Norwegian University of Life Sciences

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Acknowledgements

First and foremost, my deepest gratitude to my supervisor and co-supervisor, Åsa Frostegård and Binbin Liu, for all the guidance and support given to me during my time in the lab and the very lengthy writing process. I have learnt a great deal during my time spent under your supervision.

Sincerest thanks to Lars Bakken and Peter Dörsch for helping me with and giving advice on the non-biology side of my data. Without your aid, I would have long ago been buried under the huge amount of information that landed on my lap after each robot run.

I am immensely grateful to all the members of the UMB Nitrogen Group for welcoming me into my home-away-from-home. Extra thanks to Else and Rannei for keeping the lab running and ensuring that we do not blow up the lab by accident. ☺A very special thank you to Linda, Daniel, Junaid and Qu Zhi for putting up with the many "just a random question" moments that often followed my presence into your offices.

There are truly no words that can express my gratefulness to my wonderfully loving and caring parents. Mum and dad, as I have mentioned many times before, I would not be where I am today without the support from both of you. Thank you again for supporting my decision to stop my career to pursue a Masters degree and for the financial and emotional support supplied these last two years. Thank you also to my loving brother, Jonathan, for being there for our parents when I was half a world away. I also deeply appreciate the many non sequitur conversations we had on Skype while waiting for mum and dad. ☺

Last but definitely not least, a big "Thank you!" to all my friends here in Norway and on Facebook who have supported me in and out of the lab environment. To my fellow dancers, both Folkeswing and West Coast Swing, thanks for giving me the much-needed exercise and breaks from my lab work. To Qing Feng Zhai (清风寨), a million thanks for welcoming me into your midst, feeding me and giving me much needed writing breaks whenever I needed it.

I will forever be grateful to all who had given me assistance, big or small, during my two years here and the year leading up to my thesis submission.

~ Thank you ~ Tusen takk ~ 多谢 ~ ありがとうございます ~ 감사합니다 ~

Ås (Norway), May 2013

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Lim Yen Nee, Natalie Date received

Abstract

Denitrification is one of the main processes in the nitrogen cycle, and it is the reduction of nitrate to N_2 through a series of intermediates, removing biologically available nitrate from the biosphere. NO and N_2O are both gaseous intermediates of denitrification which influence atmospheric reactions due to the formation of reactive nitrate radicals in the atmosphere. In addition, N_2O is a potent greenhouse gas that has been on the rise in the last few decades. Thus, it is essential to the agricultural industry to explore the genetic reasons behind high N_2O emissions from cultivated soils.

Previous studies from our laboratory group utilised quantitative polymerase chain reaction (qPCR) and advanced laboratory-based gas measurements in complement to characterise denitrification gene expression and gas production/utilisation profiles. In those studies, soil pH was discovered to be a very important variable controlling the final reduction step of N_2O to N_2 in denitrification. However, further molecular work on acidic soil samples was stalled by ineffective nucleic acid extraction and DNA-contaminated RNA samples. Even with current technological advancements, successful extraction of nucleic acids from inhibitor-rich peat soil samples has been recognised as a difficult task. Often, separate extraction reactions or even extraction methods have to be used in order to achieve nucleic acids which are usable for downstream applications. However, this has created a potential source of technical bias, since the DNA and RNA extracted may not be directly comparable due to the heterogeneity of soil environments.

Thus, this study first aimed to identify a suitable nucleic acid extraction method for the above mentioned acidic peat soils. Currently available methods were assessed for their ability to co-extract DNA and RNA from acidic peat soils, but were unable to yield mRNA suitable for downstream application. A new method, NM-OSP, was then designed with the information gained from the three failed methods and with maximum flexibility and transparency in mind, unlike many commercially available products. The NM-OSP method was tested on high and low pH peat soils to test its robustness. Although the new method was unable to yield RNA samples that were free of genomic DNA from acidic soils, DNA isolated from both high and low pH soils were of amplifiable quality. Also, high quality mRNA was successfully extracted from high pH soils, reverse-transcribed and quantified using in a qPCR. Denitrification gene expression patterns of the high pH soil matched a previous study using the same soil, confirming that the new extraction method was comparable to more traditional

extraction methods and was not likely to create any new method-based bias of the samples. Furthermore, the new method yielded higher DNA and mRNA yields than one of the most commonly used methods in environmental studies.

Combining this new extraction method with the aforementioned laboratory-based robotised gas measuring incubation system, the denitrification potential of high and low pH peat soils was analysed. Nucleic acids (DNA and mRNA) were extracted from the soils at multiple time points during incubation. The transcripts of denitrification enzymes were quantified and the expression patterns were correlated with the gas production/utilisation rates. Similar to previous studies, complete denitrification to N_2 without external alteration of soil pH was possible but retarded in acidic soils. Comparison of gas profiles from soils with different pH values show a strong pH effect on denitrification and the delayed N_2O reduction in low pH soils may be indicative of dissimilar Denitrification Regulatory Phenotypes (DRP) in soils of different pH.

In conclusion, although the underlying genetic mechanisms have yet to be revealed, complete denitrification to N_2 in acidic soils is possible in closed systems. However, this does not occur *in situ* because of the delayed activation of the N_2O reductase (N_2OR). This delayed N_2OR activation may be caused by two different DRP in high and low pH soils, hinting at the extent of DRP effects on NOx gas production. The discovery of DRP possibly playing a major role in N_2 production has helped to reveal the potential of low pH soils in performing complete denitrification to N_2.

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 (UMB) in Ås, Norway.

Sammendrag

Denitrifikasjon er en av hovedprosessene i nitrogensyklusen. Ved denitrifikasjon fjernes biologisk tilgjengelig nitrat fra biosfæren ved reduksjon av nitrat til N_2, via en rekke mellomprodukter. Gassene NO og N_2O er begge mellomprodukter i denitrifikasjonen som sterkt påvirker atmosfæriske reaksjoner som en følge av dannelsen av reaktive nitrat-radikaler i atmosfæren. I tillegg er N_2O en potent drivhusgass som det har blitt registrert økende mengder av de siste tiårene. Det er derfor viktig å forsøke å finne den genetiske årsaken til de høye N_2O utslippene fra landbruksjord.

Tidligere studier gjort i vår forskningsgruppe har benyttet kvantitativ «polymerase chain reaction» (qPCR) og avanserte laboratorium baserte gassmålinger for å karakterisere uttrykket av gener involvert i denitrifikasjons og gass-kinetikk i jord. I disse studiene ble det konstatert at pH i jord er en meget viktig variabel som kontrollerer det endelige trinn i denitrifikasjonen hvor N_2O blir redusert til N_2. Ytterligere molekylært arbeid med sure jordprøver ble i midlertidig hindret på grunn av ineffektiv ekstrahering av nukleinsyrer og DNA-forurensede RNA prøver. Selv med dagens teknologiske fremskritt, er det allment kjent at det kan være svært vanskelig å ekstrahere nukleinsyrer fra inhibitor-rike jordprøver. Ofte må separate reaksjons- eller ekstraksjonsmetoder benyttes for å oppnå nukleinsyrer som er brukbare i nedstrøms applikasjoner. Dette er en potensiell kilde til teknisk bias, da det ekstraherte DNA og RNA-et kanskje ikke er direkte sammenliknbare på grunn av heterogeniteten av jordprøvene.

Første delen av denne masteroppgaven dreier seg om å finne en egnet metode for ekstraksjon av nukleinsyrer fra sur torvjord. Nåværende tilgjengelige metoder ble vurdert etter deres evne til å ko-ekstrahere DNA og RNA fra sur torvjord, men ingen av metodene var i stand til å gi mRNA egnet til videre anvendelse. En ny metode, NM-OSP, ble deretter utformet utfra informasjonen ervervet fra de tre mislykkede metodene, og med vekt på maksimal fleksibilitet og åpenhet, noe som står sterkt i kontrast i forhold til mange kommersielt tilgjengelige produkter. For å undersøke hvor robust NM-OSP metoden var, ble den testet på jord med lav og høy pH. Selv om den nye metoden ikke var i stand til å gi RNA fritt for genomisk DNA fra sur jord, var DNA isolert fra både høy og lav pH jord av amplifiserbar kvalitet. mRNA av høy kvalitet ble dessuten ekstrahert fra jord med høy pH, reverser-transkribert og kvantifisert ved hjelp av qPCR. Mønsteret for uttrykket av denitrifikasjonsgener i jord med høy pH matchet en tidligere studie utført med samme jord,

noe som bekrefter at den nye ekstraksjonsmetoden var sammenlignbar med mer tradisjonelle ekstraksjonsmetoder, og at det er lite sannsynlig at den nyutviklede metoden har en annen bias enn den tradisjonelle. Videre ga den nye metoden høyere DNA og mRNA avkastning enn en av de mest brukte metodene i miljøstudier.

Denitrifikasjons potensialet for torvjord med høy og lav pH ble videre analysert ved robotiserte gassmålinger i laboratoriet i kombinasjon med den nyutviklede ekstraksjonsmetoden. Under inkuberingen ble det på flere tidspunkter ekstrahert nukleinsyrer (DNA og mRNA) fra jordprøvene. Transkripsjonen av denitrifikasjonenzymer ble kvantifisert og samsvaret mellom dette og målt gassproduksjon ble undersøkt. I likhet med tidligere studier, var komplett denitrifisering til N_2 uten ekstern endring av jord pH mulig, men forsinket i prøvene fra surt jordsmonn. En sammenligning av gassprofilene fra jord med ulike pH-verdier viser en sterk pH effekt på denitrifikasjon, og den forsinkede N_2O reduksjon i lav pH jord kan være en indikasjon på ulike Denitrifisering Regulatoriske Fenotyper (DRP) i jord av ulik pH.

Komplett denitrifikasjon til N_2 kan forekomme i sur jordsmonn om de stenges inne i et gasstett system. Dette skjer derimot i liten gard under naturlige forhold på grunn av en forsinket aktivering av N_2O reduktase (N_2OR). Denne forsinkede aktiveringen av N_2OR kan være forårsaket av to forskjellige DRP i jord med høy og lav pH, noe som kan gi antydninger til omfanget DRP-effekter kan ha på produksjonen av NOx gasser. Oppdagelsen av at DRP muligens spiller en svært viktig rolle i produksjonen av N_2, har bidratt til å avdekke potensialet til å utføre komplett denitrifikasjon til N_2 i jord med lav pH.

Arbeidet i denne avhandlingen ble gjennomført i Miljømikrobiologi gruppen ved Institutt for Kjemi, Bioteknologi og Matvitenskap (IKBM) av Universitetet for Miljø og Biovitenskap (UMB) i Ås, Norge.

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List of abbreviations

cDNA	Complementary DNA strand to RNA
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
DRP	Denitrification Regulatory Phenotype/s
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
G2 beads	G2 lysis beads
GC	Gas chromatograph
gDCC	Genomic DNA Clean & Concentrator Kit
gDNA	Genomic DNA
GL beads	Three-size glass bead mix
MB beads	Glass beads from the PowerSoil DNA Isolation Kit
mRNA	Messenger RNA
N_2	Dinitrogen gas
N_2O	Nitrous Oxide gas
N_2OR	Nitrous oxide reductase
NA	Nucleic acids
NAP	Periplasmic nitrate reductase
NAR	Membrane-bound nitrate reductase
NIR	Nitrite reductase
NM	Newly designed method
NM-OSP	Newly designed method with OneStep PCR Inhibitor Removal Kit clean-up
NO	Nitric Oxide gas
NOA	Nitric Oxide Analyzer
NOR	Nitric oxide reductase
NOx	Nitrogen oxides (NO and N_2O)
O_2	Gaseous oxygen
OSP	OneStep PCR Inhibitor Removal Kit
P-C-I	Phenol-chloroform-isoamyl alcohol (25:24:1)

PCR	Polymerase chain reaction
PD	PowerSoil DNA Isolation Kit
PEG	Polyethylene glycol
PM	PowerMicrobiome RNA Isolation Kit
PO	Progressive Onset, a type of Denitrification Regulatory Phenotype
PS	RNA PowerSoil Total RNA Isolation Kit
PS-D	RNA PowerSoil Total RNA Isolation Kit with RNA PowerSoil DNA Elution Accessory Kit
PVPP	Polyvinylpolypyrrolidone
qPCR	Quantitative PCR; Real-time PCR
RCC	RNA Clean & Concentrator-5 Kit
RCO	Rapid Complete Onset, a type of Denitrification Regulatory Phenotype
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ssDNA	Single-stranded DNA
TEM	Traditional Extraction Method
TNA	Total nucleic acids
w/v	Weight per volume
w/w	Weight per weight

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1. Introduction

1.1. The nitrogen cycle

The Earth is a vast, inter-linked environment where a number of important biological processes are highly regulated. Of these, the cycling of nitrogen is arguably one of the most fascinating since it involves the massive atmospheric pool of inert dinitrogen gas (N_2). The nitrogen cycle is made up of several processes, both well- and little-understood, including nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonia oxidation (anammox) (Figure 1.1). Together with nitrogen fixation, these processes continuously cycle inert nitrogen from the atmosphere into biologically available nitrogen. The only aerobic process, nitrification, oxidises organic nitrogen into nitrate through a series of reaction intermediates. In contrast, there are three anaerobic processes: denitrification, DNRA and anammox, which reduce nitrate to N_2 (denitrification and anammox) or ammonia (DNRA). There is also the process of aerobic denitrification, but it is not a well-studied method and little is known other than that it is a denitrification process that is able to occur under aerobic conditions (Meiklejohn, 1940, Jetten, *et al.*, 1999, Ahn, 2006).

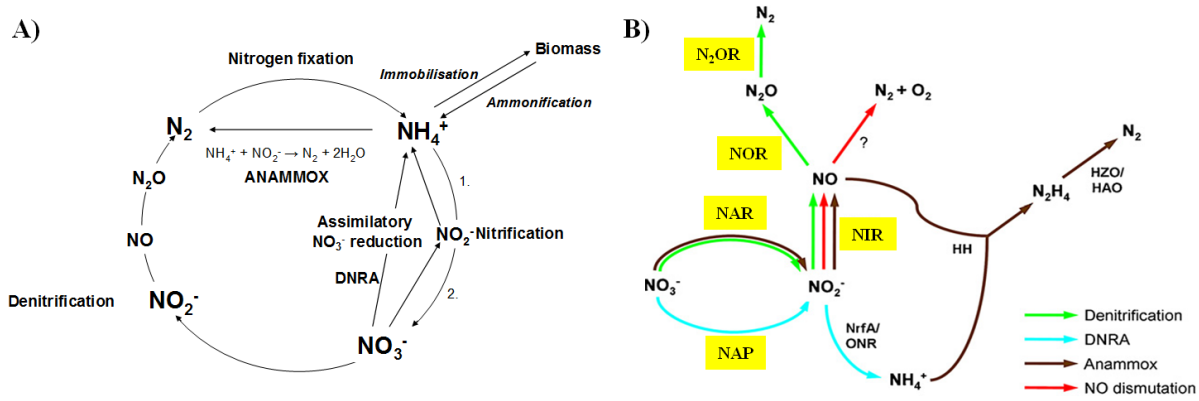

Figure 1.1 The nitrogen cycle. A) Processes in the nitrogen cycle continuously cycle nitrogen through the biologically available (NH_4^+, NO_2^- and NO_3^-) and unavailable (N_2) forms. *Figure from Bergaust, 2009.* B) Processes that involve nitrate respiration include denitrification, anammox and dissimilatory nitrate reduction to ammonium (DNRA). Denitrification enzymes are highlighted in yellow and further described in Section 1.3. *Figure adapted from Kraft* et al., *2011*.

1.2. Denitrification

Of the known processes in the nitrogen cycle, denitrification is of particular interest because it is a process where microorganisms use a series of enzymes to reduce nitrate through a series of intermediates to N_2. This means that fixed, biologically available nitrate is removed by denitrification back into N_2 (Zumft, 1997). As such, it has wide-ranging impacts on the agricultural industry, where high denitrification rates would result in increased fertiliser usage and increased crop production costs. Unlike anammox which is a strict anaerobic process, there is evidence that denitrification is an anaerobic process that tolerates low levels of oxygen under certain circumstances (Jetten, *et al.*, 1999, Ahn, 2006, Bergaust, *et al.*, 2008).

Denitrification is a biological stepwise process that involves the sequential reduction of the N-oxyanions, nitrate and nitrite, to the gaseous N-oxides (NOx), nitric oxide (NO) and nitrous oxide (N_2O), to N_2 (Figure 1.2). These individual reduction reactions involve reductases with four different substrates: periplasmic nitrate reductase (NAP) and membrane-bound nitrate reductase (NAR) reduces nitrate; nitrite reductase (NIR) reduces nitrite; nitric oxide reductase (NOR) reduces NO; and nitrous oxide reductase (N_2OR) reduces N_2O (Zumft, 1997, Moura & Moura, 2001). To further complicate matters, the end product of denitrification may either be N_2O or N_2, and the intermediate gaseous products NO and N_2O are known to 'leak' from the denitrification reaction (Firestone & Davidson, 1989, Mahne & Tiedje, 1995, Madsen, 2008). The extent of this leakage is so severely apparent that the term "denitrification sensu stricto" has been used to describe nitrite and nitric oxide reduction, and "nitrous oxide respiration" is considered a separate and optional step in the denitrification process (Zumft, 1997).

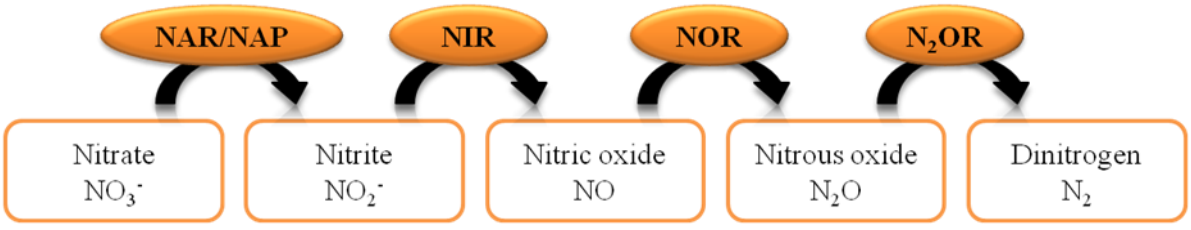

Figure 1.2 The denitrification process. Denitrification is the reduction of nitrate to dinitrogen using reductase enzymes, and with nitrite, nitric oxide and nitrous oxide as intermediate products. NAP: periplasmic nitrate reductase; NAR: membrane-bound nitrate reductase; NIR: nitrite reductase; NOR: nitric oxide reductase; N_2OR: nitrous oxide reductase.

1.2.1. NOx gas emissions

The intermediate gas products of denitrification, NO and N_2O (collectively known as NOx), play important roles in atmospheric reactions due to the reactive nitrate radicals formed in the atmosphere (Uherek, 2004, Bowman, *et al.*, 2011). One such major reaction would be the reaction of NOx gases with atmospheric water to form nitric acid, which contributes to acid rain (Uherek, 2004).

Of the two NOx gases, N_2O, is also known to be important greenhouse gases, and a lot of interest in denitrification is due to this intermediate product rather than the end product of N_2 (Madsen, 2008). This interest in N_2O emission has been gradually increasing in recent years, partly because it is over 200 times more potent than the well-known carbon dioxide (CO_2) as a greenhouse gas, and partly because atmospheric levels of N_2O have been steadily on the rise since the late 1970s (Madsen, 2008). Traditionally N_2O emissions have been measured-in-field with microcosm studies conducted in parallel, resulting in a large number of studies monitoring and exploring new ways to capture such emissions using advanced technologies (Molstad, *et al.*, 2007, Hovlandsdal, 2011, Raut, *et al.*, 2012). More importantly, the biological perspective on the issue had been largely neglected in the past, and only in recent years has there been interest to develop better molecular methods to link the genetic cause of N_2O emissions, or the lack thereof, with field observations (Jones, *et al.*, 2008, Jung, *et al.*, 2012, Jones, *et al.*, 2013). Since N_2O emissions are a concern in both water and soil systems, there has also been much work conducted to further understand these processes, and to help mitigate N_2O emissions (Dong, *et al.*, 2002, Dong, *et al.*, 2009, Hénault & Revellin, 2011). This interest has also spilled over into industrial processes and is often discussed from a biotechnology perspective, with much desire to implement knowledge gained from exploratory studies in the industry to reduce negative environmental effects (Ahn, 2006).

1.2.2. Emissions from industrial processes

Agriculture, waste water treatments and effluents, and composting are some of the largest anthropological producers of NO and N_2O worldwide, and have been some of the most active industrial processes looking to adapt denitrification processes to mitigate greenhouse gas production (Ahn, 2006, Dong, *et al.*, 2009, Maeda, *et al.*, 2010). Interestingly, denitrification has been viewed as both a highly desirable and extremely unwelcome process, depending on

the type of industry. In wastewater treatment plants, denitrification is very important because it prevents the eutrophication of water bodies, by removing nitrate from the effluent prior to release into water bodies (Zumft, 1997, Ahn, 2006). Quite the opposite, the very same process is highly detrimental to agricultural systems, removing biologically available nitrogen (nitrate) from the soil. This results in reduced crop yields and possible greenhouse gas (NO or N_2O) emissions, and translates into massive financial repercussions.

Thus, gas production is often monitored under controlled experimental systems in an effort to trace the source of such N_2O emissions and determine methods to reduce such emissions (Maeda, *et al.*, 2010, Hénault & Revellin, 2011). Also, exploratory studies are not uncommon. Specifically, the effect of fertilisation on agricultural soil has been closely monitored, since the agricultural industry is an important and currently irreplaceable source of NO and N_2O emissions (Chen, *et al.*, 2010, Raut, *et al.*, 2012).

With the growing global population, global food requirements are on the rise, and barring a completely novel way to generate food, agriculture-related emissions will only increase in the near future. As such, it is of high importance to better understand the reasons behind greenhouse gas production of agricultural fields, so that we may find a way to mitigate such emissions as far as possible. One major problem is the current fertiliser addition practices. On one hand, it is well-known that the availability of nitrogen (fertiliser) strongly affects plant growth, since nitrogen limitation increases plant stress and results in decreased plant growth and chlorophyll content (Hamonts, *et al.*, 2013). On the other hand however, only a very small amount of fertiliser is used by the plants and over-fertilisation of soils increases denitrification rates, resulting in faster organic nitrogen loss and even higher NO and N_2O emissions (Felber, *et al.*, 2012). Given that the source of these emissions (agricultural fields) affects the global food source, there is pressing need to better understand the emission patterns and the genetic reasons behind it, so that we may be able to find methods to mitigate emissions from these irremovable agricultural soils.

1.2.3. pH – the ‘master variable’ controlling denitrification

There are many factors that affect denitrification, including the presence of plant roots, flooding of soils and rate of N-addition (fertilisation) to the soil (Hamonts, *et al.*, 2013). However, empirical evidence for a direct effect of soil pH on the $N_2O/(N_2+N_2O)$ product

ratio of denitrification (high at low pH) has shown that pH is the 'master variable', and affects denitrification greatly (Firestone, *et al.*, 1980, Šimek & Cooper, 2002, Bergaust, *et al.*, 2010, Liu, *et al.*, 2010). It is generally accepted that lower pH environments either restrict denitrification completely, or cause a delayed denitrification reaction (Šimek & Cooper, 2002, Jones, *et al.*, 2011). Additionally, evidence from field observations of acidic soils has shown a propensity to emit higher levels of N_2O than neutral pH soils (Hovlandsdal, 2011, Raut, *et al.*, 2012). The mechanisms involved are still not well understood, but recent studies in our group provide strong evidence for a post-transcriptional phenomenon by which low pH interferes with the assembly of the N_2OR enzyme in the periplasm (Bergaust, *et al.*, 2010). An alternative explanation put forward by Jones and colleagues is that a large portion of known denitrifiers simply lack a functional N_2OR gene and are thereby unable to reduce N_2O to N_2 (Jones, *et al.*, 2008, Jones, *et al.*, 2013).

Although a large proportion of laboratory-based experiments is conducted with pure cultures or extracted cells in liquid media and is not directly relatable to field observations, soil-cultivation studies have no control of the pH that the microorganisms experience (Bergaust, *et al.*, 2008, Bergaust, *et al.*, 2010, Nadeem, *et al.*, 2013). Thus, there is much that may be learnt about the physiology and biochemistry of bacteria from pure culture studies. However, some researchers believe that such pure culture experiments attempt to use bacteria with higher pH optima to denitrify at suboptimal conditions, and may therefore not reflect true soil conditions (Jones, *et al.*, 2011). As such, there may be previously unknown and never before isolated soil microorganisms that play a major role in generating N_2 in lower pH soils (Jones, et al., 2011).

1.2.4. Soil denitrifiers

When trying to relate laboratory-based experiments to field observations, one of the main problems is the complexity of soil microbial communities. As far as nitrate and nitrite reduction is concerned, there are three groups of bacteria in the soil community (Figure 1.3): True denitrifiers are defined as organisms that produce N_2 or N_2O from a substrate of nitrate or nitrite and this reduction of N-oxyanions is coupled to the organisms' growth (Mahne & Tiedje, 1995); "nitrite accumulators" are microorganisms that perform only nitrate reduction and do not carry any of the enzymes required for the reduction of nitrite, NO or N_2O (Gamble, *et al.*, 1977, Zumft, 1997); and bacteria that are not involved in nitrogen cycling

and do not perform any oxidation or reduction of N-compounds. However, since these studies were conducted, the discovery of another nitrite-reducing pathway, anammox, has been discovered (Jetten, *et al.*, 1999). Thus, the term "nitrite accumulators" is no longer appropriate for this group of nitrate-reducing bacteria since DNRA and anammox bacteria reduce nitrate to nitrite and not further to NO, N_2O or N_2, but do not accumulate nitrite. For the rest of this thesis, this group will instead be referred to as "nitrate reducers".

Estimations of denitrifying bacteria in the soil environment have ranged from 5% of all soil microflora, to 10% of anaerobic microorganisms (Gamble, *et al.*, 1977, Henry, *et al.*, 2006). In contrast, nitrate reducers are more common in the environment, making up 40% of anaerobic microorganisms (Gamble, *et al.*, 1977). Although not considered true denitifiers, nitrate reducers may take part in other nitrogen cycling processes such as DNRA or anammox. When combined, these two fractions make up nearly half of all anaerobic-growing microorganisms, and play a major role in shaping the nitrogen cycling process in soils. However, of these bacteria, only the true denitrifiers result in the loss of biologically-available nitrogen, and thus their presence affects not only the microflora of a soil, but the plants growing in the same soil as well.

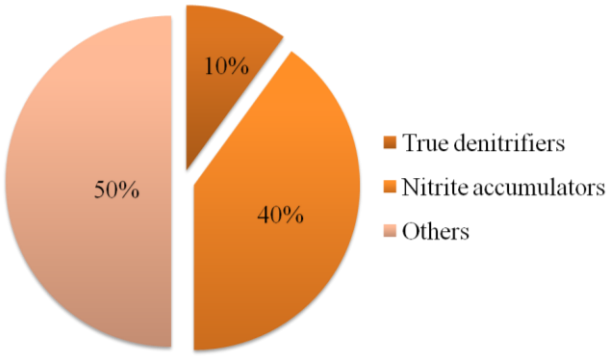

Figure 1.3 The anaerobic bacteria community in soils. True denitrifiers make up a relatively small portion of the soil microbial community, and nearly half of soil anaerobes are not involved in nitrogen cycling.

1.3. Denitrification enzymes

The enzymes in denitrification have important roles to play, providing energy to their host denitrifiers in the absence of oxygen (O_2) as the terminal electron acceptor. Additionally, these enzymes have to control the build-up of intermediate products, ensuring that the levels do not become toxic for the cells. Controlled by a large number of regulators, these enzymes thus have the dual duty of providing energy and controlling toxic intermediate build-up (Zumft, 1997). In particular, nitrite and NO concentrations have to be tightly controlled in denitrifiers so as to avoid concentrations that are toxic to the cells (Bowman, *et al.*, 2011). The concentration at which these intermediates are controlled differs according to species and their tolerance levels (Zumft, 1997, Bergaust, *et al.*, 2008).

The denitrification enzymes NAP, NAR, NIR, NOR and N_2OR are shown in their respective positions (membrane-bound or in the periplasm) in denitrifiers in Figure 1.4, and are further described in the following sections.

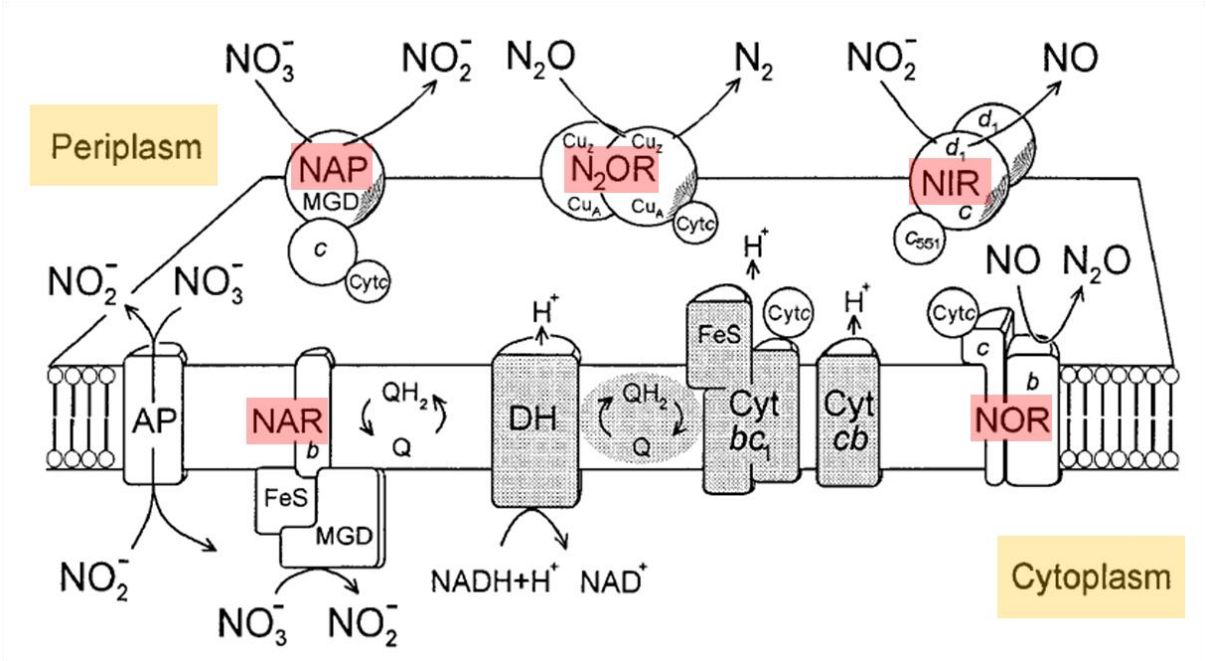

Figure 1.4 The position of denitrification enzymes in denitrifiers. Denitrification enzymes are highlighted in red. NAR and NOR are membrane-bound, whereas NAP, NIR and N_2OR are in the periplasm. NAP: periplasmic nitrate reductase; NAR: membrane-bound nitrate reductase; NIR: nitrite reductase; NOR: nitric oxide reductase; N_2OR: nitrous oxide reductase. *Figure adapted from Zumft, 1997.*

1.3.1. Nitrate reductase

The first enzymes in the denitrification process, the nitrate reductases, are classified according to their location in the cell (Figure 1.4). The NAR enzyme is membrane-bound, located on the cytoplasmic side, and is expressed and used only under fully anaerobic conditions. The NAP enzyme located in the periplasm, is oxygen-tolerant, and is expressed and active under aerobic conditions (Bell, *et al.*, 1990, Zumft, 1997). Additional to being able to function under oxic conditions, nitrate reductases are not unique to denitrifiers and large numbers are estimated in the environment, as previously described (Bothe, *et al.*, 2000, Kraft, *et al.*, 2011). Hence as mentioned previously, the terms "nitrate reducers" that perform "respiratory nitrate reduction" have been used to describe organisms that carry NAR, NAP, or both (Gamble, *et al.*, 1977, Zumft, 1997).

1.3.2. Nitrite reductase

Due to the toxicity of both the substrate (nitrite) and the product (NO), NIR enzymes are especially important in denitrifiers (Bowman, *et al.*, 2011). This enzyme has also been of great interest to the scientific community for two reasons. Firstly, the product of the NIR enzyme, NO, is the first gaseous product in denitrification, and the relative rate of NIR and NOR controls the build-up of NO, thus determining if excess NO escapes into the atmosphere. Secondly, there are two different NIR enzymes that appear to be evolutionarily distinct and which, until now, has never been found in the same organism (Zumft, 1997). Despite having different structures and prosthetic groups, NirK is a Cu(II) trimer protein and NirS is a cytochrome dimer (cytochrome cd_1), the two proteins are functionally similar, with both able to reduce nitrite to NO (Zumft, 1997).

Due to the dissimilar evolutionary history of the two proteins, complete genotypic characterisation of the proteins and the microorganisms that produce these proteins has been hampered. The NirK enzyme in particular, appears to have diverged during evolution, making a universal primer design extremely difficult (Braker, *et al.*, 1998, Hallin & Lindgren, 1999, Falk, *et al.*, 2010). Thus, there has not yet been a single primer designed that has been successful at capturing the full *nirK*-containing community. Additionally, our research group has found that different primers targeting *nirK* capture different parts of the same microbial community, and in some cases, one primer may work better in one soil, but poorly in another

(unpublished). This sentiment is also reflected in the literature, where multiple primers have sometimes been used for the same samples (Green, *et al.*, 2010). The amplification efficiencies and unspecific amplification tendencies of primers have also differed depending on the type of sample used, and optimisation of amplification conditions may not always be able to solve these problems (Binbin Liu pers. comm.).

In contrast, studies with the *nirS* gene have indicated that *nirS*-containing organisms were more prevalent in soils, and have implied that *nirK*-containing organisms play a smaller role in soil denitrification (Gamble, *et al.*, 1977, Coyne, *et al.*, 1989). Although there have been some studies that have shown that the NirK enzyme plays a stronger role than the NirS enzyme, these are relatively few, and there is general consensus in the literature that NirS is more often the main player in nitrite reduction to NO (Liu, *et al.*, 2010, Maeda, *et al.*, 2010, Hamonts, *et al.*, 2013).

1.3.3. Nitric oxide reductase

NOR is a membrane-bound enzyme that is not expressed or synthesised under fully oxic conditions (Zumft, 1997). As mentioned briefly, the substrate for NOR enzymes, NO, is toxic to bacteria at high concentrations due to its chemical properties, inhibiting the growth and metabolism of microorganisms (Zumft, 1997, Bergaust, *et al.*, 2008, Falk, *et al.*, 2010, Kraft, *et al.*, 2011). The lethality of NO can also be observed in its use as a defence mechanism in eukaryotic macrophages in removing phagocytosed pathogens. Although bacteria also carry other enzymes for NO detoxification, the importance of NOR in removing the threat of NO and converting it to harmless N_2O in denitrifiers cannot be more strongly stressed.

1.3.4. Nitrous oxide reductase

The final enzyme in denitrification is the N_2OR. The process of denitrification has previously been described as N-oxyanion reduction to N_2O or N_2, showing that nitrous oxide respiration is not considered an essential step when classifying denitrifiers or determining the occurrence of denitrification (Mahne & Tiedje, 1995). The N_2OR enzyme has been found to be sensitive to environmental factors, especially pH, and may not always be able to perform the final "nitrous oxide respiration" step if disrupted (Bergaust, *et al.*, 2010). Also, as previously

mentioned in Section 1.2.2, there is much interest in the activity of N_2OR in the environment, since it is currently the only known biological mechanism of N_2O removal to an inert product.

1.3.5. The unrelated-ness of denitrification enzymes

Although classified by scientists collectively as "denitrification enzymes" that perform successive reduction reactions, the reductase enzymes involved in denitrification are not always evolutionarily related to each other (Zumft, 1997). Additionally denitrifiers are not grouped phylogenetically and their enzymes exist across a wide range of mostly unrelated bacteria (Zumft, 1997). Even within a single genus, some bacteria may be able to perform denitrification while others may not, and the production and reduction rates of gases may be vastly different (Liu, *et al.*, 2013). Moreover, in some habitats, phylogenetically-related denitrification genes have been found in unrelated bacteria, indicating that horizontal gene transfer between unrelated members of the same community is possible (Falk, *et al.*, 2010).

1.3.6. Primers targeting denitrification enzymes

Unfortunately, denitrification enzymes have highly dissimilar sequences, with no specific variation in the 16S rRNA signalling the presence of a denitrifier and are spread out across a wide range of unrelated organisms (Philippot & Hallin, 2005, Falk, *et al.*, 2010). Thus, studies investigating denitrifier populations require the use of primers targeting genes coding for denitrification enzymes (Smith, *et al.*, 2007). However, denitrification enzymes appear to have a complicated evolutionary history and do not necessarily have well-conserved regions in the coding sequence, resulting in poor 'universal primer' constructs that are only able to capture closely-related bacteria (Jones, *et al.*, 2008). As a result, research on denitrification does not allow one to conduct comparatively simple 16S rRNA phylogenetic studies, instead requiring the design of specific primers for each denitrification enzyme.

This is also reflected in the literature, where there is a slew of available primers targeting the different denitrification genes, none of which are, unfortunately, truly universal primers capable of amplifying all organisms with the target gene (Braker, *et al.*, 1998, Hallin & Lindgren, 1999, Henry, *et al.*, 2004, Throbäck, *et al.*, 2004, Chen, *et al.*, 2010). This is

completely different from the well-established primers for the conserved 16S rRNA gene, where commonly used primers have barely deviated from the first primers designed in the early 1990s (Weisburg, *et al.*, 1991, Muyzer, *et al.*, 1993). Although there is evidence that the primers targeting the 16S rRNA gene may not be as universal as we believe them to be, the bias in those primers are nothing compared to the phylum- or clade-specificity of denitrification primers (Farris & Olson, 2007, Jones, *et al.*, 2013).

Specific primers aside, a wide variety of genes have been targeted in denitrification studies, including *narG*, *napA*, *qnorB* and *nirS* in the literature (Smith, *et al.*, 2007). As mentioned previously, many studies choose the NIR as the target because it produces the first gaseous intermediate in denitrification. Another common target is the N_2OR because it reveals the rate of complete denitrification to N_2. Many recent studies have also used this same gene combination, most probably because it is the most environmentally relevant (Maeda, *et al.*, 2010, Hamonts, *et al.*, 2013). The expression pattern of these genes could potentially reveal the speed at which gaseous intermediates easily lost to the atmosphere are produced, and the amount of time necessary for N_2OR to be expressed, turning greenhouse gases to inert N_2.

1.4. Measuring gas emissions

In many environmental studies, gas measurements are taken *in situ* at the experimental site, resulting in single field measurements over long time scales that may not paint the full picture (Dong, *et al.*, 2002, Hénault & Revellin, 2011, Hovlandsdal, 2011). Unfortunately, while it is understandable that continuous measurements are difficult to take at field sites, single measurements spread out over weeks and months does not allow one to pinpoint reactions and changes at a microbial level. Additionally, field observations are subject to many uncontrollable environmental variables, and the resulting gas measurements may be a reflection of causes other than denitrification. Thus, such experiments need to be complemented with those performed under controlled laboratory conditions so as to fully isolate microbial response and the resultant effect, from the background noise present in the field. An additional benefit to laboratory experiments is that microorganisms may be extracted immediately, and their genetic expression may be analysed as required.

However, even in the laboratory, it is often difficult to sample gases repeatedly at structured and consistent times. Many laboratory-based experiments are conducted separate from the field, not taking gas production and utilisation into account, and the conclusions are based purely on results from molecular methods (Cantera & Stein, 2007, Smith, *et al.*, 2007). Other studies see the value in corresponding gas profiles, but are only able to perform single or few gas measurements, and the resultant gas profiles are created via extrapolation (Bleakley & Tiedje, 1982, Liu, *et al.*, 2003, Henderson, *et al.*, 2010, Stremińska, *et al.*, 2012). Unfortunately, the few studies with frequent gas measurements are often short-term and are thus unable to reflect the full effect of denitrification in complex soils (Mahne & Tiedje, 1995).

In contrast, the robotised auto-sampling system designed by our research group gives us full control throughout long sampling times and also measures multiple gases including N_2 which is difficult to measure due the risk of leakage from the surrounding air (Molstad, *et al.*, 2007). Sampling frequency is fully robotised and computer controlled, ensuring that the necessary gas measurements are performed continuously through the night (and for as long as required) in a precise and repeatable manner (Section 2.4). Additionally, the system allows up to 40 flasks to be incubated at constant temperatures. The robotised incubation system has also been used in a variety of experiments, ranging from pure cultures to soil samples (Bergaust, *et al.*, 2010, Falk, *et al.*, 2010, Liu, *et al.*, 2010, Jones, *et al.*, 2011)

1.5. Nucleic acid (NA)-based analysis

In the never-ending search for knowledge in the soil sciences, analysis based on nucleic acids (NA) is a relatively new field. Able to provide precious information on the genetic basis of many physiological effects in the environment, molecular techniques have become increasingly important. However, a yet-insurmountable hurdle preventing efficient gathering of knowledge is the occurrence of 'difficult to work with' environmental samples, which will be further elaborated in Section 1.5.3.

In addition to the gas profiles mentioned in the previous section, the genetic reasons behind the gas production seen is also necessary in order to better understand the processes that go on in the soil. This is because relying on only gas kinetics to determine the presence of denitrifiers is near-impossible due to the presence of denitrification enzyme orthologues. Currently understudied and relatively unknown, orthologues have been found in nitrifying bacteria (Cantera & Stein, 2007). Additionally, with only the knowledge of the gas profile, the exact response of the microbial community cannot be established, making it impossible to determine the reason behind the observed phenotypical response (Nadeem, *et al.*, 2013). As such, there have been a number of studies that have combined the observed gas production profiles with molecular methods, chief among them are amplification based methods (Dong, *et al.*, 2009, Liu, *et al.*, 2010).

1.5.1. DNA versus mRNA

DNA is one of the most common targets of genetic analyses, and the extraction and subsequent sequencing of DNA extracted from soils enables the identification of the members of the soil community present (Jones, *et al.*, 2013). However, DNA is only able to provide the potential of the soil microbial community since not all present microorganisms are metabolically active. Also, it is well-known that DNA degradation is relatively slow in the environment, and the extracted DNA may have come from long-dead microorganisms that was left in the soil matrix (Moran, *et al.*, 2013). Another disadvantage of DNA as the target molecule is the unreliability of quantification studies. There has been recent evidence that some denitrification genes may occur in duplicate copies in some bacteria, rendering the quantification of DNA useless (Jones, *et al.*, 2008).

In contrast, mRNA is able to reveal the active players in any environment, giving us the potential to investigate how different treatments affect the microflora at a genetic level, resulting in the field observations (Moran, *et al.*, 2013). Molecules of mRNA are also extremely useful as sensitive bioassays because of their short half-lives. Unlike proteins and DNA which have longer half-lives, mRNA intracellular stocks change quickly according to environmental changes, and any measurement of mRNA would be indicative of responding cells (Philippot & Hallin, 2005, Moran, *et al.*, 2013). Protein responses are relatively slower and may last long after the environmental stimulus has ended, since the proteins themselves are not degraded immediately after the pulse, except in the case of targeted protein degradation (Moran, *et al.*, 2013). Moreover, enzymes may have multiple functions or may co-metabolise certain substrates, further complicating any conclusions that may be drawn from analysing only proteins (Figure 1.5).

Quantifying mRNA, on the other hand, would reveal expression patterns, potentially showing the importance of the synthesised protein. When used together, the presence of detectable DNA would show the potential of a community, the mRNA would show the active members under experimental conditions, and the constructed gas profiles would show the phenotypic effect.

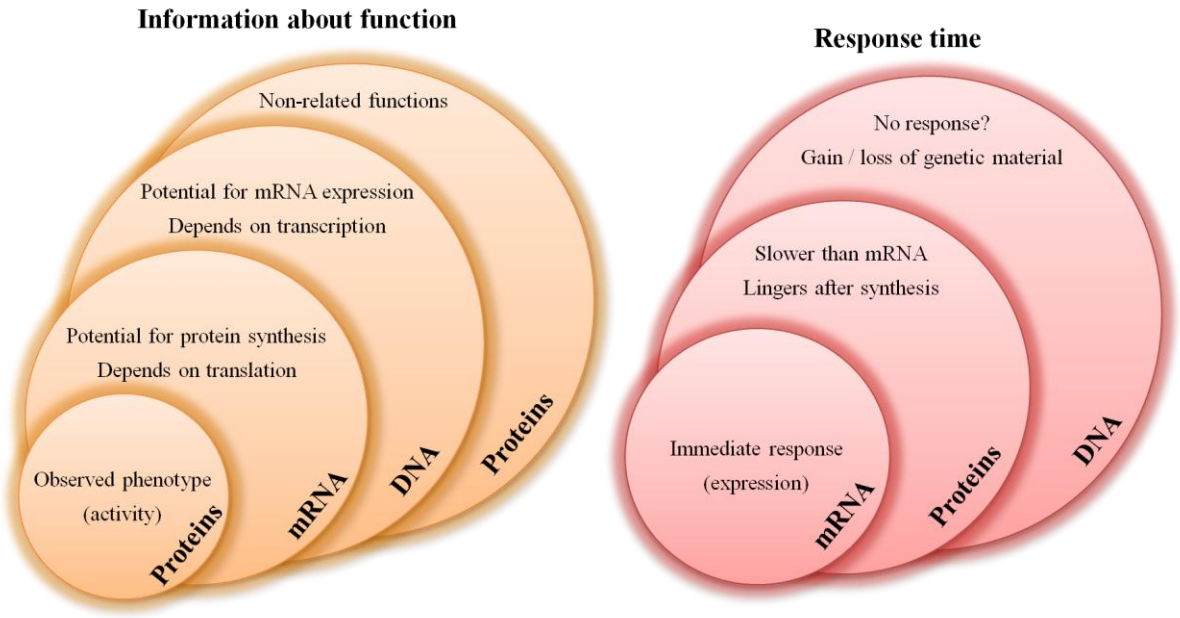

Figure 1.5 Difference between information obtained about function and the response time of molecules in bacterial cells. Proteins are able to give specific knowledge since they directly affect the phenotype, but they may have non-related functions and they also tend to linger in the cell after transcription, masking small or immediate changes. Although mRNA is not as specific, reflecting the potential for protein synthesis (but of an unknown number), it responds quickly due to its quick transcription and short half-life. DNA is only able to reflect the potential for genetic expression, and does not respond quickly to environmental changes.

1.5.2. NA extraction from soil

It is extremely important to have a good NA extraction method, since the extracted NA fraction would affect all downstream processes (Bakken & Frostegård, 2006, Smith & Osborn, 2009). Poor extraction methods result in biased DNA and RNA extracts, thereby not reflecting the true community genetic structure. It has also been recognised that difficulties faced during the extraction procedure is a major factor causing severe bias in results, especially when analysing results from sensitive downstream processes such as qPCR (Fleige & Pfaffl, 2006, Smith & Osborn, 2009, Gadkar & Filion, 2013). Consequently, extraction methods should ideally contain minimal opportunities for the introduction of bias. Three common ways of introducing bias are highlighted below.

The first method of bias introduction is by culturing organisms prior to extracting nucleic acids. In the past when downstream processes were less sensitive, it was often necessary to increase the amount of genetic material present by culturing organisms to high numbers prior to extracting total nucleic acids (TNA). However, current amplification and quantification methods are highly sensitive, requiring very low quantities of genetic material, and are a vast improvement over older methods (Gadkar & Filion, 2013). Thus, the extraction of TNA directly from bulk soil is preferable, since culturing inevitably biases the results towards the most dominant bacteria that was able to grow on the culture media (Falk, *et al.*, 2010, Hirsch, *et al.*, 2010, Jones, *et al.*, 2011). Importantly, culturing bacteria after extracting cells from soil would also change the mRNA composition, and the results would no longer be relevant to the study of the environment where it came from (Moran, *et al.*, 2013).

The second potential introduction of bias is the separate extraction of DNA and RNA. Since soil is a heterogeneous environment filled with microsite activity hotspots, there is a potential for variation between the individual extraction reactions. In order to maximise comparability of the extracted genetic material, DNA and RNA should be extracted from the same soil sample. As previously mentioned, by comparing the DNA and RNA from each soil sample to itself, the DNA and mRNA reveal the potential and active members of the soil, respectively.

Another common way to introduce bias into samples is the improper selection of the method used to lyse bacteria. Lysis is the first step of all TNA extraction methods, and is thus the most important. Inappropriate lysis methods may introduce severe bias by favouring the lysis of certain organisms and not others, thereby distorting results of downstream processes (Frostegård, *et al.*, 1999, Bakken & Frostegård, 2006).

1.5.3. Difficulties in extracting Total Nucleic Acids (TNA)

In a review published recently, DNA instead of mRNA was found to be the target molecule in most studies, despite the obvious advantages of mRNA-based observations (Gadkar & Filion, 2013). This is probably due to the difficulty of obtaining stable, high quality, and inhibitor-free RNA that has deterred many researchers from working with the more informative mRNA molecule (Gadkar & Filion, 2013). The half-lives of mRNA molecules are very short, even under laboratory RNase-free conditions and degraded RNA would affect downstream processes, interfering with conclusions that may be drawn from the experiments (Fleige & Pfaffl, 2006, Gadkar & Filion, 2013, Moran, *et al.*, 2013). Thus, current methods for RNA extraction either require the addition of RNase inhibitors or depend on the speed of extraction and minimum time spent outside of the -80°C freezer, but even then does not work successfully for all samples (Griffiths, *et al.*, 2000, Kotiaho, *et al.*, 2010, Mettel, *et al.*, 2010).

Furthermore, soil samples are well-known to be difficult to work with, in particular peat soils which tend to have high quantities of co-extracted inhibitory compounds (Gadkar & Filion, 2013). The common definition of these 'difficult to work with' samples is samples which yield NA that are unusable in downstream processes, despite being present in large quantities. Many downstream processes used in the analysis of NA are inhibited by unknown compounds co-extracted from the soil samples. The exact composition of these inhibitory compounds is often a mystery, although humic acids and polyphenolic compounds have been identified as some of the co-extracted compounds inhibiting enzymatic activity (Peršoh, *et al.*, 2008, Moran, *et al.*, 2013). Since most NA analysis downstream processes require the use of enzymes, the inability to isolate inhibitor-free NA is a major barrier in the analysis of low pH soils and other inhibitor-filled environmental samples.

A second problem is that of speed. When working with only DNA, the speed of the extraction procedure is often not as essential, since DNA is relatively stable at room temperature. However, RNases are ubiquitous in the environment and as mentioned above, mRNA degrades very quickly (Moran, *et al.*, 2013). Thus, it is not easy meeting the criteria set down in a recent review, that extracted high-quality mRNA should contain minimal amounts of genomic DNA (gDNA) contamination and should not contain any inhibitory compounds (Gadkar & Filion, 2013).

With these two considerations in mind, the number of methods suitable for working with inhibitor-rich soil samples is relatively low. The most successful extraction procedures often

utilise commercially-available kits that are often not open to optimisation, due to the secrecy surrounding kit components. Additionally, many kits on the market extract only DNA or only RNA from single reactions, and few give the option of co-extracting DNA and RNA from the same reaction. Also, the inherent problems (presence of inhibitory compounds) when it comes to extracting DNA from the soil matrix, as described above, is difficult to overcome. Thus, some have chosen instead to extract cells from the soil prior to extracting TNA (Lindahl & Bakken, 1995, Nadeem, *et al.*, 2013). However, this adds the additional factors of cell survival through the cell extraction process, additional time spent in the extraction process thereby increasing the risk of losing quickly degraded mRNA, and the risk of losing a portion of cells that are tightly bound to soil particles (Bakken & Frostegård, 2006).

1.5.4. Currently available non-kit TNA extraction methods

Commercial kits aside, there are a wide variety of methods currently used worldwide. This is partly because of the difficulty of finding a single universal method that works for all samples (Gadkar & Filion, 2013). Many of these methods are based on the method published over a decade ago by Griffiths and colleagues (2000), which was designed for the co-extraction of DNA and RNA from soil samples, and is still one of the most commonly used methods in environmental microbiological studies. Briefly, this TNA extraction method involves bead-beating the sample with glass beads in an extraction buffer that includes phenol and chloroform to help remove the released cellular proteins, and polyvinylpolypyrrolidone (PVPP) to assist in the removal of humic substances. The method, with minor variations, has previously been successfully used to extract a wide variety of environmental samples (Liu, *et al.*, 2010, Hamonts, *et al.*, 2013).

Generally, most current extraction procedures are based on Griffiths and colleagues (2000) method and involve the use of, or some variant of, the above mentioned materials (Peršoh, *et al.*, 2008, Kotiaho, *et al.*, 2010, Mettel, *et al.*, 2010). However, not all these methods yield satisfactory results for all samples, and more often than not, commercially available NA extraction kits are used instead for purposes of speed and ease of use (Falk, *et al.*, 2010, Maeda, *et al.*, 2010). Unfortunately, there appears to have been little breakthrough in this field, and the conclusion that there is still no consensus on the best method to use has not changed in almost twenty years (Lindahl & Bakken, 1995, Gadkar & Filion, 2013). The best advice at the moment is that the methods should be chosen as the samples require, but we

should be careful when comparing results from different extraction procedures, due to the different inherent biases of each method (Gadkar & Filion, 2013).

1.5.5. Quantitative polymerase chain reaction (qPCR)

With the advent of the genomic era in the 1990s, a large variety of methods have been used in the analysis of environmental microbial community composition, including the well-established method of fingerprinting using DGGE (Muyzer, *et al.*, 1993, Maeda, *et al.*, 2010, Lim, *et al.*, 2011). Early denitrification studies have also utilised traditional and less sensitive methods, such as immunological capture using polyclonal antibodies (Coyne, *et al.*, 1989). Although immunological assays are highly specific for their target enzymes and resistant to minor genetic sequence mutations which do not affect the protein's overall action, the use of polyclonal antibodies is labour-intensive and highly-specialised work, requiring large quantities of target protein and specialised equipment (and animals) for the synthesis of said antibodies. As such, many current studies have preferred the use of modern, automated and more sensitive amplification-based methods. Chief among them is quantitative PCR (qPCR), which allows quantification of NA in real-time.

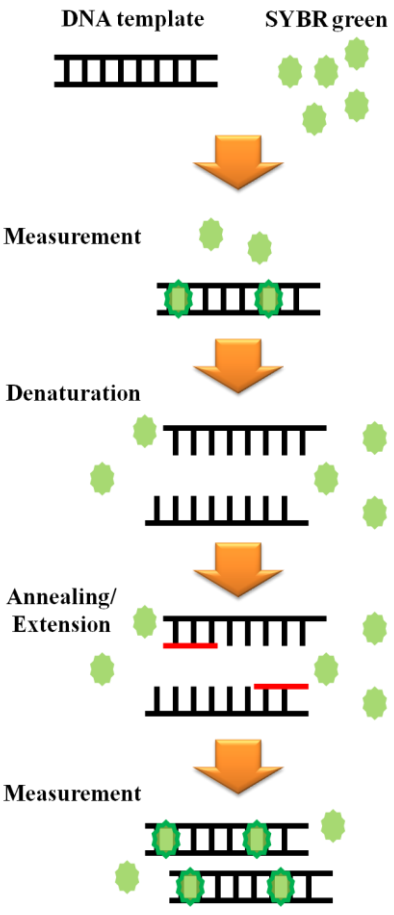

Figure 1.6 The basis of quantitative PCR (qPCR). The fluorescent dye, SYBR green, produces a light signal when it intercalates between DNA base pairs.

A commonly used method now, qPCR, is well-known and oft-used in molecular biology for the analysis of DNA and reverse-transcribed RNA (Ruijter, *et al.*, 2009, Smith & Osborn, 2009). Using a fluorescent dye (normally SYBR green) that produces a light signal when intercalated, the number of NA, or 'transcripts', are determined by calculating the signal produced during the exponential phase of amplification (Figure 1.6). Through these light signals, the original number of NA copies prior to amplification may be calculated. These general DNA-intercalating dyes are useful for complex community studies where only the conserved regions of the genetic

code are used, since probe-based methods (e.g. TaqMan probes) require additional conserved regions within the region targeted for amplification.

Unlike the traditional "end-point PCR", where samples are only analysed at the end of the amplification reaction, qPCR is more reliable as it depends only on the exponential phase (Smith & Osborn, 2009). This is because there may be biases in the amplification reaction, stopping the amplification of certain target templates before others (Smith & Osborn, 2009). Thus, by measuring the number of amplicons in the sample in real-time by way of an intercalating agent (e.g. SYBR green), the signal captured is more sensitive and reliably accurate (Smith & Osborn, 2009, Nõlvak, *et al.*, 2012). Able to analyse large quantities of samples quickly, qPCR has been found to be sensitive and highly specific, and is not as labour-intensive as other traditional methods such as Southern blots (Gadkar & Filion, 2013).

1.5.6. Quantifying NA in environmental samples

In recent years, qPCR has been used on environmental samples to varying degrees of success, but amplification efficiencies have sometimes been unexplainably low (Hamonts, *et al.*, 2013). Despite this setback, quantitative measurements of mRNA in complex communities have been found to be indispensible, allowing one to identify active fractions of the community (Smith, *et al.*, 2007, Dong, *et al.*, 2009). Allowing one to numerically determine changes in the community composition as well as increases and/or decreases in gene expression between soils or treatment types is a very powerful tool. This potentially enables the matching of field observations with the biological reason behind these observations. When mRNA expression profiles are coupled with field observations, a more complete story may be told, and the reasons behind may be determined. This linking of field and laboratory data has been applied in a number of studies to date (Dong, *et al.*, 2009, Liu, *et al.*, 2010).

1.6. Aims of this study

As mentioned in earlier sections, despite the obvious advantages of analysing mRNA instead of DNA, the use of mRNA with qPCR is uncommon in peat soil studies, largely due to the known difficulties in extracting intact mRNA from soil samples. The current study continues from a study conducted previously by our laboratory group on high pH soils (Liu, *et al.*, 2010). In the previous study using similar qPCR and gas measurement techniques, soil pH was found to play an important role in the expression of denitrification genes and the gas production/utilisation rates. However, further work on low pH soil transcription patterns was stalled by ineffective TNA extraction and DNA-contaminated RNA samples.

Thus, this study aimed to assess currently available methods, and failing that, develop a new method to co-extract DNA and mRNA from acidic peat soils. Once a method was identified, the second aim of the study was to compare the gas and denitrification gene expression profiles of high and low pH soils from the same region. Since results using different NA extraction procedures are not directly comparable, the same high pH soil also was used in this study as a standard of comparison between the two studies and extraction methods.

2. Materials and Methods

A full list of laboratory instruments, equipment, chemicals, reagents and commercially-available kits and products used in this study, including manufacturer information, is available in Appendix 1. The manufacturer for the DNase enzymes is listed in this section due to similarities in the product names.

2.1. Soils

The soils used in this study were collected from field experimental plots established 35 years ago in Fjaler, western Norway (Figure 2.1). The soil in that region is a naturally low pH peat soil. In 1978 when the plots were established, various amounts of shell sand was added to different plots, raising the pH of the soil gradually through the years (Sognnes, *et al.*, 2006). A total of six plots, three each belonging to two different shell sand additions, were chosen for sampling: Rute 6, 12 and 18 were control plots with no additions; whereas Rute 4, 5 and 21 were plots with 800 m^3 of shell sand added per hectare of soil (Figure 2.2). The soil was collected from 5 or 6 spots that were away from the edge in each plot and pooled together, to reduce edge effects. Edge effects refers to the effect of dissimilar soil (with different properties) mixing with the controlled experimental plot soil.

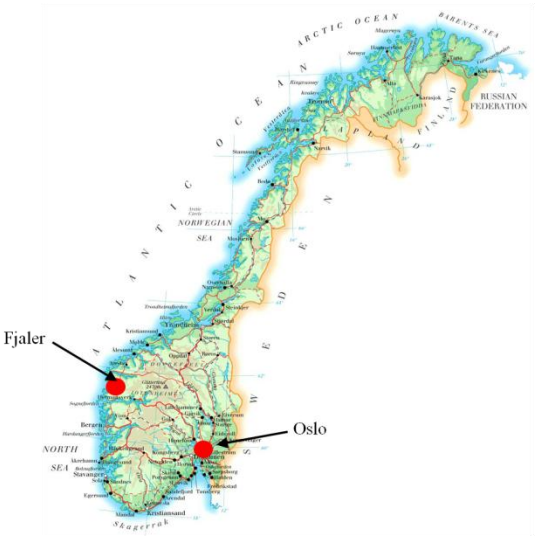

Figure 2.1 Map of Norway showing the location of the sampling site, Fjaler, in relation to the capital, Oslo. The soil samples used in this study were collected from a long-term field experiment established in Fjaler, Norway, in 1978. *Map image adapted Vidiani.com – Maps of the World*

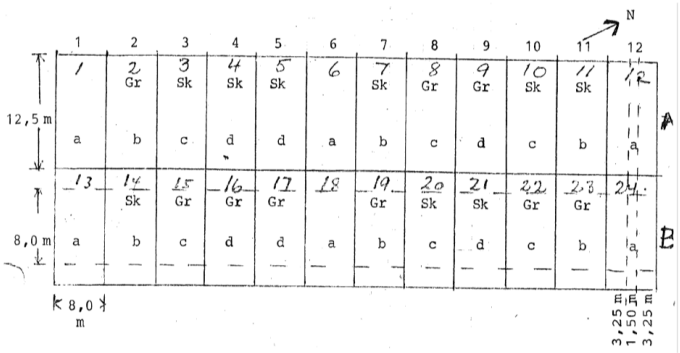

Figure 2.2 Map of the long-term field experimental plots. The plots are labelled "Rute 1" to "Rute 24", in two rows of twelve. Plots marked "a" are control plots that were unlimed. Plots marked "Sk" had shell sand added. Plots "b", "c" and "d" had 200, 400 and 800 m^3 shell sand added per hectare, respectively. In this study, samples were taken from control "a" plots (Rute 6, 12 and 18) and the heaviest limed "d" plots (Rute 4, 5 and 21).

As the soils were collected during the rainy season in autumn, the soils received from the test site were relatively wet and not possible to pass through a sieve. Excess moisture from the soils was removed by placing the soil in thin layers on 50.8 cm x 100 cm long sheets of Lab Soakers (lab bench protectors) and patted dry. The soils were dried only as much was needed to ensure that they passed through the sieve as solid particles and not as mud. Each soil was sieved through 6 mm sieves to remove large particles such as root fragments, stones and leaves. The sieved soil was stored moist at 4°C until used for incubation.

2.2. Soil measurements

Prior to incubation, soil pH was measured by adding 25 mL of MilliQ water or 0.01 M calcium chloride solution (Appendix 1.4) to 5 g of soil. The slurry mixture was shaken in 50 mL glass laboratory bottles and the sediment was allowed to settle for 5 minutes before the pH was measured using a pH meter. The pH was taken after the reading was stable for at least one to two minutes. Measurements were also taken at the end of the robot incubation to determine if anaerobic incubation affects soil pH.

For the long term pH experiment, the first pH measurement was taken after shaking the bottle. A stir bar was then added to the flasks and left on a magnetic stirrer to be stirred continuously for 10 days, with pH measurements taken periodically.

2.3. Soil preparation for method development

Soil from Rute 6 (no shell sand addition) was used in the design and optimisation of a new nucleic acid method, and was prepared as follows: The soil was mixed with 5 mg dry, powdered clover plants per gram of soil, by rolling the weighed soil and clover on aluminium foil. The soil was then incubated at 15°C for 72 hours, then transferred into 120 mL air-tight glass serum flasks and sealed with a butyl-rubber septum and aluminium crimp. The flasks were anaerobised by six cycles of gas evacuation and helium filling. The anaerobised flasks were incubated in a water bath at 15°C for 3 hours. The seal on each flask was broken and the soil was snap frozen in liquid nitrogen and stored in 50 mL disposable centrifuge tubes at -80°C until use.

2.4. Measurements of gas kinetics and nitrite concentrations

To measure the activity and gas production kinetics of the different soils, soils from all six plots were prepared and incubated in the robotised incubation system (Figure 2.3) as designed previously in the laboratory group, but without stirring in the flasks (Molstad, *et al.*, 2007).

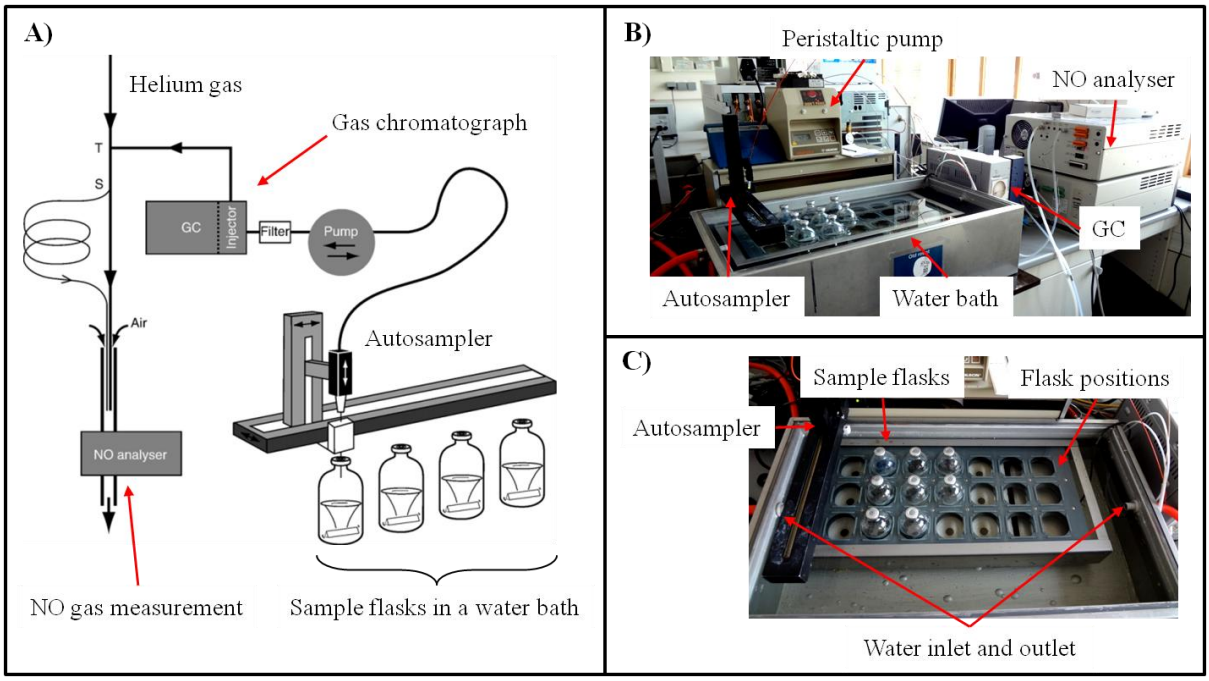

Figure 2.3 Setup of the robotised incubation and gas sampling system. A) Schematic diagram of the robotised incubation system, and the gas flow to the gas chromatograph (GC) and NO gas analyser. The gases are sampled from flasks with a peristaltic pump and are split between a GC and an NO analyser, using helium as the carrier gas. *Figure adapted from Molstad* et al., *2007*. B) Photograph of the robotised incubation system setup in the laboratory. C) Photograph of the water bath setup in the laboratory. The water is maintained at 15°C for the entire experiment with a water recirculator (not shown).

Briefly, the soils were first revived by incubating with powdered clover as described above. Following that, 10 g of each soil was weighed into 120 mL air-tight glass serum flasks and sealed with septa and crimps, as described in Section 2.3. The flasks were immediately anaerobised by evacuation and helium flushing for two cycles, and 0.5 mL of 36 mM of KNO_3 (Appendix 1.4) was sprayed evenly on the surface of the soil, using a 1 mL syringe with a 40 mm long 27 G needle. The flasks were placed into the water bath attached to the robotised gas measurement system and incubated at 15°C for the duration of the experiment (Figure 2.4). Prior to starting the gas measurements, gas overpressure in the flasks was released after placing into the water bath, using a needle attached to a 5 mL syringe with the plunger removed and filled with approximately 1 mL of water. The levels of gaseous oxygen (O_2), nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen gas (N_2) in each flask were

measured at 3 hour-intervals, for a period of 120 hours. All gas measurements were performed by a CP-4900 Micro Gas Chromatograph (GC), except for NO, which was measured by a Model 200A Chemiluminescence NOx Analyser.

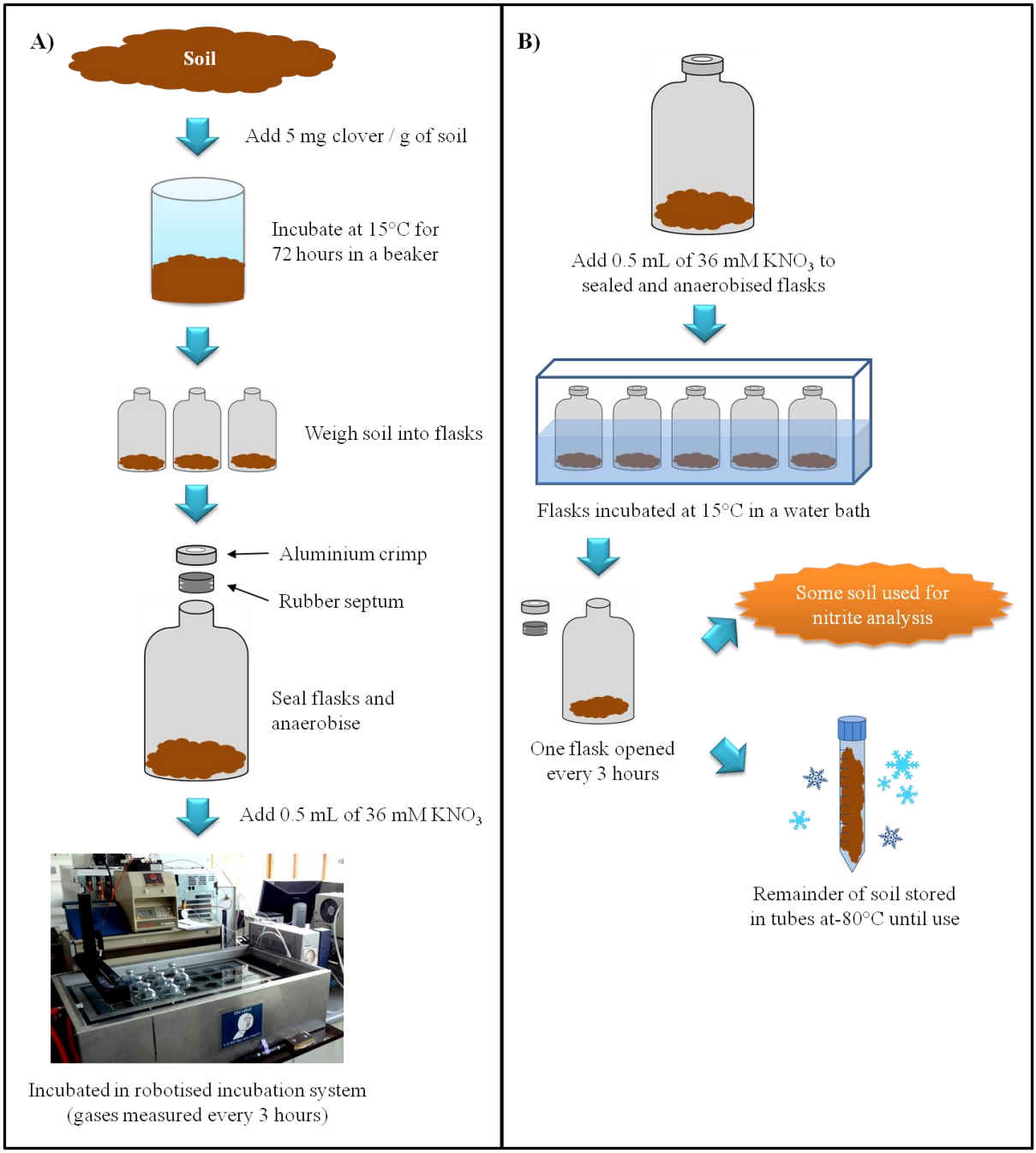

Figure 2.4 Procedure for sample preparation for the robotised incubation system and nitrite measurement. A) All soils incubated in the robotised gas autosampler system were prepared as shown in the flowchart. B) Flasks identical to those in the robotised incubation system were prepared and incubated in a water bath at 15°C. One parallel flask was sacrificed at each gas measurement for nitrite analysis. The remaining soil was stored at -80°C until use in nucleic acid extraction.

Three gas standards were used to offset the dilution due to sampling and the leaks caused by injection damage of flask septa. These leaks are not a large problem and do not normally affect the gas measurements, but are taken into account to minimise data inaccuracy and variation. The NO standard contained 25 ppm NO in N_2, and the High and Low standards contained 151 ppm and 0.585 ppm of N_2O, respectively. The High and Low standards also contained 1% carbon dioxide (CO_2) and 1% methane (CH_4), and 361 ppm CO_2 and 1.89 ppm CH_4, respectively (gases not relevant to the current study). For each gas sample that was taken, an equal amount of inert Helium (He) gas was returned to each flask. Thus, the dilution caused by sampling from each flask corresponds with the number of samples taken, and affects all flasks, including the gas standard flasks, equally. The leakage of atmospheric nitrogen and oxygen into the flasks is caused by the injection itself and the damage caused to the septa due to repeated puncturing of the septa, and also affects all flasks equally. Both the sampling dilution and injection leaks were taken into account using the gas standards, when converting the GC data from peak area into gas concentrations. Due to the unavoidable variation between flasks, the gas data shown in the Results (Section 3.2) are from single, representative flasks. The full set of gas data from all flasks can be found in the appendices (Appendix 2.1).

Additional to the flasks in the incubation robot, parallel flasks were prepared as described above, and placed in a water bath in an incubator, maintaining a flask ambient temperature of 15°C (Figure 2.4). The parallel flasks were opened at each gas sampling time point, and most of the soil was transferred to 15 mL disposable centrifuge tubes, snap frozen with liquid nitrogen, and then stored at -80°C until nucleic acid extraction. The remaining soil was used for soil nitrite content measurements.

Briefly, the soil was weighed into pre-weighed 1.5 mL microcentrifuge tubes, and 750 µL of MilliQ water was added. The tubes were centrifuged for 5 minutes at maximum speed in a tabletop MiniSpin centrifuge. Twenty microlitres of the supernatant was injected using a 100 µL glass syringe into a Nitric Oxide Analyzer NOA 280i, that was set up according to manufacturer's instructions (Figure 2.5). The purge vessel of the system contains 3 mL of the reducing agent, sodium iodide (NaI, 1% w/v in acetic acid, Appendix 1.4). Helium gas is continuously bubbled through the reducing agent to maintain an oxygen-free environment in the system. At low pH (due to the presence of acetic acid) and in an anaerobic environment, the injected nitrite is reduced to nitric oxide, which is measured by chemiluminescence using the NOA 280i (Walters, *et al.*, 1987).

The equation for the conversion of nitrite to NO is as follows:

$$I^- + NO_2^- + 2H^+ \rightarrow NO + \frac{1}{2}I_2 + H_2O$$

(from the Sievers Nitric Oxide Analyzer NOA 280i Operation and Maintenance Manual)

To calculate the amount of nitrite in the soil, the dry weight of the soil was measured by leaving the microcentrifuge tubes in a drying oven at 60°C for one week. The water content of the soil was determined and the soil nitrite content per gram of soil was calculated.

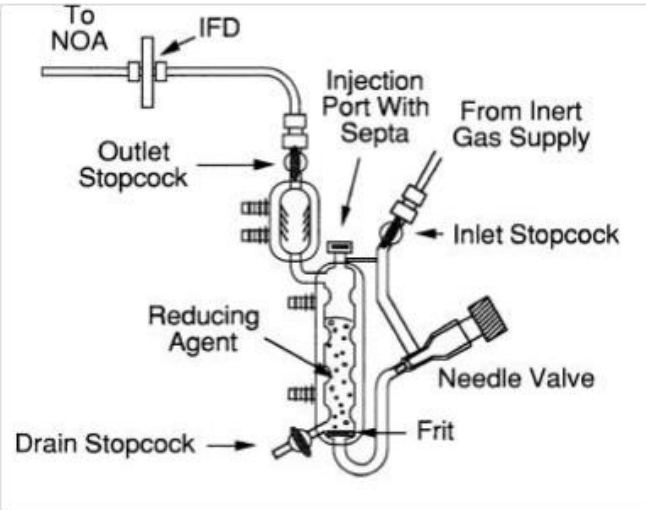

Figure 2.5 Setup for nitrite analysis. The reducing agent used is sodium iodide (1% w/v in acetic acid). Continuous helium flow bubbling through the reducing agent via the "Frit" keeps the system oxygen-free. Samples are injected into the system via the "Injection port with septa". Nitrite is reduced to nitric oxide, and is measured by the Nitric Oxide Analyzer (NOA). *Image from the Sievers Nitric Oxide Analyzer NOA 280i Operation and Maintenance Manual*.

2.5. DNA extraction optimisation

We have not been successful in obtaining mRNA from our low pH soil sample using currently available methods, due to the necessity of extracting DNA and RNA in the same procedure (Binbin Liu, pers. comm.). Although it is simpler to extract DNA and RNA from separate soil samples, performing a co-extraction is more beneficial since the fractions will be more comparable (Section 1.5.2). Thus, by sequencing the two fractions, we will potentially be able to pinpoint the active organisms in the total soil bacterial community.

For this reason, the DNA extraction method was first optimised since it is easier to obtain only amplifiable DNA, than to obtain amplifiable DNA and DNA-free RNA that is usable for downstream processes at the same time. The length of bead-beating time, the type of beads used, and the effectiveness of a commercial DNA extraction kit were tested.

2.5.1. Bead-beating lysis

The effect of varying lengths of bead-beating time was investigated. DNA was extracted from soil samples using the method previously described by Griffiths *et al.* (2000), with minor modifications. Briefly, 0.25 g of soil was weighed into 2 mL screw-capped microcentrifuge tubes containing one of three glass bead mixes: Glass beads of three different sizes (0.10-0.11 mm, 1.0 mm and 2.5-3.5 mm, GL beads), the PowerSoil DNA Isolation Kit glass beads (MB beads), or G2 lysis beads (G2 beads).

Following that, 250 µL of phenol, 250 µL of chloroform:isoamyl alcohol (24:1, Appendix 1.4) and 500 µL of hexadecyltrimethylammonium bromide (CTAB) extraction buffer (Appendix 1.4) was added to the soil and glass beads before 1-3 cycles of lysis in a FastPrep-24 Instrument, at 6.0 metres x sec^{-1} for 45 s. The samples were centrifuged at 16 000 x *g* for 5 minutes, and the aqueous phase was transferred to a new tube on ice. An equal volume of chloroform:isoamyl alcohol (24:1, Appendix 1.4) was added to remove residue phenol, and the tubes were centrifuged at 16 000 x *g* for 5 minutes. The nucleic acids were precipitated with 2 volumes of 30% polyethylene glycol (PEG) 6000 (Appendix 1.4) for 2 hours on ice, then pelleted by centrifugation at 16 000 x *g* for 20 minutes. The pellet was washed twice with 70% ethanol (Appendix 1.4), dried using a SpeedVac Concentrator, resuspended in

100 µL of nuclease-free water, and stored at -20°C until use. This method is named the Traditional Extraction Method (TEM) from here on.

2.5.2. PowerSoil DNA Isolation Kit (PD)

A commercially available kit, the PowerSoil DNA Isolation Kit (PD), is known in our laboratory group to be able to provide relatively good quality DNA with a quick and easy extraction protocol (Binbin Liu, pers. comm.). The PD kit was used according to manufacturer's instructions to extract DNA from high and low pH soils, with the following changes and additions. Each extraction contained 0.25 g soil aliquots, and the bead-beating method as described previously was used for cell lysis. The extracted DNA was stored at -20°C until use.

2.6. Total Nucleic Acids (TNA) extraction

Several methods were tested for their ability to extract total nucleic acids that are suitable for downstream processes, including amplification with functional gene primers, reverse transcription, and quantification with real-time PCR. The different extraction method procedures are summarised in Table 2.1, and the components used in the different methods are compared in Table 2.2. All centrifugation steps performed in the following extraction methods are at 4°C in a Table Top Micro Refrigerated Centrifuge 3500 with a RA-2724 rotor, or follows manufacturer's instructions, unless otherwise specified. All work involving RNA was performed using Aerosol Resistant Tips (filtered pipette tips), and all associated materials were cleaned with RNaseZap (surface decontaminant for RNases) prior to use.

Table 2.1 Summary of the procedures of extraction methods used. TEM: Traditional Extraction Method; PM: PowerMicrobiome kit; PS: RNA PowerSoil kit; PS-D: RNA PowerSoil kit with DNA accessory kit; NM: method designed in this study; N/A: not applicable.

Process	TEM	PM	PS	PS-D	NM
Lysis	Bead-beating	Bead-beating	Bead-beating	Bead-beating	Bead-beating
Protein precipitation	No	Unknown	Unknown	Unknown	Yes
Nucleic acid capture on column	N/A	Yes	Yes	Yes	N/A
Fractions split	No	No	No	No	Yes
Nucleic acid precipitation or elution	TNA precipitation	TNA eluted	RNA eluted	RNA eluted	TNA precipitation
Fractions split	Yes	Yes	No	No	N/A
DNase treatment	Yes	Yes	Yes	Yes	Yes
DNase precipitation	No	No	No	No	Yes
Nucleic acid precipitation or elution	N/A	N/A	N/A	DNA eluted	RNA precipitation
DNA and RNA clean-up with kit	Yes	Yes	No	Yes	Yes

Table 2.2 Comparison of extraction method components. TEM: Traditional Extraction Method; PM: PowerMicrobiome kit; PS: RNA PowerSoil kit; PS-D: RNA PowerSoil kit with DNA accessory kit; NM: method designed in this study; P-C-I: Phenol-chloroform-isoamyl alcohol (25:24:1); BME: β-mercaptoethanol; gDCC: Genomic DNA Clean & Concentrator; RCC: RNA Clean & Concentrator-5; OSP: OneStep PCR Inhibitor Removal.

	TEM	PM	PS	PS-D	NM
Bead material	Glass	Glass	Unknown	Unknown	Glass
Bead sizes	0.10-0.11 mm 1.0 mm 2.5-3.5 mm	0.10-0.11 mm 1.0 mm 2.5-3.5 mm	One type only, unknown size	One type only, unknown size	0.10-0.11 mm 1.0 mm 2.5-3.5 mm
Extraction buffer	P-C-I CTAB	P-C-I Solution PM1 BME	P-C-I Bead Solution Solution SR1 Solution SR2	P-C-I Bead Solution Solution SR1 Solution SR2	P-C-I CTAB
Special solutions	None	PM1 – PM7	SR1 – SR7	SR1 – SR7	MPC Protein Precipitation Reagent
Precipitation solution	PEG 6000	None	None	None	Isopropanol
Nucleic acid capture column	None	Spin Filter †	RNA Capture Column †	RNA Capture Column †	None
Inhibitors removed with	Clean-up kits	Solution PM2 Clean-up kits	Column	Column Clean-up kits	Clean-up kits
Clean-up kits used	gDCC RCC	gDCC RCC	None	gDCC RCC OSP	gDCC RCC OSP ‡

† Nucleic acid capture columns are provided in the respective kits.

‡ OSP was only used in the second trial, see Section 2.6.4 for details.

2.6.1. Extraction using phenol and chloroform

TNA from the collected previously snap-frozen soil were extracted using the TEM method, as described in Section 2.5.1, with the following changes. The extracted TNA was resuspended in 100 µL of DEPC-treated water instead of nuclease-free water, and stored at -80°C until use. For further DNA analysis, 50 µL of the extracted nucleic acids was

purified with the Genomic DNA Clean & Concentrator Kit (gDCC), according to manufacturer's instructions, and eluted with 20 µL of nuclease-free water.

Of the remaining 50 µL of nucleic acids, 15-20 µL was used in a DNase digestion using the DNase I (Sigma) or RNase-free DNase (QIAGEN), following manufacturer's instructions. The resulting DNase-digested sample was then purified using the RNA Clean & Concentrator-5 Kit (RCC), and reverse transcribed to cDNA as described in Section 2.8.

2.6.2. PowerMicrobiome RNA Isolation Kit (PM)

Total nucleic acids were extracted using the PowerMicrobiome RNA Isolation Kit (PM) according to manufacturer's instructions, with the following exceptions and additions. The amount of soil used per extraction was 0.25 g, and 250 µL of phenol and 250 µL of chloroform:isoamyl alcohol (24:1) was added to the soil in the glass bead tubes prior to lysis in a FastPrep-24 Instrument, as described in Section 2.5.1. Of the 100 µL of eluate at the end of the extraction, 16 µL was digested with DNase I (Sigma), following manufacturer's instructions. The DNase-digested sample was purified with the RCC kit, and OneStep PCR Inhibitor Removal Kit (OSP), and reverse transcribed to cDNA as described in Section 2.8. The remainder of the eluate was stored at -20°C for use as DNA template.

2.6.3. RNA PowerSoil Total RNA Isolation Kit (PS)

Due to successful DNA extraction from the current soil samples using the PD Kit, the corresponding kit designed for RNA by the same manufacturer, the RNA PowerSoil Total RNA Isolation Kit (PS), was tested. The kit was used according to manufacturer's instructions, with the following changes. The maximum recommended amount of 2.0 g of soil was used for each extraction reaction; and an additional DNase digestion after RNA isolation was performed using DNase I (Sigma) or RNase-free DNase (QIAGEN), following the individual manufacturer's instructions.

In a second trial, the RNA PowerSoil DNA Elution Accessory Kit was used successively in order to obtain DNA from the same soil sample (PS-D). Also, the following additions were added to the protocol described above, as recommended by the manufacturer. An empty 2 mL

syringe was applied to the top of the column, and positive pressure was applied, not exceeding a drip rate of one drop per second.

2.6.4. Newly designed method (NM)

A new method utilising parts of the TEM (Section 2.5.1) and some components from the MasterPure RNA Purification Kit was developed in this study to co-extract DNA and RNA from the same soil sample.

The new method (NM) follows the TEM procedure (Section 2.5.1), deviating after the aqueous phase from the chloroform:isoamyl alcohol was transferred to a new tube on ice. To this tube, 175 µL of MPC Protein Precipitation Reagent was added, and the sample was mixed by vortexing briefly. The mixture was centrifuged at 12 000 x *g* for 10 minutes, and the supernatant was separated equally into two new microcentrifuge tubes on ice. Five hundred microlitres of isopropanol was added to both tubes, which were then inverted 100 times. The tubes were centrifuged at 12 000 x *g* for 10 minutes, and the supernatant was discarded, leaving the nucleic acid pellet behind.

For the DNA tube, the nucleic acid pellet was washed twice with 70% ethanol, dried in a SpeedVac Concentrator, and resuspended in 100 µL of nuclease-free water. The resuspended total nucleic acids were purified with the gDCC kit, according to manufacturer's instructions, and eluted with 20 µL of nuclease-free water. In a second trial, the OSP kit was used to clean the total nucleic acids before purification with the gDCC kit.

For the RNA faction, the nucleic acid pellet was resuspended in 195 µL of 1X DNase Buffer and 5 µL of RNase-Free DNase I (Epicentre), before incubation at 37°C for 30 minutes. After the incubation, 200 µL of 2X T and C Lysis Solution was added to stop the digestion reaction. To remove the DNase, 200 µL of MPC Protein Precipitation Reagent was added, and the mixture was vortexed briefly before incubating on ice for 5 minutes. The reactions were pelleted at 12 000 x *g* for 10 minutes, and the supernatant was transferred to new tubes on ice. Isopropanol was used to precipitate the nucleic acids, as described above. The pellet was washed twice with 70% ethanol, dried, and resuspended as described above for the DNA. The resuspended RNA-only faction was purified with the RCC kit, then reverse transcribed as described in Section 2.8. In the second trial, the OSP kit was used to clean up the RNA before purification with the RCC kit and reverse transcription.

2.7. Confirmation of nucleic acid isolation

All isolated DNA and RNA samples were measured using either the NanoDrop Spectrophotometer ND-1000 or the Qubit Fluorometer. All measurements were made using 2 µL of each sample. For Qubit measurements, DNA and RNA measurements were made using the Qubit dsDNA BR Assay kit and the Qubit RNA Assay kit, respectively. After confirmation, all nucleic acids were stored at -20°C (short-term) or -80°C (long-term).

2.8. Nucleic acid amplification and verification

To confirm amplifiability of extracted DNA, synthesised cDNA, and complete digestion of DNA in RNA samples, 1 µL of each sample was used as the template in DNA amplification reactions using primers targeting the 16S rRNA gene. The forward primer used was 27F, and either reverse primers, 518R or 1492R, were used. Functional gene primers targeting the *nirS* and *nosZ* gene sequences were used to confirm presence and amplifiability of the less widespread denitrification genes. The sequences of all primers used in this study are listed in Table 2.3. All PCR amplifications were performed in a 2720 Thermal Cycler. The 25 µL PCR reaction mixes contained 0.4 µM of each primer, ≤ 10 ng of DNA template, 0.125 U of *TaKaRa Taq* , 2.5 µL of 10X PCR Buffer and 400 µM of each dNTP (all reagents except primers supplied in Recombinant *Taq* DNA Polymerase *TaKaRa Taq*).

Table 2.3 Sequences of primers used in this study. The primers targeting the 16S rRNA gene were used to confirm the presence of amplifiable DNA. Functional gene primers were used to determine the presence of denitrifiers in the soil samples.

Primer	Target gene	Sequence (5' → 3')	Source
27F	16S rRNA	AGA GTT TGA TCM TGG CTC AG	Weisburg *et al*., 1991
518R	16S rRNA	ATT ACC GCG GCT GCT GG	Muyzer *et al*., 1993
1492R	16S rRNA	GGT TAC CTT GTT ACG ACT T	Weisburg *et al*., 1991
cd3aF	*nirS*	GTS AAC GTS AAG GAR ACS GG	Throbäck *et al*., 2004
R3cd	*nirS*	GAS TTC GGR TGS GTC TTG A	Throbäck *et al*., 2004
ZF	*nosZ*	CGY TGT TCM TCG ACA GCC AG	Kloos *et al*., 2001
1622R	*nosZ*	CGS ACC TTS TTG CCS TYG CG	Throbäck *et al*., 2004
ZR	*nosZ*	CAT GTG CAG NGC RTG GCA GAA	Kloos *et al*., 2001

Amplification with primers targeting the 16S rRNA gene was performed according to the following protocol: Initial denaturation at 95°C for 5 minutes, 30 cycles of 30 seconds at 95°C, 30 seconds at 54°C and 40 seconds at 72°C, and a final extension at 72°C for 10 minutes. The primers targeting the functional genes (*nirS* and *nosZ*) used a touchdown-PCR protocol: 95°C for 5 minutes, 15 cycles of 95°C for 30 seconds, 57°C for 40 seconds (–0.5°C per cycle), 72°C for 1 minute, 25 cycles of 95°C for 30 seconds, 53°C for 40 seconds, 72°C for 1 minute, and a final extension of 72°C for 7 minutes. Both PCR protocols were optimised in the laboratory for use on these soil samples.

To verify that the amplification or TNA extraction was successful, the samples were analysed on 1% (w/v) agarose gels with ethidium bromide (0.5 µL per 40 mL of gel). One microlitre of 6X Gel Loading Dye was mixed with 5 µL of each reaction and loaded into individual wells. The 100 bp DNA Ladder was used as per manufacturer's instructions as the molecular weight marker. The samples were separated on the agarose gels at 110 V for 15 to 40 minutes. The gels were viewed under UV light in a Gel Doc XR system using the Quantity One 1-D Analysis Software (ver. 4.6.7).

2.9. Reverse transcription and quantification of cDNA

RNA samples that were confirmed to have no remaining amplifiable gDNA was reverse transcribed using High Capacity RNA-to-cDNA Master Mix or SuperScript VILO MasterMix. The reason for the change in reverse transcriptase is due to a discontinuation of the former product, and the latter was recommended by the manufacturer as an equal or better replacement. Briefly, regardless of the reverse transcriptase used, 5 µL of RNA template was used in each reverse transcription reaction, as per manufacturer's instructions. The reverse transcribed cDNA was stored at -20°C until further analysis. The cDNA synthesis was confirmed by amplification with primers targeting the 16S rRNA gene and/or functional genes, as described previously in Section 2.8.

The StepOnePlus Real-Time PCR System (with StepOne Software, v2.0) was used to quantify the number of mRNA transcript copies in each sample. The 20 µL reaction mixtures were made with SYBR *Premix Ex Taq* II (Tli RNaseH Plus) according to manufacturer's instructions, and contained 0.4 µM of each primer and 2 µL of template cDNA. The samples

were loaded into 96-well plates, sealed with adhesive films suitable for qPCR analysis, and spun down with a 96-well plate centrifuge before loading into the real-time PCR system.

For quantification of the 16S rRNA gene, the qPCR assay programme used was: 95°C for 30 seconds, 40 cycles of 30 seconds at 95°C, 30 seconds at 54°C, 40 seconds at 72°C and 20 seconds at 82°C. The fluorescent signals were measured during the 82°C-period, to reduce the background signals from primer dimers and unspecific PCR products. A final melting curve analysis from 60°C to 95°C was performed to determine specificity of the amplicons. The primers targeting the functional genes (*nirS* and *nosZ*) followed the same programme, except at an annealing temperature of 53°C, and the extension for 1 minute at 72°C instead.

2.10. Statistical analysis

Simple statistical analysis was performed to determine significant difference between extracted DNA and RNA amounts in high and low pH soils. The data was analysed in Microsoft Excel using two-tailed independent Student's *t*-test with unequal variances, and the difference was considered significant if the confidence level was $p \leq 0.05$.

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3. Results

As mentioned in the Introduction, inhibitor-rich soils are known to be notoriously difficult to extract NA from (Gadkar & Filion, 2013). As such, this makes it nearly impossible to study the genetic mechanisms behind the high production of the greenhouse gases NO and N_2O by the low pH soil denitrifying community. The aim of this thesis was to assess the effectiveness of several TNA extraction methods, and, if necessary, optimise the methods for the co-extraction of DNA and RNA from a low pH peat soil. This optimised method was then tested on soil samples incubated in a robotised incubation system that measures gas production from the soils, and the expression of denitrification genes would be compared to the production and utilisation rates of NOx and N_2 gases.

3.1. Optimal conditions for DNA extraction from low pH soils

Due to the nature of nucleic acid extraction and analysis methods, it is easier to extract only DNA or RNA, than to co-extract both fractions from the same soil sample. Thus, the optimal conditions for DNA extraction from low pH soil were first investigated, as the same conditions may be used in TNA extraction.

The number of cycles of bead-beating greatly affected the size of the extracted DNA, reducing the gDNA smear size with increasing cycle number (Figure 3.1A).With only one cycle of bead-beating, most of the gDNA was larger than 8 kb; whereas two or three cycles resulted in a smaller gDNA smear, 3-8 kb in size. On the other hand, the highest quantities of DNA extracted came from two cycles of bead-beating, increasing the DNA yield by more than two-fold when compared to one cycle of bead-beating. Since three cycles of bead-beating produced both slightly lower yields and more sheared gDNA, two bead-beating cycles was determined to be most optimal.

The type of beads used in the bead-beating treatment was also found to affect the size of gDNA extracted, but not the DNA yield. Although there was no apparent difference when used on high pH soil samples, the low pH soil revealed G2 beads to be harshest; giving the smallest size gDNA smears (Figure 3.1B). Although there is no observable difference between the GL beads and the glass beads provided in the PD kit, previous reviews have highlighted the importance and effectiveness of different sized beads in cell lysis (Bakken &

Frostegård, 2006). As such, the mix of three bead sizes was chosen as the most optimal bead type to use.

To test the effectiveness of the newly optimised bead-beating procedure (two cycles of bead-beating with the glass bead mix of three sizes), it was used together with the PD kit to extract gDNA from low pH soil. The mix of three bead sizes was again the most effective, showing that the lysis procedure may be used with the TEM method or in conjunction with commercial kits (Figure 3.1C). As such, the optimised bead-beating procedure of two cycles and three-size bead mix is used in all further NA extractions.

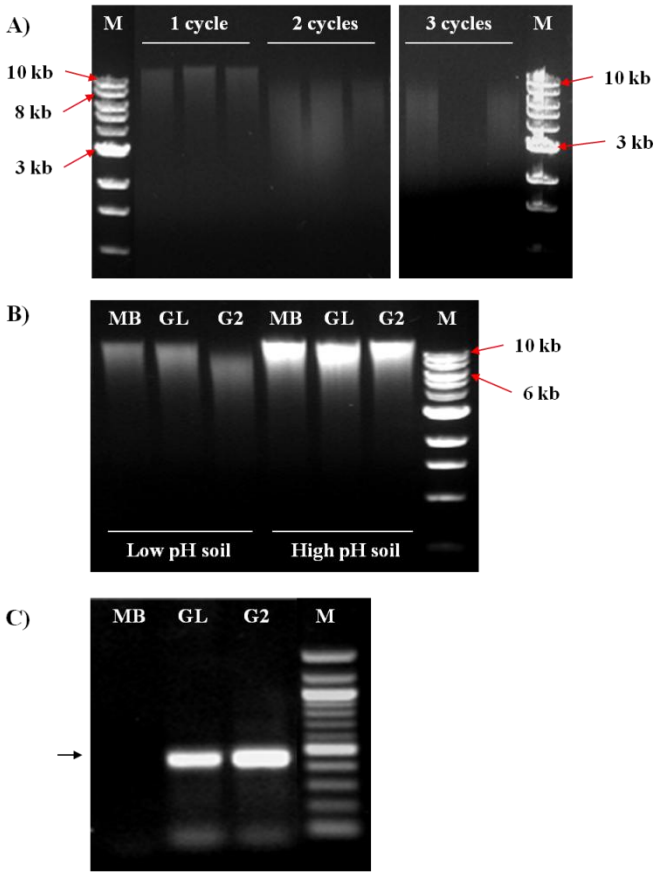

Figure 3.1 Length of bead-beating and bead type affects the size of extracted genomic DNA (gDNA). A) Longer bead-beating results in badly sheared gDNA. B) The gDNA from low pH soil was more susceptible to shearing by G2 beads. C) The three-size glass bead mix works with the PowerSoil DNA Isolation kit as well. M: 1000 bp DNA ladder; MB: glass beads from the PowerSoil DNA Isolation kit; GL: three-size glass bead mix; G2: G2 beads; black arrow indicates amplicon size of interest.

3.2. Agarose gel analysis of raw TNA extracts

The presence of gDNA causes reverse transcription reactions for downstream mRNA quantification to be useless and must thus be completely removed by DNA digestion. One of the best ways to confirm complete DNA digestion is to run a PCR or a qPCR. However, both methods are time consuming, requiring at least 1.5 hours before reaching a result. Since mRNA, which degrades relatively quickly due to the ubiquitous distribution of RNases in the environment, is one of the molecules of interest in this study, speed and efficiency is of great importance when handling TNA samples (Section 1.5). Also importantly, reverse transcription reactions are costly and time-consuming, so there is a preference not to waste resources on samples without RNA.

Consequently, a faster, if less absolute method, than gene amplification is necessary to determine the presence and/or absence of gDNA and RNA in the raw TNA extracts. The method chosen in this study is analysis with agarose gels, which is a quick (15-20 minutes) method to analyse samples for DNA and RNA. A variety of agarose gel concentrations, voltage and time combinations were tested for running gels, with a priority for quick and clear results. The best result was 1% agarose gels at 110 V for 15 minutes, which allows the clear observation of the gDNA smear, the 23S, 16S and 5S rRNA (Figure 3.2). However, the 1 kb DNA ladder is not well separated when the gels are run at high voltage and for such a short time, so the 100 bp DNA ladder is used in this study when the raw TNA extract is analysed on agarose gels. The 23S and 5S rRNA tend to migrate a little slower than the 1.5 kb and 1 kb bands, regardless of the DNA ladder used, so this is used to confirm the presence of rRNA in TNA samples (Figure 3.2).

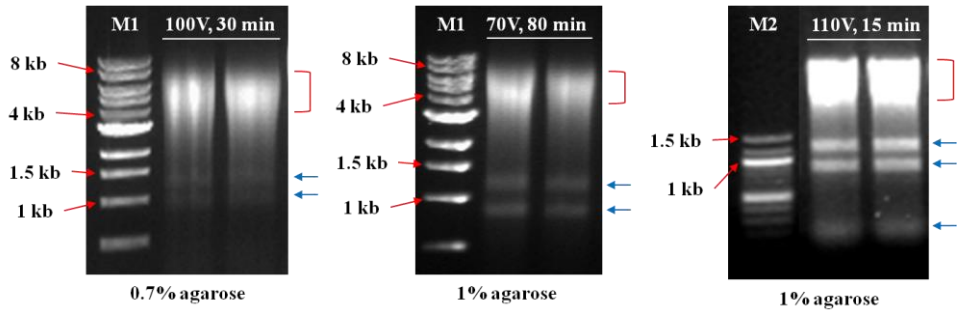

Figure 3.2 Analysis of raw total nucleic acids (TNA) extract on agarose gels. The average size of the gDNA smear when extracted with methods used in this study is between 4 kb and 8 kb. When viewed on agarose gels used for DNA analysis, the 23S and 16S rRNA migrate a little further down the gel than the 1.5 kb and 1 kb bands in the DNA ladders. M1: 1 kb DNA ladder; M2: 100 bp DNA ladder; red brackets indicate the gDNA smear; blue arrows indicate the 23S, 16S and 5S rRNA

3.3. Optimisation of TNA extraction

As previously described in the Introduction, nucleic acids extracted from inhibitor-rich soils tend to be of insufficient quality for further downstream processes such as amplification and reverse transcription (Section 1.5.3). Despite the success with the PD kit in extracting DNA (Section 3.1), RNA cannot be co-extracted with the kit and is thus useless in gene expression studies requiring mRNA analysis. As such, this study further compared several different methods to extract TNA that are able to provide useable DNA and RNA fractions from the same soil sample, and describes a newly designed method for TNA extraction from soil samples containing high amounts of inhibitory compounds. Below is a summary of the different methods used, as well as the reasons for choosing and discarding them (Figure 3.3).

Method	Reason for choice	Result
TEM	• Common method for TNA extraction • Works for other soil samples	• DNA is amplifiable • Incomplete DNA digestion • Reverse transcription inhibited
PM	• New product recommended by colleagues in GEUS • Designed for samples with large quantities of inhibitory compounds	• DNA is amplifiable • Incomplete DNA digestion • Reverse transcription efficiency unknown due to residual DNA
PS	• Good results with corresponding DNA extraction kit • Potentially removes need for DNA digestion	• Complete DNA digestion • Reverse transcription successful • cDNA is amplifiable • No DNA fraction
PS-D	• Successful trial (above) • Needed to confirm that method could be up-scaled and accelerated	• DNA is unamplifiable • Incomplete DNA digestion • Reverse transcription inhibited
NM	• Previous methods had too many unknowns • Purification of samples is necessary early in method	• DNA is amplifiable • Complete DNA digestion • Reverse transcription inhibited
NM with OSP	• Problem stems from inhibitory compounds • OSP removes PCR inhibitors (manufacturer's recommendation)	• DNA is amplifiable • Complete DNA digestion • Reverse transcription successful * • cDNA is amplifiable *

Figure 3.3 Summary of extraction methods tested and why they were chosen and/or abandoned. TEM: Traditional Extraction Method; PM: PowerMicrobiome kit; PS: RNA PowerSoil kit; PS-D: RNA PowerSoil kit with DNA accessory kit; NM: method designed in this study; OSP: OneStep PCR Inhibitor Removal kit.
'*' denotes exceptions discovered after the method was developed (see Section 3.5.6).

3.3.1. Traditional extraction method (TEM)

The TEM by Griffiths and colleagues (2000) utilising phenol, chloroform and CTAB extraction buffer was tested using different DNase enzymes and digestion times. The raw extract from soil samples contained DNA and RNA (Figure 3.4A), but the method was unable to yield satisfactorily useable DNA and RNA without further purification. Further purification of the DNA fraction with commercial kits gave amplifiable DNA (Figure 3.4B). However, this method did not allow for purification of the RNA-only fraction prior to digestion with DNase enzymes, and the digestion was always partial and incomplete, leaving amplifiable residual DNA (Figure 3.4C).

To rule out the possibility of incomplete digestion due to insufficient digestion time, the samples were digested for up to 2 hours using both DNases. The number of amplifiable DNA copies was quantified in a qPCR reaction with primers targeting the 16S rRNA gene. No difference was found between the copies of residual DNA in the RNA fraction and reverse transcribed cDNA. Selected samples were analysed on an agarose gel to confirm the amplicon size, to exclude unspecific amplification by the primers (Figure 3.4D). Additionally, the inhibitory compounds leftover in the RNA-only fraction could not be satisfactorily removed by RNA clean-up kits, resulting in an inhibited reverse transcription.

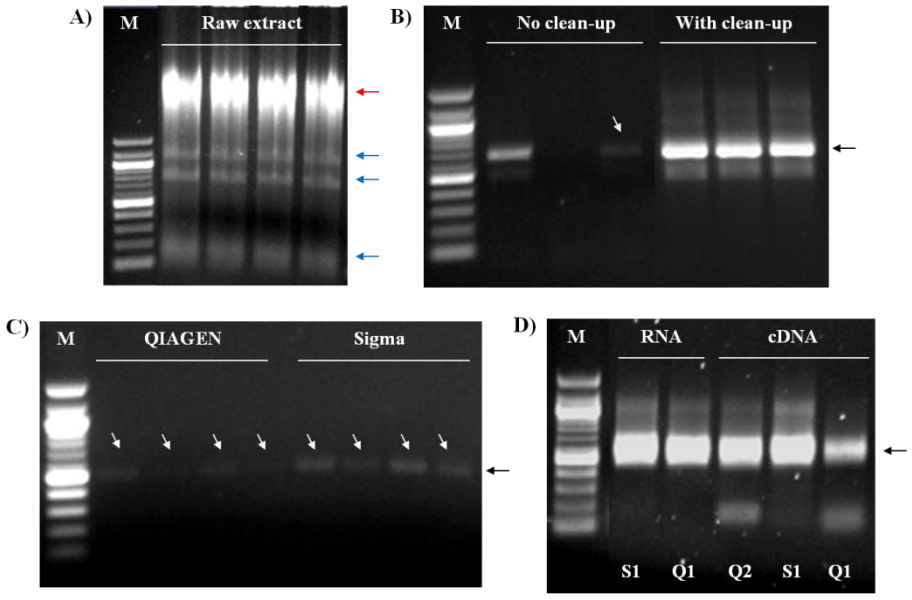

Figure 3.4 Extraction of TNA from soil samples using the traditional extraction method (TEM). A) TNA extraction from soil samples was successful. B) The use of clean-up kits gave higher PCR efficiencies and more consistent DNA amplification. C) DNase digestion was incomplete, leaving amplifiable DNA in the RNA fraction. D) Selected qPCR samples were analysed on an agarose gel. Long digestion times could not remove all amplifiable DNA (S: Sigma DNase I; Q: QIAGEN RNase-free DNase; numbers indicate length of digestion in hours). M: 100 bp DNA ladder; red arrow indicates gDNA smear; blue arrows indicate the 23S, 16S and 5S rRNA; black arrow indicates amplicon size of interest; white arrows indicate faintly visible bands on the gel.

3.3.2. Commercially available TNA extraction kits

The PM kit was used on recommendation by colleagues at Geological Survey of Denmark and Greenland (GEUS), Denmark, working on Arctic permafrost soils (Carsten Suhr Jacobsen, pers. comm.). The kit is commercially available from MO BIO Laboratories, and was originally designed for samples with large quantities of inhibitory compounds, example faecal and stool samples. The entire extraction process using the kit was faster than that of the TEM but was found to be less effective than the traditional method. The PM kit was also able to extract DNA and RNA from the soil samples, but at lower concentrations than the TEM (Figure 3.5A). This may have been due to either the inability of the kit's Spin Filter (nucleic acid capture column) to bind all the nucleic acids in the sample, or that a portion of the nucleic acids was not successfully eluted from the column.

Although the DNA extracted from the soil using the PM kit required the use of other clean-up kits, the purified DNA was easily amplifiable and produced large quantities of amplicons of the correct size (Figure 3.5B). However, although the DNA digestion appeared to be complete from analysis on agarose gels, amplification with primers targeting the 16S rRNA gene revealed residual DNA in the RNA fraction. It is likely that inhibitors were present in the raw TNA extract that prevented complete DNA digestion, resulting in the leftover DNA. As such, since the DNase digestion step comes before inhibitor clean-up in this kit, the same problem was met as in the TEM. The residual DNA in the RNA fraction also rendered the reverse transcription efficiency uncertain, given that the residual DNA was amplified as well, interfering with the RNA quantification process. Furthermore, there was a large variation between the technical replicates, showing that consistent TNA extraction with this method is not possible.

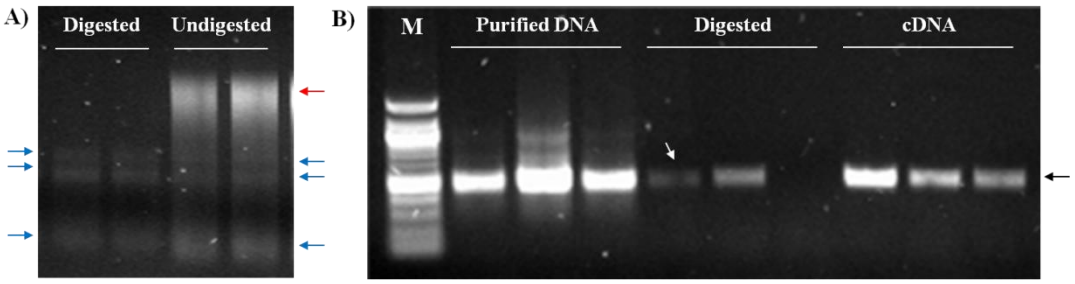

Figure 3.5 The PowerMicrobiome RNA Isolation Kit (PM) did not yield DNA or RNA of satisfactory quality. A) Both DNA and RNA were successfully co-extracted. B) Purified DNA was strongly amplifiable, but DNA digestion was incomplete, so the reverse transcription efficiency was uncertain. The consistency of results between technical replicates was also poor. M: 100 bp DNA ladder; red arrow indicates gDNA smear; blue arrows indicate the 23S, 16S and 5S rRNA; black arrow indicates amplicon size of interest; white arrows indicate faint bands on the gel.

As mentioned briefly in Section 2.5.3, the PS kit was chosen based on experience with the corresponding DNA extraction kit, PD, which yielded strongly amplifiable DNA suitable for downstream processes, and had a comparatively quick protocol (Section 3.1). In the first trial, the raw extract had very little DNA left in the RNA fraction, which was completely digested using an additional DNase step, and the reverse transcribed cDNA was of amplifiable quality (Figure 3.6). However, the entire procedure took more than seven hours for only two samples, and was not ideal for the quick extraction required to avoid possible mRNA degradation. Additionally, the PS kit is originally designed to yield only RNA, removing most, if not all, the DNA from the sample. Therefore, a second trial involving a larger sample size, an accessory kit to co-elute DNA, and an additional positive-pressure-application step was performed (PS-D).

In this second trial, positive pressure was applied as recommended by the manufacturer, increasing the rate at which the sample is passed through the RNA Capture Column (included in the kit). The DNA and RNA fractions were of poor quality (inhibitory compounds present) and neither fraction was usable: Neither the DNA fraction nor the reverse transcribed cDNA was amplifiable, even after additional clean-up. Thus, the success of the reverse transcription to cDNA could not be assessed, since the lack of amplification could be due to either the presence of inhibitory compounds preventing amplification, or that there was no cDNA present. Additionally, the contaminating DNA in the RNA fractions was much higher than without the positive pressure (Figure 3.6). The amount of time required when using this kit is also unpractical, at more than eight or nine hours for six samples.

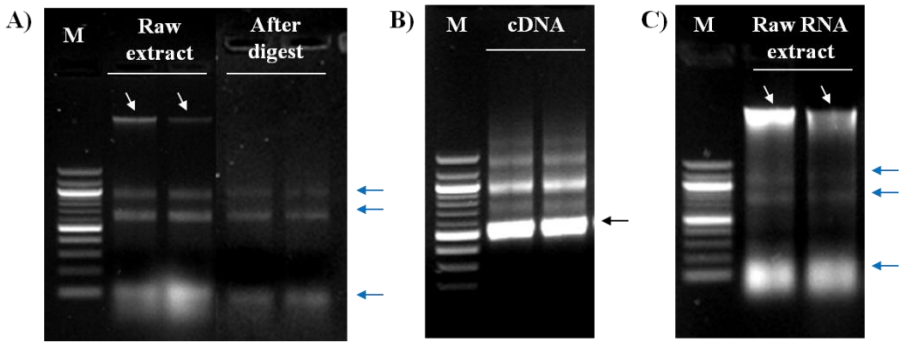

Figure 3.6 The RNA PowerSoil Total RNA Isolation Kit (PS) yielded good quality cDNA but is not suitable for large sample sizes or quick extractions. The results of the kit when working with few samples appeared to be promising (A-B), but failed to perform when the process was up-scaled (C). A) RNA samples obtained contained small amounts of genomic DNA that was removed by DNase digestion. B) Primers targeting the 16S rRNA gene (27F and 518R) successfully amplified the cDNA. C) Positive pressure applied to the elution column resulted in more contaminating gDNA in the RNA fraction. M: 100 bp DNA ladder; black arrow indicates amplicon size of interest; white arrows indicate genomic DNA in the raw extract; blue arrows indicate the 23S, 16S and 5S rRNA.

3.3.3. Method design and optimisation

Due to the results from previous experiments, the general conclusion was that both the DNA digestion by DNase and the reverse transcription were inhibited by unknown compounds that were co-extracted during TNA extraction. Additionally, the lack of transparency of commercially available products proved to be a major barrier in method optimisation. When using the kits, it was not possible to adjust or change individual steps because of the secrecy surrounding the composition and use of each component. Hence, a method was designed around the idea that only known components are used, giving the flexibility to change the method as required, since addition and/or deletion of individual steps in the procedure is possible. Also, this method was designed to at least partially purify the extracted RNA prior to DNA digestion, removing enzyme inhibiting compounds.

This relatively quick NM was able to co-extract DNA and RNA in approximately 3-4 hours. Also, gDNA in the RNA fraction was below detectable levels on the agarose gel after DNA digestion (Figure 3.7). However, even after purification, the DNA fraction performed inconsistently when amplified. Primers targeting the 16S rRNA gene were unable to consistently amplify the purified DNA, and primers targeting the denitrification gene *nosZ* gave amplicons of the wrong size (Figure 3.7). Although there was no amplifiable DNA in the RNA fraction, the reverse transcribed cDNA was not amplifiable either. Thus, it was undeterminable which reaction, the DNA digestion or reverse transcription, was inhibited.

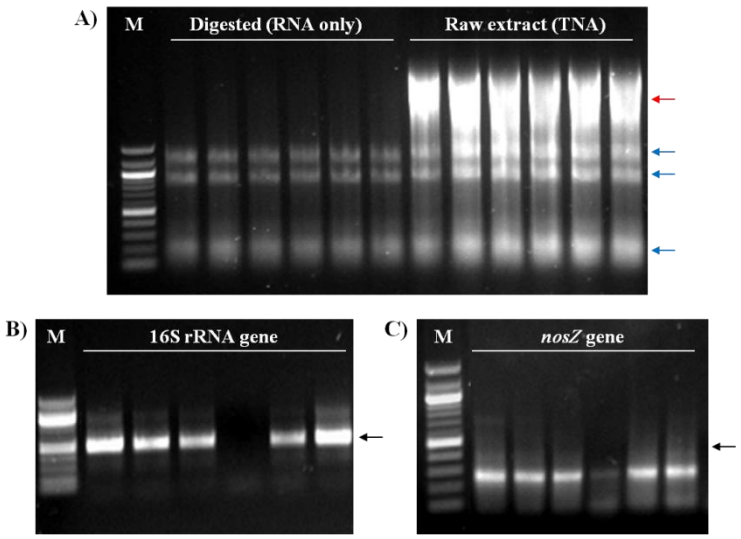

Figure 3.7 The newly designed method (NM) was able to co-extract DNA and RNA, but amplification was poor and inconsistent. A) DNase digestion reduced gDNA to below detectable levels. B) DNA amplification using primers targeting the 16S rRNA gene was inconsistent. C) PCR reactions with primers targeting the *nosZ* gene gave amplicons of the wrong size. M: 100 bp DNA ladder; red arrow indicates the genomic DNA smear; blue arrows indicate the 23S, 16S and 5S rRNA; black arrows indicate amplicon size of interest.

Despite the apparent successful digestion of DNA in the RNA fraction, the amplification of DNA was still problematic. Consequently, the presence of residual inhibitory compounds was surmised to be the main problem affecting the enzymatic activity of DNA polymerase and reverse transcriptase. Upon manufacturer's recommendation, the OSP, which comprises of a single inhibitor removal column, was used in a second trial (NM-OSP) on both the TNA and DNase-digested RNA fractions. The inclusion of the OSP allowed for more consistent and repeatable results from both the DNA and RNA fractions, with or without the use of additional purification kits (Figure 3.8). Furthermore, even with the additional handling of RNA samples (due to the extra purification and clean-up steps), any possible residual DNA left in the RNA fraction remained unamplifiable, i.e. there was no contaminating amplifiable DNA in the RNA fraction which would have affected downstream processes, including qPCR and sequencing.

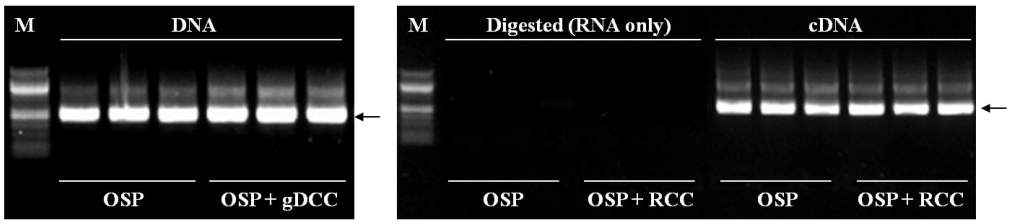

Figure 3.8 Use of the OneStep PCR Inhibitor Removal Kit (OSP) improves extraction repeatability. The clean up step with OSP give highly repeatable results, regardless of additional clean-up. The DNA and cDNA were amplifiable, and RNA samples did not contain amplifiable DNA. The use of additional clean-up kits neither improved nor worsened the quality. M: 100 bp DNA ladder; gDCC: Genomic DNA Clean & Concentrator kit; RCC: RNA Clean & Concentrator-5 kit; black arrows indicate amplicon size of interest.

3.3.4. Comparison of tested methods

Table 3.1 is a compilation of the results of total nucleic acids extraction from each method. In brief, regardless of the method used, nucleic acid purification and clean-up is crucial for downstream processes. All methods tested, except for the PS kit-based ones, were able to provide amplifiable DNA with the use of additional clean-up kits. Both the PS kit and NM procedures were able to completely digest the amplifiable DNA in RNA samples and provide amplifiable cDNA (see Section 3.5.6 for exception). However, only NM-OSP was able to extract nucleic acids quickly and efficiently. The NM-OSP method is also not limited to the number of samples that can be processed concurrently.

Thus, the current candidate method for effective co-extraction of DNA and RNA from our low pH soil samples is NM-OSP, which is the newly designed method with nucleic acid clean-up using the OSP kit. To investigate the method's performance, NM-OSP was used to co-extract DNA and mRNA from high and low pH peat soils, and the resultant nucleic acids were used in downstream processes to define the denitrification gene expression pattern of such soils after anaerobisation (Section 3.5.5).

Table 3.1 Comparison of extraction method results. Of the methods tested, only the NM-OSP method was able to yield amplifiable DNA and cDNA from our soil samples. TEM: Traditional Extraction Method; PM: PowerMicrobiome kit; PS: RNA PowerSoil kit; PS-D: RNA PowerSoil kit with DNA accessory kit; NM: method designed in this study; gDCC: Genomic DNA Clean & Concentrator; RCC: RNA Clean & Concentrator-5; OSP: OneStep PCR Inhibitor Removal kit; N/A: not applicable.

	TEM	PM	PS	PS-D	NM	NM-OSP
DNA clean-up	gDCC	gDCC	N/A	gDCC	gDCC	OSP
DNA amplifiable?	Yes	Yes	N/A	No	Yes	Yes
Residual DNA after digestion?	Yes	Yes	No	Yes	No	No *
RNA clean-up	RCC	RCC	Not required	RCC	RCC	OSP
cDNA amplifiable?	No (inhibited)	Unknown	Yes	No (inhibited)	No (inhibited)	Yes

3.4. Soil pH measurement parameters

The exact procedure for pH measurement is often overlooked and treated as non-essential. In environmental studies, pH is sometimes measured with water and may be, in some cases, stirred or shaken continuously overnight (Mor, *et al.*, 2006). The use of calcium chloride ($CaCl_2$) solution instead of water in pH measurements causes the dissociation of H^+ ions that are bound to the negatively charged soil particles. This H^+ dissociation effect creates less fluctuation and a more reliable pH reading. Since the dissociation happens relatively quickly at a molecular level, the need for prolonged sample agitation is removed, thus reducing side effects such as pH change due to continuous agitation. Reasons for such pH change include the dissolving of materials such as shell sand, which dissociates into CO_3^{2-} in water and eventually causes a rise in pH due to chemical equilibrium. In this study, the use of water versus 0.01M $CaCl_2$ solution in soil pH measurement, as well as the effect of prolonged stirring was examined.

As predicted, the measurement made with water fluctuated greatly and provided a falsely higher pH than in reality (Figure 3.9, on the next page). The measurement made with $CaCl_2$ solution was more stable, with a dip in pH only after 4 days of continuous stirring. Also as expected, the continuous stirring caused the shell sand to be broken up and dissolved, increasing the pH of soils with shell sand addition. Surprisingly, the bulk of pH increase for all soils took place overnight from Day 1 to Day 2, increasing the pH by 0.5-0.6 pH units. In comparison, the pH only increased by < 0.2 pH units over the next 8 days. The soils with no shell sand addition did not have much response to the prolonged stirring, fluctuating by 0.1 pH units in the first 4 days. This test shows that pH measurement should be done quickly with minimal agitation, and with $CaCl_2$ solution, and that there is no benefit in continuous overnight agitation. As such, further pH measurements taken in this study used $CaCl_2$ solution and quick agitation.

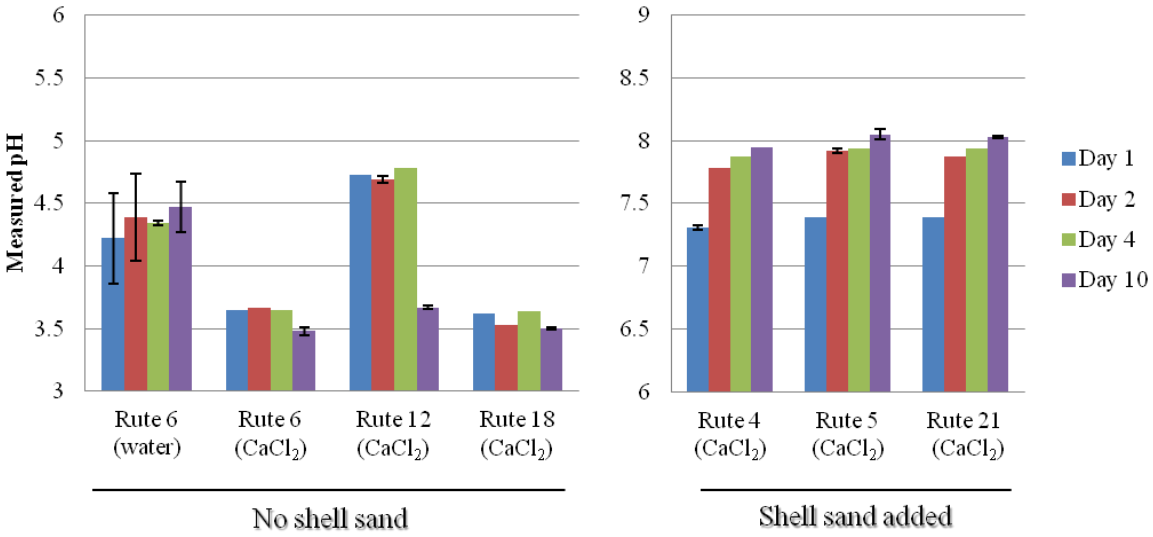

Figure 3.9 Continuous stirring when measuring soil pH may give a false high pH. Prolonged stirring caused the pH to increase over time in soils with shell sand. There is no such effect in low pH soils, except for Rute 12. Measurements made with water fluctuated greatly, stabilised slowly and gave false pH readings (~ 0.5 pH units higher). Error bars on the graph indicate the degree of pH fluctuation during measurements.

3.5. Anaerobised low and high pH peat soils

Six different field plots from the same experimental site were chosen for the analysis of NOx gas production. The soils were anaerobised and incubated in air-tight flasks in a robotised incubation system with an autosampler linked to a GC and NO analyser. Gas measurements were taken every 3 hours for the analysis of NOx gas production. Soil samples were taken simultaneously for the analysis of nitrite content and denitrification gene expression as well.

3.5.1. Soil pH

Prior to the start of the experiment conducted in the robot incubation system, the pH of all soil plots was measured. Experimental plots with no shell sand addition had notably lower pH than plots that experienced shell sand addition 35 years ago (Table 3.2). The pH of soil from Rute 12 was > 1 pH unit higher than the other plots without shell sand addition, and may be due to the location of the plot at the edge of the experimental site (Figure 2.2). Despite our best sampling efforts, the entire Rute 12 square may have been subjected to the influence of external factors, beyond the controlled conditions of the experimental field site (edge effect as described in Section 2.1). As such, Rute 12 is considered an outlier and is not considered a typical “low pH soil”. Measurements taken from that plot in this study are considered to be outside the low pH soil data set, and treated as a mid-range pH soil sample.

Table 3.2 The effect of shell sand addition on soil pH. The pH of soils from six plots was measured using 0.05 M $CaCl_2$. The addition of shell sand to the plots 35 years ago raised the pH of the soil significantly. The higher pH in Rute 12 is believed to result from plot edge effects due to its location (Figure 2.2). A: no shell sand added to soil; D: 800 m^3 shell sand added per hectare of soil.

Soil plot	Plot description	pH	pH designation
Rute 4	D	7.31	High
Rute 5	D	7.39	High
Rute 6	A	3.65	Low
Rute 12	A	4.73	Mid-range
Rute 18	A	3.62	Low
Rute 21	D	7.39	High

In addition to pre-incubation measurements, the pH of the soil was measured at the end of the anaerobic incubation in the robot system. An increase in pH was observed in the low and mid-range pH soils, and a decrease in pH was seen in the high pH soils (Figure 3.10). Aside from the trend of increased pH in acidic soils and decreased pH in alkaline soils, the magnitude of pH change did not appear to correspond to the starting pH of the soils. The reduction in pH may have been caused by the accumulation of organic acids. Reasons for pH increases include the oxidation of organic acids or the accumulation of ammonium and amines during protein degradation. Denitrification itself may also have been responsible for increasing the pH of the soil – the reduction of nitrate to NOx gases results in a rise in pH due to the uptake of H^+ or outlet of OH^-, to compensate for the uptake of nitrate and/or nitrite. Nevertheless, the change in pH is relatively small compared to the starting pH (the acidic and alkaline soils are still acidic and alkaline, respectively), and is a cumulated effect of nearly 6 days. Thus, the pH change during incubation was likely to have little, if any, effect on denitrification in these soils.

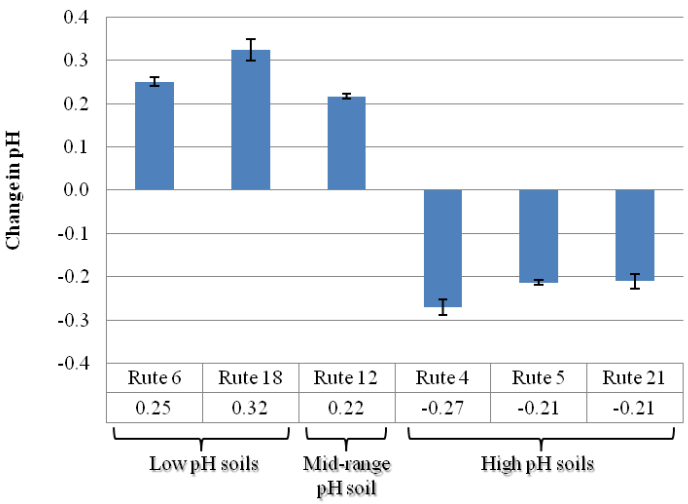

Figure 3.10 Change in soil pH after anaerobic experiments. The pre- and post-incubation pH change of the soils grouped according to the starting soil pH. The low and mid-range pH soils registered an increase in pH, whereas the high pH soils had a reduction in pH.

3.5.2. Soil water content

Based on the above pH measurements, two plots were selected as representative samples in comparing high versus low pH soils in the experiments: Rute 5 was chosen to represent the high pH, shell sand added soil plots; Rute 6 was chosen to represent the control low pH soil plots with no shell sand addition. Once chosen, the water content of the two plots representing high and low pH soils was measured for the calculation of nitrite content. The high pH soil contained much less water than the low pH soil, with 0.91 mL g^{-1} of dry soil and 2.90 mL g^{-1} of dry soil, respectively. However, the considerable difference in water content may be due to shell sand flakes in the high pH soil, making up the bulk of the dry weight.

Both soils were also monitored for water content reduction during storage at 4°C since sampling in November. The reduction in soil moisture for the Rute 5 and Rute 6 was marginal, at 17% and 1%, respectively, showing that the soils were not dried up by pre-storage treatments or during storage. This change in soil moisture is extremely minor, especially when compared to the soil moisture fluctuations *in situ*.

3.5.3. Production and usage of nitrogen compounds

As previously described in Section 2.4, the six soils were incubated aerobically with clover at 15°C for 72 hours to revive them from storage at 4°C. The soil was aliquoted into flasks and anaerobised by flushing with helium. Nitrite was added to the flasks, after which gas production was monitored with a robotised incubation system for 122 hours. Nitrite levels were measured in the parallel flasks identical to those in the robot, opening and sacrificing one flask each time gases were sampled in the GC system.

Due to uncontrollable flask-to-flask variation as well as the time required by the robot to sample the individual flasks (5-8 minutes per flask), the gas measurements from each replicate flask always differed slightly from each other (Figure 3.11). The production rate of each gas was very similar with a low standard deviation, but the utilisation rates differed greatly from flask to flask. Additionally, the N_2 measurements were further complicated by the leakage of atmospheric N_2 into some flasks. Since overlaying or averaging gas measurements did not provide more information than choosing one of the three replicate flasks to represent each soil, all gas kinetics graphs from here onwards are not overlays of multiple samples, but of single representative flasks. The full set of gas measurement data may be found in the supplementary results section (Appendix 2.2).

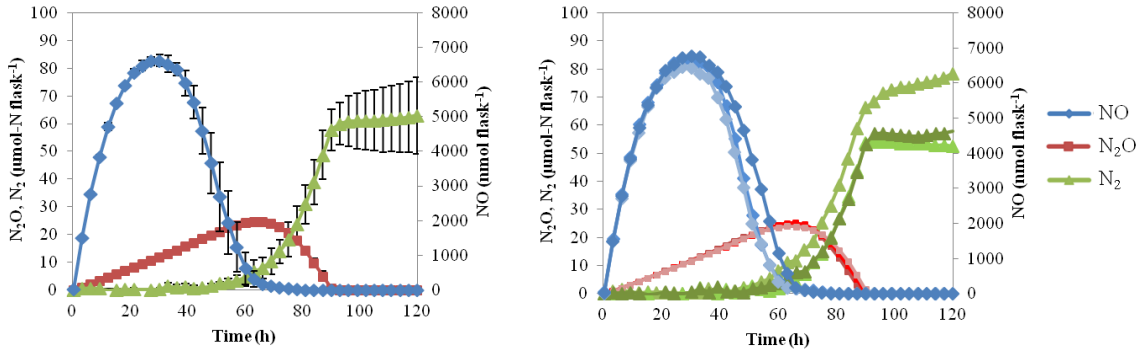

Figure 3.11 Gas measurements were affected by individual flask variations. For all soils, the standard deviations (left) for NO and N_2O measurements differed during gas utilisation but not during production. An overlay of the three replicate flasks (right) clearly shows one of the flasks performing differently, hence increasing the overall standard deviation. NO values are exaggerated (shown in nmol) due to its importance as a signalling molecule.

Nitrite measurements show that the high pH soil converted nitrate to nitrite quickly, rising to millimole per gram of dry soil levels, within the first 10 hours of incubation (Figure 3.12). After the initial peak in nitrite accumulation, the nitrite levels fluctuated around 1.5 mmol g^{-1} of soil, before dropping after 30 hours. The NO and N_2O levels in the flask headspace reflect a similar trend, with a peak and drop in NO and N_2O levels prior to the 30-hour mark (Figure 3.13). The N_2 levels increased near-exponentially from the start of the experiment, before reaching a concentration plateau at around 30 hours.

In contrast, the low pH soil accumulated little nitrite, with concentrations rising slowly and fluctuating around 40-50 μmol g^{-1} dry soil (Figure 3.12). The drop in nitrite levels back to zero was not captured due to an insufficient number of parallel flasks for nitrite measurement. The flasks in the robotised GC were opened after the experiment and tested for nitrite content. The flasks were found to contain nearly no nitrite, showing that nitrite is reduced to near-zero in the acid soil, but it evidently took longer than 45 hours and less than 122 hours (Supplementary results, A2.1). The low nitrite accumulation in the acid soil is possibly reflecting chemical decomposition of nitrite at low pH, which may have also caused the high levels of NO build-up in the headspace (Figure 3.13).

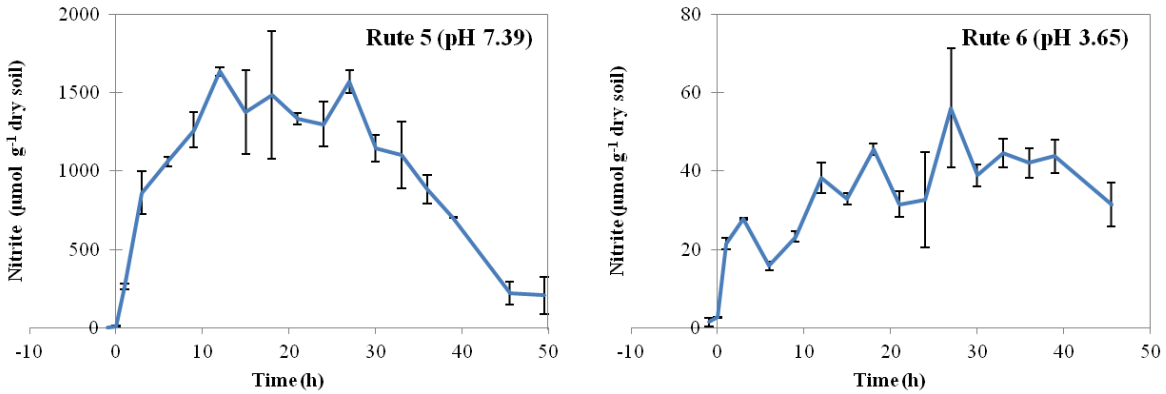

Figure 3.12 Nitrite levels in representative low and high pH soils. The amount of nitrite was measured in experimental flasks parallel to those in the robotised gas chromatograph system. The representative of high pH soils, Rute 5, had a distinct elevation of nitrite levels, to 1.5 mmol nitrite per gram of dry soil, before dropping to near zero, after 40 hours. In contrast, the low pH soil representative, Rute 6, had a slight elevation in nitrite levels, to 40 µmol nitrite per gram of dry soil, and fluctuated around that level. The reduction to zero was not captured due to insufficient parallel flasks.

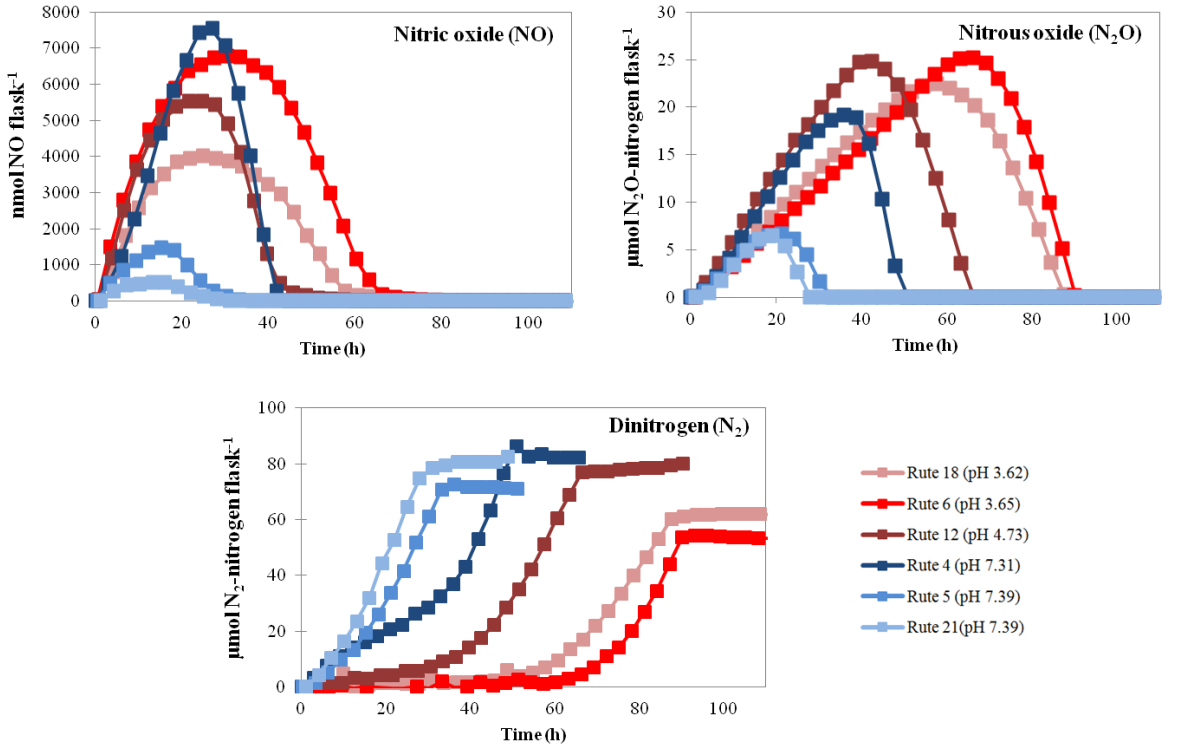

Figure 3.13 Production of NOx and N_2 gases by acidic and alkaline soils. The NO gas detected in flasks with low pH soils persisted at higher levels and for a longer time than high pH soils. Also, N_2O and N_2 were produced at a slower rate in low pH soils than high pH soils. Rute 12 (pH 4.7) displayed a gas kinetic which was an intermediate of the lower pH soils (~ pH 3.6) and higher pH soils (> pH 7). Red lines are low pH soils, blue lines are high pH soils.

Closer inspection of the gas production and utilisation rates of the two representative soils showed that in both soils, NO production declined as N_2O production increased, until there was a net reduction of NO (Figure 3.14). The peak N_2O reduction was found to occur only after maximum NO reduction, and exactly mirrored the peak in N_2 production. The N_2 production rate quickly decreased when all the available N_2O was reduced, after which there was no net production or utilisation of any of the NOx gases, confirming the completion of denitrification in both high and low pH soils.

However, there were some notable differences between the timing and production rates of NOx gases between the soils. In the high pH soil, starting production rates of NO, N_2O and N_2 were nearly identical per mole of nitrogen, unlike the low pH soil. Also, N_2O production peaked earlier than maximum NO reduction in high pH soils, unlike in the low pH soil where maximum production of N_2O mirrored the greatest reduction of NO. Additionally in high pH soils, the production of N_2 began immediately after anaerobisation, whereas in low pH soils, the production of N_2 began only after most of the NO had been reduced, which was after the maximum NO reduction rate. Together, these results argue for the two soils having two different denitrification regulation systems.

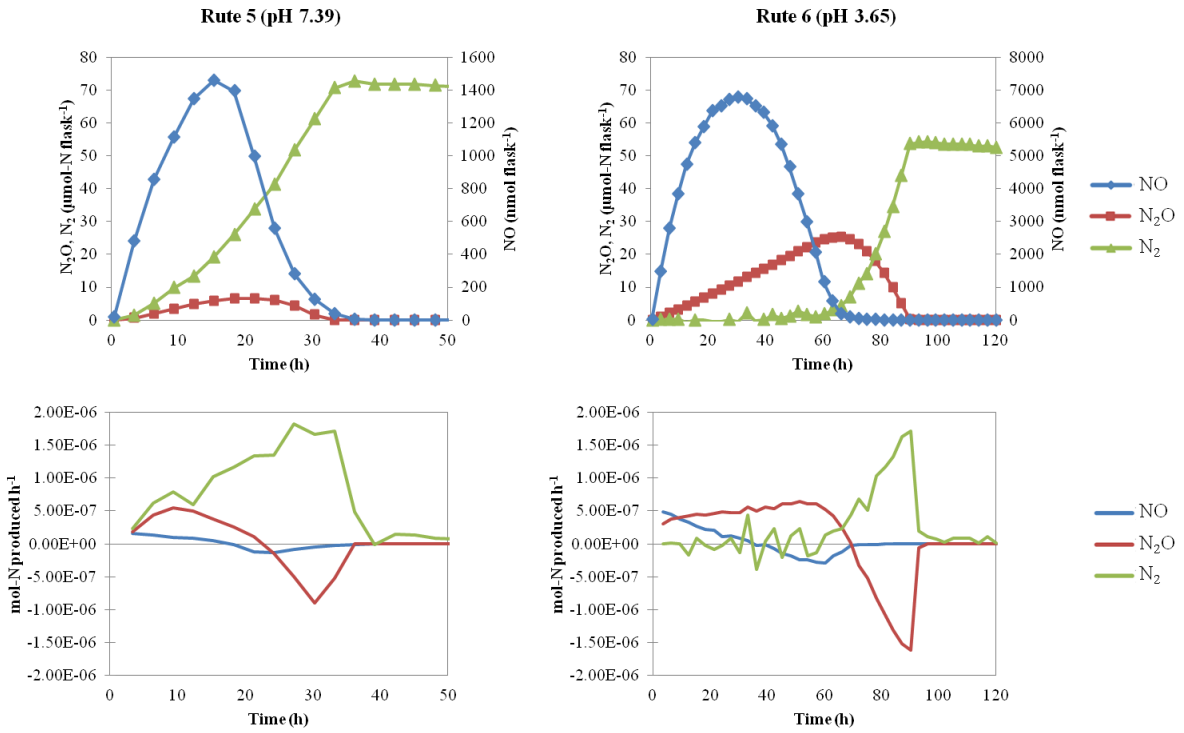

Figure 3.14 Production and reduction rates of NO, N_2O and N_2 in acidic versus alkaline soils. NO, N_2O and N_2 production started at the same rate in high pH soils. Over time, the net rate of NO production dropped to negative, where more NO was being reduced than produced. In contrast, the N_2 production rate increased steadily until N_2 levels reached a plateau. Low pH soils showed a completely different gas production profile, where N_2 production rate remained low until NO was almost completely reduced.

3.5.4. Extracted nucleic acids

The newly developed method was tested on two of the peat soils used in the robotised GC experiment, as chosen in Section 3.5.2. The extraction of nucleic acids was successful for all 18 samples from each of the two soils: For the high pH soil, 240.0 ± 50.8 ng DNA and 85.6 ± 13.7 ng RNA per gram of soil was obtained; for the low pH soil, 187.0 ± 16.3 ng DNA and 81.1 ± 6.4 ng RNA per gram of soil was obtained (measurements made with Qubit). Based on statistical analysis (Section 2.10), there is no significant difference in the amount of RNA extracted from the two soils (p = 0.0004). In contrast, there is a significant difference in the amount of DNA extracted from the two soil types (p = 0.22).

The RNA fractions were confirmed to be DNA-free by analysis of TNA on agarose gels, as well as by performing a 16S rRNA gene amplification on the RNA-only fraction and the reverse transcribed cDNA (Figure 3.15). All cDNA samples from both soils provided strong bands following the amplification, and the RNA samples were free of amplifiable DNA.

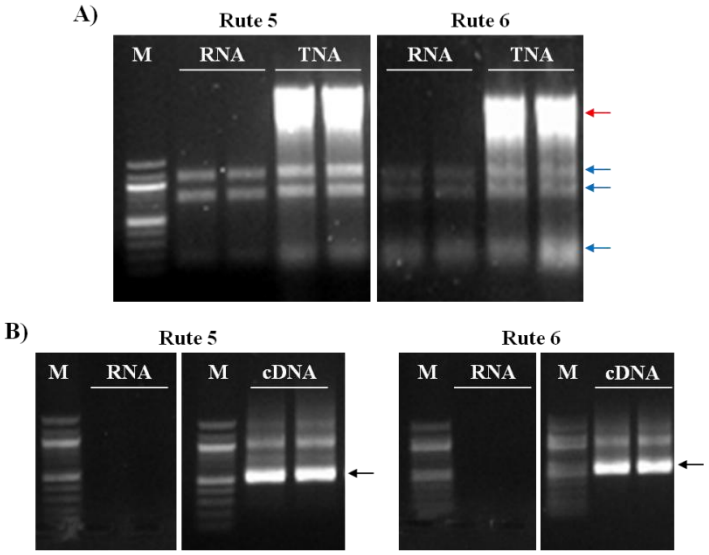

Figure 3.15 Amplifiable DNA digestion is complete in both the low and high pH soil samples. A) The smearing of gDNA was observed in undigested samples, but was below detectable levels in digested samples. B) There is no amplifiable DNA in the RNA fractions in both high and low pH soils, and the 16S rRNA gene from the cDNA is amplifiable. M: 100 bp ladder; red arrow indicates gDNA smear; blue arrows indicate the 23S, 16S and 5S rRNA; black arrows indicate amplicon size of interest.

3.5.5. Denitrification gene expression

Based on the observed gas kinetics (Figure 3.13) the biggest difference in timing between the two soils was the production of N_2 gas. Thus, the expression pattern of the N_2OR which is responsible for converting N_2O to N_2 and is encoded by the gene *nosZ*, was chosen for analysis. The efficiency of the qPCR was 83% and 81% for the high and low pH soils, respectively. A clear and distinct peak in *nosZ* expression was observed in the high pH soil, with a maximum of 2 000 copies of *nosZ* per ng of RNA extraction (Figure 3.16). In contrast, there appeared to be only a consistent low fluctuation of *nosZ* expression in the low pH soil.

To provide a second point of comparison between the two soils, the expression of the cytochrome cd_1 NIR, encoded by *nirS*, was analysed in both soils. As mentioned in the Introduction (Section 1.3.2), the NIR enzyme plays an important role in NOx gas control in denitrification, since NO is the first gaseous product in the denitrification process. The qPCR efficiency was 72% and 79% for the high and low pH soils, respectively. Similar to the *nosZ* expression, the *nirS* expression profile of the two soils differed greatly. In high pH soils, *nirS* expression increased quickly, reaching a peak maximum around 6 hours after anaerobisation (Figure 3.16). In contrast and similar to the *nosZ* expression, there was no distinct peak in *nirS* expression in low pH soils. At the point of anaerobisation, there was an apparent sudden increase in *nirS* expression, but since only one point was captured in this experiment, it is unknown if the point captured was the apex of a peak, on either side of the maximum of a very quick peak in expression, or just a flask/sample anomaly.

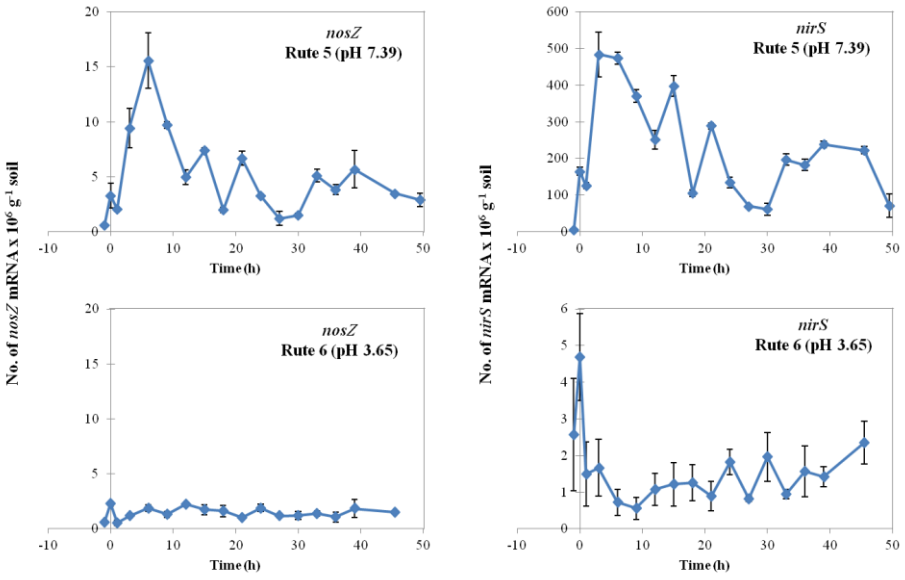

Figure 3.16 *nosZ* and *nirS* expression profiles of high and low pH soils. High pH soils revealed a peak in *nosZ* and *nirS* expression within 10 hours of anaerobisation, before dropping off to lower levels that were still higher than pre-anaerobisation. In contrast, there was no distinct peak in *nosZ* and *nirS* expression prior to 50 hours in low pH soils. Pre-anaerobisation levels are marked as '-1 hour'.

3.5.6. Quantification of TNA in low and high pH soils

To quantify and compare the amount of amplifiable DNA, cDNA and residual DNA in the RNA fraction, qPCR reactions with primers targeting the 16S rRNA, *nirS* and *nosZ* genes were performed. The purpose was to determine the baseline 'noise' inherent in the different soil nucleic acid extracts. The efficiencies for the reactions are as stated previously for the primers targeting *nirS* and *nosZ*, and 69% for the primers targeting the 16S rRNA gene. For both the high and low pH soils, all primers used resulted in the successful amplification of the DNA and cDNA fractions (Figure 3.17). The large standard deviation in cDNA copy numbers is mainly caused by different denitrification gene expression levels at different times. Multiple samples from different time points were chosen to cover the widest range possible of copy number variation.

Alarmingly, for all three primer sets, amplifiable DNA was detected in almost all the low pH soil RNA samples tested. There was considerably fewer amplifiable DNA copy numbers in the RNA samples, showing that the DNA was mostly digested, but a small amplifiable fraction was left behind. Additionally, the amount of undigested DNA in the RNA samples was highly variable and unpredictable. In contrast, the melt curve analysis showed that the low signal detected from the high pH soil RNA fraction was due to unspecific binding of primers (16S rRNA and *nosZ* genes) or part of the background noise (*nirS* gene).

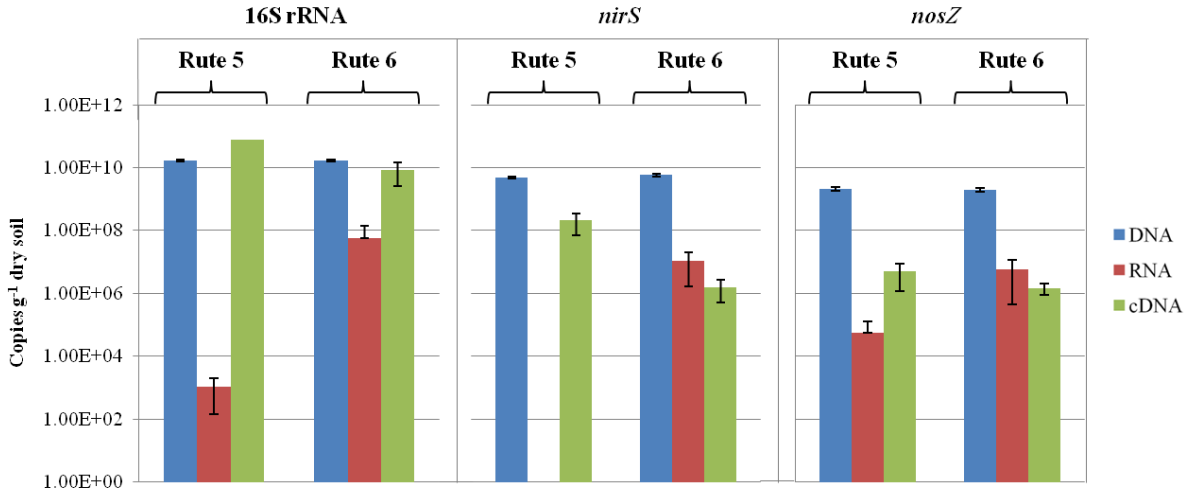

Figure 3.17 Comparison of amplifiable DNA copy numbers in DNA-only, RNA-only and reverse transcribed cDNA fractions. All DNA and cDNA fractions from both high pH (Rute 5) and low pH (Rute 6) soils were amplifiable. Amplifiable DNA was detected in the RNA-only fraction from the low pH soil, using primers targeting the 16S rRNA, *nirS* and *nosZ* genes. In contrast, the signal detected from the RNA-only fractions of the high pH soil indicated unspecific primer binding and/or background noise.

This discovery of amplifiable DNA came nearly one week after the nucleic acids were co-extracted from the soil samples, and after normal PCR amplification with primers targeting the 16S rRNA gene detected no amplifiable DNA in the RNA fraction (Figure 3.15). As yet, there is no known reason for this unexplainable phenomenon. Implications of this finding are further discussed in the following chapter (Section 4.3.3).

4. Discussion

4.1. Co-extraction of DNA and RNA from inhibitor-rich soils

As introduced at the beginning of this paper, one of the main barriers when working with soil samples is the presence of inhibitory compounds (Section 1.5). These compounds are co-extracted with NA and inhibit enzymatic activity, interfering with downstream processes. Thus, one of the aims of this study was to assess the abilities of a well-known and oft-used TNA extraction method and several commercial kits to extract both inhibitor-free DNA and RNA from low pH peat soils. When these methods failed to yield satisfactory NA, a new flexible and transparent method was designed and tested on a low pH peat soil with which we have not previously been successful in the extraction of useable RNA.

4.1.1. Strengths and weaknesses of extraction methods tested

Three different extraction methods were thoroughly tested for their ability to co-extract DNA and RNA from a difficult low pH soil sample. Of the three tested, the first is a common method used to extract TNA from environmental samples, and the other two are commercially available kits. With the information gained from the three failed methods, a new method was designed, based on the idea of maximum flexibility of and transparency in the individual procedures and the components used.

The first method tested was the TEM, based on the method published by Griffiths and colleagues (2000), and is commonly used in environmental studies. The TEM included minor changes made by our laboratory group to optimise extraction efficiency from our samples. Despite widespread success within and without our group, TEM's success on our low pH soils was poor at best: The DNA extracted was useable after clean-up, but the presence of enzyme inhibitors made the method unsuitable for the analysis of RNA due to incomplete DNA digestion (Section 3.3.1, Figure 3.4). This problem remained unsolvable in this study, since the method purified and cleaned up the nucleic acids only after the DNase digestion, which, in itself, was already inhibited. Additionally, even after clean-up, amplification of residual DNA and reverse transcribed cDNA was unsuccessful, and the compounds responsible for the inhibition of the reaction still remain unidentified.

Despite strong recommendation by our colleague, the first commercial kit tested, PM, performed poorly on our current samples. Similar to the TEM, the extracted DNA was only useable after further clean-up with other kits, and the DNA was not digested completely either (Section 3.3.2, Figure 3.5). Although the reverse transcription was no longer inhibited, the residual DNA leftover in the RNA fraction meant that the RNA was still unusable in downstream processes. Similar to above, the incomplete DNA digestion could not be overcome in this study as the inhibitory compounds were also unknown.

The following kit tested, PS, was chosen due to success of the DNA-only version of the kit, PD, on the same low pH peat soils – it was able to extract large quantities of relatively inhibitor-free DNA quickly (Section 3.1). The PS kit had the potential to clean-up most of the inhibitors from our samples, but the procedure took too long and was inefficient (Section 3.3.2, Figure 3.6). The physical properties of the low pH peat soil sample clogged up the kit's RNA Capture Column very badly, resulting in an extremely slow drip rate during the RNA purification process. This extended the procedure time from a theoretical 3 hours to well over 7 hours. Although the rRNA extracted using the kit yielded amplifiable cDNA, the reverse transcription success rate of mRNA was unknown. This is because only primers targeting the rRNA were used, as the genetic expression pattern of the soil was yet unknown. This would mean that any result showing the absence of mRNA could have been due to either unsuccessful mRNA extraction or the lack of gene expression. Additionally, the probability of mRNA surviving degradation at room temperature during a 7-hour extraction procedure is very slim, and the extracted mRNA fraction would have been heavily biased towards mRNA that was more resistant to degradation, and would not reflect true physiological mRNA concentrations.

As such, the PS kit was retested using manufacturer's recommendations for faster and larger-scale extractions – positive pressure application to the column using a syringe. Unfortunately, this addition resulted in the worst result of all the methods tested: The DNA was unamplifiable, even after repeated clean-up using multiple kits; the DNase digestion of the RNA fraction was incomplete; and the reverse transcription was inhibited (Figure 3.6). It was concluded that the above problems stemmed from the co-elution of proteins and other inhibitory compounds with the nucleic acids from the RNA Capture Column due to the applied positive pressure. Alarmingly, the inhibitory compounds eluted were either of such high concentrations or of such unique nature that no commercially-available clean-up kit was

able to undo the damage done, and the DNA and RNA fractions were entirely unusable in downstream processes.

In short, although most of the methods tested were able to extract useable DNA, they failed to extract RNA quickly and for large sample sets. Even among the commercial kits on the market, the vast majority are nearly impossible to work with in sample sets exceeding 10 – these kits are mostly used for testing occasional samples, and not for repeated large-scale characterisation experiments such as those performed regularly in our laboratory group (kit manufacturers pers. comm.). The current study requires repeatable large scale co-extraction of DNA and RNA fractions from soil samples, thus not allowing the use of the above tested kits. Even though the original plan of triplicate TNA extractions from 40 unique soil samples, not including the triplicates reverse transcription and qPCR reactions, was greatly reduced due to financial and temporal reasons, the current 36 single (non-triplicate) samples already proved to be unmanageable by commercial kit standards.

4.1.2. Problems overcome with NM

As mentioned in Section 3.3.3, the NM was designed to overcome the problems identified in the TEM and commercial kits. The main barrier was the co-extraction of inhibitory compounds, which persists in most, if not all, TNA samples from this low pH soil. These inhibitors affected all reactions that involved the use of enzymes, including amplification, nucleic acid digestion and reverse transcription. In addition, the commercially available kits tested, while effective to varying degrees, lacked transparency. This made method optimisation nearly impossible, since the individual components and their uses were unknown. The additional factor of unknown chemical composition also made it difficult to combine and coordinate the use of kits and enzymes from different sources, since some products were sensitive to the presence of certain chemicals. For example, reverse transcriptases are sensitive to the presence of EDTA, which is sometimes used in solutions to stop DNase activity.

Method and reagent transparency potentially allows the user to uncouple individual reactions within the method from each other, giving the user the choice to remove unnecessary procedures or add on additional ones as required. In some cases, time is of the essence, and the removal of dispensable procedures would be a great advantage, e.g. due to the quick

degradation of mRNA molecules. Alternatively, the addition of other procedures such as proteinase and/or RNase digestion, without having to unnecessarily clean up the sample multiple times is a potentially huge gain, since each clean up procedure results in the loss of a small fraction of NA.

Hence, the NM was designed to provide the user with as much method and reagent transparency and flexibility as possible, allowing changes to be made to the method as required. The only unknown reagent that remains in the method is the solution used to precipitate proteins, which may be removed in future method optimisations (Section 4.1.5). Also, an important part of this method is that the extracted RNA is at least partially purified prior to DNA digestion, potentially removing some of the enzyme inhibiting compounds.

Tests with the NM showed that it had a much quicker procedure than the other tested methods, in part owing to its quick purification procedures, fast nucleic acid precipitation, and short DNase digestion time. Additionally, the amount of DNA and RNA co-extracted from the samples were of sufficient quantity to ensure large amounts of genetic material to work with. By itself, the NM was unable to yield cDNA, but was capable of fully digesting amplifiable DNA in the RNA fraction (Figure 3.7). The addition of the OSP kit at the end of the NM yielded amplifiable cDNA reverse transcribed from mRNA (Figure 3.8). The use of clean-up kits for further clean up and sample concentration was found to be optional due to the effectiveness of the OSP in cleaning up the nucleic acid samples (Figure 3.8). In some cases, the quantity of extracted DNA was so great that the samples had to be diluted before amplification (genomic smears were faintly visible on the gel).

4.1.3. Nucleic acid clean-up and purification

As mentioned previously, a major problem with all extraction methods, including the method designed in this study, NM, is the presence of inhibitory compounds in the co-extracted NA. For DNA fractions, that is a relatively simple problem to solve. A wide variety of DNA clean-up kits are available from the many different manufacturers, from the basic DNA amplicon clean-ups, to the total gDNA clean-up kits which also concentrate the extracted gDNA into a smaller volume. Since there are no procedures that require the use of enzymes prior to cleaning up, the extracted DNA samples are relatively problem-free after going

through one or more rounds of clean-up using these kits – the optional RNase and proteinase digestions that involve enzymes all come after.

In contrast, RNA extraction methods and kits mostly include mandatory DNase digestion prior the use of clean-up kits, and RNA clean-up with commercial kits is not possible prior to DNase digestion. This is because the most effective RNA clean-up kits are column-based clean-up kits, but these columns bind all the NA in the sample (both DNA and RNA), as well as inhibitory compounds with similar chemical properties to NA. Given that RNA clean-up columns are typically designed to have small load capacities as there is substantially less RNA than DNA in any sample, any attempt to clean-up TNA in an RNA column prior to digestion would result in the loss of almost all RNA, since the DNA in the sample will overload the column. The large amount of DNA bound to the column results in a low RNA-binding capacity and loosely bound RNA, so the RNA easily elutes during the wash steps (kit manufacturers pers. comm.). High binding capacity DNA clean-up columns cannot be used either, since DNA clean-up kits are not RNase-free. Early trials using the gDCC to clean up RNA samples resulted in substantial loss of rRNA (data not shown), so the effect on mRNA is presumably worse. Currently, there are no kits on the market known to this author that allow the clean-up of TNA from inhibitory compounds, with the exception of the OSP kit used in this study. Still, the OSP has limits, since it is only able to clean up the so-called "PCR inhibitory compounds" with specific chemical properties, and may thus not bind all inhibitors present in soil samples.

Consequently, one of the reasons for choosing the PS kit during the method comparison phase was because the kit did not clean-up the extracted TNA after the extraction process, but instead purified TNA and preferentially eluted specific NA fractions. The basis of the PS kit is the binding of TNA to a column, and using different elution buffers to preferentially elute specific fractions of the bound material. As such, even if the inhibitory compounds were to have properties similar to all NA, unless they have properties identical to DNA and RNA, there is a much lower likelihood of co-elution. However, the kit had several unacceptable drawbacks – the kit required ten times the amount of soil required than any other method (2 g) for each extraction, and took over 7 hours for two samples. Adding positive pressure as the manufacturer suggested reduced the binding force of the column, causing DNA, RNA and inhibitory compounds to co-elute during both elution steps, resulting in large amounts of DNA in the RNA eluate, and inhibitors in both DNA and RNA eluates (Section 3.3.2).

4.1.4. Inhibitor removal

As seen in Section 3.5.6, the NM-OSP method is not without problems. The current cause for the re-emergence of amplifiable DNA in the RNA fraction is unknown. One of the most likely explanations would be that some inhibitory compounds were left in the extracted RNA, preventing complete DNA digestion and inhibiting amplification of the residual DNA. Based on this explanation, the inhibitory compounds would have degraded over time, thus allowing the amplification of residual DNA that was no longer inhibited some time after the original residual DNA amplification test. However, this degradation of inhibitory compounds would have to take place at -80°C and in less than 5 days. This is further discussed in Section 4.3.1.

Regardless of the cause, the current setback in this extraction method is the residual DNA leftover after partial DNA digestion. One immediate possibility would be to digest the RNA sample with DNases again after several days' storage at -80°C, to remove the residual DNA. This was unfortunately not tested because there was insufficient time. Alternatively, instead of struggling with leftover inhibitory compounds subsequent to TNA extraction, another method may be to remove inhibitors prior to cell lysis. Previous studies have shown that aluminium sulphate, $Al_2(SO_4)_3$, serves as an excellent inhibitory compound flocculant, allowing for the removal of inhibitory compounds from soil samples by the simple addition of flocculant and careful pH control (Peršoh, *et al.*, 2008). This method, however, is not without its shortcomings, least of all the optimisation difficulties.

Unfortunately, the use of the $Al_2(SO_4)_3$ as a flocculant is not as simple as adding a set amount into each soil sample prior to TNA extraction. Instead, different concentrations and amounts have to be used for each soil sample, depending on the starting inhibitor concentration. The main reason for this is because the overuse of $Al_2(SO_4)_3$ lowers the extracted DNA yield (Peršoh, *et al.*, 2008). There is, as yet, no explanation why this may be so, but it may be hypothesised that $Al_2(SO_4)_3$ may have an osmotic effect on intact cells, possibly lysing the population of fragile cells. However, $Al_2(SO_4)_3$ should not, in theory, have much of an effect on healthy, intact cells. Instead, this author's best hypothesis is that $Al_2(SO_4)_3$ binds to the DNA left in the soil matrix by previously lysed cells or to DNA from dead cells with undegraded/partially degraded membranes, thus lowering the "total DNA" extracted from the soil. Of course, this requires further investigation to determine the exact effect of high $Al_2(SO_4)_3$ concentrations on the soil microflora and the reason behind lower DNA yields.

If it is assumed that $Al_2(SO_4)_3$ affects the non-dead portion of cells and thus affects the DNA fraction of interest as Peršoh and colleagues did, the exact amount of flocculant used will need to be tried and tested prior to the actual experiment. Peršoh and colleagues (2008) have highlighted the high heterogeneity of the soil matrix, with its microbial hotspots and microsites, and high humus concentration variability. This means that the amount of $Al_2(SO_4)_3$ required for each TNA extraction reaction may be different, even within the same soil sample. This would require a series of soil reactions with technical triplicates per soil and treatment type to confirm repeatability of flocculation, in order to test the different $Al_2(SO_4)_3$ concentrations and determine the most suitable flocculant concentration. To a certain extent, the problem of performing such large sample series testing may be side-stepped by first testing the concentration of flocculant required on the different unincubated soil samples prior to using it for the robot incubations. Unfortunately, this would raise the question of whether or not the additional incubation, anaerobisation and/or nutrient addition steps affects the tendency for humic acids to dissociate from soil particles and dissolve in the $Al_2(SO_4)_3$ solution, thus affecting the subsequent flocculation process.

Unfortunately, despite the promising results of the successful reactions, Peršoh and colleagues also found residual humic acid, phenol and/or protein contamination in a number of their samples, and recommended the disposal of such samples. This showed that their present method with $Al_2(SO_4)_3$ was, while promising, inconsistent. Sadly, the technical replicates in his experiment were not used to depict technical consistency, but were instead used to ensure the acquisition of at least a single inhibitor-free sample with high quantities of nucleic acids. This system of TNA extraction poses a major problem when working with a large quantity of samples, such as the scale required in the current study. Nevertheless, it may be possible, with further optimisation, to use a low concentration of $Al_2(SO_4)_3$ to flocculate at least a portion of the inhibitory compounds in the soil prior to using the NM-OSP method, thus reducing the effect of inhibitors on subsequent procedures.

4.1.5. Future improvements

Possible avenues to explore for further method optimisation would be to attempt to increase the yield of DNA and RNA extracted. At present, increasing the starting soil amount does not increase the TNA yield, and may owe to insufficient salt ions for efficient NA precipitation with isopropanol – the ideal NA precipitation ratio should be 0.7 volumes isopropanol and

0.1 volumes 5M salt. However, due to the current unknown salt concentration of the MPC Protein Precipitation Reagent, it is difficult to judge if additional salt should be added to the reactions. As such, one of the possibilities that are currently under exploration is the removal of the protein precipitation step, instead proceeding straight to NA precipitation, and adding the use of an OSP column immediately after the resuspension of nucleic acids and prior to DNA digestion. This has the double benefit of potentially increasing the user's control on NA precipitation and removing most of the enzyme inhibitors before the DNA digestion step.

4.2. Anaerobic incubation of low and high pH peat soils

In order to determine the effect of NOx gas production in low and high pH soils during anoxic spells, soil from six field plots were anaerobised and incubated in air-tight flasks at 15°C in a robotised incubation system. The gases in the flask headspace were measured every 3 hours by injection into a GC and a NOA. Simultaneously, flasks identical to those in the robot were sacrificed for nitrite measurement. Unlike the implications of previous reports (Šimek & Cooper, 2002), all acidic soils were capable of reducing N_2O completely, producing N_2 as a final product, albeit at a retarded rate. The expression patterns of the NIR, *nirS*, and N_2OR, *nosZ*, observed in high pH soils in the current study are similar to those seen previously, showing that the current TNA extraction method is able to yield results similar to the TEM (Liu, *et al.*, 2010). The expression patterns of the denitrification genes in low pH soils unfortunately remain unclear, due to extraction-related issues.

4.2.1. Reduction of nitrogen compounds

Gas and nitrite measurements were made from anaerobised flasks incubated at 15°C every 3 hours until the headspace in all the flasks reached a plateau in N_2 levels.

Gas production and utilisation was found to group relatively well according to the pH of the soils (Figure 3.13): The slowing of NOx gas reduction was observed to directly vary with the pH of the soil – the lower the pH of the soil, the slower the reduction of NO and N_2O gases. Despite having a relatively close pH to the other high pH soils, with only a difference of 0.08 pH units, Rute 4 appeared to be an outlier. The reason may either be that Rute 4 is just under the critical threshold for these reduction reactions, or that Rute 4 had previously been subject to unknown environmental factors, changing the way that it behaves.

In spite of the unexpected difference, the production of N_2O and N_2 from Rute 4 soil still followed the same pH trend, having gas kinetic curves in between that of the higher pH soils (Rute 5 and 21) and the mid pH soil, Rute 12. The two highest pH soils (Rute 5 and 21) reduced NO to N_2O the quickest, followed by Rute 4 and the mid-range pH soil from Rute 12, with the slowest reduction by the two lowest pH soils, Rute 6 and 18. The only exception is the production of NO, where all plots showed immediate NO production following anaerobisation.

In the high pH soils, all three gases (NO, N_2O and N_2) were produced almost immediately, and nitrite levels accumulated to the millimole per gram of dry soil range (Figure 3.12-13). In contrast, the low pH soils appeared to suppress nitrite accumulation at very low levels, instead amassing NO and producing N_2O at a near-linear rate. The reduction of N_2O to N_2 appeared to take place only after nearly all the NO had been reduced to N_2O, after which the N_2O levels were very quickly diminished.

The trend seen in high pH soils is similar to the model denitrification organism, *Paracoccus denitrificans*, which, at near neutral pH, completely reduces nitrate to N_2 gas, and accumulates nitrite to relatively high levels (Bergaust, *et al.*, 2010). Although the same paper showed evidence that the N_2OR enzyme cannot be successfully synthesised in a low pH environment, this may not entirely contradict current results. Bergaust and colleagues (2010) showed that such synthesis is not possible in *P. denitrificans*, in a liquid culture environment. As the current study utilises a full soil microflora community in a soil matrix where substrate and nutrient transport is severely restricted, there are several possible reasons for current results. One hypothesis is that there are bacteria that have evolved or been selected naturally to tolerate low pH environments, possessing mechanisms other than those observed in *P. denitrificans* to enable the assembly of a functional N_2OR enzyme. Another reason could be that such low pH-tolerant bacteria may use an entirely different enzyme, and that such an enzyme's synthesis is pH-independent. Last but not least, the products of bacterial metabolism during incubation were found to increase the bulk pH in acidic soils slightly (Figure 3.10), implying that even higher pH increases in microsites may have occurred, providing denitrification hot spots for N_2OR production and thus N_2O reduction.

Aside from the issue of denitrification ability in acidic soils, another question remains: Why is the reduction of N_2O in acidic soils so much slower? Unfortunately, the last hypothesis of waste product build-up, thus increasing the environmental pH and allowing successful N_2OR synthesis, by itself is an insufficient explanation. Previous trials in our laboratory with the same acidic soil yielded similar gas trends regardless of the total time taken for denitrification, showing that the strongest factor is the presence of NO (unpublished). This means that the reduction of N_2O in acidic soils is affected by either the physical presence of NO or by the incomplete reduction of it. Previous studies have shown that the different denitrification enzymes are highly influenced by electron availability (provided by carbon compounds), with the N_2OR enzyme being the worst at electron scavenging when the cells are more oxidised, thereby resulting in increased N_2O emissions (Schalk-Otte, *et al.*, 2000).

Thus, it may be postulated that the low pH soils are generally carbon-starved, and that the N_2OR enzyme is unable to compete successfully for electrons until the NIR and NOR enzymes are no longer required, when there is no more substrate nitrite and NO, respectively.

An alternative explanation for this phenomenon is that the low environmental pH may cause lowered or inhibited carbon uptake due to lower levels of available dissolved organic carbon (Kalbitz, *et al.*, 2000). This low carbon uptake results in fewer electrons available in the cell for the denitrification processes, and due to electron competition, N_2OR activity becomes inhibited. As reviewed by Šimek and Cooper (2002), previous studies have shown that the amount of dissolved carbon in soils has a larger impact on denitrification than pH, and that the pH effect may be an indirect effect of dissolved carbon quantities. Thus, the phenomenon of slow N_2OR activation may be due to the low carbon availability, rather than a low concentration of total carbon in the soil. This is further backed up by previous results in our laboratory showing that acidic soils flushed with glutamic acid adjusted to the same pH as the soil completes denitrification to N_2 in as little as 30 hours (unpublished).

Another possible explanation is that the current observed denitrification trends are not caused by the presence or absence of NOx compounds or available carbon, but simply due to different Denitrification Regulatory Phenotypes, or DRP, of the soil communities (Bergaust, *et al.*, 2011). Recent research has shown that even microorganisms within the same genus may have different DRP, and that the trends of nitrite, NO, N_2O and N_2 production is heavily dependent on the organism's DRP (Liu, *et al.*, 2013). The Rapid Complete Onset (RCO) type is defined by the production of all three gaseous denitrification products (NO, N_2O and N_2) at detectable levels early on and suppressed nitrite accumulation; whereas the Progressive Onset (PO) type accumulates high amounts of nitrite, and shows sequential production and utilisation of NOx compounds (Liu, *et al.*, 2013). The high and low pH soils from the current study appear to display the typical RCO and PO types, respectively, with the exception of nitrite utilisation (Figure 3.12-14). This observed 'atypical' nitrite utilisation is likely due to the heterogeneous composition of the soil microflora, since the previous studies describing DRP used pure cultures (Bergaust, *et al.*, 2011, Liu, *et al.*, 2013). The high pH soil displayed high nitrite accumulation, but rapid production and utilisation of NOx compounds. Conversely, the acidic soil consistently kept nitrite levels at low but detectable levels, and showed a typical PO style of NO and N_2O reduction pattern. Previous results from the laboratory showed that the PO trend did not change even with glutamic acid flushing,

maintaining the same sequential reduction, but at an accelerated pace (unpublished). Together, these results give strong evidence for the soils being influenced by DRP type.

A possible hypothesis for the development of the acidic soil's PO type may be the long-term exposure to an acidic environment. When coupled with the earlier hypotheses of low dissolved organic carbon availability in acidic environments and electron competition, this strongly argues for a selective environmental pressure for microorganisms that naturally have a PO phenotype, as the N_2OR enzyme is not competitive and would normally lose out to other enzymes. Thus over time, microorganisms that naturally have a RCO phenotype would lose out to PO type organisms, by wasting energy on producing 'useless' N_2OR enzymes that are unable to reduce N_2O due to their low competitiveness for electrons. Hence, this author postulates that the N_2OR enzyme in the acidic soil PO type microflora may be synthesised at a later time than the NIR and NOR enzymes, saving the microbes' naturally low energy resource. This would ensure that the N_2OR enzyme is synthesised only when it is necessary and when there is excess carbon, guaranteeing the availability of electrons for N_2O reduction. This DRP-based explanation may also partially explain the common observation of retarded N_2O reduction by low pH soil denitrifiers in high pH media, since they have been selected for over time by the low pH environment and would not reduce N_2O any quicker, even under optimal conditions for N_2OR synthesis (Šimek & Cooper, 2002, Liu, *et al.*, 2010).

Hence, while previous research has given much explanation as to why high pH soil microflora are able to perform complete denitrification but are unable to denitrify under low pH conditions, this study furthers current knowledge by showing that the low pH microflora are also able to do so under acidic conditions, provided there is enough time. Moreover, this study supports recent studies, strongly suggesting that high and low pH soils may function under different DRP, thus at least partially explaining why high and low pH soils do not produce the same denitrification end-products *in situ*.

4.2.2. Field implications

Despite showing that complete denitrification and N_2 production is possible in low pH soils, there is irrefutable evidence that the field plots where the acidic soils came from emit almost only NO and N_2O gas (Hovlandsdal, 2011). This is in contrast to the field plots with high pH soil, where less N_2O emissions are observed (Hovlandsdal, 2011). This contradiction of

observations may be due to the fact that experiments performed in this study took place in a laboratory controlled environment with stable conditions, free from external variables, such as meteorological or physical disturbance. The anaerobised flasks in the robot were completely sealed from the outside, meaning that while the system was guaranteed anoxic (atmospheric oxygen would not get into the system), the NO and N_2O produced by the soils were not released into the atmosphere, and remained in the flask headspace. This means that the produced NOx compounds had the opportunity to slowly diffuse back into the soil (unlike in the field), where denitrification and the complete reduction of said NOx compounds was actively taking place. In a field system, the same N_2O and NO produced by the soil microflora would presumably have successfully escaped into the atmosphere, far from the denitrifiers that may be resident in the low pH soil. In contrast, the quicker N_2O reduction and N_2 production in high pH soils may be the primary reason for field observations of high N_2 and not N_2O emissions.

As such, the current study provides evidence to add on to the hypothesis of previous studies that low pH soils are unlikely to perform complete denitrification, favouring instead the production of N_2O as the final product (Šimek & Cooper, 2002, Liu, *et al.*, 2010, Raut, *et al.*, 2012). This study postulates that low pH soils have the potential to complete the denitrification process under field conditions, but are generally unable to do so due to the slow activation of the N_2OR enzymes in the soil microflora. In the time it takes for the N_2OR enzymes to begin N_2O reduction, the bulk of the N_2O gas has diffused out of the soil matrix and into the atmosphere. Consequently, field measurements are only able to pick up NO and N_2O gas emissions and not N_2 emissions.

4.2.3. Gas and nitrite measurements

As mentioned briefly in Section 3.5.3, flask-to-flask variation was apparent in this experiment (Figure 3.11). Part of this was caused by the replacing of sampled headspace gases with helium and the repeated puncturing of septa during sampling. Although the autosampler was programmed to puncture the septa at random positions, reducing the probability of creating a hole in the septa, gas leakage over time is inevitable. Despite the undeniable differences between the parallel flasks, especially in those which suffered worse septum puncture leakage, the trend observed in the flasks was surprisingly resilient (Supplementary results, A2.2). The gas production and utilisation observed in each flask

grouped together well for each soil and did not overlap despite the flask variations, confirming the reliability of the gas trend data.

Unfortunately, there appeared to be some variation between and within the parallel-incubated flasks that were not used for gas measurements (Figure 3.12, Supplementary results A2.1). Unexpectedly, a still unknown variable, possibly the leakage of headspace gases into the atmosphere and vice versa, appeared to have swung the nitrite reduction reaction equilibrium from one side of an unknown threshold to another. To elaborate, one of the parallel flasks from the low pH soil (Rute 6) was left to be opened at hour 119 of the experiment for nitrite measurements, due to the slow reduction of nitrite. The flasks in the robot were opened 3 hours later, at hour 122 of the experiment. The nitrite levels of the parallel flask stayed at around 10 μmol g^{-1} of dry soil, whereas all 3 flasks in the robot showed 0-0.3 μmol g^{-1} of dry soil (Supplementary results, A2.1). While it is possible that there was a burst of nitrite reduction during the last 3 hours of the experiment, it is more likely that there was an unknown factor in the parallel flask preventing a complete reduction of nitrite in the soil, especially since the N_2 levels in the flasks had reached a stable level around hour 93 of the experiment. An alternative explanation would be the heterogeneous presence of microsite bacterial hot spots, which is also likely to be the main reason for the variation seen in the technical triplicates when measuring nitrite (Supplementary results, A2.1).

While the value of nitrite in the parallel flasks may not be exactly the same as that in the robot-incubated flasks, both the gas and nitrite data point towards a general trend of complete denitrification apparent in both the robot-incubated and non-robot-incubated flasks. Further improvements to the method and experimentation may need to be performed to acquire more precise corresponding nitrite values, but this author believes the observed reduction of NOx compounds to be conclusive evidence of complete denitrification in the soils tested.

4.2.4. Future improvements

Due to currently available equipment restrictions, it is unfortunately not possible to include more than 30 flasks in the robot to ensure identical replicate flasks. Instead, this author proposes the following improvements and/or additions be made to future experiments of a similar nature.

Towards the end of this study, equipment for measuring nitrate became recently available in the laboratory. Although current measurements of nitrite and NOx gases have indicated complete denitrification to N_2, it is unknown how much and how quickly the nitrate in the soil has been reduced. Current calculations of total N_2 produced exceed the amount of nitrate added to the soil, suggesting that there was pre-existing nitrate in the soil, thus complicating conclusions that may be drawn regarding denitrification rates in these soils. Given the current hypothesis of DRP for the different soils, knowledge of nitrate reduction rates may be essential to formulate a more complete picture of such phenotypes. Additionally, knowledge of pre-existing nitrate quantities in the soil would allow for the potential analysis of other nitrogen cycle processes aside from denitrification, for example anammox and DNRA. To further expand the range of questions that may be answered in future investigations, accurate and precise methods for the analysis of ammonia content may also be necessary.

Another possible change may be the method of nitrate addition to soils. In this study, nitrate was added directly to the soil, and this was performed for direct comparison with a previously published study using this method (Liu, *et al.*, 2010). Alternatively, another method currently in use in our research group is the flushing of soils with nitrate solution. This has the benefit of ensuring a known homogeneous starting concentration of nitrate, as well as giving more control over the water content in the soils. However, some problems faced with this method include the possible washing out of nutrients from the soil, as well as the addition of low concentrations of buffers, since the nitrate solutions have to be buffered to match the soils' respective pH. Nevertheless, preliminary trials with the acidic soils and this method of incubation do not appear to have affected the soil's denitrification ability (unpublished).

Aside from a more accurate control of nitrate in the soil, there also needs to be a better method to standardise or measure the water content in the soil. In the current study, the primary purpose for water content measurements was for the calculation of nitrite concentrations, since the nitrite was dissolved in the soil moisture. Thus, this study recorded soil water content was "millilitre per gram of dry soil", and other factors were not taken into account, e.g. the presence of shell sand in high pH soil samples. A more accurate way to calculate water content in the field of soil sciences would be to calculate the percentage water-filled pore space (WFPS), which also gives addition information on the transport rates of gases to the liquid phase (Lars Bakken, pers. comm.). These WFPS percentages allows for the inference of the efficiency of transport of gases between the headspace and soil moisture. This may supply additional information on the reduction of headspace gases, since the

denitrification rates observed may be partially restricted by the rate of gas transfer. Hence, this method of calculation and measurement is probably essential for future studies, so that the water content data can be more relevant to the gas data.

In addition to the water content being more accurately reflected with the above method, the current "per gram of dry soil" (g^{-1} dry soil) denomination for most calculations in this study requires revision as well. This denomination does not reflect the presence of shell sand or the extra weight that it carries, thus biasing the results slightly. Previous studies with the current soil measures the organic carbon content in the low pH (unlimed) soils and high pH (limed) soils to be ~ 49% and ~40%, respectively (Liu, *et al.*, 2010). Although the soil samples were collected at different times, the organic content of the soils are unlikely to have changed drastically in a period of a few years. Hence, these figures may be taken to show that the shell sand greatly dilutes the w/w percentage of organic carbon content in the soil. Thus, this author recommends that future studies of this nature use the denomination "per organic carbon content" or something similar, which more accurately reflects the microbially active part of the soil.

4.3. Denitrification gene expression patterns

With the success of a newly developed TNA extraction procedure, the method was put to use in extracting nucleic acids from the high and low pH peat soil samples. Both soils were known to contain inhibitory compounds, and required specially optimised methods or kits to extract useable nucleic acids. Nucleic acids from the high pH soil had previously been extracted successfully in our laboratory group using an optimised version of the TEM (Liu, *et al.*, 2010). In contrast, RNA (both rRNA and mRNA) from the low pH soil had never before been extracted successfully and reverse transcribed to useable cDNA – there was always leftover amplifiable DNA in the RNA from the digestion (Binbin Liu, pers. comm.). Also, the DNA extracted from the low pH soil typically required one to two rounds of clean-up using one or more commercially available clean-up kits, and often required further dilution before the DNA was amplifiable. The dilution has so far been assumed to play a role in diluting out the effect of the inhibitor compounds. These two soils were chosen to represent two "difficult to extract TNA" soils, with the latter low pH soil considerably more difficult than the other high pH soil. To test the robustness of the extraction method to handle different soil types, the procedure was used without further optimisation after the initial development of the method.

4.3.1. Nucleic acid extraction and purification

Nucleic acids were successfully extracted from both soil samples using the NM-OSP extraction method. Relatively large amounts of DNA and RNA were successfully co-extracted from both the high and low pH soils using this method (Section 3.5.4). While the method worked perfectly for all the high pH soil samples, the DNase digestion of the RNA fraction was incomplete for the low pH soil samples (Figure 3.17). The reason for this still remains unknown; since the original residual DNA amplification tests for all the acidic soil samples were negative (Figure 3.15). Current evidence points towards incomplete DNA digestion during the extraction and purification procedures, and that the remaining inhibitory compounds inhibited the residual DNA amplification test (with primers targeting the 16S rRNA gene) that was performed immediately after the original extraction procedure, giving a false negative (Section 3.5.4). The inhibitory compounds were then degraded or rendered ineffective sometime during storage, thus allowing later amplification with the same primers to proceed, inhibitor-free (Section 3.5.6).

The alternative explanation of large-scale contamination of samples with DNA due to technical error is unlikely, especially since the high pH soils' RNA fractions remain contaminant-DNA-free even after repeated handling. The possibility of accidental introduction of reverse transcriptases is even less plausible than a mass introduction of contaminating DNA. Additionally, the contaminating DNA was positive for the functional denitrification genes tested, *nirS* and *nosZ*. Given the low occurrence of 5-10% of these functional genes in the environment, it is doubtful that random external DNA contamination would result in amplifiable denitrification genes (Gamble, *et al.*, 1977, Henry, *et al.*, 2006).

Thus, what remains is the puzzling cause of this unexplained sudden gain in amplifiability of residual DNA in such a short time, and during storage at such low temperatures. Currently, evidence appears to imply that the inhibitory compounds present in the soil samples degraded relatively quickly, allowing the previously unamplifiable DNA fractions to regain their amplifiability in less than 5 days, during storage at -80°C. Inhibitory compounds such as humic substances are known to be extremely resistant to degradation even by enzymes, and the probability of such inhibitory compounds degrading at such low temperatures without enzymatic aid seems even less plausible. However, this appears to be the most logical explanation. If it is true, this opens up possibilities for obtaining inhibitor-free samples with minimum fuss, and this author recommends testing this hypothesis and the possibility of allowing the natural degradation of inhibitory compound at -80°C prior to digestion with DNase enzymes or further nucleic acid purification procedures. Another option may be to perform a second DNA digestion the sample with DNase again, to see if the sample remains DNA-free after the second digestion.

4.3.2. Expression patterns in high pH soils

DNA digestion problems aside, this study has successfully shown that the NM-OSP is a faster method than the TEM extraction procedure, and yields higher quantities of DNA and RNA, when working with non-ideal experimental samples. Although the RNA fraction of the low pH soil was not useable in downstream processes, it was a definite improvement from not having been able to extract RNA from the soil samples at all. Additionally, the method has proven to be extremely effective with the difficult but less troublesome high pH soil, requiring no further purification or clean-up aside from the OSP kit (Figure 3.15). Furthermore, the results from the current experiment and using the NM-OSP method shows

similar expression patterns as have been observed previously in the same high pH soils where the TNA were extracted using the TEM (Liu, *et al.*, 2010). This proves that the NM-OSP method is at least equal to the TEM procedure, yielding similar results.

Excitingly, while both the TEM and the current method yielded similar expression patterns, the number of mRNA copies extracted and reverse transcribed with the NM-OSP method were much higher, nearly 100 times and 200 times for the *nirS* and *nosZ* genes, respectively (Liu, *et al.*, 2010). In addition, the expression levels fell to nearly zero in the previous study, whereas it was maintained well above detectable levels in the current study (Figure 3.16). These differences may be due to the different sampling or storage times of the soil, or an effect of the different TNA extraction methods used. Further comparisons need to be performed on identical soil samples in order to confirm or disprove the higher sensitivity of the NM-OSP method when compared to the TEM for low copy numbers of selected genes. Extraction methods that lead to higher sensitivity of downstream processes are highly desirable, especially in studies on the rare microflora of microbial communities.

4.3.3. Implications of DNA-contaminated RNA

At first glance, finding amplifiable DNA in the RNA faction appears to have rendered all the data useless. However, if there were indeed high amounts of amplifiable DNA in the cDNA fraction as well, the cDNA copy numbers should have been much higher than the present near negligible amounts (Figure 3.16-17). Additionally, if the digestion was as random as it appeared to be, then there should have been unexplained spikes in the number of copies of cDNA present in the samples, instead of the low background fluctuation currently observed in Figure 3.16. Furthermore, any contaminating DNA would contribute to, and not remove from, the copy numbers measure in the qPCR, thus resulting in higher background levels but a useable expression pattern (Figure 3.17). Thus, this author believes that while the background levels of mRNA present may be exaggerated due to the presence of residual DNA, the lack of any observable peak in *nirS* and *nosZ* expression is a true portrayal of the expression patterns in the soil. This is further backed up by the results from the high pH soil, showing that the DNA-free cDNA copy numbers were much higher than the DNA-contaminated cDNA from the low pH soil (Figure 3.16).

4.3.4. Functional gene primers used in this study

In the current study, only primers targeting the *nirS* and *nosZ* genes were used. This is partly because this is an exploration study, and there has been no available information on the expression patterns of denitrification genes of this low pH soil. Thus, two functional genes coding for the NIR and N_2OR enzymes were chosen for this study, as they had previously given strong responses in previous experiments with the high pH soil (Liu, *et al.*, 2010).

As mentioned in the Introduction (Section 1.3.2), the NIR enzymes are more complicated to analyse because the two different enzymes, *nirK* and *nirS*, have evolved independently, and there are no universal primers to capture all nitrate-reducing organisms (Zumft, 1997). Additionally, due to our previous success with amplifying the *nirS* gene and the unspecific amplification of the *nirK* gene during amplification reactions, only the *nirS* gene was analysed in this study (Liu, *et al.*, 2010). However, results indicated that there was almost no peak in *nirS* expression in low pH soils. Given that nitrite reduction was observed, this means that either there was a constant production and maintenance of NIR enzymes, or the expression of NIR was not captured with the present methods. The first reason does not seem likely, since it would be a very costly burden for nitrite-reducers to constantly synthesise and sustain enzymes that may not be useful. Thus, it is more likely that the current methods, in particular the primers, are not suitable for capturing the expression of *nirS* in low pH soils.

Unfortunately, most known denitrifier sequences are from the phylum *Proteobacteria* and, for both the *nirK* and *nirS* genes, the most widely-used primers are designed to target conserved sequences in these known denitrifiers (Braker, *et al.*, 1998, Hallin & Lindgren, 1999, Michotey, *et al.*, 2000, Throbäck, *et al.*, 2004). However, even within the *Proteobacteria*, one of the model denitrifying organisms, *Bradyrhizobium japonicum*, has an 'unamplifable' *nirK* gene despite having a near-perfect match for the primers targeting the *nirK* region, based on CLUSTAL alignment using sequences available on GenBank, (Throbäck, *et al.*, 2004). As such, the poor performance of *nirK* primers and the heavy *Proteobacteria* bias may be partly to blame for the established assumption that *nirS* plays a stronger role than *nirK* (Gamble, *et al.*, 1977, Coyne, *et al.*, 1989). Since there is the possibility that the low pH soil used in this study may not favour *Proteobacteria* denitrifiers, one may hypothesise that either the soil used in this study has non-*Proteobacteria* nitrite-reducers, or that the soil favours nitrite-reducers carrying the *nirK* gene instead. The latter hypothesis is further supported by recent research showing that NirK enzymes may be

favoured over NirS in certain environments (Maeda, *et al.*, 2010). In light of this, the original hypothesis in our research group that '*nirS* plays a stronger and dominant role than *nirK* and is dominant in soils' has been revised to include the possibility of a larger role played by *nirK*-containing microorganisms in low pH soils. This strongly highlights the importance of analysing these soils in the future for the presence and expression of *nirK*. Additionally, this problem of *Proteobacteria* bias is not unique to the NIR enzyme, and applies to many of the primers targeting denitrification genes. Thus, this author proposes the use of multiple primers for all denitrification enzymes when possible, in order to obtain a more complete picture of the denitrifying microbial community.

The primers targeting the *nosZ* gene, ZF and 1622R, are widely used in similar denitrification studies, and are effective for the amplification of *nosZ*-positive denitrifiers (Throbäck, *et al.*, 2004). However, similar to primers targeting the *nirS* and *nirK* genes, the *nosZ* primers were designed using *Proteobacteria* species, and it is known in the literature that these primers are generally unable to pick up non-*Proteobacteria* N_2O reducers (Kloos, *et al.*, 2001, Jones, *et al.*, 2011, Jung, *et al.*, 2012). Thus, even though the current study finds no *nosZ* expression with the current primers, it is plausible to hypothesise that it is the inability of the primers to pick up the entire N_2O-reducing microbial community, rather than a true lack of detectable *nosZ* expression. As was suggested above for the *nirK and nirS* genes, the design and use of primers specifically targeting conserved *nosZ* sequences in non-*Proteobacteria* phyla, for example *Firmicutes* or *Bacteroidetes*, will be essential in future studies of these soils (Jones, *et al.*, 2011, Jung, *et al.*, 2012).

Thus, the current study's results do not give evidence of the lack of expression of genes for NIR and N_2OR enzymes in low pH soils, but instead indicate the presence of a very different denitrifying community in acidic soils, possibly members of phyla other than *Proteobacteria*. This is further strengthened by the fact that nucleic acid extraction from inhibitor-rich low pH soils is not easy, and that the primers designed from available sequences tend to be those that were isolated in higher pH environments.

4.3.5. Efficiency of qPCR

In the current study, the efficiencies of the qPCR runs were relatively low, and may have been due to the plasmid constructs. Repeated tests using constructed plasmid DNA standards

with primers targeting the *nosZ* gene gave efficiencies of between 81% and 84%. Similar tests with primers targeting the *nirS* gene and their respective plasmid gave efficiencies of between 72% and 79%. Reactions with primers targeting the 16S rRNA gene were even worse, at < 70% efficiency. Similarly low efficiencies have also been recorded for primers targeting the same denitrification genes by Hamonts and colleages (2013), where efficiencies for primers targeting the *nirS* and *nosZ* genes were 76% and 84%, respectively. However, the low efficiencies observed in this study are suspected to be due to the age of the plasmid constructs used as DNA standards when performing qPCR. Due to insufficient time, this was not rectified in this study, but this author recognises and recommends the need for new plasmids to be constructed prior to future qPCR analyses.

Additionally, it was unknown in this study if there were leftover inhibitors in the extracted NA samples, further reducing the efficiency of the qPCR. As mentioned in the Introduction, qPCR analyses are generally normalised by the efficiencies calculated from the plasmid standards, and are assumed to be similar across the samples (Ruijter, *et al.*, 2009). While normally not a poor assumption, this author recognises in retrospect that PCR efficiencies are unlikely to be similar for the amplification of plasmids from pure cultures and extracted nucleic acids from inhibitor-rich soil samples. Thus, rectification of such false assumptions and correction of errors is required for inhibitor-rich samples such as those in this study. A number of methods have been suggested in the literature, including the intentional 'spiking' of samples with λ-bacteriophage DNA as internal standards, but many of these methods involve sacrificing already precious-little nucleic acid samples (Beller, *et al.*, 2002). This author believes that the most suitable alternative is to use a mathematical algorithm that allows for the estimation of individual sample PCR efficiency rates using already available qPCR data, as in the case of reanalysing samples with the LinRegPCR program (Ruijter, *et al.*, 2009, Töwe, *et al.*, 2010). Due to time constraints, qPCR data in this study is presented using baseline estimations from the software that comes with the qPCR machine, and assumes a standard PCR efficiency, based on the known DNA standards. However, future reanalysis of the current data with the LinRegPCR program is possible, and would reflect more accurate quantities and PCR efficiencies. Additionally, the PCR efficiencies would also inversely reflect the amount of inhibitors remaining in the samples, allowing a more quantitative method to analyse the effectiveness of inhibitor removal, rather than the current 'positive-negative amplicon' analysis.

4.3.6. Future improvements

One of the more pressing issues to confirm is the denitrification expression in high and low pH soils. Due to time and reagent constraints in the current study, technical replicates during TNA extraction were sacrificed in favour of extraction from multiple time points for each soil. Thus, while the peaks in *nirS* and *nosZ* expression in high pH soils are indisputable, the two subsequent minor peaks at hour 15 and hour 21 are highly suspect, and are either due to the extraction procedure or flask-to-flask variations (Figure 3.16). Additionally, the single time point spike seen in the low pH soil at 0 hour is only one point and may be considered an anomaly. Although the single data point is made up of technical replicates, the data comes from a single flask and a single time point. As such, it is unknown if the flask itself should be considered an outlier or if it is true peak. This may be easily confirmed in the future by re-extracting the TNA in triplicates from the soils, which are currently stored at -80°C, but was not repeated in this study due to the aforementioned constraints.

Another possibility to look into is more frequent sampling of each flask, allowing closer analysis of the apparent peak expression. This is because the peak in expression may last for a shorter time than the current sampling frequency of three hours, and sampling on either side of a true expression peak may lead to false conclusions. Current research monitors gene expression every two hours or more, and we do have no reason to expect quicker bursts of expression for denitrification genes due to its relatively slow response (Saleh-Lakha, *et al.*, 2009, Henderson, *et al.*, 2010). This is different from quick-responding non-denitrification genes such as those controlled by the presence of superoxides that respond in a matter of minutes (Blanchard, *et al.*, 2007). Even with the proposed hourly measurements, there will be equipment and manpower difficulties to overcome, since this would require sampling the flasks prior to complete evacuation, and there will be low amounts of oxygen left in the system. However, it may be a necessity to try such sampling methods in order to gain a more complete picture of the denitrification microbial community's immediate response to anoxia.

A more difficult problem to address is the base unit for NA quantities. A variety of units have been used, from "per gram of soil" to "per copy of 16S rRNA", each justified with their own advantages (Bergaust, *et al.*, 2008, Liu, *et al.*, 2010). The current study expresses nucleic acid concentrations with the "per gram of soil" denomination, in order for direct comparison with a previous study (Liu, *et al.*, 2010). However, this is likely to bias the high pH soils unfairly, since a large amount of the soil is comprised of inorganic shell sand. Thus, future

concentrations may be more favourably expressed as "per organic carbon content", as pointed out in Section 4.2.4. Although this author recognises that it may be most useful to express mRNA quantities as "per copies of cDNA", it is unfortunately not possible with currently available methods. There is no reliable quantification of cDNA (which are ssDNA), and the only available methods are subtractive. Current ssDNA measurement kits measure the TNA present, which means that all ssDNA, dsDNA and RNA are measured in the sample. As such, multiple measurements will be required to quantify ssDNA in each sample – dsDNA and RNA will have to be measured concurrently for subtractive purposes. Thus, one sample will have to be measured thrice in order to obtain ssDNA quantities: ssDNA measurements subtracting the dsDNA and RNA values would give the real ssDNA values. Unfortunately even then, the calculated ssDNA may not reflect cDNA numbers, if there are other sources of ssDNA present in the sample. Thus, it is the opinion of this author that the base unit used to express mRNA copy numbers depends on the questions asked in each study and is freely changeable, so long as justifications can be made as to the choice of unit.

4.4. Future directions

Aside from the previously mentioned improvements that may be made in future studies, the following are recommendations for future directions of the current study.

4.4.1. Non-sudden anaerobisation

An interesting branch of study to explore would be the non-sudden anaerobisation of soils. Currently, experimental data appears to suggest that NO and N_2O are the main gases produced by the currently used low pH soil, confirming field measurements from this plot that also show high levels of N_2O production. However, the current laboratory-based experiments simulate a sudden influx of carbon into the system (clover addition), followed three days later by a very sudden and quick complete anaerobisation, bringing the O_2 levels down to negligible levels within 10 to 15 minutes. In real world conditions, such sudden anoxic spells are highly unlikely to happen, let alone exactly three days after a sudden high carbon influx. Instead, a more realistic circumstance would be a slow decline in O_2 levels, creating a relatively, but not completely, anaerobic environment after some time. This would simulate real-world scenarios that are more directly relatable to field conditions and help to address questions asked by soil scientists, thus encouraging interdisciplinary collaboration.

4.4.2. Carbon addition to soils

In the current study, powdered clover was added according to the wet weight of the soils, which meant that the high pH soils had probably received more carbon (clover) per organic carbon present in the soil, due to the additional shell sand weight. This may have exaggerated the disparity of available carbon between the high and low pH soils, creating an even wider difference in the observed denitrification process. This argues for the flushing of equal quantities of carbon and nitrogen through the soil before each experiment, to ensure homogeneous starting conditions for each soil (Section 4.2.4). However, despite possibly offering deeper insights into the denitrification mechanisms of the soil microbiota, such ‘levelling of the playing field’ would further remove the laboratory experiment from field conditions. As such, it is in this author’s opinion that the choice to add clover and/or flood and flush soils with carbon and/or nitrate solutions is entirely dependent upon the question

asked and the type of study one undertakes. If one chooses to identify factors contributing to high N_2O emissions in the field, powdered clover may be more suitable. But if one chooses instead to investigate the denitrification potential of the soil microflora regardless of field conditions, then flushing the soil with a solution containing carefully controlled amounts of carbon and nitrate may be more suitable.

If after considering the pros and cons of each method, clover is still perceived to be the carbon source of choice, it may be necessary to measure the carbon content in soils before and after clover addition. Comparing the carbon use with non-addition negative controls would potentially allow one to determine the level of carbon starvation the soil microbial community actually faces in the natural environment.

Another point to note is that while clover is a more natural and likely source of carbon into the system than glutamic acid or simple sugars, the effect of different carbon sources remain unknown. Future studies should thus be undertaken to analyse how the different members of the soil microbial community respond to different carbon sources, or the lack of carbon addition.

4.5. Conclusion

This study has successfully developed a method, NM-OSP, for the extraction of TNA from high and low pH peat soils. While further work needs to be done to obtain DNA-free RNA fractions from low pH soils, all DNA isolated have been found to be of good and amplifiable quality. Using the NM-OSP, mRNA was obtained from high pH soils, reverse-transcribed and quantified in a real-time PCR. Moreover, the results were found to correspond with an earlier study performed using the TEM, with evidence that the NM-OSP is even more efficient at TNA extraction than the TEM. In addition, although the cause remains unclear, gas analysis of anaerobised soils indicated that complete denitrification to dinitrogen gas (N_2) is possible in low pH soils without externally altering the pH of the soil. In conclusion, although the underlying genetic mechanisms are not entirely known, the results of this study have indicated the effect of DRP on NOx gas production, and helped to reveal the potential of low pH soils in performing complete denitrification to N_2.

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Appendix

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A1. Supplementary materials and methods information

A1.1. Non-mechanical laboratory equipment

Equipment	Supplier
100 µL glass syringe	SGE Analytical Science, VIC, Australia
120 mL air-tight glass serum flasks	Matriks AS, Oslo, Norway
2 mL screw-capped microcentrifuge tubes	SARSTEDT AG & Co., Nümbrecht, Germany
Aerosol Resistant Tips (ART, various sizes)	Molecular Bioproducts, CA, USA
Aluminium crimp	Matriks AS, Oslo, Norway
Butyl-rubber septum	Matriks AS, Oslo, Norway
Disposable centrifuge tubes (various sizes)	Greiner Bio-One GmbH, Frickenhausen, Germany
G2 lysis beads	MO BIO Laboratories, CA, USA
Glass balls 2.5-3.5 mm	VWR International, PA, USA
Glass beads 0.1-0.11 mm	B. Braun Medical AG, Melsungen, Germany
Glass beads ca. 1 mm	Glaswarenfabrik Karl Hecht GmbH & Co KG – "I", Sondheim / Rhön, Germany
Glass laboratory bottles	DURAN Group GmbH, Wertheim/Main, Germany
MicroAmp Fast 96-Well Reaction Plate with Barcode	Applied Biosystems, Life Technologies, CA, USA
MicroAmp Fast Reaction Tubes	Applied Biosystems, Life Technologies, CA, USA
MicroAmp Optical 8-Cap Strip	Applied Biosystems, Life Technologies, CA, USA
MicroAmp Optical Adhesive Film	Applied Biosystems, Life Technologies, CA, USA
Microcentrifuge tubes (various sizes)	Axygen Inc., CA, USA
Needles (various sizes and lengths)	B. Braun Medical AG, Melsungen, Germany
PlastiPak syringes (various sizes)	BD Medical, NJ, USA
Versi-Dry Lab Soakers	Nalgene Nunc International, NY, USA

A1.2. Laboratory instruments (with affiliated software programs)

Instrument	Manufacturer
2720 Thermal Cycler	Applied Biosystems, CA, USA
CP-4900 Micro Gas Chromatograph	Varian Inc., Agilent Technologies Inc., CA, USA
Delta 320 pH meter	Mettler Toledo AG, Greifensee, Switzerland
Drying oven	Termaks AS, Bergen, Norway
FastPrep-24 Instrument	MP Biomedicals, CA, USA
Gel Doc XR system (with Quantity One 1-D Analysis Software, ver. 4.6.7)	Bio-Rad Laboratories, CA, USA
MiniSpin microcentrifuge	Eppendorf AG, Hamburg, Germany
Mini-Sub Cell GT or Wide Mini-Sub Cell gel electrophoresis systems	Bio-Rad Laboratories, CA, USA
Model 200A Chemiluminescence Nox Analyser	Teledyne Instruments, CA, USA
NanoDrop Spectrophotometer ND-1000	Nanodrop Technologies, Thermo Fisher Scientific, MA, USA
Nitric Oxide Analyzer NOA 280i (with NOA Firmware version 3.00)	Sievers Instruments Inc, CO, USA
Plate Spin II centrifuge	Kubota, Tokyo, Japan
PowerPac Basic 300	Bio-Rad Laboratories, CA, USA
Qubit Fluorometer	Invitrogen, Life Technologies, CA, USA
SpeedVac Concentrator	Savant Instruments Inc., NY, USA
StepOnePlus Real-Time PCR System (with StepOne Software v2.0)	Applied Biosystems, Life Technologies, CA, USA
Table Top Micro Refrigerated Centrifuge 3500, rotor RA-2724	Kubota, Tokyo, Japan

A1.3. Chemicals

Chemical	Supplier
10 mg/mL ethidium bromide	VWR International, PA, USA
96% ethanol	Kemetyl Norge AS, Vestby, Norway
Acetic acid	Merck KgaA, Darmstadt, Germany
Calcium chloride, dihydrate ($CaCl_2 \cdot 2H_2O$)	Merck KgaA, Darmstadt, Germany
Chloroform	Merck KgaA, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Sigma-Aldrich, MO, USA
Hexadecyltrimethylammonium bromide (CTAB)	Merck KgaA, Darmstadt, Germany
Isoamyl alcohol	Merck KgaA, Darmstadt, Germany
Isopropanol	A/S Vinmonopolet, Oslo, Norway
Phenol	Sigma, Sigma-Aldrich, MO, USA
Polyethelene glycol (PEG) 6000	Merck KgaA, Darmstadt, Germany
Polyvinylpolypyrrolidone (PVPP)	Sigma, Sigma-Aldrich, MO, USA
Potassium nitrate (KNO_3)	Merck KgaA, Darmstadt, Germany
SeaKem LE agarose	Lonza, ME, USA
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 ($NaNO_2$)	Merck KgaA, Darmstadt, Germany
Sodium phosphate dibasic (Na_2HPO_4)	Merck KgaA, Darmstadt, Germany
Sodium phosphate monobasic (NaH_2PO_4)	Merck KgaA, Darmstadt, Germany
Trizma base	Sigma, Sigma-Aldrich, MO, USA

A1.4. Experimental reagents

Calcium chloride (0.01 M)

- 0.3676 g $CaCl_2 \cdot 2H_2O$ (calcium chloride, 4ehydrate)
- 250 mL MilliQ water

Chloroform:isoamyl alcohol (24:1)

- 48 mL chloroform
- 2 mL isoamyl alcohol

Ethylenediaminetetraacetic acid (EDTA, 0.5 M), pH 8.0

- 186.1 g EDTA
- NaOH (sodium hydroxide) pellets and 5 M NaOH to pH 8.0
- MilliQ water to 1 L

Ethanol, 70%

- 73 mL of 96% ethanol
- 27 mL of MilliQ water

Hexadecyltrimethylammonium bromide (CTAB) extraction buffer

- 50 g CTAB
- 250 mL 0.7 M NaCl (sodium chloride)
- 250 mL 0.24 M phosphate buffer (pH 8.0)
- 5 g polyvinylpolypyrrolidone (PVPP)

KNO_3 (36 mM)

- 0.3640 g KNO_3 (potassium nitrate)
- 100 mL MilliQ water

Phosphate buffer (0.24 M), pH 8.0

- 189.4 mL 0.2 M Na_2HPO_4 (sodium phosphate dibasic)
- 10.6 mL 0.2M NaH_2PO_4 (sodium phosphate monobasic)
- 200 mL MilliQ water

Sodium iodide in acetic acid (1% w/v)

- 12.5 mL acetic acid
- 12.5 mL MilliQ water
- 0.25 g NaI (sodium iodide)

TAE, 50x *(for gel electrophoresis)*

- 242 g Tris base
- 57.1 mL acetic acid
- 100 mL 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0
- MilliQ water to 1 L

30% polyethylene glycol (PEG) 6000

- 30 g PEG 6000
- 100 mL MilliQ water

A1.5. Commercially-available products, kits and accessories

Nucleic acid extraction kits

Product	Manufacturer
MasterPure RNA Purification Kit • MPC Protein Precipitation Reagent • 1X Dnase Buffer • Rnase-Free Dnase I • 2X T and C Lysis Solution	Epicentre Biotechnologies, WI, USA
PowerMicrobiome RNA Isolation Kit	MO BIO Laboratories, CA, USA
PowerSoil DNA Isolation Kit	MO BIO Laboratories, CA, USA
RNA PowerSoil DNA Elution Accessory Kit	MO BIO Laboratories, CA, USA
RNA PowerSoil Total RNA Isolation Kit	MO BIO Laboratories, CA, USA

DNase I

Product	Manufacturer
DNase I Amplification Grade	Sigma, Sigma-Aldrich, MO, USA
RNase-free DNase Set	QIAGEN, North Rhine-Westphalia, Germany

Clean up kits

Product	Manufacturer
Genomic DNA Clean & Concentrator Kit	Zymo Research, CA, USA
OneStep PCR Inhibitor Removal Kit	Zymo Research, CA, USA
RNA Clean & Concentrator-5 Kit	Zymo Research, CA, USA

Amplification and reverse transcription

Product	Manufacturer
High Capacity RNA-to-cDNA Master Mix	Applied Biosystems, Life Technologies, CA, USA
Recombinant *Taq* DNA Polymerase *TaKaRa Taq* • *TaKaRa Taq* (5 U/µL) • 10X PCR Buffer • dNTP Mixture (2.5 mM each)	Takara Bio Inc, Shiga, Japan
SuperScript VILO MasterMix	Invitrogen, Life Technologies, CA, USA
SYBR *Premix Ex Taq* II (Tli RNaseH Plus) • SYBR *Premix Ex Taq* II • ROX Reference Dye (50X)	Takara Bio Inc, Shiga, Japan

Miscellaneous

Product	Manufacturer
All primers used in this study	Invitrogen, Life Technologies, CA, USA
100 bp DNA Ladder • 6X Gel Loading Dye, Blue • 100 bp DNA Ladder	New England BioLabs, MA, USA
DEPC-treated water	Ambion, Life Technologies, CA, USA
Nuclease-free water	Ambion, Life Technologies, CA, USA
Qubit dsDNA BR Assay kit	Life Technologies, CA, USA
Qubit RNA Assay kit	Life Technologies, CA, USA
RNaseZap	Ambion, Life Technologies, CA, USA

A2. Supplementary results

A2.1. Nitrite measurements

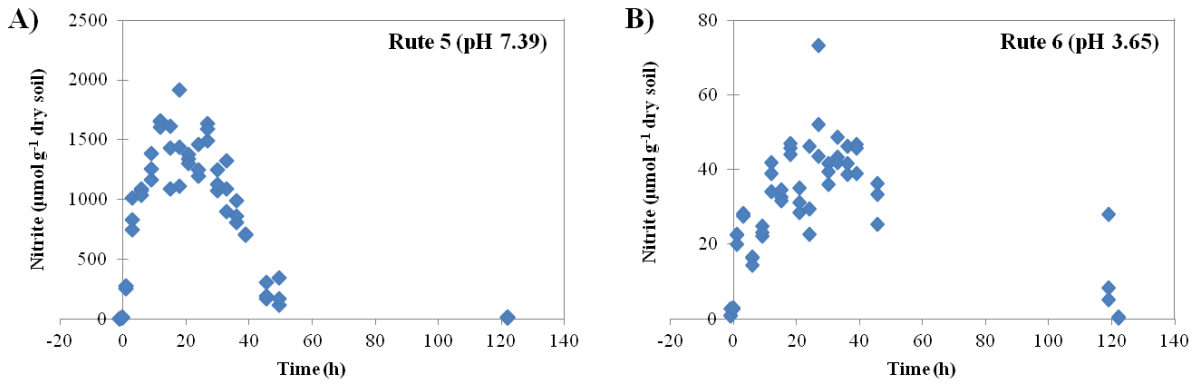


Figure A2.1 Nitrite concentrations measured in parallel flasks. Concentration of nitrite was measured thrice for each flask in high (A) and low (B) pH soils. Variation in nitrite concentration at each time point is believed to be caused by the effect of microsite hot spots.

A2.2. Gas measurements of all flasks

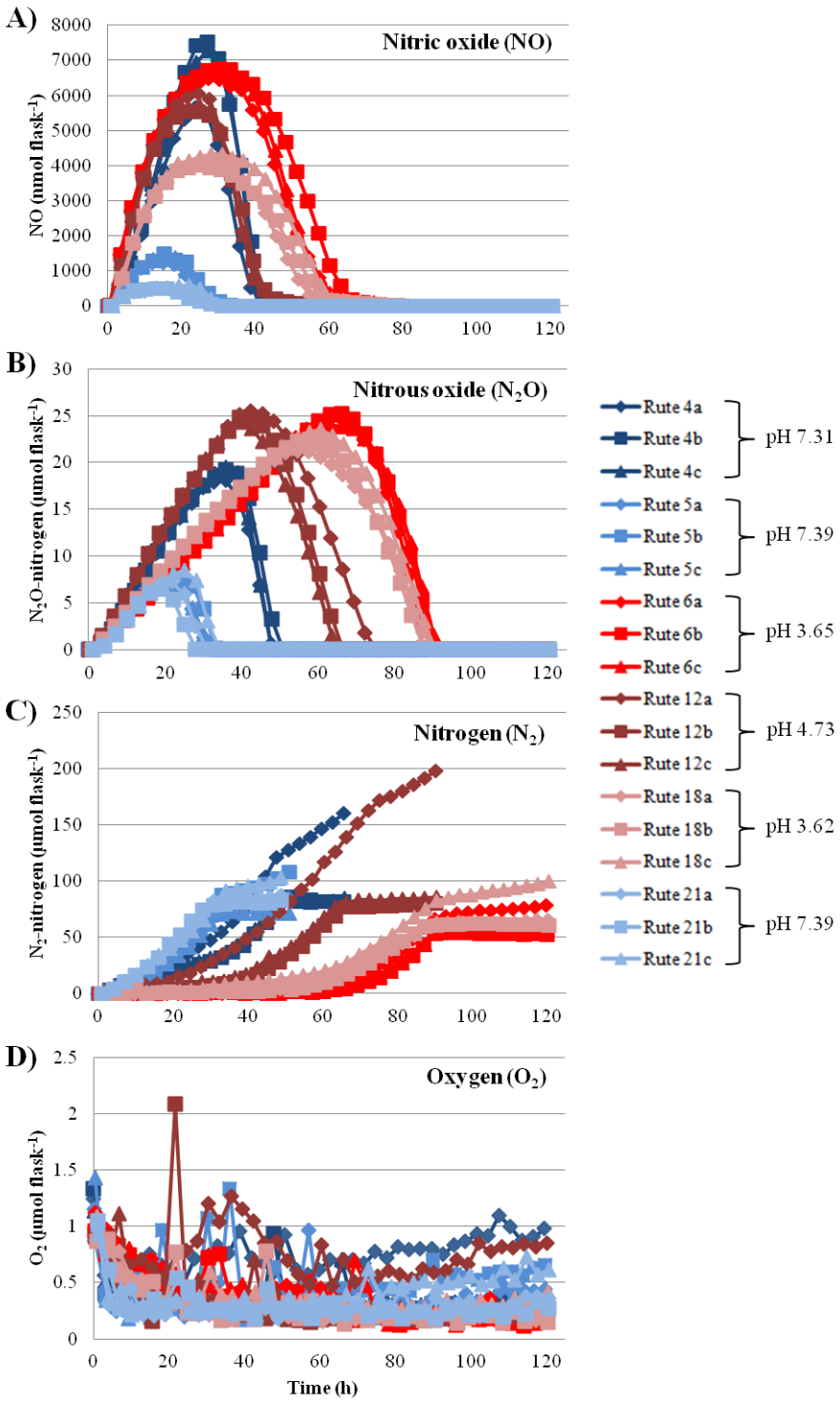


Figure A2.2 Production of NOx gases by different soils in all experimental flasks, as measured by the GC and NOA. The graphs detail the complete gas data from the robotised incubation system, of the gases NO (A), N_2O (B), N_2 (C) and O_2 (D). The grouping of the technical triplicates is close together, showing high repeatability. The flasks show little variation in gas production, although the rate of NO and N_2O use varies slightly amongst the flasks. Flask Rute 4a and Rute 12a had seal problems, with atmospheric N_2 leaking into the flasks.

A2.3. NOx gas production rates

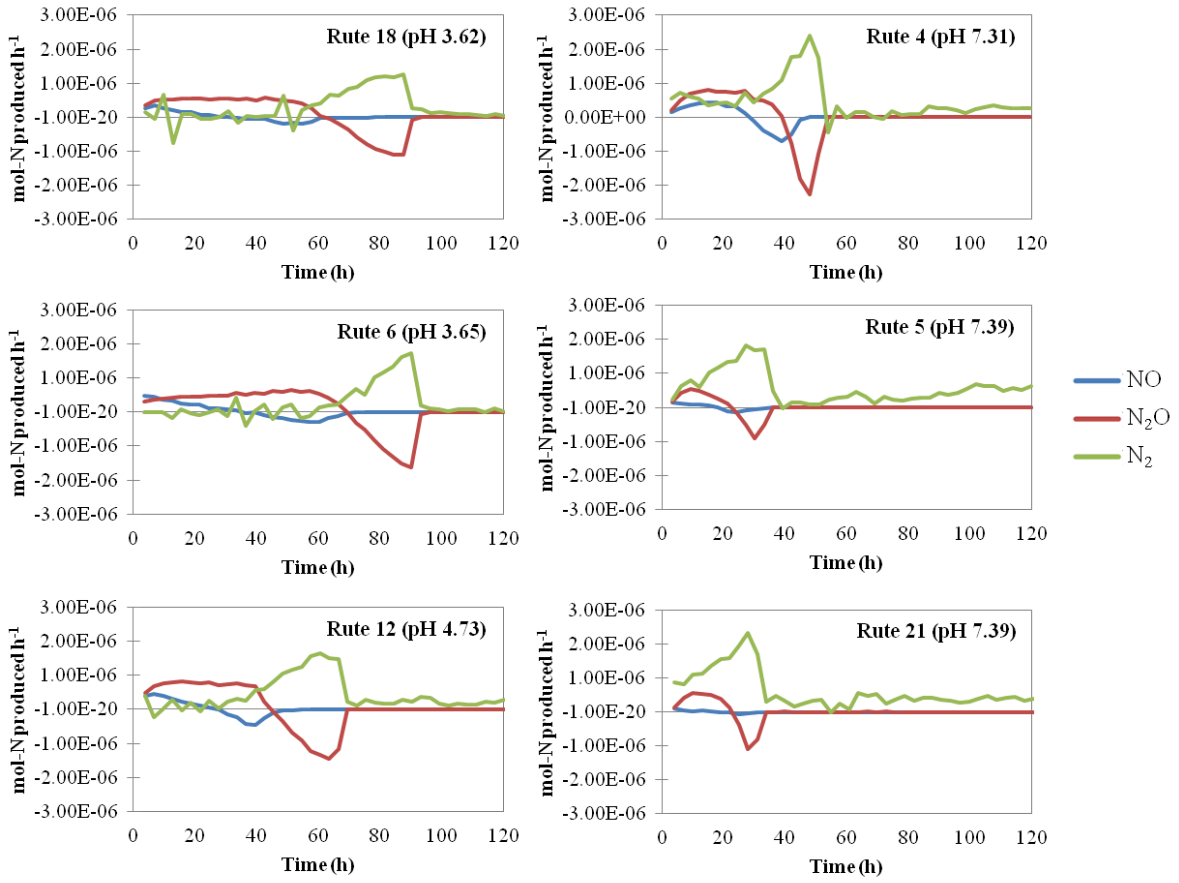


Figure A2.3 Production and utilisation rates of NOx gases in representative flasks. Positive values indicate gas production, negative values indicate net utilisation. The timing of production and utilisation of gases is dependent on the bulk soil pH.

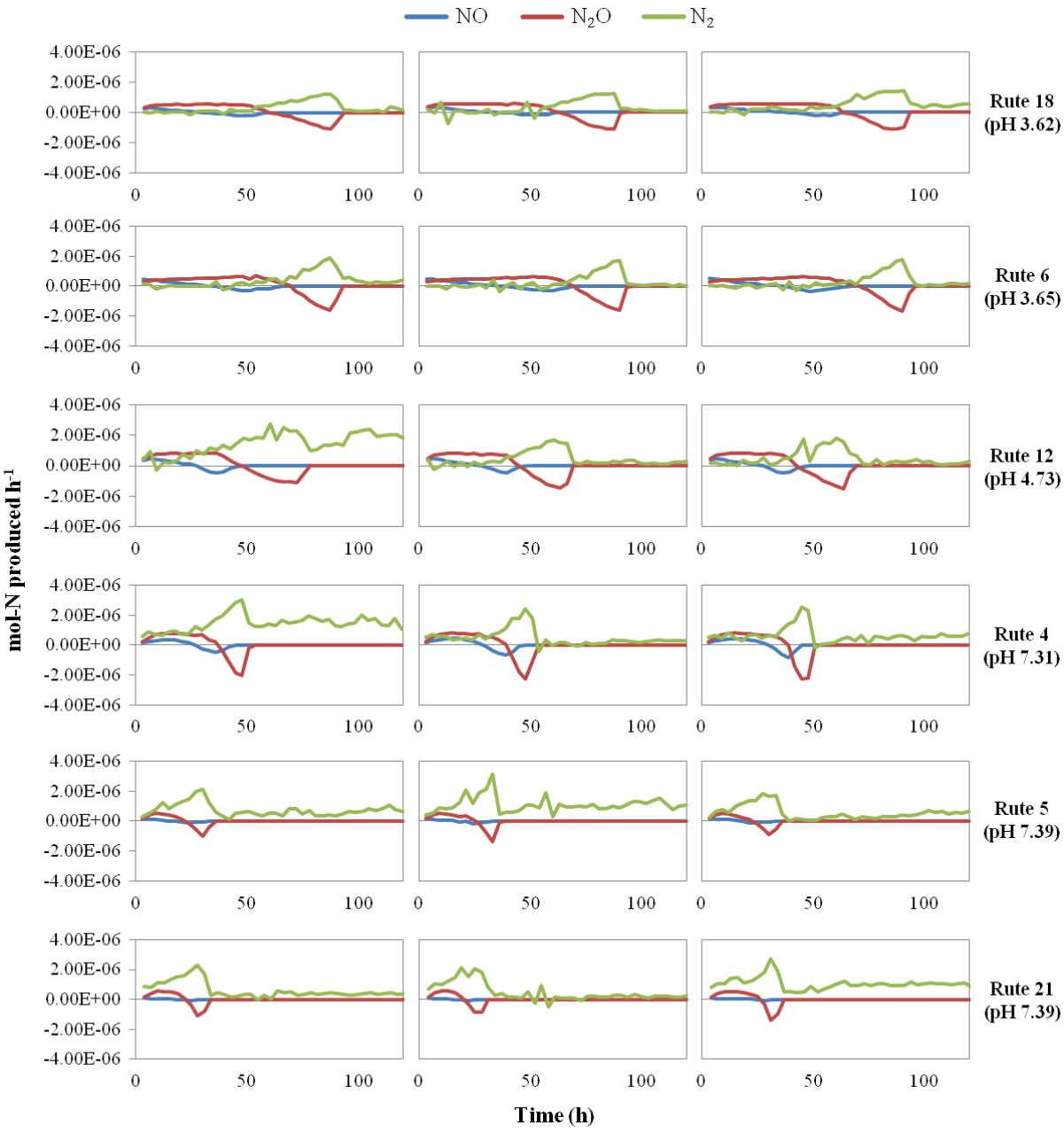
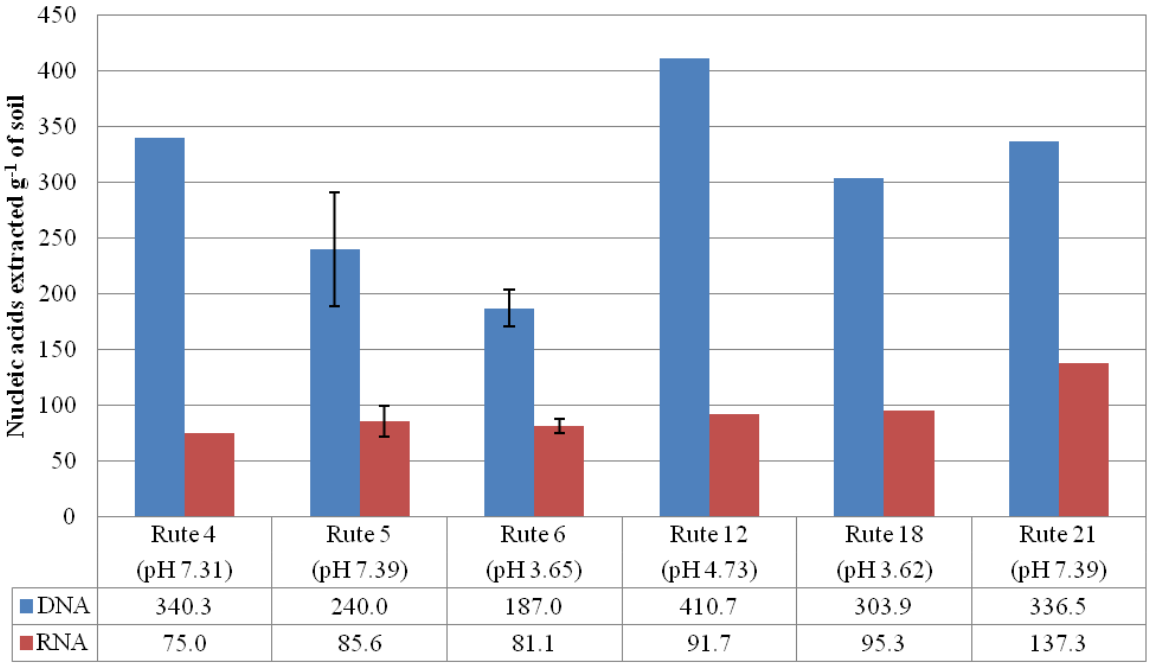


Figure A2.4 Gas production and utilisation directly varies with the pH of the soils: Higher pH equals faster rates. The production and utilisation rates are grouped according to pH, and appear to be more drawn out in lower pH soils. The continuous production of N_2 in all the flasks after a peak in N_2 production may be attributed to atmospheric N_2 leaking into the flasks.

A2.4. Total nucleic acids extracted



	Rute 4 (pH 7.31)	Rute 5 (pH 7.39)	Rute 6 (pH 3.65)	Rute 12 (pH 4.73)	Rute 18 (pH 3.62)	Rute 21 (pH 7.39)
DNA	340.3	240.0	187.0	410.7	303.9	336.5
RNA	75.0	85.6	81.1	91.7	95.3	137.3

Figure A2.5 The amount of nucleic acids extracted from each soil type. Multiple samples were taken only from Rute 5 and Rute 6; all other soils had a single DNA and RNA extraction to serve as reference samples.

~ End of thesis ~

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