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„Cultivation and Characterization of an Extremely Thermophilic Ammonia Oxidizing Archaeon"

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Abstract

Without nitrogen, life on Earth would not be possible. Therefore, the nitrogen cycle has always been of special interest and has been investigated and updated ever since. Still, many parts of it remain unclear. In this study, extremely thermophilic archaea from hot springs were cultivated in actively nitrifying enrichment cultures that produced nitrite and smaller amounts of nitrate from ammonia. Their physiology with the main focus on the ammonia oxidizing Thaumarchaeon *Candidatus* Nitrosocaldus cavascurensis was characterized. Fluorescence *in situ* hybridization (FISH) confirmed that this organism has a small coccoid shape. It grows optimally around 70°C. Although higher temperatures were tolerated, this temperature appeared to be most favorable to obtain stable cultures over longer time periods. Urea was an alternative energy source for the organism. Different cultivation conditions and inhibitors were tested in order to improve the enrichment of the ammonia oxidizer. A second archaeon of the novel phylum Aigarchaeota was detected in the cultures by molecular techniques. FISH analyses identified rod-shaped cells of this organism. It grew better under nitrite oxidizing conditions without the ammonia oxidizer, but its energy metabolism remained unclear.

1. Introduction

1.1 Archaea and the nitrogen cycle

Archaea are one of the three domains of life: Bacteria, Archaea and Eukarya. This tripartite system was proposed by Carl Woese in 1990 (Woese, Kandler and Wheelis, 1990). So the former bipartite system consisting of Prokaryotes and Eukaryotes was altered by the separation of the Prokaryotes, containing until then Bacteria and Archaebacteria (later Archaea) (Woese and Fox, 1977; Woese et al., 1990). The earlier classification into Prokaryotes and Eukaryotes was mainly based on cell morphology and structure, until 1965, when Emile Zuckerkandl and Linus Pauling proposed to classify organisms not only by their morphology or metabolisms, but by the study and comparison of their genome or specific gene sequences (Zuckerkandl and Pauling, 1965). In light of the phylogenetic studies then performed by Carl Woese and colleagues (Woese and Fox, 1977; Woese et al., 1990) it became clear, how deeply Bacteria and Archaebacteria were separated phylogenetically. Archaea in turn are divided into various phyla (like Bacteria) (for recent review see Spang et al. Science 2017) and play key roles in the main biogeochemical cycles like the carbon and nitrogen cycles - in both aquatic and terrestrial ecosystems (Offre et al., 2013). Many organisms with different energy metabolisms and physiologies have been cultivated and characterized. Nevertheless, the cultivation of novel archaeal species often remains tricky, especially if you want to obtain a pure culture. Therefore, a lot of information we know about archaea is based on genomic studies, not on cultivated strains. For obtaining a pure culture of a specific archaeon the use of antibiotics is quite popular because most antibiotics are ineffective against archaea due to the their lack of peptidoglycan in the cell wall (Khelaifia and Drancourt 2012) and their differences in ribosomes and other cellular proteins.

The nitrogen cycle is known to be one of the main cycles on Earth and plays a key role in both marine and terrestrial ecosystems. Generally, the nitrogen cycle consists of nitrification, both the aerobic and anaerobic parts of denitrification, nitrogen fixation and anammox (anaerobic ammonia oxidation). This study involved organisms of the aerobic part of the nitrogen cycle, the nitrification, which consists of two steps: first, the oxidation of ammonia to nitrite and second, the oxidation of

nitrite to nitrate.

Nitrification is a main step of the global nitrogen cycle. Whereas for its first step, the oxidation of ammonia to nitrite both ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) are known, its second step, the oxidation of nitrite to nitrate is by now only known to be performed by bacteria (NOB). NOB are divided up into three genera: *Nitrospira*, *Nitrobacter* and *Nitrotoga* (Nowka, Daims, Spieck, 2015). Although AOA, AOB and NOB coexist in a wide range of habitats where nitrification takes place, no thermophilic bacterial ammonia oxidizer and no nitrite oxidizer are known yet, only thermophilic ammonia-oxidizing archaea. The nitrogen cycle has been fundamentally revised in the past years, especially after the discovery of both anaerobic ammonia oxidation and archaeal ammonia oxidation. Earlier, ammonia oxidation was only known to be performed by *Beta*- and *Gammaproteobacteria*, bacteria that thrive chemolithoautotrophically under aerobic conditions (Purkhold et al.; 2000, Purkhold et al.; 2003; Kim et al. 2012). For a long time, archaea, on the other hand, have been seen to be obligate extremophiles, adapted to conditions where other organisms could not survive. With the discovery of mesophilic archaea as well as the improvement of molecular techniques, the omnipresence of archaea was discovered (Schleper et al., 2007). Archaea can be found in almost every environment on Earth at considerable numbers. In most habitats ammonia oxidizing archaea (of the phylum Thaumarchaeota, see below) even outnumber ammonia oxidizing bacteria (Leininger et al., 2006; He et al., 2007; Sauder et al., 2012).

1.2 Thaumarchaeota

Thaumarchaeota is a recently described phylum branching deeply in the domain *Archaea*. It belongs to the so-called TACK-superphylum (short for **T**haumarchaeota, **A**igarchaeota, **C**renarchaeota, **K**orarchaeota) (Guy and Ettema, 2011). All thaumarchaeota known to date are characterized by growing via the oxidation of ammonia to nitrite. This was based on rRNA gene phylogeny and protein phylogenies, as well as the realisation that thaumarchaeota have a unique set of information processing genes. The *amoA*-gene is used as biomarker for detecting ammonia oxidizers. It encodes the subunit A of ammonia monooxygenase that catalyses the first step of ammonia oxidation (Rotthauwe et al., 1997; Francis et al.,

2005). Based on quantitative studies of 16S rRNA and *amoA* genes, ammonia-oxidizing archaea (AOA) seem to not only be dominant, but also play a key role, especially in extreme ecosystems and in the ocean (Francis et. al, 2005; Leininger et al., 2006; Zhang et al., 2008; Jiang et al., 2010). Based on the genomes of *Cenarchaeum symbiosum* and *Nitrosopumilus maritimus* the phylum Thaumarchaeota was proposed (Preston et al., 1996; Könneke et al., 2005; Spang et al., 2010; Walker et al., 2010; Brochier-Armanet et al., 2008). In addition, the two organisms seemed to be representatives of a big widespread group and by now, the phylum Thaumarchaeota consists of four orders, *Nitrososphaerales*, *Nitrosopumilales*, *Nitrosotaleales* and *Nitrosocaldales*.

In 2005, the first pure culture of AOA was described, *Nitrosopumilus maritimus* (Könneke et al., 2005). Before the creation of the phylum Thaumarchaeota, signatures like 16S rRNA and *amoA*-genes were discovered in the environments with molecular techniques, but not via cultivation. These representatives were originally assigned to Crenarchaeota and were divided up into two clades named Group 1 crenarchaeota: Group 1.1a (marine) and Group 1.1b (soil) (Könneke et al., 2005; de la Torre et al., 2008; Spang et al., 2010; Walker et al., 2010; Brochier-Armanet et al., 2008). Only later became those formerly known groups associated with ammonia oxidation.

Ammonia-oxidizing archaea can be found in various habitats, both aquatic and terrestrial (Stahl and de la Torre, 2012). *Nitrosotalea devanaterra*, for instance, grows in soils at pH 4 or below (Lehtovirta-Morley et al., 2011). AOA are dependent on low ammonia concentrations and do not tolerate higher ones, whereas AOB, on the other hand, only grow at higher concentrations (Di et al., 2009; Di et al., 2010; Verhamme et al., 2011). Still, there are exceptions like *Nitrososphaera viennensis*. While cultivation, *N.viennensis* still grew at ammonia concentrations of 10-15 mM with the effect of an increasing lag phase (Tourna et al., 2011). Thus, AOA seem generally to be better adapted to pristine habitats, whereas AOB rather thrive in agricultural environments that obtain fertilizers. Via molecular marker genes, like the *amoA*-gene, various AOA were also detected in hot environments, for instance in hot springs (Reigstad et al., 2008; Zhang et al., 2008; Jiang et al., 2010; Cole et al., 2013). But although there is already a broad range of ammonia-oxidizing archaea described and the existence of hyperthermophilic ones known, only one was obtained in an enrichment culture until recently: *Candidatus* Nitrosocaldus

yellowstonii, which grows at a temperature of 74°C (de la Torre et al., 2008).
Thermophilic and hyperthermophilic organisms seem to play a key role in the early history of life and its further evolution. Based on 16S rRNA analysis to reconstruct the tree of life, (hyper-)thermophilic archaea form most of the basal lineages, a finding that was used to formulate the hypothesis that mesophilic archaea evolved from thermophilic ancestors. This assumption can also be expanded to the phylum Thaumarchaeota. Based on 16S rRNA analysis *Candidatus* Nitrosocaldus yellowstonii formed a sister group to mesophilic AOA (de la Torre et al., 2008), indicating that a hyperthermophilic AOA could be the common ancestor of those two sister groups. Thermophilic AOA like *Candidatus* Nitrosocaldus cavascurensis, as the organism investigated in this study, could therefore contribute to a better understanding of the early evolution of ammonia-oxidizing archaea.

1.3 Cultivation of a thermophilic ammonia-oxidizing archaeon

The cultivation of an ammonia-oxidizing archaeon has its own challenges. The first pure culture of ammonia-oxidizing archaea was obtained for the marine archaeon *Nitrosopumilus maritimus* (Könneke et al., 2005). A highly enriched culture of this archaeon (90%) was obtained by a series of transfers with a 10% inoculum after half a year, a pure culture was obtained by a combination of end-point dilutions, the use of antibiotics and filtration. In addition to the basal medium, only 1mM bicarbonate and 0.5-1mM ammonia were added as carbon and energy sources as *Nitrosopumilus maritimus* was supposed to grow chemolithoautotrophically (Könneke et al., 2005). A similar strategy, only with an extra addition of pyruvate, was used for the isolation of *Nitrososphaera viennensis*, an archaeon found in Viennese soil (Tourna et al., 2011; Stieglmeier et al., 2014). Both *Nitrosopumilus maritimus*, with optimal growth rates at a temperature of 28°C, and *Nitrososphaera viennensis*, with optimal growth rates at 42°C, are mesophilic ammonia-oxidizing archaea (Stieglmeier et al., 2014).
AOA were found in hot springs by the use of molecular marker genes (Reigstad et al., 2008, Zhang et al., 2008; Jiang et al., 2010), even before the cultivation of *Candidatus* Nitrosocaldus yellowstonii from a hot spring in Yellowstone National Park (USA). Until then the temperature limit for nitrification was in a rather moderate range, for both bacteria and archaea. So, for instance, there were bacteria known

that perform nitrification in culture at a temperature of 55°C (Lebedeva et al., 2005). However, with the discovery of *Candidatus* Nitrosocaldus yellowstonii, it was extended to higher temperatures. Its enrichment cultures were incubated at temperatures between 60-80°C, resulting in an optimal cultivation temperature of 65-74°C and a still measurable nitrite production down to 60°C. Like *Nitrosopumilus maritimus* and *Nitrososphaera viennensis*, also *Candidatus* Nitrosocaldus yellowstonii grows chemolithoautotrophically by oxidizing ammonia aerobically, apparently its sole way of gaining energy. Likewise, a regular transfer of 10% inoculum to a fresh medium seemed to be most successful for the enrichment culture as soon as 90% of the provided ammonia was turned into nitrite (de la Torre et al., 2008).

In this study, the morphology and cultivation of the extremely thermophilic ammonia oxidizing archaeon *Candidatus* Nitrosocaldus cavascurensis was investigated and its characteristics compared with those of *Candidatus* Nitrosocaldus yellowstonii. Since *Candidatus* Nitrosocaldus yellowstonii is the only cultivated thermophilic ammonia-oxidizing archaea until now, cultivation methods for *Candidatus* Nitrosocaldus cavascurensis were chosen similarly. Like with enrichment strategies, that led to the isolation of a pure culture of *Nitrosopumilus maritimus* and *Nitrososphaera viennensis*, different combinations for the enrichment cultures of *Candidatus* Nitrosocaldus cavascurensis were tested with the goal of obtaining a pure culture.

1.4 Aigarchaeota

The phylum Aigarchaeota was recently described based on the metagenomic analysis (Nunoura et al., 2005) of *Candidatus* Caldiarchaeum subterraneum. Its phylogenetic placement was rather difficult. Although the genome bore a resemblance to those of *Nitrosopumilus maritimus* and *Cenarchaeum symbiosum*, both belonging to the phylum Thaumarchaeota, its genome had unique traits and was not comparable to any other organism (Nunoura et al., 2005; Nunoura et al., 2011, Takami et al., 2015). One of those unique traits, for instance, was an intron in the 16S rRNA gene. Within the domain Archaea, such introns have so far only been found in the orders Desulfurococcales and Thermoproteales (Jay and Inskeep, 2015). Nevertheless, the status as a separate phylum of Aigarchaetoa has been

debated as the organism branched deeply within the phylum Thaumarchaeota (Nunoura et al., 2011; Rayman et al., 2014). Like Thaumarcheota, Aigarchaeota also belong to the so-called TACK-superphylum (Guy and Ettema, 2011).

Ancestors of the TACK-superphylum including Aigarchaeota were just recently discussed to have played a key role in the origin of Eukaryota. This hypothesis is based on the circumstances that solely members of the TACK-superphylum and eukaryotes have certain traits in common, not found in bacteria or other archaea. Such traits include for instance factors involved in cytokinesis (Guy and Ettema, 2011). Although Aigarchaeota seem to be related to Thaumarchaeota (Nunoura et al., 2011; Rayman et al., 2014), no genes for nitrification were discovered (Takami et al., 2015). In general, their energy metabolism remains unknown, because no organism of this phylum has yet been cultivated. Until now members were usually discovered in hydrothermal environments, although there are also moderate ones known (Hirayama et al., 2005; Nunoura et al., 2005; Nunoura et al., 2011). They live under aerobic conditions and according to their genomes gain energy chemoorganoheterotrophically with the possibility of autotrophy (Beam et al., 2016). Via fluorescence *in situ* hybridization it was shown that until now known aigarchaeota have a filamentous shape (Beam et al., 2016). In this study, the morphology and cultivation of an until now unknown aigarchaeote was investigated and its characteristics compared with *Candidatus* Caldiarchaeum subterraneum.

1.5 Aims of this study

The aim of this study was the characterization of hyperthermophilic archaea from hot springs, with a special focus on ammonia-oxidizing archaea. Enrichment cultures with nitrification activities had earlier been obtained from a hot spring of the island Ischia and have been cultivated in the lab at temperatures around and above 70°C ever since. 16S rRNA and *amo*A-gene analyses had shown earlier that the enrichment cultures contained an ammonia-oxidizing archaeon closely related to *Candidatus* Nitrosocaldus yellowstonii. Thus, it obtained the tentative name *Candidatus* Nitrosocaldus cavascurensis (named after the cave Cava Scura, Italy). Other interesting observations were measurable, like nitrite oxidation activity and the presence of 16S rRNA genes of Aigarchaeota, indicating that this group might also grow actively in the cultures.

The aim of this study was to better characterize these two thermophilic archaea in the enrichment cultures, in particular:

- to test a variety of different cultivation conditions and identify those that favor growth of the ammonia oxidizing archaea and/or the Aigarchaeote
- to determine the temperature optimum of the AOA activity
- to use fluorescence *in situ* hybridization for identification of the AOA cells and Aigarchaeota
- to determine the total community compositions in the enrichment cultures with 16S rRNA-based analyses

2.Materials & Methods

2.1 Cultivation

2.1.1 Solutions and media

Fresh water medium (FWM) was used for the cultivation of the so-called Scu-cultures. It consisted of NaCl 1gL^{-1}; $MgCl_2$ * 6 H_2O 0.4gL^{-1}; CaCl * 2 H_2O 0.1gL^{-1}; KH_2PO_4 0.2gL^{-1} and KCl 0.5gL^{-1}. It was based on the cultivation of *Candidatus* Nitrosocaldus yellowstonii (Torre et al., 2008) with the only alteration by the addition of KH_2PO_4. It was autoclaved for sterilization. The medium was stored at 4°C and only opened in the Laminar Flow in order to prevent contamination.

Modified non-chelated trace elements mixture consisted of MilliQ water 987 ml; HCl (conc. ~12.5M) 8 ml (100 mM); H_3BO_3 30 mg (0.5 mM); $MnCl_2$ x 4 H_2O 100 mg (0.5 mM); $CoCl_2$ x 6 H_2O 190 mg (0.8 mM); $NiCl_2$ x 6H_2O 24 mg (0.1 mM); $CuCl_2$ x 2 H_2O 2 mg (0.01 mM); $ZnSO_4$ x 7 H_2O 144 mg (0.5 mM); Na_2MoO_4 x 2 H_2O 36 mg (0.15 mM). The solution was autoclaved and stored in the dark at 4°C for max. 6 months.

Vitamin solution consisted of Biotin 0.02 gL^{-1}; Folic acid 0.02 gL^{-1}; Pyridoxine HCl 0.10 gL^{-1}; Thiamine HCl 0.05 gL^{-1}; Riboflavin 0.05 gL^{-1}; Nicotinic acid 0.05 gL^{-1}; DL-Pantothenic acid 0.05 gL^{-1}; P-Aminobenzoic acid 0.05 gL^{-1}; Choline chloride 2.00 gL^{-1}; Vitamine B12 0.01 gL^{-1}. The pH was adjusted to 7 with KOH. The solution was sterilized by filtration (0.2µm) and kept in the dark at 4°C for max. 6 months.

FeNaEDTA solution (7.5 mM) consisted of FeNaEDTA 2753 mgL^{-1} and was sterilized by filtration. It was stored in the dark at 4°C. The pH was adjusted to 7 with KOH and the solution was sterilized by filtration (0.2µm) and stored at 4°C for max. 6 months.

$NaHCO_3$ solution (1 M) (M=84g/mol)

4.2g $NaHCO_3$ were dissolved in 50ml of autoclaved MilliQ water.

NH_4Cl (1M)

A stock solution of 1M was prepared, autoclaved and stored at 4°C.

1M $NaNO_2$ (M=69g/mol)

69mg $NaNO_2$ were dissolved in 1ml MilliQ water, sterilized by filtration (0.2µm) and stored at 4°C.

Final composition of the medium

The final composition of the medium (18ml media + 2ml culture) consisted of 18ml FWM; 20µL NH_4Cl (1mM); 20µL modified trace elements; 20µL FeNaEDTA; 20µL vitamin solution and 40µL sodium bicarbonate (2mM). The pH was at approximately 7-7.5 and there was no need of adapting it, as well as no buffer was used. The medium was prepared in 120ml serum bottles containing 18ml media, 2ml of culture and 100ml of headspace. The final composition was prepared in the sterile laminar-air-flow-chamber FASTER BH-EN 2005 (Szabo-Scandic).

2.1.2 Incubation temperatures

Different incubation temperatures for the cultures in a range between 55°C to 80°C were tested in the following steps: 55°C, 60°C, 65°C, 70°C, 72°C, 74°C, 76°C, 80°C.

2.1.3 Filtration

Filtration was performed with a 1.2µm filter. The 2ml culture was filtered right before inoculation. Thereby, two different methods were performed: filtration once during inoculation and filtration every time during inoculation when being transferred to a fresh medium.

2.1.4 Heat shocks

After inoculation the cultures were shocked for 2 hours at the temperatures 78°C, 80°C, 82°C, 84°C, 86°C, 88°C or 90°C, before being incubated at 70°C again.

2.1.5 Antibiotics

Various antibiotics were tested in combination with different supplements according to the following table. They were additionally inserted, the media composition remained the same.

Table 1 Overview of the antibiotics and supplements supplied to fresh water medium used in this study

antibiotics + supplements
100µg/ml Gentamicin + 0.1mM Pyruvate
50µg/ml Gentamicin, 50µg/ml Sulfamethazine + 0.1mM Pyruvate
100µg/ml Sulfamethazine + 0.1mM Pyruvate
100µg/ml Gentamicin + 0.1mM Oxaloacetate
50µg/ml Gentamicin, 50µg/ml Sulfamethazine + 0.1mM Oxaloacetate
100µg/ml Sulfamethazine + 0.1mM Oxaloacetate
100µg/ml Gentamicin + 0.1mM Glyoxylate
50µg/ml Gentamicin, 50µg/ml Sulfamethazine + 0.1mM Glyoxylate
100µg/ml Sulfamethazine + 0.1mM Glyoxylate
100µg/ml Rifampicin
100µg/ml Rifampicin + 0.1mM Pyruvate
100µg/ml Rifampicin + 0.1mM Glyoxylate
100µg/ml Rifampicin + 0.1mM Oxaloacetate
100µg/ml Novobiocin
100µg/ml Novobiocin + 0.1mM Pyruvate
100µg/ml Novobiocin + 0.1mM Glyoxylate
100µg/ml Novobiocin + 0.1mM Oxaloacetate

2.1.6 Nitrite oxidizer cultures

The media composition of the so-called NO-cultures (nitrite oxidizers cultures) was as followed: 18ml FWM; 20µl $NaNO_2$ (1mM); 20µl modified trace elements; 20µl FeNaEDTA ; 20µl vitamin solution and 40µl sodium bicarbonate (2mM). Just as for the ammonia oxidizing cultures 20ml of final composition (culture+media) with a 10% inoculum was prepared in 120ml serum bottles. The original cultures were kept at 70°C, too. The following temperatures were tested for cultivation: 55°C, 60°C, 65°C, 70°C, 74°C and 77°C. A combination of 100µg/ml Sulfamethazine + 0.1mM Pyruvate was also tested, as well as 1.2µm-filtration during inoculation. Concentrations of 1mM $NaNO_2$ and 0.5mM $NaNO_2$ were investigated.

2.2 Following Growth by Photometric Measurements

The cultures were measured twice a week via photometric measurements to investigate their consumption of ammonia and their production of nitrite and nitrate,

respectively. The NO-cultures were tested for their nitrite consumption and nitrate production to get an overview of the growth of the cultures. Generally, all cultures were transferred to fresh media as soon as a production level of more than 700µM of the end product was achieved.

2.2.1 Ammonia measurement

For the measurement of ammonia two solutions were required. Those were prepared freshly on the same day or were used within a maximum of 2-3 days. The general requirements for solutions will be described subsequently. The exact amount of required solutions depended on the amount of samples and was therefore calculated before every measurement. For the colour reagent 4.25g sodium salicylate ($C_7H_5NaO_3$) and 31.95mg sodium nitroprusside dihydrate were dissolved in 25ml MilliQ water. The solution was mixed with 0.3M NaOH and MilliQ water (1:1:1). The oxidation solution consisted of 0.04g dichloroisocyanuric acid sodium salt dihydrate dissolved in 40ml of MilliQ water. For the standard curve 1mM NH_4Cl was used as the calibration solution. The exact concentration of the different steps of the standard curve were as followed:

Table 2 Different concentration steps of the standard curve for the measurement of ammonia

standard	µM	FWM (in µL)	NH_4Cl (in µL)
1	1000	0	200
2	800	40	160
3	600	80	120
4	500	100	100
5	400	120	80
6	300	140	60
7	200	160	40
8	100	180	20
9	0	200	0

A mixture of 200µl of sample or standard, 400µl FWM, 300µl colour reagent and 120µl oxidation solution was prepared in Eppendorf-tubes. The tubes were shaken manually and kept for at least 30min at room temperature. For the measurement 200µl of the solution in each tube was pipetted in one well of a microtiter plate and measured via photometer (Tecan Sunrise) at an absorbance of 660nm.

2.2.2 Nitrite measurement

For the measurement of nitrite, a solution of sulfanilamide/NED was prepared in advance according to the following protocol: Under constant stiring, 150ml ortho-phosphoric acid was dissolved in 700ml MilliQ water and mixed with 10g sulfanilamide. To dissolve it more easily, the solution was slightly heated. 0.5g alpha-naphtylethylendiamindihydrochloride was added and everything was filled up to 1L with MilliQ water. As the solution is very light sensitive, light was prevented as much as possible. For storage it was kept at 4°C and used for several weeks. For the measurement Eppendorf-tubes were filled with 780µl FWM, 20µl sample and 200µl sulfanilamide/NED reagent. The standard curve was prepared according to the following table, 1mM $NaNO_2$ was used as calibration solution:

Table 3 Different concentration steps of the standard curve for the measurement of nitrite, prepared in Eppendorf-tubes

standard	µM	FWM (in µL)	1mM NaNO2 (in µL)	FWM (in µL)
1	1000	780	20	0
2	800	784	16	20
3	600	788	12	40
4	500	790	10	50
5	400	792	8	60
6	300	794	6	70
7	200	796	4	80
8	100	798	2	90
9	0	800	0	100

The tubes were shaken manually and incubated for at least 10min in the dark at room temperature. 200µl of each tube were pipetted in a microtiter plate and the absorbance was measured at 545nm (Tecan Sunrise).

2.2.3 Nitrite measurement (second option)

The solutions were directly mixed in a microtiter plate. Each well contained 20µl of the sample, 180µl FWM and 100µl sulfanilamide/NED. The standards were prepared according to the following table. The calibration solution was 0.1mM $NaNO_2$.

Table 4 Different concentration steps of the standard curve for the measurement of nitrite, prepared directly in a microtiter plate

standard	µM	0.1mM NaNO2 (in µL)	FWM
1	100	100	0
2	80	80	20
3	60	60	40
4	50	50	50
5	40	40	60
6	30	30	70
7	20	20	80
8	10	10	90
9	0	0	100

100µl FWM and 100µl sulfanilamide/NED were added to each well. The plate was covered and incubated for at least 10min in the dark at room temperature and measured at an absorbance of 545nm (Tecan Sunrise). The samples were diluted 1:5 (20µl sample + 80µl FWM), which had to be considered in the calculations afterwards.

2.2.4 Nitrate measurement (quick analysis)

For the measurement of nitrate, two reagents were prepared in advance. Reagent 1 consisted of 400mg VCl_3 dissolved in 50ml of 1M HCl and was filtered afterwards. Reagent 2 consisted of a 1:1-mixture of 50mg N-naphtylethylenediamine dissolved in 250ml of MilliQ water and 5g sulfanilic acid in 500ml of 3M HCl. The measurement was directly prepared in a microtiter plate. Each well contained 20µl sample and 80µl FWM or 100µl standard solution, additionally 100µl of reagent 1 and 100µl of reagent 2 were added. The calibration solution consisted of 0.1mM $NaNO_3$ and the standard was prepared according to the following table:

Table 5
Different concentration steps of the standard curve for the measurement of nitrate, prepared directly in a microtiter plate

standard	µM	$NaNO_3$ (in µL)	FWM
1	100	100	0
2	80	80	20
3	60	60	40
4	50	50	50
5	40	40	60
6	30	30	70
7	20	20	80
8	10	10	90
9	0	0	100

The plate was incubated for at least 1hour at 37°C to achieve a colour change. The absorbance was measured at 540nm (Tecan Sunrise). Again, the sample was diluted 1:5, which had to be considered in the calculations. Due to the conversion of nitrate to nitrite, caused by the application of VCl_3, not only nitrate is measured but also nitrite. To get the pure amount of nitrate in the sample, the value of the nitrite measured beforehand has to be subtracted from the value measured here.

2.3 Cell counting & calculation

The cell counting was performed with the Neubauer improved cell counting chamber (Superior Marienfeld Germany, depth = 0.1mm). A sample of 5µl was taken from each culture and applied to the counting chamber. For scanning, a Nikon Eclipse 50i Microscope was used.

The average growth rate µ (day^{-1}) was calculated using the following formula:

$$\mu = (\ln xt - \ln x0) / (t - t0)$$

and the average doubling time t_d (in days) with the formula:

$$t_d = \ln 2 / \mu$$

2.4 DNA-extraction

The DNA-extraction was performed according to the lab-protocol for enriched cultures. The following solutions were prepared in advance:

DEPC-H_2O (Diethylpyrocarbonate-H_2O) was diluted in MilliQ water with a dilution of 1:1000. The Eppendorf-tubes were shaken vigorously (manually) and incubated at room temperature for 3-4hours, autoclaved and then stored at -20°C.

SDS Extraction buffer consisted of 8.18g NaCl ; 2.52 g $Na_2 SO_3$; 20 ml Tris/HCl (1M); 20 ml EDTA (0.5M); 20 ml SDS (10%) and was filled up to 200ml with MilliQ water. The solution was treated with 0.1% DEPC over night and sterilized by autoclavation afterwards.

Polyethylenglycol solution (PEG) consisted of 18.7g NaCl and 60g PEG. This was filled up to 200ml with MilliQ water, treated with 0.1% DEPC over night and sterilized by autoclaving.

For DNA-extraction 1.5ml of an enriched culture was taken and centrifuged (13,2 krpm, 4°C, 10min, Eppendorf Centrifuge 5415R). In the meantime, the SDS-buffer was pre-heated to 70°C until the solution was clear. The supernatant was discarded and the pellet was kept on ice if not immediately resuspended in 0.5ml SDS extraction buffer. The entire mixture was transferred to Lysing Matrix B tubes. For every DNA-extraction a negative control was provided that only contained SDS extraction buffer. The Lysing Matrix B tubes contained 0.5g of Bulk B Beads per tube. The following steps were performed under the fume hood. To each tube 0.5ml Phenol/Chloroform/Isoamylalcohol (25:24:1) was added and shaken in a Fast prep machine (MP FastPrep-24™) for 30 seconds at speed 4 so lysis could take place. The tubes were put on ice for 2 minutes before being centrifuged again (13,2 krpm, 4°C, 10min, Eppendorf). The tubes were kept on ice while the top phase was transferred to a new tube. To the new tube 0.5ml of Chloroform/Isoamylalcohol (24:1) was added, approximately the same amount of liquid that the tube already contained. The tubes were shaken manually for several seconds and centrifuged again (13,2 krpm, 4°C, 10min, Eppendorf), before being put on ice. The top phase was again transferred to a new tube. For a quick check, the tubes were spun shortly to control if they only contained one phase. If that was the case, 1µl of glycogen was added and mixed by pipetting. For the following steps no fume hood was required. 1ml PEG, around twice the amount of liquid that the tubes contained, was added. The tubes were again shaken manually for several seconds for DNA precipitation and were either incubated for 2hours at room temperature or overnight at 4°C. Afterwards, the tubes were centrifuged (13,2 krpm, 4°C, 10min, Eppendorf) and the supernatant discarded. The pellet was washed with 1ml 70% ice-cold ethanol. Mostly, no pellet was seen which is normal for the Scu-cultures. The pellet was resuspended by pipetting and the tubes centrifuged (13,2 krpm, 4°C, 10min, Eppendorf). The supernatant was discarded again and the tubes spun for 10 seconds in order to be able to discard as much supernatant as possible. The pellet was dried via SpeedVac (3min/30°C/AQ, Eppendorf Concentrator *plus*). If the pellet wasn't dry afterwards, this step was repeated, but it was never dried for longer than

1-2 minutes to prevent destroying the pellet. It was resuspended in 30µl of DEPC-H_2O by pipetting and incubated for at least 30 minutes at room temperature before being stored at -20°C.

2.5 Polymerase chain reaction (PCR)

Primers that were used:

Archaea: 16S Archaeal primer for general Archaea: 109F/Arch 1492R

A109F: ACK GCT CAG TAA CAC GT (Reference:Großkopf et al., 1998)

A1492R: GGT TAC CTT GTT ACG ACT T (Standard Vector Primer, Eurofins)

Bacteria: 16S Bacteria primer for general Bacteria: 27F/Bac 1492R

Eubac 27F: AGA GTT TGA TCC TGG CTC AG (Reference: Lane et al., 1991)

1492R: GGT TAC CTT GTT ACG ACT T (Reference: Lane et al., 1991)

Thermophilic AOA: ThAOA 277F/632R

ThAOA-277F: CCA TAY GAC TTC ATG ATA GTC (Reference: Alves et al., (unpublished))

ThAOA-632R: GCA GCC CAT CKR TAN GTC CA (Reference: Alves et al., (unpublished))

AOA: amoA 19F/amoA 638R

Cren-amoA19F: ATG GTC TGG YTW AGA CG (Reference: Leininger et al., 2006)

Arch-amoA-638R: GCR GCC ATC CAT CTR TA (Reference: Alves et al., 2013)

Aigarchaeota: 16S Aig Fw/ 16S Aig Rv

16S_Aig_Fw: CGGGTTGATGGCTCGGCCTCCTACC (self-designed)

16S_Aig_Rv: GGCCCATGGAAAACAGCAGCCCTCAA (self-designed)

AOB: 16S AOB amoA1F*/ amoA 2R

amoA-1F * / amoA-1F(J): GGG GHT TYT ACT GGT GGT (Reference: Stephen et al.,1999)

amoA-2R: CCC CTC KGS AAA GCC TTC TTC (Reference: Rotthauwe et al., 1997)

NOB: Nitrospira NxrB 169F / 638R

nxrB169f: TAC ATG TGG TGG AAC A (Reference:Pester et al., 2013)

nxrB638r: CGG TTC TGG TCR ATC A (Reference: Pester et al., 2013)

NOB: Nitrobacter Nxr1F / Nxr2R

NxrB-1F: ACG TGG AGA CCA AGC CGG G (Reference: Vanparys et al., 2007)

NxrB-1R: CCG TGC TGT TGA YCT CGT TGA (Reference: Vanparys et al., 2007)

2.5.1 Primer design

The primer design was performed with the help of Gene Runner (Version 3.01), a program that analyses DNA sequences. Potential sequences were between 19-21bp long and checked for their filter temperature/ melting temperature, tendencies to form hairpin/ dimers/ bulge loops/ internal loops, RNA hybrids, mismatches and GC+AT temperature and annealing temperature. They were checked by BLAST and ordered from the company Eurofins.

For the Aigarchaeota both forward and reverse primer were designed on the flanks of the 16SrRNA, so they cover a length of 2327bp and therefore the entire 16S rRNA including the intron in-between.

Aig_Fw
5‘-CGGGTTGATGGCTCGGCCTCCTACC -3‘

Aig_Rv
5’- GGCCCATGGAAAACAGCAGCCCTCAA -3’

flank (150bp) 16S rRNA + Intron flank (150bp)

2.5.2 PCR

A mastermix for the PCR was calculated and prepared for each primer pair. For one sample the compostition consisted of:

0.15µl	Taq polymerase
5µl	5x Flexi buffer (green)
2µl	$MgCl_2$ (25mM)
0.5µl	10mM dNTP
0.25µl	BSA (0,2mg/ml)
1.25µl	primer F (10µM)
1.25µl	primer R (10µM)
12.6µl	H_2O
2µl	DNA
Total:	**25µl**

PCR-Programs

16S Archaea, 16S Bacteria general

95°C	5min	
94°C	30sec	35x
55°C	30sec	
72°C	2min	
72°C	10min	
4°C	pause	

ThAOA 277F/632R

95°C	10min	
95°C	30sec	35x (25x)
55°C	45sec	
72°C	45sec	
72°C	10min	
4°C	pause	

AOA: amoA 19F/amoA 638R

95°C	5min	
94°C	30sec	35x
55°C	45sec	
72°C	45sec	
72°C	10min	
4°C	pause	

AOB amoA1F*/ amoA 2R

95°C	5min	
94°C	30sec	35x
55°C	30sec	
72°C	30sec	
72°C	10min	
4°C	pause	

NOB Nxr1F / Nxr2R;

95°C	5min	
95°C	40sec	35x
55°C	40sec	
72°C	1min	
72°C	10min	
4°C	pause	

NOB NxrB 169F / 638R

95°C	5min	
95°C	40sec	35x
55°C	40sec	
72°C	1min	
72°C	10min	
4°C	pause	

Aig. 16S Aig Fw/ 16S Aig Rv

95°C	10min	
94°C	30sec	10x
55°C	30sec	
72°C	2min	
92°C	30sec	15x
55	45sec	
72	45sec	
72	10min	
4°C	pause	

Gelelectrophoresis

A 1xTAE gel was prepared and 7µl of the PCR-product was loaded. Generally, the gel ran at 90V for 50min.

EDTA (0.5M; pH=8.0) consisted of 14.61g EDTA dissolved in 70ml MilliQ water. The pH was adjusted to pH=8.0 with 10M NaOH and the solution was filled up to 100ml with MilliQ water and was autoclaved.

50xTAE consisted of 242g Tris-Base, 57.1ml acetic acid, 100ml EDTA (0.5M; pH=8.0) and was filled up to 1L with MilliQ water.

2.6 High-throughput Sequencing

For the High-throughput Sequencing runs (Ion Torrent), separate cultures were prepared originating from the mother cultures Scu2B and Scu2C as well as Scu2B-NO. DNA-extraction took place according to the protocol in chapter „2.4 DNA-Extraction“. 16S rRNA gene products were amplified and the reads processed and controlled for quality by Romana Bittner. The data were analysed and aligned with the SILVA database (state: 2016) in cooperation with Sophie Abby and Leonarda Langsam.

2.7 Fluorescence *in situ* hybridization (FISH)

2.7.1 FISH protocol

5ml of sample was taken from each culture. The sample was centrifuged for 30 minutes at 13,2krpm in a cooling centrifuge (Eppendorf Centrifuge 5415R). The supernatant was discarded and the pellet was put on ice. Mostly, there was no pellet visible from the enrichment cultures. The pellet was washed with 1ml PBS and dissolved by slightly pipetting. The sample was centrifuged (Eppendorf) for 8 minutes at 8 krpm and afterwards the supernatant was discarded again. This washing step was repeated once. For the fixation of the cells, 1ml of 4% PFA was used. PFA is very toxic and easily volatilized, so the following steps were performed under a fume hood. The pellet was dissolved again and the sample was put on ice for one hour and then centrifuged (Eppendorf) for 10 minutes at 13krpm. The PFA was discarded and the pellet was once more washed with PBS as described earlier. A mixture of PBS : 100% EtOH = 1:1 was prepared in the meantime. The pellet was dissolved in 0.5ml of it and the sample was kept at 4°C overnight.

PBS-buffer consisted of 8gL^{-1} NaCl; 0.2gL^{-1} KCl; 1.44gL^{-1} Na_2HPO_4 and 0.24gL^{-1} KH_2PO_4. The pH was adapted to 7.4 using HCl, the solution autoclaved and stored at 4°C.

The following probes were used (d = double labelled):

Table 6 Overview of the different fluorescence *in situ* hybridization probes used in this study

Name	Target	F[a]	M[b]	Sequence
Eub338	Bacteria	FAM	603	5'-GCTGCCTCCCGTAGGAGT-3'
Arch915	Archaea	dCy3	647	5'-GTGCTCCCCCGCCAATTCCT-3'
HotT	*Ca.* N. cavascurensis	dCy3	707	5'-CCATGGCTCCTGGAATGG G-3'
Aig	Aigarchaeota of the Scu-cultures	dCy3	801	5'-TGAGAGGATCTGGAACGACCT C-3'
Cren569	most envir. Cren-/Thaumarchaeota	dCy3	313	5'-GCTACGGATGCTTTAGG-3'

[a] F - short for fluorophore
[b] M - short for the first three numbers of the molecular weight

Eub338 (Amann et al., 1990), Arch915 (Stahl and Amann, 1991) and Cren569 (Jurgens et al. 2000) were already published probes, whereas the probes HotT and Aig were specifically designed for this study. The probe design will be described in the following chapter. The first three numbers of the molecular weight of the probes

were considered in the calculations. The calculations were performed according to the following protocol.
All used archaeal probes were dCy3-conjugated (d = double-labelled), the general bacteria probe EUB338 in FAM. Stock solutions were 100µM, final working stocks were 10x stocks with 30ng/µL or 50ng/µL. In order to get the required dilution factor, the first three numbers of the molecular weight of the dCy3-labelled probes have to be divided by 30, for FAM-labelled probes by 50.

calculation in general:
(first three numbers):(weight in the final solution (in ng)) = probe dilution

calculations for ARCH915 in dCy3:
647:30= 21.56 (so 22)
This means 1µl of the stock probe ARCH915 was diluted with 21µl MilliQ water, the final solution consisted of 22µl.

calculations for EUB338:
603:50 = 12.06 (so 12)
Therefore, 1µl of EUB338 stock solution was diluted in 11µl MilliQ water.

The final concentrations for the used probe solutions were 30ng/22µl for ARCH915 and 50ng/12µl for EUB338. The diluted probes as well as the stocks are very photosensitive and were kept as dark as possible during the entire experiment. The hybridization buffer was prepared freshly on the same day in an Eppendorf-tube and consisted of 360µl 5M NaCl; 40µl 1M Tris-HCl; 798µl MilliQ water; 2µl 10% SDS (preheated at 70°C for approximately 10 minutes until the solution was completely clear) and 800µL 40% Formamide (FA), which was added at the very end under the fume hood. For each FISH slide that was used a separate buffer was prepared. The concentration of the FA varied due to the different probes. To equalize the volume of the buffer, the amount of MilliQ water was always adapted according to the following table:

Table 7 Overview of the different Formamide concentrations in the hybridization buffer

% FA	µL FA	µL MilliQ water
0	0	1598
5	100	1498
10	200	1398
15	300	1298
20	400	1198
25	500	1098
30	600	998
35	700	898
40	800	798
45	900	698
50	1000	598

Microscope slides with epoxy resin colour mask (Marienfeld) were used. Per well normally 1 drop of 0.01% Poly-L-Lysin is used to improve the attachment of the cells and is dried for 5 minutes. The rest of it was pipetted of. In the case of the Scu-cultures, this step was skipped after two attempts due to the lack of noticeable improvement. Each well was covered with 10µl of sample. *E.coli* was used as a positive control. The sample was air-dried and covered to prevent contamination. The following steps were performed under the fume hood. Each well was covered with 20µl hybridization buffer and 2µl of each probe (one for archaea, one for bacteria). To prevent destroying the sample, the tip of the pipette never touched the slide directly. The rest of the hybridization buffer was poured into a so-called hybridization chamber which consisted of a 50ml falcon tube with a piece of paper towel in it to suck up the solution. Afterwards the slide was horizontally inserted into the chamber, so the drops on the wells were not disarranged. The falcon tube was closed and put into a hybridization oven at 46°. The ideal hybridization time differs from probe to probe and was tested for 3 hours, 5 hours and overnight. In the meantime, a washing buffer was prepared. Here the stringency must be considered and should always be the same as in the hybridization buffer. So for 40% FA, for instance, the buffer consisted of 0.46ml of 5M NaCl, 1ml of 1M Tris-HCl and 0.5ml of 0.5M EDTA. This solution was then filled up to 50ml with MilliQ water. For other concentrations, the amount of NaCl and MilliQ water were adapted according to the following table:

Table 8
Overview of the different NaCl concentrations in the washing buffer

%FA	µL NaCl	mL MilliQ water
0	9000 (= 0.9 M)	40
5	6300	42.7
10	4500	44.5
15	3180	45.82
20	2150	46.35
25	1490	47.01
30	1020	47.48
35	700	47.8
40	460	48.04
45	300	48.2
50	180	48.32

The washing buffer was preheated in a water bath to 48°C. Also, a new falcon tube containing 50ml MilliQ water was prepared and put on ice. After hybridization the slides were put into the washing buffer for 10 minutes, then shortly dipped into the ice-cold MilliQ water before being carefully dried by an air-flow. The slides were sealed using Vector Shield and nail polish to fix the cover slide. A Nikon Eclipse 50i Microscope was used with a Prior Lumen 200 Fluorescence Illumination System.

2.7.2 FISH-probe-design

For the design of the FISH-probes three main points must be checked: the accessible regions of the structured rRNA, the melting temperature and the non-target-hits for the probe. First, the rRNA maps of *E.coli* and *M.sedula* (Behrens et al.; 2003) were checked for regions that were easily accessible. Only if the regions looked promising in both maps, they were considered for the probe-design. For those promising regions, the melting temperature was checked via the program Oligo Calc (Oligonucleotide Properties Calculator). Generally, a melting temperature of 57°C or above is desirable (Hugenholtz et al.; 2002), but also lower temperatures down to 55°C were considered since the general bacterial probe EUB338 works with a melting temperature of 55°C and is one of the most commonly used probes. The FISH-probes were tested on the arb-silva-homepage for testing probes. The probe sequence was inserted and the probe evaluated by checking the different amounts of mismatches starting with 0 and going up to 3 mismatches with the SILVA database (state: 10/2015). Possible probes were tested by BLAST. Non-target-hits were only tolerated if they had at least 2 mismatches in the middle of the probe.

3. Results

3.1 Cultivation of an extremely thermophilic ammonia oxidizing archaeon

Based on their sampling site the cultures are referred to as Scu-cultures (from cava scura). The continuous Scu-cultures were always kept slightly shaking at 70°C (ZWYR-2102C Labwit Shaking Incubator) and consisted of Scu2B, Scu2B-2 (replicate of Scu2B), Scu2C, Scu2C-2 (replicate), F1, F1-2 (replicate). Scu2B and Scu2C were originally inocculated from the same sampling site, but were kept separately ever since. The F-cultures originated from the Scu2C-cultures after being filtered once (1,2µm-filter). To determine the most suitable cultivation condition, the Scu-cultures were cultivated under different conditions and growth was checked via the measurement of ammonia, nitrite and nitrate as well as microscopy. After the morphology of both the hot Thaumarchaeota (AOA) and the Aigarchaeota was identified by FISH (see later chapter), cell counting was also performed.

3.1.1 Comparison of the two continuous cultures Scu2B and Scu2C

For stable long-term cultivation 70°C appeared to be the most suitable temperature because the cultures died after a while when grown at higher temperature. The different cultures Scu2B (Fig. 1) and Scu2C (Fig. 2) hardly differed in their production of nitrite neither in production rate nor time. It paralleled ammonia consumption and reached its maximum after 6-8 days. However, only culture Scu2B produced also nitrate after nitrite had reached its maximum (after about 7 days in Fig. 1), whereas Scu2C hardly seemed to harbour nitrite oxidation activity. Scu2B showed the tendency to start the nitrite production faster and more efficiently than Scu2C.

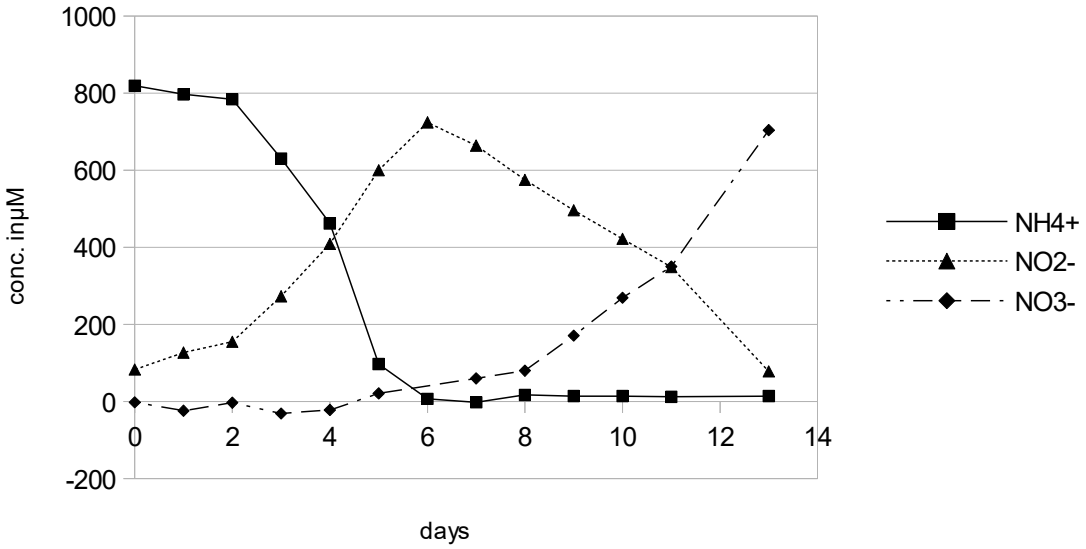

Figure 1 The culture Scu2B-2 was incubated at 70°C for 13 days and ammonia consumption, nitrite production and nitrate production was measured. Ammonia consumption and nitrite production correlated well. Nitrate production started around day 8.

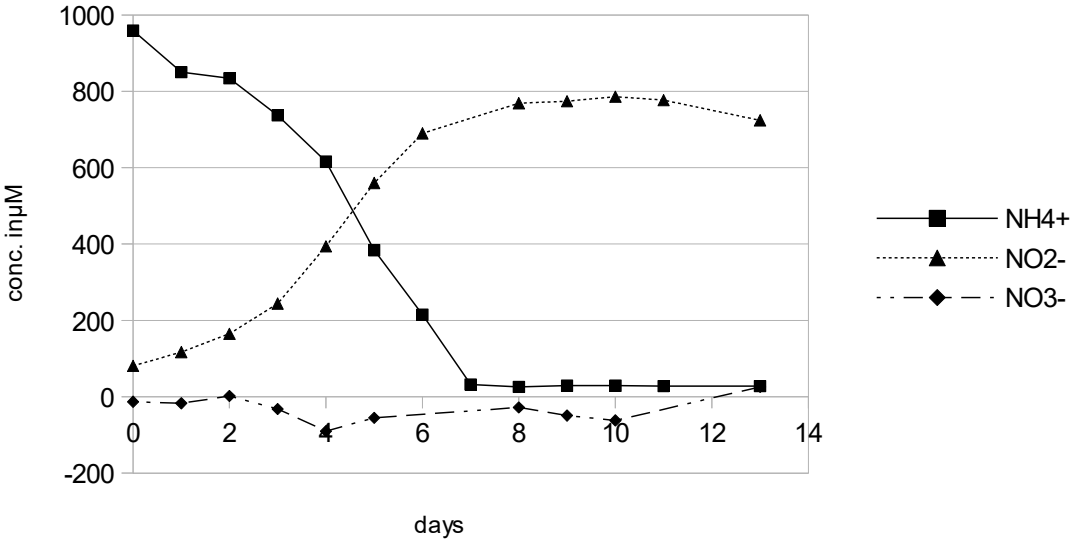

Figure 2 The culture Scu2C-2 was incubated at 70°C for 13 days and ammonia consumption, nitrite production and nitrate production was measured. Ammonia consumption and nitrite production correlated well. Nitrate production was not measurable.

3.1.2 Effect of heat shock on growth

If the AOA are more thermostable than the bacteria in the culture, then incubations at increased temperatures could eventually lead to a better enrichment of the AOA. Therefore, the effect of short incubations at higher temperatures was tested and growth was subsequently followed. Heat shocks were performed for 2 hours right after inoculation. They were tested for cultures with a basic incubation temperature of 70°C and 74°C. After heat shocks at 90°C and 88°C no nitrite production was measurable. 86°C was the highest heat shock temperature that showed nitrite production afterwards, however accompanied by a slower production rate than without a heat shock. Generally, the 74°C-cultures seemed to react more sensitively to heat shocks with a tendency to cease nitrite production afterwards. 78°C and 80°C heat shocks resulted in a stable nitrite production rate, even after being transferred to fresh media several times. In conclusion, *Candidatus* Nitrosocaldus cavascurensis can apparently tolerate temperatures up to 86°C for a short time period and heat shocks could potentially lead to an enrichment of AOA over the bacteria in the culture .

Table 9 Effect of different cultivation temperatures on Scu-cultures and the average production rate [µM/day] of nitrite

	70°C-culture[a]	74°C-culture[a]
78°C	+++	+++
80°C	+++	+++
82°C	++	-
84°C	+	-
86°C	+	++
88°C	-	-
90°C	-	-

[a] Abbreviations used for culture conditions: (+++) culture grew nicely afterwards; (++) culture grew nicely afterwards, but with a longer lag-phase until nitrite production started; (+) culture died after few transfers to a fresh medium; (-) culture died

3.1.3 Enrichment of AOA by filtration

With a 1.2µm filter, filtration was performed before inoculation to enrich preferably for small organisms in the cultures, because AOA are known to be rather small (<1µm) as is also the case for *Candidatus* Nitrosocaldus cavascurensis (see chapter 3.4). While the culture, that was filtrated every time before being transferred to new media, died after several transfers, the culture, that was only filtrated once, seemed

to benefit from it and had a slight increase in nitrite production. This culture was kept in duplicate with the names F1 and F1-2.

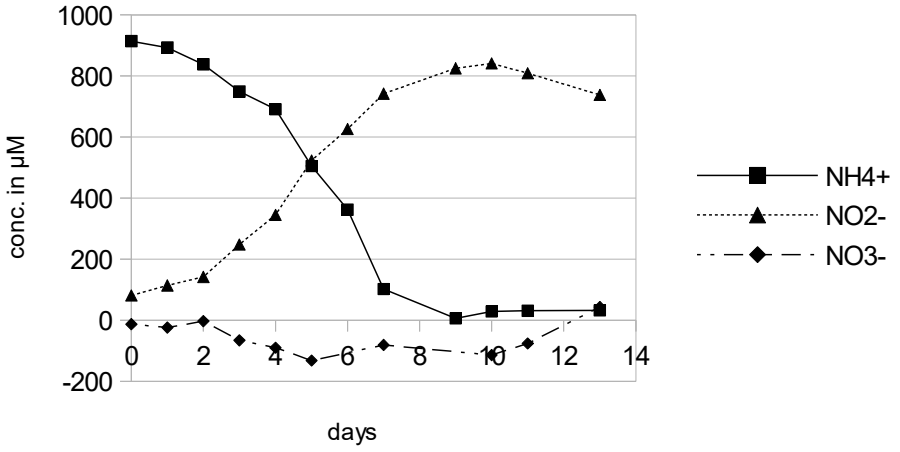

Figure 3 The culture ScuF1-2 was incubated at 70°C for 13 days and ammonia consumption, nitrite production and nitrate production was measured. Ammonia consumption and nitrite production correlated well. Nitrate production was not measurable.

3.1.4 Effect of different cultivation temperatures on growth and temperature optimum

Temperatures of 55°C, 60°C, 65°C, 70°C, 74°C, 76°C and 80°C were tested for the cultivation of the F-cultures (Fig. 4).

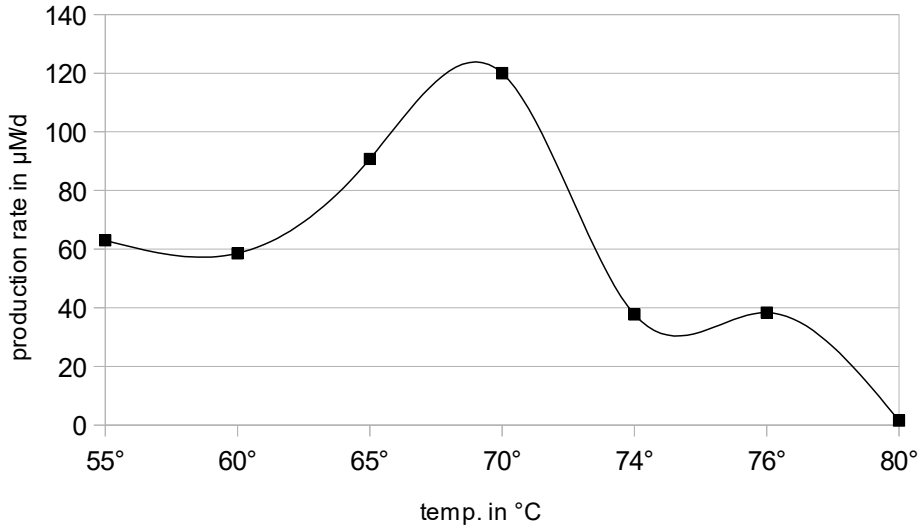

Figure 4 Average production rate in µM/day for the F-cultures (Scu2C culture that was filtrated once) at different temperatures.

There was an optimum around 70° for nitrite production. With increasing temperature the average nitrite production rate decreased. 70°C also appeared to be the most suitable temperature for long-term cultivation, because at higher temperatures the cultures did not grow reliably over longer time periods. The table below shows the average production rate in µM per day for each temperature step.

Table 10 Average production rate [µM nitrite/day] at different cultivation temperatures for the culture F1-2

Temp.	55°	60°	65°	70°	74°	76°	80°
Av. Prod. Rate	63	59	91	120	38	38	2

3.1.5 Growth on urease

Candidatus Nitrosocaldus cavascurensis carries the gene for urease. Therefore, 1mM ammonia was replaced by 0.5mM urea to investigate if it can also grow exclusively on urea. Nitrite production was observed in this culture as well as small coccoid cells were detected via microscopy. However, the nitrite production rate was lower in comparison to a culture under the same conditions based on ammonia (Fig.5). Also, after several transfers it was difficult to keep the culture stable. Still, for a short-term, urea was a suitable substrate alternative to ammonia.

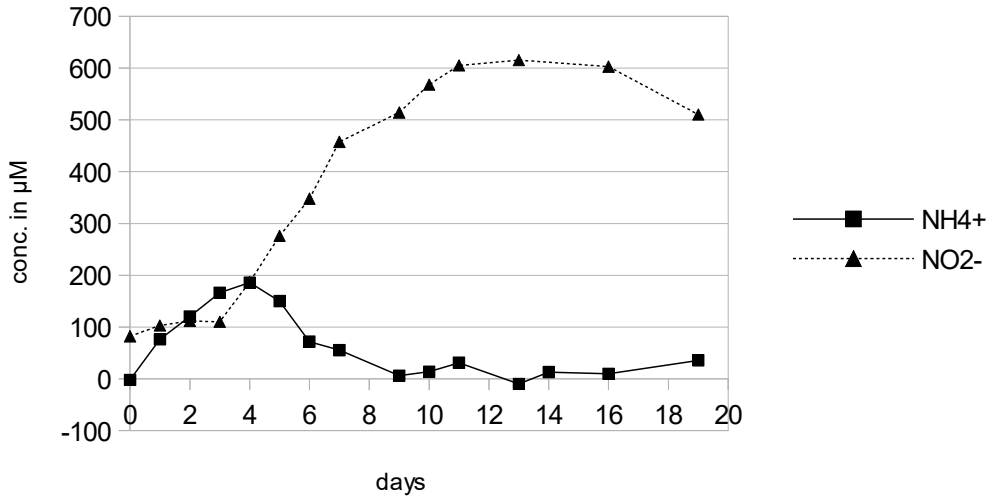

Figure 5 The Urea-culture (0.5mM Urea) was incubated at 70°C for 19 days and ammonia production/consumption, nitrite production and nitrate production measured.

3.1.6 Growth upon addition of antibiotics and supplements

In order to try to inhibit growth of bacteria in the culture and thereby enrich for the AOA, different antibiotics in combination with several supplements were tested. The choice of antibiotics and supplements was based on heat-stability as well as former experiments with Thaumarchaeota. None of the combinations described in the following table seemed to be suitable for the cultures (see table 11). Within the first days there were barely any cells detectable via microscopy and no nitrite production either. Only the combination of 100µg/ml sulfamethazine + 0.1mM pyruvate allowed some growth. Although no nitrite production was observed, an increase of short rod-shaped cells was seen within the first days. Those cells are supposed to be the nitrite oxidizer as will be explained later in chapter 3.5.

Table 11 Overview of the different antibiotics and supplement combinations added to the fresh water medium that was used for the cultivation of the Scu- and NO-cultures

antibiotics	results[a]
100µg/ml Gentamicin	-
50µg/ml Gentamicin, 50µg/ml Sulfamethazine	-
100µg/ml Sulfamethazine	-

antibiotics+supplements	results[a]
100µg/ml Gentamicin + 0.1mM Pyruvate	-
50µg/ml Gentamicin, 50µg/ml Sulfamethazine + 0.1mM Pyruvate	-
100µg/ml Sulfamethazine + 0.1mM Pyruvate	(+)
100µg/ml Gentamicin + 0.1mM Oxaloacetate	-
50µg/ml Gentamicin, 50µg/ml Sulfamethazine + 0.1mM Oxaloacetate	-
100µg/ml Sulfamethazine + 0.1mM Oxaloacetate	-
100µg/ml Gentamicin + 0.1mM Glyoxylate	-
50µg/ml Gentamicin, 50µg/ml Sulfamethazine + 0.1mM Glyoxylate	-
100µg/ml Sulfamethazine + 0.1mM Glyoxylate	-

antibiotics+supplements	results[a]
100µg/ml Rifampicin	-
100µg/ml Rifampicin + 0.1mM Pyruvate	-
100µg/ml Rifampicin + 0.1mM Glyoxylate	-
100µg/ml Rifampicin + 0.1mM Oxaloacetate	-
100µg/ml Novobiocin	-
100µg/ml Novobiocin + 0.1mM Pyruvate	-
100µg/ml Novobiocin + 0.1mM Glyoxylate	-
100µg/ml Novobiocin + 0.1mM Oxaloacetate	-

[a] Abbreviations used for results: **-** , death of the culture within 2-3days, no more cells were seen via microscopy and neither a production of nitrite nor nitrate measurable anymore; **(+)**, after 2-3 days still some cells were seen via microscopy; **+**, after 2-3 days still some cells were seen via microscopy and production of nitrite and/or nitrate was still measurable.

3.2 Community composition of the cultures with endpoint polymerase chain reaction (PCR) analyses

3.2.1 PCR analyses with the general bacteria and archaea primers

To get a first overview of the community composition of the cultures, PCR was performed on different levels starting from general domains down to phylum- or genus-specific level. General bacteria primers 27F/Bac 1492R (Lane et al., 1991) and archaea primers 109F/Arch 1492R (Großkopf et al., 1998, Eurofins) showed the presence of both archaea and bacteria in the cultures.

3.2.2 PCR analyses with the Thaumarchaeota primers amoA and ThAOA

To confirm that the hot Thaumarchaeote was still present in the cultures after almost 1.5 years of cultivation in the lab, both amoA- (Alves et al., 2013) and amoA-ThAOA-primers (Reference: Alves et al., (unpublished)) were tested. amoA-ThAOA-primers were specifically designed for thermophilic ammonia-oxidizing archaea because the already established amoA-primers rarely worked for (hyper)thermophilic archaea (R. Alves, unpublished observation). The hot Thaumarchaeote was still present in all the cultures as products with the amoA-ThAOA-primers were generated in all cases (Fig. 6).

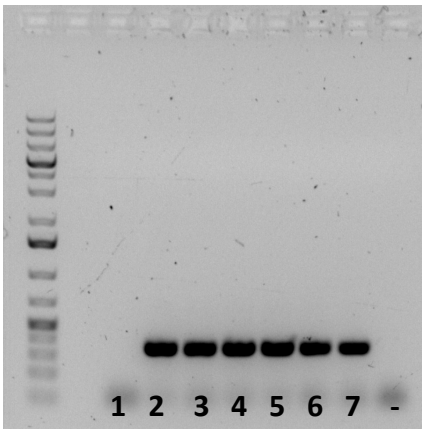

Figure 6 Agarose gel electrophoresis of PCR products generated with amoA-ThAOA primers to amplify amoA genes of thermophilic archaea from 6 Scu-cultures. Also one N.*viennensis* culture was tested in order to check if the primers do not also target moderat AOA. Negative control (-): water as template, lanes 1-5 denote the five separate cultures : (1) Vkon (N.*viennensis* with mutualistic bacteria partner inside), (2) Scu2C-B, (3) Scu2C-70°C, (4) Scu2C-70°C with 80°C heat shock, (5) Scu2C-74°C with 80°C heat shock, (6) Scu2B, (7) Scu2C-2

To be able to make a correlation between the measurement of ammonia consumption, nitrite production, growth and well-being of the hot Thaumarchaeote, it needed to be demonstrated that there is only a single strain of ammonia oxidizers in

these cultures. So AOB-specific primers (AOB amoA1F*/ amoA 2R; Stephen et al.,1999) as well as NOB-primers (Nitrospira NxrB 169F / 638R; Pester et al., 2013) (Nitrobacter Nxr1F / Nxr2R; Vanparys et al., 2007) were tested and both showed a negative result for all cultures (Fig.7).

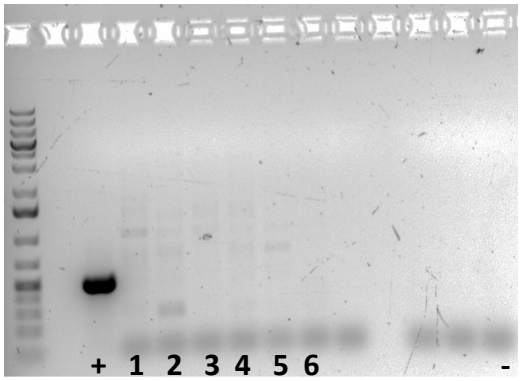

Figure 7 Agarose gel electrophoresis of PCR products generated with AOB primers to amplify amoA genes of ammonia oxidizing bacteria from 5 Scu-cultures. Negative control (-): water as template, positive control (+) *N.multiformis* as template, lanes 1-5 denote the five separate cultures : (1) Scu2C-B, (2) Scu2C-70°C, (3) Scu2C-70°C with 80°C heat shock, (4) Scu2C-74°C with 80°C heat shock, (5) Scu2B, (6) Scu2C-2

3.2.3 PCR analyses with self-designed Aigarchaeota primers

Specific PCR-primers were designed for the Aigarchaeota 16S rRNA gene sequences that were discovered earlier in the culture by high throughput sequence analyses. These primers were used to examine, if any aigarchaeota were successfully propagated over time within the cultures. PCR products were obtained from all 6 cultures tested indicating that aigarchaeota might indeed still be present in all cultures (Fig.8).

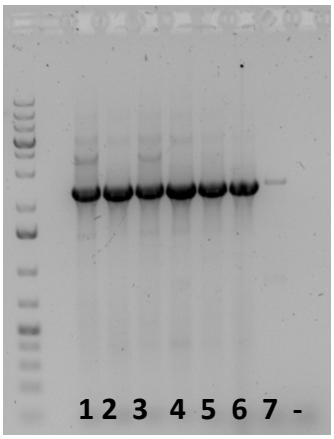

Figure 8 Agarose gel electrophoresis of PCR products generated with Aig primers to amplify 16S rRNA genes of the Aigarchaetoa from 6 Scu-cultures. Negative control (-): water as template, there is no positive control available for Aigarachaeota, lanes 1-7 denote the five separate cultures : (1) Scu2C-B, (2) Scu2C-70°C, (3) Scu2C-70°C with 80°C heat shock, (4) Scu2C-74°C with 80°C heat shock, (5) Scu2B, (6) Scu2C-2, (7) Vkon (N.*viennensis* with mutualistic bacteria partner inside)

3.3 Community composition of the cultures based on 16S rRNA gene analyses

Already in February 2014, approximately half a year after sampling, 16S rRNA gene analyses were performed with high throughput sequencing on DNA of the Scu-cultures. Already back then assigned genes for Thaumarchaeota were found, as well as potentially of an Aigarchaeote. During this study again two 16S rRNA gene diversity studies were performed on a high throughput sequencing machine (Ion Torrent) for the Scu-cultures (November 2015 and May 2016), as well as one for the nitrite-oxidizer culture Scu2B-NO (May 2016) which originated from the Scu2B-culture, but was propagated on nitrite instead of ammonia as energy source.

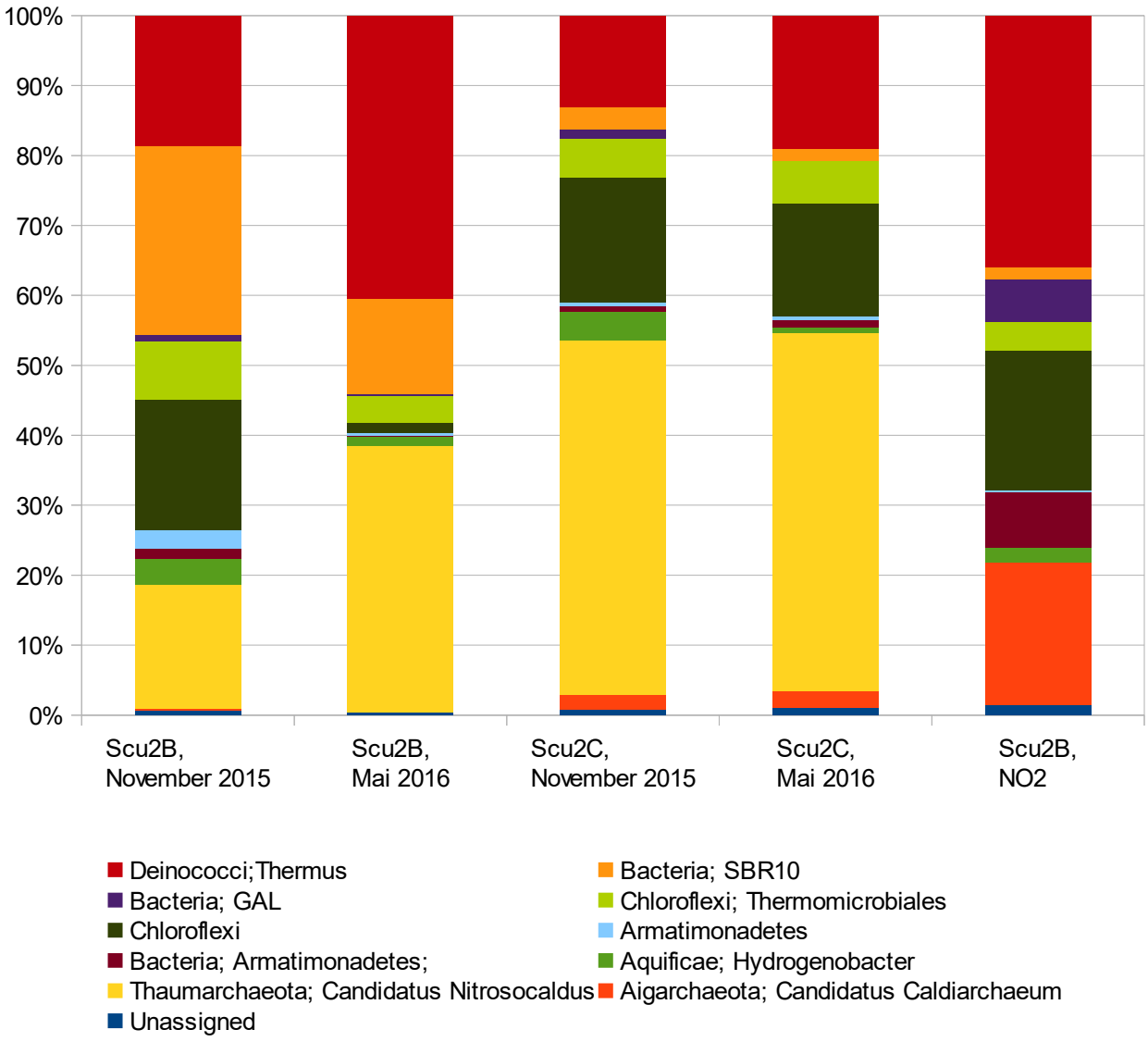

Figure 9 16S rRNA gene diversity studies of the cultures Scu2B, Scu2C and NO2 (originated from Scu2B) in November 2015 and May 2016, respectively. The reads were taxonomically assigned and the community composition calculated.

For this purpose, primers that cover bacteria and archaea 16S rRNA genes were used to amplify the products. The reads were then processed, controlled for quality and then taxonomically assigned. In the six months between those two runs (approximately 13 transfers of the culture) the community composition of Scu2C stayed rather stable with a dominance of *Candidatus* Nitrosocaldus spp. of about 50%. The relative abundance of *Candidatus* Nitrosocaldus spp. in the Scu2B on the other hand increased from 17% up to almost 38%. The Aigarchaeota on the other hand stayed rather stable in Scu2C with around 2% and seemed to disappear in the Scu2B cultures which declined from 0.3% to 0%. Remarkably, the Aigarchaeote significantly increased up to 20% in the Scu2B-NO2-culture, whereas it represented only 0.3% of the community in the Scu2B-culture it originated from. In the Scu2B-NO2-culture, the feed of 1mM ammonia was replaced with 1mM nitrite, so it is not surprising that the hot Thaumarchaeota disappeared in this culture. Besides, the community composition stayed rather the same with some changes in the abundances, with Aigarchaeota and *Thermus spp*. of the class Deinococci increasing. Furthermore, also GAL, Chloroflexi and Armatimonadetes increased although to a lesser extent. With the hot Thaumarchaeote gone, the Aigarchaeote is the only archaeon left in this culture at this detection limit. Nevertheless, it is important to also consider that unassigned genes still remain from all of the analysed cultures.

3.4 Visualization of *Ca.* Nitrosocaldus cavascurensis by fluorescence *in situ* hybridization (FISH)

3.4.1 FISH analyses with the probes Arch915/Eub338

The general archaeal probe Arch915 (Stahl and Amann, 1991) and the general bacterial probe Eub338 (Amann et al., 1990) were used to distinguish bacteria from archaea and to get a first idea about the possible morphology of the hot Thaumarchaeote, which was supposed to be the dominant archaeon in the cultures. Most of the coccoid cells were targeted by the general archaea probe, whereas the majority of the rod-shaped cells were targeted by the general bacteria probe (Fig. 10). Some cells remained unlabelled, which might be due to the fact that the probes do not match their rRNA sequences.

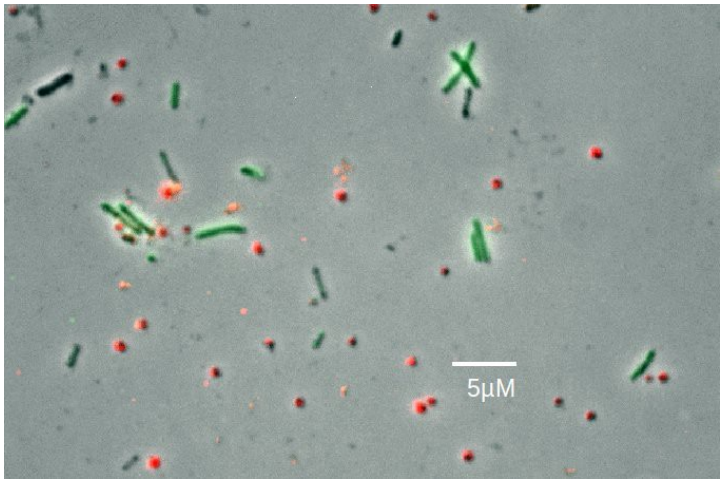

Figure 10 Overlay of Fluorescence Microscopy images taken after hybridization of cells from culture Scu2C with probes Eub338 in FAM (stained in green) and Arch915 in dCy3 (stained in red).

3.4.2 FISH analyses with the probes HotT/ Eub 338

Because a lot of unassigned genes remained in the culture that could potentially represent other archaea, specific FISH-probes for *Candidatus* Nitrosocaldus cavascurensis were designed to have a very exclusive targeting for this species. After testing those under several formamide-concentrations, as well as various hybridization temperatures and times, it became clear that they do not work (data not shown).

3.4.3 FISH analyses with the probes CREN569/ Eub338

Since our newly designed specific probes did not produce results, the already published FISH-probe Cren569 (Jurgens et al. 2000) was used to target the Thaumarchaeote in the cultures. The probe was aligned to all sequences obtained from the cultures. It exhibited no mismatches to the 16SrRNA of *Candidatus* Nitrosocaldus cavascurensis and did not target any other known organisms in these cultures. Only the Aigarchaeota, with two mismatches in the middle of the probe, could have been possibly targeted, although this was considered rather unlikely. To rule out this cross reaction, the probe was also tested on the NO-culture in which the Aigarchaeota were more abundant, i.e. about 20% as compared to around 2% in the Scu-cultures. No hybridization was seen in this culture, indicating that the probe was indeed specific for thaumarchaeota. The data will be shown in the following chapter

on Aigarchaeota. As already seen with the general archaea probe, Cren569 hybridized to the small coccoid cells in the culture.

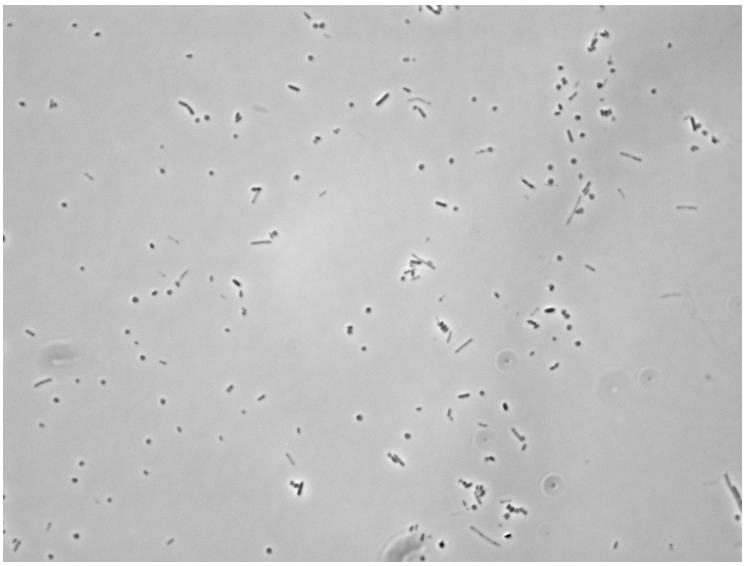

Figure 11
Lightmicroscopy (phase contrast) image (100x magnification) of cells from the culture Scu2C

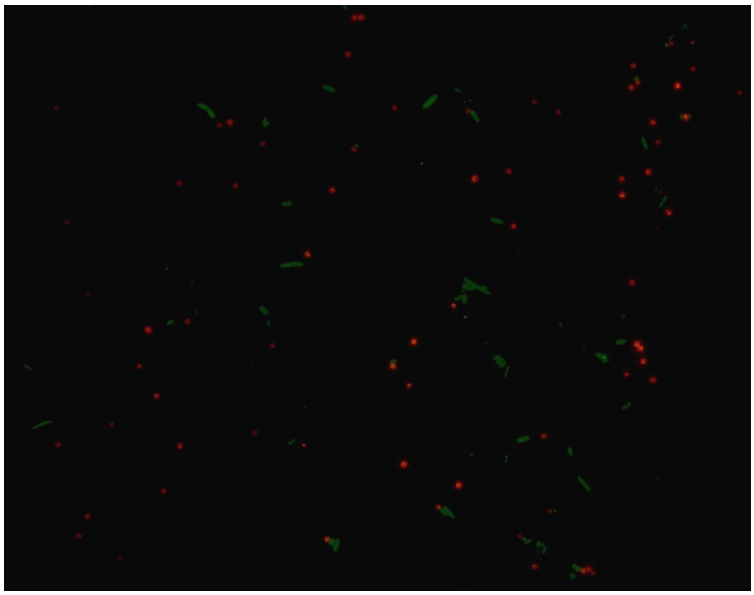

Figure 12
Fluorescence Microscopy image taken after hybridization of cells from the culture Scu2C with probes Eub338 in FAM (stained in green) and Cren569 in dCy3 (stained in red)

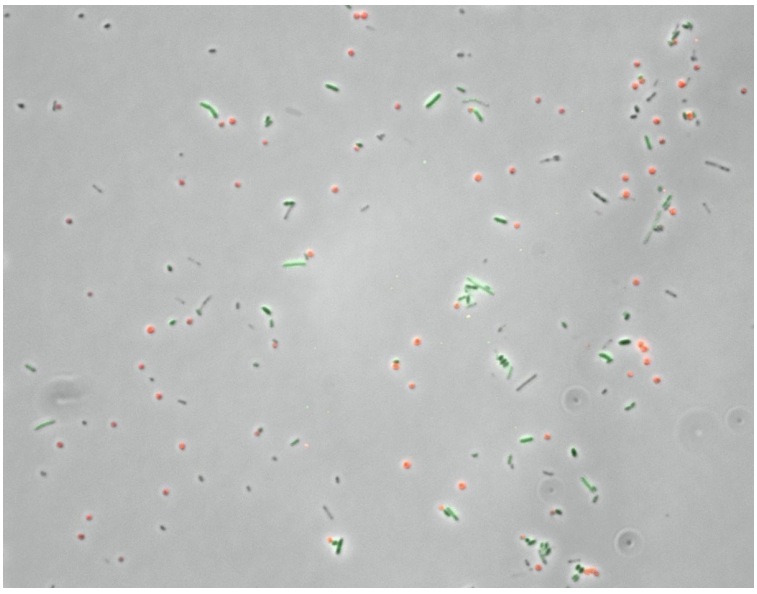

Figure 13
Overlay of phase contrast and Fluorescence Microscopy images taken after hybridization of cells from the culture Scu2C with probes Eub338 in FAM (stained in green) and Cren569 in dCy3 (stained in red).

3.5 Visualization of the Aigarchaeote by fluorescence *in situ* hybridization

3.5.1 FISH analyses with the probes Arch915/ Eub338

According to the community analyses, the Aigarchaeota were the only detectable archaea left in the NO-cultures. Therefore, the general bacterial probe, Eub338 in FAM, and the general archaeal probe, Arch915 in dCy3, were tested on those cultures to get a better knowledge of the morphology of the Aigarchaeota. According to the FISH-pictures the Aigarchaeota are short rod-shaped archaea (see red cells in Fig. 15 and 16).

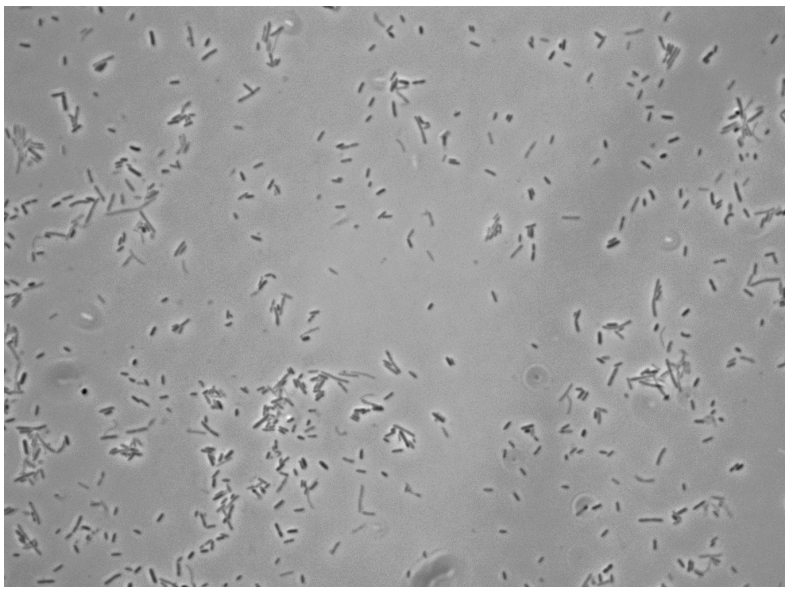

Figure 14
Lightmicroscopy image (100x magnification) of cells from the NO-culture

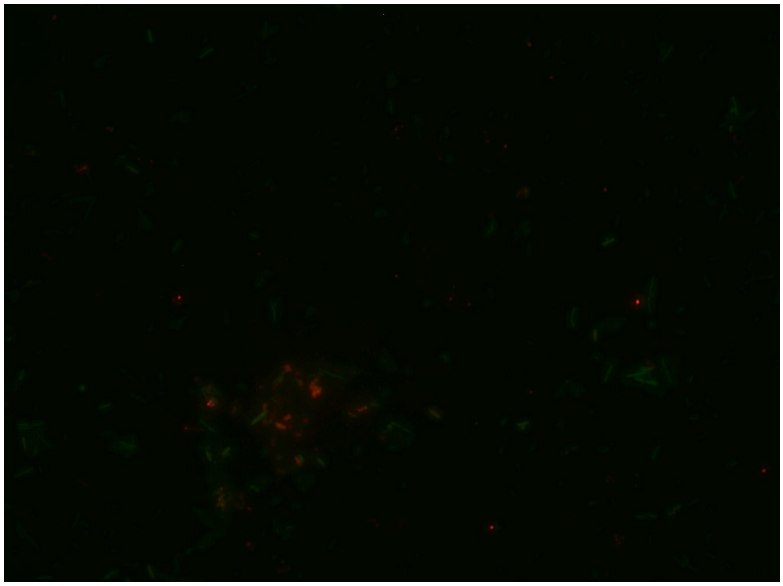

Figure 15
Fluorescence Microscopy image taken after hybridization of cells from the NO-culture with probes Eub338 in FAM (stained in green) and Arch915 in dCy3 (stained in red)

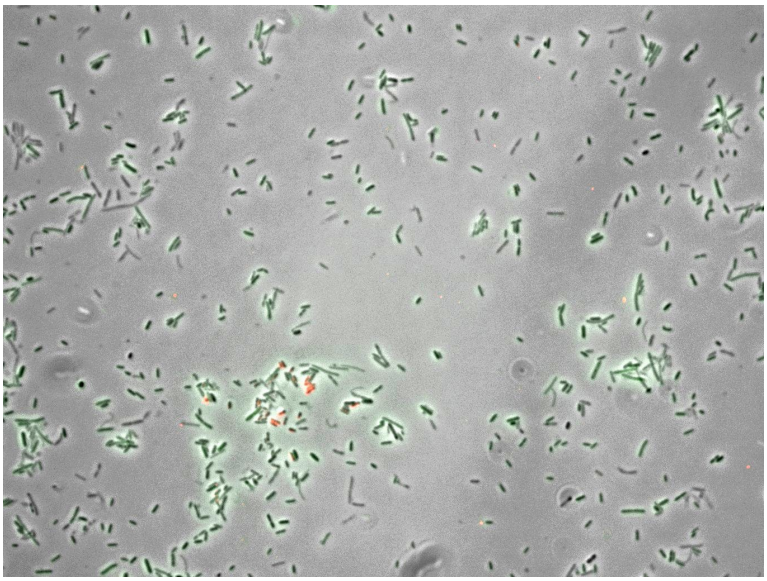

Figure 16
Overlay of phase contrast and Fluorescence Microscopy images taken after hybridization of cells from the NO-culture with probes Eub338 in FAM (stained in green) and Arch915 in dCy3 (stained in red).

3.5.2 FISH analyses with the probes Cren569/ Eub338

To make sure that the Cren569-probe does not target the Aigarchaeota, another FISH, with the combination of Cren569 in dCy3 and Eub338 in FAM, was performed on the NO-cultures. Only the Eub338 showed a positive result on the culture, the Cren569 did not target any cells at all. Therefore, the probe Cren569 does not target the Aigarchaeota, so the Cren569 could be used to identify *Candidatus* Nitrosocaldus cavascurensis in the Scu-cultures.

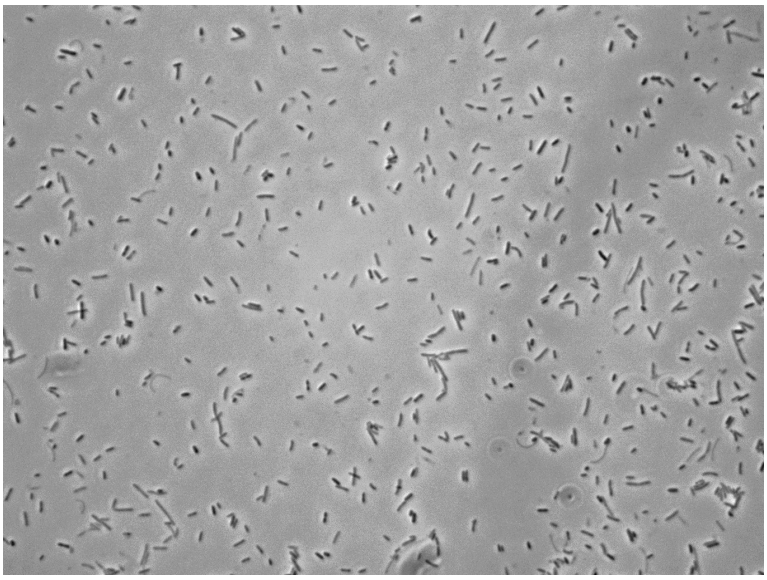

Figure 17
Lightmicroscopy image (100x magnification) of cells from the NO-culture

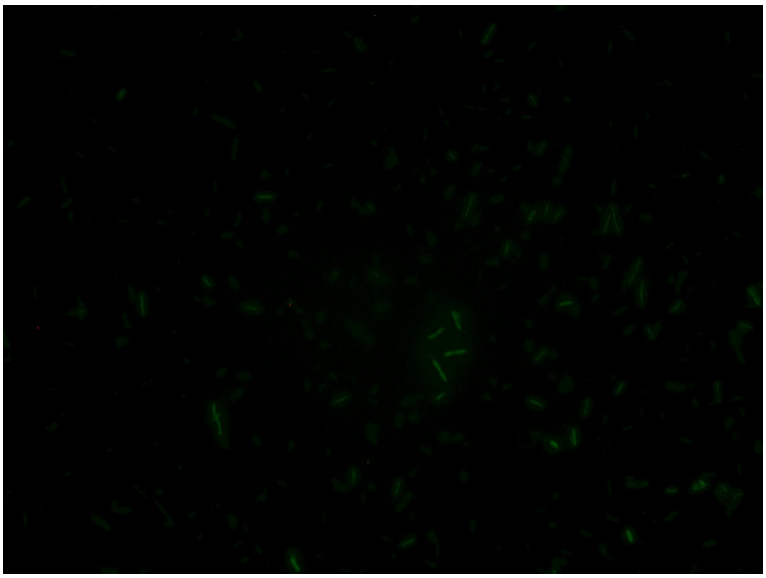

Figure 18
Fluorescence Microscopy image taken after hybridization of cells from the NO-culture with probes Eub338 in FAM (stained in green) and Cren569 in dCy3 (stained in red)

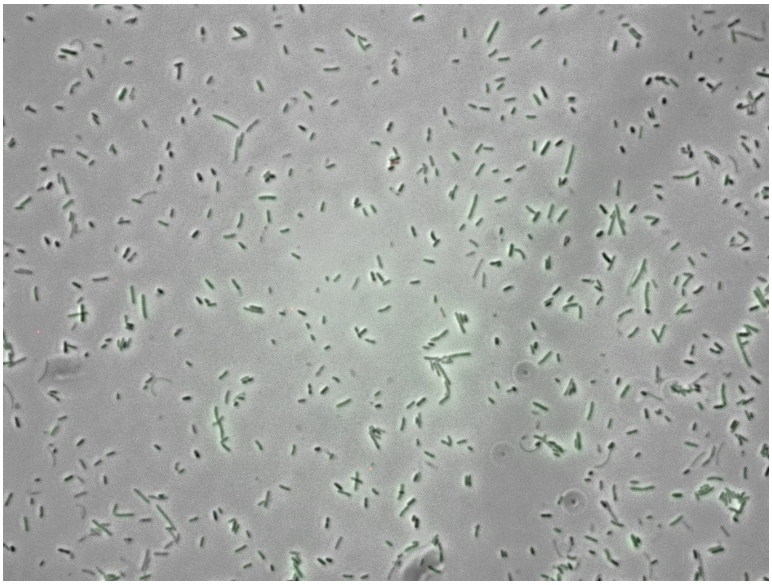

Figure 19
Overlay of phase contrast and Fluorescence Microscopy images taken after hybridization of cells from the NO-culture with probes Eub338 in FAM (stained in green) and Cren569 in dCy3 (stained in red).

3.5.3 FISH analyses with the probes Aig/ Eub338

Specific FISH-probes were designed for the Aigarchaeota based on their 16S rRNA gene sequence. They were tested in FISH under various conditions, but did not show any hybridization, similar to the HotT-probe (see above).

3.6 NO-cultures

The NO-cultures were set up with nitrite in this study, in order to separate the AOA and the potential nitrite oxidizers. The NO-cultures were cultivated at 70°C and 74°C. They originated from the Scu2B-culture and were fed with 1mM $NaNO_2$ instead of 1mM NH_4Cl. Nitrite consumption, as well as nitrate production were regularly measured. Both cultures started the production of nitrate after approximately 10 days with a concomitant consumption of nitrite, but a higher amount of nitrate was produced at 74°C in a shorter time. So 74°C was the preferred cultivation temperature (Fig. 20 and 21).

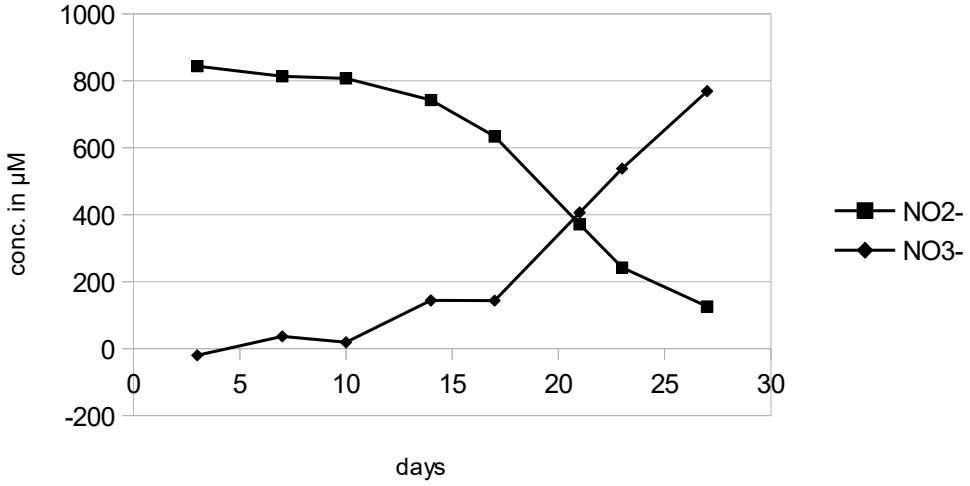

Figure 20 The NO-culture was incubated at 74°C, the medium contained 1mM $NaNO_2$. Nitrite consumption and nitrate production was measured for 27 days and correlated well.

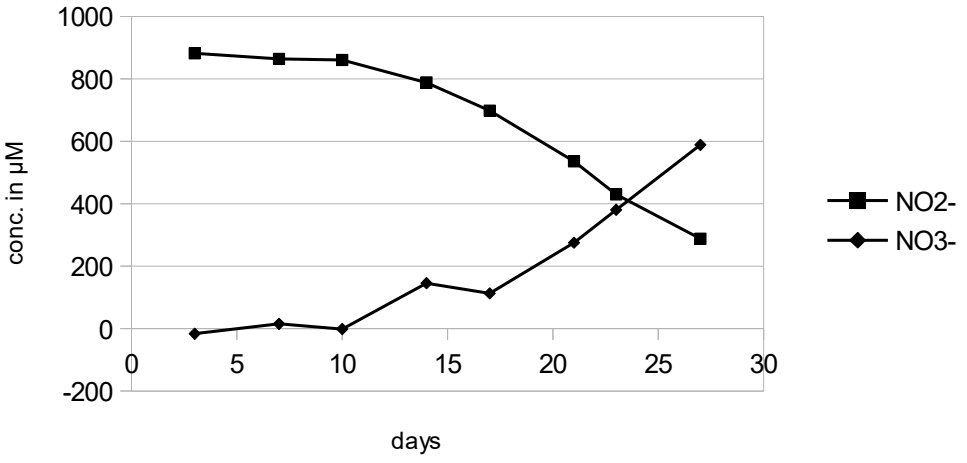

Figure 21 The NO-culture was incubated at 70°C, the medium contained 1mM $NaNO_2$. Nitrite consumption and nitrate production was measured for 27 days and correlated well.

To determine if nitrate could also be produced through a chemical reaction, catalysed by the high temperatures, a negative control was incubated. It contained pure medium of the same composition, but without inoculum and it was incubated for 30 days. Neither nitrite consumption nor nitrate production was measurable in this control (Fig.22).

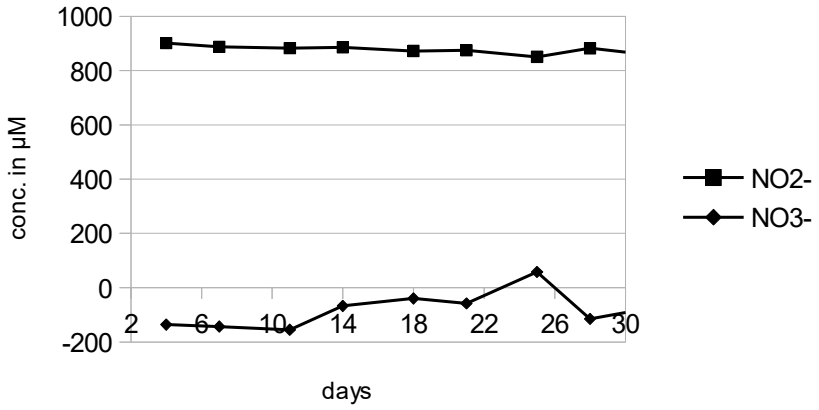

Figure 22
Pure medium (without culture) with 1mM $NaNO_2$ was incubated at 70°C for 30 days as a negative control. Neither nitrite consumption nor nitrate production was measurable.

The NO-cultures were also incubated at lower temperatures down to 55°C. Although 65°C showed a rather long lag phase (ca. 30 days), a rather high amount of nitrate was produced (Fig. 23). A similar behaviour was also observable at 60°C (Fig.24). On the other hand, 55°C did not show any nitrate production after more than 30 days.

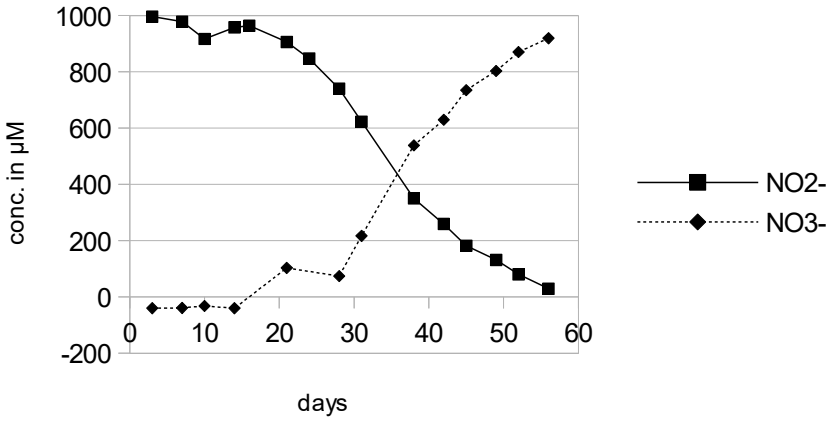

Figure 23 The NO-culture was incubated at 65°C, the medium contained 1mM $NaNO_2$. Nitrite consumption and nitrate production was measured for 57 days and correlated well.

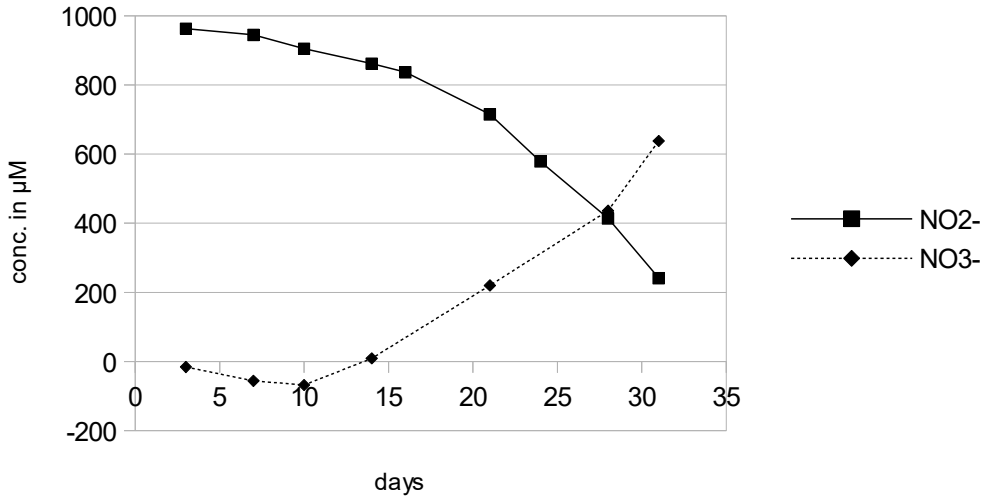

Figure 24 The NO-culture was incubated at 60°C, the medium contained 1mM $NaNO_2$. Nitrite consumption and nitrate production was measured for 27 days and correlated well.

Under the assumption that the nitrite oxidizers might be small rods, a combination of sulfamethazine and pyruvate was added to the basic media. This was the combination which had shown an increase of small rods earlier (see above chapter 3.1.6). This combination resulted in a longer lag phase of about 23 days, but still nitrate production was observable (Fig. 25).

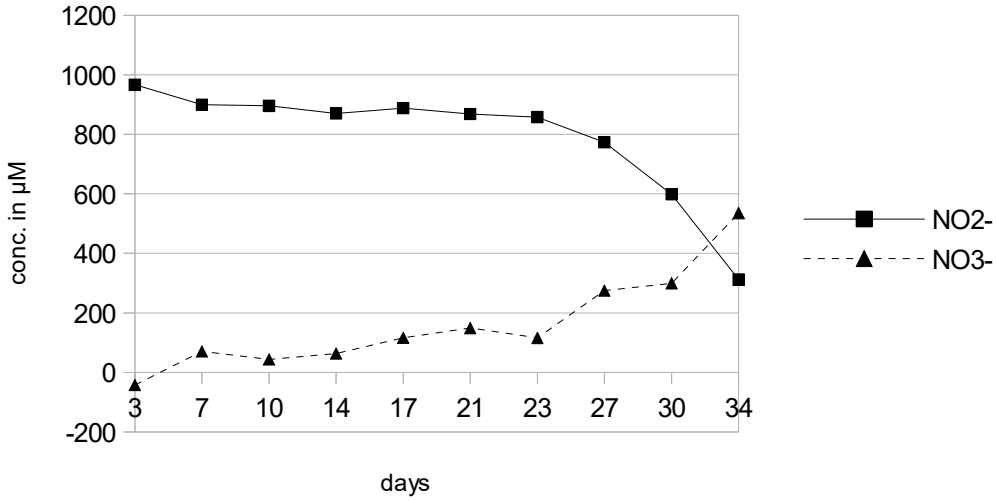

Figure 25 The NO-culture was incubated at 70°C, the medium contained 1mM $NaNO_2$and a combination of sulfamethazine and pyruvate. Nitrite consumption and nitrate production was measured for 34 days and correlated well.

3.7 Correlating cell growth and ammonia oxidation

The growth rate was compared between the cultures Scu2C, cultivated at 70°C, and Scu2C_70°, 78Sch, cultivated at 70°C with two hours of heat shock at 78°C after inoculation. Although the growth rate was higher for the heat shock culture and the average doubling time shorter, a permanent cultivation at 70°C was more favourable, as previously mentioned in chapter 3.1.2. During the exponential phase, the growth rate μ was around 0.7.

Table 12 Average growth rate μ per day and average doubling time in days for the cultures Scu2C and Scu2C_70°C, 78Sch

	Scu2C	**Scu2C_70°, 78Sch**
average growth rate μ (day^{-1})	0,722	0,774
average doubling time t_d (in days)	0,96	0,895

Based on the Ion Torrent data only two archaea – the hot Thaumarchaeota and the Aigarchaeota – seem to be present in the Scu-cultures. As described in the former chapter, the coccoid cells were archaeal ones, whereas the majority of rod-shaped cells were bacteria, as well as the Aigarchaeota. So the Thaumarchaeota seemed to make up the coccoid cells in the Scu-cultures. Based on this, cell counting of all the coccoid cells was performed in order to distinguish if the amount of coccoid cells and both the consumption of ammonia, as well as the production of nitrite parallelled each other. According to the cell counts, the growth of the Thaumarchaeota in the culture matched nicely both the consumption of ammonia and the production of nitrite (Fig. 26 and 27). Maximal cell counts were 7,76 x 10^6 cells/ml.

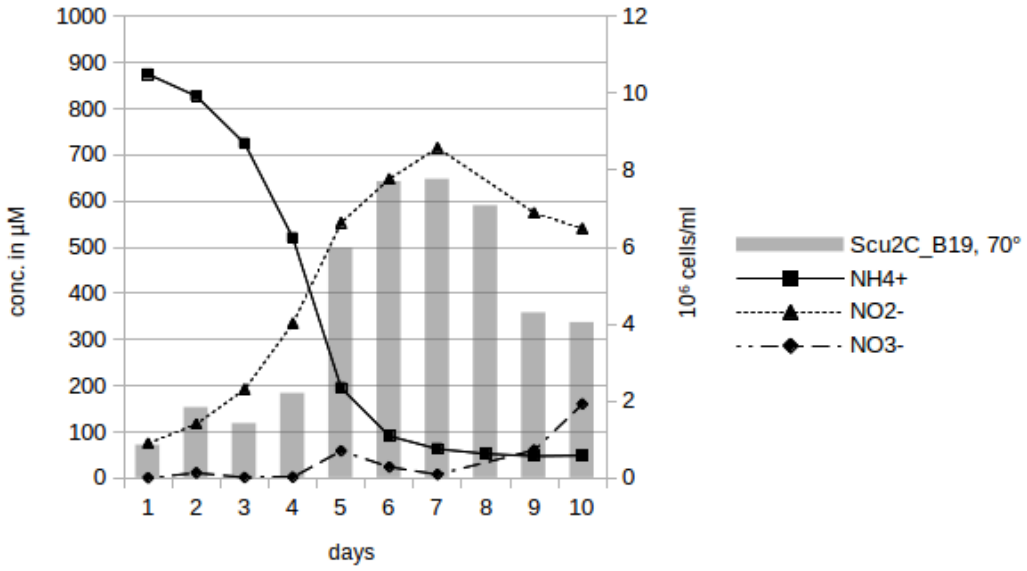

Figure 26 The culture Scu2C-B19 was incubated at 70°C and ammonia consumption, nitrite production and nitrate production were measured each day over a period of ten days and cell counts were performed. The amount of coccoid cells correlated well with both ammonia consumption and nitrite production. Nitrate production started around day 9 when cell counts and nitrite production decreased.

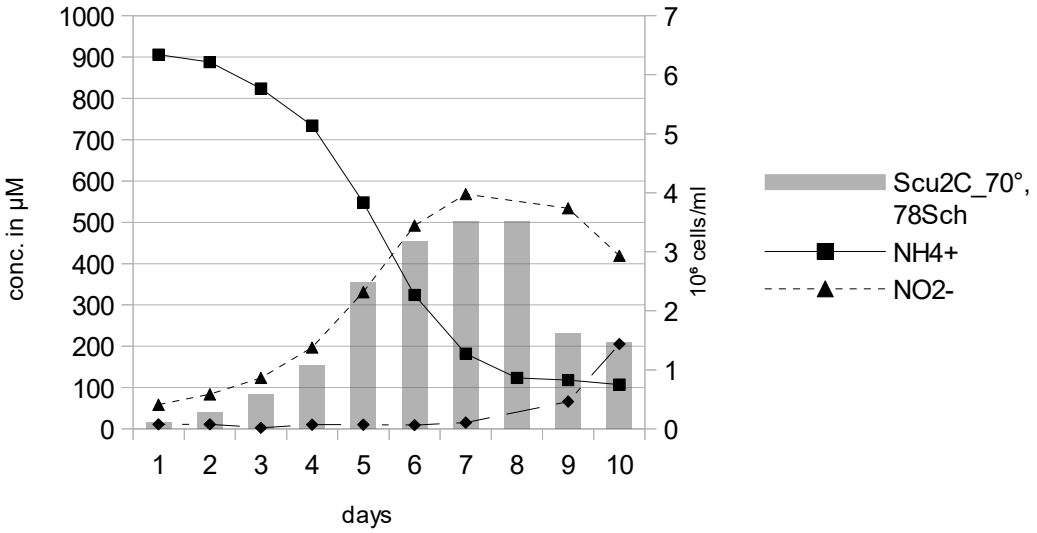

Figure 27 The culture Scu2C was incubated at 70°C after a 2 hour heat shock at 78°C. Ammonia consumption, nitrite production and nitrate production were measured each day over a period of ten days and cell counts were performed. The amount of coccoid cells correlated well with both ammonia consumption and nitrite production. Nitrate production started around day 9 when cell counts and nitrite production decreased.

4. Discussion

4.1 Cultivation of the ammonia oxidizing archaeon *Ca.* Nitrosocaldus cavascurensis

The Scu-cultures with ammonia oxidizing activities were tested under various growth conditions with the goal to improve culture conditions for the AOA. Regarding temperature, the hyperthermophilic thaumarchaeote in the Scu-cultures had a growth optimum at around 70°C, which was also the optimal temperature for stable long-term cultivation. To obtain a better enrichment of the archaeon several conditions and treatments were tested.

A production of nitrite was detectable at a temperature range between 55-76°C for *Candidatus* Nitrosocaldus cavascurensis, which is a slightly broader range in comparison with its closest known relative, *Candidatus* Nitrosocaldus yellowstonii, which has a still measurable nitrite production between 60-74°C. Nevertheless, both share the same production optimum at temperatures between 65-74°C, as well as a coccoid cell form and preferences in the medium composition, like the feeding of 1mM ammonia. During the exponential phase, the growth rate μ of *Candidatus* Nitrosocaldus cavascurensis was around 0.7 and therefore only slightly lower compared to *Candidatus* Nitrosocaldus yellowstonii *(*μ=0.8) (de la Torre et al., 2008). However, in the enrichment cultures of *Candidatus* Nitrosocaldus yellowstonii up to 90% of the fed ammonium was converted into nitrite (de la Torre et al., 2008), whereas in comparison, the enrichment cultures of *Candidatus* Nitrosocaldus cavascurensis seemed to react more sensitively to the accumulation of nitrite, as a concentration of more than 0.7mM nitrite seemed to inhibit *Candidatus* Nitrosocaldus cavascurensis. This, on the other hand, might explain how the dependancy of *Candidatus* Nitrosocaldus cavascurensis and the nitrite oxidizer in the cultures has evolved. Nitrite oxidation in the Scu-cultures usually started as soon as 0.7mM of nitrite was produced.

Unexpectedly, the AOA in the culture were very sensitive to antibiotics. Consequently, no ammonia oxidation was detectable even several days after inoculation into a medium containing any of the tested antibiotics. For obtaining a pure culture of *Nitrosopumilus maritimus*, streptomycin was used (Könneke et al., 2005) and also for *Nitrososphaera viennensis*, the addition of streptomycin turned out to be efficient in cleaning up the enrichment culture in order to get a pure one,

as well as a combination of several different antibiotics like carbenicillin, ampicillin and kanamycin (Stieglmeier et al., 2014). Although various combinations of (heat-stable) antibiotics were tested for cleaning up the enrichment cultures in favour of *Candidatus* Nitrosocaldus cavascurensis, none was successful and all led to the extinction of coccoid cells in the cultures within a few days. This could essentially be attributed to two reasons: First, the AOA itself was sensitive to the antibiotics, or second, a bacterial partner in the culture that is essential for growth of the AOA died due to the antibiotics. Furthermore, the addition of several supplements did not change this reaction. Heat shocks, on the other hand, of up to 86°C, were an efficient method for cleaning up the culture. At higher preincubation temperatures no nitrite production was measurable. The switch from ammonia to urea as energy source showed that *Candidatus* Nitrosocaldus cavascurensis can survive on urea, at least for a brief period. In case there were other ammonia oxidizers in the culture that cannot process urea, this could be used as a selective enrichment condition. However, there are no indications of such organisms based on the 16S rRNA community analysis, although up to 1% of the 16S rRNA sequences of the high throughput sequencing data could not be assigned to known taxa. Rather beneficial for cleaning up the culture was the filtration via a 1.2µm-filter before inoculation. Although this resulted in a short lag phase for nitrite production, the culture reached higher final nitrite concentrations. Nevertheless, when the culture was filtrated every time, while being transferred to fresh media, this resulted in the death of the culture. So in conclusion, filtration seemed to be an efficient tool as long as it was not used too often. While filtration and heat shocks might potentially help the enrichment of the AOA, all of the tested antibiotics resulted in the death of the entire culture within days. In summary, the findings indicate that a mixture of heat shocks, adaption of cultivation temperatures and filtration seem to be the most successful treatment to eventually achieve a pure culture of *Candidatus* Nitrosocaldus cavascurensis.

4.2 Aigarchaeota and nitrite oxidizer

The only Aigarchaeota, *Candidatus* Caldiarchaeum subterraneum, that has so far been analysed by FISH stemmed from a subsurface hot water stream in Japan (Nunoura et al., 2011) and exhibited a filamentous shape (Beam et al., 2016). However, there were no such filamentous shapes detected in the Scu-cultures. The

cultures solely consisted of cocci and rods. While the majority of cocci could be assigned to *Candidatus* Nitrosocaldus cavascurensis the hypothesis was formed that the Aigarchaeota in those cultures are rod-shaped. This was confirmed by fluorescence *in situ* hybridization. Remarkably, there was an increase of Aigarchaeota cells within six months in the Scu2B-NO2-culture, i.e. a culture that was only fed with nitrite and no more ammonia and therefore produced nitrate from nitrite. Originating from the Scu2B-culture with 0.3% of the cells being assigned to Aigarchaeota, 16S rRNA sequences belonging to Aigarchaeota made up 20% of the total community in the Scu2B-NO2-culture. Furthermore, microscopy confirmed this observation, as the Scu2B-NO2-culture then solely consisted of rods. No cocci could be found in the cultures anymore without ammonia oxidation, which supports the hypothesis that *Candidatus* Nitrosocaldus cavascurensis represented the cocci in the Scu-cultures. Therefore, two assumptions can be formulated: first, the AOA in the culture have an inhibiting effect on the Aigarchaeota, therefore their relative numbers increase when ammonia is omitted. Or second, the Aigarchaeota themselves are nitrite oxidizers in this enrichment culture. In this context it is interesting to note that the community composition of the Scu-cultures showed similarities to the community composition that was detected in a bacterial 16S rRNA gene amplification study of the cultures from *Candidatus* Nitrosocaldus yellowstonii (de la Torre et al., 2008). In both *Thermus*, *Thermomicrobium* and *Aqificales spp.* were abundant, but none of those lineages are known for nitrification. Additionally interesting is also the fact, that those cultures showed exclusively nitrite, but no nitrate production. Therefore, one could conclude that none of these organisms is the nitrite oxidizer in the Scu2B-NO-culture, which would further support the hypothesis that the Aigarchaeota might be the nitrite oxidizers in the Scu-cultures. Again, also in the 16S rRNA community analysis of the Scu2B-NO-culture around 1,4% of the 16S rRNA sequences of the high throughput sequencing data could not be assigned to known taxa. However, considering the nitrite production and the comparably high increase of 16S rRNA sequences belonging to Aigarchaeota (about 20% of the total community), the Aigarchaeote of the culture seems to be a more likely candidate for being the nitrite oxidizer. Not surprising is the circumstance, that the Aigarchaeote of the Scu2B-NO-culture grows nicely at a temperature of 70°C, due to the fact that also *Candidatus* Caldiarchaeum subterraneum was discovered in a subsurface environment at a temperature of

70°C (Hirayama et al., 2005). In any case, the observation of an increase in Aigarchaeota in our cultures should be very useful in the future for an identification of their metabolism.

4.3 Failure of self-designed FISH probes

For this study special FISH probes (HotT- and Aig-FISH) were designed for a most exclusive targeting of the Thaumarchaeote and the Aigarchaeote in the cultures. Both probes were tested under various hybridization conditions, but did not result in any signal. A reason for that could be the targeted region. Those regions were chosen according to the rRNA maps from the bacterium *E.coli* and the archaeon *M.sedula* (Behrens et al.; 2003), due to their easy accessibility. Although this applies to *E.coli* and *Metallosphaera sedula*, it most likely did not for *Candidatus* Nitrosocaldus cavascurensis and the Aigarchaeota of the Scu-cultures. Alternatively, the hybridization conditions for these probes might not have been well optimized yet, although different formamide concentrations and hybridization temperatures were tested.

Eventually, the morphology of *Candidatus* Nitrosocaldus cavascurensis was identified via Cren569, a probe that perfectly matched its 16S rRNA gene and none of the other microorganisms in the culture. The morphology of the Aigarchaeote, on the other hand, was detected by the general archaeal probe Arch915. This was possible because the Aigarchaeote was, according to the 16S rRNA gene data, the only remaining archaeon in the NO-cultures. Cren569 was also tested for the NO-cultures to ensure that it really does not target the Aigarchaeota, which was the case.

5. Conclusion and Outlook

In this study hyperthermophilic archaea from hot springs were investigated. The main focus was on the Thaumarchaeon *Candidatus* Nitrosocaldus cavascurensis. *Candidatus* Nitrosocaldus cavascurensis has a growth optimum around 70°C. For the purpose of obtaining a pure culture, different cultivation conditions were tested. Especially heat shocks and filtration appeared to be useful tools to clean up the culture, whereas all tested antibiotics resulted in the death of the entire culture within days. Therefore, a combination of heat shocks and filtration is a good starting point for obtaining a pure culture, but the method still needs to be refined. *Ca.* N. cavascurensis will be an excellent study object for physiology and for evolutionary analyses. For example, its genome sequence might reveal, if AOA arose originally in hot springs and later radiated into moderate environments, or if extremely thermophilic AOA are rather secondary adaptations that derive from moderate archaea.

Fluorescence *in situ* hybridization revealed that *Candidatus* Nitrosocaldus cavascurensis is of a small coccoid shape. The Aigarchaeote, on the other hand, is rod-shaped. There seems to be some kind of inhibiting interaction between *Candidatus* Nitrosocaldus cavascurensis and the Aigarchaeota, because as soon as Thaumarchaeota were absent from the culture, the abundance of Aigarchaeota increased drastically. In addition, according to diversity analysis, the Aigarchaeote was the dominant organism in the nitrite oxidizing cultures. Furthermore, none of the other detected microorganisms in these cultures are known for nitrite oxidation.
Therefore, the hypothesis was formed that the Aigarchaeote might be a nitrite oxidizer. Future studies of these cultures should focus on the metabolism of the Aigarchaeota. Both, optimizing its cultivation conditions to obtain a pure culture, as well as screening for genes that indicate nitrite oxidation are necessary to prove the metabolic hypothesis.

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7. Zusammenfassung

Ohne Stickstoff wäre Leben auf unserer Erde nicht möglich. Daher ist es nicht verwunderlich, dass vor allem der Stickstoffkreislauf für die Wissenschaft seit jeher von besonderem Interesse ist. Doch, trotz intensiver Forschung, verbleiben noch viele seiner Aspekte im Unklaren. Ein umfassendes Verständnis über ihn ist jedoch unabdingbar, wollen wir die Welt, in der wir leben, verstehen.

Diese Studie wurde hyperthermophilen Archeen aus heißen Quellen gewidmet mit einem besonderen Fokus auf *Candidatus* Nitrosocaldus cavascurensis, ein Thaumarchaeon. Es galt eine optimale Kultivierungsmethode für *Candidatus* Nitrosocaldus cavascurensis zu bestimmen, ebenso wie seine Morphologie und Physiologie zu untersuchen. Des Weiteren wurde die gesamte Zusammensetzung der Kulturen analysiert. In früheren Untersuchungen wurde ein Aigarchaeon in den Kulturen gefunden. Seine Morphologie, sowie seine Rolle in den Kulturen wurde untersucht. Außerdem, ob es eine Interaktion zwischen den hier vorkommenden Thaumarchaeen und Aigarchaeen gibt.

Mittels Fluoreszenz *in situ* Hybridisierung wurde bestätigt, dass es sich bei *Candidatus* Nitrosocaldus cavascurensis um ein kokkenförmiges Archaeon handelt, wohingegen das Aigarchaeon eine kurze Stäbchenform hat. Als optimale Kultivierungstemperatur für *Candidatus* Nitrosocaldus cavascurensis wurde 70°C ermittelt. Für das Erreichen einer Reinkultur sollten eine Kombination aus Hitzeschocks und Filtration am effizientesten sein. Der Einsatz von Antibiotika hingegen führte in allen Versuchen zu einem Tod der kompletten Kultur innerhalb weniger Tage. Weiters wurden Thaumarchaeen und Aigarchaeen getrennt und fortan in separaten Kulturen kultiviert. Dabei war erkennbar, dass die Dichte an Aigarchaeen ohne Thaumarchaeen deutlich zunahm. Somit könnte man daraus schließen, dass *Candidatus* Nitrosocaldus cavascurensis inhibierend auf die Aigarchaeen in diesen Kulturen wirken. Außerdem war in den Kulturen, die noch Aigarchaeen aber keine Thaumarchaeen mehr besaßen, immernoch eine deutliche Produktion an Nitrat messbar. Da noch kaum etwas über den Metabolismus der Aigarchaeen bekannt ist, könnte es ein, dass diese die Nitritoxidierer in den Kulturen sind. Alle weiteren Mikroorganismen in den Kulturen, die durch Ion Torrent

Untersuchungen ermittelt wurden, sind nicht für eine nitritoxidierende Aktivität bekannt.

Zusammenfassend heißt dies, dass für *Candidatus* Nitrosocaldus cavascurensis bei 70°C eine stabile Langzeitkultivierung möglich ist. Für eine Reinkultur sind eine Kombination aus Hitzeschocks und Filtration, jedoch ohne Antibiotika, ein guter Ansatz, der jedoch noch weiter verfeinert werden muss. Weiterhin wurde ein Hinweis darauf gefunden, dass Aigarchaeen möglicherweise nitritoxidierende Funktionen besitzen, jedoch sind weitere Untersuchungen, für genauere Aussagen diesbezüglich, erforderlich.