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Biological vs. chemical regulation of nitrite concentrations in acidic and neutral pH soils: A study of kinetics and bacterial denitrifier communities

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Sincerely, Heidi Therese Hillier
August 15th 2015

Abstract

Denitrification is an anaerobic microbial respiratory process, which takes part in the global biogeochemical nitrogen cycle. Microbial denitrifiers contribute to the global recirculation of nitrogen, by stepwise reduction of nitrate (NO_3–) to dinitrogen gas (N_2), via nitrite (NO_2^-) and the gaseous intermediate products nitric oxide (NO) and nitrous oxide (N_2O). These gasses have a severe impact on the atmospheric chemistry. NO is involved in the formation of acid rain and of undesired ozone in the troposphere, while nitrous oxide (N_2O) is the third most important greenhouse gas, and also the main destructor of stratospheric ozone. One major controller of denitrification in soil is the pH. Previous studies by NMBU Nitrogen group on Norwegian peat soils showed that NO_2^- concentrations were kept consistently low in low pH soils, while NO_2^- accumulated in neutral pH soils. Inversely, low pH soils accumulated large amounts of gaseous NO and N_2O. While this has largely been attributed to the non-biological reduction of N-compounds to gaseous products, i.e. chemodenitrification, some form of biological control has not been completely excluded. The overarching goal of this thesis was to determine the significance of chemodenitrification compared to the enzymatic reduction of NO_2^- in both acidic and neutral pH peat soils. To gain insight into the community composition of the bacterial denitrifiers, I also quantified genes coding for denitrification reductases.

Agricultural peat was sampled from an experimental site in Fjaler, on the west coast of Norway. I compared the production and consumption/apparent disappearance of NO_2^- and nitrogenous gases in three gamma- irradiated peat samples (pH 3.2, pH 5.2 and pH 7.2), and three non-sterilized peat samples (pH 3.8, pH 5.7 and pH 6.8). Gas kinetics was measured using an innovative robotized incubation system, and an ozone-based chemiluminescence approach was used to measure NO_2^- loss and production in peat. The chemiluminescence method for quantification of NO_2^- ,which has scarcely been used to measure NO_2^- in soils, was found to be a rapid and sensitive method that can measure NO_2^- in the nM range, and arguably more precise than the more commonly used spectrophotometric assays, based on the Griess reaction. The method was therefore successfully used to measure the minute by minute loss of NO_2^- in peat. Accurate gas and NO_2^- data allowed for mathematical modelling of NO_2^- loss in sterilized peat, and further determination of the rate contribution of chemical degradation contrary to the enzymatic reduction of NO_2^- to NO.

I found that partitioning of NO_2^- to peat particles occurs regardless of pH, within one minute of adding NO_2^- to sterilized and non-sterilized peat. Further, results show that NO_2^- protonates in acidic environments to produce mainly NO and some N_2O. By modelling the nitrite kinetic of sterilized peat soils, I determined the rate of chemical decomposition in all peat soils. As expected, chemodenitrification rates were highest in the pH 3.8 peat soils, reducing all added nitrite within 5 hours, whereas nitrite

was stable in pH 6.8 peat soils. Taking these NO_2^- decay rates into account, I found comparable nitrite reductase activities in both neutral and acidic peat soils, and verified that the biological reduction of NO_2^- in acidic peat soils is significant and more important than generally assumed. Gas kinetics from non-sterilized peat showed that acidic peat had a progressive onset of the denitrification gasses (NO, N_2O and N_2), while the pH neutral peat had an immediate production of all denitrification gasses. In accordance with earlier studies, the measurements on the bioactive peat showed that NO_2^- accumulated in the neutral pH peats, but not in the acidic peat. This may possibly be caused by a different regulation of denitrification in response to a strong selective pressure from an acidic environment. The abundance of the genes *nirS* (coding for nitrite reductase) relative to the *16S* rRNA gene copies increased with increasing pH, while the opposite was true for the ratio of the *nosZ*/*16S* rRNA genes. The *nosZ*/*nirS* ratio was thus highest in the low pH soil, suggesting that the delayed reduction of N_2O in the low pH soil was not due to a low genetic potential.

Together, the results of this thesis paint a more complex picture of nitrite interactions in soil than previously hypothesized, where ion exchange, chemodenitrification and denitrification by bacterial communities play important and inter-linking roles in regulation.

Sammendrag

Denitrifikasjon er en anaerobisk mikrobiell prosess som deltar i den globale nitrogensyklusen. Mikrobielle denitrifiserere bidrar ved å stegvis redusere nitrat (NO_3^-) til dinitrogengass (N_2), via mellomproduktene nitrogenmonoksid (NO) og dinitrogenmonoksid (N_2O). Disse gassene er spesielt kjent for å ha negative virkninger på kjemien i Jordens atmosfære. Det er derfor av stor miljømessig interesse å lære mer om utslipp av disse gassene. NO er involvert i dannelsen av surt regn og uønsket ozon (O_3) I troposfæren, mens N_2O er den tredje mest viktige drivhusgassen, men også hoved nedbryteren av troposfærisk O_3. En kontrollerende faktor for denitrifikasjon i jord er pH. Tidligere undersøkelser utført av Nitrogengruppen ved Norges universitet for miljø- og biovitenskap (NMBU) av norsk torvjord viste at NO_2^--konsentrasjoner ble holdt konsekvent lav, i lav-pH jordprøver, mens NO_2^- akkumulerte i nøytral-pH jordprøver. Motsatt, i lav-pH jordprøver ble store mengder gassformet NO og N_2O akkumulert.Selv om dette hovedsakelig har blitt forklart som en ikke-biologisk reduksjon av N-forbindelser (kjemodenitrifikasjon), så har ikke biologisk kontroll blitt helt avskrivet.Hovedmålet for denne oppgaven har vært å bestemme viktigheten av kjemodenitrifikasjon sammenlignet med enzymatisk reduksjon av NO_2^- i både sur- og nøytral-pH torvjordsprøver. For å få bedre forståelse for samfunnsstrukturen til denitrifiserere mikroorganismer, kvantifiserte jeg også gener som koder for denitrifikasjonsreduktaser.

Torvjord fra jordbruk ble samlet inn fra et eksperimentelt felt i Fjaler kommune, på vestkysten av Norge. Produksjonen og et tilsynelatende tap av NO_2^- ble sammenlignet i tre gammabestrålte torvjordsprøver (pH 3,2, pH 5,2 og pH 7,2), og tre ikke-steriliserte torvjordsprøver (pH 3,8, pH 5,73 and pH 6,8). Gasskinetikk ble målt ved bruk av et innovativt, robotisert inkubasjonssystem, laget av Nitrogen gruppen ved NMBU. NO_2^- i torv ble målt ved bruk av en ozon-basert kjemiluminiscens metode, som normalt sett ikke har blitt brukt til slike målinger i jord. Metoden var svært rask og sensitiv, og tapet av NO_2^- kunne der med bli målt minutt-for-minutt, i konsentrasjoner helt ned i nM-området. Kjemiluminiscensmetoden er dermed mer følsom en de mer tradisjonelle spektrofotometriske metodene, slik som Griess-reaksjonen. Nøyaktige målinger av NO_2^- , NO, N_2O og N_2 gjorde det mulig å matematisk modellere tap av NO_2^- i sterilisert torvjord, og dermed bestemme de ratene som kjemiske og enzymatiske prosesser degraderer NO_2^- med.

Jeg har funnet at NO_2^- binder seg til torvjordpartikler via ionebytting, uavhengig av de testede pH verdiene i torvjorden. Videre viser resultatene at NO_2^- protoneres til HNO_2 i surt miljø, som fører til produksjon av NO og noe mindre N_2O. Modellering av kjemiske og mikrobiologiske gassproduksjons- og gass nedbrytningsrater for NO_2^- i både sterilisert og ikke-sterilisert torvjord, viser at det er hovedsakelig enzymatiske reaksjoner som er ansvarlige for produksjonen av NO i både surt og alkalisk miljø. Gasskinetikk viser at sur torvjord har en progressiv utvikling av denitrifikasjonsgasser (NO, N_2O og

N_2, mens alkalisk jord produserer alle gasser med en gang. Det ble også vist at NO_2^- akkumulerer i alkalisk jord, mens konsentrasjoner i sur jord ikke øker. Antall kopier av denitrifikasjonsgenet *nirS* var også funnet til å avta i sur torvjord, relativt til antall kopier av *16S* rRNA genet, mens det motsatte var funnet for antall kopier av *nosZ*. Disse resultatene viser at biologiske prosesser er viktige i sur torvjord, og at det sannsynligvis er en annen form for regulering av denitrifikasjonsreduktaser med hensyn til pH.

Contents

List of Abbreviations

Abbreviation	Explanation
cDNA	Complementary DNA
CTAB	Hexadecyltrimerthylammonium bromide
DNA	Deoxyribonucleic acid
dsDNA	Duble stranded DNA
GC	Gaschromatograph
mRNA	messenger RNA
N_2	Dinitrogen
N_2O	Nitrous oxide
NO	Nitric oxide
NOPS	Nitric oxide purge vessel system
N_2OR	Nitrous oxide reductase
NA	Nucleic acid
NAP	Nitrate reductase (periplasmic)
NAR	Nitrate reductase (membrane bound)
NIR	Nitrite reductase
NO	Nitric oxide
NO_x	Nitrogen oxides (NO and/or N_2O)
NOR	Nitric oxide reductase
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
qPCR	Quantitative PCR; Real-time PCR
RNA	Ribonucleic acid
SB	Sodium Borate Buffer

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Introduction

1.1 The nitrogen cycle

Nitrogen recirculation is one of the largest biogeochemical cycles on Earth. The main pool of nitrogen is found as dinitrogen gas (N_2), and makes up 78 % of our atmosphere. N_2 is an inert molecule due to the triple bond between the two nitrogen atoms, and thus unavailable to most organisms. That is, with the exception of a few specialized microorganisms that can fixate N_2 to form NH_3. Fixated nitrogen is then used in various oxidation and reduction reactions, that gives rise to nitrogen compounds with oxidation states that range from +5 (NO_3^-) to -2 (NH_3). Without this recirculation, nitrogen would never be available to animals, as plants depend on the availability of nitrates to produce biomass (Madsen 2008). Since the industrialization era, humans have contributed the global nitrogen cycle through the use of fertilizers produced by the Haber-borsch process (Gruber et al. 2008). The Harber-borsch method converts N_2 to NH_3, and thus introduces reactive nitrogen which we have used as fertilizers. Although the process has successfully increased food production, it has also caused a doubling of reactive nitrogen species in the biosphere (Vitousek et al. 1997). Additionally, fertilization on agricultural fields have been shown to acidify the soil, which impacts the microbial processes that recirculate nitrogen (Cuhel et al. 2010; Raut et al. 2012). The microbial pathways that are responsible for the recirculation of nitrogen, includes aerobic nitrification, and the four anaerobic pathways: nitrogen fixation, denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonia oxidation (anammox) (Bouwman 1998; Jetten 2008; Kraft et al. 2011) (Fig. 1.1).

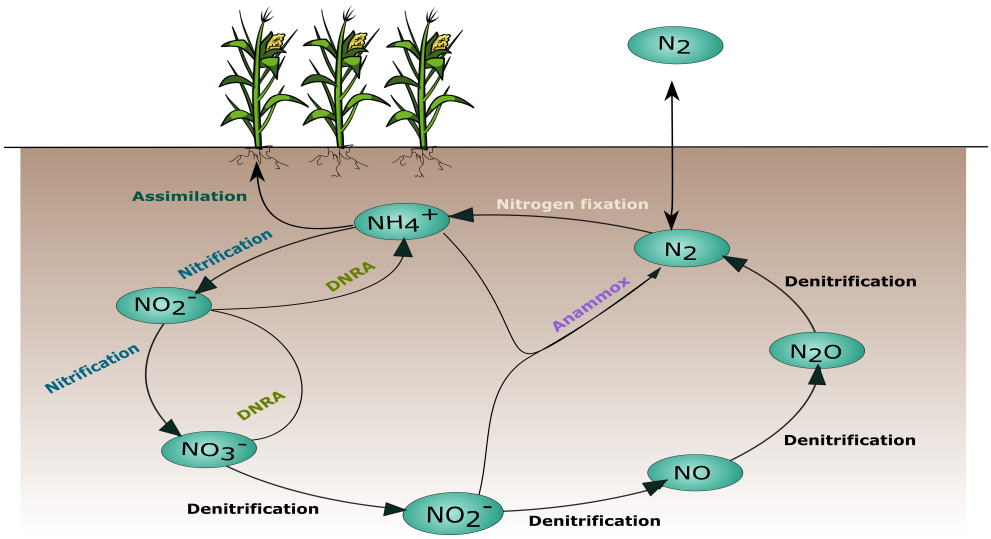


Figure 1.1. Nitrogen recirculation, a simplified illustration. Microbial pathways include nitrification, nitrogen fixation, denitrification, DNRA and anammox

1.2 Environmental impact

Some of the molecules produced through nitrogen recirculation can negatively impact our environment. This particularly concerns nitric oxide (NO) and nitrous oxide (N_2O), which in this thesis will collectively be referred to as to as NO_x. The N_2O is a greenhouse gas that reacts in the second most inner layer of our atmosphere, called the stratosphere. The stratosphere contains the ozone layer which is a thick blanket of mainly O_3 molecules. When N_2O from the biosphere reaches the stratosphere it reacts in two ways: as an ozone depleting radical, and as a major greenhouse gas. N_2O is a very potent greenhouse gas due to it's global lifetime estimated to 131 years (Hartmann et al. 2013). The global warming potential of N_2O is estimated to be 300-fold more potent than that of CO_2 (Bothe et al. 2007; Portmann et al. 2012; Ravishankara 2009). In addition, NO can be oxidized to HNO_2 and HNO_3, that are importnat contributors to acid rain (Bothe et al. 2007).

The main source of anthropogenic NO_x are agricultural soils. It is estimated that 40-60% of the atmospheric N_2O is contributed from soils, and 60% of this fraction a direct result of fertilization (Olivares et al. 2013; Schlesinger 2007). The Intergovernmental Panel on Climate Change (IPCC) estimated that the abundance of N_2O in our atmosphere has increased by 20% since the pre-industrialization period (Hartmann et al. 2013).

The chemistry that surrounds nitrite also has an effect of the troposphere where we find hydroxyl radicals (HO). The HO molecules oxidize greenhouse gasses, and thus reduce the amount of reactive gasses that reach the stratosphere, and the ozone layer. NO_x has also been shown to be a great source of HO radical, which have a positive effect on our environment. However, NO_x also is a source of ozone formation in the troposphere, which is inhaled by humans, and especially an issue in rural, industry-heavy and trafficated areas (Allen 2002). Studies have shown that nitrite that protonates to HNO_2^-, can further produce the gaseous molecule HONO (Equation. C1.1) (Su et al. 2011), and thus be a source of HO radicals .

$$NO_2^-(aq) + H^+(aq) \longleftrightarrow HNO_2(aq) \longleftrightarrow HONO(g) \tag{C1.1}$$

Nitrite in soils thus acts as an addition pathway for gaseous N-compounds to enter the atmosphere, and a strong source of (HO) molecules that react in the lower troposphere. This highlights that non-biological reactions also contribute to the recirculation of nitrogen molecules from soils to the atmosphere (Fig. 1.2) (Su et al 2011).

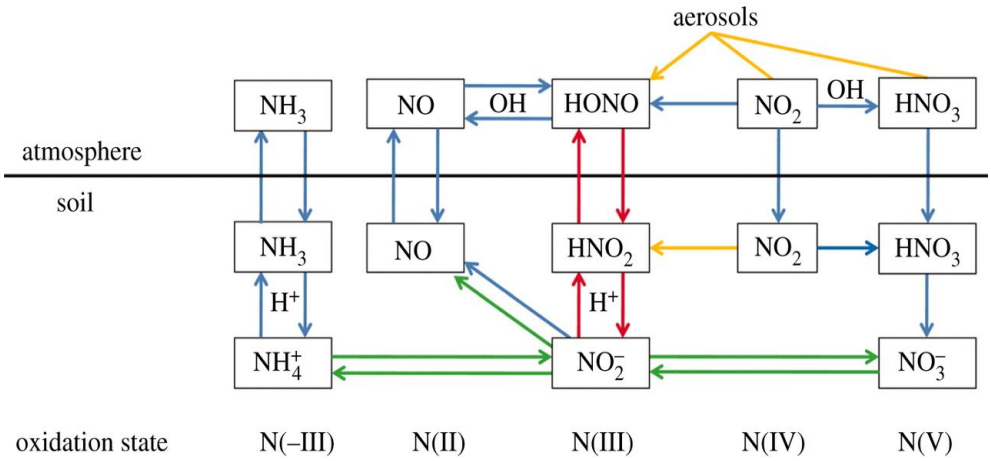


Figure 1.2. "Coupling of atmospheric HONO with soil nitrite. Red arrows represent the multiphase processes linking gaseous HONO and soil nitrite (acid–base reaction and phase partitioning), green arrows represent biological processes, orange arrows represent heterogeneous chemical reactions converting NO2 and HNO3 into HONO and blue arrows represent other related physicochemical processes in the N cycle" (Su et al. 2011)

The NO_x molecules are mainly produced by the two opposing microbial processes: nitrification and denitrification. The terme "hole- in-a-pipe" was coined by Firestone and Davidson [1989], to describe the production and consumption of NO_x. The model states that NO_x "leaks" from both processes, but of the two, denitrification is considered to be a greater cause of NO_x emission from agricultural soil (Bouwman 1998) (Fig. 1.3). The model is a simplification of NO_x production. Each reduction or oxidation step is strictly controlled by enzymes, and some organisms may i.e. lack some of the enzymes required for a full denitrification proteome. Others might for some reason inhibit the expression of nitrogen metabolizing enzymes under certain environmental conditions e.g. start to accumulation nitrite due to only expressing enzymes that reduce NO_3^- to NO_2^- (Liu et al. 2013; Mania et al. 2014). Environmental conditions affect microorganisms differently, and might cause partial expression or unsuccessful folding of proteins, that results in NO_x emissions from soils (section 1.3.4).

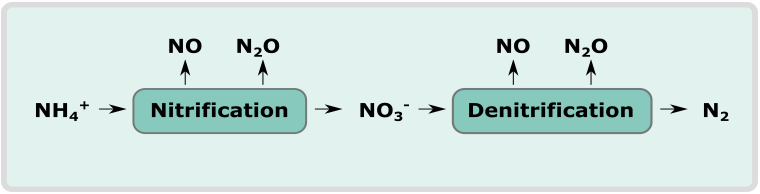


Figure 1.3. Hole-In-A-Pipe model suggest NO and N_2O escape from nitrification and denitrification. Modified from Madsen 2008.

1.3 Denitrification

In agricultural fields, microbial abundance is estimated to 10^{10} bacterial cells per gram soil(Henry et al. 2004). Many of these organisms take part in the global nitrogen recirculation, where denitrification constitutes one of the main groups of organisms that anaerobically recirculate nitrogen. Denitrification is one of the most common pathways within the microbial nitrogen cycle, found within all the domains of life: prokaryotes, archaea, and even eukaryotes, although most denitrifiers are found within the phylum of proteobacteria (Zumft 1997).

The denitrification pathway is an alternative form of respiration, expressed when oxygen levels are low. Nitrogen oxides are then the second most favorable electron acceptors for the generation of energy through the electron transport chain. A molecule with a higher oxidation state is a stronger electron donor, and when oxygen is not present the preferred order of electron donors from nitrogen species becomes: NO_3^- (+5), NO_2^- (+3), NO (+2) and finally N_2O (+1), which can be reduced to N_2 (0) (Fig. 1.4) (Madsen 2008). Denitrification in the strict sense, in the reduction of the ion NO_2^- to the gaseous intermediate NO and N_2O, but still the term "complete denitrification" is therefore used to describe the reduction of NO_3^- to N_2.

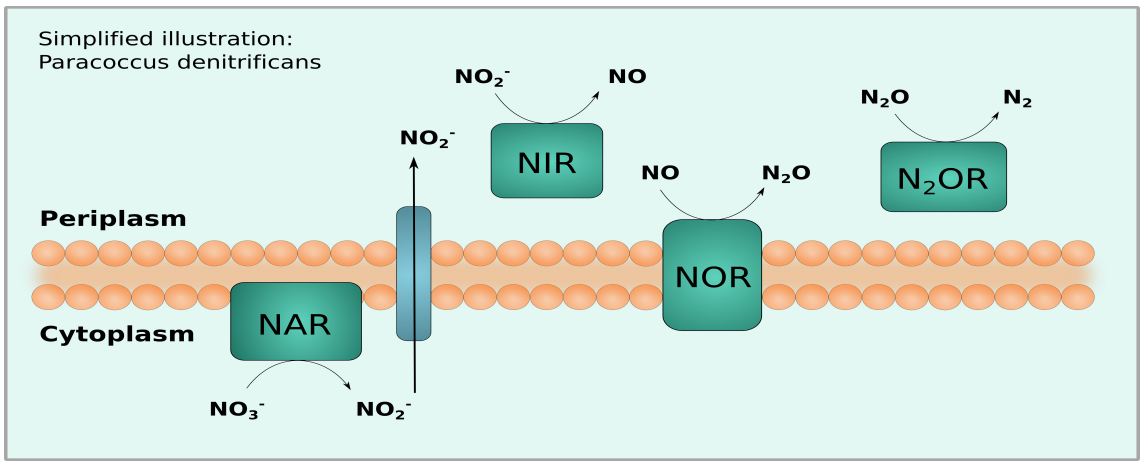


Figure 1.4. Denitrification proteome in *Paracoccus denitrificans* includes NAR, NAP [not shown], NIR, NOR and N_2OR.

Denitrification is found amongst a wide range of bacterial taxa, and amongst others, in the model organisms *Paracoccus denitrificans*. The model organism has the following four categories of reductases: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N2OR). It has two types of NAR encoded by *napA* and *narG* (Fig.1.4) (Kraft et al. 2011)

1.3.1 Denitrification enzymes

The enzymes that catalyze the oxidation or reduction of nitrogen molecules are found in many distantly related taxa of microorganism. To detect denitrifiers in the environment, specific primers targeting NAR, NIR, NOR or N_2OR are commonly used. Universal primers that bind to *16S* rRNA of the prokaryotic ribosome, are a challenge to use - as there are few universally conserved sequences in the gene amongst denitrifiers (Philippot 2005).

Complete denitrification begins with the reduction of NO_3^-. Although *P. denitrificans* is a model organism for the denitrification process, nitrate respiration is well known amongst many non-denitrifying organisms, and particularly well studied in *Escherichia coli* (Zumft 1997). Denitrifiers and nitrate reducers catalyze the reduction of NO_3^- by nitrate reductase (NAR), a membrane-bound complex, which is also found in several non-denitrifying bacteria. The complex consists of three subunits: NarG, NarH and NarI, transcribed from the nar gene cluster. *narG* encodes the active binding site for NO_3^-, and faces the cytoplasm. NAR is expressed anaerobically through transcriptional regulators induced by NO_3^- or NO_2^- (section 1.3.2). Some bacteria either exclusively or additionally have the isofunctional periplasmic Nitrate Reductase complex (NAP) encoded by the *nap* gene cluster. The enzyme consists of two subunits NapA and NapB, where NapA contains the active binding site for NO_3^- (Kraft et al. 2011; Zumft 1997). The Nap complex can be expressed regardless of anaerobiosis and, therefore, serves to produce NO_2^- under aerobic conditions (Philippot 2005). The presence of Nap might help organisms to transition from aerobic to anaerobic respiration.

Two isofunctional yet unrelated NIR enzymes, NirS and NirK, are located in the periplasm of denitrifying bacteria, encoded by the *nir* gene cluster. However, denitrifiers either encode NirS (I and II) or NirK, but never both in the same organism. Although the two enzymes are isofunctional, they are structurally different and contain different prosthetic metals in the active binding site. NirS is homodimeric with cytochrome *cd1* in the active site, while NirK is homotrimeric with copper in the active site (Kraft et al. 2011; Zumft 1997). The regulation and reduction of NO_2^- by NIR is important for organisms, as HNO_2 can diffuse across the cell membrane into the organism and be potentially lethal (Conrad et al. 1997).

NOR catalyzes the reduction of the second intermediate of denitrification, NO to N_2O. However, NO metabolizing enzymes are also found in many non-denitrifying organisms (Zumft 1997). Three main groups of NOR enzymes are found in prokaryotes: long chain qNOR (only denitrifiers), short chain cNOR and qCuNOR (Zumft 2005). NOR is important to remove or detoxify NO concentrations before it becomes lethal for the cell. A large variety of microorganisms have the Nor enzymes, showing the importance for microorganisms to have an NO detoxification mechanism (Braker et al. 2003; Henry et al. 2006).

1.3.2 Regulation of denitrification

Denitrifying organisms live in fluctuating environments, exposed to anaerobic spells and scarce resources. Hence, there is a need to regulate metabolic genes to thrive in such altering environments. Denitrifying organisms switch from aerobic respiration to anaerobic respiration when oxygen levels become low. The switch is assisted by transcription regulators in the Crp-Fnr superfamily (Körner et al. 2003). Pure culture studies with the model organisms *P. denitrificans* have three main regulators of gene expression: NarR, NNR and FrnP, that all belong to the Crp-Fnr superfamily (Spanning et al. 1997; Wood et al. 2001).

The transcriptional regulator NarR, signaled by nitrate or NO2- in the organism's environment, initiates the transcription of narG and napA that code for nitrate reductase (Wood et al. 2001). NarR deficient mutants are unable to perform denitrification when supplied with NO3-, even under anaerobic conditions, although denitrification is resumed when supplied with NO2-.NarR is therefore unlikely involved in any other regulatory steps in the denitrification process (Bergaust et al. 2012).

The transcriptional regulator NNR, signaled by low oxygen levels and NO, induces the transcription of nirS and norC promoters that code for NO2- and NO reductase (Spanning et al. 1999). NNR is also regulated by a feedback loop from the production of NO, triggering the transcription of the nosZ promoter to produce N2OR (Bergaust et al. 2012). The third transcriptional regulator, FnrP, is signaled by low oxygen levels, and induces the expression of nitrate reductase and N2OR (Spanning et al. 1997).

1.3.3 Denitrification regulatory phenotypes

The term Denitrificaiton regulatory phenotype (DRP) was coined in order to create descriptive phenotypes of microbial processes (Bergaust et al. 2011). T]. The characterization is based on a few basic traits of either complex communities or pure cultures. The traits include amongst others: accumulation of denitrificaiton intermediates . The traits include amongst others: accumulation of denitrification intermediates (NO_2^-, NO and N_2O),transition of these intermediat and onset of denitification as a response to oxygen depletion. Two examples of DRP include Rapid Complete Onset (RCO) and progressive onset (Liu et al. 2013),characterized within in the *Thauera* genus. The RCO exhibits a rapid production of all denitrification product as soon as O_2 is depleted, with no detectible amounts of nitrite. In contrast, PO exhibits a progressive production of denitrification products with nitrite accumulation. Characterization of DRP is at a starting point, but can potentially produce realistic models of NO_x production in different environments - as more information is gained for characterization of organisms and communities.

1.3.4 pH as a regulator of denitrification

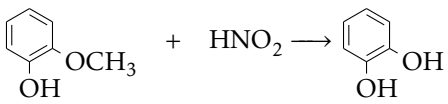
The major environmental variables that affect microbial respiration rates in soils includes amongst others moisture and carbon content, pH and temperature (Hënault et al. 2012; Lesschen et al. 2011). Of these, pH has in particular been called a "master variable" of denitrification. Simek and Copper (2002) summarize in a review article that soils with low pH (typically below pH 5) are observed to increases the N2O/(N2 + N2O) product ratio, compared to higher pH soils. The pH-value is a long-term selective pressure, which is found to alter the microbial community structure. Metagenomic sequencing along a pH gradient in soil has indicated shifts in and between the microbial phyla following a change in pH-value (Bartram et al. 2014). Molecular work by Bergaust (2010) and Liu (2009) also indicates that the low pH might cause a posttranscriptional problem in the assembly of the reductase that reduces N_2O to N_2.

1.4 Other forms of denitrification: chemodenitrification

Although biological processes are regarded as the main actors in nitrogen recirculation, it has been noted that chemical dissociation, particularly of nitrite, may play a role in the production of NO_x((Kappelmeyer et al. 2003; Nömmik et al. 1971). The non-biological reaction able to produce NO_x is termed chemodenitrification. The contribution of chemodenitrification has been shown to correlate with the pH value of the soil (Kappelmeyer et al. 2003). In general, NO_2^- is believed to accumulate under alkaline conditions, as chemical stability is favored. However, below pH 5.47 the chemical equilibrium between HNO_2 and NO_2^- shifts towards HNO_2. The shift towards HNO_2 is favored under acidic conditions due to the low pK_a value of NO_2^- (Eq.C1.2) (Cleemput et al. 1996).

$$NO_2^- + 2\,H^+ \longleftrightarrow 2\,HNO_2 \longleftrightarrow NO + NO_2 + H_2O \tag{C1.2}$$

The nitrous acid (HNO_2) molecules are chemically unstable under acidic conditions, and thus decompose to NO (Cleemput et al. 1996; Kappelmeyer et al. 2003). Chemodenitrification rates have been found to correlate with organic matter in soils, to which it has been shown that nitrogen compounds bind (Kappelmeyer et al. 2003). The formation of gaseous methyl (CH_3ONO), from reactions with methyl groups, is also suggested as a source of N_2O (Bremner 1997; Chalk et al. 1983; Cleemput 1998) (Eq. C1.3 and C1.4) (Chalk et al. 1983).



(C1.3)

$$CH_3ONO \longrightarrow CH_2O + N_2O + H_2O \tag{C1.4}$$

Phenolic compounds are also postulated to react with NO_2^- to produce N_2O (Chalk et al. 1983), a process called nitrosation. During nitrosation, organic compounds bond to the nitroso functional group

(C–N=O). Such organic compounds are called nitroso- (C–NO) and oximino (C–NOH) compounds. The chemistry of these compounds is not fully understood, but oximino compounds are suggested to further react with excess HNO_2, producing N_2O (Eq. C1.5) (Chalk et al. 1983).

$$C{=}NOH + HNO_2 \longrightarrow C{=}O + N_2O + H_2O \tag{C1.5}$$

NO_2^- that cannot be accounted for by biological processes, is assumed to be chemically decomposed through chemodenitrification, and particularly in reactions with phenolic and humic substances. Few studies have attempted to quantify the significance of chemodenitrification, but one study was done by Nömmnik and Thorin (Nömmik et al. 1971), who investigated loss of NO_2^- in steam-sterilized raw humus during an anaerobic incubation. They added NO_2^- to raw humus and calculated recovery rates after one-, two-, four- and seven days, in three humus samples with the subsequent pH values of 4.3, 6 and 7.3. Interestingly, they found that NO was produced in all three humus samples, where the two lower pH samples had NO production accounting for 55 to 60% of the added N. The humus samples with a pH of 7.3 produced significantly less NO, accounting for 40% of the added NO after a total of 7 days (Fig. 1.5). The investigation showed that all added NO_2^- could not be accounted for in sterilized humus, regardless of pH. Nömmik and Thorin suggested the formation of nitroso compounds with soil organic matter might account for undetected N. In summary, the reactions that cause N "disappearance" are not well understood (Cleemput 1998; Cleemput et al. 1996; Nömmik et al. 1971).

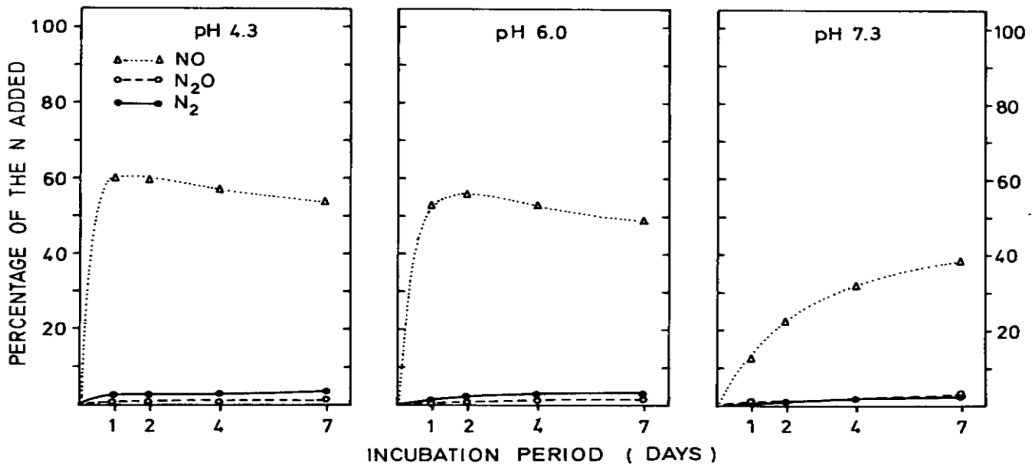


Figure 1.5. "Amounts and quantitative composition of nitrogen gases formed from added nitrite during incubation of steam-sterilized raw humus of different pH; nitrogen addition rate 400 ppm." (Nömmik et al. 1971)

1.5 Method to study nitrogen recirculation in soils

Production of nitrogenous gases from soils in response to pH, is often studied in soils from long-termed experimental field sites, adjusted to different pH-values (Hovlandsdal 2011). One such site is located on the west coast of Norway, in Fjaler. The site consists of peat, which is a loosely structured soil type of organic matter that has decomposed under very moist conditions. The Von-Post scale of humification is used to range peat on a scale of 1-10 to characterize the decomposition of the organic matter ((Grønlund et al. 2013)). Peat is usually thought of as a bog; however, it is also used in agriculture where peat bogs have been drained and established as meadows (Sognnes et al. 2006).

Peat has a very high carbon content, where humus or humic substances (HS) are the main bulk of soil organic matter. This makes the material particularly tricky to work with in downstream molecular methods. Humic substances (HS) cause problems during nucleic acid extraction from soil. They are released from soil particles and co-extracted with nucleic acid due to their structural and chemical similarities. HS are thought to interfere with a multitude of molecular methods, including enzyme activity of DNase, RNase and restriction endonuclease, DNA-DNA hybridization, PCR reactions, transformation of competent cells and measurements of nucleic acid concentrations (Wang et al. 2012).

1.5.1 Analyzing NO_2^-

Denitrification in the strictest sense starts with the reduction of NO_2^-, and as previously mentioned NO_2^- can also dissociate to HNO_2 and potentially take many different directions from there through chemical reactions or biological reactions. As NO_2^- is involved in multiple processes, both chemically and biologically, there is a keen interest in measuring (NO2-).

There are a several standardized methods used to measure NO_2^- in liquid. These methods are commonly used in medical research, due to the role of NO as a signaling transduction molecule and cytotoxic effect in the human body. NO has routinely been measured indirectly through NO_2^-, most commonly through a spectrophotometric assay using the Griess reagent (Ridnour et al. 2000). The principle of the Griess reaction is that NO_2^- reacts under acidic conditions, to produce a chromophoric azo product. The azo product strongly absorbs light at 545 nm - and can thus be measured (Giustarini et al. 2008). The spectrophotometric method, however, lacks the sensitivity required when measuring concentrations of NO_2^- under 1 µM. A more sensitive method that can detect NO_2^- concentrations in the nanomolar range, is an ozone-based chemiluminescence approach (Nagababu et al. 2010). The ozone based method uses reducing agents to produce NO gas that reacts with ozone molecules, to produce NO_2. A nitric oxide analyzer then measures the amount of NO_2 produced,and has a detection limit of 1 picomole (Eq.C1.6) (*Nitric Oxide Analyser NOA^{TM} 280i - Operation and Maintenance Manual* 2001).

$$NO + O_3 \longrightarrow NO_2 + O_2 \tag{C1.6}$$

1.5.2 Analyzing nitrogen gases

The production of NO, N_2O or N_2, can be measured both in laboratories or at field sites. The choice is a tradeoff between the variables one can control in the laboratory, and the external validity of the results gained from gas measurements in the field. Both field and laboratory-based methods have been conducted with peat from the experimental field site in Fjaler, on the west coast of Norway (Hovlandsdal 2011; Liu et al. 2010). Studies of gas emission in the field are affected by fluctuating moisture content, fertilization, temperature and uncontrolled variability between areas of sampling (Hovlandsdal 2011). Although results of such studies can have a more direct application to agricultural practices. In contrast, results produced in the laboratory cannot always be applied to real life situations, but instead reveal underlying causes at molecular levels with high internal validity (Liu et al. 2010).

A robotized incubation system developed by Molstad et al. (2007) (Molstad et al. 2007) was developed to allow sensitive and controlled measurements of gas production. The main components of the computerized incubation system include a gas chromatograph (GC), nitric oxide analyzer, water bath and an auto-sampler. The system detects amongst other gases N_2O, NO, N_2, O_2 and CO_2. The system has been used to monitor gas kinetics from both complexed communities and pure-cultures, from amongst other samples peat from the experimental site at Fjaler (Bergaust et al. 2011; Liu et al. 2013).

1.5.3 Extraction of nucleic acids

To work with the molecular aspects of nitrogen recirculation, nucleic acid (NA) from crude soil or peat samples must be purified for accurate downstream molecular methods. The choice of purification method depends on the type of environmental sample and contaminants present (protein, humic acid, clay, etc.). A commonly used method was developed by Griffiths et al., (2000) to co-extracts DNA and RNA from natural environments. To obtain NA, cells are lysed either chemically or physically. Griffiths et al. (2000) selected bead beating in combination with the chemical extraction buffer hexadecyltrimethylammonium (CTAB) to lyse cells, and also included phenol-chloroform-isoamyl alcohol (25:24:1) to separate the organic phase (containing proteins) from the aqueous phase (containing NA). The size of the beads, velocity of beating and duration of the physical treatment affect the lysis of cells, and are adjusted to avoid shearing NA. During the co-extraction of RNA and DNA, it is important to use RNase-free equipment as RNA is easily degraded. Griffiths et al.(2000) then purified the aqueous phase to remove residual phenol, by adding Chloroform-isoamyl alcohol (24:1). The solution is then mixed and centrifuged to separate the organic and aqueous phase, and NA is precipitation from the

aqueous phase using polyethylene glycol 6000 at room temperature for 2h. Finally, the NA is washed in 70% ethanol and air dried before resuspension in RNase free water (Griffiths et al. 2000).

1.5.4 Quantitative polymerase chain reaction (qPCR)

To quantify specific genes or their transcripts from purified DNA or cDNA, the qPCR method is commonly used. It is an adaptation of the common PCR method, for quantification of a selected gene in an environmental sample. The qPCR method, also termed real-time PCR, is a common tool within the microbial ecology field. Similar to PCR, primers are designed to target a gene of interest which is then amplified through cycles of denaturation, annealing and DNA-synthesis, but unlike PCR the results can be used to compare the relative abundance of genes or gene expression through amplification of cDNA. A qPCR-machine detects the amplification of each PCR product through a fluorescent signal, produced after each PCR cycle. SYBR green®dye is a commonly used to produce the florescent signal which is detected during the anealing step of each qPCR cycle, as the dye flources when bound to dsDNA (Fig. 1.6) (Henry et al. 2004; Smith et al. 2008).

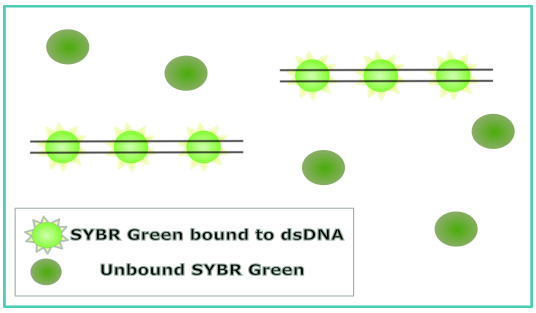


Figure 1.6. The SYBR green dye fluoresces when bound between two DNA base pairs, allowing quantification of gene copy numbers in a real-time PCR machine

1.6 Aim of Study

Nitrite is a key molecule in the nitrogen cycle. It is formed as an intermediate product during both nitrification and denitrification, and may occasionally accumulate in soils and wastewaters. Under anoxic conditions, NO_2^- is formed from the reduction of nitrate and may then react chemically with organic compounds, or be used by microorganisms in enzymatically catalyzed reactions, resulting in the production of gaseous compounds such as NO and N_2O. Low pH soils generally seem to accumulate little or no NO_2^-, and it is a common notion that this is mainly due to chemical reactions with soil components. Another explanation could be that NO_2^- concentrations are regulated by the microorganisms in low pH soils in order to avoid toxic effects by NO_2^- or its reaction products. In this thesis, I wanted to determine the significance of chemodenitrification compared to the enzymatic reduction of NO_2^- that takes place during microbial respiration, in peat soils of different pH. To gain

further insight into the denitrifier communities in these peats, I also wanted to quantify genes coding for denitrification reductases.

2

Materials and Method

A list of laboratory equipment, kits, media, buffers and chemicals can be found in Appendix A.

2.1 Field work

Peat was sampled from a long-term liming experiment established between 1976 and 1977 on the west-coast of Norway, at Fureneset in Fjaler district (61°17'41.2N 5°03'0.2.2E). The experimental site was originally drained and established as a meadow, to study the physical characteristics of biomass production when adding shellsand to peat (Sognnes et al. 2006). The site consists of 24 plots, where each plot is 96 m^2 in size. The peat range from H5-H9 on the Van Post scale of humification, which is moderate to highly decomposed peat (Grønlund et al. 2013). The site experiences a typical oceanic climate with mild temperatures and high moisture levels all year around (Sognnes et al. 2006).

Figure 2.1. Experimental field site at Fureneset, Fjaler district, Norway

Nine plots were sampled early December 2014, and were comprised of the following: three untreated field replicates (A1, A6, and A18); three field replicates treated with 200 m^2 shellsand pr. hectare (B7, B11, and B14); and, three field replicates treated with 800 m^2 shellsand pr. hectare (C4, C5, and C21).

Peat was pooled from five sampling spots located diagonally across each plot at least 1 m away from the edges. Each spot captured a column of peat where the top 2 cm containing vegetation was removed. Two kilograms of peat from each plot was transported back to Ås in plastic bags and stored at 4 °C. Peat samples were air-dried until they could pass through a 8 mm sieve followed by a 4 mm sieve, and again stored at 4 °C. Larger particles including root hairs and grass were removed while air drying, and samples were regularly tilted during the drying process to avoid edge effects.

2.2 Characterization of peat

The pH value of the peat samples was measured using $CaCl_2$. Negatively charged peat particles preferentially bind to Ca^{2+}, which releases H^+, thereby giving accurate measurements of the pH value. The following method was used to prepare and measure pH in peat samples. Five milliliters $CaCl_2$ was added to a 15 mL centrifuge tube with 2 g of peat. The suspension was shaken for one minute and left to settle for one hour before measuring the pH value of the supernatant.

The water holding capacity (WHC) was calculated for each of the peat samples to normalize the moisture content in subsequent experiments (section 2.9). The following method was used to prepare and measure pH in peat samples. Peat from each plot was placed in a funnel with filter paper and immersed with water. The samples were covered with parafilm to avoid water loss by condensation. The WHC was reached after five hours. The top layer (approximately 0.5 cm) of peat was used to measured the dry weight, and the WHC was calculated through equation 2.1.

$$\theta = \frac{mass_{wet} - mass_{dry}}{mass_{dry}} \tag{2.1}$$

2.2.1 Sterilization of five peat samples

To determine the loss of NO_2^- due to biological processes in peat, we first investigated the NO_2^- loss in sterilized peat. Five peat samples from the experimental field site in Fjaler (two low pH (~3.2), one mid (~ 5.2) and two high pH (~ 7.2) were sent for sterilization by gamma irradiation in spring 2014. The five sterilized peat samples were left to settle in storage at 4 °C for four months, to reduce the effect of radiolysis in subsequent experiments [Natalie Lim pers. comm].

Confirming sterility of gamma-irradiated peat

The five sterilized peat samples were monitored in the robotized incubation system to confirm the sterility (section 2.6). Ten grams of peat from each of the five samples were prepared in glass-serum flasks, sealed with rubber septa and aluminum crimps, and made anaerobic as described. The flasks were then placed in the water bath of the robotized incubation system and kept at 15 °C. To each of the five flasks, glutamate was added to a total concentration of 1 µmol N per gram of peat, as described. Over-pressure was after that released, and 5 mL O_2 was injected with a gas syringe. The gas-kinetics were monitored continuously every fourth hour for five days, and O_2 was re-injected into the flasks when concentrations were low.

Immediately after measuring gas-kinetics, the sterilized samples were further examined by plating onto Malt Agar (MA) and tryptic soy agar (TSA) to confirm sterility. The serum flasks previously

used to measure gas-kinetics were opened, and 50 mL sterilized MilliQ water was added to each peat sample. The flasks were shaken to produce a slurry and left to settle. Ten milliliters of supernatant was transferred to a 15 mL centrifuge tube, and left to settle further. A 10-fold dilution series was prepared from the supernatant of the settled slurries, and used to plate on TSA and MA plates. The MA was prepared with streptomycin to select for fungi, and TSA was prepared with cycloheximide to select for bacteria. A pour-plate and a spread-plate series were prepared with the MA and TSA. 100 µL of each dilution was added to the spread plates while 100 µL of each dilution was added to the pour plates, and all plates were incubated at 20 °C. The plates were monitored continuously, and a final count was made after four days.

2.3 Methods for working with NO_3^- and NO_2^- in peat

2.3.1 Measuring NO_3^- and NO_2^-

NO_3^- and NO_2^- were quantified using the nitric oxide purge system (NOPS) (Sievers), which consisted the following main components: a glass purge-vessel for the reduction of NO_3^- or NO_2^-; carrier gas; a filter; gas bubbler; and, a nitric oxide analyzer (NOA)(Sievers)(Fig. 2.2). The system was kept oxygen-free by a continuous flow of the carrier gas N_2. The purge vessel was filled with 4 mL reducing agent: either 1% w/v NaI in 50% w/v acetic acid to quantify NO_2^-, or VCl_3 in 1 M HCl to quantify nitrate. To quantify NO_3^-, the reducing agent was additionally heated to 95 °C by the heating jacket. To prevent hydrochloric vapor from damaging the NOA, the gas was passed through a condenser and additionally bubbled through a NaOH solution to neutralize any residual vapor. To quantify NO_3^- or NO_2^-, 10 µL of liquid sample was injected through the teflon septum. Nitrite or nitrate were then subsequently reduced nitric oxide by the reducing agent.

$$NO_2^- + 2H^+ + e^- \longrightarrow NO + H_2O \tag{C2.1}$$

To quantify the detected amount of nitric oxide, a standard curve was obtained with a 10-fold dilution series from 0.01 mM to 10 mM of nitrate. Ten microliters of each dilution was injected into the NOPS for quantification. The reducing agent was replaced when the ions in the reducing agents were exhausted. A new standard curve was obtained each time the reducing agent was replaced.

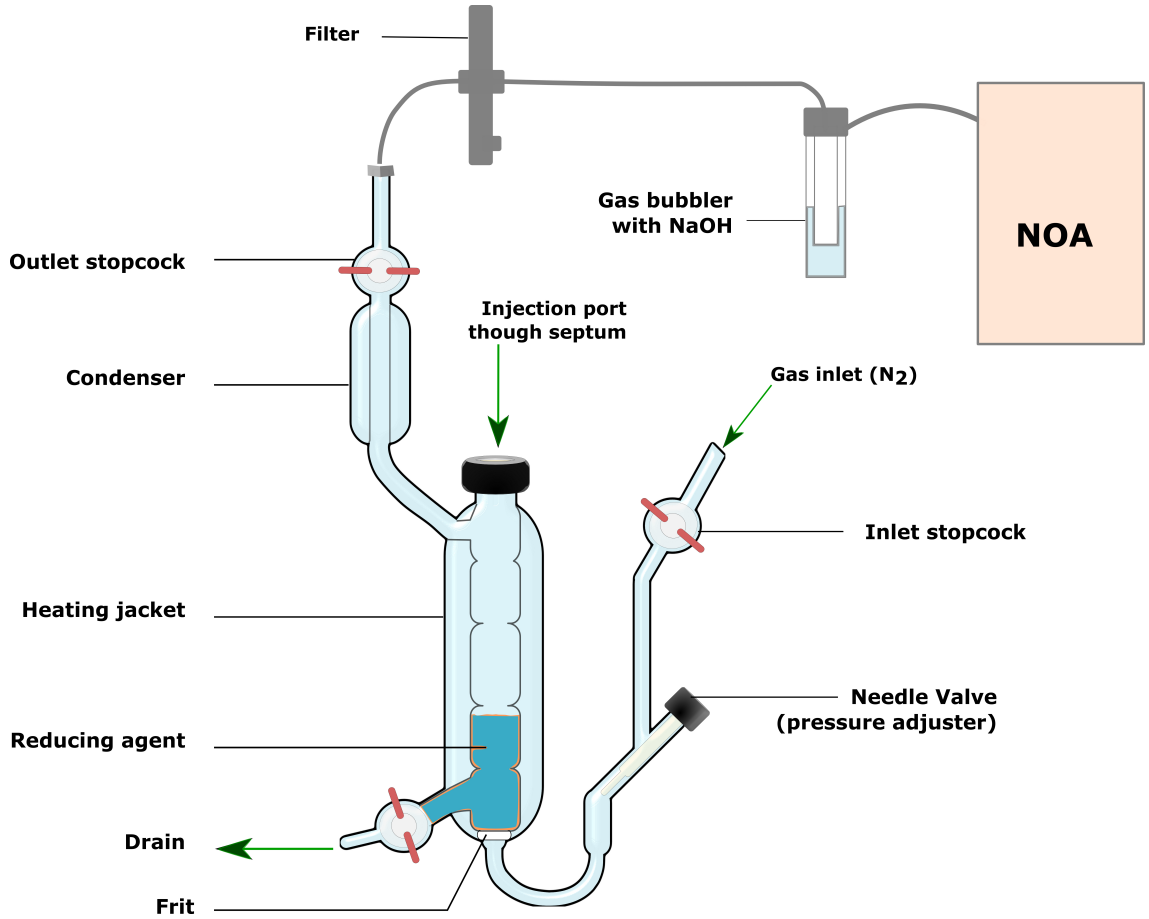


Figure 2.2. Nitric oxide purge vessel for quantification of nitrate or nitrite, pressure adjusted with the needle valve. Liquid samples are injected through the septum, and reduced to NO

2.3.2 Dosing peat with NO_3^- and NO_2^-

Some samples were amended with either 100 µL or 10 µL of 10 mM NO_3^- or NO_2^-, before incubation at 15 °C. The solute were in this case spread on the peat using a syringe, to rotate the needle in small and larger circles, while pulling the needle from the bottom of the tube to the top, to achieve as even spread of the solute as possible. The solute was added to achieve a total concentration or 1 µmol N per gram of peat.

2.3.3 Quantifying NO_3^- and NO_2^- in peat

To inject samples into the NOPS, the solutes in intact peat (previously sieved) had to be brought out into liquid. Either 5 mL or 500 µL MilliQ water was added to 2 g or 0.2 g of intact peat, and vortexed to produce a peat slurry. The slurry was centrifuged at 14 000 x *g* for 2 minutes to pellet peat particles,

and 10 µL of the supernatant was injected into the purge vessel of the NOPS to quantify NO_3^- or NO_2^- present in the sample.

2.3.4 Evaculation of samples for anaerobic treatments

The glass-serum flasks for anaerobic treatments, were sealed with rubber septa and aluminum crimp, and made anoxic by six or twelve cycles of evacuation (60 seconds) and helium filling (30 seconds). Over pressure was released after flasks were placed in a water bath at 15 °C .

2.4 Measuring loss of NO_2^- in sterilized peat, over 50 h

Chemodenitrification was investigated in the five sterilized peat samples, by comparing NO_2^- loss between sterilized samples with three different pH values (section 2.2.1).

Two grams of peat from the five different samples were prepared in 12 mL glass serum flasks, with one aerobic and one anaerobic treatment. Due to the destructive nature of the NO_2^- quantification method, NO_2^- could only be quantified once from the same flask. Eleven replicate flasks were therefore prepared for each peat sample, where each flask was incubated five more hours than the previous replicate, to quantify NO_2^- loss every fifth hour over a period of 50-hours. To quantify NO_2^- under different environmental conditions, parallel aerobic and anaerobic flasks were prepared for each time point, creating a total of 22 flasks for each peat samples (11 aerobic and 11 anaerobic). (Fig.2.3).

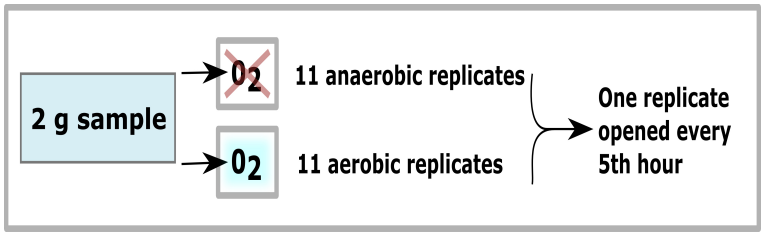


Figure 2.3. Experimental design for quantifying NO_2^- loss over time in five sterilized peat samples

Flask preparation and NO2- quantification

For each of the five-peat samples: 22 replicate flasks (11 anaerobic and 11 aerobically incubated) were prepared with two grams of peat, sealed with septa and aluminum crimps. The anaerobically treated replicates were evacuated as described in section 2.3.4, before 100 µL NO_2^- was added to all flasks as described in section 2.3.2. All flasks were incubated in a water bath at 15 °C (Fig. 2.2). NO_2^- was quantified every fifth hour from one replicate in each treatment, using the previously described method (2.3.2). A maximum of three flasks was prepared at once, to avoid resuspension and degradation of NO_2^- after centrifugation before quantification. Peat was transferred from the 12 mL flasks to 1.5 mL

microcentrifuge tubes, by adding 5 mL MilliQ water and shaking to produce a peat slurry. The slurry was transferred to a 1.5 mL microcentrifuge tube, and NO_2^- was quantified as described in section 2.3.3.

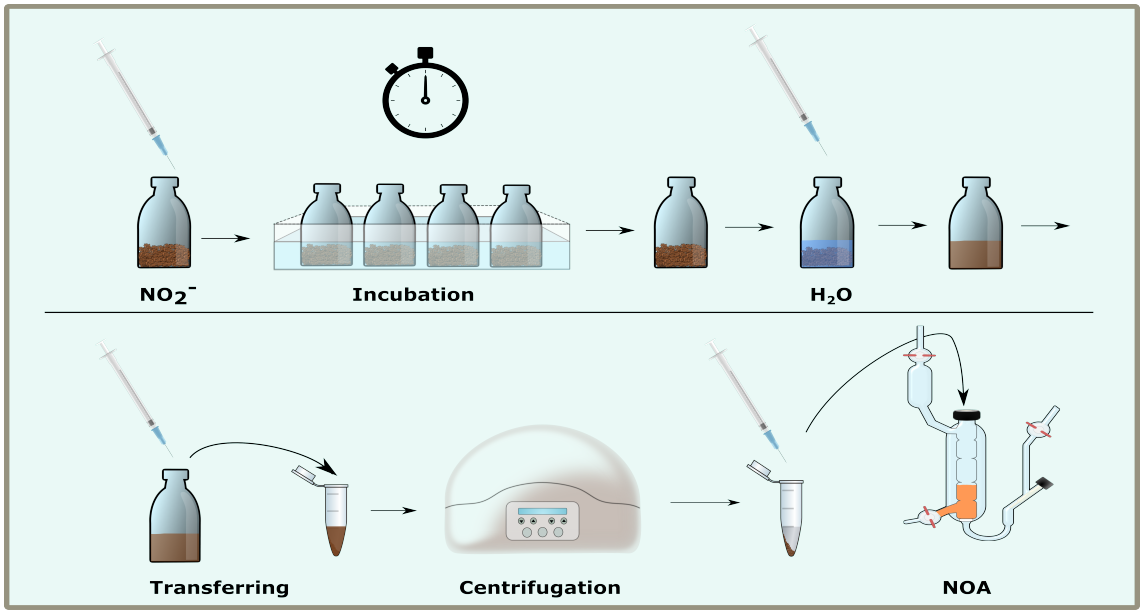


Figure 2.4. Quantification of NO_2^- from intact peat. Five mL water was added to intact peat incubated in 12 mL glass serum flasks. Peat slurry was transferred to microcentrifuge tubes, centrifuged at 14 000 x *g* to pellet peat, 10 µL of the supernatant was measured for NO_2^- quantified using the NOPS.

2.5 The five hour loss of NO_2^- in sterilized peat

The prior investigation showed that NO_2^- could not be recovered after five hours in the sterilized low pH peat, and most NO_2^- was lost in the high pH peat within the first few hours of the incubation. A second experiment was therefore designed to capture the rapid loss of NO_2^- and further understand the kinetics at hand.

Peat was prepared aerobically as no difference in the loss NO_2^- was found between the anaerobic and aerobic treatments in the previous experiment. To understand the kinetics within a much shorter time frame, a method was developed to prepare samples with a minimal amount of time between addition and quantification of NO_2^-. All of the five sterilized peat samples were included (section 2.2.1), to evaluate pH as a variable affecting the kinetics.

Preparation of samples:

For each of the five-peat samples, 0.2 grams of peat was prepared directly in 1.5 mL microcentrifuge tubes. One tube at a time was dosed with 10 µl NO_2^- as described (section 2.3.2). The incubation was tracked with a stopwatch from the moment NO_2^- was added and stopped by preparing the sample for quantification as described (section 2.3.2). Only three minutes passed from the addition of NO_2^- till quantifying NO_2^- using the NOPS (Fig. 2.5).

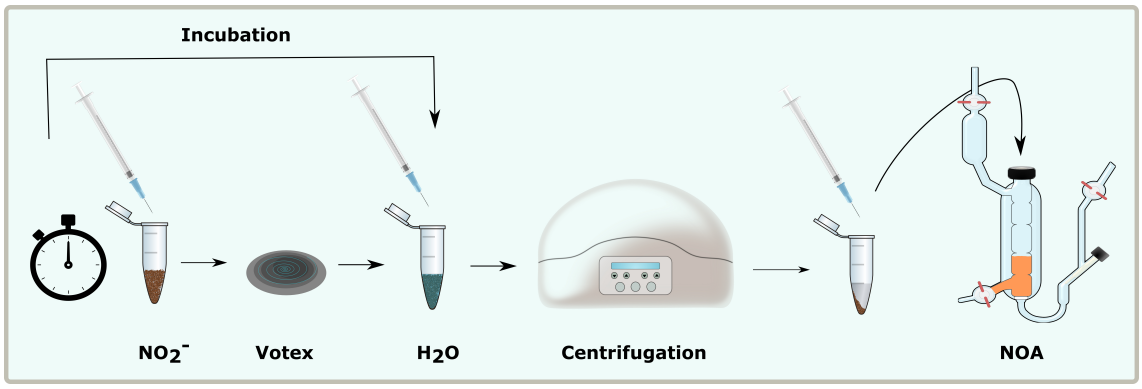


Figure 2.5. Rapid quantification of NO_2^- from intact peat in 1.5 mL microcentrifuge tubes. Nitrite added to peat was incubated and mixed. Water was added to stop the incubation and peat pelleted by centrifugation. Ten µl of supernatant was injected to the NOA purge vessel to quantify NO_2^-

To get a proper time point zero, 10 µl of 10 mM NO_2^- was added with 500 µl autoclaved MilliQ water, and NO_2^- was immediately quantified as previously described in section 2.3.2 and Fig. 2.5.

2.6 Investigating the change in rate of NO_2^- loss

Three experiments were carried out to further characterize the kinetics involved in loss of NO_2^- in peat. The high and low pH peat samples were compared in all of the three following investigations.

2.6.1 Sterilization as a factor

A rapid loss of NO_2^- was found to occur during the first 10 minutes of each incubation experiment, with a indication of two separate kinetics of NO_2^- loss, one preliminary rate which was faster then the secondary rate. To evaluate if this could have been an artifact of gamma-irradiation, NO_2^- loss was compared between unsterilized and sterilized high and low pH samples. Each of the four peat samples, two unsterilized and two sterilized, were aerobically prepared in 1.5 mL microcentrifuge tubes. The tubes containing 0.2 g of peat were dosed with 10 µL of NO_2^- as previously described (section 2.3.2). All four peat samples were prepared in tubes that received the following different incubation times: 0-6 minutes, 8 minutes, 10 minutes, 15 minutes, 20 minutes and 30 minutes. The previously described method (Fig. 2.5) was used to quantify NO_2^- in samples after incubation.

2.6.2 Concentration of NO_2^- as a factor

To investigate if the concentration of NO_2^- added to gamma-irradiated peat affected the rate of NO_2^- loss, a 10 minute incubation experiment was preformed where both high and low pH peat samples were dosed with NO_2^- concentration ranging from 10 nM up to 100 mM NO_2^- per gram of peat. Only aerobic samples were prepared, and unsterilized peat was used, as no difference was found in the previously described investigations. Ten 1.5 mL microcentrifuge tubes with 0.2 g of peat were prepared for the unsterilized high and low pH peat samples. One tube for each of the two samples were dosed with the following NO_2^- concentration (N per gram of peat): 10 nM, 100 nM, 0.01 µM, 1 µM, 2.5 µM, 5 µM, 10 µM, 25 µM, 50 µM, 75 µM and 100 µM. All tubes with 0.2 g of peat were incubated for 10 min before NO_2^- was quantified using the previously described method (Fig. 2.5).

2.6.3 Ion-exchange as a factor, by measuring NO_3^-

To investigate if ion-exchange from peat particles was a cause of the preliminary NO_2^- loss, 0.2 g of unsterilized peat from both high and low pH samples were prepared aerobically in 1.5 mL microcentrifuge tubes. Peat aliquoted into tubes were dosed with 10 µL of NO_3^- as previously described (section 2.3.2). The peat samples were prepared and incubated as described in section 2.6.1. Two methods were used to quantify NO_3^-, the first method added 500 µL water, while the second added 500 µL of 0.01M KCl_2 to stop the incubation time before quantifying NO_3^- with the previously described method (section 2.3.2).

2.7 Using the robotized incubation system

To measure the gas kinetics while incubating intact peat anaerobically, we used the robotized incubation system developed by Molstad et al. (2007). The main components of the computerized incubation system include a gas chromatograph (GC), NOA (sievers), peristaltic pump, water bath and an autosampler. The system accommodates up to 44 glass serum flasks and detects amongst other gases N_2O, NO, N_2, O_2 and CO_2. Three standard gases are included in each experiment to calculate for dilution in the flask headspace over time: a high standard with 10 000 ppm CO_2, 10 000 ppm CH_4 and 150 ppm N_2O; a low standard with 361 ppm CO_2, 585 ppb N_2O, 1.84 ppm CH_4, 210 000 ppm O_2 and 78 000 ppm N_2; and, a NO standard with 25 ppm NO. The autosampler removes gas out of headspace using a peristaltic pump, followed by injection of He gas back into the flask following each sampling to account for pressure change. Sampled gas was pumped to a 6-port valve injector, which leads most of the gas to the GC and a smaller portion to the NOA. The autosampler waits for the GC and NOA to finish analyzing, before collecting gas from next flask. Each time the autosampler samples from the same flask position, it punctures the septum at a slightly different point to avoid septum failure and leakage over time.

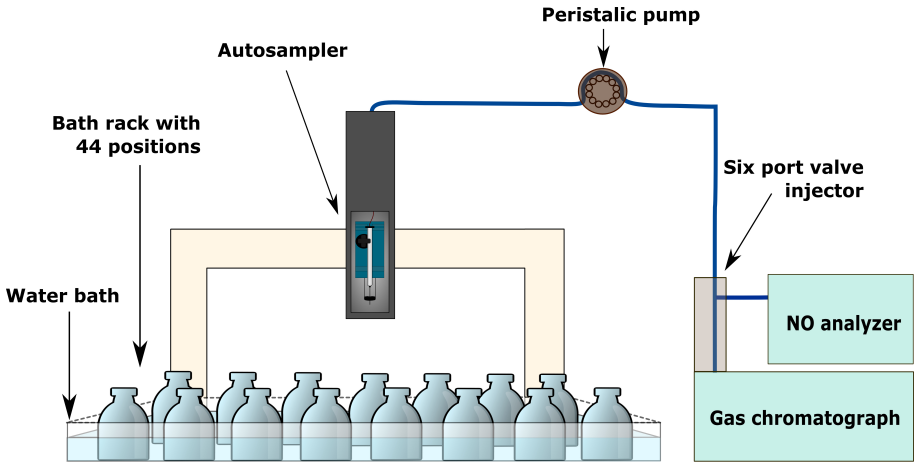


Figure 2.6. Robotized incubation system with autosampler and water bath. The system was connected to a gas chromatograph and a separate NO analyzer (Molstad et al. 2007).

2.8 Gas kinetics of sterilized peat

To evaluate the significance of chemodenitrification, we used the robotized incubation system to monitor gas kinetics from the five gamma- irradiated peat samples (section 2.2.1). Three 120 mL glass serum flasks with five grams of peat were prepared, for each of the five-peat samples, sealed with rubber septa and aluminum crimps. The flasks were made anaerobic with 12 cycles of evacuation as previously described (section 2.3.4), and placed in the water bath of the robotized incubation system. Right before gas was sampled from the flask headspace, 250 µL NO_2^- was dosed into each flask NO_2^- as previously described (section 2.3.2) followed by the release of overpressure. A control treatment with three flasks for each of the five samples were prepared as described above, except that only water was added instead of NO_2^-. The robotized incubation system was set to sample gas every fourth hour over a total period of 100 hours.

2.9 Gas-kinetics of non-sterilized peat

Non-sterilized peat from the three plots, A6, B7 and C5, were monitored by the robotized incubation system. Monitoring of gas production was combined with quantification of NO_2^-, and for timing the snap freezing of samples with liquid nitrogen for subsequent mRNA extraction.

The experimental design was as following: peat from plots A6, B7, and C5 were prepared in glass serum flasks as described in section 2.9. The flasks were then placed in the robotized incubation system at 15 °C to measure gasses. Parallel flasks for NO_2^- measurements, and for snap freezing of peat for subsequent mRNA extraction, were incubated in a separate water bath at 15 °C. Each time the robotized incubation system measured gas from the flasks, one parallel from each sample was opened to measure NO_2^-, and freeze with liquid nitrogen for storage as described in section 2.9. The robotized incubation system waa set to measure gas every third hour over a total period of 80 hours.

Preparing flasks

Before measuring gasses, peat was aerobically revived using clover to mimic a natural carbon source (Liu et al. 2010). Clover was dried then pulverized by a 1 mm shredder prior to use. Peat from plots A6, B7 and C5, were individually mixed with 5 mg clover per gram of wet weight peat on a sheet of aluminum foil, and tilted from side to side to achieve an even mixture. Then incubated aerobically for 72 hours at 15 °C to activate the microbial community.

When preparing the peat for measurement in the robotized incubation system, carbon, moisture and NO_3^- was also normalized between samples by controlling the amount of peat and concentration of NO_3^- in each flask. The amount of peat was normalized based on the carbon content from each of the

Table 2.1. Pereparation of peat to normalize carbon, moisture and NO_3^-

Peat sample	A6	B7	C5
Initial WHC	82 %	78 %	68 %
WHC after drying, for normalization of moisture	61 %	59 %	46 %
Target WHC after added liquid	90 %	90 %	90 %
Grams of fresh weight to achieve 1.5g SOC vial-1	8	7.5	5.8
Liquid added (of nitrate solution)	1.03 mL	0.85 mL	0.92 mL
Concentration of nitrate	17.19 mM	3.95 mM	5.75 mM
Total NO3- concentration in liquid	5 mM	5 mM	5 mM

three plots (Liu et al. 2010). The initial NO_3^- concentration in each peat type was used to customize the additional NO_3^- added to each flask, to achieve a normalized NO_3^- concentration in the prepared flask samples. The NOPS was used to find the initial NO_3^- concentrations, using 0.2 grams of peat from each plot and the previously described method (section 2.5).

To avoid adding a small volume of NO_3^-, peat was additionally dried to approximately 60% of the total WHC. Dried peat was then aliquoted into flasks amended with the calculated volume and concentration of NO_3^- , that gave a moisture content at 80% of the WHC and total nitrate concentration of 5 mM N $vial^{-1}$. The concentration of NO_3^- in peat was kept below 10 mM per gram of peat, as some microorganisms can experience higher concentrations as toxic [Åsa Frostegård. pers comm.].

NO_3^- was added to each flask before evacuation, and flasks were additionally shaken to mix, prior to sealing with septa and aluminum crimps. The flasks were then made anaerobic as described in section 2.3.4, and placed in a water bath at 15 °C, before over-pressure was released. Table **??** summarizes the standardized conditions of carbon, moisture and NO_3^- concentrations.
From peat sample A6, a total of 36 replicate flasks were prepared. Three flasks were used to measure gas kinetics, while the remaining flasks were stored in an external incubator to measure NO_2^- and prepare snap frozen peat sample every third hour throughout the incubation. A total of 18 replicate flasks were prepared from peat sample B7, and 15 replicate flasks were prepared from peat sample C5.

Quantifying NO_2- and snap freezing peat

Every third hour when the robotized system sample gas, one parallel flask from each peat type was opened and content transferred into a 15 mL centrifuge tube. The tube was pre-cooled and filled with 5 mL liquid nitrogen, which immediately froze the peat. Frozen samples were immediately stored at −80 °C and later used to extract mRNA (section 2.12). Each time a parallel flask was opened, 0.2 grams

of peat was also weighed into a 1.5 mL microcentrifuge tube to quantify NO_2^- as described in section 2.3.1.

2.10 Simulating NO_2^- disappearance in sterilized peat

With the use of the statistical program Microsoft Excel, the measured amounts of NO_2^- in sterilized peat was plotted by it's natural logarithm to which a linear regression line was drawn to determine the decay rate constants (Kd). The Kd value was calculated for each of the five sterilized peat samples.

Professor Lars Bakken, in the NMBU nitrogen cycle research group, then further modeled the NO_2^- data, and gave Equations 2.2 and 2.3 to describe the loss of NO_2^- in sterilized peat. Equation 2.2 simulates loss of NO_2^- in sterilized peat, with the following parameters: the decay constant (K), fraction of NO_2^- in liquid (DNO2- w), partitioning factor (P) that gives fraction of NO_2^- that binds to peat through ion-exchange, the chemical decay rate constant (Kd) for each of the five-peat sample, concentration of NO2- in water (W), concentration of NO_2^- bound to solid phase of peat (S). P is also used to calculate the water fraction factor (WF), which gives the fraction of NO_2^- in the liquid phase after equilibrium is established through ion-exchange [= P/(P+1)].

$$\frac{\Delta NO_{2-w}^-}{\Delta t} = -k \cdot (W - S \cdot P) - k_d \cdot NO_2^- \tag{2.2}$$

Equation 2.3 determines the NO_2^- concentration in the water phase of peat, with the following parameters: the concentration of NO_2^- in liquid phase ([NO2-]w), the amount of water present in the peat analyzed for NO_2^-, (Vw, given in mL per gram peat) and the fraction of NO_2^- in liquid phase after ion-exchange has reached equilibrium (WF).

$$[NO_2^-]_s = [NO2-]w \cdot \frac{Vw}{WF} \tag{2.3}$$

Denitrification is the sum of both microbial respiration (V_{NIR}) and chemical decomposition (V_{NOchem}). Equations 1.4 to 1.6 as given by Lars Bakken, were used to determine the contribution of respiratory and chemical NO production. First we determined the total gross rate of NO production (GR_{NO}), which is the summmuarized net rates of subsequent denitrification gases e.g. $GR_{NO} = R_{NO} + R_{N2O} + R_{N2}$. Secondly, V_{NOchem} was calculated from Equation 2.4, with the following paramteres: first order decay constant (Kd), concentration of NO_2^- in liquid phase of each sample ($[NO_2^-]$), and fraction of NO_2^- recovered as NO in sterilized peat (F_{NO}). Thirdly, the contribution of nitrogen gas production from V_{NIR} was calculated from Equation 2.5. And lastly, equation 2.6, to define the contribution of V_{NOchem} to denitrification.

$$V_{NOchem} = [NO2-] \cdot K_d \cdot F_{NO} \tag{2.4}$$

$$V_{NIR} = GR_{NO} - V_{NOchem} \tag{2.5}$$

$$V_{NOchem} = [NO_2^-] \cdot K_d \tag{2.6}$$

2.11 Optimization of primers

The primer-template annealing step in PCR reaction was optimized for the peat samples from Fjaler, using a gradient-master cycler for the following genes: NapA, NarG, NirK, NirS, nosZ and 16S (Table 2.2).

Table 2.2. *List of primers*

Gene	Primer name	Sequence	Primer type	Amplicon size	Reference
narG	narG-f	TCGCCSATYCCGGCSATGTC	Forward		Bru, Sarr, Philippot, 2007
narG	narG-r	GAGTTGTACCAGTCRGCSGAYTCSG	Reverse	173bp (narG-f, narG-r)	Bru, Sarr, Philippot, 2007
napA	V17m	TGGACVATGGGYTTYAAYC	Forward		Bru, Sarr, Philippot, 2007
napA	4r	ACYTCRCGHGCVGTRCCRCA	Reverse	152bp (v17m and 4r)	Bru, Sarr, Philippot, 2007
nosZ	Z-F	CGYTGTTCMTCGACAGCCAG	Forward		Kloos, Mergel, Rösch, Bothe, 2001
nosZ	1622R	CGSACCTTSTTGCCSTYGCG	Reverse	453bp (Z-F,1622R)	Kloos, Mergel, Rösch, Bothe, 2001
nirK	F1aCu	ATCATGGTSCTGCCGCG	Forward		Hallin, Lindgren, 1999
nirK	R3Cu	GCCTCGATCAGRTTGTGGTT	Reverse	473bp (F1aCu,R3Cu)	Hallin, Lindgren, 1999
nirK	517F	TTYGTSTAYCACTGCGCVCC	Forward		Chen, Luo, Hu, Wu,Wei, 2010
nirK	1055R	GCYTCGATCAGRTTRTGGTT	Reverse	539bp (417F,1055R)	Chen, Luo, Hu, Wu, Wei, 2010
nirS	1F	CCTAYTGGCCGCCRCART	Forward		Braker, Fesefeldt, Witzel, 1998
nirS	6R	CGTTGAACTTRCCGGT	Reverse	890 (1F,6R)	Braker, Fesefeldt, Witzel, 1998
nirS	cd3aF	GTSAACGTSAAGGARACSGG	Forward		Throbäck, Enwall, Jarvis, Hallin, 2004
nirS	R3cd	GASTTCGGRTGSGTCTTGA	Reverse	425bp (cd3aF,R3cd)	Throbäck, Enwall, Jarvis, Hallin, 2004
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	Forward		Weisburg et al. 1991
16S rRNA	518R (P2)	ATTACCGCGGCTGCTGG	Reverse	492bp (27F,518R)	Muyzer, de Waal, Uitterlinden, 1993

2.12 Nucleic acid extraction

A method developed by Natalie Lim [submitted] based on the Griffith extraction protocol (Griffiths et al. 2000) was used to co-extract nucleic acid from sample plots A6, B7 and C5 sampled in 2014. The samples were previously incubated and snap froze with liquid nitrogen at timed moments to capture the genetic information (section 2.12). The method yields a nucleic acid fraction that contains both DNA and RNA, and is therefore referred to as total nucleic acid (TNA). A fraction of the TNA was further purified to gain DNA and RNA.

A combination of a chemical and physical method was used to lyse cells in 0.25 g of wet weight peat. This included bead beating using *FastPrep-24 Instrument*(MP Biomedicals) with 0.25 mL each of three sizes of glass beads (0.10 mm, 1 mm and one 2.5-4.5 mm bead), and 500 mL CTAB extraction buffer. Proteins were removed using phenol:chloroform:isoamylalcohol (24:24:1), and additional chloroform wash steps were used to remove residual phenol. TNA was precipitated at room temperature in isopropanol with 3 M sodium acetate, adjusted to pH 5.1 with acetic acid. The TNA pelleted was washed with ethanol to remove residual inhibitors, and dried in a SpeedVac Concentrator. The pellet was resuspended and purified with OneStep PCR inhibitor Removal Kit (ZymoResearch). TNA was stored at −80 °C.

Fifty microliters of TNA was used to purify DNA using Genomic DNA clean & Concentrator Kit (ZymoResearch) . DNA was stored at −20 °C. Fifty microliters of TNA was used to purify RNA, which was first treated with a DNase digest step using Turbo DNase Kit (Life Technologies), and thereafter purified with ZymoResearch RNA Clean & Concentrator kit.

Thirteen microliters of RNA was reverse transcribed to cDNA, using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen) as described by manufacturer. Remaining RNA was stored at −80 °C , and cDNA was stored at −20 °C.

2.12.1 Quantification of nucleic acid

The benchtop fluorometer Qubit®was used to quantify DNA, RNA, mRNA samples. Qubit master mix was prepared as described by the manufacturer, and 2 µL undiluted sample was added to 198 µL of the Qubit®master-mix, before quantification by the fluorometer. A high standard (10 µg/mL) and a low standard (0 µg/mL) was used to calibrate the fluorometer before quantifying samples.

2.12.2 PCR conditions

Target DNA was amplified with Applied Biosystems®2720 Thermal Cycler. Amplification of target genes were performed using the following protocol: Initial denaturation at 95 °C for 1 minute, 30 cycles of 30 seconds at 95 °C, 30 seconds at optimized annealing temperature, 40 seconds at 72 °C, and a final 10 minutes at 72 °C to fully extent any remaining single-stranded DNA.

Amplification of target genes were confirmed on 1% agarose gels, with sodium borate (SB) buffer. One microliter of 6X loading dye and 4X GelRed was pre-mixed with 5 µL PCR product before loading into the individual wells on the agarose gel. Products were separated on the agarose gels between 90 to 120 V, for 20 to 50 minutes.

2.13 Producing plasmid DNA standards for qPCR

Standards for quantification by qPCR, were produced by cloning target genes (*narG*, *napA* and *nirS*) into plasmid DNA using the pCR2.1®vector (Invitrogen). The vector contained an ampicillin resistance gene, and subsequent lysogeny broth (LB) therefore contained ampicillin (100 µg/mL) to select for Escherichia coli containing the vector.

To produce the plasmids, target genes were first amplified by PCR and then ligated overnight to pCR2.1®vectors (Invitrogen), followed by transformed into One shot®TOP10 (Invitrogen) Chemically Competent E.coli as described by manufacturer. E.coli were then incubated overnight in 5 mL LB media at 37 °C, while horizontal shaken at 225 rpm for optimal growth. The cultures were spread on LB agar plates with 40 µL X-Gal, and incubated overnight at 37 °C. Colonies that turned white had successfully replaced the lacZ gene in the pCR2.1®vector, with the targeted denitrification gene. Ten single white colonies from each culture was therefore streaked onto new LB agar plates, incubated overnight at 37 °C, and sent for Sanger sequencing. The nucleotide sequence was analyzed by performing a BLAST search on the NCBI database site, to confirm the presence of the targeted gene.

$$\text{number of copies (molecules)} = \frac{(Xng) \cdot (6.0221 \cdot 10^{23} \text{ molecules / mole})}{(N \cdot 660 \text{ g / mole}) \cdot (1 \cdot 10^{9} \text{ ng/g})} \tag{2.7}$$

The isolated colonies were after that inoculated into 5 mL LB media, and incubated overnight. One milliliter of the culture was used to make glycerol stocks for long term storage, with 1 mL of bacterial culture and 300 µL of 87% glycerol, and stored at −80 °C. One to two milliliters of the culture was used to isolate the plasmid DNA using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) as described

by the manufacturer.

2.14 Quantification by Real-Time Polymerase Chain Reaction (qPCR)

For absolute quantification of genes copies, in DNA, cDNA and mRNA samples, we used the StepOnePlus Real-Time PCR machine (with StepOne software, v2.0). A reaction mixture of 20 µL was made with, 0.4 µM of each primer; 2 µL of template nucleic acid; 10 µL SYBR *premix Ex Taq II* (Tli RNaseH PLus); and, 0.4 µL ROX dye. A 96-well qPCR plate was used to load the samples, sealed with adhesive film, and centrifuged for two minutes before loading intro the real-time PCR machine.

2.15 Statistical analysis of molecular data

Statistical analysis was performed in R®, with the exception of qPCR efficiencies. A *tukeys test of variance* was performed to determine if the amount of DNA and RNA extracted were significantly different as an effect of pH. Confidence level was set to $p \leq 0.05$ for all statistical analyzes.

3

Results

The overall aim of this thesis was to determine the significance of chemodenitrification compared to the enzymatic reduction of NO_2^- in both acidic and neutral pH peat soils. Also, to further understand what happens to microbial communities in acidic environments by quantifying genes coding for denitrification reductases. This thesis has evaluated the significance of chemodenitrification, through dissociation of NO_2^- in sterilized peat, to find how much this can contribute to N gas production relative to microbial respiration. This question has not been thoroughly investigated in earlier studies, and thus it is unknown how much of the gross nitrogen gas production in soils, is due to chemical or biological reactions. In this work, I have found that NO_2^- is chemically degraded to produce mainly NO. A portion of the NO_2^- also binds to peat particles through ion-exchange, and is thus not recovered as nitrogen gas. Through simulation of NO_2^- decay and measurement of the gross nitrogen gas production in non-sterilized peat, I have also estimated the rate at which chemical or enzymatic reactions contribute to NO production. From these calculations I have found that microbial respiration is important even in acidic environments. I have also shown that there are differences between the microbial denitrifier communities in low, mid and high pH peat samples, were the abundance of the genes *nirS* and *nosZ*, coding for denitrification reductases, is decreasing with pH.

3.1 Characterization of peat, collected on December 3rd 2014

The pH value of peat was determined after sieving the samples. Instead of water, the pH was determined using 0.01 M $CaCl_2$ which preferentially binds to peat particles causing H+ to be released into the liquid. Peat samples from plots C4, C5 and C21 were approximately at a neutral pH (7), peat from the naturally low pH plots (A1, A6, and A18) had an average pH of 3.4, and peat samples from the mid pH plots (B7, B14, and B21) had an average pH value of 5.32. Peat samples from plot C5, A6 and B7 were chosen to represent the respective pH group in subsequent quantification of gasses and extraction of nucleic acids.

Table 3.1. *Characterization of peat*

Peat Plot	shellsand per hectare peat	pH-value	Denoted in text	WHC
A1	0	3.24	Low	91%
A6	0	3.80	Low	89%
A18	0	3.16	Low	89%
B7	200 m^3	5.73	Mid	93%
B11	200 m^3	5.89	Mid	93%
B14	200 m^3	4.34	Mid	88%
C4	800 m^3	6.77	High	84%
C5	800 m^3	6.80	High	90%
C21	800 m^3	6.80	High	84%

The water holding capacity (WHC) was determined after sieving the samples. The initial WHC (3.1), was used to design subsequent experiments with unsterilized peat. Peat was characterized as highly decomposed, forming a relatively uniform paste when squeezing, with root hairs as the only recognizable structure.

3.2 Evaluating the sterility of gamma-irradiated peat

The sterility of the gamma-irradiation peat samples was evaluated by plating. Malt agar (MA) with streptomycin was prepared to select for fungi, and tryptic soy agar (TSA) with cycloheximide was prepared to select for bacterial cells. Ten grams of each peat sample was amended with 1 µmole filter sterilized glutamate g^{-1} to optimize microbial growth conditions, before monitoring gas development with the robotized incubation system. Glutamate was inteded to stimulate growth of microorganims by serving as a carbon source, however, gas production was inconclusive due to maintance errors with the robotized incubation system. The prepared samples were therefore mixed with 40 mL water to produce a slurry, and serial diluted to deduice sterility though plating.

One milliliter from each dilution level was used to inoculate TSA and MA pour plates, while 100 µL of each dilution level was used to make MA and TSA spread plates. The plates were monitored at intervals for four days (Table. 3.2).

Table 3.2. *Colony forming units g^{-1} peat. Malt agar (MA) with 100 mg/mL streptomycin, and tryptic soy agar (TSA) with 100mg/mL cyclohexiamide. Incubated at for four days at* 15 °C.

Media and Method	Low pH 1	Low pH 2	Mid pH	High pH 1	High pH 2
MA spread plate	$4*10^2$	$2.2*10^4$	$7.2*10^3$	$2.9*10^4$	$6.8*10^3$
MA pour plate	$9.6*10^1$	$5.6*10^3$	$1.5*10^3$	$2*10^4$	$2.4*10^3$
TSA spread plate	$<4*10^1$	$1.2*10^2$	$1.6*10^3$	$<4*10^1$	$4*10^1$
TSA pour plate	$<4*10^0$	$2.8*10^2$	$1.2*10^4$	$1.2*10^1$	$4*10^0$

The number of cells was brought down seven to ten orders of magnitude (Henry et al. 2004), depending on sample type and plating method. The bacterial cell count was mostly below or near the detection limit. The fungal CFU numbers were in most cases 1-3 orders of magnitude higher. Considering that our experiments were conducted in less favorable environments that these plates where incubated with (without glutamate addition and four days of optimal incubation), we concluded that microbial metabolisms was therefore unlikely to affect our results.

3.3 Chemodenitrification in sterilized peat

3.3.1 Loss of NO_2- in sterilized peat

Nitrite loss over a 50-hour period was investigated in five sterilized peat samples, to compare the kinetics of NO_2^-loss between three pH values. From each peat sample, two batches of 11 replicate flasks with a total of 1 g dry weight peat was prepared, one batch was prepared aerobically while the other was made anaerobic. Each flask was amended with 1 µmole NO_2^- per gram dry weight peat. All flask were incubated at 15 °C, and NO_2^- was measured every fifth hour.

A substantial decrease in NO_2^- was measured in all five samples at the first time point (five hours), and no difference was found between anaerobic and aerobic treatments. Nitrite was lost in a pH dependent pattern, i.e. the amount NO_2^- in the five different peats, regardless of treatments, grouped together according to pH over the 50-hour incubation period. At the 50-hour time point, the low and mid pH samples had negligible amount of NO_2^-, while the high pH samples had around 40-50% NO_2^- loss (Fig. 3.1).

Over half of the initially added NO_2^- was lost after only five hours of the incubation (in the flasks with low or mid- pH peat), and a second experiment was therefore performed to measure NO_2^- loss within the first five-hour time frame. All five-peat samples were used to prepare separate batches, with replicate flasks, than contained 0.2 g dry weight peat. Each flask was then amended with 1 µmole NO_2^- per gram dry weight peat, and incubated aerobically. Nitrite was measured at short intervals over the five-hour period (Fig. 3.1).

For the five hour incubation experiment, NO_2^- showed a pH-dependent pattern of loss; the low pH samples had negligible amounts of NO_2^- after four hours, the mid pH was found to lose NO_2^- in an intermediate pattern of the low and high pH samples, while the high pH samples fluctuated around 40-50% loss after the first 10 minutes and throughout both the five-hour and 50-hour incubation.

Interestingly, 20 to 30% of the initially added NO_2^- was lost within one minute in all five-peat samples, before a slower rate of loss initiated. The apparent change in rate after only one minute could tentatively be explained as an artifact of gamma irradiation - due to radiolysis. To test this hypothesis, NO_2^- loss over 10 minutes was measured in unsterilized peat (both high- and low pH samples), and was found to have the same change in the rate of NO_2^- loss, after only one minute. The two apparently different rates must consequently be a result of reactions that occur in the peat regardless of sterilization.

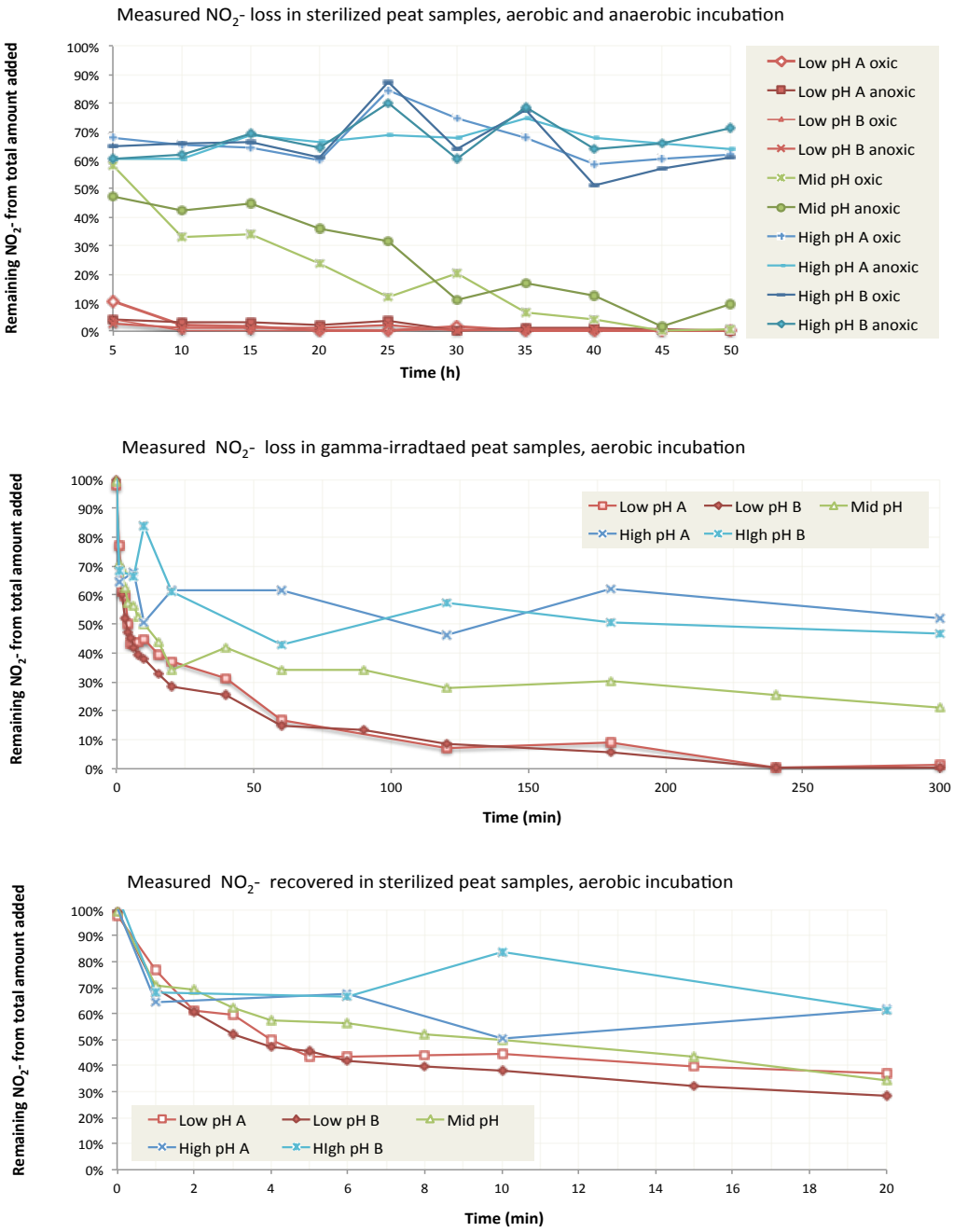


Figure 3.1. Loss of NO_2^- in sterilized peat over 50 hours, in five-peat samples. Each peat sample was used to prepare two patches of flasks with 5 g fresh weight peat, with a total of 2.5µmol NO_2^-. One batch from each peat sample was anaerobically incubated, while the other was anaerobically incubated. NO_2^- was measured every fifth hour over a period of 50-hours. Note the different time scales, where the top, middle and bottom panel are shown on a 50-hour, 300 min and 20 min time scale, respectively

3.3.2 Sterilization as a cause of rate change

I compared the rate at which NO_2^- was lost in sterilized and non-sterilized peat, and found the same change in rate after one minute. The observed change in rate, at which NO_2^- was loss, was therefore found to be independent of sterilization.

3.3.3 Concentration of NO_2- as a cause of rate change

I further investigated if the rate of NO_2^- loss could be a consequence of the initial concentration of NO_2^- added to the peat. As the change in rate of NO_2^- loss was found to be independent of sterilization, only non-sterilized peat was used. Ten replicate 1.5 mL microcentrifuge tubes with intact peat (0.1 g dry weight) was prepared in two batches, one from a low pH peat sample, and one from a high pH peat sample. Different concentrations of NO_2^- was added to the ten replicate tubes from each of the two batches, and incubated for 10 minutes before quantifying the amount NO_2^-. The NO_2^- concentrations were: 0.01 µmole, 0.1 µmole, 1 µmole, 2 µmole, 2.5 µmole, 5 µmole, 7 µmole and 10 µmole N g^{-1}. The amount of NO_2^- loss was found to be approximately 50%, regardless of NO_2^- concentrations and pH value of the peat.

3.3.4 Ion-exchange as a cause of rate change

I proceeded to test whether the observed rate change at which NO_2^- was lost, could be explained by ion-exchange with peat particle i.e. due to the establishment of equilibrium between the peat particles and the liquid present in the peat. In the previous two incubation experiments, I measured NO_2^- loss by extracting the anion from the intact peat into a liquid form, using milliQ water. To confirm the occurrence of ion-exchange, I swapped the milliQ water with 0.01M KCl, which neutralized the anion binding capabilities of the peat, i.e. eliminating the possibility of ion-exchange. Additionally, I amended the peat with NO_3^- instead of NO_2^-, due to the unstable nature of NO_3^- which is prone to self-degradation. The immediate one-minute rapid rate of loss was found when measuring NO_3^- with milliQ water, while measurements with KCl did not change the concentrations of NO_3^- in the liquid for neither low nor high pH samples.

3.4 Nitrogen gasses produced in sterilized peat

The robotized incubation system was used to detect the nitrogen gasses NO, N_2O and N_2 from the five sterilized peat sample. Three replicate 120 mL flasks with 5 g fresh weight peat (2.5 g dry weight) were prepared from each of the five-peat samples. The flasks were made anaerobic in He-atmosphere and NO_2^- was added to a total of 2.5 µmole NO_2^- per flask. The flasks were measured for gas development every fifth hour, over a period of 135-hours.

Most of the NO_2^- was recovered in the form of NO. The low, mid and the high pH samples produced on average 1.31, 1.24 and 0.94 µmol NO per vial, respectively. The accumulated amounts of N_2O-N were notably different between the peat samples, where the mid pH accumulated on average more than 10-times the amount of the low or high pH samples. The amounts of N_2-N produced was close to negligible in all five-peat samples, with a gross average of 0.029 µmole N per via (Table. 3.3).

Table 3.3. *Nitrogen gasses measured from 120 mL flasks with sterilized peat, incubated anaerobically for 135-hours. Each flask contained a total of 2.5 g dry weight peat, and 2.5 µmole NO_2^-. Gases are presented as µmole N per flask*

Sterilized peat samples	NO [µmol flask^{-1}]	N_2O-N [µmol flask^{-1}]	N_2-N [µmol flask−1]
Low pH A	1.31	0.15	0
Low pH B	1.24	0.13	0.04
Mid pH	0.96	1.61	0.11
High pH A	0.12	0.19	0
High pH B	0.06	0.17	0

The relationship between the apparent NO_2^- disappearance and recovery through nitrogen gases, i.e. the product stoichiometry, was not only calculated for the low and mid pH samples as the NO_2^- was completely degraded and, therefore, easier to deduce. The product-stoichiometry from decay of NO_2^- for the low pH sample was: 60% NO-N and 12% N_2O-N, and for the mid pH samples: 34% NO and 24% N_2O-N. However, if we disregard the reduction of NO gas that occurred in the mid-pH sample after 50-hours, the NO product stoichiometry become 40% instead of 34%. For all five-peat samples, there is still an unaccounted amount of N that amounts to 28% and 26% for the low and mid-pH sample, respectively.

3.5 Simulating NO_2- disappearance in sterilized peat

The apparent loss of NO_2^- in peat was found to be the results of two processes: ion exchange with peat particles (i.e. establishment of equilibrium), and chemical NO_2^- decay. A set of parameters were defined with the help from Lars Bakken, to simulate mathematically the loss of NO_2^-. These parameters include; a partitioning factor (P) for binding of NO_2^- to peat, a first order decay rate constant (K_d) for each peat sample, the fraction of NO_2^- in water (WF), and the constant for sorption of NO_2^- from liquid to peat particles (K). The parameters for the model are summarized in Table. 3.4.

Table 3.4. *Parameters for mathematical modeling of NO_2^- loss in five sterilized peat samples. Parameters include a P = partitioning factors of NO_2^- bound to peat particles through ion-exchange, WF = fraction of NO_2^- in water surrounding peat particles, K_d = first order decay rate and K = rate of sorption to peat particles*

Sterilized peat samples	~pH	P	WF	$K_d(min^{-1})$	K (min^{-1})
Low pH (A)	3.2	0.77	0.44	0.0146	0.245
Low pH (B)	3.2	0.96	0.49	0.0139	0.214
mid pH	5.1	0.74	0.43	0.0014	0.166
High pH (A)	7.2	1.37	0.58	7 · 10-6	0.355
High pH (B)	7.2	1.06	0.52	4 · 10-5	0.050

The fitness of the NO_2^- decay model was determined by comparing the simulated and measured NO_2^- amounts. The simulated values were shown to be in good agreement with the measured NO_2^- loss, as shown in Figure 3.2 for one of the low pH samples (R=0.99). The simulated values follow the measured amounts of NO_2^-, and also takes into account the rapid and initial rate of loss due to ion exchange.

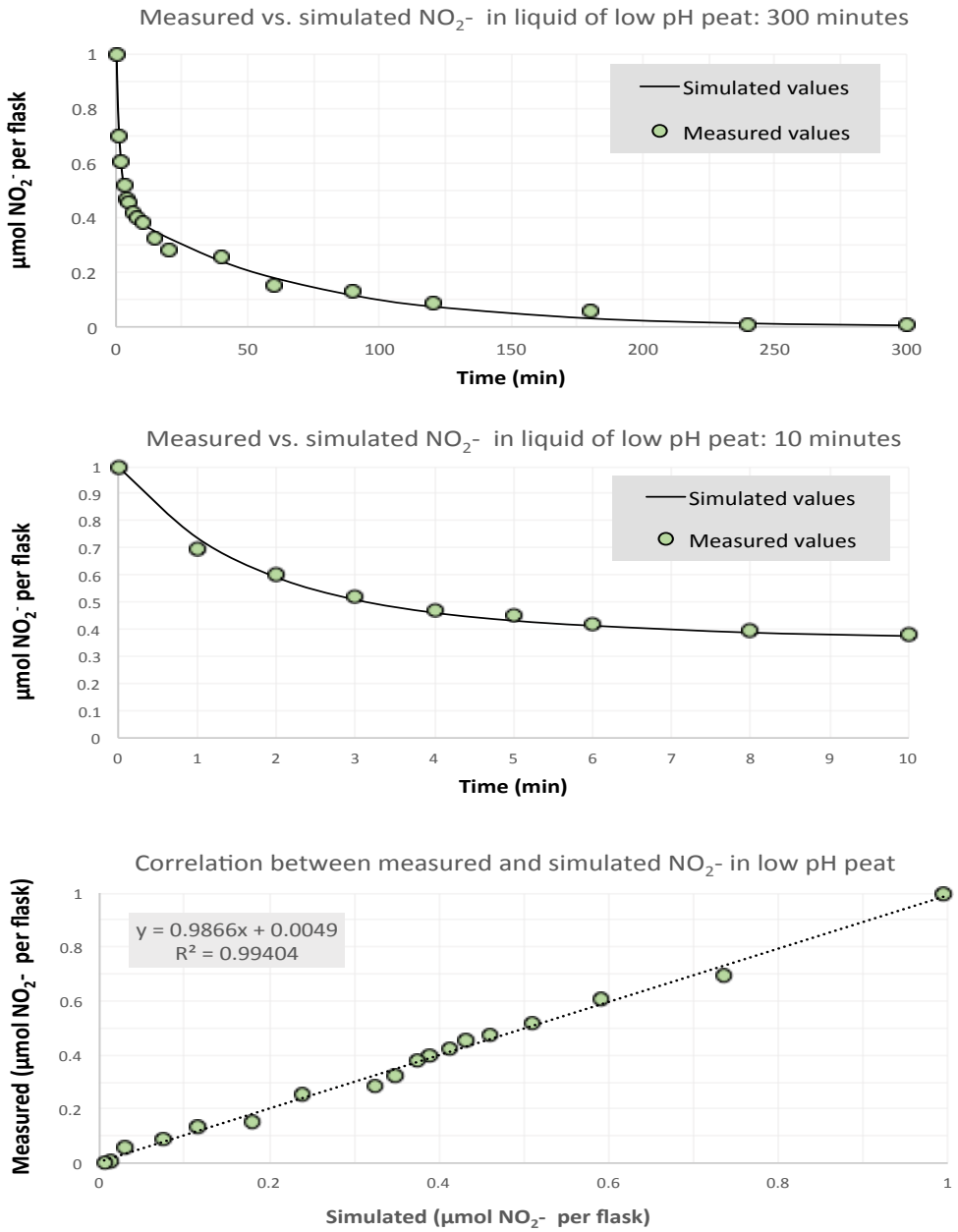


Figure 3.2. Measured versus simulated NO_2^- loss in low pH peat. The initial concentration of NO_2^- was 1 µmol NO_2^-. The parameters of the simulated decay in low pH peat are: K_d = 0.0146 (min^{-1}), and P = 0.77. Note the different scales on the graphs where the top and middle panel are shown on a 300 min and 10 min scale, respectively. The correlation between the measured and simulated NO_2^- loss is shown in the bottom panel where R^2 = 0.99.

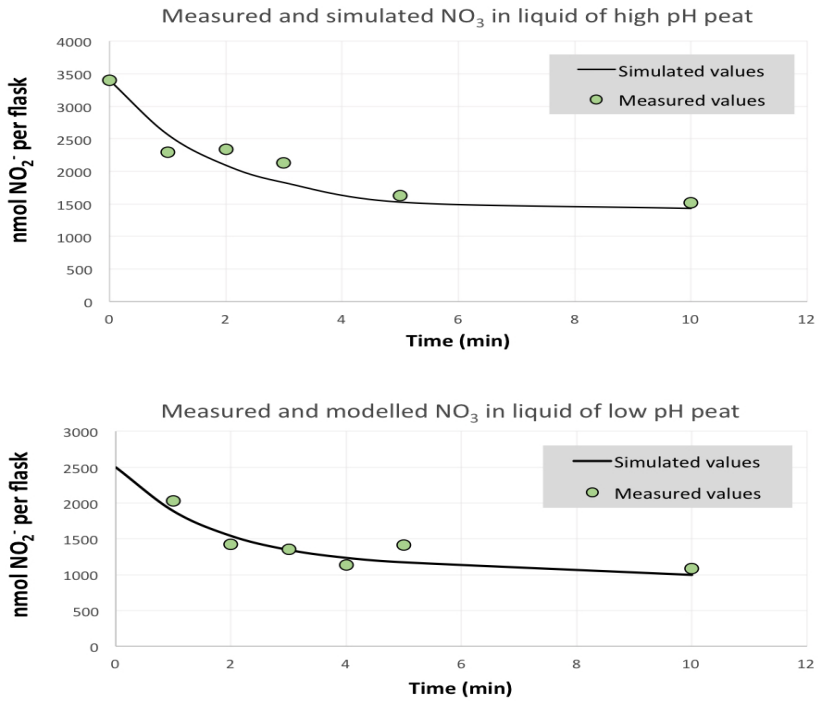


Figure 3.3. Measured NO_3^- compared to simulated NO_3^- loss non-sterilized low pH peat

As the model fits the measured values, we can use the simulated decay model to compare NO_2^- loss to NO production, as NO was the main product of chemodenitrifiation. As shown in figure 3.4, the production of NO follows the decay of NO_2^- in the low and mid pH samples.

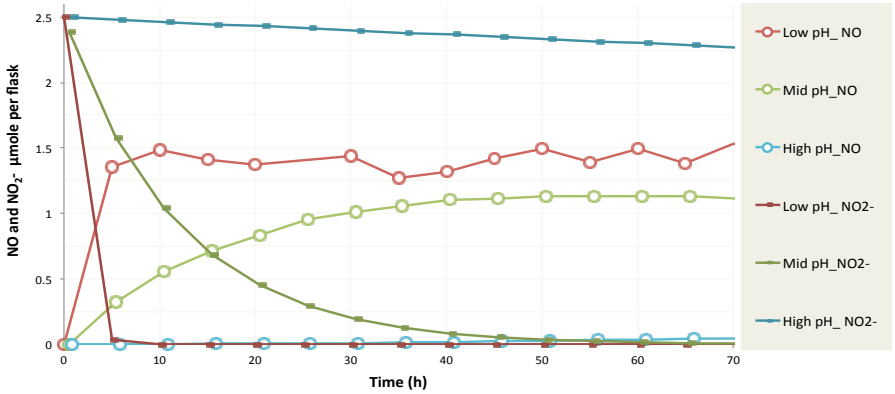


Figure 3.4. Simulated NO_2^- decay and measured NO production in sterilized peat, anaerobically incubated over a total period of 135-hours [enitre period not shown]. The measured NO gas is graphed from three representative flasks with one low pH sample, one mid-pH sample and one high pH sample (amended with 2.5 µmol NO_2^- per vial).

3.6 Nitrogen gas production in non-sterilized peat

Gas production in non-sterilized peat is the sum of chemical and biological reduction of NO_2^-. Simulated NO_2^- decay in sterilized peat can be used to determine the rate of which chemical decay, relative to microbial respiration, contributes to the total rate of nitrogen gas production. Thus, I proceeded to anaerobically incubated and monitored non-sterilized peat over a period of 135-hours, to determine the total production of nitrogen gas. Also, NO_2^- was measured every third hour throughout the incubation, to account for its production from NO_3^-, and compare to the relative differences between a low (A6), mid (B7) and high (C5) pH peat sample.

Three non-sterilized peat samples from plot A6, B7, and C5 gathered at the experimental site at Furene-set in Fjaler, were prepared in flasks to measure nitrogen gas production and NO_2^-. Prior to incubation, each peat sample was characterized in order to standardize the peat in each flask to the following; a water content of 90 %, a NO_2^- concentration of 5 mM, and a total Soil Organic Cabon Content (SOC) of 1.5 g per flask. Between 15 and 24 replicate flasks were prepared from each peat sample: three flasks from each peat were monitored for gas production, while the remaining flasks were stored in an external incubator to measure NO_2^- (destructive method), and snap freezing in liquid nitrogen for downstream mRNA extractions (continued work by Natalie Lim in the Nitrogen cycle research group). All flasks were incubated aerobically in He-atmosphere at 15 °C.

The low pH sample maintained NO_2^- concentrations below 1 µmol N per flask throughout the incubation, with a minor NO_2^- maximum accumulated concentrations after nearly 44 h. In contrast, the mid and high pH samples had an immediate production of NO_2^- that reached maximum accumulated after nearly 20 h around 10 and 8 µ mol N per flask, respectively. The production of NO gas was immediate in all three sample, and reached maximum accumulated concentration after about 20 h. The maximum accumulated concentration of NO from the low pH sample reached about 6 µmol N per flask, compared to the mid and high pH sample where maximum accumulated concentration reached 6 and 5 µmol N per flask, respectively. The production of N_2O was also immediate in all samples, although N_2O from the low pH sample reached maximum accumulated concentration 15 h later than N_2O from the mid and high pH peat. The maximum accumulated concentrationsof N_2O were also higher in the low pH sample, which peaked around 8 µmol N per flask. In contrast, N_2O from the mid and high pH sample peaked around 6 and 2 µmol N per flask, respectively. Other than peak concentrations of gas and time frame for complete denitrification, the production of NO and N_2O was found to follow a similar pattern in all three sample. The final denitrification product, N_2, was not produced in the low pH peat before NO concentrations were below 2 µmol N per flask - and plateaued after 48 h. In comparison, N_2 was immediately produced in the mid and high pH samples and plateaued after only 36 h. Overall, the gas production in the mid and pH samples were found to be similar, where all gasses were produced

from the onset of denitrification. In contrast, the low pH peat demonstrated a different regulation of denitrification, with delayed gas production.

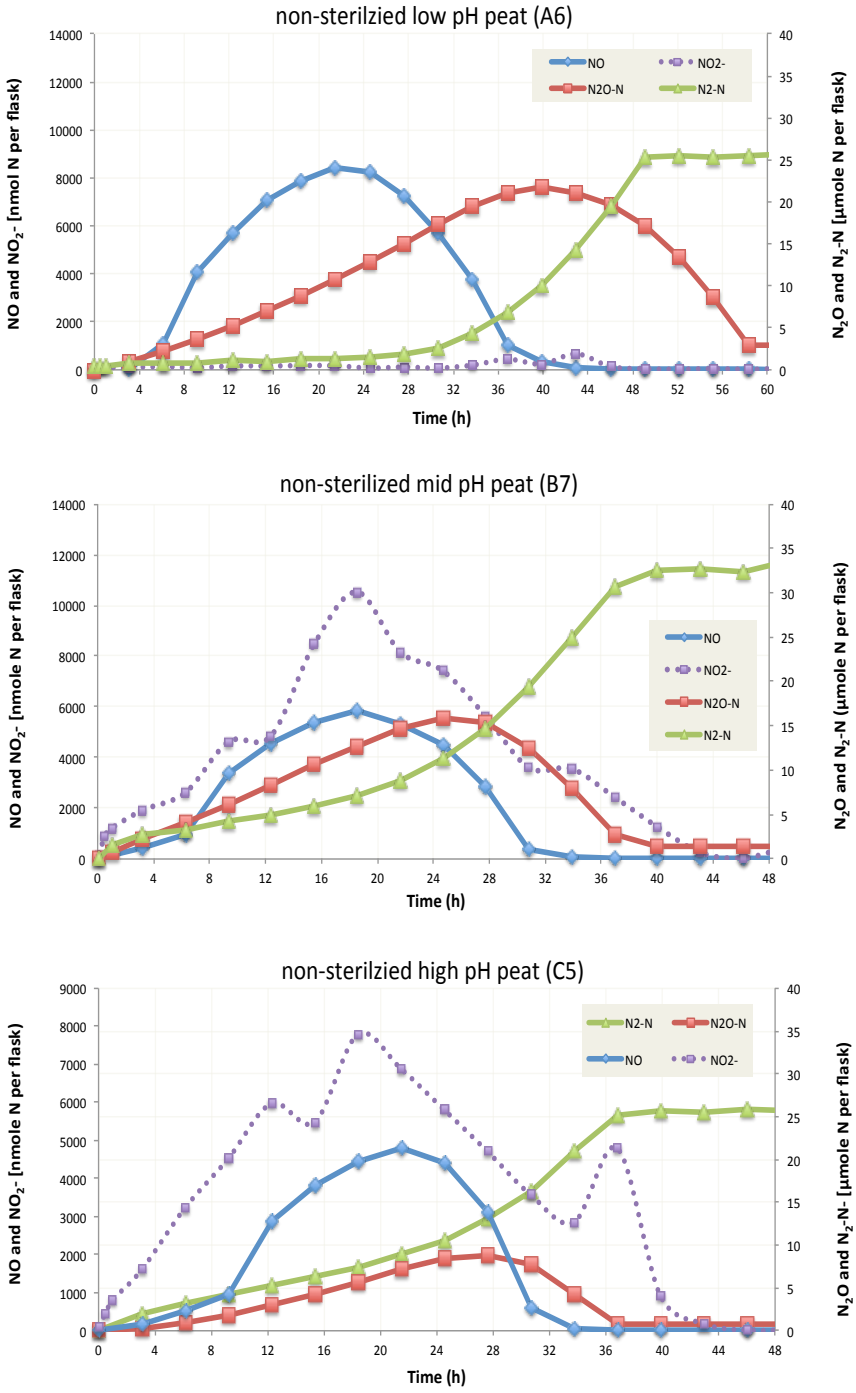


Figure 3.5. Gases produce in non-sterilized peat, from a low (A6), mid (B7) and high (C5) pH sample gathered at the experimet field site in Fjaler

The similareties between the mid-and high pH sample is further demonstrated, when comparing the concentrations of NO_2^- reative the the amount of liquid in each sample (Fig. 3.6). The mid and high pH sample than reach maximum accumulated concentration around 5 µM after 15 h, while the low pH sample only reached a minor peak after nearly 44 h.

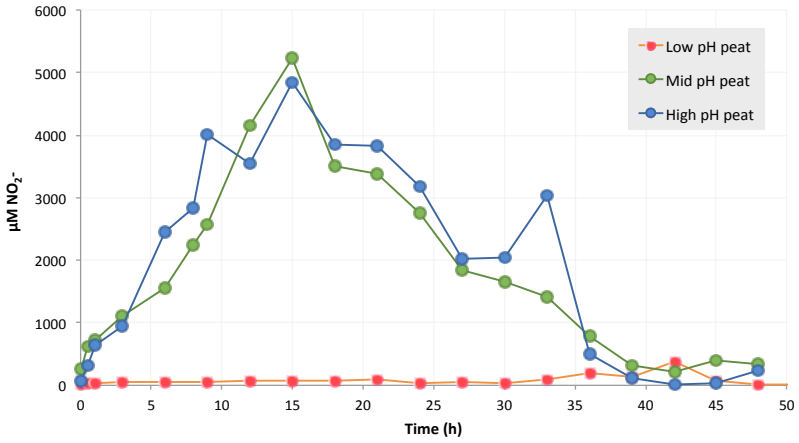


Figure 3.6. The concentration of NO_2^- per liquid in non-sterilized peat, produced through the reduction of NO_3^-. Compared between a low (A6), mid (B7) and high (C5) pH peat samples, gathered at the experimental field site in Fjaler

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3.7 Chemical vs. biological contribution to NO_2- decay

Denitrification is the sum of both microbial respiration (V_{nir}) and chemical decomposition (V_{chem}) of NO_2^-. The NO_2^- decay rates (Section 2.10), and the gross rate of NO production (GR_{NO}) from non-sterilized peat, can thus be used to define V_{nir} (Equation. 3.1).

$$V_{NIR} = GR_{NO} - V_{NOchem} \tag{3.1}$$

The rate to which N_2O and N_2 are produced in non-sterilized peat are compared to the rate contribution of VNIR and VNO2Chem, in Figure 3.7.In the low pH peat, V_{nir} keeps NO_2^- at a very low level throughout denitrification. During the first 30 h of incubation, the fraction of chemical decomposition (i.e. $V_{NO2-chem}/[V_{NO2-chem} + V_{nir}]$) accounts for an average of 19% (± 2.8%) of the total NO_2^- decay. V_{chem} is not negligible, but enzymatic activity is the main contributor to denitrification in low pH peat. In the mid pH sample, the fraction of chemical decomposition during the first 30-hours accounts for an average of 42% (± 0.4%) of the degraded no_2^-. Thus, chemical decomposition of NO_2^- contributes significantly in mid-pH samples, and the rates are comparable to the enzymatic reduction of NO_2^- throughout the incubation. In the high pH sample, the fraction of chemical decomposition during

the first 30-hours accounts for an average of 1.8% (± 0.05%) of the degraded no_2^-. Thus, chemical decomposition of NO2- is negligible in high pH samples as demonstrated by the rates.

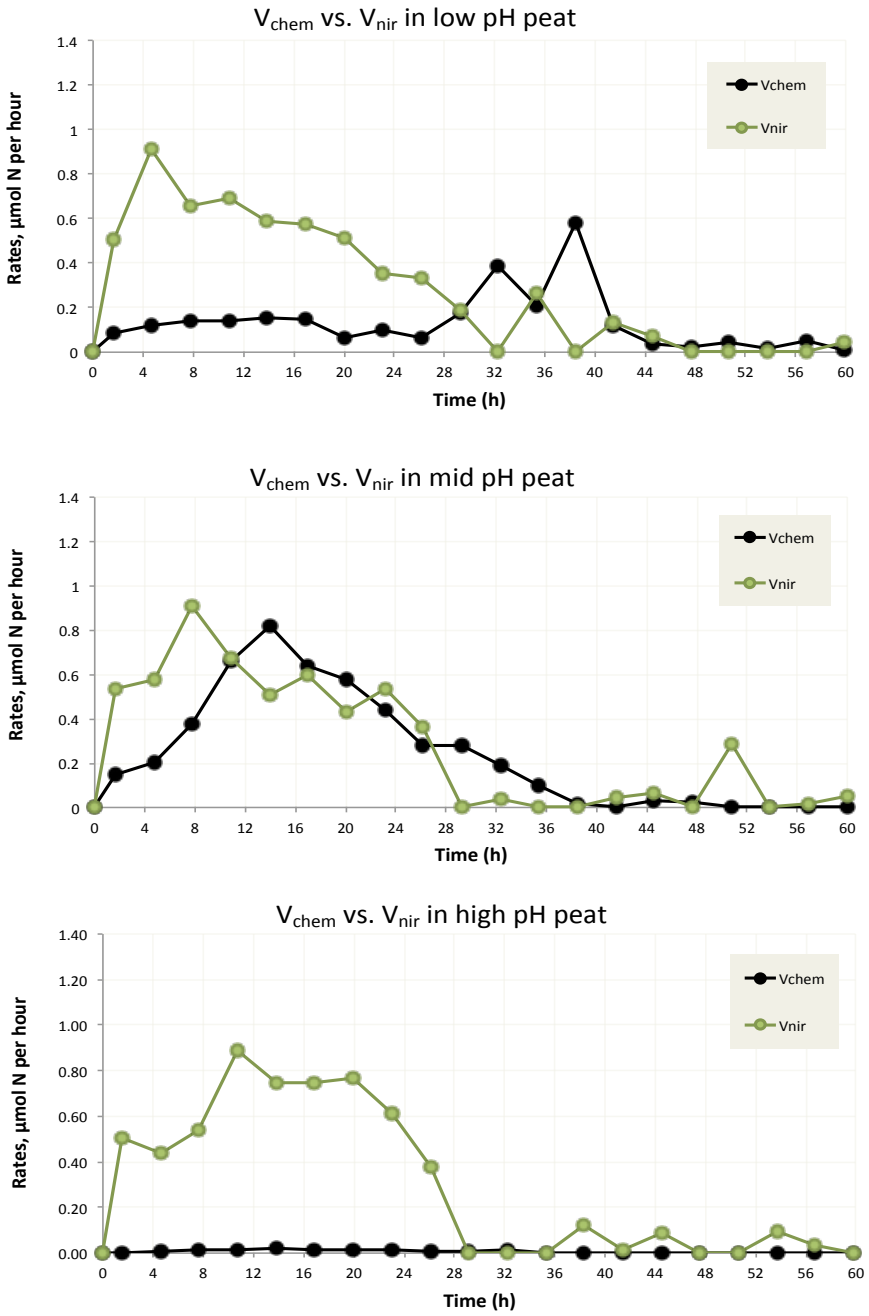

Figure 3.7. Comparison of chemical vs. enzymatice NO_2^- decay reates in low, mid and high pH peat

3.8 Optimization of primers

An optimal annealing temperature for specific binding of primer pairs to target DNA (Table. **??**), were determined with a gradient thermal cycler to secure strong and specific amplification of target genes. Primers were prepared in PCR reaction mixtures with DNA previously extracted and purified from peat and amplified. Primers that annealed non-specifically from 50 to 60 °C were re-amplified using a temperature gradient from 58 to 68 °C. Results showed that most primer pairs bound specifically to target DNA around 60 °C. However, we were unable to optimize the amplification of *nirK* (1F, 1R) and *cnorB* (1F, 6R) gene fragments. Most primer pairs had an optimal annealing temperature around 60 °C.

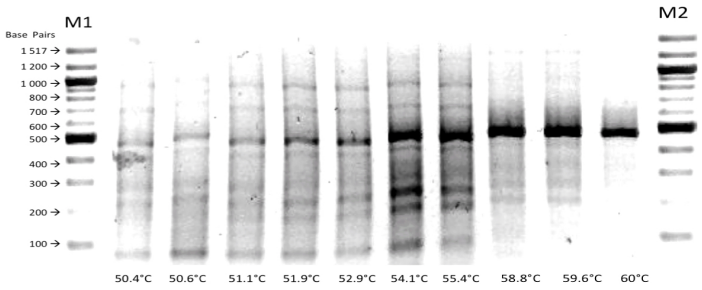

Figure 3.8. Experimental determination of optimized annealing temperature. Shown here is an example of optimization of primer pair ZF and 1622R (453bp) annealing to *nosZ* with a temperature range from 50 to 60 °C. Binding of primers to *nosZ* was determined optimal at 60 °C. M1 and M2: 100 bp ladder.

Of the available primer pairs, 3.5, we were unable to optimized the binding of *nirK* (1F,1R) and *cnorB* (1F,6R) due to unspecific binding from 50 to 68 °C. We were able to optimize amplification of *qnorB* (2F.7R), however, the amplification with the available primer pairs was weak and therefore unsuited for quantification with real-time PCR.

Table 3.5. *Experimentally determined annealing temperature of available primer pairs*. Gradient PCR was performed as described (Fig.3.8), to optimized amplification of target targeted genes in nucleic acid extracted from peat. Optimal binding was not found for *cnorB* (1F,6R) and *nirk* (1F,1R), in addition to *qnorB* which bound weakly to template DNA

Gene	Primer pair	Annealing temperature
napA	V17m, 4R	60 °C
narG	narG-f, narG-r	60 °C
nirK	1F, 1R	undefined
nirK	517F, 1055R	65 °C
nirK	FlaCu, R3Cu	65 °C
nirS	cd3aF, r3cd	61 °C
cnorB	1F, 6R	undefined
qnorB	2F, 7R	53 °C*
nosZ	ZF, 1622R	60 °C
nosZ	II-F, II-R	60 °C
16S	518R, 27F	60 °C

3.9 Extraction of nucleic acids

Nucleic acid was extracted from peat sample A6, B7 and C5, using Natalie Lim's optimized protocol. Extraction of nucleic acids was successful for all three samples. The amounts of DNA and RNA extracted from the low (A6), mid (B7) and high (C5) pH plots (hereby referred to as A6-, B7- and C5-DNA/RNA) were as following; 91 ± 24 ng A6-DNA and 20 ± 4 ng A6-RNA g^{-1} peat, 138 ± 35 ng B7-DNA and 19 ± 11 ng B7-RNA g^{-1} peat, and 125 ± 12 ng C5-DNA and 26 ± 12 ng C5-RNA g^{-1} peat (as calculated from measurements using Quibit®). From statistical analysis using *one-way ANOVA*, there was found to ba a significant difference between the samples (p=0.0168). However, *Tukey's test of pairwise comparisons*, only found a significant difference between the B7- and A6-DNA ($\alpha = 0.05$). No sigificant different was found between the amounts of extracted RNA (p = 0.408).

Crude genomic DNA and RNA extracts were visualized on agarose gels (Figure 3.9), to confirm presence of rRNA bands and DNA smears, and additionally give a rough estimation of extraction purity. Smears within the RNA extract indicates that the DNA digest was incomplete. DNA contamination can give false-positive results when quantifying gene copy number through qPCR. Purity control are therefore crucial, when working with rRNA. In the RNA extract in Figure. 3.9, both rRNA bands (23S and 16S) are detected, although some variation of band strength is observed between the replicate extracts. There are stronger rRNA bands in the A6- and C5-RNA, relative to the B7-RNA. This problem could potentially have been resolved through practice and more familiarity with the extraction and purification protocol. Since no smears were detected in the rRNA extract, it seems to be free for DNA contaminants. rRNA was therefore reverse transcribed to cDNA. Although efforts were made to improve extraction quality, there was not enough time to work on downstream processses in the course of this thesis.

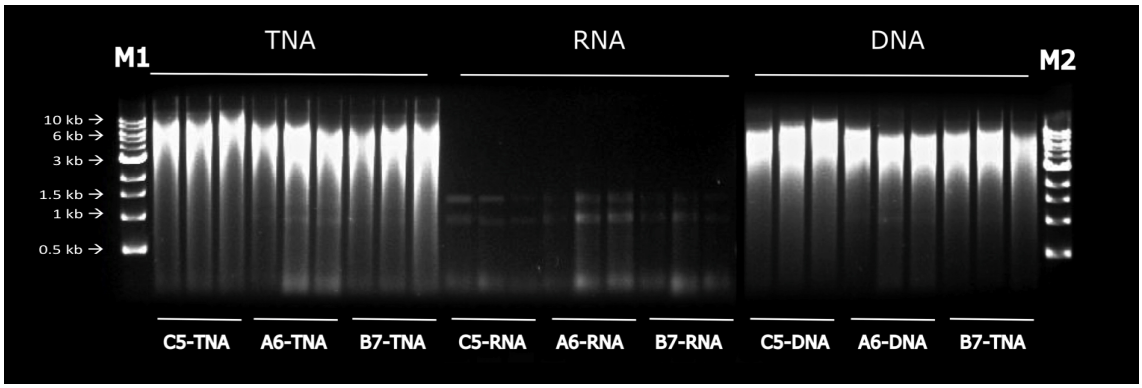

Figure 3.9. Confirming extraction and quality of TNA, RNA and DNA. Nucleic acid was separated on a 1% agarose gel with SB-buffer, at 90 V for 50 min. TNA and DNA smear were observed between 3 and 8 kb, while two ribosomal RNA bands (23S and 16S) were observed between 1 and 1.5 kb. M1 and M2: 1 Kb DNA ladder

3.10 Gene abundance in peat, evaluated through gel electrophoressis

The metabolic denitrification genes *nirS*, *nirk* and *nosZ*, were visualized on agarose gels. The strength and width of the bands were used to give a rough estimation of the abundance of these genes in the low-, mid-, and high pH peat, before quantification of gene copies using qPCR. DNA extracted from peat (A6-DNA, B7-DNA and C5-DNA) was amplified through PCR to detect 16S rRNA and metabolic genes (Fig. 3.6). The abundance of *nirS* genes was detected with the cd3aF and R3cd primer pair. The abundance of *nirK* was detected with the 517F and 1055R primer pair. The abundance of *nosZ* was detected with the F1aCu and R3Cu primer pair.

Table 3.6. *Abudance of respiratory denitrification genes was roughly indicated through visualization on an agarose gel. The abundance was roughly divided intro three categories: 1) DNA band barely or not visualized on gel (-), DNA band was visualized on gel (+) and strong DNA band visualized on gel (++).*

Gene and primer pair	Low-pH (C6-DNA)	mid-pH (B7-DNA)	high-pH (C5-DNA)
nirS (cd3aF,r3cd)	-	++	+
nirK (517F,1055R)	+	++	+
nirK (FlaCu,R3Cu)	+	++	+
nosZ (ZF,1622R)	+	+	++
nosZ (II-F,II-R)	+	+	+

3.11 Quatification of gene copies, through qPCR

The nitrogen cycle research group has a collection of plasmid DNA, with metabolic gene fragments of the denitrification process in *P. denitrificans*. However plasmid DNA with *napA* and *narG* gene fragments were not available, and therefore produced by cloning.

The gene fragments were amplified by PCR and ligated into vectors, then chemically transformed into *E.coli* for harvesting and extraction of plasmid DNA. The gene copy number was calculated for each sample of plasmid DNA, and serial diluted to 10^1 from 10^8 gene copies to asses the amplification efficiency in qPCR runs. The aplification efficiencies for plasmids with *napA* and *narG* gene fragments were subsequently 95% and 93%.

As previously stated, there was not enough time to complete quantification of all genes (Table. 3.5). I thus only quantified the gene copy numbers of *nosZ*, *nirS* and *16S* rRNA in extracted C5-, B7- and A6-DNA. As the available primers for these genes have shown strong binding to target, and previously amplified genes in DNA extracted from a similar environment. Plasmid DNA, produced by colleagues

Table 3.7. Ratio between the number of quantified genes in low, mid and high pH peat

	pH 3.8 (low)	pH 5.73 (mid)	pH 6.8 (high)
nirS / *16S* rRNA	0.36 %	13.14 %	10.14 %
nosZ / *16S* rRNA	12.30 %	0.09 %	0.37 %
nosZ / *nirS*	3410.59 %	0.75 %	3.65 %

in the nitrogen cycle group, were used as standards for quantification of *nosZ*, nirS and 16 S rRNA by qPCR. The amplification efficiency of plasmid DNA with *nosZ*, *nirS* or *16S* rRNA gene fragments were 98%, 97% and 98%, respectively. Quantification gene copy numbers were successful in both the A6-, B7- and C5-DNA.

The mean *nirS* gene copy numbers per gram peat were: 3.02×10^8 in A6-DNA,1.1×10^{10} in B7-DNA, and 8.57×10^9 in B7-DNA. The mean *nosZ* gene copy numbers per gram peat were: 1.03×10^8 in A6-DNA, 8.23×10^7 in B7-DNA and 3.18×10^8 in C5-DNA. The mean *16S* rRNA gene copy numbers per gram peat were: 8.37×10^{10} in A6-DNA, 8.37×10^{10} in B7-DNA and 3.57×10^{10} in C5-DNA

Ratio comparison showed that: *nirS* gene copies relative to the abundance of *16S* rRNA copies, increased with pH. In contrast, the abundance of *nosZ* increased with decreasing pH, relative to the abundance of *16S* rRNA copies. The relative ratio of *nosZ* to *nirS* gene copies increased with decreasing pH.

3.12 Further analysis

As my time on this project was limited, I did not manage to work on mRNA extraction and quantification. Further extractions for metagenomics sequencing will be performed by Natalie Lim, and will shed some more light on the regulation of microbial communities in acid, compared to neutral environments.

4

Discussion

The work presented in this thesis is a part of a larger project with Ph.D. candidate Natalie Lim, that aims to further understand the gas kinetics and regulation of microbial communities in acidic and pH neutral environments. Here I have aimed to understand what role chemodenitrification plays in acidic environments relative to microbial respiration, such as denitrification. Through this thesis, I have managed to evaluate the relative rate of chemical decay compared to an enzymatic reduction of NO_2^-, to determine the significance of chemodenitrification. The molecular work has also highlighted the differences between microbial communities in low, mid and high pH peat, as well as contributed to future work on metagenomics sequencing.

Chemodenitrification is the non-biological process that produces nitrogenous gasses, through acid-based reactions. Although these reactions are not thoroughly defined, they involve reactions with organic compounds and humic substances in soils (Chalk et al. 1983). A key molecule in these reaction is NO_2^-, which is known to protonate to HNO_2 under acidic conditions (Cleemput et al. 1996). NO_2^- is also a key molecule in denitrification, although the actual contribution to production of nitrogenous gas production from chemodenitrification - compared to the enzymatic reactions, is not well defined.

Nömmik and Thorin conducted one of the few studies that focused on the role of chemodenitrification (Nömmik et al. 1971). They compared an apparent NO_2^- loss over seven days, in both acidic, neutral and alkaline sterilized raw humus. In this thesis I have further investigated the loss of NO_2^- in sterilized peat with different pH-values, by repeating and further investigating the sterilization study by Nömmnik and Thorin. The measured gas from sterilized peat was found to be similar to Nömmnik and Thorin's findings: they recovered close to 60% of their added N in sterilized humus with pH 4.3, whereas I recovered about 72% of added N in sterilized peat with pH 3.2, and 74% of added N in sterilized peat with pH 5.2. In addition, accurate measurements from a chemiluminescent based method have shown that partitioning through ion-exchange, occurs in all peat samples included in this study. Further, I have used the accurate gas and NO_2^- kinetic data to simulate and determine the rate at which NO_2^- decays/ is reduced by chemical or enzymatic reactions.

4.1 Challenges when determining NO_3^- and NO_2^- concentrations in peat soils

Working with intact peat poses different challenges than pure-culture work, as the material contains a complex microbial community, and an environment that is heterogeneous. Amending peat with NO_3^- or NO_2^- demands extra precaution - to achieve an even distribution of solutes. In the two incubation experiments designed to measure NO_2^- loss in sterilized peat, I added NO_2^- using a syringe with large and small circular motions. The weakness of this method is the small volume of NO_2^- amended to the

peat (100 µL), which is difficult to distribute completely homogeneously. Therefore, I later increased the probability of a homogenous distribution of solute, by first drying the peat so that a larger liquid volume could be added. This improvement was later used when amending non-sterilized peat with NO_3^- to measure gas kinetics and NO_2^-.

When investigating the loss of NO_2^- sterilized peat, two different scales have been used: 2 g peat in the 50 h incubation, and 0.2 g peat in the 5 h incubation. An apparent weakness when using these volumes is that root hairs, pebbles, twigs or other impurities, if present, occupy a relatively large volume of the 2 or 0.2 g of peat prepared in tubes. However, when combining the measured NO_2^- loss from the 50 h and 5 h incubations experiments that used different scales, the data fits well with each other, showing that small volumes, and scaling, has not interfered with the measurements of NO_2^-. Additionally, parallel high and low pH samples were measured for NO_2^- loss. and gave similar results, indicating that variations due to heterogeneous peat did not affect the overall loss of NO_2^- in sterilized peat.

In the field of microbial ecology, there is a general lack of appropriate method for measuring NO_2^-. Spectrophotometric assays are commonly applied, although variable results are given as NO_2^- decays rapidly. The present chemiluminescent method measured NO_2^- within approximately 3 minutes of adding NO_2^- to peat. Using this method (with no incubation time), I was able to measure approximately 100% of the added NO_2^-. Also, it allowed me to measure the minute-by-minute loss of NO_2^-. This approach is therefore found to be highly accurate and appropriate for the study of NO_2^- and kinetics in soils.

4.2 Measuring the apparent disappearance of NO_2^- in sterilized peat

A clear difference was found between the low, mid and high-pH samples when measuring the loss of NO_2^- in the sterilized peats. Only the low and mid-pH samples had an apparent loss all of the added NO_2^-. This is not surprising, as NO_2^- is expected to protonate to HNO_2 under acidic conditions, and thus produce NO (cite). It was therefore not expect that NO_2^- concentrations in the pH neutral sample would decrease rapidly, however, our measurement found that approximately half the added NO_2^- was apparently lost in the high-pH sample within a 10-minute time frame.

Interestingly, all samples had an apparent loss of NO_2^- within a the first 10-minute time frame. By measuring the minute-by-minute decrease in NO_2^- concentration, the immediate decrease before a slower rate of decay initiated was found to amounted to approximately 30% of added NO_2^-. Logically, this could have been due to an after effect of sterilization. However, a comparison of NO_2^- loss in non-sterilized peat confirmed that the immediate loss occurred regardless of sterilization. As the rate of which NO_2^- was apparently lost cannot be accounted for by any known biological processes, I investigated the loss of NO_3^- in sterilized peat, and found that approximately 30% of NO_3^- was also lost

within the first minute. This is curious, as NO_3^- is not expected to protonate nor dissociate under acidic conditions, and indicates that the immediate apparent loss was due to a chemical reaction in the peat, more specifically, ion-exchange with peat particles. To confirm this, I measured NO_3^- using 0.0 1M $CaCl_2$, which resulted in no measured loss of NO_3^-. Thus, I have found that partitioning of NO_2^- does occur in peat regardless of pH, and further confirms that protonation of NO_2^- does occur under acidic conditions.

4.3 Production of gas and and NO_2^- in sterilized peat

Chalk and Smith (Chalk et al. 1983) stated that chemodenitrification could be a major source NO from soils and, in accordance, I found that chemical decay of NO_2^- is mainly recovered as NO and some N_2O in the low and mid pH samples (pH ~3.2 and ~ 5.2, respectively). The results also support that a pH around 5 is a transitional value for the protonation of NO_2^-, as hardly any gasses were produced in the high-pH samples (pH ~ 7.2), similar to Nömmik and Thorin's findings.

From the entire 135 h of gas data, NO is produced at the same rate at which NO_2^- is lost. The production of N_2O on the other hand, continues after the complete loss of NO_2^- and throughout the incubation. This indicates that N_2O might be a secondary product of NO, through some unknown chemical reaction. The results also support that NO is the main product of chemodenitrification (Chalk et al. 1983).

4.4 Production of gas and and NO_2^- in non-sterilized peat

Gas was measured in non-sterilized peat to compare the gas kinetics in a low, mid and high-pH peat, and to determine the gross production rates of each denitrification product - to further simulate and define the contribution of chemical vs. biological production of nitrogen gases.

Gas and NO_2^- production was found to be different between the low and high-pH sample, with the mid-pH peat showing a trend similar to that of the high-pH sample. NO_2^- did not accumulate in the low pH sample, and was maintained under 1 µmol N per flask throughout the incubation. In contrast, the mid and high-pH sample immediately started to accumulate NO_2^-. The concentrations of N_2O in nM reached the same peak concentrations at approximately the same time in the mid and high pH samples. Gas production in the low pH sample was staggered: NO and N_2O were immediately produced although NO reached a peak approximately 20 h before N_2O, and N_2 production was suppressed until NO concentrations were decreased to at least half of the peak concentration. The mid and high pH sample immediately produced all denitrification gasses (NO, N_2O and N_2), and also completed denitrification approximately 20 h before the low-pH sample.

These NO_2^- measurements show that denitrification in low-pH peat can completely reduce N_2O to N_2 given enough time. The results highlight that different regulatory systems might be present in the low-pH peat compared to peat with higher pH. One reason could be that the microbial community composition differs between high- and low pH soils, and that the organisms in these communities differ with respect to their regulation of NIR expression. A different regulation of NIR could be the results of a different microbial community structure, which need to be further investigated through metatranscriptomics. Since an acidic environment poses an entirely different selective pressure to the microorganisms, such as NO_2^- in its protonated form (HNO_2) and toxicity due to NO, it is thus reasonable to think that microorganism developed a strict regulation of NO_2^-.

The difference in the timing of the onset of gas production between the low compared to the mid and high pH sample, also supports that there is a different regulation of denitrification genes. Although the term denitrification regulatory phenotypes (DRP) is more appropriate to describe a particular microorganism, it can also be used to describe the collective behavior of microbial communities (Bergaust et al. 2011). One apparent trait is the immediate onset of gas production in the mid and high pH samples, while the staggered production of the denitrification gasses in the low-pH samples. This phenotypical trait has previously been termed RCO and PO, respectively (Liu et al. 2013), and support that a different regulation must be in place - that results in these phenotypes.

4.5 Chemical vs. biological contributions to nitrogen gas production

To understand the relationship between denitrification and pH, we must also understand how to microbial community relative to chemical reactions, contribute to gas production. Through mathematical modeling, thanks to the help from Professor Lars Bakken, I have been able to show the relative rate of which chemical and enzymatic reactions contribute to nitrogen gas production in peat. I found that in the low-pH samples, enzymatic reactions does account for the main contribution, although chemical reactions are still present at a low rate. Surprisingly, chemical reactions were found to contribute at about the same rate as enzymatic reactions in the mid-pH sample, for reasons still unknown. In the high-pH sample, only enzymatic reactions were found to account for nitrogen gas production. These results confirm that denitrification plays a role in acidic environments. However, microbial respiration was shown to be as important in the acidic environment. Thus, a different regulation of NO_2^- production in acidic environments, should not be entirely contributed to chemodenitrification. The gas production profiles between acidic and neutral pH, can thus be the results of a shifts in the microbial community due to a strong selective pressure - and not only due to chemical reactions in the low pH peat.

4.6 Molecular findings

Plasmid DNA with *napA* and *narG* gene fragments were successfully produced and will contribute to further research in the NMBU Nitrogen Group. Optimization of available primers was also completed, and was thus used in this thesis and with further work on the agricultural peat from Fjaler.

Co-extraction of DNA and RNA was complicated by inhibitory compounds, particularly in the low pH peat. These compounds can amongst other substances be humic acids, which are known to cause problems in downstream processes (Wang et al. 2012). DNA was successfully extracted, but due to time constraints, molecular work with RNA and metatranscriptomics was not possible within the scope of this thesis and will be completed by Ph.D. candidate Natalie Lim in the NMBU Nitrogen Group. Although I successfully extracted RNA, particular care has to be taken with the purity of the extracted RNA to avoid false-positive results when working with downstream processes such as qPCR.

A roughly estimated abundance of *nirS*, *nirK*, *nosZ* and *16S* rRNA gene copies using gel electrophoresis, indicated that there is a decreasing abundance of gene with decreasing pH. However, quantification of *nirS*, *nosZ* and *16S* rRNA gene copies with qPCR showed a clear increase in the abundance of *nirS* gene copies relative to the abundance of *16S* rRNA copies, and the exact opposite relation for *nosZ*: where the abundance increased with decreasing pH, relative to the abundance of *16S* rRNA copies . Interestingly, the relative ratio of *nosZ* to *nirS* gene copies was highest in the low pH peat. By comparing these results with the gas kinetics, it shows that the delayed/staggered reduction of N_2O in the low pH peat, should not have been due to a lack of nosZ genes. These results also support that a different regulation in the microbial community might be the cause of the observed gas and *NO2-* kinetics.

4.7 Conclusion

Through the work presented in this thesis, I have shown that partitioning of NO_2^- does occur in peat, regardless of pH. NO_2^- was also found to protonate in peat with a pH value of 5 or lower, and produce mainly NO. N_2O was also found to be a likely secondary product of NO production, through chemodenitrification. Gas production and regulation of NO_2^- in non-sterilized peat were found to distinctly different between the acidic (pH 3.1) and alkaline (pH 7.2) peat, and indicative of different regulatory systems, particularly of NIR. The rate that NO_2^- decays due to chemodenitrification was found to be low in the acidic peat, and non-exciting in the alkaline peat. Additionally, enzymatic reduction of NO_2^- was found to have similar rates in the acidic and pH neutral peats. Thus, I have shown that microbial respiration is important in acidic peat, and that NO_x production is, for the most part, a result of microbial respiration.

A

Appendices

A.1 Equipment

A.1.1 Kits

Table A.1. *Kits*

Name	Supplier	Purpose
GeneJET Plasmid MiniPrep Kit	Thermo Science	Plasmids Extraction
DNA Clean & Concentrator™-5	Zymo Research	DNA Purifiation
RNA Clean & Concentrator™-5	Zymo Research	RNA Purification

A.1.2 Laboratory equipment and intruments

Table A.2. *Laboratory Equipment*

Equipment	Supplier
100 µL glass syringe	SGE Analytical Science, Australia
120 mL air-tight glass serum flasks	Matriks AS, Norway
12 mL air-tight glass serum flasks	Matriks AS, Norway
Aerosol resistant Tips (ART)	Molecular BIoproduct, USA
Aluminium crimp	Matriks AS, Norway
Butyl-rubber sptum	Matriks AS, Norway
Disposable centrifuge tubes	Greiner bio-one GmbH, Germany
Glass beads 2.5-3.5 mm	VWR Laboratories, USA
Glass beads 0.1-0.11 mm	B. Braun Medical AG, Germany
Glass beads ca. 1 mm	Glaswarenfabrik Karl Hecht GmbH & Co KG , Germany
MicroAmp Fast 96-well reaction Plate with barcode	Applied biosystems, Life technologies
MIcroAmp Optical Adhesiv Film	Applied biosystem, Life technologies
MIcrocentrifuge tubes	Axygen Inc. USA
Needles	B. Braun Medical AG, Germany
Plasic syringes	BD Medical, USA
Pipette tips	Thermo scientific

Table A.3. *Scientific instruments*

Instrument	Supplier	Details
2720 thermal cycler	Applied Biosystems, USA	
Delta 320 pH meter	Mettler Toledo AG, Greif	
Incubator	Provocell	
FastPrep-24 Instrument	MP Bioomedical, USA	
Gel Doc XP system	Bio-Rad Laboratories, USA	
MiniSpin microsentrifuge	Eppendorf AG, Germany	
Wide Mini-Sub Cell electrophoresis system	Bio-Rad Laboratories, USA	
Nanodrop Spectrophotometer ND-1000	Nanodrop Technologies, USA	
Plate Spin II centrifuge	Kubota, Japan	
Qubit Flourometer	Invitrogen, Life technologies, USA	
SpeedVac Concentrator	Savant Instruments Inc., USA ’	
StepOnePlus Real-Time PCR System	Applied Biosystems, Life Technologies, USA	
Table Top Micro Refrigerated Centrifuge 3500	Kubota, Japan	RA-2724 Rotor
Nitric Oxide (NO) Analyzer	Sievers, USA	

Table A.4. *List of chemicals*

Substance	Supplier
100% ethanol	kemytyk Norge AS, Norway
96% ethanol	Kemytyl Norge AS, Norway
Acetic acid	Merck KgaA, Germany
Ampicillin	Sigma, USA
Agar	Merck KgaA, Germany
Agarose	Lonza, USA
Calcium choride, dihydrate ({CaCl2 * 2H2O})	Merck KgaA, Germany
Chloroform	Merck KgaA, Germany
Cycloheximide	Sigma, USA
Gel red	Biotium
Hexadecyltrimethylammonium bromide (CTAB)	Merck KgaA, Germany
Isoamyl alcohol	Merck KgaA, Germany
Isopropanol	Vinmonopolet, Norway
Phenol	Sigma, USA
Polyvinylpolypyrrolidone (PVPP)	Sigma, USA
Potassium nitrate ({KNO3})	Merck KgaA, Germany
Sodium hydroxide	Merck KgaA, Germany
Sodium iodide ({NaI})	Merck KgaA, Germany
Sodium nitrite ({NaNO2})	Merck KgaA, Germany
Sodium phosphate dibasic ({Na2HPO4})	Merck KgaA, Germany
Sodium phosphae monobasic ({NaH2PO4})	Merck KgaA, Germany

A.2 Chemicals, buffers and solutions

Table A.5. *Media and solutions*

Media or solution	Components	Amount
S.O.C	BactoTM Tryptone	2.0 g
	BactoTM yeast extract	0.5 g
	NaCl [1M]	0.057 g
	kCl [1M]	0.019 g
	$MgSO_4$	0.247 g
	H_2O	60 mL
L.B.	Tryptone	10g
	Yeast extract	5g
	NaCl	10g
	MilliQ water	950 mL
CTAB extraction buffer	CTAB	50g
	Sodium Chloride (NaCl) [0.7M]	250 mL
	Phosphate buffer [pH 8] [0.24M]	250 mL
	Polyvinylpolyoyrrolidone (PVPP)	5 g
Phosphate buffer (component of CTAB)	Sodium phosphate dibasic [0.2M]	189.4 mL
	Sodium phosphate monobasic [0.2 M]	10.6 mL
	MilliQ water	200 mL
Calcium chloride [0.01M]	Calcium chloride	0.3676 g
	MilliQ water	150 mL
Sodium iodide in acetic acid (1% w/v)	Acetic acid	12.5 mL
	Sodium iodide	0.25 g
Vanadium(III) chloride - filter twice	Vanadium(III) chloride	0.4 g

A.3 Peat collected on december 3rd 2014, from experimental plot X22

Table A.6. *Plot X22: Soil samlped in 2014*

Patch Number	Treatment	pH
1	none	low
6	none	low
12	none	low
13	none	low
18	none	low
24	none	low
2	Limed (20)	mid
7	Limed (20)	mid
11	Limed (20)	mid
14	Limed (20)	mid
19	Limed (20)	mid
23	Limed (20)	mid
3	Limed (40)	mid
8	Limed (40)	mid
10	Limed (40)	mid
15	Limed (40)	mid
18	Limed (40)	mid
20	Limed (40)	mid
22	Limed (40)	mid
4	Limed (80)	high
12	Limed (80)	high
13	Limed (80)	high
18	Limed (80)	high
24	Limed (80)	high
2	Limed (80)	high
7	Limed (80)	high
11	Limed (80)	high
14	Limed (80)	high

5

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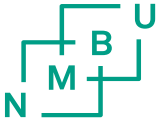

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