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DIPLOMARBEIT

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Molecular and Cultivation-based Analyses of Nitrifying Communities from Mesophilic and Thermophilic Environments

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Verfasserin / Verfasser:	Nathalie Schuster
Studienrichtung /Studienzweig (lt. Studienblatt):	Genetik - Mikrobiologie
Betreuerin / Betreuer:	Prof. Dr. Michael Wagner

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Chapter 1

Introduction

1.1 Nitrification

Nitrification is a fundamental step of the global biogeochemical nitrogen cycle (Fig. 1.1). This two-step process is catalyzed by microorganisms, which use the oxidation of reduced inorganic nitrogen compounds as their major energy source (Bock and Wagner 2006). Nitrification is long known to be catalyzed by two physiologically distinct groups of microorganisms: aerobic, chemolithoautotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (Juretschko 2000; Koops et al. 2003; Adamczyk 2005). Ammonia oxidizers catalyze the first and rate-limiting step of nitrification, the oxidation of ammonia to nitrite, whereas nitrite oxidizers perform the second step of nitrification, the oxidation of nitrite to nitrate (Bock and Wagner 2006). These two functional groups are widely distributed in various environments, including fresh water, seawater and salt lakes, soils, rocks, masonry and wastewater treatment systems and buildings (Kowalchuk and Stephen 2001; Bock and Wagner 2006).

Besides aerobic ammonia oxidation, anaerobic ammonium oxidation (Anammox) to dinitrogen gas with nitrite as electron acceptor was described (Van de Graaf et al. 1990; Mulder et al. 1995) and the responsible bacteria a few years later identified as members of the phylum *Planctomycetes* (Strous et al. 1999). These obligate anaerobic organisms are now known to be important players in the marine nitrogen cycle (Dalsgaard et al. 2005; Kuypers et al. 2003; Schmid et al. 2007; Lam et al. 2007), freshwater ecosystems (Penton et al. 2006) and wastewater treatment plants (Jetten et al. 2001).

In addition to autotrophic nitrification, various chemoorganotrophic bacteria, fungi and algae are capable of ammonia oxidation. This so called heterotrophic nitrification is a cometabolism that is not coupled to energy conservation (Wood 1988; Bock and

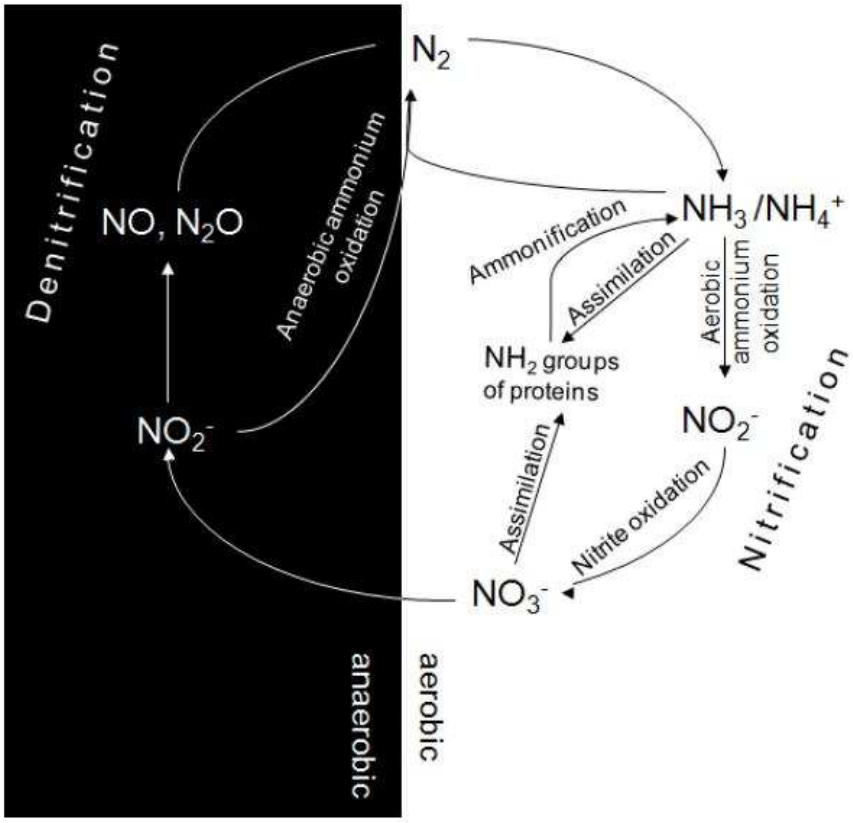

Figure 1.1: The biogeochemical nitrogen cycle.

Wagner 2006).

Since the first isolation of nitrifying microorganisms in the late 19th century, it has been assumed that all autotrophic ammonia oxidizers are within the *Bacteria* (Jetten 2008). However, metagenomic surveys of soil and marine samples provided the first genetic hints that some crenarchaeotes might be capable of ammonia oxidation (Venter et al. 2004; Treusch et al. 2005; Schleper et al. 2005). Finally, Könneke et al. (2005) could prove that a member of this phylum is capable to aerobically oxidize ammonia. This radically changed the view on the microbial players involved in aerobic ammonium oxidation, as reviewed by Nicol and Schleper (2006) and Jetten (2008). Until now, no nitrite-oxidizing archaea have been discovered.

Ammonia- and nitrite-oxidizing microorganisms (AOM, NOM) often thrive in the same environment, but are adapted to separate niches. In general, they are adapted to different ammonium and nitrite levels (Schramm et al. 1999; Maixner et al. 2006; Hatzenpichler et al. 2008) and often differ in their affinity to the respective substrate. Among other factors that are responsible for the distribution of AOM and NOM are temperature, pH, oxygen and salinity (Alawi et al. 2007; Santoro et al. 2008; Nicol et al. 2008; Erguder et al. 2009).

1.1.1 Ammonia oxidation

Ammonia oxidation is a two-step process. In AOB, ammonia monooxygenase (Amo), a membrane-associated enzyme (Hyman and Wood 1985), catalyzes the oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH) (Fig. 1.2). Amo was found to share many similarities with the particulate monooxygenase (Pmo) of methane-oxidizing bacteria (MOB) and the enzymes are evolutionary related (Holmes et al. 1995). The second step is performed by hydroxylamine oxidoreductase (Hao), which oxidizes hydroxylamine to nitrite (Fig. 1.2), and is located in the periplasmic space but anchored in the cytoplasmic membrane (Olson and Hooper 1983; Bock and Wagner 2006).

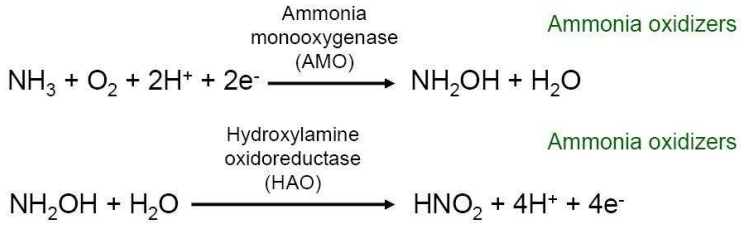

Figure 1.2: Ammonia oxidation reactions catalyzed by Amo and Hao enzymes of AOB.

The ammonia monooxygenase consists of three subunits, AmoA, AmoB and AmoC which are encoded by genes *amoA*, *amoB* and *amoC* which are organized in an operon. The AmoA protein is assumed to contain the active site of Amo (Hyman and Arp 1992) and *amoC*, which is located in the region upstream of the genes *amoA*/*amoB*, might encode a chaperone helping the AmoA and AmoB complex to integrate into the membrane (Klotz et al. 1997).

Ammonia-oxidizing archaea were shown to encode homologs of the three bacterial Amo-subunits, however, phylogenetic analysis showed that the novel archaeal proteins form a separate, distinct group within the Amo/Pmo family (Schleper et al. 2005; Treusch et al. 2005). AmoA can be used as a phylogenetic and functional marker for the analysis of both AOB and AOA (Purkhold et al. 2000; Schleper et al. 2005).

Amo uses free ammonia (NH_3) as substrate. Free ammonia and ionized ammonium (NH_4^+) represent two forms of reduced inorganic nitrogen, which exist in equilibrium depending upon pH, temperature and salinity. If the pH is low, the equilibrium shifts to NH_4^+, thus less substrate is available. Amo does not possess high substrate specificity and is additionally able to oxidize several apolar compounds such as methane, carbon monoxide and some aliphatic and aromatic hydrocarbons (Hooper et al. 1997). The broad substrate range of Amo also is responsible for inhibition of ammonia oxidizers by a variety of substances, like for example ethylene (Bock and Wagner 2006).

1.1.1.1 Ammonia-oxidizing bacteria (AOB)

The known AOB, characterized by the prefix *Nitroso-*, are members of the β- or γ-subclass of *Proteobacteria* (Bock et al. 1991; Bock and Wagner 2006). The genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* form a closely related monophyletic group within the β-subclass of *Proteobacteria*, whereas the genus *Nitrosococcus* is affiliated to a separate branch within the γ-subclass of *Proteobacteria* (Purkhold et al. 2000). Ammonia oxidizers have a slow growth rate and the shortest generation time measured in laboratory experiments was around 7 h for *Nitrosomonas* (Bock et al. 1990; Bock and Wagner 2006). Until now, AOB of the γ-subclass have only been detected in the marine environment (Kowalchuk and Stephen 2001; Koops et al. 2003), whereas AOB of the β-subclass of the *Proteobacteria* have a much broader environmental range (Kowalchuk and Stephen 2001; Koops et al. 2003).

1.1.1.2 Ammonia-oxidizing archaea (AOA)

For years, archaea were characterized as obligate extremophiles and to be metabolically constrained to a few environmental niches. However, this picture changed within the last two decades, when it was discovered that mesophilic members of the crenarchaeota are of crucial importance in many moderate environments. Most importantly, marine crenarchaeotes were shown to represent up to 40% of the bacterioplankton in deep ocean waters (Karner et al. 2001) and to account for 1-5% of 16S rRNA genes in soil (Ochsenreiter et al. 2003).

Phylogenetic analysis revealed distinct mesophilic crenarchaeotal lineages, which are mostly defined from sequences of marine plankton (group I.1A) or soils (group I.1B), but many other lineages exist (Schleper et al. 2005).

In 2005, Könneke et al. (2005) reported the isolation of "*Candidatus* Nitrosopumilus maritimus"that grows chemolithoautotrophically by aerobically oxidizing ammonia to nitrite, being the first observation of nitrification within the *Archaea*. The isolation of this marine crenarchaeote provided definitive proof that members of this phylum are capable of catalyzing the first step of nitrification (Könneke et al. 2005).

The potential for crenarchaeotal ammonia oxidation has also been confirmed by enrichment of a member of the Group 1.1b "soil"lineage, "*Candidatus* Nitrososphaera gargensis"(Hatzenpichler et al. 2008), and enrichment of *Nitrosocaldus yellowstonii*, an ammonia-oxidizing archaeon from a terrestrial hot spring (De la Torre et al. 2008).

Besides, the sponge symbiont *Cenarchaeum symbiosum*, affiliated to the marine group I.1A of crenarchaeota, is likely capable of ammonia oxidation (Hallam et al. 2006).

Until now, archaeal *amoA* gene sequences have been discovered in a wide range of habitats, like hot springs (Reigstad et al. 2008; De la Torre et al. 2008), marine ecosystems (Karner et al. 2001; Francis et al. 2007; Beman et al. 2008), estuaries/fresh water (Caffrey et al. 2007; Santoro et al. 2008), soil (Leininger et al. 2006; Tourna et al. 2008) and wastewater treatment plants (Park et al. 2006). Additionally, some studies demonstrate that archaeal *amoA* copies are more abundant than bacterial *amoA* copies in terrestrial (Leininger et al. 2006) and marine environments (Francis et al. 2005; Wuchter et al. 2006; Mincer et al. 2007) or indicate a higher transcriptional activity of archaeal over bacterial ammonia oxidizers in soil (Treusch et al. 2005; Leininger et al. 2006; Tourna et al. 2008), suggesting that AOA play a major role in the global nitrogen cycle. Recently, Jetten (2008) postulated that due to increasing indications for the importance of archaeal ammonium oxidizers in the global nitrogen cycle the contribution of ammonium-oxidizing bacteria has to be reassessed.

Important factors that are responsible for the distribution of AOA are substrate concentration and temperature. Archaeal *amoA* genes were detected in environments which often contain only low amounts of ammonia, such as open-ocean and hot springs (Wuchter et al. 2006; Reigstad et al. 2008). Recently, Martens-Habbena et al. (2009) found that the NH_3 concentration required for growth of "*Candidatus* Nitrosopumilus maritimus"is more than 100-fold lower than the minimum concentration required by cultivated AOB. The affinity of "*Candidatus* Nitrosopumilus maritimus"for NH_3 is among the highest affinities reported for microbial substrates and is more than 200-fold higher than that of AOB (Martens-Habbena et al. 2009). As stated by Martens-Habbena et al. (2009), their findings provide evidence for the existence of oligotrophic ammonia oxidizers among the Crenarchaeota and their ability to compete for ammonia in the nutrient-low ocean water.

The existence of nonthermophilic (i.e. *Nitrosopumilus maritimus* and *Cenarchaeum symbiosum*) and thermophilic (i.e. "*Candidatus* Nitrosocaldus yellowstonii"and "*Candidatus* Nitrososphaera gargensis") members of the ammonia-oxidizing crenarchaeota, and the detection of archaeal *amoA* genes at sites with very low (down to 0.2 °C) to high (up to 97 °C) temperatures provides an example of the broad distribution and diversity of AOA and the niches that they can occupy (Erguder et al. 2009).

Recently, Brochier-Armanet et al. (2008) stated that mesophilic crenarchaeota should be considered as members of a third archaeal phylum, which they named *Thaumarchaeota*. Their suggestion is based on the genome sequence of the mesophilic crenar-

chaeote *Cenarchaeum symbiosum*, which differs from hyperthermophilic crenarchaeota and branches deep in the archaeal tree. Features of the genomes of "*Candidatus* Nitrososphaera gargensis" and "*Candidatus* Nitrosopumilus maritimus" indicate that these two organisms might be affiliated to *Thaumarchaeota* (Spang et al., unpublished).

1.1.2 Nitrite oxidation

Nitrite oxidation is a reversible process performed by the membrane-bound nitrite-oxidoreductase (Nxr). This enzyme catalyzes the oxidation of nitrite to nitrate (NO_3^-; Fig. 1.3) and, in the absence of oxygen, the reduction of nitrate to nitrite (Bock and Wagner 2006; Sundermeyer-Klinger et al. 1984). Nxr of *Nitrobacter* is a heterodimer containing α (NxrA) and β (NxrB) subunits and is evolutionary related to the Nar-type dissimilatory nitrate reductase (Kirstein and Bock 1993).

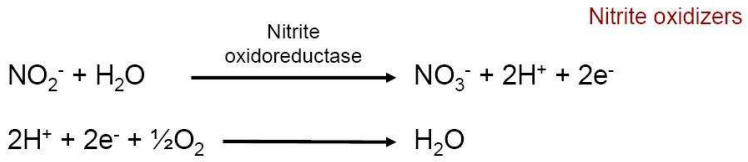

Figure 1.3: Nitrite oxidation as catalyzed by the Nxr enzyme of NOB.

1.1.2.1 Nitrite-oxidizing bacteria (NOB)

The second step of nitrification is catalyzed by aerobic nitrite-oxidizing bacteria (NOB), which are named with the prefix *Nitro-*, and catalyze the oxidation of nitrite to nitrate. Nitrite oxidizers belong to a broad range of phylogenetic groups and until now comprise the genera *Nitrobacter* (α-subclass of *Proteobacteria*), *Nitrococcus* (γ-subclass of *Proteobacteria*), *Nitrospina* (tentatively assigned to the δ-subclass of *Proteobacteria*), the recently discovered *Nitrotoga* (β-subclass of *Proteobacteria*) (Teske et al. 1994; Alawi et al. 2007) and *Nitrospira* (phylum *Nitrospirae*) (Ehrich et al. 1995).

Due to the polyphyletic distribution of NOB in several classes of *Proteobacteria* and in the *Nitrospirae* phylum, it remains difficult to investigate the diversity of NOB using the 16S rRNA gene as phylogenetic marker. For the *Nitrobacter* genus, which contains highly similar 16S rRNA gene sequences, it was recently found that the highly variable *nxrA* gene provides a good marker gene for studying molecular diversity of *Nitrobacter* (Poly et al. 2007).

Like AOB, NOB have slow growth rates. The shortest generation time measured in laboratory experiments was around 10 h for *Nitrobacter* (Bock and Wagner 2006).

While species of the genus *Nitrobacter* have been isolated from a variety of environments, including soil and fresh water, it was long assumed that the other genera were confined to marine environments (Bock and Koops 1992). However, bacteria affiliated to the genus *Nitrospira* were detected in many non-marine habitats and were shown to be the dominant nitrite oxidizers in wastewater treatment plants (Juretschko et al. 1998; Schramm et al. 1999; Daims et al. 2001).

An example for niche differentiation among NOB provides the different adaptation to nitrite of *Nitrospira* and *Nitrobacter*. It is known that *Nitrospira*-like bacteria have a higher affinity for nitrite and represent K strategists, while *Nitrobacter* species are r strategists which depend on higher nitrite concentrations (Schramm et al. 1999).

1.2 Aims of this work

1.2.1 Physiological characterization of "*Candidatus* Nitrososphaera gargensis"enriched from the Garga hot spring

In 2005, Lebedeva and colleagues enriched thermophilic nitrifiers from a hot spring in Russia and showed that the highest nitrite production was found between 46° and 50°C. In 2008, Hatzenpichler and coworkers identified an archaeon responsible for oxidizing ammonia chemoautotrophically in this enrichment. It was affiliated to the soil group 1.I.b of *Crenarchaeota* and named "*Candidatus* Nitrososphaera gargensis"(identifying the sampling site, the Garga hot spring). Mesophilic crenarchaeota are abundant in various, often nutrient-deprived, environments (Martens-Habbena et al. 2009). Prosser and Nicol (2008) stated that possible alternative substrates for archaeal Amo have to be considered and that mesophilic crenarchaeota might use potential alternative metabolisms and growth strategies.

Therefore, the aim of this study was to characterize the physiology of "*Candidatus* Nitrososphaera gargensis"by determining whether this chemolithoautotrophic AOA has a potential for mixotrophic growth by using microautoradiography (MAR). Enrichments were kindly provided by cooperation partners from Moscow (Elena Lebedeva) and Hamburg (Eva Spieck).

1.2.2 Identification of bacterial contaminants in the Garga hot spring enrichments

The enrichment containing "*Candidatus* Nitrososphaera gargensis" has been maintained for several years, but no pure culture is so far available. This enrichment contained around 5-7 microbial contaminants, which were assumed to be heterotrophic bacteria. One of the contaminants was identified as a novel putative AOB (Hatzenpichler 2006). The aim was to identify all bacteria in order to obtain a pure culture of the AOA, since information about their identity might help to select against them. In this study, these stable bacterial contaminants in the enrichment should be identified, further characterized and if possible, visualized directly by fluorescence *in situ* hybridization (FISH).

Additionally, Elena Lebedeva could enrich the putative novel AOB in a separate culture ("culture 7.3"). As proof of ammonia oxidation was still missing, cultures were incubated with NH_4^+ and ^{13}C-labeled sodium bicarbonate and sent to Vienna to investigate the samples by using Raman-microscopy.

1.2.3 Attempts to enrich novel nitrifying microorganisms from environmental samples

The first ammonia-oxidizing archaeon, "*Candidatus* Nitrosopumilus maritimus", was recently isolated by Könneke and coworkers (2005), but until now no archaea performing the second step of nitrification, the oxidation of nitrite to nitrate, have been found. Enrichment cultures were performed in order to selectively enrich nitrite-oxidizing archaea.

Incubation media were inoculated with soil sampled near the Vienna Ecology Center and sludge from an industrial WWTP located near the Humber estuary (UK). Soil and sludge enrichments were performed according to a protocol established by Simon and colleagues (2005), who successfully enriched soil crenarchaeota within a relatively short time. In a second attempt, enrichment cultures were performed using biofilm growing on filter installations obtained from a pool of the "Haus des Meeres" (Vienna). Biofilm was inoculated directly in aquarium water. By using aquarium water as inoculum, Könneke et al. (2005) had managed to enrich "*Candidatus* Nitrosopumilus maritimus" from an aquarium tank.

The aim was the phylogenetic identification and *in situ* detection (using FISH) of new nitrifiers.

Chapter 2

Material and methods

In this study, all buffers, media and solutions were prepared using double distilled and filtered water (H_2O_{bidist}) produced by a water purification facility (MQ Biocel, Millipore Corporation, Billerica, MA, USA). They were autoclaved in a watervapour-high pressure autoclave (Varioclav 135S, H+P, München Germany) for 20 min at 121°C and 1.013×105 Pa pressure and stored at room temperature (RT), if not stated otherwise. Chemicals were purchased and used in p.a. quality, if not stated otherwise. Substances and solutions unstable at high temperatures were sterile filtered and added after autoclaving.

2.1 Technical equipment

Table 2.1: Technical equipment used

Equipment	**Company**
Beadbeater BIO 101	Carlsbad, CA, USA
Capillary electrophoresis for sequencing and T-RFLP: 3130xl Genetic Analyzer	Applied Biosystems Lincoln, USA
Centrifuges: Mikro 22 R Rotina 35 S	 Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Confocal Laser Scanning Microscope LSM 510 Meta	Zeiss, Jena, Germany
DCodeTM system for DGGE	Biorad, München, Germany
Elektroporator MicroPulserTM	Biorad, München, Germany
Gelcarriage: HoeferTM HE 33 - gel running tray (7 × 10cm) Sub-Cell GT UV-Transparent Gel Tray (15 × 15 cm)	 Amersham Biosciences (SF) Corp., USA Biorad, München, Germany
Gel electrophoresis: HoeferTM HE 33 Mini Horizontal submarine unit Sub-Cell® GT	 Amersham Biosciences (SF) Corp., USA Biorad, München, Germany
Gel Dokumentationsystem MediaSystem FlexiLine 4040	Biostep, Jahnsdorf, Germany
Heatblock VWR Digital Heatblock	VWR international, West Chester, PA, USA
Hybridisation oven UE-500	Memmert GmbH, Schwabach, Germany

LabRAM HR800 UV confocal Raman microscope	Jobin-Yvon, UK
Laminar flow hood Safe 2010 Modell 1.2	Holten, Jouan Nordic, Allerød, Dänemark
Microwave MD6460	Microstar
Mixing block MB-102	Biozym Scientific GmbH, Oldendorf, Germany
PCR thermocyclers: Icycler Mastercycler gradient	 Biorad, München, Germany Eppendorf, Hamburg, Germany
pH-Meter WTW inoLab Level 1	Wissenschaftl.-Techn. Werkstätten GmbH, Weilheim, Germany
Photometers: NanoDrop® ND-1000 Spectralphotometer SmartSpecTM 3000	 NanoDrop Technologies, Wilmington, USA Biorad, München, Germany
Power device for gelelectrophoresis PowerPac Basic	Biorad, Munich, Germany
Orbital shaker Innova 2300	New Brunswick Scientific Co., Inc., Madison NJ, USA
Power device for gelelectrophoresis	PowerPac Basic Biorad, München, Germany
Transilluminator UST-30M-8E (312 nm)	Biostep GmbH, Jahnsdorf, Germany
tubing pumps IPC-N-4	Ismatec®, Glattbrugg-Zürich, Switzerland
Ultrasonic homogenizer HD 2070	Bandelin Sonopuls, Bandelin Electronics, Berlin, Germany
Vortex Genie 2	Scientific Industries, New York, USA
Water purification facility: MQ Biocel	Millipore Corporation, Billerica, MA, USA
Waterbaths: DC10 GFL Typ 1004	 Thermo Haake, Karlsruhe, Germany Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Water purification facility	Ultra ClearTM, Barsbüttel, Germany
Watervapour high pressure autoclaves: Varioclav 135S Varioclav 25T	 H+P, München, Germany H+P, München, Germany

2.2 Software

Table 2.2: Software used

Program	**URL**	**Reference**
analySIS getIT	http://www.soft-imaging.net/	Olympus Soft Imaging Solutions
e-seq	http://www.licor.com/bio/eSeq/DNASeq1.jsp	Licor Inc., Lincoln, NE, USA
ARB software-package	http://www.arb-home.de/	Ludwig et al. 2004
Basic Local Alignment Search Tool	http://www.ncbi.nlm.nih.gov/BLAST/	Altschul et al. 1990
daime	http://www.microbial-ecology.net/daime/	Daims et al. 2006
FinchTV	http://www.geospiza.com/finchtv/	Geospiza, USA
Labspec software	http://www.jobinyvon.de/pressreleases/0611labspec.htm	Jobin-Yvon
Peak Scanner	http://appliedbiosystems.com/peakscanner	Applied Biosystems, USA
Phylip	http://evolution.genetics.washington.edu/phylip.html	Felsenstein 1993
probeBase	http://www.microbial-ecology.net/probebase/	Loy et al. 2003
probeCheck	http://www.microbial-ecology.net/probecheck/	Loy et al. 2008
Ribosomal Database Projekt	http://rdp.cme.msu.edu/	Cole et al. 2003
TRF-CUT	http://www.arb-home.de/	Ricke et al. 2005

2.3 Expendable items

Table 2.3: Expendable items used

Expefndable item	**Company**
Alukappen Rotilabo®	Carl Roth GmbH & Co., Karlsruhe, Germany
Citifluor AF1	Agar Scientific Limited, USA
Cover slips 24 × 50 mm	Paul Marienfeld, Bad Mergentheim, Germany
Elektroporations-Küvetten, 0.2 cm	Biorad, München, Germany

Eppendorf Reaktionsgefäße (ERT), various sizes	Eppendorf AG, Hamburg, Germany
Erlenmeyer-Kolben DURAN®, various sizes	Schott Glas, Mainz, Germany
Glass bottles	Ochs GmbH Glasgerätebau & Laborfachhandel, Bovenden, Germany
Glascapillaries (50 µl in 5.1 cm)	Idaho Technology Inc., Salt Lake City, UT, USA
Injekt® - F syringe	Braun Melsungen AG, Melsungen, Germany
IsoporeTM membrane filters, various sizes	Millipore, Ireland
Microseal "A"Film	MJ Research, Waltham, MA, USA
Microplate 6 Well/ Flat Bottom	Iwaki/Asahi Technoglass, Gyouda, Japan
Mikrotitterplatte MicrosealTM 96, V-Boden	MJ Research, Waltham, MA, USA
Nitrate test strips	Merck KGaA, Darmstadt, Germany
Nitrite test strips	Merck KGaA, Darmstadt, Germany
Petri dishes 94/16	Greiner Bio-one GmbH, Frickenhausen, Germany
pH indicator sticks	Macherey-Nagel GmbH & Co, Düren, Germany
Pipet tips, various volumes	Carl Roth GmbH & Co., Karlsruhe, Germany
Plasticcuvettes, Halb-Mikro	Greiner Bio-One GmbH, Frickenhausen, Germany
Rotilabo®-Schlauchverbinder	Carl Roth GmbH & Co., Karlsruhe, Germany
Sampling vessels, 50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Schott DURAN® glass bottles, various sizes	Labware Schott AG, Mainz, Germany
slides, 10 Well	Paul Marienfeld, Bad Mergentheim, Germany
Sterican® insulin needle, various sizes	Braun Melsungen AG, Melsungen, Germany
Tygon®-Schlauch ST	Carl Roth GmbH & Co., Karlsruhe, Germany

2.4 Chemicals

Table 2.4: Chemicals used

Chemicals	**Company**
4'-6'-diamidino-2-phenylindole (DAPI)	Lactan Chemikalien und Laborgeräte GmbH, Graz, A
Acetic acid	Carl Roth GmbH & Co., Karlsruhe, Germany
Acrylamide/Bisacrylamide (40%, 37.5:1)	Biorad, Munich, Germany
Agar	Fluka Chemie AG, Buchs, Switzerland
Agarose, Electrophoresis Grade	Invitrogen Corporation, Carlsbad, CA, USA Cambrex
Ammonium chloride (NH_4Cl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Ammonium molybdate tetrahydrate $(NH_4)6Mo_7O_{24} \times 4H_2O$	Carl Roth GmbH & Co., Karlsruhe, Germany
Ammonium peroxydisulfate (APS)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Amphotericin B	Biochemika, Fluka, Steinheim, Germany)
Ampicilline	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Blocking reagent	Roche Diagnostics Wien GmbH, Vienna, Austria
Boric acid (H_3BO_4)	Fluka Chemie AG, Buchs, Switzerland
Bromphenol blue	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Calcium chloride dihydrate ($CaCl_2 \times 2H_2O$)	Mallinckrodt Baker BV, Deventer, Holland
Calciumcarbonat ($CaCO_3$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Chloramphenicol	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Cobalt(II) chloride hexahydrate ($Cl_2Co \times 6H_2O$)	Fluka Chemie AG, Buchs, Switzerland
CopperII sulfate 5-hydrate ($CuSO_4 \times 5H_2O$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Cycloheximide	Sigma-Aldrich, Steinheim, Germany
Dichloroisocyanuric acid sodium salt dihydrate	Sigma-Aldrich, Steinheim, Germany
Di-Sodiumhydrogenphosphate (Na_2HPO_4)	J. T. Baker, Deventer, Holland
Ethanol absolute	Merck KGaA, Darmstadt, Germany
Ethidium bromide (EtBr)	Fluka Chemie AG, Buchs, Switzerland
Ethylenediamine tetra-acetic acid (EDTA), di-sodium salt	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Iron II sulfate hepta-hydrate ($FeSO_4 \times 7H_2O$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Formamide (FA)	Fluka Chemie AG, Buchs, Switzerland
Hydrochloric acid (HCl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Hydrogen peroxide (H_2O_2) 30%	Carl Roth GmbH & Co., Karlsruhe, Germany
Isopropanol (2-propanol)	Carl Roth GmbH & Co., Karlsruhe, Germany
Isopropyl-b-D-Thiogalactopyranoside (IPTG)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Magnesiumsulfate heptahydrate ($MgSO_4 \times 7H_2O$)	Merck KGaA, Darmstadt, Germany
Manganese(II)-sulfate monohydrate ($MnSO_4 \times H_2O$)	Carl Roth GmbH & Co., Karlsruhe, Germany
N-(1-Naphthyl)-ethylendiamindihydrochlorid	Carl Roth GmbH & Co., Karlsruhe, Germany
N-Allylthiourea	Fluka Chemie AG, Buchs, Switzerland
N,N,N',N'-Tetra-methyl-ethylenediamine (TEMED)	Fluka Chemie AG, Buchs, Switzerland
N,N-Di-methylformamide (DMF)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Nessler's reagent	Fluka Chemie AG, Buchs, Switzerland

Nickel(II) chloride hexahydrate ($NiCl_2 \times 6H_2O$)	Riedel-de Haen, Seelze, Germany
NuSieve® 3:1 Agarose (low-melting-point)	Bio Science Rockland, Inc., Rockland, ME, USA
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Poly-L-Lysin solution	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Potassium acetate (KCl)	J. T. Baker, Deventer, Holland
Potassium dihydrogen phosphate (KH_2PO_4)	Mallinckrodt Baker BV, Deventer, Holland
Sodium bicarbonate ($NaH^{12}CO_2$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium bicarbonate ($NaH^{13}CO_3$)	Cambridge Isotope Laboratories, Inc., Andover ,USA
Sodium chloride (NaCl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium dodecyl sulphate (SDS)	Fluka Chemie AG, Buchs, Switzerland
Sodium-di-hydrogenphosphate (NaH_2PO_4)	J. T. Baker, Deventer, Holland
Sodium hydroxid (NaOH)	J. T. Baker, Deventer, Holland
Sodium nitrite ($NaNO_2$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium nitroprusside dihydrate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Sodium salicylat	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Streptomycin	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Sulfanilamid	Carl Roth GmbH & Co., Karlsruhe, Germany
SYBR® Green I	Cambrex Bio Science, Rockland, Inc., Rockland, ME,USA
Tetracycline hydrochloride	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Tris	Carl Roth GmbH & Co., Karlsruhe, Germany
Triton X-100	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Tryptone	Oxoid LTD., Hampshire, England
Urea	USB Corp., Cleveland, USA
X-Gal(5-brom-4-chlor-3-indolyl-β-D-galactopyranoside)	Carl Roth GmbH & Co., Karlsruhe, Germany
Xylencyanol	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Yeast extract	Oxoid LTD., Hampshire, England
Zinc sulphate heptahydrate ($ZnSO_4 \times 7H_2O$)	Carl Roth GmbH & Co., Karlsruhe, Germany

2.5 Buffers, media and solutions

2.5.1 General buffers

a) TE buffer

Tris 10 mM

EDTA 5 mM

pH was adjusted to 8.0 using HCl.

b) PBS

- **PBS stock solution**

NaH_2PO_4	200 mM	35.6 g/l
Na_2HPO_4	200 mM	27.6 g/l

pH of NaH_2PO_4 solution was adjusted to 7.2-7.4.

- **1 × PBS**

NaCl	130 mM	7.6 g/l
PBS stock solution	10 mM	50 ml/l
H_2O_{bidist}		ad 1000 ml

pH 7.2–7.4

- **3 × PBS**

NaCl	390 mM	22.8 g/l
PBS stock solution	30 mM	150 ml/l
H_2O_{bidist}		ad 1000 ml
pH 7.2 –7.4		

2.5.2 Media and solutions for enrichment of nitrifiying bacteria and archaea

2.5.2.1 Medium for AOA enrichment

- **Stock solution (10 ×), autoclaved**

KH_2PO_4	0.54 g
KCl	0.74 g
$MgSO_4 \times 7\ H_2O$	0.049 g
NaCl	5.84 g
H_2O_{bidist}	ad 1000 ml

- **Trace element solution (TES), sterile filtered**

$MnSO_4 \times H_2O$	0.0338 g
H_3BO_4	0.0494 g
$ZnSO_4 \times 7\ H_2O$	0.0431 g
$(NH_4)\ 6Mo_7O_{24} \times 4\ H_2O$	0.0394 g
$FeSO_4 \times 7\ H_2O$	0.937 g
$CuSO_4 \times 5\ H_2O$	0.022 g
H_2O_{bidist}	ad 1000 ml

- **AOA medium**

  100 ml stock solution

  0.5 ml cresol red (0.05 % in 0.01 N HCl), for monitoring pH

  1 ml TES

0.144 g $CaCl_2 \times 2\ H_2O$

(0.0267 g NH_4Cl)

H_2O_{bidist} ad 1000 ml

2.5.2.2 Medium and solutions for NOM enrichments

- **Stock solution**

$CaCO_3$	0.07 g
NaCl	5.0 g
$MgSO_4 \times 7H_2O$	0.5 g
KH_2PO_4	1.5 g
H_2O_{bidist}	ad 1000 ml

- **Trace element solution (TES)**

$MnSO_4 \times H_2O$	0.0338 g
H_3BO_3 (BO_4)	0.0494 g
$ZnSO_4 \times 7\ H_2O$	0.0431 g
(NH_4) 6Mo7O24 $\times$ 4 H_2O	0.0371 g
$FeSO_4 \times 7\ H_2O$	0.937 g
$CuSO_4 \times 5\ H_2O$	0.025 g
H_2O_{bidist}	ad 1000 ml

- **Nickel Cobalt solution (NiCo)**

$NiCl_2 \times 6\ H_2O$	3.8 µM
$COCl_2 \times 6\ H_2O$	7.6 µM

- **NOM medium**

100 ml stock solution

1 ml TES

1 ml NiCo

ad 1000 ml MQ

pH 8.6 (the pH was adjusted with NaOH)

The medium was stored for 1 month at room temperature, after which the pH should be 7.0. Because pH was 8.4, it was adjusted by adding 2 N HCl and 0.2 N NaOH. 2 × 500 ml of NOM medium were sterile filtered trough a 0.2 µM pore filter and stored at 4°C.

- **$NaNO_2$ solutions**

  2.5 mM, 6.25 mM, 12.5 mM, 25 mM

  The appropriate amount of $NaNO_2$ was weighted and each time diluted in 200 ml of media (1/4 water, 3/4 aquarium water) and sterile filtered.

- **0.3 M $NaH^{12}CO_3$ and $NaH^{13}CO_3$**

  $NaHCO_3$ was diluted in aquarium water and sterile filtered.

2.5.3 Buffers, solutions and standards for gel electrophoresis

a) TAE buffer

- **50 × TAE**

Tris	2 M
Sodium acetate	500 mM
EDTA	50 mM

pH was adjusted to 8.0 with pure acetic acid.

- **1 × TAE**

50 × TAE	20 ml/l
H_2O_{bidist}	ad 1000 ml

b) TAE buffer, modified (Millipore)

- **50 × TAE, modified**

Tris	2 M
EDTA	5 mM

pH was adjusted to 8.0 with pure acetic acid.

- **1 × TAE, modified**

50 × TAE	20 ml/l
H_2O_{bidist}	ad 1000 ml

c) TBE buffer

- **10 × TBE**

Tris	890 mM	162.0 g/l
Boric acid	890 mM	27.5 g/l
EDTA	20 mM	9.3 g/l
H_2O_{bidist}	ad 1000 ml	
pH 8.3 – 8.7		

- **1 × TBE**

10 × TBE	100 ml/l
H_2O_{bidist}	ad 1000 ml

d) Loading buffer

Ficoll	25% (w/v)
Bromphenol blue	0.05% (w/v)
Xylencyanol	0.05% (w/v)
EDTA	50 mM

e) Ethidium bromide solution

- **Ethidium bromide stock solution**

  10 mg/ml Ethidium bromide (EtBr) in H_2O_{bidist}

- **Ethidium bromide staining solution**

  EtBr-stock solution diluted 1:10,000 in H_2O_{bidist}

f) SYBR® Green I solution

- **SYBR® Green I stock solution**

  SYBR® Green I 10,000 × concentrate in DMSO

- **SYBR® Green I staining solution**

  SYBR® Green I stock solution diluted 1:10,000 in H_2O_{bidist}

g) DNA ladder (KbL)

GeneRulerTM 1kb (Fermentas, St. Leon-Rot, Germany)

2.5.4 Culture media for *Escherichia coli* (E. coli) strains

a) Luria Bertani medium (LB medium)

Tryptone	10.0 g/l
Yeast extract	5.0 g/l
NaCl	5.0 g/l
H_2O_{bidist}	ad 1000 ml

pH 7.0-7.5. For solid media 15 g/l agar were added before autoclaving.

b) SOC medium

Tryptone	2 % w/v
Yeast extract	0.5 % w/v
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM

2.5.5 Antibiotics

To solid media antibiotic stock solution was added after the temperature of the autoclaved media dropped to ∼50°C. Media were stored at 4°C. Antibiotics were added to liquid media right before use.

a) Amphotericin B (Amph B)

Amphotericin B	25 mg/ml

Amph B was added to the medium to reach a final concentration of 25 µg/ml.

b) Ampicillin stock solution (Amp)

Ampicillin	100 mg/ml

Amp was dissolved in 50% $EtOH_{abs}$ and added to the medium to reach a final concentration of 100 µg/µl.

c) Chloramphenicol stock solution (Camp)

Chloramphenicol	170 mg/ml

Camp was dissolved in 50% $EtOH_{abs}$ and added to the medium to reach a final concentration of 70 µg/ml.

d) Cycloheximide

Cycloheximide	100 mg/ml

Cycloheximide was added to the medium to reach a final concentration of 100 µg/ml.

e) Streptomycin (Str)

Streptomycin	100 mg/ml

Str was dissolved in H_2O_{bidist} and added to the medium to reach a final concentration of 100 µg/ml.

f) Tetracycline (Tet)

Tetracyclin	50 mg/ml

Tet was dissolved in 50% $EtOH_{abs}$ and added to medium reaching a final concentration of 100 µg/ml.

2.5.6 Solutions for selection and induction

a) X-Gal stock solution

X-Gal is used for blue/white screening after cloning and allows identification of insert-positive cells.

X-Gal (5-brom-4-chlor-3-indolyl-β-D-galactopyranoside)	40 mg/ml

X-Gal was dissolved in di-methylformamide (DMF) and sterile filtered using 0.22 µm pore size filters (Millipore). The solution was stored in the dark at -20°C. To identify insert-positive cells, before cells were plated, 40 µl of X-Gal solution were spread on a LB-agar plate containing 100 µg/ml ampicillin.

b) IPTG stock solution

Isopropyl-β-D-thiogalactopyranosid (IPTG)	1 M

IPTG was dissolved in H_2O_{bidist}, sterile filtered (using 0.22 µm pore size) and stored at -20°C. IPTG was used to induce *in vivo* transcription of 16S rRNA genes which were cloned into plasmids and transferred into *E. coli* expression strain JM109 (DE3) (see 2.15 and 2.24). Therefore, 100 µl of IPTG stock solution were added to an actively growing 100 ml culture (OD_{600} = 0.334).

2.5.7 Solutions for plasmid isolation

a) P1 buffer

Tris-HCl, pH 8.0	50 mM
EDTA	10 mM
RNAse A	100 µg/ml

b) NaOH/SDS solution

H_2O_{bidist}	8 ml
NaOH (2 M)	1 ml
10% SDS	1 ml

c) Potassium acetate/acetate solution

KCl (5 M)	6 ml
H_2O_{bidist}	2.85 ml
Acetic acid (pure)	1.15 ml

2.6 Enrichment of nitrifying microorganisms

2.6.1 Enrichment of nitrifying microorganisms from soil and sludge

2.6.1.1 Sampling and sample preparation

Two 50 ml Greiner tubes were filled with soil sampled near the Vienna Ecology Center from a meadow at a depth of approximately 15 cm. The soil was pounded and plant roots, big fungal hyphae and small stones were removed with sterilized forceps.

Sludge originated from an industrial wastewater treatment plant located near the Humber, an estuary on the east coast of England, and was kindly provided by Marc Mussmann.

2.6.1.2 Cell separation and inhibition of bacterial growth

Different methods for cell separation were combined with chemical and physical treatments to inhibit growth of bacteria. The methods were based on a protocol by Simon et al. (2005), who enriched crenarchaeotes in cultures inoculated with plant root extract. Crenarchaeotes accounted for up to 20% of total microorganisms after only two months. Thus, in this study a medium resembling the one reported by Simon et al. (2005) was used and cell separation methods were performed in accordance to their study, but different antibiotics were supplemented and incubations were not shaken. The following steps were performed:

- washing

  20 g of soil were dissolved in 15 ml of 1 × PBS (autoclaved) and shaken for 1 h 20 min at 220 rpm at room temperature in order to wash the cells from soil particles. The remaining soil was stored at –80°C.

  For sludge enrichments, 19.3 g of sludge were dissolved in 15 ml of 1 × PBS.

- sonication

  After the washing step, microorganisms were displaced from soil particles and sludge aggregates by sonication for 30 sec at 20% power.

- freeze-thawing

In addition to sonication, in one of the enrichments a freeze-thaw cycle was performed to break open cell clusters. Therefore, samples were frozen in a dry ice-ethanol bath for 5 min and quickly thawed in a water bath (48°C) for 5 min.

- centrifugation

  To remove the coarse particles, samples were centrifuged and pelleted for 1 min at 520 g (pellet 1), 1170 g (pellet 2) and 2090 g (pellet 3) using a Rotina 35S centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Each time the supernatant was collected. Then the cells were centrifuged at 15,820 g for 5 min and the SN discarded (SN 4).

- lysozyme treatment

  Lysozyme damages bacterial cell walls by catalyzing hydrolysis of glycosidic bonds in peptidoglycan, which is not present in most archaeal cell walls. To restrict the growth of bacteria, the pellet was dissolved in 4.5 ml TE-buffer containing lysozyme (0.5 g in 5 ml of TE-buffer) and incubated for 15 – 30 min at 37°C.

- freeze-thawing

  After lysozyme usage, freeze/thaw treatments should even more effectively destroy the already weakened bacterial cell walls. Therefore, freeze-thaw cycles were performed by three consecutive rounds of quick-freezing in a dry ice-ethanol bath for 5 min, followed by thawing at 48°C for 5 min. Subsequently, cells were centrifuged again at 15,820 g for 5 min and the SN discarded (SN 5). The pellet was dissolved in 0.9 ml NOM-medium (enriched biomass).

2.6.1.3 Inoculation

The culture media prepared for enrichment of NOM was a mineral medium, which consisted of salts and trace elements. The only carbon source was bicarbonate.

Enrichment 1: soil

200 µl of cells (enriched biomass) were inoculated into 20 ml of NOM-medium (1:100 dilution). To each culture amphotericin B was added to inhibit fungal growth. Streptomycin and tetracycline were added to prevent bacterial growth.

The following incubations were performed:

1. Incubations with NO_2^-: Cultures were supplemented with NO_2^- to the final

concentration of either 75 µM or 750 µM. Thus, 20 µl and 200 µl of the 75 mM NO_2^- stock solution were added, respectively.

2. NO_2^- control: Medium was incubated without cells to test for abiotic conversion of NO_2^-. The control was supplemented with NO_2^- to the final concentration of 750 µM.

3. Incubations with NH_4^+ : Cultures were supplemented with NH_4^+ to the final concentration of either 75 µM or 750 µM. Thus, 20 µl and 200 µl of the 75 mM NH_4^+ stock solution were added, respectively.

4. NH_4^+ control: Medium was incubated without cells to test for chemical loss of NH_4^+ via evaopration. The control was supplemented with NH_4^+ to the final concentration of 750 µM.

The flasks were each filled with only one-fifth of its volume. A large head space was left in order to guarantee oxygen supply. Cultures were incubated without shaking at 20°C in the dark. The remaining 100 µl of cells (enriched biomass) were fixed with PFA (see 2.21.1.1).

Enrichment 2: soil and sludge

In another approach, 5 ml of NOM-medium (1:25 dilution) was inoculated with 200 µl of cells in order to obtain a more concentrated culture. Each culture was supplemented with cycloheximide, which inhibits translation in eukaryotes and was utilized to avoid fungal growth.

The following incubations were performed:

1. Incubations with NO_2^-/NH_4^+: The setup consisted of 3 incubations supplemented with NO_2^- and NH_4^+ to the final concentration of 5, 50 and 500 µM, therefore 0.35, 3.5 and 35 µl of substrates were added, respectively.

2. NO_2^- /NH_4^+ control: No cells were added to the medium. The control was supplemented with NO_2^- and NH_4^+ to the final concentration of 500 µM.

A 6 well microplate (Iwaki/Asahi Technoglass, Gyouda, Japan) was used for incubations, which were carried out without shaking at 20°C in the dark. The remaining 100 µl of cells (enriched biomass) were fixed with PFA (see 2.21.1.1).

2.6.2 Enrichment of nitrifying microorganisms from biofilm of aquarium filters

2.6.2.1 Sampling in the "Haus des Meeres"

Two autoclave bags full of filter balls overgrown with biofilm and 10 l of aquarium water (artificial seawater "Reef crystals", Aquarium Systems) were collected from the filter installations of the big shark pool in the "Haus des Meeres", Vienna. The filter balls were made of polypropylene and had a diameter of approximately 5 cm. The pool comprised 300 000 l of water and is permanently run at a temperature of 27°C and a pH of 8.4.

2.6.2.2 Sample preparation for pre-incubations

In the beginning, different pre-incubations were performed in order to test if any nitrifying activity could be observed. First, big particles like shells and plastic stripes were removed from filter balls with forceps. The incubations were supplied with either ammonia or nitrite, in order to support growth of ammonia and nitrite oxidizers, respectively. To select for archaea, streptomycin and tetracycline were added and to inhibit fungal growth all cultures were supplemented with amphotericin B (final concentration: 25 µg/ml). To test if the nitrifying community was present in the water or in the biofilm, incubations with aquarium water (without biofilm) and with differently treated biofilm were performed. For the incubations with biofilm, the biofilm was washed off from three filter balls with 20 ml of aquarium water. For each incubation 20 ml of aquarium water were used, except for the incubation with the whole filter balls, in which more volume was necessary to cover the balls. For the incubations 100 ml Schott flasks were used. They were filled with only one-fifth of its volume, guaranteing oxygen supply, and covered with aluminium foil.

The following incubations were performed:

1. aquarium water only, supplemented with NO_2^-: To test if the nitrifying community was present in the water or in the biofilm, aquarium water without biofilm was incubated. The control was supplemented with NO_2^- to the final concentration of 500 µM, thus 133 µl of the 75 mM NO_2^- stock solution were added.

2. NO_2^- + biofilm: Biofilm was washed off the filter balls without further treatment. Therefore, an Erlenmeyer flask containing aquarium water and a filter ball was shaken well until the biofilm went off the ball. The procedure was repeated with

two more filter balls. All cultures were supplemented with NO_2^- to the final concentration of 500 µM.

3. NO_2^- + antibiotics + biofilm: Antibiotics targeting bacteria were added to select for archaea. Streptomycin and tetracycline were added to reach the final concentration of 100 µg/ml and 50 µg/ml, respectively. Thus, 20 µl were added from each stock solution.

4. NO_2^- + antibiotics + crushed biofilm: Biofilm was crushed by vortexing, in order to allow antibiotics to more easily penetrate into the biofilm.

5. NO_2^- + filtrate: After washing the biofilm off the filter balls, aquarium water containing biofilm was filtered (Millipore) in order to inoculate medium only with biofilm flocs bigger than 12 µm. After filtration, the flocs were scraped off the filter and added to the medium.

6. NO_2^- + balls: Filter balls as a whole were incubated in aquarium water.

Approaches 1. to 5. were also performed supplemented with NH_4^+. Therefore, these cultures were supplemented with NH_4^+ to the final concentration of 500 µM. Thus, 500 µl of the 20 mM NH_4^+ stock solution were added.

All cultures were incubated in the dark without shaking at 27°C.

These pre-incubations were performed in order to test if any nitrifying activity could be observed. To test for nitrification, samples were taken after four and five days to measure NH_4^+ and NO_2^- concentrations.

10 filter balls of the original aquarium biofilm sample obtained from "Haus des Meeres" were washed in 100 ml of aquarium water and fixed with EtOH and PFA (see 2.21.1) for later FISH analysis.

2.6.2.3 Main incubations

For each incubation biofilm was washed off 3 filter balls using 20 ml of aquarium water. Every incubation except the chemical control (autoclaved H_2O_{bidist}) was supplemented with 20 µl of sterile filtered 1 M $NaHCO_3$ (final concentration: 1 mM) to support autotrophic growth, and cycloheximide to prevent fungal growth. To some incubations streptomycin was added to the final concentration of 100 µg/ml (cultures labeled AB). The incubations were supplemented with either 500 µM of sterile filtered NH_4^+ or 500 µM of sterile filtered NO_2^-, therefore, 100 µl of a 100 mM NH_4^+ and NO_2^- stock solution were added, respectively.

The following incubations (27°C in the dark) were performed to enrich the nitrifying community:

1. NO_2^- + aquarium water only
2. NO_2^- + biofilm (in duplicates; abbreviation: NO_2^- bf I, II)
3. NO_2^- + antibiotic + biofilm (in duplicates; abbreviation: NO_2^- AB bf I, II)
4. NH_4^+ + aquarium water only
5. NH_4^+ + biofilm (in duplicates; abbreviation: NH_4^+ bf I, II)
6. NH_4^+ + antibiotic + biofilm (in duplicates; abbreviation: NH_4^+ AB bf I, II)
7. H_2O_{bidist} (autoclaved) + NH_4^+ + NO_2^- (as chemical control)

Samples were taken regularly to measure substrate concentrations and control the pH. Additionally, samples were collected for FISH analysis of the cultures. Therefore, 1-2 ml of biomass were collected and PFA-fixed as described in 2.21.1.1. After sampling for FISH, cultures were refilled with aquarium water.

2.6.2.4 Dilution of incubations

After approximately every two months the incubations were diluted 1:10 to select for the nitrifying community and to dilute non-target organisms. Therefore, 2 ml of the respective old incubations were inoculated in 18 ml of fresh sterile filtered aquarium water (stored at 4°C) and supplemented as described above (see 2.6.2.3).

2.6.2.5 Stable isotope probing

With the intention to perform stable isotope probing combined with Raman microscopic analysis, four of the nitrite consuming cultures (NO_2^- bf I, II and NO_2^- AB bf I, II) were connected to an automatical feeding and aeration system. This was done to provide a more effective culturing and faster growth of biomass.

Setting up of pumps, preparation of tubes and feeding solutions

Tygon tubes for feeding and aeration were cut into appropriate length, connected with adapters (Rotilabo®-Schlauchverbinder, Carl Roth GmbH & Co., Karlsruhe, Germany) and applied to the pumps (IPC-N-4, Ismatec®, Glattbrugg-Zürich, Switzerland) or connected to an aerator. The tubes for feeding were washed three times with

70% EtOH and afterwards three times with sterile filtered aquarium water. The pumps were adjusted in such a way that the flow in each tube was the same. Finally, in 8.6 sec a volume of 100 µl was pumped through. Pumps were pumping at power 6 and feeding was set for every 3 h. Depending on the amount of µM NO_2^- added, pumping periods were set between 2.5 sec and 17.2 sec. The aerator was set up to change every two h the head space in cultures with fresh air. For feeding, 2.5 mM, 6.25 mM, 12.5 mM and 25 mM $NaNO_2$ solutions were prepared. $NaNO_2$ was diluted in one quarter of autoclaved water and three quarters of sterile filtered aquarium water. This was done due to evaporation of the medium in cultures: the aquarium water was used to avoid dilution of the cultures, while the water was used in order to prevent too high salt concentration in the incubation medium.

Setup for incubation

For incubations 200 ml syringe bottles were used. For each culture, three different incubations were performed:

1. culture + ^{12}C bicarbonate + NO_2^-
2. culture + ^{13}C bicarbonate + NO_2^-
3. culture + ^{12}C bicarbonate w/o NO_2^- (negative control)

The incubation volume was 50 ml and 1:10 dilutions were performed. Thus, 5 ml of the respective culture (incubating since January 2009) were added to 45 ml of sterile filtered aquarium water. After this, flasks were closed with aluminium caps (Rotilabo®, Carl Roth GmbH & Co., Karlsruhe, Germany) and the following additions were done using needles (Sterican® insulin needle, Braun Melsungen AG, Melsungen, Germany). Cultures were supplemented with cycloheximide and streptomycin as before. ^{12}C incubations were supplemented with sterile filtered 0.3 M $NaHCO_3$ and ^{13}C incubations were supplemented with sterile filtered 0.3 M $NaHCO_3$, leading to a final concentration of about 5 mM ^{12}C or ^{13}C bicarbonate. Flasks were incubated in a water bath at 27°C with mild shaking and tubes from the NO_2^- solution and the aerator were connected to them (Fig. 2.1). Tubes were covered with aluminium foil to inhibit phototrophic growth. Between aeration tubes and flasks 0.25 µm pore filters were added, to reduce possible contamination from air. At the beginning, NO_2^- was added every 3 h to the final concentration of 5 µM. When no NO_2^- accumulated, the feeding rate was increased. This was done either by increasing the feeding time or by replacing the NO_2^- solution with a more concentrated one. Incubations were carried out in the dark. Prior to PFA-fixation (2.21.1.1) cultures were shaken and 1 ml of culture was sampled with syringe and needle.

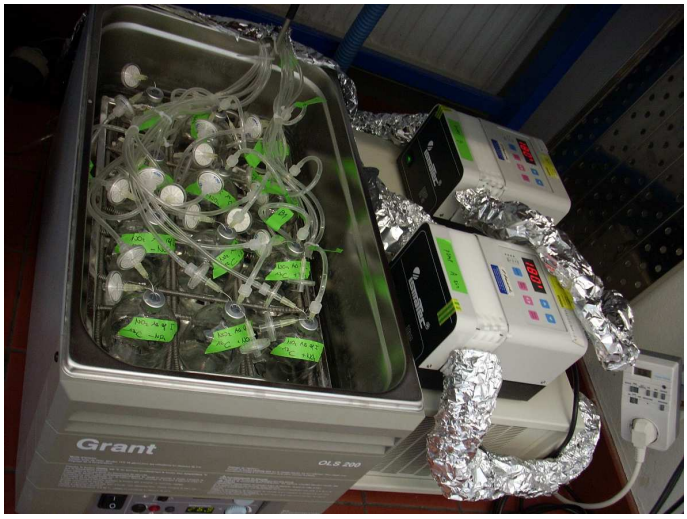

Figure 2.1: Setup for stable isotope probing. On the left side, the incubations in the waterbath are shown. On the right side, the two pumps are visible, which are connected to cultures trough tubes covered with aluminium foil.

The different conditions for the respective enrichment cultures are summarized in Table 2.5.

Table 2.5: Conditions for nitrifying enrichment cultures.

	sample material	**cell separation**	**medium**	**antibiotics**	**nutrients**	**incubation volume**
enrichment 1	soil	washing (PBS) sonication centrifugation lysozyme treatment	NOM	tetracycline streptomycin amphotericin B	75, 750 µM NO_2^-, NH_4^+	20 ml
enrichment 2	soil	washing (PBS) sonication centrifugation lysozyme treatment freeze-thawing	NOM	cycloheximide	5, 50, 500 µM NO_2^-, NH_4^+	5 ml
	sludge	washing (PBS) sonication centrifugation lysozyme treatment freeze-thawing	NOM	cycloheximide	5, 50, 500 µM NO_2^-, NH_4^+	5 ml
enrichment 3	biofilm from aquarium filters	washing (seawater)	aquarium water	streptomycin cycloheximide	500 µM NO_2^-, NH_4^+	20ml, 50ml

2.7 Enrichment cultures from the Garga hot spring

2.7.1 "*Candidatus* Nitrososphaera gargensis"enrichment culture from the Garga hot spring

2.7.1.1 Sample material

One ERT with a thick brown cell pellet was sent by cooperation partners from Hamburg. Additionally, four 50 ml Greiner tubes filled with NH_4^+-free and bicarbonate-free AOA-medium were received. The enrichment mainly consisted of the ammonia-oxidizing archaeon (AOA) "*Candidatus* Nitrososphaera gargensis"(∼70% of biomass) and about 5-7 estimated heterotrophic contaminants (∼30%). One of the contaminants had already been described as a putative AOB (Hatzenpichler 2006).

2.7.1.2 Activation and maintaining of enrichment culture

After resuspending the cell pellet in a minimal volume of medium, it was added to a 250 ml Erlenmeyer flask containing 80 ml of the AOA-medium. 2 ml of a 20 mM NH_4Cl stock solution were added, leading to a final ammonium concentration of 0.5 mM. 500 µl of this culture were immediately taken and frozen at -20°C (zero time point for NH_4^+ and NO_2^- measurements). The culture was incubated in the dark at 46°C without shaking. NH_4^+ and NO_2^- concentrations were monitored daily by photometric methods (see 2.8) and pH adjusted if necessary by addition of 0.1% NaOH, until the medium was slightly pink. After the culture became highly active (NH_3 oxidation to NO_2^- per day), 65 ml of medium containing 0.5 mM NH_4^+ was added and the culture was divided into two approximately equal volumes (∼70 ml). Later on, cultures were diluted further to avoid accumulation of NO_2^-.

NH_4^+, NO_2^- concentration measurements and pH control

Samples (500 µl) were collected daily for measurement of NH_4^+ and NO_2^- concentrations in order to follow the oxidation of NH_3 to NO_2^- and to gain information about the activity of the cells. Because of the production of acidic substances by ammonia oxidizers pH was controlled regularly with pH stripes. When all NH_3 was converted to NO_2^-, the culture was fed with new NH_4^+.

2.7.1.3 Temperature experiment with Garga enrichment culture

To investigate the temperature dependence of NH_3 oxidation, a temperature series experiment was performed. Incubations were done in duplicates at following temperatures: 37°C, 46°C, 56°C and 66°C. 3 ml of an active culture containing 0.5 mM NH_4^+ at adjusted pH (∼7.5-8) were pipetted into 10 ml autoclaved glass-vials, which were put into closed 50 ml Greiner tubes and incubated at the respective temperature. For negative control, H_2O_{bidist} supplemented with 0.5 mM NH_4^+ was incubated at 46°C. Samples (200 µl) were taken to measure NH_4^+ and NO_2^- values at time point zero. Every day the tubes were opened shortly close to a flame, to guarantee that enough oxygen was available. After every 3-4 days, samples (200 µl) were taken for NH_4^+ and NO_2^- concentration measurement, and stored at -20°C. In addition to this, cultures were fed with 75 µl of 20 mM new NH_4^+ (0.5 mM end concentration). Additionally, pH was controlled in some of the cultures. After two weeks, the experiment was stopped. NH_4^+ and NO_2^- were measured photometrically as described in 2.8.

2.7.2 Enrichment culture containing a putative AOB from the Garga hot spring

An enrichment culture ("culture 7.3") highly enriched with putative AOB was maintained by Elena Lebedeva. Cultures were supplemented with $NaH^{13}CO_3$ and $NaH^{12}CO_3$ and incubated for 72 h (performed by Elena Lebedeva in Hamburg). During the incubations, cells consumed around 2 mM of NH_4^+. Samples were taken at different time points and were both PFA- and EtOH-fixed. Finally, they were sent to Vienna. The aim was to perform Raman spectroscopic analysis combined with FISH in order to proof autotrophic growth in the presence of ammonia as energy source at the single cell level.

2.8 Measurement of Ammonium, Nitrite, Nitrate and pH

2.8.1 Ammonium

2.8.1.1 Colorimetric determination of ammonium concentration in potassium chloride and water extractions

The principle of the method is the oxidation of ammonia to chloroamine by sodium dichloroisocyanuric acid, which subsequently forms a green indophenol in the presence of phenolic compounds under alkaline conditions. The absorbance is measured at 660 nm.

Solutions

0.3 M NaOH	
Sodium salicylat solution	8.5 g sodium salicylat and 63.9 g sodium nitroprusside dihydrate dissolved in 50 ml H_2O_{bidist}.
Color reagent	0.3 M NaOH solution, sodium salicylate solution and H_2O_{bidist} mixed 1:1:1; freshly prepared before use
Oxidation solution	50 mg of dichloroisocyanuric acid sodium salt dihydrate dissolved in 50 ml H_2O_{bidist}.

For calibration an ammonium nitrogen stock standard solution (1,000 mg/l) and an ammonium nitrogen working standard solution (5 mg/l; 0.5 ml of stock diluted in 99.5 ml H_2O_{bidist}) were prepared.

Procedure

Prior to each measurement, standard dilutions for calibration were prepared. Each time, 10 standard dilutions were prepared, starting with a concentration of 357 µM nitrogen-NH_4^+ and diluted 1:2 with H_2O_{bidist}. 300 µl of standards and 300 µl of samples were pipetted into 1.5 ml ERT and 150 µl of color reagent and 60 µl of oxidation solution were added. If samples were expected to contain higher concentrations of NH_4^+ than the most concentrated NH_4^+ standard, appropriate dilutions were performed. Then, samples were shaken on a mixing block (Biozym Scientific GmbH, Oldendorf, Germany) at 1,000 rpm for 30 min. The color intensity was measured with a spectrophotometer (SmartSpecTM 3000, Biorad, München, Germany) at 660 nm.

Calculation

The measured adsorption of standards was plotted against concentration and a linear

regression was performed using Excel (Microsoft). If a straight line was obtained for standards, its equation was used to determine the NH_4^+ concentration of the samples.

2.8.1.2 Nessler's reagent

To quickly find out if NH_4^+ was present in the cultures, Nessler's reagent, an alkaline solution of potassium tetraiodomercurate(II) ($K_2(HgI_4)$), was used. NH_4^+ reacts with the mercury to form a complex of $HgO{\cdot}Hg(NH_2)I$, which leads to a yellow or brown (at higher NH_4^+ concentrations) coloration. To 40 µl of samples 40 µl of Nessler's reagent was added and after vortexing the color was controlled by eye.

2.8.2 Nitrite

2.8.2.1 Photometric Measurement of NO_2^- concentration

For photometric measurement of the NO_2^- concentration the Griess reagent was used. The Griess reagent system uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride under acidic (phosphoric acid) conditions to detect NO_2^- in a variety of samples.

Solutions

0.1% N-1-napthylethylenediamine dihydrochloride (NED)

1% sulfanilamide in 5% phosphoric acid

Procedure

Determination of NO_2^- concentration in samples was performed following the manufacturers protocol (Promega, Madison, USA).

2.8.2.2 Nitrite test stripes

For a rough estimation of the concentration of NO_2^-, test stripes (Merck KGaA, Darmstadt, Germany) were used.

2.8.3 Nitrate

For a rough estimation of the concentration of NO_3^-, test stripes (Merck KGaA, Darmstadt, Germany) were used.

2.8.4 pH measurement

To control the pH value test stripes sensitive in a range between 7.5 to 9 (Macherey-Nagel GmbH & Co, Düren, Germany) were used.

2.9 Cultivation and maintenance of recombinant *E. coli* strains

2.9.1 Culture conditions and cell harvesting

Solutions

LB medium

Amp stock solution

Procedure

Recombinant *E. coli* cells were either plated on solid media or inoculated into liquid media. LB media contained 100 µg/ml Amp in order to avoid growth of cells lacking plasmid. To culture cells in liquid media, a single white colony was picked under sterile conditions from plates with a toothpick, inoculated in a tube containing 5 ml of LB medium and incubated at 37°C o/n on an orbital shaker (Innova 2300; New Brunswick Scientific Co., Inc., Madison NJ, USA) at 200 rpm. For harvesting cells, 2 × 2 ml of the culture were centrifuged at 13,000 rpm for 1 min at RT (Mikro 22R, Hettich, Tuttlingen, Germany) in sterile ERT.

When cells were cultured for Clone-FISH (see 2.24.3), 1 ml of an o/n culture was inoculated into 200 ml Erlenmeyer flasks containing 100 ml of LB medium and incubated at 37°C on an orbital shaker at 200 rpm until the final OD was reached. For OD measurement, sterile LB medium was used as blank control. OD was measured in plastic cuvettes (Halb-Mikro, Greiner) using a spectrophotometer (SmartSpecTM 3000, Biorad) at a wavelength of 600 nm.

2.9.2 Maintenance

For maintenance single clones were applied to LB-Amp masterplates, incubated o/n at 37°C and plates stored at 4°C.

2.10 DNA isolation

2.10.1 Extraction of genomic DNA from environmental samples

2.10.1.1 Isolation of genomic DNA from biofilm

For DNA isolation biofilm was washed off 10 filter balls with 100 ml of aquarium water and subsequently divided into 2 Greiner tubes. The biofilm samples were centrifuged at 15,820 g for 15 min at RT (Rotina 35S). After centrifugation the supernatant (SN) was discarded and the pellets were stored on -20°C and on 4°C for DNA extraction. DNA was extracted from biofilm sample using the Ultra CleanTM Soil DNA Kit (MoBio Laboratories). The cells were disrupted by bead beating two times for 20 sec at 4.5 m/s (Beadbeater BIO 101, Carlsbad, CA, USA) using bead beating vials. The extraction was performed following the manufacturer's protocol. As negative control H_2O_{bidist} was used. At the end the isolated DNA was dissolved in 40 µl H_2O_{bidist} and stored at –20°C.

2.10.1.2 Isolation of genomic DNA from Garga hot spring enrichment

a) Kit

Genomic DNA was extracted from 2 ml of enrichment culture using the Power SoilTM DNA Kit (MoBio Laboratories). The cells were mechanically disrupted by bead beating 2 times for 20 sec at an intensity of 4.5 m/s. The isolation was done according to the manufacturer's protocol. A negative control (H_2O_{bidist}) was used to test for contamination of the reagents. After the isolation DNA was dissolved in 50 µl H_2O_{bidist} and stored at –20°C.

b) Phenol-Chloroform-DNA extraction

DNA was extracted from 2 ml of culture using the Phenol-Chloroform-DNA extraction method similar as performed by Lueders et al. (2004).

Solutions

120 mM $NaPO_4$ buffer, pH 8	112.87 mM Na_2HPO_4, 7.12 mM NaH_2PO_4, filter sterilize, autoclave
TNS-solution	500 mM Tris-HCl pH 8.0, 100 mM NaCl, 10% SDS (w/v), adjust pH with HCl, filter sterilize, autoclave

PEG precip. solution	30% (wt/vol) polyethylene glycol 6000 in 1.6 M NaCl, prepared with RNAse free water, autoclave
EB buffer	10 mM Tris-HCl, pH 8.5, prepare with RNAse free water, filter sterilze, autoclave

Procedure

750 µl of 120 mM NaPO4 buffer and 250 µl of TNS were filled into lysing matrix A vials (Bio 101, Carlsbad, CA, USA). Then the sample (maximum 500 µl) was added to the vial so that it was maximally filled to the top of the gripping ring. Next, samples were bead beaten for 45 sec at 6.5 m/s and placed on ice. After centrifugation for 4 min at maximum speed at 4°C 800 µl of SN were pipetted into a 2 ml vial on ice. DNA was extracted with 1 vol of Phenol/Chloroform/Isoamylalcohol (25:24:1) at pH 8 to increase DNA and decrease RNA yield, mixed and centrifuged again for 4 min (max. speed, 4°C). 700 µl of SN were taken and placed into a 2 ml "Phase Lock Gel Heavy"tube. Then, 1 Vol. of Chloroform/Isoamylalcohol (24:1) was added and samples were centrifuged for 4 min at maximum speed. After this, 650 µl of SN were mixed thoroughly with 2 volumes of PEG and precipitated by centrifugation for 30 min (max. speed, 4°C). The liquid was removed and 500 µl of cold 70% EtOH were added to wash the pellet. After centrifugation (4 min, max. speed, 4°C) the EtOH was removed carefully and DNA was dried briefly at RT (max. 5 min). The DNA precipitate was dissolved in 50 µl H_2O_{bidist} and stored at -20°C. A negative control was performed to test for potential contamination of reagents.

2.10.2 Extraction of plasmid DNA from recombinant *E. coli* cells

The plasmid isolation was done according to the principle of alkaline lysis, precipitation of proteins and precipitation of plasmid DNA by 2-propanol.

Solutions

P1 buffer

NaOH/SDS solution

Potassium acetate/acetate solution

2-propanol

70% EtOH

Procedure

2 × 2 ml of an o/n culture were centrifuged in a sterile ERT for 1 min at 13,000 rpm (Mikro 22R) and the SN was discarded. The cell pellet was resuspended in 100 µl of buffer P1 and incubated for 5 min at RT to digest RNA. To lyse cells, 200 µl of NaOH/SDS solution were added. Tubes were inverted several times and incubated for 5 min on ice (meanwhile inverted several times). For protein precipitation, 150 µl of potassium acetate/acetate solution were added, the tubes vortexed briefly, and put on ice for 5 min. Then, the proteins were separated by centrifugation (13,000 rpm, 1 min) and the obtained SN (=450 µl) transferred to a new sterile ERT. DNA was precipitated by adding one volume of 2-propanol, inverting the tubes several times and incubating them for 10 min at RT. After this, the tube was centrifuged (13,000 rpm, 2 min), the SN removed, and the DNA-pellet washed in 500 µl of ice-cold 70% EtOH. After a final centrifugation step (13,000 rpm, 1 min) SN was removed and the pellet dried on air at 46°C in a hybridization oven. The DNA was dissolved in 40 µl of H_2O_{bidist} and stored at -20°C.

2.11 Quantitative and qualitative analysis of nucleic acids

2.11.1 Quantitative, photometric analysis of nucleic acids

DNA concentrations were quantified using a NanoDrop® ND-1000 spectrophotometer (Wilmington, USA). Before measurement the spectrophotometer was blanked with 1.5 µl of H_2O_{bidist}. 1.5 µl of the extracted nucleic acid solution were pipetted onto the end of a fiber optic cable and the nucleic acid concentration was measured at λ=260nm.

2.11.2 Qualitative analysis of nucleic acids using agarose gel electrophoresis

Qualitative analysis of nucleic acids was performed by using gel electrophoresis, in which an electric current is applied to a gel matrix. The gel matrix provides a network in which DNA fragments are separated dependent on their size. Migration of fragments is obtained by applying an electric field, in which DNA moves due to its negative charge to the positive electrode.

Solutions

1-2.5 % (w/v) agarose in 1 × TBE buffer

Loading buffer

DNA-ladder (KbL)

EtBr staining solution

Procedure

Different amounts of agarose were weighted, mixed with 1 × TBE buffer and heated in a microwave until the solution was homogenous. For control of successful PCR concentrations 1-2% agarose gels were used, whereas for RFLP analysis concentrations >2% were used. The gel was cooled under running water and poured into a gel-tray containing a comb. After polymerization of the gel the carriage was transferred to the electrophoresis apparatus. PCR-products and fragments derived from RFLP analysis were mixed with loading buffer in a ratio of 2:1 and pipetted into the pockets of the gel. A DNA marker (KbL) with fragments of known length was run to compare the size of the DNA fragments with. Dependent on the type of use electrophoresis was run between 60 and 90 min applied with an electric current between 90 and 120V. After electrophoresis, DNA was stained in an ethidium bromide bath for ∼30 min. EtBr intercalates into nucleic acids and emits visible light after excitation with UV light, making DNA observable. With a transilluminator emitting UV-light (λ=312nm) band patterns were visualized and recorded with a gel-documentary system.

2.12 *In vitro* amplification of DNA fragments with Polymerase Chain Reaction (PCR)

To amplify gene fragments of interest polymerase chain reaction and gene specific primers (see Table 2.6) were used. The reaction is initiated by a single denaturation step, followed by 20-40 temperature-cycles. The cycling starts with a DNA denaturation step, in which hydrogen bonds between complementary bases of double stranded DNA templates are broken. After this, primers, oligonucleotides with typical length between 15 and 25 nucleotides, anneal to single-stranded DNA templates in a sequence specific manner takes place. The last step is the extension/elongation, in which DNA polymerase synthesizes a new DNA strand complementary to the DNA template. A single final extension/elongation step is performed to attach dATP overhangs to the 3' ends of the fragments, which are needed later for ligation of the amplicons into

Table 2.6: Primers used for the amplification of plasmids carrying 16S rRNA gene fragments

Primer name[a]	Sequence (5'-3')[b]	T [°C][c]	Binding position[d]	Specificity	Reference
616V	AGA GTT TGA TYM TGG CTC	54	7 – 24	most *Bacteria*	Juretschko et al. 1998
630R	CAK AAA GGA GGT GAT CC	54	1528 – 1542	most *Bacteria*	Juretschko et al. 1998
1492R	GGY TAC CTT GTT ACG ACT T	56	1492 – 1510	most *Bacteria* and *Archaea*	Loy et al. 2002
Arch21F	TTC CGG TTG ATC CYG CCG GA	56	7 – 26	most *Archaea*	DeLong 1992
C341F[e]	CCT ACG GGA GGC AGC AG	60 – 57[f]	341 – 357	most *Bacteria*	Muyzer et al. 1993
907R	CCG TCA ATT CMT TTG AGT TT	60 – 57[f]	907 – 926	most *Bacteria*	Muyzer et al. 1993
RHG1148R	AGT GCC CAC CTC TCG CGT	61	1130 – 1148	putative AOB	Hatzenpichler 2006
Arch912R[g]	GTG CTC CCC CGC CAA TTC CTT TA	56	912 - 934	most *Archaea*	Lueders and Friedrich 2002
Ntcoc84F	CGG AAA GGT GGC TGG CGA	54	84 – 102	*Nitrococcus mobilis*	Juretschko 2000

[a] F, forward primer; R, reverse primer
[b] abbreviations according to IUPAC: K = G/T, M = A/C, Y = C/T
[c] annealing temperature of the primer
[d] according to *E. coli* 16S rRNA (Brosius et al. 1981)
[e] primer was used for DGGE analyses with a GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') at its 5'-end
[f] primers were used in a touchdown PCR
[g] modification of the original Arch915R primer, which carries a 3-bp elongated 3' end and was designed by Lueders and Friedrich (2002)

TOPO TA-vectors (see 2.15). Primers described in this study were obtained from Thermo Electron GmbH (Ulm, Germany). PCR reactions were performed using the Icycler (Biorad, Munich, Germany) or the Mastercycler gradient PCR cycler (Eppendorf, Hamburg, Germany). PCR amplification from the "Haus des Meeres"enrichment cultures were performed directly with the biomass and without prior DNA-extraction.

2.12.1 Amplification of 16S rRNA gene fragments

Solutions:

$MgCl_2$ (25 mM) (Fermentas Inc., Hanover, MD, USA)

$MgCl_2$ (25 mM) (QIAgen, Hilden, Germany) when using hot start Taq

10 × Ex Taq polymerase-buffer (Fermentas Inc., Hanover, MD, USA)

10 × hot start Taq polymerase-buffer (QIAgen, Hilden, Germany)

Nucleotide-Mix (2 mM/dNTP) (Fermentas Inc., Hanover, MD, USA)

Forward primer (25-100 pmol/µl)

Reverse primer (25-100 pmol/µl)

Bovine Serum Albumine (BSA; 20 mg/ml) (New England BioLabs Inc., Beverly, MA, USA)

Taq DNA-polymerase (5 units/µl) (Fermentas Inc., Hanover, MD, USA)

hot start Taq DNA-polymerase (5 units/µl) (QIAgen, Hilden, Germany)

H_2O_{bidist}

Standard reaction mix:

$MgCl_2$	2-4 µl (dependent on polymerase used)
Buffer (10 x)	5 µl (dependent on polymerase used)
dNTP-mix	5 µl
Forward primer	0.5-1 µl
Reverse primer	0.5-1 µl
BSA	0.5 µl
hot start/standard Taq DNA polym.	0.25 µl
Template	1-3 µl
H_2O_{bidist}	ad 50 µl

For every PCR a master mix (without template) was prepared and, dependent on how much template was used, 47-49 µl of the master mix were pipetted into each tube. Additionally, for every PCR a positive control and a negative control (H_2O_{bidist} instead of template) were included.

The conditions used for the amplification of 16S rRNA gene fragments are shown in Table 2.7.

Table 2.7: Conditions for the amplification of 16S rRNA gene fragments

PCR-step	**Temp.**[$°C$]	**Time**	**Number of cycles**
Denaturation	95	5, 15 min[a]	1
Denaturation	95	30 sec	35
Annealing	54 – 60[b]	30 sec	
Elongation	72	1 min	
final elongation	72	10 min	1

[a] Taq or hot start Taq polymerase, respectively
[b] for the respective annealing temp. of the primers see table 2.5.

To amplify 16S rRNA genes of bacterial contaminants in the "*Candidatus* Nitrososphaera gargensis"enrichment, a PCR with primers 616F/630R combined with RHG1148Rlock was performed. RHG1148Rlock is specific for a putative AOB but lacks the OH-group on the 3' end, inhibiting the addition of nucleotides by the polymerase. The primer was used with the aim to select against the amplification of putative AOB 16S rRNA gene fragments. For PCR, the following combinations were used: 616F/630R/RHG1148Rlock and 2×616F /630R/RHG1148Rlock. To test if the intact primer RHG1148R works, PCR was performed with 616V/RHG1148R only.

2.12.2 Touchdown PCR for the amplification of 16S rRNA gene fragments for Denaturing Gradient Gel Electrophoresis (DGGE)

For amplification of 16S rRNA gene fragments for DGGE a touchdown PCR was performed (Tab. 2.8), in which the annealing temperature is decreased during cycles.

Table 2.8: Conditions for the amplification of 16S rRNA gene fragments for DGGE analysis

PCR-step	**Temp.** $[°C]$	**Time**	**Number of cycles**
Denaturation	95	5 min	1
Denaturation	95	40 sec	12
Annealing	60-57[a]	40 sec	
Elongation	72	1 min	
Denaturation	95	40 sec	23
Annealing	57	40 sec	
Elongation	72	1 min	
final elongation	72	10 min	1

[a] the annealing temperature was decreased by 0.5°C every second cycle until a temperature of 57°C was reached

2.12.3 Amplification of *amoA* gene fragments

Table 2.9: Conditions for the amplification of *amoA* gene fragments

PCR-step	**Temp.** $[°C]$	**Time**	**Number of cycles**
Denaturation	95	5, 15 min[a]	1
Denaturation	95	40 sec	35
Annealing	50-53[b]	40 sec	
Elongation	72	1 min	
final elongation	72	10 min	1

[a] Taq or hot start Taq polymerase, respectively
[b] for the respective annealing temp. of the primers see Table 2.8.

Table 2.10: Primers used for the amplification of *amoA* gene fragments

Primer name[a]	Sequence (5'-3')[b]	T [°C][c]	Binding position[d]	Specificity	Reference
*amoA*1F	GGG GTT TCT ACT GGT GGT	50	332 – 349	β-proteobacterial AOB	Rotthauwe et al. 1997
*amoA*2R	CCC CTC TGC AAA GCC TTC TTC	50	802 – 822	β-proteobacterial AOB	Rotthauwe et al. 1997
Arch-*amoA*F	STA ATG GTC TGG CTT AGA CG	53	-	AOA	Francis et al. 2005
Arch-*amoA*R	GCG GCC ATC CAT CTG TAT GT	53	-	AOA	Francis et al. 2005

[a] F, forward primer; R, reverse primer
[b] abbreviations according to IUPAC: S = C/G
[c] annealing temperature of the primer

2.12.4 Amplification of *nxrB* gene fragments

Table 2.11: Primers used for the amplification of *nxrB* gene fragments

Primer name[a]	Sequence (5'-3')[b]	T [°C][c]	Binding position[d]	Specificity	Reference
nxrBF169	TAC ATG TGG TGG AAC A	56,2	169 - 184	all known *Nitrospira*-like *nxrB*	Maixner et al., in prep.
nxrBR638 (707)	CGG TTC TGG TCR ATC A	56,2	638 - 653	all known *Nitrospira*-like *nxrB*	Maixner et al., in prep.
nxrBF706	AAG ACC TAY TTC AAC TGG TC	56	706 - 725	*Nitrobacter, Nitrococcus*	Maixner et al., in prep.
nxrBR1431	CGC TCC ATC GGY GGA ACM AC	56	1411 - 1430	*Nitrobacter, Nitrococcus*	Maixner et al., in prep.

[a] F, forward primer; R, reverse primer
[b] (abbreviations according to IUPAC)
[c] annealing temperature of the primer

Table 2.12: Conditions for the amplification of *nxrB* gene fragments

PCR-step	Temp.[°C]	Time	Number of cycles
Denaturation	95	5, 15 min[a]	1
Denaturation	95	40 sec	35
Annealing	54-56.2[b]	30 sec	
Elongation	72	1 min	
final elongation	72	10 min	1

[a] Taq or hot start Taq polymerase, respectively
[b] for the respective annealing temp. of the primers see Table 2.10.

2.13 Gel Purification of PCR products

Solutions

2 % (w/v) Nusieve 3:1 agarose (low-melting-point) in 1 × TAE buffer

Loading buffer

DNA ladder (KbL)

SYBR® Green I staining solution in TAE

Procedure

Low-melting-point agarose gels were stained with SYBR® Green I staining solution for 20 – 30 min and DNA-bands of correct size were cut out from gels with 50 µl glass capillaries (Idaho Technology Inc., Salt Lake City, UT, USA) and transferred into a sterile ERT. 50 µl H_2O_{bidist} were added and the mixture was melted at 75°C for 10 min on a heating block (VWR international, West Chester, PA, USA), before cloning was performed.

2.14 Cloning of gene amplicons with the TOPO TA Cloning® kit

PCR products were cloned into vector pCR®II-TOPO. Cloning was performed using the TOPO TA Cloning® kit (Invitrogen Corporation) following the protocol provided by the manufacturer. For transformation chemical-competent *E. coli* TOP10 cells were used. The vector pCR®II-TOPO carries dTTP overhangs and a covalently bound topoisomerase that catalyzes the ligation with dATPs located at the end of the PCR-products. Successful transformation and presence of the insert was tested via blue/white screening of the colonies. The vector comprises two antibiotic resistance genes (ampR and kanR) and the *lacZα* fragment, which is interrupted by the insertion site. Cells containing a vector without insert complement the *lacZ* gene and are able to cleave X-Gal into a blue product, whereas cells with an insert will be white. Cells without vector are killed due to their sensitivity against the antibiotics used.

2.14.1 Ligation

Standard reaction mixture: 6 µl

PCR-product	4 µl
Salt solution	1 µl
Vector	1 µl

The mix was centrifuged briefly and incubated for 20 min at RT before transformation. In order to achieve a higher cloning output, more PCR-product and salt solution were used.

2.14.2 Transformation

The entire ligation reaction mix was added to the competent *E. coli* TOP10 cells and the mixture was stired gently with a tip. After incubation for 30 min on ice, the cells were heat-shocked for 30 sec at 42°C and immediately placed on ice. 250 µl of SOC medium were added and the cells incubated for 1 h at 37°C on an orbital shaker (200 rpm). On each plate (LB-Amp) 40 µl of sterile X-Gal were spread out. For each cloning approach 100 µl and 150 µl of pre-incubated cells were plated and incubated o/n at 37°C.

2.14.3 Identification of recombinant clones

After o/n-incubation plates were put on 4°C for ∼30 min to enhance coloration of the colonies. White colonies were picked using sterile toothpicks and transferred to a masterplate (LB-Amp). At the same time, clones were used for a M13-screening PCR to check for correct insert size.

2.14.4 Insert size verification via M13-screening PCR

M13-primers binding positions flank the TOPO cloning vectors multiple cloning site (Tab. 2.13). Thus, a PCR (see Tab. 2.14) with this primer pair enables to amplify the insert and to verify its correct size by agarose gel electrophoresis.

The reaction mix was the same as for a standard PCR described above except that no BSA was added to the master mix. Colonies were picked using sterile toothpicks and suspended in the reaction mix, provided in the cavities of a 96-well microtiterplate, which was kept on ice. Prior to PCR, the microtiterplate was sealed with thermostable foil. PCR-products were analyzed by using a 1% agarose gel.

Table 2.13: Primers used for M13-screening PCR

Primer name[a]	**Sequence (5´-3´)**	**T [°C]**[b]	**Reference**
M13F	GTA AAA CGA CGG CCA G	60	TOPO cloning kit (Invitrogen
M13R	CAG GAA ACA GCT ATG AC	60	Corporation, Carlsbad, CA, USA)

[a] F, forward primer; R, reverse primer
[b] annealing temperature of the primer

Table 2.14: Conditions for M13-screening PCR

PCR-step	**Temp.**[$°C$]	**Time**	**Number of cycles**
Denaturation	95	5	1
Denaturation	95	40 sec	35
Annealing	60	40 sec	
Elongation	72	40 sec	
final elongation	72	10 min	1

M13 PCR-products from clones carrying a correct-sized insert were inoculated into LB-Amp liquid medium and incubated at 37°C o/n at 200 rpm. The plasmid DNA was isolated from the recombinant *E. coli* cells as described in 2.11.2.

2.15 RFLP

Restriction fragment length polymorphism (RFLP) analysis were performed with the M13-screening PCR-products in order to estimate the sequence diversity represented in a clone library. In RFLP, DNA is cut by restriction enzymes (restriction endonucleases) at specific short recognition sites. The resulting DNA fragments are then separated by length with agarose gel electrophoresis after which specific band patterns are obtained. Since different sequences have different restriction sites, RFLP as a fingerprinting technique can be used as a method for rapid comparison of DNA and thus for estimating the sequence diversity of a clone library.

2.15.1 Restriction and inactivation of enzymes

Standard reaction mix for RFLP:

PCR product	5 µl
restriction-enzyme (10 U/µl)	0.3 µl
restriction-buffer	1 µl
H_2O_{bidist}	3 µl

For each restriction a mastermix of enzymes, buffer and H_2O_{bidist} was prepared and 4,3 µl of the mix was pipetted into each reaction tube. The used enzymes are listed in Table 2.15. Then, the PCR-product was added and the reaction was incubated for 3 hours at 37°C. Enzymes were inactivated for 20 min at 65°C and reactions were put on 4°C before they were applied to >2% agarose gels and run at 90 V for 1.5 h. The plasmids of at least two representative clones of each banding pattern obtained were isolated (2.11.2) and the inserts of the purified plasmids were sequenced (see 2.19).

Enzyme	Restriction site[a]	Buffer	T_{inc} [°C][b]	Company
AluI	AG↓CT	Tango	37	Fermentas Life Sciences Inc., Hanover, MD, USA
MspI (HpaII)	C↓CGG	Tango	37	Fermentas Life Sciences Inc., Hanover, MD, USA
RsaI	GT↓AC	Tango	37	Fermentas Life Sciences Inc., Hanover, MD, USA

[a] arrow indicates site of restriction
[b] incubation temperature

Table 2.15: Restriction enzymes used for RFLP and T-RFLP analysis

2.16 T-RFLP

Terminal-restriction fragment length polymorphism (T-RFLP) is based on PCR amplification of a target gene with one or both the primers having their 5' end labeled with a fluorescent molecule. Like in RFLP, amplicons are subjected to a restriction reaction (see 2.16.1). After this, the mixture of fragments is separated using capillary electrophoresis and the sizes of the different terminal fragments are determined by a fluorescence detector. Only the sizes of the terminal restriction fragments (T-RFs), that is the labeled ends, are determined. The result of a T-RFLP profiling is a graph called electropherogram. What appears as a band after separation by gel electrophoresis appears as a peak on the electropherogram. T-RFLP can provide a sensitive and rapid means to assess community diversity and obtain a distinctive fingerprint of a microbial community (Liu et al. 1997).

Primers were labeled at 5' ends with fluorescent dye phosphoramidite fluorochrome 5-carboxyfluorescein (6-FAM) or oxazole yellow (YO) from Thermo Electron GmbH Ulm, Germany. For bacteria, 616F-FAM/1492R-YO were used, and for archaea 21F/Arch912-FAM and 21F/1492-YO were used. PCR conditions used for T-RFLP can be found in Table 2.5 and 2.6, respectively. PCR-products were purified with the QIAquick® PCR Purification Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol and dissolved in 50 µl DNA-free H_2O_{bidist}. Then, the concentration of each purified PCR product was determined on a Nanodrop photometer (see 2.12.1). Total amount of PCR products in the restriction mixture was 100 ng. Restriction was done using endonucleases AluI, MspI and RsaI (Tab. 2.15), to obtain a higher resolution as

compared to the use of one enzyme. For each restriction a mastermix of enzyme and buffer was prepared and 2.5 µl of the mix were added to each PCR tube. Digestion and inactivation of the enzyme was carried out as described for RFLP in 2.16.1. After restriction samples were wrapped in aluminium foil and stored at -20°C, before further analysis.

Standard reaction mix for T-RFLP:

PCR product	100 ng
restriction-enzyme (10 U/µl)	0,5 µl
restriction-buffer	2 µl
H_2O_{bidist}	ad 20 µl

2.17 DGGE

Denaturing gradient gel electrophoresis (DGGE) can be used to evaluate the diversity of complex microbial systems (Muyzer et al. 1993). The technique reveals sequence variation in a mixture of PCR-fragments of equal length. This separation is based on the sequence-dependent melting behavior of DNA fragments in a polyacrylamide (PAA) gel containing a concentration gradient of an increasing denaturant (Muyzer and Smalla 1998), for example urea. An artificial GC-rich sequence, a so-called GC-clamp, is added to the 5'-end of primers to prevent complete melting of the DNA fragments and to detect differences in their melting behavior.

The conditions of touchdown PCR for the amplification of 16S rRNA gene fragments for Denaturing gradient gel electrophoresis (DGGE) are listed in Table 2.7. DGGE analysis were performed using 6% PAA solution (for the separation of fragments of about 600 bp length). With acrylamide solutions containing either 20% and 80% of urea, or 40% and 80% of urea a gradient was formed.

2.17.1 Preparation of acrylamide stock solutions

Stock solution with 0% urea (6% acrylamide)

Acrylamide/bisacrylamide solution 40% (37.5:1)	70 ml
50 × TAE buffer	10 ml
H_2O_{bidist}	ad 500 ml

The solution was stored in the dark at 4°C.

Stock solution with 80% urea (8% acrylamide)

168 g urea were added slowly to 100 ml H_2O_{bidist}.	
Acrylamide/bisacrylamide Solution 40% (37.5:1)	70 ml
50 × TAE	10 ml
Formamide	160 ml
H_2O_{bidist}	ad 500 ml

The solution was stored in the dark at 4°C.

2.17.2 Preparation of PAA gels

Solutions

10% SDS

Acrylamide stock solution with 0% urea

Acrylamide stock solution with 80% urea

Ammonium peroxydisulfate (APS)

N,N,N',N'-tetra-methyl-ethylene-di-amine (TEMED)

Preparations

First, glass plates and spacers were cleaned with 10% SDS solution, washed with H_2O_{bidist} and dried at RT. Then, the apparatus for the preparation of the gel was assembled. 11 ml of acrylamide solutions for the respective lowest and highest urea-concentration, 4-5 ml 0% solution for the collecting gel (sup), and 2 ml 80% solution for the stop gel (seal) were prepared.

Procedure

After addition of 25 µl APS and 8 µl TEMED to the 80% stop solution, it was carefully pipetted between the glass plates and let stand for polymerization. The stop gel was prepared to impede DNA fragments from running into the buffer during gel separation. Meanwhile, the gradient solutions with 20 and 80% of urea (first gel) and 40 and 80% of urea (second gel) were poured into the gradient mixer (Econo model EP-1 gradient pump Biorad, München, Germany). Air bubbles were removed from solutions. To each solution 30 µl of APS and 8 µl of TEMED were added. The gel was gently poured under constant mixing of the solutions by a magnetic stirrer. After this process 20

µl APS and 4 µl TEMED were supplemented to the 0% solution which was carefully pipetted on top of the gel. In the end, the comb was applied and the gel was incubated for ∼1,5 h at RT to let the acrylamide polymerize. If the gel was not used immediately after polymerization, it was stored at 4°C over night.

2.17.3 Electrophoresis

Preparations

The DGGE chamber (Biorad, München, Germany) was filled with 1 × TAE buffer (modified) and the buffer was pre-heated for 1,5 h at 63 °C prior to gel electrophoresis. After application of the gel, the comb was removed and the gel-pockets were rinsed with buffer. The gel was pre-run for 15 min at a voltage of 150 V.

Procedure

The PCR-products were mixed with 6 × loading buffer and pipetted into the gel-pockets. DGGE analysis was performed with amplified genes from extracted DNA and clones. 50 µl of amplicons from environmental DNA were mixed with 10 µl of loading dye and 4 µl of clonal DNA were mixed with 1 µl of loading dye, respectively. DGGE was run for 4.5 h at 150 V and at a temperature of 60°C. Finally, the gel was stained with SYBR® Green I staining solution for 45-60 min. Evaluation was done as described for agarose gels (see 2.12.2).

2.17.4 Excision and purification of DNA from PAA gels

Stained DNA-bands of interest were cut out with a scalpell, transferred to a sterile 1.5 ml ERT and diluted in 30 µl H_2O_{bidist}. Excised bands were stored at -20 °C. Later on, reamplification of PCR-products with DGGE primers (Tab. 2.6) was performed under the conditions listed in Table 2.8. The PCR-products were purified using QIAquick® PCR Purification Kit (QIAgen) following instructions of the protocol and DNA concentration was measured on a Nanodrop photometer (see 2.12.1). Finally, these amplificates were used for sequencing.

2.18 Sequencing

Sequencing was performed after the di-deoxy mediated chain termination method (Sanger et al. 1977) combined with PCR including a DNA-polymerase (Saiki et al.

1988).

Procedure

PCR was performed wit a reaction mix that contains both dNTPs and ddNTPs. ddNTPs lack the 3' OH group needed by the polymerase for adding nucleotides. Therefore, after the integration of a ddNTP the elongation of the PCR product stops. Each of the four ddNTPs is labeled with a fluorescent dye, each with different emission spectrum. Thus, the identity of the 3' end of the nucleotide at which the PCR stopped is revealed by the labeled PCR product. PCR products of different length and differently labeled are separated by capillary electrophoresis in the DNA sequencer. Sequencing was carried out using the DNA Sequencer Applied Biosystems 3130 following the manufacturer protocol. For sequencing of plasmid inserts vector-specific primers TopoSeq-F or TopoSeq-R were used, whereas for direct sequencing of DGGE-bands the respective forward-primer was used as sequencing primer (Tab. 2.6). After capillary electrophoresis DNA sequence chromatograms are obtained. Sequences were determined using the software program e-seq (Licor Inc., Biotechnology Devision, Lincoln, NE, USA) and exported in FASTA-format.

2.19 Sequence analyses

2.19.1 Sequence proofreading

Proofreading of sequences was performed using the FinchTV software. Sequences were imported into ARB (2.20.3) and bases wrongly identified by automatic inspection were manually corrected.

2.19.2 Quick analyses of sequences

To quickly analyze their phylogenetic affiliation the obtained sequences were compared against existing online databases using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). BLAST is a sequence comparison algorithm optimized for speed and used to search sequence databases for optimal local alignments. The most similar sequences were imported as reference sequences into the software program ARB for a detailed comparison.

2.19.3 Comparative sequence analysis using the ARB software package

Sequence data were further analyzed using the software program ARB (Ludwig et al., 2004). The ARB software is a graphically oriented package comprising various tools for sequence database handling and data analysis, like data import and export, sequence editing, sequence alignment, filter calculation, phylogenetic treeing and primer and probe design and evaluation.

2.19.3.1 Alignment of sequences

FASTA-files with sequences of interest were imported into ARB. After deleting vector nucleotides, sequences were automatically aligned by using the fast aligner function. With the fast aligner new sequences can be compared to either an already existing dataset or to the selected reference sequences. In the process, homologous regions are aligned. After this, sequence alignment was manually improved. Sequences of protein-coding genes (*nxr*, *amoA*) were translated in silico and amino acid sequences was aligned.

2.19.3.2 Oligonucleotide probe design targeting 16S rRNA

A 16S rRNA targeted oligonucleotide probe for fluorescence *in situ* hybridization (FISH) studies was designed manually with the ARB software. Probes were designed in a way that mismatches to non-target-organisms were located in the center of the probe. First, sequences of the target organisms were screened for a site at which all had exactly the same nucleotide sequence. Then the site was compared to *E. coli* 16S rRNA gene sequence to control if the probe targeted a conserved or variable region. The aim was to find a variable region which would not be found in non-target sequences. Thus, additional sequences from different phyla were used for comparison of the selected site. To control if the probe could hybridize to any non-target organisms, the probe sequence was matched against SILVA database using the programme probeCheck, and against the Ribosomal Database Project using the tool probe match. The length of the designed probe was 18 nucleotides and the GC content was ∼60% (see Tab. 2.14).

2.20 Phylogenetic analyses

Phylogenetic trees were calculated by analysis of nucleic acid (16S rRNA) and protein (*nxrB*) sequences and were performed using the software package ARB (Ludwig et al. 2004). Calculation of phylogenetic trees was performed using conservation filters, in which highly variable base pairs and sequence ends were excluded. In all calculations unrelated sequences were used as outgroup, allowing the rooting to the position of the outgroup. For each nucleotide tree, only sequences longer than 1,100 nt were used. Therefore, 16S rRNA genes were sequenced from both sides and merged using ARB.

Two important methods for phylogenetic reconstruction included in ARB are described shortly below. Treeing methods are classified according to how data are treated and how trees are constructed. Comparative 16S rRNA analysis is a most widely used technique in identification, classification and systematics of organisms today.

2.20.1 Neighbour joining

Neighbour joining (NJ) is a distance-based method, in which aligned sequences are converted into a pairwise distance matrix, which provides the basis for the calculation of the tree. The distance between each pair of two sequences is used to evaluate branch lengths in trees. Since neighbour joining underestimates the actual distance between sequences, correction models (e.g. Jukes-Cantor (DNA), Kimura (protein), ...) are needed. NJ trees are assembled by clustering, in which one sequence after the other is joined to the group with the lowest divergence, resulting in a gradually growing tree. However, final tree distances often do not reflect the actual distance matrix distances. An advantage of NJ is that the time needed for calculation of trees is short and large data sets can be analyzed. A disadvantage is the loss of original sequence information, due to the use of distance values for treeing calculation.

2.20.2 Maximum likelihood

Maximum likelihood (ML) is a method, in which each single nucleotide site is considered directly. For a set of data and an underlying evolutionary probability model, ML tries to find the tree that maximizes the probability of observing the input data (a tree that makes the data "more likely"). Trees are constructed on the basis of optimality methods, which try to find the tree that concurs best with a model of evolution. This is achieved by heuristically searching a subset of all trees, which is then expected to

contain the optimal tree with the maximum likelihood. An advantage is that real sequences are analyzed and tree topology and branch lengths are considered. A drawback is that the method is very time consuming.

2.21 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization uses rRNA-targeted fluorescent oligonucleotide probes and permits the phylogenetic identification and quantification of individual cells without the need for prior cultivation. After cell fixation and immobilization to a slide, hybridization under stringent conditions and a washing step are performed. After this, analysis of samples can be achieved using fluorescence microscopy (Amann et al. 1995; Llobet-Brossa et al. 1998).

2.21.1 Cell fixation

Due to the different cell wall composition of gram-positive and gram-negative bacterial cells as well as archaeal cells, different fixation methods had to be used.

2.21.1.1 Cell fixation with paraformaldehyde (PFA)

PFA crosslinks terminal amino groups and therefore stabilizes the cell wall. 3 Vol. 4% PFA were added to 1 Vol. of sample and incubated for 2-3 h at 4°C (enrichment cultures) or for 1 h at RT (*E. coli* cells heterologously expressing cloned 16S rRNA genes). When samples were fixed o/n, 0.5% PFA was used. To stop fixation the sample was centrifuged (21,250 g, 15 min, 4°C; Rotina 35S) and the SN was discarded. The pellet was resuspended in 1 × PBS and centrifuged again. Finally, SN was discarded and the pellet resuspended in 1 Vol 1 × PBS and 1 Vol. $EtOH_{abs}$. Fixed cell samples were stored at -20°C.

2.21.1.2 Fixation with EtOH

1 Vol $EtOH_{abs}$ was added and samples were stored at -20°C. Samples were centrifuged, SN was discarded and the cell pellet washed in 1 × PBS. After another centrifugation step the pellet was resuspended in 1 Vol 1 × PBS. Then, 1 Vol $EtOH_{abs}$ was added and samples were stored at -20°C.

2.21.2 *In situ* hybridization

2.21.2.1 16S rRNA targeted oligonucleotide probes

To select appropriate probes the online database probeBase (Loy et al. 2003) was used. The oligonucleotide probes used (Thermo Electron GmbH, Ulm, Germany) are described in Table 2.16. From stock solutions, working solutions with a final concentration of 50 ng/µl for Fluos-labeled probes and 30 ng/µl for Cy3- and Cy5-labeled probes were made. Stock and working solutions were stored in the dark at -20°C.

2.21.2.2 Immobilizing cells on glass slides

When small volumes (<10 µl) were used for FISH, 10 µl 1 × PBS were pipetted on the well of a slide to guarantee an even distribution of the cells on the slide. Depending on the respective cell concentration of the sample, 0.5-30 µl of sample were pipetted on the slide and dried at 46°C for 10 min in a hybridization oven.

2.21.2.3 Dehydration of the sample

After immobilization, an increasing EtOH series was performed. Therefore, slides were put into 50%, 80% and 96% EtOH for 3 min, respectively, and air dried afterwards.

2.21.2.4 Probe hybridization

Hybridizations were performed at 46°C between 1.5 and 3 h in a hybridization oven. Appropriate stringency was achieved by addition of formamide to the hybridization buffer and NaCl to the washing buffer. Formamide increases the reaction stringency by destabilizing hydrogen-bondings between nucleic acids, while Na+-ions stabilize the negative nucleic acid duplex due to its positive charges.

Solutions

5 M NaCl

1 M Tris/HCl, pH 8.0

0.5 M EDTA, pH 8.0

10 % (w/v) SDS

Formamide (FA)

Table 2.16: Characteristics of oligonucleotide probes used for FISH analysis

Probe name	**Sequence (5'-3')**	**[FA][a]**	**Binding position[b]**	**Specificity[c]**	**Reference**
EUB338[d]	GCT GCC TCC CGT AGG AGT	0 - 50	338 - 355	most *Bacteria*	Amann et al., 1990
EUB338II[d]	GCA GCC ACC CGT AGG TGT	0 - 50	338 - 355	*Planctomycetales*	Daims et al., 1999
EUB338III[d]	GCT GCC ACC CGT AGG TGT	0 - 50	338 - 355	*Verrucomirobiales*	Daims et al., 1999
ALF968	GGT AAG GTT CTG CGC GTT	20	968 - 985	*α-Proteobacteria0*	Neef 1997
BET42a	GCC TTC CCA CTT CGT TT	35	1027 - 1043	*β-Proteobacteria*	Manz et al., 1992
GAM42a	GCC TTC CCA CAT CGT TT	35	1027 - 1043	*γ-Proteobacteria*	Manz et al., 1992
PLA46	GAC TTG CAT GCC TAA TCC	30	46 - 63	Planctomycetales	Neef et al., 1998
Nscoc1248	TGC TTG GCC ACC CTC TGT	35	1248 - 1265	*Nitrosococcus oceani*, *Nitrosococcus halophilus*	Juretschko, 2000
Nso1225	CGC CAT TGT ATT ACG TGT GA	35	1224 - 1243	*β*-proteobacterial AOB	Mobarry et al., 1996
NEU	CCC CTC TGC TGC ACT CTA	40	653 - 670	most halophilic and halo-tolerant *Nitrosomonas spp.*	Wagner et al., 1995
FGall178a	TCC CCC TCA GGG CAT ATG	35	178 - 195	family *Gallionellaceae*	Lücker, unpublished
Ntoga221	TAT CGG CCG CTC CGA AAA	35	221 - 238	genus *Nitrotoga*	Lücker, unpublished
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	50	712 - 732	most members of the phylum *Nitrospirae*	Daims et al., 2001
Ntspa712Comp	CGC CTT CGC CAC CGG TGT TCC	50	712 - 732	competitor for Ntspa712	Daims et al., 2001
Ntspa662	GGA ATT CCG CGC TCC TCT	35	662 - 679	genus *Nitrospira*	Daims et al., 2001
Ntspa662comp	GGA ATT CCG CTC TCC TCT	35	662 - 679	competitor for Ntspa662	Daims et al., 2001
Ntspa1151	TTC TCC TGG GCA GTC TCT CC	35 - 40	1151 - 1170	sublin. I of *Nitrospirae*	Maixner et al., 2006
Ntspa1431	TTG GCT TGG GCG ACT TCA	35	1431 - 1148	sublin. II of *Nitrospirae*	Maixner et al., 2006
NIT3	CCT GTG CTC CAT GCT CCG	40	1035 - 1052	*Nitrobacter* spp.	Wagner et al., 1996
NIT3comp	CCT GTG CTC CAG GCT CCG	40	1035 - 1052	competitor for NIT3	Wagner et al., 1996
Nctoc84	TCG CCA GCC ACC TTT CCG	10	85 - 101	*Nitrococcus mobilis*	Juretschko, 2000
Ntspn693	TTC CCA ATA TCA ACG CAT TT	10	694 - 713	*Nitrospina gracilis*	Juretschko, 2000
RHG1130	AGT GCC CAC CTC TCG CGT	35	1130 - 1148	putative AOB	Hatzenpichler, unpubl., 2006
GaBI830	GGT CAA ACC CAC CCA CAC	35	830 - 848	*Thermaerobacter* spp.	this study

ARCH915	GTG CTC CCC CGC CAA TTC CT	10	915 - 934	most *Archaea*	Stahl, 1991
CREN512	CGG CGG CTG ACA CCA G	10	512 - 527	most Crenarchaeota	Jurgens et al., 2000
RHGA702	GTG GTC TTC GGT GGA TCA	10	702 - 719	"*Candidatus* Nitrososphaera gargensis"	Hatzenpichler et al., 2008
NonEUB	ACT CCT ACG GGA GGC AGC	0 - 30	-	control (complementary to EUB338)	Wallner et al., 1993

[a] formamide concentration in the hybridization buffer
[b] according to *E. coli* 16S rRNA (respective 23 rRNA for probes BET42a and GAM42a) (Brosius et al., 1981)
[c] for newly designed probes only full length sequences detected are listed
[d] these probes were mixed to detect all Bacteria; this mix is referred to below as EUBmix

Hybridization buffer (1 ml):

5 M NaCl	180 µl
1 M Tris/HCl pH 8,0	20 µl
Formamide	× µl
10% (w/v) SDS	1 µl
H_2O_{bidist}	ad 1 ml

Washing buffer (50 ml, pre-warmed in 48°C):

5 M NaCl	y µl
1 M Tris/HCl pH 8,0	1 ml
0.5 M EDTA pH 8,0	500 µl*
10% (w/v) SDS	50 µl
H_2O_{bidist}	ad 50ml

*EDTA was used to bind bivalent cations and used for NaCl concentrations $\leq$ 225 mM only.

Table 2.17: Volumes of FA, NaCl and EDTA used in hybridization and washing buffers

Hybridization buffer (1 ml)			**Washing buffer (50 ml)**		
Formamide [%]	**Formamide [µl]**	**H20bidest [µl]**	**NaCl [mM]**	**5 M NaCl [µl]**	**0.5 M ETDA [µl]**
0	0	800	900	9.000	-
10	100	700	450	4.500	-
20	200	600	225	2.150	500
25	250	550	159	1.490	500
30	300	500	112	1.020	500
35	350	450	80	700	500
40	400	400	56	460	500
45	450	350	40	300	500
55	550	250	20	100	500
70	700	100	0	0	500

Procedure

10 µl of hybridization buffer and 1 µl of the fluorescently labeled probe (final concentration: 5 ng/µl and 3 ng/µl for Fluos and Cy3 probes, respectively) were pipetted onto the well and the solution was gently stirred without scratching the surface. The slide was then put into a 50 ml tube (Greiner Bio-One GmbH, Frickenhausen, Germany) with a tissue soaked in remaining hybridization buffer. The tube was closed and incubated for 1.5-3 h at 46°C in a hybridization oven UE500 (Memmert GmbH, Schwabach, Germany). After hybridization, the slide was washed in pre-warmed washing buffer for exactly 10 min. After washing, the slide was dipped into ice-cold H_2O_{bidist} and immediately dried using compressed air. Slides were stored at -20°C in the dark until microscopic analysis.

The fluorescence intensity of newly designed probes was tested by FA-series of *E. coli* cells that heterologously expressed the cloned 16S rRNA gene of interest (see 2.24) by increasing concentrations of FA.

2.21.3 Staining with 4'-6'-di-amidino-2-phenylindole (DAPI) and Sybr Green

Before microscopic analysis samples were stained with 4'-6'-di-amidino-2-phenylindole (DAPI), which binds to double stranded DNA, making cells more easily observable during microscopic analyses. The DAPI stock solution (1 mg/ml) was diluted 1:1,000 in H_2O_{bidist}. 10 µl of this working solution were pipetted carefully onto the sample. After incubation of 3-10 min in the dark, DAPI was removed. The sample was washed gently with 10 µl of H_2O_{bidist} to remove remaining DAPI and slides were dried for 15 min at RT in the dark.

For Sybr Green staining, the stock solution was diluted 1:1,000 in Citifluor. The working solution was then carefully pipetted between samples and distributed over the biomass by applying a coverslip.

2.21.4 Confocal Laser Scanning Microscopy (CLSM)

Confocal Laser Scanning Microscopy (CLSM) has the advantage to collect the light emitted or reflected by a single plane of a sample examined. Light that is coming from objects outside the plane gets stopped by a pinhole, in order that only light from objects in focus can reach the detector. Specimen are scanned with a laser and reconstructed

by software. Besides, CLSM allows the analysis of the 3-dimensional structure of a sample.

2.21.4.1 Detection of fluorescently labeled cells

Before the coverslip was applied, samples were embedded in Citifluor AF1 (Agar Scientific Limited) to reduce bleaching during microscopic analysis. Sample analysis was performed using a Confocal Laser Scanning Microscope LSM 510 Meta (Zeiss, Jena, Germany) that was equipped with an Argon-laser (430-514 nm) for excitation of the Fluos-fluorophore) and two Helium-Neon-lasers (543 nm and 633 nm) for excitation of Cy3 and Cy5, respectively). DAPI was excitated by UV light at around 350-365 nm. For magnification, 40 ×, 63 × and 100 × Plan-Neoflar objectives were used and combined with a 10 × ocular. Documentation was done by using the provided software.

2.21.4.2 Evaluation of formamide (FA) series

Signal intensity for each FA concentration was evaluated using 63 × magnification by CLSM. Settings for optimal signals were adjusted to 10% FA concentration and saved for all following recordings. The mean signal intensity for different FA concentrations and the best fitting sigmoid curve was calculated for 5-10 fields of view (for at least about 100 cells per view) using the daime software (Daims et al. 2006). Finally, the highest FA concentration yielding a bright FISH signal of the probe was determined to be the optimal stringency condition for the probe.

2.22 CARD-FISH

Fluorescence *in situ* hybridization (FISH) was hailed as a breakthrough for microbial ecology. Nevertheless, methodological improvements are important to overcome for example the difficulties in samples other than from highly eutrophic systems. Many microorganisms are slow growing, starving or in a dormant stage in environmental samples, and the signal intensities are frequently below the detection limit or lost in high background fluorescence. One alternative to improve the sensitivity of FISH was the combination with catalyzed reporter deposition (CARD) (Morita 1998; Schönhuber et al. 1997; Pernthaler et al. 2002). The hybridization involves an oligonucleotide that is covalently crosslinked to a horseradish peroxidase (HRP). Amplification of the signal is achieved by the radicalization of multiple tyramide molecules by a single horseradish

peroxidase. The molecular weight of HRP is approximately 40 kDa, which makes cell permeabilization an important issue (Amann and Fuchs 2008).

2.22.1 Preparation of poly-L-lysine coated slides

Due to many washing steps during CARD-FISH, slides were coated with poly-L-lysine to improve cell immobilization .

Solutions

1% HCl in 70% EtOH

0.01% poly-L-lysine solution

Procedure

10 well slides (Paul Marienfeld, Bad Mergentheim, Germany) were washed in 1% HCl in 70% EtOH for 5 min, followed by coating with 0.01% poly-L-lysine solution for 5 min. Coated slides were dried for 1.5 h at 60°C in a Heraeus T20 drying oven (Kendro Lab. Products, Hanau, Germany) and stored in a dustfree box at RT.

2.22.2 Cell immobilization

Sample immobilization on poly-L-Lysine coated slides and EtOH series were done as described for standard FISH (2.22).

2.22.3 Embedding

To guarantee better immobilization (due to the high number of washing steps in CARD-FISH) the slide was dipped into 0,2% agarose (in H_2O_{bidist}) and dried at RT.

2.22.4 Permeabilization of the cell wall

Because the HRP is a high molecular weight enzyme the samples had to be treated with proteinase K or lysozyme to permeabilize cell walls.

2.22.4.1 Proteinase K treatment

For the stock solution 5 mg of proteinase K (Sigma-Aldrich Chemie GmbH, Steinhausen, Germany) were dissolved in 1 ml TE (0.1 M Tris, 0.01 M EDTA). Then, 15 µl of the proteinase K working solution (15 µg/ml) were pipetted onto each well and the slide was incubated at RT between 5 and 10 min, depending on the sample to be analyzed. After that, samples were washed in 50 ml H_2O_{bidist} for 1 min.

Inhibition of proteinase K

The slide was incubated in 0.01 M HCl for 15 min to to inactivate remaining proteinase K and to bleach endougenous peroxidases to avoid a false positive CARD-FISH signal.

2.22.4.2 Lysozyme treatment

Instead of proteinase K, lysozyme treatment was performed for non-archaeal cells. Besides, for some samples both proteinase K and lysozyme treatment were used to compare hybridization signals. 5 mg lysozyme (Fluka Chemie GmbH, Buchs, Switzerland) were solved in 1 ml TE buffer and 20 µl of this solution were pipetted onto the sample. After an incubation for 15-60 min in a humid chamber (50 ml tube with tissue soaked in water) at 37°C, the lysozyme was removed by washing the slide in H_2O_{bidist} two times for 1 min. Afterwards, the slides were dried with compressed air.

2.22.4.3 Inactivation of endogenous peroxidases

The slide was put into methanol containing 0.15% H_2O_2 for 30 min to inactivate intracellular peroxidases and to reduce autofluorescence of sample material. Samples were washed again two times for 1 min in H_2O_{bidist} and air dried.

2.22.5 Probe hybridization

Hybridizations were done according to 2.21.2, but with the following differences. The hybridization buffer was the same as used for standard FISH except for the presence of dextran sulfate (1 g/10 ml; Sigma, D 8906), a substance known to stabilize nucleic acid duplexes, and blocking reagent (Roche Diagnostics Vienna GmbH), yielding a final concentration of 1% (w/v). 10 µl of hybridization buffer and 1 µl of HRP-labeled probe (50 ng/µl) were pipetted onto the wells so that a final probe concentration of 0.17 ng/µl was reached. The slide was put into a humid chamber (tissue soaked in

H_2O_{bidist} with respective FA concentration) and incubated at 46°C for 3-18 h.

2.22.6 Washing

For washing, all constituents in the washing buffer were the same as described for standard FISH in Tab. 2.17, except for the use of EDTA (0.5 M) also at NaCl concentrations ≥225 mM (to obtain a 450 mM NaCl concentration in the washing buffer, 4400 µl of 5 M NaCl were used). After hybridization slides were put into pre-warmed washing buffer and incubated for 15 min at 48°C. After washing the slide was dipped shortly into ice-cold H_2O_{bidist} to remove the salt and then incubated in 1 × PBS for 10 min at RT. The slide was dabbed on blotting paper and quickly dried with compressed air.

2.22.7 Tyramide signal amplification

The tyramide signal amplification solution was freshly prepared each time. Cy3- or Fluos-tyramide stock solution was first diluted 1:10 in H_2O_{bidist} and afterwards in amplification buffer, which consisted of following substances:

PBS (20 ×)	2 ml
Blocking reagent (10%)	0.4 ml
NaCl (5 M)	16 ml
H_2O_{bidist}	ad 40 ml
Dextrane sulfate	4 g

The solution was heated up to 40-60°C.

Tyramide was diluted 1:10 in amplification buffer and 1 µl of 0.15% H_2O_2 was added per 100 µl of solution (final concentration: 0,0015%). The solution was kept on ice in the dark until use. From this mixture 10 µl were pipetted onto each well and the slide was incubated in a humid chamber in the dark at 46°C for 1 h. Afterwards, the slide was dabbed on tissue paper and washed in 1 × PBS for 10 min at RT in the dark. Finally, samples were washed in H_2O_{bidist} (RT, dark) for 1 min, the slide was air dried and samples were stained with DAPI as described in 2.21.3 and stored at -20°C in the dark until microscopic analysis.

2.23 FISH of *E. coli* expressing cloned 16S rRNA genes (Clone-FISH)

To validate the specificity of newly designed probes, clone-FISH was performed. In this method 16S rRNA genes of interest are cloned into an expression vector containing a T7 RNA polymerase promoter and transferred into *E. coli* host cells with an IPTG-inducible T7 RNA polymerase. By addition of IPTG the *in vivo* transcription of plasmid inserts is induced and by the use of Camp, which increases plasmid copy numbers and leads to an accumulation of RNA, high numbers of target rRNA can be generated (Schramm et al. 2002). Finally, cells are fixed and used for FISH analysis.

2.23.1 Amplification of 16S rRNA genes and cloning

16S rRNA genes were amplified (B.13.1.) and PCR products were cloned into an expression vector (B.15.). After transformation of chemical competent *E. coli* TOP10 cells clones were screened via M13 PCR and inserts were sequenced. The respective plasmid was isolated as described in 2.11.2.

2.23.2 Electroporation of *E.coli*

Solutions

SOC medium

LB medium

Kan stock solution

Procedure

During this step, electro-competent *E. coli* cells are transformed with isolated plasmids via electroporation. An aliquot of *E. coli* JM109 (DE3) cells was thawed on ice. Then, 1 µl of plasmid solution (containing insert of clone GaBI27) was added and the solution mixed gently. Cells were transferred carefully to a pre-cooled electroporation cuvette (0.2 cm; Biorad, München, Germany) using a 1 ml pipette tip to minimize shearing stress. The cuvette was inserted into the electroporator MicroPulserTM (Biorad, München, Germany) and electroporation was performed at a voltage of 2.49 kV for 4.8 ms (condenser 25 mF, resistor 200 Ω). 250 µl SOC-medium were added immediately and the solution was transferred to a sterile ERT. The cells were incubated at

37°C for 1 h at 200 rpm. Afterwards, 10 µl and 50 µl of the cells were spread out on LB-Kan plates and incubated over night at 37°C. Plates were then stored at 4°C.

2.23.3 *In vivo* transcription of plasmid encoded 16S rRNA genes

Preparations

3 clones were picked from the LB-Kan plate and a M13-screening PCR was carried out to test for the correct insert size (see 2.14.4). After this, 5 ml LB-Kan medium was inoculated with one positively tested clone and incubated o/n at 37°C at 200 rpm.

Solutions

LB-Kan medium

1 M IPTG

Camp stock solution

Procedure

1 ml of pre-culture was inoculated into 100 ml LB-Kan medium in a 200 ml Erlenmeyer flask (Schott Glas, Mainz, Germany) and grown at 37°C at 200 rpm until an OD_{600} of 0.334 (see 2.11.1). After the appropriate cell density had been reached, *in vivo*0 transcription was started by adding 100 µl of 1 M IPTG and incubating the culture for 1 h. After that, 170 µl Camp were added and the cells were incubated for 4 h. Finally, cells were harvested by centrifugation for 5 min at 3270 g (Rotina 35S).

2.23.4 Cell fixation, test on successful in vivo transcription and FISH

Cells were fixed with PFA for 45 min at RT as described in 2.21.1.1. FISH and FA-series were then performed under identical conditions for all hybridizations as described in 2.21.2. After hybridizations, samples were embedded in CF and 6-10 pictures were recorded for each of the 10 slides (FA concentrations from 0 to 70%) using CLSM (2.21.4).

2.24 Raman-spectroscopy and Raman-FISH

Raman spectroscopy is a technique based on the measurement of the vibrational energy of chemical bonds. It can be applied also for microbial cells on single cell level to study their ecophysiology. Samples are illuminated with monochromatic light generated by a laser and inelastically scattered light following excitation is measured. Fingerprints of abundant cellular components such as nucleic acids, proteins, lipids and carbohydrates are clearly visible in the Raman spectra. Anabolic incorporation of ^{13}C stable isotope tracers causes significant changes in the observed resonance spectra due to modification of bond vibrational states through the increased molecular mass contributed by the heavier isotopes. This modification is termed a "red shift". Since these spectral shifts correlate with the tracer content of the cells, Raman microspectroscopy offers a quantitative approach. Isotope-labeling techniques combined with molecular detection tools are frequently used by microbial ecologists to directly link structure and function of microbial communities and to monitor metabolic properties of uncultured microbes at the single cell level. The combination of Raman spectroscopy with FISH offers the simultaneous cultivation-independent identification and metabolic characterization of microbial cells. (Huang et al. 2004; Huang et al. 2007; Wagner 2009).

Raman microscopy was performed using a LabRAM HR800 UV confocal Raman microscope (Jobin-Yvon, UK) based on an Olympus BX-41 microscope. The Olympus microscope chassis was modified with a 100-W Xenon lamp and standard FITC, Cy3 and Cy5 filter blocks and an F-View camera (Soft Imaging Systems). For combined Raman-FISH analysis, cells displaying oligonucleotide hybridization signals were located using epifluorescence imaging on the modified Raman (Huang et al. 2007), and their Raman spectra recorded.

2.24.1 Raman spectroscopy with putative AOB

2.24.1.1 Cell immobilization on calcium fluoride slides

Due to the very highly enriched culture of putative AOB ("culture 7.3"), Elena Lebedeva), Raman analysis without FISH was carried out. 1.5 µl of PFA-fixed cells from ^{13}C and ^{12}C incubations lasting 72 h were spotted onto a calcium fluoride (CaF_2) slide and dried. CaF_2 slides were used to reduce background peaks. After this step, the slide was dipped for 2 sec in ice-cold H_2O_{bidist} and dried rapidly by compressed air. Raman analysis was performed without mounting media or coverslips.

2.24.1.2 Raman spectroscopic analysis

For calibration and Raman spectra recording, the Labspec software (Jobin-Yvon) was used.

Calibration

Prior to analysis, the system was calibrated using a silicon Raman reference (520 cm^{-1}). Once the right focus plane was reached, a single spectrum from the silicon coated slide was acquired with an NdYAG laser. Therefore, the image acquisition was switched to the inbuilt Raman CCD detector, the laser was started and the option "autocalibration multigradients"was chosen. The exact settings for recording spectra are described below.

Spectral analysis of single cells

For Raman spectral analysis of a chosen cell, the laser was focused directly on the middle of a single cell. Raman scattering was excited by the laser at 532.09 nm and the incident laser power was adjusted to D1, in which damage of cells should be avoided. The pinhole of the Peltier cooled (-70°C) CCD Raman detector was set to 250 µm and spectra were obtained by acquisition at 1250 cm^{-1} and 60 sec duration each. For ^{12}C- and ^{13}C-incubations, spectra for 32 and 30 cells were recorded, respectively. After this, Raman spectra with little background noise were processed for baseline correction and normalized and smoothed using Labspec software. The spectra were then exported to Excel (Microsoft) for peak determinations and calculations of the red shift ratio (RSR). The red shift ratio of a specific peak is calculated as the intensity of wavelength of the unlabeled peak divided by the wavelength of the labeled peak and was shown to correlate with the ^{13}C-content in cells by Huang et al. (2007).

Analysis of spectra with Excel

With the diagram assistant, 32 spectra were recorded for cultures fed with ^{12}C and 30 spectra were recorded for cultures fed with ^{13}C bicarbonate, and averaged. Significant peaks of the mean spectra like phenylalanine and cytochrome C were visually compared and further investigated for presence of shifts. The RSR was calculated for phenylalanine for each single ^{12}C and ^{13}C spectrum by dividing peak intensities at 965 cm^{-1}(labeled) through peak intensities at 1001 cm^{-1} (unlabeled). The RSR of ^{12}C and ^{13}C peaks were spotted in a diagram and compared.

2.24.2 Raman-FISH with *Nitrococcus* enrichment from "Haus des Meeres"

Before taking samples, incubations were shaken and 1 ml of ^{12}C culture and 1 ml of ^{13}C culture were used for PFA-fixation (2.21.1.1). For Raman-FISH, 15-30 µl of biomass were pipetted onto CaF_2 slides and FISH was carried out as described in 2.21.2 with the EUBmix probe in Fluos and Ntcoc84 in CY3. Additionally, Raman-FISH was carried out with a PFA-fixed sample of a pure culture of *Nitrococcus mobilis*. For FISH analysis, the Raman microscope was upgraded with an epifluorescence light source, epifluorescence filter blocks and an extra fluorescence imaging camera. To take pictures, the programme analySIS getIT was used. Exposure time and intensity of light were adjusted until single cells exhibited a distinct signal. Then, pictures were taken and single cells, which were identified as *Nitrococcus*, were chosen for spectral analysis, which was performed as delineated for putative AOB in 2.24.1.2. In contrast to Huang et al. (2007) no background fluorescence was observed when cells hybridized with Cy3-labeled probes, thus no bleaching step with the incident NdYAG laser prior to spectra acquisition was necessary. 20 spectra of cells fed with ^{12}C bicarbonate and 33 spectra of cells fed with ^{13}C bicarbonate were recorded. For the pure culture, 7 spectra were recorded.

2.25 MAR

Microautoradiography (MAR) is a technique that enables direct visualization of active cells and their metabolic capabilities without prior enrichment or cultivation. When combined with fluorescence *in situ* hybridization using oligonucleotide probes for identification of the microorganisms, it is possible to link key physiological features to the identity of microorganisms (Nielsen 2003). MAR is based on incubation with radioactively labeled substrates and relies on the emission of β-particles during radioactive decay. Cells that have incorporated the radioactive labeled compound will lead to silver grain formation in a photographic emulsion on top of them and can thus be distinguished from metabolically inactive cells.

Solutions

NH_4Cl 1M stock solution	20 mM working solution, final concentration: 0.5 mM
NaOH 0,1% w/v	
allyl-thiourea (ATU) 200 µM	40 µl added to 2 ml medium

Table 2.18: Substrate concentrations used for MAR incubations

Substrate	with NH_4^+	w/o NH_4^+	Substrate [µM]	Substrate added [µl]	Radio-activity added [µCi]	^{14}C-labeled Substrate [µM]	^{14}C-labeled Substrate added [µl]	ATU[a] [µM]	ATU added [µl]
Pyruvate	yes	yes	79	23	1.8	79	20	/	/
Amino acid mixture[b]	yes	yes	25	57.5	2.5	25	55	/	/
Phenol	yes	yes	104	11.5	3	104	6.6	/	/
Benzoic acid	yes	yes	620	7.7	1	620	1.1	/	/
Bicarbonate	yes	yes	26	23	3	26	13.2	/	/
Phenol + ATU	no	yes	104	11.5	3	104	6.6	200	20
Benzoic acid + ATU	no	yes	1860	7.7	1	1860	1.1	200	20
Dead control Bicarbonate	yes	no	26	23	3	26	13.2	/	/

[a] ATU, allyl-thiourea
[b] amino acid mixture containing all amino acids (in different concentrations)

2.25.1 Pre-incubations

From a culture enriched with "*Candidatus* Nitrososphaera gargensis"containing 0.5 mM NH_4^+ and with adjusted pH (7.0), 2.3 ml were pipetted into 12 autoclaved 10 ml glas-vials each, representing 10 different experiments containing NH_4^+ (5 experiments in duplicates and negative control). From a second culture (obtained by splitting the culture), in which NH_3 had been completely oxidized to NO_2^-, 2.3 ml each were pipetted into 14 10 ml glas-vials each, representing the 14 different experiments without NH_4^+ in the medium (7 experiments in duplicates). As a test for chemographic uptake and chemical loss of NH_4^+, a dead control was prepared. Therefore, 4 ml of the culture were fixed o/n with 1% PFA at 4°C. On the following day, dead cells (resuspended in 1 × PBS) were resuspended in 600 µl of NH_4^+-free medium and 300 µl were added with 2 ml of medium in 10 ml glas-vials (final volume 2.3 ml). NH_4^+ concentration was adjusted to 0.5 mM. To all glas-vials the different (non-radioactive) heterotrophic substrates or bicarbonate and ATU were added (Table 2.18). After this, the vials were inserted into 50 ml closed tubes (Greiner), to avoid loss of NH_4^+ via evaporation of water, and the cultures were incubated for 5 h at 46°C without shaking.

2.25.2 Incubations with radioactively labeled substrates

Before starting the main incubation, 300 µl from each vial was removed to measure NH_4^+ and NO_2^- concentrations (vials were shaken before sampling; 2.8). After this, NH_4^+ was adjusted to 0.5 mM for experiments containing NH_4^+ and the pH was

adjusted by adding 220 µl of 0.1% NaOH, which later turned out to have increased pH to 9. Nevertheless, the vials were transferred to the isotope-lab, in which the ^{14}C-labeled substrates were added. The incubations were performed o/n at 46 °C without shaking in a hybridization oven. In Table 2.18 the substrate concentrations and the amount of radioactivity added are listed.

2.25.2.1 PFA-fixation of cells and supernatant collection for downstream analysis

After the incubation with radioactive substrates, approximately 2 ml of each incubation was transferred to 2 ml ERT and centrifuged for 10 min at 21,250 g at 4°C (Rotina 35S). The SN was transferred into new 2 ml ERT. 300 µl of the SN was pipetted into 1.5 ml ERT for subsequent NH_4^+ measurement and 50 µl of the SN was put into 0.5 ml caps for subsequent NO_2^- measurement.

For PFA-fixation of the cells a 3% PFA solution was used. PFA was added to the cell pellets and incubated for 3 h at 4°C and processed as described in 2.21.1.1. Finally, pellets were diluted in 200 µl PBS:EtOH (1:1) and stored at -20°C until further analysis.

2.25.3 Test on activity dependence on pH

Because MAR experiments were performed at a too high pH, the activity of cells was compared at neutral and alkaline pH. Therefore, incubations in duplicates supplemented with 0.5 mM NH_4^+ were performed. 2 ml of the culture was added to each of four glass vials. Two of the vials were supplemented with 25 µl of 0.1% NaOH and 175 µl of H_2O_{bidist}, leading to a pH of around 7.5, whereas the other two veils were supplemented with 200 µl of 0.1% NaOH, leading to a pH of 8.5-9, at which MAR experiments were performed. After a short incubation, samples to measure NO_2^- production were taken.

2.25.4 MAR protocol

2.25.4.1 Sample preparation

6 spots of 1 × PBS were pipetted on coverslips and 1-2 µl of biomass were distributed. On each coverslip, 3 duplicates were spotted, yielding 6 spots per coverslip. Coverslips were dried at 46°C before covering of samples with film emulsion took place.

Solutions:

Kodak D19	40 g/l	mix for 300 ml: 12 g	stored at RT in the dark
Stopping solution: H_2O_{bidist}			stored at - 4°C
Fixative: Sodiumthiosulfate 30%		mix for 300 ml: 90 g	stored at - 4°C

2.25.4.2 Covering with film emulsion and exposition

The autoradiographic film emulsion (LM-1 emulsion, Amersham) was heated for 10 min at 45°C and after that carefully stirred with a glass rod. All working steps were performed in a dark room. Some of the film emulsion was poured into a dipping vessel. To cover the slides, each slide was dipped for approximately 7 sec into the film emulsion. Then, sildes were dried on the backside with tissue paper. Finally, slides were fixed horizontally in a box, which was wrapped into aluminium foil, together with a package of dry silica gel. The box was stored on 4°C and slides were exposed from several days to weeks.

2.25.4.3 Development of film

In the dark room slides were taken out of the box and transferred into a plastic rack. Subsequently, slides were submerged into the developing solution for 1-5 min, depending on the expected signal intensity. Afterwards, slides were submerged in the ice-cold stopping solution (H_2O_{bidist}) for 1 min and into the ice-cold fixative solution (Sodiumthiosulfate) for 4 min. Finally, slides were submerged into ice-cold H_2O_{bidist} for 2 min and dried and stored in the box at 4°C until further analysis. Before investigation of the slides on a CLSM they were stained with Sybr green in CF (see B.21.3.). Pictures were taken with same settings using CLSM.

Chapter 3

Results

3.1 "*Candidatus* Nitrososphaera gargensis"enrichment from the Garga hot spring

3.1.1 "*Candidatus* Nitrososphaera gargensis"

3.1.1.1 Activity of the enrichment

Only some hours after the culture was inoculated, near-stoichiometric conversion of ammonia to nitrite by AOA was observed: after o/n incubation NH_4^+ concentration had decreased by ∼320 µM and NO_2^- concentration had increased by ∼300 µM. After some weeks, activity of the cultures decreased until they finally were not active any more.

3.1.1.2 Microautoradiography (MAR)

The first exposure time examined was 3 weeks, the second exposure time was around 9 weeks. In all cultures, except for cultures supplemented with amino acids, "*Candidatus* Nitrososphaera gargensis"was forming very dense aggregates, in which single cocci were not recognizable any more. Results are summarized in Table 3.1 and 3.2. Heterotrophic bacteria were always present as single rods and never showed any MAR-signal, thus only AOA are described.

For the samples exposed around 9 weeks no differences to samples exposed shorter could be observed, except for the incubations supplemented with phenol and supplemented with NH_4^+, in which big aggregates were MAR positive (but were not before).

In general, slides that were exposed longer were more difficult to analyze because some slides were covered with a black layer (which was not composed of silver grains but leftovers of film). In Figure 3.2 cultures supplemented with and w/o NH_4^+ and incubated with different radioactively labeled substrates are shown. In nearly all cultures AOA form dense aggregates, in contrast to cultures supplemented with amino acid mix, in which single cocci are visible. It can be observed that silver grains accumulated close to and over clusters of the AOA, and often accumulated only in the middle of clusters. PFA-fixed cells that should serve as dead control (C) revealed silver grain formation.

In Table 3.3 NH_4^+ and NO_2^- concentrations before and after the MAR incubation are listed. It could be observed that NH_4^+ consumption in cultures supplemented with NH_4^+ was quite high. However, NO_2^- production was not very high in cultures supplemented with bicarbonate, as well as in most of the other cultures. Only cultures supplemented with amino acid mix (both with NH_4^+ and w/o NH_4^+) were producing a lot of NO_2^-.

Test for activity dependence on pH

Since MAR incubations were performed at a too high pH (9.0), NO_2^- production was tested at pH 7.5 and 9.0. No difference in activity was observed: in both cultures around 250 μM of NO_2^- was produced over two days.

Table 3.1: Sample description and MAR results for incubations with NH_4^+.

	sample description (+NH_4^+)	**MAR signal (+NH_4^+)**
Bicarb I	cocci form dense aggregates	yes: most of the aggregates MAR positive, centre very active, border less active
Bicarb II	small & big aggregates of cocci	yes: strong MAR signal in middle of aggregates, big aggregates strong MAR positive
Benz I	aggregates	no
Benz II	small & middle-sized aggregates, one big aggregate	no
AA I	very big loose aggregates, single cocci still visible	no
AA II	like AA I	no
Phe I	no biomass (washed off)	not determined
Phe II	cocci in aggregates (middle-sized & small)	no
Pyr I	some small aggregates, middle-sized & big aggregates	yes/no: small aggregates not active; middle-sized low activity; big aggregate partly active
Pyr II	middle-sized & big aggregates	yes: aggregates strong & medium active in middle
Dead I (Bicarb)	middle-sized & small aggregates (no big aggregates)	yes/no: small aggregates no signal; some with signal
Dead II (Bicarb)	middle-sized & small aggregates (no big aggregates)	yes/no: small aggregates no signal; some with signal

Table 3.2: Sample description and MAR results for incubations w/o NH_4^+.

	sample description (-NH_4^+)	**MAR signal (-NH_4^+)**
Bicarb I	small, middle-sized & big aggregates	yes/no: small aggregates low signal; some aggregates very strong signal
Bicarb II	a lot of middle-sized aggregates	yes: signal in middle of aggregates
Benz I	a lot of small aggregates, middle-sized aggregates, one big aggregate	no
Benz II	small, middle-sized aggregates	no
Benz ATU I	broken slide	not determined
Benz ATU II	broken slide	not determined
AA I	huge loose aggregates, cocci visible; in middle more dense	no
AA II	huge loose aggregates, cocci visible; in middle more dense	no
Phe I	big & small aggregates	yes: big aggregate with a lot of signal (middle); small aggregates no strong signal; some no signal
Phe II	big & small aggregates	yes: huge aggregate with signal; small aggregate without signal
Phe ATU I	small, middle-sized & one big aggregate	yes/no: big aggregate black in middle; small aggregates little/no signal
Phe ATU II	middle-sized aggregates	yes/no: active & not active aggregates
Pyr I	small, middle-sized & two big aggregates	yes/no: active (at certain areas) & not active aggregates
Pyr II	aggregates	yes/no: active & not active aggregates

3.1.1.3 Temperature experiment

To evaluate the temperature dependence of NO_2^- formation, cultures (in duplicates) were incubated at four different temperatures for 14 days and NH_4^+ and NO_2^- concentration were measured regularly by photometric methods (2.8). In Figure 3.1 NO_2^- concentrations over time at the respective temperature are shown. It was observed that AOA were still producing NO_2^- at a temperature of 66°C. A list of measured NH_4^+ and NO_2^- concentrations at the different temperatures and sampling points and a list of the NO_2^- production (at each temperature) at each sampling point, per day and within two weeks is added on the enclosed CD ("Temperature experiment").

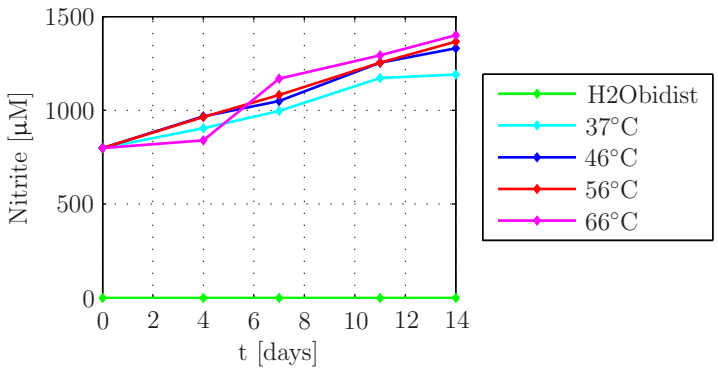

Figure 3.1: NO_2^- production at four different incubation temperatures over a time span of 14 days. For graph calculations, mean values of the duplicates were used.

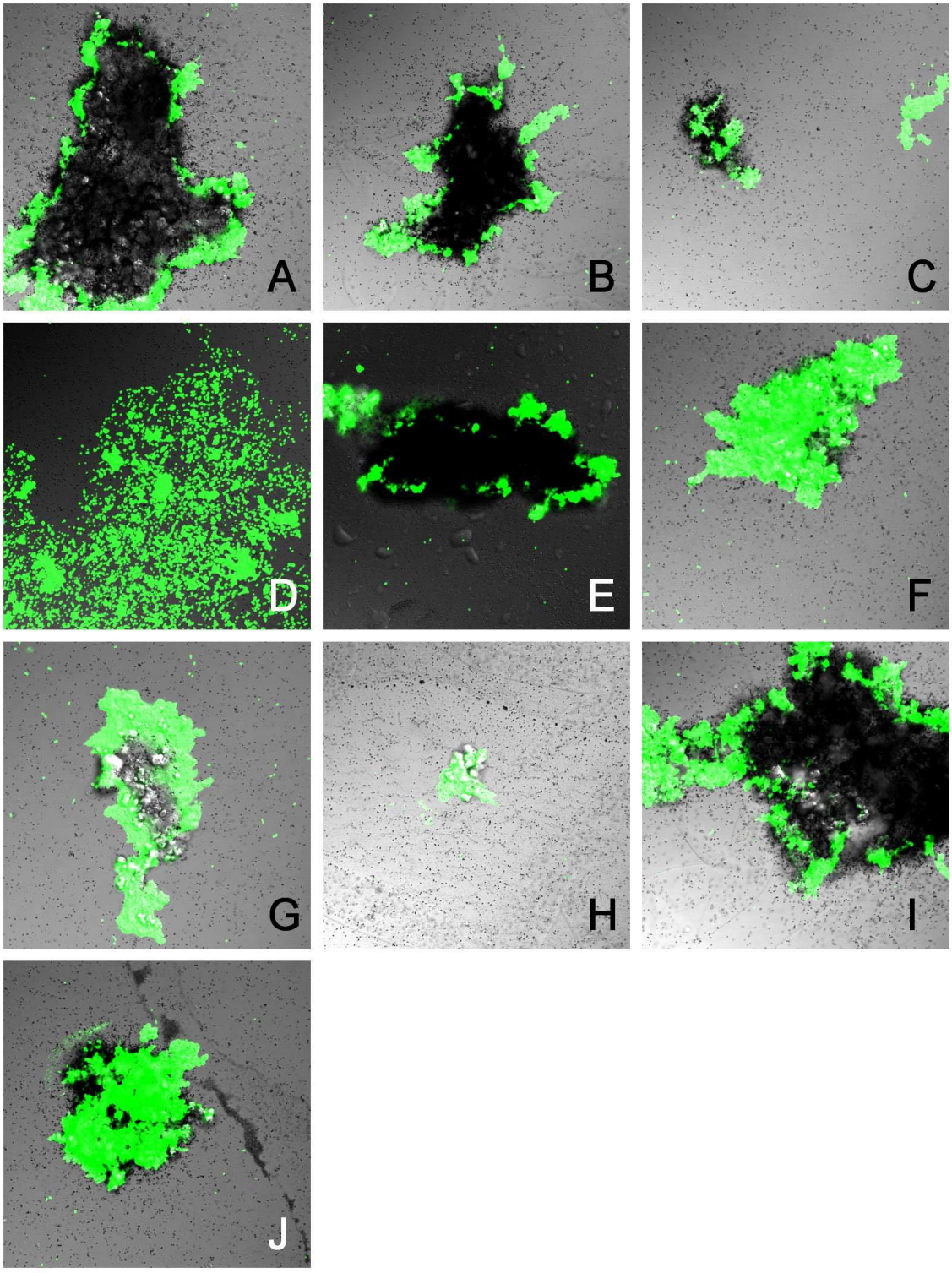

Figure 3.2: MAR performed with an enrichment culture of "*Candidatus* Nitrososphaera gargensis" after exposure for three weeks. Cells are stained with Sybr green. (A) AOA microcolony with uptake of ^{14}C bicarbonate supplemented with NH_4^+ and (B) w/o NH_4^+. (C) PFA-fixed dead control incubated with ^{14}C bicarbonate, showing a microcolony with a MAR signal. (D) Loose aggregate supplemented with NH_4^+ showing no uptake of amino acid mix. (E) Microcolony with uptake of ^{14}C pyruvate supplemented with NH_4^+ and (F) with poor uptake of substrate w/o NH_4^+. (G) Microcolony with no uptake of ^{14}C benzoate supplemented with NH_4^+. (H) Microcolony with no uptake of ^{14}C phenol supplemented with NH_4^+. (I) MAR-positive microcolony incubated w/o NH_4^+ with ^{14}C phenol and (J) with ^{14}C phenol and ATU.

Table 3.3: NH_4^+ and NO_2^- concentrations pre and post the MAR incubations. Cultures that were supplemented with NH_4^+ are marked with "+", those that were not fed are marked with "-".

	NH_4^+				NO_2^-		
	Pre	Pre(fed)	Post	**consumption**	Pre	Post	**production**
AA I+	381	500	410	**90**	23	322	**299**
AA II+	384	500	410	**90**	37	315	**278**
Benz I+	229	500	361	**139**	771	802	**31**
Benz II+	221	500	369	**131**	831	872	**41**
Bicarb I+	222	500	356	**144**	747	807	**60**
Bicarb II+	217	500	344	**156**	756	805	**49**
Phe I+	269	500	320	**180**	674	678	**4**
Phe II+	285	500	379	**121**	706	755	**49**
Pyr I+	231	500	358	**142**	763	791	**28**
Pyr II+	234	500	363	**137**	779	767	**-12**
Dead I+	414	500	355	**145**	0	-3	**-3**
Dead II+	387	500	403	**97**	-1	-2	**-1**
	NH_4^+				NO_2^-		
	Pre		Post	**consumption**	Pre	Post	**production**
AA I-	22		18	**4**	54	252	**198**
AA II-	23		21	**3**	67	445	**378**
Benz I-	2		3	**-1**	580	697	**117**
Benz II-	1		5	**-4**	574	579	**5**
Benz ATU I-	1		5	**-4**	592	615	**23**
Benz ATU II-	1		5	**-4**	589	598	**9**
Bicarb I-	2		3	**-1**	585	614	**29**
Bicarb II-	0		5	**-5**	600	611	**11**
Phe I-	1		4	**-4**	874	616	**-258**
Phe II-	1		4	**-2**	589	653	**64**
Phe ATU I-	2		5	**-3**	587	593	**6**
Phe ATU II-	2		5	**-3**	596	579	**-17**
Pyr I-	2		3	**-1**	579	584	**5**
Pyr II-	1		3	**-2**	588	609	**21**

3.1.2 Bacterial contaminants of the "*Candidatus* Nitrososphaera gargensis"enrichment

3.1.2.1 Amplification of bacterial 16S rRNA gene fragments

DNA was extracted using the Power SoilTM DNA Kit (MoBio Laboratories) and Phenol-Chlorofotm DNA extraction (2.10.1.2) from the "*Candidatus* Nitrososphaera gargensis"enrichment.

For the amplification of bacterial 16S rRNA genes the general primer pair 616F/630R (Table 2.6) was used, leading to an amplicon length of 1535 bp. The PCR-product was cloned and insert-positive clones were identified via M13 screening PCR (Table 2.13). A total of 120 clones were picked.

In another PCR, primers 616F/630R were combined with the primer RHG1148Rlock, which lacked the OH group at 3' end. This was done in order to try to inhibit amplification of 16S rRNA genes of a putative AOB and to amplify genes belonging to other contaminants in the enrichment. Amplicons had a length of 1535 bp. As a control, primer 616F was used with the intact primer RHG1148R for PCR, which resulted in an amplicon length of 1141 bp. After cloning of PCR-products, 71 clones were picked and tested for correct insert via M13-screening PCR.

3.1.2.2 RFLP

a) RFLP with clones from 616F/630R PCR

RFLP screening was carried out for 90 bacterial clones using enzymes AluI and MspI (Table 2.15). Four different patterns were obtained, two of them were very abundant. At least one clone representing each pattern was sequenced. Finally, 24 sequences were obtained (named GaB).

b) RFLP with clones from 616F/630R/RHG1148Rlock PCR

RFLP analysis was done for 107 bacterial clones using enzymes AluI and MspI. Four patterns could be observed, among them two that were not observed for clones using primer pair 616F/630R. A total of 21 clones, representing all different patterns, were sequenced. Clones were named GaBI.

3.1.2.3 Sequence analysis

The 24 GaB-clone sequences were compared to online databases using BLAST. All sequences showed high similarity to putative AOB, which were already known to be present in the enrichment (termed RHG; (Hatzenpichler et al. 2008; Hatzenpichler 2006)). Thus, no further analysis was performed.

Among 21 GaBI-clone sequences 9 were identified as putative AOB clones. 9 of the sequences (GaBI3, 5, 14, 17, 26, 27, 28, 33, 36, 58 and 69) had high similarity to *Thermaerobacter subterraneus*, and 2 (GaBI5, 69) were highly similar to the 16S rRNA gene of an uncultured low GC gram positive bacterium clone and to *Thermaerobacter* sp. Another sequence (GaBI25) was highly similar to an uncultured cyanobacterium clone and to *Microcoleus chtonoplastes*.

3.1.2.4 DGGE analysis

Touchdown DGGE PCR was performed as described in Table 2.8 with primers C341F and 907R (Tab. 2.6). As template, DNA extracted from the "*Candidatus* Nitrososphaera gargensis"enrichment, RHG-clones (which were the least similar to each other), GaB-clones and GaBI-clones were used.

In the first DGGE gel (20-80% gradient) (Fig. 3.3) one strong band was observed for each clone. The fragments of clone RHG27 and 28 migrated a little further than fragments of the other clones. DGGE performed with DNA extracted from the enrichment resulted in three distinct bands. All samples revealed additional faint bands. The upper band obtained from enrichment DNA was the only band which was represented in the clone library by putative AOB (GaB- and RHG-clones).

In order to obtain a higher resolution, a second DGGE gel was performed containing a 40-80% gradient (Fig. 3.4). DGGE PCR using the enrichment sample as a template was carried out in replicates, pooled and applied to the gel. Like in the first DGGE gel, clones produced a prominent band and some weak bands. The fragment of clone RHG36 migrated a little slower than fragments of the other clones. In the enrichment DNA sample, three characteristic bands and some faint bands were present. Two of the fragments observed in the environmental sample were represented in the clone library: the upper band represented the putative AOB (RHG clones), while to the lowest band the two *Thermaerobacter*-clones (GaBI27 and 33) could be assigned. Clone GaBI25 revealed a single band which could not be detected in the enrichment DNA. The enrichment DNA fragments (designated as band 1, 2 and 3) were cut out, reamplified,

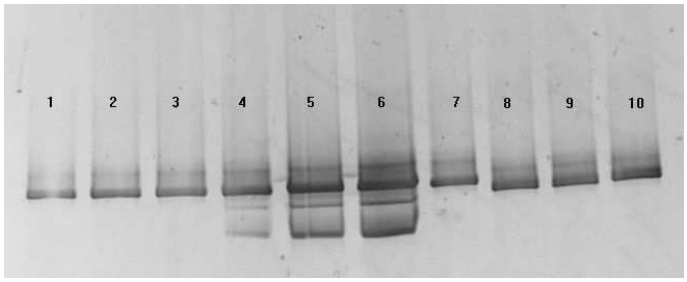

Figure 3.3: DGGE gel containing an urea gradient from 20-80% showing the low diversity of bacterial 16S rRNA genes amplified from the Garga hot spring enrichment (lane 5, 6) and clones (GaB35, 42, 51 and 56: lane 1-4; RHG16, 27, 28 and 30: lane 7-10). Due to a pipetting mistake in lane 5 some of the material applied ran through lane 4. Only one fragment observed for the environmental sample is represented in the clone library.

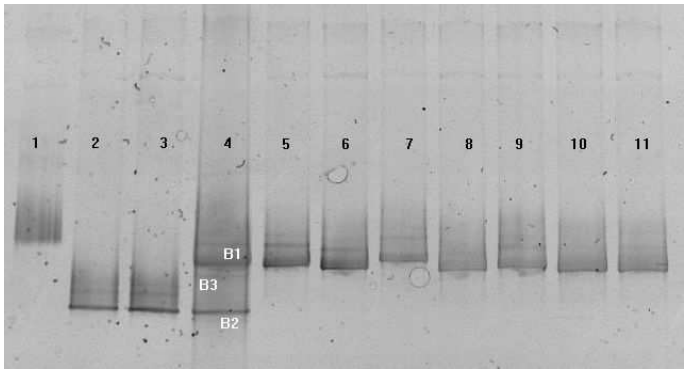

Figure 3.4: DGGE gel containing an urea gradient from 40-80% showing bacterial 16S rRNA gene fragments amplified from the Garga hot spring enrichment (lane 4) and clones (GaBI25, 27 and 33 (latter two: *Thermaerobacter*) : lane 1-3; RHG16, 26, 36 and 40: lane 5-8; GaB35, 42, 51: lane 9-11). Two of three fragments observed for the environmental sample are represented in the clone library. Letters B1-B3 indicate the three obtained fragments that were cut out, reamplified and sequenced.

purified and sequenced.

DGGE sequence analysis

Sequences of reamplified DGGE bands were compared to other sequences using BLAST. Band 1 was most similar to putative AOB clones (RHG). Band 2 and 3 both could be identified as *Thermaerobacter*-related sequences.

3.1.2.5 T-RFLP

For PCR, 5'-labeled primers 21V/Arch912-FAM and 21V/1492R-YO were used to amplify archaeal 16S rRNA genes, while for bacterial genes primers 616V-FAM/1492R-YO (30 cycles; Tab. 2.6) were used as described under the conditions in Table 2.7. After

PCR-products had been purified (QIAquick® PCR Purification Kit, QIAgen, Hilden, Germany) their concentration was determined. Total amount of PCR products in the restriction mixture was 100 ng. Restriction was done using three different endonucleases, AluI, MspI and RsaI (Tab. 2.15), to obtain a higher resolution (see 2.16).

The *in silico* predicted T-RF length from 16S rRNA sequences of *Thermaerobacter* (GaBI clones), putative AOB (GaB and RHG clones) and "*Candidatus* Nitrososphaera gargensis" are listed in Table 3.4.

Table 3.4: *In silico* predicted T-RF lengths for bacterial and archaeal clones.

	T-RF length (bp)		
	AluI	MspI	RsaI
Thermaerobacter (616V)	69	147	488
Thermaerobacter (1492R)	443	33	119
putative AOB (616V)	234	123	471
putative AOB (1492R)	443	129	85
"*Candidatus* N. gargensis" (1492R)	81	519	613
"*Candidatus* N. gargensis" (912R)	129	142	43

The resulting electropherograms for bacteria are shown in Figure 3.5, 3.6 and 3.7. For bacteria primers 616V-FAM/1492R-YO were used. In every electropherogram, peaks could be assigned either to *Thermaerobacter* sp. or the putative AOB. Assignment was performed using the program TRF-CUT, which is implemented in the ARB software and which links clonal sequences to T-RF peaks. With all enzymes, except for AluI and the terminal fragments amplified with 1492R, two single peaks could be assigned either to *Thermaerobacter* sp. or putative AOB, respectively. Using AluI only one peak was assigned to both *Thermaerobacter* and putative AOB. The peak at 20 bp, which was present in all electropherograms, represented the primer. Next to peaks that could be assigned to either *Thermaerobacter* sp. or putative AOB, in some electropherograms some more small peaks were visible, probably representing pseudopeaks (see Discussion 4.2.4)

The archaeal profiles were obtained by using 21V/Arch912V (Fig. 3.8) and 21V/1492R (Fig. 3.9) and the same restriction enzymes as for bacteria. In each profile obtained using reverse primer 912R one major peak and smaller ones are visible, whereas only one peak was obtained using primer 1492R.

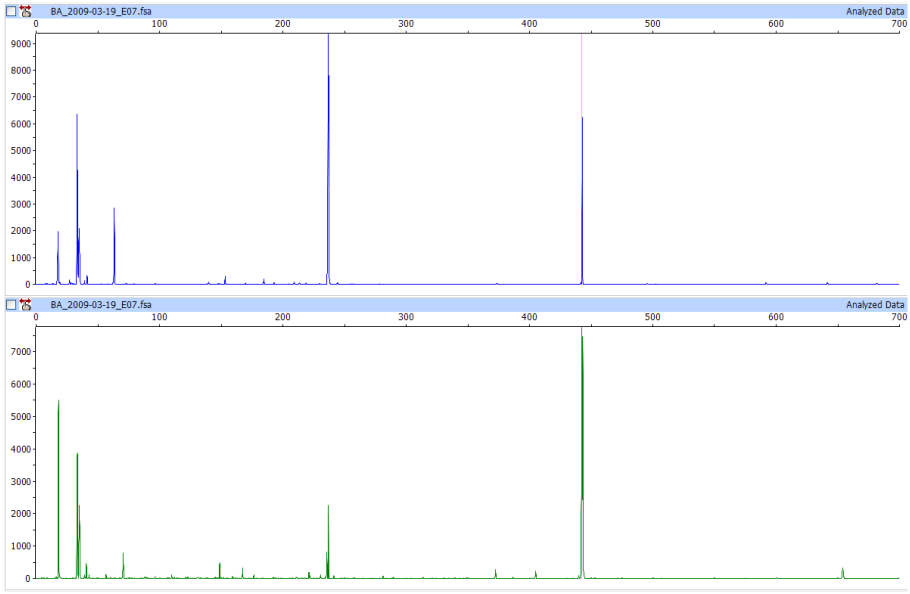

Figure 3.5: Electropherograms of the bacterial community in the enrichment sample derived after digestion with AluI. TRFs amplified with labeled 616V (above) and with labeled 1492R (below) are shown. y-axis: relative fluorescence units, x-axis: fragment size (bp). Abbreviations of organisms: Th, *Thermaerobacter*, pAOB, putative AOB. T-RF lengths above: 69=Th, 234=pAOB, below: 443=Th, pAOB

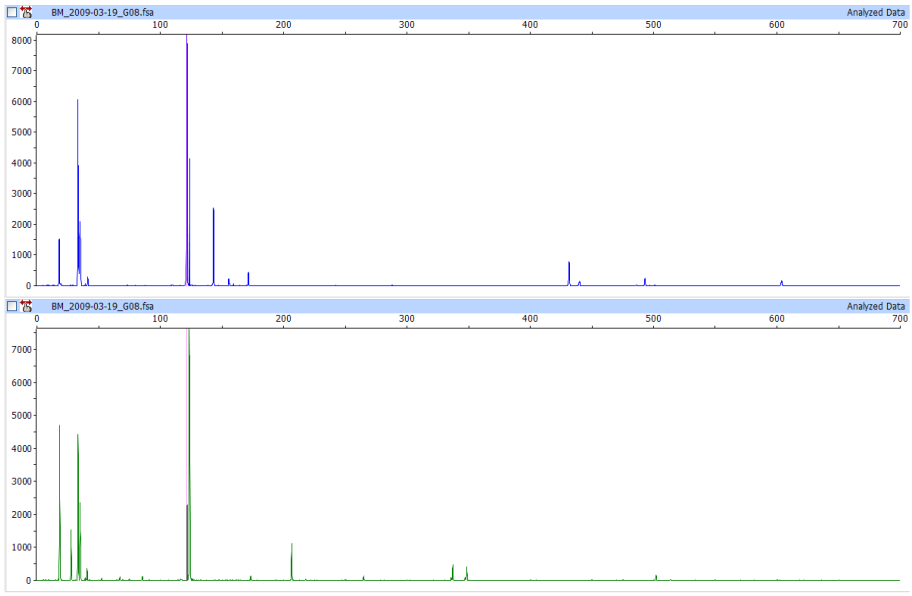

Figure 3.6: Electropherograms of the bacterial community in the enrichment sample derived after digestion with MspI. TRFs amplified with labeled 616V (above) and with labeled 1492R (below) are shown. y-axis: relative fluorescence units, x-axis: fragment size (bp). Abbreviations of organisms: Th, *Thermaerobacter*, pAOB, putative AOB. T-RF lengths above: 147=Th, 123=pAOB, below: 33=Th, 129=pAOB

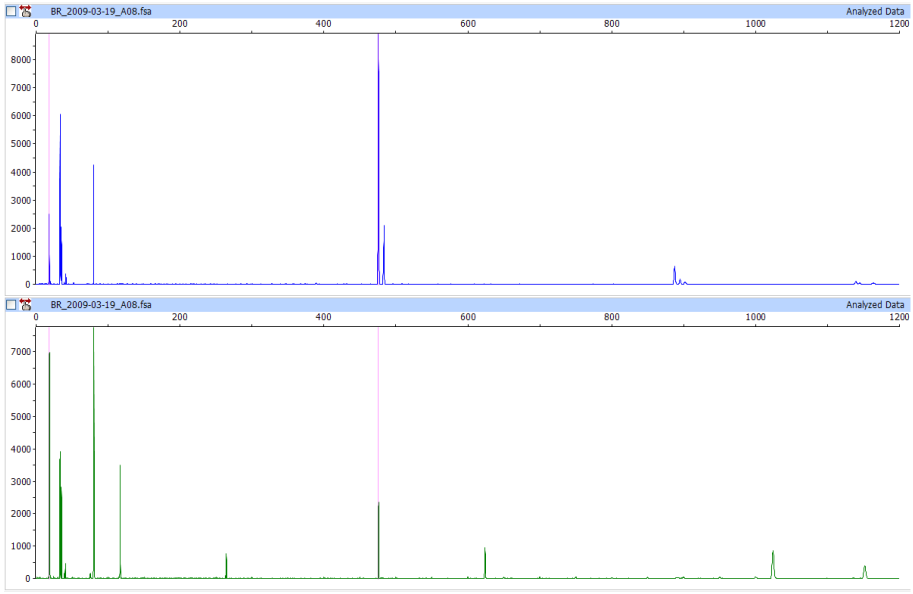

Figure 3.7: Electropherograms of the bacterial community in the enrichment sample derived after digestion with RsaI. TRFs amplified with labeled 616V (above) and with labeled 1492R (below) are shown. y-axis: relative fluorescence units, x-axis: fragment size (bp). Abbreviations of organisms: Th, *Thermaerobacter*, pAOB, putative AOB. T-RF lengths above: 488=Th, 471=pAOB, below: 119=Th, 85=pAOB

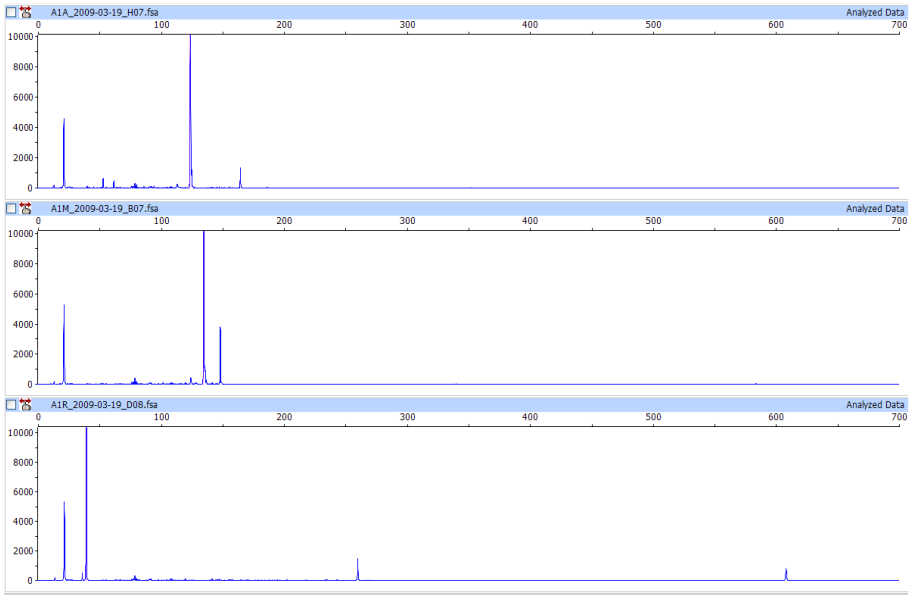

Figure 3.8: Electropherograms of "*Candidatus* Nitrososphaera gargensis" in the enrichment sample derived after digestion with AluI, MspI, RsaI (from above). For T-RFLP analyzing archaea, the reverse primer (912R) was labeled. N.g., "*Candidatus* Nitrososphaera gargensis". T-RF length above: 129=N.g., middle: 142=N.g., below: 43=N.g.

Figure 3.9: Electropherograms of "*Candidatus* Nitrososphaera gargensis" in the enrichment sample derived after digestion with AluI and MspI (from above). For T-RFLP analyzing archaea, the reverse primer (1492R) was labeled. N.g., "*Candidatus* Nitrososphaera gargensis". T-RF length above: 81=N.g., below: 519=N.g.

3.1.2.6 Phylogeny

For phylogenetic analysis only full length sequences were used. All obtained sequences, not affiliated to putative AOB, formed a monophyletic branch outside *Thermaerobacter* spp (Fig. 3.10). The sequences of the enrichment showed high similariy (>99%) to each other, except for sequence GaBI58F, which was >97% similar to the other clones. The closest relative was *Thermaerobacter marianensis* (or *Aerothermobacter marianus*) with a similarity between 88-89% (∼87% similarity for GaBI58F).

3.1.2.7 *In situ* detection of *Thermaerobacter* spp. in the enrichment sample

a) FISH-probe design

To detect *Thermaerobacter* spp. in the enrichment sample a FISH-probe (GaBI830), specific for all obtained GaBI-clones (Tab. 2.20), was designed using ARB as described in 2.20.3.2.

b) Clone-FISH

For evaluation and validation of the newly designed probe the 16S rRNA of the clone

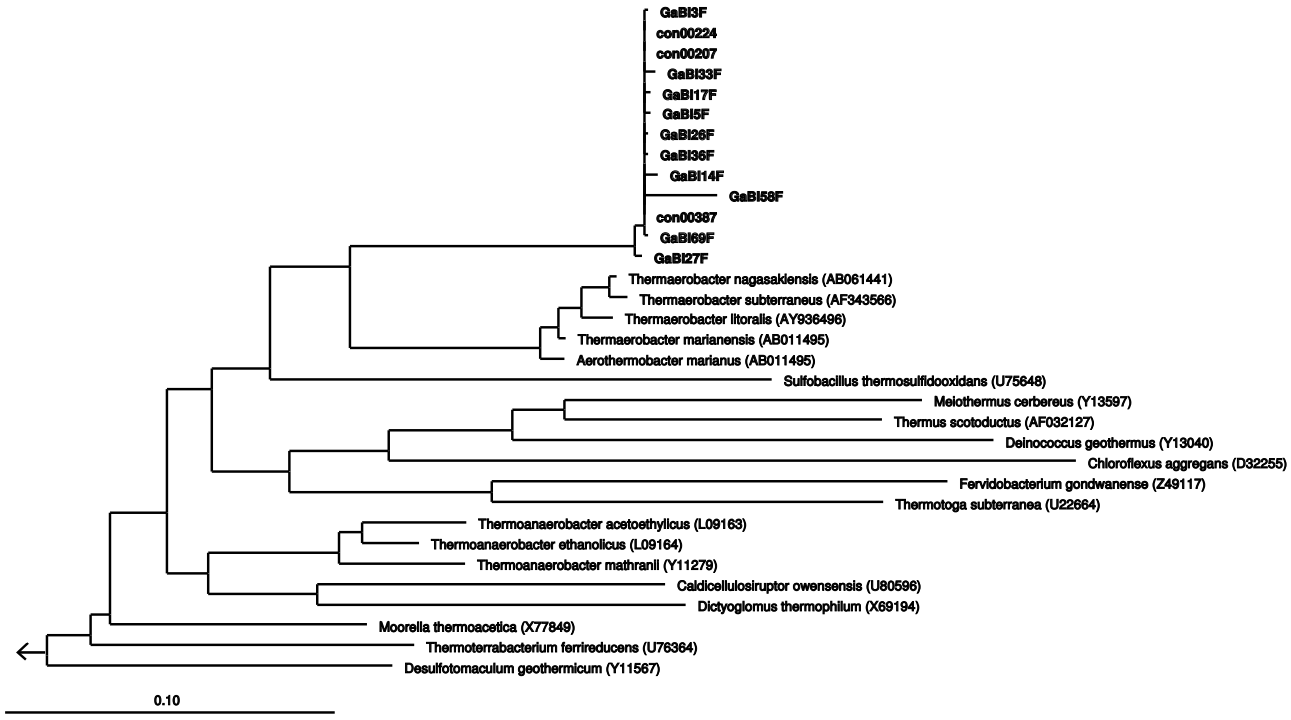

Figure 3.10: 16S rRNA based ML tree, rooted against *Chlamydia psicatti*, showing the phylogenetic positioning of *Thermaerobacter*-related sequences obtained from the Garga hot spring enrichment (in bold). The tree was calculated by analysis of sequences >1,400 nt. Only positions conserved in at least 50% of all bacteria were used for tree determination. Short sequences from metagenomic analysis (con; Hatzenpichler, unpublished) were subsequently added without changing the overall tree topology. All clones clustered together and showed highest similarity to *Thermaerobacter marianensis*. The scale bar represents 10% estimated sequence divergence.

GaBI27 was heterologously expressed in *E. coli* cells JM109.

Test of new probe on clone-FISH sample and enrichment

Before FA series were performed, the specific probe GaBI30 was used for FISH with *E. coli* expressing 16S rRNA genes of *Thermaerobacter* spp. and with the Garga enrichment sample. The aim was to test if the newly designed probe hybridizes to *Thermaerobacter* 16S rRNA, therefore an FA concentration of only 10% was used. For the clone-FISH sample, probes Gam42a/HGC and EUBmix/GaBI830 (Tab. 2.13) were used as described in 2.22.2. Since *Thermaerobacter marianensis* is a high-G+C-content bacterium (Takai et al., 1999), a probe that targets high-G+C-content bacteria (HGC) was used. All *E. coli* cells revealed a signal with the probe specific for γ-*Proteobacteria*, but no signal was obtained with the HGC probe. The negative control did not show any unspecific signals. EUBmix showed signals for all cells, while intensity of the signal varied for GaBI830.

Additionally, FISH was performed with the PFA- and EtOH fixed enrichment sample. HGC/EUBmix probe and EUBmix/GaBI30 probe were used for FISH analysis. Generally, the signals were stronger when PFA-fixed cells were used. When the Fluos-labeled EUBmix probe was used, most of the rods showed weak FISH-signals. These rods revealed also signals when using Cy3-labeled HGC probe. After the EUBmix/GaBI830 hybridization, EUBmix signal was only rarely detectable and no signal was obtained using the *Thermaerobacter* spp. specific probe.

FA series

For the newly designed probe, FA-series from 0% to 70% were performed to evaluate the best FA concentration for FISH-hybridization. After this, a FA concentration of 35% was found to provide the optimal stringency (Fig. 3.11).

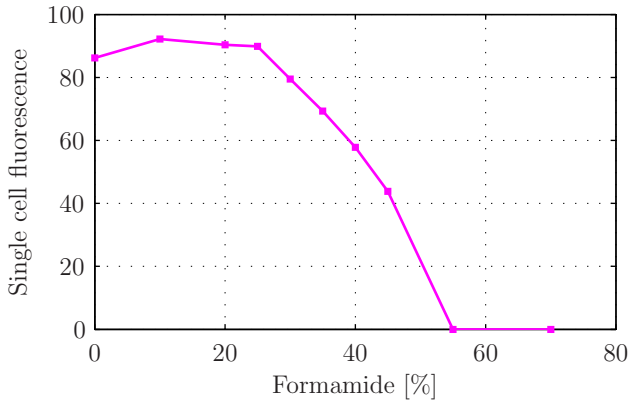

Figure 3.11: Dissociaton profile of *Thermaerobacter*-specific probe GaBI830 under increasing FA concentrations. The mean fluorescence intensity of at least 100 cells was determined for each data point.

c) FISH with the enrichment sample

FISH was carried out with cells from the enrichment that had been fixed with EtOH and PFA immediately after cultures were started using probes EUBmix/GaBI30. Additionally, a younger (diluted) culture, which was not very active, was used to test the specific probe for *Thermaerobacter* spp. In PFA- as well as EtOH-fixed samples of the original culture a lot of cell could be identified as bacteria (using EUBmix). Among them, aggregates of *Thermaerobacter* spp. could be detected. In the diluted culture a lot of bacteria were present, but no *Thermaerobacter* spp. were detectable.

In an attempt to identify and quantify *Thermaerobacter*-related species, probes GaBI30, RHG1130 (specific for putative AOB in the enrichment) and EUBmix were used. Therefore, a culture was used on which metagenomic analysis had been carried out (Hatzenpichler, unpublished). Sybr green staining (see 2.21.3) showed that cocci

(AOA) were very abundant. For FISH, combinations of following probes were used: GaBI830/EUBmix, RHG1130/EUBmix and RHG1130/GaBI830. Some rods could be identified as *Thermaerobacter*-related species, but only weak signals were obtained. Putative AOB revealed brighter FISH signals and were more abundant in the sample than *Thermaerobacter*-related species. Finally, bacteria were either identified as *Thermaerobacter* or putative AOB and no other contaminants were observed.

3.2 Enrichment containing a putative AOB from the Garga hot spring

3.2.1 FISH

To quantify putative AOB and contaminants in the enrichment, a sample (which was taken 24 h after starting the incubation) was investigated with FISH using probe RHG1130 specific for the putative AOB combined with EUBmix and DAPI staining. A lot of cells were detectable with DAPI and one dominant morphological type, short rods, was observed. A very minor population was probable represented by cocci, but they were not easily distinguishable from the short rods. Nearly all of the cells hybridized to the EUBmix probe. All short rods showed a FISH signal with the specific probe RHG1130 and thus were identified as the putative AOB. To test if the cocci might represent "*Candidatus* Nitrososphaera gargensis", CARD-FISH with EUBmix and probe RHGA702, specific for the archaeon, was performed. After DAPI staining, some cocci could be detected, which were present in clusters and did not give a signal with the EUBmix probe. When using probe RHGA702, bright FISH signals were detected only for very densely packed clusters of cocci. In contrast, in loosely packed aggregates the FISH signal was weak or not detectable.

3.2.2 Raman spectroscopic analysis

In order to show ammonia-oxidizing activity of the putative AOB, they were cultured in ammonia oxidizer medium with ^{12}C or ^{13}C bicarbonate as sole carbon source and supplemented with ammonia as energy source (performed by Elena Lebedeva). For Raman analysis, samples incubated for 72 h were used. Confocal Raman microscopy produced accurate and highly reproducible Raman vibrational spectra of microorganisms. Key peaks determined from the literature for major cellular constituents such as proteins, lipids, nucleic acids and carbohydrates were present in each Raman spectrum

of a single cell.

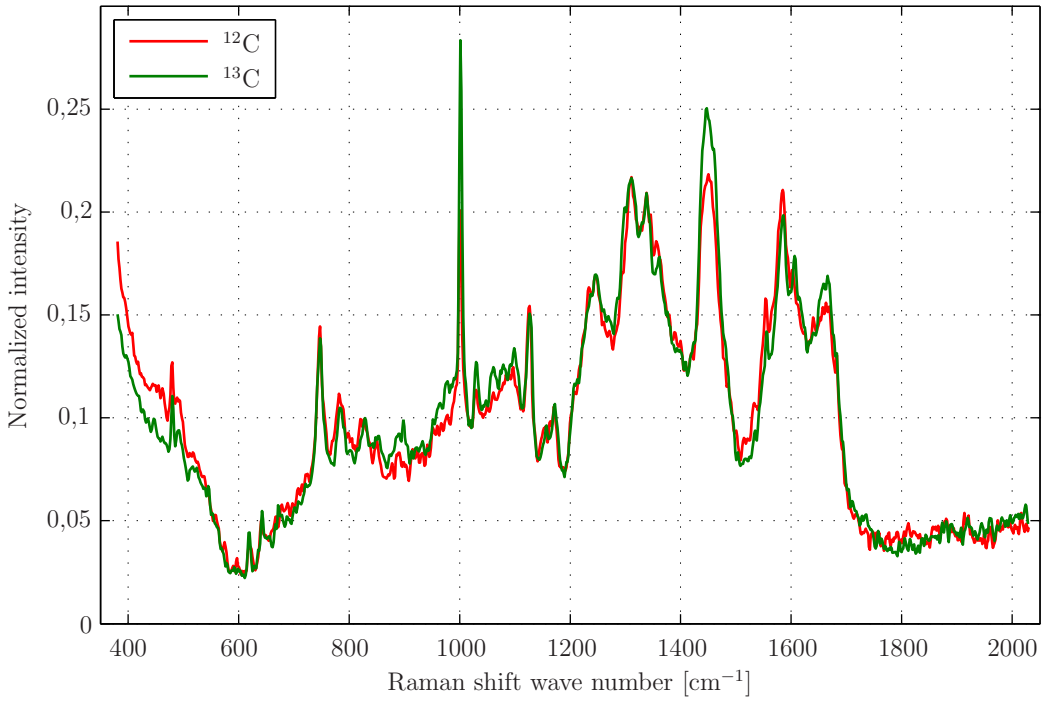

Figure 3.12: Raman mean spectra obtained for PFA-fixed putative AOB grown in media containing either ^{12}C- or ^{13}C-labeled bicarbonate as sole carbon source. For ^{12}C- and ^{13}C-incubations, 32 and 30 cells were analyzed and their spectra averaged, respectively. Spectra were acquired over 60 s for an individual cell and baseline was corrected, normalized and smoothed. The strong resonance peak at 1001 cm^{-1} shows phenylalanine and one pronounced cytochrome c peak at 747 cm^{-1}. Assignment of spectral regions to compound classes was performed according to previously published data (Naumann et al. 2001, Huang et al. 2004, Pätzold et al. 2008).

Comparing the mean spectra from cultures grown on ^{12}C or ^{13}C bicarbonate (denoted as ^{12}C and ^{13}C, respectively) did not reveal any red-shifts in characteristic regions of the spectrum (Fig. 3.12). The characteristic peak of the amino acid phenylalanine at 1001 cm^{-1} was analyzed in detail by calculating the red shift ratio (RSR). The RSR of each ^{12}C and ^{13}C spectrum was calculated and plotted on a diagram.

The RSR of the ^{13}C spectra fell, with one exception, within the range of all ^{12}C spectra, which showed that cells were not labeled sufficiently for detection by Raman microspectroscopy (Fig. 3.13). In 2007, Huang et al. demonstrated a linear relationship between RSR and ^{13}C content in bacterial cells. If the upper RSR of ^{12}C is considered as outlier, four 13 spectra with RSR between 0.63 and 0.74 would be above ^{12}C RSR values and indicative of around 15-30% labeling, according to the study of Huang et al. (2007). The average RSR of ^{12}C spectra was <0.5, which indicated that cells were not

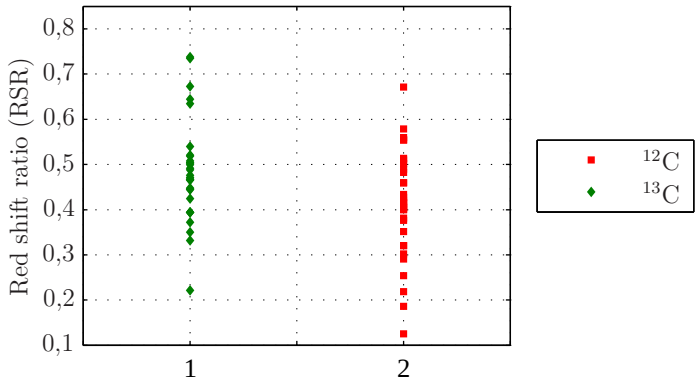

Figure 3.13: Red shift ratios (RSR) of the phenylalanine peak of ^{13}C and ^{12}C spectra of putative AOB.

labeled.

3.3 Enrichment of nitrite-oxidizing microorganisms from soil and sludge

3.3.1 Enrichment 1: soil

3.3.1.1 Determination of cell numbers in inoculum

During the cell separation procedure (2.6.1.2) samples were taken between the centrifugation steps to check for cell loss and cell density. Samples were stained with Sybr green (2.21.3) and examined under the microscope. First, three centrifugation steps at low speed were performed to remove big particles. After the first centrifugation, biomass (pellet 1) contained only some cells and after the second centrifugation step, the biomass (pellet 2) consisted of a low amount of cells which formed aggregates. After the third centrifugation step the pellet (pellet 3) contained more cells. Then, samples were centrifuged two times at high speed. This time the supernatant was controlled for cell loss. After the first high speed centrifugation, a lot of cells were detected in the supernatant (SN 4). After another centrifugation, the supernatant (SN 5) contained a high number of mainly small cells, indicating that during the separation procedure loss of biomass occurred. The enriched biomass sample, which was used as inoculum, contained a lot of cells and some smaller particles which were probably sediment. There were no aggregates and filaments visible and all cells had the same morphology.

3.3.1.2 Control of NH_4^+, NO_2^- and NO_3^-

During the incubations, NH_4^+ concentration was determined photometrically and NO_2^- and NO_3^- concentrations were estimated by using test stripes (Merck KGaA) (see 2.8). In Table 3.5 the values at the different time points are listed. NH_4^+ and NO_2^- concentrations at day 0 differed from the intended starting concentrations. Since the photometrical measurement of NH_4^+ should be accurate, for cultures supplemented with NH_4^+ probably a wrong working solution had been prepared. However, since knowing the exact NH_4^+ concentration at the start of the incubations in order to observe NH_4^+ decrease was more important than obtaining the wanted concentration, this error was not corrected. For measurement of NO_2^- concentrations test stripes were used which are not as accurate as the photometric determination of NO_2^- levels. Thus, the NO_2^- concentration should have been measured again photometrically. On the other hand, the aim was not to know the exact NO_2^- level but to test for NO_2^- decrease. Therefore, no effort was made to determine NO_2^- concentrations by a more accurate method or to obtain the originally wanted starting levels of NO_2^-.

After 28 days, NH_4^+ was only slightly decreased in cultures supplemented with NH_4^+, whereas NO_2^- could not be detected at any time point. In cultures supplemented with NO_2^-, nitrite concentrations did not decrease until day 28, where no nitrite could be measured.

Table 3.5: NH_4^+, NO_2^- and NO_3^- concentrations measured in soil incubations.

Start: day 0				Day 14			Day 28	
	NH_4^+ µM	NO_2^- µM	NO_3^- µM	NH_4^+ µM	NO_2^- µM	NO_3^- µM	NO_2^- µM	NO_3^- µM
NH_4^+ control	310	0	0	330	0	0	0	0
NH_4^+ 75 µM	120	0	0	50	0	0	0	0
NH_4^+ 750 µM	380	0	0	300	0	0	0	0
NO_2^- control	0	1700	1600-4000	10	1700	4000-8000	1700	1600-4000
NO_2^- 75 µM	80	40-100	160	60	100-200	160	0	0
NO_2^- 750 µM	80	1700	1600-4000	50	1700	4000-8000	0	0

	Day 29 NH_4^+ µM
NH_4^+ control	490
NH_4^+ 75 µM	60
NH_4^+ 750 µM	120
NO_2^- control	30
NO_2^- 75 µM	70
NO_2^- 750 µM	70

As a rough estimate of cell density OD_{600} was regularly measured with a photometer (Biorad). No evidence for increased cell density was obtained.

3.3.1.3 Detection of cells in inoculum and incubations using FISH and CARD-FISH

EUBmix, Arch915 and Cren512 probes were used for FISH in a PFA-fixed sample that was used as inoculum for enrichment of nitrite oxidizers from soil (Tab. 3.6). After each hybridization DAPI staining was performed. There were no aggregates visible but a lot of cocci could be detected with EUBmix.

The different incubations were screened via FISH to test for enrichment of bacteria and archaea (Tab. 3.7).

After 29 days of incubation (Tab. 3.8) cultures were overgrown by fungal hyphae. In order to remove them, cultures were filtered, but after some days the filtered incubations became overgrown again and the cultures were discarded.

Table 3.6: CARD-FISH performed with soil inoculum. Samples were treated either with lysozyme (Lys) or proteinase K (PK) in order to test for the best permeabilization method.

CARD-FISH inoculum: day 0	
	FISH signal
EUBmix (Lys 15')	small single cells, some cocci, little rods; low signal intensity
EUBmix Lys 30'	more cells detected than Lys 15', coccoid, irregular; stronger signal
EUBmix Lys 60'	similar to Lys 30'
EUBmix PK 10'	a lot of small cocci (not all DAPI stained cells); the strongest signal (as compared to Lys 15', 30' and 60')
Arch915 (Lys 15')	no signal
Arch915 Lys 30'	no signal
Arch915 Lys 60'	no signal
Arch915 PK 10'	no signal
Arch915 PK 5'	no signal
Cren512 PK 10'	no signal
Cren512 PK 5'	no signal

Table 3.7: CARD-FISH with soil enrichments after 15 days of incubation. PK, proteinase K

CARD-FISH: day 15	
	Arch915 PK 10'
NH_4^+ control	few cells; no signal
NH_4^+ 75 µM	few cells, rarely big dividing cells, bacterial ghosts? lysed cells?; no clear signal
NH_4^+ 750 µM	few cells: small cocci, big cells, bacterial ghosts? lysed cells?; signal for big cells
NO_2^- control	few cells, some small cocci, big cells; signal for big cells
NO_2^- 75 µM	more cells: rods, filaments, a lot of sediment; signal for rods and in cell cluster
NO_2^- 750 µM	few cells; signal for some small cocci
Inoculum	signal for big dividing cell
	general observation: big dividing cells in some cultures

Table 3.8: FISH and CARD-FISH with soil enrichments after 29 days of incubation. PK, proteinase K

FISH: day 29	
	EUBmix(Fl.)/Arch915(Cy3)
NH_4^+ control	low number of cells; some EUBmix signal, probably some Arch signal
NH_4^+ 75 µM	a lot of hyphae (fungi?); no signal
NH_4^+ 750 µM	a lot of cells; no signal (some small cells maybe EUBmix positive)
NO_2^- 75 µM	a lot of hyphae, some small cells; maybe some EUBmix and Arch signal
NO_2^- 750 µM	a lot of hyphae; rarely EUBmix signal, some Arch signal
	Bet42a/Fl.)/Gam42a(Cy3)
NH_4^+ control	low number of cells; no signal
NH_4^+ 75 µM	a lot of hyphae; no signal
NH_4^+ 750 µM	some cells, no hyphae; no signal
NO_2^- 75 µM	fungal hyphae
NO_2^- 750 µM	a lot of cells; no signal
	EUBmix(Fl.)/EUK(Cy3)
	SYBR green in CF staining
NO_2^- 750 µM	EUK signal for hyphae
CARD-FISH: day 29	
	EUBmix PK 10'
NH_4^+ control	some single cells, weak DAPI staining, some big dividing cells strong DAPI staining; some small cells signal
NH_4^+ 75 µM	a lot of hyphae, some single cells (rods); no signal
NH_4^+ 750 µM	a lot of single cells, rods dominant; no signal
NO_2^- control	some single cells; some signal, big dividing cell: signal
NO_2^- 75 µM	some hyphae, some cells, sediment; no signal
NO_2^- 750 µM	some hyphae, some cells, sediment; no signal
	Arch PK 10'
NH_4^+ control	some single cells; rarely signal
NH_4^+ 75 µM	some cells, low number of hyphae; no signal
NH_4^+ 750 µM	a lot of cells (rods), strong DAPI staining; (nearly) no signal
NO_2^- control	some cells, some clusters, some big dividing cells; some signal
NO_2^- 75 µM	some cells, sediment; no signal, signal in cluster
NO_2^- 750 µM	rods, filaments; clusters; signal in clusters

3.3.2 Enrichment 2: soil and sludge

3.3.2.1 Control of NH_4^+, NO_2^-, NO_3^- and pH

Substrate concentrations were controlled in weekly intervals by photometric methods (NH_4^+, NO_2^-) and via test stripes (NO_3^-; Merck KGaA). Additionally, pH was controlled (∼5.5 in each culture at both days) using test stripes (Macherey-Nagel GmbH & Co; see Tab. 3.9).

Table 3.9: NH_4^+, NO_2^- and NO_3^- concentrations measured in soil/sludge incubations. Data are rounded.

	Day 8			Day 14		
	NH_4^+ µM	NO_2^- µM	NO_3^-- µM	NH_4^+ µM	NO_2^- µM	NO_3^-- µM
soil 5 µM NH_4^+, NO_2^-	5	10	0	10	0	0
soil 50 µM NH_4^+, NO_2^-	20	50	160	0	0	0
soil 500 µM NH_4^+, NO_2^-	0	470	1600	0	480	1600
soil control 500 µM NH_4^+, NO_2^-	10	530	1600	0	540	1600
sludge 5 µM NH_4^+, NO_2^-	15	10	0	0	10	0
sludge 50 µM NH_4^+, NO_2^-	20	60	160	0	60	160
sludge 500 µM NH_4^+, NO_2^-	10	500	1600	10	480	1600
sludge control 500 µM NH_4^+, NO_2^-	10	500	1600	0	540	1600

3.3.2.2 Detection of cells in inoculum and incubations using FISH and CARD-FISH

EUBmix and Arch915 probes were used for identification of PFA-fixed cells that were used as inoculum for enrichment of NOM from soil and sludge. Additionally, samples were stained with Sybr green and DAPI (Tab. 3.10).

Table 3.10: FISH and CARD-FISH performed with soil and sludge. The same samples were used as inoculum for cultures. For CARD-FISH samples were treated either with lysozyme (Lys) or proteinase K (PK).

FISH: inoculum		
	sludge	**soil**
SYBR green	a lot of cells: short rods, some long rods, no clusters, sludge particles with a lot of single cells	a lot of single cells, filaments
DAPI	high number of cells	high number of cells
EUBmix	some cells in clusters (sediment), some filaments; weak signal	(nearly) no signal
Arch915	signal	rarely signal (more than EUBmix)
NonCy3, NONEUB Fluos	no signal, some cells with autofluorescence (thick cocci)	no signal
CARD-FISH: inoculum		
	sludge	**soil**
Arch915 PK 10'	high number of sludge particles and rods; in and around particles a lot of signal (DAPI: much more signal)	sediment, little AF; very rarely signal
EUBmix Lys 1h	a lot of signal (nearly all DAPI stained cells)	some cells signal, compared to DAPI ∼10%
EUBmix PK 10'	a lot of signal (not all DAPI stained cells), more signal with Lys	less signal than with Lys

After 14 days putative fungal hyphae were visible in the cultures. In order to test for fungal contamination, FISH was carried out using probe EUK combined with EUBmix (Tab. 3.11). Since hyphae revealed a strong signal with the EUK probe, incubations were stopped.

Table 3.11: FISH with soil and sludge enrichments after 14 days of incubation.

FISH: day 14	
	EUBmix(Fl.)/EUK(Cy3)
Soil 5 µM NH_4^+, NO_2^-	single cells, cluster; some single cells EUBmix signal, a lot of hyphae EUK signal
Soil 50 µM NH_4^+, NO_2^-	cell cluster EUBmix signal, some hyphae EUK signal
Sludge 5 µM NH_4^+, NO_2^-	single cells in flocs EUBmix signal, hypahe EUK signal
Sludge 50 µM NH_4^+, NO_2^-	hyphae with EUK signal

3.4 Enrichment from biofilm growing on aquarium filters

3.4.1 Preparatory work

3.4.1.1 FISH with biofilm used as inoculum for enrichment of NOM

To screen the composition of the biofilm, FISH was performed with PFA- and EtOH-fixed cells, using probes EUBI, EUBII, EUBIII, EUBmix, Alf968, Bet42a, Gam42a, Nso1225, Ntspa712; Ntspn693, Ntcoc84, Nit3, Pla46, Arch915, EUK and NonEUB combined with Nonsense under the conditions described in 2.21.2. Description of the sample and observed FISH signals are summarized in Table 3.12. DAPI staining showed the presence of a high number of single cells and cells grown in clusters, among them rods and cocci, but also filaments. A majority of DAPI-stained cells were detected by EUBmix. In general, a much better FISH signal was obtained with Cy3-labeled probes than with Fluos-labeled probes. Overall, signals were rarely detectable for single cells (except with EUBmix), and cells in clusters often showed a better FISH-signal. For rods located in clusters signals were obtained with probes Bet42a, Gam42a, Nso1225, Ntspn693, Ntcoc84 and Nit3. Strong signal and different cell morphologies were detected when using Alf968. In many cases, the nonsense probe showed a lot of signal, mainly for rods in clusters, making it possible that FISH signals using other probes were unspecific. Signals for some cocci in clusters were detected using probe Ntspa712 and Pla46. Archaea were not or only rarely detected using FISH. Thin filaments which were present in the biofilm did not reveal any signal using EUK probe.

3.4.1.2 DNA extraction and PCR with biofilm inoculum

After DNA extraction with the Ultra Clean Soil DNA kit (2.11.1.1), the DNA concentration was 15 ng/µl for the sample. 2.4 ng/µl DNA were measured in the negative

Table 3.12: Description of community composition of aquarium water biofilm revealed after FISH and DAPI staining with PFA- and EtOH-fixed samples. Probes were Cy3-labeled and used in combination with EUBmix (Fluos), if not stated otherwise. Fl., Fluos

	sample: biofilm	
	PFA	**EtOH**
EUBI	single cells and clusters: signal; Fluos weak signal; not all DAPI-stainded cells with signal	in clusters lot of signal, weaker signal with Fluos
EUBII	signal in clusters: cocci	single rods and in clusters: signal
EUBIII	signal in clusters: rods	signal in clusters: rods
EUBmix	single cells and clusters: signal; Fluos weak signal; not all DAPI-stainded cells with signal	in clusters a lot of signal; signal better with Cy3
Alf968	no single cells with signal, cluster: a lot of rods with signal, single cells weak/no EUBmix signal	many clusters with signal: rods, cocci; EUBmix signal in aggregates
Alf968(Fl.)/EUBmix(Cy3)	weak or no Alf signal; many cells EUBmix signal	no Alf signal
Bet42a	no single cells with signal; in clusters: rods with (unspecific?) signal	some single rods with signal thick filaments: EUBmix signal
Bet42a(Fl.)/EUBmix(Cy3)	no signal; a lot of EUBmix in clusters	weak signal, some rods signal
Gam42a	short rods in clusters with signal	some single rods with signal; clusters: rods with signal
Gam42a(Fl.)/EUBmix(Cy3)	some rods in cluster with signal; unspecific signal in clusters	weak signals, not clear if cells
Nso1225	in clusters some rods with signal	in clusters some rods with signal
Ntspa712	in clusters signal for cocci (or AF?)	no description
Ntspn693	a lot of rods with signal, mainly in clusters	cocci in clusters
Ntcoc84	signal for rods, mainly in clusters	signal for rods, mainly in clusters
Nit3	rods in clusters with signal (unspecific?)	a lot of rods with signal
Pla46	a lot of cocci (attached on filamentous structures) with signal	cocci (cells?) in clusters with signal
Arch915	most single cells no Arch and EUBmix signal; clusters: some rods with signal (unspecific?)	in cluster: cocci with signal
EUK/EUBmix	thin filaments no signal; EUBmix weak	some signals (some thick filaments, tetrads), no signal for long filaments
Nonsense/NONEUB	a lot of Cy3 signal in aggregates, often rods; signal similar with Ntcoc84 , Ntspn693 probe	a lot of autofluorescence (Cy3); in clusters: (unspecific) signal for rods, not as much as with Alf968

control. These concentrations were used undiluted for PCR. For amplification of bacterial and archaeal 16S rRNA gene fragments, primers 616V/630R and 21V/1492R were used under the conditions described in Table 2.7, respectively. Bacterial 16S rRNA amplicons had a length of 1535 bp, whereas archaeal 16S rRNA amplicons were 1503 bp long. Both bacterial and archaeal *amoA* gene fragments were amplified (see Tab. 2.9) when using the primers amoA1F/amoA2R and ArchamoAV/ArchamoAF (Tab. 2.10), respectively, and resulting in an amplicon length of 490 bp for bacterial *amoA* and of 595 bp for archaeal *amoA*. To amplify parts of the *nxrB* gene (see Tab. 2.12), primers nxrBF169/nxrBF638 were used (Tab. 2.11), resulting in an amplicon length of 485 bp.

3.4.1.3 pre-incubations: NH_4^+, NO_2^- and NO_3^- measurements

In pre-incubations, substrate concentrations were measured (see Tab. 3.13) to test for nitrifying activity. In some of the cultures supplemented with NH_4^+ and NO_2^-, a lot of NH_3 and NO_2^- was consumed after some days, respectively. Substrates concentrations

differed significantly between cultures without and with antibiotics.

Table 3.13: NH_4^+, NO_2^- and NO_3^- measured during pre-incubations with biofilm from "Haus des Meeres". As starting concentration around 500 µM of NH_4^+ and NO_2^- were added.

	start		**after 4 days**		**after 5 days**	
	NH_4^+ µM	NO_2^- µM	NH_4^+ µM	NO_2^- µM	NO_2^- µM	NO_3^- µM
NH_4^+ aquarium water only			431	2	1	
NH_4^+ bf	453	4	35	8	4	400
NH_4^+ AB bf			42	343	385	
NH_4^+ AB crushed bf			286	53	70	
NH_4^+ filtrate			438	0	1	
NO_2^- aquarium water only			25	655	501	
NO_2^- bf	46	508	25	6	310	400
NO_2^- AB bf			39	265	254	
NO_2^- AB crushed bf			37	309	283	
NO_2^- filtrate			56	514	482	
NO_2^- balls			31	16	61	

3.4.2 Main incubations

3.4.2.1 Control of NH_4^+, NO_2^-, NO_3^- concentration and pH

NH_4^+, NO_2^- concentration and pH were regularly measured whereas NO_3^- was only rarely tested. Culture dilutions (1:10) were performed approximately every two to three months. In the following chapters, "original culture"refers to the initial cultures that were started, while "diluted culture"refers to cultures that originate from one or more dilutions of the initial culture. Cultures were prepared on 23.10. (referred to as original cultures). On 26.11., 26.01., 17.04. and 22.07. 1:10 dilutions were performed.

a) Observations with incubations supplemented with NH_4^+

In the original cultures, NH_4^+ was nearly completely consumed in approximately 4 days. Results, if not stated otherwise, were consistent between duplicates. After the first dilution, NH_4^+ was consumed slowly, and in later cultures, in which only Nessler's reagent was used to test for presence or absence of NH_4^+ (see 2.8.1.2), NH_4^+ started to accumulate. In the original and diluted NH_4^+ cultures without antibiotics (NH_4^+ bf I, II), NO_2^- concentrations always remained very low and most of the time no NO_2^- could be detected. In the two original cultures supplemented with antibiotics (NH_4^+ AB bf I, II), NO_2^- concentrations slowly increased over time and reached a concentration of 3-4 mM after three months. This was not observed in diluted cultures supplemented with antibiotics. NO_2^- also accumulated in the culture which was supplemented with

NH_4^+ and only contained filtered aquarium water as medium. NO_3^- was constantly measured only in the original cultures. In the cultures with no antibiotics added, NO_3^- concentrations did not change significantly over time, whereas in the cultures containing antibiotics and in the NH_4^+ control culture, NO_3^- concentration increased up to 8 mM. pH decreased in all cultures over time, but more dramatically in cultures to which no antibiotics were added, and was readjusted regularly.

b) Observations with incubations supplemented with NO_2^-

In the original cultures, NO_2^- accumulated in the cultures to which antibiotic had been added. The microorganisms in cultures without antibiotic completely oxidized NO_2^- in the first week. Then, NO_2^- started to accumulate. If it is not stated otherwise, results were consistent between duplicates. After the first dilution was performed, NO_2^- was oxidized in all four NO_2^- cultures after some weeks. After this, in all cultures and following dilutions that were performed, NO_2^- was consumed in approximately seven days (NO_2^- concentrations were always measured after 7-10 days, after which NO_2^- could never be detected using test stripes). The NO_2^- concentration in the aquarium water control was stable for most of the time. From the start of the incubations on, NO_3^- concentrations in all four NO_2^- cultures were very high (0.8 to 8 mM) and changed over time. The NO_3^- concentration in the aquarium water control was constant. NH_4^+ concentrations were measured only in original cultures and cultures obtained after the first dilution. They remained low (between 0 and 0.5 mM) and alternately increased or decreased. pH was relatively stable and was readjusted if necessary in cultures without antibiotics. For a detailed list of all concentrations measured and observations recorded over a 10 month period see the accordant sheet (substrate concentrations and pH of the "Haus des Meeres"enrichments) on the attached CD.

3.4.2.2 Detection of cells using FISH and CARD-FISH

For FISH experiments, the following probes and combination of these probes were used at the conditions described in 2.21.2 and 2.22: EUBmix, Arch915, Alf698, Bet42a, Gam42a, Nso1225, Nscoc1248, Ntspa712, Ntspa662, Ntspa1431, Ntspa1151, Ntcoc84, Ntspn693, Nit3, FGall178a, Ntoga221 and Nonsense (Cy3) combined with NonEUB probe (Fluos).

a) NH_4^+ incubations

In the two cultures without antibiotic, high numbers of cocci and rods could be detected with DAPI. They were either single cells or formed differently sized clusters embedded in exopolymeric substance. Nearly all of the DAPI-stained cells showed signals with

the EUBmix probe. Arch915 did not reveal any signal when using standard FISH protocol, whereas with CARD-FISH a very small amount of cells showed a specific signal. When using probe Gam42a, signals were obtained for a large proportion of rods, but no signals were observed using Nscoc1248. Many small cocci showed signals when using probe Ntspa712, but no signal could be observed when using genus specific probe Ntspa662, and sublineage specific probes Ntspa1431 and Ntspa1151. In cultures NH_4^+ bf II, a signal with Nso1225 was observed only in one small aggregate.

In one original culture supplemented with antibiotic, a lot of cells could be detected with DAPI staining, whereas in the diluted cultures only a low number of cells could be seen. Some of them showed FISH signals using EUBmix, but no archaea could be detected using FISH and CARD-FISH. Many cells were detected using Gam42a, and one big aggregate in one NH_4^+ bf I culture contained some rods which hybridized to probe Nso1225.

In summary, no FISH signals were observed with probes specific for ammonia and nitrite oxidizers. Signals were only obtained using EUBmix, Gam42a and Ntspa712, although FISH was performed several times and PFA-fixed cells from different cultures and different amounts of biomass were used.

b) NO_2^- incubations

In cultures without antibiotic, a lot of cells were present as revealed by DAPI staining. Most of the cells showed signals using EUBmix probe. Using CARD-FISH, archaea were not or only rarely detectable. γ-*Proteobacteria* were observed mainly in clusters. In both original duplicates, signals were obtained for thick cocci located in clusters using probe Ntcoc84 as well as probe Gam42a. In all NO_2^- bf II cultures cells identified as *Nitrococcus* were dominant and densely packed in small and large clusters but also present as single cells. In contrast, in culture NO_2^- bf I *Nitrococcus* microcolonies became less abundant after some dilutions or could not be detected any more. In Fig. 3.14 *Nitrococcus* growing as single cells and in clusters is depicted.

In one NO_2^- bf I culture, a very low number of *Nitrospira* (using probe Ntspa662) could be observed in one big cluster, but signals were bleaching fast. No enriched population of NOB was detected with other probes targeting known nitrite oxidizers. Thus, more general probes were tested. Using Alf968, a lot of cells, mainly rods which were present as single cells and in clusters, showed FISH signals and probe Pla46 revealed many cells to be members of the *Planctomycetes*.

In NO_2^- cultures supplemented with antibiotic, a lot of cells were present but nearly all of the detected single cells and clusters showed FISH signals with EUBmix. Signals

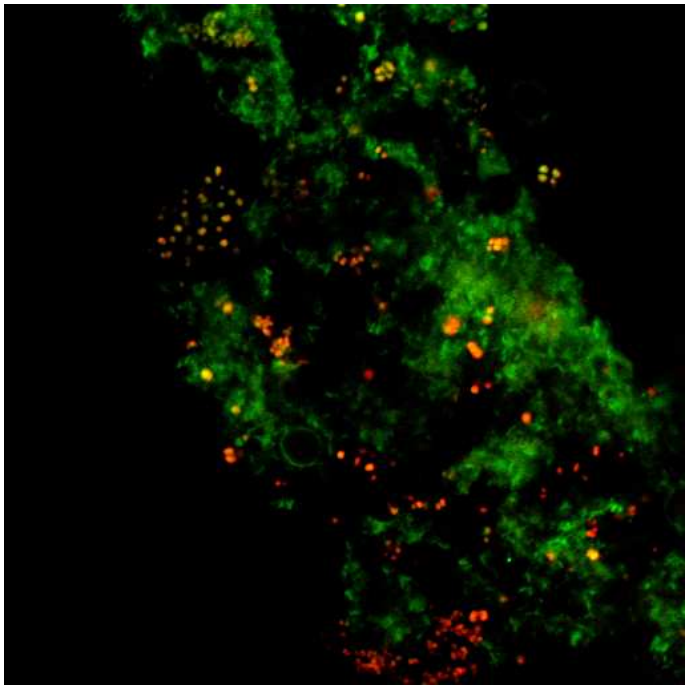

Figure 3.14: *In situ* analysis of *Nitrococcus* within biofilms of enrichment NO_2^- bf II. The FISH picture shows *Nitrococcus* as single cells and in microcolonies (detected by probe Ntcoc84). Green color results from binding of probe-mix EUBmix to bacterial cells. *Nitrococcus* cells appear yellow, due to simultaneous binding of EUBmix (Fluos) and Ntcoc84 (Cy3) probe.

could be seen using Ntspa712, but no signals were obtained using more specific probes Ntspa662, Ntspa1431 and Ntspa1151. Gam42a showed signals in culture I. At a certain time point original cultures were very highly dominated by cocci (EUBmix), whereas diluted cultures did not contain cocci in such high amounts any more. The cocci did not show FISH signals with known NOB probes or probes Alf698, Bet42a and Pla46.

3.4.2.3 Molecular investigation of the NO_2^- cultures: clone libraries for bacterial 16S rRNA and *nxr* genes

a) Amplification of bacterial 16S rRNA genes, cloning and RFLP analysis, sequencing and BLAST analysis

PCR was performed directly with cells from four active cultures supplemented with NO_2^- (NO_2^- bf I, II, NO_2^- AB bf I, II). For amplification of bacterial 16S rRNA genes primers 616V/630R and a hot start Taq polymerase were used. Bacterial 16S rRNA gene fragments were obtained from all four cultures, having an amplicon length of approximately 1535 bp. 16S rRNA clone libraries were constructed for cultures NO_2^- bf I, II and NO_2^- AB bf I. After cloning, RFLP analysis was performed. Since many

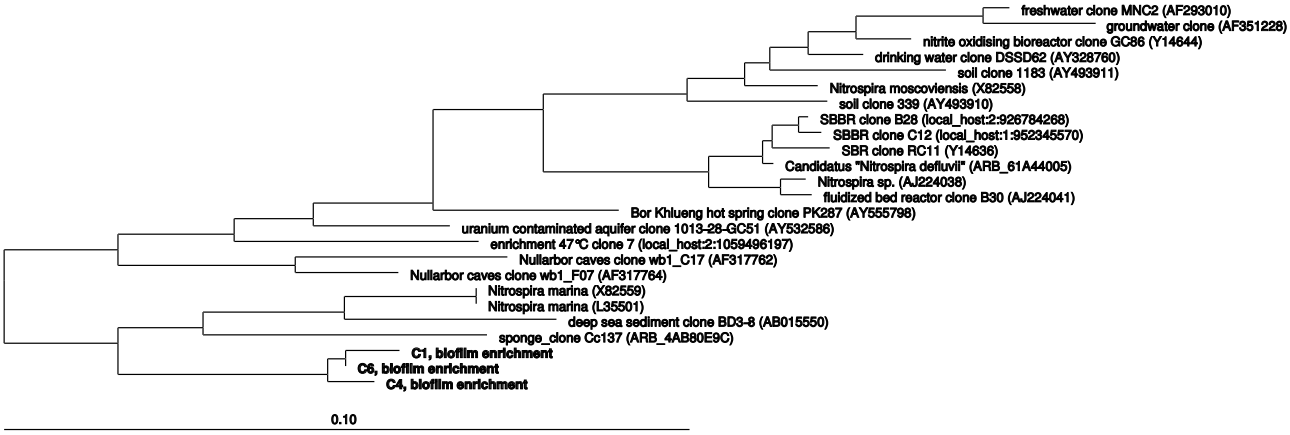

Figure 3.15: Unrooted phylogenetic tree of the phylum *Nitrospira* based on comparative analysis of 16S rRNA sequences, showing the positioning of sequences obtained in this study from biofilm enrichment NO_2^- AB bf I from "Haus des Meeres"(in bold). The tree topology was determined by maximum-likelihood analysis of sequences longer than 1,300 nucleotides. Only positions conserved in at least 50% of all *Nitrospira* were used for tree inference. The scale bar represents 10% estimated sequence divergence.

different patterns were detected, a high number of clones was sequenced. Finally, for culture NO_2^- bf I, culture NO_2^- bf II and culture NO_2^- AB bf I, 20, 31 and 20 16S rRNA sequences were obtained, respectively.

Sequences were compared against online databases using BLAST, which showed that none of the 20 sequences obtained from culture NO_2^- bf I had any similarity to known nitrite oxidizers. Seven sequences were similar to γ-*Proteobacteria* and eight sequences were similar to uncultured α-*Proteobacteria*. Three sequences were identified as uncultured *Chloroflexi*. From 31 sequences from NO_2^- bf II, 18 were closely related to α-*Proteobacteria* and six sequences derived from uncultured members of *Planctomycetes*. For culture NO_2^- AB bf I, 21 16S rRNA sequences were received. Three sequences were closely related to *Nitrospira marina* 16S rRNA genes. Most of the sequences (eight) were similar to uncultured γ-proteobacterial clones and six sequences were closely related to uncultured α-proteobacterial clones. The remaining four sequences were similar to uncultured planctomycetes. The *Nitrospira marina* related sequences (C1, C4 and C6) were further analyzed using ARB and their phylogenetic affiliation determined (Fig. 3.15). The three sequences (C1, C4 and C6) were 97.6-99.2% similar to each other and showed 92.2-93.9% similarity to *Nitrospira marina*.

b) Amplification of *nxrB* genes from the "Haus des Meeres"enrichment, cloning and sequencing

To amplify *Nitrospira nxrB* and *Nitrobacter nxrB* gene fragments, primers nxrBF169/ nxrBR707 and nxrB706/nxrBR1431 were used, respectively (Tab. 2.11). *Nitrospira*

nxrB gene fragments with a length of 485bp were amplified in all four cultures and clone libraries were constructed from cultures NO_2^- bf I, II and NO_2^- AB bf I. Only in culture NO_2^- bf II *Nitrobacter nxrB* fragments with a length of 725 bp were amplified. Finally, for culture NO_2^- bf I seven *nxrB Nitrospira* sequences (Anxr1-7) were obtained. For culture NO_2^- bf II eight *nxrB Nitrobacter* (*Nitrococcus*, BnxrB1,3-9) sequences and three *nxrB Nitrospira* (Bnxr2,3,10) sequences were obtained and for culture NO_2^- AB bf I six *nxrB Nitrospira* sequences (Cnxr1,2,5-8) were received. After analyzing the *nxrB* sequences using ARB, phylogenetic trees were calculated for *Nitrococcus nxrB* (Fig. 3.16) and *Nitrospira nxrB* sequences (Fig. 3.17), respectively. *Nitrococcus nxrB* sequences were 97.4-99.6% similar to each other and had a similarity of 97.4-98.7% to *Nitrococcus mobilis*, but only 61.8-63.2% similarity to *Nitrobacter hamburgensis nxrB* sequences on amino acid level. *Nitrospira nxrB* sequences from culture NO_2^- bf I (Anxr clones) were 97.3-100% similar to each other and had the highest similarity to *Nitrospira marina nxrB* sequences (98.0-98.7%). BnxrB clones (culture NO_2^- bf II) were 98.0-100% similar to each other and between 98.0 and 99.3% similar to *Nitrospira marina nxrB* sequences. Cnxr clones (culture NO_2^- AB Bf I) were 98.7-100% similar to each other and had a similarity of 97.3-98.7% to *Nitrospira marina nxrB* sequences.

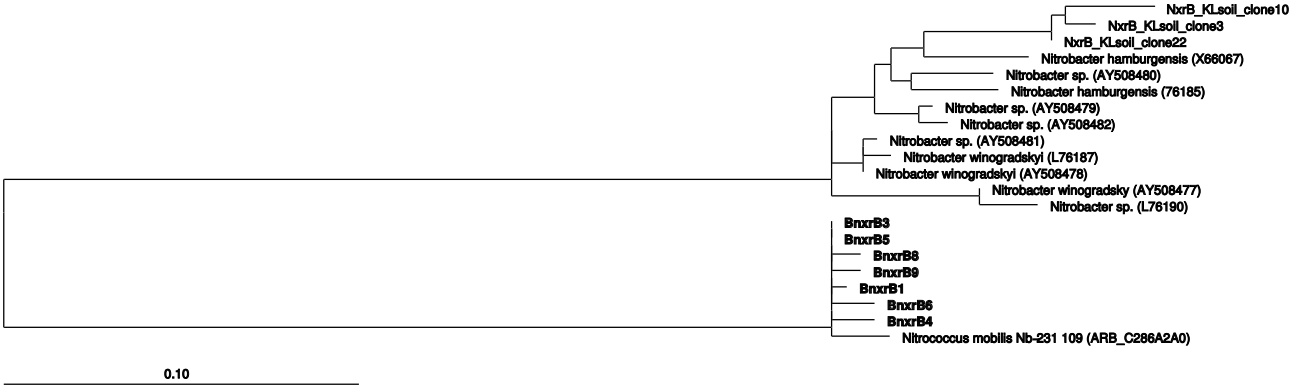

Figure 3.16: Unrooted *NxrB* based phyML tree of *Nitrococcus* and *Nitrobacter*, showing the positioning of sequences obtained from biofilm enrichment NO_2^- bf II using primers nxrBF706/nxrBR1431. Scale bar represents 10% estimated sequence divergence.

3.4.2.4 Amplification of 16S rRNA genes of *Nitrococcus mobilis*, cloning and sequencing

Because no *Nitrococcus* 16S rRNA sequences could be found in the 16S rRNA clone library from culture NO_2^- bf II, a semispecific PCR with primers Ntcoc84F/630R and Ntcoc84F/1492R was carried out to specifically amplify 16S rRNA genes of *Nitrococcus*. As a positive control, PFA-fixed cells of a pure culture of *Nitrococcus mobilis* were used. Primers Ntcoc84F/630R did not yield a product with a pure culture of *Nitrococcus*

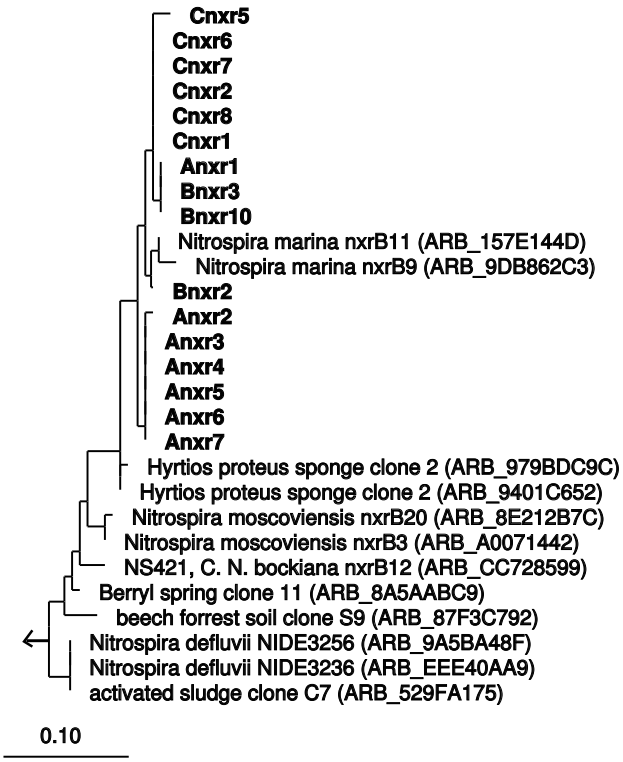

Figure 3.17: *NxrB* based phyML tree of *Nitrospira*, showing the positioning of sequences obtained from biofilm enrichment NO_2^- bf I (Anxr), NO_2^- bf II (Bnxr) and NO_2^- AB bf I (Cnxr) using primers nxrBF169/nxrBR707. The tree was rooted against *Nitrococcus mobilis nxrB*. The scale bar represents 10% estimated sequence divergence.

mobilis. When using primes Ntcoc84F/1492R, amplicons with the expected length of approximately 1408 bp were obtained. After cloning and sequencing, 18 full sequences were obtained. Sequences were imported into ARB and aligned to the deposited 16S rRNA gene of *Nitrococcus mobilis*. All 18 sequences revealed a similarity of 100% to each other. The sequence similarity to the 16S rRNA gene of *Nitrococcus mobilis* was 100%.

3.4.3 Stable Isotope Probing (SIP)-incubations

3.4.3.1 Consumption of NO_2^-

The four NO_2^- cultures incubated each with ^{12}C and ^{13}C bicarbonate became stable within some weeks and after this, constantly consumed the added NO_2^-. The most active culture was NO_2^- bf II, which was fed with approximately 1.5 mM NO_2^- per week after around six weeks of incubation. NO_2^- bf I (the duplicate culture) was not as active as NO_2^- bf II, but oxidized 800 µM NO_2^- per week. The cultures with added antibiotic were less active and consumed roughly 400 µM NO_2^- per week after six

weeks of incubation. NO_3^- concentrations did not change significantly and were in the range between 400 and 800 µM. pH was stable (between 8.4 and 8.6) and never had to be readjusted. For a detailed description of the data see the attached CD ("SIP"). Unfortunately, due to an error in the heating system of the waterbath, cultures were killed and had to be discarded after around 2 months.

3.4.3.2 Investigation of cultures (staining, FISH) and confirmation of activity using Raman-FISH

The composition of cultures supplemented with antibiotic and incubated either with ^{12}C- or ^{13}C-bicarbonate (NO_2^- AB bf I, NO_2^- AB bf II) was analyzed using DAPI staining. In cultures NO_2^- AB bf I ^{13}C or ^{12}C, mostly single cells and only a few clusters were detected and rods were the dominant morphological type. Cultures NO_2^- AB bf II ^{13}C and ^{12}C contained not so many single cells and cells were clustering together. Mainly rods were observed. Cultures without antibiotic (NO_2^- bf I, II) were screened by FISH using probes Ntcoc84/EUBmix and DAPI staining to screen the composition of cultures and the enrichment of *Nitrococcus mobilis*. In culture NO_2^- bf I incubated with ^{13}C or ^{12}C, one morphology was dominating, namely very short single rods. Only few small clusters could be observed, in which rarely some *Nitrococcus* cells were detected. Composition of cultures differed between NO_2^- bf II cultures incubated with ^{12}C and ^{13}C. NO_2^- bf II ^{12}C was highly dominated by short rods, which also could be observed in NO_2^- bf I cultures. Only one cluster containing some *Nitrococci* was observed. The only heterotrophs seemed to be the highly abundant short rods. The ^{13}C culture contained a lot of cells of unknown identity. Most of the unknown bacteria were very short rods, but they were not as abundant as in the ^{12}C incubation and in NO_2^- bf I cultures. Differently sized clusters were present. Each cluster contained colonies and single cells of *Nitrococcus mobilis*, which were embedded in a matrix.

Raman-FISH was performed with culture NO_2^- bf II, in which *Nitrococcus mobilis* was very abundant and the most NO_2^- was consumed. Raman microscopy produced accurate and reproducible Raman vibrational spectra of *Nitrococcus* mobilis. Characteristic peaks determined from the literature for dominant cellular component such as proteins, lipids, nucleic acids and carbohydrates were clearly visible in the spectra of a single cell (Fig. 3.18).

Comparing the ^{12}C- and ^{13}C mean spectra did reveal "red-shifts"in characteristic peaks of the spectra, like cytochrome or phenylalanine. The characteristic peak of phenylalanine was specifically analyzed by calculating the red shift ratio (RSR). The RSR of each ^{12}C spectrum was compared to the RSR of each ^{13}C spectrum and plotted on

a diagram (see Fig. 3.19). Nearly all RSR of the ^{13}C spectra fell above RSR of ^{12}C spectra, indicating that nearly all cells from the ^{13}C experiment were labeled. The average RSR of ^{13}C spectra was 0.72, which indicated labeling of around 25%, according to the study of Huang et al. (2007). The cell that showed the most labeling had a RSR of 0.81 and was labeled ∼40%. The two cells which had a RSR within the range of ^{12}C incubated cells were probably not labeled. All in all, *Nitrococcus mobilis* cells in culture NO_2^- bf II were labeled up to 40% after an incubation of around 6 weeks with stable isotopes.

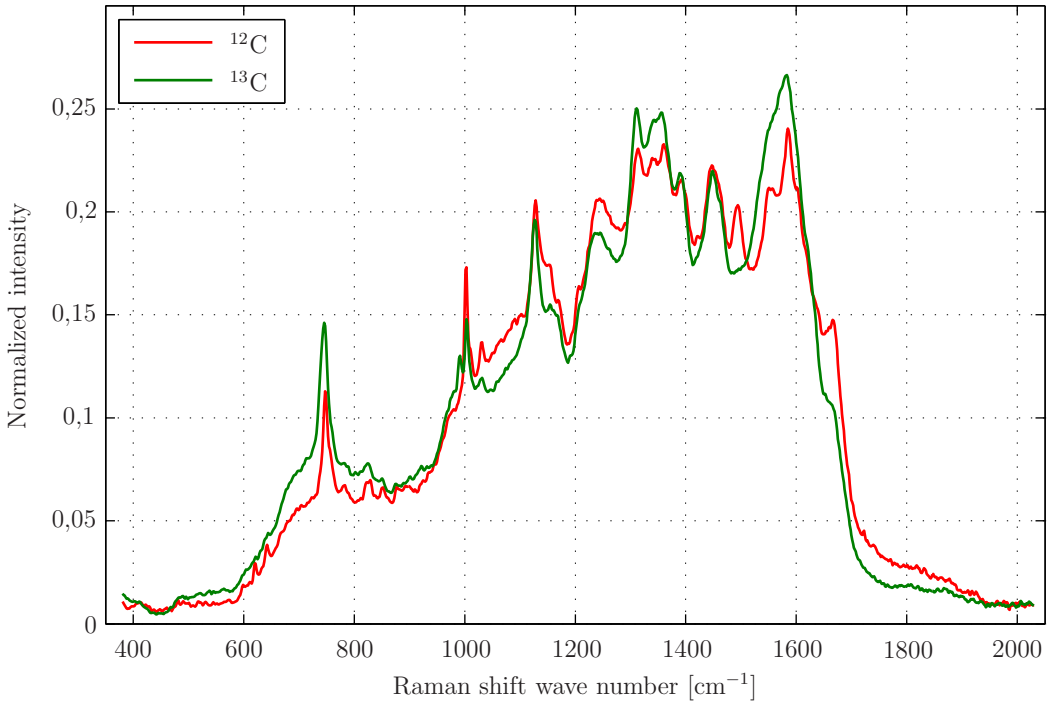

Figure 3.18: Raman mean spectra obtained for PFA-fixed *Nitrococcus mobilis* enrichments grown in aquarium water containing either ^{12}C- or ^{13}C-labeled bicarbonate as sole carbon source. For ^{12}C- and ^{13}C-incubations, 20 and 33 cells were analyzed and their spectra averaged, respectively. Spectra were acquired over 60 s for an individual cell and in each case baseline corrected, normalized and smoothed. The strong resonance peak at 1003 cm^{-1} shows phenylalanine, and at 747 cm^{-1} a pronounced peak of cytochrome c is visible. Assignment of spectral regions to compound classes was performed according to previously published data (Naumann et al. 2001, Huang et al. 2004, Pätzold et al. 2008).

Originally, the aim was to perform Raman microscopic analysis with all active cultures. Since no known NOB (except for *Nitrococcus*) could be identified, the intention was to test for autotrophic growth of cells showing a characteristic morphology. Since all cultures were killed after 2 months, these experiments could not be performed.

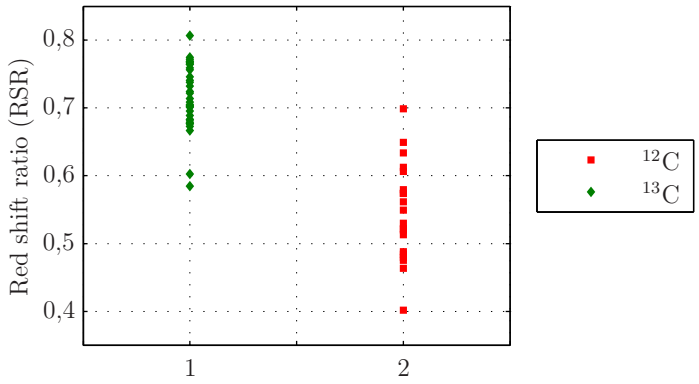

Figure 3.19: Red shift ratios (RSR) of the phenylalanine peak of ^{13}C and ^{12}C spectra.

Chapter 4

Discussion

4.1 "*Candidatus* Nitrososphaera gargensis"enrichment from the Garga hot spring in Russia

4.1.1 "*Candidatus* Nitrososphaera gargensis"

A thermophilic, nitrifying enrichment originating from the Garga hot spring in Russia had been maintained for years under nitrifying conditions at a temperature of 46°C (Lebedeva et al. 2005). In 2006, Hatzenpichler screened for AOM and found that the enrichment consisted of a dominant population of a single archaeon, which was then shown to be an aerobic, chemolithoautotrophic ammonia oxidizer and named "*Candidatus* Nitrososphaera gargensis"(Hatzenpichler et al. 2008), and a minor population of bacterial contaminants. "*Candidatus* Nitrososphaera gargensis"is a moderately thermophilic (46°C optimum of NH_3 oxidation) archaeon affiliated to the I.1b soil cluster and adapted to very low ammonium concentrations (partial inhibition of AOA was observed with 3.08 mM ammonium) (Hatzenpichler et al. 2008).

Background

Bacterial Amo is known to be a multifunctional enzyme which oxidizes methane, carbon monoxide and a range of organic compounds, but these do not provide growth substrates in laboratory culture (Prosser and Nicol 2008). Presently, little is known of the alternative substrates for archaeal Amo but the sequences are only distantly related to bacterial Amo which might have consequences for functional diversity. Additionally, Prosser and Nicol (2008) suggested that potential alternative metabolisms and growth strategies for mesophilic crenarchaeota have to be considered. Taken to-

gether, their abundance may not result only from ammonia oxidation but, for example, from mixotrophic or heterotrophic growth. Therefore, higher crenarchaeal *amoA* gene abundance may not be linked to archaeal ammonia oxidation (Prosser and Nicol 2008). Wuchter et al. (2003) demonstrated the uptake of bicarbonate using marine surface water *in situ*, and that the majority of autotrophically incorporated carbon is found in crenarchaeal isoprenoids. However, Ingalls et al. (2006) showed that at a depth of 670 m in the Pacific Ocean, the 14C inside archaeal membrane lipids could not be explained by incorporation of dissolved inorganic carbon and calculated that 17% of the lipid carbon was derived from heterotrophic consumption of organic carbon. Their data suggest that either the marine archaeal community consists of both autotrophs and heterotrophs or is represented by a single population with a mixotrophic metabolism. Indeed, Ouverney and Fuhrman (2000) found indications for heterotrophy of planktonic archaea, as they demonstrated their uptake of amino acids, but it was not determined whether these archaea were crenarchaeota. Agogué et al. (2008) determined contributions of putative AOA and AOB to the total archaeal and bacterial community in subsurface waters to 4000 m depth in the North Atlantic and could show that most bathypelagic crenarchaeota were not autotrophic ammonia oxidizers. They observed a significant decrease of archaeal *amoA* copies with depth and very low fixation rates of carbon dioxide and concluded therefore a heterotrophic lifestyle of bathypelagic crenarchaeota. However, these studies aimed for marine archaea and their growth strategies. Comparable studies for soil archaea do rarely exist.

In this study, it was determined whether the chemolithoautotrophic AOA "*Candidatus* Nitrososphaera gargensis" has a potential for mixotrophic growth using MAR. Since MAR only gives information about the potential C-source, chemical measurements of NH_4^+ and NO_2^- concentrations before and after the MAR-incubation were performed to test if energy for growth or maintenance derived from ammonia oxidation.

4.1.2 Microautoradiography (MAR)

Biomass, stained with Sybr green, consisted of differently sized microcolonies of "*Candidatus* Nitrososphaera gargensis" and rarely planktonic rods which were bacterial heterotrophic contaminants. The heterotrophic bacteria had morphologies from thick to short rods but also longer filaments could be rarely seen. The only exception was cultures supplemented with amino acid mix, in which colonies could not be detected but only single AOA cells were present.

In general, silver grains accumulated close to and over clusters of the AOA, but often were observed to accumulate only in the middle of clusters or only at specific sites.

No single colony was detected at which silver grains coincided exactly with the AOA microcolonies. Since microcolonies of AOA were very dense, it can not be excluded that heterotrophic bacteria resided within the aggregates and were active. However, FISH did not give any hint for the presence of bacteria inside the AOA microcolonies. Unfortunately, experiments were performed at the wrong pH (pH 9 instead of 7.5), making it obligatory to repeat the experiment. The original sample from the Garga hot spring had a pH of 7.9 (Lebedeva et al. 2005), while the enrichments were maintained at a pH of 7.5. During the experiment it was observed that cells mostly formed aggregates (but single cells before), which were probably caused by this pH change. It is possible that cells on the border of aggregates were not so active compared to cells in the middle of the aggregates, in which they could have been protected from the high pH and thus more active. However, cultures that were incubated at a pH 9 showed no difference in activity to ones at 7.5, which does not substantiate this hypothesis.

A negative control with PFA-fixed biomass supplemented with NH_4^+ and 14C-labeled bicarbonate was performed to test for possible adsorption phenomena and chemography of the 14C-bicarbonate and potential chemical loss of NH_4^+ through evaporation. No negative controls were performed with the other 14C-labeled substrates. Since different substrates might have different adsorption characteristics, a negative control for each substrate is a necessary part of the experiment in order to get conclusive results. Unfortunately, microcolonies from the negative control showed a positive MAR signal, making it difficult to interpret the MAR results from cultures supplemented with bicarbonate. In the discussion it always has to be kept in mind that cells possibly did not incorporate the substrates and that MAR signals were caused by substrate adsorption on the cell surface. Lee et al. (1999) used pasteurized samples as a control for possible adsorption phenomena, since they observed enhanced adsorption with paraformaldehyde-treated biomass that was probably caused by fixative-induced chemical modification of bacterial cell surfaces. Besides, they postulate that once chemical adsorption is observed for a specific substrate, extra washing steps must be used. Thus, it is possible that during the incubation labeled bicarbonate adsorbed on PFA-treated cells of the dead control. On the other hand, it is possible that an old PFA-solution was used. It was stated that in unbuffered solutions (used in this study) the pH might drop and PFA might decompose (product information by Sigma-Aldrich). However, in the dead control NH_4^+ concentration decreased, but no NO_2^- was produced, and since no nitrite oxidizers were present, it is more likely that cells were dead and not active, and that MAR signals were caused by adsorption of substrate onto the biomass.

NH_4^+ decrease is no proof of ammonia oxidation, since ammonia can also evaporate (more pronounced at elevated temperatures), used as nitrogen source (for assimila-

tion) or can be stored intracellularly. However, it must be noticed that accumulation of NH_4^+ inside the cell might, above a certain level, lead to intracellular concentrations that negatively influence the cytoplasmic pH and thus could result in inhibition (Hatzenpichler et al. 2008). Therefore, the storage of high amounts of NH_4^+ is unlikely. Focus will lie on NO_2^- production, which can only result from ammonia oxidation.

Most microcolonies were heavily coated with silver grains in bicarbonate cultures with and without NH_4^+ addition. In cultures without NH_4^+ addition MAR-positive cells were present, which could indicate that cells consumed intracellularly stored NH_3. However, only negligible amounts of NO_2^- were produced. In the sample supplemented with NH_4^+ only a little bit more NO_2^- was produced compared to the sample to which no NH_4^+ was added, demonstrating that AOA in general had not been very active during the experiment.

In the sample supplemented with benzoate no silver grain coated microorganisms were observed, which showed that benzoate was not used as heterotrophic substrate. In benzoate cultures that were not fed with NH_4^+ negligible amounts of NO_2^-, like in bicarbonate cultures, were produced. Some NO_2^-, but smaller amounts than in the bicarbonate cultures supplemented with NH_4^+, was produced in benzoate cultures supplemented with NH_4^+. This could indicate that NH_3 was used for maintenance. Cultures that were not supplemented with NH_4^+ probably used intracellularly stored NH_3 as energy source.

Samples that were incubated with phenol and NH_4^+ did not give any MAR signal after three weeks of exposure, but longer exposure lead to silver grain formation, indicating that only small amount of phenol was assimilated. Microcolonies in phenol cultures without NH_4^+ addition and without NH_4^+ addition but ATU addition were covered with silver grains. In the incubation supplemented with NH_4^+ only little NO_2^- was produced. In the incubation without NH_4^+ addition either intracellularly stored NH_3 was used as energy source and/or phenol was oxidized itself, which should then result in absence of NO_2^- production. However, in one duplicate, some NO_2^- was formed. To test if Amo oxidizes phenol, cultures without NH_4^+ were supplemented with allylthiourea (ATU), a substance known to inhibit bacterial Amo (Adamczyk et al. 2003) and, to a minor extent, archaeal Amo (Hatzenpichler et al. 2008). Hatzenpichler et al. (2008) observed after addition of ATU in the presence of ammonium and bicarbonate a strongly decreased MAR signal of the AOA microcolonies. In incubations with ATU addition, no NO_2^- was produced. Nevertheless cells were MAR positive, which suggests that they used phenol not only as heterotrophic source of carbon but also as energy source. Since ATU is a known inhibitor of Amo was, it is assumed that the enzyme was

not catalyzing the oxidation of phenol. After addition of ATU the MAR signal did not decrease, which favors the conclusion that phenol was used as energy source. Besides, the results show that NH_4^+ presence partly inhibited either uptake or utilization of phenol, possibly indicating that the AOA prefer NH_4^+ over phenol as source of energy. The utilization of aromatic compounds by certain bacteria is well known for a long time (Evans 1946) and recently, it also has been shown that a thermophilic archaeon (*Sulfolobus solfataricus*) can efficiently grow on phenol as the sole source of carbon and energy by the action of monooxygenases (Izzo et al. 2005).

No silver grains could be observed in incubations that were fed with amino acid mix, regardless if NH_4^+ was added or not. This was not expected, since amino acids are ubiquitous, and provide an important source of both C and N that can be used directly for biosynthesis as well as metabolized for energy (Suttle et al. 1991). Interestingly, contrary to all other incubations, AOA in the presence of amino acids formed no microcolonies, but were present as planktonic cells. Additionally, the NO_2^- production in amino acid cultures both with and without NH_4^+ addition was much higher than in any other culture, including the positive control (bicarbonate). Compared to the other incubations, the NH_4^+ decrease in amino acid cultures was quite low. These results led to the assumption that NH_3 released by the hydroxylation of amino acids was used as energy source. Besides, in no other incubation similar amounts of NO_2^- were produced. It is possible that the high pH and high concentration of amino acids caused adverse effects and led to dispersion of aggregates. Hallab et al. (1995) reported the relation between cellular adhesion and surface charge, surface roughness, adsorbed protein and cell morphology and although they tested cell adsorption on different materials in can be assumed that some of these factors also influenced the adherence of AOA in this study.

Since "*Candidatus* Nitrososphaera gargensis" formed very dense aggregates, it can be assumed that EPS supported biofilm formation. EPS like extracellular DNA is suggested to function as intercellular adhesion (Izano et al. 2008). EPS is known to be important in flocculation or biofilm formation (Sobeck and Higgins 2002). At high pH, acidic groups in EPS become negatively charged, leading to repulsion between the negatively charged EPS (Comte et al. 2006). Such structures need to be stabilized by the presence of cations to neutralize the negative charge. It is possible that amino acids in the medium chelated positively charged metal ions, which prevented biomass from dispersion. Chelators are usually negatively charged oxygen or nitrogen containing molecules that interact with positively charged metal ions to form a stable complex. For chelation, at least two electron pair donor groups are needed to form a complex with a positively charged metal ion, which acts as electron pair acceptor.

A well known chelating agent is EDTA. With EDTA it was possible to make calcium ions inaccessible to activated sludge, which resulted in reduction of floc strength and increase of solubility of EPS (Sanin and Vesilind 2000). Amino acids are known to function as chelators, which are used e.g. in plant nutrition (DeWayne 1986). Glycine is used in crop production as a chelating agent for micronutrients. Other amino acids used to complex or chelate cation micronutrients include lysine, glutamic acid, cysteine and histidine. It is likely, that with increasing pH chelating properties of some amino acids increase, since they have more negatively charged groups. Besides, since glycine is the simplest amino acid, it is likely that some amino acids having complex side chains represent very efficient chelators. In conclusion, it can be assumed that amino acids caused cell dispersion due to their function as chelators. This effect was enhanced by the high pH. Since dispersed aggregates were not observed in culture supplemented with other substrates, it is likely that the amino acid mix combined with a high pH led to dispersion of aggregates. None of the other substrates is likely to have chelating properties since they have only one group that can serve as an electron pair donor.

Microcolonies with attached silver grains could also be detected in cultures fed with pyruvate. Only very low amounts of NO_2^- were produced in all cultures, indicating that pyruvate was used and oxidized to CO_2 via the citric acid cycle, during which energy is generated. Genomic data indicate that *Cenarchaeum symbiosum*, a member of the marine group I.1a of crenarchaeota, harbors besides an ammonia-based chemolitoautotrophic energy metabolism a nearly complete citric acid cycle and therefore may be able to grow mixotrophically (Hallam et al. 2006). The MAR results suggest that "*Candidatus* Nitrososphaera gargensis" may be able to use pyruvate as carbon source and therefore has the possibility to grow mixotrophically.

In summary, there are hints that "*Candidatus* Nitrososphaera gargensis" is able to grow mixotrophically. However, since the dead control supplemented with 14C-labeled bicarbonate showed MAR signals and since no negative controls were performed for the other 14C-labeled substrates, it can not be ruled out that substrates adsorbed unspecifically onto biomass. On the other hand, it is possible that unspecific MAR signals only took place in the dead control, since incubations were done with PFA-fixed biomass, and Lee and colleagues (1999) observed enhanced levels of unspecific adsorption with PFA-treated biomass. Nevertheless, experiments need to be redone. In this study no washing steps were performed after MAR incubations and prior to PFA-fixation of cells. Zang et al. (2008) reported that the radioactivity in the pasteurized control was reduced through an additional washing step from 62% to 20%. Thus, when repeating the experiments, a pasteurized control instead of PFA-fixed biomass could be used as negative control, but extra washing steps should be done prior to PFA-fixation

(after all incubations) to guarantee the removal of excess unincorporated substrates. In addition, for each substrate used, a dead control should be performed to test for possible adsorption phenomena, since they can differ between different substrates.

4.1.3 The thermophilic ammonia oxidizer "*Candidatus* Nitrososphaera gargensis"

One of the most important factors regulating nitrification is temperature, and temperatures between 30°C and 36°C are estimated as optima for nitrifying bacteria (Belser 1979). However, Lebedeva et al. (2005) could demonstrate the presence of nitrifiers in the Garga hot spring enrichment at temperatures between 45°C and 59°C, with maximum oxidation rates at 46 and 50°C. Although temperature experiments already had been performed in 2005, they were repeated in this study because the community composition and the amount of AOA changed over the years. Thus, the "*Candidatus* Nitrososphaera gargensis" enrichment was incubated at four different temperatures and nitrite production was measured. Although the culture was not very active, nitrite was produced at all four temperatures, the most at higher temperatures of 56°C and 66°C. Interestingly, in the early enrichment ammonia oxidation was already inhibited at 60°C (Lebedeva et al. 2005). It is shown that "*Candidatus* Nitrososphaera gargensis" even is active at temperatures above 66°C. The moderately thermophilic "*Candidatus* Nitrososphaera gargensis" represents the first organism within group I.1b for which ammonia oxidation has been shown and all previously described AOA and AOB were mesophiles (Hatzenpichler et al. 2008). In the meantime, Reigstad et al. (2008) proofed archaeal ammonia oxidation in terrestrial hot springs at high temperatures and De la Torre et al. (2008) described an ammonia-oxidizing member of the crenarchaeota that grows up to 74°C. These findings are in accordance with studies which reported nitrite oxidation (Lebedeva et al. 2008), nitrogen fixation (Mehta and Baross 2006) and denitrification (Stetter 1998) in thermophilic environments. A thermophilic origin was also proposed for the anaerobic oxidation of ammonium (Canfield et al. 2006). Barns et al. (1996) hypothesized that mesophilic crenarchaeota are descendants of ancestral thermophiles and Hatzenpichler et al. (2008) concluded that archaeal ammonia oxidation evolved under thermophilic conditions and that mesophilic AOA might represent adaptations to lower temperatures.

4.2 Bacterial heterotrophic contaminants in the Garga hot spring enrichment

Since the start of the enrichment in 2001, no pure culture of "*Candidatus* Nitrososphaera gargensis"has been obtained. It was always known that heterotrophic bacteria were present in the culture, but their identity and function remained unknown. With more information on the nature of the contaminants, our cooperation partners hoped to be able to select against them and obtain a pure culture of the AOA. The Garga enrichment consisted of ∼70% AOA and ∼30% bacterial heterotrophic contaminants when experiments were done in this study. The screening for bacterial contaminants was started before metagenomic analysis had been performed. Thus, at this time point it was estimated that around 5-7 different bacterial contaminants were living in the enrichment. So far, only one contaminant had been identified as a putative AOB (Hatzenpichler 2006), which will be discussed in detail later (see 3.3).

For identification of bacterial contaminants the full cycle 16S rRNA gene approach was used, which consists of DNA extraction, PCR, cloning, sequencing, phylogenetic analysis, probe design and FISH with the original sample do identify microorganisms *in situ*.

4.2.1 Amplification of bacterial 16S rRNA gene fragments with PCR

Bacterial 16S rRNA genes were amplified using general primers. Additionally, another PCR was performed using general primers in combination with a primer specific for the putative AOB, which was lacking the 3' OH group and bound downstream of the 630R primer. This was done in order to inhibit amplification of 16S rRNA genes of the putative AOB, which were the only identified bacteria at that time. The aim was to amplify 16S rRNA genes of other contaminants in the enrichment, which seemed not to be amplified due to a DNA extraction or PCR bias, or not detected due to a too low number of analyzed clones. Since DNA polymerase adds a nucleotide onto only a preexisting 3'-OH group, it needs a primer with a 3'-OH group at which it can add the first nucleotide. This "inhibiting primer"would not be elongated by polymerase. Since the Taq polymerase used has a low 5'-3' exonuclease activity, it should not degrade inhibiting primers from its 5' end with high efficiency, and therefore the amplification of putative AOB 16S rRNA genes should be less efficient.

4.2.2 RFLP and sequence analysis

After the amplification of bacterial 16S rRNA gene fragments in the enrichment culture, cloned genes were analyzed using RFLP to get information about the diversity of sequences in the clone library.

a) RFLP with clones after general 16S rRNA PCR

RFLP screening was carried out with 90 bacterial clones and 24 were sequenced, representing the four different patterns obtained. However, after BLAST, all 24 sequences were found to be most similar to already identified putative AOB (RHG clones; Hatzenpichler 2006). Since different patterns turned out to represent the same species, it is likely that different strains of putative AOB were present or that sequence divergence was caused by the Taq polymerase, which lacks proofreading activity and causes one mismatch per 1,000 bp. Due to BLAST hits it was assumed that putative AOB were positively selected. When they were identified by Hatzenpichler (2006), PCR was performed directly with the cell pellet. In this study, DNA extraction was performed prior to PCR, but nevertheless no new bacterial contaminant could be identified. A reason could be that cells of certain contaminants were not cracked efficiently and, thus, only putative AOB 16S rRNA genes could be amplified during PCR. However, bead beating should crack open cells very effectively. Therefore, it was assumed that due to a PCR bias only the 16S rRNA genes of putative AOB were amplified. It is possible that secondary structures in ssDNA of other bacteria led to lower amplification of their 16S rRNA genes. On the other hand, high temperatures and reduced $MgCl_2$ concentration should prevent the formation of secondary structures. A second possibility is that other contaminants lacked primer binding sites, although general primers were used.

b) RFLP with clones after PCR selecting against putative AOB

RFLP analysis was performed for 107 bacterial clones. Four patterns were obtained, but in contrast to the general 16S rRNA gene PCR, two new patterns could be observed. 21 clones, representing all different patterns, were sequenced. This time, bacteria could be identified that had the highest similarity to *Thermaerobacter subterraneus* or an uncultured low GC gram positive bacterial 16S clone and to *Thermaerobacter* sp. One sequence (GaBI25) was highly similar to an uncultured cyanobacterium clone and to *Microcoleus chtonoplastes*, a cyanobacterium. Although some of the clones were affiliated to the putative AOB, it was the first time that another bacterial contaminant could be detected. Although it is not clear how exactly and if the PCR using an "inhibiting"primer worked at all, the only difference to the first PCR was this extra primer. In addition, in the first attempt to get bacterial sequences, a total of 90 clones

revealed only four patterns, and after sequencing all turned out to be putative AOB 16S rRNA genes. As revealed by BLAST, the new obtained sequences were similar to a species named *Thermaerobacter*. The *Microcoleus chtonoplastes*-related sequence seemed to be a chimaera and never could be identified again (see DGGE, Fig. 3.4). Although the original sample was taken from a microbial mat which was dominated by a filamentous cyanobacterium and a phototrophic bacterium (Lebedeva et al. 2005), it is not very likely that a cyanobacterium was able to survive over years in cultures which were maintained in the dark and transferred via plates occasionally.

4.2.3 Phylogeny of *Thermaerobacter*-related sequences

All obtained 16S rRNA sequences were highly similar to each other and thus it can be assumed that they derived from a single species. Short fragments of putative 16S rRNA genes of *Thermaerobacter* obtained during metagenomics clustered together with clones obtained in this study. Cut out DGGE bands were not used for phylogeny because only full length sequences should be used for tree calculations.

After phylogenetic tree calculations the sequences were shown to form a monophyletic branch closest to a cluster formed by *Thermaerobacter* spp., which falls into the order *Clostridiales* within the phylum *Firmicutes*. The clones showed the highest similarity to *Thermaerobacter marianensis* (88-89%), a high-GC-content bacterium related to grampositive, low-GC-content anaerobic thermophilic bacteria within the *Bacillus-Clostridium* subphylum isolated by Takai et al. (1999) from the deep sea. Members of the genus *Thermaerobacter* occur in the world's deepest sea-floor but also in hydrothermal vent environments. *Thermaerobacter marianensis* is described as strictly aerobic heterotroph capable of utilizing as sole energy and carbon source different organic compounds like yeast extract, peptone, cellulose, sugars and amino acids (Takai et al. 1999). The bacteria are not known to be able of denitrification, which can be performed by a wide range of microorganism and includes the subsequent reduction of nitrate via nitrite to nitric oxide, nitrous oxide and finally dinitrogen gas under anoxic conditions (Jetten 2008). Incubations were maintained under aerobic conditions but possible niches with very low oxygen concentrations have to be considered. However, no hint for denitrifying activity of *Thermaerobacter* was found, suggesting that *Thermaerobacter*-related bacteria in the enrichment thrived on excretion products of "*Candidatus* Nitrososphaera gargensis", putative AOB or degraded biomass. The strictly aerobic lifestyle of *Thermaerobacter marianensis* and the optimum pH for growth between 7 and 7.5 are both in accordance with the conditions under which the enrichment was maintained. However, the temperature range for growth was reported

to be 50 to 80°C, with an optimum at 75°C. Cultures have been kept at 46°C, which is below the temperature range reported and far below the optimum growth temperature of *Thermaerobacter marianensis*. However, it is likely that the contaminating bacteria inside the enrichment do have a different temperature range for growth than *Thermaerobacter marianensis*, due to high sequence divergence. On the other hand it is also possible that one of the reasons why these bacteria were not abundant and never overgrew AOA was suboptimal temperature. In conclusion, it can be assumed that the *Thermaerobacter*-related contaminants were not able to denitrify but could live on dead biomass and end-products of the AOA metabolism. Besides, for none of the other described species (*Thermaerobacter nagasakiensis*, *Thermaerobacter subterraneus* and *Thermaerobacter litoralis*) the capability for denitrification has been reported (Nunoura et al. 2002; Spanevello et al. 2002; Tanaka et al. 2006).

4.2.4 DGGE and T-RFLP as fingerprint analysis of the community

To obtain more information on the number of bacterial contaminants in the sample, the diversity was screened using DGGE and T-RFLP.

a) DGGE

DGGE fragments were also amplified from the clonal 16S rRNA gene sequences, to test which bands in DGGE gels were represented in the clone library. The diversity was, as expected, very low. However, only three fragments could be observed. This result differed from the estimated 5-7 bacterial contaminants. A reason might be that primers did not target all bacterial contaminants, although the primers used should target most of bacteria (Muyzer et al., 1993). Since DGGE is a PCR based method, the general bias of molecular techniques in microbial ecology, e.g. produced by sample handling, uneven cell lysis and PCR, have to be kept in mind (Von Wintzingerode et al. 1997; Muyzer and Smalla 1998). It can not be excluded that certain genes had little accessibility due to secondary structures and thus were not or only in minor amounts amplified during PCR. It was reported that amplification of parts of the 16S rRNA gene using universal primers underestimates biodiversity (Vallaeys et al. 1997). In addition to the limited sensitivity of detection of rare community members it is generally known that the detection limit of PCR is highly increased when attaching a GC clamp to the forward primer, which is very disadvantageous for detection of low abundance community members. However, there are strategies to overcome these problems, as for example the application of a group-specific PCR or a nested PCR (Heuer et al.

1997; Vanbroekhoven et al. 2004). Vanbroekhoven et al. (2004) could reduce the detection limit from 10^4 cells/g soil to 10 cells/g soil using a nested PCR approach. Thus, it is likely that using a nested PCR instead of a direct PCR approach in this study would result in an increase in sensitivity. However, in this study the aim was to identify all of the bacterial contaminants, thus no group-specific primers could be used. Two of three obtained fragments were represented in the existing clone library, one representing *Thermaerobacter* (GaBI) clones and the other one representing putative AOB (RHG) clones. Nevertheless, all three fragments were cut out, purified and sequenced. Comparison of the sequences using BLAST showed that band 1 (see Fig. 3.4) indeed was the most similar to putative AOB clones. Band 2 and 3 both could be identified as *Thermaerobacter* sequences, which led to the assumption that probable different strains of *Thermaerobacter* were present, resulting in different migration of their 16S rRNA gene fragments. DGGE is highly accurate and a single base substitution already leads to a different melting behavior of double-stranded DNA molecules (Wu et al. 1998; Myers et al. 1987). Myers et al. (1985) reported that DGGE can detect up to 95% of all possible single base substitutions amongst sequences of up to 1000 bp in length. Thus, it is also possible that no different *Thermaerobacter* strains were present but that two DGGE bands affiliated to *Thermaerobacter* resulted from PCR-associated artifacts (inaccuracy of the Taq polymerase). However, misincorporation by Taq polymerase had to happen in the early cycles of the PCR, otherwise this fragment would not be amplified to the amount detectable by DGGE. On the other hand, a limitation of DGGE is the detection of molecules produced by different rRNA operons of the same organisms (Nuebel et al. 1996). Thus, it can not be ruled out that different 16S rRNA gene operons of the same *Thermaerobacter* species resulted in two different bands. One strong band and additional faint bands were observed for each clone (Fig. 3.4). DGGE with enrichment DNA also resulted in three prominent bands and, at close distance, weak bands. Since these bands could be seen in both enrichment and clonal DNA, they likely represent artifacts. Janse et al. (2004) reported the observation of artifactual "double bands"which were also occurring with cloned sequences. However, the authors were able to significantly decrease the intensity of artifactual bands by extending the final elongation step. Their explanation was that Taq polymerase was probably hindered by secondary structures which could be disrupted by elongated incubation at a high temperature and allow the enzyme to complete the elongation.

b) T-RFLP

T-RFLP was performed as an additional method for community analysis and because it is considered to be more sensitive than DGGE (Moeseneder et al. 1999; Nunan et al.

2005). To guarantee the best resolution, forward and reverse primer were labeled, each with different a fluorescent dye, and three different restriction enzymes were applied. Phylogenetic identification of T-RFLP peaks was based on clonal sequences and their *in silico* predicted TR-F lengths. In all electropherograms prominent peaks could be assigned to *Thermaerobacter* and putative AOB, when bacteria-specific primers were used for PCR. One primer combined with restriction enzyme AluI resulted in a T-RF with the same length for *Thermaerobacter* and putative AOB. This serves as an example in which the observed diversity was lower than expected due to conserved regions in both of the bacterial contaminants, showing that more than one restriction enzyme should be used. Since T-RFLP is a PCR based method it is limited by inherent biases of this technique. In consequence, the results were interpreted not quantitatively but only qualitatively. Although T-RFLP is a semiquantitative method (Sipos et al. 2007; Sánchez et al. 2006), for quantitative studies PCR bias and the number of operons and the potential differences between them have to be considered (Moeseneder et al. 1999). Since no information is available for numbers of operons in the contaminants and PCR bias was not evaluated, only the number of peaks was interpreted. In all electropherograms additional peaks next to *Thermaerobacter* and putative AOB-peak were visible. One peak could be seen in all diagrams and probably was representing primer dimers or nonspecific PCR products. However, due to the high formamide concentration double stranded primer dimers are not very likely. In general, other peaks observed could represent additional members of the bacterial community in the enrichment or PCR artifacts. Sánchez et al. (2006) observed additional T-RFs due to formation of chimeric molecules and pseudo-T-RFs derived from partly single-stranded 16S rRNA amplicons. However, T-RFLP in contrast to DGGE still gave the hint for the possible presence of additional contaminants in the Garga enrichment, which might have been overlooked due to undersampling of clones.

T-RFLP using archaeal primers was performed to test if "*Candidatus* Nitrososphaera gargensis"was the only archaeon present in the enrichment. However, some electropherograms revealed not a single peak as expected, but one additional smaller peak. The additional small peaks may represent a minor population of another archaeon, different strains of "*Candidatus* Nitrososphaera gargensis"or pseudopeaks. However, metagenomics proofed that "*Candidatus* Nitrososphaera gargensis"was the only archaeon in the enrichment and no hint for different "*Candidatus* Nitrososphaera gargensis"strains was obtained (Hatzenpichler, unpublished). Thus, it can be assumed that PCR led to the formation of pseudopeaks. Since no more bacterial contaminants could be shown to be present in the enrichment by DGGE, and T-RFLP showed one or two additional peaks, which probably represented artifacts, it was assumed from

this data that no other contaminants were present in the enrichment culture. Finally, after metagenomic analysis was finished, seven bacterial rRNA genes were partially sequenced. All of them were assigned to *Thermaerobacter* or the putative AOB. Thus, the characterization of the bacterial community in the Garga enrichment can be seen as completed.

4.2.5 Clone-FISH and detection of *Thermaerobacter*-like bacteria in the enrichment

For evaluation and validation of the newly designed probe Clone-FISH was carried out, in which the 16S rRNA of *Thermaerobacter* clone GaBI27 was heterologously expressed in *E. coli* cells.

Before FA series were performed, the probe was tested on the clone-FISH sample and the Garga enrichment sample under non-stringent conditions. Since *Thermaerobacter marianensis* is a high-GC content bacterium (Takai et al. 1999), probe HGC was used. Unexpectedly, no signal was obtained with the HGC probe using the clone-FISH cells. Probably the rRNA was folded differently in *E. coli* so that probes had no access to their binding site. When using GaBI30 probe, signal for different cells varied, indicating that the cloned 16S rRNA genes of *Thermaerobacter* were expressed in different amounts in *E. coli*. In the enrichment sample a lot of bacterial rods were present. In contrast to the *E. coli* clone-FISH cells, most of the rods could be identified as high-GC content bacteria but no *Thermaerobacter*-related bacteria were detected. An explanation why *Thermaerobacter* could not be found is that probably the 16S rRNA genes of these bacteria have secondary structures that need higher FA concentrations to get denatured, which can be referred as low accessibility of the target sites. Indeed, after using 35% FA concentration, *Thermaerobacter* spp. were detected (see below). In the *E. coli* cells, however, the specific probe showed signals. It is possible, that ribosomal RNA were differently folded when heterologously expressed and revealed a better accessibility to probes despite non-stringent conditions.

After this, FA series were performed to determine the optimal hybridization conditions of the new designed probe, which was a concentration of 35% FA (Fig. 3.11).

After Clone-FISH and FA series were completed, the specific probe was used for *in situ* detection and visual quantification of *Thermaerobacter*-like contaminants in the enrichment. Besides, the aim was to compare the quantity of *Thermaerobacter* and putative AOB. In the oldest culture, which was also used for MAR experiments, a lot of bacteria could be detected and among them, aggregates of *Thermaerobacter*-

related bacteria were visible. Signals were obtained for straight to slightly curved rods, which was the morphology of *Thermaerobacter marianensis* as described by Takai and colleagues (1999). Since genome sequencing was performed with an earlier culture that was not maintained in this study, FISH was done in more detail with PFA-fixed cells of this sample. Some AOA were present as planktonic cells but most of them formed very dense clusters, in which no cells could be distinguished (and which also could be observed during MAR experiments). For AOM it is known that they tend to form clusters (Sliekers et al. 2005) and AOA clusters possibly were also embedded in EPS, which could provide the substrates for bacterial heterotrophs. In this sample some bacteria were detected, which formed loose aggregates. Among the rod-shaped bacteria that were detected more could be identified as putative AOB than as *Thermaerobacter*-related species. Besides, signals for putative AOB were brighter than for *Thermaerobacter*-like bacteria. It is likely that the ribosomal content of *Thermaerobacter* was low, since the temperature possibly was not optimal for their growth (see 4.2.3). Besides, no energy and carbon source was added to support heterotrophic growth. Thus, *Thermaerobacter*-related organisms were dependent on the activity of the AOA or possibly putative AOB inside the enrichment. An explanation why putative AOB were more abundant than *Thermaerobacter*-related species and why signals were stronger is that, assuming they are indeed ammonia oxidizers, they were supplemented with their energy source. On the other hand it is possible that abundance of *Thermaerobacter* was underestimated since it was reported that *Thermaerobacter marianensis* is Gram variable and cells stain slightly Gram-positive in the exponential growth phase but Gram-negative in the stationary growth phase (Takai et al. 1999). For Gram-positive bacteria an additional cell wall degradation step like lysozyme treatment might be necessary to ensure that probes can enter the cell through their thick cell walls (Shockman and Barett 1983; Bidnenko et al. 1998).

4.3 A putative AOB enriched from the Garga hot spring

4.3.1 Origin and first discovery of putative AOB

One of the stable contaminants in the enrichment described above was described as putative AOB (Hatzenpichler 2006). First hints for putative AOB in the enrichment were obtained by Lebedeva et al. (2005) who performed immunofluorescence labeling using AmoA and AmoB antibodies and could detect curved rods with AmoB antibodies

(confirmed by Hatzenpichler, unpublished). The morphology of the cells was most similar to vibroid members of the genus *Nitrosospira* (Lebedeva et al. 2005). Additionally, the enrichment was unsuccessfully screened for *amoA* and *amoB* genes of β- and γ-proteobacterial AOB and for 16S rRNA genes of β-proteobacterial AOB (Hatzenpichler 2006; Hatzenpichler et al. 2008). Using general 16S rRNA primers, sequences were obtained that formed a monophyletic group outside the β-proteobacterial AOB. Metagenomics of the enrichment revealed four 16S rRNA gene fragments of putative AOB, but no *amoA* genes could be found. Besides, no known AOB could be detected in the sample using AOB- and nitrite oxidizer-specific FISH probes (Hatzenpichler et al. 2008). A newly designed specific probe (RHG1130) showed signals for cone-like shaped microorganisms. Furthermore, cells were located in close proximity to cells of the genus *Nitrospira* (Hatzenpichler 2006). Using FISH-MAR, Hatzenpichler (unpublished) observed signals for putative AOB, but also incubations without added NH_4^+ showed MAR signals (although weaker).

Recently, Elena Lebedeva (unpublished) was able to highly enrich the putative AOB in a separate culture, in which ammonia-oxidizing activity was observed in incubations run at 3 mM NH_4^+. Since a specific probe had been designed to detect putative AOB (Hatzenpichler 2006) and proof of their capability of ammonia oxidation was still missing, Raman-FISH combined with stable isotope probing was performed to link identity and function of the microorganisms. Therefore, Elena Lebedeva incubated enrichment cultures containing putative AOB (Hamburg) with ^{13}C bicarbonate for 72h, fixed cells at different time points and sent the samples to Vienna.

First, FISH with the specific probe RHG1130 combined with EUBmix and DAPI staining was performed to get information about the composition and density of the enrichment. The nonsense probes did not give any unspecific signal, thus unspecific binding of the probes could be excluded. After DAPI staining one dominant morphology was detected, which were curved short rods. In original enrichments of the Garga hot spring, Lebedeva et al. (2005) reported the presence of two different cell types of putative AOB in the enrichment: small pleiomorphic cells and curved rods, and that curved rods were characterized by intracellular membrane-stacks, which is typical for *Nitrosospira* ssp. The cone-like morphology observed by Hatzenpichler (2006) might reflect the pleiomorphy of this cells and represent one of the possible cell shapes. However, in the recent enrichment culture putative AOB cells were curved small rods and looked the same as described by (Lebedeva et al. 2005). Nearly all of the cells in the enrichment hybridized to the EUBmix probe and all short rods showed a FISH signal with the specific probe RHG1130, and thus could be identified as putative AOB. Of course, some rods not representing the putative AOB might have been overlooked.

A very minor population was represented by cocci. They were only detectable using DAPI staining and thus they were assumed to be archaea. In conclusion, FISH results showed that the culture was highly enriched with putative AOB.

4.3.2 Do cocci represent "*Candidatus* Nitrososphaera gargensis"?

Putative AOB have been representing one of the bacterial contaminants and have been coexisting very stably for many years in the enrichment culture containing the AOA "*Candidatus* Nitrososphaera gargensis". In the new enrichment of putative AOB, some cocci were observed after DAPI staining, and thus were assumed to most likely be "*Candidatus* Nitrososphaera gargensis". In order to test if the cocci might represent AOA, CARD-FISH with EUBmix and RHGA702 probe, specific for the archaeon, was performed. Some cocci forming aggregates could be seen with DAPI, which was already observed in the AOA enrichment during MAR experiments. Cocci did not give any signal with the EUBmix probe, which indicated that cells were archaea. Using the probe specific for "*Candidatus* Nitrososphaera gargensis", bright FISH signals were detected but only in the very dense clusters. Some loose aggregates revealed only weak or no FISH signal. A reason for the different intensities of FISH signal could be that tight aggregates were metabolically more active and thus had a higher ribosome content. It is commonly referenced that the preferred mode of growth for bacteria is a high density community described as a biofilm (Costerton et al. 1995), which might be true for archaea as well. In a study by Schleheck et al. (2009) it was reported that planktonic aggregates have characteristics of surface associated biofilms, thus "*Candidatus* Nitrososphaera gargensis"clusters can be viewed as biofilm. It has been reported in a lot of studies that cells forming biofilms benefit over planktonic single cells in respect of protection from various environmental or artificial stresses and antibiotic treatments, nutrient availability and metabolic cooperativity, enhanced cell-cell interaction and horizontal gene transfer (e.g. Schleheck et al. 2009, Davey and O'toole 2000). Since "*Candidatus* Nitrososphaera gargensis"was numerically dominated by putative AOB and, assuming that both are ammonia oxidizers, they had to compete for NH_3. However, incubations were run at a concentration of 3 mM NH_4^+, and partial inhibition of the AOA was observed with 3.08 mM NH_4^+ (Hatzenpichler et al. 2008). A reason why putative AOB did not form clusters might be that they were optimally supported, were highly enriched and were not exposed to environmental stress. Thus, the need to form clusters to enhance metabolic cooperativity was not given.

4.3.3 Stable isotope labeling combined with Raman spectrometric analysis: are putative AOB indeed ammonia oxidizers?

In order to prove ammonia-oxidizing activity of putative AOB, they were incubated with ^{12}C or ^{13}C bicarbonate as sole carbon source and supplemented with NH_4^+ as energy source. For Raman spectroscopic analysis, only samples incubated for 72 h (maximum incubation time) were used. In the 12C mean spectrum and the ^{13}C mean spectrum characteristic peaks described in literature were detectable, corresponding to proteins (e.g. phenylalanine, cytochrome c), nucleic acids, lipids and carbohydrates (Naumann 2001; Maquelin et al. 2002; Pätzold et al. 2008) (Fig. 3.12). No shifts of these peaks were visible, indicating that cells were not labeled with ^{13}C. In 2008, Pätzold and coworkers used the resonance Raman effect of cytochrome c (Cyt c) and were able to record the microbial distribution of nitrifiers and anammox bacteria directly in their natural environment. The authors were able to group bacteria down to strain level based on the heterogeneity of the resonance Raman spectra of the heme containing protein Cyt c (Pätzold et al. 2008). Cyt c is a part of the respiratory chain of most bacteria (Bertini et al. 2006) and nitrifiers are known to have a high content of the heme-containing protein Cyt c (Pätzold et al. 2008). Because of the variation of the protein content between different bacterial strains, it should allow the identification of bacteria with a high Cyt c-content, although in literature concerning the identification of microorganisms resonant Raman spectra of Cyt c are currently not considered (Pätzold et al. 2008). The difficulty is to assign the spectrum to a specific bacterial species due to the limited amount of references and the lack of a spectral database of unknown microorganisms (Pätzold et al. 2006). Thus, if putative AOB are indeed ammonia oxidizers, a high Cyt c amount would provide a hint for a nitrifying lifestyle and spectra could be compared to *Nitrosomonas* and *Nitrobacter* strains (Pätzold et al. 2008). A pronounced Cyt c peak for putative AOB was obtained at 747 cm$^-$1, which was one of four cytochrome C peaks observed for *Nitrosomonas*. Two additional cytochrome c peaks could be observed, but were not as pronounced as the peak at 747 cm$^-$1.

Finally, the peak of the essential amino acid phenylalanine was analyzed in detail by calculating the red shift ratio (RSR). Wei et al. (2008) performed surface-enhanced raman spectroscopy (SERS) and compared SERS spectra of three cysteine-containing aromatic peptides to their respective normal Raman spectra. The authors observed that the 1003 cm^{-1} peak of the ring breathing mode in phenylalanine was the most intense peak of all the aromatic modes in both SERS and Raman spectra. The final

conclusion of their study was that aromatic amino acid residues provide the dominant features of peptides and proteins when present and make spectral interpretation easier. Thus, the easily assigned peak of phenylalanine was used to determine ^{13}C labeling. The RSR of nearly all ^{13}C of putative AOB spectra fall within the range of ^{12}C spectra, which confirms that phenylalanine of cells is not labeled (see Fig. 3.12). In 2007, Huang et al. demonstrated a linear relationship between RSR and ^{13}C content of cells. According to their study, an average RSR of ^{12}C spectra <0.5 indicates absence of label (Huang et al. 2007).

There are three possibilities why putative AOB were not labeled:

First, it can be concluded that "putative AOB"are not able to oxidize NH_3 and that they were not responsible for ammonia oxidation in the enrichment and did not fix labeled inorganic carbon. There are some observations that disfavor this conclusion, like the detection of *amoB* using antibodies and FISH-MAR, in which cells were active, despite cells grown without NH_4^+ also showed some MAR signal. Putative AOB were shown in one culture to live in close proximity to *Nitrospira*, which is another hint that they might be ammonia oxidizers. In sea- and freshwater environments as well as in soil, nitrite produced by the ammonia oxidizers is immediately consumed by nitrite oxidizers (El-Demerdash and Ottow 1983; Schmidt 1982), and consequently AOB and NOB are colocalized. Close associations between ammonia oxidizers and nitrite oxidizers were reported in several studies (Schramm et al. 1998; Maixner et al. 2006). The inability to amplify *amoA* and AOB-specific 16S rRNA gene fragments can be consequences of primers being designed on the basis of known AOB and, thus, might easily underestimate the true diversity. This is a common problem in microbial ecology, as for example reported by Treusch et al. (2005), who presented genes encoding a potential novel nitrite reductase and Amo-related proteins that had not been identified earlier by PCR from environmental samples because they were too divergent from already known genes. Sequences detected by PCR amplification are limited due to the nature of the primers used (Treusch et al. 2005). A possible solution for this drawback is metagenomics, as the discovery of archaeal *amoA* genes had shown. Analyzing metagenomic data obtained from the Sargasso Sea (Venter et al., 2004) and soil, (Treusch et al. 2005) first revealed putative archaeal *amoA*-like genes. The findings of archaeal *amoA* genes increased interest of researchers and finally led to the isolation of the first marine crenarchaeote that grows chemolithoautotrophically by aerobically oxidizing ammonia (Könneke et al. 2005). Finally, pronounced Cyt c peaks observed after Raman analysis could be another hint that putative AOB are indeed nitrifiers (Pätzold et al. 2008).

Second, the incubation of cultures for 72 h was probably too short to detect any labeling in the cells. The sensitivity of Raman-FISH was reported not to be suitable below a labeling level of 10 atom% ^{13}C (Huang et al. 2007), which was unfortunately not considered before planning the experiment. Although the enrichment culture was very active within this time and oxidized around 2 mM of NH_3, NH_3 is an energetically poor compound and thus carbon fixation rate probably was low. Theoretically, it is also possible that cells only used energy for maintenance. Maintenance energy is the energy consumed during activities that allow the cell to survive without biomass production (Geets et al. 2006). However, it is unlikely that no biomass was produced at all, since both energy and carbon source were available. In order to detect labeling in the cells, turnover of cellular components has to occur. However, it is generally known that nitrifiers are slow-growing organisms because their cell growth is inefficient (Bock and Wagner 2006). For cell division in natural environments, most nitrifier species even need a generation time of several days to weeks depending on substrate and oxygen availability, temperature and pH (Bock and Wagner, 2006). However, cells do not necessarily have to grow to get labeled, but carbon has to be utilized or incorporated, for example in the form of storage compounds. It is possible that each single cell did oxidize only small amounts of NH_4^+ and did not fix enough carbon to get sufficiently labeled for Raman analysis. How much energy is generated in an enrichment culture is relative to the density of cells in the enrichment. In summary, it is very likely that putative AOB were not incubated for a long enough time for detection of carbon fixation, at least not in such amount that it could be detected using Raman spectroscopy. A more realistic incubation time therefore would be three weeks, with taking samples after one, two and three weeks. However, cross-feeding could be a problem since the culture probably consisted of another ammonia oxidizer, "*Candidatus* Nitrososphaera gargensis". Summarized, cells might have been labeled but under the detection limit of Raman spectroscopy. As an alternative method and without the need to repeat incubations the more sensitive method using nanoSIMS could be applied. NanoSIMS instruments are significantly more sensitive than Raman microspectroscopy and allow the quantification of several isotopes in a microbial cell simultaneously (Wagner 2009). Lechene et al. (2006) reported that the absolute sensitivity of SIMS is among the highest of current chemical analysis methods and 1000 times more sensitive than 14C autoradiography, which by itself is ∼10-100 times more sensitive than Raman. Thus, if putative AOB were indeed actively fixing carbon, but not enough to be detectable via Raman spectroscopy, nanoSIMS would be the method of choice.

Third, it cannot be excluded that "*Candidatus* Nitrososphaera gargensis", which was detected as contaminant in the enrichment, was oxidizing NH_3. Although only a small

amount of cells in very dense clusters could be detected, it cannot be ruled out that contrary to the putative AOB each single cell was extremely active. However, Raman data for AOA did not indicate any labeling (performed by Kilian Stoecker), however, it can also not be excluded that 72 h incubation time was too short to observe labeling. On the other hand, incubations were performed with 3 mM NH_4^+ and Hatzenpichler et al. (2008) reported partial inhibition of the AOA at 3.08 mM NH_4^+, which supports the hypothesis that AOB and not AOA were oxidizing NH_3. Recently, a study showed that a strain of "*Candidatus* Nitrosopumilus maritimus"was inhibited by similar NH_4^+ concentrations as reported for "*Candidatus* Nitrososphaera gargensis", suggesting that both represent ammonia oxidizers thriving in oligotrophic environments (Martens-Habbena et al. 2009).

Concluding, the incubation of cultures was probably too short to detect any labeling in the cells using Raman spectroscopy. Therefore, besides Raman spectroscopy combined with stable isotope labeling to proof NH_3 oxidation, more sensitive methods like MAR-FISH or nanoSIMS should be used.

4.4 Enrichment of nitrifiers from soil and sludge

Both enrichments were performed according to a study by Simon and colleagues (2005), who were able to enrich crenarchaeota from soil within 2 month up to 20%. However, different antibiotics were used because in cultures supplemented with antibiotics used by Simon and coworkers *Pseudomonas fluorescens* had been enriched, which was found to be able to grow on the antibiotics (Großpraktikum).

For soil enrichments sampling was performed at a depth of 15 cm. This depth was chosen due to the light-sensitivity of nitrifiers: Mueller-Neugück and Engel (1961) observed that *Nitrobacter winogradskyi* was completely inhibited by light and Schön and Engel (1962) and Bock (1965) found strains of *Nitrobacter* and Nitrosomonas to be sensitive to light and suggested that photo-oxidation of cytochrome c was the mechanism for inhibition. Besides, Leininger et al. (2006) reported a high abundance of archaeal *amoA* copies and transcripts in different soil types between 10 and 70 cm depth. The coexistence of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) is well founded (Schramm et al. 1998; Maixner et al. 2006). In order to find nitrite-oxidizing microorganisms (NOM) a depth at which AOA are supposed to be abundant (Leininger et al. 2006) was chosen.

Since efforts to enrich nitrifiers were unsuccessful, only a short discussion will follow. The inoculum samples in the enrichments contained a lot of biomass, showing that the

separation procedure worked well.

In the soil enrichment (enrichment 1), NH_3 and NO_2^- were consumed in all cultures after some weeks, however, at that time cultures were overgrown by fungal hyphae and enrichments had to be discarded. Fungi are very dominant in terrestrial ecosystems and play an important role in soil ecosystems (Smit et al. 1999; Le Calvez et al. 2009). Since fungi are known to possess the potential for heterotrophic nitrification (De Boer and Kowalchuk 2001) it is thus likely that substrates were consumed by fungi and not by ammonia- and nitrite oxidizers using organic compounds from dead biomass. Besides, many fungi can perform denitrification (Shoun et al. 1992) and are known to assimilate nitrate and nitrite.

Despite using high concentrations of amphotericin B, an antibiotic which binds to ergosterol, the primary sterol in the fungal cell membrane, fungi were growing in the enrichments (amphotericin B leads to a disruption of the membrane and oxidative proteins in the target cells; (Ellis 2002)). Although resistance to amphotericin is quite rare, there are reports on fungal resistance to this antibiotic (Sutton et al. 1999; Ellis 2002). Ellis (2002) stated that resistance is caused by a decrease in the amount of ergosterol in the fungal membrane or a change in the target lipid, which leads to a decrease in the binding of amphotericin B.

CARD-FISH revealed that most of the cells in the inoculum and the cultures were bacteria. However, some larger cells, which seemed to be dividing (not stained by EUBmix but with Arch915), probably have represented archaea. In the late stage, hyphae could be seen by eye.

In enrichment 2 (soil, sludge) some modifications were introduced. Additional freeze-thaw cycles during cell separation were performed to kill bacteria more efficiently, cycloheximide was used (instead of amphotericin B) to inhibit protein synthesis in eukaryotic cells (Krokhina et al. 1991). However, only low numbers of archaeal cells were detectable in the cultures and already after two weeks they were overgrown by cycloheximide-resistant fungi, a well known problem in field soils (Ali-Shtayeh and Abu Ghdeib 1999).

Finally it was found that the group of Simon also had to deal with fungal contamination. However, they performed much more cultures and were able to maintain cultures not contaminated by fungi. In conclusion, a lot of different cultures supplemented with different antibiotics, substrate concentrations and regular dilutions would most likely result in successful enrichment of archaea. The fungus could be identified by Sylvia Klaubauf as *Gliocladium roseum*.

4.5 Enrichment using biofilm from aquarium filters of the "Haus des Meeres"

4.5.1 Amplification of 16S rRNA and functional genes from biofilm growing on aquarium filters

Bacterial and archaeal 16S rRNA genes were amplifiable as well as bacterial and archaeal *amoA* gene fragments and *nxrB* gene fragments of *Nitrospira*. Though the mere presence of a gene is not a proof of the enzymatic activity *in vivo*, the ability to amplify general 16S rRNA genes and functional genes of nitrifiers from inoculum provides a good basis for trying to enrich nitrifiers from that sample.

4.5.2 A closer look at the biofilm using FISH

The amount of biomass in the biofilm was high and the composition within the biofilm was very diverse: planktonic cells and clusters were present and different cell morphologies could be detected. Most of cells showed signals using EUBmix probe but archaea were not or only rarely detectable, suggesting that archaea were present in the biofilm only in a minor amount. However, it can not be excluded that some archaea were overlooked using standard FISH protocol. It was reported that archaea are important members of marine microbial communities (Reed and Colwell 2002; Rusch et al. 2003) and are known to possess unusual cell wall structures (Wagner et al. 2003). Therefore, standard permeabilization protocols may not be effective for archaea, which are characterized by diverse cell walls and possess compounds that often differ from those typical for bacteria (Kandler and König 1993). Additionally, they might have a ribosomal content under the detection limit of standard FISH. Thus, to improve the sensitivity of FISH a combination with catalyzed reporter deposition (Schönhuber et al. 1997; Schönhuber et al. 1999; Pernthaler et al. 2002) can be used. (Ishii et al. 2004) used protease for permeabilization of cell walls and reported improved detection of rare organisms such as archaea using CARD-FISH.

Due to fungal filaments dominating the former soil incubations, filaments in the biofilm sample were tested for signals using probe EUK, but no signals were detected and only some rare thick filaments and tetrads showed signals. Fungi are regarded as key organisms in terrestrial ecosystems which play an important role in the soil ecosystems (Smit et al. 1999; Le Calvez et al. 2009), however, there are studies about marine fungal species (Schoch et al. 2007; Le Calvez et al. 2009).

In general, Cy3-labeled probes yielded a much better FISH signal than Fluos-labeled probes. Overall, signals for planktonic cells were rarely detectable, whereas cells in clusters often showed a strong FISH-signal. It often has been reported that biofilm forming cells benefit over planktonic cells, among other reasons due to metabolic co-operativity (Schleheck et al. 2009; Davey and O'toole 2000), and therefore might have shown a brighter FISH signal due to higher ribosome content.

Unfortunately, the nonsense probe showed signals for rods located in clusters. Only probe Alf968 revealed much more signal than the nonsense probe, suggesting that *α-Proteobacteria* were probably present. In summary, FISH did not yield a lot of information about the identity of microorganisms in the sample.

4.5.3 Nitrifying activity in pre-incubations

Before starting the main-incubations, pre-incubations were performed for five days to test for nitrifying activity. To some cultures antibiotics were added with the aim to select for archaea. All incubations were run under fully aerobic conditions in the dark at 27°C without shaking.

In incubations supplemented with NH_4^+ (NH_4^+ bf, NH_4^+ AB bf; see Tab. 3.13) nearly all of the added NH_4^+ was oxidized after 4 days, indicating that ammonia oxidizers had been active. There was no difference between the cultures with and without antibiotic, indicating that either both AOB and AOA or AOB resistant to streptomycin were present. In the culture to which no antibiotic was added (NH_4^+ bf), no NO_2^- was present after 4 days, indicating that nitrite oxidizers oxidized NO_2^- to NO_3^-, which was present. Despite aerobic conditions denitrification, the anaerobic reduction of NO_3^- and NO_2^- to N_2, can not be completely excluded, because the existence of anaerobic niches remains possible. Denitrifiers are found in almost all phylogenetic bacterial groups and among archaea (Zumft 1997). However, NO_3^- was detectable and denitrifiers use NO_2^- and NO_3^- as substrate, thus under denitrifying conditions NO_3^- should not be present. In the culture supplemented with antibiotics (NH_4^+ AB bf) NO_2^- was accumulating after four and five days. This probably was indicating that no nitrite-oxidizing archaea and no antibiotic-resistant NOB were present in the culture. In the culture to which antibiotic and crushed biofilm was added (NH_4^+ AB crushed bf), only little activity was observed, suggesting that microorganisms living as biofilm were more shielded from antibiotic compared to planktonic cells. Finally, a culture in which biofilm was crushed and filtered to only incubate big particles (NH_4^+ filtrate), revealed no ammonia oxidation and leads to the assumption that microorganisms were lost during filtration. In the aquarium water control (NH_4^+ aquarium water only) no

NO_2^- production could be observed, which leads to the conclusion that both AOB and AOA were present in the biofilm and not in the aquarium water. The measured NO_2^- and NO_3^- concentrations indicate that ammonium was not lost through evaporation but oxidized by microorganisms. Decrease of NH_4^+ concentration could also originate from assimilation or intracellular storage. However, NH_3 can not be assimilated without energy and AOM gain energy by NH_3 oxidation. On the other hand, heterotrophic microorganisms use different sources of energy for NH_3 assimilation, but it is not very likely that NH_4^+ decrease is mainly caused by assimilation of NH_3 by heterotrophs. The storage of high amounts of the toxic substrate NH_3 might negatively affect the cells and is unlikely. Consequently, the most probable reason for ammonia decrease is ammonia oxidation by AOM.

In cultures fed with NO_2^-, differences between the culture with biofilm (NO_2^- bf) and between cultures with crushed biofilm (NO_2^- crushed bf) and the biofilm supplemented with antibiotics (NO_2^- AB bf) were observable (see Tab. 3.13). Whereas in the culture with biofilm all of the NO_2^- added was oxidized after four days, a lot of NO_2^- was still present in the culture with biofilm and antibiotics and with crushed biofilm and antibiotics. This led to the conclusion that assumed nitrite-oxidizing archaea or antibiotic-resistant bacteria were not present. It is possible that some antibiotic resistant NO_2^- oxidizers consumed some of the NO_2^- or that denitrifiers used NO_2^-. In the culture to which biofilm that was crushed and filtered was added (NO_2^- filtrate), no decrease of NO_2^- was detectable, indicating that a lot of microorganisms had been lost by filtration. In one culture whole filters balls with intact biofilm were used for incubations (NO_2^- balls) and showed high activity, but were not used due to easier handling and sampling of washed off biofilm. The NO_2^- incubation with aquarium water showed no change of NO_2^- concentration, indicating that NOM were localized only in the biofilm but not in the surrounding aquarium water.

There is strong evidence that NO_2^- decrease was caused by NOM. First, incubations were performed under aerobic conditions, and denitrification is an anaerobic respiration process, although it is possible that some anaerobic niches formed within aggregates and provided conditions for denitrifiers. In the culture with biofilm (NO_2^- bf), NO_3^- could be detected, which suggests that NOB formed NO_3^- and denitrifiers were not present, since NO_3^- provides a substrate but was detectable. A hint that NO_2^- decrease was not caused by abiotic factors is provided by the aquarium water control, in which NO_2^- did not decrease. In summary, differences between cultures treated with and without antibiotic were observed, providing evidence that antibiotic treatment influenced culture composition and/or activity. Additionally, NOM seemed to be present in biofilm but absent from the aquarium water.

4.5.4 Substrate utilization patterns in main incubations

4.5.4.1 Incubations supplemented with ammonia

NH_3 was consumed constantly in cultures with and without antibiotic, indicating that ammonia-oxidizing bacteria as well as ammonia-oxidizing archaea were active.

Ammonia is, in addition to lithotrophic nitrifiers, oxidized by various heterotrophic bacteria, fungi and algae to nitrate (Focht and Verstraete 1977; Killham 1986; Papen et al. 1989), but contrary to lithotrophic nitrification the so called heterotrophic nitrification is not coupled to energy conservation and therefore, heterotrophic nitrifiers are dependent on the presence of organic carbon (Bock and Wagner 2006). It cannot be completely excluded that decrease of NH_3 concentration partly resulted from heterotrophic nitrification. Although the sole supplemented carbon source in the medium was inorganic carbon (added in the form of bicarbonate), the aquarium water probably contained high amounts of organic substrates, which unfortunately could not be circumvented while enrichment cultures were maintained. It is likely that these substrates were used and that organic substrate concentration decreased with time, but for each dilution fresh aquarium water was used, providing again new organic carbon source. Furthermore, it is possible that organic matter was present due to death and subsequent degradation of microorganisms and subsequent release of organic compounds, which supplemented growth of heterotrophic nitrifiers. Bacterial mortality is known to be an important factor that influences both growth and abundance of microorganisms (Cottrell and Kirchman 2003). Besides, heterotrophic microorganisms might have thrived from by-products of nitrifiers located in the biofilm. It was demonstrated that autotrophic nitrifiers produce and release soluble microbial products (SMP) into solution from substrate metabolism (usually with biomass growth), and decay biomass (Rittmann and Stahl 1994). In conclusion, it is not known if heterotrophs obtained their carbon source from the aquarium water or from SMPs of nitrifiers or dead biomass. But it is likely that heterotrophs were supplemented with organic carbon source. On the other hand, it was reported that nitrification rates of heterotrophic nitrifiers are low compared to those of autotrophic nitrifiers (Robertson and Kuenen 1988) and that heterotrophic nitrification is thought to occur preferentially under conditions unfavorable for autotrophic nitrification, e.g. in acidic environments (Killham 1986). Therefore, it is unlikely that NH_3 was oxidized primarily by heterotrophic nitrifiers.

These observations support the hypothesis that ammonia oxidizers and possibly, in minor amounts, heterotrophic nitrifiers were responsible for ammonia decrease. In conclusion, most of ammonia was used as energy source for maintenance, biosynthesis

and growth.

Besides aerobic oxidation of ammonia by nitrifiers, ammonia can also be oxidized anaerobically by obligate anaerobic ammonium-oxidizing (Anammox) microorganisms (Strous et al. 1999), which use ammonia and nitrite to produce dinitrogen gas (N_2). These organisms, members of the *Planctomycetales*, are now known to be key players in the marine nitrogen cycle as they were found in a range of marine environments including sediments, sea ice and anoxic water columns (Dalsgaard et al. 2005), but thrive only under strictly anaerobic conditions. Incubations in this study were run under fully aerobic conditions. Thus it can be ruled out that anaerobic ammonia oxidizers were present and anammox process can be excluded.

Finally, ammonia volatilization can be a reason for ammonium loss. Ammonia is a degradation product of organic nitrogen compounds and dissolves in water in the ionized form (NH_4^+) at neutral pH and in the unionized form (NH_3) at high pH (Sasaki et al. 2002), at which it will evaporate. It was reported that increasing temperature and/or soil pH markedly increased NH_4^+ volatilization (Ernst and Massey 1960). Incubations were performed at 27°C and a pH of around 8.5, at which a lot of nitrogen in form of NH_4^+ should be present and might evaporate. However, studies also have shown that ammonia and not ammonium is the substrate for ammonia oxidation in *Nitrosomonas* (Suzuki et al. 1974; Schmidt and Bock 1998). Thus with higher pH more substrate is available. On the other hand, pH sometimes dropped to around 5 and was not immediately adjusted. It is not known how many days cultures were maintained at a low pH, however, activity did not decrease. These AOB possibly possessed an active ammonia/um uptake system, which was reported already for *Nitrosomonas europaea* (Schmidt et al. 2004; Weidinger et al. 2007). Concluding, under pH of around 8.5, at which cultures were mostly maintained, the NH_4^+ pool will decrease faster due to both higher ammonia loss and ammonia-oxidizing activity. However, since in water controls ammonia was stable, evaporation was not assumed to be the main cause of ammonia decrease. In summary, it can be assumed that ammonia was oxidized by AOB and AOA. Cultures were active from the start of the incubations, suggesting that they had optimal growth conditions. They consumed around 500 µM of ammonia in 1-8 days.

pH dropped in cultures, which is an indication for ammonia-oxidizing activity, since the oxidation of ammonia results in an acidification of the environment (Meincke et al. 1989). pH dropped more often in cultures without antibiotics. An explanation for that is that in cultures without antibiotics both AOB and AOA are supposed to be present, whereas in cultures supplemented with antibiotic only AOA and possibly some resistant AOB were active. After the first dilution of cultures, ammonia was not consumed

as fast any more as in original cultures, and in diluted cultures ammonia started to accumulate. Serial dilutions were performed in order to enrich nitrifiers further and to reduce the amount of heterotrophic contaminants. In general, serial dilution and subsequent recultivation from the most diluted sample is the classical approach for isolation of nitrifying bacteria (Bartosch et al. 2002; Bock and Koops 1992). Spieck et al. (2006) were successfully enriching *Nitrospira*-like bacteria using a novel enrichment protocol, in which they performed dilutions after every 2-3 months, which was also used for the incubations described here. However, their enrichments were much more discriminative, as for example the use of different nitrite concentrations, to enrich NOB adapted to different niches, showed. It is important that dilutions are not performed too early, because the target group has to be enriched already. In particular, ammonia oxidizers are known to grow very slowly. Thus, it is possible that ammonia oxidizers were not enriched enough yet and that they were rapidly outcompeted and overgrown by heterotrophs after dilutions, which e.g. used degraded biomass for growth. It might also be possible that dilutions were performed after a too long incubation time, which would also favor heterotrophic growth. It was shown that at low dilution rates the amount of dead biomass increases, which would support heterotrophs with organic substrate. Sinclair and Topiwala (1970) modeled cell viability in a chemostat culture and assumed that cell viability is a function of the dilution rate. Thus, at low dilution rates, like in this study, the amount of dead biomass may represent an appreciable proportion of the total biomass (Veillux 1980). It can be assumed that at low dilution rates heterotrophs rely on activity of ammonia oxidizers and their release of organic by-products, and/or on a high mortality rate, since organic substrates from aquarium water most likely were used in a short time. Dead biomass favors heterotrophs and their growth is not dependent any more only on excretion of organic substances from ammonia oxidizers. Besides, it was reported that in the presence of organic carbon, nitrifiers are usually outcompeted by heterotrophs due to the low growth rate and low growth yield (Kindaichi et al. 2004).

Another possibility why cultures became less active with time might be inhibition of ammonia oxidation by toxic compounds. For example, toxic intermediates such as hydroxylamine in high concentrations were shown to inhibit exponentially growing cells of *Nitrosomonas europaea* (Abeliovich and Vonshak 1993). Because in the cultures discussed here no AOB were grown exponentially and community was composed of a lot of other species, it can be excluded that hydroxylamine accumulated and assumed that it was subsequently oxidized further to NO_2^-. Besides, hydroxylamine is not stable at RT. Accumulation of NO_2^- was not observed in cultures without antibiotic. Since no accumulation of NO_2^- was observed, it can be excluded that high NO_2^- concentrations

were the reason for less activity. It is likely that NO_2^- was oxidized further to NO_3^- by nitrite oxidizers. In cultures with antibiotic, NO_2^- concentrations slowly increased over time. Here it can be assumed that NO_2^- inhibited and killed AOM, although in the first month nitrite was not present in very high amounts (around 300 µM). An explanation why nitrite accumulated in cultures with antibiotics is that NOB were killed and NOA not present.

An important factor that definitively influences marine communities are bacteriophages because they are numerous and important components of oceanic food webs (Paul 2008). Fuhrmann (1992) stated that viral lysis provides a density-dependent agent of bacterial mortality, resulting in higher growth rates at higher levels of bacterial abundance. Besides, Paul (2008) observed that environmental conditions that favor host growth activated lytic phage genes, resulting in phage production and host lysis and infection of more rapidly growing host cells. Since environmental conditions were chosen that constantly favored growth of ammonia oxidizers, it is possible that, after they had been enriched, cell lysis by phages was increased.

4.5.4.2 Incubations supplemented with nitrite

In the original cultures supplemented with NO_2^-, NO_2^- accumulated in the cultures with antibiotic. The aim was to enrich archaea involved in nitrite oxidation, which have not been detected until now. The accumulation of nitrite in cultures supplemented with antibiotics led to the assumption that nitrite-oxidizing archaea were not present. On the other hand, they could be present but inhibited by other microorganisms. The microorganisms in cultures without antibiotic consumed the added NO_2^- within the first week. Then, NO_2^- also started to accumulate, suggesting that NOB were present but eventually inhibited by toxic intermediates of other organisms.

Very interestingly, after the first dilution was made, NO_2^- was used after some weeks in all four NO_2^- cultures, and from then on was constantly consumed. Since all cultures were active, it was assumed that in incubations supplemented with antibiotics either archaea or resistant bacteria oxidized nitrite. Since substrate concentrations, oxygen, temperature and pH were not changed, these factors can not be the reason for sudden activity of NOB. Activity was observed some weeks after the first dilutions were performed. Thus it is likely, that a minor population of slow growing, possibly resistant (in cultures supplemented with antibiotics) NOM became enriched after this long time.

The original cultures very likely contained a very diverse and dense microbial commu-

nity. Probably, nitrite oxidizers were outcompeted at the beginning by fast-growing heterotrophs, which could use organic compounds from aquarium water or degraded biomass. It was reported that in the presence of organic carbon, nitrifiers are outcompeted by heterotrophs due to their low growth rate and low growth yield of the former organisms (Okabe et al. 1996; Kindaichi et al. 2004). Cultures became active after dilutions were performed. Thus, it can be assumed that dilutions favored growth of NOB. From the time at which NOB became active, it is very likely that the competition between heterotrophs and nitrifiers for dissolved oxygen and space started, which was reported for biofilm models and is a well described process (Ohashi et al. 1995; Nogueira et al. 2002). Okabe et al. (1996) described that the presence of organic substrates in waste water creates competition between nitrifiers and heterotrophs. Organic substrates were available and originating from aquarium water, dead biomass and by-products of nitrifiers.

In general, it is more likely that nitrification was the reason for NO_2^- decrease, because cultures were run under aerobic conditions. However, it is possible that anaerobic niches were present that enabled denitrification. If denitrification is assumed to be the reason for nitrite decrease, nitrite should be consumed constantly from the beginning on. On the other hand, it can not be excluded that denitrifiers had to adapt to environmental conditions. However, in the cultures supplemented with NO_2^-, the NO_3^- concentrations were high and in most cultures increased over time, whereas NO_3^- in the aquarium water control was stable. This indicated that denitrification was not the major process in the incubations, but that NO_3^- was formed by NOB.

To rule out the possibility of denitrification in the cultures, pool dilution technique could be performed. Isotope pool dilution techniques enable gross rates of nitrification (or mineralization) to be determined by monitoring the decline in the 15N abundance in a nitrate (or ammonium) pool. This technique enables not only to test for nitrification but also to distinguish between autotrophic and heterotrophic nitrification (Barraclough and Puri 1995). However, for this method, nitrite oxidation rates and nitrate production rates have to be evaluated. Unfortunately, substrate concentrations could not be measured on high performance liquid chromatography (HPLC) due to the high salt concentration in the aquarium water, which has the same retention time as nitrite and nitrate. Thus, no final proof of nitrification was obtained, although it was very probable.

It has to be mentioned that the chance to enrich nitrifiers was quite low from the beginning, because incubations were not very discriminative. Only one substrate concentration and only one antibiotic to suppress growth of bacteria was used. To increase the

outcome of enrichments, different substrate concentrations selecting differently adapted NOB, different antibiotics and incubation volumes should be used and much more parallel cultures should be started. The cultivation of nitrifying microorganisms requires a lot of time and space, which both were not available for this study.

4.5.5 Inability to detect ammonia oxidizers in cultures supplemented with ammonia

FISH was performed with cultures that were actively consuming ammonia over some weeks to months using general and AOB-specific probes. Archaea were present in very small amounts and could only be detected using CARD-FISH. Since only low numbers of archaea were detected, it can be concluded that AOA were not dominant in cultures to which antibiotic was added. The observation with FISH that archaea were only rarely detectable leads to the assumption that AOB were responsible for ammonia oxidation, although it cannot be excluded that a low number of AOA were highly active. In all cultures no hint for enrichment of any known AOB was observed. In cultures without antibiotic, contrary to cultures with antibiotic, NO_2^- concentration remained always low, indicating that nitrite was oxidized further by NOB. However, also NOB could not be detected. In a culture without antibiotic very low signal was observed using probe Nso1225, specific for β-proteobacterial AOB, which include *Nitrosomonas* (and *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* (Purkhold et al., 2000). However, no signal was obtained using Bet42a, suggesting that signals were unspecific and providing another confirmation that no *β-Proteobacteria* were enriched. The only probe that revealed signal was Gam42a, but no signal was obtained using the probe specific for *Nitrosococcus oceani*, which is affiliated with the γ-subclass of the *Proteobacteria* (Watson 1965; Purkhold et al. 2000). Although FISH was performed several times using different cultures and different amount of biomass, no AOB were identified.

It is likely that biofilm was attached to the surface of the glass flasks, which was unfortunately not controlled during the whole study. Underhill and Prosser (1987) investigated surface attachment of nitrifying bacteria and demonstrated that growth rate of *Nitrosomonas* and *Nitrobacter* cells colonizing glass surfaces was greater than that of suspended cells. A hint for their attachment to the surface is that the glass surface of some flasks probably was turbid. On the other hand, it is possible that only a low number of AOB were present and have been overlooked, however it is more likely that, since cultures were consuming ammonia, AOB constituted a certain amount of the community and thus should be visible with FISH. Additionally, it was

reported that nitrifiers in general prefer living in aggregates (Sliekers et al. 2005), which comprise a lot of cells and should not be easily overlooked. Neither can it be ruled out that PFA-fixation of cells was performed when cells did not contain a high amount of ribosomes. However, for AOB it is known that even under starving conditions they sustain their ribosome content and thus, if AOB really are present in the sample, should be detectable by FISH (Wagner et al. 1995; Morgenroth et al. 2000). Since no AOB could be identified, another plausible explanation is that known AOB were not present in the enrichment sample and therefore could not be detected with probes designed on the basis of known AOB. In summary, attachment of nitrifiers to the glass surface is the most probable reason for the inability to detect AOB.

4.5.6 *Nitrococcus* in cultures supplemented with nitrite

In the culture supplemented with NO_2^- (NO_2^- bf II) and all diluted cultures that followed, thick cocci were identified as *Nitrococcus mobilis*, a nitrite oxidizer belonging to the γ-subclass of *Proteobacteria*. *Nitrococcus mobilis* was isolated from marine environments and described as a large coccus (Watson and Waterbury 1971). *Nitrococcus* in the enrichments had a characteristic coccoid, but frayed morphology. They were present as planktonic cells but mostly formed characteristic microcolonies in the form of dense aggregates, which easily could be distinguished from other cells in the enrichment cultures. The dense microcolonies were surrounded by layers of extrapolymeric substance (EPS). Interestingly, cells organized in tetrads and the formation of dense microcolonies embedded in EPS has been reported for *Nitrospira*-like cells (Bartosch et al. 2002; Spieck et al. 2006), and similar observations were made in this study for cultures containing *Nitrococcus*. Daims et al. (2001) observed cavities and a network of channels inside *Nitrospira* microcolonies, which they suggested provides a better diffusion of nitrite, gases, and metabolic waste compounds throughout the aggregates. Thus, it can be assumed that also *Nitrococcus* microcolonies were forming a complex network.

There are many studies that reported on the coexistence of heterotrophs with nitrifiers in autotrophic nitrifying biofilms cultured without an external organic carbon supply (Okabe et al. 1996; Okabe et al. 1999; Okabe et al. 2002). Some studies have shown that extracellular exopolymeric substances and soluble microbial products (SMP) produced by nitrifying bacteria are utilized by heterotrophic organisms (Rittmann and Stahl 1994; Kindaichi et al. 2004). Thus, they also interact with other microorganisms through the exchange of organic matter. Some of the SMP have been identified as humic and fulvic acids, polysaccharides, proteins, amino acids, nucleic acids, and

structural components of cells (Barker and Stuckey 1999). Since heterotrophic microorganisms grew to a considerable proportion in the cultures reported here, even after dilutions series were performed, it can be assumed that organic substrates were present in high amounts. No external organic carbon was added, however, the incubation medium (aquarium water) provided organic substrates. Thus, next to SMP produced by nitrifiers, aquarium water and degraded biomass provided organic substrates for heterotrophic bacteria. However, since nitrifiers were supplemented constantly with their carbon and energy source, SMPs likely served as constant carbon and energy source for heterotrophic bacteria in the cultures. It is worth to mention that heterotrophic contaminants did not seem to be very diverse. In addition, the microbial community in some (also duplicate) cultures developed completely different, showing the need to perform a lot of replicates. In some incubations it even seemed as if only one contaminant was present. This indicates that only a low number of species could be stably maintained in the cultures and compete with NOB. Although morphology is not a good characteristic to distinguish microorganisms, it was in certain cultures quite noticeable that rods having exactly the same length and shape were present as major contaminants.

Spieck et al. (2006) noticed *Nitrospira*-like bacteria forming aggregates or living a planktonic lifestyle, which was also observed in this study for *Nitrococcus*. They speculated that clusters establish while nutrients are available in high amounts and planktonic cells when resources are limited. The authors stated that this switch is an efficient strategy to escape severe conditions and to colonize new habitats. Additionally, they stated that variable cell morphology may be a common adaptation mechanism to changing growth conditions (Spieck et al. 2006). Since *Nitrococcus* cultures were regularly supplemented with NO_2^-, starving periods did no longer take place. Indeed, most of the cells were forming clusters, a support of this theory. The irregular shape of cells, organization into tetrads surrounded by layers of EPS and the change from clusters to a planktonic lifestyle, observed in enrichments of *Nitrospira*-like bacteria from soil and activated sludge, were also observed for enrichments of *Nitrococcus* of aquarium biofilm. These observations probably represent common features of nitrite-oxidizing bacteria.

In summary, it can be assumed that there was an efficient food web in the autotrophic nitrifying biofilm community, in which heterotrophic bacteria lived from organic compounds abundant in water and produced by nitrifiers, as shown in a study by Kindaichi et al. (2004).

4.5.7 Unidentified decrease of NO_2^- in some cultures

In one culture supplemented with NO_2^- (NO_2^- bf I) probably low numbers of *Nitrospira* were present (as shown by FISH), but they could not be detected in diluted cultures. It is possible that they became diluted or were attached to the glass surface of the flask and overlooked. In NO_2^- cultures supplemented with antibiotic nearly all of the detected single cells and clusters were bacteria, indicating that streptomycin-resistant NOB were oxidizing nitrite. In general, no known NOB were detectable, but a lot of cells, mainly rods, turned out to belong to the *α-Proteobacteria*. The only so far known nitrite oxidizer that belongs to the α subclass of *Proteobacteria* is *Nitrobacter*, a rod-shaped bacterium, which could not be detected. However, soils are regarded as the privileged habitats of *Nitrobacter* (Degrange et al. 1998; Grundman and Normand 2000), and *Nitrospira* is regarded as more important than *Nitrobacter* in, for example, wastewater treatment plants and aquaria (Vanparysa et al. 2007). Thus, *α-Proteobacteria* were presumably heterotrophic contaminants. Some of the older cultures supplemented with antibiotic were very highly dominated by planktonic coccoid bacteria, which first were thought to represent NOB. However, probes targeting known NOB did not show a signal. After dilutions were performed, the community of microorganisms changed and planktonic rods were dominating. Since nitrite consumption did not change over time, it can be assumed that these abundant rods did not represent NOB. Finally the question arises who is oxidizing nitrite in cultures supplemented with antibiotic and the culture with no antibiotics added (NO_2^- bf I), in which only in some cases *Nitrococcus* had been observed.

Although NOB could not be identified, it is likely that NOB were present, since NO_2^- was oxidized. The planktonic cell community changed over time and after dilutions had been performed, but nitrite oxidation rates were stable, suggesting that planktonic cells were not responsible for nitrite oxidation and that NOB were located mainly in aggregates. Formation of aggregates was also observed for *Nitrococcus* cultures in this study and for *Nitrospira*-like bacteria in a study by Spieck et al. (2006). In cultures supplemented with antibiotics a lot of aggregates were present. They, however, looked different from aggregates formed by *Nitrococcus* and no characteristic tetrad formation or morphology was observed within the aggregates. According to Spieck et al. (2006), nitrite oxidizers were present in clusters since they were supplemented with nutrients in high amounts.

In conclusion, it is possible that some new and antibiotic resistant NOB were oxidizing the NO_2^-. However, it is more likely that NOB could not be identified because they were attached to the glass surface of the flasks. In the case of new members of the

NOB, RNA-SIP would provide a helpful method to gain information about the identity of the active population.

4.5.8 16S rRNA gene sequences

16S rRNA clone libraries were performed for cultures supplemented with NO_2^- (NO_2^- bf I, II) and one culture supplemented with NO_2^- and antibiotics (NO_2^- AB bf I). RFLP analysis showed that the community composition was diverse, since many different patterns were obtained.

None of the sequences obtained from culture with NO_2^- added (NO_2^- bf I) showed any similarity to known nitrite oxidizers. This is in accordance with FISH results, because only rarely some *Nitrococcus* colonies could be detected. Out of 20 sequences eight belonged to the α-subclass of *Proteobacteria*, seven sequences were affiliated to γ-*Protebacteria* and three sequences were similar to *Chloroflexi*. This was comparable to a study about ecophysiological interaction between nitrifying and heterotrophic bacteria in autotrophic nitrifying biofilms (Kindaichi et al. 2004), in which α-*Proteobacteria* followed by γ-*Proteobacteria* were the numerically dominant heterotrophs. In another study (Okabe et al. 2005) *Chloroflexi* were the most dominant members of heterotrophic bacteria, followed by α-, β- and γ-*Proteobacteria*.

In the culture supplemented with NO_2^- (NO_2^- bf II), in which *Nitrococcus* was very abundant, no 16S rRNA genes of *Nitrococcus* were obtained. Most of the 31 sequences (18) were closely related to α-*Proteobacteria*, which was in agreement with FISH results. Six sequences were similar to *Planctomycetes* and only two affiliated to the γ-*Proteobacteria*. This result shows that the microbial community might have developed differently in duplicate cultures (NO_2^- bf I and II), showing the need to perform many duplicates. An explanation why *Nitrococcus* 16S rRNA genes were not amplified is that primer binding sites were possibly lacking, although the general primers 616V and 630R should target *Nitrococcus* (Juretschko et al. 1998). As discussed later (4.5.10), *Nitrococcus* 16S rRNA genes could be amplifies using a semispecific PCR with primer pair Ntcoc84V/1492R.

Interestingly, from the culture with antibiotic added (NO_2^- AB bf I), three out of 21 sequences were closely related to *Nitrospira marina* 16S rRNA genes. Most of the remaining sequences belonged to γ-*Proteobacteria* and some sequences to uncultured α-*Proteobacteria*. Eilers et al. (2000) depicted that γ-*Proteobacteria* are typically not abundant members of marine bacterial communities, but they have the potential for rapid growth which enables them to overgrow other bacteria in enrichment cultures.

Thus, it is possible that *γ-Proteobacteria* were not very abundant in the original samples. The phylum *Nitrospira* currently consists of three main monophyletic lineages and the genus *Nitrospira* forms monophyletic sublineages (Daims et al. 2001; Spieck et al. 2006; Lebedeva et al. 2008). Within a lineage, sequences share 16S rRNA similarities of at least 94.9%, while, based on a suggestion by (Stackebrandt and Goebel 1994), the similarities of sequences that belong to different sublineages are always below 94.0%. Sequences showed the highest similarity to *Nitrospira marina* (∼93%). However, since this value is below 94%, they were not falling into sublineage IV, which hosts the cultivated species *Nitrospira marina* (Daims et al. 2001). Thus, sequences obtained in this study were possibly not members of any of the four sublineages. However, these sequences are incomplete (∼900 bp) and their exact phylogenetic affiliation cannot be determined with certainty. Since their similarity is close to 94% it might be possible that full length sequences would affiliate to sublineage IV of *Nitrospira*.

It is interesting that no *Nitrospira* were detectable using FISH and that no cells were observed that showed their characteristic morphology. However, it is still possible that low numbers of *Nitrospira* cells were present and overlooked. Even if *Nitrospira* have been overlooked by FISH it is still not likely that such a small amount was actively oxidizing nitrite and not getting enriched, despite their slow growth rate. Besides, nitrite consumption in cultures in which *Nitrococcus* was abundant was comparable to cultures supplemented with antibiotics. Although different NOB are adapted to different substrate concentrations, it is not likely that a very small, overlooked population of NOB oxidizes the same amount of nitrite as a highly abundant population of *Nitrococcus*. Therefore, a more likely possibility is that the majority of NOB was attached to the glass surface of the flasks.

Nevertheless, *Nitrospira* 16S rRNA gene sequences were found in a culture supplemented with streptomycin. *Nitrospira*-like bacteria which fell into sublineage I of *Nitrospira* were reported to be resistant to low concentrations of ampicillin (50 μg/ml), and this was used to suppress growth of *Nitrobacter* and heterotrophic bacteria (Spieck et al. 2006). However, they reported that tetracycline and streptomycin (50-100 μg/ml) caused decrease of *Nitrospira*-like bacteria. In this study streptomycin was added to cultures with a final concentration of 100 μg/ml, which makes it unlikely that *Nitrospira* was inside the cultures. On the other hand, it is possible that *Nitrospira* were located inside the biofilm. It is known that microbial biofilms provide protection against antibiotics probably due to a high binding capacity of charged molecules or degradation of antibiotics by extracellular enzymes (Foley and Gilbert 1996). However, although *Nitrospira*-like bacteria reported by Spieck et al. (2006) were surrounded by dense layers of EPS, they still were sensitive to streptomycin. However, the Spieck and coworkers

investigated *Nitrospira*-like bacteria from activated sludge, and results are probably not comparable to marine ecosystems. It is possible that the microorganisms detected here were resistant to streptomycin, as they differed in their 16S rRNA genes from members of sublineage I. It could be that *Nitrospira*-like bacteria identified in this study gained genetic material from other organisms by horizontal gene transfer. Horizontal gene transfer plays an important role for survival and persistence of microorganisms (Coughter and G.J. 1989).

4.5.9 *NxrB* sequences

Nitrospira nxrB gene fragments were obtained from all four nitrite cultures but clone libraries were performed only for cultures supplemented with NO_2^- (NO_2^- bf I, II) and supplemented with NO_2^- and antibiotics (NO_2^- AB bf I). Only in culture NO_2^- bf II *nxrB* fragments could be amplified using primers for *Nitrobacter*, which also target *nxrB* of *Nitrococcus*. BLAST hits did not reveal the definite origin of the sequences, since nitrite-oxidoreductase has high similarity to respiratory nitrate reductase (Kirstein and Bock 1993; Poly et al. 2007; Starkenburg et al. 2008).

However, tree calculation using ARB showed that sequences indeed grouped together with *Nitrococcus mobilis nxrB*. Interestingly, *nxrB* gene sequences showed 97.4-98.7% similarity to *nxrB* of *Nitrococcus mobilis*, while their 16S rRNA genes were 100% similar to each other (see below). This suggests that *nxrB* is more variable than 16S rRNA genes of *Nitrococcus*. Poly et al. (2007) stated that, in contrast to the highly similar 16S rRNA genes of NOB, the more variable *nxrA* gene provides a good marker gene for studying molecular diversity of NOB (Poly et al. 2007). It is likely, that this is also true for *nxrB* genes of NOB.

Interestingly, in culture I, in which some *Nitrococcus* had been detected in early cultures, no *Nitrococcus nxrB* could be amplified, indicating that *Nitrococcus* probably were overgrown by other NOB or heterotrophs by then.

4.5.10 16S rRNA gene sequences of *Nitrococcus mobilis*

Interestingly, *Nitrococcus* 16S rRNA genes were only obtained after a semispecific PCR using Ntcoc84V/1492R, but not with primers Ntcoc84V/630R. This shows that primer 630R was the reason why *Nitrococcus* 16S rRNA genes had not been amplified before. 630R binds at position 1528-1542 and targets most of bacteria (Juretschko et al. 1998), 1492R binds position 1492-1510 and targets most of bacteria and archaea (Loy et al.

2002). The 18 full sequences had a similarity of 100% to each other and to *Nitrococcus mobilis*. Thus, according to 16S rRNA gene similarity, neither a new species nor a new strain had been enriched. However, *nxrB* genes showed more diversity. Although it is very unlikely, it cannot be completely excluded that the positive control (pure culture of *Nitrococcus mobilis*) was cloned.

4.5.11 Effective SIP

Cultures were incubated with stable isotopes to perform Raman microscopic analysis in order to test for autotrophic growth. For SIP incubations, newly diluted cultures were connected to peristaltic pumps, automatically aerated and fed with either ^{12}C or ^{13}C bicarbonate as the sole carbon source and nitrite as energy source. This system had the advantage that cultures were fed constantly with low amounts of nitrite and optimally supported with oxygen. Besides, this more controlled cultivation method was used in order to enhance growth of biomass, since a sufficient amount of biomass is necessary to perform SIP. Indeed, using this system, increased nitrite consumption was observed in the cultures without antibiotic. Finally, the most active culture (NO_2^- bf II), in which *Nitrococcus* had been identified, oxidized approximately 1.5 mM nitrite per week after around six weeks of incubation compared to 0.5 mM in manually fed cultures. The duplicate culture (NO_2^- bf I) was not active, which probable contained a low number of *Nitrococcus*, oxidized around 800 µM nitrite per week. The cultures with added antibiotic were less active and consumed nitrite in the range of the manually fed cultures. Unfortunately, microorganisms were killed after around 2 months, due to an error in the heating system of the waterbath. Within this time, Raman microscopic analysis could only be performed with culture NO_2^- bf II. Since *Nitrococcus mobilis* could be identified in this culture, Raman microscopic analysis combined with FISH was performed.

4.5.12 Stable isotope labeling combined with Raman-FISH confirms activity of *Nitrococcus mobilis*

Before Raman spectroscopic analysis, *Nitrococcus* cultures were tested for successful enrichment of *Nitrococcus mobilis*. In NO_2^- bf I *Nitrococcus* was rarely detected and only present in some small colonies. This suggests again that in culture I *Nitrococcus* was not the dominant nitrite oxidizer. In NO_2^- bf II supplemented with ^{13}C bicarbonate, *Nitrococcus mobilis* had been highly enriched. A lot of microcolonies had formed which were embedded in layers of EPS. A possible reason why *Nitrococcus* was more

abundant in cultures that were automatically supplemented with NO_2^- than in manually fed cultures is that they were constantly fed with nitrite which increased their growth rate. Raman-FISH was performed with this culture. Besides, from this culture *nxrB* and 16S rRNA sequences of *Nitrococcus mobilis* were already available.

In the ^{12}C mean spectrum and the ^{13}C mean spectrum dominant peaks of cellular compounds were visible, like proteins (e.g. phenylalanine, cytochrome c), nucleic acids, lipids and carbohydrates (Naumann 2001; Maquelin et al. 2002; Pätzold et al. 2006). The ^{12}C- and ^{13}C mean spectra showed "red-shifts" for characteristic peaks, like cytochrome or phenylalanine (see Raman analysis with putative AOB, 4.3.3). In both mean spectra, one pronounced peak of cytochrome c at 747 cm$^-$1 is clearly visible (Fig. 3.18). The phenylalanine peak (1003 cm$^-$1)was used for determination of ^{13}C labeling by calculating the red shift ratio (RSR). The RSR of nearly all cells incubated with ^{13}C was higher than the RSR of cells incubated with ^{12}C. Since the RSR is linearly correlated with the ^{13}C content of cells (Huang et al. 2007), most of the cells were labeled with ^{13}C. According to Huang et al. (2007), cells were labeled on average around 25% up to 40%. In conclusion, incubations with stable isotope for six weeks led to an average labeling of *Nitrococcus mobilis* of around 25%.

Chapter 5

Conclusion

Nitrification is a key part of the global nitrogen cycle and until recently was thought to be catalyzed only by two distinct groups of microorganisms, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). However, the recent detection of ammonia-oxidizing archaea (AOA) in various terrestrial and marine habitats changed the view of the potential key players in nitrogen cycling. For functional and phylogenetic analysis of nitrifying microorganisms, the genes for the key metabolic enzyme of ammonia-oxidizing microorganisms (AOM), ammonia monooxygenase (Amo), and of NOB, nitrite-oxidoreductase (Nxr) are used as markers.

In this study, the physiology of the moderately thermophilic chemoautotrophic AOA "*Candidatus* Nitrososphaera gargensis", a member of the soil group I.1b of crenarchaeota, was investigated for potential mixotrophic growth using microautoradiography (MAR). Since mesophilic crenarchaeota are very abundant and possibly are capable of mixotrophic or heterotrophic growth, studies suggest that potential alternative metabolisms and growth strategies for mesophilic crenarchaeota have to be considered. Unfortunately, only a dead control was performed for bicarbonate, which showed a positive MAR signal. Therefore it is impossible to tell whether MAR signals resulted from substrate adsorption on the cell surface or from incorporation of substrates.

Additionally, the nitrifying thermophilic enrichment culture of "*Candidatus* Nitrososphaera gargensis"was screened for contaminants. The aim was to identify contaminants and use this knowledge also to optimize cultivation strategies. Former MAR experiments identified the contaminants as heterotrophs with a rod-shaped morphology, and one contaminant already has been identified as a putative AOB. One new member of the contaminating population could be identified by its 16S rRNA sequences. Despite several attempts, it was not possible to detect other contaminants in the enrichment

by the 16S rRNA approach, and DGGE or T-RFLP. This is in agreement with other analysis, such as metagenomics.

To proof autotrophic growth of the putative AOB, stable isotope probing with labeled bicarbonate and Raman microscopic analysis were performed. However, no labeling could be detected, suggesting that the incubation time was too short to detect labeling via Raman spectroscopy or that "putative AOB"are not able to oxidize ammonia.

Furthermore, in order to find novel nitrifying microorganisms, enrichment cultures with soil and sludge from a WWTP were performed. No nitrifiers could be enriched, since despite antibiotics cultures were rapidly overgrown by fungi.

In a second attempt to enrich nitrifying microorganisms, biofilm growing on filter balls from an aquarium pool in the "Haus des Meeres"was used as inoculum. It was possible to amplify bacterial and archaeal 16S rRNA genes as well as the functional genes *amoA* and *nxrB* from the biofilm. In addition, decrease of ammonia and nitrite could be observed in the enrichments. In none of the cultures high numbers of archaea could be detected. No known AOB could be identified, however, in some cultures *Nitrococcus mobilis*, a member of NOB, was detected. A clone library and sequence analysis confirmed that the enriched NOB were indeed 100% similar on 16S rRNA level to the isolated *Nitrococcus mobilis*. However, in other active cultures no NOB could be detected and in clone libraries no 16S rRNA genes of nitrifiers were found, except for one culture, in which three clones were most similar to *Nitrospira marina* from "sublineage IV"of the genus *Nitrospira*. In order to show autotrophic growth of *Nitrococcus mobilis* on a single-cell level, a culture that was incubated with ^{13}C-labeled bicarbonate was investigated using Raman microscopy. Finally, Raman-FISH analysis provided proof of autotrophic growth of *Nitrococcus mobilis*. Taken together, the obtained results show that biofilm from aquarium water provided a good sample for enrichments of nitrifying microorganisms and that in some cultures *Nitrococcus mobilis* was successfully enriched. Nitrite-oxidizing cultures, in which no NOB could be identified, possibly contained only a very low amount of nitrifiers or harbored new microorganisms involved in nitrification. Another plausible explanation is that NOB attached to the glass surface of the incubation flasks and were overlooked.

Chapter 6

Zusammenfassung

Nitrifikation stellt einen Schlüsselprozess des globalen Stockstoffkreislaufes dar und wird von zwei verschiedenen Gruppen von Mikroorganismen katalysiert: ammoniak-oxidierenden Bakterien (AOB) und nitrit-oxidierenden Bakterien (NOB). Die kürzliche Entdeckung von ammoniak-oxidierenden Archaeen (AOA) in diversen terrestrischen und marinen Habitaten veränderte das Bild der potentiell beteiligten Mikroorganismen im Stickstoffkreislauf. Das metabolische Schlüsselenzym der ammoniak-oxidierenden Mikroorganismen (AOM) stellt die Ammoniak Monooxygenase (Amo) dar, und jenes der NOB die Nitrit Oxidoreduktase (Nxr). *Amo* und *nxr* Gene werden zur funktionellen und phylogenetischen Analyse von nitrifizierenden Mikroorganismen als Markergene eingesetzt.

In dieser Studie wurde die Physiologie des moderat thermophilen chemoautotrophen AOA "*Candidatus* Nitrososphaera gargensis", einem Mitglied der Gruppe I.1b der Crenarchaeota, auf potentielles mixotrophes Wachstum mittels Mikroautoradiographie (MAR) getestet. Mesophile Crenarchaeoten sind sehr abundant und es gibt Hinweise auf ihr mixotrophes oder heterotrophes Wachstum, weshalb diverse Studien alternative Metabolismen und Wachstumsstrategien mesophiler Crenarchaeoten in Betracht ziehen. Bedauerlicherweise wurde in der Bicarbonat-Totkontrolle des Experiments ein positives MAR Signal beobachtet und für die weiteren verwendeten Substrate keine jeweils eigene Totkontrolle vorbereitet. Daher war es unmöglich zu unterscheiden, ob die MAR Signale aus der Adsorption der Substrate an die Zelloberfläche oder aus der Inkorporation der Substrate in die Zelle resultierten.

Zusätzlich sollten die bakteriellen Begleitorganismen in der nitrifizierenden, thermophilen Anreicherung von "*Candidatus* Nitrososphaera gargensis"identifiziert und nachgewiesen werden, unter anderem um die Kultivierungsstrategie verbessern zu können. Früher

durchgeführte MAR Experimente gaben bereits Hinweise auf heterotrophe Bakterien mit stäbchenförmiger Morphologie. Ein Kontaminant war bereits vor der Studie als putativer AOB identifiziert worden. Im Laufe der Arbeit konnte ein weiterer Kontaminant anhand seiner 16S rRNA Sequenz identifiziert werden. Trotz mehrfacher Ansätze und Versuche war es nicht möglich, die Identität weiterer Kontaminanten anhand des 16S rRNA Ansatzes und mit Fingerprint-Methoden wie DGGE und T-RFLP in der Anreicherung zu bestimmen. Dieses Ergebnis wurde auch durch metagenomische Analyse der Anreicherung unterstützt.

Um autotrophes Wachstum der putativen AOB nachzuweisen, wurden Zellen mit isotopenmarkiertem Bicarbonat inkubiert. Die Inkubationen wurden mittels Raman-Mikrospektroskopie untersucht und ausgewertet. Da keine markierten Zellen entdeckt wurden ist es möglich, dass die Inkubation der AOB mit stabilen Isotopen zu kurz war um markierte Zellen mittels Raman-Mikrospektroskopie zu detektieren, oder, dass die "putative AOB"kein Ammoniak oxidieren können.

Ein zweiter wichtiger Teil der Arbeit beinhaltete den Versuch, neue nitrifizierende Mikroorganismen aus verschiedenen Umweltproben anzureichern. Dazu wurden Kulturen mit Erde und Klärschlamm angesetzt. Allerdings konnten keine Nitrifikanten angereichert werden, da trotz der Zugabe von Antibiotika die Kulturen nach kurzer Zeit von Pilzen überwachsen waren.

In einem weiteren Versuch nitrifizierende Mikroorganismen anzureichern wurde Biofilm, der auf den Filteranlagen eines Aquariums des Haus des Meeres wuchs, als Inokulum für die Anreicherungskulturen verwendet. Hier konnten sowohl bakterielle und archaeelle 16S rRNA Gene als auch die funktionellen Gene *amoA* und *nxrB* amplifiziert werden. Außerdem wurde in den Anreicherungen ein Verbrauch von Ammoniak und Nitrit beobachtet. In keiner der Kulturen waren Archaeen abundant. Weiters konnten keine bekannten AOB gefunden werden, in einigen Kulturen wurde jedoch *Nitrococcus mobilis*, ein Nitrit Oxidierer, identifiziert. Die Erstellung einer Klonbibliothek und anschließende Sequenzanalysen bestätigten die Identität der angereicherten NOB als *Nitrococcus mobilis* basierend auf einer 16S rRNA Ähnlichkeit von 100%. Allerdings konnten in anderen Kulturen, die ebenfalls aktiv Nitrit konsumierten, weder NOB identifiziert, noch ihre 16S rRNA Gene in den dazugehörigen Klonbibliotheken gefunden werden. Eine Ausnahme stellten drei Klone dar, die in einer Kultur gefunden wurden und große Ähnlichkeit zu *Nitrospira marina* aus der Untergruppe IV des Genus *Nitrospira* aufwiesen. Um autotrophes Wachstum von *Nitrococcus mobilis* auf Einzelzellebene nachzuweisen, wurden die Zellen mit ^{13}C-markiertem Bicarbonat inkubiert und anschließend mittels Raman-Mikrospektroskopie untersucht.

Mit Raman-FISH konnte schließlich autotrophes Wachstum von *Nitrococcus mobilis* gezeigt werden. Zusammenfassend zeigen die Ergebnisse dieser Kultivierungen, dass Biofilm aus Aquarium-Filteranlagen eine geeignete Umweltprobe für Anreicherungen von nitrifizirenden Mikroorganismen darstellte und in einigen Kulturen *Nitrococcus mobilis* erfolgreich angereichert werden konnte. Mikrobielle Biofilme in Kulturen, die Nitrit oxidierten, in denen jedoch keine NOB identifiziert werden konnten, bestanden möglicherweise nur zu einem sehr geringen Anteil aus Nitrifikanten oder aber enthielten neue und unbekannte nitrifizierende Mikroorganismen. Eine andere plausible Erklärung dafür, dass keine NOB gefunden werden konnten, ist die Möglichkeit, dass sich die NOB an die Wände der Glasflaschen, in denen sie inkubiert wurden, anlagerten und somit übersehen wurden.

Chapter 7

Abbreviations

16S rRNA	small subunit of rRNA
α	alpha
β	beta
γ	gamma
ε	molar extinction coefficient
λ	lambda, unit of wavelength
µ	micro (10-6)
Ω	Ohm
°C	degree Celsius
%	percent
A	adenine
AB	antibiotic
abs	absolute
Amo	ammonium monooxygenase
amoABC	genes coding for subunits A, B and C of Amo
Amp	ampicillin
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
AOM	ammonia-oxidizing microorganisms
APS	ammonium peroxy-di-sulfate
ARB	software package for phylogenetic analysis (from lat. arbor, "tree")
ATU	allyl-thiourea
bf	biofilm
bidist	double-distilled and filtered
BLAST	basic local alignment search tool

bp	base pair(s)
c	centi ($10^{-}2$)
C	cytosine
Camp	chloramphenicol
CARD	catalyzed reporter deposition
CLSM	confocal laser scanning microsope (or microscopy)
CO_2	carbon di-oxide
Cy3	5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy3.18-derivative N-hydroxysuccimidester
Cy5	5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy5.18-derivative N-hydroxysuccimidester
D	Dalton (1,66018x10-24 g)
DAPI	4'-6'-di-amidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DMF	N,N-di-methylformamide
DMSO	di-methylsulfoxid
DNA	desoxyribonuleic acid
dNTP	desoxy-nucleotide-tri-phosphate
E.	*Escherichia*
EDTA	ethylene-di-amine-tetra-acetic acid
e.g.	exempli gratia (lat., "example given")
EPS	extrapolymeric substance
ERT	Eppendorf reaction tube
et al.	et alteri (lat., "and others")
EtBr	ethidium bromide
EtOH	ethanol
F	forward (used for labeling of primers)
FA	formamide
Fig.	figure
FISH	fluorescence *in situ* hybridization
Fluos	5,(6)-carboxyfluorescein-N-hydroxysuccimidester
g	gram(s)
G	guanine
GC%	mol % guanine and cytosine
h	hour(s)
H^+	Proton
Hao	hydroxylamine oxidoreductase
H_2O	water

H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
i.e.	id est (lat., "that is")
IPTG	isopropyl-â-D-thiogalactopyranoside
IUPAC	International union of pure and applied chemistry
k	kilo (103)
K	guanine or thymine
Kan	kanamycin
KBL	kilobase-ladder (DNA length standard)
KCl	potassium acetate
l	liter(s)
lacZ	gene coding for â-galactosidase
lacZα	α-subunit of lacZ
LB	Luria Bertani
m	milli (10-3); meter(s)
M	molar; adenine or cytosine
MAR	microautoradiography
Max.	maximum
min	minute(s)
ML	maximum likelihood
MOB	methane-oxidizing bacteria
n	nano (10-9)
N	adenine, thymine, guanine or cytosine
NaCl	sodium chloride
NaOH	sodium hydroxide
NH_2OH	hydroxylamine
NH_3	ammonia
NH_4^+	ammonium
NJ	neighbor joining
N_2	dinitrogen
NO_2^-	nitrite
NO_3^-	nitrate
NOA	nitrite-oxidizing archaea
NOB	nitrite-oxidizing bacteria
NOM	nitrite-oxidizing microorganisms
nt	nucleotide(s)

Nxr	nitrite oxidoreductase
nxrAB	genes coding for subunits A and B of Nxr
O_2	molecular oxygen
o/n	overnight
ODx	optical density, measured at a wavelength of x nm
p.a.	*pro analyticum* (lat., "for analysis"), grade of purity
Pa	Pascal
PAA	poly-acrylamide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	para-formaldehyde
PHYLIP	phylogeny interference package (software package for phylogenetic analysis)
Pmo	particulate methane monooxygenase
R	reverse (used for labeling of primers); adenine or guanine
RDP	ribosomal database project
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal RNA
RSR	red shift ratio
RT	room temperature
S	cytosine or guanine
SDS	sodium dodecyl sulfate
sec	second(s)
SN	supernatant
sp.	species (singular)
spp.	species (plural)
T	thymine
T_a	annealing temperature
Tab.	table
TAE	Tris-acetate-EDTA
Taq	thermostable DNA-polymerase from *Thermus aquaticus*
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-tetra-methyl-ethylene-di-amine
Temp.	temperature
T_{inc}	incubation temperature
T-RFLP	Terminal restriction fragment length polymorphism

T-RF	Terminal restriction fragment
U	uracil; unit(s)
UV	ultraviolet
V	forward (used for labeling of primers); Volt
Vol	volume(s)
W	adenine or thymine
w/v	weight per volume
X-Gal	5-brom-4-chlor-3-indolyl-â-D-galactopyranoside

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Appendix A

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Thanks for everything.

Appendix B

Curriculum vitae

Personal data

Name	Nathalie Schuster
Date of birth	16.01.1983
Place of birth	Vienna
Citizenship	Austria
Family status	unmarried

Education

1997 - 2002	Bundes-Oberstufenrealgymnasium für Studierende der Musik, Vienna, 2002 high-school diploma
1997 - 2002	study of oboe at the University of Music and Performing Arts, Vienna
since Mai 2002	study of biology at the University of Vienna with focus on genetics and microbiology
since Mai 2008	diploma work at the Department of Microbial Ecology, Vienna Ecology Center, University of Vienna

Temperature experiment

cultures were incubated at 37°C, 46°C, 56°C and 66°C; duplicates (I, II) were performed; MQ=negative control (H2Obidist); T=temperature

	NH4 and NO2 concentrations measured									
incubations	**08/28/09**		**09/01/09**		**09/04/09**		**09/08/09**		**09/11/09**	
	NH4 µM	**NO2 µM**	**NH4 µM**	**NO2 µM**	**NH4 µM**	**NO2 µM**	**NH4 µM**	**NO2 µM**	**NH4 µM**	**NO2 µM**
start culture	701	798								
37 I			539	910	1010	948	1212	1133	1585	1141
37 II			510	897	927	1045	1153	1210	1455	1241
46 I			479	906	950	1070	1072	1267	1455	1368
46 II			491	1029	953	1029	1167	1238	1571	1293
56 I			314	986	718	1069	938	1259	1305	1416
56 II			372	941	732	1093	993	1246	1339	1317
66 I			162	1041	441	1163	649	1269	1117	1432
66 II			246	636	589	1174	802	1317	1225	1,368
MQ I	601	2	427	1	968	0	1345	0	1703	1
MQ II			454	1	931	1	1257	1	1631	0

	NO2 production						
	28.8.-1.9.	**1.9.-4.9.**	**4.9.-8.9.**	**8.9.-11.9.**	**in 2 weeks**	**per day**	**average per T**
37 I	112	38	185	8	343	25	28
37 II	99	148	165	31	443	32	
46 I	108	164	197	101	570	41	38
46 II	231	0	209	55	495	35	
56 I	188	83	190	157	618	44	41
56 II	143	152	153	71	519	37	
66 I	243	122	106	163	634	45	43
66 II	-162	538	143	51	570	41	
MQ I	-2	-1	0	1			
MQ II	-1	0	0	-1			

Substrate concentrations and pH of the "Haus des Meeres" enrichments

Incubations	10/23/08 NH4	NO2	NO3		10/24/08 NH4	NO2	NO3		10/27/08 NH4	NO2	NO3		10/30/08 pH
	µM	µM	mg/l	µM	µM	µM	mg/l	µM	µM	µM	mg/l	µM	
NH4	534	1	25	403	147	0	25	403	86	0	25	403	
NH4, bf 1	805	26	25	403	161	162	50	806	117	2	25	403	7.9
NH4, bf 2					197	191	50	806	109	0	50	806	
NH4, AB, bf 1					175	21	25	403	93	62	50	806	8,4-8,6
NH4, AB, bf 2					150	385	100	1613	69	264	100	1613	
NO2	1	460	100-250	1613-4032	109	484	100-250	1613-4032	73	499	100-250	1613-4032	
NO2, bf 1	14	aus	100-250	1613-4032	136	440	100-250	1613-4032	74	0	50	806	8.2
NO2, bf 2					145	332	100	1613	74	0	50	806	
NO2, AB, bf 1					179	411	100	1613	65	468	100-250	1613-4032	8,4-8,6
NO2, AB, bf 2					224	OVER	250-500	4032-8060	68	OVER	500	8060	
MQ													
MQ													

OVER=not enough diluted and therefore not measurable

Incubations	10/30/08 NH4	NO2	NO3		11/5/08 NO3		11/7/08 NH4	NO2	11/10/08 pH	11/13/08 pH	11/14/08 NH4	NO2	NO3		11/24/08 pH	NH4	NO2	NO3		11/26/08	12/9/08 NH4	NO2	pH	01/09/09 pH	01/23/09 NH4	01/26/09 some cultures with fungi (c pH	NO2	NO3	
	µM	µM	mg/l	µM	mg/l	µM	µM	µM			µM	µM	mg/l	µM		µM	µM	mg/l	µM		µM	µM			µM		µM	mg/l	µM
NH4	68	3	25	403	25	403	51	21		8	234	35	25	403	<7,5	527	OVER	<500	8060		177	1824			22		2886	>500	>8060
NH4, bf 1	58	3	50	806	<50	806	44	-1		5	211	1	<50	806	<7,5	314	1	50	806		132	0			6		0	50	806
NH4, bf 2	41	0	>50	806	100	1613	76	123	<7,5	5	289	133	>100	1613	<7,5	393	0	100	1613		176	0	>7,2		33		0	100-250	1613-4032
NH4, AB, bf 1	13	117	>50	806	100	1613	23	253		7-7,5	198	312	50	806	8.2	329	422	100-250	1613-4032		101	850			8	7.5	3275	>500	>8060
NH4, AB, bf 2	14	365	100	1613	100-250	1613-4032	13	704		7-7,5	197	913	250-500	4032-8060	7.5	241	1266	500	8060		75	2188			10	7.5	3865	>500	>8060
NO2	7	504	100-250	1613-4032	100	1613	2	516		8	193	498	100-250	1613-4032	8.4	128	425	100-250	1613-4032		27	2		8.4	66	<7,5	0	25	403
NO2, bf 1	9	x0	<100	1613	100-250	1613-4032	30	OVER		5	294	1186	>500	8060	<7,5	586	1289	500	8060		150	1148		8.2	0	~7,5	0	>100	>1613
NO2, bf 2	10	x0	<100	1613	250	4032	33	OVER	<7,5	5	257	1170	500	8060	<7,5	520	1222	<500	8060		160	1		8.2	27	<7,5	0	100-250	1613-4032
NO2, AB, bf 1	9	567	100-250	1613-4032	100-250	1613-4032	3	746		7.5	203	875	250	4032	8.4	280	1028	<500	8060		1	957		8.4	-5	8.6	0	100-250	1613-4032
NO2, AB, bf 2	17	OVER	500	8060	500	8060	4	OVER		7-7,5	207	2300	>500	8060	8.2	353	3228	>500	8060		3	3767		8.4	-8	8.6	OVER	>500	>8060
MQ 29.10.	487	495	100	1613	100-250	1613-4032	199	578	8.2		494	574	100-250	1613-4032	<7,5	655	606			MQ	637	654			553	<7,5	789	~250	4032
MQ 30.10.	490	499	100	1613																									

02/02/09		pH
NH4		<7,5
NH4, AB, bf 1		8.2
	NO2 test stripes fro	
NO2	present	
NO2, bf 1	absent	7–7,5
NO2, AB, bf 2	present	8–8,5

	02/14/09 NO2 mg/l	pH	02/26/09 NO2 mg/l	pH	03/06/09 all original cultures discarded	pH
NH4	/	<7,5	/	<7,5	/	<7,5
NH4, AB, bf 1	/	8.6	/	fungi		
NO2	20	<7,5	20	<7,5	15	<7,5
NO2 bf I	0	8.4	0	8.6	fungi	
NO2 AB bf II	>80	8.6	>80	8.8	>80	8.8
MQ	80		>80			

11/26/08
new dilutions

	11/26/08 NH4	NO2	12/9/08 NH4	NO2	pH	01/09/09 pH	NH4	01/26/09 pH	NO2	NO3	
	µM	µM	µM	µM			µM		µM	mg/l	µM
MQ	129	511	123	536	8.5		283	8.2	574	100-250	1613-4032
NH4	168	182	-6	804	8.2		303	<7,5	2688	>500	>8060
NH4, bf 1	196	-1	127	601	8.2		295		0	~100	1613
NH4, bf 2	190	-1	-5	707	8.2		341		0	~100	1613
NH4, AB, bf 1	110	45	96	107	8.4		220		OVER	>500	>8060
NH4, AB, bf 2	92	143	78	372	8.4		253		OVER	>500	>8060
NO2	-6	472	5	494	8.4	8.2	174	8.4	472	~100	1613
NO2, bf 1	3	562	4	542	8.4	8.4	212	8.8	0	~50	806
NO2, bf 2	0	523	2	284	7.9	8.4	194	<7,5	2	~50	806
NO2, AB, bf 1	-5	554	-9	568	8.8	8.2	157	8.8	7	<50	<806
NO2, AB, bf 2	2	734	-4	782	8.6	8.4	171	8.8	0	<50	<806

	02/02/09 NO2 mg/l	
NH4		<7,5
NH4, bf 1		<7,5
NH4, bf 2		<7,5
NH4, AB, bf 1		<7,5
NH4, AB, bf 2		<7,5
NO2	20	8.2
NO2, bf 1	0	8.4
NO2, bf 2	0	<7,5
NO2, AB, bf 1	20	8.6
NO2, AB, bf 2	~5	8.6

	02/14/09 NO2 mg/l	pH	02/26/09 NO2 mg/l	pH	03/06/09 NO2 mg/l	pH	03/13/09 NO2 mg/l	pH	NH4 (Nessler's reagent fr	03/24/09 NO2 mg/l	pH	NH4	04/02/09 NO2 mg/l	pH	NH4	04/17/09 NO2 mg/l	pH	NH4	NO3 mg/l
MQ	~40	/	~40	/	~40	/	~40	/	yellow to clear	~40	/	yellow to clear	~40	/	yellow	/	/	yellow	
NH4	/	7.5	/	<7,5	/	<7,5	/	<7,5	yellow	/	<7,5	yellow	/	7.5	yellow	/	7.5	clear	
NH4, bf 1	/	<7,5	/	<7,5	/	<7,5	/	<7,5	orange	/	<7,5	orange	/	<7,5	orange	/	<7,5	yellow	
NH4, bf 2	/	<7,5	/	<7,5	/	<7,5	/	<7,5	orange	/	<7,5	yellow	/	<7,5	yellow	/	<7,5	yellow	
NH4, AB, bf 1	/	7.5	/	7.5	/	7.5	/	7.5	yellow	/	8.2	yellow	/	7,5-7,9	yellow	/	8.6	yellow	
NH4, AB, bf 2	/	<7,5	/	<7,5	/	7.5	/	7.5	yellow	/	<7,5	yellow	/	<7,5	yellow	/	7.9	clear	
NO2	20	8,2-8,4	20	8.2	20	7.9	20	7.9	/	20	8.2	/	20	7.9	/	20	7.9	/	100-250
NO2, bf 1	0	8,6-8,8	0	8.8	0	8,6-8,8	0	8.8	/	0	9.1	/	0	9.1	/	0	9.2	/	100-250
NO2, bf 2	0	<7,5	0	<7,5	0	<7,5	0	<7,5	/	0	<7,5	/	0	<7,5	/	0	<7,5	/	100-250
NO2, AB, bf 1	0	8,6-8,8	2–5	8.8	0	8,6-8,8	0	8.8	/	0	8.8	/	0	8.8	/	0	8.8	/	>100
NO2, AB, bf 2	0	8,6-8,8	0	8.8	0	8,6-8,8	0	8.8	/	0	8.8	/	0	9.1	/	0	8.8	/	100-250

	04/27/09 NO2 mg/l	pH	NH4	NO3 mg/l	05/06/09 NO2 mg/l	pH	NH4	NO3 mg/l	05/13/09 NO2 mg/l	NO3 mg/l	05/20/09 pH	05/27/09 pH
MQ	40	/	/	100-250								
NH4	/	<7,5	clear		/	<7,5	clear					
NH4, bf 1	/	fungi	orange									
NH4, bf 2	/	<7,5	clear		/	<7,5	clear					
NH4, AB, bf 1	/	fungi	yellow									
NH4, AB, bf 2	/	7.5	yellow		/	<7,5	yellow					
NO2	10–20	7.9	/	100	5–10	7.9	/	~250				
NO2, bf 1	0	8.8	/	100-250	0	8.8	/	>500	0	250		
NO2, bf 2	0	<7,5	/	100-250	0	<7,5	/	500	0	~500	<7,5	<7,5
NO2, AB, bf 1	0	8.8	/	100-250	0	8.6	/	>500	0	~500		
NO2, AB, bf 2	0	8.8	/	100	0	8.6	/	250	0	~500		

	06/26/09 NO2 mg/l	07/03/09 NO2 mg/l	pH	07/29/09 NO2 mg/l	pH
NO2, bf 1	0	0		0	8.6
NO2, bf 2	0	fungi	<7,5		
NO2, AB, bf 1	0	0		0	8.6
NO2, AB, bf 2	0	0		0	8.6

02/14/09
new dilutions

	02/14/09		02/26/09		03/06/09		03/13/09			03/24/09			04/02/09			04/17/09			
MQ	40	/	40	/	40	/	40	/	yelllow	40	/	yellow	40	/	yellow	40	/	yellow	
NH4	/	8.6	/	8.4	/	8.4	/	8.2	clear	/	7.9	clear	/	7,5-7,9	clear	/	7.9	clear	
NH4, bf 1	/	8.6	/	8.8	/	8.6	/	8.8	orange	/	9.1	orange	/	8.8	orange	/	9.1	yellow	
NH4, bf 2	/	7.5	/	<7,5	/	<7,5	/	<7,5	yellow	/	<7,5	yellow	/	<7,5	clear	/	<7,5	clear	
NH4, AB, bf 1	/	8.6	/	8.8	/	8.8	/	8.8	yelllow	/	9.1	yellow	/	8.8	orange	/	8.8	yellow	
NH4, AB, bf 2	/	8.6	/	8.4	/	8.2	/	8.4	nearly clear	/	8.2	clear	/	7.9	clear	/	8.2	clear	
NO2	~20	8.6	~20	8.6	~20	8.6	~20	8.6	/	20-40	8.6	/	20-40	8.4	/	20	8.6	/	250
NO2, bf 1	0	8.6	0	8.8	0	8.8	0	8.8	/	0	9.1	/	0	8.8	/	0	9.1	/	100
NO2, bf 2	0	8.6	0	8.8	0	8.8	0	8.8	/	0	9.1	/	0	8.8	/	0	8.8	/	100
NO2, AB, bf 1	0	8.6	0	8.8	10	8,6-8,8	0	8.6	/	0	8.6	/	0	8.6	/	0	8.8	/	100
NO2, AB, bf 2	0	8.6	0	8.8	0	8.8	0	8.8	/	0	9.1	/	0	8.8	/	0	8.8	/	100

	04/27/09				05/06/09				05/13/09 used for SIP NO2	
MQ	/	/	yellow							
NH4	/	<7,5	clear		/	<7,5	clear			
NH4, bf 1	/	9.1	yellow		/	9.1	yellow			
NH4, bf 2	/	<7,5	orange		fungi					
NH4, AB, bf 1	/	9.1	yellow		/	9.1	yellow			
NH4, AB, bf 2	/	7.5	clear		/	7.5	clear			
NO2	40	8.6	/	100	20-40	8.6	/	100		
NO2, bf 1	0	8.8	/	100	0	8.8	/	100-250	0	~250
NO2, bf 2	0	8.6	/	50	0	8.6	/	250	0	250
NO2, AB, bf 1	0	8.8	/	50	0	8.8	/	250	0	250
NO2, AB, bf 2	0	8.8	/	50	0	8.6	/	250	0	250

04/17/09
new dilutions

	04/27/09		05/06/09			05/13/09			05/20/09	05/27/09
NO2, bf 1	10	8.8	100	0	8.8	100-250	0	100-250	8.8	
NO2, bf 2	0	8.6	25	0	8.8	100-250	0	100-250		8.6
NO2, AB, bf 1	5–10	8.6	50	0	8.8	100-250	2–5	100-250	8.8	
NO2, AB, bf 2	0	8.8	25	0	8.8	100-250	0	100-250		8.6

	06/26/09 NO2 mg/l	07/03/09 NO2 mg/l	07/29/09 NO2 mg/l	
NO2, bf 1	20	0	0	
NO2, bf 2	0	0	0	8.6
NO2, AB, bf 1	0	0	0	
NO2, AB, bf 2	~10	0	0	

07/22/09
new dilutions

	07/29/09	08/06/09 NO2 mg/l	08/14/09 NO2 mg/l
NO2, Bf 1	5–10	0	0
NO2, Bf 2	5–10	0	0
NO2, AB, Bf 1	20	0	0
NO2, AB, Bf 2	2	0	0

26.6. SIP START	05/27/09			05/29/09			06/01/09	06/02/09			06/03/09		06/04/09			06/05/09	
feeding: 50µM NO2/3h		NO2 mg/l morning	afternoon	feeding: deconnected from pumps		NO2 mg/l	NO2 mg/l	NO2 mg/l	NO3 mg/l	pH	NO2 mg/l	NO3 mg/l	NO2 mg/l morning	afternoon	feeding: 50µM NO2/24h	NO2 mg/l	feeding: 5µM NO2/3h
NO2 AB bf I	13C	10			NO2 AB bf I	13C	10–20	5	50		2–5	25-50	2	>5		5	
	12C	10	<20			12C	~5	~10	50	8,4-8,6	10		10	~20		20	
NO2 AB bf II	13C	10			NO2 AB bf II	13C	~5	2–5	~50		2		2	5		5	
	12C	10	10–20			12C	5	5	~50		5		2–5	<10		5	
NO2 I	13C	10			NO2 I	13C	2–5	2–5	25-50		2		0	5		5	
	12C		10–20			12C	10–20	10	50	8.6	5	50	5	10–20		2–5	
NO2 II	13C	<5			NO2 II	13C	0	2–5	50		0	25-50	2–5	5		0	
	12C					12C	0	~10	25-50		0	25-50	0	5		20	
							manually feeding										

		06/12/09	06/19/09	06/22/09				06/23/09		06/24/09	06/25/09	06/26/09		06/29/09				
		NO2 mg/l	NO2 mg/l	NO2 mg/l morning	NO3 mg/l	NO2 mg/l afternoon	feeding: 10µM NO2/3h	NO2 mg/l	NO3 mg/l	NO2 mg/l	feeding: 16µM NO2/3h	NO2 mg/l	feeding: 14µM NO2/3h	NO2 mg/l morning	NO3 mg/l	feeding: 13µM NO2/3h	NO2 mg/l afternoon	NO2 mg/l late afternoon
NO2 AB bf I	13C	2	0	0	25	0		0	25	0		2–5		~10 (disconn.)			10 (disconn.)	
	12C	20	20	10	50	10 (disconnected)		10 (disconn.)	25	10 (disconn.)		<10 (disconn.)		>5 (disconn.)			5 (disconn.)	
NO2 AB bf II	13C	2	0	0	25	0		0	25	0		2–5		5–10 (disconn.)			~10 (disconn.)	
	12C	0	0	0	25	0		0	25	0		2–5		10 (disconn.)			10 (disconn.)	
NO2 I	13C	0	0	0	25	0		0	25	0		2–5		<5			2–5 (disconn.)	2–5
	12C	0	0	0	25	0		0	25	0		2–5		5			<5 (disconn.)	2–5
NO2 II	13C	0	0	0	25	0		0	25	0		2		0	>25			
	12C	0	0	0	25	0		0	25	0		2		0				

		06/30/09			07/01/09	07/02/09	07/03/09	07/04/09	07/06/09		07/07/09		07/09/09		07/10/09		07/13/09	
		NO2 mg/l morning	NO2 mg/l afternoon	feeding: 13µM NO2/3h	NO2 mg/l	NO2 mg/l	NO2 mg/l	feeding:	NO2 mg/l	feeding:	NO2 mg/l	feeding:	NO2 mg/l	feeding:	NO2 mg/l	feeding:	NO2 mg/l	feeding:
NO2 AB bf I	13C	5–10 (disconn.)			~5 (disconn.)	5	<5	disconn.	2 (disconn.)		0–2	7µM NO2/3h	2–5 (disconn.)		2 (disconn.)		0	10µM NO2/3h
	12C	5 (disconn.)			~5 (disconn.)	5	5	disconn.	<5 (disconn.)		<5 (disconn.)		2–5 (disconn.)		2–5 (disconn.)		2–5 (disconn.)	
NO2 AB bf II	13C	5–10 (disconn.)			~5 (disconn.)	<5	<5	disconn.	0	10µM NO2/3h	0–2	10µM NO2/3h	2 (disconn.)		0	10µM NO2/3h	0	10µM NO2/3h
	12C	5–10 (disconn.)			5–10 (disconn.)	5–10	5	disconn.	<5 (disconn.)		2–5 (disconn.)		2 (disconn.)		0–2	10µM NO2/3h	2 (disconn.)	
NO2 I	13C	2 (disconn.)	0		0–2	0–2	0–2	9µM NO2/3h	0	10µM NO2/3h	0	10µM NO2/3h	0	11µM NO2/3h	0	11µM NO2/3h	0	13µM NO2/3h
	12C	2–5 (disconn.)	2		2	>2 (disconn.)	0	9µM NO2/3h	0	10µM NO2/3h	0	10µM NO2/3h	0	11µM NO2/3h	0	11µM NO2/3h	0	13µM NO2/3h
NO2 II	13C	0			0			17µM NO2/3h	0	20µM NO2/3h	0–2	20µM NO2/3h	0	23µM NO2/3h	0	23µM NO2/3h	0	26µM NO2/3h
	12C	0			0			17µM NO2/3h	0	20µM NO2/3h	0	20µM NO2/3h	0	23µM NO2/3h	0	23µM NO2/3h	0	26µM NO2/3h

		07/15/09		07/17/09
		NO2 mg/l	feeding:	all cultures killed
NO2 AB bf I	13C	0–2	10µM NO2/3h	
	12C	2–5 (disconn.)		
NO2 AB bf II	13C	0	10µM NO2/3h	
	12C	0	10µM NO2/3h	
NO2 I	13C	0	13µM NO2/3h	
	12C	0	13µM NO2/3h	
NO2 II	13C	0	26µM NO2/3h	
	12C	0	26µM NO2/3h	