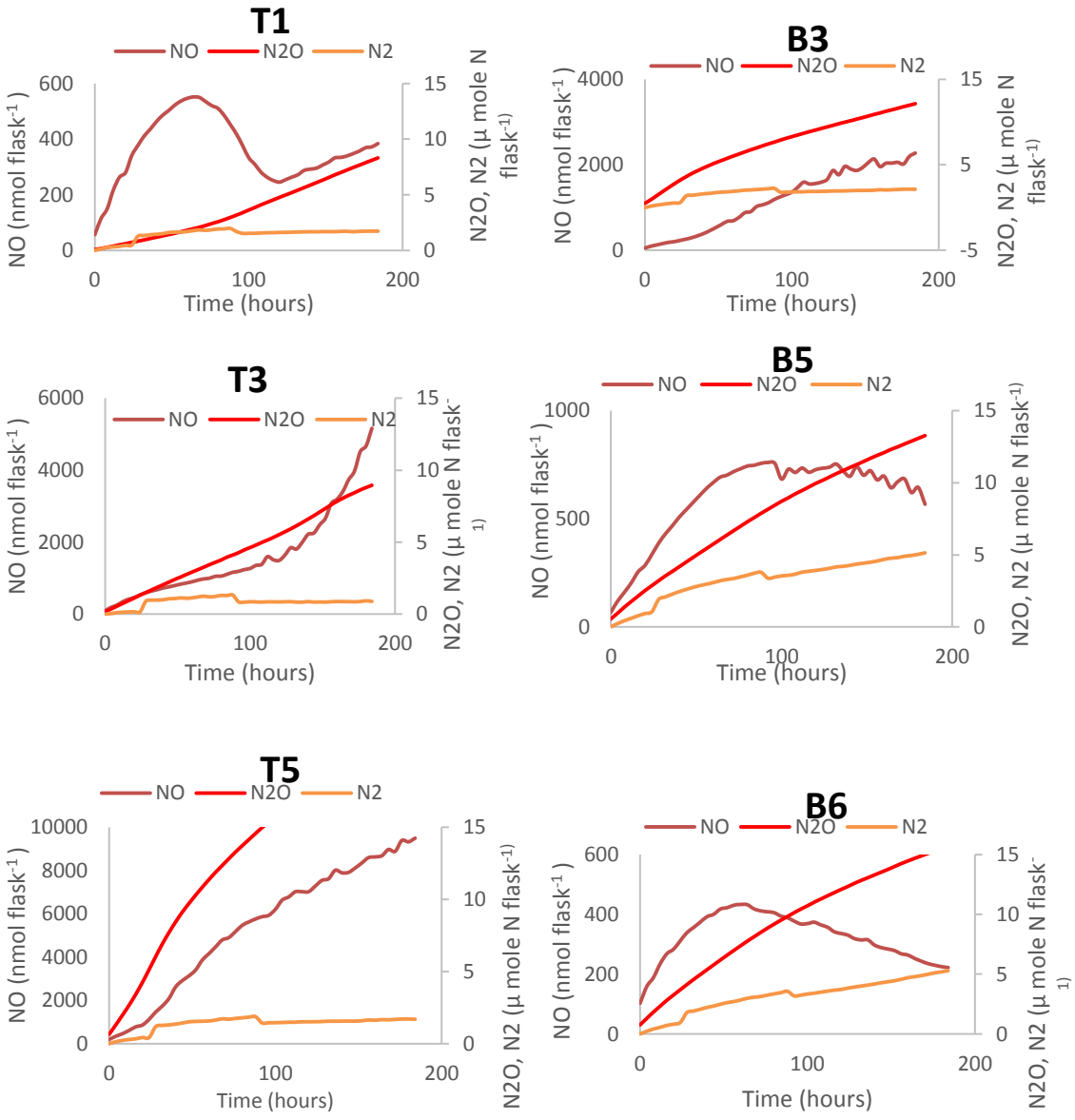


Figure 4.11 **Gas kinetics Graphs** for NO, N_2O and N_2 during incubation of soil under anoxic conditions. NO and N_2O reduction was minimum on the hillslope (HS) as compared to ground discharge zone (GDZ).Emission of N_2 was observed to be higher in GDZ as compared to HS. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ.

5. Discussion:

We investigated the abundance of functional genes involved in ammonium oxidation (*amo*A of AOB and AOA), nitrite oxidation (*nxr*B) and denitrification (*nir*K, *nir*S, *nos*Z) in top soils from 8 locations along the flow path spanning from hilltop to the outlet in the GDZ. 16S rRNA abundance was studied to estimate the total bacterial community present. 16S rRNA abundance was found to be higher in GDZ which was against the expectations. As on GDZ organic carbon concentration was low, and regular stream of water present. But a slightly higher pH was observed in GDZ in previous studies by Zhu (Zhu, Mulder et al. 2013). All functional genes were normalized to 16S rRNA (appendix).

As mentioned earlier that the emission of N_2O from soil to the atmosphere by microbes is directly or indirectly affected by the factors which influence the rate of nitrification or denitrification. From previous studies it has been found that in the emission of N_2O denitrifiaction plays a significant role as compared to nitrification. More than 71% of N_2O emissions are from the process of denitrification (Zhu, Mulder et al. 2013). The exchange of gases between soils and atmosphere is due to the microbial activities and chemical process. It is not well understood that how the gas flux interact. The emission of N_2O from soil is influenced by many environmental factors, soil conditions, pH, presence or absence of oxygen.

5.1. Nitrification:

Previous functional studies indicate that nitrite (NO_2^-) oxidation was retarded at HS samples. This was the reason that we quantified the abundance of ammonia oxidizers (microbes that produce nitrite) and nitrite oxidizer (microbes that consume nitrite) by quantification of the relevant functional genes, gene *amoA* coding for for bacterial and archaeal ammonia monooxygenase and gene *nxrB* coding for the beta-subunit of nitrite oxidoreductase. The ratio between gene *amoA* and gene *nxrB* for the different samples showed that gene *amoA* (archaea ammonia oxidizers) is highr as compared to *nxrB* (nitrite oxidizers) at HS than at GDZ result shown in graph 4.3.1. Higher gene *amoA* abundance as compared to gene *nxrB* could be a reason for NO_2^- accumulation at the HS.

For ammonia oxidizers, we studied the gene *amoA* both in archaea and in bacteria. We found out that AOA is more abundant as compared to AOB (figure 4.2) this could be because our soil was acidic and there is evidence that AOA play more important role in oxidizing ammonia in acidic soils as compared to AOB (Prosser and Nicol 2008).

Rate of nitrification is controlled by several environmental factors, as explained in section 1.3. Nitrification is an aerobic process and is affected by the presence of oxygen (Schurgers, Dörsch et al. 2006). In previous studies higher N_2O emissions were observed in lower O_2 levels (Zhu, Mulder et al. 2013), this is because in lower O_2 levels, nitrification genes are not fully expressed and intermediate products (NO,N_2O) are emitted. Nitrification rate is also affected by the availability of substrate ammonia (NH_3) rather than ammonium (NH_4^+) (De Boer and Kowalchuk 2001). Yet another important controller of nitrification is pH. Nitrification is absent in highly acidic soils (although recently nitrification activity by AOA group has been observed in acidic soils) because low pH results in formation of NH_4^+ leading to unavailability of NH_3. The optimal pH range for nitrification is 4.3 (at this low pH it is the archaeal nitrification that is important) to 7.5 (Yao, Gao et al. 2011).

NO and N_2O emission by the process of nitrification are suggested to be due to incomplete oxidation of NH_2OH or NO_2^- or there accumulations. As explained earlier that higher ammonia oxidizers (gene *amoA*) abundance as compared nitrite oxidizer to (gene *nxrB*) could be a reason for NO_2^- accumulation at the HS.

5.2. Denitrification:

In nitrogen rich soils when there is deficiency of oxygen the bacteria can switch to nitrate respiration. This happens in intermediate steps of nitrogen cycle. The disadvantage of this is that during this process the bacteria can produce environmentally hazardous gases like NO and N_2O.Researchers are interested in N_2O gas because N_2O is known to be a potent green house gas; it is known to account for 0.03% of green house gas emission. Due to its radiative capacity its global warming potential is 300 times more as compared to carbon gas (Richardson, Felgate et al. 2009).

As mentioned in section (1.4) denitrification is the step wise reduction of nitrate or nitrite to N_2 several enzymes are involved and the intermediate steps are an important source of N_2O emission. The *nosZ* gene encodes N_2OR which is the most important enzyme that converts N_2O to N_2 (Bergaust, Bakken et al. 2011). *nosZ* abundance was observed to be higher at the GDZ as compared to the HS. One possible explanation for this could be that the N_2OR is sensitive to the presence of oxygen (Bergaust, *et al.*, 2011). On HS there is a frequent transition between oxic and anoxic states. incoming rain makes the HS slope anoxic and the water floats from the HS to the GDZ leading to oxic condiotions again. while the GDZ has stable anoxic conditions.

Another important factor which can affect the process of denitrification is the pH, the soil pH in GDZ was found to be 0.5-0.6 units higher as compared to the HS. pH has a direct affect on denitrification (Liu, Morkved et al. 2010). Diffusion limitation in the denser GDZ soil can result in high dissolved N_2O concentrations and promot *nos*Z expression.This was in accordance with our results i.e. higher denitrification activity at the GDZ as compared to the HS(Zhu, Mulder et al. 2013).

N_2O emissions in previous study were found to be higher from the hillslope than from the GDZ, which at first thought seems counterintuitive as HS is rich in organic material. Our results are in accordance with the previous study. We assumed that the nos/nir ratio was higher in the GDZ, but our result showed the opposite case. So this hypothesis was rejected.

The ratio of *nos/nir* was declining from HS towards the GDZ. Thus, we can say that more bacteria harbor the gene *nosZ* then the gene *nirK* as seen in graph 5. The gene *nos*Z is higher in GDZ as compared to the HS (graph 4.5), but the *nos/nir* ratio indicates opposite case this ratio does not relate to the functional activity of the gene as seen in graph 4 a, c. One possible reason for this is that there is frequent transition between oxic and anoxic conditions at the HS, meaning that genes are not fully expressed. For genes to be completely expressed stable conditions are required. These unstable transitions between oxic and anoxic conditions influence the regulation of the genes at either the transcription level or at the post-trasncriptional levels, as discovered in early studies (Bergaust 2009). As mentioned earlier, N_2OR is sensitive to the presence of oxygen. On HS there is frequent transition between oxic and anoxic states while the GDZ have stable anoxic conditions due to which the N_2OR activity is higher at GDZ.

Toxic intermediate products (NO_2^-, NO, and N_2O) of denitrification are emitted less at GDZ as compared to HS, because favorable conditions (stable anoxic conditions) for denitrification are present in the GDZ. Due to which all denitrification enzymes (NAR, NIR, NOR, N_2OR) are fully expressed and reduction nitrate (NO_3^-) or nitrite (NO_2^-) to nitrogen gas (N_2) is favored.

Denitrification and nitrification rate was expected to be higher in HS as compared to GDZ but opposite case happened. This could be explained that stable anoxic conditions are present at GDZ. GDZ serves like a funnel where all the rain water from the HS is collected. In addition to water different microbes and nutrients (upper layer of soil) from the HS gather in GDZ, due to which the activity is higher in GDZ.

As mentioned in Section 1.5 DNRA and denitrification are in competition for available NO_3^-. Both this process takes place under the same environmental conditions (anoxic environmet), so it is difficult to decide which process is responsible for N_2O emissions. DNRA could be dominant at the HS, the where organic carbon is present in excess, while denitrification is dominant at GDZ where organic carbon is limited. For future studies, it will be interesting to see the genes which encode the key enzyme for DNRA (respiratory cytochrome *c* NO_2^- reductase known as NrfA) and compare its abundance with the denitrification genes.

5.3. Anammox:

The reason to quantify anammox, was to predict if anammox are responsible for the disappearance of NH^3. We tried to study the abundance of anamox in the soil, but did not get any success. This could be due to the reason that the soils samples were not collected from anoxic area; we collected the soil samples approximately 15cm deep. Anammox are strict anaerobes, to study the anammox we should have collected the soil samples from further deep layers. Ammonia is disappearing along the HS; anammox study can possibly explain the disappearance of ammonia along the HS. It will be very interesting for future studies to collect the soil sample from deep soil layers to study the anammox.

5.4. Sulphate reducing bacteria:

The main reason to study the sulphate reducing bacteria (SRB) was to understand where the reductive power in the groundwater discharge zone at TSP comes from to drive the observed strong NO_3^- sink. At the site there was a strong smell of H_2S at the GDZ, indicating the presence of a strong reducing force. There was a strong observed sink for sulfate, so we speculated whether sulfide could play a role as reducing agent, somehow releasing electrons when ground water table moves up and down.

As seen from our results that all the activities are higher at the GDZ as compared to the HS slope. It is being thought that the reducing force present in GDZ could be responsible for higher activities. Opposite was expected, as HS is rich in carbon or nutrient content so higher activity was expected at HS. This is something different from the typical thinking that every microbial activity is controlled by carbon, as we have seen that there is a high reducing force at the GDZ and low carbon content (DOC) but higher activity. Furthermore there is lots of iron present at the site, predicted from the soil color. The sulfur reacts with the iron and forms pyrites, Iron is also a source of electron donor at the site.

5.5. Robotized experiment (semi-automated robotic incubation system)

We studied the process of denitrification also by robotized incubation, from robot study we can predict that rate of denitrification is higher in GDZ as compared to HS. This result is in accordance with the molecular part studied in the current study.

With time there was seen a decrease in nitrite (N_2O^-) concentrations. Overall the nitrite concentrations were very low, and there was no significance difference in HS and GDZ, there was observed a slightly higher nitrite concentration in GDZ as compared to the HS, this could be because nitrate reduction rate is higher in GDZ as compared to HS. Higher nitrite at GDZ is also in accordance with the dentrification results (higher denitrification at GDZ) from both molecular and functional part.

Slight increase in nitrate NO_3 concentration was observed over time, this can be due to several reasons including that soil was vortexed (probably accessing NO_3 not in equilibrium with the soil solution in situ) also soil dries out during storage leading to increase in concentration furthermore soil accumulates NO_3 by mineralisation even at 4^oC and soil was sieved quite a while ago, inducing extra mineralization.

There was observed a slightly higher nitrite concentration in GDZ as compared to the HS, this could be because nitrate reduction rate is higher in GDZ as compared to HS. Higher nitrite at GDZ is also in accordance with the dentrification results (higher denitrification at GDZ) from both molecular and functional part. To get more accurate results more replicates are needed to be measured after regular intervals.

In the previous kinetics studies slurries were used while in the current study we used soil. Gas kinetics Graphs for NO, N_2O and N_2 during incubation of soil under anoxic conditions showed as expected. N_2O and NO reduction was less on the hillslope (HS) as compared to ground discharge zone (GDZ). The reason for this as explained earlier could be that N_2OR is sensitive to the presence of oxygen. On HS there is frequent transition between oxic and anoxic states while the GDZ have stable anoxic conditions due to which the N_2OR activity is higher at GDZ. Emission of N_2 was observed to be higher in GDZ as compared to HS as seen in figure 4.12. This could be due to that at GDZ as mentioned earlier balanced denitrification is taking place i.e. all enzymes (NAR, NIR, NOR, N_2OR) are regulated and expressed leading to reduction nitrate (NO_3^-) or nitrite (NO_2^-) to nitrogen gas (N_2) emission.

The fluctuations seen in kinetics of NO, N_2O and N_2 could be due to that water content was forgotten to be adjusted while measuring the respiration, due which the rate of respiration was low, the water content was adjusted after about 36 hours and increase in rate of respiration was observed. Another reason could be that the conditions were made anoxic by removing oxygen and helium washing. There was seen slight oxygen leakage, as shown in gas kinetic graphs in appendix. Presence of small amount of oxygen might have affected the rate of denitrification.

5.6. Methodological effort

Soil is a complex matrix, containing diverse microbial community, it is the environmental factors e.g. oxic or anoxic conditions or presence of nutrients, pollutants or toxic chemicals which decide the survival strategy of microbes in a specific environment (Almås, Mulder et al. 2005).This study was conducted to quantify the microbial community involved in emission of N_2O in the atmosphere from a subtropical forest ecosystem in southwest China. We do realize that the sampling, shipment and storage of the soil affect the microbial community or their

activity, but we assume that this influence is same for all the samples, and the comparison of the abundance of the functional groups is still valid

The extraction and purification of DNA from soil was not an easy task because of the impurities like humic acid, phenol, protein and low pH. Purification step was difficult due to the similarities between the nucleic acid and humic acid.To get good quality DNA several attempts were made and different purification kits were tried, DNA was lost in the purification step. We used direct DNA extraction method in which cells were lysed in soil by using glass beads in a bead beater. The purity of the extracted and purified DNA was measured by using a nano drop spectrophotometer and Qubit fluorometer as results can be seen in appendix, only good quality of DNA was considered, quality of DNA was judged by ratio of absorbance at 260 nm and 280nm.

qPCR :

We aimed to get the efficiencies between 90-100%, in some cases 80% or above were also considered. For some of the primers like those primers targeting the *nosZ* gene we had to repeat the experiment several times to get good efficiencies. Emphasis on higher efficiency was given because efficiency represents the amount of the increase in PCR product after every cycle. Low efficiencies indicate that the sample might contain inhibitor, poor primer design or pipetting errors. Another important factor which was considered during a qPCR was the R^2 which represents the linearity, only 0.9 or greater values for R^2 was considered. If the efficiency or R^2 were low then the experiment was repeated.

In this study we had three biological replicates, from eight different spots. To test the repeatability and reliability of the qPCR, we made technical replicates from two spots for the primer *amoA* result showed that the technique was repeatable and reliable.

Agarose gel:

The agarose gel was used for separating different size of DNA fragments, the DNA in the gel is only visible under UV if appropriate dye is added or if the gel is stained with dye. The dye being used in our lab was ethidium bromide (Etbr) but it was banned in the lab because it is toxic and is

known to be carcinogen and mutagen. Due to its toxicity, the dye was replaced by gel red a less toxic dye. The problem with the gel red was separation. We had to struggle a lot get appropriate conditions i.e. voltage, time, % of gel and conc. of the gel red.

Conclusion:

It was observed that abundance of all functional genes (ammonium oxidation (*amo*A of AOB and AOA), nitrite oxidation (*nxr*B) and denitrification (*nir*K, *nir*S, *nos*Z) sulphate reducing bacteria (*dsr*A)16S rRNA) were higher in GDZ as compared to HS, one of the reason could be due to the presence of stable anoxic condition at the GDZ and another reason could be that it is being thought that the reducing force present in GDZ could be responsible for higher activities. The emission of N_2O by denitrification is observed to be higher as compared to nitrification process. In denitrification the study abundance of gene *nos*Z play important role in determining the emission of N_2O.

The N cycling gene copy numbers do not explain the whole story, to understand the complete story we need the functional studies along with the molecular studies. We can hypothesize that in the GDZ, the communities are more abundant as compared to the HS.

Future work :

Study of anammox of can probably provide the answer to the disappearance of ammonia along the HS. In addition the whole community can be studied by using more advanced technologies like finger printing (DGGE of functional genes) or sequencing (amplicons sequencing or metagenome) by this advanced techniques we will know about fungus, archea, and all other genes involved in nitrification and denitrification.

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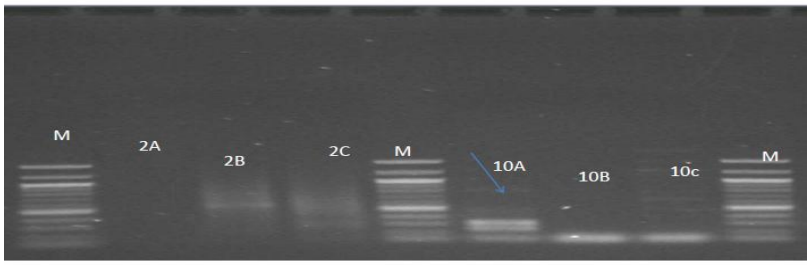
Appendix

Appendix:

1. Gel pics:

Gel for primer 2 (DSR1-F+,DSR-4R) size 221bp and 10 (A6842, A438f) size 246 bp dedicated, gel conc 1% v-110 time 40 mins.



Primer 2

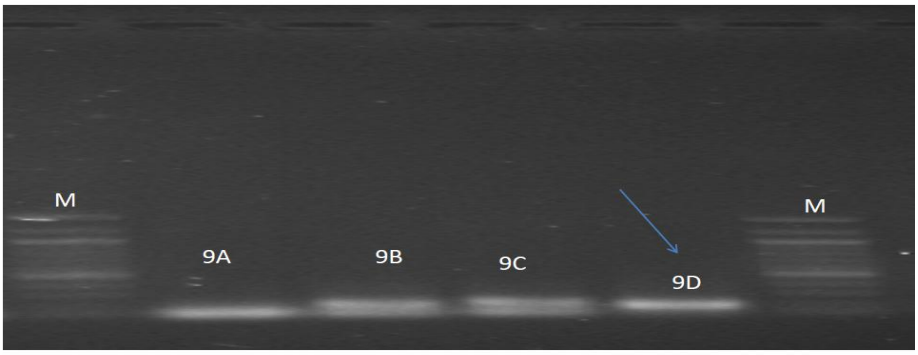
M= ladder
2A- DNA from my sample
2B-DNA from binbin`s sample
2C-DNA from nateliez sample

Primer 10

M-ladder
10A-DNA from my sample
10B-DNA from binbin`s sample
10C-DNA from nateleiz sample

For anammox primers (A6842, A438f), it was difficult to get a band from the soil samples collected from Tieshanping (TSP). I used other soil available in our laboratory (Binbin soil samples and Nateleiz soil sample), but did not get any successful result.

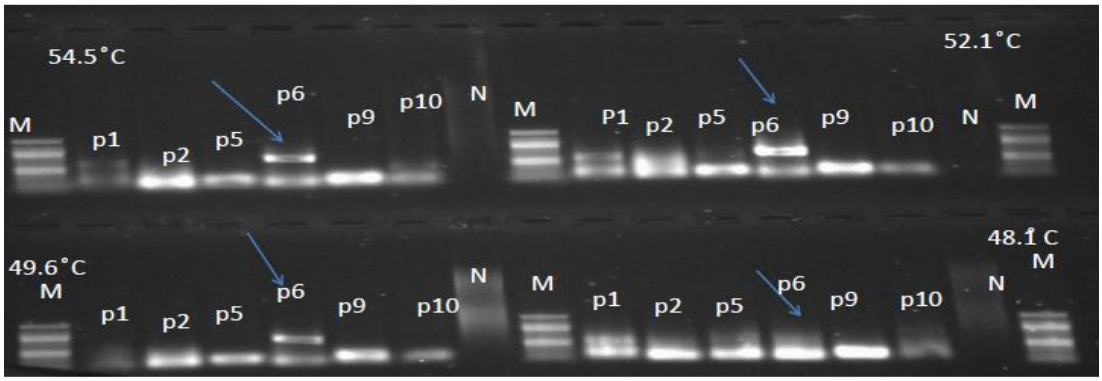
Gel for band 9 (NSR1113F,NSR264R) size 151bp,different anneling temperatures are compared and the sharp band appeared at 48.1 ° C , gel 1% V-110 for 40 mins



M-ladder
9A-54.5 °C ,9C-49.6°C
9B- 52.1 °C ,9D-48.1°C

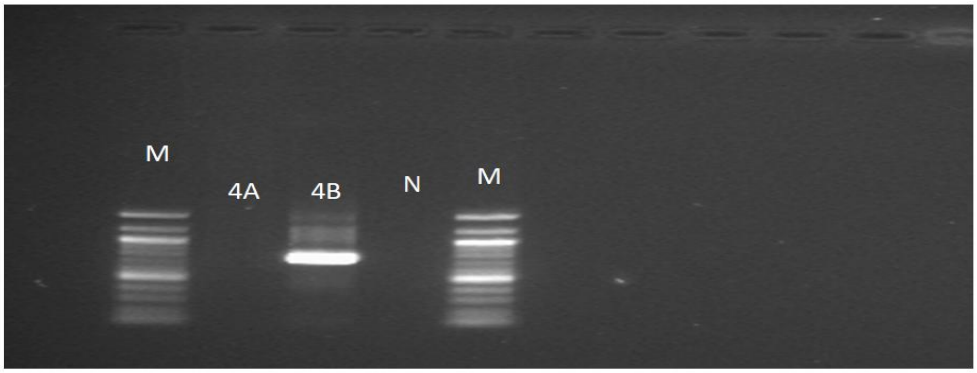
The above figure shows the band for nitrite oxidizers.

Pcr run at diiferent temperature gradient,Band for primer 6 (518 F, Nbacter))size 532bp at pcr anneling temp 52.1.gel 1% 0.8 In 80 µl of buffer v=110 for 20 mins



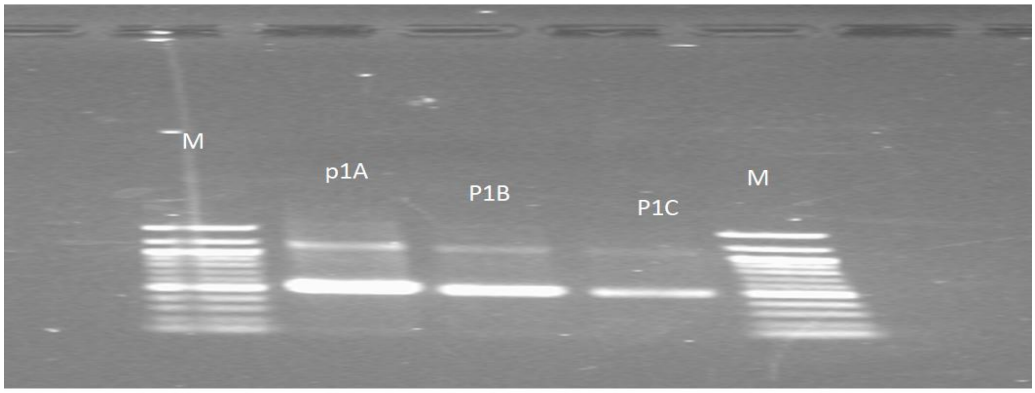
Above figure showing band for primer nitrite oxidisers

Gel for primer 4 (cren.amoF Ia, Arch.amo AR) band size, pcr anneling temp 55 C. Gel 1% V-110, for 40 mins.



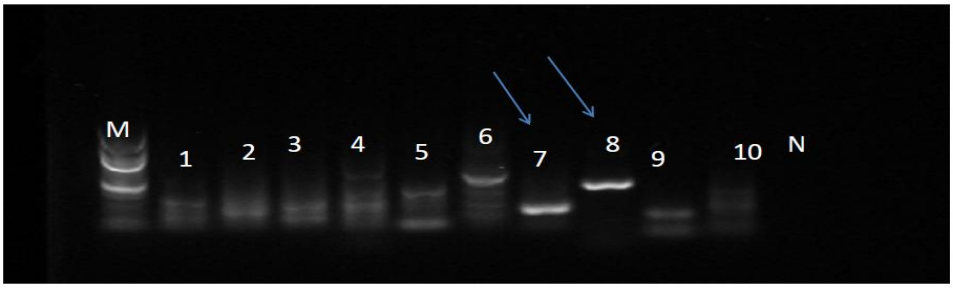
M- ladder
N- negative control
4A-DNA from my sample
4B-DNA from binbins sample

Gel showing band for primer 1 (amoA 1F, amoA 2R) , size 491 bp, with different concentartion of gel red. Gel 1% v-110, for 40 mins



M- ladder, p1A- 1µl gel red + 5 µl of pcr
P1B-5µl diluted 100 times gel red + 5 µl pcr
P1C-2µl diluted 100 times gel red + 5 µl pcr

Gel showing band for primer 7 (518F,Nbacter) and 8 (FGPS 872, FGPS 1269)
Anneling temperature 52-56 C.Gel 1% 0.4 in 40 ml for 40 mins = 80



M-ladder
N-negative ccontrol

2. DNA quality and quantity

Table (1)DNA yield obtained after extraction and quantification by Nanodrop and Qubit method for replicate 1

Sampling site	DNA by Nanodrop ng/µl	Ratio 260/280	Ratio 260/230	DNA by Qubit ng/µ
T0	35.2	1.71	1.58	19.2
T1	30.4	1.68	1.48	18.0
T3	21.0	1.69	1.56	12.6
T5	27.3	1.94	1.98	21.8
B2	37.0	1.85	1.32	24.8
B3	32.6	1.96	2.21	33.8
B5	27.7	1.96	2.31	22.8
B6	86.8	1.90	2.08	47.5

Table (2)DNA yield obtained after extraction and quantification by Nanodrop and Qubit method for replicate 2

Sampling site	DNA by Nanodrop ng/µl	Ratio 260/280	Ratio 260/230	DNA by Qubit ng/µ
T0	123.2	1.49	1.06	38.5
T1	66.8	1.54	0.98	19.8
T3	72.1	1.64	1.28	32.5
T5	50.9	1.64	1.37	29.5
B2	61.3	1.79	1.64	42.1
B3	62.1	1.82	1.87	48.6
B5	68.1	1.90	1.91	48.2
B6	84.6	1.87	1.94	52.0

Table (3)DNA yield obtained after extraction and quantification by Nanodrop and Qubit method for replicate 3

Sampling site	DNA by Nanodrop ng/µl	Ratio 260/280	Ratio 260/230	DNA by Qubit ng/µ
T0	47.5	1.59	1.25	17.6
T1	32.0	1.46	1.06	9.3
T3	58.6	1.66	1.32	23.2
T5	99.4	1.66	1.26	42.4
B2	67.2	1.83	1.66	46.4
B3	47.4	1.67	1.49	33.9
B5	59.7	1.88	2.11	52.0
B6	64.3	1.89	2.02	47.3

3. Ration of specific gene/16s gene:

16s rRNA abundance was assessed as a general marker for bacterial abundance. Ratio of functional genes to 16S rRNA was analyzed. This was done to see the abundance of functional community to the total microbial community. The results showed that the abundance of functional genes were higher in ground discharge zone (GDZ) as compared to the hillslope (HS).

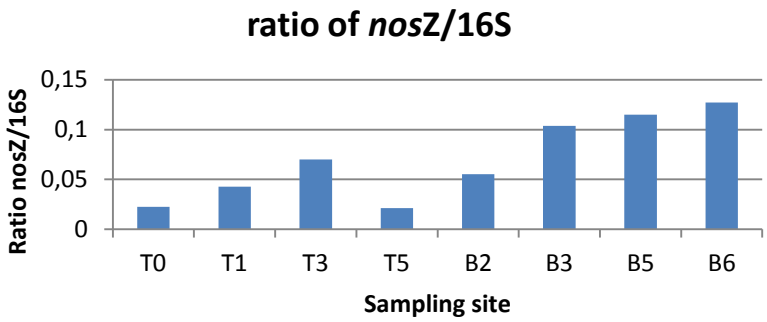


Figure 1 Ratio between gene *nosZ* and gene *16S*. There is an increase in ratio of *nosZ/16S* from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

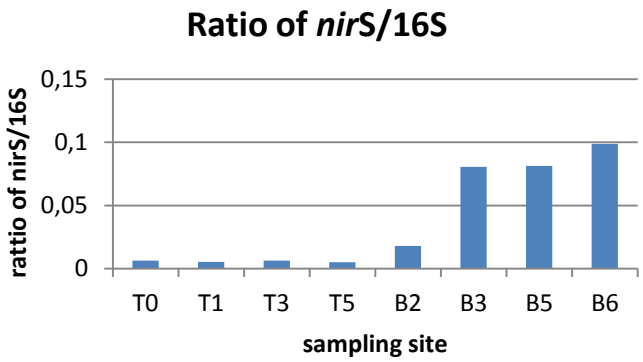


Figure 2 Ratio between gene *nirS* and gene *16S*. There is an increase in ratio of *nirS/16S* from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

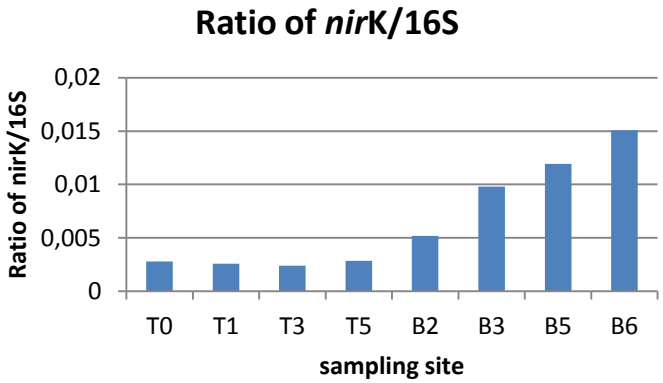


Figure 2 Ratio between gene *nirK* and gene *16S*. There is an increase in ratio of *nirK/16S* from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

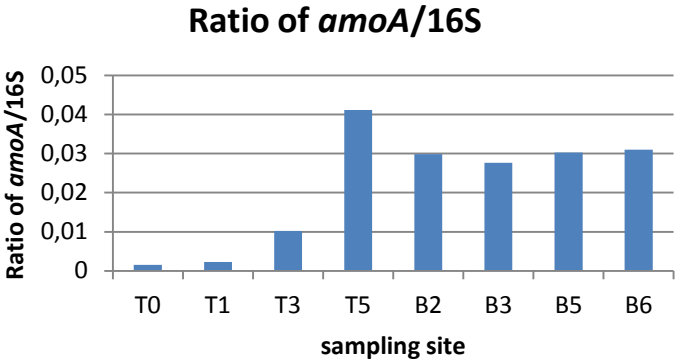


Figure 3 Ratio between gene *amoA* for archaea and gene *16S*. There is an increase in ratio of *amoA/16S* from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

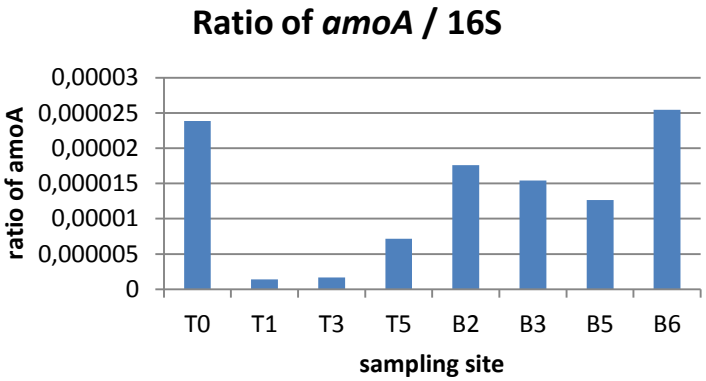


Figure 4 Ratio between gene *amoA* for bacteria and gene *16S*. There is an increase in ratio of *amoA/16S* from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

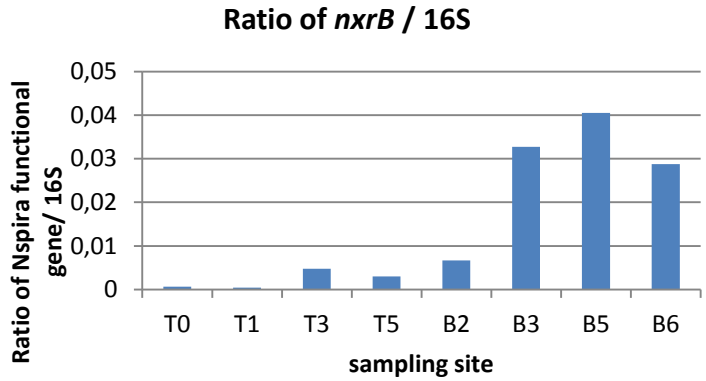


Figure 5 Ratio between gene *nxrB* and gene *16S*. There is an increase in ratio of *nxrB/16S* from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

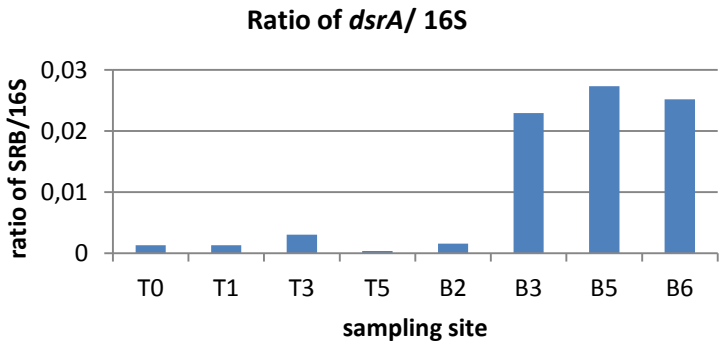


Figure 6 Ratio between gene *dsrA* and gene *16S*. There is an increase in ratio of *dsrA/16S* from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

4. Each gene copy number Normalized to ng^{-1} DNA:

Ratio of functional genes to ng^{-1} was analyzed. This was done to see the abundance of functional community to ng^{-1} DNA. The results showed that the abundance of functional genes were higher in ground discharge zone (GDZ) as compared to the hillslope (HS).

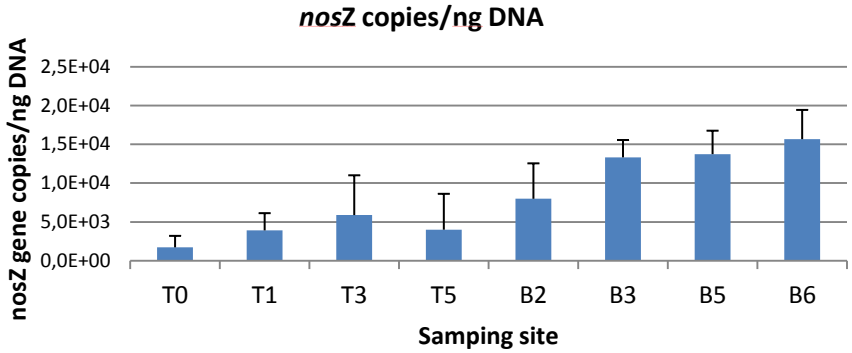


Figure 7 Ratio between gene *nosZ* and *ng^{-1} DNA*. There is an increase in ratio of *nosZ*/ng DNA from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

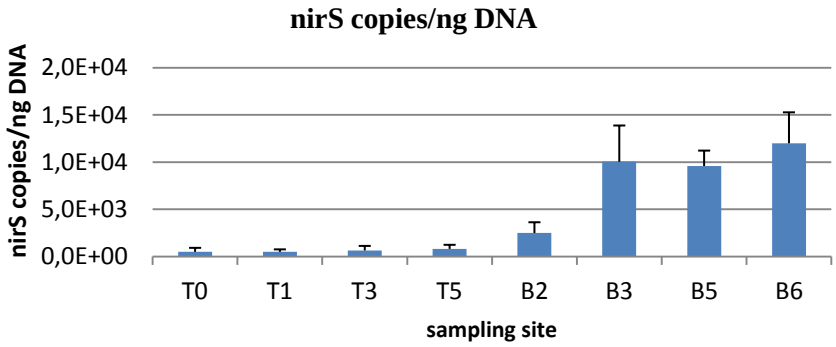


Figure 8 Ratio between gene *nirS* and *ng^{-1} DNA*. There is an increase in ratio of *nirS*/ng DNA from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

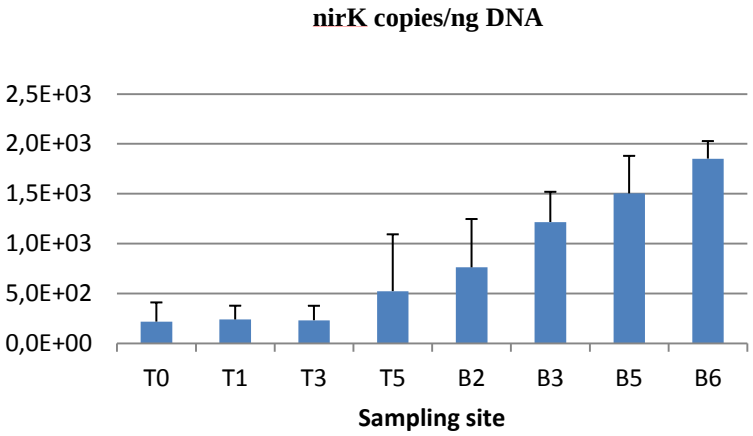


Figure 9 Ratio between gene *nirK* and *ng^{-1} DNA*. There is an increase in ratio of *nirK*/ng DNA from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

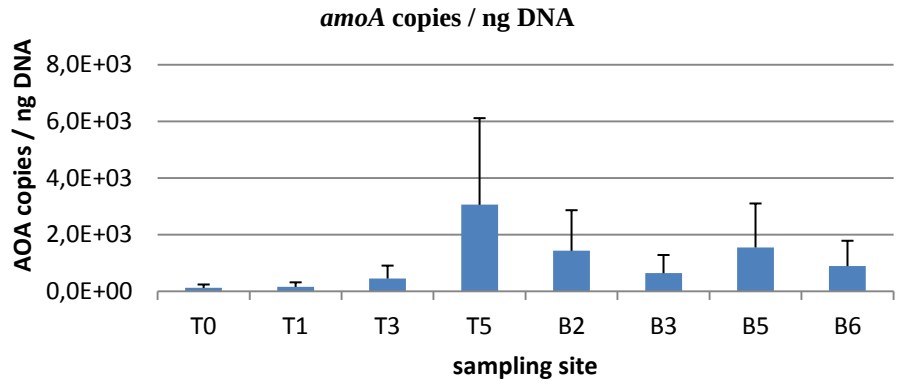


Figure 10 Ratio between gene *amoA* and *ng^{-1} DNA*. There is an increase in ratio of *amoA*/ng DNA from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

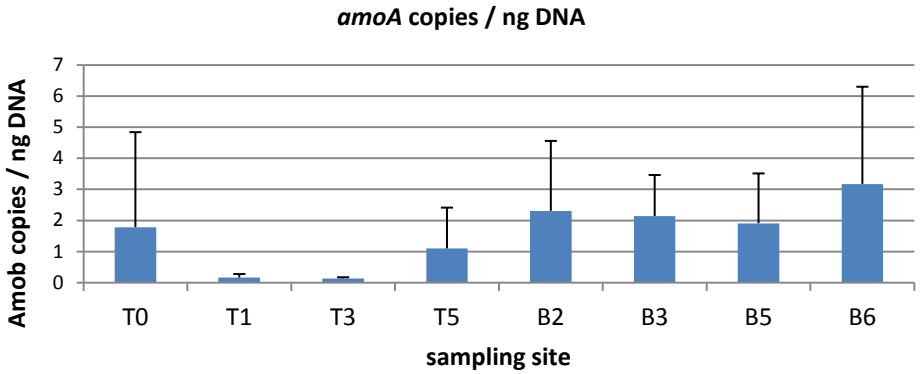
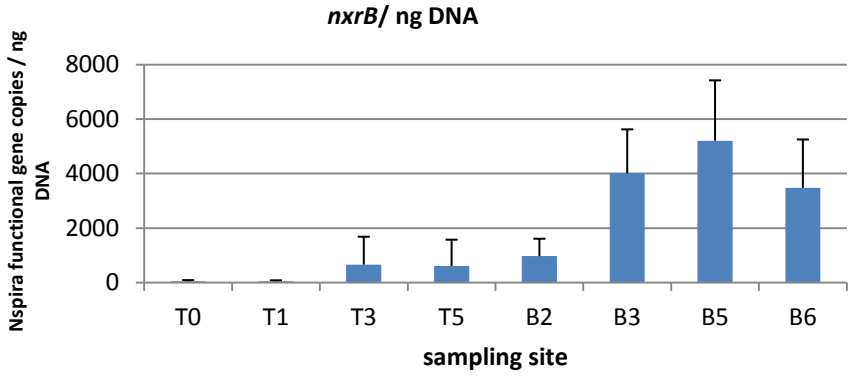


Figure 11Ratio between gene *amoA* and *ng^{-1} DNA*. There is an increase in ratio of *amoA*/ng DNA from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ



Figur 12 Ratio between gene *nxrB* and *ng^{-1} DNA*. There is an increase in ratio of *nxrB*/ng DNA from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

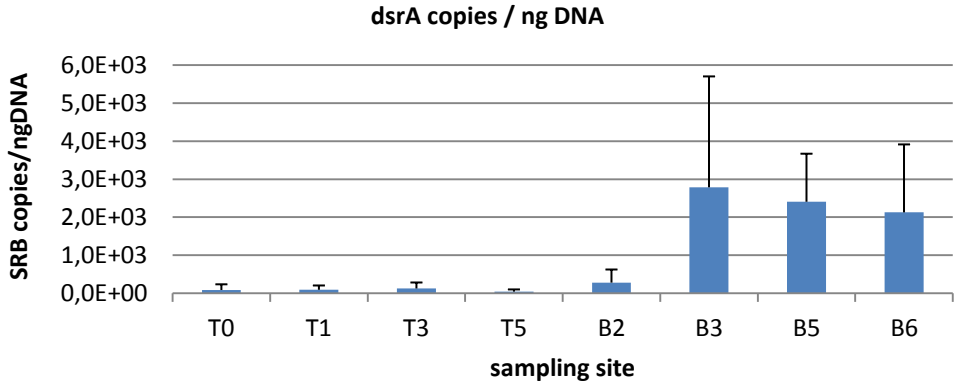
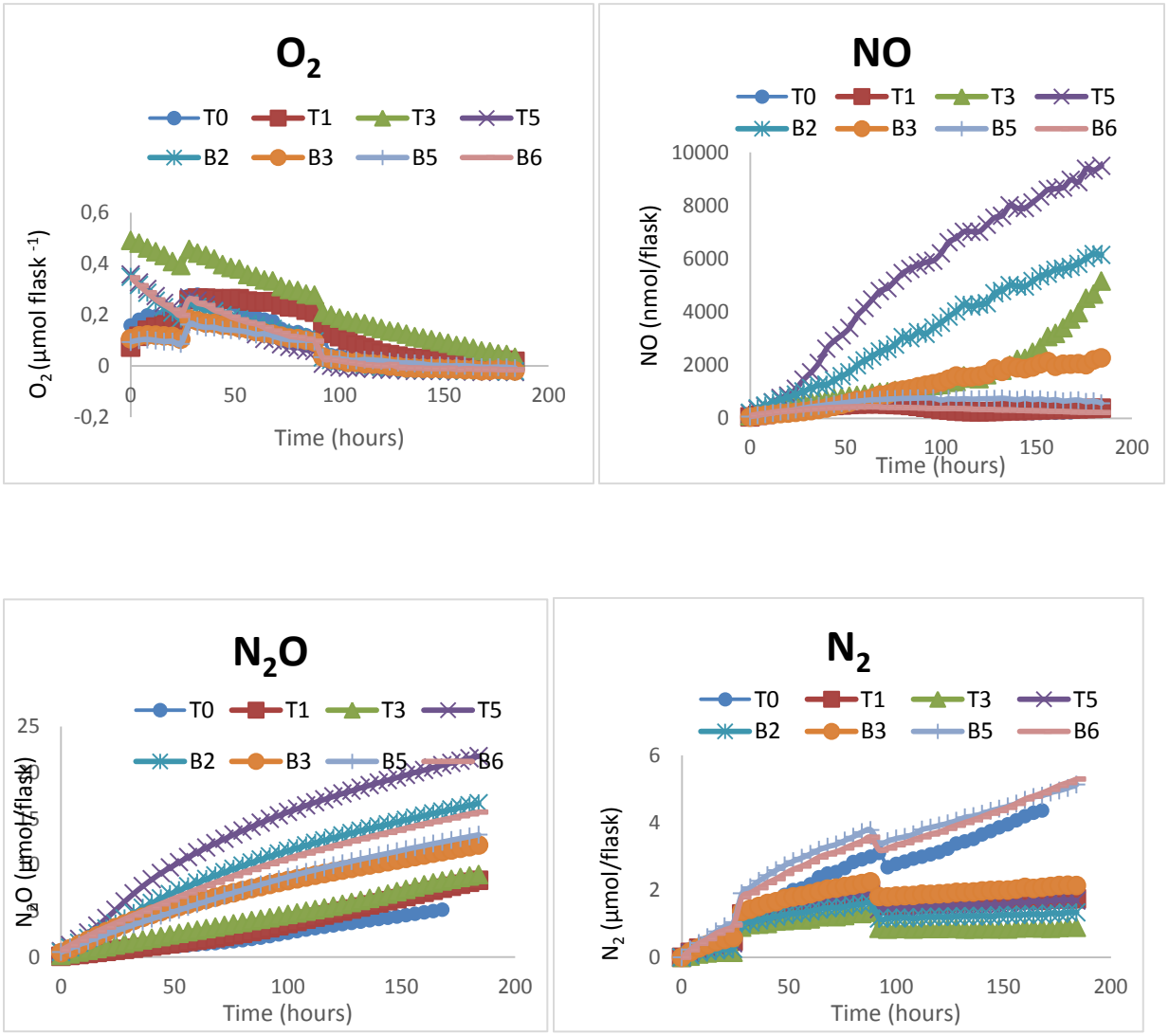


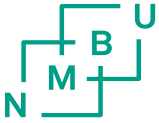
Figure 13 Ratio between gene *dsrA* and *ng^{-1} DNA*. There is an increase in ratio of *dsrA*/ng DNA from HS to GDZ. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ

Gas kinetics Graphs:

Gas kinetics Graphs for NO, N_2O and N_2 during incubation of soil under anoxic conditions showed as expected that N_2O and NO reduction was less on the hillslope (HS) as compared to ground discharge zone (GDZ). Emission of N_2 was observed to be higher in GDZ as compared to HS



Figur 14 Gas kinetics Graphs for NO, N_2O and N_2 during incubation of soil under anoxic conditions. NO and N_2O reduction was minimum on the hillslope (HS) as compared to ground discharge zone (GDZ).Emission of N_2 was observed to be higher in GDZ as compared to HS. T0 T1 T3 and T5 symbolize the different sampling point along the HS while B2, B3, B5 and B6 symbolize the different sampling point along the GDZ.



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