universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Initial Characterization of *Candidatus Nitrosopila Arctica*, an Ammonia Oxidizing Archaeon from Arctic Soils“

verfasst von

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 444
Studienrichtung lt. Studienblatt:	Diplomstudium Ökologie
Betreut von:	Univ.-Prof. Dipl.-Biol. Dr. Christa Schleper

ABSTRACT

Nitrogen is an essential element on earth, it has different chemical forms, which go through different oxidation and reduction reactions. One particularly important reaction in nature is the biological ammonia oxidation, because it is the first step of the nitrification process, which plays a crucial part in the global nitrogen cycle. At the end of the 19th century the first nitrifying microorganism was isolated, since then it was supposed that all ammonia oxidisers were bacteria. However, in 2005 this has changed with the isolation of the first ammonia oxidizing archaea (AOA) and the recognition that AOA occur in larger numbers in all environments. However, as only two AOA have so far been obtained in pure cultures, their physiology is very little explored. Therefore, it is of high importance to study ammonia oxidizing archaea and their characteristics.

The aim of this work was to explore the metabolic capabilities and growth requirements of a new ammonia oxidizing archaeon stemming from soils of Spitsbergen, and to improve its growth in enrichment cultures. The growth of the enrichment cultures was followed by measuring ammonium consumption and nitrite production. Additionally, for some cultures growth was followed by quantitative PCR. Furthermore, the archaeon was characterized microscopically and its 16S rRNA gene was sequenced for phylogenetic placement.

All enrichment cultures were incubated at 20 °C in the dark. Ammonium and urea were used as substrate for ammonia oxidation. The highest 16S rRNA gene copies/mL and the highest NO_2^- production rate were obtained with urea as energy source. Growth was observed with an initial ammonium concentration of 0.5 mM to 20 mM but with an extension of the lag phase at the highest ammonium concentration and a nitrite end concentration of maximally 900 µM in the medium. With an initial 1 mM urea concentration in the medium a maximal end concentration of nitrite up to 2 mM was obtained and a shorter lag phase compared to the control culture with an initial 0.5 mM ammonium concentration. Growth was sustained between pH 6 and 7.5 in the enrichment cultures.

Supplementation of the medium with glyoxylate but not pyruvate, yielded higher 16S rRNA gene copy numbers in the enrichment cultures. However, no significant effect of glyoxylate was observed on the nitrite production rate.

The maximal NO_2^- production rate of 16.1 µM/day in the exponential phase was obtained in an enrichment culture with 1 mM initial urea concentration. The organism needed on average 137 days to convert 1 mM ammonium compared to *Nitrososphaera viennensis* (10 days). It seems that the Arctic strain is either miss-

ing some important substrate for its optimal growth or it is an extremely slow growing organism.

The greenhouse gas nitrous oxide (N_2O) was produced in our AOA enrichment cultures. However, the yield of 0.012 % N_2O per NO_2^- was about 10 fold lower than for other AOA. Acetylene inhibited growth of enrichment cultures and nitrous oxide production.

The total culture volumes were changed to 100 mL in order to increase biomass yields. This resulted in an extension of the lag phase but comparable maximal nitrite production rates of 7.4 μM/day for 100 mL and 7.9 μM/day for 20 mL total culture volumes.

Different antibiotics against bacteria were used to further enrich for archaea. Culture growth with 100 μg/mL ampicillin, carbenicillin and kanamycin was observed. However, this resulted in a significantly lower nitrite production in enrichment cultures supplemented with carbenicillin or kanamycin, potentially by inhibiting growth of bacteria in the enrichments that have positive growth effects on the AOA.

Fluorescence *in situ* hybridisation (FISH) and light microscopy was performed to identify and morphologically characterize this new archaeon. With archaea-specific probes a coccoid cell morphology with approximately 2 μm in diameter was identified. These cells tended to grow in suspended aggregates of different sizes and also as single cells.

Archaeal 16S rRNA gene sequences from various enrichment cultures of different soils of Spitsbergen were 99.6 % identical to each other over a region of 759 nucleotides and differed by 3.82 % from the sequence of *Nitrososphaera viennensis* EN76.

We tentatively named the organism *Candidatus Nitrosopila arctica* (Latin masculine adjective nitrosus, nitrous - here intended to mean nitrite producer; Latin feminine noun pila, ball, ball shaped; Latin feminine arctica, Arctic, northern)

ZUSAMMENFASSUNG

Stickstoff ist ein für die Erde wesentliches Element. Dieses Element besitzt verschiedene chemische Formen, die wiederum verschiedene Oxidations- und Reduktionsreaktionen durchlaufen. Die biologische Oxidation von Ammoniak ist der erste Schritt der Nitrifikation, welche eine entscheidende Rolle im globalen Stickstoffkreislauf hat. Der erste nitrifizierende Mikroorganismus wurde Ende des 19. Jahrhunderts isoliert. Seitdem wurde angenommen, dass alle Mitglieder der Gruppe der Ammoniak-oxidierenden Mikroorganismen Bakterien seien. Erst im Jahr 2005 hat sich diese Ansicht verändert: Einerseits durch die Isolierung des ersten Ammoniak oxidierendem Archaeons (AOA), andererseits durch die Erkenntnis, dass AOA in größerer Abundanz in allen Habitaten auftreten. Allerdings wurde bisher nur von zwei Ammoniak-oxidierenden Archaea eine Reinkultur erreicht, weshalb ihre Physiologie bis heute auch wenig erforscht ist. Aus diesem Grund ist die Untersuchung von Ammoniak-oxidierenden Archaea und ihrer Eigenschaften von großer Bedeutung. Ziel dieser Arbeit war es die verschiedenen, möglichen Stoffwechselwege und Wachstumsbedürfnisse eines neuen Ammoniak-oxidierenden Archaeons, welches aus Bodenproben von Spitzbergen (Arktis) isoliert wurde, zu erforschen. Außerdem wurde versucht das Wachstum der Archaea in den Anreicherungskulturen zu steigern. Hierbei wurde das Wachstum durch Messung des Verbrauchs von Ammoniak und der Nitrit Produktion in den Anreicherungskulturen bestimmt. Zusätzlich wurde das Wachstum von manchen Kulturen mit quantitativer PCR verfolgt. Darüber hinaus wurde das neue Archaeon mittels Mikroskopie morphologisch charakterisiert. Um eine phylogenetische Zuordnung zu ermöglichen, wurden außerdem die 16S rRNA Gene des Archaeons sequenziert.

Die Inkubation aller Anreicherungskulturen erfolgte im Dunkeln bei 20 °C. Ammoniak und Harnstoff wurden als Substrat für die Ammoniak Oxidation verwendet. Die höchste Anzahl an 16S rRNA Gen-Kopien pro mL und die höchste NO_2^- Produktionsrate wurde mit Harnstoff als Substrat gemessen. Es wurde Wachstum bei einer Anfangs-Konzentration von 0.5 mM bis 20 mM Ammoniak beobachtet, wobei es allerdings zu einer Verlängerung der lag Phase bei der höchsten Konzentration kam. Außerdem wurde bei den höheren Konzentrationen im Medium eine maximale Endkonzentration von 900 µM Nitrit gemessen. Bei einer Anfangs-Konzentration von 1 mM Harnstoff wurde eine Endkonzentration von bis zu 2 mM Nitrit und eine kürzere lag Phase im Vergleich zu den Kontroll-Kulturen mit 0.5 mM Ammoniak beobachtet. Für pH Werte zwischen pH 6 und 7.5 zeigte sich anhaltendes Wachstum in den Anreicherungskulturen. Eine Ergänzung des Medi-

ums mit Glyoxylat führte zu einer höheren Anzahl an 16S rRNA Kopien in den Anreicherungskulturen. Allerdings konnte kein signifikanter Effekt von Glyoxylat auf die Nitrit Produktionsrate festgestellt werden.

Die maximale NO_2^- Produktionsrate von 16.1 μM pro Tag wurde in Kulturen mit 1 mM Anfangs-Konzentration an Harnstoff gemessen. Der untersuchte Mikroorganismus benötigte 137 Tage um 1 mM Ammoniak zu oxidieren. Im Vergleich dazu benötigt *Nitrososphaera viennensis* nur 10 Tage. Zwei denkbare Erklärungen wären, dass entweder diesem Stamm aus der Arktis ein wichtiger Nährstoff für sein optimales Wachstum fehlt oder dass es sich um einen extrem langsam wachsenden Organismus handelt. Die Produktion des Treibhausgases Distickstoffmonoxid (N_2O) konnte in unseren Anreicherungskulturen nachgewiesen werden. Allerdings wurden nur 0.012 N_2O Moleküle pro 100 NO_2^- Moleküle gebildet, was um das zehnfache weniger liegt als bei anderen AOA. Acetylen inhibierte das Wachstum und die Distickstoffmonoxid Produktion von den Kulturen.

Das Gesamtvolumen der Kulturen wurde auf 100 mL erhöht um eine Steigerung an Biomasse zu erhalten. Dies resultierte in einer Verlängerung der lag Phase, wobei es aber zu vergleichbaren maximalen Nitrit Produktionsraten von 7.4 μM pro Tag für 100 mL und 7.9 μM pro Tag für 20 mL Gesamtvolumen kam. Um die Archaea weiter anzureichern kamen verschiedene Antibiotika gegen die bakteriellen Kontaminanten zum Einsatz. Es konnte ein Wachstum der Kulturen mit 100 μg/mL Ampicillin, Carbenicillin und Kanamycin beobachtet werden. Die mit Carbenicillin und Kanamycin behandelten Anreicherungskulturen produzierten allerdings signifikant weniger Nitrit, was eventuell durch Inhibierung der Bakterien in den Kulturen, welche einen positiven Wachstumseffekt auf die AOA ausübten, zu erklären ist.

Fluoreszente in situ Hybridisierung und Lichtmikroskopie wurden eingesetzt, um dieses neue Archaeon zu identifizieren und morphologisch zu charakterisieren. Mittels Archaea spezifischer Sonden wurden Zellen in Form von Kokken mit einem ungefähren Durchmesser von 2 μm als *N. arctica* identifiziert. Diese Zellen neigten dazu, in Aggregaten verschiedener Größen und auch als Einzelzellen zu wachsen.

Die 16S rRNA Gen Sequenzen der Archaea von diversen Anreicherungskulturen aus den verschiedenen Bodenproben aus Spitzbergen, waren über eine Region von 759 Basenpaaren zu 99.6 % identisch und unterschieden sich zu 3.82 % zu der Sequenz von N. viennensis EN76.

Der vorläufige Name dieses Mikroorganismus ist *Candidatus Nitrosopila arctica* (Latein männlich Adjektiv nitrosus, nitros hier im Sinne von Nitrit-Produzent; Latein weiblich Nomen pila, Ball, wie ein Ball geformt; Latein weiblich arctica, Arktis, nördlich)

ACKNOWLEDGMENTS

First of all, I greatly thank Prof. Christa Schleper for being a wonderful supervisor. In addition to giving me the opportunity to write my thesis at her department she has always supported me in all possible ways and encouraged my enthusiasm for the cultivation of archaea.

Many thanks go to my co-supervisor Ricardo Alves, for his patience, motivation and his advice through my work. Further, for introducing me to the fascinating world of cultivation at our lab.

I also want to thank all my colleagues at the Archaea Biology and Ecogenomics division, who made my lab life more productive and fun at the same time: Amir, Andi, Anja, Andrea, Andrea M., Bella, Clarissa, Dani, Isa, Julia, Lisa, Melina, Niko, Rita, Silvia, Simon, Tim and Ziga. Furthermore, I thank Micha, who helped me a lot with the N_2O cultures. I am grateful to Nika and Steffi for their help with FISH and to Pierre for his advice in the field of DNA Extraction. I would like to thank Romana for her support and friendship during my work.

I wish to thank Chrisi, Ina, Jules and Tina for their company, friendship and their encouragement since the beginning of my studies. I have very much enjoyed the numerous learning-sessions, which were not only fun but also helped to improve my grades.

I am very grateful to my family, in particular my mother and brother, for their love and support since ever. I owe my deepest gratitude to my father, who passed away far too early, but rose my interest for the amazing world of microorganisms already in my childhood.

Finally, I want to thank my partner Andreas for always being there for me, for many great adventures all over the world, and last but not least for his ongoing 24/7 computer support.

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ABBREVIATIONS

µ	micro
µg	microgram(s)
µL	microlitre(s)
µm	micrometer(s)
µM	micromolar
°C	degree Celsius
%	percent
abs	absolute
AMO	ammonia monooxygenase
Amp	ampicillin
AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
bp	base pair(s)

Ca.	candidatus
Cb	carbenicillin
CO_2	carbon dioxide
conc.	concentration
Cy3	5,5′-di- sulfo-1,1′-di-(X- carbopentynyl)-3,3,3′,3′-tetramethylindol-Cy3.18-derivative N-hydroxysuccimidester
d	day(s)
DAPI	$4' - 6'$—di-amidino-2-phenylindole
DNA	desoxyribonuleic acid
dNTP	desoxy-nucleotide-tri-phosphate
EDTA	ethylene-di-amine-tetra-acetic acid
EtOH	ethanol
FA	formamide
FISH	fluorescence *in situ* hybridisation
Fluos	5,(6)-carboxyfluorescein-N-hydroxysuccimidester
FWM	fresh water medium
g	gram(s)
g.	glyoxylate
h	hour(s)
HAO	hydroxylamine oxidoreductase
H_2O	water
HCl	hydrochloric acid
K	kelvin
Kan	kanamycin
KCl	potassium chloride
L	litre(s)
M	molar

mL millilitre

mM millimolar

mod. modified

NaCl sodium chloride

NaOH sodium hydroxide

NH_2OH hydroxylamine

NH_3 ammonia

NH_4^+ ammonium

nm nanometer

NOB nitrite oxidizing bacteria

N_2 dinitrogen

N_2O nitrous oxide

NO_2^- nitrite

NO_3^- nitrate

Nxr nitrite oxidoreductase

PBS phosphate buffered saline

PCR polymerase chain reaction

PFA para-formaldehyde

pyr. pyruvate

RNA ribonucleic acid

rpm rotations per minute

rRNA ribosomal RNA

RT room temperature

SDS sodium dodecyl sulfate

S-EC standard enrichment conditions

sec second(s)

SNP single nucleotide polymorphism

spp species

TAE tris-acetate-EDTA

Taq polymerase *Thermus aquaticus* DNA polymerase

TE tris-EDTA

T temperature

qPCR quantitative PCR

u unit(s)

v/v volume concentration

× times

1 INTRODUCTION

Every living organism depends on nitrogen, an essential element on earth, which controls, e.g., diversity, functioning and species composition in many ecosystems (Vitousek et al. 1997). Furthermore, elemental nitrogen, a diatomic gas (N_2), is the major component of the Earth's atmosphere.

Nevertheless, nitrogen is often the limiting nutrient in primary production (Elser et al. 2007, Vitousek and Howarth 1991), because animals and plants are not able to use elemental nitrogen. Di-nitrogen gas (N_2) has to be converted to ammonia (NH_3) to become biologically available for primary producers. This process is called nitrogen fixation and is mostly carried out by prokaryotes. However, there are two other processes known for nitrogen fixation, lightning and since the early 20th century the Haber-Bosch process (Fields 2004).

In the last years, the flux of nitrogen through the global nitrogen cycle has undergone drastic changes (Galloway and Cowling 2002). More than half of the fixed nitrogen per year has its origin in anthropogenic processes, e.g., the Haber-Bosch process, which is used to produce organic fertilizers (Galloway and Cowling 2002, Nevison and Holland 1997). An excessive use of ammonia-based fertilizer and cultivation of nitrogen-fixing crops lead to eutrophication and the production of the strong greenhouse gas N_2O. These consequences may increase global climate change, which endangers the Arctic region. Negative effects of the climate warming are thawing of the Arctic permafrost soils and an increase of the active-layer thickness (Anisimov et al. 2007). This, in turn, could lead to shifts in function and structure of this ecosystem and can further increase the climate change. Therefore, the detailed understanding of the microorganisms which are involved in the global nitrogen cycle (Figure 1), especially in the process of nitrification, has become increasingly important.

This work focuses on archaea from Arctic environments involved in ammonia oxidation which is a crucial step in the nitrification process.

1.1 NITRIFICATION

Nitrification is one of the most important processes in the nitrogen cycle. It is a two step process, which is catalysed by different microorganisms. These microorgan-

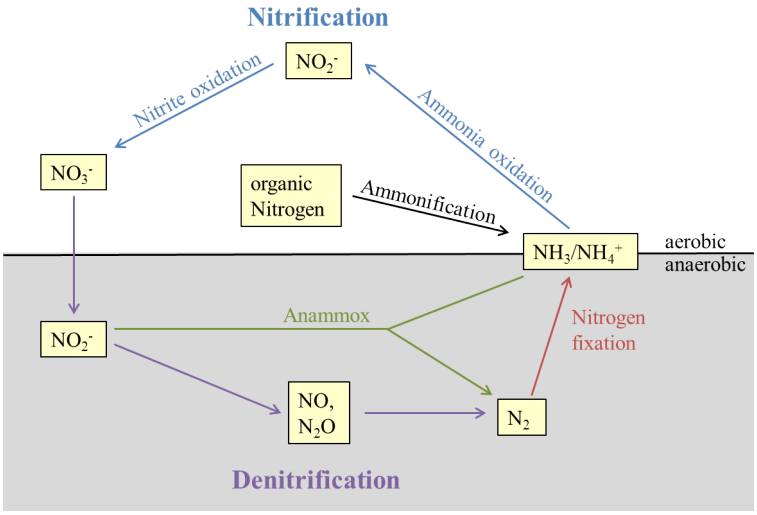


Figure 1: Major transformations in the global nitrogen cycle: The most prevalent chemical forms of nitrogen together with the corresponding oxidation and reduction reactions.

isms use the oxidation of reduced inorganic nitrogen compounds as their energy source.

For more than 100 years it was known that two groups of microorganisms catalyse this process, aerobic chemolithoautotrophic ammonia oxidizing bacteria (AOB) on the one hand and nitrite oxidizing bacteria (NOB) on the other hand (De Boer and Kowalchuk 2001, Koops et al. 2006, Purkhold et al. 2000). The first step, the oxidation of ammonia to nitrite via hydroxylamine is the rate limiting step, which is catalysed by ammonia oxidisers (Stieglmeier et al. 2013). The second step, the oxidation of nitrite to nitrate, is catalysed by nitrite oxidisers (Prosser 1990). So far no microorganism has been discovered that is able to perform the complete oxidation of NH_3 to NO_3^- (Kowalchuk and Stephen 2001, Teske et al. 1994).

1.1.1 Archaea

In the 1970s a new domain of life, the so-called *Archaea*, were discovered by Carl Woese and colleagues (Woese and Fox 1977, Woese et al. 1978). In the beginning only microorganisms living in extreme environments such as submarine hydrothermal vents (Stetter et al. 1990, Stetter et al. 1983) and salt lakes as well as strictly anaerobic methanogens were recognized to belong to this new domain (Woese et al. 1990). Metagenomic analyses of marine and soil samples revealed first insights that archaea are capable of ammonium oxidation in aerobic, non extreme environments (Schleper et al. 2005, Treusch et al. 2005, Venter et al. 2004). Currently *Archaea* consist of six phyla: *Crenarchaeota*, *Euryarchaeota*, *Nanoarchaeota*, *Korarchaeota*, *Aigarchaeota* and *Thaumarchaeota* (Guy and Ettema 2011). The genome

analysis of *Ca. Cenarchaeum symbiosum* led to the proposal of the phylum *Thaumarchaeota* in 2008 (Brochier-Armanet et al. 2008). This proposal was confirmed when more genome sequences became available (Spang et al. 2010). Until now, *Thaumarchaeota* represent the deepest branching lineage in the archaeal phylogeny. Furthermore, Thaumarchaeota are among the most abundant archaea on earth, because they comprise all potential ammonia oxidizing archaea, that reside in huge numbers in terrestrial and marine environments (Nicol et al. 2008, Stahl and de la Torre 2012). Presently, the only physiology known for the members of the *Thaumarchaeota*, is chemolithotrophic growth on ammonia (Hatzenpichler 2012). However, giant thaumarchaeotes were discovered that may be involved in sulphur cycling (Muller et al. 2010), which demonstrated our lack of knowledge of this phylum.

1.1.2 Ammonia oxidation

Ammonia oxidation is the first step of nitrification. It consists of two reactions. In ammonia oxidizing bacteria, a membrane-associated enzyme, called ammonia monooxygenase (AMO), catalyses the oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH) (Hyman and Wood 1985). Then a periplasm-associated enzyme hydroxylamine oxidoreductase (HAO) oxidises hydroxylamine to nitrite (Hooper et al. 2004, Olson and Hooper 1983) (Figure 2).

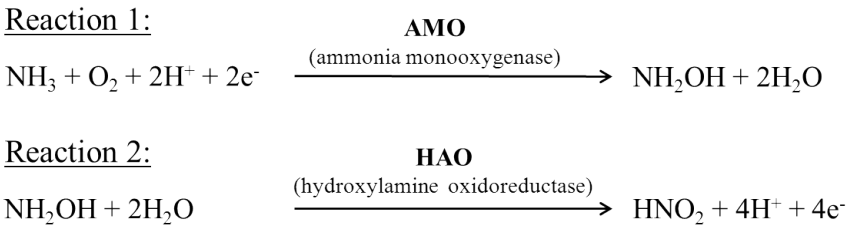


Figure 2: Ammonia oxidation formulas of AOB and the corresponding enzymes.

The enzyme AMO consists of three subunits: AmoA, AmoB and AmoC. Also in ammonia oxidizing archaea (AOA), homologous genes of these three subunits were found (Schleper et al. 2005, Treusch et al. 2005). Furthermore, the gene *amoA* can be used as phylogenetic and functional marker for both AOA and AOB (Purkhold et al. 2000, Schleper et al. 2005).

The enzyme AMO can use reduced inorganic nitrogen, in the form of free ammonia (NH_3), as substrate. There are, however, two forms of reduced inorganic nitrogen, ammonia (NH_3) and ionized ammonium (NH_4^+) in the environment. Both exist in equilibrium depending on salinity, pH and temperature. As pH is decreasing, the equilibrium shifts to NH_4^+, thereby less substrate is available for ammonia oxidisers (Burton and Prosser 2001).

1.1.3 Nitrite oxidation

Nitrite oxidation is the second step of nitrification, which is a reversible process, performed by the membrane-bound nitrite oxidoreductase (Nxr). The enzyme Nxr catalyses the oxidation of nitrite (NO_2^-) to nitrate (NO_3^-) (Bock and Wagner 2006, Sundermeyer-Klinger et al. 1984) (Figure 3).

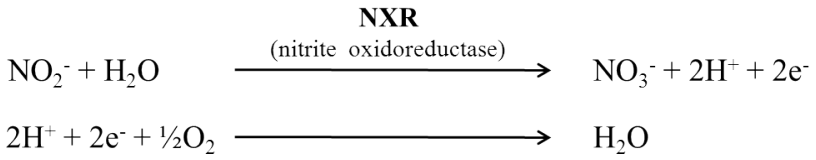


Figure 3: Nitrite oxidation of NOB.

The process of nitrite oxidation is performed by aerobic nitrite oxidizing bacteria (NOB). Nitrite oxidizing bacteria are widely distributed within the proteobacteria and belong to several phylogenetic groups (Teske et al. 1994).

1.2 AMMONIA OXIDIZING ARCHAEA (AOA)

The first nitrifying microorganism was isolated at the end of the 19th century (Winogradsky 1890). In the following decades it was supposed that all autotrophic ammonia oxidisers were bacteria (Jetten 2008).

The isolation of the archaeon *Nitrosopumilus maritimus* has changed this view and was a major breakthrough. It was the first isolated aerobic ammonia oxidizing archaeon coming from a mesophilic environment (Könneke et al. 2005). However, only two pure cultures (Könneke et al. 2005, Tourna et al. 2011) and few enrichment cultures (De La Torre et al. 2008, French et al. 2012, Hatzenpichler et al. 2008, Jung et al. 2011, Kim et al. 2012, Lehtovirta-Morley et al. 2011, Mosier et al. 2012) of ammonia oxidizing archaea have been obtained, since the isolation of *Nitrosopumilus maritimus*.

For bacteria the process of ammonia oxidation is relatively well characterized. The oxidation of ammonia to nitrite via an intermediate product is mechanistically similar for AOB and AOA. A recent study could show that the marine archaeon *Nitrosopumilus maritimus* used the same intermediate product, hydroxylamine, as ammonia oxidizing bacteria. However, biochemically they seem to differ in the second step of ammonia oxidation. Bacteria use the enzyme HAO whereas archaea possibly use a novel enzyme complex, because no gene encoding a bona fide HAO can be found in the genomes of Thaumarchaeota (Vajrala et al. 2013).

Ammonia oxidizing archaea and bacteria are widely distributed in various ecosystems. Different characteristics allow them to populate different niches. A crucial factor is the substrate concentration, which controls the relative distribution of AOA compared to AOB. AOA are considered to be generally adapted to lower ammonium concentrations and AOB to high ammonium concentrations (Lehtovirta-Morley et al. 2011, Martens-Habbena et al. 2009, Verhamme et al. 2011). However, different studies could show that AOA are adapted to ammonium concentrations between 10 mM to 20 mM (Jung et al. 2011, Tourna et al. 2011). This is comparable to what is known about the most oligotrophic AOB, but still low compared to the highest NH_4^+ tolerance of ammonia oxidizing bacteria (Koops et al. 2006).

Another factor which influences the community structure of ammonia oxidisers is temperature. Ammonia oxidizing archaea are adapted to a broad range of temperatures (Zhalnina et al. 2012). It was shown that AOA can oxidise ammonia to nitrite at temperatures from $-1\,^{\circ}C$ in Arctic coastal waters to $97\,^{\circ}C$ in hot springs of Iceland (Kalanetra et al. 2009, Reigstad et al. 2008). So far no AOB were detected in environments with constant temperatures over $40\,^{\circ}C$ (Hatayama et al. 1999, Hatzenpichler 2012).

In 2011, a study demonstrated that, among seven measured physicochemical parameters pH, carbon, nitrogen, C:N ratio, organic matter content, vegetation and soil moisture, that pH is the major driver of the AOA community structure (Gubry-Rangin et al. 2011). Some AOA are more tolerant to low pH than AOB. Further, AOA are known to grow under different pH values, which range from pH 4.5 to 7.5 (Lehtovirta-Morley et al. 2011, Nicol et al. 2008). Furthermore, archaeal *amoA* genes were found at a pH between 2.5 and 9 in hot springs (Reigstad et al. 2008, Zhang et al. 2008). It is described that at pH values lower than 7 ammonia is ionized to ammonium. Different mechanisms have been proposed to explain ammonia oxidation at low pH values, e.g, pH-neutral microenvironments, biofilm and aggregate formation (Allison and Prosser 1993, De Boer et al. 1991, Lehtovirta-Morley et al. 2011). Furthermore, some ammonia oxidisers have the possibility to change the substrate and use, e.g., urea as ammonia source at lower pH values (Burton and Prosser 2001).

Another difference in the physiology of AOA and AOB might be that AOA can also use organic carbon for growth (Tourna et al. 2011). Although use of organics has also been demonstrated for AOB (Hommes et al. 2003, Krümmel and Harms 1982, Martiny and Koops 1982, Wallace et al. 1970), environmental studies indicate alternative metabolisms for AOA (Jia and Conrad 2009, Ke et al. 2013, Mußmann et al. 2011)

In pure cultures of *Nitrososphaera viennensis* it was observed that additional pyruvate, an organic carbon source, stimulated its growth (Tourna et al. 2011).

1.2.1 The ecological role of AOA

AOA and AOB have "conquered" aquatic and terrestrial environments all over the world, e.g., soils, marine environments, salt lakes, freshwater and wastewater treatment systems (Fernàndez-Guerra and Casamayor 2012, Hatzenpichler 2012, Li et al. 2011, Schleper and Nicol 2010). However, they differ in their abundance, activity and distribution (Martens-Habbena et al. 2009, Prosser and Nicol 2008, Sauder et al. 2011, Verhamme et al. 2011). This can be caused by the differences in the structure of the enzyme AMO between AOA and AOB, archaeal AMO has a higher affinity for substrates (Martens-Habbena et al. 2009). Furthermore, as mentioned above AOA might be able to use organic carbon, they are adapted to a broad range of temperatures and pH values (Hallam et al. 2006, Lehtovirta-Morley et al. 2011, Zhalnina et al. 2012). It was shown that archaeal *amoA* copies are more abundant in soils (Leininger et al. 2006) and in marine environments (Wuchter et al. 2006) than those of ammonia oxidizing bacteria.

The conversion of ammonia to nitrite via ammonia oxidisers and to nitrate via nitrite oxidisers can lead to nitrogen loss in soils. This is due to the fact that nitrate is more soluble than ammonium. Nitrate leaching from soils can have several consequences, e.g., groundwater contamination and water eutrophication (Kowalchuk and Stephen 2001). Therefore, ammonia oxidizing archaea are partially involved in nitrogen loss from ecosystems.

A significant source of nitrous oxide production in soil is autotrophic nitrification (Colliver and Stephenson 2000, Shaw et al. 2006). Recent studies have shown that AOA and AOB can produce nitrous oxide (N_2O) (Jung et al. 2011, Shaw et al. 2006). Nitrous oxide is a strong greenhouse gas, which has a warming potential 310 times higher than carbon dioxide (CO_2). Nitrous oxide is after carbon dioxide and methane the third most important greenhouse gas (Susan 2007). AOB produce N_2O from NH_2OH during nitrification or from NO_2^- during nitrifier-denitrification (Shaw et al. 2006, Kool et al. 2010). For AOA the pathway of N_2O production is not yet understood (Hatzenpichler 2012, Löscher et al. 2012).

The high abundance of AOA in different ecosystems and the production of the strong greenhouse gas N_2O are some reasons why it is particularly important to investigate ammonia oxidizing archaea.

1.3 ARCTIC SOIL

The Arctic is a polar region and consists of the iced-covered Arctic ocean, parts of North America, Europe and Asia and all of Greenland and Spitsbergen.

Figure 4: Map of the Arctic region. (Ritz 2013)

Arctic and boreal regions have an important role, as they occupy 22 % of the terrestrial surface (Chapin et al. 2000). The climate in the Arctic is characterized by short cool summers and long cold winters. The vegetation is made up of mosses, lichens, herbs, dwarf shrubs and graminoids. All these plants are forming the so called tundra. The characteristics of tundra are a cold climate with low biotic diversity and a simple vegetation structure. Further, the tundra is characterized by a short season of growth and reproduction, and nutrients are in the form of dead organic material. In the Arctic tundra the growing season is limited to 50-60 days per year. Moreover, the soil is formed slowly and there is a layer of permanently frozen subsoil which is called permafrost. Arctic soils play also an important role in the global carbon cycle, because they contain huge carbon pools (Tarnocai et al. 2009).

The Arctic region is endangered by the climate change. Shifts in function and structure of this ecosystem can have a great impact on the atmosphere and can further increase the climate change. Consequences of climate warming can be degradation of permafrost, an increase of the active-layer thickness and earlier

snowmelt (Anisimov et al. 2007). At the top of the permafrost layer, the temperatures have increased by up to 3 °C since the 1980s (Pachauri and Reisinger 2007).

Not much is known yet about how the terrestrial and marine ecosystems will respond to the first-order climate changes. An example of a physical change that will affect the terrestrial ecosystem is thawing of permafrost (McGuire et al. 2006). Also the effect of alterations in the nitrogen cycle on the nutrient cycling in the Arctic is not completely understood (Nemergut et al. 2005).

In Arctic soils nitrogen is the major limiting nutrient. Further, some soil microorganisms have to compete with plants for available nitrogen (Anisimov et al. 2007, Nordin et al. 2004).

One group of these soil microorganisms are ammonia oxidizing archaea, which - as mentioned before - convert ammonia to nitrite. The produced nitrite can further be oxidized to nitrate by nitrite oxidizers. However, nitrate is more soluble, which can lead to nitrogen loss in soils. Therefore, ammonia oxidizing archaea are indirectly involved in nitrogen loss from Arctic soils. Further, AOA are known to produce, N_2O, a strong greenhouse gas.

Due to the above mentioned impact of AOA on the nitrogen cycle it is crucial to learn more about these organisms in arctic environments, as they are frequently involved in nitrate leaching from soils and production of greenhouse gases. The need for a better understanding of their physiology and metabolism prevails. In particular, it is important to learn more about how AOA will react to environmental changes, and to evaluate the consequences on the global climate as well as for the global nitrogen cycle.

1.4 ARCTIC SOIL ENRICHMENTS

In August 2009, my colleague Ricardo J. Eloy Alves collected soil samples of different sites in Spitsbergen (78 °N). These samples came from Arctic tundra landscapes: fen wetlands, moss, shrub and tussock dominated tundra and cryoturbated soils (frost boils)(Alves et al. 2013). Back then, he had started 48 initial enrichment cultures. The aim was to enrich for different ammonia oxidizing archaea.

The cultures were inoculated with the soil samples from the top layer of all Spitsbergen sites. For each soil four enrichment cultures were initiated by inoculating 1 g soil in 20 mL fresh water medium (Tourna et al. 2011) containing 0.5 mM NH_4^+. The ammonium and nitrite concentrations in the enrichment cultures were measured at different time-points. The cultures where ammonia was consumed were sub-cultured in a second stage with 20 % inoculum in 20 mL fresh water medium. In the second and third stage of archaeal enrichment cultures he tried different temperatures, such as 4, 14, 17, 20, 28, 32 and 37 °C. Further, he used

different treatments, e.g., ampicillin and lysozyme, to further enrich for archaea (Alves et al. 2013). Only in the enrichment cultures at 20 °C he observed a stable and continuous NH_4^+ consumption and NO_2^- production.

At the beginning of this work the best known growth conditions of *N. arctica* were 20 mL total culture volume, 16 mL fresh water medium (FWM) (80 %, v/v), 4 mL inoculum (20 %, v/v), pH ~ 7 and an ammonium concentration of 0.5 mM. For the inhibition of bacterial contaminants streptomycin 100 µg/mL was used as default antibiotic.

1.5 AIMS OF THIS WORK

The aim of this work was to characterize the arctic ammonia oxidizing archaea (AOA) enrichment cultures, which were started by my co-supervisor Ricardo J. Eloy Alves and to obtain stable cultures of these ammonia oxidizing archaea. For this purpose different additional substances were tested to increase the growth rate which was followed by measuring ammonia consumption and nitrite production. Additionally, enrichment cultures were treated with antibiotics to further enrich for AOA. Fluorescence *in situ* hybridisation was used to identify archaea in the enrichment cultures. We also investigated the cultures with light microscopy to morphologically characterize these new AOA.

Another important aim was to obtain a sufficient amount of biomass of an Arctic ammonia oxidizing archaeon to be able to sequence the whole genome.

2

MATERIALS AND METHODS

2.1 EQUIPMENT

Equipment used for my studies is listed in Table 1.

Table 1: Equipment

Equipment	**Company**
Photometers:	
NanoDrop ND-1000	PeqLab
Qubit 2.0 Fluorometer	Invitrogen
CM Sunrise	Tecan
PCR Cyclers:	
TP Gradient 96 Thermocycler	Biometra
Mastercycler epgradientS realplex^2	Eppendorf
Centrifuges :	
Zentrifuge 54152	Eppendorf
Universal 320R	Hettich
Ministar silverline	VWR
Autoclaves :	
VX-120 snb:3206	Systec
CE-EL 18L	CertoClav
Sample containers :	
Polystyrene tubes $216-2637$ (30 ml)	VWR
Polypropylene tubes (50 ml)	Greiner Bio-One
Glass bottles (250 ml)	SCHOTT DURAN
Indicator sticks (pH) :	
Indicator sticks (4.5 to 10.0)	ROTH
Indicator sticks (1 to 14)	Fisherbrand

Refrigerated Incubator KB 240	Binder
Laminar Flow BH-EN 2005 S	Faster
UV3 HEPA PCR Workstation	UVP
Gel Doc XR System	BioRad
Gelchamber Easy Phor MS	Biozym
Eclipse 50i Microscope	Nikon
FastPrep® - 24	MP Biomedicals
Shaking Waterbath 1085	GFL
Water purification facility MQ Elix 10	Millipore
HB-1000 Hybridizer	UVP
Thermomixer comfort 5355	Eppendorf
Rotamax 120 horizontal shaker	Heidolph
Lab dancer vortexer	VWR

2.2 SOFTWARE

All software was running under Windows XP (Microsoft).

- Microsoft Excel 2010
- Realplex Eppendorf Software Version 2.2.0.84
- Tecan XFluor4
- SigmaPlot version 11.0
- NIS-Elements F 3.2
- BioEdit v7.0.9
- MEGA5.2
- ATGC PhyML 3.0

2.3 CHEMICALS

In Table 2 all chemicals, which were used in my studies, are listed.

Table 2: Chemicals

Chemicals	Company
6 × DNA loading dye	Fermentas
4'-6'-di-amidino-2-phenylindole (DAPI)	Sigma
Acetic acid	ROTH/Lactan
Agarose (LE)	Biozym
Ammoniumchloride	Sigma
Ampicillin sodium salt	ROTH/Lactan
Biotin	Sigma
Boric acid	ROTH/Lactan
Calciumchlorid-dihydrate	ROTH/Lactan
Carbenicillin di-sodium salt	ROTH/Lactan
Cefoxitin sodium salt	ROTH/Lactan
Cephalexin hydrate	Sigma
Chloramphenicol	ROTH/Lactan
Cobalt(II) chloride hexahydrate	Sigma
Copper(II) chloride dihydrate	ROTH/Lactan
Dichloroisocyanuric acid	Fluka
Disodium hydrogen phosphate	ROTH/Lactan
Ethylenediamine tetra-acetic acid (EDTA)	ROTH/Lactan
Ethanol absolut	Merck
Ethidium bromide 1 %	ROTH/Lactan
Formaldehyde 37 %	ROTH/Lactan
Formamide 99.5 %	ROTH/Lactan
Glycogen (20 mg/ml)	Fermentas
HEPES	ROTH/Lactan
Hydrochloric acid 37 %	ROTH/Lactan
Incidin	Ecolab
iQ SYBR Green Supermix	Bio-Rad
Isoamyl alcohol	ROTH/Lactan
Isopropanol Mol. Biol.Grade	AppliChem
Kanamycin sulfate	ROTH/Lactan
Magnesium chloride	Sigma
Magnesium chloride hexahydrate	Sigma

Manganese(II) chloride tetrahydrate	Merck
Monosodium phosphate	Fluka
N-1-Naphtylethylendiamindihydrochloride	ROTH/Lactan
Nickel(II) chloride hexahydrate	Sigma
Ortho phosphoric acid (> 85%)	ROTH/Lactan
Phenol:chloroform:isoamyl alcohol 25 : 24 : 1	Fisher Scientific
Polyethylenglycol PEG 8000	ROTH/Lactan
Poly-L-Lysine solution	Sigma
Potassium chloride	AppliChem
Potassium di-hydrogen phosphate	ROTH/Lactan
Potassium hydroxide	AppliChem
Sodium bicarbonate	Sigma
Sodium chloride	AppliChem
Sodium dodecyl sulfate	ROTH/Lactan
Sodium glyoxylate	Sigma
Sodium hydroxide	ROTH/Lactan
Sodium molybdate dihydrate	Merck
Sodium nitrite	ROTH/Lactan
Sodium nitroprusside dihydrate	Fluka
Sodium pyruvate	ROTH/Lactan
Sodium salicylate	Sigma
Spectinomycin dihydrochloride pentahydrat	Sigma
Streptomycin sulfate	Sigma
Sulfanilamide	Sigma
TAE-buffer (50 ×, Rotiphorese ®)	ROTH/Lactan
Trichloroacetic acid	ROTH/Lactan
Trichloromethane/Chloroform 99%	ROTH/Lactan
Tris hydrochloride	ROTH/Lactan
Zinc sulfate heptahydrate	ROTH/Lactan

2.4 MEDIA, SOLUTIONS AND BUFFERS

All media, buffers and solutions used in my studies were prepared with double distilled and filtered water (MilliQ by Millipore Corporation 18.2 MΩ, filter size

0.22 µm). In the next step they were autoclaved for 20 minutes at a temperature of 121 °C (Systec VX-120), if not stated otherwise. Solutions and substances (e.g. antibiotics) which are unstable at high temperatures were sterile filtered (filter size 0.2 µm) and added after autoclaving. Furthermore, if not stated otherwise, all media, buffers and solutions were stored at 4 °C.

2.4.1 Media and solutions for AOA enrichment cultures

- Basalmedium for FWM (1 ×)

NaCl	1.0 g
$MgCl_2 \cdot 6H_2O$	0.4 g
$CaCl_2 \cdot 2H_2O$	0.1 g
KH_2PO_4	0.2 g
KCl	0.5 g
MilliQ	1000 mL

- Fresh water medium (FWM)

Modified Trace Elements	1.0 mL
FeNaEDTA Solution	1.0 mL
Vitamin Solution	1.0 mL
Sodium Bicarbonate (1 M)	2.0 mL
Energy source (1 M)	x mL
Streptomycin (50 mg/mL)	2.0 mL

  These solutions had to be added to 1 L basalmedium. The energy source, which was used, was depending on the experiment. In the standard enrichment cultures a concentration of 0.5 mM ammonia was used.

- Modified Non-chelated trace element mixture (1 L)

HCl	8.0 mL (100 mM)
H_3BO_3	30 mg (0.5 mM)
$MnCl_2 \cdot 4H_2O$	100 mg (0.5 mM)
$CoCl_2 \cdot 6H_2O$	190 mg (0.8 mM)
$NiCl_2 \cdot 6H_2O$	24 mg (0.1 mM)
$CuCl_2 \cdot 2H_2O$	2 mg (0.01 mM)
$ZnSO_4 \cdot 7H_2O$	144 mg (0.5 mM)
$Na_2MoO_4 \cdot 2H_2O$	36 mg (0.15 mM)
MilliQ	987 mL

- FeNaEDTA solution (1 L, 7.5 mM)

FeNaEDTA	2753 mg
MilliQ	1000 mL

- Vitamin solution (1 L, adjust to pH 7 with KOH and sterile filtered)

Biotin	0.02 g
Folic acid	0.02 g
Pyridoxine HCl	0.10 g
Thiamine HCl	0.05 g
Riboflavin	0.05 g
Nicotinic acid	0.05 g
DL Pantothenic acid	0.05 g
P Aminobenzoic acid	0.05 g
Choline Chloride	2.00 g
Vitamine B12	0.01 g

- Sodium bicarbonate solution (1 M, 80 mL)

$NaHCO_3$	6.7208 g
MilliQ	80 mL

- Ammoniumchloride solution(1 M, 60 mL)

NH_4Cl	3.21 g
MilliQ	60 mL

- Urea solution (1 M, 10 mL, sterile filtered (0.2 µm))

CH_4N_2O	0.6006 g
MilliQ	10 mL

- Sodium pyruvate (1 M)

Sodium pyruvate	0.55 g
MilliQ	5 mL

2.4.2 Antibiotics for AOA enrichment cultures

- Streptomycin (50 mg/mL, sterile filtered (0.2 µm))

Streptomycin	0.5 g
MilliQ	10 mL

- Kanamycin (100 mg/mL, sterile filtered (0.2 µm))

Kanamycin	1.0 g
MilliQ	10 mL

- Ampicillin (100 mg/mL, sterile filtered (0.2 µm))

Ampicillin	1.0 g
MilliQ	10 mL

- Carbenicillin (50 mg/mL, sterile filtered (0.2 µm))

Streptomycin	0.5 g
MilliQ	10 mL

2.4.3 Buffers and solutions for gel electrophoresis

All buffers and solutions for gel electrophoresis were stored at room temperature.

- 50 × TAE buffer (1 L)

Tris	242 g
Acetic acid conc.	57.1 mL
EDTA (0.5 M, pH 8)	100 mL
MilliQ	842.9 mL

- 1 × TAE buffer (1 L)

50 × TAE	20 mL
MilliQ	980 mL

- 0.5 × TAE buffer (1 L)

50 × TAE	10 mL
MilliQ	990 mL

- Ethidium bromide solution (dilution 1 : 40)

Ethidium bromide (1 %, 10 mg/mL)	1 mL
MilliQ	39 mL

- DNA Ladder (Fermentas)
  GeneRuler™ 50 bp DNA Ladder, ready-to-use, SM0373
  GeneRuler™ 100 bp DNA Ladder, ready-to-use, SM0243
  GeneRuler™ 100 bp Plus DNA Ladder, ready-to-use, SM0321
  GeneRuler™ 1 Kb DNA Ladder, ready-to-use, SM0313
  GeneRuler™ 1 Kb Plus DNA Ladder, ready-to-use, SM1333

- Agarose gel (1.5 %, 50 mL)

Agarose	0.75 g
1 × TAE buffer	50 mL

2.4.4 Buffers and solutions for DNA extraction

All buffers and solutions with the exception of EtOH and glycogen, which were stored at -20 °C, were stored at room temperature.

- SDS 10 % (not autoclaved)

SDS	4.325 g
MilliQ	150 mL

- EDTA (0.5 M, pH 8)

EDTA	21.918 g
MilliQ	150 mL

- Tris/HCl (1 M, pH 7.5)

Tris/HCl	23.64 g
MilliQ	150 mL

- SDS extraction buffer

NaCl	8.18 g
Na_2SO_3	2.52 g
SDS 10 %	20 mL
EDTA (0.5 M, pH 8)	20 mL
Tris/HCl (1 M, pH 7.5)	20 mL
MilliQ	200 mL

- PEG solution

NaCl	18.7 g
PEG 8000	60 g
MilliQ	200 mL

- 100 × TE buffer

Tris/HCl (2 M, pH 7.5)	50 mL
EDTA (0.5 M, pH 8)	20 mL
MilliQ	30 mL

- Chloroform/Isoamyl alcohol 24 : 1

Chloroform	24 mL
Isoamyl alcohol	1 mL

- Ethanol (70 %, 50 mL)

$Ethanol_{abs}$	35 mL
MilliQ	15 mL

- Sodium chloride (5 M)

Sodium chloride	8.766 g
MilliQ	30 mL

2.4.5 Buffers and solutions for Fluorescence *in situ* hybridisation (FISH)

All buffers and solutions except EtOH, which was stored at -20 °C, were stored at room temperature. These buffers and solutions were not autoclaved.

- Na_2HPO_4 (200 mM)

Na_2HPO_4	8.9 g
MilliQ	250 mL

- NaH_2PO_4 (200 mM)

NaH_2PO_4	2.3996 g
MilliQ	100 mL

- PBS stock solution (250 mL)
  To adjust the pH value of PBS stock solution to 7.2-7.4 approximately 50 mL of NaH_2PO_4 was added to 200 mL Na_2HPO_4.

- 1 × PBS buffer (200 mL)

NaCl	1.52 g
PBS stock solution	10 mL
MilliQ	190 mL

- PFA solution (4 %, 50 mL)

Formaldehyde (37 %)	5.4 mL
MilliQ	44.6 mL

- SDS 10 %

SDS	4.325 g
MilliQ	150 mL

- Tris/HCl (1 M, pH 8)

Tris/HCl	23.64 g
MilliQ	150 mL

- EtOH (50 %, 50 mL)

$EtOH_{abs}$	25 mL
MilliQ	25 mL

- EtOH (70 %, 200 mL)

$EtOH_{abs}$	140 mL
MilliQ	60 mL

- EtOH (80 %, 50 mL)

$EtOH_{abs}$	40 mL
MilliQ	10 mL

- EtOH (96 %, 50 mL)

$EtOH_{abs}$	48 mL
MilliQ	2 mL

- Alcohol solution (1 %HCl in 70 % EtOH)

HCl (37 %)	1.35 mL
EtOH (70 %)	48.65 mL

- Poly-L-Lysin (0.01 %)

Poly-L-Lysin (0.1 %)	0.2 mL
MilliQ	1.8 mL

- EDTA (0.5 M, pH 8)

EDTA	9.305 mL
MilliQ	50 mL

- Hybridisation buffer (FA 30 %, 1 mL)

NaCl 5 M	180 µL
Tris/HCl (1 M, pH 8)	20 µL
Formamide	300 µL
MilliQ	500 µL
SDS 10 %	1 µL

- Washing buffer (for FA 30 %, 50 mL)

NaCl 5 M	1020 µL
Tris/HCl (1 M, pH 8)	1000 µL
EDTA (0.5 M, pH 8)	500 µL
MilliQ	37.48 mL
SDS 10 %	1 µL

2.4.6 Buffers and solutions for ammonium and nitrite determination

- 0.3 M NaOH solution (stored at room temperature)

NaOH	1.2 g
MilliQ	100 mL

- Sodium salicylat solution (10 mL)

Sodium salicylat	1.7 g
Sodium nitroprusside dihydrate	12.7 mg
MilliQ	10 mL

- Colour reagent (30 mL)

Sodium salicylat solution	10 mL
0.3 M NaOH solution	10 mL
MilliQ	10 mL

- Oxidation solution (10 mL)

Dichloroisocyanuric acid sodium salt dihydrate	0.1 g
MilliQ	10 mL

- Ammoniumchloride solution (0.85 mM, 50 mL)

NH_4Cl (1 M)	0.0425 mL
MilliQ	49, 9575 mL

- Griess reagent (Sulfanilamide/NED) (1 L, stored at 4 °C in the dark)

Ortho phosphoric acid	150 mL
Sulfanilamide	10 g
N1-Naphtylethylendiamindihydrochloride	0.5 g
MilliQ	850 mL

- Sodium nitrite solution (1 mM, 50 mL)

$NaNO_2$	3.45 mg
MilliQ	50 mL

2.5 ENRICHMENT CULTURES

2.5.1 Ammonia oxidizing archaea enrichment cultures

The standard conditions for the enrichment cultures were: 20 mL culture volume, 16 mL FWM (Tourna et al. 2011), 4 mL inoculum (20 %, (v/v)) and 0.5 mM ammonium. For the inhibition of bacterial contaminants streptomycin 100 µg/mL was

used as default antibiotic. The enrichment cultures were incubated at 20 °C in the dark, in a refrigerated incubator.

Setup of cultures:

The whole equipment was cleaned with Incidin and dried under the laminar flow cabinet. All working steps had to be done in the laminar flow cabinet. 20 % (v/v) of a stationary culture was pipetted into a sterile flask and 80 % (v/v) fresh water medium (Tourna et al. 2011) was added. Then all the remaining substances like the energy source, additional antibiotics or carbon sources were added. As a control, flasks without inoculum were prepared in parallel to the enrichment cultures under the same conditions. All cultures were then gently mixed and 200 µL (20 µL) were taken for ammonium (nitrite) measurements, respectively. Every 10 to 50 days samples were taken to measure the concentration of ammonium and nitrite. When all the ammonia was converted to nitrite, cultures were either subcultured or stored at 4 °C.

2.5.2 Culture flasks used for enrichment cultures

Three different sample containers with screw caps were used:

1. VWR 216 – 2637, material: polystyrene (30 mL), for 20 mL culture volume.
2. Greiner Bio-One, material: polypropylene tubes (50 mL volume), for 40 mL culture volume.
3. Glass bottles (250 mL volume), for 100 mL culture volume.

2.6 STATISTICAL ANALYSIS

To find out which treatments had an effect on the enrichment cultures, statistical analysis of NO_2^- production values were performed. For each culture NO_2^- production in the exponential phase was calculated from log-linear plots of nitrite concentration against days. NO_2^- production rates were analysed by One Way Analysis of variance (ANOVA) using Sigmaplot version 11.0. As post hoc test Holm Sidak was performed. Before NO_2^- production values had to pass a normality test and an equal variance test. If they passed, ANOVA was performed. For all analysis standard parameters of SigmaPlot 11.0 were used.

NO_2^- production:

$$\frac{N2 - N1}{t_2 - t_1} = x\,[\mu M/\,day]$$

Generation time:

Generation time in the exponential phase was once calculated for 16S rRNA gene copies per mL. Therefore N1 and N2 have to be replaced by gene copies time point 1 and gene copies time point 2.

$$\frac{(\mathrm{LOG}(N2) - \mathrm{LOG}(N1))}{\mathrm{LOG}(2)} = n$$

$$\frac{(t_2) - t_1)}{n} = x\,[\mathrm{days}]$$

N1 ... NO_2^- concentration at time point 1

N2 ... NO_2^- concentration at time point 2

t_1 ... time point 1

t_2 ... time point 2

2.6.1 N_2O - production of *Nitrosopila arctica*

Inocula were grown with either 1 mM ammonium or 0.5 mM ammonium and 100 µg/mL kanamycin. The cultures were grown in 120 mL glass serum bottles sealed with sterile butyl rubber stoppers and aluminium crimp caps. At several time points during growth gas samples were taken using a sterile syringe. 15 mL gas sample was taken and 12 mL gas was then transferred to 10 mL sealed (Baysilone paste, Bayer) and evacuated glass vials. These glass vials where stored at 4 °C until analysis by gas chromatography (AGILENT 6890N, Vienna, Austria; injector: 120 °C, detector: 350 °C, oven: 35 °C, carrier gas: N_2) connected to an automatic sample-injection system (DANI HSS 86.50, Headspace-Sampler, Sprockhövel, Germany). N_2O concentrations were detected with a 63Ni-electron-capture detector. Standard gases (Inc. Linde Gas, Vienna, Austria) contained 0.5, 1 and 2.5 µL/L N_2O. Analysis was performed by Barbara Kitzler (BFW, Federal Forest Office). At five time points also a glass vial with sterilized air was prepared as a control. In order to prevent a vacuum in the cultures the removed gas was replaced immediately with 15 mL fresh sterilized (filter size 0.2 µm) air. The inhibitor acetylene was added to the cultures during growth in an end concentration of 0.01%. After taking samples from the gas phase of these cultures the inhibitor was again added to the gas phase.

N_2O - Calculations:

N_2O values given in ppm, received from Barbara Kitzler, were converted to µM, using the following equations.

$$\frac{((x \cdot (y - 15\,mL)) + (15\,ml \cdot z))}{y} = a_1$$

where a_1 denotes the N_2O value in ppm corrected for 15 mL gas addition and a is the uncorrected N_2O value in ppm.

Step 1 and 2 were calculated for both N_2O values: corrected (for 15 mL new gas) and uncorrected.

(1) $$a \cdot y \cdot \left(\frac{273\,K}{(273\,K + 25\,°C)}\right) = b$$

(2) $$\frac{b}{V_m} = c$$

Average of all three c for each time point was calculated.

(3) $$c_{avg,t1} + (c_{avg,to} - c_{avg,corr.,to}) = d$$

For time point 0 the uncorrected value was taken to convert to µM.

(4) $$\frac{d}{y} = N_2O\ \mu M$$

a_1 ... N_2O value in ppm corrected for 15 mL gas addition

a ... N_2O value in ppm uncorrected

b ... N_2O value in nL total gas volume at 25 °C

c ... N_2O value in nmoL

v_m ... molar volume at 0 °C is 22.41 mmoL

c_{avg} ... average of N_2O value of the triplicates in nmoL for each time point

$c_{avg,corr.}$ ... average of corrected N_2O value of the triplicates in nmoL for each time point

t ... time point

d ... N_2O value in nmoL per gas volume

x ... N_2O values, in ppm

y ... gas volume (mL) in the glass serum bottles, which changes when samples for nitrite and ammonium were taken

z ... average N_2O of the air control samples in ppm

N_2O yield:

N_2O yield was calculated for each time point. Then the average was calculated.

$$\frac{N_2O\,\mu M}{NO_2^-\,\mu M} = N_2O\,yield$$

2.7 MEASUREMENT OF AMMONIUM AND NITRITE

2.7.1 Colourimetric determination of ammonium (NH_4^+)

The principle of the method is the oxidation of ammonium to chloroamine by sodium dichloroisocyansure acid which forms a green indophenol in presence of phenolic compounds in an alkaline medium. The absorbence will be measured photometrically at 660 nm (modified protocol of what is described in (Kandeler and Gerber 1988)).

Procedure:

Standard dilutions from 0.5 mM to 0.0078 mM were prepared for a calibration curve (Table 3).

Table 3: NH_4^+ Standards

Standard (µM)	**FWM** (µL)	0.85 mM **NH_4Cl** (µL)
500	600	0
250	300	300
125	450	150
62.5	525	75
31.25	562.5	37.5
15.625	581.25	18.75
7.8125	590.63	9.37
0	600	0

200 µL samples of the enrichment cultures were collected in 1.5 mL Eppendorf tubes and 400 µL FWM was added. 300 µL colour reagent and 120 µL oxidation solution were added to standards and samples. All samples were shaken on a horizontal shaker at 200 rpm for 30 minutes. With a spectrophotometer the colour intensity at 660 nm was measured.

Calculation:

A calibration curve was plotted from measured absorption against NH_4^+ concentration and a linear regression was performed using Excel. The equation of the linear regression line was used to determine the NH_4^+ concentration of the samples.

2.7.2 Colourimetric determination of nitrite (NO_2^-)

Procedure:

Standard dilutions (20 µM - 0 µM) were prepared for a calibration curve (Table 4).

Table 4: NO_2^- Standards

Standard (µM)	**FWM** (µL)	1 **mM** $NaNO_2$ (µL)
20	780	20
16	784	16
12	788	12
10	790	10
8	792	8
6	794	6
4	796	4
2	798	2
0	800	0

20 µL were collected from the enrichment cultures and 780 µL FWM was added. 200 µL Griess reagent was added to standards and samples. Thereafter samples were shaken and incubated in the dark for at least 15 minutes at room temperature. With a spectrophotometer the absorbence at 545 nm was measured.

Calculation:

A calibration curve was plotted with measured absorbence against NO_2^- concentration and a linear regression was performed using Excel. The equation of the linear regression line was used to determine the NO_2^- concentration of the samples.

2.8 DNA EXTRACTION OF ENRICHMENT CULTURES

The DNA extraction protocol is a modified version of those described previously (Griffiths et al. 2000).

2.8.1 Phenol-Chloroform Extraction

All centrifugation steps were done at maximum speed in an Eppendorf centrifuge, at 4 °C.

Protocol:
1 or 2 mL of the enrichment cultures was centrifuged for 20 minutes to collect the cells. The supernatant was carefully removed and 500 µL SDS extraction buffer was added, which was pre warmed to 65 °C. The cell pellet was re-suspended in the extraction buffer and the mixture was transferred to a Lysing Matrix tube E (MP Biomedicals). Afterwards 500 µL Phenol/Chloroform/Isoamyl alcohol (25 : 24 : 1, pH 8) was added. The cells were bead beaten for 30 seconds at 4 m/s in a FastPrep®- 24 machine. Then the tubes were cooled on ice for 2 minutes and centrifuged for 10 minutes. The supernatant was transferred into a new tube. An equal volume of Chloroform/Isoamyl alcohol (24 : 1) was added and the tube was mixed by hand. Samples were centrifuged for 10 minutes and the supernatant was transferred in a new tube. Then 2 volumes of PEG solution were added, mixed by hand and incubated overnight at 4 °C for precipitation. Samples were centrifuged for 30 minutes and the supernatant was carefully removed. Samples were washed with 1 mL of cold 70 % EtOH and centrifuged for 10 minutes. Supernatant was removed and the DNA pellet was dried at 60 °C. At last, the DNA pellet was re-suspended in 50 µL nuclease-free water.

For N_2O and pH Experiment: Instead of PEG precipitation the DNA was precipitated with 40 µL NaCl, 1 µL glycogen (20 mg/ml) and 500 µL isopropanol. After incubation for 1 hour at room temperature, samples were centrifuged for 35 minutes and the supernatant was carefully removed. Then the procedure was continued as above.

2.9 QUANTITATIVE AND QUALITATIVE ANALYSIS OF DNA

2.9.1 Quantitative analysis of DNA using a photometric assay

2.9.1.1 NanoDrop

To quantify the amount of DNA from the DNA extractions a Nano Drop spectrophotometer was used. First, it was blanked with 1 µL nuclease-free water. Second, 1 µL of the extracted nucleic acid was measured at 260 nm.

2.9.1.2 Qubit™ 2.0 Fluorometer

To quantify small amounts of DNA the Qubit™ dsDNA HS Assay Kit was used.

Protocol: Before measurement all the solutions were equilibrated to room temperature. For preparation of the working solution, the Qubit™ dsDNA HS reagent was diluted 1 : 200 with the Qubi™ dsDNA HS buffer. For the calibration two standards were prepared. In each tube 190 µL of working solution was pipetted and 10 µL of each Qubit™ standard. Carefully the standards were vortexed without creating bubbles. For the samples 199 µL working solution was added to 1 µL sample and vortexed for 2 to 3 seconds. The standards and samples were incubated at room temperature for 2 minutes. On the screen of the Qubit 2.0 Fluorometer DNA was pressed and then dsDNA High Sensitivity. For a new calibration the first standard was measured and then the second standard. At last, the samples were measured.

For the calculation of concentration of the samples:

$$\text{QF value} \cdot \left(\frac{200}{1}\right) = x\,(\text{ng/mL})$$

2.9.2 Qualitative analysis of DNA by agarose gel electrophoresis

Gel electrophoresis was used to do a qualitative analysis of DNA. It is a method to separate e.g. DNA fragments based on their size, by applying an electric field to a gel matrix. This gel matrix provides a network to separate the fragments and through the electric field DNA fragments move due to their negative charge to a positive electrode. EtBr is used to detect DNA because it intercalates into nucleic acids and emits visible light after excitation with UV light.

Agarose was mixed with 1 × TAE buffer and then heated up in a microwave until it was completely dissolved. Before three drops of EtBr (dilution 1 : 40,

concentration (conc.) 0.025 %) were added, the gel had to be cooled under running water. Afterwards, the gel was mixed and poured into a gel tray containing a comb. After polymerization of the gel, the gel tray was transferred into a chamber which was filled up with 0.5 × TAE buffer until the gel was completely covered. The comb was removed and the DNA-ladder as well as the polymerase chain reaction (PCR)/quantitative PCR (qPCR) products mixed with loading dye in a ratio 5 : 1 were loaded. The electrophoresis was performed for 30 minutes at 120 V and then 15 minutes at 100 V to obtain a better resolution of the band patterns. At last, DNA bands were illuminated with a Gel Doc XR System.

2.10 IN VITRO AMPLIFICATION OF DNA FRAGMENTS BY POLYMERASE CHAIN REACTION (PCR)

Gene fragments of interest were amplified by polymerase chain reaction with gene specific primers. The PCR started by a single denaturation step, which was followed by $20 - 40$ temperature-cycles. These cycles consisted of three steps:

1. Denaturation:

   Hydrogen bonds between complementary bases of double stranded DNA templates are broken.

2. Primer annealing:

   Primer are oligonucleotides with a length between 15 - 25 nucleotides, which anneal to single stranded DNA. Each Primer has a specific annealing temperature, which depends on length and nucleotide composition.

3. Elongation:

   The DNA polymerase synthesizes a new complementary strand to the DNA template.

For all amplifications a negative control without DNA template was performed. As positive control for amplification of archaeal 16S rRNA and *amoA* gene fragments genomic DNA from *Nitrososphaera viennensis* was used. For bacterial 16S rRNA and *amoA* gene fragments genomic DNA from *Nitrosomonas europaea* was used. All primers are listed in Table 20 and solutions used for PCR in Table 5.

For all PCR reactions TP Gradient 96 Thermocycler (Biometra) was used.

Table 5: PCR solutions

PCR solutions	**Company**
Go Taq DNA Polymerase (5 units/µL)	Promega
Dream Taq DNA Polymerase (5 units/µL)	Thermo Scientific
Go Taq Flexi Buffer (5 ×)	Promega
Magnesium chloride ($MgCl_2$) (25 mM)	Promega
dNTPs (10 mM)	Fermentas
Bovine serum albumin (20 mg/mL)	Thermo Scientific

2.10.1 Amplification of *amoA* gene fragments from Archaea and Bacteria

The standard reaction mix and the PCR program for the amplification of *amoA* gene fragments from archaea and bacteria are listed in the Tables 6 and 7.

Table 6: *amoA* PCR reaction mix

Solutions	**Volume/reaction** (µL) [final conc.]
Go Taq DNA Polymerase (5 u/µL)	0.125
Go Taq Flexi Buffer (5 ×)	5 [1 ×]
$MgCl_2$ (25 mM)	2 [2 mM]
dNTPs (10 mM)	0.5 [0.2 mM]
BSA (20 mg/mL)	0.25 [0.2 mg/mL]
Forward primer (10 µM)	1.25 [0.5 µM]
Reverse Primer (10 µM)	1.25 [0.5 µM]
DNA template	1
PCR H_2O	13.625
Total	25

2.10.2 Amplification of amoA gene fragments from *Nitrososphaera viennensis* for qPCR standards

As DNA template genomic DNA from *Nitrososphaera viennensis* was used. The standard reaction mix and the PCR program are listed in the Tables 8 and 9.

Table 7: *amoA* PCR program

PCR step	**Temperature** [°C]	**Time**	**Number of cycles**
Denaturation	95	5 min	1
Denaturation	95	45 sec	
Primer annealing	55	45 sec	35
Elongation	72	45 sec	
Final elongation	72	10 min	1
Pause	4	-	1

Table 8: *amoA* PCR reaction mix for qPCR standards

Solutions	**Volume/reaction** (µL) [final conc.]
Go Taq DNA Polymerase (5 u/µL)	0.25
Go Taq Flexi Buffer (5 ×)	10 [1 ×]
$MgCl_2$ (25 mM)	4 [2 mM]
dNTPs (25 mM)	0.4 [0.2 mM]
BSA (20 mg/mL)	0.5 [0.2 mg/mL]
NV-LamoA-F(10 µM)	2.5 [0.5 µM]
NV-LamoA-R (10 µM)	2.5 [0.5 µM]
DNA template	2
PCR H_2O	27.85
Total	50

2.10.3 Amplification of 16S rRNA gene fragments from Archaea and Bacteria

This amplification protocol was used to prepare 16S rRNA qPCR standards from genomic DNA of *Nitrososphaera viennensis* for archaea and for bacteria from *Nitrosospira tenuis*.

The standard reaction mix for 16S rRNA gene fragments from archaea and bacteria and for 16S rRNA qPCR standards is listed in Table 10. The PCR program for 16S rRNA gene fragments from archaea and for 16S rRNA qPCR standards is listed in Table 11. The PCR program for bacterial 16S rRNA gene fragments is listed in Table 12.

Table 9: *amoA* PCR program for qPCR standards

PCR step	**Temperature** [°C]	**Time**	**Number of cycles**
Denaturation	95	5 min	1
Denaturation	95	45 sec	
Primer annealing	56	45 sec	30
Elongation	72	2 sec	
Final elongation	72	10 min	1
Pause	4	-	1

Table 10: 16S rRNA PCR reaction mix

Solutions	**Volume/reaction** (µL) [final conc.]
Go Taq DNA Polymerase (5 u/µL)	0.125
Go Taq Flexi Buffer (5 ×)	5 [1 ×]
$MgCl_2$ (25 mM)	2 [2 mM]
dNTPs (10 mM)	0.5 [0.2 mM]
BSA (20 mg/mL)	0.25 [0.2 mg/mL]
Forward primer (10 µM)	1.25 [0.5 µM]
Reverse Primer (10 µM)	1.25 [0.5 µM]
DNA template	1
PCR H_2O	13.625
Total	25

Table 11: 16S rRNA PCR program

PCR step	**Temperature** [°C]	**Time**	**Number of cycles**
Denaturation	95	5 min	1
Denaturation	95	30 sec	
Primer annealing	55	30 sec	35
Elongation	72	2 min	
Final elongation	72	10 min	1
Pause	4	-	1

Table 12: bacterial 16S rRNA PCR program

PCR step	**Temperature** [°C]	**Time**	**Number of cycles**
Denaturation	95	5 min	1
Denaturation	95	45 sec	
Primer annealing	55	45 sec	35
Elongation	72	45 sec	
Final elongation	72	10 min	1
Pause	4	-	1

2.11 IN VITRO AMPLIFICATION OF DNA FRAGMENTS BY QUANTITATIVE POLYMERASE CHAIN REACTION

Two different qPCR Master Mix were used: Sybr green Mix 2 × (Quiagen) and Go Taq Master Mix qPCR 2 × (Promega). Standards were prepared in duplicates or triplicates from 10^8 - 10^1 copies/µL. The standards for 16S rRNA genes were purified genomic amplicons (PCR primers: A109F, A1492, Eubac27F and 1492R) from *Nitrososphaera viennensis* for archaea and for bacteria from *Nitrosospira tenuis*. The standard for archaeal *amoA* genes were genomic amplicons (Primers: NV-LamoA-F and NV-LamoA-R (Alves, RJE, unpublished)) from *Nitrososphaera viennensis*.

This sign ↯ in the qPCR program represents the time point when the emitted fluorescence was detected.

2.11.1 *amoA* - Archaea

The standard reaction mix and the qPCR program for amplification of archaeal *amoA* gene fragments are listed in Tables 13 and 14.

Table 13: archaeal *amoA* qPCR reaction mix

Solutions	**Volume/reaction** (µL) [final conc.]
PCR H_2O	3,8
GoTaq qPCR Mix (2 ×)	10 [1 ×]
19F (10 µM)	2 [1 µM]
TamoA632R-4 (10 µM)	2 [1 µM]
BSA (20 mg/mL)	0.2 [0.2 mg/mL]
DNA template	2
Total	20

2.11.2 16S rRNA Archaea

The standard reaction mix and the qPCR programs for amplification of archaeal 16S rRNA gene fragments are listed in Tables 15, 16 and 17.

2.11.3 16S rRNA Bacteria

The standard reaction mix and the qPCR program for amplification of bacterial 16S rRNA gene fragments are listed in Tables 18 and 19.

Table 14: archaeal *amoA* qPCR program

PCR step	**Temperature** [°C]	**Time**	**Number of cycles**
Denaturation	95	10 min	1
Denaturation	95	15 sec	
Primer annealing	58	45 sec	40
Elongation	60	45 sec	
	78	10 sec	↯
Melting curve	95	15 sec	
	60	15 sec	1
		20 min	↯
	95	15 sec	
Final elongation	60	10 min	1

Table 15: archaeal 16S rRNA qPCR reaction mix

Solutions	**Volume/reaction** (µL) [final conc.]
PCR H_2O	4,6
qPCR Mix (2 ×)	10 [1 ×]
Cren-771F (10 µM)	1.6 [0.8 µM]
Cren-957R (10 µM)	1.6 [0.8 µM]
BSA (20 mg/mL)	0.2 [0.2 mg/mL]
DNA template	2
Total	20

Table 16: archaeal 16S rRNA qPCR program (Quiagen qPCR Mix)

PCR step	**Temperature** [°C]	**Time**	**Number of cycles**
Denaturation	95	15 min	1
Denaturation	95	30 sec	
Primer annealing	54	30 sec	40
Elongation	72	30 sec	↯
Melting curve	95	15 sec	
	60	15 sec	1
		20 min	↯
	95	15 sec	
Final elongation	72	5 min	1

Table 17: archaeal 16S rRNA qPCR program (Promega qPCR Mix)

PCR step	**Temperature** [°C]	**Time**	**Number of cycles**
Denaturation	95	10 min	1
Denaturation	95	15 sec	
Primer annealing	54	30 sec	40
Elongation	60	30 sec	
	78	10 sec	↯
Melting curve	95	15 sec	
	60	15 sec	1
		20 min	↯
	95	15 sec	
Final elongation	60	5 min	1

Table 18: bacterial 16S rRNA qPCR reaction mix

Solutions	**Volume/reaction** (µL) [final conc.]
PCR H_2O	5.8
Sybr Mix (2 ×)	10 [1 ×]
P2 (10 µM)	1 [0.5 µM]
P3 (10 µM)	1 [0.5 µM]
BSA (20 mg/mL)	0.2 [0.2 mg/mL]
DNA template	2
Total	20

Table 19: bacterial 16S rRNA qPCR program

PCR step	**Temperature** [°C]	**Time**	**Number of cycles**
Denaturation	95	15 min	1
Denaturation	95	15 sec	
Primer annealing	55	45 sec	40
Elongation	72	40 sec	↯
Melting curve	95	15 sec	
	60	16 sec	1
		20 min	↯
	95	15 sec	
Final elongation	72	5 min	1

2.12 SEQUENCING OF 16S RRNA GENES

From eight different cultures, two stemmed from Tundra fen soil, four from Frost boil soil and two from Moss tundra soil, purified archaeal 16S rRNA PCR amplicons were sequenced. All cultures had reached the stationary phase, when the DNA sample was taken. 16S rRNA PCR (Primer: 109F and 1492R) in triplicates and for qualitative analysis a 1.5 % gel was performed. The PCR products were combined and cleaned with NucleoSpin Extract II PCR Clean up (Macherey-Nagel GmbH and Co. KG). The amount of DNA was measured with NanoDrop. For each culture 10 µL with either 5 µM forward (109F) or reverse (1492R) primer were prepared and sent to LGC Genomics (LGC 2013).

2.13 LIGHT-MICROSCOPY

AOA enrichment cultures were visually investigated by phase contrast - microscopy using the Eclipse 50i Microscope (Nikon). Pictures were taken using the program NIS-Elements F 3.2. For cultures in the lag phase 300 µL was taken and centrifuged for 20 minutes at full speed. Next, 270 µL supernatant were removed and the cell pellet was resuspended in the remaining medium. When the cultures were in the stationary phase 30 µL were taken and directly investigated. 10 µL of the samples were pipetted onto a glass slide and a cover slip was placed on top. Next, a drop of immersion oil was pipetted onto the cover slip and the samples were investigated with 1000 × magnification.

2.14 FLUORESCENCE IN SITU HYBRIDISATION (FISH)

All centrifugation steps were done at 4°C and at maximum speed (13, 200 rpm). An optimal formamide concentration of 30 % (pers. comm. Stefanie Aiglsdorfer) was used for all probes.

2.14.1 Cell fixation

In order to find the optimal cell fixation protocol for the enrichment cultures we compared two different methods, using either ethanol or paraformaldehyde to fix the cells. After cell fixation the samples were stored at 4°C.

500 µL enrichment culture was harvested and centrifuged for 40 minutes. The supernatant was discarded and the cell pellet was washed with 1 mL 1× PBS. After that, the sample was centrifuged for 15 minutes and the supernatant was discarded.

EtOH-Fixation:

The cells were dissolved in 45 µL 1× PBS and 45 µL ice cold $EtOH_{abs}$ and incubated on ice for two hours.

Paraformaldehyde(PFA)-Fixation:

The cells were re-suspended in 50 µL 1× PBS and 150 µL PFA 4 % was added to the sample. Next, samples were incubated on ice for two hours and centrifuged for 15 minutes. The supernatant was discarded and the sample was washed with 1 mL 1× PBS and again centrifuged for 15 minutes. At last, cells were dissolved in 45 µL 1× PBS and 45 µL ice cold $EtOH_{abs}$.

2.14.2 Slide preparation

All slides had to be washed with alcohol solution before they were coated with Poly-L-lysine (0.01 %) for at least 5 minutes. Then they were dried for 1 hour at 60°C.

2.14.3 *In situ* hybridisation

Three different oligonucleotide probes were used and are described in Table 21. For the probes working solutions were prepared and were stored in the dark at −20°C as well as stock solutions.

For immobilisation of the cells 1 to 20 µL of fixated cells were pipetted onto the coated glass slides and dried.

An ethanol series with increasing percentage was performed for dehydration of the cells. Slides were placed one after the other into 50 %, 80 % and 96 % ethanol for 3 minutes. At last, the glass slides were air dried.

Probe hybridization was done at 46°C over night in a hybridization oven. Proper stringency was achieved by adding sodium chloride to the washing buffer, because positive sodium ions stabilize the negative nucleic acid duplex. Formamide was added to the hybridization buffer, which positively affects reaction stringency, by destabilizing hydrogen bonds between nucleic acids.

Lysozyme treatment:

20 µL 1 × PBS was pipetted on each well with dried sample for 1 minute and was then removed. Next, 20 µL lysozyme (5 ng/50 mL) was put on the samples, incubated for 10 minutes and was then removed. Then, 20 µL 1 × PBS was pipetted on each well, incubated for 1 minute and was removed. At last, samples were dried at 37°C for 1 minute.

After this, 10 µL hybridisation buffer and 1 µL fluorescence-labelled probes were pipetted on the samples and gently mixed without scratching the surface.

Next, the slide was placed into a 50 mL falcon tube containing a paper sheet soaked in remaining hybridisation buffer. The tubes were closed and slides were incubated in the dark, over night (16 hours) at 46°C in the hybridisation oven. After hybridisation, the slides were placed into 50 mL falcon tubes with washing buffer (pre-heated to 48°C) for 10 minutes in a 48°C warm water bath. Then once dipped into ice cold MilliQ and immediately dried in an air stream.

2.14.4 4'-6'-di-amidino-2-phenylindole (DAPI) staining

Afterwards, the samples were stained with $4' - 6'$—di-amidino-2-phenylindole (DAPI), which binds to double stranded DNA. DAPI stock solution (5 mg/mL) was diluted 1 : 1000. Then 20 µL DAPI (1 : 1000) was pipetted on the samples and incubated for 10 minutes. After removing the DAPI, samples were washed with 20 µL 1 × PBS for 5 minutes. PBS was removed and the slides were dried in the dark. At last, some drops of Vectashield were put around the wells onto the slide and a cover slip was placed on top.

2.14.5 Fluorescence microscopy

Fluorescence microscopy was done using Eclipse 50i Microscope and the software NIS-Elements F 3.2. A drop of immersion oil was pipetted onto the cover slips and the samples were investigated with 1000 × magnification.

2.15 PHYLOGENY

16S rRNA gene sequences obtained from the different cultures were analysed and processed using the programs BioEdit v7.0.9 (Hall 1999), MEGA 5.2 (Tamura et al. 2011) and ATGC PhyML 3.0 (Guindon et al.2010).

First forward and reverse sequences were aligned against a reference sequence and then against each other. Then the sequences were cut with BioEdit and combined. 16S rRNA gene sequences from the different cultures were analysed together with the 16S rRNA gene sequences from the clone library of the N_2O experiment, which was done by my co-supervisor Ricardo J. Eloy Alves (Alves et al. 2013). Second, they were aligned with MUSCLE using MEGA 5.2. A sequence identity matrix was performed using BioEdit v7.0.9.

Due to the fact that the sequences were very similar, one representative was chosen and combined with 16S rRNA gene sequences from different AOA clusters (GenBank), which enable a proper classification. Next, 16S rRNA gene sequences were aligned with MUSCLE. To find the best DNA model and to calculate a Maxi-

mum likelihood tree, a model test was performed using MEGA 5.2. At last, a Maximum likelihood tree including 100 bootstrap support values was calculated with GTR model (General Time Reversible) (Tavaré 1986) with Gamma-distributed site rate variation and invariable sites using ATGC PhyML 3.0 (Guindon et al. 2010).

Table 20: Primers

Primer name	Sequence ($5' - 3'$)	T[°C]	Specificity	Reference
P2	ATT ACC GCG GCT GCT GG	55	**most bacteria**	Muyzer et al. 1993
P3	CCT ACG GGA GGC AGC AG			
Eubac27F	AGA GTT TGA TCC TGG CTC AG	55	**most bacteria**	Weisburg et al. 1991
1492R	GGT TAC CTT GTT ACG ACT T			
A109F	ACK GCT CAG TAA CAC GT	55	**most archaea**	Großkopf et al. 1998
A1492R	GYY ACC TTG TTA CGA CTT			Nicol et al. 2008
Cren-771F	ACG GTG AGG GAT GAA AGC T	54	**most archaea**	Ochsenreiter et al. 2003
Cren-957R	CGG CGT TGA CTC CAA TTG			
amoA1F	GGG GTT TCT ACT GGT GGT	55	**most AOB**	Rotthauwe et al. 1997
amoA2R	CCC CTC KGS AAA GCC TTC TTC			
Arch-amoA-7F-1	ATG GTC TGG BTD AGA CG	55	**most AOA**	Alves et al. 2013
Arch-amoA-638-R	GCR GCC ATC CAT CTR TA			
Cren-amoA-19F	ATG GTC TGG YTW AGA CG	55	**most AOA**	Tourna et al. 2008
Cren-amoA-616R	GCC ATC CAT CTG TAT GTC CA			
NV-LamoA-F	CGC ATG ATC GGC CGC AGA GT	56	**most AOA**	Alves, RJE, unpub.
NV-LamoA-R	GCC TAG TAG CGA CCC GCC CT			

Table 21: Oligonucleotide probes used for FISH

Probe name	**Sequence** $5' - 3'$	**[FA]** %	**Binding position**	**Specificity**	**Reference**
EUB338	GCT GCC TCC CGT AGG AGT	30	338 – 355	*most Bacteria*	Amann et al. 1990
EUB338II	GCA GCC ACC CGT AGG TGT	30	338 – 355	*Planctomycetales*	Daims et al. 1999
EUB338III	GCT GCC ACC CGT AGG TGT	30	338 – 355	*Verrucomicrobiales*	Daims et al. 1999
ARCH915	GTG CTC CCC CGC CAA TTC CT	30	915 – 934	*Archaea*	Stahl and Amann 1991

3

RESULTS

3.1 ENRICHMENT CULTURES OF AMMONIA OXIDIZING ARCHAEA

The starting point of this thesis was to continue AOA enrichment cultures, which my co-supervisor Ricardo J. Eloy Alves has initiated in 2009 (Alves et al. 2013). The enrichment cultures had been transferred 11 times since their initial inoculation. The standard conditions for the enrichment cultures were: 20 mM culture volume, 16 mL FWM (Tourna et al. 2011), 4 mL inoculum (20 %, v/v) and an initial ammonium concentration of 0.5 mM. For the inhibition of bacterial contaminants streptomycin 100 µg/mL was used as antibiotic. The enrichment cultures were incubated at 20 °C in the dark in a refrigerated incubator. The cultures were incubated until all ammonia was converted to nitrite. Culture growth was followed by measuring nitrite concentrations.

In the frame of my work over 400 enrichment cultures were grown. In most cultures near stoichiometric conversion of ammonia to nitrite was observed (Figure 5). Ammonium and nitrite concentrations were measured every 5 to 60 days depending on their growth. Dependent on the inoculum, initial NH_4^+ and NO_2^- concentrations varied between AOA enrichment cultures at day 0. In this work the abbreviation S-EC stands for standard enrichment conditions. S-EC conditions were: 20 % (v/v) inoculum, 80 % (v/v) FWM, 0.5 mM ammonium as energy source calculated for total volume, 100 µg/mL streptomycin, pH ~ 7 and 20 mL total culture volume in a 30 mL flask. On average S-EC cultures needed 128 days to reach the stationary phase and the average of the generation time was 34 days.

Statistical analysis was performed for culture treatments where we had enough replica. For all these enrichment cultures the NO_2^- production rate (NO_2^- mM per day) in the exponential phase was calculated as an approximation for growth. All phases of growth mentioned in this work are related to the nitrite production. These enrichment cultures were statistically analysed with One Way Analysis of variance (ANOVA) using SigmaPlot version 11.0. Holm Sidak was additionally performed as post hoc test. The Figures for the statistically analysed cultures contain only selective representatives for the different treatments. Further, the different letters in the figures indicate a significant difference in NO_2^- production in the exponential phase between treatments ($p \leqslant 0.05$).

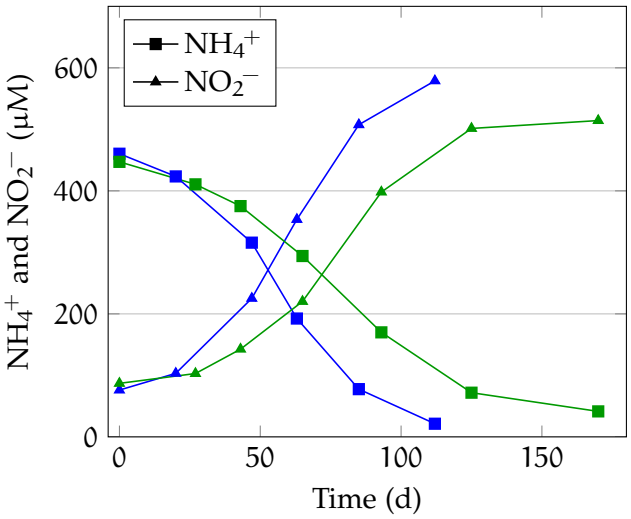


Figure 5: Ammonia consumption and nitrite production of S-EC cultures: Different line colours represent cultures from different inocula, which were grown under standard conditions.

The preliminary name for the studied ammonia oxidizing archaeon is *Ca. Nitrosopila arctica* ("the ball shaped nitrifier from the Arctic").

3.1.1 Enrichment cultures of different Arctic soils

Enrichment cultures from three different Arctic soils: Frost boil, Moss tundra and Tundra fen were compared. There was no statistically significant difference (p value = 0.413) in NO_2^- production between the different cultures (Table 24 in Appendix A.1). This was the first hint that in all enrichment cultures from these soils the same ammonia oxidizing archaea were growing. This was confirmed by sequencing amplified 16S rRNA genes of different enrichment cultures from the three soils. These 16S rRNA sequences showed a 99.6 % nucleotide identity (see section 3.4). Therefore, for all other statistical analysis, enrichment cultures from these soils were treated as replica.

3.1.2 Influence of different culture volumes on nitrite production

To obtain more biomass of *N. arctica* we inoculated cultures with larger volumes (20 mL, 40 mL and 100 mL). There was a significant difference in NO_2^- production between total culture volume 20 mL and 40 mL at two ammonium concentrations (0.5 mM (p value = 0.019) and 1 mM ammonium (p value = 0.004)). Enrichment cultures with 20 mL culture volume had a significantly faster NO_2^- production rate. Furthermore, a significant difference (p value = 0.021) was detected between total culture volume 40 mL and 100 mL with 1 mM ammonium (Table 25 in Ap-

pendix A.1). 100 mL culture volumes with 1 mM ammonium had a significantly faster NO_2^- production rate. There was no significant difference (p value = 0.736) between 20 mL and 100 mL total culture volume with 1 mM ammonium.

The NO_2^- production rate of standard enrichment cultures (20 mL) was significantly faster (6.012 µM/day) as for cultures with 40 mL total volume (4.369 µM/day). The lag phase of the cultures with 40 mL total volume was up to 60 days longer than from the standard cultures (20 mL). Further, more nitrite was produced by the standard cultures. On average standard cultures needed 124 days to reach stationary phase, whereas cultures with 40 mL culture volume needed 153 days (Figure 6).

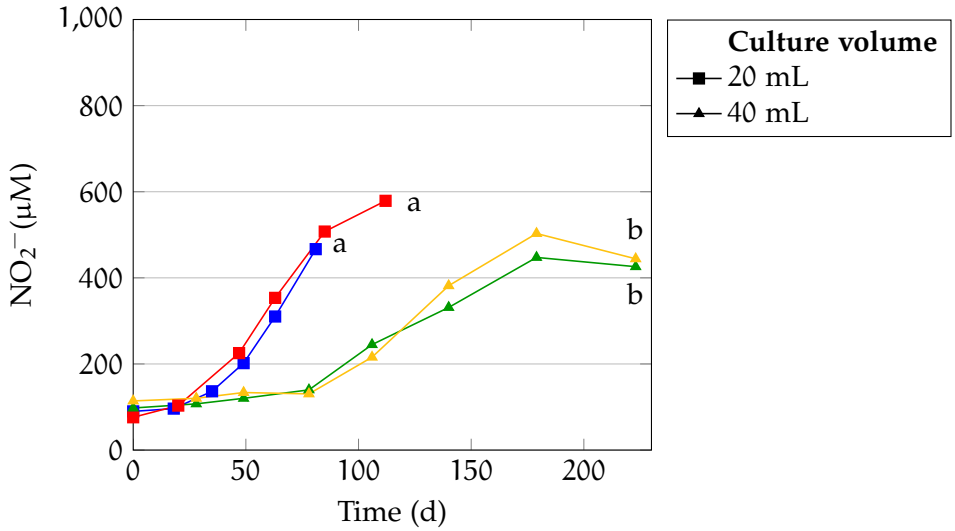


Figure 6: Growth of *N. arctica* with 0.5 mM ammonium comparing 20 mL and 40 mL culture volumes: Different line colours represent cultures from different inocula. All inocula were grown under standard conditions. The different letters indicate a significant difference in the exponential phase between treatments ($p \leqslant 0.05$).

There was a significant difference (p value = 0.004) when we compared NO_2^- production of enrichment cultures with 20 mL and 40 mL total volume, with 1 mM ammonium. The NO_2^- production rate was 7.886 µM NO_2^-/day for S-EC cultures (20 mL) and 4.817 µM/day for cultures with 40 mL total volume. We observed a longer lag phase for the cultures with 40 mL culture volume. Further, enrichment cultures with 40 mL culture volume never produced as much nitrite as cultures with 20 mL volume. To reach the stationary phase cultures with 20 mL volume needed on average 137 days and cultures with 40 mL culture volume 200 days (Figure 7).

There was no significant difference (p value = 0.431) observed between the culture volumes 20 mL and 40 mL with 0.25 mM urea as sole energy source. The NO_2^- production rate was 6.798 µM/day for 20 mL cultures and 6.301 µM/day for 40 mL with 0.25 mM urea (Table 25). Cultures with 20 mL volume needed on

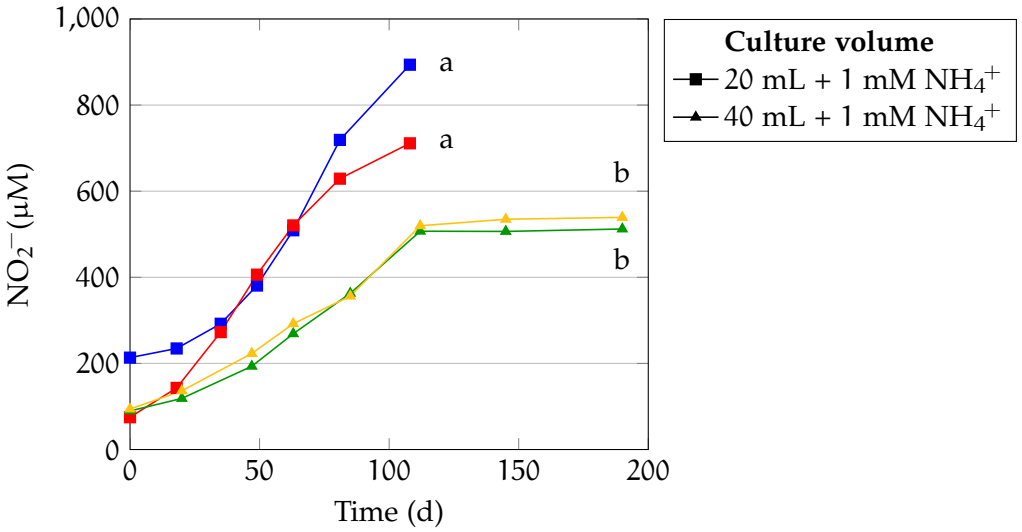


Figure 7: Growth of *N. arctica* with 1 mM ammonium comparing 20 mL and 40 mL culture volumes: Different line colours represent cultures from different inocula. Inoculum culture conditions were: standard conditions (yellow and green), additionally yeast extract (0.01 %) (blue) and additionally ampicillin (100 µg/mL) and lyzosyme treatment (red). The different letters indicate a significant difference in the exponential phase between treatments ($p \leqslant 0.05$).

average 158 days and cultures with 40 mL volume 160 days to reach the stationary phase.

NO_2^- production rates were significantly different (p value = 0.021) between enrichment cultures with 40 mL and 100 mL total culture volumes, with 1 mM ammonium. The NO_2^- production rate was 4.817 µM/day for enrichment cultures with 40 mL and 7.444 µM/day for 100 mL volume. The lag phases of the cultures with 40 mL and 100 mL total culture volume were similar (Figure 8).

No significant difference (p value = 0.736) in NO_2^- production between 20 mL and 100 mL cultures was observed with 1 mM ammonium (Table 25). The NO_2^- production rate was 7.886 µM/day for enrichment cultures with 20 mL and 7.444 µM/day for 100 mL volume. However, the lag phase of the cultures with 100 mL volume was much longer. Enrichment cultures with 100 mL volume needed on average 191 days to reach the stationary phase, whereas cultures with 20 mL volume needed only 137 days.

In summary cultures with 20 mL and 100 mL total volume had a significantly faster NO_2^- production than cultures with 40 mL volume, with ammonium as energy source.

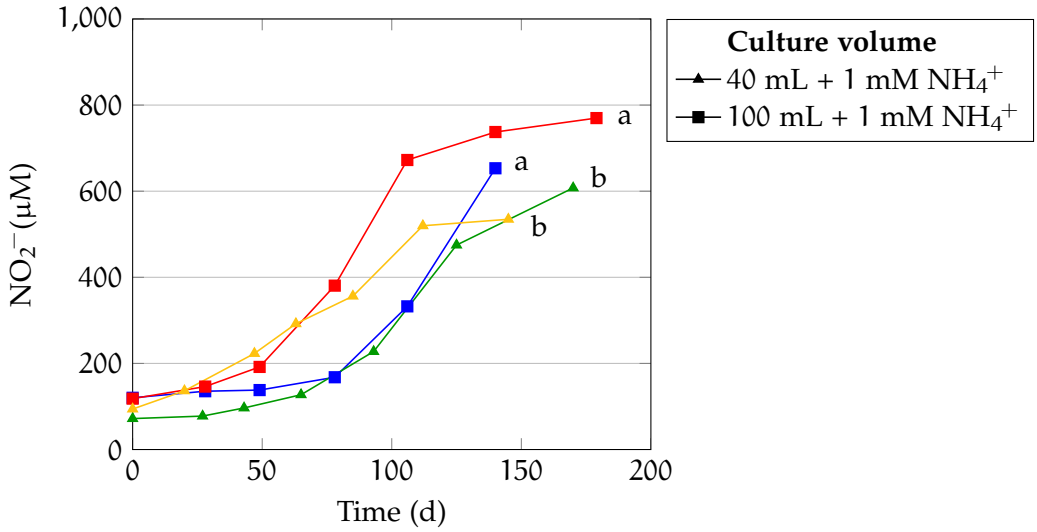


Figure 8: Growth of *N. arctica* with 1 mM ammonium comparing 40 mL and 100 mL culture volumes: Different line colours represent cultures from different inocula. Inoculum culture conditions were as following: 1 mM ammonium in 40 mL (red and blue), standard conditions (yellow) and additional 0.5 mM pyruvate (green). The different letters indicate a significant difference in the exponential phase between treatments ($p \leqslant 0.05$).

3.1.3 Influence of different antibiotics on nitrite production

Growth of enrichment cultures was tested in the presence of 100 µg/mL ampicillin, carbenicillin and kanamycin.

NO_2^- production of enrichment cultures supplemented with different antibiotics were statistically analysed. No significant difference (p value = 0.138) in NO_2^- production rate was detected between standard enrichment cultures (6.012 µM/day) and cultures with 100 µg/mL ampicillin (4.467 µM/day) (Table 26 in Appendix A.1). The same result was obtained with cultures supplemented with 50 µg/mL kanamycin (p value = 0.054) (Table 27 in Appendix A.1). However, we observed that enrichment cultures with 50 µg/mL kanamycin had an extended lag phase of around 50 days and needed on average 222 days to reach the stationary phase. The NO_2^- production rate in the exponential phase was 6.012 µM/day for S-EC cultures and 4.336 µM/day for cultures with 50 µg/mL kanamycin (Figure 9).

A significant difference (p value = 0.006) was observed in cultures with 100 µg/mL carbenicillin (Table 26 in Appendix A.1). These cultures had a significantly lower NO_2^- production than S-EC cultures (Table 26). The NO_2^- production rate was 6.012 µM/day for S-EC cultures and 3.102 µM/day for cultures with 100 µg/mL carbenicillin. Furthermore, we observed in these cultures an extension of the lag phase, they needed in average 165 days to reach the stationary phase.

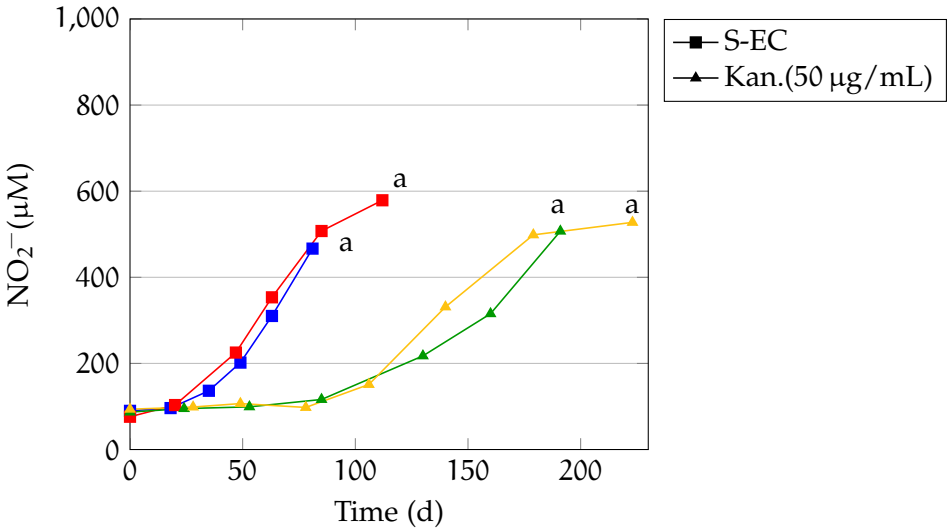


Figure 9: Influence of 50 µg/mL kanamycin on growth of *N. arctica* compared to S-EC: Different line colours represent cultures from different inocula. Inocula growth conditions were: standard condition (blue and red), 100 µg/mL kanamycin (yellow) and 50 µg/mL kanamycin (green). The different letters indicate a significant difference in the exponential phase between treatments ($p \leqslant 0.05$).

In comparison standard enrichment cultures reached the stationary phase on average in 137 days (Figure 10).

A significant difference (p value = 0.016) was observed between S-EC cultures and cultures with 100 µg/mL kanamycin (Table 27 in Appendix A.1). In the enrichment cultures with 100 µg/mL kanamycin we observed a small extension of the lag phase (Figure 11). These enrichment cultures had a significantly lower NO_2^- production than S-EC cultures. The NO_2^- production rate was 6.012 µM/day for S-EC cultures and 4.166 µM/day for cultures with 100 µg/mL kanamycin (Table 27 in Appendix A.1).

We did not observe a significant difference (p value = 0.316) in nitrite production between S-EC cultures with 50 µg/mL kanamycin compared to cultures with 0.25 mM urea and 50 µg/mL kanamycin. The NO_2^- production rate was 4.446 µM/day for S-EC cultures with 50 µg/mL kanamycin and 5.537 µM/day for cultures with 0.25 mM urea and 50 µg/mL kanamycin (Table 28 in Appendix A.1). However, enrichment cultures with urea needed on average 152 days to reach the stationary phase, whereas S-EC cultures with 50 µg/mL kanamycin needed 222 days.

Furthermore, there was no significant difference (p value = 0.074) in the nitrite production rate between enrichment cultures with 0.25 mM urea (6.798 µM/day) and cultures with 0.25 mM urea and 50 µg/mL kanamycin (5.537 µM/day). Same result was observed by comparing enrichment cultures with 0.25 mM urea and

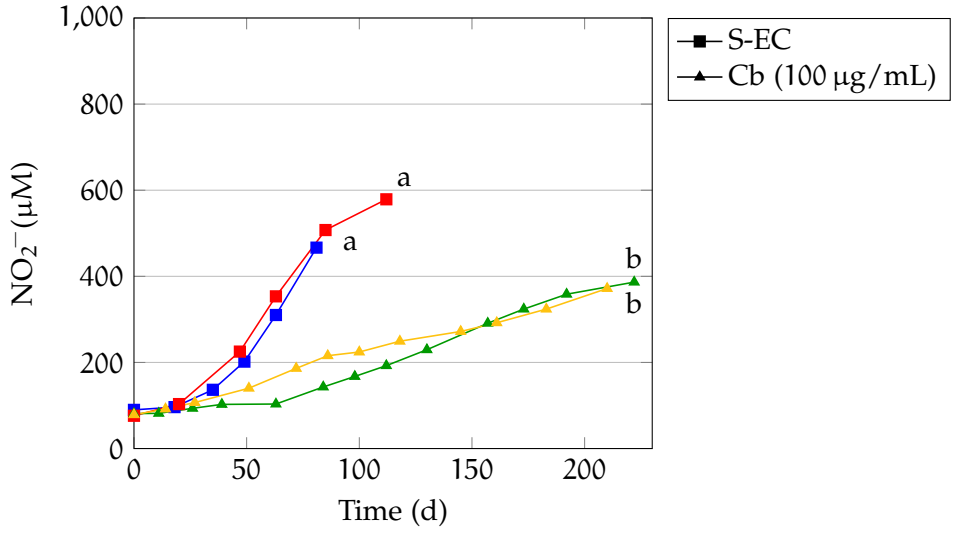


Figure 10: Influence of 100 µg/mL carbenicillin on growth of *N. arctica* compared to S-EC: Different line colours represent cultures from different inocula. Inocula growth conditions were: standard condition (blue, red and yellow) and 100 µg/mL carbenicillin and 0.5 mM pyruvate (green). The different letters indicate a significant difference in the exponential phase between treatments ($p \leqslant 0.05$).

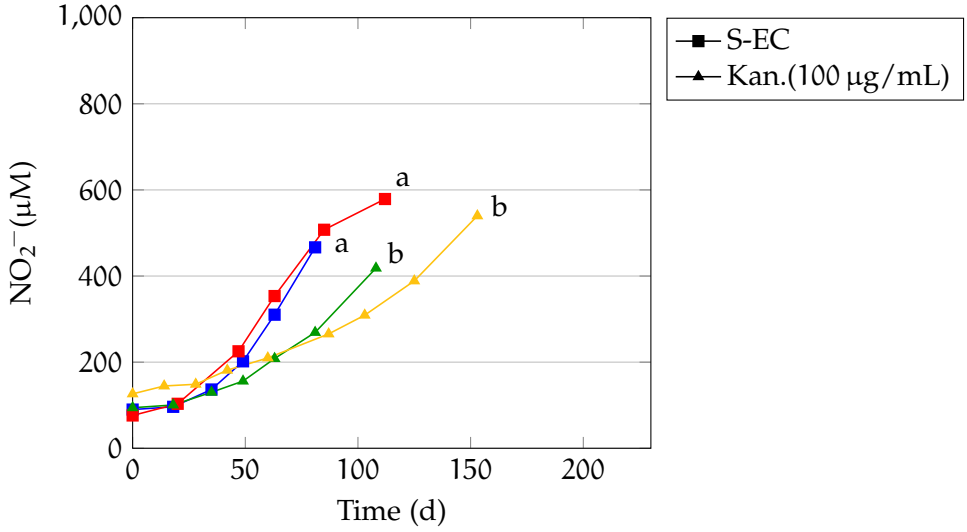


Figure 11: Influence of 100 µg/mL kanamycin on growth of *N. arctica* compared to S-EC: Different line colours represent cultures from different inocula. All inocula had grown under standard conditions. The different letters indicate a significant difference in the exponential phase between treatments ($p \leqslant 0.05$).

50 µg/mL kanamycin growing with 20 mL (5.537 µM/day) volume or 40 mL (6.301 µM/day) total culture volume(p value = 0.245) (Table 28 in Appendix A.1).

To sum up, only enrichment cultures with 100 µg/mL carbenicillin or kanamycin have shown a significantly lower NO_2^- production in the exponential phase.

3.1.4 Influence of different ammonium concentrations on ammonia oxidation

Enrichment cultures with different ammonium concentrations 0.5 mM (S-EC), 1 mM and 10 mM were performed. NO_2^- production rates in the exponential phase were calculated and statistically analysed.

In enrichment cultures with 1 mM ammonium we observed a significantly faster NO_2^- production than at 0.5 mM (p value = 0.047). The NO_2^- production rate was 6.012 µM/day for S-EC cultures and 7.886 µM/day for cultures with 1 mM ammonium (Table 29 in Appendix A.1). In some enrichment cultures with 1 mM ammonium we noticed an extension of the lag phase (Figure 12).

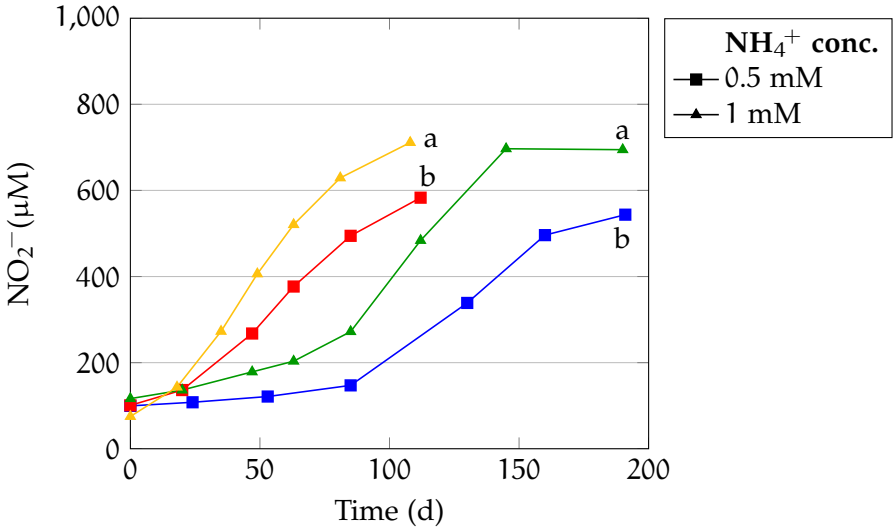


Figure 12: Growth of *N. arctica* at standard condition 0.5 mM compared to 1 mM ammonium: Different line colours represent cultures stemming from different inocula. Inoculum culture conditions were: standard conditions (blue, red and green) and yellow had additionally ampicillin (100 µg/mL) and lyzosyme treatment. The different letters indicate a significant difference in the exponential phase between treatments ($p \leqslant 0.05$).

No significant difference (p value = 0.556) was determined in NO_2^- production rates between standard enrichment cultures (6.012 µM/day) and cultures with 10 mM ammonium (5.321 µM/day). The same result was obtained (p value

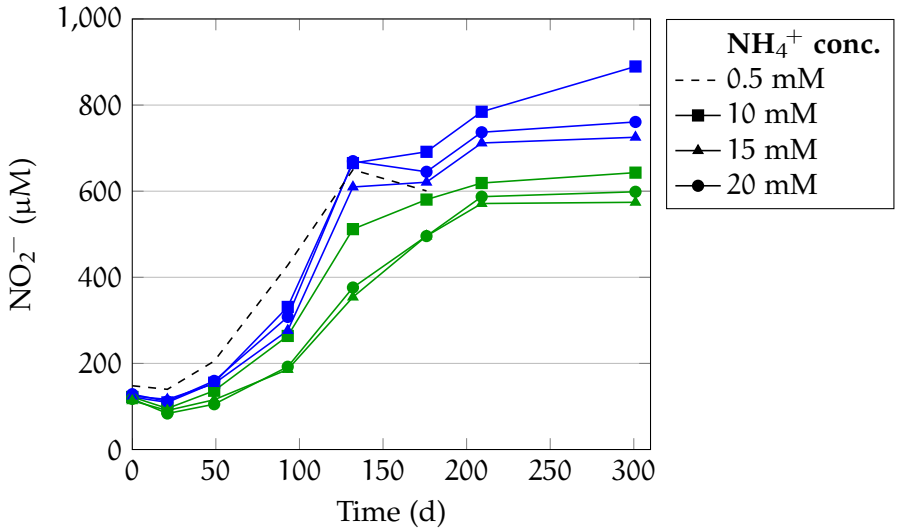


Figure 13: Growth of *N. arctica* at high ammonium concentrations: The lines with the same colours represent cultures from the same inoculum, which were pre-grown under standard conditions (0.5 mM NH_4^+). Dashed line: standard culture (S-EC) grown under standard conditions.

= 0.312) when we compared S-EC cultures (6.012 µM/day) with 0.25 mM urea (6.798 µM/day). Further, no significant difference (p value = 0.075) was observed between 1 mM (7.886 µM/day) and 10 mM ammonium (5.321 µM/day) (Table 29 in Appendix A.1).

An experiment with biological duplicates at three different ammonium concentrations (10 mM, 15 mM and 20 mM) was performed. It was not possible to perform a statistic analysis, due to too few cultures. Growth was observed for all tested ammonium concentrations. However, nitrite was only produced up to 900 µM maximum, although more substrate was available (Figure 13). The NO_2^- production rate on average was 6.012 µM/day for S-EC cultures, 7.464 µM/day for cultures with 10 mM ammonium, 6.996 µM/day for 15 mM ammonium and 6.427 µM/day for cultures with 20 mM ammonium. The S-EC culture with 0.5 mM ammonium had a lag phase of 49 days. We observed an extension of the lag phase of up to 44 days in the cultures with 10 mM to 20 mM ammonium. At day 132 the S-EC culture reached the stationary phase, whereas the cultures with the high ammonium concentrations reached the stationary phase between 132 and 200 days.

Another observation was that the cultures with 10 mM ammonium produced more nitrite than the cultures with 15 mM and 20 mM ammonium. We noticed differences in nitrite production between the biological duplicates due to the different inocula.

Taken together we determined culture growth up to 20 mM ammonium and a nitrite production up to 900 µM.

3.1.5 Influence of different urea concentrations on ammonia oxidation

Growth on different concentrations of urea as energy source was tested in another experiment. It was not possible to perform a statistic analysis, due to too few cultures. The inoculum was pre-grown with 0.5 mM urea and 50 µg/mL kanamycin. Enrichment cultures were supplemented with five different urea concentrations 0.5 mM, 1 mM, 3 mM, 5 mM and 10 mM.

Cultures with 0.5 mM and 1 mM urea converted most of the ammonia to nitrite as well as the control culture. Further, we observed a shorter lag phase up to 36 days, in the cultures with 0.5 mM and 1 mM urea compared to the control culture (0.5 mM NH_4^+). The enrichment cultures with 3 mM, 5 mM and 10 mM urea stopped nitrite production between day 58 and 85 (Figure 14).

The highest NO_2^- production rate in the exponential phase was 16.099 µM/day for the culture with 1 mM urea. The culture with 0.5 mM urea had a nitrite production rate of 12.195 µM/day and the control culture 7.128 µM/day. The generation time in the exponential phase was 25 days for 1 mM urea, 29 days for 0.5 mM urea and 40 days for the control culture.

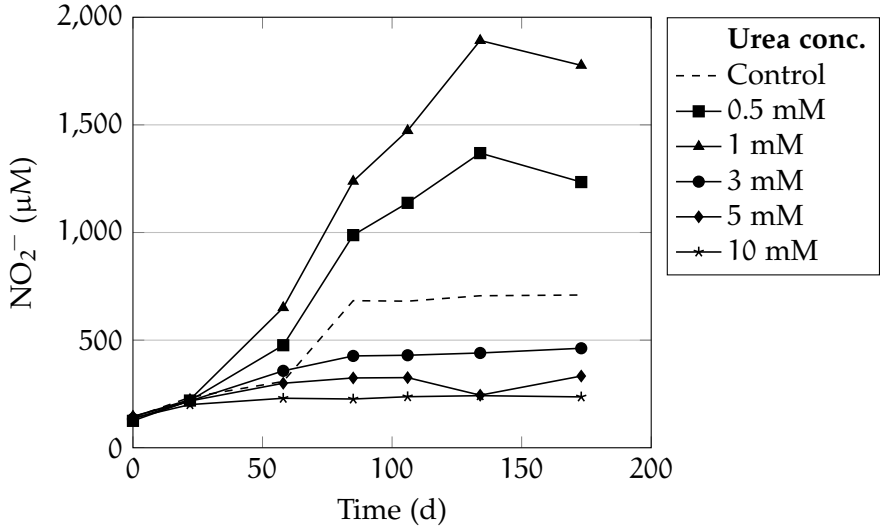


Figure 14: Growth of *N. arctica* at different urea concentrations: Growth at 0.5 mM and 1 mM urea was observed. All cultures stemmed from the same inoculum, which was grown with 0.5 mM urea and 50 µg/mL kanamycin. Dashed line: control culture grown under standard conditions (0.5 mM NH_4^+).

All enrichment cultures were started at a pH of approximately 7. However, the fresh water medium of the cultures in this experiment was not buffered. For this reason pH was measured at day 106 for all cultures. The pH for the control culture and the enrichment cultures with 0.5 mM and 1 mM urea was ~ 7. The pH for the enrichment cultures with 3 mM, 5 mM and 10 mM urea was between 8 - 9.

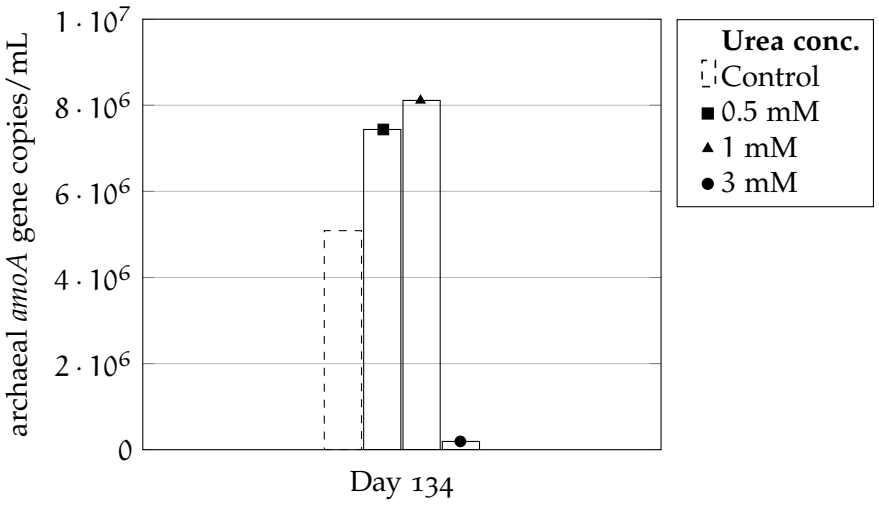


Figure 15: qPCR of archaeal *amoA* genes of enrichment cultures with different urea concentrations: All enrichment cultures stemmed from the same inoculum, which was grown with 0.5 mM urea and 50 µg/mL kanamycin. Dashed bar: represents control culture grown under standard conditions (0.5 mM NH_4).

At day 134, 1 mL of each culture, except for the cultures with 5 mM and 10 mM urea, was sampled to perform DNA extraction and quantitative PCR. In Figure 15 the results of quantitative PCR of archaeal *amoA* genes can be seen. The enrichment culture with 1 mM urea had the highest archaeal *amoA* gene copy number per mL. Further, the enrichment culture with 3 mM urea had the lowest archaeal *amoA* gene copy number. Our qPCR results confirm what can be inferred from the nitrite production of the cultures (Figure 14). However, there is not much difference in the archaeal *amoA* gene copy number between the cultures with 0.5 mM and 1 mM urea. We observed a stimulation of urea on *amoA* gene copies compared to the control culture.

3.1.6 Influence of organic carbon sources on ammonia oxidation

The effect of two different organic carbon sources, pyruvate and glyoxylate, were tested on growth of *N. arctica*. There was no significant difference in NO_2^- production with or without additional 0.5 mM pyruvate (p value = 0.559) or 0.1 mM glyoxylate (p value = 0.439) detectable. The NO_2^- production rate in the exponential phase was 6.012 µM/day for S-EC cultures, 5.304 µM/day for cultures with 0.1 mM sodium glyoxylate and 6.620 µM/day for cultures with 0.5 mM pyruvate (Table 30 in Appendix A.1).

An experiment was performed where we compared biological duplicates of S-EC with biological duplicates of enrichment cultures with additional 0.25 mM sodium glyoxylate. All cultures stemmed from the same inoculum, which was grown with 0.1 mM sodium glyoxylate in 40 mL culture volume. Due to too few

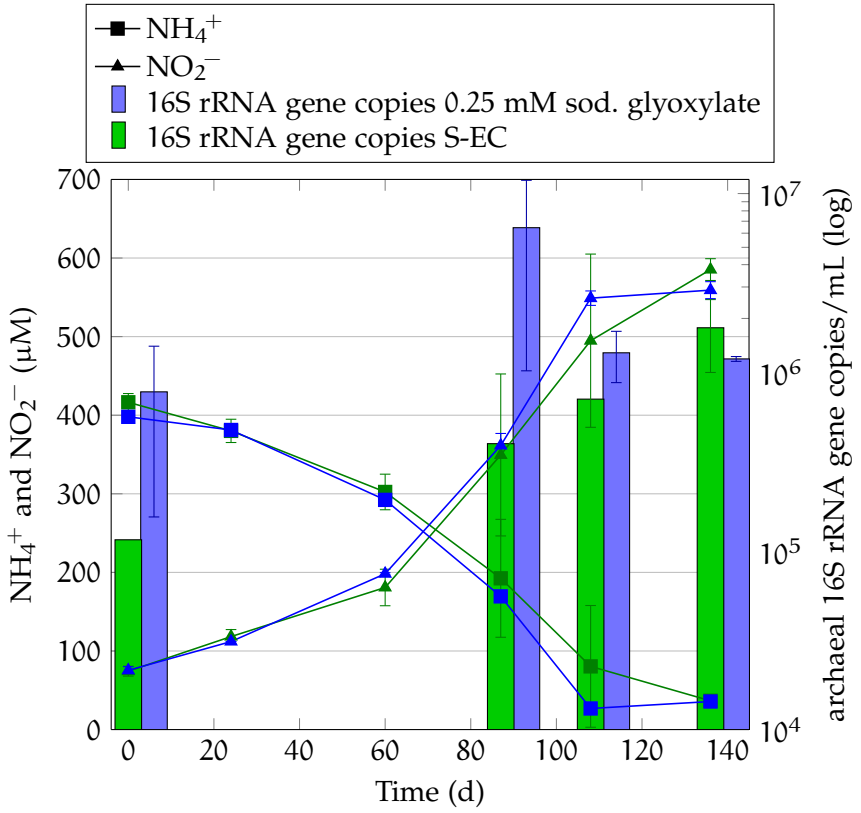


Figure 16: Average with standard deviation of ammonia consumption and nitrite production of S-EC replicates (green) and replicates with 0.25 mM sodium glyoxylate (blue), with archaeal 16S rRNA gene copies from four different time points 0, 87, 108 and 136 (bars).

cultures, it was not possible to perform a statistic analysis. The growth of the cultures were followed by measuring nitrite production and archaeal 16S rRNA gene copies (Figure 16).

The lag phase was 60 days long in all cultures. However, the cultures with additional glyoxylate reached the stationary phase of NO_2^- production at day 108, whereas the control cultures did not reach the stationary phase at day 136 (Figure 16). On average the NO_2^- production rate in the exponential phase was for the S-EC duplicates 7.509 µM/day and 8.930 µM/day for duplicates with 0.25 mM sodium glyoxylate. The generation time in the exponential phase calculated on the basis of the nitrite production and 16S rRNA gene copies/mL, were similar for the S-EC culture.

At four different time points (day: 0, 87, 108 and 136) 1 mL DNA of each culture was sampled for quantitative PCR. Green and blue bars in Figure 16 represent the results of quantitative PCR on archaeal 16S rRNA gene. However, we measured only one time point (day 136) for one of the S-EC duplicates (green), whereas for the other duplicate we measured all four time points.

For the enrichment cultures with sodium glyoxylate we observed the highest archaeal 16S rRNA gene copy number ($1.03 \cdot 10^7$) at day 87. After this time point copy numbers were decreasing.

3.1.7 Influence of pH on growth of the AOA enrichment cultures

To find the optimal pH for this new ammonia oxidizing archaeon *N. arctica*, an experiment was performed were we had three different enrichment cultures which we used for inoculation of biological triplicates for each pH. The inocula had different treatments: standard conditions or 1 mM ammonium or 0.5 mM ammonium and 100 µg/mL kanamycin. We tested growth at pH 4, 5, 6 and 7. The enrichment cultures were inoculated with the standard conditions except for the pH value. Notice that, the fresh water media was not buffered in this experiment. Only for the enrichment cultures at pH 7 we observed nitrite production. Further, the lag phase of these cultures was 30 days long and stationary phase was not reached at day 135. The cultures at pH 4, 5 and 6 did not produce any nitrite. However, the nitrite concentration in the cultures with pH 6 was stable, whereas nitrite concentration in the cultures with pH 4 and 5 was decreasing between day 0 and 30 and then concentrations were nearly stable (Figure 17).

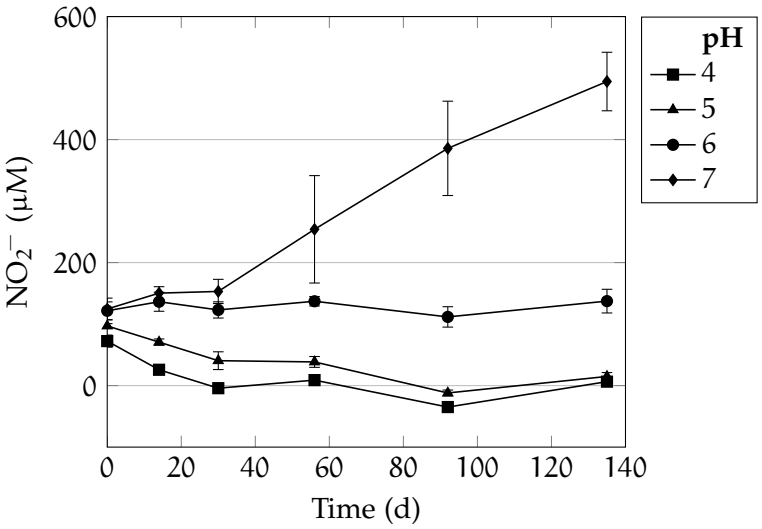


Figure 17: Average of nitrite production of *N. arctica* at four different pH values with ammonium: Nitrite production only at pH 7 was observed.

In a second experiment enrichment cultures from four different inocula were tested with 0.5 mM urea as energy source. The inocula had different conditions: three inocula had 1 mM ammonium in 40 mL culture volume and the fourth one had 0.5 mM ammonium and ampicillin 100 µg/mL in 40 mL culture volume. Six different pH values were tested: 4, 5, 6, 6.5, 7 and 8. Notice that, the FWM for the

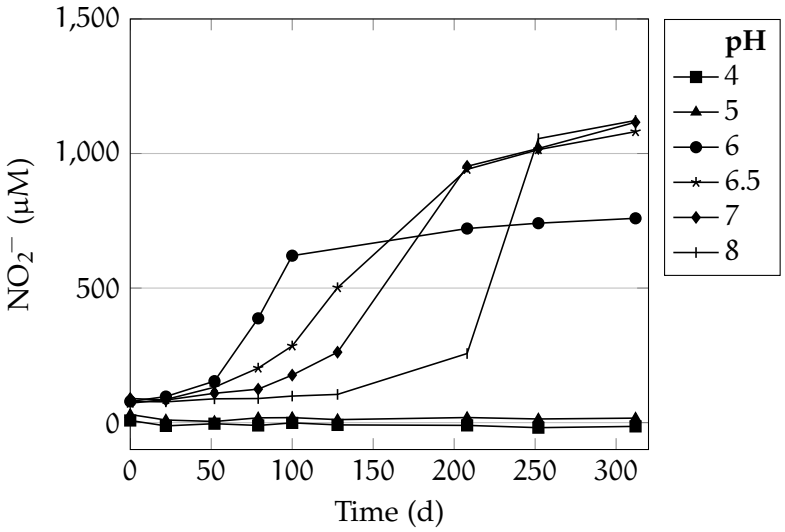


Figure 18: Growth of *Ca. N. arctica* at six different pH values with 0.5 mM urea as energy source: Enrichment cultures produced nitrite between pH values from 6 to 7. At day 128 also the enrichment culture at pH 8 started to produce nitrite. All cultures were stemming from the same inoculum, which was grown with 1 mM ammonium in 40 mL culture volume.

enrichment cultures was not buffered, therefore the pH value was tested at day 100. Growth was observed between pH values 6 - 7 in a time span of 208 days from the inoculum which was grown with 1 mM ammonium in 40 mL culture volume (Figure 18). The shortest lag phase of nitrite production was detected at pH 6 with 52 days. Cultures at pH 6.5 and 7 had a lag phase of 79 days. However, culture with pH 6 did not produce as much nitrite as the cultures with pH 6.5 and 7. The nitrite measurement at day 128 for the enrichment culture at pH 6 was left out. Enrichment cultures at pH 4 and 5 did not produce any nitrite. Another observation was that after 128 days also the enrichment culture at pH 8 started to produce nitrite. The nitrite production rate in the exponential phase was 11.089 µM/day for pH 6, 7.735 µM/day for pH 6.5, 8.633 µM/day for pH 7 and 18.149 µM/day for pH 8.

At day 100 pH was measured and except for pH 8 which decreased, an increase of 0.5 pH value was observed. This means that the pH values changed from 4, 5, 6, 6.5, 7 and 8 to 4.5, 5.5, 6.5, 7, 7.5 and 7.5, respectively.

Enrichment cultures from two other inocula (1 mM ammonium in 40 mL and 0.5 mM ammonium with 100 µg/mL ampicillin in 40 mL) showed the same trend, though with an extension of the lag phase (Figure 19). The highest nitrite concentration was reached with the cultures at pH 7. On average the nitrite production rate was 7.983 µM/day for cultures at pH 6, 10.894 µM/day at pH 6.5 and 11.555 µM/day for cultures at pH 7. The enrichment cultures of the fourth inoculum (1

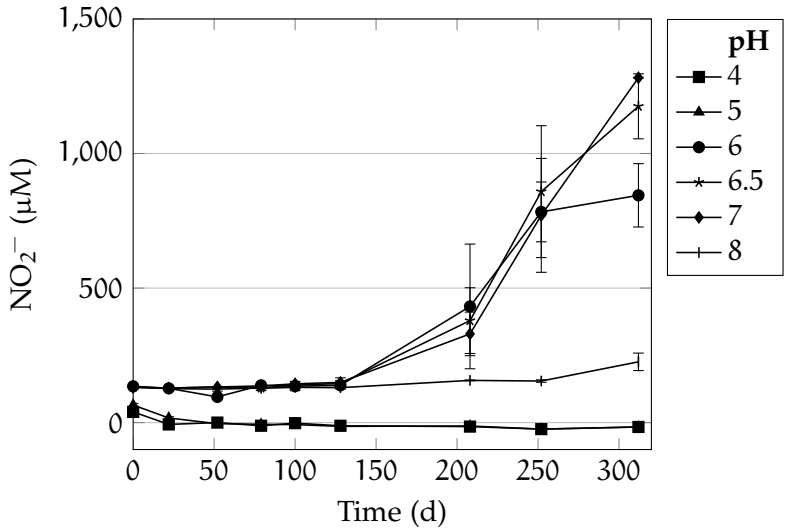


Figure 19: pH variation with 0.5 mM urea as energy source. Average with standard deviation of nitrite production of biological duplicates at six different pH values: Enrichment cultures produced nitrite between pH values 6-7. Inocula where grown with 1 mM ammonium in 40 mL culture volume or ampicillin 100 µg/mL in 40 mL culture volume.

mM ammonium in 40 mL culture volume) did not start to produce nitrite at day 208 (data not shown).

3.1.8 N_2O - production of Nitrosopila arctica

An experiment to test for N_2O production was performed with two groups of quadruplicates (A and B) from two different inocula and one control culture without inoculum. Inocula were pre-grown with 1 mM ammonium or with 0.5 mM ammonium and kanamycin 100 µg/mL. At day 76 acetylene was added to one enrichment culture, at a final concentration of 0.01 %. We used acetylene to inhibit nitrite and nitrous oxide production in the cultures. In the biological triplicates (A and B) we observed an ammonia consumption and a nitrite and nitrous oxide production (Figure 20). Cultures A and B reached the stationary phase after 105 days. In both A and B nitrous oxide production occurred in parallel to nitrite production. In cultures A the highest N_2O value was at day 105 and in cultures B at day 86.

The average of N_2O yield (N_2O/NO_2^-) was 0.007 % N_2O per NO_2^- for the cultures A and 0.012 % for the cultures B.

After addition of 0.01 % acetylene at day 76 we observed that nitrite and nitrous oxide production stopped almost completely. Further, ammonia consumption was still decreasing, however, very slowly in comparison to the cultures without acetylene (Figure 21). Until day 76 the ammonia consumption, nitrite and nitrous oxide

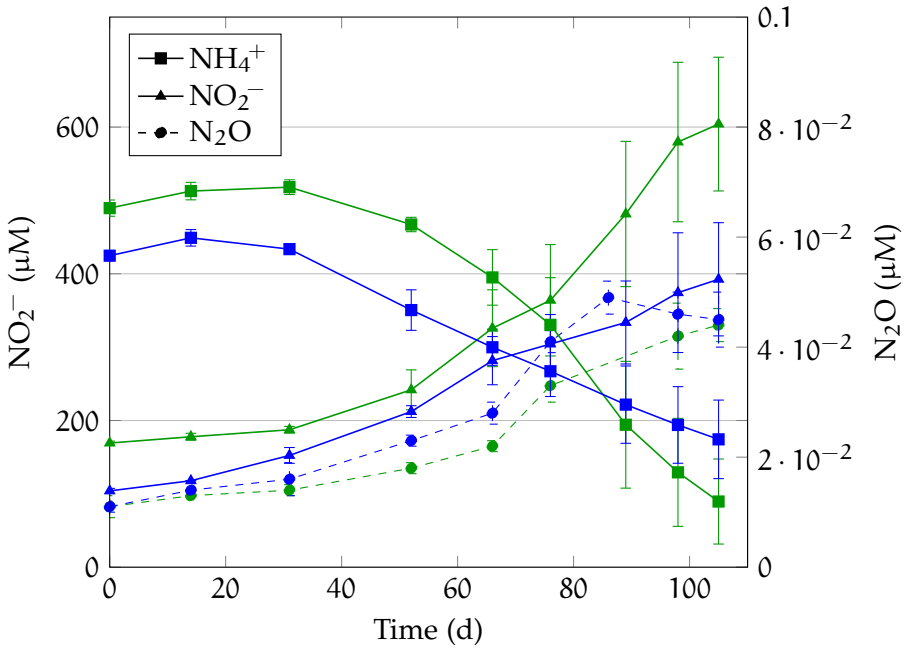


Figure 20: Experiment A (green) and B (blue) for N_2O production of *N. arctica*: Average with standard deviation of NH_4^+ consumption, NO_2^- and N_2O production of three replicate cultures (A) and three replicate cultures (B) at standard conditions. All cultures from Experiment A stemmed from the same inoculum, which was grown with 1 mM ammonium. All cultures from Experiment B stemmed from the same inoculum, which was grown with 0.5 mM ammonium and kanamycin 100 µg/mL.

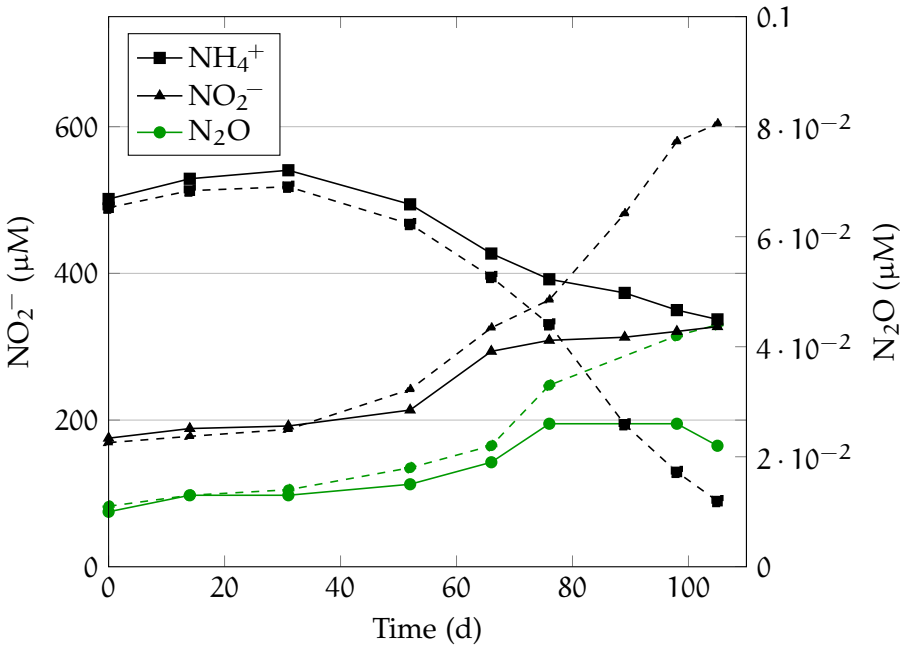


Figure 21: NH_4^+ consumption, NO_2^- and N_2O production of an enrichment culture with standard conditions, to which 0.01 % acetylene was added at day 76: Inoculum was grown with 1 mM ammonium. Dashed Line: Average of NH_4^+ consumption, NO_2^- and N_2O production of triplicate enrichment cultures (Experiment A), which were grown at standard conditions stemming from the same inoculum (1 mM ammonium).

production was nearly the same for the cultures (Experiment A) with and without acetylene.

Bacterial and archaeal *amoA* PCR (Primer bacteria: amoA1F and amoA2R, archaea: Cren-amoA19F and Cren-amoA616R) was performed to control that nitrous oxide was not produced by contaminating AOB. We could not detect bacterial *amoA* genes in the cultures, while a product was obtained in the control reaction using genomic DNA from *Nitrosospira tenuis* as a template. In all cultures archaeal *amoA* genes were detected (Figure 22).

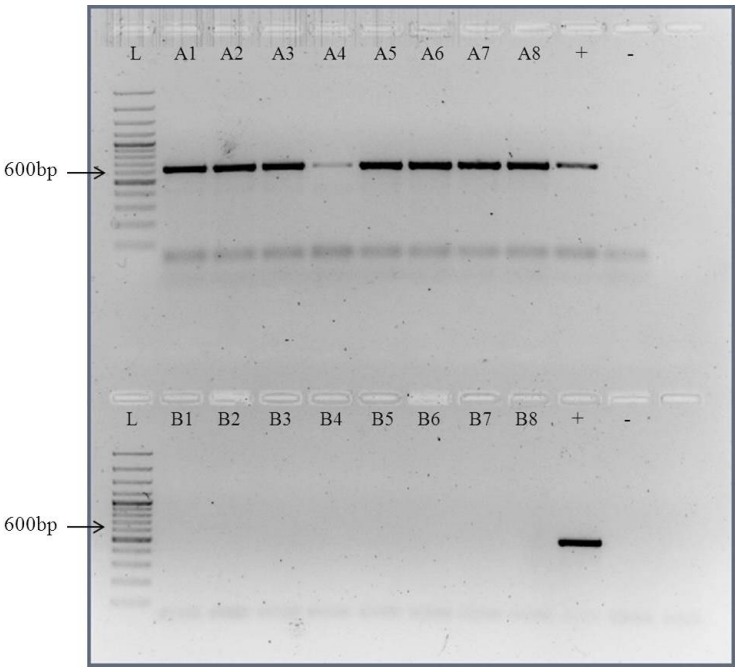
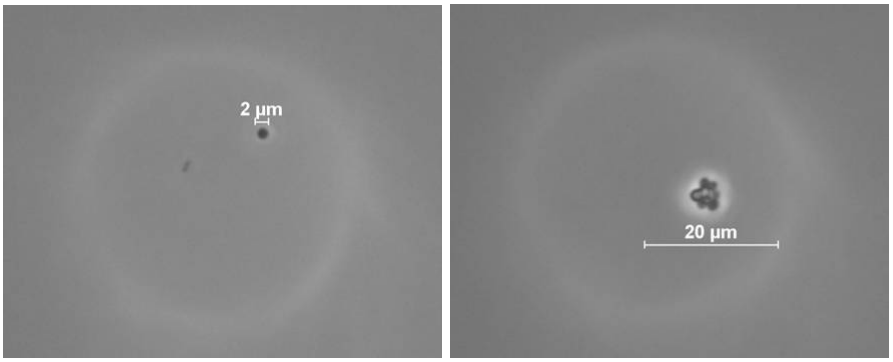


Figure 22: Gel picture of *amoA* PCR products (day 76) for AOA and AOB: L (ladder 100bp plus). In lane 10 the positiv control (+) and in lane 11 the negative control (-) was loaded. Samples from A1 to A4 were archaeal *amoA* amplicons from the enrichment cultures (Experiment A). The samples A5 to A8 were archaeal *amoA* amplicons from enrichment cultures (Experiment B). The samples B1 to B4 (Experiment A) and samples B5 to B8 (Experiment B) were bacterial *amoA* PCR reactions.

3.2 LIGHT-MICROSCOPY OF NITROSOPILA ARCTICA

Enrichment cultures were investigated by light microscopy. Samples of the enrichment culture were taken in the lag phase, end of exponential phase and stationary phase. We observed cells of different morphology including cocci, rods and filamentous cells in different sizes. In all cultures we observed cocci with approximately 2 µm in diameter, which formed clusters (Figures 23a, 23b, 24). Most of these clusters were approximately 5 to 10 µm, however, we also found bigger clusters (Figure 24). In some cultures we noticed white flocks, which were visible to the naked eye. We investigated these flocks by light microscopy and observed many cocci attached to filamentous cells (Figure 26, 27). The enrichment cultures consisted not only of cocci, also rods in different sizes were found (Figure 25).



(a) (b)

Figure 23: AOA Enrichment culture investigated by phase contrast (1000 × magnification): In image (a) we can see a coocus shaped cell with approximately 2 µm in diameter. In image (b) a cluster of cocci was observed.

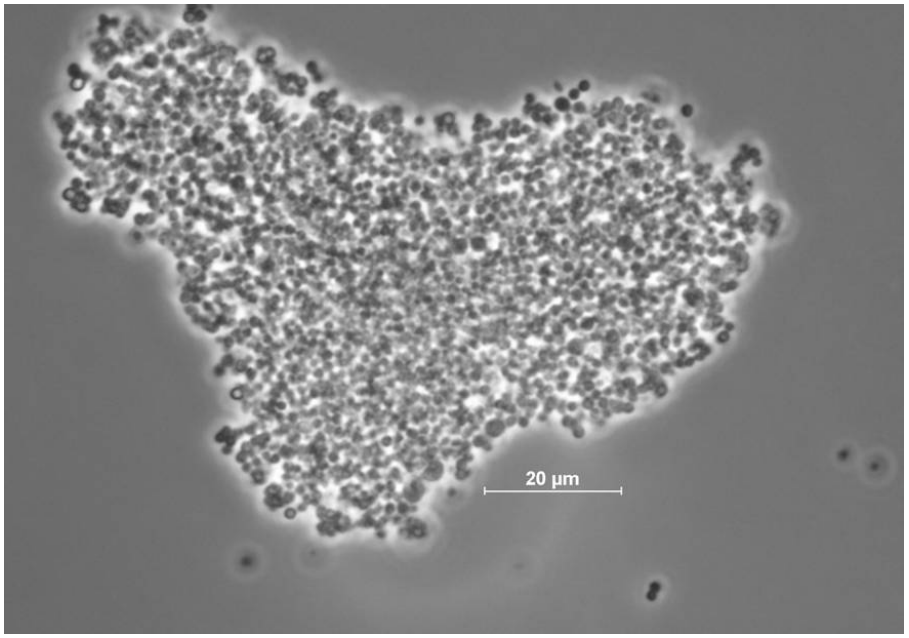


Figure 24: AOA Enrichment culture investigated by phase contrast (1000 × magnification): We observed a big cluster of cocci shaped cells.

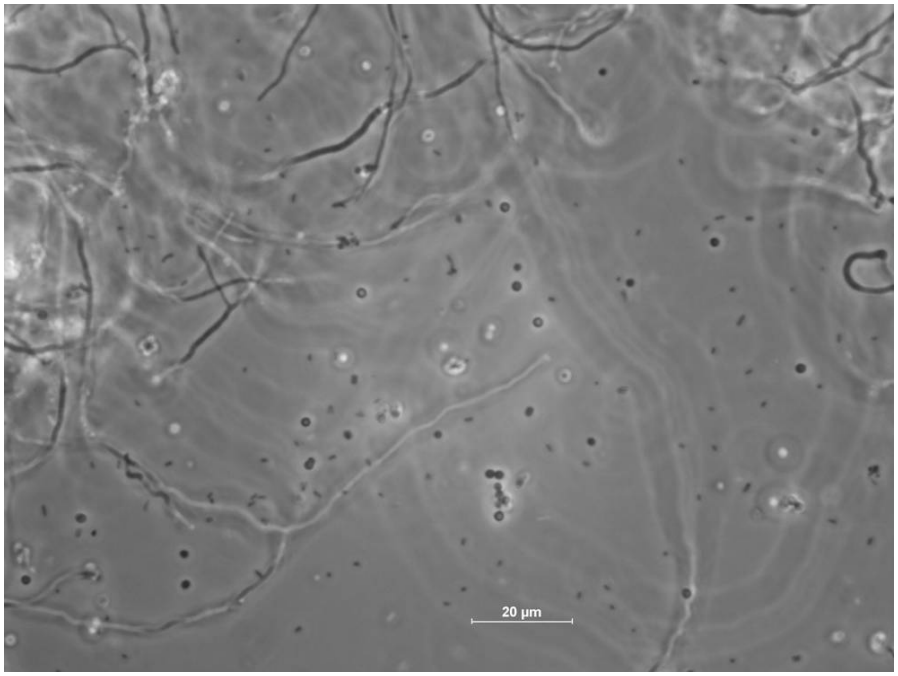


Figure 25: AOA Enrichment culture investigated by phase contrast (1000 × magnification): We observed rod shaped cells, differing in their size, cocci and filamentous shaped cells.

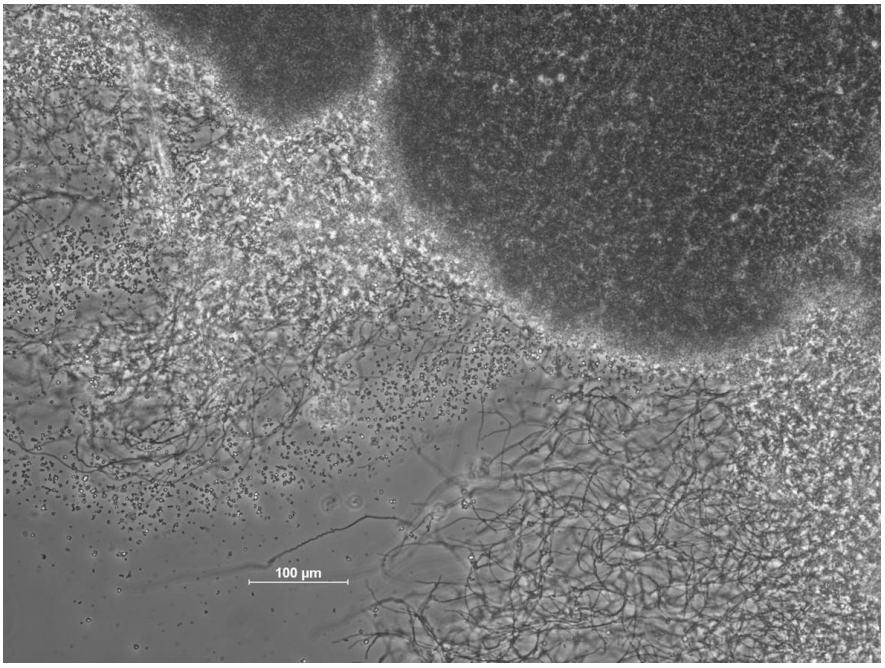


Figure 26: AOA Enrichment culture investigated by phase contrast (400 × magnification): We observed many cocci shaped cells attached to filamentous cells.

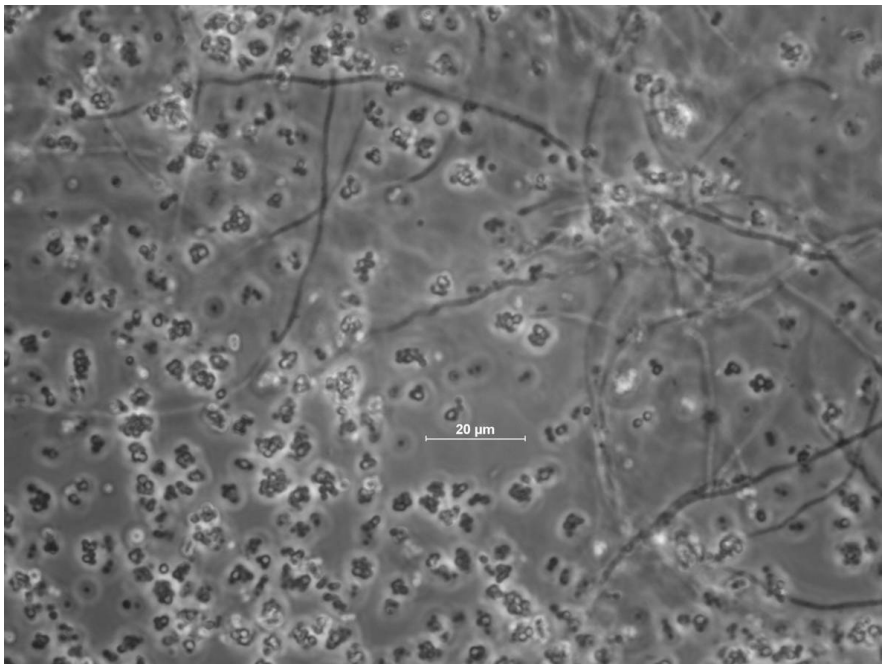
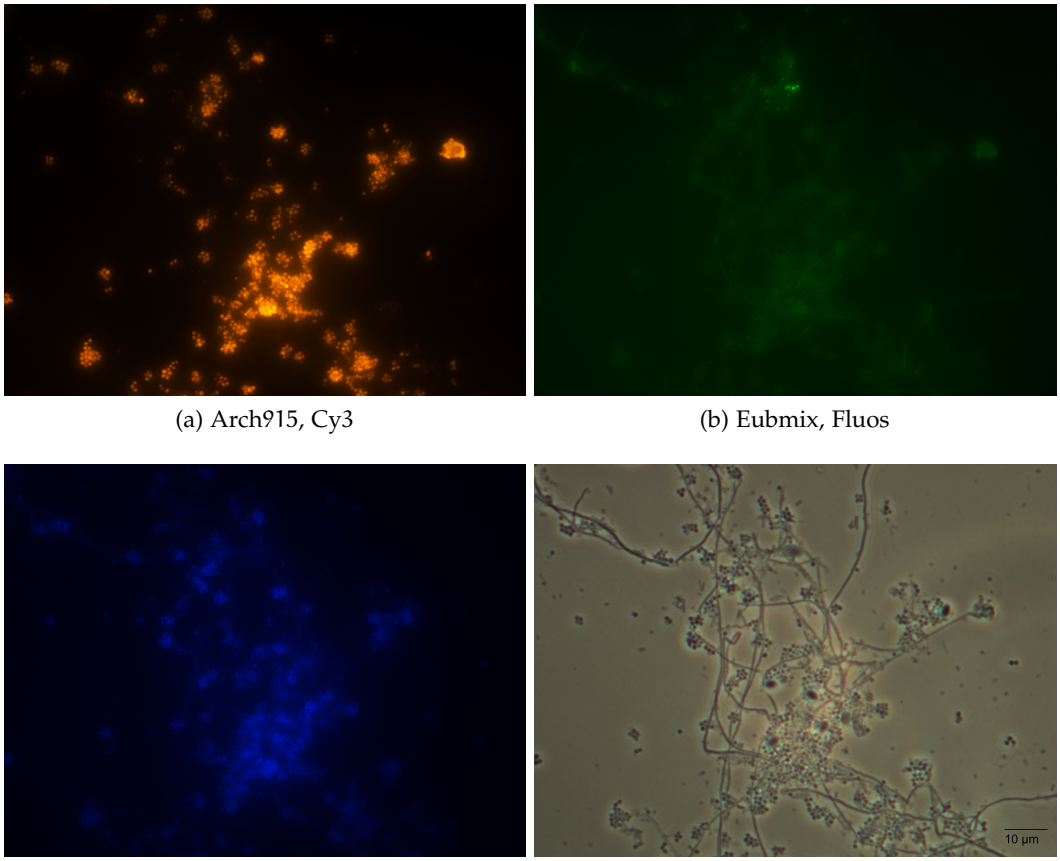


Figure 27: AOA Enrichment culture investigated by phase contrast (1000 × magnification): We observed many cocci cluster next to filamentous cells.

3.3 FLUORESCENCE IN SITU HYBRIDISATION OF ENRICHMENT CULTURES

Enrichment cultures were investigated by FISH using two different probes, for archaea Arch915 in orange (Cy3) and for bacteria Eubmix in green (Fluos). Cells were additionally stained with DAPI (blue). The cells were fixed with EtOH. We observed again a lot of cocci shaped cells attached to filamentous cells and some small rods (Figure 28d). In Figure 28a the bright signals obtained with the archaeal-specific probe Arch915 are visible. Further, we obtained too faint signals with the bacterial probe (Figure 28b). With DAPI we did not observe all cells, the signals for the filamentous cells where not as bright as for the cocci shaped cells and the small rods didn't even show a signal (Figure 28c). We also tried PFA for cell fixation, however, we could not improve the results.

In summary we observed that these (approximately 2 µm) cocci, which formed clusters, were probably the ammonia oxidizing archaea (*Nitrosopila arctica*).



(a) Arch915, Cy3

(b) Eubmix, Fluos

(c) DAPI

(d) Light microscopy

Figure 28: AOA Enrichment culture investigated with FISH (1000 × magnification): The cocci showed bright signals with the archaea-targeting probe Arch915 in Cy3 (orange) (Image (a)). With the probe Eubmix in Fluos (green) we observed only very faint signals (Image (b)). We noticed that not all cells were stained with DAPI (blue), the filamentous cells showed only faint signals with DAPI (Image (c)). In Image (d) the same sample is showed by phase contrast.

3.4 PHYLOGENETIC ANALYSIS

From eight different cultures, two were coming from Tundra fen soil, four from Frost boil soil and two from Moss tundra soil, purified archaeal 16S rRNA PCR amplicons were forward and reverse sequenced. All cultures had reached the stationary phase, when the DNA sample was taken.

The DNA amounts of the cleaned PCR products of the enrichment cultures can be seen in the Table 22. A gel was performed for qualitative analysis of the triplicate PCR reactions. For each PCR reaction of the enrichment culture we obtained a specific band at the expected size of ~ 1390bp (Figure 29).

The obtained sequences were aligned and cut in BioEdit to a fragment length of 759 nucleotides.

Table 22: amount DNA

enrichment cultures	**amount DNA** [ng/µl]
S12 – 2 (Tundra fen)	14.33
S17 – 4 (Frost boil)	17.70
S18 – 8.2 (Frost boil)	39.68
S18 – 4 (Frost boil)	23.59
S20 – 12 (Moss tundra)	17.40
S21 – 16 (Moss tundra)	48.32
S24 – 4 (Tundra fen)	22.98
S25 – 16 (Frost boil)	21.81

I received 20 16S rRNA clones from the clone libraries of the N_2O enrichment cultures Experiment A and B, these clone libraries were done by my co-supervisor Ricardo Alves (Alves et al. 2013). All archaeal 16S sequences, including the eight 16S rRNA PCR amplicons and the 20 16S rRNA clones, were aligned in BioEdit and a sequence identity matrix was calculated. The sequences showed a 99.6 % nucleotide identity. Of these 20 16S rRNA clone sequences 14 were identical, five differed between 1 to 2 positions and one had a 1 nucleotide gap. Of the eight sequenced 16S rRNA amplicons, six were identical and two had the same SNP at position 399. All 16S rRNA sequences of *Ca. N. arctica* and 16S rRNA sequences of *Ca. Nitrososphaera viennensis* were aligned in BioEdit, they differed in 29 positions over a region of 759 nucleotides, *Ca. N. arctica* and *Ca. N. viennensis* EN 76 showed a 96.17 % nucleotide identity over 759 nucleotides (Figures 31, 32, 33 and 34 in Appendix A.1)

One representative of the 16S rRNA sequences of *N. arctica* was chosen and a maximum-likelihood tree including 100 bootstrap support values was calculated with GTR model with Gamma-distributed site rate variation and invariable sites

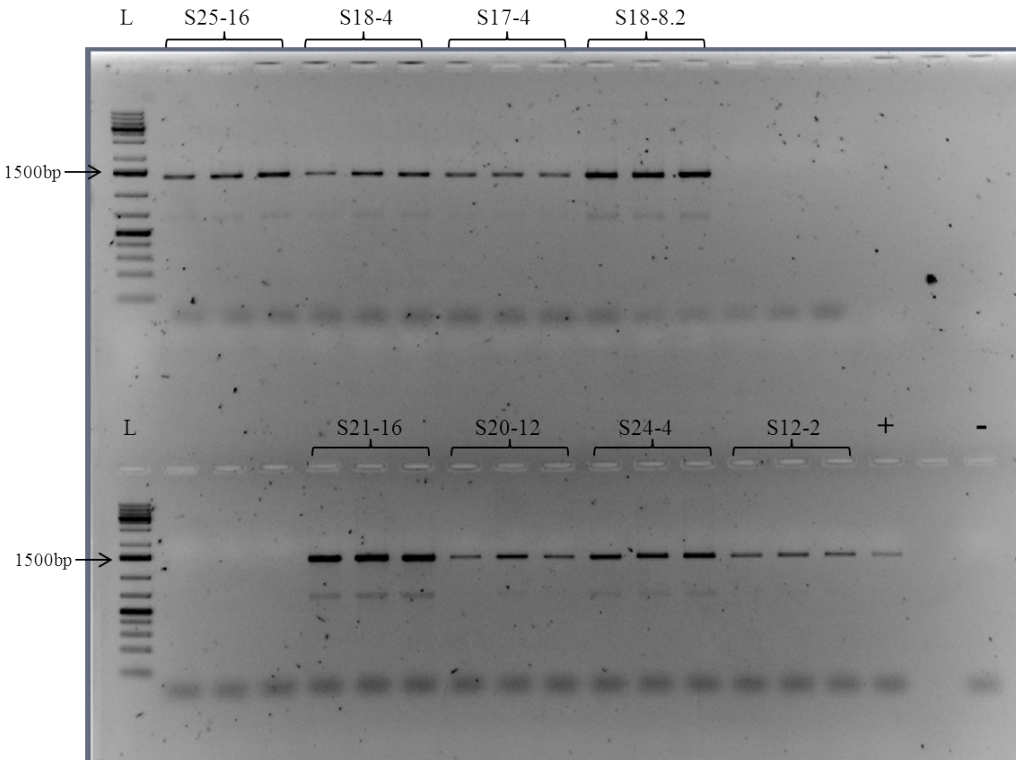


Figure 29: Gel picture of the archaeal 16S rRNA PCR amplicons for sequencing: L (ladder 1kb plus), positive control (+) and negativ control (-). Bands for the 16S rRNA PCR amplicons from each culture, can be seen on their expected size.

using ATGC PhyML 3.0. The representative sequence of *N. arctica* (red framed) clustered together with the fosmid clone 29i4 and belongs to an uncharacterised sister lineage of *Nitrososphaera viennensis* (Figure 30). The 16S rRNA gene showed 98.5 % nucleotide identity to the fosmid clone 29i4 over 753 nucleotides.

In summary *Ca. Nitrosopila arctica* belongs to the phylum *Thaumarchaeota* within an uncharacterised group next to *Nitrososphaera viennensis*.

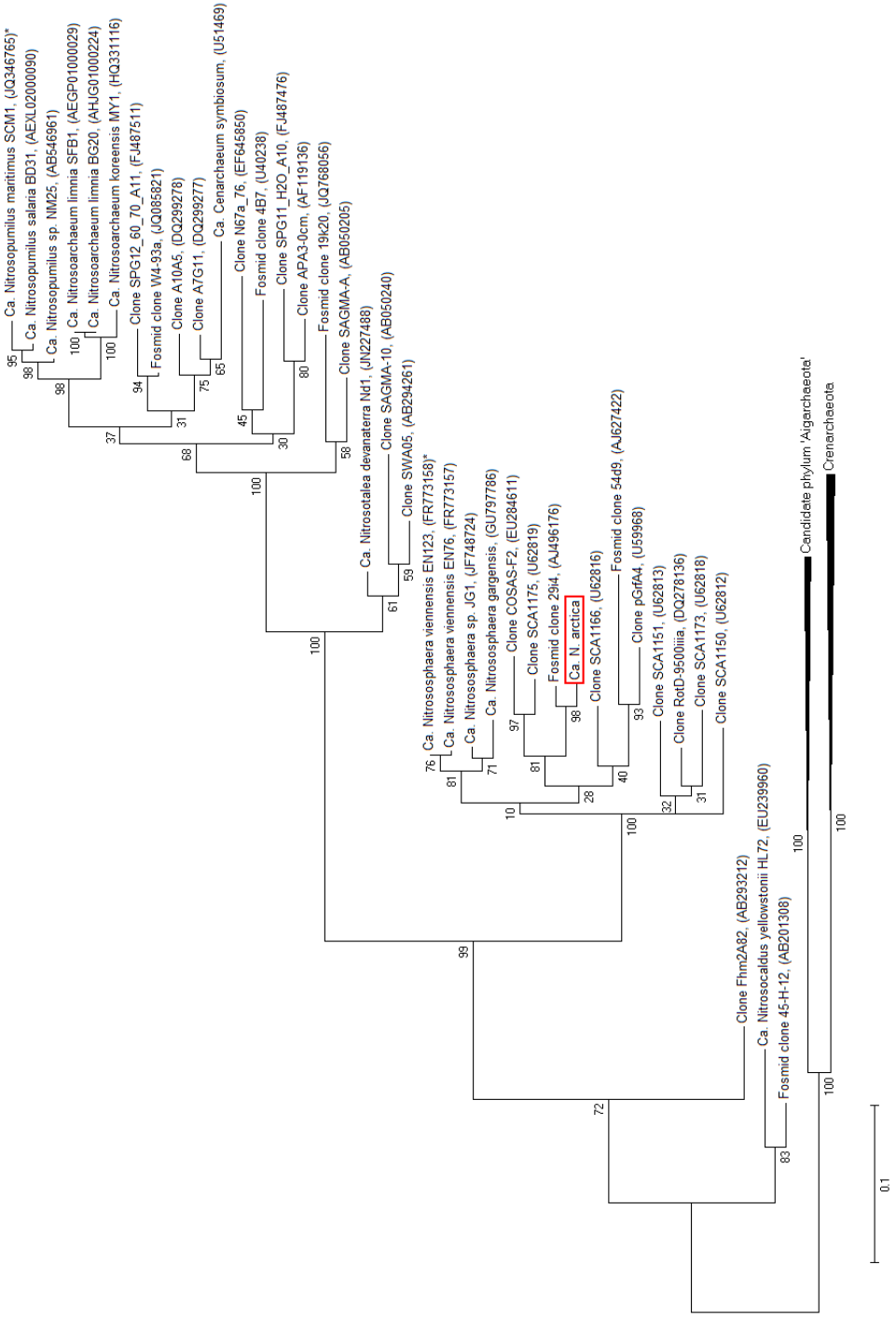


Figure 30: Maximum-likelihood phylogenetic tree of archaeal 16S rRNA sequences, including one representative of *Ca. N. arctica*. Tree was calculated based on the GTR model with Gamma-distributed site rate variation and invariable sites. The percentage of 100 bootstrap replicates are illustrated close to the nodes. Scale bar indicates substitutions per site. The representative sequence of *N. arctica* (red framed) clusteres together with the fosmid clone 29i4. The 16S rRNA gene is 98.5 % identical to this clone over 753 nucleotides. Furthermore, *Ca. N. arctica* belongs to a sister cluster of *Nitrososphaera viennensis*.

4

DISCUSSION

4.1 ENRICHMENT CULTURES OF AMMONIA OXIDIZING ARCHAEA

In this study enrichment cultures of a new ammonia oxidizing archaeon were characterized. Only two pure cultures and few enrichment cultures of ammonia oxidizing archaea have been obtained since their discovery. This study should help to gain more insight into the physiology of ammonia oxidizing archaea and to characterize the first such organism from an arctic environment.

The different growth phases and generation times described in this study are all related to the nitrite production of the enrichment cultures, because no pure culture exists yet. These enrichments may vary in their viability or in their degree of enrichment for the archaeon, which might have lead to variability in our results.

4.1.1 Influences of different culture volumes on nitrite production

Due to the low final cell yields of the organism (cultures remain clear even in stationary phase), experiments with larger total culture volumes were performed to obtain more biomass of *N. arctica* for physiological studies and for sequencing the whole genome.

Cultures with 40 mL culture volume never produced as much nitrite as cultures with 20 mL volume. The significant difference in growth between the culture volumes 20 mL and 40 mL might be due to the fact that we used containers consisting of different materials. Enrichment cultures were cultivated for 20 mL volumes in polystyrene tubes, for 40 mL in polypropylene tubes and for 100 mL in glass bottles. Possibly polypropylene affects the growth of *N. arctica*. However, I could not find studies where this effect was observed for archaea and bacteria.

Another possibility could be the variance in headspace. The enrichment cultures with 20 mL and 40 mL volumes had each a 10 mL headspace. The headspace for the 100 mL cultures was 150 mL (60 %, percent of total volume). Enrichment cultures with 20 mL volumes had $33.\overline{3}$ % of total volume whereas enrichment cultures with 40 mL volumes only had 20 %. It could be that oxygen was limiting. However, nitrite production was still possible because of gas exchange when cultures were opened for sampling.

No significant difference in nitrite production in the exponential phase was observed between the culture volume 20 mL and 100 mL with 1 mM ammonium. This could be a hint that it makes no difference between $33.\overline{3}$ % to 60 % headspace for the NO_2^- production rate. The extension of the lag phases of the enrichment cultures with 100 mL volume could be due to an adaptation time to bigger volumes or the use of glass bottles.

To sum up 20 mL culture conditions are currently the best for this ammonia oxidizing archaeon. To obtain more biomass, 100 mL total culture volume should be used, although an extension of the lag phase was observed. The polypropylene containers for 40 mL total volume should not be used anymore, because there was an extension of the lag phase and these enrichment cultures produced less nitrite during the exponential phase than cultures with 20 mL or 100 mL total culture volume. Further investigations are necessary with quantitative PCR to confirm these results. Furthermore, cultures with 40 mL total volume should be cultured in glass or polystyrene containers to confirm if these observed effects are only caused by the polypropylene containers.

4.1.2 Influence of different antibiotics on nitrite production

Different antibiotics were tested to inhibit bacterial contaminants, to investigate their effect on growth (nitrite production) of *N. arctica* and to further enrich this AOA.

We tested three different antibiotics ampicillin, carbenicillin and kanamycin in different concentrations. Ampicillin and carbenicillin are both β-lactam antibiotics affecting bacterial cell wall synthesis, where ampicillin belongs to the aminopenicillin family and carbenicillin to the carboxypenicillin subgroup. Streptomycin and kanamycin belong to the aminoglycoside bactericidal antibiotics, they both inhibit protein synthesis. Different studies have shown that archaea are insensitive to these antibiotics (Andronopoulou and Vorgias 2003, Hilpert et al. 1981, Kandler and König 1998, Tourna et al. 2011).

The decrease in NO_2^- production and the extension of the lag phase upon addition of carbenicillin and kanamycin could be due to the fact that the bacterial contaminants were affected by these antibiotics. Possibly the AOA depend on these contaminants. This dependency could be based on the fact that the contaminants may use some by-products, made by these AOA which would be toxic to them in higher concentrations. Another alternative is that the contaminants have a positive growth effect on the ammonia oxidizing archaea by supporting them with substrates they lack in the medium.

A further explanation for the extension of the lag phases in cultures with high antibiotic concentration is that these AOA are inhibited by the accumulating antibiotics in the medium. The lag phase of enrichment cultures with different antibiotics was between 40 and 100 days. The increasing nitrite production after the long lag phase may be caused by the decreased antibiotic concentration due to their decomposition. This enables the AOA and the bacterial contaminants to grow.

Enrichment cultures with kanamycin, which were grown with urea as energy source did not show a significant effect on nitrite production compared to the S-EC cultures with kanamycin (Table 28). This observation could be due to the fact that the enrichment cultures with urea and kanamycin more often stemmed from inocula with additional antibiotics. Possibly the bacterial contaminants in these cultures adapted to these antibiotics.

Furthermore, there was no significant difference (p value = 0.074) in the nitrite production rate between enrichment cultures with 0.25 mM urea (6.8 µM/day) and cultures with 0.25 mM urea and 50 µg/mL kanamycin (5.5 µM/day). However, the p = value is very low. The number of enrichment cultures with 0.25 mM urea and 50 µg/mL kanamycin which were statistically analysed was only 4. With more replicates there will may be a significant difference detectable between treatments.

Taken together, additional 100 µg/mL carbenicillin and kanamycin led to an extension of the lag phase in the enrichment cultures. Whereas cultures with urea as energy source were not as much affected by these antibiotics as cultures with ammonium as energy source. Enrichment cultures with ampicillin did not show a significant effect on nitrite production. To verify these observations investigations using quantitative PCR to estimate the percentage of enrichment are advisable.

4.1.3 Influence of different ammonium concentrations on ammonia oxidation

Experiments with different ammonium concentrations were performed to detect the concentration for the optimal growth and the adaptation limit of these AOA for ammonium.

In all enrichment cultures with different ammonium concentrations (0.5 mM, 1 mM, 10 mM, 15 mM and 20 mM) we determined nitrite production. However, in some enrichment cultures with ammonium concentrations higher than the standard conditions (0.5 mM) we observed extended lag phases. A recent study also described an extension of the lag phase for AOA at higher ammonium concentrations (French et al. 2012). This could not be shown for ammonia oxidizing

bacteria (AOB). They suggested that AOB have an advantage at higher ammonium concentrations, which is supported by the detection of high abundances of AOB in environments with high ammonium concentrations (French et al. 2012, Verhamme et al. 2011). Nevertheless, the extension of the lag phases in our study could also be caused by differences in quality of the inocula.

As described above, these outcomes have been described before in ammonia oxidizing archaea (French et al. 2012, Jung et al. 2011, Tourna et al. 2011). Ammonia oxidizing archaea are considered to be generally adapted to lower ammonium concentrations and ammonia oxidizing bacteria to higher ammonium concentrations. However, quite recent studies showed that some AOA are also adapted to ammonium concentrations between 3 mM and 20 mM. This is comparable with what is known about most oligotrophic AOB. Nevertheless, this is still lower than compared to the highest NH_4^+ tolerance of AOB (Hatzenpichler et al. 2008, Jung et al. 2011, Koops et al. 2006, Lehtovirta-Morley et al. 2011, Martens-Habbena et al. 2009, Tourna et al. 2011, Verhamme et al. 2011).

Another observation was that nitrite was only produced up to an end concentration of 900 µM in the medium with ammonium as energy source, also when more substrate was available. Different studies have shown that high nitrite concentrations can be toxic for ammonia oxidizing bacteria (Stein and Arp 1998, Tan et al. 2008). Therefore, AOA may be not adapted to higher nitrite concentrations. Further, ammonia oxidation was completely inhibited at 0.35 mM nitrite in *Nitrosopumilus maritimus* (Könneke et al. 2005). The enzyme nitrite reductase has been proposed to be involved in nitrite detoxification in *Nitrosomonas europaea*, an ammonia oxidizing bacterium (Beaumont et al. 2002). This enzyme was also found in ammonia oxidizing archaea (Bartossek et al. 2010, Treusch et al. 2005), but still very little is known about nitrite detoxification in AOA. In an experiment with different urea concentrations we observed a nitrite production up to 2 mM (Figure 14), which indicated that this AOA was not inhibited by 2 mM nitrite. The question why nitrite production stopped at 900 µM nitrite with ammonium as energy source while we do not observe such a behaviour with urea remains unanswered.

In summary, we obtained a significantly faster NO_2^- production rate in enrichment cultures with 1 mM ammonium (7.9 µM/day) than in S-EC cultures (6.0 µM/day). Further, we detected growth at 20 mM ammonium, with an extension of the lag phase. This could indicate a longer adaptation time to high ammonium concentrations.

Further investigations are necessary to detect the tolerance level of ammonium for this ammonia oxidizing archaeon. Analysis with qPCR is advisable to verify the results and to estimate the percentage of enrichment.

4.1.4 Influence of different urea concentrations on ammonia oxidation

Experiments with different urea concentrations were performed to investigate if growth of *N. arctica* was possible with urea as alternative substrate for ammonia oxidation and to detect the tolerance level.

For a member of group I.1.b, *Nitrososphaera viennensis*, it was described that it has the capability to use urea instead of ammonium as sole energy source (Tourna et al. 2011). Further, growth on urea was also predicted for *Ca. N. gargensis* (Spang et al. 2012). We could confirm ammonia oxidation in our enrichment cultures with urea as sole energy source. Different urea concentrations (0.5 mM, 1 mM, 3 mM, 5 mM and 10 mM) were tested to determine the optimal substrate concentration. However, the pH for the enrichment cultures with 3 mM, 5 mM and 10 mM urea changed from approximately 7 to 8 - 9. These enrichment cultures stopped nitrite production between day 58 and 85 (Figure 14). This could be an effect of the increasing pH, which could indicate that *N. arctica* cannot grow at a pH higher than 8. Furthermore the highest nitrite production rate (16.1 µM/day), 16S rRNA gene copies/mL ($8.11 \cdot 10^6$) and fastest generation time (25 days) was in this experiment obtained with 1 mM urea. However, this is only an assumption due to the missing control culture with 2 mM ammonium.

In summary 1 mM urea is currently the best known concentration for urea to stimulate nitrite production of *N. arctica*. Furthermore, we obtained the highest archaeal *amoA* copy number per mL with 1 mM urea, i.e. the highest cell densities. Further investigations must be done using buffered fresh water media and more replicates to investigate the tolerance level and the optimal growth conditions of this AOA for urea. Furthermore, qPCR on archaeal and bacterial 16S rRNA genes over the growth curve should be done for a better comparison between ammonium and urea as energy source and to estimate the enrichment level.

4.1.5 Influence of organic carbon sources on ammonia oxidation

Two different organic carbon sources, pyruvate and glyoxylate, were tested to optimize the growth rate of *N. arctica*. For ammonia oxidizing archaea it was shown that some can take up organic carbon sources (Hallam et al. 2006, Tourna et al. 2011), whereas others may be inhibited by organic carbon (Zhalnina et al. 2012). For *Nitrososphaera viennensis* additional pyruvate in the fresh water medium stimulated the culture growth. *N. viennensis* is able to grow chemolithoautotrophically, however, considerable growth rates were only obtained when low amounts of pyruvate where added to the medium or when grown in coculture with bacteria (Tourna et al. 2011).

We could not observe a positive or negative effect of additional pyruvate on NO_2^- production in the enrichment cultures. Further investigations with quantitative PCR for AOA and bacteria are necessary to investigate if pyruvate has an effect on cell density of these AOA and to obtain their enrichment level.

Sodium glyoxylate, an organic carbon, was tested because it appears that some archaea lack the enzymes isocitrate lyase and malate synthase which are necessary for the glyoxylate cycle (Berg et al. 2007). Only for halophilic archaea high activity of both enzymes was observed (Oren and Gurevich 1995, Serrano et al. 1998). Furthermore, for *Methylobacterium extorquens* glyoxylate is toxic (Okubo et al. 2010). Therefore, glyoxylate is may be one possibility to inhibit the bacterial contaminants and stimulate growth of the AOA in the enrichment cultures.

An experiment was performed, where we compared standard enrichment cultures with enrichment cultures with additional glyoxylate via quantitative PCR. For the cultures with glyoxylate we observed the highest 16S rRNA gene copy numbers ($1.03 \cdot 10^7$) at day 87, afterwards the copy numbers decreased. A possible explanation for this observation could be that these cultures had reached the stationary phase and began to die. The generation time in the exponential phase based on nitrite production and 16S rRNA gene copies/mL were similar for the S-EC culture. Furthermore, the generation time between day 108 and 136 was 60 days based on NO_2^- production and 35 days based on 16S rRNA gene copies/mL. It seems that growth of *N. arctica* based on nitrite correlates not completely with the cell numbers. For this reason, it will be important in the future to perform always a quantitative PCR. It seems that either *N. arctica* is missing some important substrate for its optimal growth or it is an extremely slow growing organism. The positive effect of glyoxylate on the cell number is a hint that may be some substrate, e.g., carbon is missing for the optimal growth of *N. arctica*.

Taken together, it seemed that glyoxylate had a stimulating effect on these AOA, because 16S rRNA gene copies and nitrite production reached the stationary phase 28 days earlier than the control cultures. However, quantitative PCR for the bacterial 16S rRNA gene should be done to estimate the percentage of enrichment. In contrast, we could not observe a significant effect on nitrite production in the exponential phase with additional organic carbon sources, which is another hint that nitrite production and cell numbers did not completely correlate under these growth conditions. Further investigations are necessary to verify the results by performing experiments with more replicates and in parallel archaeal and bacterial qPCR.

4.1.6 Influence of pH on growth of the AOA enrichment cultures

Different pH values ranging from 4 to 8 were tested to investigate the optimal pH for *N. arctica*. Ammonia oxidizing archaea are known to grow under different pH values, which range from pH 4.5 to 7.5 (Lehtovirta-Morley et al. 2011, Nicol et al. 2008). Furthermore, archaeal *amoA* genes were found at a pH between 2.5 and 9 in hot springs (Reigstad et al. 2008, Zhang et al. 2008).

Nitrite production was only observed at pH 7 in the enrichment cultures with ammonium as sole energy source. However, as pH is decreasing the equilibrium between ammonia and ammonium changes to ammonium (Allison and Prosser 1991, Burton and Prosser 2001, Kadam and Boone 1996). Although this might not be a problem, because it is known that AOA and AOB have genes for ammonium transporter (Yan et al. 2012).

Nevertheless in a study about ammonia oxidisers they noticed no growth and nitrite production below pH 7 with ammonium as energy source, whereas with urea growth between pH 4 and 7.5 was observed (Burton and Prosser 2001). We obtained similar results for this new archaeon, with ammonium as substrate nitrite production occurred only at pH 7. With urea growth occurred between pH 6 and 7.5. It seems that ureolysis provides a mechanism for nitrification in acidic environments (Burton and Prosser 2001). In the beginning no nitrite production was observed in the culture at pH 8. However, between day 128 and 250 we noticed nitrite production in these cultures, due to the poorly buffered medium pH decreased to 7.5, which enables growth. It seems that *N. arctica* is not able to grow above pH 8.

In summary enrichment cultures only grew at pH 7 with ammonium as energy source whereas with urea they were able to grow between pH 6 and 7.5. The enrichment culture at pH 6 with urea as substrate had the shortest lag phase. However, these cultures did not produce as much nitrite as the cultures at pH 6.5 and 7. The pH decline to pH 7.5 in the enrichment culture with pH 8 is an explanation for the detected nitrite production after day 128. Further investigations using buffered media to determine the optimal pH value for *N. arctica* are on the agenda as future work.

4.1.7 N_2O - production of *N.arctica*

Autotrophic nitrification is a significant source of nitrous oxide production in soils (Colliver and Stephenson 2000, Shaw et al. 2006). Recent studies have shown that AOA and AOB can produce nitrous oxide (N_2O) (Jung et al. 2011, Shaw et al. 2006). We could confirm a nitrous oxide production for *N. arctica*. However,

the average of the nitrous oxide yield (N_2O per NO_2^-) was only 0.007 % (Experiment A) and 0.012 % (Experiment B). In comparison with *Ca. Nitrosoarchaeum koreensis*, which has an N_2O yield of 0.13 % with 2 mM ammonium (Jung et al. 2011), *N. arctica* has a 10 fold lower N_2O yield. To confirm if nitrite and nitrous oxide was only produced by the AOA in the cultures, PCR on bacterial and archaeal *amoA* genes was performed. No bacterial *amoA* genes were observed in the enrichment cultures. This verifies that the AOA are producing N_2O and NO_2^-.

We added 0.01 % acetylene to one of the cultures (Experiment A) to confirm if nitrous oxide is produced concomitantly with nitrite. Acetylene inactivates the ammonia monooxygenase (Hyman and Wood 1985), which is the key enzyme for the first step of ammonia oxidation. Therefore, acetylene inhibits nitrification in ammonia oxidizing archaea and bacteria (Hyman and Arp 1992, Offre et al. 2009, Taylor et al. 2010, Tourna et al. 2011). We could confirm these observations in our enrichment culture. Nitrite production as well as nitrous oxide production nearly stopped after addition of acetylene. This experiment also demonstrated that acetylene is perhaps an inhibitor for growth of *N.arctica*. However, qPCR on archaeal 16S rRNA genes should be performed to verify this result.

To sum up this new AOA produced nitrous oxide, however, in lower amounts than other ammonia oxidizing archaea. The production of a potent greenhouse gas indicates how important it is to study ammonia oxidizing archaea.

4.2 LIGHT-MICROSCOPY AND FISH OF NITROSOPILA ARCTICA

We investigated enrichment cultures by light microscopy and fluorescence *in situ* hybridisation to identify and morphologically characterize the AOA. *N.arctica* was identified by archaea-specific probes as spherically shaped cells of approximately 2 µm in diameter. These cells often tended to form clusters, which differ in size, however, also single cells were observed. The samples for microscopy were taken in the lag phase, exponential phase and in the stationary phase. Although the organism grows extremely slowly, we never observed drastic changes in their morphology, which would have suggested stress or a lack of growth substrate.

In some cultures we noticed white flocks, which were visible to the naked eye. These flocks were filamentous cells and a lot of AOA clusters were attached to them. This indicates on the one hand that *N.arctica* prefers to be attached on solid surfaces. On the other hand it can be that these filamentous cells and *N.arctica* depend on each other. Due to the production or consumption of substrates, which then positively affect growth.

Further investigations are necessary to explore, if AOA like to attach to solid surfaces. The development of fluorescence *in situ* hybridisation as another method for quantifying AOA would give an alternative to qPCR or an additional chance to calculate cell numbers.

5

CONCLUSION

In this work we were able to demonstrate stable growth of *N. arctica* in enrichment cultures and to improve and extend our knowledge on its growth requirements. We obtained maximal cell numbers of *N. arctica* when we used 1 mM urea as energy source and when we added 0.25 mM sodium glyoxylate to the enrichment cultures. The NO_2^- production rate in the exponential phase was 6.0 µM/day for standard enrichment cultures (0.5 mM NH_4^+), 16.0 µM/day with 1 mM urea and 8.9 µM/day with 0.25 mM sodium glyoxylate and 0.5 mM NH_4^+. No significant difference in NO_2^- production compared to the standard conditions (p value = 0.736) was observed when we used 100 mL total culture volume. However, an extension of the lag phase occurred when we used the larger culture volume.

According to this work the best currently known growth conditions for *N. arctica* are: 20 mL total culture volume, 20 % (v/v) inoculum, 80 % (v/v) FWM, 100 µg/mL streptomycin, 1 mM urea, pH between 6 - 7.5 and incubation at 20 °C in the dark. Under these conditions, the organism has a generation time of approximately 25 days assuming that nitrite production directly parallels growth, as observed for other AOA.

Ca. Nitrosopila arctica, *Nitrososphaera viennensis* (Tourna et al. 2011) and *Ca. Nitrososphaera gargensis* (Hatzenpichler et al. 2008) belong to group I.1.b within the phylum *Thaumarchaeota*. The best known growth conditions are compared in Table 23.

Table 23: Growth conditions

	Ca. N. arctica	*Ca. N. viennensis*	*Ca. N. gargensis*
Medium	FWM	FWM	mod. mineral medium
Buffer	-	HEPES	-
Energy source (mM)	1 urea	1 or 3 NH_4^+	0.5 NH_4^+
Organic carbon (mM)	-	0.5 pyruvate	-
pH	6 - 7.5	7.5	7.8
Total volume (mL)	20 or 100	20 or 100	4500
Temperature (°C)	20	37	46

N. arctica grows well in media containing initial ammonium concentrations up to 20 mM and urea concentrations up to 1 mM. In contrast *Nitrososphaera viennensis* is inhibited by 20 mM ammonium and for *Ca. Nitrososphaera gargensis* a partial inhibition at 3.08 mM ammonium was observed (Hatzenpichler et al. 2008, Tourna et al. 2011). Furthermore, *Nitrososphaera viennensis* is also able to use urea as sole energy source (Tourna et al. 2011). In a recent genome study growth on urea was also predicted for *Ca. N. gargensis* (Spang et al. 2012).

In our study we could not observe an inhibiting effect of nitrite up to 2 mM. For *N. viennensis* no nitrite inhibition up to 3.5 mM was noticed (Tourna et al. 2011). Further, we could not detect a positive growth effect with pyruvate and glyoxylate on nitrite production. However, we observed a positive effect with glyoxylate on archaeal 16S rRNA gene copy numbers compared to enrichment cultures without glyoxylate. In comparison, *N. viennensis* is able to grow chemolithoautotrophically, however, considerable growth rates were only obtained when low amounts of pyruvate where added to the medium or when grown in coculture with bacteria (Tourna et al. 2011). *N. arctica* needs on average 137 days to convert 1 mM ammonia to nitrite, whereas *N. viennensis* needs only 10 days (Tourna et al. 2011). Further, the shortest generation time we observed for *N. arctica* is 25 days. In contrast, *N. viennensis* has an about 10 fold lower generation time (Tourna et al. 2011). It seems that *N. arctica* is either missing some crucial substrate for its optimal growth or it is an extremely slow growing organism. The positive effect of glyoxylate on the cell number is one hint that may be some important substrate, e.g., carbon is missing for the optimal growth of *N. arctica*. Another indication is that enrichment cultures supplemented with carbenicillin or kanamycin show a significantly lower nitrite production, potentially by inhibiting growth of bacteria in the enrichments that have positive growth effects on *N. arctica*.

With archaeal-specific probes we identified spherically shaped cells of approximately 2 µm in diameter as *N. arctica*. These cells tended to grow in single cells and also in clusters of different sizes. We never observed drastic changes in their morphology, which could suggest a lack of substrates. However, in some cultures we noticed a lot of these cell aggregates attached to filamentous cells, which indicates on the one hand that they prefer to be attached on solid substrate. On the other hand it can be that these filamentous contaminants have some positive growth effects on *N. arctica*.

We observed nitrous oxide production by *N. arctica*, however, the highest yield (N_2O per NO_2^-) was on average 0.012 % and this is about 10 fold lower than observed for *Ca. Nitrosoarchaeum koreensis* (Jung et al. 2011) and 8 fold lower than for *Nitrososphaera viennensis* (Stieglmeier pers.comm.)

Cultivation of ammonia oxidisers is a challenging task. However, stable enrichment cultures of a new ammonia oxidizing archaeon, which belongs to group I.1.b within the *Thaumarchaeota*, were obtained. This extremely slow growing archaeon helped to gain further insights into the fascinating world of ammonia oxidizing archaea. Nevertheless, to verify these results, experiments with more replicates combining the methods fluorescence *in situ* hybridisation and quantitative PCR for analysis of these cultures are advisable.

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APPENDIX

A.1 TABLES AND FIGURES

Table 24: Statistical results - soils

Experiment	Name	N	Mean	St Dev	p	α	Holm-Sidak
Soils - NO_2 production	Fen wetland S-EC	4	4.734	1.116	0.413	0.050 : 0.049	-
	Frost boil S-EC	31	6.026	2.007			
	Moss tundra S-EC	32	6.159	2.077			

Table 25: Statistical results - volumes

Experiment	Name	N	Mean	St Dev	p	α	Holm-Sidak
Volume - NO_2 production	S-EC	67	6.012	2.006	0.019	0.050 : 0.573	Significant
	S-EC + 40 mL	9	4.369	1.071			
Volume - NO_2 production	1 mM NH_4 + 20 mL	5	7.886	1.920	0.004	0.050 : 0.867	Significant
	1 mM NH_4 + 40 mL	11	4.817	1.572			
Volume - NO_2 production	1 mM NH_4 + 20 mL	5	7.886	1.920	0.736	0.050 : 0.049	-
	1 mM NH_4 + 100 mL	3	7.444	1.226			
Volume - NO_2 production	1 mM NH_4 + 40 mL	11	4.817	1.572	0.021	0.050 : 0.618	Significant
	1 mM NH_4 + 100 mL	3	7.444	1.226			
Volume - NO_2 production	0.25 mM Urea + 20 mL	7	6.798	1.039	0.431	0.050 : 0.048	-
	0.25 mM Urea + 40 mL	4	6.301	0.777			

Table 26: Statistical results - antibiotics

Experiment	Name	N	Mean	St Dev	p	α	Holm-Sidak
Ampicillin - NO_2 production	S-EC	67	6.012	2.006	0.138	0.050 : 0.183	-
	S-EC + Amp (100 µg/mL)	4	4.467	1.951			
Carbenicillin - NO_2 production	S-EC	67	6.012	2.006	0.006	0.050 : 0.762	Significant
	S-EC + Cb (100 µg/mL)	4	3.102	1.281			

Table 27: Statistical results - antibiotics 2

Experiment	Name	N	Mean	St Dev	p	α	Holm-Sidak
Kanamycin - NO_2 production	S-EC	67	6.012	2.006	0.054	0.050 : 0.365	-
	S-EC + Kan (50 µg/mL)	6	4.336	2.087			
Kanamycin - NO_2 production	S-EC	67	6.012	2.006	0.016	0.050 : 0.602	Significant
	S-EC + Kan (100 µg/mL)	8	4.166	1.970			
Kanamycin - NO_2 production	S-EC + Kan (50 µg/mL)	6	4.336	2.087	0.878	0.050 : 0.048	-
	S-EC + Kan (100 µg/mL)	8	4.166	1.970			
Kanamycin - NO_2 production	S-EC + Kan (100 µg/mL)	8	4.166	1.970	0.949	0.050 : 0.048	-
	1 mM NH_4 + Kan (100 µg/mL)	3	4.244	0.558			
Kanamycin - NO_2 production	1 mM NH_4 + Kan (100 µg/mL) + 20 ml	3	4.244	0.558	0.835	0.050 : 0.050	-
	1 mM NH_4 + Kan (100 µg/mL) + 40 mL	3	4.062	1.300			

Table 28: Statistical results - antibiotics 3

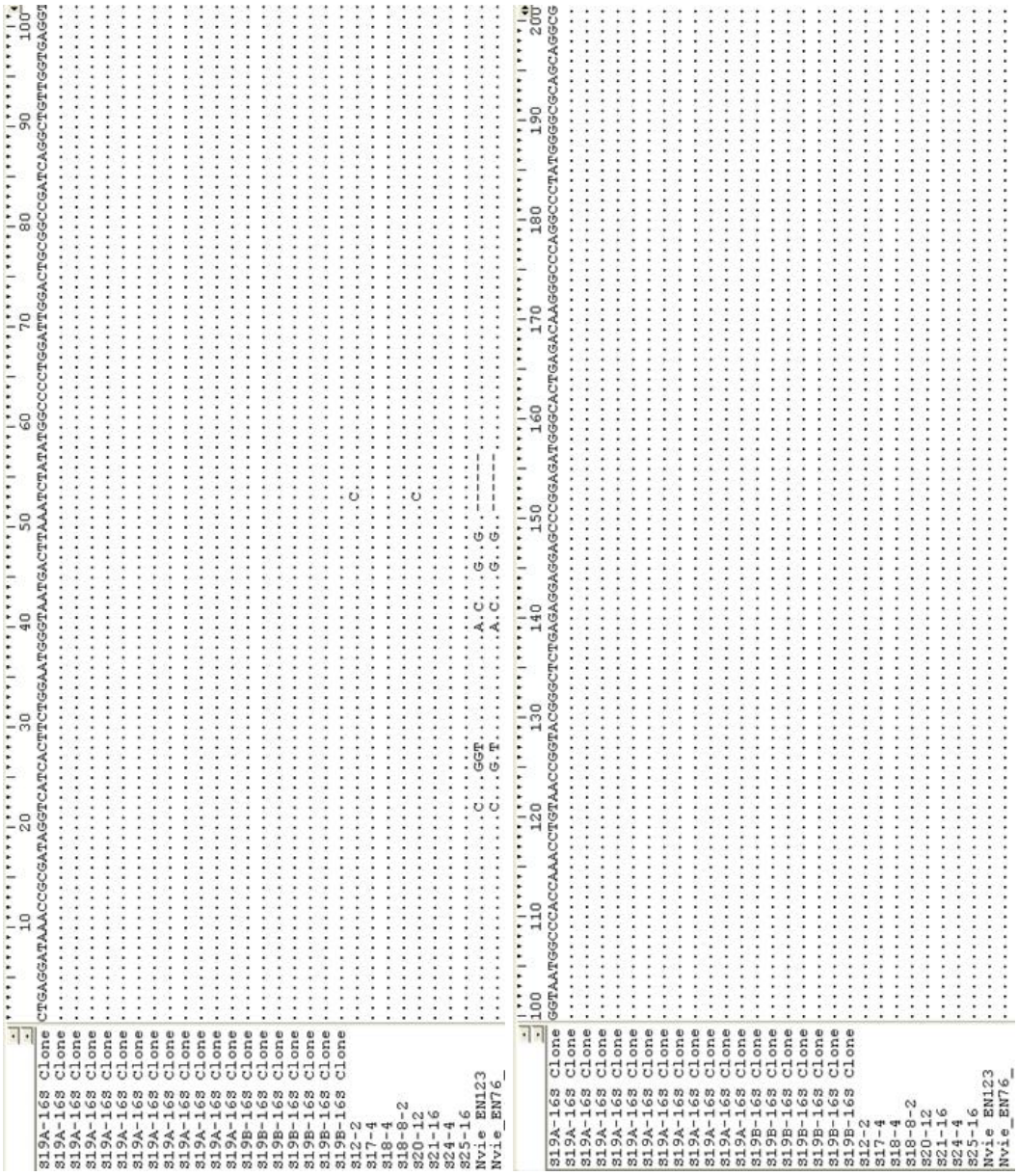
Experiment	Name	N	Mean	St Dev	p	α	Holm-Sidak
Kanamycin - NO_2 production	S-EC + Kan (50 µg/mL)	6	4.336	2.087	0.316	0.050 : 0.061	-
	0.25 mM Urea + Kan (50 µg/mL)	4	5.537	0.898			
Kanamycin - NO_2 production	0.25 mM Urea	7	6.798	1.039	0.074	0.050 : 0.339	-
	0.25 mM Urea + Kan (50 µg/mL)	4	5.537	0.898			
Kanamycin - NO_2 production	0.25 mM Urea + Kan (50 µg/mL) + 20 ml	4	5.537	0.898	0.245	0.050 : 0.102	-
	0.25 mM Urea + Kan (50 µg/mL) + 40 mL	4	6.301	0.777			

Table 29: Statistical results - energy source

Experiment	Name	N	Mean	St Dev	p	α	Holm-Sidak
Energy source conc. - NO_2 production	S-EC	67	6.012	2.006	0.047	0.050 : 0.395	Significant
	1 mM NH_4	5	7.886	1.920			
Energy source conc. - NO_2 production	S-EC	67	6.012	2.006	0.556	0.050 : 0.047	-
	10 mM NH_4	3	5.321	0.811			
Energy source conc. - NO_2 production	1 mM NH_4	5	7.886	1.920	0.075	0.050 : 0.353	-
	10 mM NH_4	3	5.321	0.811			
Energy source conc. - NO_2 production	S-EC	67	6.012	2.006	0.312	0.050 : 0.051	-
	0.25 mM Urea	7	6.798	1.039			

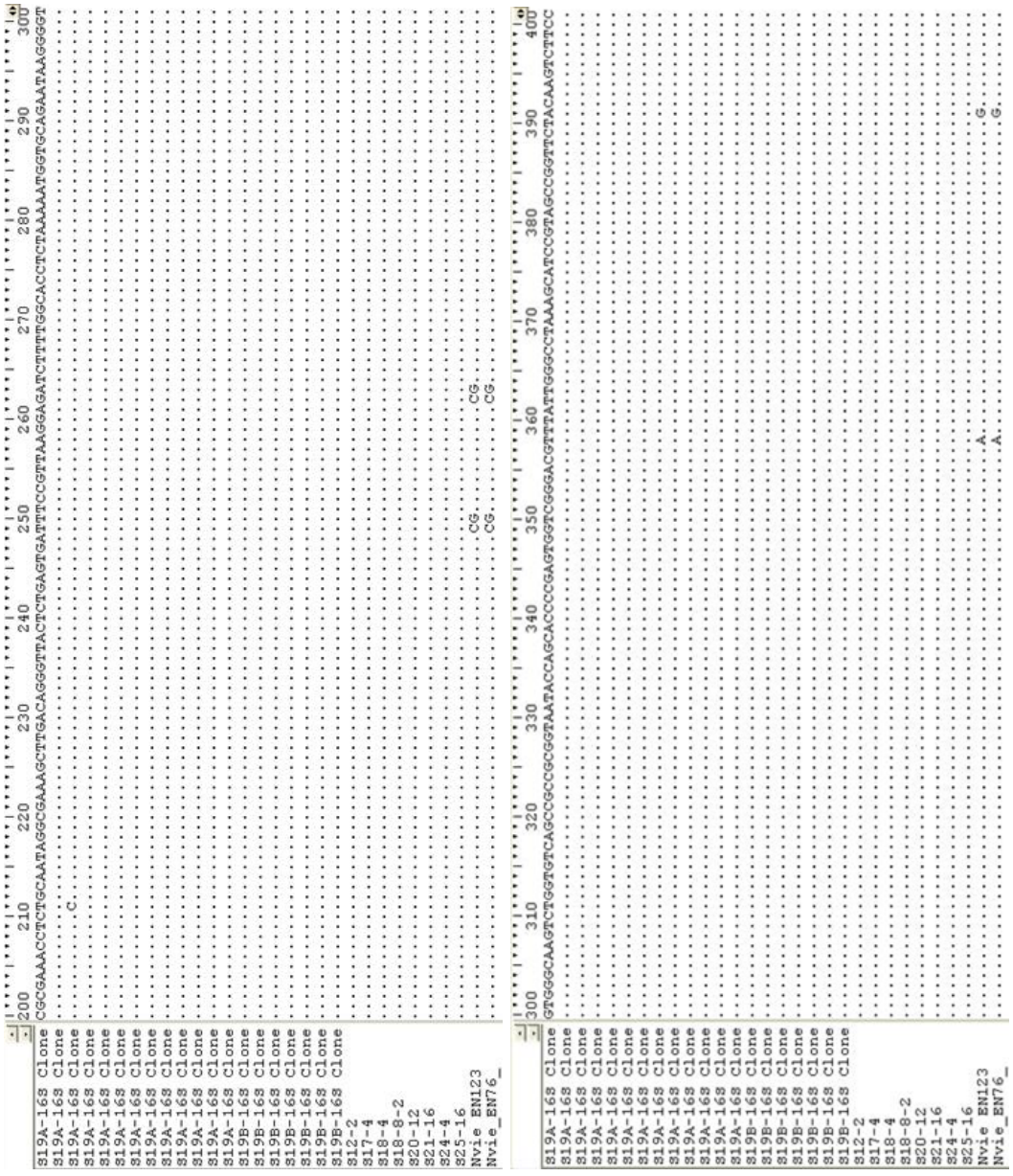
Table 30: Statistical results - carbon source

Experiment	Name	N	Mean	St Dev	p	α	Holm-Sidak
Carbon source - NO_2 prod.	S-EC	67	6.012	2.006	0.559	0.050 : 0.047	-
	0.5 mM Pyruvate	4	6.620	2.136			
Carbon source - NO_2 prod.	S-EC	67	6.012	2.006	0.439	0.050 : 0.047	-
	0.1 mM Sodium glyoxylate	5	5.304	0.965			
Carbon source - NO_2 prod.	0.25 mM Urea	7	6.798	1.039	0.072	0.050 : 0.349	-
	0.25 mM Urea + 0.1 mM Sodium g.	3	5.492	0.325			
Carbon source - NO_2 prod.	0.1 mM Sodium glyoxylate	5	5.304	0.965	0.761	0.050 : 0.049	-
	0.25 mM Urea + 0.1 mM Sodium g.	3	5.492	0.325			
Carbon source - NO_2 prod.	0.1 mM Sodium glyoxylate	5	5.304	0.965	0.254	0.050 : 0.095	-
	0.5 mM Pyruvate	4	6.620	2.136			



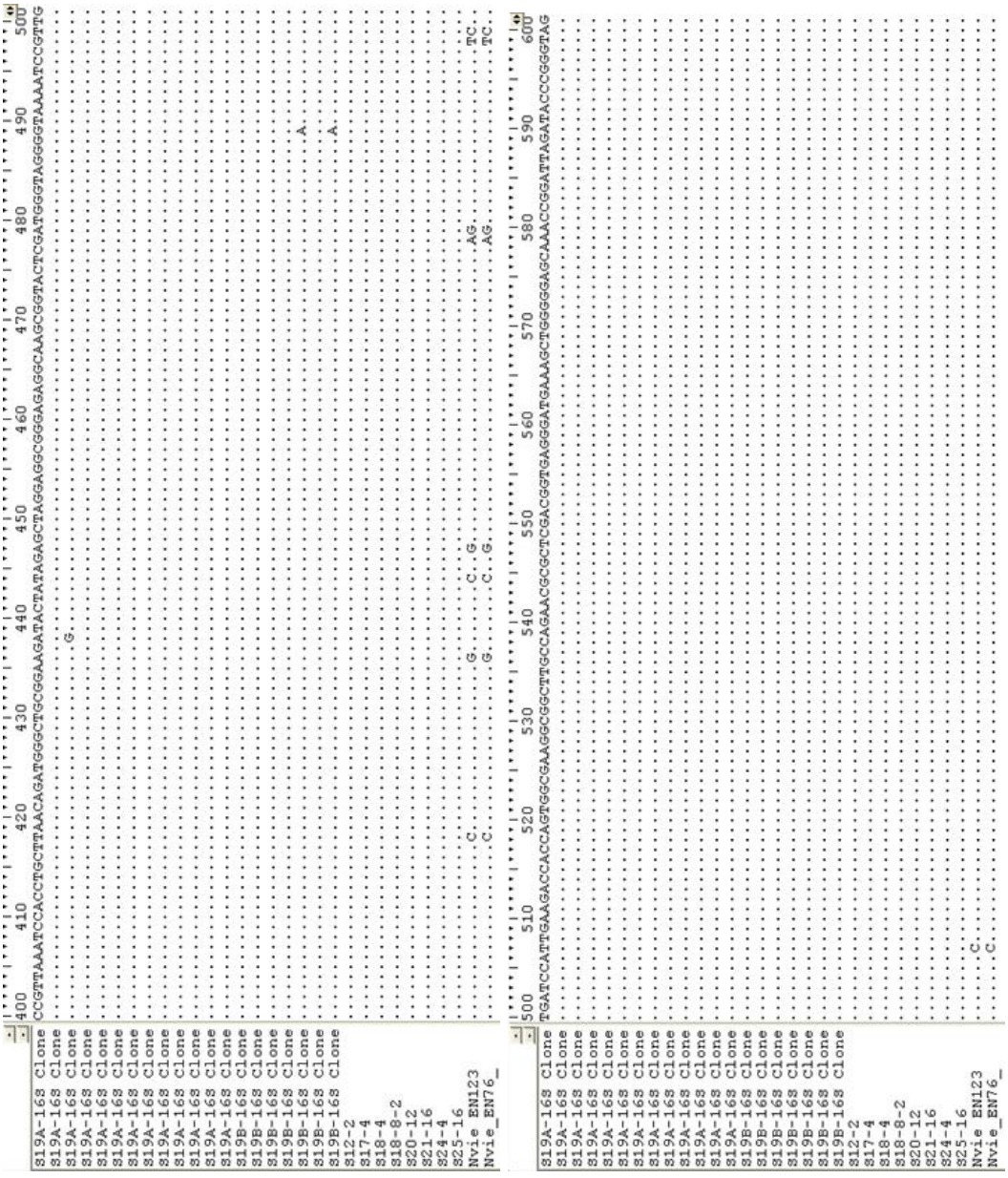
(a) (b)

Figure 31: 16S rRNA sequence alignment (BioEdit): 20 16S rRNA clones sequences and eight 16S rRNA amplicon sequences of *Ca. N. arctica* and 16S rRNA sequences of *Ca. N. viennensis* EN67 and EN123 were aligned in BioEdit.



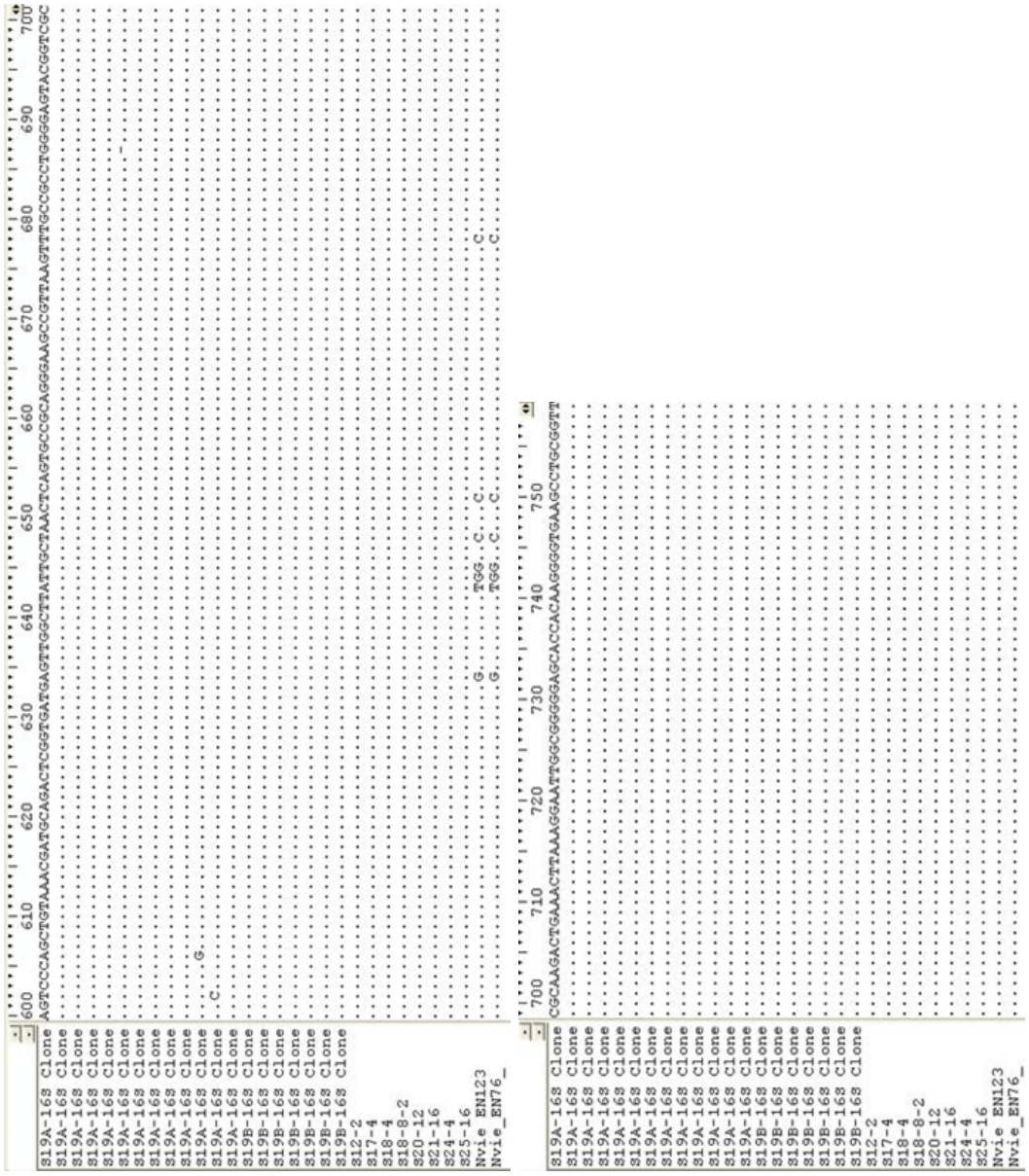
(a) (b)

Figure 32: 16S rRNA sequence alignment (BioEdit) continued.



(a) (b)

Figure 33: 16S rRNA sequence alignment (BioEdit) continued.



(a) (b)

Figure 34: 16S rRNA sequence alignment (BioEdit) continued.

A.2 WISSENSCHAFTLICHER LEBENSLAUF

ANNA ZAPPE

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Leistungsstipendium für das Diplomstudium Ökologie

VORTRÄGE

März 2012 Science Day des Fakultätszentrums Ökologie

Vortrag beim Science Day des Fakultätszentrums Ökologie.
Titel: Cultivation of Ammonia Oxidizing Archaea from Arctic Soils

PUPLIKATIONEN

März 2013 Nitrification rates in Arctic soils are associated with functionally distinct populations of ammonia-oxidizing archaea

The International Society for Microbial Ecology Journal, Nitrification rates in Arctic soils are associated with functionally distinct populations of ammonia-oxidizing archaea.
Authors: Ricardo J Eloy Alves, Wolfgang Wanek, Anna Zappe, Andreas Richter, Mette M Svenning, Christa Schleper and Tim Urich