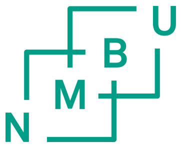

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I would also like to thank my co-supervisors Lars Bakken and Binbin Liu, without them all the results would have been in parts per million, and the phylogenetic trees would have been a bush, not trees.

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To the rest NMBU Nitrogen Group, and IKBM enviornmental microbiology group: Thank you for all nice lunches, for answering weird questions, for always being willing to help.

To my parents, who understood better than me that I needed to go home, who has always been supportive of me. To my sisters, thank you for being there to talk to and for helping me set up the trees with families, orders and classes.

# Abstract

Denitrification is an anaerobic, microbial, step-wise process, reducing nitrate through the intermediates nitrite, NO and $N_2O$ to $N_2$. Because NO and $N_2O$ are gases, they can escape the soil into the atmosphere. This is particularly bad for $N_2O$ , which is a green house gas with over 200 times the potency of $CO_2$. There are a lot of different factors at play when it comes to why soil bacteria not always complete denitrification all the way to N2, but pH seems to be a major player. When soil pH sinks, the emission of $N_2O$ rises.

Even though molecular methods are capable of extract both DNA and RNA from soil to analyse the different denitrification genes, there is still need to study of how one species express denitrification. Studies of pure cultures are the best way to see whether or not the transcription of a gene to mRNA leads to the translation of a functioning reductase.

This thesis has as a goal to isolate bacteria from soil with high (7.4) and low (3.7) pH to build a local collection of denitrifying bacteria for our research group. It also became the start of the development of a new method for identifying nitrate reduction, denitrification and phenotypes within the denitrification process, all in the same bottle.

To get an as wide isolation as possible, diluted soil slurry was plated on 1/10 Tryptic Soy Agar, and incubated aerobically for 14 days at 20 °C. Colonies were picked based on colour, size, consistency and shape, isolated on agar as well as inoculated into small flasks for gass analysis containing 1/10 Tryptic Soy Broth with 1mM nitrate and 1mM nitrite. At turbidity, the flasks were capped and sealed with a rubber septa and an aluminium cap. The headspace was rinsed and made anaerobic with helium. 1% $N_2O$ and 1% $O_2$ was added to the headspace.

After 7 days the amount of $N_2$ and $N_2O$ was quantified by gas chromatography, with an auto sampling system developed by the research group. Nitrite and NO was quantified in through the use of a Nitric Oxide Analyser. Isolates capable of reducing one or more of the compounds were sent to 16S rRNA sequencing.

Out of 99 isolates from the pH 7.4 soil, 41 isolates were capable of reducing one or more of nitrate, nitrite, NO and $N_2O$. 8 isolates were able to reduce nitrate/nitrite to $N_2$. 11 were only capable of reducing nitrate. There were isolated bacteria from19 genera from 13 families, 10 orders and 7 classes.

The isolation of bacteria from the pH 3.7 soil was split in two. 50 isolates were grown on medium with pH 7.5 (same medium as the isolates from the pH 7.4 soil grew on) while 33 isolates were grown on medium with pH 5.7. Out of 50 isolates on pH7.5 medium, 25 were positive for reduction of nitrate, nitrite, NO and/or $N_2O$. None of the isolates were able to reduce nitrate or nitrite all the way to $N_2O$. One isolate was able to reduce $N_2O$ to $N_2$, and 3 isolates reduced nitrate and nitrite to $NH_4^+$ through dissimilatory nitrate reduction to ammonium (DNRA.). Bacteria were isolated from 6 families, 4 order and 3 classes. A whole of 18 of 33 isolates grown on acidic medium were capable of reducing nitrate, nitrite, NO and/or $N_2O$. Ten isolates reduced nitrate/nitrite all the way to $N_2$.

# Samandrag

Denitrifikasjon er ein anaerob mikrobiologisk prosess som reduserar nitrat via mellomprodukta nitritt, NO og $N_2O$ til $N_2$. Sidan NO og $N_2O$ er gassar, er både i stand til å sleppa ut i atmosfæren. Særleg ille er $N_2O$, som er over 200 gonger meir potent som drivhusgass enn $CO_2$. Det er mange ulike faktorar som spelar inn når det gjeld kvifor mikroorganismane i jorda ikkje alltid er i stand til å fullføra denitrifikasjonen heilt til $N_2$, men pH ser ut til å spela ein stor rolle. Når pH i jordsmonnet synk, stig utsleppet med $N_2O$.

Sjølv om molekylære metodar er i stand til å trekkje ut både DNA og RNA frå jord for analysering av dei ulike gena som er involvert i denitrifikasjon, har ein likevel behov for å kunna studera korleis enkeltorganismar utfører denitrifikasjon. Studiar av organismen i reinkultur er den sikraste måten ein kan seie kor vidt transkripsjonen av eit gen til mRNA fører til translasjon av eit fungerande protein.

Denne oppgåva hadde som mål å isolere bakteriar frå høg-pH og låg-pH jord til ei lokal samling for forskingsgruppa. Det vart òg starten på utviklinga av ein ny metode for å identifisere nitratreduksjon, denitrifikasjon og andre fenotypar innan denitrifikasjonsprosessen samstundes, i same flaske.

For å få ei så brei isolering som mogleg, vart fortynna jord/vatn-blanding platespreidd på 1/10 Tryptic Soy Agar (TSA) og inkubert aerobt 14 dagar ved 20 °C. Koloniar vart så utvalde på bakgrunn av utsjånad (farge, storleik, konsistens), isolerte på ny agar, men også inokulert i flytande medium i små flasker for gassanalye. Flaskene innehald 1mM nitrat og 1 mM nitritt, og flytekulturane vart inkubert til vekst var synleg. Då vart flaskene kaplsa med gummisepta og aluminiumshette og lufta i flaskene vart bytta om med heliumsgass, 1% $O_2$ og 1% $N_2O$.

Etter 7 dagar vart mengda $N_2$ og $N_2O$ i flaskene fastsett ved hjelp av gaskromatografi, i eit system som er utvikla av gruppa. NO og nitritt vart måla på ein «Nitric oxide analyzer». Bakteriar positive for reduksjon av eitt eller fleire av dei tilsette komponentane vart teke vidare til 16S rRNA sekvensering.

Av 99 isolat isolerte frå jord med pH 7.4 var 41 isolat positive for reduksjon av nitrat, nitritt, NO og/eller $N_2O$. 8 isolat var i stand til å redusera nitrat/nitritt til $N_2$, 11 reduserte berre nitrat. Det vart isolert bakteriar frå 19 slekter, 13 familiar, 10 ordenar og 7 klassar.

Isolasjonen av bakteriar frå jord med pH 3.7 var delt i to. 50 isolat vart dyrka på og i medium med pH 7.5 (same vekstmediet som bakteriane frå pH 7.4 jorda hadde), medan 33 isolat vart dyrka på medium med pH 5.7. Det var 25 isolat isolert på det nøytrale vekstmediet som var positive for reduksjon av nitrat, nitritt, NO og/eller $N_2O$. Ingen var i stand til å redusera nitrat/nitritt til $N_2$, eitt isolat kunne redusera $N_2O$ til $N_2$ og 3 reduserte nitrat og nitritt til $NH_4^+$ gjennom «dissimilatory nitrate reduction to ammonium» (DNRA). Det vart isolert bakteriar frå 6 familiar, 4 ordenar og 3 klassar. Det var 18 av 33 isolat dyrka på surt medium som var i stand til å redusere nitrat, nitritt, NO og/eller $N_2O$. 10 av dei reduserte nitrat/nitritt heile vegen til $N_2$.

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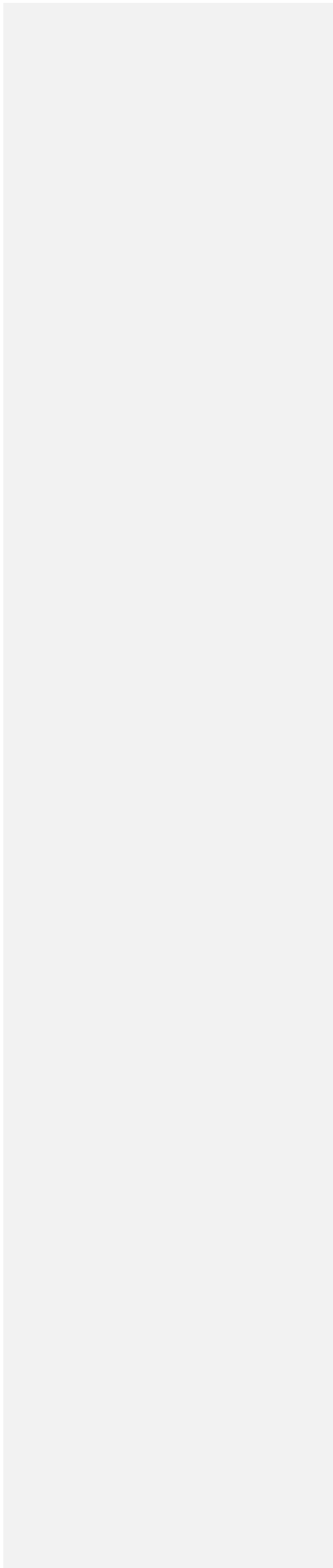

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# 1. Introduction

## 1.1 General background and aims

This master thesis is the first, initial work within a large project financed by the Marie Sklodovska-Curie-program. The project is called NORA- Nitrous Oxide Research Alliance, and within one of the work packages is the isolation of a large number of denitrifying bacteria. The aim of the thesis was, through a broad isolation program, to build a collection of bacteria from both high and low pH soil, with as wide a variety in taxonomy as possible within the parameters, and a large variation of phenotypic characteristics from complete denitrifiers (reducing nitrate to dinitrogen (N2) gas ), to those that are capable of only some of the steps.

Microbes are everywhere. Any surface we touch, the air we breathe, the water we drink, the food we eat, our insides and our outsides have microbial life. Environmental microbiology seeks to learn which organisms live in local ecosystems, how it connects to the larger ecosystems that is our planet, understand their needs, how they work, and what we can gain from this knowledge.

## 1.2 The Nitrogen cycle

The Nitrogen cycle describes the biochemical cycle of Earth's nitrogen. Most of the Earth's nitrogen reservoir is not readily available to its inhabitants, trapped as atmospheric dinitrogen gas ($N_2$), or in the Earth's crust. Because so much of the nitrogen is unavailable to most life, nitrogen often becomes a limiting factor for plant and microbial growth, leading some bacteria to use energy to fix it through nitrogen fixation. The nitrogen cycle includes amongst other the microbial prosesses: nitrogen fixation, nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), assimilatory nitrate reduction (nitrite is incorporated into the biomass), anaerobic ammonium oxidation (anamox). Nitrification is an aerobe process, while denitrification, DNRA, and anamox are anaerobic. (Maier 2009)

## 1.3 Denitrification

Denitrification is an anaerobic, microbial, step-wise process, reducing nitrate through the intermediates nitrite, nitric oxide (NO), and nitrous oxide ($N_2O$) to $N_2$. Nitrate, nitrite, NO and $N_2O$ all act as final electron acceptors in the electron transport chain when dioxygen ($O_2$) is unavailable to the bacteria. This makes denitrification a very interesting process, as fixed nitrogen is released back out in the atmosphere, creating a loss of available nitrogen in the biosphere in exchange for energy. (Zumft 1997)

Each step of the denitrification is catalysed by an enzym, a reductase named after the substrate it reduces. Nitrate is reduced to nitrite either by membrane bound nitrate reductase (NAR), or periplasmic nitrate reductase (NAP). Nitrite is reduced by nitrite reductase (NIR) to NO. NO in turn is reduced by nitric oxide reductase (NOR) to $N_2O$. $N_2O$ is reduced by nitrous oxide reductase ($N_2OR$) to $N_2$.

Denitrification is most often referred to as the reduction of nitrate through the intermediates nitrite, NO, $N_2O$, to $N_2$, although it could also be described as respiratory nitrate reduction, denitrification sensu stricto (nitrite and nitric oxide respiration) and nitrous oxide respiration. (Zumft 1997) In this thesis however full-fledged or complete denitrification is used about the reduction of nitrate to $N_2$, and any other phenotype will be described.

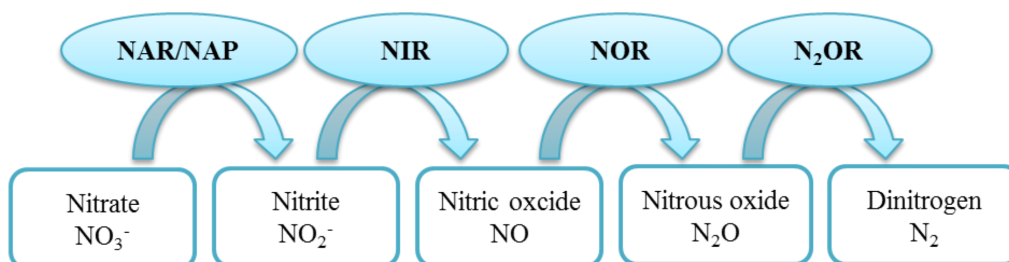



**Figure 1.1 Denitrification.** Denitrification is the step-wise process of reducing nitrate to dinitrogen with reductase enzymes. Membrane bound nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase ($N_2OR$).

## 1.4 Culturing

Microbes are everywhere. Any surface we touch, the air we breathe, the water we drink, the food we eat, our insides and our outsides have microbial life. Environmental microbiology seeks to learn which organisms live in local ecosystems, how it connects to the larger ecosystems that is our planet, understand their needs, how they work, and what we can gain from this knowledge.

We have for a long time known that the number of bacteria able to grow on plate agar medium is not the same as bacteria in the soil from which we try to isolate it. With an estimated $>10^9$ in a gram of soil, around 1% culturablility for soil bacteria is seen as the norm, with 5% as an upper limit. (Janssen et al. 2002) (Davis et al. 2005) This leaves us with quite the amount of bacteria deemed unculturable and nonculturable. While there has been a developement in culturing techniques, like creating a diffusion growth chamber in a simulated natural enviornment, (Kaeberlein et al. 2002) but compaired to plate spreading, it seems specialised and time consuming. And while the limits of culturability seem thight, molecular techniques opened up for the seemingly endless possebilities of culture-independent studies.

There have been done large isolations of denitrifying bacteria from soil(Ishii et al. 2011) earlier, with various methods for isolating them as well as how to decide whether the bacteria were capable of denitrification. Denitrification is an anaerobe process and many use the ability to grow anaerobically on, or in, a nitrate medium as a first screening, and continue only with those that grew on plates, or produced gas in medium (Cheneby et al. 2004; Dandie et al. 2007; Gamble et al. 1977; Ishii et al. 2011), although some isolate aerobically (Falk et al. 2010)

## 1.5 Molecular techniques

The discovery of the DNA structure in the 1950s , the use of 16S ribosomal RNA (16S rRNA) to determine phylogeny in the late 1970s (Woese & Fox 1977) and the development of the polymerase chain reaction (PCR) in 1983 opened the door for molecular methods and tools to microbial ecology. The last 20 years or so has seen a raise in culture-independent methods based on sequencing. The ability to extract DNA directly from an environmental sample

through chemical or mechanical lysis of the cells gives us a way to study whole microbial ecosystems without having to isolate a single culture.

Through DNA fingerprinting, one could easily see differences between two

Denautering gradient gel electrophoresis (DGGE) separates DNA based on its content, not its lenght. While initially developed for medicinal purposes, DGGE have proven effective for microbial ecologist to study diversity in a sample community DNA. (Muyzer et al. 1993; Newby et al. 2009)

The idea behind this thesis was to isolate denitrifying soil bacteria, as the number of model organisms within the field is somewhat limited, and while molecular methods might reveal the community composition, pure cultures give the chance to study how different organisms solve similar problems.
There are methods for detecting nitrate respirators and denitrifying bacteria that has been in use for many years. Some base themselves on simply detecting the absence or presence of nitrate, nitrite and gas, other methods quantify the amounts of nitrite, some measures the amount of $N_2O$ through gas chromatography. This method does the latter, although it is one of few that also quantify $N_2$.
(Newby et al. 2009)

## 1.6 Gas measurements

Gas chromatography is a way to analyse the gases in a sample, both to determine which they are and relatively quantify the content of them. As with other chromatography techniques, gas chromatography separates the different molecules in a sample from each other based on the speed they travel through, in the case of a GC, a long column. To lead the gases through, an inert carrier gas is used. Based on the time the different gases hits the sensors, we know which gases and quantified based on the signals given by known standards.

Working with $N_2$ is not easy due to the high abundance of it in the air around us it is impossible to avoid some leakage. A system needs to be as air-tight as possible. In denitrification studies one has often avoided having to measure nitrogen by inhibiting $N_2OR$ with acetylene ($C_2H_2$).

The robot system used in this thesis was similar to the one in Molstad (Molstad et al. 2007) developed for use in our research group, but used mostly for

# 2. Materials

## 2.1 Laboratory equipment

| *Laboratory equipment* | *Supplier* |
|---|---|
| | |
| Aluminium caps | Matriks |
| Automatic pipettes, various sizes | Thermo |
| Centrifuge tubes, 15 and 50 ml | |
| Disposable cuvettes | Brand |
| Drigalski spatula, glass | |
| Glass flask, 12 ml | Matriks |
| Glass flask, 50 ml | Matriks |
| Glass gas syringes with pressure lock, 5 ml | VICI Precision Sampling |
| Laboratory bottles with blue caps | |
| Microcentrifuge tubes:<br>-Regular 1,5 ml<br>-PCR tubes | <br>Axygen<br>Axygen |
| Parafilm | |
| Petri dishes, 9 cm | |
| Rubber septa | Matriks |
| Sterile filters, 0,20 µm and 0,40 µm pore size | Sarstedt |
| Syringes, 1-50 ml | BD Plastipak |
| Various glass equipment | |
| Various glass equipment | |
| Glass syringe, 10 µL | |

| *Instruments* | |
|---|---|
| | |
| 2720 Thermal Cycler | Applied Biosystem |
| Autosampler | |
| Centrifuges<br>Kubota 3500<br>Eppendorf minispin microsentrifuge | <br>Kubota<br>Eppendorf |
| Evacuation system | |
| Gas Chromatograph<br>AgilentTG Technologies, 7890A GC systems | Aglient |
| Nitric oxide analyzer NOA 280i | Sievers |
| Delta 320 pH meter | Mettler |
| WPA spectrawave s800 Diode array Spectrophotometer | |
| Gel Doc XR system (with Quantity One 1-D Analysis Software, ver. 4.6.7) | Bio-Rad Laboratories |
| Mini-Sub Cell GT or Wide Mini-Sub Cell gel electrophoresis systems | |
| NanoDrop Spectrophotometer ND-1000 | |
| SpeedVac Concentrator | |

| *Software* | |
|---|---|
| | |
| R version 2.15.2<br>-R commander | R |
| EZchrome elite | |
| NOA Firmware Version 3.00 | |
| Phyton | |
| MEGA 5.0, Molecular Evolutionary Genetics Analysis | |
| BioEdit Sequence Alignment Editor, version 7.2.0 | |
| | |

## 2.2 Chemicals

| **Chemicals** | **Supplier** |
|---|---|
| | |
| 100 X TRIS EDTA Buffer Concentrate | Fluka |
| Acetic acid, CH3COOH | |
| Bacto agar | |
| Cycloheximide | |
| Disodium hydrogen phosphate, $Na_2HPO_4$ | |
| EDTA, $C_{10}H_{16}N_2O_8$ | |
| Ethanol, $C_2H_6OH$ | Kemetyl, Norge |
| Ethidium bromide, EtBr | |
| SeaKem LE agarose | Lonza |
| Sodium dihydrogen phosphate, $NaH_2PO_4$ | |
| Sodium iodie, NaI | JT Baker |
| Sodium nitrate, $NaNO_3$ | |
| Sodium nitrite, $NaNO_2$ | |
| Sulphuric acid, $H_2SO_4$ | Norsk medisinaldepot |
| Tris Base, $C_4H_{11}NO_3$ | |
| Triton X100 | Sigma |
| | |

## 2.3 Standards

| *Standard* | *Components* |
|---|---|
| High GC standard | 150 ppm $N_2O$<br>1% $CO_2$<br>1% $CH_4$ |

| | |
|---|---|
| Low GC standard | 5 ppm $N_2O$<br>100 ppm $CH_4$<br>5 ppm $H_2$<br>250 ppm Sulphur Hexafluoride, $F_6S$<br>2000 ppm $CO_2$ |
| NO standard | 25 ppm NO in $N_2$ |
| Nitrite standard | 50 µM $NaNO_2$ in MilliQ water |

## 2.4 Media

| *Media* | *Supplier* |
|---|---|
| 1/10 TSB (Tryptic soy broth), pH 7,5<br>Medium:<br>3 g Tryptic soy broth<br>Tap water to 1 liter<br>Sterilized by autoclaving 15 minutes at 121°C<br>Agar (TSA): 1/10 TSB medium with 1,5% agar<br>Cycloheximide was added after the media had cooled to ~60°C and transferred to petri dishes. | Merck |

| 1/10 TSB, pH 5,7<br>Medium:<br>3g Tryptic soy broth<br>15 g agar<br>1,4 mL 1M $H_2SO_4$<br>6,5 mL 0,2 M $Na_2HPO_4$<br>93,5 mL 0,2 M $NaH_2PO_4$<br>Add tap water to 1 liter<br>Sterilized by autoclaving 15 minutes at 121°C<br>Agar (TSA): 1/10 TSB medium pH 5,7 with 1,5% agar<br>Cycloheximide was added after the media had cooled to ~60°C and transferred to petri dishes. | |
|---|---|
| 1/10 TSB, pH 7,5 for gas analysis<br>Medium:<br>3 g Tryptic soy broth<br>1,0 mL 1M $NaNO_3$<br>1,0 mL 1M $NaNO_2$<br>Tap water to 1 liter<br>4 mL added to 12mL flasks and sterilized by autoclaving: 15 minutes at 121°C<br><br>Cycloheximide was added at time with inoculation of the flasks | |

| 1/10 TSB, pH 5,7 for gas analysis<br>Medium:<br>3g Tryptic soy broth<br>15 g agar<br>1,4 mL 1M $H_2SO_4$<br>6,5 mL 0,2 M $Na_2HPO_4$<br>93,5 mL 0,2 M $NaH_2PO_4$<br>1,0 mL 1M $NaNO_3$<br>1,0 mL 1M $NaNO_2$<br>Tap water to 1 liter<br>4 mL added to 12mL flasks and sterilized by autoclaving: 15 minutes at 121°C<br><br>Cycloheximide was added at time with inoculation of the flasks | |
|---|---|

## 2.5 Solutions and buffers

| *Solutions/buffer* | |
|---|---|
| 0,2 M phosphate buffer pH 5,7: | 6,5 mL 0,2 M $Na_2HPO_4$<br>93,5 mL 0,2 M $NaH_2PO_4$ |
| Lysosyme solution, 20 mg/mL | 20 mg Lysozyme<br>20 µL100 x TRIS EDTA Buffer (1M Tris-HCl, 0,1M EDTA)<br>12 µL Triton X-100<br>968 µL milliQ water |
| Cycloheximide, 10 mg/mL<br>. | 100 mg cycloheximide<br>3 mL ethanol<br>7 mL milliQ water<br>Cycloheximide was dissolved in ethanol, water was added. Sterile filtered through a 0,2 µM filter. |
| TAE, 50 x | 242 g Tris Base<br>57,1 mL acetic acid<br>100 mL 0,5 M EDTA, pH 8<br>Final volume 1 l |

## 2.6 DNA

| *DNA* | |
|---|---|
| dNTP-mix, 2,5 mM | |
| DNA standards<br>1kb DNA ladder | New Englad Bio Lab |
| | |
| | |

## 2.7 Primers

| Name | Sequence | Source |
|---|---|---|
| 27F | AGAGTTTGATCMTGGCTCAG | (Weisburg et al. 1991) |
| 1492R (s) | GGTTACCTTGTTACGACTT | (Weisburg et al. 1991) |

## 2.8 Kits

| | Supplier |
|---|---|
| M0267S DNA polymerase and buffer<br>*Taq* DNA Polymerase<br>10X Buffer | New England BioLab |
| Qubit® dsDNA BR Assay<br>Qubit® dsDNA BR reagent<br>Qubit® dsDNA BR buffer<br>Qubit® dsDNA BR standard #1 (0 ng/ µL)<br>Qubit® dsDNA BR standard #2 (100 ng/ µL) | Invitrogen |
| QIAamp® DNA Mini kit<br>QIAamp Mini spin column in a 2 mL collection tube<br>Lysis buffer AL<br>Protenase K<br>Wash buffer AW 1<br>Wash buffer AW 2<br>2 mL collection tubes | Qiagen |
| E.Z.N.A.™ Gel Extraction Kit<br>HiBind® DNA column with 2 ml collection tubes<br>Wash buffer<br>Binding buffer<br>Elution buffer | Omega Bio-tek |
| | |

# 3. Methods

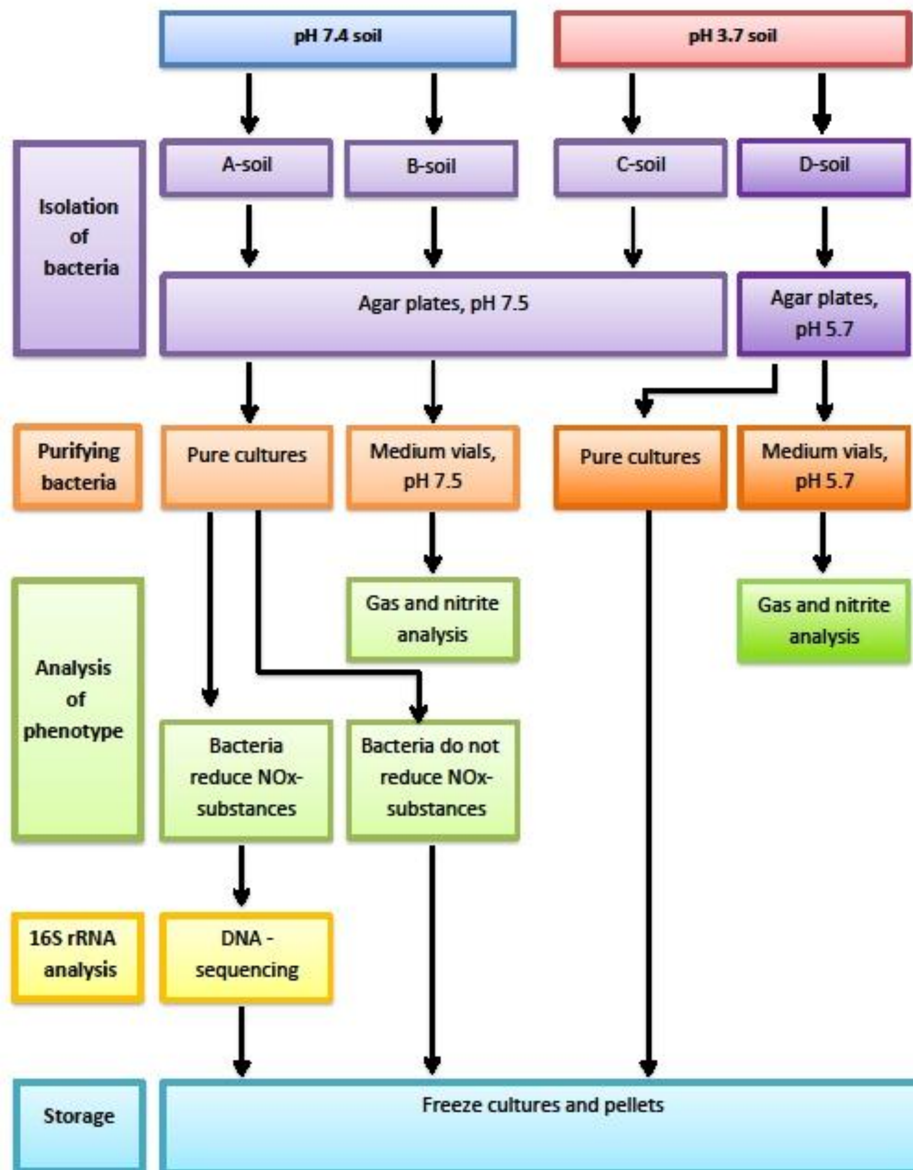



**Figure 3.1 From soil to storage, a quick overview of the setup.** Soils from the same area, but with different pH were mixed with water and plated onto agar plates. Bacteria were isolated from the plates and cultured in liquid medium as well as being purified on new plates. After gas and nitrite analysis the bacteria that could reduce NOx-substances from the A-, B- and C-soils had their 16S rRNA-gene sequenced. No bacteria from the D-soil were sequenced. All the isolates, both positive and negative, were stored at -80° C in 15% glycerol and as pellets.

**Comment [AF1]:** Dette er en kjempefin figur! Men eg er litt usikker på det oransje steget; kva mener du med Medium vials; og hvordan skiller det frå «Pure cultures»?

Og en annen ting: «Soil» er densamme i A-B og i C-D. Bruk annen betegneing, f eks «Series» eller A samples, B samples…..

## 3.1 Isolation of bacteria

Soil samples were collected from a long-term research field in Fjaler, Sogn og Fjordane, Norway (61°18'N, 5°03'E). In 1978 the field, consisting of peat soil, was divided into plots and the pH in the soil was increased from pH 3.62 to pH levels ranging from 4.7 to 7.4 by adding shell sand (Lim 2012; Sognnes et al. 2006). The high pH soil (pH 7.38) used for the A- and B- series was sampled from plot 21d in May 2012 and the isolations were done at two occasions in August/September 2012. The low pH soil (pH 3.65) was sampled from plot 6 in November 2012, and isolations were done in November 2012 (C-series) and in the last week of February 2013 (D-series).

Tryptic Soy Agar (TSA), diluted to 1/10 of normal concentration, was chosen for the isolation. The medium is considered to support growth of a wide variety of soil bacteria, and is commonly used for viable counts of these. Since soil is a nutrient poor environment, a ten times diluted TSA was considered to resemble the situation in soil more than full concentration medium. Moreover, it slows down the growth of fast growing bacteria, thus allowing more slowly growing bacteria to form visible colonies. To avoid fungal growth, all media throughout the study contained 100 mg $L^{-1}$ cykloheximide which is a commonly used antibiotic against fungi in general.

Isolation of bacteria was done according to the following protocol:

1. The bacteria were mechanically separated from the soil through blending in an omnimixer. For each of the 4 isolations A-D, 20 grams of soil was added to 200 mL autoclaved MilliRo water and the slurry was mixed in an omnimixer (3 x 1 minute at speed 7-8). The container was cooled on ice during and between mixing to avoid overheating.
2. The soil slurry was poured into an Erlenmeyer-flask and allowed to settle for 10 minutes letting soil particles sink to the bottom. Bacteria attached to those particles were thus lost.
3. Portions from the soil solution were diluted 10-fold, down to a dilution of $1/10^7$.
4. 100 µL of the dilutions between $1/10^3$ - $1/10^7$ was spread on 1/10 TSA with 100 mg $L^{-1}$ cykloheximide. Bacteria from each dilution were spread on 5-10 plates. The A-, B-, and C-series were spread on agar, pH 7.5. No adjustments were done. The D-series

was spread on agar in which the pH had been adjusted to 5.7 using a 0.02 M phosphate buffer and 1 M $H_2SO_4$.

5. The plates were wrapped in parafilm to avoid drying, and incubated aerobically at 20°C – 24 °C for at least 14 days. Colonies were counted after 14 days of incubation. After incubation, bacteria were picked from selected colonies (about 2-8 colonies per plate) with an inoculation loop and spread on new plates. Colonies were chosen based on diversity in colour, size, shape, texture, and growth time. A total of 182 colonies were isolated: 39 colonies from the A samples, 60 from the B samples, 50 from the C samples and 33 from the D samples.
6. No further purification of bacterial isolates was done at this stage since it would be more efficient to wait until after gas analyses and then only purify those that gave positive results with respect to NOx reduction.

## 3.2 End point analysis: gas and nitrite measurements

The experimental set-up was designed to identify "full-fledged" denitrifying bacteria capable of reducing $NO_3^-$ to $N_2$, as well as partial denitrifiers, which can perform one or more of the reduction steps in denitrification (i.e. reduction of $NO_2^-$, NO and/or $N_2O$). In addition, the design allowed identification of nitrate reducers and organisms performing dissimilatory reduction of nitrate to ammonium (DNRA).

Bacteria picked from single colonies were inoculated into small serum flasks with TSB, nitrate and nitrite. The cultures were grown aerobically to avoid denitrification, and first at turbidity they were sealed and made anaerobic through an evacuation cycle of helium filling and evacuation, after which a small amount of $O_2$ was added to aid the transition from an aerobic environment to an anoxic one. By adding $N_2O$ to the headspace, the bacteria had access to 3 of the 4 electron acceptors used in denitrification.

After incubation the amounts of $N_2O$ and $N_2$ in the headspace of the flasks were determined by gas chromatography using a gas chromatograph (GC) with an auto sampler (see chapter 3.2.2). NO (also in head space) and nitrite (in liquid) concentrations were measured separately using a NO-analyser (see chapter 3.2.3). The quantity of the different gases and compounds were calculated based on known standards.

The A-series was run with only one sample, as a first try, while the other series were run in duplicates. The plan was to re-run any A-isolate which seemed interesting, but it was postponed due to schedule difficulties. The other samples were run as duplicates, as the preliminary results seemed to add up rather well. This is further discussed in chapter 5.

### 3.2.1 Preparation of samples

1. The selected colonies were inoculated into 12 mL serum flasks with 4 mL autoclaved, sterile 1/10 Tryptic Soy Broth containing 1 mM $NaNO_3$, 1 mM $NaNO_2$, and 100 µg/mL cycloheximide. The flasks were wrapped in sterile aluminium foil and incubated at 20°C till turbidity. The A, B and C series were inoculated into TSB medium of pH 7.5, while the D-series was inoculated into TSB medium of pH 5.7.
2. At turbidity the flasks were sealed with sterile rubber septa and capped with aluminium caps. An anoxic environment was created in the flasks by repeatedly evacuating the air and pumping helium into the flask in a cycle of 120 s evacuation followed by 30 s of helium filling. The cycle was repeated 3 times, and ended with additional 40 s of helium filling. A sterile filter with pore size of 40 µm was used between the needle and the gas system to avoid contamination.
3. The pressure in the flasks was adjusted to atmospheric pressure by letting out the excess helium through a dismantled syringe. By having some water in the syringe, gas flow became visible, and air from the atmosphere was prevented from entering the vials.
4. 0.075 mL pure $O_2$ and 0.075 mL pure $N_2O$ was added to the flasks.
5. The flasks were incubated at 20°C and analyses were conducted after 7 and 14 days.

### 3.2.2 Gas chromatography (GC)

**Figure 3.2 Gas chromatograph (GC) and autosampler.** To the left is a schematic overview of the autosampler and the GC, to the right is a photograph of the setup. The photograph shows the autosampler with its arm to the left, the pump is on top of the autosampler and the GC is to the right side. The flasks are in a rack giving known positions for the robot to sample from. Helium is fed through the pump. (The figure is modified from that found in Molstad et al. 2007)

The headspace of the samples was analysed on an Agilent 7890A GC, containing a Molsieve column to separate $N_2$ from $O_2$, a thermal conductivity detector (TCD), an electron capture detector (ECD) and a flame ionization detector (FID). The GC was connected to an auto sampler which was controlled by a program written in Python, while the GC was controlled through the program EZchrome elite. (Molstad et al. 2007)

$O_2$, $N_2$, $CO_2$ and higher levels of $N_2O$ were measured with the TCD and lower levels of $N_2O$ were measured with the ECD. The samples were placed in a rack with 2 slots for standards in 120 mL serum flasks and 129 slots for small serum flasks. This set-up allowed us to monitor 65 samples at a time. By sampling air every other sample, one tried to avoid $N_2O$ carry-over between the samples.

*Procedure:*

1. Samples were placed in a rack for small serum flasks, with every other slot empty to allow air sampling to avoid residue $N_2O$ in the column from one measurement contaminate the next. Two standards, one high and one low (see Materials). The autosampler was programmed to take samples from a rack for small flasks, taking

samples from the appropriate number of flasks, pumping up for 45 seconds, a runtime of 720 seconds including 45 seconds of pumping helium back down into the flasks. Sampling from the 2 standards was done every 10 samples.

2. The samples were set up on the EZchrome elite with a prewritten method that gave the GC a runtime of 720 seconds, further instructing it to run one sequence, sampling standards every 10 samples, thus making it equal in setup as the auto sampler. Helium was used as the inert carrier gas.

### 3.2.3 NO-analysis

The relative amount of NO was quantified on a Sievers Nitric oxide analyzer NOA 280i with a connected purge chamber. NO was transported from the purge chamber to the analyser with a carrier gas ($N_2$). The NO reacted with ozone ($O_3$) in a chemiluminescent reaction creating red light that was detected by a photomultiplier tube. (Sievers 2001)

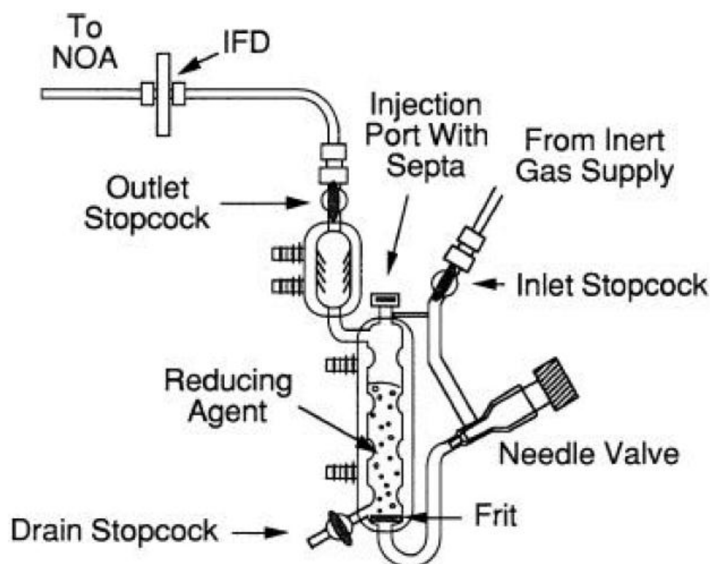



**Figure 3.3 Nitric oxide analyser-setup, the purge chamber.** $N_2$ comes in through the gas supply, acting as a carrier gas. The needle valve is used to regulate the pressure of the gas flowing in. If the sample is liquid, it's injected through the injection port septa down into the reducing agent, if it's gas it's injected into the headspace above the reducing agent (if there is any). The NO is carried by the $N_2$ to the NOA. (Figure from Sievers (2001))

*Procedure:*

1. The instrument was set up with a measuring time of 60 seconds and the gas pressure was regulated so that the cell pressure inside the analyser was equal to the air pressure in the laboratory.
2. The amount of NO in the flasks was measured by manual injection of 0.2 mL headspace gas to the purge chamber. The syringe was "washed" by pumping air from the laboratory in and out in between samplings.
3. Quantification was done based by comparing peak areas to those of a standard containing 25 ppm NO, which was was measured 5-10 times to set up a standard deviation for variation in the instrument.

### 3.2.4 Nitrite analysis

Nitrite was measured using the same instrument as for NO measurements, except that 5 mL 1% NaI in 50% acetic acid was added to the purge chamber. Nitrite was reduced to NO by the iodide, and carried by nitrogen to the NO-analyser where it reacted with ozone ($O_3$) in a chemiluminescent reaction creating red light that was detected by a photomultiplier tube. To be within the linear range of the instrument, samples were diluted from 1 mM to 50 µM $NaNO_2$

*Procedure:*

1. 0.1-0.2 mL of medium was taken from the flasks with a syringe and put in autoclaved microcentrifuge tubes.
2. Media was diluted 20 times by adding 10 µL media to 190 µL MilliQ in new microcentrifuge tubes.
3. The NO analyser was set up with approximately 5mL NaI in 50% acetic acid in the purge chamber, 60 seconds measuring time and cell pressure equal to air pressure in the lab.
4. 5 µL of standard 50 µM $NaNO_2$ was added to the purge chamber with a glass syringe. This was repeated 5-10 times.
5. 5 µL of the diluted sample was added to the purge chamber with a glass syringe.
6. In samples with apparently low nitrite concentrations, undiluted samples were analysed.

### 3.2.5 Data analysis

The quantity of the gases was calculated by converting the area of the respective peaks to ppm using the formula: (Standard ppm/standard area)*sample area = sample ppm. For nitrogen and oxygen, air was used as standard with 780000 ppm nitrogen and 210000
ppm oxygen. This was further converted to mole as described by Molstad et al. (2007)(Molstad et al. 2007). For NO and nitrite, the average of several measurements of the standard was used.

Nitrite concentrations were determined by converting the area of the NO peak to molar concentrations using the formula: (Sample concentration=Standard concentration/standard area)*sample peak area. To determine the amount of nitrite per flask, the concentration was multiplied by the volume of liquid medium in the flask (4 ml).

Deciding which isolates to sequence for identification of taxonomic affiliation was done by setting up an unpaired t-test. The average amounts of nitrite and gases was calculated in samples, and controls, for which a standard deviation and a 95% confident interval based on a unpaired t-test was set up. An unpaired t-test was set up with:
- $H_0$= No difference from zero-value.
- $H_1$= Change from zero-value.

Calculations were done in the statistical program R, with the graphical user interface R Commander.

Final determination of significance was done using R. Average values and standard deviations were calculated for isolates with two or more samples, as well for all controls. For the isolates with only one sample (thus no replication), one assumed the same standard deviation as for the controls. This is described in chapter 3.4

## 3.3 Taxonomic and phylogenetic analysis of the isolated bacteria

All bacterial isolates that showed significant reduction of one or more of the nitrogen oxides used as electron acceptors in denitrification (nitrate, nitrite, NO or $N_2O$) were identified based on their 16S rRNA gene sequences. Each isolate was cultured in liquid medium, DNA was extracted and purified, and the 16S rRNA gene was amplified using the universal primers 27F and 1492R, resulting in fragments of approximately 1500 base pairs. After gel purification, using Omega bio-tek's E.Z.N.A. ™ Gel Extraction Kit, the samples were concentrated using a vacuum centrifuge, packed, and sent to a commercial sequencing company (Macrogen, the Netherlands) for sequencing.

### 3.3.1 DNA-extraction

In this setup the DNA was extracted from the cells with the QIAamp® DNA Mini kit. To lyse the cells the procedure for Gram Positive bacteria was used. The procedure for "Tissues" was used from the booklet's point 4, to rinse the DNA.

1. Cells were inoculated into flasks with 20 mL autoclaved 1/10 Tryptic Soy Broth and allowed grow to an $OD_{660}$ of 0.5 - 1.
2. 1 mL bacterial culture was into a 1.5 mL micro centrifuge (Microcentrifuge) tube and was centrifuged for 10 min at 5000 x g (7500 rounds per minute (rpm)).
3. The pellet was suspended in a 180 µL enzyme solution with 20 mg/mL Lysozyme, 20mM Tris-HCl, 2mM EDTA, 1.2% Triton.
4. The solution was incubated at 37° C for at least 30 minutes.
5. 20 µL proteinase K and 200 uL Buffer AL was added to the solution before it was vortexed and incubated at 56° C for 30 minutes and then for a further 15 min at 95°C.
6. The samples were spun down by centrifuging for a few seconds to remove drops from the lid.
7. To the solution there was added 200 µL Buffer AL, it was mixed by pulse-vortexing for 15 s and incubated at 70°C for 10 min.
8. The samples were centrifuged for a few seconds to remove droplets from the lid.

9. 200 µL 96% ethanol was added and the sample was vortexed for 15 s, so that the solution was homogenous, and centrifuged for a few seconds to remove droplets from the lid.
10. The solution was carefully applied to the QIAamp Mini spin column in a 2 ml collection tube. The cap was closed and the sample was centrifuged at 6000 x g (8000 rpm) for 1 minute. The column was placed in a new collection tube and the old collection tube with filtrate was discarded.
11. 500 µl Buffer AW1 was added to the column, the cap was closed and the sample was centrifuged at 6000 x *g* (8000 rpm) for 1 min. The column was placed in a new 2 mL collection tube and the old collection tube with the filtrate was discarded.
12. 500 µl Buffer AW2 was added to the column, the cap was closed and the sample was centrifuged at full speed (13 900 rpm) for 3 min.
13. The collection tube and filtrate were discarded; the column was placed in a clean 1.5 ml centrifuge tube and centrifuged at full speed for 1 minute to eliminate any carryover AW2.
14. The old micro centrifuge tube was discarded, the column was placed in a new micro centrifuge tube and 200 µl distilled water or TE-buffer was added to the column. The sample was incubated for 1 minute and then centrifuged at 6000 x *g* (8000 rpm) for 1 min.
15. Repetition of step 14.

To get a more concentrated DNA-sample, although smaller DNA yield, step 14 and 15 could be done with less water or TE-buffer.

### 3.3.2 Polymerase chain reaction (PCR)

To amplify the 16S rRNA gene a polymerase chain reaction (PCR) was set up. PCR amplifies DNA through a three step process; denaturation, annealing and elongation. During the denaturation the temperature is high enough for the two strands of DNA to separate. The temperature is lowered for the annealing of the primers. They will bind to their specific sites. During the elongation, the temperature is taken up again to activate the DNA polymerase which will run along the DNA-strands, starting from the primers, adding nucleotides making

the DNA once again double stranded. Theoretically, the amount of amplified DNA is doubled every time the cycle is repeated.

Materials:

*Taq* DNA Polymerase from New England BioLabs Inc.

10X ThermoPol Reaction Buffer

dNTP-mix

Primers (see Materials, section XX)

Procedure:

The reagents were mixed in PCR-tubes accordingly to New England Biolabs instructions, with a reaction shown as in Table 3.1. This happened on ice.

**Table 3.1 PCR reagents**

| ***Reagent*** | ***Volume*** | ***Final concentration*** |
|---|---|---|
| $H_2O$ | To 50 µl | |
| 10X ThermoPol Reaction Buffer | 5 µl | 1X |
| 2,5 mM dNTP | 4 µl | 200 µM |
| 10 µM Forward primer, 27F | 1 µl | 0.2 µM |
| 10 µM Reverse primer, 1492R | 1 µl | 0.2 µM |
| Template DNA | 1 µl | <1000 ng |
| *Taq* DNA Polymerase | 0.25 µl | 1.25 units/50 µl PCR |

The samples were placed in a thermal cycler and a cycle was set up as shown in Table 3.2. PCR- settings

**Table 3.2 PCR-settings**

<table>
<tr><th>Temperature</th><th>Action</th><th>Time</th><th>Cycles</th></tr>
<tr><td>94 ° C</td><td>Initial denaturation</td><td>5 minutes</td><td>1</td></tr>
<tr><td>94 ° C</td><td>Denaturation</td><td>1 minute</td><td rowspan="3">35</td></tr>
<tr><td>50 ° C</td><td>Annealing</td><td>1 minute</td></tr>
<tr><td>72 ° C</td><td>Extension</td><td>1 minute</td></tr>
<tr><td>72 ° C</td><td>Final extension</td><td>10 minutes</td><td>1</td></tr>
<tr><td>4 ° C</td><td>Storage</td><td>∞</td><td></td></tr>
</table>

### 3.3.3 Agarose gel electrophoresis

To determine if the PCR amplicons were of the expected size,, 5 µl of the PCR-reaction was analysed by gel electrophoresis on a 1% agarose gel.

1) 0.5 g agarose was added to 50 1X TAE buffer in an Erlenmeyer flask and the solution was heated in a microwave until all the agarose was melted. For larger gels, 0.8 g agarose and 80 ml 1X TAE buffer was used.
2) The solution was cooled to around 60°C, and a drop of 0.7% ethidium bromide was added and the solution was thoroughly mixed.
3) The solution was added to a moulding chamber and a comb was put down. The gel was set to cool.
4) After the gel was cooled, it was moved to a gel running chamber and covered in 1X TAE buffer.
5) 5 µl of the PCR product was mixed with loading dye on parafilm before added to the gel together with a 1kb ladder.
6) The gel was run at 90-110 V for about 30-45 minutes.
7) The gel was taken from the gel running chamber and photographed under UV light.

### 3.3.4 Gel extraction and purification

PCR-purification was done by using the Omega bio-tek's E.Z.N.A. ™ Gel Extraction Kit, using spin columns.

1.) Up to 50 µl PCR-product was run on an agarose gel, and the bands were highlighted under UV-light.
2.) The band representing the amplified 16 S rRNA gene fragment was cut out of the gel with a scalpel and put into a clean pre-weighed 1.5 ml micro centrifuge tube, and the gel bit was weighed. Volume was decided by assuming a density of 1g/ml to the gel, giving 0.3 g gel a volume of 0.3 mL.
3.) An equal volume of Binding Buffer (XP2) was added. The mixture was incubated at 55°C until the gel was completely melted. The mix was shaken every 2-3 minutes.
4.) 700 µL DNA/agarose solution was added to a HiBind® DNA column in a 2 ml collection tube and centrifuged 1 minute at 10000 x *g*.

5.) Flow through was discarded and step 4 was repeated until all DNA/agarose solution had been centrifuged.
6.) 300 µL Binding Buffer (XP2) was added to the column and it was centrifuged 1 minute at 10000 x *g*. Flow through was discarded.
7.) 700 µL SPW Wash Buffer was added to the column and it was centrifuged 1 minute at 10000 x *g*. Flow through was discarded.
8.) The column matrix was dried by centrifuging for 2 minutes at 13000 x *g*.
9.) The column was placed in a clean 1.5 ml microcentrifuge tube. 50 µL Elution Buffer was added directly to the column matrix. Incubated for 1 minute. Centrifuged for 1 minute at 13000 x *g* to elute DNA. Column was discarded.

**3.3.5 Concentrating DNA**

The company sequencing the samples wished to have about 50ng/µL DNA in each sample. To achieve this, some of the samples had to be concentrated using a vacuum centrifuge. The vacuum lowered the evaporation temperature of the solution, allowing water to evaporate at room temperature

**3.3.6 DNA quantification**

DNA was quantified after extraction from cells, after gel extraction and after concentrating it. Two methods were used, Qubit® fluorometer and NanoDrop™.

Qubit® uses florescent dyes that specifically bind to DNA, RNA or proteins, depending on what one want to quantify. When bound they are activated and start emitting a light which is measured by the fluorometer. For example: the more DNA there is in the sample, the more dye binds to it and the stronger the light signal becomes and one can quantify the amount of sample.

Nanodrop is a spectrophotometric method, measuring how much light with wavelength 260 and 280nm the DNA and proteins in a sample absorb.

Both methods calculate the amount of DNA based on standard measurements. While Nanodrop measurements are faster, Qubit measurements are more accurate. While Nanodrop

will measure both DNA and proteins in a sample, as both would absorb light at the same wave lengths, Qubit measures the light emitted from the DNA which the florescent dye has been bound to. The dye used for DNA will not bind to proteins.

Qubit® procedure:

1. For every reaction,1 µL Qubit® dsDNA BR reagent was added to 199 µL Qubit® dsDNA BR buffer, making a master mix.
2. In Qubit assay tubes the 2 standards (0 and 100 ng/ µL) were prepared by adding 10 µL standard to 190 µL master mix. The standards were vortexed briefly, spun down, and incubated for 5 minutes at room temperature.
3. In Qubit assay tubes, 2 µL of the sample was added to 198 µL master mix. The samples were vortexed briefly, spun down and incubated for 5 minutes.
4. The instrument was set to BR dsDNA assay, the two standards were measured and the samples were measured.
5. The amount of DNA in the samples equals to QF value * (200/2).

NanoDrop™ procedure:
Procedure:

1) The spectrophotometer was calibrated with 2 µL nuclease free water.
2) The spectrophotometer was blanked with 2 µL TE buffer.
3) The samples were measured by using 2 µL of the sample.

## 3.4 Statistics

The results were analysed statistically using the program R and the graphical user interface (GUI) R Commander, with packages from the NBMU statistics group.

All gas measurements were done in duplicates, except for the A-samples which were only done as single measurements. . For all duplicate samples, the averages and standard deviations were determined. Samples in which leakage had occurred were removed and the remaining sample was treated the same way as the A-samples. The controls were grouped and averages and standard deviations were calculated for these as well.

A two sample t-test on the summarized data was performed with *mu* being the controls and *mv* being sample, testing $mu - mv = 0$ (two-sided) for nitrite, NO and $N_2O$, and $mu < mv$ (one-sided) for $N_2$ one-sided. The tests were done at 0.05 and 0.01 significance.

## 3.5 Storage

All bacterial strains are being stored in 15% glycerol at -80° C. To prepare this, the isolates were inoculated into flasks containing 20 mL 1/10 TSB (pH 7.5 for A, B and C- series and pH 5.7 for D-series) and incubated until turbidity was observed. Then, 500 µL portions of the cultures were mixed with 500 µL 30% glycerol in cryo-tubes and placed in the -80° C freezer. In addition, 1 mL portions of the cultures were centrifuged at 13 400 rpm for 2 minutes, the supernatant was removed and the cell pellets were frozen as described for the glycerol stocks.

# 4 Results

## 4.1 Isolation of bacteria

Bacterial colonies were visible after 3 days, and enumerated after 14 days, at which point the number of colonies were counted, and a total viable count was calculated for each of the 4 soil spreads (See Table 4.1). The A- and B-spread were from the same pH 7.4 soil, plated on pH 7.5 agar plates, the C-spread was from the pH 3.7 soil and spread on pH 7.5 agar, and the D-spread was from the pH 3.7 soil and spread pH 5.7 agar.

**Table 4.1 Total viable counts** Colony forming units (CFU) after 14 days of incubation from the different soils,

| Isolation serie | A (pH 7.4 soil, pH 7.5 agar) | B (pH 7.4 soil, pH 7.5 agar) | C (pH 3.7 soil, pH 7.5 agar | D (pH 3.7 soil, pH 5.7 agar) |
|---|---|---|---|---|
| **Colony forming units** | $5.7*10^7$ | $4.27*10^7$ | $3.14*10^7$ | $3.88*10^7$ |
| **CFU / g soil** | $2.9*10^6$ | $2.14*10^6$ | $1.57*10^6$ | $1.94*10^6$ |

All the counted plates had a $1:10^6$ dilution, with about between 30-50 colonies on each plate, growing mainly as individual colonies, making them fairly easy to count and to isolate from. The plates with lower dilutions ($1:10^5$) had many very small colonies, and the larger ones would sometimes merge with other colonies, or completely overtake them, making counting more difficult. Several of the plates with lower dilutions were overgrown with *Bacillus cereus var. myocides*, a bacterium often found in soil, recognisable by the characteristic hairy-looking spirals covering the agar surface. (Bergey's 2009) These plates were for the most part discarded before isolation started, except in one case where a colony inhibited the growth of it, seen as a clear zone (Sample C41). *Bacillus cereus var. mycoides* was not isolated. Fungal growth was generally sparse, a few colonies were observed, but this did not interfere with the isolation.

There were clear differences between the A- and B-plates, the C-plates, and the D-plates. The A- and B-plates were similar to each other and had mainly white and yellow colonies of

different shapes and sizes, as well as some large, dry, peach-coloured ones and small orange hard ones. The C-plates had a distinct difference between the lower dilutions and higher, as the higher dilutions showed several large orange colonies. The orange colonies were also present at the lower dilutions, but they were not as large as the one seen in Figure 4.1.1 and there were again several white and yellow colonies. The D-plate had many translucent colonies, for the most part yellow, white/grey, as well as opaque yellow and white. There were some water-coloured, transparent large colonies, but isolation of those was unsuccessful. The difference between the C- and D-spread can be seen in Figure 4.1.1, although of different dilutions.

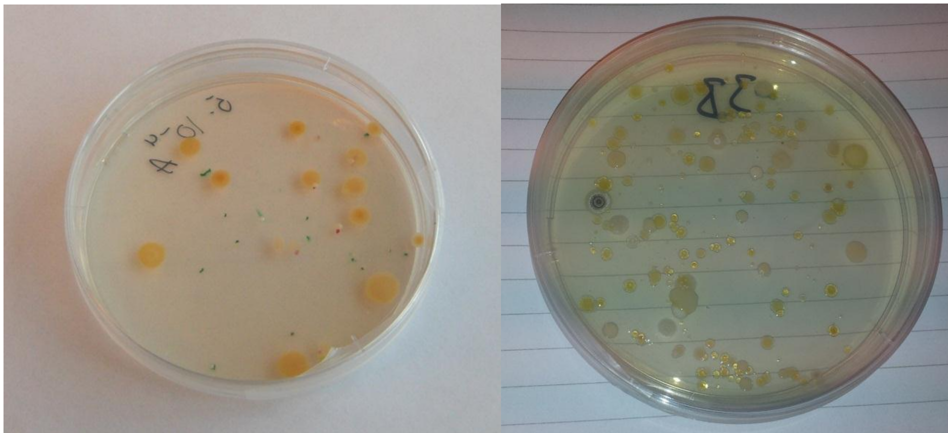


Figure 4.1.1 **Agar plates, C- and D-spread.** Plate to the left is a C-spread, the red and green dots on the plate were used for counting colonies. The large orange colonies dominated many of the plates, with smaller white and yellow colonies in between. The plate to the right is a D-spread, with mostly yellow and white colonies, some opaque, some translucent. The plates are of different dilutions.

### 4.1.2 Isolation

Bacteria were isolated from a total of 182 colonies, with 39 from the A-spread, 60 from the B-spread, 50 from the C- spread and 33 from the D- spread. The isolates were named after which spread they came from. Most of the isolates were white or yellow, but orange, peach coloured and red colonies were also isolated. Descriptions of the isolates can be found in the appendix, Tables A.01- A.05.

## 4.2 End point analysis

The end point analysis of which compounds were reduced and which compounds were accumulated in the flasks made it possible to propose a phenotype for each of the isolates and divide them into groups based on that phenotype, as seen in figure 4.2. Although not all of the groups could be distinguished between, due to the lack of nitrate measurements, the isolates have been sorted into 6 groups depending on their end-point analysis. In Figure 4.2 the different groups that was detectable have been colour coded, showing groups that were not distinguishable from each other as the same.

The results from the end point analysis are presented in two tables for each group, one table for the A- and B-isolates (from pH 7.3 soil) and one table for the C- and D-isolates (from pH 3.7 soil), even though the last two were grown on medium with different pH. All results are in µmole nitrogen N per flask, both as the total amount of the different nitrogen compounds in the vial, and the change compared to the initial amount present in the vial.
Based on nitrogen mean balance calculations (amount added *vs* amount recovered), a genotype is proposed for each of the isolates.

| | Nitrate reductases (NAR/NAP) | Nitrite reductases (NIR) | Nitric Oxide Reductase (NOR) | Nitrous Oxide Reductase (NOS) |
|---|---|---|---|---|
| Full fledged (all reductases) | Accumulates $N_2$ | | | |
| Only Nitrate reductase | Accumulates $NO_2^-$ | | | |
| Only NIR | | Accumulates NO | | |
| Only NOR (Not tested) | | | Accumulates $N_2O$ | |
| Only NOS | | | | Accumulates $N_2$ |
| NAR/NAP and NIR | Accumulates NO | | | |
| NIR and NOR | | Accumulates $N_2O$ | | |
| NOR and NOS (read as only NOS) | | | Accumulates $N_2$ | |
| NAR/NAP, NIR and NOR | Accumulates $N_2O$ | | | |
| NIR, NOR, NOS | | Accumulates $N_2$ | | |
| DNRA | $NO_3^-$ and $NO_2^-$ reduced to $NH_4^+$ | | | |

**Figure 4.2. An overview of the observed phenotypes.** Observed phenotypes and the genetic setup of the respective phenotypes as deduced from the end point analysis. Groups with the same colour appear with the same end products, since nitrate was not measured and lack of NO. The “only NOR”-group was not tested. DNRA (dissimilatory nitrate reduction to ammonium) organisms had reduced the $NO_2^-$ to NH4+, without production of $N_2O$ or $N_2$.

### 4.2.1 Full-fledged denitrification, $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

The samples able to carry out full-fledged denitrification were able to reduce all the presented nitrogen ($NO_3^-$, $NO_2^-$ and $N_2O$) to $N_2$. As nitrate concentrations were not measured, one cannot say for sure if nitrate was reduced, but based on the mean balance calculations of nitrogen, a genotype has been proposed for each of the isolates. A total of 18 of the 182

isolates (10%) showed full-fledged denitrification, 8 isolates from the pH 7.4 soil, and 10 isolates from the pH 3.7 soil.

The isolates have been separated into two tables based on the pH in the soil they were isolated from. The A-and B-isolates from the pH 7.4 soil are in Table 4.2.1, and the D-isolates from the pH 3.7 soil are in table 4.2.2. There were no full-fledged isolates found in the C-isolates.

Table 4.2.1. **Full-fledged denitrification, isolates from soil with pH 7.4, A- and B-samples.** Results from end point analysis and a proposed phenotype for bacteria isolated from pH 7.4 soil that reduced nitrite, $N_2O$, and possibly nitrate to $N_2$. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in µmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was believed present. Isolates with significant marking * or ns (not significant) had a p-value above 0.10

| **Sample** | **$NO_3^-$** | **$NO_2^-$** | **$\Delta NO_2^-$** | **NO** | **ΔNO** | **$N_2O$** | **$\Delta N_2O$** | **$\Delta N_2$** | **NAR** | **NIR** | **NOR** | **$N_2OR$** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | 4.0 | 4.1 | 0.0 | 0.0 | 0.0 | 8.0 | 0.0 | 0.0 | | | | |
| A25 | | 0.0 | -4.1 | 0.0 | 0.0 | 0.0 | -7.9 | 8.2†† | | + | + | + |
| A37 | | 0.0 | -4.1 | 0.0 | 0.0 | 0.0 | -7.9 | 17.6†† | + | + | + | + |
| B03 | | 0.0 | -4.1 | 0.0 | 0.0 | 0.4 | -7.2 | 14.0†† | + | + | + | + |
| B07 | | 0.0 | -4.5 | 0.0 | 0.0 | 0.0 | -7.3 | 6.5† | | + | + | + |
| B21 | | 0.0 | -4.5 | 0.0 | 0.0 | 0.0 | -7.3 | 14.5† | + | + | + | + |
| B24 | | 0.0 | -4.5 | 0.0 | 0.0 | 0.0 | -7.3 | 11.6† | | + | + | + |
| B33 | | 0.0 | -4.1 | 0.0 | 0.0 | 0.0 | -7.4 | 14.8** | + | + | + | + |
| B39 | | 0.0 | -4.1 | 0.0 | 0.0 | 0.0 | -7.4 | 17.9†† | + | + | + | + |

Significance marking: †† $p < 0.01$; †p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns $p > 0.10$

**Table 4.2.2. Full-fledged denitrification, isolates from pH 3.7 soil.**

Results from the end point analysis and a proposed phenotype for bacteria isolated from pH 3.7 soil grown on pH 5.7 medium (D-isolates) that reduced nitrite, $N_2O$, and possibly nitrate to $N_2$. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in μmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present. Isolates with significant marking * or ns (not significant) had a p-value above 0.10.

| **Sample** | **$NO_3^-$** | **$NO_2^-$** | **$\Delta NO_2^-$** | **NO** | **ΔNO** | **$N_2O$** | **$\Delta N_2O$** | **$\Delta N_2$** | **NAR** | **NIR** | **NOR** | **$N_2OR$** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | 4.0 | 2.8 | 0.0 | 0.0 | 0.0 | 7.6 | 0.0 | 0.0 | | | | |
| D02 | | 0.0 | -2.8 | 0.0 | 0.0 | 0.0 | -7.5 | $3.2^{\dagger\dagger}$ | | + | + | + |
| D10 | | 0.0 | -2.8 | 0.0 | 0.0 | 0.0 | -7.6 | 17.0* | + | + | + | + |
| D12 | | 0.0 | -2.8 | 00 | 0.0 | 0.0 | -7.6 | $6.9^{\dagger\dagger}$ | | + | + | + |
| D19 | | 0.0 | -2.8 | 0.0 | 0.0 | 0.0 | -7.6 | $12.1^{\dagger}$ | | + | + | + |
| D20 | | 0.0 | -2.8 | 0.0 | 0.0 | 0.0 | -7.6 | 9.1* | | + | + | + |
| D21 | | 0.0 | -2.8 | 0.0 | 0.0 | 0.0 | -7.6 | $9.7^{\dagger}$ | | + | + | + |
| D22 | | 0.0 | -2.8 | 0.0 | 0.0 | 0.0 | -7.6 | 12.5* | + | + | + | + |
| D23 | | 0.0 | -2.8 | 0.0 | 0.0 | 0.0 | -7.6 | $9.6^{\dagger\dagger}$ | | + | + | + |
| D25 | | 0.0 | -2.8 | 0.0 | 0.1 | 0.1 | -7.5 | $3.0^{\dagger}$ | | + | + | + |
| D28 | | 0.0 | -2.8 | 0.0 | 0.0 | 0.0 | -7.6 | $16.2^{\dagger\dagger}$ | + | + | + | + |

Significance marking: $^{\dagger\dagger}$ $p< 0.01$; $^{\dagger}$p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns $p > 0.10$

## 4.2.2 Nitrate reduction only, $NO_3^- \rightarrow NO_2^-$

Reduction of nitrate to nitrite without further reduction of the nitrite, was the most common of the phenotypes in this study, with a total of 40 isolates (22%) displaying this phenotype and thus accumulating nitrite (Tables 4.2.3 and 4.2.4). Another 14 showed results that indicated that they have nitrate reductase as well as one or more of the other reductases. 26 of the 40 isolates originated from the pH 7.4 soil and 14 from the pH 3.7 soil, with 12 C-isolates, grown on pH 7.5 medium, and 2 D-isolates, grown on pH5.7 medium. The proposed genotype is based on the recovery of nitrogen as nitrite accumulation, not nitrate measurements.

The isolates have been separated based on the pH in the soil they were isolated from, the A- and B-isolates from the pH 7.4 soil, and the C-and D-isolates from the pH 3.7 soil.

**Table 4.2.3. Nitrate reduction only, isolates from pH 7.4 soil.**

Results from the end point analysis for bacteria isolated from pH 7.4 soil (A- and B-isolates) that reduced nitrate to nitrite. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in µmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present. Isolates with significant marking * or ns (not significant) had a p-value above 0.10

| Sample | $NO_3^-$ | $NO_2^-$ | $\Delta NO_2^-$ | NO | ΔNO | $N_2O$ | $\Delta N_2O$ | $\Delta N_2$ | NAR | NIR | NOR | $N_2OR$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | 4.0 | 4.5 | | 0.0 | | 7.3 | | | | | | |
| A04 | | 8.7 | 4.6†† | 0.1 | 0.1 | 8.9 | ns | ns | + | | | |
| A08 | | 9.6 | 5.5†† | 0.1 | 0.1 | 9.7 | ns | ns | + | | | |
| A13 | | 9.4 | 5.3†† | 0.0 | 0.0 | 8.7 | ns | ns | + | | | |
| A20 | | 8.3 | 4.2†† | 0.0 | 0.0 | 7.4 | ns | ns | + | | | |
| A22 | | 7.4 | 3.3†† | 0.0 | 0.0 | 8.0 | ns | ns | + | | | |
| A24 | | 9.7 | 5.6†† | 0.0 | 0.0 | 8.0 | ns | ns | + | | | |
| A26 | | 6.9 | 2.8†† | 0.0 | 0.0 | 8.6 | ns | ns | + | | | |
| A28 | | 8.0 | 3.9†† | 0.0 | 0.0 | 8.3 | ns | ns | + | | | |
| A32 | | 9.2 | 5.1†† | 0.0 | 0.0 | 8.2 | ns | ns | + | | | |
| A34 | | 9.4 | 5.4†† | 0.0 | 0.0 | 8.6 | ns | ns | + | | | |
| A39 | | 6.3 | 2.2†† | 0.1 | 0.1 | 8.8 | ns | ns | + | | | |
| B04 | | 8.5 | 4.4†† | 0.0 | 0.0 | 6.9 | ns | ns | + | | | |
| B18 | | 9.2 | 4.7†† | 0.0 | 0.0 | 7.4 | ns | ns | + | | | |
| B20 | | 6.3 | 1.8†† | 0.0 | 0.0 | 7.2 | ns | ns | + | | | |
| B25 | | 9.2 | 4.7†† | 0.0 | 0.0 | 7.6 | ns | ns | + | | | |
| B26 | | 9.3 | 4.8† | 0.0 | 0.0 | 8.0 | ns | ns | + | | | |
| B28 | | 9.9 | 5.4†† | 0.0 | 0.0 | 7.5 | ns | ns | + | | | |
| B29 | | 8.8 | 4.2† | 0.0 | 0.0 | 7.8 | ns | ns | + | | | |
| B32 | | 8.3 | 4.2†† | 0.0 | 0.0 | 7.4 | ns | ns | + | | | |
| B35 | | 7.7 | 3.6†† | 0.0 | 0.0 | 7.7 | ns | ns | + | | | |
| B47 | | 6.8 | 2.7†† | 0.0 | 0.0 | 7.1 | ns | ns | + | | | |
| B52 | | 8.5 | 4.4† | 0.0 | 0.0 | 7.7 | ns | ns | + | | | |
| B54 | | 6.4 | 2.3† | 0.0 | 0.0 | 7.5 | ns | ns | + | | | |
| B56 | | 8.6 | 4.6** | 0.0 | 0.0 | 7.7 | ns | ns | + | | | |
| B58 | | 8.5 | 4.5†† | 0.0 | 0.0 | 7.7 | ns | ns | + | | | |
| B59 | | 8.5 | 4.5†† | 0.0 | 0.0 | 7.6 | ns | ns | + | | | |

Significance marking: †† $p < 0.01$; † p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns $p > 0.10$

**Table 4.2.4. Nitrate reduction only, isolates from pH 3.7 soil.**

Results from the end point analysis for bacteria isolated from pH 3.7 soil (C- and D-isolates) that reduced nitrate to nitrite. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in μmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present. Isolates with significant marking * or ns (not significant) had a p-value above 0.10.

| Sample | $NO_3^-$ | $NO_2^-$ | $\Delta NO_2^-$ | NO | ΔNO | $N_2O$ | $\Delta N_2O$ | $\Delta N_2$ | NAR | NIR | NOR | $N_2OR$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C initial | 4.00 | 4.0 | 0.0 | 0.0 | 0.0 | 7.3 | | | | | | |
| C02 | | 9.0 | 4.8†† | 0.0 | 0.0 | 7.2 | ns | ns | + | | | |
| C06 | | 8.6 | 4.4†† | 0.0 | 0.0 | 7.5 | ns | ns | + | | | |
| C07 | | 8.5 | 4.3†† | 0.0 | 0.0 | 7.3 | ns | ns | + | | | |
| C11 | | 8.2 | 4.0† | 0.0 | 0.0 | 7.6 | ns | ns | + | | | |
| C14 | | 8.1 | 3.9† | 0.0 | 0.0 | 7.3 | ns | ns | + | | | |
| C16 | | 8.6 | 4.5† | 0.0 | 0.0 | 7.2 | ns | ns | + | | | |
| C19 | | 8.8 | 4.6† | 0.0 | 0.0 | 7.3 | ns | ns | + | | | |
| C34 | | 5.8 | 1.6† | 0.0 | 0.0 | 9.6 | ns | ns | + | | | |
| C35 | | 8.9 | 4.8† | 0.0 | 0.0 | 7.6 | ns | ns | + | | | |
| C44 | | 9.0 | 4.8† | 0.0 | 0.0 | 8.7 | ns | ns | + | | | |
| C48 | | 8.1 | 3.9† | 0.0 | 0.0 | 7.8 | ns | ns | + | | | |
| C50 | | 9.4 | 5.2* | 0.0 | 0.0 | 7.6 | ns | ns | + | | | |
| D initial | 4.0 | 2.8 | | 0.0 | | 7.6 | | | | | | |
| D07 | | 3.3 | 4.1†† | 0.0 | 0.0 | 7.5 | ns | ns | + | | | |
| D24 | | 6.9 | 4.1† | 0.0 | 0.0 | 7.5 | ns | ns | + | | | |

Significance marking: †† $p < 0.01$; †p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns $p > 0.10$

### 4.2.3 Nitrate to $N_2O$: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$

The samples that lacked functional nitrous oxide reductase ($N_2OR$), but had the other reductases, were able to reduce the given nitrate and nitrite to $N_2O$. Since nitrate was not measured, one cannot say for sure if nitrate was reduced as well, but based on mean balance calculations; a genotype has been suggested for each sample. A total of 13 isolates (7%) accumulated $N_2O$, with 3 from the pH 7.4 soil and 10 from the pH 3.7 soil. The isolates from the pH 3.7 soil split into 8 C-isolates and 2 D-isolates.

The samples have been separated based on the pH in the soil they were isolated from, the A- and B-samples from the pH 7.4 (Table 4.2.5) soil and the C-and D-samples from the pH 3.7 soil (Table 4.2.3).

**Table 4.2.5 Nitrate to $N_2O$, isolates from pH 7.3 soil**
Results from the end point analysis and a proposed phenotype for bacteria isolated from pH 7.4 soil grown (A- and B-isolates) that reduced nitrite, and possibly nitrate to $N_2O$. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in µmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present. Isolates with significant marking * or ns (not significant) had a p-value above 0.10

| **Sample** | **$NO_3^-$** | **$NO_2^-$** | **$\Delta NO_2^-$** | **NO** | **ΔNO** | **$N_2O$** | **$\Delta N_2O$** | **$\Delta N_2$** | **NAR** | **NIR** | **NOR** | **$N_2OR$** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | 4.0 | 4.1 | | | | 7.3 | | | | | | |
| A02 | | 0.0 | -4.1 | 0.0 | 0.0 | 10.3 | $2.4^{\dagger\dagger}$ | ns | | + | + | |
| A23 | | 0.0 | -4.1 | 0.0 | 0.0 | 14.3 | $6.4^{\dagger\dagger}$ | ns | + | + | + | |
| B22 | | 0.1 | -4.5 | 0.0 | 0.0 | 15.2 | $7.9^{\dagger}$ | ns | + | + | + | |

Significance marking: $^{\dagger\dagger}$ p< 0.01; $^{\dagger}$p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns p > 0.10

**Table 4.2.6 Nitrate to $N_2O$, isolates from pH 3.7**
Results from the end point analysis and a proposed phenotype for bacteria isolated from pH 3.7 soil grown on pH 7.5 medium (C-isolates) and pH 5.7 medium (D-isolates) that reduced nitrite, $N_2O$, and possibly nitrate to $N_2$. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in µmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present. Isolates with significant marking * or ns (not significant) had a p-value above 0.10.

| **Sample** | **$NO_3^-$** | **$NO_2^-$** | **$\Delta NO_2^-$** | **NO** | **ΔNO** | **$N_2O$** | **$\Delta N_2O$** | **$\Delta N_2$** | **NAR** | **NIR** | **NOR** | **$N_2OR$** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C initial* | | *4.2* | | *0.0* | | *7.3* | | | | | | |
| C01 | | 0.5 | $-3.7^{\dagger}$ | 0.0 | 0.0 | 13.3 | 6.0* | ns | + | + | + | |
| C04 | | 2.2 | $-2.0^{\dagger}$ | 0.0 | 0.0 | 9.6 | $2.2^{\dagger}$ | ns | | + | + | |
| C10 | | 3.4 | -0.8* | 0.0 | 0.0 | 8.3 | $1.0^{\dagger\dagger}$ | ns | | + | + | |
| C18 | | 3.8 | -0.4* | 0.0 | 0.0 | 7.7 | $0.3^{\dagger\dagger}$ | ns | | + | + | |
| C20 | | 2.0 | $-2.2^{\dagger\dagger}$ | 0.0 | 0.0 | 9.3 | $1.9^{\dagger\dagger}$ | ns | | + | + | |
| C30 | | 0.3 | $-3.9^{\dagger}$ | 0.1 | 0.1 | 10.2 | $2.9^{\dagger}$ | ns | | + | + | |
| C38 | | 0.0 | $-4.2^{\dagger\dagger}$ | 0.0 | 0.0 | 12.2 | $4.9^{\dagger}$ | ns | (+) | + | + | |
| C39 | | 2.5 | $-1.7^{\dagger\dagger}$ | 0.0 | 0.0 | 8.8 | $1.4^{\dagger}$ | ns | | + | + | |
| *D initial* | | *2.8* | | *0.0* | | *7.6* | | | | | | |
| D01 | | 0.0 | $-2.8^{\dagger\dagger}$ | 0.0 | 0.0 | 10.5 | 2.9* | ns | | + | + | |
| D31 | | 0.0 | $-2.8^{\dagger\dagger}$ | 0.3 | 0.0 | 9.9 | 2.4* | ns | | + | + | |

Significance marking: $^{\dagger\dagger}$ p< 0.01; $^{\dagger}$p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns p > 0.10

### 4.2.4 Nitrite reduction $NO_2^- \rightarrow NO$

Nitrite reduction is the reduction of nitrite to NO and the process was performed by one sample from the pH 3.7 soil, isolated on pH 7.5 medium. Nitrate was present in the medium, but not measured. Based on nitrogen mean balance, a genotype has been suggested. Since NO was measured separately after the gas chromatography, there has been some dilution of the gas.

**Table 4.2.7 Nitrite reduction, isolate from pH 3.7 soil**

Results from the end point analysis and a proposed phenotype for bacteria isolated from pH 3.7 soil grown on pH 7.5 medium (C-isolates) that reduced nitrite t NO. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in µmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present. Isolates with significant marking * or ns (not significant) had a p-value above 0.10

| Sample | $NO_3^-$ | $NO_2^-$ | $\Delta NO_2^-$ | NO | ΔNO | $N_2O$ | $\Delta N_2O$ | $\Delta N_2$ | NAR | NIR | NOR | $N_2OR$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Initial* | 4 | 4.1 | | | | 7.3 | | | | | | |
| C13 | | 2.0* | -2.2 | $0.5^{\dagger\dagger}$ | 0.5 | 7.9 | ns | ns | | + | | |

Significance marking: $^{\dagger\dagger}$ p< 0.01; $^{\dagger}$p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns p > 0.10

### 4.2.5 Nitrous oxide reduction, $N_2O \rightarrow N_2$.

Some of the samples were only able to reduce $N_2O$ to $N_2$, not reducing nitrite. Whether or not these samples are able to reduce NO is not known. Only three samples went into this group, two from the pH 7.4 soil and one from the pH 3.7 soil.

**Table 4.2.8 Nitrous oxide reduction, isolates from both soils**

Results from the end point analysis and a proposed phenotype for bacteria isolated both soils that only reduced $N_2O$ to $N_2$. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in µmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present. Isolates with significant marking * or ns (not significant) had a p-value above 0.10

| Sample | $NO_3^-$ | $NO_2^-$ | $\Delta NO_2^-$ | NO | ΔNO | $N_2O$ | $\Delta N_2O$ | $\Delta N_2$ | NAR | NIR | NOR | $N_2OR$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | 4.0 | 4.1 | | 0.0 | | 7.3 | | | | | | |
| B1 | | 3.3 | ns | 0.0 | 0.0 | 0.0 | -6.2†† | 6.0†† | | | | + |
| B14 | | 4.3 | ns | 0.0 | 0.0 | 0.6 | -6.7†† | 5.9* | | | | + |
| C12 | | 4.7 | ns | 0.0 | 0.0 | 0.0 | -7.3†† | 9.9† | | | | + |

Significance marking: †† p< 0.01; †p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns p > 0.10

### 4.2.6 Dissimilatory nitrate reduction to ammonium (DNRA) $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$

DNRA is a process reducing nitrate via nitrite to ammonia. Eight of the isolates from the pH 3.7 soil were tentatively classified as DNRA bacteria since no nitrite was found after incubation, and no nitrogen was recovered as NO, $N_2O$, or $N_2$. However, since nitrate was not measured, it is unknown whether nitrate has been reduced or not.

**Table 4.2.9 Dissimilatory nitrate reduction to ammonium (DNRA), isolate from pH 3.7 soil**

Results from the end point analysis and a proposed phenotype for bacteria isolated from pH 3.7 soil grown on pH 7.5 medium (C-isolates) and on pH 5.7 medium (D-isolates) with no nitrite left, and no NO, $N_2O$, or $N_2$ produced. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in μmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present.

| Sample | $NO_3^-$ | $NO_2^-$ | $\Delta NO_2^-$ | NO | ΔNO | $N_2O$ | $\Delta N_2O$ | $\Delta N_2$ | NAR | NIR | NOR | $N_2OR$ | DNRA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C initial | 4 | 4.1 | | | | 7.3 | | | | | | | |
| C26 | | 0.0†† | -4.2 | 0 | 0 | 7.4 | ns | ns | | | | | + |
| C27 | | 2.0†† | -2.2 | 0 | 0 | 9.1 | ns | ns | | | | | + |
| C37 | | 0.0†† | -4.1 | 0 | 0 | 7.2 | ns | ns | | | | | + |
| C40 | | 0.0†† | -4.2 | 0 | 0 | 7.3 | ns | ns | | | | | + |
| D09 | | 2.4* | -0.4 | 0 | 0 | 7.4 | ns | ns | | | | | + |
| D06 | | 0.0†† | -2.8 | 0 | 0 | 7.0 | ns | ns | | | | | + |
| D26 | | 0.0†† | -2.8 | 0 | 0 | 3.8 | ns | ns | | | | | + |
| D30 | | 2.0† | -0.8 | 0 | 0 | 7.9 | ns | ns | | | | | + |

Significance marking: †† $p < 0.01$; † p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns $p > 0.10$

### 4.2.7 Non-classified phenotypes

Some isolates could not be classified into the defined phenotype groups, either due to high variation in the gas measurements (or low significance values) or simply because they may not have had the time to finish the reduction of NOx. These isolates are named "non-classified" in the following tables as well as in the discussion. In total 9 samples, all from the pH 3.7 soil, were classified as non-classified. Since nitrate was not measured, we cannot say for sure if nitrate has been reduced as well, but based on nitrogen recovery and significance levels, a genotype has been suggested for each sample.

**Table 4.2.10 Non-classifed isolates from pH 3.7 soil**

Results from the end point analysis and a proposed phenotype for bacteria isolated from pH 3.7 soil grown on pH 7.5 medium (C-isolates) and on pH 5.7 medium (D-isolates) that did not fit within any of the earlier presented groups. Numeric results, both total amounts and difference (Delta, Δ) from initial amounts, are presented in µmole nitrogen per flask. The proposed phenotype is marked with +, indicating whether or not the reductase was present.

| Sample | $NO_3^-$ | $NO_2^-$ | $\Delta NO_2^-$ | NO | ΔNO | $N_2O$ | $\Delta N_2O$ | $\Delta N_2$ | NAR | NIR | NOR | $N_2OR$ | DNRA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C initial | 4 | 4.2 | | 0 | | 7.3 | | | | | | | |
| C22 | | 6.0†† | 1.8 | 0 | 0 | 10.1† | 2.8 | -0.3* | + | + | + | | |
| C33 | | 3.8†† | -0.4 | 0 | 0 | 7.6* | 0.2* | 0.3* | | | | | + |
| C36 | | 1.5* | -2.6 | 0.8†† | 0.8 | 8.0†† | 0.6 | -0.2* | | + | + | | |
| C45 | | 4.3* | 0.2 | 0 | 0 | 6.8†† | -0.6 | -0.7* | + | | | | |
| C47 | | 4.8* | 0.6 | 0 | 0 | 7.0†† | -0.3 | -0.2* | + | + | + | | |
| C49 | | 4.5* | 0.4 | 0 | 0 | 7.6† | 0.3 | -0.2* | + | + | + | | |
| D initial | 4 | 2.8 | | 0 | | 7.6 | | | | | | | |
| D05 | | 2.3†† | -0.4 | 0 | 0 | 7.4† | -0.1 | -2.8* | | | | | + |
| D17 | | 0.0†† | -2.8 | 0 | 0 | 7.8* | 0.2 | 2.5† | | + | | | + |
| D32 | | 0.0†† | -2.8 | 0 | 0 | 0.0†† | -7.6 | 4.3* | | + | + | + | |

Significance marking: †† p< 0.01; †p= [0.01, 0.05]; ** p= [0.05, 0.10]; */ns p > 0.10

## 4.3 Sequence analysis

A wide range of taxonomically different bacteria representing both Gram negative and Gram positive bacteria was sequenced. While there were some isolates that were closely related, many of them was either alone or had one close relative.

Of the isolates that showed some form of reducing any of the compounds, around 70 grew to an $OD_{660}$ between 0.3 and 1 in a liquid culture and had their DNA extracted. After PCR, gel electrophoresis and gel extraction, 64 samples were sent to Macrogen Corporations, Amsterdam, the Netherlands for sequencing. Of these, 60 sequences were readable, although for 12 of them only one of the primers had worked during the sequencing, giving them a sequence between 500 and 700 base pairs to decide species by. This caused problems with aligning the samples and reference sequences, but after removing all sequences under 1000 base pairs, one was able to align them and build the phylogenetic trees seen in Figures 4.3.1, 4.3.2. and 4.3.3

Some of the short sequences were for some reason not removed in the screening, but based on the database search; they are correctly placed in the tree. All sequences were put through Blast, and the highest scores for cultured and uncultured matches are presented in Table A.05, in the appendix.

The trees shows the taxonomic distributions of organisms in each of the two soils, with A-and B-samples coming from the same pH 7.4 soil in one tree and the C-samples coming from the pH 3.7 soil in a separate tree. There is a higher variety between the isolates from the high pH soil, while the low pH soil shows 5 groups, and only one Gram positive sample (other Gram positive samples were found, but were removed at the aligning due to short sequences. See Table A.05 in the appendix). As we know from the gas results, there were not any full-fledged denitrifiers in the C-series from the low pH soil, but there were DNRA organisms. The isolates from the D-soil were not sequenced.

### 4.3.1 Sequence analysis of isolates from the pH 7.3 soil

From the pH 7.3 soil, 27 sequenced isolates were put in the tree found in figure 4.3.1 and 4.3.2. There were 13 Gram negative, and 14 Gram positive isolates. There were 4 phenotypes present: nitrate/nitrite to $N_2$, nitrate to nitrite, nitrate/nitrite to $N_2O$ and $N_2O$ to $N_2$. For the most part, closely related isolates presented the same phenotypes.

The first half of the tree consists of Gram negative bacteria from the two phyla, *Bacteroidetes* and *Proteobacteria*. The *Bacteroidetes* are separated into the classes *Cytophagia* and *Flavobacteriia*. Starting from the top of the tree, Isolate B1 was an *Algoriphagus*, order *Cyclobacteriaceae*, and the only sequenced isolate to reduce $N_2O$ to $N_2$. The two *Flavobacterum* sequenced had different phenotypes, one only reducing nitrate to nitrite (Isolate A8), the other reducing nitrate/nitrite to $N_2O$ (Isolate A2). (Bergey's 2011)
The *Proteobacteria* are represented by the three classes *Alpha*-, *Gamma*- and *Betaproteobacteria.* There were two isolates from the *Alphaproteobacteria*, both from the order *Rhizobiales.* Isolate B33 was identified as *Ensifer adhaerens,* the only isolate from the family *Rhizobiaceae*, and it reduced nitrate/nitrite to $N_2$, as well as Isolate B56, a nitrite accumulating *Afipia* from the family *Bradyrhizobiaceae.* There were two isolates from the *Gammaproteobacteria,* Isolates B39 and A25, both of which reduced nitrate/nitrite to $N_2$. They were *Pseudomonas,* of the order *Pseudomonadales.*

The *Betaproteobacteria* was the largest group of the Gram negatives, with 6 isolates. They were all from the order *Burkholderiales,* but while 4 of the isolates fell into the family *Comamonadaceae,* the isolates B22 and A20 are of genuses that have yet to be placed in a family. Isolate B22 reduced nitrate/nitrite to $N_2O$ and was identified as *Methylibium,* closely related to *Piscinibacter*. Isolate A20 reduced nitrate to nitrite and identified as an *Inhella inkyongensis*. Isolates B3 and A37 were both complete denitrifiers, and identified as *Hydrogenophaga taeniospiralis*. Isolate B26 reduced nitrate to nitrite and was identified as a *Variovorax*. Isolate A23 reduced nitrate/nitrite to $N_2O$ and was identified as a *Polaromonas*.

There were 14 sequences that represented Gram positive isolates. All of them had the same phenotype, nitrite accumulation from nitrate reduction. The isolates were distributed between the phyla *Firmicutes* and *Actinobacteria,* orders *Bacilli* and *Actinobacteria*. There were two

isolates belonging to the order *Bacillales*; Isolate B25 belonging to the genus a *Paenibacillus,* and Isolate B28 belonging to the genus a *Bacillus.*

Within the *Actinobacteria,* there were 4 orders. *Micromonosporales* contained Isolate B4, a *Micromonospora.* Order *Micrococcales* was split between the families *Microbacteriaceae* and *Micrococcaceae.* Isolate A22 was identified as a *Microbacterium* of the former order, while Isolates B32, B47, B57, B58 and B59 were identified as genus *Arthrobacter.* From order *Corynebacteriales,* family *Nocardiaceae*, Isolate B54 was identified as a *Rhodococcus.* Within the *Propionibacteriales,* the family *Nocardioidaceae* 4 isolates were identified as 3 genuses. Isolate B35 was identified as a *Marmoricola,* Isolate B52 and A24 were identified as *Actinobacterium,*while Isolate B18 was identified as an *Aeromicrobium*.

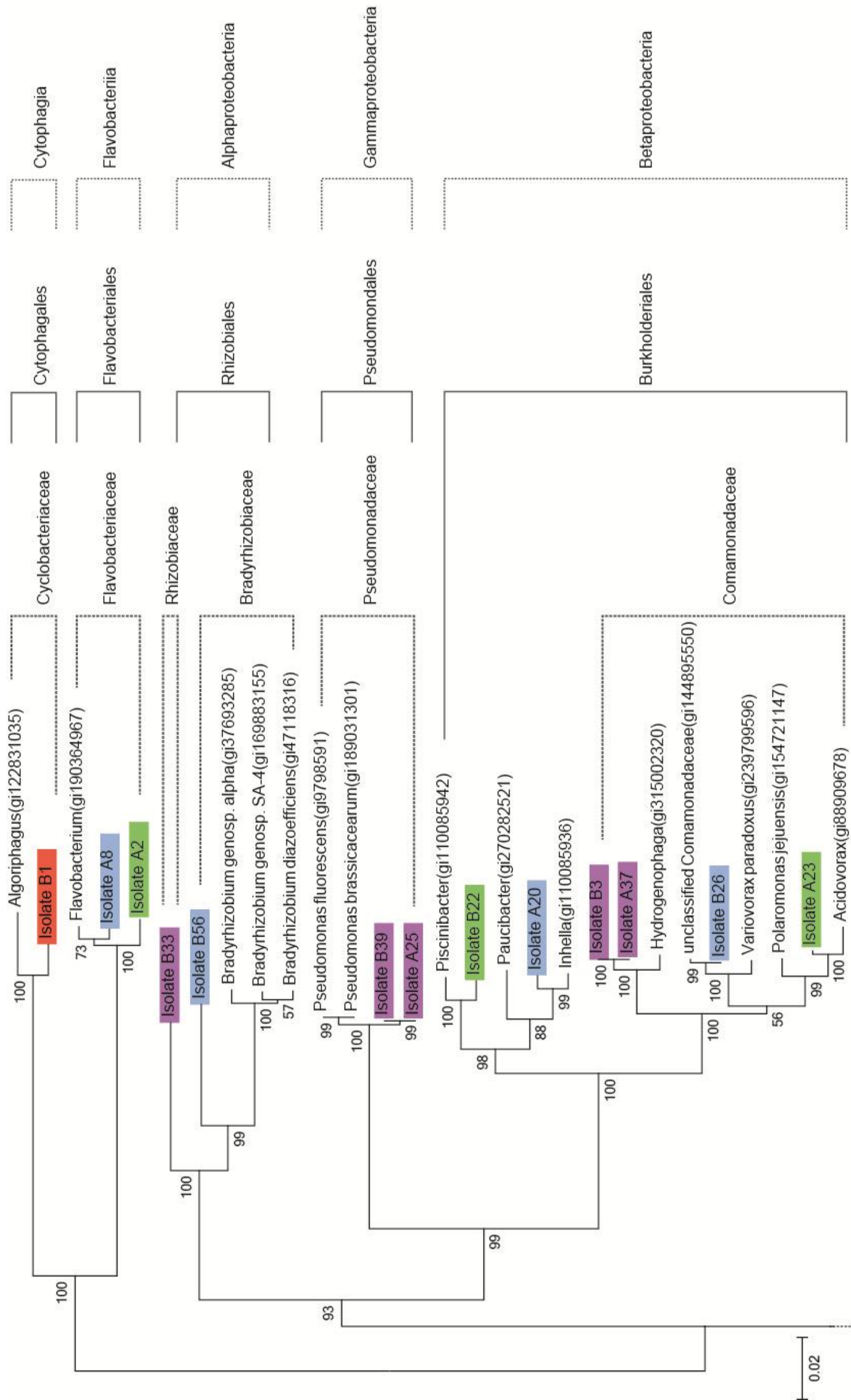
**4.3.2 Sequence analysis from the pH 3.7 soil**

From the pH 3.7 soil, 17 sequenced isolates were put in the tree found in figure 4.3.3. There were 16 Gram negative isolates and 1 Gram positive. There were 4 phenotypes present: nitrate to nitrite, nitrate/nitrite to $N_2O$, nitrate/nitrite to $NH_4^+$ (DNRA) and nitrate to NO. For the most part, closely related isolates presented the same phenotypes.

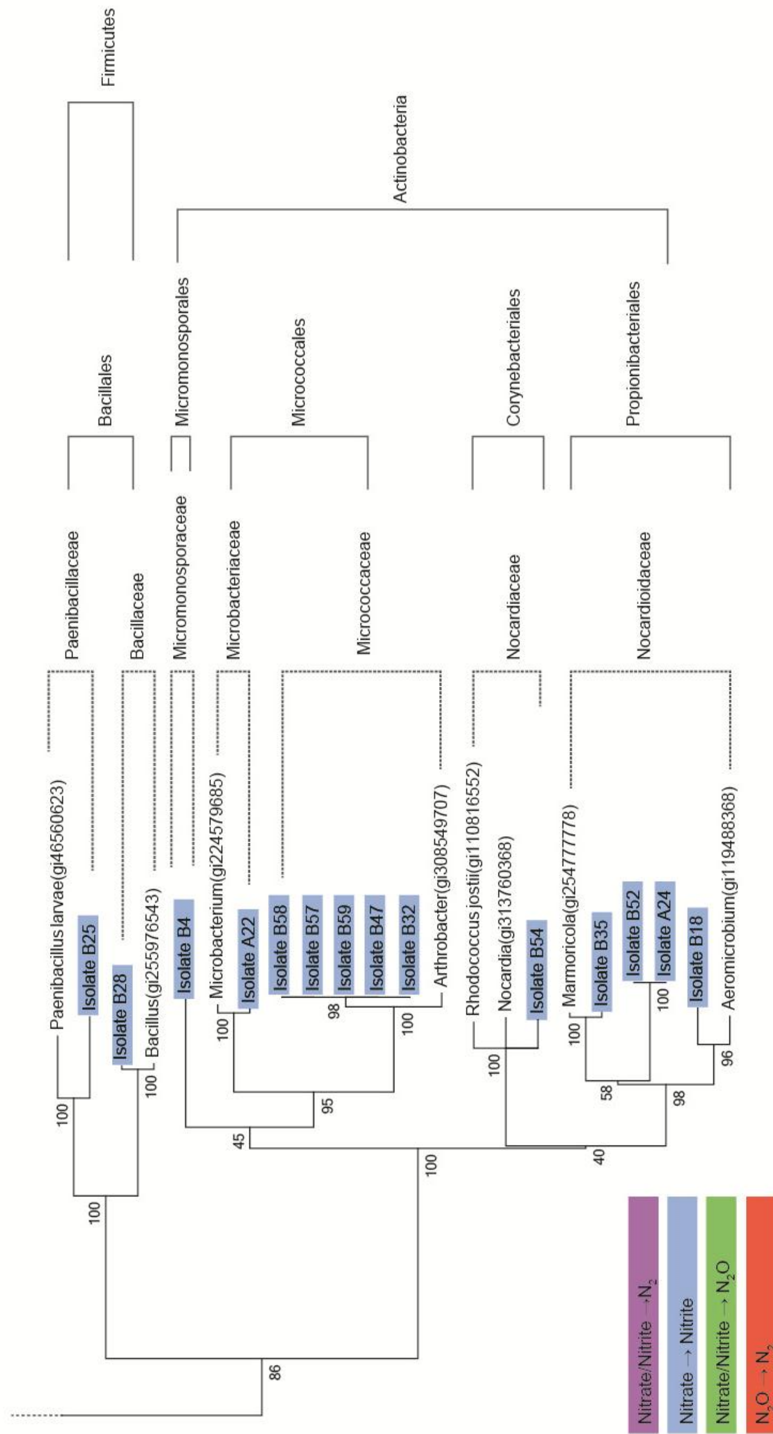
All the Gram negative isolates were from the phylum *Proteobacteria,* and split into *Betaproteobacteria* and *Gammaproteobacteria.* The *Betaproteobacteria* were all of the order *Burkholderiales,* but split into the families *Comamonadaceae* and *Burkholderiaceae*. In *Comamonadaceae* there were three isolates. Isolate C11 and C34 reduced nitrate to nitrite, while isolate C22 reduced nitrate/nitrite to $N_2O$. The isolates in family *Burkholderiaceae,* were all found to be *Burkholderia,* and all had nitrate reductase.

The *Gammaproteobacteria* split into two orders, *Enterobacteriales* and *Xanthomonadales.* In the former family, Isolate C37 was identified as a *Rhanella,* belonging to the family *Enterobacteriaceae.* This was one of two sequenced DNRA-bacteria. All isolates belonging to *Xanthomonadales* were of family *Xanthomonadaceae,* genus *Rhodanobacter*. All of the isolates, except Isolate C13, reduced nitrate/nitrite to $N_2O$. Isolate C13 accumulated NO from nitrite reduction.

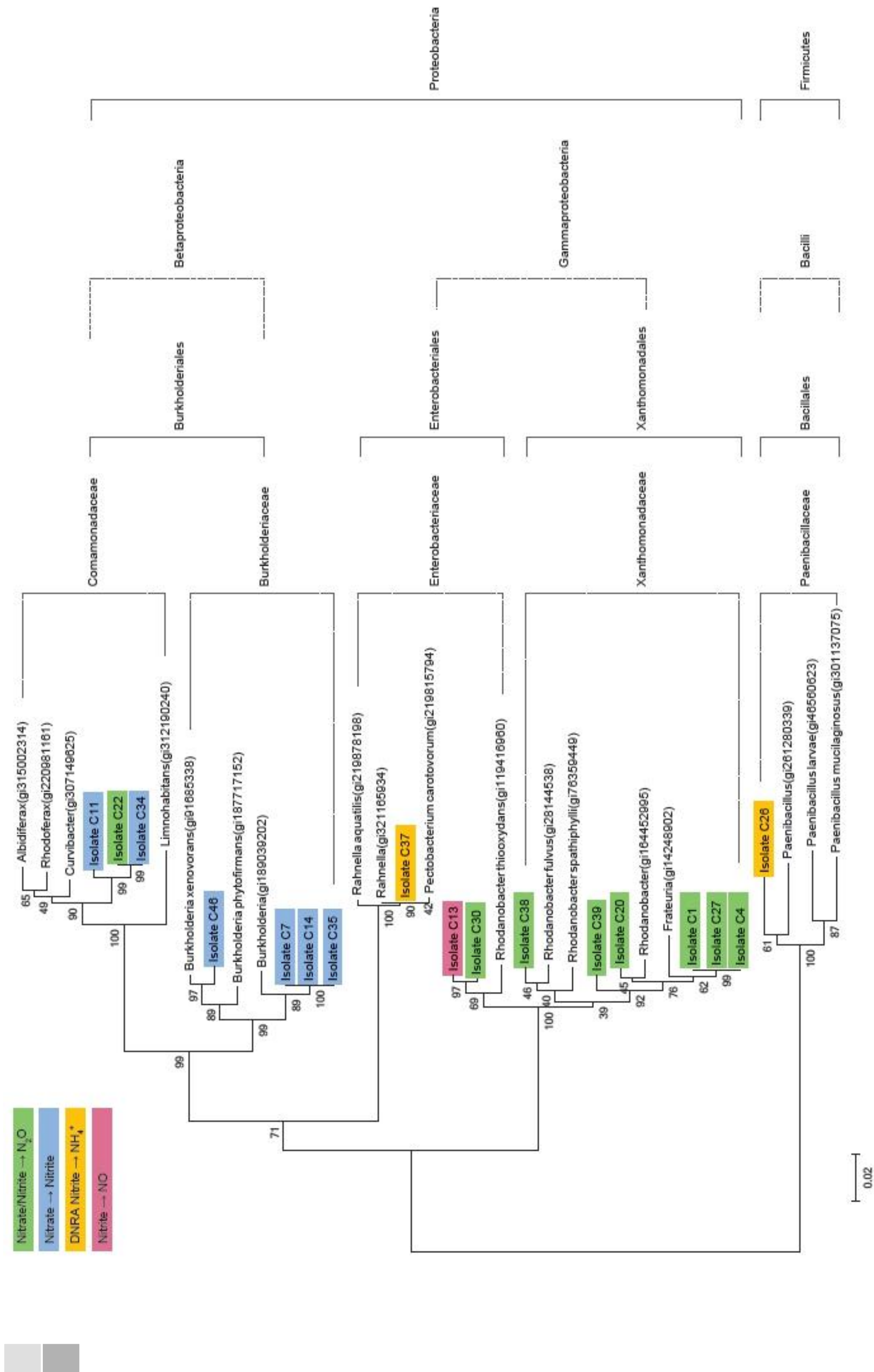
The Gram positive isolate, Isolate C26, belonged to the phylum *Firmicutes, Bacilli*, order of *Bacillales* in the family of *Paenibacillaceae.* It reduced nitrite to $NH_4^+$, and was classified as a DNRA bacterium.



Algoriphagus(gi122831035)
Isolate B1
Flavobacterium(gi190364967)
Isolate A8
Isolate A2
Isolate B33
Isolate B56
Bradyrhizobium genosp. alpha(gi37693285)
Bradyrhizobium genosp. SA-4(gi169883155)
Bradyrhizobium diazoefficiens(gi47118316)
Pseudomonas fluorescens(gi9798591)
Pseudomonas brassicacearum(gi189031301)
Isolate B39
Isolate A25
Piscinibacter(gi110085942)
Isolate B22
Paucibacter(gi270282521)
Isolate A20
Inhella(gi110085936)
Isolate B3
Isolate A37
Hydrogenophaga(gi315002320)
unclassified Comamonadaceae(gi144895550)
Isolate B26
Variovorax paradoxus(gi239799596)
Polaromonas jejuensis(gi154721147)
Isolate A23
Acidovorax(gi88909678)
Cyclobacteriaceae
Flavobacteriaceae
Rhizobiaceae
Bradyrhizobiaceae
Pseudomonadaceae
Comamonadaceae
Cytophagales
Flavobacteriales
Rhizobiales
Pseudomonondales
Burkholderiales
Cytophagia
Flavobacteriia
Alphaproteobacteria
Gammaproteobacteria
Betaproteobacteria
0.02
Continues on the next page



Firmicutes
Actinobacteria
Bacillales
Micromonosporales
Micrococcales
Corynebacteriales
Propionibacteriales
Paenibacillaceae
Bacillaceae
Micromonosporaceae
Microbacteriaceae
Micrococcaceae
Nocardiaceae
Nocardioidaceae
Paenibacillus larvae(gi46560623)
Isolate B25
Isolate B28
Bacillus(gi255976543)
Isolate B4
Microbacterium(gi224579685)
Isolate A22
Isolate B58
Isolate B57
Isolate B59
Isolate B47
Isolate B32
Arthrobacter(gi308549707)
Rhodococcus jostii(gi110816552)
Nocardia(gi313760368)
Isolate B54
Marmoricola(gi254777778)
Isolate B35
Isolate B52
Isolate A24
Isolate B18
Aeromicrobium(gi119488368)
100
100
100
86
100
45
100
95
98
100
100
100
40
100
58
100
98
96
Nitrate/Nitrite → $N_2$
Nitrate → Nitrite
Nitrate/Nitrite → $N_2O$
$N_2O$ → $N_2$
0.02



Nitrate/Nitrite → N$_2$O
Nitrate → Nitrite
DNRA Nitrite → NH$_4^+$
Nitrite → NO
Proteobacteria
Firmicutes
Betaproteobacteria
Gammaproteobacteria
Bacilli
Burkholderiales
Enterobacteriales
Xanthomonadales
Bacillales
Comamonadaceae
Burkholderiaceae
Enterobacteriaceae
Xanthomonadaceae
Paenibacillaceae
Albidiferax(gi315002314)
Rhodoferax(gi220981161)
Curvibacter(gi307149625)
Isolate C11
Isolate C22
Isolate C34
Limnohabitans(gi312180240)
Burkholderia xenovorans(gi91685338)
Isolate C48
Burkholderia phytofirmans(gi187717152)
Burkholderia(gi189039202)
Isolate C7
Isolate C14
Isolate C35
Rahnella aquatilis(gi219878198)
Rahnella(gi321165934)
Isolate C37
Pectobacterium carotovorum(gi219815794)
Isolate C13
Isolate C30
Rhodanobacter thiooxydans(gi119416960)
Isolate C38
Rhodanobacter fulvus(gi28144538)
Rhodanobacter spathiphylli(gi78356449)
Isolate C39
Isolate C20
Rhodanobacter(gi164452995)
Frateuria(gi14248902)
Isolate C1
Isolate C27
Isolate C4
Isolate C26
Paenibacillus(gi261280339)
Paenibacillus larvae(gi46560623)
Paenibacillus mucilaginosus(gi301137075)
0.02

**Figures 4.3.1 and 4.3.2 on pages 48 and 49 Phylogenetic tree for isolates from the high pH soil**

Phylogenetic tree based on 16S rRNA gene sequences, showing the relationships among the different isolates from the pH 7.3 soil and recognized species of the same families, orders and class. The respective isolates denitrifying phenotype is shown through colour coding. Tree was constructed by the neighbour-joining method using MEGA version 5. Bootstrap values (based on 500 replications) are shown at each node.

**Figure 4.3.3 on page 50 Phylogenetic tree for isolates from the low pH soil**

Phylogenetic tree based on 16S rRNA gene sequences, showing the relationships among the different isolates from the pH 3.7 soil and recognized species of the same families, orders, class and phylum. The respective isolates denitrifying phenotype is shown through colour coding. Tree was constructed by the neighbour-joining method using MEGA version 5. Bootstrap values (based on 500 replications) are shown at each node.

# 5 Discussion

## 5.1 The isolation process

With the intention of making this a broad isolation study within the timeframe of a master thesis, it was decided to use 1/10 strength TSA, incubate aerobically for 14 days at 20° C. By doing the initial isolation with air supply, we hoped that more bacteria would grow than when faced with anoxic conditions. By waiting 14 days, and marking which bacteria grew when, the slow growing bacteria would also have a chance. Using a low concentration of nutrients would also slow down the fast growing bacteria that otherwise might have dominated the plates.

By letting the bacteria grow in aerobe conditions, it varies from many of the large isolation studies, as they cultured bacteria on/in anaerobic nitrate medium from the start. (Cheneby et al. 2004; Dandie et al. 2007; Gamble et al. 1977; Ishii et al. 2011)

The big difference between the method used in this thesis and most other isolation study of denitrifying bacteria is the ability to quantify all of the intermediates, as well as adding those substrates to the flasks.

One expects there to be more than $10^9$ bacteria in a gram of soil, of which only about 1 % are culturable(Davis et al. 2005) . By letting the plates incubate aerobically and for 14 days, one hoped to capture as many and as a diverse group of bacteria.

Growth on the agar plates showed several different colonies, although the spread of the C-soil showed more similar looking colonies than the other plates, and reported the lowest number of cell forming units, though still in the number of millions per gram soil. One of the reasons for this could be the pH difference between what the bacteria was used to in the soil and the pH in the medium. The C-culture was spread on and grew on plates and in un-buffered medium with a pH of 7.5, while the soil from which they came had a pH of 3.7.

The D-spread came from this soil as well, but the medium used for the D-series was buffered down to pH 5.7, thus closer to what the bacteria lived in, most likely making it easier from the bacteria to grow there, as the plates did not seem dominated by one bacteria. The soil had been stored at 4 C for 3 months between the C- and D-spread, but the effect of this is not known.

## 5.2 End point analysis

The isolated bacteria were inoculated into small flasks with of tryptic soy broth with nitrate and nitrite, and set to incubate until turbidity was observed. This would take from 1-6 days. Flasks that did not show within a week were discarded. At turbidity the flasks were sealed with a rubber septum and an aluminium cap, and the headspace was anaerobised though helium rinsing, after which 1% $N_2O$ and 1 % $O_2$ were added. The oxygen was added as to not shock the isolates with the sudden lack of oxygen, giving them the chance to adapt. After another 7 days of incubation the amount of $N_2O$ and $N_2$ was quantified through gas chromatography, while NO and nitrate was quantified using a Nitric Oxide Analyser.

There was 4 µmole nitrogen from nitrate, 4 µmole nitrogen from nitrite, and about 7.3 µmole nitrogen from $N_2O$ in each flask before incubation.

For the most part the gas and nitrite measurements were stable. There were few outliers. Due to all the $N_2$ in the air, contamination through leakage could have been a huge problem, but the method developed in the NMBU Nitrogen Group (formerly known as UMB Nitrogen Group) and described in Molstad et al. (2007) (Molstad et al. 2007) is quite airtight. With a study as large as this (about 450 flasks were measured), there were a few samples that had leakages, and some weaknesses became apparent.

### 5.2.1 Controls

The negative controls were stable throughout the whole experiment, and such gave good indications when it came to where the levels of the compounds ought to be if the bacteria could not use them. Oxygen levels were higher and $CO_2$ levels were lower than for flasks with growing bacteria, as would be expected. The amount of oxygen and $C_2O$ as well as no visible growth of bacteria was a good indication for this process having been aseptic, as cross contamination between the flasks when measuring the gas, and evacuating the flasks was

feared as a possibility. Twice mould was seen growing on the surface of the controls, and these flasks were discarded. Unlike the samples, cycloheximide had not been added to the controls.

There ought to have been positive controls as well as the negative ones, an oversight that has been rectified in the further analysis of the isolates.

**5.2.2 Nitrate**

Nitrate was not measured, nor was there made any attempts at trying to decide whether nitrate was still present in the samples. This is one of the major faults in this study. Medium samples of the vials were frozen for later testing, but this was never done, as due to time constraint the instruments were never set up.

The plan was to use the nitric oxide analyser by setting up a second purge chamber next to the purge chamber used for nitrate measurements and do parallel measurements. In the first purge chamber nitrite would have been measured as earlier described. In the second purge chamber, instead of using NaI as the reducing agent, one would use a reducing agent capable of reducing both nitrate and nitrite to NO. Calculating the amount of nitrate would then simply be a matter of subtracting the amount of nitrite from the total amount of nitrate and nitrite.

A colorimetric test for nitrate could have been performed by reducing nitrate to nitrite and then used a nitrite indicator. In such a case one would first have to reduce all the nitrite in the samples (in those cases where nitrite had not been completely reduced), so that it would not give a false positive. This was unfortunately not thought of, as it would have been a good supplement to the results.

That being said, by calculating the total amount of nitrogen recovered one could for the most part deduce whether nitrate had been reduced or not. Nitrate respiration to nitrite was fairly easy due to the accumulation of nitrite. Nitrate to nitric oxide was not observed, the samples accumulating NO did not accumulate more than parts of the nitrite. Nitrate reduced to $N_2O$ was usually not too difficult to differentiate from when only nitrite had been reduced.
The main problem was the $N_2$-measurements, as the standard deviation in nitrogen in the flask from the start was rather large.

**5.2.3 Nitrite**

Nitrite was measured with a Nitric Oxide Analyser (NOA) which had a purge chamber connected, into which nitrite

Nitrite measurements were the most stable measurements when measuring the controls, something that is not very surprising, as the concentration of nitrite was the same in all flasks before autoclaving. Depending on evaporation during and after autoclaving, the loss of water from some flasks will have been larger than from others, giving it some variation, as well as variations when the medium was diluted for nitrite measurements and variations when measuring nitrite.

Nitrite is unstable in acidic solutions, and that was a problem when it came to analysing the D-samples, although not as bad as feared. While the concentration of nitrite in the medium ought to have been 1mM, in the controls it was down to 0.67 mM, giving a nitrite loss of 30%. An increase in NO was noted, but it was minimal: 3 nmole in controls, some samples had around 30 nmole NO.

**5.2.4 Nitric Oxide (NO)**

The amount of nitric oxide in the flasks was quantified by using a nitric oxide analyser (NOA). NO was the only compound not added to the flasks, putting the default value of NO in the flasks to 0 µmole. The NO measurements were taken after the gas chromatography to avoid $N_2$ leakage. The measurements were thus diluted, and that dilution was not calculated into the results.

The NO measurements were for the most part small, and practically 0. Only a 2 isolates accumulated any NO of significance, 0.5 and 0.8 µmole. NO is toxic to bacteria. Therefore, NO accumulating bacteria need to have other bacteria in their close vicinity, which can reduce or remove NO, or they have to live in an environment from which NO easily diffuses from the system, so that the organisms are not exposed to high concentrations. In a closed environment, as in the flasks, NO would rise until a point where the bacteria could no longer survive.

### 5.2.5 Nitrous oxide ($N_2O$)

$N_2O$ was added to the samples after helium rinsing and measured on the GC. The levels were fairly stable in vials where no biological N2O reduction took place.

An increase in $N_2O$ without any reduction of nitrite would be easily detected, but depending of the amount extra $N_2O$ being injected, which reductases the bacteria had, one could get a much higher $N_2$ than thought possible, and thus reject the finding.

### 5.2.6 Dinitrogen ($N_2$)

Dinitrogen was the end product that showed the largest variation in this study. Working quantitatively with $N_2$ measurements is difficult, because it's so abundant in the air around us and it is impossible to completely avoid some leakage into the vials from the surrounding air during sampling. Therefore, , there was about as much nitrogen in the flasks at the end of the incubation, as there was $N_2O$ (~1%). The amount of dinitrogen in the control flasks varied quite a lot, giving it the largest standard deviation. Detecting leakages of nitrogen was rather easy, as there would be 10-100 times more nitrogen in those flasks than in any other, and thus several times more nitrogen than could possibly have been produced based on how much nitrogen was available in the medium.

## 5.3 Statistics

Having statistically significant results is of the utmost importance when evaluating and interpreting scientific results. Statistics is the language used to explain whether an observation is likely to be true, or not. If measurements show that one flask has more $N_2$ than another one, statistics is helpful when judging whether that is a coincidence. Thorough statistical analyses of data are often necessary for scientists to make others believe in the results and interpretations presented. And yet, in biology it can be difficult to get results stable enough to obtain that level of significance wanted.

In this thesis, the statistics has for the most part made sense, and results that looked significant were often significant. One would expect so when the amount of a compound in a flask has dropped from 4 µmole to 0 µmole, or increased from 4 to 8 µmole. For the most part this would hold true, but when working with microbiology or $N_2$ it is not always that easy.

The samples were tested with an unpaired t-test on summarized (grouped) data. There were two statistical assumptions used on the final results, depending on whether the measurements had been done on single flasks or in duplicates. The first one assumed that the standard deviation of a sample was equal to that in the control flasks, the second assumed that the controls had one standard deviation and each pair of flasks had another. The first assumption was used on the A-isolates and on all isolates where one of the samples had such high values on $N_2$ that a leakage was assumed to be present, giving them only one measurement. The second method was used for the flasks with duplicates and the few triplicates.

Both assumptions had strong and weak sides. The first, assuming the same standard deviation for the isolates as for the controls was easier to use; one would get an interval of which anything outside it was significant, either it being reduced or being produced. The problem was that since one assumed the same standard deviation for all samples, it would be the same whether the amount of the compound was the same as the control samples, if it had increased or decreased, giving a false example of the variation within the measurements at those levels. For example: The A-isolate nitrite controls contained about 4.1 µmole nitrite with a standard deviation of 0.48 µmole. This was about 12% of the value, and a reasonable standard deviation. The A-isolates that accumulated nitrate averaged at 8.4 µmole, which with a standard deviation of 0.48 seems very stable, it's less than 6%. On the other side, the samples that reduced all the nitrite also have the standard deviation of 0.48 µmole, which seems unreasonably high.

The other, calculating a standard deviation for each pair of flasks that came from the same isolate, while being a more correct method, had other limitations. If the two flasks had very similar measurements, there would be a very small standard deviation, which would skewer the results towards a higher significance. If the difference between the flasks were large, the standard deviation would be so as well, and results would be of a lower significance.  This led to some samples having significant results for reduction of nitrite and $N_2O$, with nothing left of either of them, did not have a significant result for production of nitrogen. An example of this is Isolate D22, for which both nitrite and $N_2O$ has been reduced, but the amount of dinitrogen in the flasks so different that even though it is several times higher than the amount of nitrogen in the controls.

## 5.4 Phenotype and phylogeny

All in all, based on end point analysis and sequencing, the isolation set-up was a success. Out of 182 isolates, 83 isolates have been split between 6 different phenotypes, with another 9 isolates having been given a non-classified phenotype. There were seldom more than two sequences within the same genus, giving indication to a broad isolation. There were clear phylogenetic differences between the two soils, and phenotypic differences between the A- and B-isolates, the C-isolates, and the D-isolates.

### 5.4.1 The A- and B-isolates

The A- and B-isolates originated from the pH 7.4 soil and were isolated on pH 7.5 TSA-plates. The A- and B-isolates were from the same soil, they were spread and isolated on the same medium, within a couple of weeks, and therefore the results for these isolates are presented as if they were of the same isolation. Out of 99 samples, 41 were involved in nitrate reduction or denitrification. Of those 41 strains, a total of 34 strains were successfully sequenced and 27 of these were part of the tree built for the A-and B isolates. The isolates that were not in the tree were excluded either because the sequenced gene fragments were too short (unsuccessful sequencing), or because the results from the end point analysis were not significant.

The 27 isolates presented in the tree belong to 19 genera from 13 families, 10 orders and 7 classes.

The isolates from the high pH soil showed 4 phenotypes: full-fledged denitrification, nitrate respiration only, reduction of $N_2O$ to $N_2$, and nitrate reduction to $N_2O$. The most common phenotype was nitrate respiration, with 28 isolates expressing only this phenotype, mainly within the Gram positive isolates.

Eight full-fledged denitrifiers were detected, as seen in table 4.2.1, of which 5 were successfully sequenced. Two of these (A25 and B39) clustered with *Pseudomonas* sp., two (A37 and B3) with *Hydrogenophaga taeniospiralis*, and one (B33) with *Ensifer adherens*. They are all Gram-negative proteobacteria. *Hydrogenophaga taeniospiralis* and *E. adhaerens* are known as denitrifying organisms (Casida 1982; Willems et al. 1989). Denitrification is also reported for some species of the genus *Pseudomonas (Bergey's 2005).*

All of the isolates, except A25, B07 and B24 reduced ≥ 80% of the nitrate to dinitrogen and were considered to possess all 4 of the reductases. Only 50% of the nitrogen from nitrate, nitrite, and $N_2O$ was recovered as $N_2$ for A25. There are two possible explanations for this. It does not have a nitrate reductase, or as a human error, $N_2O$ was not added, in which case it is wrongly classified and does indeed have nitrate reductase. Human error is less likely for B07, which was run in duplicates, and while one would have expected a higher numbers as both nitrite and $N_2O$ was depleted completely, the production of nitrogen is lower than expected. B24 has a higher nitrogen recovery, around 75%, lacking about the same amount of nitrogen as was in the flasks as nitrate.

The $N_2$-measurements of Isolate B33 (*E. adhaerens)* has a lower significance than the others denitrifiers had, due to a large standard deviation between the two flasks. Its phenotype is still considered solid, as all nitrate and $N_2O$ was depleted, and the nitrogen recovery was so high.

The most common phenotype was «nitrate reduction only». Nitrate respirators reduce nitrate to nitrite, but are unable to reduce the nitrite to nitric oxide, nor to ammonium. I found a total of 26 isolates of this phenotype in the high pH soil. Since nitrate was not measured, classification of isolates into this phenotype group was based on a statistically significant increase in nitrite. Of the 26 isolates, 18 isolates were added to the tree shown in figures 4.2.1 and 4.3.2. The other 8 were either not sequenced, the sequence was bad, or their sequence was too short to be a part of the alignment. The latter was the case with Isolate B29, for which the forward primer gave two signals from the 180$^{th}$ base pair. It was identified as *Bacillus aquimaris* when run through the BLAST algorithm program.

Of the bacterial isolates capable only of nitrate reduction to nitrite, only 4 were gram negative, spread amongst *Flavobacteria, Alphaproteobacteria* and *Betaproteobacteria.* All of the others were Gram-positive *Firmicutes* or *Actinobacteria*.

Nitrate respiration is a common trait within genus *Flavobacterium,* found in about half of its species. (Bergey's 2011), making the two nitrate respirators (A4 and A8) normal. Isolate A2 on the other hand reduces nitrite to $N_2O$. *Flavobacterium* .(Horn et al. 2005)

Isolate B56 is in family *Bradyrizobium* and the highest similarity is with genus *Afipia* (see table A.05 in appendix). Afipia is a small genus, but has one nitrate reducing species, *Afipia felis (Bergey's 2005)*

**5.4.2 The C-isolates**

The C-isolates were isolated from the pH 3.7 soil and was spread on pH 7.5 TSA-plates. Out of 50 isolates 22 isolates were involved nitrate reduction or denitrification, 4 were classified as DNRA organisms, and 6 isolates were non-classified. A total of 26 C-isolates were successfully sequenced, and 17 were part of the tree built for the C-isolates. The isolates that were not included in the tree had either a too short sequence, they did not have significant results from the end point analysis, or they were among the non-classified.

There were 11 isolates that showed a statistically significant ($p < 0.05$) nitrite accumulation from nitrate reduction. The phylogenetic tree (Figure 4.3.2) shows that the nitrate to nitrite respirators belong to the class *Betaproteobacteria,* order *Burkholderiales* and families *Comamonadaceae* and *Burkholderiaceae*.

There are 4 isolates that identify as the genus *Burkholderia. Burkholderia* is a versatile genus of chemoorganothropic bacteria, able to use a variety of organic compounds for carbon and energy. Usually favouring oxygen as final electron acceptor, some species are also able to respire nitrate under anoxic conditions (Bergey's 2005). Bacteria belonging to this genus are tolerant to a wide pH range, and are reported to grow on media ranging from pH 4.5 to 7.5, with some species and strains growing on pH as low as 3.5.(Stopnisek et al. 2013).

The isolates C02, C16, C19, C44, C48 and C50 were not included in the tree, since they all had too short sequences due the forward primer giving two signals from basepair 180. When running the 500-750 base pairs long sequences through the Blast algorithm program, all came up as *Bacillus aquimaris*: a Gram positive bacterium isolated from a tidal flat of the Yellow Sea, Korea. While all of the isolates are positive for nitrate reduction, it was not part of the initial description of the species (Yoon et al. 2003). *Bacillus aquimaris* was also identified from the high pH soil, with the same problem there as observed here.

Only one of the C-isolates was able to reduce $N_2O$ to $N_2$, while there among the D-samples, from the same soil, but grown in medium with a lower pH, were several that reduced $N_2O$ to $N_2$. On the other hand, several of the C-samples accumulated $N_2O$. Why did none of them reduce $N_2O$?

**5.4.3 The D-isolates**

The D-isolates originated from the low pH soil, but in contrast to the C-samples the medium on which they were spread was set to pH 5.7. The D-isolates were more variable with respect to shape and colour, compared to the C-isolates from the same soil. Out of 33 isolations, 14 isolates were capable of nitrate respiration or denitrification, 4 isolates were classified as DNRA-organisms and 3 were non-classified. Due to time constraints, the D-isolates were not sequenced.

The amount of isolates that carried out full-fledged denitrification was what made the D-isolates interesting. Ten of the isolates reduced nitrate/nitrite to $N_2$ (See table 4.2.2). That is more than twice as many as from the high pH soil. Because nitrite was not measured, it is difficult to say how many reduced nitrate, but based on nitrogen recovery, at least 3 of the samples did. Others, like D25, had a very low nitrogen recovery, less than the amount of $N_2O$ added to the flasks, although all that $N_2O$ was gone. The cause of this is not known.

Unlike the isolations done on higher pH medium, there were almost no nitrate reducers (unable to reduce the produced nitrite) among the D-isolates (Table 4.2.4). , and while the C-isolates stopped their denitrification on $N_2O$, only 2 of the D-isolates did so (Table 4.2.6). The rest carried on to $N_2$.

Three of the D-isolates could not be classified into a phenotype (Table 4.2.10). Isolate D05 showed a slight, but significant reduction of nitrite. There was no increase in NO, $N_2O$, and $N_2$, but it is possible that this is a slow-growing DNRA-organism. D17 has consumed all the nitrite. It showed no significant reduction of $N_2O$, but a significant increase in $N_2$. The increase in $N_2$ was not large, about 2.5 µmole. It could be a very small leak, making it a DNRA organism. D32 had, like D25 of the full-fledged denitrifiers, no nitrite and no $N_2O$ left, but the increase in $N_2$ was not statistically significant. The significance was much poorer than for D25, with a $p$-value $> 0.1$ compared to D25's $p$-value of $< 0.01$., but the nitrogen recovery

was slightly higher. One could argue that since the numbers are so alike, they should be in the same group, but statistically, one has a statistical significant increase, the other has not. And while the choice of including such an organism has been done earlier, that was with a higher nitrogen recovery.

The fact that the D-isolates were not sequenced is another large drawback with this study. If the same bacterial strains have been captured by both high and low pH medium, there is no way to tell whether they behaved different or not.

## 5.5 The road ahead

The isolated bacteria that grew in the liquid medium are currently stored as triplicates in 15% glycerol at -80° C, as well as cell pellets for DNA extractions. With this work as a starting point, those isolates positive for nitrate reduction, denitrification, DNRA or with uncertain results have been thawed, re-plated to ensure the purity of the samples and Gram-stained. They are currently being re-run through the method, in triplicates and with nitrate measurements to confirm the findings of this thesis. So far the results are promising.

The D-isolates are being sequenced, as they had not been so before, giving one a better and broader look at the differences between the two soils, as well as what the pH in the medium had to say when comparing the C- and the D-series.

## 5.6 Conclusion

This study has successfully isolated a wide range of nitrate reducers and denitrifying bacteria from both high and low pH soil, building a collection to be further studied. Through the isolation work, a method for deciding which components isolated bacteria can reduce has been developed.

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# APPENDIX

Table A.01 Colony description of isolates from the A-series

| | |
|---|---|
| A01 | white, glistening, large, Circular |
| A02 | yellow, dry, spreads |
| A03 | white, fat, Circular |
| A04 | pale yellow, wet |
| A05 | Small, yellow, dry |
| A06 | brown under, white at the top, brown secretion |
| A07 | yellow, wet, rounf |
| A08 | fat, Circular, wet, spreading |
| A09 | small, red/orange |
| A10 | small, red/orange, rund |
| A11 | blank, Circular |
| A12 | white, fat, Circular |
| A13 | white, dry, small |
| A14 | yellow, fat |
| A15 | yellow, dry, spreads, looks like a snowflake |
| A16 | Wet, white/pale peach |
| A17 | yellow |
| A18 | light, small dots |
| A19 | red/orange, dry, Circular |
| A20 | white, uneven edges |
| A21 | white on top, hard shell |
| A22 | small, pale yellow, Circular |
| A23 | small, pale yellow, Circular |
| A24 | small, pale yellow, Circular |
| A25 | brownish yellow, Circularish, fat |
| A26 | dry, pale peach, flowerformed colony |
| A27 | black, dry, light inside |
| A28 | yellow, small, Circular |
| A29 | yellow, flowerformed, dry |
| A30 | fat, Circular, small |
| A31 | white, dry, shell |
| A32 | yellow, fat ,Circular |
| A33 | orange/red, dry, Circular |
| A34 | white, fat, Circular |
| A35 | yellow |
| A36 | blanc |
| A37 | small, yellow |
| A38 | white, fat |
| A39 | white, black top |

Table A.02 Colony description of isolates from the B-series

| | |
|---|---|
| B07 | yellow, runny colony |
| B08 | small, pale, Circular |
| B09 | white, fat, white |
| B10 | orange, flower formed, fat, runny, |
| B11 | small, yellow |
| B12 | yellow, umbonate |
| B13 | yellow, translucent |
| B14 | yellow, convex |
| B15 | small, yellow/orange, sticky consistency |
| B16 | orange, circular, wet |
| B17 | yellow, fat, large, circular |
| B18 | light brown/beige, round, wet |
| B19 | white, round, dry |
| B20 | white/cream, round dry |
| B21 | Translucent, yellow, irregular shape, umbonate |
| B22 | light beige, round |
| B23 | small, punctiform, white/gray |
| B24 | large, white, dry |
| B25 | White, large, dry, flat, rhizoid |
| B26 | Yellow, large, round, dry |
| B27 | White, large, fat, round |
| B28 | Peach, opaque, dry, grows together with B29 |
| B29 | Orange, translucent, grows out of B28, wet |
| B30 | Punctuate, blanc |
| B31 | yellow, irregular shape, umbonate |
| B32 | pale yellow, rhizoid, curled |
| B33 | white/gray, irregular shape, very "runny", pulvinate |
| B34 | white, punctiform, dry, convex shape |
| B35 | pale yellow, translucent, flat, irregular shape,curled margin |
| B36 | yellow, irregular shape, flat, lobate margin |
| B37 | white punctiform colonies, |
| B38 | Colourless, umbonate circular colonies |
| B39 | brown, circular, erose margin, flat |
| B40 | yellow |
| B41 | irregular, opaque, pale yellow, flat, |
| B42 | whiteish, circular, small, convex |
| B43 | Yellow, opaque, irregular, unfulate margin, flat |
| B44 | yellow |
| B45 | yellow, glistening, circular, translucent, umbonate, |
| B46 | brown |
| B47 | White/gray |
| B48 | White/gray |

| | |
|---|---|
| B49 | Blanc |
| B50 | Blanc |

Table A.03 Colony description of isolates from the C-series

| | |
|---|---|
| C1 | Yellow, dry |
| C2 | Blanc, flat, round |
| C3 | Yellow |
| C4 | Yellow, wet |
| C5 | White, sticky |
| C6 | Light orange, fat |
| C7 | White |
| C8 | White, convex |
| C9 | Gray/black atop, orange under |
| C10 | Yellow, wet |
| C11 | White, punctuate |
| C12 | Orange, small |
| C13 | Yellow, translucent, irregular shape |
| C14 | White, small, round, wet |
| C15 | Light colony, comes apart |
| C16 | White/gray, large |
| C17 | White |
| C18 | Yellow |
| C19 | Orange, large, dry |
| C20 | Yellow, translucent |
| C21 | Yellow |
| C22 | Light peach, |
| C23 | Translucent, wet |
| C24 | Yellow |
| C25 | Cream and brown, wet |
| C26 | peach, flat |
| C27 | Yellow, wet |
| C28 | Brown, translucent |
| C29 | yellow, yellow secret |
| C30 | Light/gray, wet |
| C31 | White, large, dry |
| C32 | Yellow, translucent |
| C33 | Yellow, "sticky" |
| C34 | Brown/orange |
| C35 | White, dry |
| C36 | Yellow |
| C37 | White |
| C38 | Blanc |
| C39 | Yellow, fat, "sticky" |

| | |
|---|---|
| C40 | Dark, |
| C41 | Yellow, clear sone around it. |
| C42 | blanc |
| C43 | Orange, small |
| C44 | Orange, large |
| C45 | White |
| C46 | Light peach, round, umbonate |
| C47 | Pale yellow, rubbery texture |
| C48 | Blanc, punctuate, translucent |
| C49 | white |
| C50 | Blanc, translucent |

Table A.4 Colony description of isolates from the D-series

| | |
|---|---|
| D1 | Small red |
| D2 | Small red |
| D3 | Light peach, opaque, medium size |
| D4 | Yellow, small, translucent. |
| D5 | small, white |
| D6 | Yellow, opaque, large |
| D7 | Peach, punctuate |
| D8 | Orange, opaqe, small, dry |
| D9 | White, opaque, small |
| D10 | Peach, opaque, small |
| D11 | Yellow, opaque |
| D12 | Yellow, translucent, large |
| D13 | White, opaque, large, wet |
| D14 | White, opaque, medium size |
| D15 | White, opaque, small |
| D16 | Peach, opaque, small |
| D17 | Yellow, brown in he middle, opaque, small |
| D18 | White, opaque, small |
| D19 | Pale orange, small |
| D20 | Yellow, opaque, large |
| D21 | Yellow, opaque, small |
| D22 | Orange, glistening |
| D23 | Yellow, opaque, large |
| D24 | Pale peach, opaque, |
| D25 | Yellow, glistening |
| D26 | Yellow, large |
| D27 | White, punctuate |
| D28 | Peach, small |
| D29 | White, opaque, flowershaped |

| | |
|---|---|
| D30 | Yellow, opaque, small |
| D31 | Yellow, glistening, small |
| D32 | Peach, opaque |
| D33 | White, large, wet and runny |

**Table A.05 16S ribosomal RNA sequencing results.** The highest uncultured and cultured matches for the isolates that were successfully sequenced based on BLAST-search. The highest matches are presented with GenBankID, similarity, description of habitat or isolation source.

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **A2** | 1379 | JF747919.1 | 99% | Uncultured bacterium - Frasassi cave system, sulfidic spring (unpublished) | Flavobacterium sp. CL1.152 AM934685.1 | 99% | Flavobacteriia – tufa-core, Germany (unpublished) |
| **A4** | 518 | JQ867292.1 | 100% | uncultured Bacteroidetes bacterium- Dinaric Karst subterranean stream (Kostanjsek et al. 2013) | Flavobacterium sp. 71_d HG313644.1 | 100% | Flavobacteriia- Skin of midwife toad (Alytes obstetricans,)Switzerland (unpublished) |
| **A8** | 1255 | AF534192.1 | 99% | Uncultured bacterium, soil, Ithaca, N.Y, USA (Padmanabhan et al. 2003) | Flavobacterium pectinovorum AB681003.1 | 100% | Flavobacteriia- (Unpublished) |
| **A20** | 1395 | HM129806.1 | 98% | Uncultured bacterium - Nam Co Lake, Tibet (Zhang et al. 2013) | Inhella inkyongensis strain IMCC1713 NR_043920.1 | 99% | β-proteobacteria, Burkholderiales, -artificial freshwater pond, Inkyong Reservoir, Korea (Cho 2009) |
| **A22** | 1347 | DQ513008.1 | 99% | Uncultured bacterium Ridge flank crustal fluids, Pacific Ocean (Huber et al. 2006) | Cellulomonas sp. Z0-YC6814 GQ369082.1 | 99% | Actinobacteria -Rice field rhizosphere, zero-tillage practice, South Korea (unpublished) |
| **A23** | 1393 | KC620632.1 | 99% | Uncultured bacterium - acid mine drainage, TongLing pyrite mine, Anhui Province, China (unpublished) | Polaromonas sp. BAC25 EU130990.1 | 99% | Betaproteobacteria; Burkholderiales; - granular activated carbon water treatment filters (Magic-Knezev et al. 2009) |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **A24** | 1291 | KC554648.1 | 99% | Uncultured bacterium -soil of Yanshan Mountain, Hebei Province, China (unpublished) | Actinobacterium F3H1_a10 KF641678.1 | 100% | Actinobacteria -Hydrolysed polluted soil, Denmark (unpublished) |
| **A25** | 1370 | JF500973.1 | 99% | Uncultured Pseudomonas -rye-grass rhizosphere, United Kingdom (Gougoulias & Shaw 2012) | Pseudomonas sp. S8-130 EF044365.1 | 99% | Gammaproteobacteria (Frapolli et al. 2007) |
| **A32** | 675 | KC993355.1 | 98% | Uncultured bacterium -Shorebird feces, Delaware, USA (unpublished) | Bacillus sp. MB81 AB518991.1 | 99% | Bacilli -sediments from the South Korean Yellow Sea.(Velmurugan et al. 2011) |
| **A34** | 761 | KF494798.1 | 99% | Uncultured bacterium -permafrost soil Qinghai-Tibet Plateau, China (unpublished) | Arthrobacter sp. DCY81 KF212463.1 | 99% | Actinobacteridae -Ginseng soil, South Korea (unpublished) |
| **A37** | 1380 | KC255316.1 | 99% | Uncultured bacterium -calcium carbonate (moonmilk) where beetles feed, Grotta Genziana cave, Italy (unpublished) | Hydrogenophaga taeniospiralis AB681846.1 | 99% | Betaproteaobacteria (unpublished) |
| **B1** | 1337 | JF703533.1 | 99% | Uncultured Algoriphagus -root and rhizophere soil, QiXiaShan, NanJing, China (Zhang et al. 2012) | Algoriphagus sp. M45 KC464852.1 | 99% | Cytophagia -natural Euphrates poplar forest rhizosphere, Xinjiang, China Unpublished |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **B3** | 1385 | KC255316.1 | 99% | Uncultured bacterium -calcium carbonate (moonmilk) where beetles feed, Grotta Genziana cave, Italy (unpublished) | Hydrogenophaga taeniospiralis AB681846.1 | 99% | Betapro KC255316.1teobacteria (unpublished) |
| **B4** | 1328 | N/A | N/A | N/A | Micromonospora AY360152.1 | 99% | Actinobateridae -marine environment (unpublished) |
| **B12** | 1353 | GU325825.1 | 99% | Uncultured bacterium -thermophilic sludge, wastewater faciliteis, Ireland (Piterina et al. 2010) | Microbacterium sp. BA47 HQ398383.1 | 99% | Actinobacteridae -marine sponge, Scopalina ruetzleri, Bahamas (Tabares et al. 2011) |
| **B18** | 1352 | JQ407928.1 | 99% | Uncultured bacterium -subsurface flow in constructed wetland, Tunisia (Bouali et al. 2013) | Aeromicrobium panaciterrae NR_041382.1 | 99% | Actinobacteridae -soil from a ginseng field, South Korea (Cui et al. 2007) |
| **B19** | 1360 | KC554683.1 | 98% | Uncultured bacterium - soil of Yanshan Mountain, Hebei Province, China (direct submission) | Solirubrobacter sp. KF551107.1 | 99% | Actinobacteria, Rubrobacteridae -Stem of Phytolacca acinosa, China (unpublished) |
| **B22** | 1385 | JX271960.1 | 99% | Uncultured bacterium - “activated sludge in lab-scale reactor with dissolved oxygen above 2.5 mg/l”, China -(Zheng et al. 2013) | Methylibium sp. UKPF16 AB769223.1 | 99% | Betaproteobacteria; Burkholderiales - rice paddy field soil, Kumamoto, Japan (unpublished) |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **B25** | 1365 | HQ120652.1 | 99% | Uncultured bacterium -loamy sand from tomato field, Califiornia, USA (Williamson et al. 2011) | Paenibacillus sp. DSM 1482 AJ345019.1 | 99% | Bacilli, -contaminated agar plate , Göttingen, Germany (Uetanabaro 2003) |
| **B26** | 1383 | DQ158118.1 | 99% | Uncultured bacterium soil, Ithaca, NY, USA -(DeRito et al. 2005) | Variovorax ginsengisoli strain: S32319 AB649026.1 | 99% | Betaproteobacteria; Burkholderiales; -paddy field soil, Miyagi, Japan (Gorlach et al. 1994) |
| **B28** | 1386 | GQ472804.1 | 99% | Uncultured bacterium -surface water, northern Bering Sea (Zeng et al. 2011) | Bacillus sp HF678943.1 | 99% | Bacilli -biofilm, Lake Baikal, Russia (unpublished) |
| **B29** | 699 | JX872374.1 | 99% | Uncultured bacterium -water, enviornmental sample (unpublished) | Bacillus aquimaris strain BGR11 KC789770.1 | 99% | Bacilli -soil isolate from Shule river, Gansu province, China (unpublished) |
| **B32** | 1349 | KC541072.1 | 100% | Uncultured bacterium, river sediment, (unpublished) | Arthrobacter scleromae strain OS260 KF424312.1 | 100% | Actinobacteria - agricultural field, South Korea (unpublished) |
| **B33** | 1348 | FM866282.1 | 99% | Uncultured alpha proteobacterium -uranium mill tailings, Bulgaria (unpublished) | Ensifer adhaerens strain NBRC 100387 AB681162.1 | 99% | Alphaproteobacteria (unpublished) |
| **B35** | 1358 | JF429005.1 | 99% | Uncultured bacteria - Potassium rich rhizosphere soil, Anhui province, China (unpublished) | Marmoricola sp. GQ339892.1 | 99% | Actinobacteridae -ginseng field soil, South Korea (unpublished) |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **B39** | 1389 | JF500973.1 | 99% | Uncultured Pseudomonas sp. - rye-grass rhizosphere, sandy loam soil, United Kingdom (Gougoulias & Shaw 2012) | Pseudomonas sp. DQ453821.1 | 99% | Gammaproteobacteria, fluorescent (Frapolli et al. 2007) |
| **B41** | 1366 | KC541072.1 | 100% | Uncultured bacterium, river sediment, (unpublished) | Arthrobacter scleromae strain OS260 KF424312.1 | 100% | Actinobacteria - agricultural field, South Korea (unpublished) |
| **B47** | 1365 | KC541072.1 | 99% | Uncultured bacterium, river sediment, (unpublished) | Arthrobacter scleromae strain OS260 KF424312.1 | 99% | Actinobacteria - agricultural field, South Korea (unpublished) |
| **B52** | 1349 | KC554648.1 | 99% | Uncultured bacterium -Soil of Yanshan Mountain, Hebei province, China (unpublished) | Actinobacterium KF641678.1 | 100% | Actinobacteria, hydrolysed polluted soil, Denmark (unpublished) |
| **B54** | 1348 | KC554594.1 | 98% | Uncultured bacterium soil of Yanshan Mountain, Hebei province, China (unpublished) | Rhodococcus maanshanensis strain: GMC121 AB741451.1 | 99% | Actinobacteria grassland soil, Miyagi, Japan (unpublished) |
| **B56** | 1326 | FJ712611.1 | 99% | Uncultured bacterium -Sediments from Kazan mud volcano, East Mediterranean Sea (Pachiadaki et al. 2010) | Afipia sp GU377117.1 | 99% | Alphaproteobacteria - Shapotou region, China (unpublished) |
| **B57** | 1366 | KC541072.1 | 100% | Uncultured bacterium, river sediment, (unpublished) | Arthrobacter scleromae strain OS260 KF424312.1 | 100% | Actinobacteria - agricultural field, South Korea (unpublished) |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **B58** | 1362 | KC541072.1 | 100% | Uncultured bacterium, river sediment, (unpublished) | Arthrobacter scleromae strain OS260 KF424312.1 | 100% | Actinobacteria - agricultural field, South Korea (unpublished) |
| **B59** | 1366 | KC541072.1 | 100% | Uncultured bacterium, river sediment, (unpublished) | Arthrobacter scleromae strain OS260 KF424312.1 | 100% | Actinobacteria - agricultural field, South Korea (unpublished) |
| **C1** | 1390 | FN391822.1 | 99% | Uncultured bacterium -sediments from acid mine drainage, Provance, France (Bertin et al. 2011) | Rhodanobacter sp. GR24-2 FJ821731.1 | 99% | Gammaproteobacteria, negative for nitrate reductase -Ginseng field, South Korea (Kim et al. 2013) |
| **C2** | 765 | JX872374.1 | 99% | Uncultured bacterium -water (unpublished) | Bacillus aquimaris KF054895.1 | 99% | Bacilli -Wheat rhizosphere, Indore, India (unpublished) |
| **C4** | 1393 | FN391822.1 | 99% | Uncultured bacterium -sediments from acid mine drainage, Provance, France (Bertin et al. 2011) | Rhodanobacter sp. GR24-2 FJ821731.1 | 99% | Gammaproteobacteria, negative for nitrate reductase -Ginseng field soil, South Korea (Kim et al. 2013) |
| **C7** | 1378 | JF500995.1 | 99% | uncultured Burkholderia sp. - rye-grass rhizosphere, sandy loam soil, United Kingdom (Gougoulias & Shaw 2012) | Burkholderia sp. PO-04-17-25 JF763863.1 | 99% | Betaproteobacteria -Alpine Soil, Pico de Orizaba, Mexico (Weber & King 2012) |
| **C11** | 1380 | AB294319.1 | 99% | uncultured bacterium -stream, Hokkaido, Japan (Shimizu et al. 2007) | Denitrifying bacterium W99 AB162104.1 | 99% | Betaproteobacteria upland soil, Japan (Hashimoto et al. 2005) |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **C13** | 1387 | AB809948.1 | 99% | uncultured gammaproteobacterium -acidophilic nitrifying activated sludge, Japan (unpublished) | Rhodanobacter sp. 2APBS1 NR_102497.1 | 98% | Gammaproteobacteria -uranium and nitrate contaminated subsurface, US DOE Oak Ridge Integrated Field Research Center, TN, USA (unpublished) |
| **C14** | 1262 | JF500995.1 | 99% | uncultured Burkholderia sp. - rye-grass rhizosphere, sandy loam soil, United Kingdom (Gougoulias & Shaw 2012) | Burkholderia sp. PO-04-17-25 JF763863.1 | 99% | Betaproteobacteria -Alpine Soil, Pico de Orizaba, Mexico (Weber & King 2012) |
| **C16** | 756 | JX872374.1 | 99% | Uncultured bacterium -water (unpublished) | Bacillus aquimaris KF054895.1 | 100% | Bacilli -Wheat rhizosphere, Indore, India (unpublished) |
| **C19** | 711 | JX872374.1 | 99% | Uncultured bacterium -water (unpublished) | Bacillus aquimaris KF054895.1 | 100% | Bacilli -Wheat rhizosphere, Indore, India (unpublished) |
| **C20** | 739 | JQ919644.1 | 99% | uncultured gamma proteobacterium -gasoline-polluted soil, France (unpublished) | Rhodanobacter sp. NAR7(11) JX154289.1 | 99% | Gammaproteobacteria -non-limed oak rhizosphere, acidic. (Lepleux et al. 2013) |
| **C22** | 1379 | AB294319.1 | 99% | uncultured bacterium -stream, Hokkaido, Japan (Shimizu et al. 2007) | Denitrifying bacterium W99 AB162104.1 | 99% | Betaproteobacteria upland soil, Japan (Hashimoto et al. 2005) |
| **C26** | 1386 | HQ120464.1 | 99% | Uncultured bacterium -loamy sand from tomato field, Califiornia, USA (Williamson et al. 2011) | Paenibacillus amylolyticus AB115960.1 | 99% | Bacilli -soil (Tezuka et al. 2004) |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **C27** | 1393 | FN391822.1 | 99% | Uncultured bacterium -sediments from acid mine drainage, Provance, France (Bertin et al. 2011) | Rhodanobacter sp. GR24-2 FJ821731.1 | 99% | Gammaproteobacteria, negative for nitrate reductase -Ginseng field soil, South Korea (Kim et al. 2013) |
| **C28** | 1394 | KC620909.1 | 99% | Uncultured bacterium -Acid mine drainage, TongLing pyrite mine, Anhui Province, China (unpublished) | Rhodanobacter sp. LnR5-47 EU332829.1 | 99% | Gammaproteobacteria negative for nitrate reductase -Ginseng field, Liaoning, China (Wang et al. 2011) |
| **C30** | 1388 | AB809948.1 | 98% | Uncultured gamma proteobacterium --acidophilic nitrifying activated sludge, Japan (unpublished) | Rhodanobacter sp. 2APBS1 NR_102497.1 | 98% | Gammaproteobacteria -uranium and nitrate contaminated subsurface, US DOE Oak Ridge Integrated Field Research Center, TN, USA (unpublished) |
| **C34** | 1386 | AB294319.1 | 99% | uncultured bacterium -stream, Hokkaido, Japan (Shimizu et al. 2007) | Denitrifying bacterium W99 AB162104.1 | 99% | Betaproteobacteria upland soil, Japan (Hashimoto et al. 2005) |
| **C35** | 1382 | JF500995.1 | 99% | uncultured Burkholderia sp. - rye-grass rhizosphere, sandy loam soil, United Kingdom (Gougoulias & Shaw 2012) | Burkholderia sp. KKSM1 JF327643 | 99% | Betaproteobacteria -soil, South Korea (unpublished) |
| **C36** | 765 | HM565342.1 | 99% | Mittivakkat glacier front ghoetite precipitate, Greenland (unpublished) | Rhodanobacter sp. THG-DD7 KF532124.1 | 99% | Gammaproteobacteria -soil, Mt. Daemo, Seoul, South Korea (unpublished) |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **C37** | 1368 | HM142075.1 | 100% | uncultured gamma proteobacterium -Bursaphelenchus xylophilus (pine wood nematode), Chongqing, China (unpublished) | Rahnella aquatilis HX2 NR_074921.1 | 100% | Gammaproteobacteria -vineyard soil, Beijing, China (Guo et al. 2012) |
| **C38** | 1389 | DQ125620.1 | 99% | uncultured bacterium -uranium contaminated soil, NABIR FRC Area 2 Oak Ridge, TN, USA (Brodie et al. 2006) | Rhodanobacter sp. THG-DD7 KF532124.1 | 98% | Gammaproteobacteria -soil, Mt. Daemo, Seoul, South Korea (unpublished) |
| **C39** | 1394 | HM049675.1 | 99% | uncultured bacterium -soil (unpublished) | Rhodanobacter sp. THG-DD7 KF532124.1 | 99% | Gammaproteobacteria -soil, Mt. Daemo, Seoul, South Korea (unpublished) |
| **C44** | 598 | JX872374.1 | 99% | Uncultured bacterium -water (unpublished) | Bacillus aquimaris KF054895.1 | 99% | Bacilli -Wheat rhizosphere, Indore, India (unpublished) |
| **C46** | 1379 | GU179655.1 | 99% | Uncultured beta proteobacterium -oil well, Alsaska, USA (unpublished) | Burkholderia sediminicola AB740929.1 | 99% | Betaproteobacteria -grassland soil, Miyagi, Japan (unpublished) |
| **C48** | 720 | JX872374.1 | 99% | Uncultured bacterium -water (unpublished) | Bacillus aquimaris KF054895.1 | 100% | Bacilli -Wheat rhizosphere, Indore, India (unpublished) |

| Isolate | Sample size (bp) | Best uncultured hit | Similarity | Description -habitat/isolation source | Best cultured hit | Similarity | Description- habitat/isolated from |
|---|---|---|---|---|---|---|---|
| **C49** | 1333 | EF516285.1 | 99% | Uncultured bacterium -grassland soil, Angelo Coast Range Reserve, CA, USA (Cruz-Martinez et al. 2009) | Bradyrhizobium sp. R-46210 FR753090.1 | 100% | Alphaproteobacteria -Root nodules, Lotus penduncolatus 14 (De Meyer et al. 2011) |
| **C50** | 721 | JX872374.1 | 99% | Uncultured bacterium -water (unpublished) | Bacillus aquimaris KF054895.1 | 100% | Bacilli -Wheat rhizosphere, Indore, India (unpublished) |

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