universität wien

DIPLOMARBEIT

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"Identification and characterization of the nitrifying microorganisms in a temperate beech forest and in enrichment cultures originating from hot springs at Lake Baikal and Kamchatka"

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TABLE OF CONTENTS

A. INTRODUCTION

A.1. Microbial Nitrification

Nitrification, the aerobic process of conversion of ammonia (NH_3) via nitrite (NO_2^-) to nitrate (NO_3^-), is a key process in the global nitrogen cycle (Figure A.1.). It has crucial impact on human activities both beneficial, as in the case of wastewater treatment, as well as detrimental, since the process is responsible for nitrogen loss from agricultural soil.

As shown in Figure A.1. the oxidation process (in blue) comprises two steps, carried out by two distinct groups of organisms, so-called ammonia- and nitrite oxidizers, respectively. These organisms, which are to a large extent not cultivable, maintain a chemolithoautotrophic life-style. They use these inorganic nitrogen compounds as their major energy source (Bock and Wagner 2006) and fix carbon dioxide (CO_2), mainly via the Calvin-Benson-Bassham cycle. Nevertheless the capability of mixotrophic growth was reported for some organisms (Bock and Wagner 2006). The organisms, gaining their energy by oxidation of NH_3 or NO_2^-, are characterized by the suffixes *Nitroso-* and *Nitro-*, respectively (Bock and Wagner 2006).

Until recently it was assumed that these processes are only mediated by bacteria. However, mesophilic as well as thermophilic members of the phylum *Crenarchaeota* were also found to be able to oxidize NH_3 to NO_2^- (Könneke et al. 2005, de la Torre et al. 2008, Hatzenpichler et al. 2008) and might play a significant role in global nitrogen cycling (Prosser and Nicol 2008).

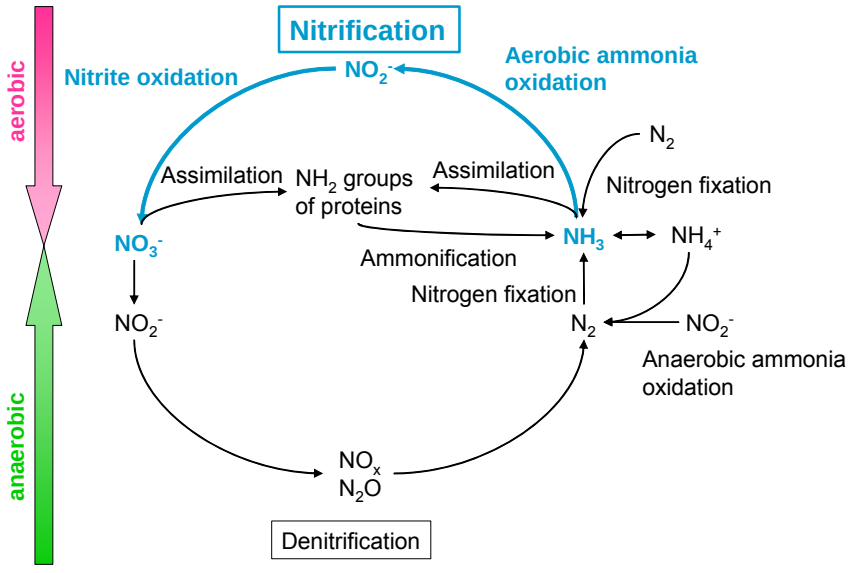


Figure A.1. The biogeochemical nitrogen cycle showing the diverse aerobic and anaerobic conversions of nitrogen. The steps of nitrification are depicted in blue.

Besides these groups, several heterotrophic organisms, bacteria as well as fungi and algae, are also able to oxidize NH_3 as well as organic nitrogen to NO_2^- or NO_3^- (Wood 1988). However, this process, referred to as "heterotrophic nitrification", is not coupled to energy conservation (Wood 1988).

Additionally to aerobic oxidation of NH_3, the possibility of anaerobic ammonium oxidation (ANAMMOX) was suggested as early as 1977 (Broda 1977), but the organisms responsible for the process were just discovered 10 years ago (Strous et al. 1999). These bacteria, phylogenetically affiliated with the *Planctomycetes*, perform oxidation of ammonium (NH_4^+) directly to N_2, with NO_2^- as electron acceptor (Strous et al. 2006).

The following chapters will describe (i) the process of autotrophic nitrification and its key players, which are also shown in figure A.2. and (ii) nitrification in thermal springs since the investigated organisms originate from this habitat (Section A.2.2.).

A.1.1. Ammonia oxidation

The first step of autotrophic nitrification is the oxidation of NH_3 to NO_2^- via hydroxylamine (NH_2OH). This is a two step reaction, mediated by two different enzymes (Figure A.3.) and has been considered to be the rate limiting process (Prosser 1989).

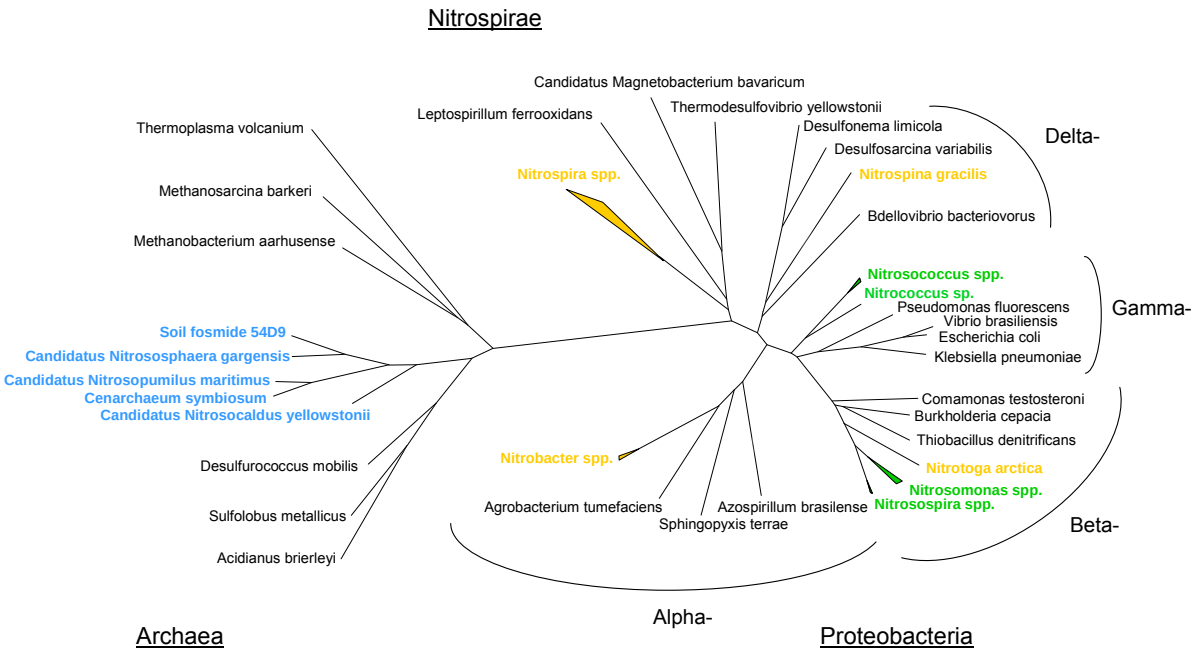


Figure A.2: Schematic phylogenetic tree depicting all known autotrophic nitrifying prokaryotes. Ammonia-oxidizing bacteria are in green, ammonia-oxidizing archaea in blue and nitrite-oxidizing bacteria in yellow.

The conversion is initiated by the ammonia monooxygenase (AMO), which catalyzes the oxidation of NH_3 to hydroxylamine (NH_2OH) in an endergonic reaction (Hollocher et al. 1981). Subsequently the hydroxylamine oxidoreductase (HAO) catalyzes the oxidation of the intermediate NH_2OH to NO_2^- (Figure A.3.), which is the energy gaining reaction (Bock and Wagner 2006). Due to the low energy yield of the overall reaction, ammonia-oxidizing bacteria (AOB) are slow growing organisms with generation times of several hours to days (Jiang and Bakken 1999, Bock and Wagner 2006).

Ammonia monooxygenase: $NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$

Hydroxylamine oxidoreductase: $NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$

Figure A.3. Reactions catalyzed by the AMO and HAO of AOB, respectively.

The AMO, a homologue to the particulate methane monooxygenase (pMMO) (Holmes et al. 1995), has only low substrate specificity. Therefore it oxidizes, among others, also methane or carbon monoxide, which can serve as competitive inhibitors (Bock and Wagner 2006). The enzyme consists of three subunits, AmoA, AmoB and AmoC and additionally, two more ORFs are probably involved in the NH_3 catabolism of AOB (Arp et al. 2007). The bacterial *amoA*, encoding for the α-subunit of the membrane-associated AMO, serves as a well established functional marker in studies of AOB (Rotthauwe et al. 1997, Purkhold et al. 2000) and its crenarchaeal homologue is also used extensively for investigation of the ammonia-oxidizing archaea (AOA) community (e.g. Nicol et al. 2008, Shen et al. 2008, Zhang et al. 2008).

A.1.1.1. Ammonia-oxidizing bacteria

Genera characterized as AOB are restricted to two phylogenetically distinct groups of the *Proteobacteria*. The genera *Nitrosomonas* and *Nitrosospira*, both present in a wide variety of habitats (Purkhold et al. 2000, Koops et al. 2003), are affiliated with the *β-Proteobacteria*, *Nitrosococcus* spp., marine representatives of AOB, are affiliated with the *γ-Proteobacteria* (Purkhold et al. 2000, Koops et al. 2003) (Figure A.2.).

All AOB, as well as the nitrite-oxidizing bacteria (NOB) *Nitrobacter* spp. and *Nitrococcus* spp. are characterized by intracytoplasmic membranes (ICM), which indicates a close relationship with and a putative ancestry from phototrophic bacteria (Teske et al. 1994).

A.1.1.2. Ammonia-oxidizing archaea

Just recently, members of the phylum *Crenarchaeota* were found to be able to oxidize NH_3 (Könneke et al. 2005, de la Torre et al. 2008, Hatzenpichler et al. 2008). Despite the fact that crenrchaeotes were found to be abundant and ubiquitous in mesophilic habitats (e.g. Karner et al. 2001, Ochsenreiter et al. 2003, Kemnitz et al. 2007), their function remained unclear for a long time. A role in nitrification was indicated when a marine ammonia-oxidizing crenarchaeote could be isolated from an aquarium (Könneke et al. 2005) and a crenarchaeal genomic fragment could be associated with *amoA*- and a*moB*-like genes in a metagenomic study in soil (Treusch et al. 2005). Since then, crenarchaeal *amoA* genes were found in various environments, both marine as well as terrestrial (Francis et al. 2005) and several lineages of *Crenarchaeota* could be potentially associated with ammonia oxidation by enrichment or isolation of members of these lineages (Figure A.2.). The presence of genes encoding for an crenarchaeal AMO has been confirmed for two members of marine group I.1a, "*Candidatus* Nitrosopumilus maritimus" and sponge symbiont "*Candidatus* Cenarchaeum symbiosum" (Hallam et al. 2006), for the thermophilic member of group I.1b, "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler et al. 2008) and recently for the thermophile "*Candidatus* Nitrosocaldus yellowstonii", growing at 72°C (de la Torre et al. 2008). The latter organism is affiliated with the "Hot Water Crenarchaeal Group (HWCG) III", now designated "Thermophilic Ammonia-Oxidizing Archaea" (ThAOA) group. All AOA isolated or enriched up to date grow chemolithoautotrophically and their physiological characteristics seem to be similar to those of AOB (Tourna et al. 2008).

Crenarchaeal *amoA* genes are not only ubiquitous (Francis et al. 2005), but have also been shown to be more abundant than bacterial *amoA* genes in various studies in terrestrial (e.g. Leininger et al. 2006, Nicol et al. 2008, Shen et al. 2008) as well as in marine habitats (e.g. Wuchter et al. 2006, Mincer et al. 2007). Furthermore, several studies reported transcriptional activity of crenarchaeal *amoA* genes in soil (Treusch et al. 2005, Leininger et al. 2006, Nicol et al. 2008, Tourna et al. 2008).

Nevertheless it remains questionable whether all of these newly discovered mesophilic *Crenarchaea* are involved in autotrophic nitrification, since there are indeed hints for a heterotrophic lifestyle (Ouverney and Fuhrman 2000, Herndl et al. 2005). For example, a role in carbon cycling was suggested for group I.1c (Kemnitz et al. 2007), a group that was repeatedly found in coniferous forest (Jurgens et al. 1997, Bomberg et al. 2003) and grassland soil ecosystems (Nicol et al. 2003, Nicol et al. 2005, Nicol et al. 2007) besides group I.1b.

The phylogenetic affiliation of the mainly mesophilic "Group I" *Crenarchaeota* remains unclear. Originally they were proposed as mesophilic lineages of the *Crenarchaeota* subdivision, but just recently it was proposed they might form a new lineage designated "*Thaumarchaeota*" (Brochier-Armanet et al. 2008).[1]

A.1.2. Nitrite oxidation

Nitrite oxidation, the second step in nitrification, is carried out by nitrite-oxidizing bacteria (NOB) via the enzyme nitrite oxidoreductase (NXR), converting NO_2^- to NO_3^- (Figure A.4.). In *Nitrobacter* spp. this reaction is reversible in the absence of oxygen (O_2) (Sundermeyer-Klinger et al. 1984, Bock and Wagner 2006). Organisms using NO_2^- as an electron donor are so far only known within the domain *Bacteria* (Figure A.2.).

Nitrite oxidoreductase: $NO_2^- + H_2O \leftrightarrow NO_3^- + 2H^+ + 2e^-$

Figure A.4. Reaction catalyzed by the NXR of *Nitrobacter* spp.

A.1.2.1. Nitrite-oxidizing bacteria (NOB)

Described NOB belong to the genera *Nitrobacter* (α subclass of *Proteobacteria*), *Nitrococcus* (γ subclass of *Proteobacteria*), *Nitrospina* (δ subclass of *Proteobacteria*) and *Nitrospira* (phylum *Nitrospirae*) (Abeliovich 2006). Additionally, an organism designated "*Candidatus* Nitrotoga arctica", constituting a new genus, was isolated just recently and is affiliated with the *β-Proteobacteria* (Alawi et al. 2007). This results in five only distantly related phylogenetic groups of NOB (Bartosch et al. 1999, Abeliovich 2006; Figure A.2.).

While *Nitrospina* sp. and *Nitrococcus* sp. seem to be strict chemolithotrophs, strains of *Nitrobacter* and *Nitrospira* have also been shown to grow under heterotrophic or mixotrophic conditions (Daims et al. 2001, Bock and Wagner 2006).

Due to cultivation biases it was assumed for a long time that *Nitrobacter* spp. is the dominating nitrite-oxidizing organism in terrestrial and freshwater environments (Bock and Koops 1992), which might hold true for some habitats (Cebron and Garnier 2005), but cultivation independent methods revealed that the genus *Nitrospira* is prevalent in a wide range of habitats, such as freshwater (Regan et al. 2002, Altmann et al. 2003), aquaria (Hovanec et al. 1998), wastewater treatment plants (Juretschko et al. 1998, Schramm et al. 1998, Daims et al. 2000) and soil (Smith et al. 2001, Bartosch et al. 2002, Freitag et al. 2005, Urich et al. 2008). Following an ecological concept proposed by MacArthur and

[1] Despite that, in this study the designation as *Crenarchaeota* and crenarchaeal (*amoA*) will be maintained.

Wilson (MacArthur and Wilson 1967) Schramm et al. hypothesized that *Nitrobacter* spp. and *Nitrospira* spp. might occupy different niches, since *Nitrospira* spp. might be K-strategists with high substrate affinity and low growth rate, while *Nitrobacter* spp. might be r-strategists (Schramm et al. 1999).

The residual nitrite-oxidizing genera *Nitrospina* and *Nitrococcus* are restricted to marine habitats (Watson and Waterbury 1971, Abeliovich 2006). For *Nitrotoga* sp., which was isolated from soil, only little information is available up to date, but there is evidence for its occurrence in WWTP (Alawi et al. 2007).

It has been shown that AOB often thrive in close proximity to NOB (Grundmann et al. 2001, Maixner et al. 2006) and maintain a mutualistic relationship, since on the one side AOB are dependent on NOB to remove the toxic metabolic product, but on the other side also NOB benefit due to the direct supply of substrate for energy generation. Similar interactions could be expected for AOA. A study by Mincer and colleagues found the depth distribution of *Nitrospina* sp. to correlate with AOA, therefore supporting the idea of interactions between these organisms (Mincer et al. 2007), but no study addressing this question in more detail has been published yet.

Since up to date the genus *Nitrospira* is the only group of NOB found in thermal habitats (Section A.1.3.), a habitat that was of special interest in this study (Section A.2.2.), it will be described in more detail in the next section.

A.1.2.1.1. The genus *Nitrospira*

Although 16S rRNA gene analysis reveals high diversity within the genus *Nitrospira*, up to date, only members of sublineages IV and II of the genus *Nitrospira* are available in pure culture: *Nitrospira marina*, a marine representative (Watson et al. 1986), and *Nitrospira moscoviensis*, isolated from an heating system in Moscow (Ehrich et al. 1995). Additionally, it was possible to enrich two members of this genus. "*Candidatus* Nitrospira defluvii", affiliated with sublineage I, was enriched from a full scale wastewater treatment plant (Spieck et al. 2006) and "*Candidatus* Nitrospira bockiana", which establishes a new lineage, originated from a Russian hot spring (Lebedeva et al. 2008). Completely missing are representatives of sublineage III, which solely consists of environmental sequences (Holmes et al. 2001), and non-thermophilic representatives of sublineage II, which can be found in various terrestrial habitats with molecular methods. This has also led to the speculation that sublineages I, III and IV mainly consist of specialized NOB, adjusted to distinct environmental conditions, while sublineage II contains more euryoecious NOB

(Daims et al. 2001). A comparable niche differentiation, as described for *Nitrobacter* spp. and *Nitrospira* spp. above, might also determine competition within the genus *Nitrospira*. There is strong evidence that the NO_2^- concentration is a critical factor for the distribution of *Nitrospira* sublineage I and sublineage II (Kim and Kim 2006, Maixner et al. 2006).

A.1.2.2. NXR - the enzyme catalyzing nitrite oxidation

In *Nitrospira* spp. as well as in *Nitrobacter* spp. and *Nitrococcus* sp. nitrite oxidation is catalyzed by the enzyme nitrite oxidoreductase (NXR). The NXR of *Nitrobacter* spp. belongs to the molypterin-binding (MopB) protein-superfamily and is associated with the inside of cytoplasmic and intracytoplasmic membranes (Tanaka et al. 1983, Sundermeyer-Klinger et al. 1984, Spieck et al. 1996). It consists of three subunits, designated NxrA, NxrB and NxrX, encoded by the genes *nxrA*, *nxrB* and *nxrX*, (Kirstein and Bock 1993), of which at least two (NxrA and NxrB) are involved in the catalytic reaction (Meincke et al. 1992), while NxrX might assist in the folding of the NXR (Starkenburg et al. 2006).

The NXR of *Nitrospira* spp. also consists of three subunits, but differs from that of *Nitrobacter* spp. not only by its molecular weight and cytochrome content, but also by the location of the nitrite-oxidizing system, which is located at the periplasmic side of the cytoplasmic membrane in *Nitrospira* spp. (Ehrich et al. 1995). This might actually be advantageous for the organism since no energy expense needs to be made for import of NO_2^- into the cell and accumulation of toxic NO_2^- inside the cytoplasm is avoided (Spieck et al. 1998). On a molecular level analyzing the genome of "*Candidatus* Nitrospira defluvii" showed that its NXR is not closely related to the NXR of *Nitrobacter* spp.. This might indicate a convergent evolution of similar enzymatic systems in two different nitrite-oxidizing systems (Maixner et al., in preparation).

Similar to AMO genes for AOA and AOB, the genes of the NXR might be used as phylogenetic markers in molecular studies of NOB.

While *amoA* is well established as a functional molecular marker investigating ammonia-oxidizing communities in diverse habitats, NXR genes have just recently been addressed in this respect. Poly and colleagues and Wertz and colleagues used the *nxrA* gene of *Nitrobacter* spp., encoding the α-subunit of the NXR, to establish a functional-marker gene based approach (Poly et al. 2008, Wertz et al. 2008). Due to the close evolutionary relationship of *Nitrobacter* species, this may be useful as a more-discriminatory target gene

than the 16S rRNA gene (Grundmann et al. 2000). This was also confirmed by Vanparys et al. (2007), who used the *nxrX* gene and a fragment of *nxrB* as molecular markers.
However, all of these studies were restricted to investigation of the genus *Nitrobacter*, since no molecular data for the NXR of *Nitrospira* sp are available in public databases and the enzymes are not closely related as pointed out above.

A.1.3. Nitrification in hot springs

One example of relevance in this study is nitrification in hot springs. Just recently it has been shown that nitrification takes place in hot springs of up to 85°C, indicating nitrogen cycling in high-temperature environments (Reigstad et al. 2008). Since the occurrence of AOB in hot springs could not be confirmed up to date, while crenarchaeal *amoA* genes can be found globally (Reigstad et al. 2008, Zhang et al. 2008), especially *Crenarchaeota* might be responsible for ammonia oxidation in hot springs. The actual activity of thermophilic and hyperthermophilic AOA could be confirmed by cultivation of such organisms, growing at 46°C (Hatzenpichler et al. 2008) and 72°C, respectively (de la Torre et al. 2008).
So far only *Nitrospira*-like bacteria are potential candidates for the oxidation of NO_2^- in hot springs. They have been observed in and enriched and isolated from thermal environments up to 60°C (Lebedeva et al. 2005, Lebedeva et al. 2008, Weidler et al. 2008). No hyperthermophilic organisms using NO_2^- as energy source are known up to date, even though complete nitrification (from NH_3 to NO_3^-) suggests the existence of such organisms (Reigstad et al. 2008).

A.2. Aims of this study

All projects pursued in this thesis are linked as all of them should contribute to a better understanding of autotrophic nitrifying microorganisms. While the first project (i) aims at characterizing an enrichment culture of nitrifying microorganisms from two hot springs in Russia, the second project (ii) concentrates on investigating the nitrifying community in an acidic forest soil and leaf-litter. Additionally (iii), it was attempted to establish a second molecular marker for the nitrite-oxidizing genus *Nitrospira*, which could help to provide more phylogenetic information concerning the taxonomy of this genus. In the following they will be described in more detail:

(i) Identification and characterization of the nitrifying organisms in four hot spring enrichment cultures

As described in section A.1.3., nitrification is known to take place in hot springs, but only a few actors are known yet. Therefore, this project attempted to identify and characterize the nitrifiers in hot spring enrichment cultures from two different hot springs at Lake Baikal (Russia) and Volcano Uzon (Kamchatka) by diverse molecular methods. Beside the classical amplification and cloning approach, techniques like FISH, CARD-FISH, FISH-MAR and immunofluorescence were applied to characterize the organisms and their physiological traits *in situ*.

(ii) Molecular diversity analysis of nitrifying organisms in acidic forest soil and leaf-litter

This project was loosely linked to the MICDIF project (http://www.micdif.net/), a project that aims "at elucidating the significance of microbial diversity on ecosystem functioning by explicit coupling of microbial ecology and community structure to biogeochemistry"[2] in a forest ecosystem. In this study, as part of this "ecosystem functioning", the community structure of nitrifying organisms in soil and leaf-litter was investigated. Consequently, to get a first insight into the nitrifying microbial community the up to date known groups of nitrifiers were assessed by PCR and cloning followed by phylogenetic analysis.

(iii) Establishment of *nxrB* as a functional marker for nitrite-oxidizers of the genus *Nitrospira*

This project is closely linked to the previous two since the marker was also applied for analysis and characterization of the hot spring enrichment cultures (i) and the nitrifying community in acidic forest soil horizons (ii). The development of this marker was possible due to the fact that during the genome analysis of "*Candidatus* Nitrospira defluvii", the genes encoding NXR in this organism could be identified and primers specific for *nxrB* could be designed (F. Maixner, personal communication). So in the attempt to establish NxrB as a functional marker, diverse samples, from pure cultures of *Nitrospira moscoviensis* to environmental samples, were screened for the gene to expand the database and to investigate the correlation of 16S rRNA- and *nxrB*-based phylogeny of *Nitrospira*-like bacteria.

In other studies *nxrA*, not *nxrB*, was used for the phylogenetic analysis of Nitrobacter (Poly

[2] http://nfn.oberwalder.info/NFN/index.php?page=10634&f=1&i=5597&s=10634

et al. 2008, Wertz et al. 2008). However, since several *Nitrobacter* species as well as "*Candidatus* Nitrospira defluvii*"* possess more than one operon encoding NXR, where *nxrA* is quite dissimilar (only 81.5% in "*Candidatus* Nitrospira defluvii"; F. Maixner, personal communication), this might additionally complicate the phylogenetic analysis. On the contrary, *nxrB* is more conserved at least for "*Candidatus* Nitrospira defluvii" (99.9%) and it might probably be better suited for phylogenetic analyses than *nxrA*. Therefore it was deployed as a functional marker gene in this thesis with the aim to determine whether *nxrB* could be a useful additional functional marker for the phylogenetic analysis of the genus *Nitrospira*.

B. MATERIAL AND METHODS

Water used in this study for buffers and solutions was double distilled and filtered (H_2O_{bidist}) using a water purification facility (Millipore GmbH, Vienna, Austria), unless stated otherwise. Chemicals were purchased and used in *p.a.* quality. Sterilization of all buffers and media was done in a water vapour-high pressure autoclave (Varioclav 135S, H+P, München Germany) for 20 min at 121°C and 1.013×105 Pa pressure, except for substances and solutions unstable at high temperature (e.g. antibiotics), which were added after autoclaving by sterile filtration (0.22 µm pore size, Asahi Techni Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan). Centrifugation was performed using a table-top centrifuge (Mikro 22R or Rotina 35S, Hettich, Tuttlingen, Germany) at room temperature (RT), unless stated otherwise.

B.1. Technical equipment

Table B.1. Technical equipment used.

Equipment	Company
Agarose gel electrophoresis gel carriage:	
Sub-Cell® GT UV-Transparent Gel Tray (15x15 cm)	Bio-Rad Laboratories GmbH, Munich, Germany
Gel running tray, UVT and casting tray (7x10 cm)	Amersham Biosciences, UK
Agarose gel electrophoresis:	
Sub-Cell® GT	Bio-Rad Laboratories GmbH, Munich, Germany
HE Mini Submarine Unit	Amersham Biosciences, UK
Bead beater Fast Prep FP 120	Savant Instruments Inc. Holbrook, NY, USA
CCD camera AxioCam HRc	Carl Zeiss MicroImaging GmbH, Jena, Germany
Centrifuges:	
Mikro 22 R	Andreas Hettich GmbH & Co. Kg, Tuttlingen, Germany
Rotina 35S	Andreas Hettich GmbH & Co. Kg, Tuttlingen, Germany
Mikro 20	Andreas Hettich GmbH & Co. Kg, Tuttlingen, Germany
DNA Sequencer Applied Biosystems 3130	Applied Biosystems, Lincoln, USA
Electrophoresis power supply:	
PowerPac Basic	Bio-Rad Laboratories GmbH, Munich, Germany
EC-105	E-C Apparatus Corporation
Gel documentation system:	
Media System FlexiLine 4040	Biostep, Jahnsdorf, Germany
Heat block VWR Digital Heat block	VWR International, West Chester, PA, USA
Hybridization oven UE-500	Memmert GmbH, Schwabach, Germany
Laminar flow hood, model 1.8	Holten, Jouan Nordic, Allerod, Denmark
Microscopes:	

Inverse microscope Axiovert 25	Carl Zeiss MicroImaging GmbH, Jena, Germany
Epifluorescence microscope Axioplan 2 imaging	Carl Zeiss MicroImaging GmbH, Jena, Germany
Confocal laser scanning microscope LSM 510 Meta	Carl Zeiss MicroImaging GmbH, Jena, Germany
Microwave MD6460 Microstar	
Magnetic stirrer:	
Variomag® Maxi	Variomag®, Dayton Beach, FL, USA
Magnetic stirrer RCT basic	IKA® Werke GmbH & Co.KG, Staufen, Germany
Mixing Block MB-102	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
PCR thermocyclers:	
iCycler Thermal cycler	Bio-Rad Laboratories GmbH, Munich, Germany
iCycler iQ Real-Time PCR Detection	Bio-Rad Laboratories GmbH, Munich, Germany
System Mastercycler Gradient	Eppendorf AG, Hamburg, Germany
pH meter inoLab pH Level 1	Wissenschaftlich technische Werkstätten (WTW) GmbH & Co.KG, Weilheim, Germany
Pipettes:	
Eppendorf Research® pipettes 1 - 1000µl	Eppendorf AG, Hamburg, Germany
Pipetteman® P2 - P1000	Gilson International, Wien, Austria
Photometer:	
NanoDrop® ND-1000 UV/Vis spectrophotometer	NanoDrop Technologies Inc., Wilmington, DE, USA
Platform shaker Innova 2300	New Brunswick Co., Inc., Madison NJ, USA
Scales:	
OHAUS® Analytical Plus balance	Ohaus Corporation, Pine Brook, NJ, USA
Sartorius BL 3100	Sartorius AG, Göttingen, Germany
Sonicator Bandelin Sonoplus HD2070	Bandelin electronic GmbH & Co.KG, Berlin, Germany
Sonotrode Bandelin Sonoplus UW 2070	Bandelin electronic GmbH & Co.KG, Berlin, Germany
Transilluminator	Biostep GmbH, Jahnsdorf, Germany
Ultrasound Cleaner USC100T	VWR International, West Chester, PA, USA
UV sterilizing PCR workstation	PeqLab Biotechnologie GmbH, Erlangen, Germany
Vortex Genie 2	Scientific Industries, New York, USA
Water baths:	
DC10	Thermo Haake GmbH, Karlsruhe, Germany
GFL® type 1004	Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Water purification system MILLI-Q® biocel	Millipore GmbH, Vienna, Austria
Watervapour high pressure autoclaves:	
Varioclav® 135 S h+P	H+P Labortechnik GmbH, Oberschleißheim, Germany
Varioclav® 25 T H+P	H+P Labortechnik GmbH, Oberschleißheim, Germany

B.2. Expendable items

Table B.2. Expendable items used.

Expandable item	Company
Cover glasses 24 x 60 mm	Paul Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany
Culturing vials	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Eppendorf twin.tec PCR plate 96	Eppendorf AG, Hamburg, Germany
Eppendorf PCR film	Eppendorf AG, Hamburg, Germany
Erlenmeyer bulbs DURAN®, various sizes	Schott Glas, Mainz, Germany
Greiner sampling vessels (15 ml, 50 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Lysing Matrix A, bead beating tubes	MP Biomedicals, Germany
Parafilm M Laboratory film	American National Can, Chicago, IL, USA
Petri dishes 94/16	Greiner Bio-One GmbH, Frickenhausen, Germany
Reaction tubes: PCR softtubes (0.2 ml) Reaction tubes (0.5 ml) Reaction tube (1.5 ml, 2 ml)	 Biozym Scientific GmbH, Hessisch Oldendorf, Germany Biozym Scientific GmbH, Hessisch Oldendorf, Germany Greiner Bio-One GmbH, Frickenhausen, Germany
Pasteur pipettes 230 mm	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Plastic pipettes (10 ml, 2 ml), single use, sterile	Barloworld Scientific Ltd., Staffordshire, UK
Microscope slides (76 x 26 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Microscope slides, 10 well	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Syringe (1 ml) Inject® - F 1ml, single use, sterile	B.Braun Melsungen AG, Melsungen, Germany
Syringe (5 ml) Omnifix® single use, sterile	B.Braun Melsungen AG, Melsungen, Germany
Syringe filter, single use, sterile, 0.20 µm pore size	Asahi Techni Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
Tips: MacroTips (1 ml) SafeSeal Tips Premium, various sizes Tips, various volumes	 Biozym Scientific GmbH, Hessisch Oldendorf, Germany Biozym Scientific GmbH, Hessisch Oldendorf, Germany Carl Roth GmbH & Co KG, Karlsruhe, Germany
Twist Top vials (2 ml), conical	Sorenson, Bioscience Inc, Salt Lake City, UT, USA

B.3. Chemicals

Table B.3. Chemicals used

Chemical	Company
4´,6-diamidino-2-phenylindole (DAPI)	Laktan Chemikalien und Laborgeräte GmbH, Graz, Austria
5-brom-4-clor-3-indolyl-ß-D-galactopyranoside (X-Gal)	Carl Roth GmbH & Co., Karlsruhe, Germany

Acetic acid	Carl Roth GmbH & Co., Karlsruhe, Germany
Agar	Fluka Chemie AG, Buchs, Switzerland
Agarose:	
LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Sieve Genetic Pure Agarose (low-melting)	Biozym Scientific GmbH, Hessiche Oldendorf, Germany
Ammonium chloride (NH_4Cl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Ammonium molybdate	Merck GmbH, Vienna, Austria
Ampicillin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
[^{14}C]bicarbonate	Hanke Laboratory Products, Vienna Austria
Blocking reagent	Roche Diagnostics Vienna GmbH, Vienna, Austria
Boric acid heptahydrate (H_3BO_2*$7H_2O$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH & Co., Karlsruhe, Germany
Bromphenol blue	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Calcium carbonate ($CaCO_3$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Calcium chloride ($CaCl_2$)	J. T. Baker, Deventer, Holland
Cetyltrimethylammoniumbromid (CTAB)	Carl Roth GmbH & Co., Karlsruhe, Germany
Citifluor AF1	Agar Scientific Ltd., Stansted, UK
Developer Kodak D19	Kodak GmbH, Vienna, Austria
Dextran sulfate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Di-ethyl-pyrocarbonate (DEPC)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Di-methylsulfoxide (DMSO)	Fluka Chemie AG, Buchs, Switzerland
Di-sodiumhydrogen phosphate (Na_2HPO_4)	Carl Roth GmbH & Co., Karlsruhe, Germany
Di-potassium hydrogen phosphate (K_2HPO_4*$3H_2O$)	J. T. Baker, Deventer, Holland
DNA away™	Molecular BioProducts, Inc., San Diego, CA, USA
Ethanol absolute	AustrAlco Österreichische Alkoholhandels GmbH, Spillern, Austria
Ethidium bromide (EtBr)	Fluka Chemie AG, Buchs, Switzerland
Ethylenediamine-tetraacetic acid (EDTA), di-sodium salt	Carl Roth GmbH & Co., Karlsruhe, Germany
Ficoll® 400	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Film emulsion Hypercoat M-1	Amersham Biosciences, UK
Formamide (FA)	Fluka Chemie AG, Buchs, Switzerland
Glycerol, 87% (w/v)	Carl Roth GmbH & Co., Karlsruhe, Germany
Hydrochloric acid (HCl), 37% (w/w)	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
Iron sulfate pentahydrate ($FeSO_4$*$5H_2O$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Kanamycin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Cresolred	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Lysozyme	Fluka Chemie AG, Buchs, Switzerland
Magnesiumsulfate heptahydrate ($MgSO_4$*$7H_2O$)	Merck GmbH, Vienna, Austria
Maleic acid	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Manganese sulfate ($MnSO_4*H_2O$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Methanol	Carl Roth GmbH & Co., Karlsruhe, Germany
Moviol 4-88	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Nesslers reagent	Fluka Chemie AG, Buchs, Switzerland
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Polyethylen glycol (PEG) 6000	Merck GmbH, Vienna, Austria
Poly-L-Lysine	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Potassium acetate	J. T. Baker, Deventer, Holland
Potassium chloride (KCl)	Merck GmbH, Vienna, Austria
Potassium di-hydrogen posphate (KH_2PO_4)	J. T. Baker, Deventer, Holland
Proteinase K	Merck GmbH, Vienna, Austria
RNAse A	Roche Applied Science, Vienna, Austria
RNase away™	Molecular BioProducts, Inc., San Diego, CA, USA
Roti®-Chloroforn/Isoamly-alcohol	Carl Roth GmbH & Co., Karlsruhe, Germany
Roti®-Phenol/Chloroform/Isoamyl-alcohol	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium acetate	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	J. T. Baker, Deventer, Holland
Sodium dodecly sulfate (SDS)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	J. T. Baker, Deventer, Holland
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium nitrite ($NaNO_2$)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium thiosulfate ($Na_2S_2O_3$)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
SYBR® Green I	Cambrex Bio Science, Rockland, Inc., Rockland, ME, USA
Trishydroxymethlaminomethane (Tris)	Carl Roth GmbH & Co., Karlsruhe, Germany
Tryptone	Oxoid Ltd., Hampshire, England
Tween™ 20	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Xylene cyanole FF	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Yeast extract	Oxoid Ltd., Hampshire, England
Zinc sulfate heptahydrate ($ZnSO_4*7H_2O$)	Carl Roth GmbH & Co., Karlsruhe, Germany

B.4. Kits

Table B.4. Kits used.

Kit	Company
Power Soil™ DNA Kit	MOBio Lab. Inc., Salana Beach, CA, USA
TOPO TA Cloning® Kit	Invitrogen Corporation, Carlsbad, CA, USA
QIAprep Spin Miniprep Kit	QIAgen, Hilden, Germany,

B.5. Buffers, media and solutions

B.5.1. General Buffers

a) PBS

(i) PBS stock solution (Na_xPO_4)

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.

(ii) 3xPBS

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

(iii) 1xPBS

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

b) TE-Buffer:

Tris	10 mM
EDTA	1 mM

The pH was adjusted to 8.0 using HCl.

B.5.2. Solutions and media for cultivation of nitrifying bacteria after E. Lebedeva

a) AOB Stock solution 20x:

KH_2PO_4	1.088 g
KCl	1.488 g
$MgSO_4*7H_2O$	0.986 g
NaCl	11.680 g
$H_2O_{bidest.}$	fill up to 1000 ml

b) Trace elements:

$MnSO_4$	44.6 mg
$H_3BO_3*7H_2O$	49.4 mg
$ZnSO_4*7H_2O$	43.1 mg
$(NH_4)_6Mo_7O_{24}*4H_2O$	37.1 mg
$FeSO_4*5H_2O$	24.9 mg
$HCl_{konz.}$	2.5 ml
$H_2O_{bidest.}$	fill up to 1000 ml

c) Cresolred

0.05% cresolred in 0,01 N HCl (50 ml)

d) 2% $NaHCO_3$ (50 ml)

e) Ammonium solution

NH_4Cl	2.5 g
$H_2O_{bidest.}$	fill up to 50 ml

f) Medium with $CaCl_2$

AOB stock solution	50 ml
NH_4Cl (solution above)	0.5 ml
$CaCl_2*2H_2O$	147 mg
Cresolred	1 ml
Trace elements	1 ml
$H_2O_{bidest.}$	fill up to 1000 ml
pH	7.6

g) Medium with $CaCO_3$

AOB stock solution	50 ml
NH_4Cl (solution above)	0.5 ml
$CaCO_3$	4 g
Cresolred	1 ml
Trace elements	1 ml
$H_2O_{bidest.}$	fill up to 1000 ml

pH	7.6

h) NOB stock solution 10x

$CaCO_3$	0.07 g
NaCl	5 g
$MgSO_4*7H_2O$	0.5 g
KH_2PO_4	1.5 g
$H_2O_{bidest.}$	fill up to 1000 ml

i) Nitrite solution:

$NaNO_2$	7 mg/ml

j) NOB medium

NOB stock solution	100 ml
trace elements	1 ml
($NaNO_2$	20 mg)
$H_2O_{bidest.}$	fill up to 1000 ml

The pH is adjusted with NaOH to 8.6. After autoclaving it will drop to approximately 7.6 after two to four days.

B.5.3. Solutions and buffers for DNA/RNA isolation

For inactivation of RNases all solutions for simultaneous DNA/RNA extraction were treated with 0.1% DEPC and incubated at RT over night prior to autoclaving.

B.5.3.1. DNA/RNA isolation after Lueders et al. (2008)

a) 120mM $NaPO_4$ buffer

Na_2HPO_4	112.87 mM,
NaH_2PO_4	7.12 mM

b) TNS-solution

Tris-HCl pH 8.0	500 mM
NaCl	100 mM
10% SDS (w/v)	

c) PEG precipitation solution

NaCl	9.35 g
PEG8000	30 g

B.5.3.2. DNA/RNA isolation after Urich et al. (2008)

a) Extraction Buffer (5% CTAB/120 mM KPO_4, pH8):

K_2HPO_4 x $3H_2O$	5.15 g
KH_2PO_4 (anhydrous)	0.20 g
CTAB	10 g
NaCl	4.09 g

b) PEG precipitation solution

See section B.5.1. c)

B.5.4. Buffers, standards and solutions for agarose gel electrophoresis

a) TAE buffer

(i) 50 x TAE

Tris	2 M
Sodium acetate	500 mM
EDTA	50 mM

pH was adjusted to 8.0 with pure acetic acid.

(ii) 1 x TAE

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

b) TAE buffer, modified (Millipore)

(i) 50 x TAE, modified

Tris	2 M
EDTA	5 mM

pH was adjusted to 8.0 with pure acetic acid.

(ii) 1 x TAE, modified

50 x TAE, modified	20 ml/l
H_2O_{bidist}	ad 1000 ml

c) TBE buffer

(i) 10 x 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

(ii) 1 x TBE

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

d) Loading buffer

Ficoll	25% (w/v)
Bromphenol blue	0.5% (w/v)
Xylencyanol	0.5% (w/v)
EDTA	50 mM

e) Ethidium bromide solution

(i) Ethidium bromide stock solution

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

(ii) Ethidium bromide staining solution

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

f) SYBR® Green I solution

(i) SYBR® Green I staining solution

SYBR® Green I stock solution diluted 1:10 000 in TAE, modified

g) DNA ladder (KbL)

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

B.5.5. Culture media for Escherichia coli (E. coli) strains

a) Luria-Bertani-medium (LB-medium)

Tryptone	10.0 g
Yeast extract	10.0 g
NaCl	5.0 g
$Aqua_{bidist}$	ad 1000 ml
pH	7.0 - 7.5

For solid media 15 g/l agar was added before autoclaving

b) SOC medium (component of the TOPO TA Cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA))

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

B.5.6. Antibiotics

For solid media, the stock solution of the respective antibiotic was added after cooling of the autoclaved medium to approximately 50°C. Media containing antibiotics were stored at 4°C.

For liquid media the antibiotic stock solution was added directly before usage.

a) Ampicillin stock solution

Ampicillin 100 mg/ml

Ampicillin was dissolved in H_2O_{bidist} and added to medium to a final concentration of 140 µg/ml.

b) Kanamycin stock solution

Kanamycin 100 mg/ml

Kanamycin was dissolved in H_2O_{bidist} and added to medium to a final concentration of 140 µg/ml.

B.5.7. Selection and induction solutions

a) X-Gal stock solution

The X-Gal stock solution was used for TOPO-TA cloning and allowed blue/white-screening of transformed cells on LB-agar plates (see also Section B.7.4.). Preparation was done as specified below:

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

X-Gal was dissolved in di-methylformamide (DMF), sterile filtrated (0.22 µm pore size) and stored in the dark at -20°C.

For the screening of insert-positive cells, 40 µl of X-Gal were distributed on the antibiotic containing LB-agar plate before plating the transformed cells.

B.5.8. 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

B.5.9. CARD – FISH solutions

a) Blocking reagent solution

Maleic acid	116 g
5 M NaCl	1.5 ml
H_2O_{bidist}	fill up to 100 ml
Blocking reagent	10 g
pH	7.5

The Blocking reagent needs to be dissolved with shaking and heating in a water bath

a) Hybridization buffer

5 M NaCl	3200 µl
1 M Tris/HCl	400µl
Dextran sulfate[a]	2 g
Formamide	x µl
Blocking reagent (10%)	2000 µl
SDS (10%)	20 µl
H_2O_{bidist}	fill up to 20 ml

[a] keep the mixture in the water bath (48 – 60°C) until the Dextran sulfate dissolves.

Table B.5. FA concentration used for hybridization buffer

FA [%]	FA [ml]	H_2O_{bidist} [ml]
10	2	12
20	4	10
25	5	9
30	6	8
35	7	7
40	8	6
45	9	5
50	10	4
55	11	3
60	12	2
65	13	1
70	14	0

From this solution aliquots of 200 µl were made and stored at -20°C until usage.

B.5.10. Solutions for FISH-Microautoradiography (MAR)

a) Developer

Kodak D19	12 g
H_2O_{bidist}	ad 300 ml

b) Fixative

Sodiumthiosulfate	90 g
H_2O_{bidist}	ad 300 ml

B.5.11. Immunofluorescence solutions

a) Blocking solution

BSA (3%)	0.3 g
1 x PBS	ad 10ml

b) Antibody dilution solution

BSA (0.05%)	10 mg
Tween 20 (0.025%)	5 µl
1 x PBS	ad 20 ml

B.6. Samples and sampling sites

B.6.1. Thermal springs at Lake Baikal and in Kamchatka

Enrichment cultures designated A and C_a originate from the Gorjackinsk hot spring, located in the north eastern part of Baikal rift zone (Buryat Republic, Russia), which was sampled in March 2006. The water temperature at the sampling site was 48°C, the pH was 8.6. For further site description see Lebedeva et al. (in preparation).

Enrichment cultures designated 4 and 8 were sampled in August 2006 at the Caldera of volcano Uzon (Kamchatka, Russia). The water temperature at the sampling site was between 40-45°C (E. Lebedeva, personal communication).

Enrichment cultures were maintained growing on inorganic media containing 0.3 mM $NaNO_2$ (enrichment cultures A, C_a and 4) and 0.3 mM NH_4Cl (enrichment culture 8), respectively, and at a temperature of 46°C and a pH of 7.6. Accumulation of NO_3 could be observed for all of them (Table B.6.). The composition of the media is described in Section B.5.2. All enrichment cultures were maintained adding sodium nitrite ($NaNO_2$) and ammonium chloride (NH_4Cl), respectively, up to the start concentration at least once a week and pH was adjusted with sodium carbonate ($NaHCO_3$) and hydrochloric acid (HCl) using cresolred as an indicator.

Table B.6. Characteristics of enrichment cultures analyzed in this study.

enrichment	origin	growing on	growth temperature	pH	Accumulation of nitrate?
A	Lake Baikal, Russia	Nitrite	46°C	7.6	Yes
C_a	Lake Baikal, Russia	Nitrite	46°C	7.6	Yes
4	Vulcano Uzon, Kamchatka	Nitrite	46°C	7.6	Yes
8	Vulcano Uzon, Kamchatka	Ammonia	46°C	7.6	Yes

B.6.2. Acidic forest soil (MICDIF project)

The MICDIF project sampling site is situated in the Vienna Woods, near Klausenleopoldsdorf (KL), Austria, at coordinates 48°07′ N 16°03′ E. The site is characterized by woodruff-beach vegetation growing on a dystric cambisol. The soil has a relatively low pH of 4. (Kitzler et al. 2006). For a more detailed site description please refer to Kitzler et al. (2006) or Hackl et al. (2005).

In the sampling procedoure three different soil horizons could be distinguished and were sampled separately, namely L_v, F_{zomy} and A_{hb} (characterization after Hackl 2001) in this study referred to as “Litter N”, “Litter A” and “soil” sample, respectively (Figure B.1.). Samples for DNA isolation were taken in February 2008, samples for FISH fixation were

taken in April 2008, using sterile sampling vessels. Samples for FISH were fixed on site and all samples were transported at 4°C and stored at -20°C.

Figure B. 1. Pictures (A) of the samping site and (B) of the different soil horizons with the respective designation in this study (in brackets).

B.6.3. Other samples screened for *nxrB* genes

Further samples screened for *nxrB* genes included biomass of *Nitrospira marina*, kindly provided by E. Spieck, Hamburg, a nitrite-oxidizing enrichment from Beryl Spring (44°, 40'N 110°, 44'W Gibbon Geyser Basin, Yellowstone National Park, USA) and environmental samples, namely cDNA reversely transcribed from DNA isolated from the main full-scale wastewater treatment plant in Vienna, Austria, and DNA material from *Hyrtios proteus* sponge material. The cDNA, which was kindly provided by F. Maixner and H. Koch, was isolated after Griffiths et al. (2000b). Sponge DNA was kindly isolated (after Webster et al. 2008) and provided by F. Behnam (Table B.7.).

Table B.7. Samples screened for *nxrB* genes.

Sample	origin	DNA isolation protocol	provided by
Nitrospira marina	isolate	-[a]	E. Spieck
cDNA HKA	environmental sample	Griffiths et al. (2000)	F. Maixner
BerylSpring DNA	environmental sample	-[a]	E. Spieck
Sponge DNA	environmental sample	Webster et al. (2008)	F. Behnam

[a] for PCR biomass material was heated to 95°C for 5 min

Further *nxrB* sequences obtained from two wastewater treatment plants, soil of the native Rothwald forest, *Nitrospira moscoviensis*, "*Candidatus* Nitrospira bockiana", "*Candidatus* Nitrospira defluvii", sponge material and marine sequencing batch reactor (SBR) enrichment cultures were kindly provided by F. Maixner.

B.7. Phylogenetic Characterization via Polymerase Chain Reaction and Cloning

B.7.1. DNA/RNA Isolation from soil and leaf-litter samples

Environment samples, such as soil, are known to contain high amounts of PCR inhibitors, like humic substances. Therefore different protocols were tested to find a suitable protocol for DNA isolation. Cell disruption, done via bead beating, was applied with varying speed and duration to find a balance between insufficient cell disruption at lower speed and shearing of DNA at harsher conditions (More et al. 1994, Frostegard et al. 1999, Martin-Laurent et al. 2001). Subsequently, the isolated DNA/RNA of each protocol was pooled prior to amplification.

B.7.1.1. DNA/RNA Isolation after Lüders et al. (2004)

Solutions

120mM $NaPO_4$ buffer

TNS-solution

Roti®-Phenol/Chloroform/Isoamylalcohol (1:24:25)

Roti®-Chloroform/Isoamylalcohol (24:25)

PEG precipitation solution

2-propanol

70% $EtOH_{abs}$

Procedure

About 0.5 g of soil or approximately 0.15 g of shredded leaf-litter sample were weight into a Lysing Matrix A bead beating cap (MP Biomedicals, Germany) and 750 µl 120 mM $NaPO_4$ buffer and 250 µl TNS solution were added. Duplicates were homogenized by bead beating 30 s at 4,5 m/s, 40 s at 5,5 m/s and 45 s at 6,5 m/s, respectively, later also referred to as “low”, “medium” and “high” and placed on ice afterwards to avoid degradation of RNA. The samples were spinned down for 4 min at 13 000 rpm in a precooled centrifuge at 4°C and 900 µl of the supernatant was transferred to a new vial and placed on ice. DNA/RNA was extracted with 1 Vol Phenol/Chloroform/Isoamylalcohol (25:24:1) (pH 8), centrifuged again at 4 min at 13 000 rpm and the upper, aqueous phase (800 µl) was transferred afterwards. Subsequently 1 Vol Chloroform/Isoamylalcohol (24:1) was added and centrifuged at the same conditions as above. 650 µl of the supernatant were then mixed with 2 volumes of PEG precipitation solution (1300 µl) and DNA/RNA was precipitated by

spinning 30 min at 4°C (13 000 rpm). The supernatant was removed with a pipette and the pellet was washed with 500 µl ice-cold 70% EtOH. After centrifugation (4 min, 13 000 rpm) and removal of the EtOH DNA/RNA was briefly dried at room temperature for a maximum of 5 min and subsequently resuspended in 50 µl of DEPC treated water.

B.7.1.2. DNA/RNA Isolation after Urich et al. (2008)

Solutions

Extraction Buffer (5% CTAB/120 mM KPO_4, pH8)

Roti®-Phenol/Chloroform/Isoamylalcohol (1:24:25)

Roti®-Chloroform/Isoamylalcohol (24:25)

PEG precipitation solution

2-propanol

70% $EtOH_{abs}$

Procedure

0.5 g of soil or approximately 0.15 g of shredded leaf-litter sample was weight into a Lysing Matrix bead beating tube A (MP Biomedicals, Germany) and for cell lysis 0.5 ml Extraction Buffer (CTAB/KPO_4) and 0.5 ml Phenol/Chloroform/Isoamylalcohol (25:24:1) pH 8 (Sigma) were added. The samples were homogenized by bead beating in duplicates as described in Section B.7.1.1. and centrifuged for 5 min at 13 000 rpm at 4°C afterwards. The upper aqueous phase was transferred into a new 1.5 ml tube and 1 Vol of Chloroform/Isoamylalcohol (24:1) was added and mixed by mix by inverting the tube. After a 5 min centrifugation step at 13 000 rpm the upper phase was again transferred to a new tube and 2 Vol of 30% PEG 8000/1.6 M NaCl were added and incubated for 2 h on ice or over night (ON) at 4°C. Precipitated DNA was spun down for 10 min at 13 000 rpm and the supernatant was taken away carefully. The resulting pellet was washed with 0.5 ml ice cold 70% $EtOH_{abs}$ (made with DEPC treated H_2O), dried for 5 min at 45°C to remove residual ethanol and resuspended in 50 µl sterile DEPC treated water.

B.7.1.3. DNA Isolation with MoBio Power Soil DNA Extraction Kit

Isolation of DNA with Power Soil™ DNA Kit (MOBio Lab. Inc., Salana Beach, CA, USA) was done using 0.5 g of soil or approximately 0.15 g of shredded leaf-litter sample, according to the manufacturer's instructions in duplicates. Alternatively to 10 min vortexing, a 45 s bead beating step at speed 6.5 m/s was applied to the samples.

B.7.2. DNA Isolation from hot spring enrichment cultures

DNA from hot spring enrichment cultures was isolated from 2 ml of enrichment cultures 4 and 8 following the instructions of the Power Soil™ DNA Kit (MOBio Lab. Inc., Salana Beach, CA, USA). For enrichment cultures A and C_a DNA was not isolated, but a cell extract of cells heated to 95°C for 5 min was used as a template for gene amplification. This was also done for 4 and 8 and both, isolated DNA and cell lysate, were used for gene fragment amplification separately.

B.7.3. Quantitative and qualitative analyses of nucleic acids

The efficiency of extraction, thus the optimal balance between amount of DNA extracted and the removal of inhibitory substances, of nucleic acids was evaluated directly by optical inspection via photometric measurements and gel electrophoresis, but also indirectly by testing the performance of the 16S rRNA gene amplification.

B.7.3.1. Photometric analyses of nucleic acids

For quantitative and qualitative photometric analysis of the extract 1 µl of nucleic acid sample was dropped onto the end of a fiber optic cable of the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) and optical density (OD) was measured at a wavelength of 230 nm, 260 nm and 280 nm following the manufacturer's instructions. The OD at a wavelength of 260 nm, the absorption maximum of DNA, is used to quantify DNA concentration, while the ratios of 260/230 and 260/280 give an indication of contamination with humic acids and proteins respectively.

B.7.3.2. Qualitative analyses of nucleic acids using agarose gel electrophoresis

Additionally, the nucleic acid extract was loaded onto an agarose gel to determine the quality of extracted DNA, especially the state of degradation.

Solutions

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

Loading buffer

DNA-ladder (KbL)

EtBr staining solution

Procedure

Depending on application 1% - 2.5% agarose (Invitrogen Corporation, Carlsbad, CA, USA Cambrex) was dissolved in the corresponding volume of 1 x TBE buffer and heated in a microwave oven for melting of the agarose. After cooling the melted solution down to 50°C, the solution was poured into a gel tray (Sub-Cell GT UV-Transparent Gel Tray 15x15 cm, Bio-Rad Laboratories GmbH, Munich, Germany) with combs and was allowed to polymerize for 15 min. Subsequently, the tray was transferred into an electrophoresis unit (Sub-Cell GT, Bio-Rad Laboratories GmbH, Munich, Germany) filled with 1 x TBE buffer and 10 µl of nucleic acid solution, mixed with an equal volume of loading buffer, were loaded into one pocket of the gel. For estimation of fragment size the size marker GeneRuler^{TM} 1kb DNA ladder (Fermentas, St. Leon-Rot, Germany) was applied to at least one pocket. Nucleic acids were separated applying a voltage of 100 V – 130 V for 50 min – 80 min, depending on the agarose concentration of the gel and the expected size of the fragment to be analyzed. The gel was stained in an EtBr-bath for 45 min and detection and documentation of DNA bands was done with a transilluminator (Biostep GmbH, Jahnsdorf, Germany) emitting UV-light (λ = 312 nm) and the affiliated gel-documentation system (Biostep, Jahnsdorf, Germany).

B.7.4. In vitro Amplification of DNA fragments via Polymerase Chain Reaction (PCR)

The PCR technique amplifies DNA regions of interest, which can serve as phylogenetic (16S rRNA gene) or functional (*amoA*, *nxrB*) molecular markers, by enzymatic replication with primers specific for these regions. The procedure consists of three steps: (i) denaturation at 95°C, which leads to the disassociation of the two DNA strands, (ii) annealing at a temperature at which primers attach specifically to the DNA strands of the gene of interest and (iii) elongation for creation of the complementary strand by a DNA polymerase.

All primers were purchased from Thermo Electron GmbH (Ulm, Germany). They were delivered lyophilized and eluted in a volume of H_2O_{bidist} that resulted in stock concentrations of 100 pmol/µl. PCRs were performed in an Icycler (Biorad, Munich, Germany) or the Mastercycler gradient PCR cycler (Eppendorf, Hamburg, Germany).

Since the MoBio Power Soil^{TM} DNA Kit was only used at a later time point all amplifications from soil and leaf-litter not using general 16S rRNA gene primers were done from DNA extracted after Urich et al. (2008).

B.7.4.1. Amplification of 16S rRNA gene fragments

Solutions

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

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

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

Forward primer (50 pmol/µl)

Reverse primer (50 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)

H_2O_{bidist}

Standard reaction mix (50 µl):

template	1-2 µl
buffer (10 x)	5 µl
dNTP-mix	5 µl
Taq	0.25 µl
Forward primer	1 µl
Reverse primer	1 µl
$MgCl_2$ (25 mM)	4 µl
BSA	0.5-1.5 µl
H_2O_{bidist}	ad 50 µl

Since several reactions are needed for PCR, a so called master mix without template was prepared. Subsequently 48 – 49 µl of this mix were transferred to each Eppendorf reaction tube (ERT) and 1-2 µl of template was added. Regularly the template was extracted DNA, but for the enrichment cultures (A, C_a, 4, 8) PCR amplification was also performed directly from the biomass, which was heated to 95°C for 5 min prior to amplification. To check the performance of PCR and for possible contaminations each run included a positive control, where 1 µl of a plasmid, containing the respective insert, was added and a negative control, without any template, respectively.

When additionally the PCR enhancer DMSO was used, this was done according to literature (Kovarova and Draber 2000).

Quality and quantity control of PCR products was done separating, staining and documenting 10 µl of PCR product on an 1.5% agarose gel described previously (Section B.7.3.2.)

Amplification of bacterial and archaeal 16S rRNA gene fragments was done with primers 616V/1492R and 21F/1492R or 21F/958R, which are specific for these domains, respectively (Table B.8.). Since this study was focusing on nitrifying bacteria, also the 16S rRNA gene of *Nitrospira*-like bacteria was targeted with the general bacterial primer 616V and specific *Nitrospira* primer 1158R or using the oligonucleotide probe S-*-Ntspa-0712-a-A-21, designed by Daims et al. (2001) for *in situ* detection of most members of the phylum *Nitrospirae*, as reverse primer (Table B.8.). Conditions for PCR are specified in table B.9.

Table B.8. Primers used for amplification of bacterial or archaeal 16S rRNA gene fragments

Primer name[a]	Sequence (5´-3´)[b]	T_a [°C][c]	Binding position[d]	Specificity	Reference
616V	AGA GTT TGA TYM TGG CTC	54	7 - 24	16S rRNA most *Bacteria*	Juretschko et al. (1998)
1492R	GGY TAC CTT ACG ACT T	56	1492 - 1510	16S rRNA most *Bacteria* and *Archaea*	Lane (1991)
Ntspa1158R	CCC GTT MTC CTG GGC AGT	58	1158 - 1175	16S rRNA most *Nitrospira*	Maixner et al. (2006)
Ntspa712R	CGC CTT CGC CAC CGG CCT TCC	-[e]	712-732	most *Nitrospirae*	Daims et al. (Daims et al. 2001)
Arch21F	TTC CGG TTG ATC CYG CCG GA	56	7 - 26	most *Archaea*	DeLong (1992)
Arch958R	YCC GGC GTT GAM TCC AAT T	56	958 - 976	most *Archaea*	DeLong (1992)

[a] F/V...forward primer, R...reverse primer
[b] abbreviations according to IUPAC: M = A/C, Y = C/T
[c] annealing temperature of the primer
[d] according to E. coli 16S rRNA (Altschul et al. 1990)
[e] to be determined by gradient PCR

Table B.9. Conditions during the amplification of 16S rRNA gene fragments

PCR step	Temp. [°C]	Time	No. of cycles
Denaturing	95	5 min	1
Denaturing	95	40 sec	
Annealing	54-58[a]	40 sec	35
Elongation	72	60-90 sec[b]	
Final Elongation	72	10 min	1

[a] depending on the annealing temperature of the respective primers (Table B.8.)
[b] depending on the length of fragment to be amplified

B.7.4.2. Amplification of *amoA* gene fragments

Bacterial and crenarchaeal *amoA* gene fragments were amplified as described in section B.7.4.1., but with primers specific for the respective gene fragments (Table B.10.) and under PCR conditions described in table B.11.

Table B.10. Primers used for amplification of bacterial and crenarchaeal *amoA* gene fragments

Primer name[a]	Sequence (5´-3´)[b]	Ta [°C][c]	target gene	Specificity	Reference
CrenAmoA1F	AAT GGT CTG GCT WAG ACG C	56	*amoA*	many crenarchaeal AOA	Könneke et al. (2005)
CrenAmoA1R	GAC CAR GCG GCC ATC CA	56	*amoA*	many crenarchaeal AOA	Könneke et al. (2005)
Arch-amoAF	STA ATG GTC TGG CTT AGA CG	53	*amoA*	many crenarchaeal AOA	Francis et al. (2005)
Arch-amoAR	GCG GCC ATC CAT CTG TAT GT	53	*amoA*	many crenarchaeal AOA	Francis et al. (2005)
AmoA1F	GGG GTT TCT ACT GGT GGT	50	*amoA*	β-proteobacterial AOB	Rotthauwe et al. (1997)
AmoA2R	CCC CTC TGC AAA GCC TTC TTC	50	*amoA*	β-proteobacterial AOB	Rotthauwe et al. (1997)

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

Table B.11. Conditions for amplification of *amoA* gene fragments

PCR step	Temp. [°C]	Time	No. of cycles
Denaturing	95	5 min	1
Denaturing	95	40 sec	
Annealing	50-56[a]	40 sec	35
Elongation	72	60 sec	
Final Elongation	72	10 min	1

[a] depending on the annealing temperature of the respective primers (Table B.10.)

B.7.4.3. Amplification of *nxrB* gene fragments

The functional gene *nxrB*, encoding the β-subunit of the nitrite oxidoreductase (NXR) was amplified with primers designed specifically for *nxrB* of *Nitrospira* spp. and *Nitrobacter* spp., respectively (Table B.12.). Conditions for amplification are listed in table B.13.

B.7.4.4. Gradient PCR

Primers, for which the optimal annealing temperature was unknown (newly designed primers) or needed to be determined individually for each sample (*nxrB*), were subjected to a gradient PCR, where several ERT containing the same reaction mix and template were heated to different annealing temperatures. Residual conditions were retained as specified for the respective fragments above.

Table B.12. Primers used for amplification of *nxrB* gene fragments

primer name[a]	Sequence (5´-3´)[b]	Ta [°C][c]	targent gene	Specificity	Reference
NxrBF706	AAG ACC TAY TTC AAC TGG TC	50-62[d]	*nxrB*	*Nitrobacter*	F. Maixner (unpublished)
NxrBR1431	CGC TCC ATC GGY GGA ACM AC	50-62[d]	*nxrB*	*Nitrobacter*	F. Maixner (unpublished)
NxrBF14	ATA ACT GGC AAC TGG GAC GG	-[e]	*nxrB*	*Nitrospira*	F. Maixner (in preparation)
NxrBR1239	TGT AGA TCG GCT CTT CGA CC	-[e]	*nxrB*	*Nitrospira*	F. Maixner (in preparation)
NxrBF19	TGG CAA CTG GGA CGG AAG ATG	-[e]	*nxrB*	*Nitrospira*	F. Maixner (in preparation)
NxrBR1237	GTA GAT CGG CTC TTC GAC CTG	-[e]	*nxrB*	*Nitrospira*	F. Maixner (in preparation)
NxrBF916	GAG CAG GTG GCG CTC CCG	-[e]	*nxrB*	*Nitrospira*	F. Maixner (in preparation)
NxrBF196	TAC ATG TGG TGG AAC A	-[e]	*nxrB*	*Nitrospira*	F. Maixner (in preparation)

[a] F...forward primer, R...reverse primer
[b] abbreviations according to IUPAC: M = A/C, Y = C/T
[c] annealing temperature of the primers
[d] temperatures used in a gradient PCR (Section B.7.3.4.)
[e] needed to be determined individually for each sample

Table B.13. PCR conditions for amplification of *nxrB* gene fragments

PCR step	Temp. [°C]	Time	No. of cycles
Denaturing	95	5 min	1
Denaturing	95	40 sec	
Annealing	50-68[a]	40 sec	35
Elongation	72	90 sec	
Final Elongation	72	10 min	1

[a] depending on the gradient chosen for gradient PCR (Section B.7.3.4.)

B.7.4.5. Control-PCR for isolated DNA, checking for potential contamination

This PCR was performed to check for potential plasmid contamination of the isolated DNA. Therefore the plasmid specific primers M13F and M13R were used (Table B.14.).

Table B. 14. Conditions for amplification of a potential plasmid contamination

Primer[a]	Sequence (5´-3´)	T_a [°C][b]	Specificity	Reference
M13F	5'-GTAAAACGACGGCCAG-3'	60	plasmid	TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA)
M13R	5'-CAGGAAACAGCTATGAC-3'	60	plasmid	

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

PCR was carried out in a 24 µl reaction mix, adding 1 µl of extracted DNA. Conditions during PCR are specified in Table B.15.

Table B.15. Conditions for amplification of a potential contamination

PCR step	Temp. [°C]	Time	No. of cycles
Denaturing	95	5 min	1
Denaturing	95	30 sec	
Annealing	60	30 sec	35
Elongation	72	60 sec	
Final Elongation	72	7 min	1

B.7.5. Cloning of gene amplificates with the TOPO TA cloning® kit

To verify the positive results of PCR and to identify the amplified fragment, it was ligated into a vector and transferred into recombinant *E. coli* TOP10 cells following the manufacturers instructions of the TOPO TA Cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA). TA cloning employs the fact that the *Taq* polymerase adds a single dATP at the end of a PCR product. Catalyzed by a topoisomerase I, which is bound to the linearized vector possessing a single dTTP overhang, PCR product and vector can be ligated. When no fresh PCR product was available, dATP was added in an additional step prior to cloning (Section B.7.5.1.).

Table B.16. Characteristics of *E. coli* strain used.

Strain	Type of transformation	Genotype	Topt. [°C]	Medium
E. coli TOP10	chemical	F'[lacIq Tn10(tetR)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 λ-	37	LB

The vector pCR®II-TOPO contains two antibiotic resistance genes, amp^R and kan^R and the *lacZα* fragment, which is crucial for blue/white screening. When a gene fragment is inserted into the vector, the *lacZα* fragment is interrupted. Therefore it cannot complement the *lacZ* gene, possessed by the *E. coli* cells, so the cell is not able to produce its blue product from X-Gal cleavage. Thus only cells harbouring an insert will appear white.
When it was necessary to cut out PCR product from a gel, the protocol was slightly modified as specified in section B.7.5.4.

B.7.5.1. Addition of a dATP-overhang to the PCR product for cloning

PCR products that were not cloned within 2 days after PCR were frozen until cloning. However, repeated freezing and thawing of the PCR product leads to a loss of the dATP-overhang of the PCR product, which is crucial for cloning. Therefore prior to cloning the PCR product was incubated in a Thermocycler at 72°C for 30 min to allow the Taq-polymerase to add the dATP-overhang in following reaction mix (30 µl):

PCR product	20.5 µl
$MgCl_2$ (25 mM)	3 µl
Buffer (10 x)	3 µl
dATPs (25 µM)	3 µl
Taq DNA polymerase (5u/µl)	0.5 µl

B.7.5.2. Ligation

Standard reaction mix for ligation:

PCR-product	4 µl
Salt solution	1 µl
Vector (pCR®II)	1 µl

Following the manufacturer's instructions fresh or dATP overhang-added (Section B.7.5.1.) PCR product was added to the ligation mixture and ligated at room temperature for 15 min.

B.7.5.3. Transformation

Preparations

After thawing chemical competent *E. coli* TOP10 cells, two LB-Kan or LB-Amp agar plates were dried at 46°C for 30 min for each reaction. SOC medium, a component of the TOPO TA cloning kit, was thawed and warmed to RT. Additionally, as preparation for the heat shock of the competent cells a water bath (Gesellschaft für Labortechnik GmbH, Burgwedel, Germany) was heated to 42°C.

Procedure

2 µl of the ligation reaction mixture were added carefully to one vial of competent cells and incubated on ice for 20 min. Subsequently the competent cells are heat-shocked for 30 sec at 42°C and placed back on ice for 1 min immediately afterwards. Thereafter the cells were supplied with 250 µl of SOC medium and incubated at 37°C for 1h on an orbital shaker at 200 rpm. After spreading 40 µl of X-Gal, 100 µl and 150 µl of the pre-incubated cells were also spread on each agar plate, respectively. The plates were then incubated at 37°C over night.

While the antibiotic selects for those cells containing a plasmid with the respective resistance, additionally blue/white screening allows the differentiation between cells possessing a plasmid with and without insert. White, thus insert containing, colonies were

picked using sterile tooth picks, transferred to the so-called "master plate" (Section B.7.6.2.) and simultaneously checked for the right insert by M13-screening PCR (Section B.7.7.).

B.7.5.4. Cloning with previous gel separation

Solutions

2% (w/v) Nusieve 3:1 agarose (low-melting-point) in 1 x modified TAE buffer
Loading buffer
DNA ladder (KbL)
SYBR® Green I staining solution

Procedure

The fresh or elongated (Section B.7.5.1.) PCR product was separated by gel electrophoresis on a 2% low-melting-point agarose gel at 100 mA for 90 min in an electrophoresis unit (Amersham Biosciences, UK), stained with SYBR® Green I staining solution and the band of the right size was cut from the gel with 50 µl glass capillaries (Idaho Technology Inc., Salt Lake City, UT, USA). The gel piece was melted at 70°C on a heat block (VWR international, West Chester, PA, USA) and after complete dissolution of 80 µl of H_2O_{bidist} was added and incubated further at 70°C for 2 min.
Ligation was done as described above (section B.7.5.2.) but using 1.5 µl of vector (pCR®II) and salt solution and ligating the mixture for 20 min. For transformation the complete ligation reaction mix is used.

B.7.6. Culturing and maintenance of recombinant E. coli strains

B.7.6.1. Culturing and cell harvesting

Solutions

LB medium
Kanamycin or Ampicillin stock solution (100 mg/ml)

Procedure

Recombinant *E. coli* cells were cultured on plates with solid, or in 5 ml tubes with liquid, LB-medium at 37°C. To select for growth of plasmid harboring cells and avoid the growth of cells with out plasmid the medium contained 100 µg/ml of Ampicillin or Kanamycin.

For culturing of cells in liquid medium, a test-tube containing 5 ml LB-medium was inoculated with a single colony from a plate under sterile conditions and incubated at 37°C on an orbital shaker (Innova 2300; New Brunswick Scientific Co., Inc., Madison NJ, USA) ON, shaking at 200 rpm. Cells were harvested the following day by centrifugation (13 000 rpm, 1 min) of 2 x 2 ml in an ERT or used for glycerol stocks (Section B.7.6.2.).

B.7.6.2. Maintenance

Short-term maintenance was achieved by transferring cells from single colonies to a antibiotic containing LB-agar plate (the so-called "masterplate"), which was incubated at 37°C ON and stored at 4°C.

For long-term strain maintenance, all clones with the right insert were kept in a 15% glycerol stock, which was stored at -80°C. Therefore 700 µl of ON grown culture was transferred to a 2 ml screw cap (Sorenson, Bioscience Inc, Salt Lake City, UT, USA), mixed with 300 µl of 50% sterile glycerol solution, incubated for 1 h at room temperature and finally frozen at -80°C.

B.7.7. Identification and screening for recombinant clones

Clones harboring a plasmid with insert could be identified due to selection pressure on the *E. coli* cells by antibiotic-containing medium and blue/white screening (Section B.7.5.). The M13-pimer pair (Table B.14.), which is a component of the TOPO-TA cloning kit, is binding to the flanks of the multiple cloning site within the TOPO-TA cloning vector and was used together with subsequent agarose gel electrophoresis (Section B.7.3.2.) to check for the size of the insert in blue/white screening-positive *E. coli* cells.

Procedure

The PCR reaction mixture was prepared as described in section B.7.4.1. but without addition of BSA. Cells of a single colony were picked using sterile tooth picks and transferred to a "masterplate" for short term maintenance (Section B.7.6.2.) and simultaneously to a well of a 96-well PCR plate (Eppendorf AG, Hamburg, Germany) already containing 25 µl of the PCR master mix. The microtiterplate was sealed with thermostable PCR film (Eppendorf AG, Hamburg, Germany) and PCR was performed under conditions described in table B.15. PCR-products were separated on a 1% agarose gel, stained with EtBr and documented as described in section B.7.3.2.

B.7.8. Restriction fragment length polymorphism (RFLP)

RFLP uses restriction endonucleases to cut DNA at enzyme-specific recognition sites. PCR-products in the expected size were subjected to a RFLP analysis to cut down the number of plasmids to sequence, as clones similar in sequence should have a similar RFLP-pattern. For all RFLP analysis the restriction enzyme *MspI* (Fermentas Life Sciences Inc., Hanover, MD, USA) was used (Table B.17.). The resulting RFLP-pattern was visualized on an agarose gel (Section B.7.3.2.).

Table B.17. Characteristics of restriction enzyme used for RFLP analysis of PCR products.

Enzyme	Restriction site[a]	Buffer	T_{inc} [°C][b]	Company
MspI	C↓CGG	Tango	37	Fermentas Life Sciences Inc., Hanover, MD, USA

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

Standard reaction mix (10 µl):

Restriction enzyme (10 U/µl)	1 µl
Buffer	1 µl
H_2O_{bidist}	3 µl
PCR-product	5 µl

The reaction mix was incubated for 3 h at 37°C. Subsequently, loading buffer was added to the reaction mixture to stop restriction and the fragments were separated on a 2% agarose gel (Section B.7.3.2.).

For each pattern obtained, the plasmid of at least one clone was isolated and the inserts of the purified plasmids were sequenced as described in the following sections.

B.7.8.1. Plasmid preparation

For sequencing and short-time storage the plasmid was isolated from the *E. coli* cells. This was done with the standard E. coli plasmid-miniprep protocol or with the Quiaprep Spin Miniprep Kit (QIAgen, Hilden, Germany), both applying the principle of alkaline lysis.

a) *E. coli* Miniprep

Solutions

P1-buffer

NaOH/SDS solution

Potassium acetate/acetate solution

2-propanol

70% EtOH

Procedure

2x2 ml of an ON grown culture were transferred to a sterile 2 ml ERT and centrifuged (Mikro 20, Andreas Hettich GmbH & Co. Kg, Tuttlingen, Germany) at 13 000 rpm for 1 min at RT. The supernatant was discarded and the pellet was completely resuspended in 100 µl P1-buffer to digest the RNA. After incubating the mix at RT for 5 min, 200 µl of NaOH/SDS solution were added for cell lysis and mixed by inverting the tube several times. The suspension was incubated on ice for 5 min and mixed again during this time. For precipitation of proteins, 150 µl potassium acetate/acetate solution were added, mixed and incubated on ice for another 5 min. Separation of proteins from the DNA was done by centrifugation (13 000, 1 min, RT) and the supernatant (≤ 450 µl) was transferred to a new ERT. DNA was precipitated adding 1 Vol of 2-propanol, inverting the tube several times and incubating the mixture for 10 min at room temperature. After centrifugation for 1 min (13 000 rpm, RT) the supernatant was removed and the DNA pellet was washed with 500 µl of ice cold 70% $EtOH_{abs}$. Following a last centrifugation step (13 000 rpm, 1 min, RT), the supernatant was removed carefully and the pellet was dried shortly at 46°C. Finally the DNA was resuspended in 50 µl $H_2O_{dest.}$ and stored at -20°C. Quantitative analysis of the plasmid extracts was done with the NanoDrop® ND-1000 (Section B.7.3.1.).

b) Quiaprep Spin Miniprep Kit (QIAgen, Hilden, Germany)

This kit separates the plasmids employing the principle of adsorption of DNA onto silica in the presence of high salt concentrations. Extraction of plasmids was done following the manufacturers instructions. Plasmids were finally eluted in 50 µl H_2O_{bidist} and stored at -20°C after quantitative analysis (Section B.7.3.1.).

B.7.9. Sequencing

For sequencing the principle of cycle sequencing was applied. This technique is a combination of the classical di-deoxy mediated chain termination method (Sanger et al. 1977) and a DNA-polymerase including PCR (Saiki et al. 1988).

Sequencing was performed using the DNA Sequencer Applied Biosystems 3130 (Applied Biosystems, Lincoln, USA) following the instructions of the manufacturer with either primer Topo-SeqF or Topo-SeqR (Table B.18.).

Sequences were visualized with the software program Finch TV (Version 1.4; http://www.geospiza.com/Products/finchtv.shtml), vector sequence parts and parts of bad sequence quality were removed. The corrected sequences were then subjected to a BLAST search (Section B.7.10.1.) and imported into the software program ARB (Ludwig et al. 2004).

Table B.18. Primers used for sequencing.

Primer[a]	Sequence (5´-3´)	T_a [°C][b]
Topo-Seq F	AGC TTG GTA CCG AGC T	60
Topo-Seq R	GTA AAA CGA CGG CCA GT	60

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

B.7.10. Sequence analysis

B.7.10.1. Preliminary analysis of sequences

For the first assignment of sequences to a phylogenetic group, they were compared against existing databases using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI, USA; http://www.ncbi.nlm.nih.gov/). The search algorithm employed here, scans the datasets of several different databases to find similar sequences and ranks them according to their significance (Altschul et al. 1990).

B.7.10.2. Comparative sequence analysis using the software ARB

ARB is a software package that includes different programs and tools for sequence database maintenance and analyses. The package comprises tools for import and export of sequence data, sequence alignment, primary and secondary structure editing, filter calculation, phylogenetic analyses as well as primer and probe design (Ludwig et al. 2004).

Automated and manual alignment of sequences

Obtained sequences were imported into the respective ARB database and automatically aligned using the fast aligner function of the editor tool, followed by manual inspection and, if necessary, improvement of the alignment. Protein-coding gene sequences were translated into amino acid (aa) sequences before alignment.

In case partial sequences needed to be assembled, all parts were loaded into a new group in the sequence editor and the assembled sequence was generated from the consensus

sequence using the "create new species from consensus" tool of the ARB editor. Aligned and assembled sequences were subsequently proof read in combination with the software program Finch TV (Version 1.4; http://www.geospiza.com/Products/finchtv.shtml).
To obtain a rough estimation of the phylogenetic position of the new sequence, it was calculated into an existing tree comprising the whole dataset using the "Add species to existing tree"-"Quick add marked" function.

B.7.10.3. Design of 16S rRNA targeted oligonucleotide probes

The design of 16S rRNA targeted oligonucleotide probes used for fluorescence *in situ* hybridization (FISH) studies (Section B.8.) was done using the "Probe design" and "Probe match" tools of ARB. The "Probe design" tool detects possible target regions highly specific for a specified group within sequences, contained in a server-database (the PT-server). Potential probes were evaluated using the "Probe match" tool, in which the probe sequences is aligned to the whole ARB database and the online tool Probe Check (http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home), where the sequence can be aligned to different databases (RDP II, SILVA, Greengenes) (Loy et al. 2008). As a general rule an optimal probe mismatch to non-target groups should be located in the middle of the sequence, rather than on the ends (Stahl and Amann 1991).

B.7.10.4. Detection of chimeric sequences

Chimeric sequences can be a bias introduced by PCR and are a frequent problem in public databases (Hugenholtzt and Huber 2003). Therefore obtained 16S rRNA gene sequences were checked for chimeras three-ways.

a) Test on chimeric sequences in ARB

Analysis of all 16S rRNA gene sequences was done subjecting base positions 1-513, 514-1026 and 1027-1539 (numbering according to *E. coli* positions; Brosius et al. 1981) to phylogenetic analysis using the neighbor joining tool of ARB (Section B.7.10.5.). Inconsistent affiliation to phylogenetic groups within the three different trees was interpreted as being caused by potential chimeric sequences.

b) Test on chimeric sequences using the online tool Bellerophon

The online tool Bellerophon (http://foo.maths.uq.edu.au) calculates partial trees of independent parts of alignments of submitted sequences and compares the distance

matrices. Checking of the sequences was performed using the Huber-Hugenholtz correction and a window size of 300 bp.

c) Test on chimeric sequences using the online tool Chimera check

The online tool Chimera Check of the RDP (Cole et al. 2003) determines if a sequence is composed of two halves, exhibiting different phylogenetic affiliation by moving a hypothetical breaking point through the submitted sequences and checks halves for their phylogenetic affiliation. This tool should just be used as additional indicator for chimeric origin of sequences.

Only if the sequence was detected as chimera with all three programs, the sequence was classified as chimera and removed. If it was only detected by one or two programs the sequence was inspected manually and classification as a chimera was decided from case to case.

B.7.10.5. Phylogeny

For phylogenetic analysis of the obtained sequences, phylogenetic trees were calculated after treeing methods described below and in further details in Ludwig et al. (1998) and Hall (2001) using the software package ARB (Ludwig et al. 2004). In all tree calculations a termini filter was used to exclude primer biased positions and 16S rRNA trees were also calculated with a 50% conservation filter for exclusion of highly variable positions. AmoA phylogeny was based on amino acid sequences, *nxrB* phylogeny was based on nucleic acid sequences. Trees (except AmoA tree) were rooted manually according to the position of the outgroup, containing unrelated or only distantly related but homologous sequences. For the tree calculation only sequences >1 300 bp (16S rRNA), >138 aa positions (Bacterial AmoA), >169 aa positions (Crenarchaeal AmoA) >1 200 bp (*nxrB* of *Nitrospira* spp.) and >1 000 bp (*nxrB* of *Nitrobacter* spp.) were used. Shorter sequences were added afterwards using the "Parsimony interactive" tool of ARB.

a) Neighbour joining and Fitch-Margoliash

As well Neighbour joining (NJ), applied for nucleic acid sequence data, as Fitch-Margoliash, used for amino acid data, are algorithmic distance methods which convert the aligned sequences into a distance matrix of pairwise differences between sequences. Subsequently, sequences with lowest divergence are clustered and the matrix is

recalculated, treating the joined sequences as one, thereby reducing the data set in size at each step. Finally the measured distances are transformed into estimated phylogenetic distance values according to models of evolution (e.g. Jukes-Cantor, Felsenstein, Kimura).
Major advantage of these methods is a low computing time, which allows handling of large data sets, but there are also several drawbacks, as in contrast to all other methods described here, distance methods are algorithmic, not tree-searching methods. Only one tree is calculated in a stepwise manner, other possible trees are already dismissed during calculations, so the optimal tree might not be found, while in tree searching a lot of trees are calculated and the best is chosen with a set of criteria. Additionally, while maximum parsimony or maximum likelihood methods use the original sequence information, comparing characters column by column (character-based methods), distance methods only use the data indirectly via the distance matrix.

b) Maximum parsimony and bootstrapping

Maximum parsimony (MP) trees are based on a model of evolution that assumes that a change in sequence is less likely than preservation. A tree is most parsimonious with the minimum number of changes and therefore minimal branch length. The minimum number of changes is calculated for each column of each tree with an algorithm and this number is the score for the tree. The tree with the lowest score is the most parsimonious one.
This method can lead to wrong tree topologies since at highly variable sites all trees might be equally parsimonious and due to its assumption of equal rates of evolution for different lineages, which is hardly the case. Additionally, it does not account for multiple substitutions that might have taken place at more variable regions of a sequence.

A possibility for validation of the output tree topology is the bootstrapping approach, which pseudoreplicates data collecting to estimate the reliability of the tree up to a thousand times. Each time a clade, present in the original tree, is also present in the pseudoalignment tree a score of 1 is assigned. Confidence values are then assigned to each node of the resulting tree.
Bootstrap analysis was done with a 50% conservation filter and 100 cycles of bootstrapping were performed. With bootstrap values ≥90% a node was considered significantly supported.

c) Maximum likelihood

Based on a heuristic search, maximum likelihood (ML) looks for the tree that, under some model of evolution, maximizes the probability of observing the data, assuming that mutations at different sites occur independently. Starting with a random tree of one site of the alignment, rearrangement of 1branches is done until the tree with the highest likelihood is found. This procedure is repeated with all possible tree topologies and for all sites of the alignment The tree with the highest probability (likelihood) for all sites is finally taken.

d) Tree puzzle

Tree puzzle is a ML-based method, which employs quartet puzzling, an algorithm, wich first randomly orders all the sequences in quartets and afterwards calculates the best tree from these quartets. To create a full tree all remaining sequences are added to this best quartet tree. This procedure is repeated for all sets of four sequences in a heuristic search. The output is a consensus tree that represents the consensus of all resulting "intermediate trees". Also to each node a reliability value is assigned.

e) Construction of a consensus tree

Given that every treeing method has advantages and disadvantages, trees obtained were consolidated in one consensus tree. This was done by merging branches differing in branching order of different treeing methods to multifurcations.

The archaeal 16S rRNA consensus tree for enrichment culture 8 (Figure C.1.) was constructed considering ML- (AxML, RAxML, PhyML) and MP-tree topologies, *nxrB* consensus tree of *Nitrospira*-like sequences (Figure C.3.B) was based on NJ-, ML- (AxML, RAxML, PhyML) and MP-tree topologies.

B.8. *In situ* detection of microbial cells

B.8.1. Fluorescence *In Situ* Hybridization (FISH)

FISH employs the fact that a cell harbors many ribosomes, which can be targeted by short oligonucleotide probes specific for parts of the small subunit of the ribosomal rRNA (16SrRNA). As there are huge public databases of 16S rRNA sequences available, probes specific for a determined phylogenetic group can be designed. These probes, labeled with a fluorescent dye, are brought into fixed cells and hybridized to the 16S rRNA under stringent conditions, so the presence of these phylogenetic groups can be visualized via

epifluorescence microscopy in their natural surrounding (*in situ*) (Amann et al. 1995, Wagner et al. 2003, Daims et al. 2005).

B.8.1.1. Sample fixation

B.8.1.1.1. Cell fixation with paraformaldehyde (PFA)

a) Production of a 4% PFA solution

Solutions

3 x PBS

1 M NaOH

1 M HCl

Procedure

33 ml H_2O_{bidist} were heated up to 60-65°C and 2-3 g of PFA were added. 1 N NaOH was added drop wise until PFA was dissolved completely and the solution cleared up. Subsequently 16.6 ml 3 x PBS were added and the solution was cooled to room temperature. pH was adjusted with HCl to 7.2-7.4. Finally the solution was sterile filtrated and stored at -20°C.

b) Cell fixation with 4% PFA

All samples were fixed directly after sampling by adding 3 Vol of 4% PFA solution to 1 Vol of sample. Dry samples were taken up in some 1 x PBS first. The sample was incubated for 1 h - 12 h on ice. For final fixation samples were centrifuged at 5 000 rpm for 15 min at 4°C, the supernatant was discarded, the pellet washed in 1 x PBS and again sedimented by centrifugation as described above. Finally the pellet was resuspended in 1 x PBS and 1 Vol $EtOH_{abs}$ was added. Samples were stored at -20°C.

B.8.1.1.2. Sample fixation with EtOH

Since PFA fixation leads to high degree of cross-linking in the cell wall of gram-positive bacteria, thus making the cell wall impermeable for the oligonucleotide probes, samples were also fixed with EtOH. Therefore 1 Vol of sample was mixed with 1 Vol of $EtOH_{abs}$ and stored at -20°C.

B.8.1.2. *In situ* hybridization

B.8.1.2.1. 16S rRNA targeted oligonucleotide probes

Appropriate probes were selected via the online database probeBase (Loy et al. 2007). The characteristics of the fluorescent dyes used and applied probes are listed in table B.19. and B.20. Oligonucleotide probes were obtained from Thermo Electron GmbH (Ulm, Germany) High Performance Liquid Chromatography (HPLC)-purified and lyophilized. For a stock solution probes were eluted in 100 µl H_2O_{bidist}. For a working solution it was further diluted to a final concentration of 50 ng/µl for Fluos-probes, and of 30 ng/µl for Cy3- and Cy5-probes. Mixes of probes with the same fluorophore (e.g. EUB-Mix) were made as working solutions with an overall concentration of 50 ng/µl for all probes. Both stock and working solutions were stored at -20°C in the dark.

Table B.19. Fluorescent dyes used for labeling of oligonucleotide probes.

Fluorescence dye	Max. of absorption [nm]	Max. of emission [nm]	ε [l/mol×cm][a]
Fluos	494	518	7.5×10^4
Cy3	554	570	1.3×10^5
Cy5	650	667	$\geq2\times10^5$

[a] molecular extinction coefficient

Table B.20. Probes used for FISH analysis.

Probe name	Sequence (5´-3´)	FA_{conc}[a]	Binding position[b]	Specificity	Reference
EUB338[c]	GCT GCC TCC CGT AGG AGT	0 - 70	338 - 355	most *Bacteria*	Amann et al. (1990)
EUB338 II[c]	GCA GCC ACC CGT AGG TGT	0 - 70	338 - 355	*Planctomycetales*	Daims et al. (1999)
EUB338 III[c]	GCT GCC ACC CGT AGG TGT	0 - 70	338 - 355	*Verrucomirobiales*	Daims et al.(1999)
ARCH915	GTG CTC CCC CGC CAA TTC CT	35	915 - 934	*Archaea*	Stahl and Amann (1991)
Ntspa662	GGA ATT CCG CGC TCC TCT	35	662 - 679	genus *Nitrospira*	Daims et al. (2001)
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	35	712-732	most *Nitrospirae*	Daims et al. (2001)
NonEUB	ACT CCT ACG GGA GGC AGC	0 - 30	-	control (complementary to EUB338)	Wallner et al. (1993)
NonSense	AGA GAG AGA GAG AGA	0	-	control	-
CREN512	CGG CGG CTG ACA CCA G	10	512 - 527	most *Crenarchaeota*	Jurgens et al. (2000)
CArch-EL180	TCC AGG CAT CGT GGT CTA	10	180 - 197	crenarchaeal clones of enrichment culture 8	this study
Ntspa-EL187	GCT TTC CCC CCG GTC CCG TG	10	187 - 206	*Nitrospira*-like clones of enrichemt cultures 4 and 8	this study
Ntspa-EL197	CGT GCC CTC GGC ACA GCT	10	197 - 214	*Nitrospira*-like clones of enrichemt cultures 4 and 8	this study
Ntspa-EL446	TTG CCC GGT CCA TCT TCC	35	446 - 463	*Nitrospira*-like clones of enrichemt cultures 4 and 8	this study

[a] formamide concentration in the hybridization buffer
[b] according to *E. coli* 16S rRNA (Brosius *et al.*, 1981)
[c] these probes were mixed to dectect all *Bacteria*; in the following to this mix is reffered to as EUB-Mix

B.8.1.2.2. Sample preparation

a) Detachment of cells from the soil or leaf-litter matrix

In order to get rid of autofluorescent soil- or large leaf-litter particles in the sample it was necessary to detach cells, possibly embedded in extra polymeric substances (EPS), from the soil or leaf-litter matrix. Therefore, the sample was subjected to sonication treatment.

3 ml of fixed sample were mixed with 3 ml 50% EtOH in a 50 ml sampling vessel and the detergents SDS (10%) and Tween 80 were added to a concentration of 0.1% and 0.05% respectively. Subsequently the sample was sonicated with a sonication probe (Sonotrode Bandelin Sonoplus UW 2070, Bandelin electronic GmbH & Co.KG, Berlin, Germany) for 3x30 s at 10% power. To remove soil particles the sample was centrifuged at 500 rpm for 2 min and the supernatant was transferred to an ERT. After repetition of the centrifugation step again the supernatant was transferred to a fresh ERT and in order to precipitate the remaining biomass it was centrifuged at 14000 rpm for 2 min. The pellet was washed with 1 ml 50% EtOH and finally taken up in 200 µl 50% EtOH and stored at -20°C until further use.

b) Immobilization

0.5-3 µl of sample or cell suspension, depending on the cell density in the respective sample, was pipetted on a roughed well of the slides (Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany) and dried in a hybridization oven (Memmert GmbH, Schwabach, Germany) at 46°C for ~10 min.

c) Dehydration

For dehydration of the cells, the samples were subjected to an increasing EtOH series. Therefore slides were transferred in succession to 50%, 80% and 96% EtOH for 3 min each and dried under an air stream afterwards. Treated like this, slides could be stored at -20°C.

B.8.1.2.3. Probe hybridization

Stringency is crucial for probe hybridization to the target organism. It can be adjusted via temperature, salt concentration and formamide (FA) concentration. As all hybridizations were done at a temperature of 46°C, stringency was achieved by addition of FA to the hybridization buffer (HB) and the concentration of salts in the washing buffer (WB). FA on the one hand weakens hydrogen-bondings between nucleic acids, leading to an increased

stringency, Na^+ ions on the other hand stabilize the rRNA/DNA duplex, due to masking of the negative backbone of the nucleic acids.

Solutions

5 M NaCl

1 M Tris/HCl, pH 8.0

0.5 M EDTA, pH 8.0

10% (w/v) SDS

Formamide (FA)

Hybridization buffer (HB, 1 ml):

5 M NaCl	180 µl
1 M Tris/HCl	20 µl
10% (w/v) SDS	1 µl
FA	x µl
H_2O_{bidist}	ad 1 ml

Washing buffer (WB, 50 ml):

5 M NaCl	x ml
1 M Tris/HCl	1 ml
10% (w/v) SDS	50 µl
0.5 M EDTA	500 µl[a]
H_2O_{bidist}	ad 50 ml

[a] EDTA was used to bind bivalent cations, and added at NaCl concentrations ≤225 mM only. By adding EDTA to the WB, its NaCl concentration increased by 0.01 M.

Table B. 21. Volumes and concentrations of FA, NaCl and EDTA used for hybridization and washing buffer, respectively.

Hybridization buffer			Washing buffer		
FA [%]	**FA [µl]**	**H_2O_{bidist} [µl]**	**NaCl [mM]**	**5 M NaCl [µl]**	**0.5 M EDTA [µl]**
0	0	800	900	9000	-
10	100	700	450	4500	-
20	200	600	225	2140	500
25	250	550	159	1490	500
30	300	500	112	1020	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

9 µl of hybridization buffer and 1µl of each selected fluorescently labeled probe were applied to each well, leading to a final probe concentration of approximately 5 ng/µl for Fluos- and 3 ng/µl for Cy3- and Cy5-labeled probes. Solutions were mixed by pipetting the liquid up and down, without scratching the surface of the slide. Remaining hybridization buffer was transferred to a tissue-containing 50 ml vessel (Greiner Bio-One GmbH, Frickenhausen, Germany). The slide was inserted into the vessel and incubated for 1.5 h - ON at 46°C in a hybridization oven (Hybridization Oven UE500, Memmert GmbH, Schwabach, Germany). After hybridization the slide was washed in the pre-warmed washing buffer for 10 min, followed by a short dipping into ice-cold H_2O_{bidist} and immediate drying with compressed air. Slides were stored at -20°C in the dark until microscopic analysis.

B.8.1.3. Staining with 4'-6'-di-amidino-2-phenylindole (DAPI)

Slides were additionally stained with DAPI, a fluorescent dye, binding double stranded DNA to make all cells visible. DAPI has an adsorption maximum at 358 nm and an emission maximum at 461 nm.

Solutions

DAPI working solution

Procedure

10 µl of DAPI solution were pipetted on each well of the slide and incubated for 5 min in the dark. After disposal of the solution each well was washed with 20 µl of H_2O_{bidist} for 10 min, to remove remaining DAPI and the slide was dried at RT for 20 min in the dark.

B.8.1.4. Confocal Laser Scanning Microscopy (CLSM)

One key feature of the CLSM is a pin hole in the beam path, which only allows the light, reflected or emitted by a single plane, to enter the detector. Therefore, fluorescence emitted by objects outside of the focal point and possible background noise is suppressed. An other key feature is that the specimen are scanned by lasers, the reflected light is detected with a photomultiplier tube and reconstructed by software instead of detection through an eyepiece.

Detection of fluorescently labeled cells

Samples were embedded in Citiflour AF1 (Agar Scientific Limited) to decrease bleaching during microscopic analysis and covered with a coverslip (Paul Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany). Hybridizations were evaluated using the Confocal Laser Scanning Microscope LSM 510 Meta (Zeiss, Jena, Germany) and the associated software. The fluorophore Fluos was excited with an argon laser, with an excitation range of 430-514 nm, fluorophores Cy3 and Cy5 were excited with two helium neon lasers, with an excitation at 543 nm and 633 nm, respectively. DAPI was detected by exposure to UV light. The microscope possessed plan-neoflar objectives with 40 x, 63x and 100 x magnification and a 10 x ocular. Documentation was done using the CLSM associated software.

B.8.2. Catalyzed Reporter Deposition (CARD)-FISH

Common problems observed for FISH are high background, due to autofluorescent particles in environmental samples such as soil or plant material or a weak signal caused by a low ribosomal content of the target cells. Therefore CARD-FISH (Amann et al. 1992) combined with tyramide signal amplification (Schonhuber et al. 1997) was developed. Instead of a fluorescent dye, rRNA-targeted oligonucleotide probes are conjugated with the enzyme horseradish peroxidase (HPR) and used for FISH. The HPR radicalizes added fluorophore-labeled tyramide, which is thereby immobilized in the cell. Thereby the signal intensity is increased about a 10-20 fold (Schonhuber et al. 1997), newer data even suggest an 26 – 41 fold increase (Hoshino et al. 2008).
The following protocol used in this study is also described in Hatzenpichler et al. (2008).

a) Coating of slides with poly-L-lysine

Slides were coated with poly-L-lysine to improve cell immobilization for samples with only low amount of biomass available. As a cationic surfactant, poly-L-lysine permits electrostatic coupling of the sample to the slide and it is suited for microscopy as it shows no autofluorescence under laser excitation.

Solutions

1% HCl in 70% EtOH

0.01% Poly-L-lysine solution

Procedure

10-well slides (Paul Marienfeld, Bad Mergentheim, Germany) were cleaned with 1% HCl in 70% EtOH for 5 min and transferred to 0.01% solution of poly-L-lysine for 5 min afterwards. Coated slides were dried at 60°C for 90 min in a hybridization oven (Memmert GmbH, Schwabach, Germany) and stored in a dry and dustless box at RT.

b) Sample immobilization and dehydration

Sample immobilization and dehydration was done as described in section B.8.1.2.2. b) and c).

c) Embedding

The sample was embedded in 0.2% agarose for treatment of the sample with cell-wall digesting enzymes, which is necessary due to the high molecular size of the HPR-labeled oligonucleotide probes (~40 kDa).

Procedure

0.1 g of low-melting-point agarose (Sieve Genetic Pure Agarose (low-melting), Biozym Scientific GmbH, Hessiche Oldendorf, Germany) was molten in 50 ml of H_2O_{bidist} or 1 x PBS in a microwave oven and kept at a temperature of 46°C. The slides with the immobilized samples were dipped into the solution, leaving a thin film of agarose on the slide and were air dried afterwards.

d) Permeabilization and inactivation of peroxidases

Permeabilization for HRP-labeled probes was done with different cell-wall digesting enzymes, such as Lysozyme or Proteinase K, depending on the organism to investigate.

Solutions

Proteinase K (15 µg/ml) or

Lysozyme solution (10 mg/ml)

0.01 M HCl

MetOH + 0.15% H_2O_2

$H2O_{bidist}$

Procedure

20 µl of Proteinase K, solved in TE-buffer (0.1 M Tris, pH 8, 0.01 M EDTA, pH 8) were pipetted on each well and incubated for 5 – 15 min at RT or 20 µl of Lysozyme solution (in 0.5 M EDTA, 1 M Tris/HCl) for 1 h at 29°C in a moist chamber and washed in H_2O_{bidist} afterwards.

Following the permeabilization treatment, the slide was incubated in 0.01 M HCl for 15 min, to inactivate the Proteinase K. After washing the slide in H_2O_{bidist} for 1 min, it was incubated in methanol (MetOH) containing 0.15% H_2O_2, to inactivate endogenous peroxidases, which would also process the tyramide. Subsequently the slide was again washed in H_2O_{bidist} twice to remove remaining MetOH.

e) Hybridization

Hybridization buffer (Section B.5.9.) and probe working solution were mixed with the probe of choice (Table B.20.) to a final concentration of 0.17 ng/µl and 20 µl were applied to each well. The slide was incubated in a humid chamber, containing a soft tissue and 1 ml of FA in the appropriate concentration, for 3 h at 46°C and treated according to standard FISH protocol (Section B.8.1.2.3.) except that EDTA was also added to the washing buffer below a FA concentration of 20% and the volume of NaCl added was adjusted to that. After the washing step the slide was dipped into 50 ml H_2O_{bidist} to remove remaining salts.

f) Tyramide Signal Amplification

Preparations

Tyramide amplification mix was prepared freshly before incubation, adding 1 µl of 1:10 with H_2O diluted fluorescine-labeled tyramide (final concentration 1:1000; synthesized and kindly provided by R. Hatzenpichler and M. Mussmann after Pernthaler et al. (2004)) and 1 µl of 0.15% H_2O_2 (final concentration 0.0015%) to 100 µl amplification buffer.

Procedure

After incubating the slide in 1 x PBS for 15 min, it was dabbed on paper to remove excess PBS without letting it run dry and 10 µl of tyramide substrate mix was applied to each well immediately. Incubation was done at 46°C for 60 min in a moist chamber and subsequently the slide was again dabbed on paper and washed in 1x PBS for 10 min and in H_2O_{bidist} for 1 min, to remove free tyramide. All incubation steps were done in the dark. The slide was

air dried, counterstained with DAPI, as described in section B.8.3.1. and analyzed by microscopy (Section B.8.1.4.).

B.8.3. FISH – Microautoradiography (MAR)

FISH-MAR provides the opportunity to correlate the *in situ* identification of microorganisms with their activity, by combination of the concept of microautoradiography, using inorganic or organic radioactive-labeled substrates taken up by microorganisms, with FISH (Lee et al. 1999).

Therefore the sample is incubated with radioactive-labeled substrate, such as [^{14}C]bicarbonate, or [^{14}C]acetate, for several hours, followed by fixation of the sample and hybridization with fluorescently labeled oligonucleotide probes. For visualization of the incorporated substrate the sample is covered with photographic film emulsion, which is thereby exposed to the radioactive substrate and forms a latent image on the film that is finally developed.

Since autotrophic nitrification is a dissimilatory process, the activity of nitrifying microorganisms can only be monitored indirectly by adding $^{14}CO_2$ to a medium containing only NH_3 or NO_2^- as energy source, thus only cells able to grow on NH_3/ NO_2^- should be actively metabolizing and taking up the labeled substrate. Advantages of this approach are a very short incubation time of a few hours and the low substrate concentration required to obtain sufficient labeling of the cells.

B.8.3.1. Sample preparation and preincubation

3 ml of enrichment culture 8 were washed with growth medium without NO_2^- (Section B.5.2.) and resuspended in 250 µl fresh medium. To overcome a possible lag-phase of the microorganisms and to avoid false positive signals from microorganisms that could metabolize on storage compounds, the 250 µl biomass were taken up in 2 ml medium, containing 0.3 mM NO_2^-, and were preincubated in vials without addition of $^{14}CO_2$ under aerobic conditions for 90 min at 43°C shaking in a water bath,. The incubations were done in biological replicates and two control experiments (i) without addition of NO_2^-, to check for non-nitrite-oxidation-related activity, and (ii) with biomass, sterilized by addition of 4% PFA for 1 h at RT, to check for chemography, using 3 ml and 1 ml of enrichment culture, respectively, were included.

B.8.3.2. Incubation with $^{14}CO_2$

The preincubated biomass was then amended with 20 µl 500 µCi $^{14}CO_2$ (Hanke Laboratory Products), resulting in a final concentration of 5 µCi, under conditions described above for 14 h and 20 h. Prior to incubation 250 µl of the supernatant were taken for later chemical analysis.

The incubated samples were centrifuged at 13 000 rpm for 10 min and while the supernatant was frozen away at -20°C for subsequent chemical analysis, the remaining samples were fixed with 4% PFA or 50% EtOH as described in section B.8.1.1. The fixed samples were stored at -20°C until further use.

B.8.3.3. Fluorescence *In Situ* Hybridization

FISH was done as described in section B.8.1.2., except that, instead of 10-well slides, coverslips, coated with Poly-L lysine (see section B.8.2. a)) were used.

Following oligonucleotide probes were applied: EUB-Mix, specific for *Bacteria*, Ntspa712 and Ntspa662, specific for the phylum and the genus *Nitrospira(e)* and the newly designed probe Ntspa-EL446, specific for the new lineage of *Nitrospira*-like bacteria found in enrichment cultures 4 and 8 in this study (Section C.1.4.3.).

Additionally the fluorescent stains DAPI or SybrGreen were applied for staining. DAPI was applied as described before (Section B.8.1.3.) prior to FISH, SybrGreen was applied together with Citiflour AF1 directly before microscopic analysis (see also section B.8.1.4.).

B.8.3.4. Microautoradiography

Solutions

Film emulsion (Hypercoat M-1)

Developer

Fixative

Procedure

The film emulsion (Hypercoat M-1, Amersham Biosciences, UK) was molten in a water bath at 48°C for 10 min and diluted with 2 ml of H_2O_{bidist} to gain optimal viscosity. The molten film emulsion was stirred carefully with a glass rod, avoiding air bubbles and poured into a dipping vessel afterwards. Each slide was dipped into the emulsion for approximately 7 sec and dried on the backside carefully to avoid sticking to the ground.

The slides were exposed at 4°C for 12 days and 21 days, respectively, stored in a box horizontally with a package of silica gel.

For development of the film emulsion the slides were placed in the developer solution for 4 min, followed by 1 min incubation in H_2O_{bidist} to stop the development and 4 min incubation in the fixative. To remove the remaining salts from the fixative, finally the slide was washed in H_2O_{bidist} for 2 min. All steps were performed in the dark and all steps, except film development were done on ice.

Developed slides were embedded in Citiflour AF1 for microscopic analysis and stored at 4°C afterwards.

B.8.4. Immunofluorescence

Immunofluorescence was done following a protocol developed by Bartosch et al. (1999), with the monoclonal antibody Hyb153-3 (Mouse IgG_1) (Aamand et al. 1996), which was reported to react with the β-subunit of the nitrite oxidoreductase of *Nitrospira*-like bacteria, as well as all other NOB before (Bartosch et al. 1999, Alawi et al. 2007).

Solutions

1 x PBS

Lysozyme solution (1mg/ml)

Blocking solution

AB-dilution solution

a) Sample preparation

1-3 µl of PFA-fixed cells (see section B.8.1.1.) were pipetted on a 10-well slide and dried in the hybridization oven for 10 min and dehydrated in an Ethanol series as described in section B.8.1.2.2. c).

b) Blocking

To avoid unspecific binding of the antibody 10 µl of blocking solution, containing 3% BSA (Section B.5.11.), was pipetted onto each well and incubated in a moist chamber for 30-60 min. Afterwards the slide was rinsed with H_2O_{bidist} to wash away the blocking solution and was dried under air stream.

c) Primary antibody hybridization

10 µl of antibody dilution solution (Section B.5.11.) was mixed on well with 1 µl of monoclonal primary antibody Hyb153-3 (Mouse IgG_1), targeting the β-subunit of the NXR, resulting in a final concentration of 1:10. The slide was incubated in a moist chamber for 1 h and washed in 1 x PBS for 15 min and air dried afterwards.

d) Secondary antibody hybridization

After adding 5% goat serum to the antibody dilution solution, 10 µl were applied to each well and 1 µl of the 1:10 diluted anti-goat secondary antibody, labeled in Cy3, was added, resulting in a final dilution of 1:100. The slide was incubated for 1 h in a moist chamber in the dark and washed for 15 min in 1 x PBS. Subsequently, the slide was rinsed with H_2O_{bidist} and air dried.

Control experiments without primary antibody, to check for unspecific binding of the secondary antibody, and without application of any antibody, to check for autofluorescence, were included in each experiment.

e) Detection of fluorescently labeled cells

For microscopy the slide was mounted with Moviol 4-88 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and analysis of immunofluorescently labeled cells was done as described in section B.8.1.4.

C. RESULTS

C.1. Characterization of nitrifying enrichment cultures obtained from hot springs at Lake Baikal and in Kamchatka

C.1.1. DNA extraction

DNA extraction from enrichment cultures 4 and 8 using the Power Soil™ DNA Kit yielded 5.9 ng/µl and 4.7 ng/µl of high molecular weight DNA, respectively.

C.1.2. Amplification, cloning, RFLP analysis and phylogenetic identification of bacterial 16S rRNA gene fragments

In a first assessment of the microbial communities of the enrichment cultures, the bacterial 16S rRNA gene was amplified with the general bacterial 16S rRNA gene primers 616V/1492R under standard conditions (Tables B.8. and B.9.) as described in section B.7.4.1. The resulting products in a size of 1501 - 1504 bp were cloned and subjected to an RFLP analysis (Sections B.7.5. and B.8.). 6 and 5 clones were sequenced, respectively.

Despite various RFLP patterns, all sequenced clones of enrichment culture A could be identified as *Nitrospira*-like sequences in a BLAST analysis (Section B.7.10.1.). For enrichment culture C_a 2 out of 5 sequenced clones had an insert similar to *Nitrospira*-like 16S rRNA gene (Appendix table I.1.). For enrichment cultures 4 and 8, 15 and 9 clones were picked, respectively, and diverse RFLP patterns were observed. At least one clone of each pattern was sequenced. In a BLAST analysis none of the sequences obtained from enrichment culture 4 were potentially related to known nitrifying organisms, while 3 of the sequences obtained from enrichment culture 8 were closest related to *Nitrospira*-like sequences (Appendix table I.1.). However, one of these sequences was found to be a chimera in several checks (Section B.7.10.4.) and one was of bad quality, so they were dismissed from further phylogenetic analysis. The BLAST results for the residual, non-*Nitrospira*-related sequences are also shown in appendix table I.1.

C.1.3. Identification of the ammonia-oxidizing organism(s) (AOO) in an enrichment from Kamchatka (enrichment culture 8)

Since enrichment culture 8 was growing on a medium containing NH_3, which was converted to NO_3^- (Table B.6.), it was examined for the presence of ammonia-oxidizing organisms (AOO).

C.1.3.1. Testing for the presence of AOB via amplification of bacterial *amoA* genes

Even though there is only little evidence at all that bacterial ammonia oxidation might take place at higher temperatures (Golovacheva 1976, cited after Lebedeva et al. 2005), the possibility of an bacterial AOO thriving in the enrichment was evaluated. Therefore a PCR using primers, specific for *amoA* of β-proteobacterial AOB (Table B.10.) using standard conditions for amplification of bacterial *amoA* genes (Table B.11.) was performed. However, no PCR product could be obtained from enrichment culture 8.

C.1.3.2. Amplification, cloning and RFLP analysis of the archaeal 16S rRNA gene fragments

In a first run archaeal 16S rRNA gene fragments could be amplified from the cell lysate (CL), as well as from the extracted DNA (D) of enrichment culture 8 with the primers 21F/1492R and were cloned into *E. coli* as described in section B.7.5. In RFLP analysis several patterns were observed and clones from each pattern were sequenced (Appendix table I.5.).

To check for more archaeal diversity in enrichment culture 8 in a second run the primer pair 21F/958R was used to amplify 16S rRNA gene fragments under conditions listed in section B.7.4.1. This yielded a fragment in the expected size of 912 bp, which was cloned and screened for insert positive clones (Section B.7.7.). 23 clones were subjected to RFLP analysis and 5 patters were observed and subsequently sequenced (Appendix table I.5.).

C.1.3.3. Amplification, cloning and RFLP analysis of crenarchaeal *amoA* genes

Crenarchaeal *amoA* genes amplified from a cell lysate of enrichment culture 8, using the primers CrenAmoA1F/1R (Table B.10.) under standard conditions (Section B.7.4.2.), were cloned following standard procedure (Section B.7.5.) and retrieved clones were screened via M13 PCR (Section B.7.7.). Clones with an insert in the expected size of 635 bp were subjected to RFLP analysis and clones of each pattern were sequenced (Appendix table I.5.).

C.1.3.4. Phylogeny of (putative) AOA in an hot spring enrichment

a) Archaeal 16S rRNA phylogeny

Retrieved sequences were imported into ARB and comparative nucleic acid sequence analysis was performed (Section B.7.10.). Several phylogenetic trees were calculated with different treeing methods and a consensus tree was created (Section B.7.10.5.). Despite

different RFLP patterns, 16S rRNA sequences, retrieved using 1492R as a reverse primer, as well as sequences retrieved using 958R as reverse primer, were ≥99% similar to each other and formed a cluster together with clone sequences from geothermal water (accession number AB113625) and South African gold mine water (accession number AB050238) (Figure C.1.). Full length sequences (>1 300 bp) were on average 99.5% and 97.2% similar to these clones. The sequences formed a separate lineage in between the marine group I.1a and the South African gold mine crenarchaeotic group (SAGMCG)-1, containing mainly sequences retrieved from South African gold mine water. The clustering with different

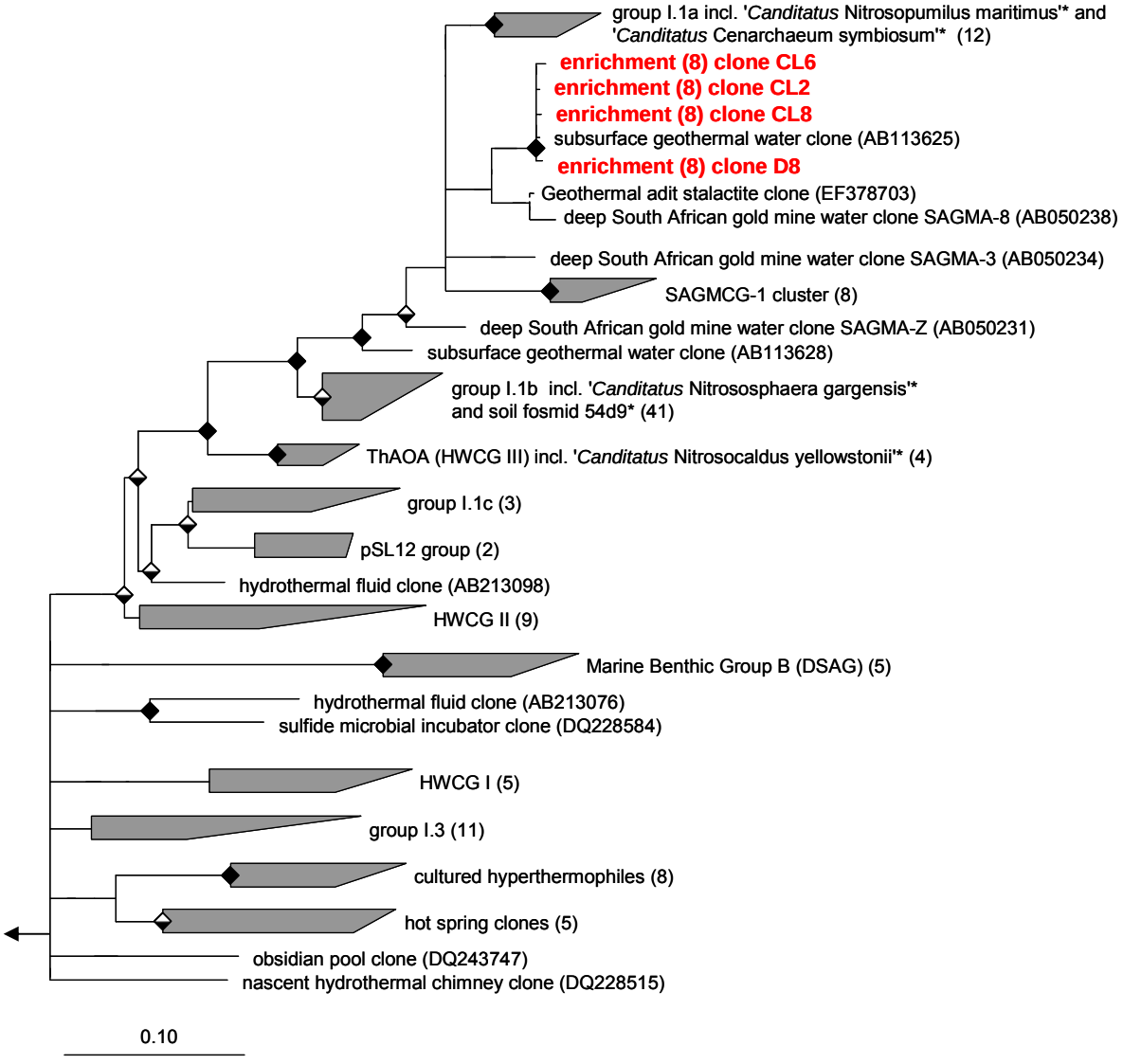


Figure C.1. 16S rRNA-based phylogenetic consensus tree showing the position of sequences retrieved from enrichment culture 8 (bold and in red). The consensus tree is based on Maximum likelihood and Maximum parsimony tree topologies, calculated with sequences >1 300 bp and a 50% conservation filter. Nodes with filled squares and half-filled squares indicate parsimony bootstrap support >90% and quartet puzzling reliability values >70%, respectively. Asterisks mark the organisms/gene fragments for which the presence of an *amoA* has been demonstrated. Accession numbers are written in brackets. The arrow indicates the outgroup, consisting of euryarchaeal sequences. The scale bar represents 10% estimated nucleotide sequence difference.

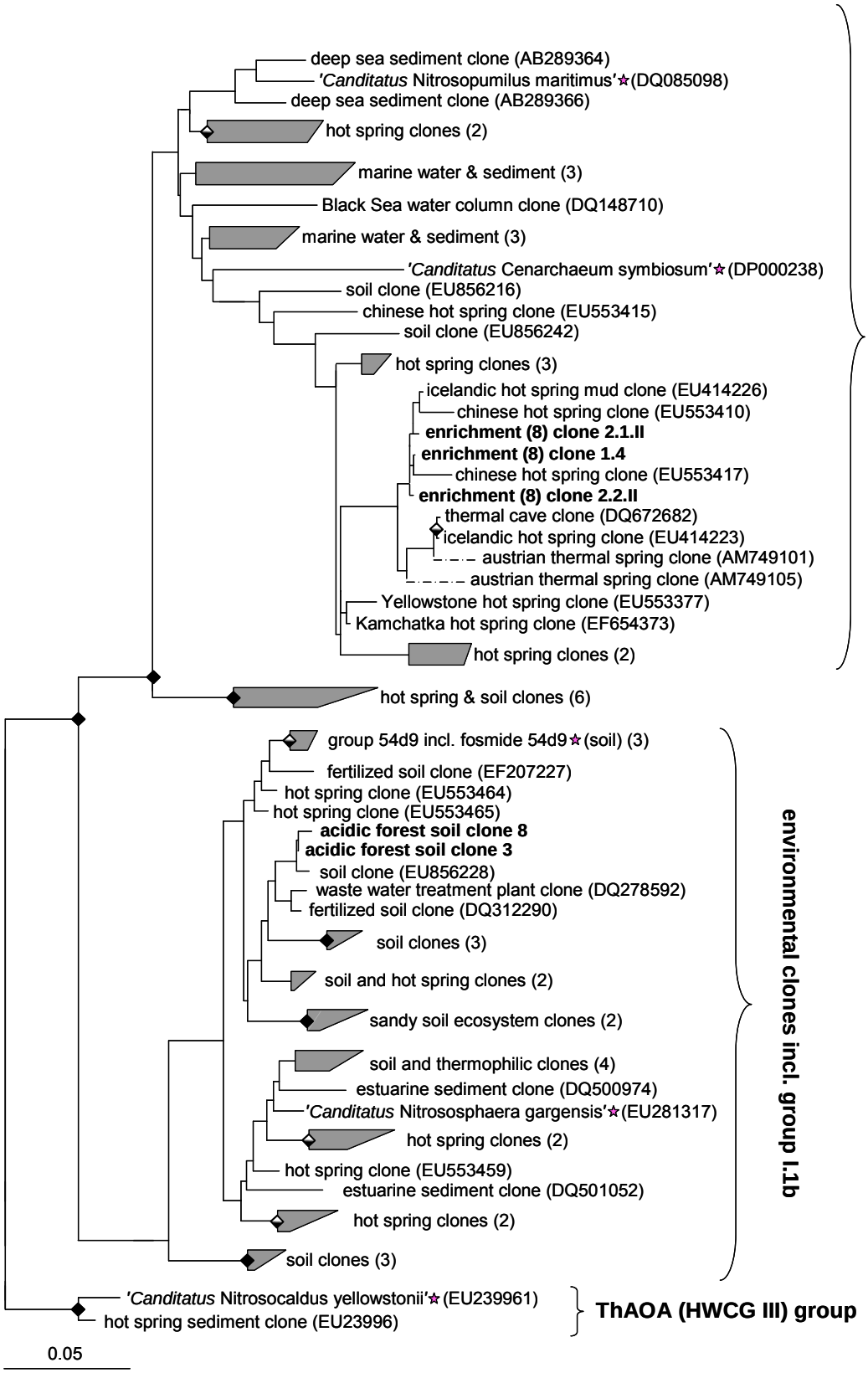


Figure C.2. Unrooted evolutionary distance (Fitch) phylogenetic tree of crenarachaeal AmoA sequences. Tree showing the position of sequences retrieved from hot spring enrichment culture 8 and acidic forest soil (in bold). Phylogenetic calculations were done with sequences >169 aa positions, shorter sequences were added afterwards and are indicated by dashed lines. Nodes with filled squares and half filled squares indicate bootstrap support >90% and >70%, respectively. Accession numbers are written in brackets. Stars indicate *amoA* sequences that can be related to the 16S rRNA gene of *Crenarchaeota*. The scale bar represents 5% estimated amino acid sequence difference.

treeing methods was not consistent concerning the evolutionary succession of marine group I.1a, SAGMCG-1 and the sequences obtained in this study. 4 out of 6 treeing methods (NJ, PhyML, AxML, Tree Puzzle) grouped the new lineage with marine group I.1a, 2 out of 6 treeing methods (MP, RaxML) grouped it outside of marine group I.1a and SAGMCG-1. Therefore a multifurcation had to be implemented (Figure C.1.).

The full length sequences were on average 93% and 92% similar to members of marine group I.1a, "*Candidatus* Nitrosopumilus maritimus" and "*Candidatus* Cenarchaeum symbiosum", respectively, which hence are the closest relatives for which the presence of an *amoA* has been demonstrated. Sequences of SAGMCG-1 were on average approximately 88% similar to sequences retrieved in this study.

b) Crenarchaeal AmoA phylogeny

A single clone with a unique RFLP pattern was 99.9% similar to the marine sponge clone PlakorGrA obtained in a study by Steger et al. (2008) done in our lab, it was considered a contaminant and dismissed from phylogenetic analysis. The residual sequences were imported into ARB and aligned to alignments published previously (Treusch et al. 2005) by comparative nucleic acid and amino acid sequence analysis (Section B.7.10.).

Additionally, further *amoA* sequences obtained in studies of hot spring environments (Reigstad et al. 2008, Weidler et al. 2008, Zhang et al. 2008) were imported and also included in the analysis. Phylogenetic calculations were done with sequences ≥169 aa positions, shorter sequences were added afterwards with the "Parsimony Interactive" tool of ARB. All AmoA sequences obtained in this study were ≥98.9% similar to each other. They grouped together with other sequences amplified from various hot spring habitats, in one of several thermal habitat clusters found within what is generally suggested to represent marine group I.1a on the phylogenetic level of AmoA (Figure C.2.). The closest related AmoA sequences of known origin belonged to the marine sponge symbiont "*Candidatus* Cenarchaeum symbiosum" and "*Candidatus* Nitrosopumilus martimus", which were on average 92.4% and 91.8% similar.

C.1.4. Identification and characterization of the nitrite-oxidizing organisms in a hot spring enrichment

In all enrichment cultures the presence of *Nitrospira*-like bacteria was observed via light microscopy (E. Lebedeva, personal communication) and confirmed by preliminary FISH results (F. Maixner, personal communication). Up to date, it is also the only lineage of

NOB known to be present and thriving at elevated temperatures (Lebedeva et al. 2005, Lebedeva et al. 2008, Weidler et al. 2008, Lebedeva in preparation). Therefore the enrichment cultures were only screened for 16S rRNA genes and *nxrB* genes of *Nitrospira*-like bacteria.

C.1.4.1. Amplification, cloning and RFLP analysis of *Nitrospira* 16S rRNA genes

For enrichment cultures A and C_a amplification with primers 616V/1158R, the reverse primer being specific for the 16S rRNA gene of *Nitrospira*-like bacteria (Table B.8.), yielded a PCR product in the size of 1163 bp, which was cut out from the gel and cloned as described B.7.5.4. For each of the enrichment cultures 9 clones were screened, all of them had an insert in the correct size and exhibited identical RFPL patterns. Out of these, 5 clones were picked and sequenced for both enrichment cultures (Appendix table I.5.).

Even though it was already confirmed with FISH that enrichment cultures 4 and 8 contained *Nitrospira*-like bacteria (F. Maixner, personal communication) and amplification with general bacterial primers 616V/R1492 (Section B.7.4.1.) yielded *Nitrospira*-like sequences, at least for enrichment culture 8 (Section C.1.2.), PCR with the *Nitrospira* specific reverse primer 1158R did not result in a PCR product.

Therefore the probe S-*-Ntspa-0712-a-A-21, specific for the phylum *Nitrospira*, was used as a reverse primer instead of 1158R, which resulted in a PCR product in the expected size of 721 bp. Again the product was cut out and cloned (Section B.7.5.4.) and fragment diversity of 11 and 10 clones, respectively, was analyzed with RFLP (Section B.7.8.).

RFLP analysis of clones obtained from enrichment culture 8 produced two differing patterns, one being identical with the pattern that was observed for all clones of enrichment culture 4 and clones of each pattern were sequenced (Appendix table I.5.)

C.1.4.2. Amplification, cloning and RFLP analysis of *nxrB*

Testing the primer pairs F14/R1239 and F19/R1237 (Table B.12.) for amplification of *nxrB* fragments from *Nitrospira*-like bacteria from enrichment cultures A and C_a, a product in the expected size of about 1 200 bp was obtained with the primer pair F19/R1237. The product was cloned, but clones were only retrieved from enrichment culture C_a. Seven out of 8 clones screened possessed an insert in the expected size and were sequenced (Appendix table I.5.).

NxrB gene fragments from enrichment cultures 4 and 8 were only amplified with the primers F19/R1237 and resulted in a product in the expected size of ~1 240 bp for

enrichment culture 8 but not for enrichment culture 4. The PCR product obtained from enrichment culture 8 was cut out, cloned and screened for insert-positive clones (Sections B.7.4.4. and B.7.6.). 5 out of 11 insert-positive clones could be identified, which exhibited 2 varying RFLP patterns. Finally all clones were sequenced (Appendix table I.5.).

C.1.4.3. Phylogenetic analysis of the nitrite-oxidizing organisms on the 16S rRNA and *nxrB* level

a) 16S rRNA phylogenetic analysis

All sequences were identified as similar to known *Nitrospira*-like sequences with BLAST and imported into ARB for further comparative nucleic acid sequence analysis. In all phylogenetic analysis (Section B.7.10.5.), all obtained sequences, except for clones N1 and N11 of enrichment culture 8, which already showed a different RFLP pattern, formed a cluster separate from known *Nitrospira*-sublineages (Figure C.3.A). Within this cluster two separate subclusters for sequences obtained from enrichment cultures A and C_a, originating from a hot spring at Lake Baikal, and 8 and 4, originating from a hot spring in Kamchatka, respectively, could be distinguished (Figure C.3.A). Clones N1 and N11 formed a cluster within the sublineage II of the genus *Nitrospira* (Figure C.3.A).

Full length sequences (>1 400 bp) of the two respective clusters were on average 96.1% similar to each other (Appendix table I.5.) and all sequences within these two clusters showed a similarity of >99% to each other. The closest enriched relative for both clusters was the thermophile "*Candidatus* Nitrospira bockiana", which had on average 93.3% (A and C_a) and 93.6% (4 and 8) 16S rRNA gene sequence similarity (Appendix table I.5.). The remaining two clones of enrichment culture 8, N1 and N11, were affiliated with other clone sequences retrieved from hot springs in Austria (accession number AM039549) and Russia (accession number AY796333) (Figure C.3.A) and were with 98.3% closely related to the only cultivated representative of *Nitrospira* sublineage II, *Nitrospira moscoviensis* (Table G.2.).

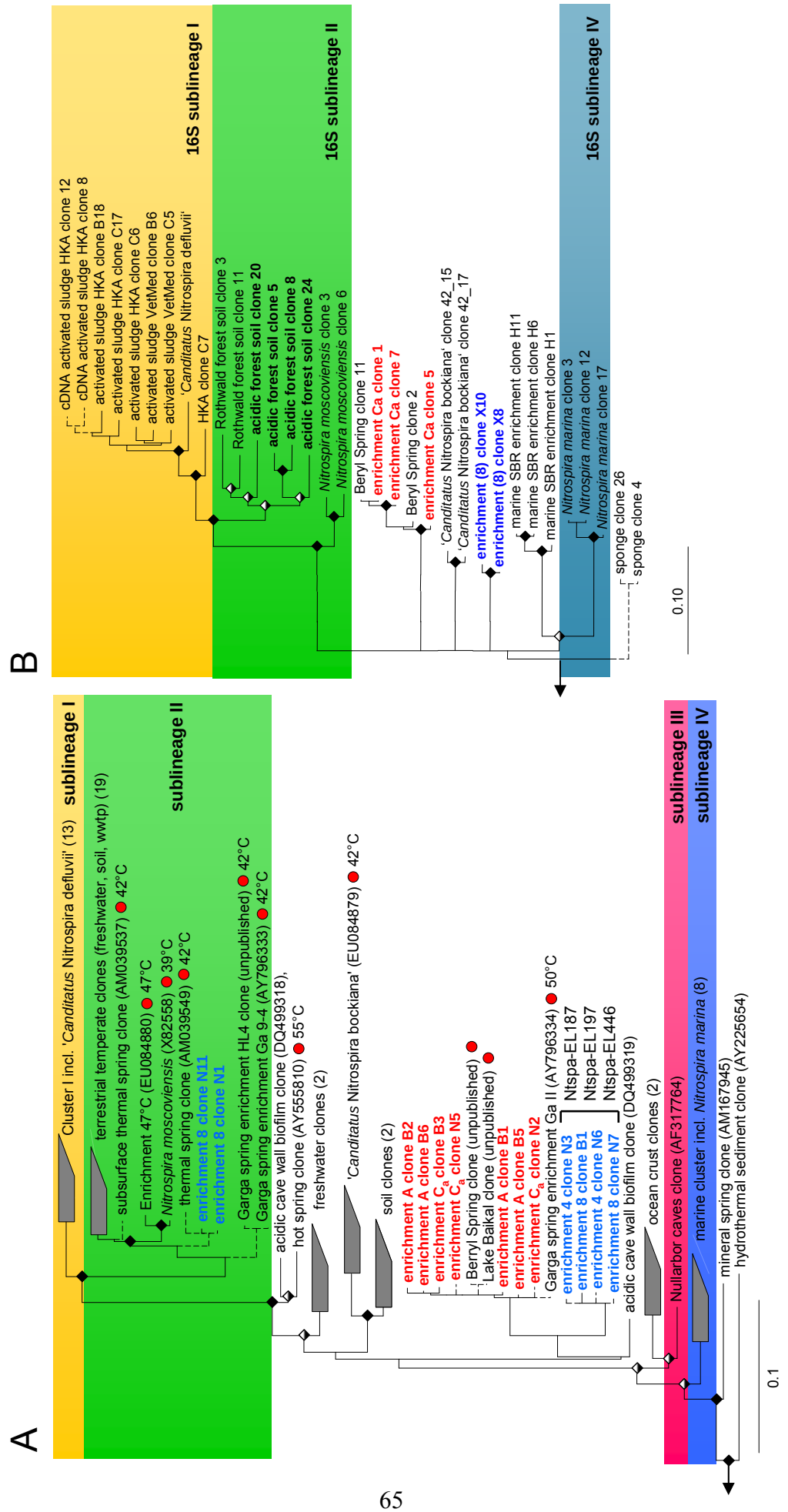
b) Phylogenetic analysis of *nxrB*

The retrieved sequences were subjected to BLAST (Section B.7.10.1.) and were closest related to the sequence of a "*Candidatus* Kuenenia stuttgartiensis" *narH*, which is wrongfully annotated in Genbank (http://www.ncbi.nlm.nih.gov/Genbank/) and also is an *nxrB* sequence (H. Daims, personal communication). Since there are no *Nitrospira*-like *nxrB* sequences deposited in GenBank up to date this might be the closest hit possible and

the retrieved sequences could therefore be considered as *nxrB* genes. These sequences were imported into ARB for further comparative nucleic acid and amino acid phylogenetic analysis (B.7.10.). Additionally, sequences obtained from other enrichment cultures and environmental samples (Table B.7.) and sequences from further samples, kindly provided by F. Maixner and H. Koch (Appendix table I.3.), were imported in ARB and included in the phylogenetic analysis.

The consensus tree for *nxrB* of *Nitrospira*-like bacteria was based on NJ-, MP- and ML-tree topologies. These trees were calculated with nucleic acid sequences >1 200 bp, shorter sequences obtained from sponge DNA and cDNA retrieved from a wastewater treatment plant were added afterwards and are indicated by dashed lines. The outgroup consists of other genes also belonging to the molypterin-binding (MopB) protein-superfamily, such as *narH* and *nxrB* of *Nitrobacter* spp. All sequences obtained from the respective enrichment cultures were more than 98% similar to each other, so it seems that no *nxrB* sequences for the sublineage II *Nitrospira*-like bacteria, found in the 16S rRNA gene clone library of enrichment culture 8, were obtained. Nevertheless, it cannot be excluded that the *nxrB* sequences of both groups *Nitrospira*-like bacteria found at the 16S rRNA-level are highly similar to each other and grouped within the obtained cluster. Like 16S rRNA, *nxrB* genes of the enrichment cultures were on average most similar to *nxrB* of "*Candidatus* Nitrospira bockiana" with 91% (8) and 90.4% (C_a) nucleic acid similarity, respectively. Similar to the 16S rRNA phylogeny, the retrieved sequences formed two distinct clusters. The branching order of the different groups is not clear as indicated by low bootstrap support in 16S rRNA gene- as well as *nxrB* -based phylogenetic analysis (<70%; figure C.3.A and B) and a multifurcation was introduced for the phylogenetic *nxrB* sequence tree (Figure C.3.B).

Figure C.3. Phylogenetic trees showing the positioning of 16S rRNA genes and *nxrB* sequences retrieved from enrichment cultures A and Ca (in red) and 4 and 8 (in blue). Arrows indicate the respective outgroups. The scale bar represents 10% estimated nucleotide sequence difference. (A) Maximum Likelihood tree of 16S rRNA sequences, phylogenetic calculations were done using a 50% conservation filter and only sequences with more than 1300 informative positions of the 16S rRNA genes, shorter sequences were added afterwards and are indicated by dashed lines. Nodes with filled squares and half-filled squares indicate parsimony bootstrap support >90% and quartet puzzling reliability values >70%, respectively. Red dots and temperatures behind designate sequences retrieved from thermal ecosystems and the temperature at the sampling point or enrichment temperature. Accession numbers are written in brackets. New probes designed for sequences obtained from enrichment cultures 4 and 8 are indicated behind the squared bracket. Outgroup consists of sequences affiliated with the phylum *Nitrospirae.* (B) Consensus tree for *nxrB* of *Nitrospira*-like bacteria based on NJ-, MP- and ML-tree topologies, trees were calculated with sequences >1 200 bp, shorter sequences were added afterwards and are indicated by dashed lines. Nodes with filled squares and half filled squares indicate parsimony bootstrap support >90% and >70%, respectively. The denoted sublineages are referring to the sublineages of the 16S rRNA tree. Outgroup consists of other genes also belonging to the molypterin-binding (MopB) protein-superfamily, such as *narH* and *nxrB* of *Nitrobacter* spp.. All sequences not obtained in this study were kindly provided by F. Maixner and H. Koch.


A
Cluster I incl. 'Canditatus Nitrospira defluvii' (13)
sublineage I
terrestrial temperate clones (freshwater, soil, wwtp) (19)
subsurface thermal spring clone (AM039537) 42°C
Enrichment 47°C (EU084880) 47°C
Nitrospira moscoviensis (X82558) 39°C
thermal spring clone (AM039549) 42°C
enrichment 8 clone N11
enrichment 8 clone N1
Garga spring enrichment HL4 clone (unpublished) 42°C
Garga spring enrichment Ga 9-4 (AY796333) 42°C
sublineage II
acidic cave wall biofilm clone (DQ499318),
hot spring clone (AY555810) 55°C
freshwater clones (2)
'Canditatus Nitrospira bockiana' (EU084879) 42°C
soil clones (2)
enrichment A clone B2
enrichment A clone B6
enrichment Ca clone B3
enrichment Ca clone N5
Berryl Spring clone (unpublished)
Lake Baikal clone (unpublished)
enrichment A clone B1
enrichment A clone B5
enrichment Ca clone N2
Garga spring enrichment Ga II (AY796334) 50°C
enrichment 4 clone N3
enrichment 8 clone B1
enrichment 4 clone N6
enrichment 8 clone N7
Ntspa-EL187
Ntspa-EL197
Ntspa-EL446
acidic cave wall biofilm clone (DQ499319)
ocean crust clones (2)
Nullarbor caves clone (AF317764)
sublineage III
marine cluster incl. Nitrospira marina (8)
sublineage IV
mineral spring clone (AM167945)
hydrothermal sediment clone (AY225654)
0.1
B
cDNA activated sludge HKA clone 12
cDNA activated sludge HKA clone 8
activated sludge HKA clone B18
activated sludge HKA clone C17
activated sludge HKA clone C6
activated sludge VetMed clone B6
activated sludge VetMed clone C5
'Canditatus Nitrospira defluvii'
HKA clone C7
16S sublineage I
Rothwald forest soil clone 3
Rothwald forest soil clone 11
acidic forest soil clone 20
acidic forest soil clone 5
acidic forest soil clone 8
acidic forest soil clone 24
Nitrospira moscoviensis clone 3
Nitrospira moscoviensis clone 6
16S sublineage II
Beryl Spring clone 11
enrichment Ca clone 1
enrichment Ca clone 7
Beryl Spring clone 2
enrichment Ca clone 5
'Canditatus Nitrospira bockiana' clone 42_15
'Canditatus Nitrospira bockiana' clone 42_17
enrichment (8) clone X10
enrichment (8) clone X8
marine SBR enrichment clone H11
marine SBR enrichment clone H6
marine SBR enrichment clone H1
Nitrospira marina clone 3
Nitrospira marina clone 12
Nitrospira marina clone 17
16S sublineage IV
sponge clone 26
sponge clone 4
0.10

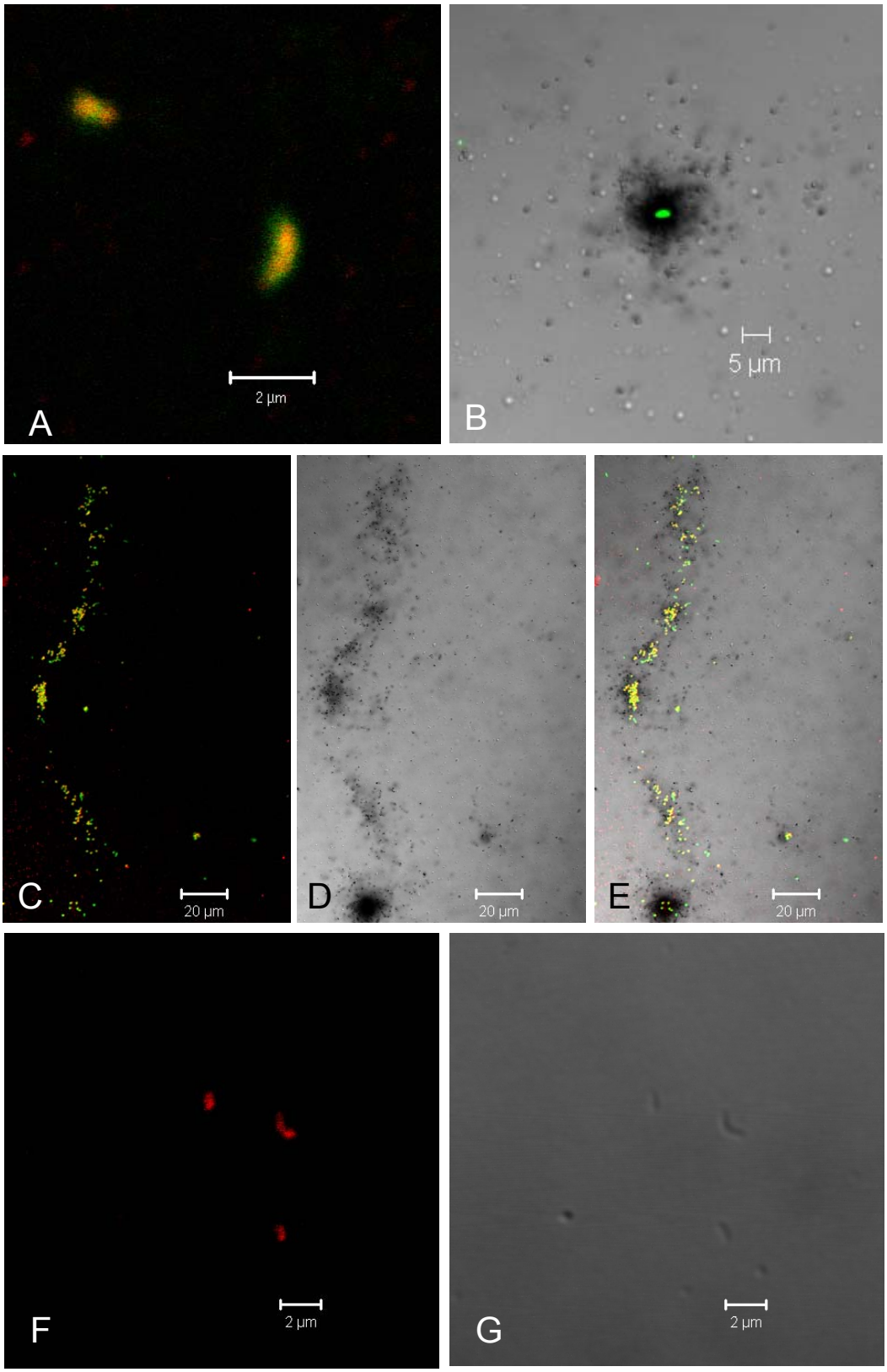


Figure C.4. *In situ* detection of nitrifying organisms in enrichment culture 8. A: FISH showing vibrio-shaped *Nitrospira*-like bacteria targeted with probes Ntspa662 and Ntspa712 (both Fluos) and the specific probe Ntspa-EL446. B: Organism found in FISH-MAR incubations with/without 0.3 mM NO_2^- and in pasteurized samples, stained with SybrGreen C-E: FISH-MAR incubation with 0.3mM NO_2^- and $^{14}CO_2$ showing actively metabolizing *Nitrospira*-like bacterial cells (probe labeling as described for A, thus cells targeted by both probes are appearing yellow) F-G: Immunofluorescence labeling of vibrio-shaped cells with the monoclonal antibody Hyb153-3, targeting the β-subunit of the nitrite oxidoreductase and a secondary antibody labeled in Cy3.

C.1.5. *In situ* detection of nitrifying microorganisms in hot spring enrichment cultures from Kamchatka

The putative AOA of enrichment culture 8 were addressed by using FISH and CARD-FISH, potential nitrite-oxidizers of the genus *Nitrospira* were addressed by using FISH. Additionally, further characterization of the NOB was attempted by FISH-MAR and immunofluorescence targeting NXR.

C.1.5.1. Attempts for *in situ* detection of putative AOA via FISH and CARD-FISH

FISH with probes Arch915 and Cren512, specific for *Archaea* and *Crenarchaea,* respectively (Table B.20.), did not result in a detectable signal. Therefore CARD-FISH was applied using the HP-labeled probes Cren512 or Arch-EL180. The latter one was designed specifically for the 16S rRNA sequences obtained from enrichment culture 8. For both probes a signal was detected within clusters in low abundance (data not shown). A Proteinase K time series incubating from 5 min – 12 min did not change this finding. However, when biomass of enrichment culture 8 (fixed at a later time point) was hybridized with the HP-labeled probe Ntspa662 or the Nonsense-probe this resulted in the same signal. An addition of blocking reagents such as BSA or salmon sperm, to prevent binding of the probe to the particles, did not have any impact. Neither it was possible to separate the cells from the particles by mechanical shearing or sonication.

C.1.5.2. *In situ* detection of *Nitrospira*-like bacteria with FISH

For *in situ* detection of the putative nitrite-oxidizing *Nitrospira*-like bacteria thriving in enrichment cultures 4 and 8, three FISH-probes specific for the *Nitrospira*-like sequences obtained, were designed with ARB as described in section B.7.10.3.

Evaluation of probes specific for the new *Nitrospira*-like bacteria of enrichment cultures 4 and 8

No signal could be detected with the newliy designed probes Ntspa-EL187 and Ntspa-EL197. However, for the third designed probe, Ntspa-EL446, a weak but recordable signal could be detected from cells in enrichment culture 8 (Figure C.4.A), but not from cells in enrichment culture 4. It should be noted that optimal FA-concentration for probe hybridization was not determined, due to the lack of a mismatch organism to test with. Nevertheless, in none of the hybridizations a signal from the negative control, *E. coli* cells heterologous expressing the 16S rRNA of "*Candidatus* Nitrospira defluvii", which has two

central mismatches (kindly provided by F. Maixner), was detected. Attempts for improvement of signal intensity included elongation of hybridization time from 1.5 h to 5 h or ON and increase of FA-concentration from 10% to 35%, both resulting in a slightly brighter signal (data not shown).

Either way, in both enrichment cultures the *Nitrospira*-like organisms could be targeted with EUB-Mix, Ntspa662 and Ntspa712 (Ntspa-Mix), the latter two specific for the genus and the phylum *Nitrospira(e)*, respectively. Nevertheless only a very weak signal could be detected – constraining the recording of pictures. Under the microscope in enrichment culture 4 *Nitrospira*-like bacteria could be observed that were up to 5 µm long, curved cells (data not shown).

Despite the high sequence similarity of sequences obtained from enrichment cultures 4 and 8 (Section C.1.4.3. and Figure C.3.A), cells detected in enrichment culture 8 showed a quite different morphology. Cells detected were at most 2 µl long and vibrio-shaped (Figure C.4.A). The cells seem to be freely distributed in the medium, no cluster formation or affiliation of labeled cells with clusters that may hypothetically contain AOAs could be observed. Additionally, as can be observed from figure C.4.C, also cells only targeted with probes Ntspa712 and Ntspa662 (in Fluos), but not with the specific probe Ntspa446 (in Cy3) could be detected in enrichment culture 8.

C.1.5.3. Immunofluorescence

Immunofluorescence using the monoclonal antibody Hyb153-3, targeting the β-subunit of the NXR, and a secondary antibody labeled in Cy3, did not result in a signal from cells of enrichment culture 4. In enrichment culture 8, a signal from cells strikingly showing the same *Vibrio*-like cell-morphology as cells observed with FISH, could be detected (Figure C.4.F-G). Under the microscope also a vague halo-shape could be observed, but not recorded. No signal could be detected from negative controls without primary or primary and secondary antibody.

C.1.5.4. *In situ* detection and FISH-MAR

Incubation of biomass from enrichment culture 8 with 0.3 mM NO_2^- and $^{14}CO_2$ for 20 h could show that the *Nitrospira*-like bacteria, forming the new lineage, were actively metabolizing CO_2 when NO_2^- was present. MAR signals corresponded with the respective FISH signal of these organisms (Figures C.4.C-E). No MAR signal could be detected for the *Nitrospira*-like bacteria in the negative controls without NO_2^- or in pasteurized samples.

Additionally, a strong signal was observed from an organism, which was only stained with DAPI or SybrGreen, respectively, therefore taking up $^{14}CO_2$ (Figure C.4.B). This signal was also detected in the negative control, where no NO_2^- was present and even more surprisingly in the negative control pasteurized by addition of PFA.

C.2. Molecular identification of the nitrifying community in an acidic forest soil

C.2.1. Quantitative and qualitative comparison of different DNA/RNA extraction protocols

For qualitative and quantitative comparison DNA/RNA was isolated with three different protocols, two using classic phenol/chloroform extraction, the third following a kit, to compare quantity and quality of nucleic acid extraction (Section B.7.1.). Testing different protocols was necessary because humic substances, co-extracted in the nucleic acid isolation procedure, have an inhibitory effect on DNA amplification (Tebbe and Vahjen 1993). Bead beating was applied for cell disruption in all protocols and was varied to compensate for the differing susceptibility of microorganisms to the treatment (More et al. 1994, Frostegard et al. 1999, Martin-Laurent et al. 2001).

C.2.1.1. Quantitative comparison via measurement of optical density

The quantity of extracted DNA/RNA was done in an optical density measurement via NanoDrop (Section B.7.2.1) and mean values were determined. First of all, it should be noted that in general the amount of extracted DNA/RNA was quite variable between replicates (standard derivation bars in figure C.5.) and no significant differences were found between the different treatments applied for each protocol. However, comparison of the protocols showed that on average DNA/RNA concentration measured by photometry (NanoDrop) was highest for DNA/RNA extracted after Lüders et al. (2004) for all bead beating treatments of soil and "Litter N" samples, and for the "Medium" treatment of "Litter A". For soil and "Litter N" DNA/RNA concentration was about 3 fold compared to the concentration of DNA/RNA extracted after Urich et al. (2008) for all treatments (Figure C.5.). However, for several DNA/RNA extracts from "Litter A", isolated after the Lüders protocol, DNA concentration could not be determined via Nano Drop due to high turbidity of these samples. The low quality of DNA extracts from "Litter A" was also indicated by the brownish color of the DNA extracts, visible by eye.

The DNA concentration of extractions with the MoBio Power Soil™ DNA Kit, the only protocol that solely extracts DNA and not both, DNA and RNA, ranged between 6.5 ng/µl for the "Low" treatment of "Litter N" and 25.9 ng/µl for the "High" treatment of the soil sample (Figure C.5.).

No significant differences between the different protocols could be observed for ratios of 260/230 and 260/280, which give an indication of contamination with humic acids and proteins respectively (data not shown).

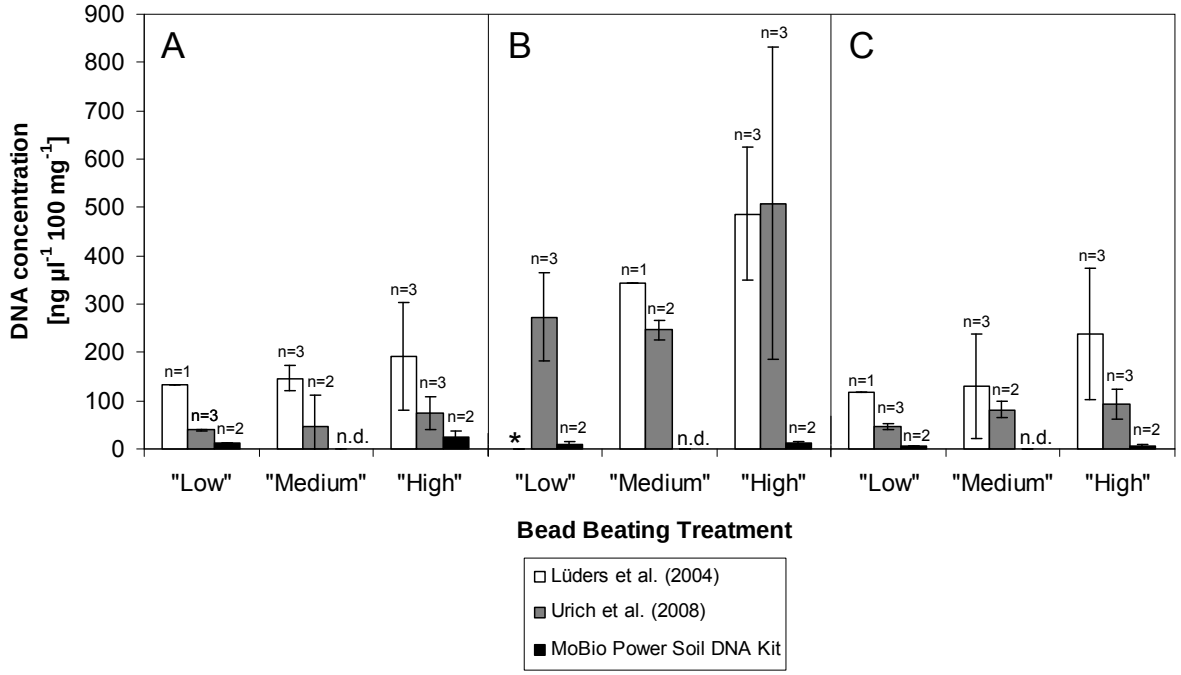


Figure C. 5. Concentration of DNA extracted from acidic forest (A) soil, (B) "Litter A", and (C) "Litter N" after different protocols. Terms "Low", "Medium" and "High" refer to the different bead beating treatments at 4.5 m/s for 30 s, 5.5 m/s for 40 s and 6.5 m/s for 45 s, respectively for DNA/RNA extracted after Lüders et al. (2004) and Urich et al. (2008) and vortexing for 10 min ("Low") and bead beating at 6.5 m/s for 45 s ("High") for MoBio Power Soil™ DNA Kit (see also section B.7.1.). Values are mean values; n is indicated by the number above the columns. No "Medium" treatment was done for Power Soil™ DNA Kit. n.d….not determined. *…the DNA concentration for the "Low" treatment of "Litter A" (B) could not be determined due to a very turbid DNA extract. Bars indicate standard deviation.

C.2.1.2. Quality control via agarose gel electrophoresis

A quality control via agarose gel electrophoresis (Section B.7.3.2.) showed that RNA and high molecular weight DNA was extracted with both non-commercial protocols (Figure C.6.A and B). Nevertheless, especially DNA extracted from soil with the Lüders protocol shows intensive smears, indicating high amounts of fragmented DNA (Figure C.6.B). For the DNA/RNA extracted after Lüders et al. (2004) as well as for Urich et al. (2008) the amount of extracted high molecular weight DNA was lower for "Litter A" than for the soil sample. (Figure C.6.A and B), therefore contradicting the results obtained by optical density measurement (C.2.1.1.).

DNA extraction with the MoBio Power Soil™ DNA Kit yielded high molecular weight DNA for all samples and all bead beating treatments (Figure C.6.C).

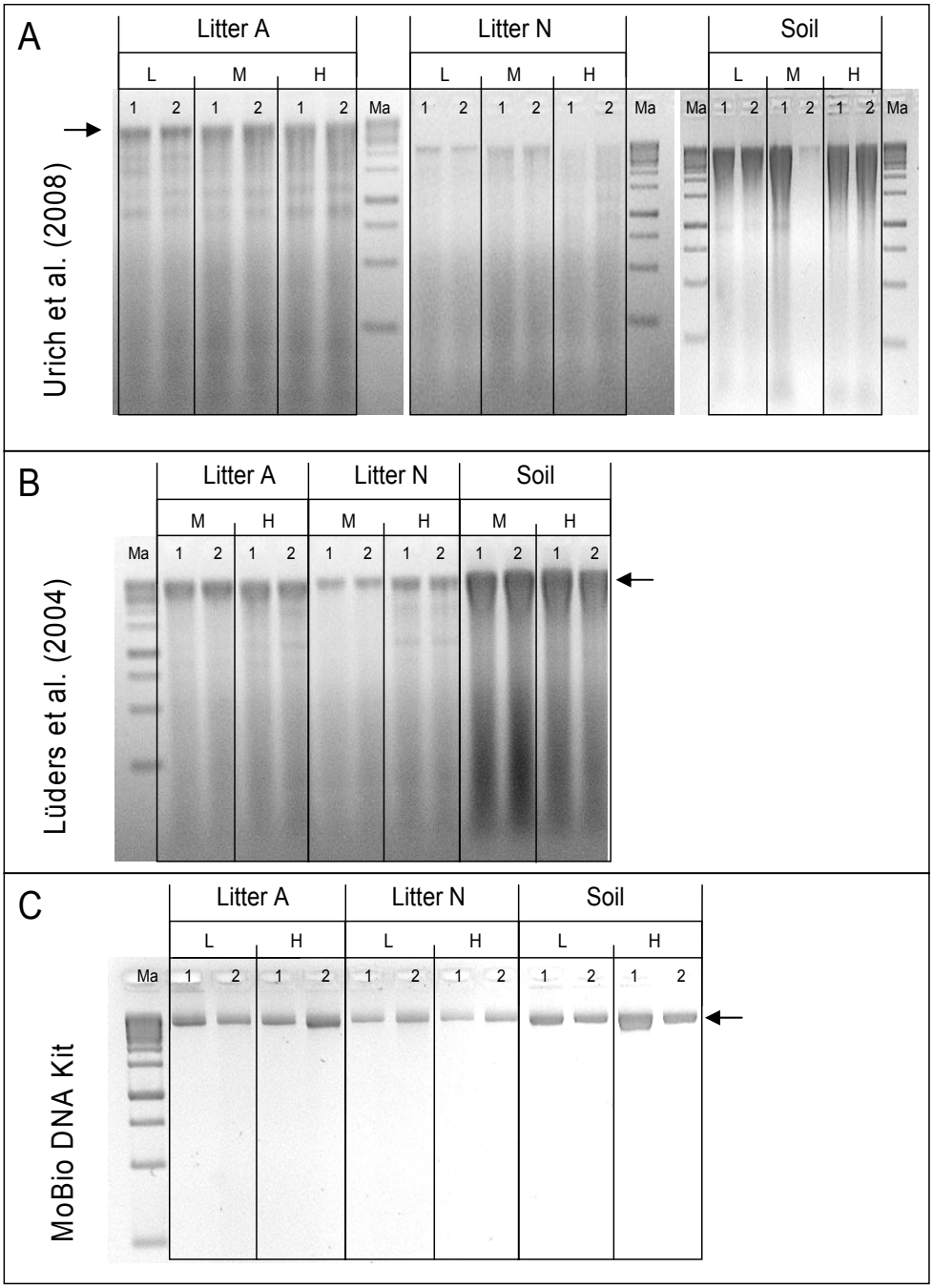


Figure C. 6. Examples for the qualitiy control of DNA extracted from soil, "Litter A" and „Litter N" after protocol of (A) Lüders et al. (2004), (B) Urich et al. (2008) and (C) MoBio Power Soil™ DNA Kit via agarose gel elecrophoresis. L, M and H are abbreviations for the different bead beating treatments "Low", "Medium" and "High", respectively; for specification of these terms see figure C.5. or section B.7.1. Replicates are numbered (1,2). Ma indicates the marker (GeneRuler™ 1kb DNA ladder). Arrows indicate high molecular weight DNA. Clearly harsher bead beating conditions lead to an increase in fragmented DNA.

C.2.1.3. PCR performance of isolated DNA

To test for potentially remaining inhibitory substances, a PCR amplifying the bacterial 16S rRNA gene with the primers 616V/1942 under standard conditions (Section B.7.4.1.) was carried out. For the soil and the "Litter N" sample DNA extracted with the protocol of Urich et al. (2008) and the MoBio Power Soil™ DNA Kit could be amplified without difficulty, but a PCR product with DNA isolated after the protocol of Lüders et al. (2004) was only obtained after an 1:10 dilution for soil and „Litter N", respectively (Table C.1.). This indicates the disposition of inhibitory substances within the isolated DNA. Amplification of DNA from "Litter A" was not possible from any of the DNA extracts without dilution. DNA extracted after Urich et al. (2008) could be amplified after a dilution of 1:10, DNA extracted after Lüders et al. could only be amplified after a dilution of 1:100 (Table C.1.).

Table C.1. Amplification efficiency (PCR performance) of DNA extracted after different protocols. Table shows the minimum template dilution neccessary to obtain a PCR product. The different beat beating treatments were pooled before amplification. Undil. indicates that dilution was not necessary.

protocol	dilution		
	"Litter A"	"Litter N"	soil
Lüders et al. (2004)	1:100	1:10	1:10
Urich et al. (2008)	1:10	undil.	undil.
MoBio DNA Kit	undil.	undil.	undil.

C.2.2. Identification of nitrifying microorganisms through amplification and cloning of gene fragments

To identify potential nitrifiers in the different litter and soil layers of the study site, phylogenetic and functional marker genes were amplified and cloned. Since the MoBio Power Soil™ DNA Kit was only used at a later time point, all PCR products obtained in the following sections were amplified from DNA extracted after Urich et al. (2008) (Section B.7.1.). Table C.2. gives an overview of the results of amplification of the different gene fragments and the dilution used for the respective sample. It shows that nitrifying microorganisms could only be amplified from soil, but not from "Litter A" and "Litter N", despite several attempts. Therefore the following chapters will only describe results from the Klausenleopoldsdorf soil sample.

For the exact numbers of clones picked, clones with an insert in the expected size, RFLPs, clones sequenced and numbers of retrieved sequences please refer to appendix table I.6.

C.2.2.1. Ammonia-oxidizing organisms in an acidic forest soil

The bacterial and crenarchaeal ammonia-oxidizing communities in acidic forest soil samples were investigated via PCR on a functional marker level, using primers specific for *amoA*, and, for *Archaea*, also on the 16S rRNA level. Products obtained were cloned, subjected to a RFLP analysis, sequenced and phylogenetically analyzed.

Table C. 2. PCR performance. Results of amplification of diverse gene fragments in leaf-litter and soil samples from Klausenleopoldsdorf performed with DNA extracted after protocol of Urich et al. (2008).

target	primers[a]	Product?		
		"Litter A"	"Litter N"	Soil
Bacterial 16S rRNA gene	616V/1492R	1:100[b]	undil.[b]	undil.
Archaea 16S rRNA gene	21F/1492R bzw. 21F/958R	n.d.[c]	n.d.	undil.
Crenarchaeal *amoA*	Arch-amoAF/R (Francis et al.)	1:100	undil.	undil.
Bacterial *amoA*	AmoA1F/2R	1:100	undil.	undil.
Nitrospira 16S rRNA gene	616V/1158R	1:50[a]	undil.	undil.
Nitrospira n*xrB*	F19/R1237 and F14/R1239	1:50	undil.	undil.
Nitrobacter nxrB	NxrBF706/NxrBR1431	1:100	n.d.	undil.

…product was obtained …no product was obtained

[a] for a detailed description of primers see section B.7.4.
[b] 1:100; 1:50; undil. indicates that template was diluted 1:100, 1:50 or was used undiluted
[c] n.d. … not determined

C.2.2.1.1. Amplification and cloning of bacterial *amoA* genes

The bacterial *amoA* was amplified using primers AmoA1F/AmoA2R (Table B.10.) under conditions described in table B.11. and a product in the expected size of 453 bp was obtained. The product was cloned as described in section B.7.5. and 20 clones were picked for screening. 11 out of these 20 clones had an insert in the expected size. Due to the low number of clones no RFLP analysis was done but all clones were sequenced (Appendix table I.6.).

C.2.2.1.2. Phylogeny of AOB in an acidic forest soil analyzing AmoA

8 of the sequenced clones were closest related to a bacterial *amoA* in a BLAST analysis, imported into the ARB database and aligned with comparative nucleic acid and amino acid sequence analysis (Section B.7.10.).

All AmoA sequences were closely related and more than 95% similar to each other. They clustered together with several other soil and sediment clones within the genus *Nitrosospira* (Figure C.7.) and were on average 98.8% similar to the next cultivated species *Nitrosospira*

sp. En13, an organism also isolated from acidic soil (pH 4.5). Due to the low resolution of AmoA (Purkhold et al. 2003) no assignment to a specific cluster could be made.

C.2.2.1.3. Amplification, cloning and RFLP analysis of the archaeal 16S rRNA genes

Archaeal 16S rRNA genes were amplified with primers 21F/1492R or 21F/958R under conditions specified previously (Table B.9.) and a product in the size of 1 438 bp and 912 bp, respectively, was obtained. In total, after cloning as described in section B.7.5., 18 and 25 clones had an insert in the expected size. From each RFLP pattern at least 3 clones were sequenced (See also appendix table I.6.).

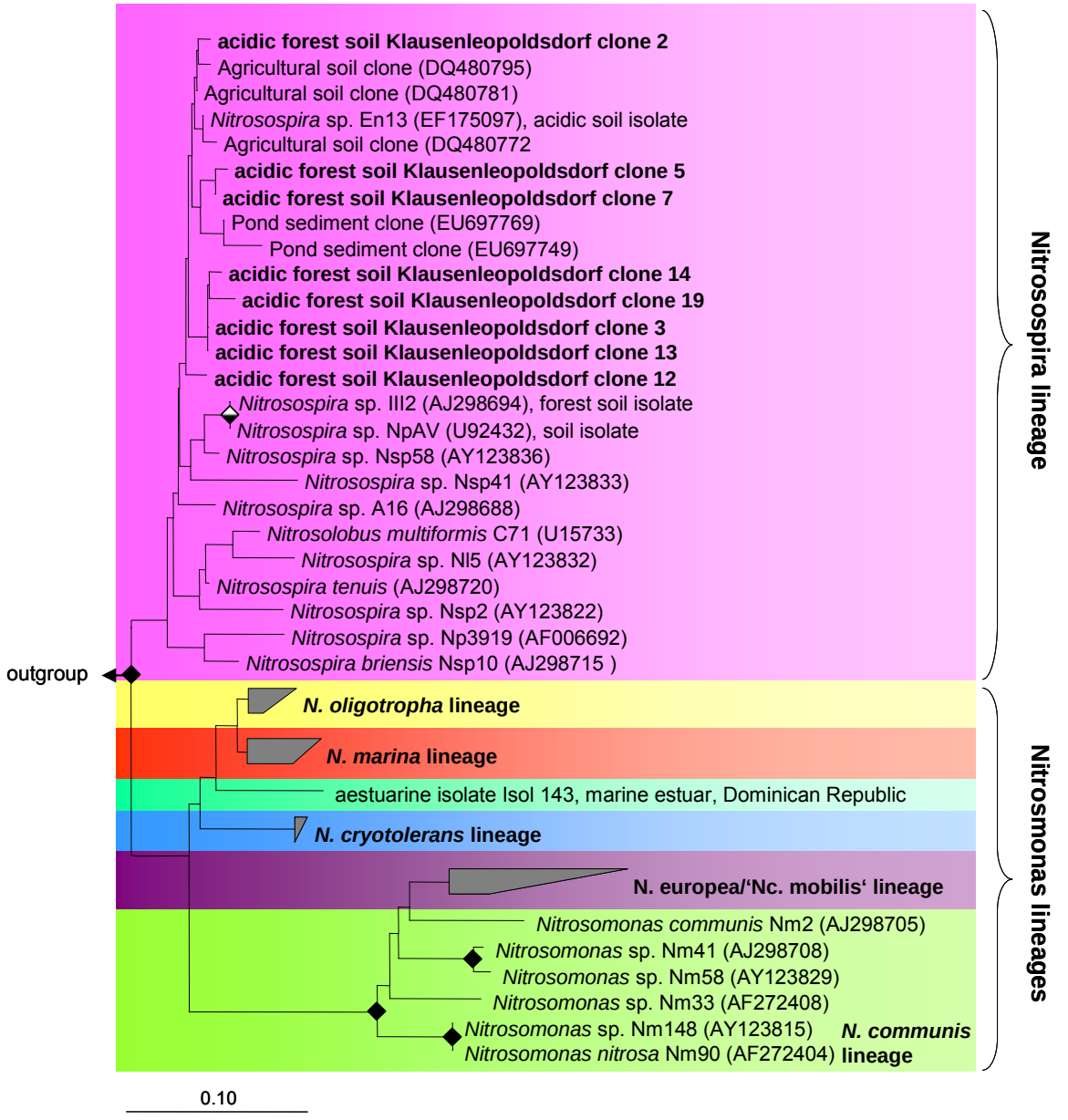


Figure C.7. AmoA based evolutionary distance (Fitch) tree of β-proteobacterial ammonia-oxidizers, showing the positioning of sequences obtained from Klausenleopoldsdorf acidic forest soil (in bold). For tree calculation sequences >138 aa positions were taken into consideration. Nodes with filled squares and half filled squares indicate bootstrap support >90% and >70%, respectively. Scale bar represents 10% estimated sequence divergence.

C.2.2.1.4. Amplification and cloning of crenarchaeal *amoA* genes

Using primers CrenAmoA1F/1R designed by Könneke at al. (2005) (Table B.10.) with DNA extracted from "Litter N" a PCR product in the size of ~640 bp was obtained under amplification conditions described in table B.11., cloned and sequenced (Sections B.7.5. and B.7.9.), but none of the sequenced clones was affiliated with crenarchaeal *amoA* genes. Analysis of retrieved sequences in ARB showed that the reverse primer bound to both ends, amplifying a random gene fragment that is not similar to any sequence deposited in public databases.

Therefore for amplification of *amoA* from soil, primers Arch-amoAF/R, designed by Francis et al. (2005) (Table B.10.), were used. A gene fragment in the size of 634 bp was obtained and cloned (Section B.7.5.). 3 clones contained an insert in the expected size and were sequenced (Appendix table I.6.).

C.2.2.1.5. Phylogeny of AOA in an acidic forest soil

a) Archaeal 16S rRNA phylogeny

In total 6 long (primers 21F/1492R) and 4 short (primers 21F/958R) archaeal 16S rRNA sequences could be identified with BLAST and were imported into ARB for further phylogenetic analysis (Section B.7.10.). In either case only sequences exhibiting the dominant RFLP pattern turned out to be of archaeal origin. The ML-tree was calculated with sequences longer than 1 300 bp and a 50% conservation filter. Shorter sequences were added afterwards using the "Parsimony interactive" tool of ARB.

In tree calculation, with exception of sequences KL14-1, KL14-15, KL14-16 and KL14-22, which all were ≥99.5% similar to each other and formed a monophyletic cluster, the obtained sequences formed at least three separate clusters within the group I.1b, close to other clones obtained from soil habitats (Figure C.8.). However, none of the clones was closely related to the two sequences in group I.1b for which the presence of an *amoA* has already been shown: the thermophilic AOA "*Candidatus* Nitrososphaera gargensis", was closest related with a range from 91.9% to 93.6%, the soil fosmid 54d9, harboring an *amoA*, was at the most 92.2% similar to the clone sequences.

b) Crenarchaeal AmoA phylogeny

Due to very bad cloning efficiency, despite of several attempts, only 3 sequences could be obtained, out of which only 2 were closest related to crenarchaeal *amoA* genes in a BLAST search. AmoA sequences retrieved from these two clones were more than 99% similar to

each other and clustered together with other soil and freshwater clones within group I.1b (Figure C.2.). Additionally, they were on average 94.6% similar to the soil fosmid 54d9, one of the few AmoA sequences that can be related to a 16S rRNA gene.

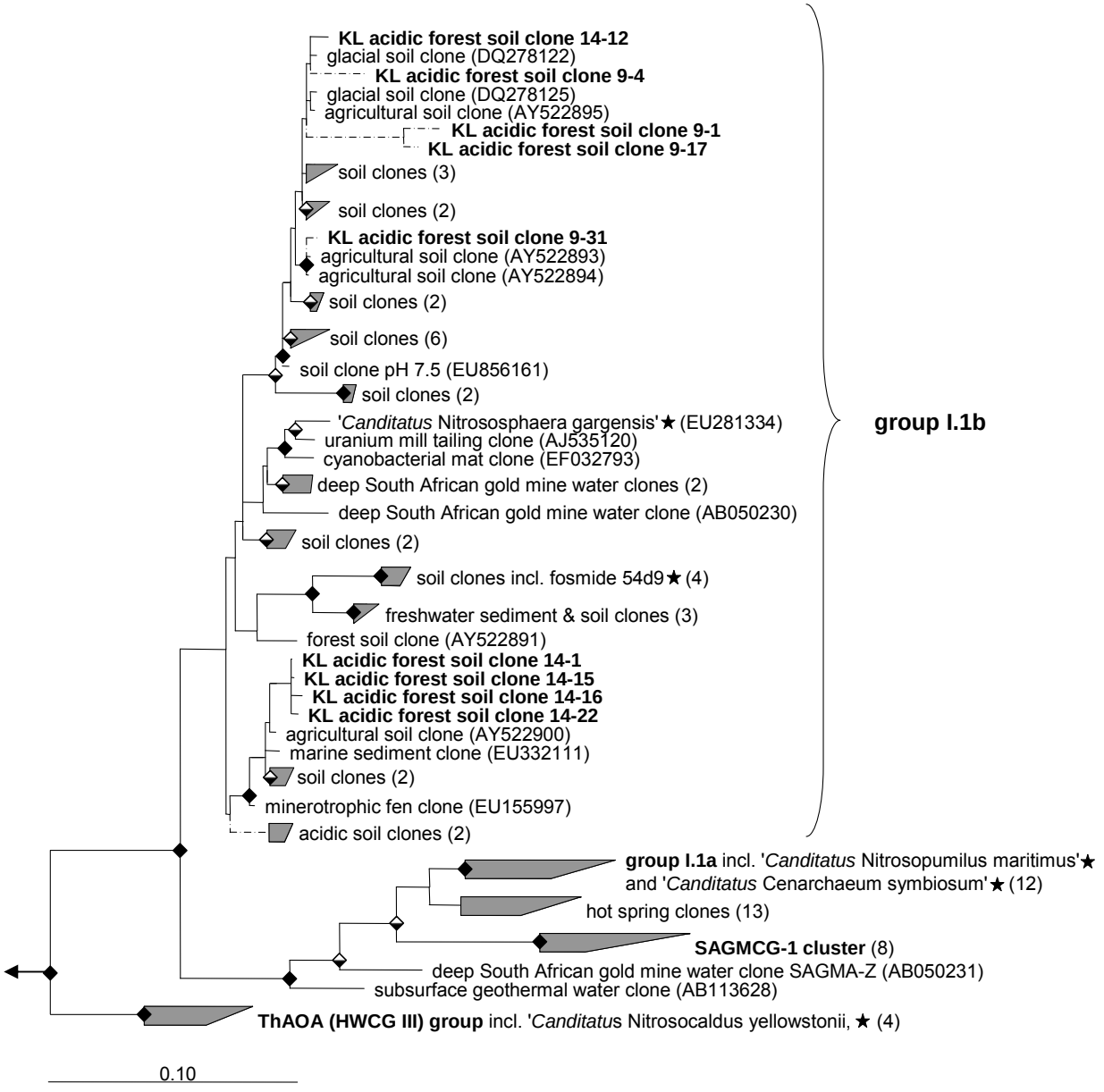


Figure C.8. Maximum Likelihood phylogenetic tree of crenarchaeal 16S rRNA gene sequences showing the position of sequences retrieved from Klausenleopoldsdorf acidic forest soil. For tree calculation sequences >1 300 bp were taken into consideration. Shorter sequences were added afterwards and are indicated by slashed lines. Nodes with filled squares and half-filled squares indicate parsimony bootstrap support >90% and quartet puzzling reliability values >70%, respectively. Asterisks mark the organisms/gene fragments for which the presence of an *amoA* has been demonstrated. Accession numbers are written in brackets. In this tree only groups relevant for depiction of positioning of obtained sequences are shown, for a more complete tree please refer to figure C.1. The scale bar represents 10% estimated nucleotide sequence difference.

C.2.2.2. Nitrite-oxidizing bacteria

Since as well *Nitrobacter* spp. as *Nitrospira* spp. are known to be abundant in soil environments (Bartosch et al. 2002, Cebron and Garnier 2005, Poly et al. 2008, Urich et al. 2008), the presence of both nitrite-oxidizing organisms was investigated based on the functional gene *nxrB* and for *Nitrospira*-like bacteria also based on the 16S rRNA gene.

C.2.2.2.1. Amplification, cloning and RFLP analysis of 16S rRNA gene fragments of *Nitrospira*-like bacteria

The presence of *Nitrospira*-like bacteria was investigated using primers specific for most *Nitrospira* (Table B.8.) for amplification of 16S rRNA gene fragments under conditions specified in section B.9. This resulted in a product of 1 165 bp, which was cloned. 16 clones with an insert in the expected size could be identified and were subjected to RFLP. For each pattern at least 2 clones were sequenced (Appendix table I.6.).

C.2.2.2.2. Amplification, cloning and RFLP analysis of *nxrB* gene fragments of *Nitrospira*-like bacteria

Primers F14/R1237 (Table B.12.) were used in a gradient PCR (Section B.7.4.4.) to amplify the *nxrB* gene fragment of *Nitrospira*-like bacteria. A PCR product in the expected size of 1 244 bp was obtained at relatively high annealing temperatures. The highest annealing temperature tested, 68°C, still yielded a product. The PCR product was cloned as described in section B.7.5. and subjected to RFLP analysis. Several RFLP patterns were observed and all clones were sequenced (Appendix table I.6.).

C.2.2.2.3. Phylogeny of *Nitrospira*-like bacteria in an acidic forest soil

a) 16S rRNA phylogeny

8 out of 10 sequences (3 patterns) were most similar to *Nitrospira*-like bacterial 16S rRNA gene sequences and were imported into ARB for further analysis. Prior to tree calculations the database was updated with all *Nitrospira*-like sequences >1 300 bp retrieved from the newest release of the SILVA database (release 96; SILVA rRNA database project, http://www.arb-silva.de/).

All of the retrieved sequences could be identified as *Nitrospira* sublineage II sequences and all clones except clone 22 were more than 99% similar to each other. Clone 22 being on average only 98.1% similar to the rest of the other sequences and positioned more distantly to other clones in the tree (Figure C.5.). Sublineage II mainly consists of sequences

obtained from mesophilic habitats and also in this case sequences clustering together with sequences obtained in this study were derived from various soil and freshwater habitats. The currently sole cultivated member of sublineage II, the thermophilic *Nitrospira moscoviensis* was only distantly related (95.5%).

Figure C.9. Phylogenetic Maximum Likelihood tree of 16S rRNA gene sequences of the genus *Nitrospira*. Sequences obtained from Klausenleopoldsdorf acidic forest soil are written in bold. The tree was calculated using a 50% conservation filter and only with sequences >1 300 bp, dashed lines indicate shorter sequences that were added afterwards. Nodes with filled squares and half-filled squares indicate parsimony bootstrap support >90% and quartet puzzling reliability values >70%, respectively. Accession numbers are written in brackets. Arrow indicates the outgroup, containing sequences of the phylum *Nitrospirae*. The scale bar represents 10% estimated nucleotide sequence difference.

b) Phylogeny of *Nitrospira*-like bacteria analyzing *nxrB*

All sequences could be identified as *nxrB* gene fragments and were imported into ARB and aligned by comparative nucleic acid and amino acid sequence analysis. For a description of sequences used in tree calculation see section C.1.4.3. b).

Sequences obtained were between 98.7% up to 99.4% similar to each other on nucleic acid level and established a cluster together with sequences obtained from another forest soil, but interestingly not with *nxrB* sequences obtained from *Nitrospira moscoviensis*. Thus, *Nitrospira* sublineage II is not a monophyletic group in the phylogenetic analysis of *nxrB* (Figure C.3.B). This is also reflected in the fact that the sequences obtained were on average with 89.1% slightly more similar to *nxrB* of "*Candidatus* Nitrospira defluvii" than to *Nitrospira moscoviensis* (88.4%) on the nucleic acid level. These sequences were also included in the tree as shown in figure C.3.B.

C.2.2.2.4. Amplification, cloning and RFLP analysis of *nxrB* gene fragments of *Nitrobacter*-like bacteria

NxrB of *Nitrobacter* sp. was amplified in a gradient PCR (Section B.7.3.4.) with the primers NxrBF706/NxrB1431 (Table B.12.), which yielded a product of ~725 bp of length. It was not possible to determine an optimal annealing temperature via gradient PCR. Several bands, including one in the right size, were detected at all temperatures from 50-62°C (data not shown). Nevertheless, the product was cut out from a gel and cloned (Section B.7.5.4.). 20 clones with an insert in the expected size were subjected to an RFLP analysis, in which 2 distinct patterns could be identified. 3 clones of each pattern were sequenced but only one type of pattern showed similarity to known *nxrB* sequences of *Nitrobacter* sp. Thus, 3 sequences could be imported into ARB for further sequence analysis (Appendix table I.6.).

C.2.2.2.5. Phylogeny of *Nitrobacter*-like bacteria analyzing *nxrB*

For phylogenetic analysis of Nitrobacter-like bacteria on the basis of *nxrB* a new ARB database was established. It included all *Nitrobacter*-like *nxrB* sequences found in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and all sequences retrieved in this study. They were imported into ARB and aligned on the basis of comparative sequence analysis (Section B.7.10.). Sequences shorter than 1 000 bp were not included in tree calculations, but added afterwards, with the "Parsimony Interactive" tool integrated in ARB (Section B.7.10.5.).

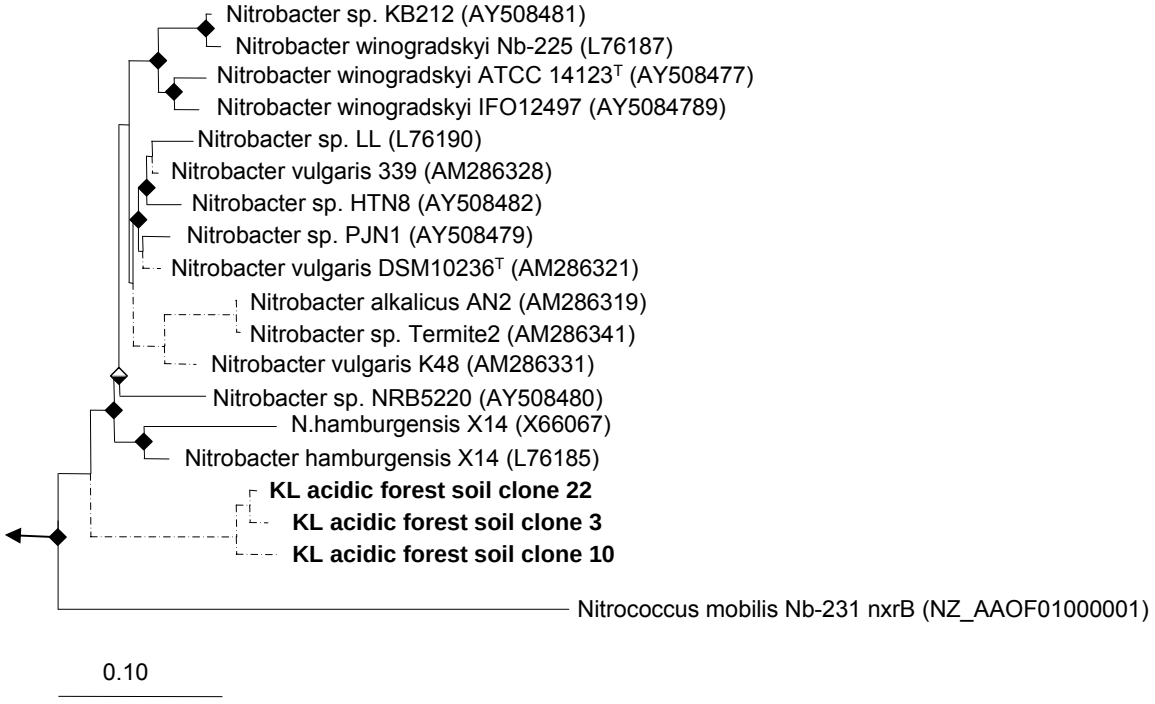


Figure C.10. Phylogenetic Maximum Likelihood tree of *nxrB* sequences of the genus *Nitrobacter*. Sequences obtained from Klausenleopoldsdorf acidic forest soil are written in bold and form a monophyletic cluster outside of the *nxrB* sequences of all known *Nitrobacter* species. The tree was calculated with sequences >1 000 nt, dashed lines indicate shorter sequences, which were added afterwards. Nodes with filled squares and half filled squares indicate bootstrap support >90% and >70%, respectively. Accession numbers are written in brackets. Arrow indicates the outgroup consisting of several *narH* and *narY* sequences, which are the closest related protein sequences to *nxrB* of *Nitrococcus*. The scale bar represents 10% estimated nucleotide sequence difference.

All *nxrB* sequences derived from acidic forest soil were more than 96% similar to each other on the nucleic acid level. They formed a monophyletic cluster outside of all other *nxrB* sequences of cultivated *Nitrobacter* species (Figure C.10.) and were only distantly related. The nucleic acid similarity to *Nitrobacter hamburgensis* X14^T (accession number X66067) and *Nitrobacter winogradski* ATCC 14123^T (accession number AY5084789) was 86.3% and 83.4%, respectively. Additionally, the sequences were on average 68% similar to the putative *nxrB* of *Nitrococcus mobilis* Nb-231 (Accession number NZ_AAOF01000001).

C.2.3. *In situ* detection of Nitrospira-like bacteria using CARD-FISH

Since it is known that application of FISH working with soil samples is challenging due to high background fluorescence (Figure C.11.A), CARD-FISH, which greatly enhances the signal intensity was applied to the soil sample to detect *Nitrospira*-like bacteria *in situ*.

Applying the probe Ntspa662, which is specific for the genus *Nitrospira*, a few labeled cells could be observed under the microscope (Figure C.11.B). The morphology of some of them could be described as long spiral-shaped rods, typically found for *Nitrospira* spp.

(arrow in figure C.11.B). Additionally a less bright signal was observed from rod-shaped objects, which did not show a DAPI signal as well in the sample, hybridized with probe Ntspa662, as in the negative control.

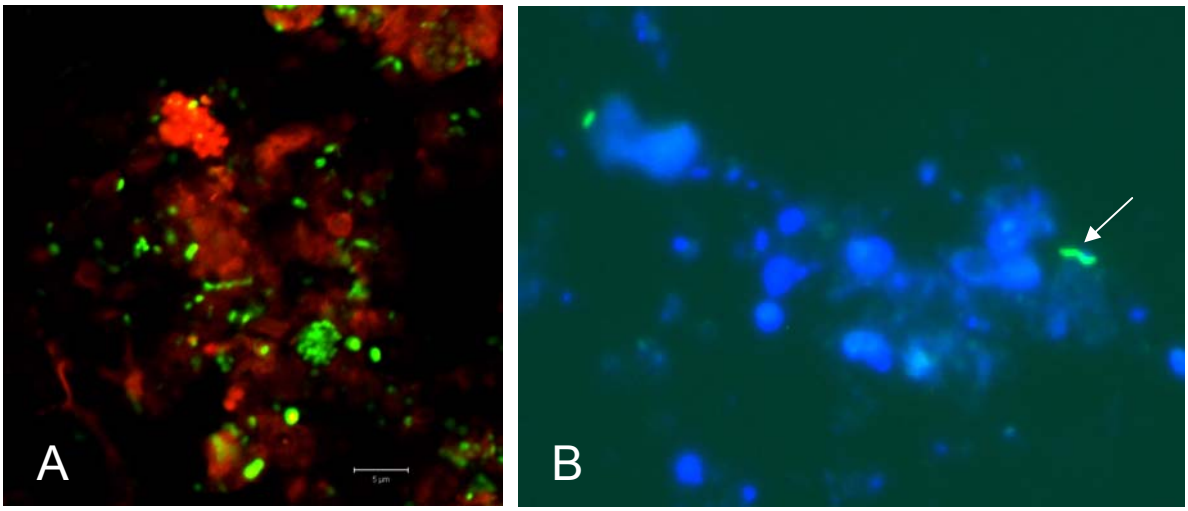


Figure C.11. CARD-FISH in soil. (A) Autofluorescence. Bacteria are labeled with probe EUB (in Fluos). Autofluorescence of soil particles in red. (B) 2 cells labeled with probe Ntspa662, specific for the genus *Nitrospira* (in Fluos), one with spiral shape (arrow). The sample was also labeled with DAPI, but signals mainly originate from autofluorescent soil particles.

C.3. Expanding the *nxrB* sequence dataset

To check the suitability of *nxrB* of *Nitrospira* as a functional marker, several samples were screened for this gene. Among these samples was a pure culture of *Nitrospira marina*, an enrichment from Beryl Spring (Yellowstone National Park, USA) and sponge material (Section B.6.3.). Further sequences were provided by F. Maixner and H. Koch from a SBR enrichment, pure cultures of "*Candidatus* Nitrospira bockiana" and *Nitrospira moscoviensis*, originating from an waste water treatment plant (WWTP).

Additionally, to investigate if this gene is also actively expressed in the environment, mRNA was isolated from a WWTP and subsequently cDNA, was produced with *nxrB* specific primers and kindly provided by H. Koch (Section B.6.3.).

C.3.1. Establishing PCR with *nxrB* specific primers

To establish PCR with *nxrB* as a phylogenetic marker, two primer pairs were designed by F. Maixner and tested on diverse samples. However results showed that neither the primers F14/R1239 nor F19/R1237 were able to amplify the *nxrB* fragment of all samples (Appendix table I.3.). This problem could also not be solved by design of a new forward

primer (F916). Additionally, it was not possible to determine a general optimal annealing temperature for these primers. While *nxrB* of *Nitrospira marina* could only be amplified at quite low temperatures (52.3°C), *nxrB* from Klausenleopodsdorf soil samples could only be amplified at relatively high temperatures (62-68°C; data not shown).

C.3.2. Amplification, cloning and RFPL analysis of *nxrB* sequences from various samples and environments

a) *Nitrospira marina*

The *nxrB* gene fragment was amplified using the primers F19/R1237 under standard conditions (Tables B.12. and B.13.) using an annealing temperature of 52.3°C and a product of 1 218 bp was obtained and cloned into competent *E. coli* cells (Section B.7.5.). As only one insert positive clone could be identified, no RFLP analysis was done and the clone was sequenced.

b) cDNA from a wastewater treatment plant

No PCR product could be obtained with the long range primers F14/F19 and R1237/R1239 (F. Maixner, personal communication). Therefore, forward primer F916, amplifying a shorter fragment, was used together with reverse primer R1237. A product of 321 bp was obtained from cDNA BI_2, but not from the DNAse digested control (Figure C.12.). The PCR product was cut from the gel and cloned (Section B.7.5.4.). 15 out of 19 clones contained an insert in the expected size and were subjected to RFLP analysis. Two patterns, one occurring only a single time, were identified and 2 and 1 clone respectively were sequenced.

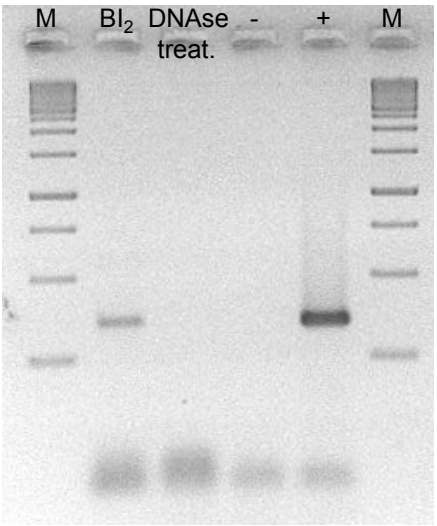


Figure C.12. PCR product obtained from transcribed cDNA (BI_2), confirming the active transcription of NxrB in a wastewater treatment plant. "M" indicates the marker (GeneRuler™ 1kb DNA ladder), "+" indicates the positive control; "-" the negative control.

c) Beryl Spring

To amplify *nxrB* from an enrichment from Beryl Spring, a hot spring in Yellowstone National Park, USA, PCR was performed using the primers F19/R1237 under standard conditions (Table B.13.) and with an annealing temperature of 59.9°C. The product of approximately 1 200 bp was cut out from the gel and cloned as described in section B.7.5.4. 11 out of 16 screened clones carried an insert in the expected size and two patterns, one occurring only a single time, were identified during RFLP analysis, so 3 and 1 clone(s), respectively, were sequenced.

d) Sponge

No PCR product could be obtained with primer pair F14/R1239, with primer pair F19/R1237 (Table B.12.) or with the newly designed forward primer F196 in combination with reverse primer R1237 (Appendix table I.3.). Later on, *nxrB* sequences could be obtained with forward primer F196 and a newly designed reverse primer, amplifying a shorter fragment of *nxrB* (F. Maixner, personal communication).

C.3.3. Phylogenetic analysis

Sequences identified as *nxrB* genes with BLAST were imported into ARB and included in phylogenetic analysis done for *nxrB* sequences obtained from Klausenleopoldsdorf acidic forest soil and hot springs at Lake Baikal and in Kamchatka (Sections C.1.4.3.b); C.2.2.2.3.b); Figure C.3.B). Due to sequence length of only 321 bp, sequences derived from cDNA from a wastewater treatment plant were added after tree calculations with the "Parsimony Interactive" tool of ARB.

Over all, the phylogenetic tree calculated from *nxrB* sequences of *Nitrospira*-like bacteria resembles the tree obtained from analysis of the 16SrRNA gene (Figures C.3.A and B). Nearly all clusters obtained 16S-based, could also be obtained *nxrB*-based. The only exception is the affiliation of soil clones, belonging to sublineage II in the 16S rRNA gene tree, which are closer related to "*Candidatus* Nitrospira defluvii" (sublineage I), than to *Nitrospira moscoviensis* (sublineage II) in the *nxrB* tree. In general, available members of published sublineages (Daims et al. 2001), were between 81.8% and 90% similar to each other (Appendix table I.4.). Additionally, it was not possible to resolve the exact branching order of the thermophilic clusters at the bottom of the tree and a multifurcation was introduced (Figure C.3.B).

D. DISCUSSION

D.1. New lineages of nitrifiers from hot springs in Russia and Kamchatka

D.1.1. Identifying the ammonia-oxidizing organism(s) of enrichment culture 8

D.1.1.1. Still no evidence for thermophilic AOB

Up to date there is no evidence for thermophilic AOB, except for one cultivation study done by Golavacheva in 1976. In that study, it was claimed that the organism responsible for ammonia oxidation in an ammonia-oxidizing enrichment culture is of bacterial origin, but this could not be confirmed since the final isolation and characterization failed (Golovacheva 1976, cited after Lebedeva et al. 2005).

Also in this work, it was not possible to amplify bacterial *amoA* from hot spring ammonia-oxidizing enrichment cultures thriving at 46°C (Section C.1.3.1.). This is in accordance with other studies that failed to amplify bacterial *amoA* from environmental samples (Hall et al. 2008, Reigstad et al. 2008) or ammonia-oxidizing enrichment cultures, originating from hot springs (de la Torre et al. 2008, Hatzenpichler et al. 2008). Even though it can't be excluded that a potential AOB was not targeted with the primers used, it seems likely that no AOB is thriving in enrichment culture 8 and the obtained AOA (Section C.1.3.4.) is responsible for the observed oxidation of NH_3.

D.1.1.2. Phylogeny of a novel putative AOA

Despite extensive screening of the archaeal 16S rRNA gene and *amoA* clone libraries established from enrichment culture 8, all obtained 16S rRNA gene and AmoA sequences were highly similar to each other (Section C.1.3.4.). The high similarities suggest that both genes were derived from a single organism and since no additional sequences were obtained, it might be concluded that there is only a single AOA present in enrichment culture 8. However, phylogenetic affiliations of 16S rRNA gene and crenarchaeal AmoA sequences look quite diverging at the first sight. The 16S rRNA gene sequences form a new lineage between SAGMCG-1 and the marine group I.1a, together with other sequences derived from thermal habitats (Takai et al. 2001, Nunoura et al. 2005) (Figure C.1.). AmoA sequences were also affiliated with other hot spring sequences derived from various studies (Spear et al. 2007, Reigstad et al. 2008, Weidler et al. 2008, Zhang et al. 2008), but built a thermophilic cluster within marine group I.1a (Figure C.2.). This raised the questions if (i) these genes were derived from the same or two different organisms, which could be

possible if the 16S rRNA gene of the putative AOA was not be amplified with the PCR performed in this study or (ii) horizontal gene transfer (HGT) (Doolittle 1999) took place, as for example also observed in the case of the phylogenetic marker *dsrAB* (Zverlov et al. 2005). However, the positioning of AmoA sequences within group I.1a is not reliable, since there is only very low bootstrap support (illustrated by the need to implement a multifurcation in a consensus tree of distance- (Fitch) and MP-tree topologies; Appendix figure I.1.). Consequently, AmoA-based phylogeny might not be able to resolve the evolutionary succession of the novel cluster and groups of marine group I.1a and it is still be possible that the AmoA phylogeny is in congruence with the 16S rRNA gene-based phylogeny.

Additional evidence for the derivation of both genes from a single species comes from a study published by Spear and colleagues (Spear et al. 2007). Most studies investigating microorganisms in thermal habitats only investigate 16S rRNA gene (Takai et al. 2001, Nunoura et al. 2005) or – in cases they are specifically looking for AOA - only *amoA* sequence diversity (Reigstad et al. 2008, Zhang et al. 2008). Just one study, done by Spear and colleagues did not only investigate both, but also found a low sequence diversity allowing them to speculate about origin of both genes from the same organism (Spear et al. 2007). Since 16S rRNA genes as well as *amoA* sequences obtained in that study are clustering together with sequences obtained from enrichment culture 8 (Figures C.1. and C.2.), this might bring further evidence to the assumption that both genes originate from a single organism.

So even though cloning or PCR biases, which might have led to non-amplification of the 16S rRNA gene of the ammonia-oxidizing crenarchaeote, cannot be excluded, these results, together with the observations made by Spear and colleagues (Spear et al. 2007), strongly suggest that the 16S rRNA gene and *amoA*, obtained from enrichment culture 8 in this study, do indeed originate from the same organism. Nevertheless, to gain certainty about the origin of both genes from a single species, a pure culture, as done for "*Candidatus* Nitrosopumilus maritimus" (Könneke et al. 2005) or sequencing of the enrichment in a metagenomics approach, which for example revealed the linkage of proteorhodopsin to an uncultured γ-proteobaterium (Beja et al. 2000), would be necessary.

D.1.1.3. *In situ* detection of a novel putative AOA

FISH-MAR would be a suitable tool to confirm that the organism obtained by cloning and sequencing from enrichment culture 8 (Section C.1.3.4.) is responsible for the observed oxidation of NH_3. However, it was not possible to establish FISH or CARD-FISH for the

novel putative AOA of enrichment culture 8. The unsuccessful attempt to establish FISH with the general archaeal probe Arch915 as well as with the crenarchaeal probe Cren712 (Section C.1.5.1.) could be caused by failure in cell wall permeabilization, inaccessibility of the target site or low ribosome content of cells.

Standard FISH probes are of low molecular weight (500 – 800 Da, (Amann and Fuchs 2008)) and in an aquatic sample 70-90% of all microscopically visible cells were permeabilized by simple fixation methods (Amann et al. 1995). Additionally, only general archaeal and crenarchaeal probes were used, which were successfully hybridized to other members of the respective groups before (Stahl and Amann 1991, Burggraf et al. 1994, Jurgens et al. 2000). Consequently, it seems implausible that the hybridization failed due to cell wall impermeability or low target site accessibility, but more likely that failure was caused by low ribosome content of these archaeal cells (Amann et al. 1995, Hatzenpichler 2006, Amann and Fuchs 2008). Ribosome content in cells can range from a few hundred to 100,000 per cell (Amann and Fuchs 2008), but to obtain a visible FISH signal at least approximately 1,400 rRNA copies per cell are required (Hoshino et al. 2008). Consequently, the number of ribosomes in these archaeal cells might lie below the detection limit of FISH. Low ribosomal content can be a result or of slow growth rate (Schaechter et al. 1958, cited after Amann et al. 1992), as it was also reported for "*Candidatus* Nitrosopumilus maritimus" (Könneke et al. 2005), or of inactivity of the targeted cells. The low ribosomal content might be a general characteristic of the crenarchaeal "group I", but there are only few cultivated or enriched members (Könneke et al. 2005, de la Torre et al. 2008, Hatzenpichler et al. 2008) and only few facts about the physiology of the members of this group are known yet. Therefore no general conclusions can be drawn concerning this issue and further research would be needed.

Application of CARD-FISH, an approach that has been shown to be able to visualize populations that were not detected by standard FISH (e.g. Pernthaler et al. 2002), was not successful either. When a NonSense probe (5'-AGA GAG AGA GAG AGA–3') was applied, bright FISH signals were detected (Section C1.5.1), indicating non-specific binding of the probes to the matrix of the cell clusters. The false positive signal made it impossible to identify signals originating from hybridized cells and consequently to decide if hybridization was successful. This could be caused by (i) probe oligonucleotides binding to the particles (ii) the enzyme binding to the particles or (iii) mechanic reasons (see below). Since unspecific binding of oligonucleotides was not detected with standard FISH, binding of CARD-FISH probe oligonucleotides seems also unlikely. Additionally, binding

of DNA or the enzyme would be highly hindered by the high molecular weight of the enzyme (40 kDA; Amann and Fuchs 2008). These assumptions are also supported by the lacking effects of addition of salmon sperm, which should prevent the probe from binding to DNA, or BSA, used as a blocking reagent. Consequently, the most plausible explanation remains that some probes might be caught mechanically within the observed inorganic matrix. Due to the tyramide amplification only one probe getting trapped might be enough for a detectable signal. To establish a successful CARD-FISH protocol it might therefore help to destroy the cell clusters. This would not eliminate the false positive signals (which might not easily be possible), but it could separate the target cells from the matrix and consequently allow to obtain true positive signals offside the matrix.

D.1.2. NOB

D.1.2.1. A novel thermophilic sublineage of *Nitrospira*-like bacteria enriched from hot springs in Russia and Kamchatka

Daims and colleagues proposed a threshold of 94.9% for sequences of one lineage and 94% for sequences of different lineages of the genus *Nitrospira*, based on experimental data (Daims et al. 2001). Phylogenetic analysis of 16S rRNA and *nxrB* genes showed that the sequences obtained from enrichment cultures A and C_a (Lake Baikal, Russia) were closest related to *Nitrospira*-like bacteria and highly similar to each other (Section C.1.4.3.). Similar, 16S rRNA gene sequences obtained from enrichment culture 4 and the dominant *Nitrospira*-like clone sequences of enrichment culture 8 (Kamchatka, Russia) and *nxrB* sequences form a monophyletic group, separate from sequences of enrichment cultures A and C_a and other hot spring clone sequences (Figure C.3.A). Still, enrichment cultures from Lake Baikal and Kamchatka were found to be 96.1% similar to each other, distinctly separated from the closest related organism "*Candidatus* Nitrospira bockiana" (Approx. 93.6% similarity; Appendix table I.1. and Section C.1.4.3.). So according to Daims et al. (2001) these organisms would constitute one new lineage. Therefore, after the enrichment of "*Candidatus* Nitrospira bockiana" (Lebedeva et al. 2008), the obtained data, support at least a second thermophilic phylogenetic sublineage within the genus *Nitrospira*. Nevertheless, as can be seen in Figure C.3.A, all *Nitrospira*-like bacteria, which (i) have their optimal growth temperature at elevated temperatures, (ii) were enriched at elevated temperatures, or (iii) were isolated from habitats with elevated temperatures, were obtained or have their (optimal) growth temperature at only moderately elevated temperatures (Figure C.3.A; red dots and temperatures indicated behind). No representative growing at

temperatures >55°C is known yet, even though Reigstad and colleagues reported complete nitrification in hot springs at temperatures up to 85°C (Reigstad et al. 2008). However, since NO_2^- is not stable at high temperatures and low pH, which was the case for the sites Reigstad and colleagues investigated, oxidation of NO_2^- could be mediated chemically not biologically. So it remains questionable if there is a niche for nitrite-oxidizing organisms and, if yes, who is carrying out nitrite oxidation at these high temperatures. Therefore, further research should be done to address this question and if possible to identify the key players in nitrite oxidation in (hyper)thermophilic environment.

D.1.2.2. Two different lineages of *Nitrospira*-like bacteria in enrichment culture 8?

The established 16S rRNA gene clone library revealed the presence of a second *Nitrospira*-like bacterium, closely related to *Nitrospira moscoviensis* and thus affiliated with *Nitrospira* sublineage II (Section C.1.4.3.a) and Figure C.3.A). This result was also supported by FISH as described in section C.1.5.2. Cells, only showing a signal with a *Nitrospira* probe-mix (Ntspa662 + Ntspa712), but not with the probe Ntspa-EL446, specific for the *Nitrospira*-like clones obtained from enrichment cultures 4 and 8 (for a discussion see section D.1.2.3.), could be detected (Figure C.4.C). The mix of probes, using different probes with the same fluorophore, was applied to gain a brighter signal; however, this has the disadvantage that it could not be distinguished if only one of the probes or both were binding to the target cells. This implicates that the signal might theoretically not originating from *Nitrospira*-like bacteria, but bacteria affiliated with other genera within the phylum *Nitrospirae*. To exclude this possibility and confirm *Nitrospira* sublineage II bacteria in the enrichment culture also *in situ*, probe Ntspa662 separately, or probes specific for sublineage II should be applied.

D.1.2.3. Fluorescence *In Situ* Hybridization - Evaluation of specific probes

Three probes, specific for the *Nitrospira*-like bacteria of enrichment cultures 4 and 8 were designed, as described in section B.7.10.3. Hybridization with two out of three probes, namely Ntspa-EL187 and Ntspa-EL197, did not result in a detectable signal (Section C.1.5.2.). This could be caused by (i) the combination of a low ribosome content of this organism, since also the EUB-Mix and Ntspa-Mix only gave a weak signal, (For a discussion of that problem see section D.1.1.3), with quenching (Torimura et al. 2001) or (ii) low accessibility of the target site, even though according to the 16S rRNA probe-accessibility schemes published (Fuchs et al. 1998, Behrens et al. 2003) both binding sites

were classified as brightness class II and should therefore be accessible. However, since *E. coli* and *Nitrospira* spp. are only very distantly related this classification might not be of high significance in this case. This is also underlined by the fact that probe Ntspa-EL446, which was hybridized successfully, is only classified as class III probe, but still a signal was observed (Figure C.4.A and C). Nevertheless, since the obtained signal was rather faint, application of CARD-FISH, which can increase signal intensity about 26 to 41-fold (Hoshino et al. 2008), or double-labeled oligonucleotide probes, which not only increase fluorescence signal intensity but also accessibility of the target site, (Stoecker et al. in press) would be possibilities for enhancement.

In enrichment culture 4 FISH-positive cells could only be detected with probes EUB-Mix and Ntspa-Mix, but not with the specific probe Ntspa446 (Section C.1.5.2.). The reasons mentioned above might also apply here, increased by the fact that enrichment culture 4 was nearly inactive when it arrived in Vienna and sample for FISH was taken (F. Maixner, personal communication).

As mentioned in section C.2.5.5., optimal hybridization conditions, thus the balance between specificity and stringency, was not tested for the probes, therefore results can only be preliminary. For this purpose the probe should be evaluated using the Clone-FISH approach described by Schramm and colleagues (Schramm et al. 2002).

D.1.2.4. Detection of NXR with the monoclonal antibody Hyb153-3

The immunofluorescence approach is an alternative way to show the presence of functionally related organisms. Studies have been done investigating the AMO of AOB (Pinck et al. 2001, Fiencke and Bock 2004, 2006) and the NXR of NOB (Bartosch et al. 1999, Alawi et al. 2007). In this study enrichment cultures 4 and 8 were hybridized with the monoclonal antibody Hyb153-3, targeting the NXR of NOB, and a signal was detected with samples of enrichment culture 8 (Figure C.4. F-G). This strongly suggested (i) that this organism is actively expressing NXR, indicating its involvement in nitrite oxidation and (ii) that this antibody also targets the NXR-system of this novel lineage of thermophilic *Nitrospira*-like bacteria, confirming that the antibody targets all known NOB (Bartosch et al. 1999, Alawi et al. 2007). This might indicate similar active sites (Spieck et al. 1998) or at least a common binding motif (Maixner et al. in preparation), which function as epitope for the antibody. Nevertheless, it should be noted that there is no evidence that the organisms targeted by immunofluorescence are identical with the organisms identified with cloning and FISH (Sections C.1.4. and C.1.5.2.). Combination of FISH and immunofluorescence should be performed for this purpose. Still, similarity of cell-shape

and size and abundance of the organism in FISH and immunofluorescence experiments (Figures C.4.A and F-G) strongly suggest that the same organism is targeted.

The lack of a signal from enrichment culture 4 (Section C.1.5.3.) might be caused by nearly non-active state of the enrichment. One could speculate that the expression of NXR, which is down regulated in response to starvation stress in "*Candidatus* Nitrospira defluvii" (Maixner et al. in preparation), was below the detection limit. However the organism was also found to maintain a relatively high "ground level" of expression (Maixner et al. in preparation), not supporting the possible explanation given above. Additionally, similar results were found by Pinck and colleagues, investigating the levels of AmoB in *Nitrosomonas europaea*. They detected high amounts of AmoB, even after starvation of cells for one year (Pinck et al. 2001). Therefore it might be more likely that the failure in the detection of NXR might be caused by changes in cell wall permeability, hindering the antibody from penetration into the cell or changes in epitope structure, which is consequently not targeted by the antibody anymore.

D.1.2.5. The novel lineage of *Nitrospira*-like bacteria is metabolically active with NO_2^- as energy source – linking phylogeny and physiology via FISH-MAR

The positive result obtained with FISH-MAR, thus the fixation of CO_2 under nitrite-oxidizing conditions (Section C.1.5.4.), strongly suggests a direct link between the oxidation of NO_2^- and this novel lineage of *Nitrospira*-like bacteria. This is in accordance with results found for other members of this genus. Fixation of inorganic carbon was demonstrated for pure cultures of *N. marina* and *N. moscoviensis* grown under laboratory conditions (Watson et al. 1986, Ehrich et al. 1995) and for *Nitrospira*-like bacteria in a WWTP (Daims et al. 2001). Similar to its relative "*Candidatus* Nitrospira bockiana", where a "probably pure culture" was able to oxidize NO_2^- at 42°C (Lebedeva et al. 2008), this novel lineage was capable of nitrite oxidation up to at least 43°C (and probably even above; see section D.1.3.).

No MAR-signal could be observed for *Nitrospira*-like bacteria just hybridizing with the genus and/or phylum specific probe (Section C.1.5.4.). Here, it should be taken into account that this might be caused by the relatively low, scattered abundance of these cells, so MAR signals couldn't be distinguished from the background properly. However, assuming the result was truly negative, lack of a MAR-signal for either indicates that (i) these cells were no NOB or (ii) were not active under incubation conditions. Either way, no conclusions about the activity/inactivity or the nitrite-oxidation capability of these organisms could be drawn and further investigations would be needed.

Additionally, MAR signals were obtained from cells in incubations with NO_2^- as well as without NO_2^- as well as from sterilized incubations (Section C.1.5.4. and figure C.4.B). These cells were not targeted by any of the applied probes, but were just stained with DAPI and SybrGreen, respectively, so no conclusion about their phylogenetic affiliation could be drawn. Especially the signal obtained in the sterilized treatment can hardly be explained. On one hand, this could be an artifact caused by unspecific binding or precipitation of $^{14}CO_3^{2-}$ to these cells, a process also called chemography. On the other hand, assumed the observed phenomenon is not just an artifact, this organism seems to survive fixation with 4% PFA and might metabolize storage compounds, but seems not to be involved in nitrite oxidation, since the same strong MAR signal occurred in all incubations.

D.1.3. Nitrification in hot springs

Nitrification was shown to take place in hot springs up to 85°C (Spear et al. 2007, Reigstad et al. 2008, Weidler et al. 2008, Zhang et al. 2008) and *amoA* (Lebedeva et al. 2005, Weidler et al. 2008) and *Nitrospira*-like 16S rRNA gene sequences (Reigstad et al. 2008, Zhang et al. 2008) can be found in hot springs globally. Therefore it was suggested as a process relevant in thermal environments (Takai et al. 2001, Nunoura et al. 2005, Spear et al. 2007, Reigstad et al. 2008, Zhang et al. 2008).

The potential AOA obtained here, is the third thermophilic crenarchaeote cultured with NH_3 as sole energy source, and is opening a third lineage of (cultured) thermophilic AOA, besides "*Candidatus* Nitrosocaldus yellowstonii", thriving at 72°C, and "*Candidatus* Nitrososphaera gargensis", exhibiting a growth temperature (46°C) similar to the putative AOA obtained here. This novel putative AOA can be found in various thermal ecosystems globally, at variable (elevated) temperatures and pH values (Bruns et al. 1999), suggesting at least some kind of relevance in this ecosystems.

Nevertheless, further confirmation for the active oxidation of NH_3 by this microorganism should be gained, for example by FISH-MAR, as done by Hatzenpichler and colleagues for "*Candidatus* Nitrososphaera gargensis" (Hatzenpichler et al. 2008).

Comparably, the nitrite-oxidizing organisms found in this study seem not to be locally restricted. Phylogenetic analysis of 16S rRNA gene and *nxrB* sequences of enrichment cultures A and C_a showed affiliation with other sequences retrieved from Beryl hot spring (Yellowstone National Park, USA) (16S rRNA and *nxrB*) and a hot spring at Lake Baikal (Russia) (16S rRNA) (Figure C.3.A and C.3.B) and also the sequences of enrichment culture 8, affiliated with sublineage II, grouped with other hot spring sequences (Figure

C.3.A). Nevertheless it should be mentioned that analysis of 16S rRNA and *nxrB* genes might lack the necessary level of resolution, since with higher resolution techniques for other hot spring organisms such geographic barriers have been found indeed (Papke et al. 2003, Whitaker et al. 2003), which seems plausible due to the "island-like nature of thermal environments" (Burgess et al. 2007).

Still, apart from biogeographical considerations, other environmental factors, such as oxygen availability, pH, temperature or nitrite concentration might also influence the abundance of these nitrite oxidizers, as it has been shown for other *Nitrospira*-like bacteria before regarding NO_2^- concentration and oxygen concentration (Schramm et al. 1999, Schramm et al. 2000, Maixner et al. 2006). These factors might also shape the communities of nitrite oxidizers within thermal springs, which currently solely consist of *Nitrospira*-like bacteria. Since hot springs, due to their extreme characteristics, provide steep gradients within a small scale (Burgess et al. 2007), the occurring different sublineages of *Nitrospira* might each occupy different niches. An observation supporting this hypothesis regarding temperature was made by E. Lebedeva. She reported that raising the temperature of continued enrichment culture 8, the morphologically different (long spiral shape) sublineage II *Nitrospira*-like bacteria disappeared, thus seemed not to be able to grow at temperatures above 50°C, but members of the novel lineage described here are able to thrive at these temperatures (E. Lebedeva, personal communication). So the newly detected sublineage of *Nitrospira* seems to be "true thermophilic" (following the definition of Reysenbach and Shock 2002), and is thus most likely not introduced from surrounding soil, but originates from within the thermal habitat. This is also at least partially supported by FISH-MAR results (since FISH-MAR was done at 43°C, which is by definition not considered "thermophilic" (Reysenbach and Shock 2002)). It was shown that it is actively metabolizing CO_2 in the presence of NO_2^-. Therefore, it might be a relevant player in this thermal ecosystem. However, to confirm that this organism is thermophilic, FISH-MAR should be applied at higher temperatures.

Even though no observations of activity could be made for the *Nitrospira* sublineage II organisms, due to their "scattered" distribution or due to inactivity, it still can be assumed to be "genuine" to thermal habitats, even though their obviously lower temperature optimum (E. Lebedeva, personal communication), since similar sequences also have been found by Weidler and colleagues in an Austrian thermal spring (2008).

D.1.4. Conclusion

Here, the enrichment of at least one new sublineage of thermophilic *Nitrospira*-like bacteria, isolated from a hot spring and growing at 46°C (and above, >50°C, E. Lebedeva, personal communication) could be confirmed by amplification and cloning of ribosomal and functional marker genes. One enrichment culture, designated enrichment culture 8, was further investigated functionally. It could be shown by immunofluorescence that this new lineage of *Nitrospira*-like bacteria is actively expressing the NXR, the enzyme catalyzing nitrite oxidation. Additionally, FISH-MAR data showed that these organisms are actively metabolizing with NO_2^- as the sole energy source. These data strongly suggest that the newly characterized *Nitrospira*-like bacteria are responsible for nitrite oxidation in the enrichment.

Furthermore, the AOO thriving in enrichment culture 8 was identified as a crenarchaeote by amplification of the 16SrRNA gene and crenarchaeal *amoA* of this organism. Phylogenetic analysis showed that it is affiliated with an uncultivated lineage of *Crenarchaeota* in between marine group I.1a and SAGMCG-1, which has not been linked to ammonia oxidation yet.

Future research should further investigate the (eco-)physiological characteristics of these newly obtained organisms and their ecological relevance in moderate thermal ecosystems, for example via FISH/NANO-SIMS (Musat et al. 2008).

D.2. Nitrifying community in an acidic forest soil

All known groups of AOO and NOB, potentially involved in nitrification in soil horizons L, F and A at the investigated study site, were accessed via amplification, cloning and comparative phylogenetic analysis of ribosomal (16S rRNA) and functional (*amoA*, *nxrB*) marker genes.

D.2.1. Testing different protocols for DNA/RNA extraction – Quality or quantity

PCR performance with DNA isolated from soil and leaf-litter is challenging due to the high humic substance and phenolic compound content of such samples. These compounds are co-extracted with the nucleic acids due to their similar chemical properties and inhibit successful amplification of target gene fragments (Tebbe and Vahjen 1993, von Wintzingerode et al. 1997, Wilson 1997). To overcome these limitations various protocols have been developed for DNA extraction from soil (e.g. Tsai and Olson 1991, Zhou et al.

1996, Griffiths et al. 2000a, Martin-Laurent et al. 2001, Arbeli and Fuentes 2007, Wang et al. 2009), but only two studies were available on DNA extraction from leaf-litter (Aneja et al. 2004, Zhang et al. 2009). Here the protocol of Lüders et al. (2004), of Urich et al. (2008) and a commercial kit, the MoBio Power Soil™ DNA Kit were tested on soil as well as on leaf-litter (Section C.2.1.).

Comparing three different protocols for soil and "Litter N" (soil horizon L) quantitatively the protocol of Lüders et al. (2004) performed most successful, since the highest DNA/RNA concentration was measured (Figure C.5.). However, performance in PCR was poor for all samples extracted after this protocol. It was not possible to amplify bacterial 16S rRNA gene fragments, without previously diluting the DNA template at least 1:10 (Table C.1.). Therefore, this protocol seemed not to be able to remove PCR inhibiting substances and supply sufficiently pure DNA for amplification. This interpretation was also supported by a brownish color of the DNA extract, pointing towards a high amount of co-extracted humic substances. Following the protocol of Urich et al. (2008) DNA/RNA concentration was only about a third compared to the protocol of Lüders et al. (2004) (Figure C.5.). Nevertheless, it was possible to amplify gene fragments from undiluted DNA (Table C.1.), so the removal of humic substances seemed more successful. These results suggest that effective removal of humic substances takes place at the expense of the yield in DNA/RNA. Additionally, one should also keep in mind that the recommended DNA concentration for a PCR template is around 100 ng/µl, while the DNA concentration of DNA exctracted after Lüders et al. (2004) might have been up to 5-fold of that (Figure C.5.). Therefore, independently from inhibition by humic acids, this might also be a crucial factor for the inhibition of the PCR with undiluted DNA.

The high content of humic substances was especially problematic for "Litter A" (soil horizon H) for both protocols mentioned above. Since this layer is mainly composed of half degraded leaves and wood (Soil Classification Working Group 1998), it is even richer in humic substances, which are, amongst others, produced from degrading lignin (Martin and Haider 1986, cited after Miltner and Zech 1998). Due to turbidity of the samples, it was not possible to measure DNA/RNA concentration via NanoDrop for several replicates (C.2.1.1.) and dilution before PCR was necessary with the Lüders protocol as well as with the Urich protocol (Section C.2.1. and table C.1.).

The only protocol with sufficient PCR performance for all samples was the MoBio Power Soil™ DNA Kit. Nevertheless it yielded only a very low concentration of DNA (Figure C.5.). Despite the fact that this might mainly originate from the fact that the Kit is the only

protocol solely extracting DNA, not RNA, a higher yield would be desirable for the recovery of low-abundance microorganisms, which nevertheless might have a huge ecosystem impact as it has been shown nicely exemplarily in a limnic habitat via FISH/NANO-SIMS just recently (Musat et al. 2008).

Consequently, this suggests that the protocol by Urich et al. (2008) as well as the MoBio Power Soil™ DNA Kit are preferable and might be more suitable for extraction of DNA from the investigated samples than the protocol by Lüders et al. (2004). Nevertheless none of these protocols seems optimal, especially not for DNA extraction from leaf-litter with its high concentration of humic acids. Since, as mentioned above, a higher yield would be desirable, further optimization should be considered.

D.2.2. No autotrophic nitrification in leaf-litter?

All attempts to amplify phylogenetic or functional marker genes specific for bacterial or crenarchaeal nitrifiers from soil horizon L ("Litter N") or soil horizon F ("Litter A") remained without a result (Section C.2.2. and Table C.2.). Consequently, numbers of autotrophic nitrifiers seem to be below detection limit of PCR, even though technical shortcoming leading to this result, like out-dilution of the template (at least for "Litter A") or PCR inhibiting factors cannot be excluded. However, the latter seems unlikely since it was possible to amplify 16S rRNA gene fragments (Table C.2.). Additionally, the hypothesis of absence of autotrophic nitrification within the leaf-litter layers was also supported by an acetylene inhibition assay done by F. Maixner. Acetylene inhibits bacterial and – as has been shown recently – also archaeal autotrophic ammonia oxidation (Bedard and Knowles 1989, Offre et al. 2009). No change in the rate of nitrification was measured when acetylene was added to leaf-litter samples (F. Maixner, personal communication), suggesting that nitrification in the leaf-litter layers of this forest site is mainly mediated heterotrophically. Heterotrophic nitrification can be carried out by bacteria, fungi or algae (Wood 1988) and there are several studies that found heterotrophic nitrification to be important in conifer forest soils (Schimel et al. 1984, Pedersen et al. 1999, Jordan et al. 2005) and grassland soil (Laughlin et al. 2008). For example Kurakov and colleagues estimated the participation of heterotrophic nitrification to the total production of nitrates in diverse native soils to be 25-46% (Kurakov et al. 2001).

However, studies investigating nitrification and nitrifying communities at forest sites usually focus on the soil layers. The vertical spatial distribution and therefore the leaf-litter layers are hardly considered, even though characteristics of the different layers might vary

(Kanerva and Smolander 2007). Consequently only few studies could be found for comparison. However, all studies are more or less not in agreement with the results found here. The two studies looking at most probable number (MPN) counts in a tropical pine forest (Krave et al. 2002) and a Douglas fir forest (Deboer et al. 1992) as well as one study, investigating the occurrence of autotrophic nitrifiers or more precisely AOB in leaf-litter samples from two pine forest sites (Laverman et al. 2001), found nitrifying bacteria and AOB, respectively, to be present, even though the AOB, investigated in the latter study were not present throughout the year. However, the characteristics of a needle forest might be quite different to the beech forest investigated in this study and the comparability remains questionable.

Since there are nearly no studies available considering the different forest floor layers and especially leaf-litter layers in forest ecosystems, and since there are indeed hints that ongoing processes might vary substantially in different forest floor layers (Kanerva and Smolander 2007), investigations should focus on the question if the absence of autotrophic nitrification is a general and/or also a temporally constant characteristic of leaf-litter of temperate deciduous forests. This could contribute to understand biological processes and nutrient fluxes in this environment.

D.2.3. Ammonia-oxidizing organisms in acidic forest soil

D.2.3.1. Phylogenetic affiliation of AOB

All *amoA* sequences obtained were affiliated with the *Nitrosospira* lineage (Figure C.7.), a lineage frequently found to be the prevalent group of AOB in soil ecosystems (Kowalchuk et al. 2000, Avrahami et al. 2002, Mendum and Hirsch 2002, Avrahami et al. 2003, Webster et al. 2005, Nugroho et al. 2007, Dell et al. 2008). It has been shown that the resolution power of *amoA* is too low to allow further assignment to any specific cluster (Koops et al. 2003, Purkhold et al. 2003). However, in general, sequences of cultured species being closest related to sequences obtained here, are assigned to *Nitrosospira* cluster 3 on a 16S rRNA gene level. This is not consistent with other studies, since several studies found *Nitrosospira* cluster 2 to be dominant in acidic soils, while cluster 3 seems to me more frequent in neutral, agricultural soils (Stephen et al. 1998, Bruns et al. 1999, Kowalchuk et al. 2000). However, these attributions of ecophysiological characteristics to the respective clusters found in literature probably cannot be generalized. Several studies reported the detection of cluster 3 affiliated sequences in acidic soil (Webster et al. 2002, He et al. 2007) and it seems cluster 3 can at least be divided into two sublineages with

diverging preferred habitats (Webster et al. 2005). Additionally, some cluster 3a organisms (in some studies also referred to as cluster 10 (Avrahami et al. 2003, Avrahami and Bohannan 2007)) were isolated from acidic soils, among them the closest related organism *Nitrosospira* En13, which was isolated from acidic soil with a pH of 4.5 (Purkhold et al. 2003, Avrahami and Bohannan 2007).

Since all AmoA sequences obtained were highly similar to each other (>98%), they might as well (but not necessarily) belong to a single species, given that Purkhold et al. defined a threshold of <85% AmoA aa similarity as indicative for a new species (Purkhold et al. 2003).

D.2.3.2. The *Crenarchaeota* in acidic forest soil belong to group I.1b

Since, besides AOB, also AOA are commonly found in soil and are considered to be important players (e.g. Leininger et al. 2006, Nicol et al. 2008, Tourna et al. 2008), analysis of the 16S rRNA gene and crenarchaeal *amoA* was performed. This revealed affiliation of the obtained sequences with group I.1b, but also the only distant relation to "*Candidatus* Nitrososphaera gargensis", the sole known member of this group (Figures C.8. and C.2.). While the retrieved 16S rRNA sequences were diverse and widely distributed within group I.1b, only two *amoA* sequences could be retrieved in total, implying substantial undersampling. So to confirm the involvement of the retrieved 16S rRNA phylotypes in ammonia oxidation, additional *amoA* sequences should be obtained. Assuming that the obtained 16SrRNA gene sequences are retrieved from AOA, these results would be in accordance with other studies investigating the AOA community in soil ecosystems. Several studies found group I.1b *Crenarchaea* to be the dominant group in soil (Buckley et al. 1998, Ochsenreiter et al. 2003, Sliwinski and Goodman 2004, Prosser and Nicol 2008), including acidic soils (Nicol et al. 2008). Therefore, Ochsenreiter and colleagues suggested that this might be the group most capable of competition with the highly diverse community of bacteria in soil (Ochsenreiter et al. 2003). However, there are also a few studies that found group I.1c to be dominant in soil (Nicol et al. 2005, Kemnitz et al. 2007, Nicol et al. 2007, Hansel et al. 2008), but so far there is no evidence that they are involved in nitrification and their importance in nitrogen cycling remains uncertain.

Studying AOA, among diverse environmental factors such as soil succession (Nicol et al. 2005) or fertilizer (He et al. 2007), pH has been proposed to influence AOA abundance in soil (He et al. 2007, Nicol et al. 2008). Nonetheless, in this study the obtained sequences

throughoutly grouped with sequences from soil closer to a neutral pH (as far as data were available), suggesting this is not the primary determining factor in this case.

D.2.4. Diversity of nitrite-oxidizing bacteria in acidic forest soil

D.2.4.1. Unspecific binding of the primers developed for amplification of *nxrB* of *Nitrobacter* spp.

Unspecific binding of the primer was observed up to an annealing temperature of 62°C and despite cutting the PCR product from a gel. This suggests that the primers might not be optimal for amplification of *Nitrobacter*-like bacteria *nxrB* genes. Therefore, even though of course higher annealing temperatures should be tested first, maybe the design of new, more specific primers should be considered.

D.2.4.2. An uncultured lineage of *Nitrobacter* spp.

Using the *nxrB* gene for phylogenetic analysis of *Nitrobacter* spp. might be advantageous since they are all closely related (Abeliovich 2006, Vanparys et al. 2007) and this functional marker might provide a higher resolution power (Grundmann et al. 2000). However, all nucleic acid sequences obtained with primers specific for *nxrB* of *Nitrobacter*-like bacteria were highly similar to each other and formed a monophyletic cluster outside of all *nxrB* sequences of cultivated *Nitrobacter* species in the calculated tree (Figure C.10.). This result suggests that these sequences originated from yet uncultivated *Nitrobacter* species. It can be assumed that the obtained sequences are gene fragments encoding for the β subunit of the NXR for two reasons: (i) they are still quite closely related to other *Nitrobacter nxrB* genes (>83%) and (ii) the genes closest related to the *nxr* genes of Nitrobacter (*nxrA* as well as *nxrB*) within the MopB protein-superfamily are the *nxr* genes of *Nitrococcus* sp., suggesting a conservation of function for genes within this cluster. This is in accordance with other studies, investigating the diversity of *nxrA* gene in different soils. There, the authors also found that a considerable number of obtained sequences were only distantly related to sequences of known *Nitrobacter* species (Poly et al. 2008, Wertz et al. 2008).

Since the two copies of *nxrB* on the nucleic acid level were found to be 96% and 97% similar to *nxrB* in the genomes of *Nitrobacter hamburgensis* $X14^T$ and *Nitrobacter winogradski* Nb-255, respectively (data not shown), sequence similarity for sequences obtained in this study (>96%) seems to be in the range of intraspecies sequence divergence

occurring for this gene. This indicates that probably only one "species" of *Nitrobacter* spp. was detected in the investigated samples, suggesting low diversity or low primer coverage.

D.2.4.3. Detection of *Nitrospira*-like bacteria

Besides *Nitrobacter* spp., *Nitrospira*-like bacteria are also commonly found in soil habitats (Bartosch et al. 2002, Cebron and Garnier 2005, Poly et al. 2008, Urich et al. 2008). The 16S rRNA gene-based comparative sequence analysis done in this study showed that all obtained sequences were affiliated with the highly diverse *Nitrospira* sublineage II and were closest related to soil or rhizosphere soil clones (Figure C.9.). The sequences formed two clusters within this lineage, even though one only consisted of one clone, this suggests further niche differentiation within sublineage II. However, since only one study could be found, investigating the diversity of *Nitrospira*-like bacteria in soil (Freitag et al. 2005), too few data are available to correlate diversity with environmental factors.

Analyzing the *nxrB* sequences, comparable results were obtained (C.3.B). So it could be shown that *Nitrospira*-like bacteria are present and might also contribute to nitrification processes in this acidic forest ecosystem. Further evidence for the occurrence of *Nitrospira*-like bacteria in soil came from application of CARD-FISH with *Nitrospira*-specific oligonucleotide probes, where it was possible to detect spiral-shaped cells, a shape typical for *Nitrospira*-like bacteria (Figure C.11.). Nevertheless this result should be taken with caution, since also autofluorescent rod-shaped material, showing a, less than CARD-FISH, but still bright signal, could be detected. Differentiation of "true" signals and autofluorescence was possible due to the lack of a DAPI signal of the autofluorescent material, but the two might not always be clearly distinguishable.

D.2.5. Conclusion

In a first assessment of the nitrifying microbial community in acidic forest soil, it was not possible to amplify phylogenetic or functional marker genes from soil horizons L ("Litter N") and F ("Litter A"). These results suggest that mainly heterotrophic nitrification is taking place in the leaf-litter layers of the forest floor. However, all major terrestrial groups of nitrifying organisms, known up to date, could be detected in soil horizon A (soil). For AOA and *Nitrospira* spp. additional diversity within these phylogenetic groups was observed, altogether pointing towards a high diversity of nitrifiers within this soil ecosystem. Nevertheless, no conclusions about the abundance or their respective contribution to the nitrogen cycling, thus about the key players in this environment, can be drawn, since the data obtained simply rely on clone libraries.

Nitrification in acidic soils is of special interest, since no existing pure culture of AOB or NOB is able to nitrify below a pH of 5.5 (De Boer and Kowalchuk 2001). This might be partially caused by cultivation biases, since there are, for example, no pure cultures of *Nitrospira* spp. or AOA occurring in soil and their physiological properties remain largely unknown. Nevertheless, low pH is definitely a challenge for nitrifying organisms, especially AOO, since at low pH most of the utilizable substrate NH_3 is ionized and reduced to NH_4^+ (Frijlink et al. 1992, cited after Nicol et al. 2008), for which the membranes of the ammonia oxidizers are not permeable (Bock and Wagner 2006). Possible mechanisms to cope with this problem have been proposed, such as biofilm formation (Allison and Prosser 1991, cited after Nicol et al. 2008) or urease activity (Burton and Prosser 2001). However, in this study the affiliation of obtained sequences seemed not to be highly correlated with pH, since for example AOA 16S rRNA sequences were not closely related to any of the sequences known to be obtained from acidic soil. Other factors such as water content, oxygen partial pressure, pH, carbon and nutrient content (Hansel et al. 2008) might be more influential.

Apart from that, looking into the history of microbial ecology research, one should maybe be careful with generalizations and the correlation of single environmental factors with specific groups of microorganisms. On one hand there might be a high degree of niche differentiation and the defined groups might be too simplicistic anyway. On the other hand additionally not all potential players might even been known yet, the best example for this being the discovery of AOA (Francis et al. 2005, Könneke et al. 2005, Treusch et al. 2005) and *Nitrotoga* sp. (Alawi et al. 2007). Moreover, the question remains if the tools available are powerful enough yet.

The identification of key players within this ecosystem as well as the key environmental factors shaping a (nitrifying) microbial community seem difficult to determine. An attempt to address the first question in a very general way, was using different inhibitors, to determine the contribution of heterotrophic and autotrophic nitrification, respectively (De Boer and Kowalchuk 2001). But this seems hardly possible on a more downscale level, since for example there is no known inhibitor, effectively discriminating against AOB or AOA. The most promising approaches that probably could shed light onto both questions at the moment seem to be metatranscriptomics (Frias-Lopez et al. 2008) or even metabolomics (Ram et al. 2005) or – as the most recent and advances technique – FISH/NANO-SIMS (Musat et al. 2008). However, at the moment application of these

methods to soil ecosystems remain a challenge due to the high biodiversity commonly observed in soil and also have their limitations, since unknown microorganisms or functions most likely will remain undetected.

D.3. Establishing *nxrB* as a phylogenetic marker for the genus *Nitrospira*

Using functional genes as markers often gives the opportunity to address functional groups that are otherwise only distantly related, but horizontal gene transfer complicates the correlation of functional and phylogenetic markers (Doolittle 1999). They can be well conserved as in the case of bacterial *amoA* (Aakra et al. 2001, Koops et al. 2003), but might not necessarily reflect the phylogeny obtained on 16S rRNA gene basis as in case of the functional markers *narG* (Gregory et al. 2003) or *nirK* and *nirS* (Gregory et al. 2003, Heylen et al. 2006).

This study suggests that 16S rRNA gene and *nxrB*-based phylogeny of *Nitrospira*-like bacteria is congruent to a high degree. All clusters obtained 16S rRNA gene-based, could also be obtained *nxrB*-based with one exception. The soil clones, affiliated with sublineage II in the 16S rRNA gene tree, are closer related to "*Candidatus* Nitrospira defluvii" (sublineage I), than to *Nitrospira moscoviensis* (sublineage II) in the phylogenetic tree of *nxrB*. Problems to resolve the furcations of the thermophilic lineages of the tree also hampered 16S rRNA gene phylogenetic analysis (Figures C.3.A and B). So the hypothesis by Zhou and colleagues stating that functional genes might posses a higher resolution in differentiating various microorganisms but give less robust information about phylogenetic relationships (Zhou et al. 2008), which can for example be observed for AOB 16S rRNA and AmoA-based phylogeny (Koops et al. 2003), only holds true partially. Indeed a higher resolution within clusters was observed, but the information about branching order of these organisms in the lower part of the tree is equally bad for both genes.

Nevertheless, using *nxrB* of *Nitrospira*-like bacteria for phylogenetic analysis could provide additional information and can be considered as a useful functional marker.

E. SUMMARY AND CONCLUSION

Nitrification, a key process in the global nitrogen cycling, is mainly mediated by nitrifying microorganisms that can be discovered in nearly every habitat around the world. In this study nitrifying enrichment cultures originating from two hot springs at Lake Baikal (Russia) and Kamchatka (Russia) and nitrifying microorganisms in soil and leaf-litter (soil horizons L and F) of a temperate acidic forest site were investigated.

The phylogeny of AOO and NOB, metabolizing NH_3 (enrichment culture 8) or NO_2^- (enrichment cultures A, C_a and 4) to NO_3^- in the enrichment cultures, isolated from the hot springs and growing at 46°C, was analyzed via amplification, cloning and sequencing of diverse molecular markers. Subsequently, FISH with probes specific for the detected microorganisms was applied for *in situ* detection and the NOB were additionally characterized functionally by application of immunofluorescence, targeting the nitrite oxidoreductase (NXR), and FISH-MAR. Thereby, the AOO thriving in enrichment culture 8 could be identified as crenarchaeotes. Phylogenetic analysis showed that they are affiliated with an uncultivated lineage of crenarchaeotes, in between marine group I.1a and SAGMGC-1, which hadn't been linked to ammonia oxidation yet. The dominant NOB could be confirmed as a novel sublineage of *Nitrospira*-like bacteria that were actively expressing the NXR, the enzyme catalyzing nitrite oxidation, as could be shown by immunofluorescence and actively metabolizing at 43°C in the presence of NO_2^- as the sole energy source.

The nitrifying community at a temperate acidic forest site was also assessed by amplification and cloning of phylogenetic and functional marker genes. Contrary to available literature, it was not possible to amplify any gene linked to known nitrifiers from leaf-litter layers, suggesting that nitrification is mainly mediated by heterotrophic microorganisms. However, genes of all known nitrifiers common in terrestrial habitats could be detected in soil horizon A (soil). For AOA and *Nitrospira* spp. additional diversity within these phylogenetic groups was observed, pointing towards a high diversity of nitrifiers within this soil ecosystem. Nevertheless, no conclusions about the abundance or their respective contribution to the nitrogen cycling, thus about the key players in this environment, can be drawn, since the data obtained simply rely on clone libraries.

For the investigation of *Nitrospira*-like bacteria *nxrB*, the gene encoding the β-subunit of the NXR, was assessed for the use as an additional molecular marker. Therefore a database

from cloned and sequenced *nxrB* genes of available cultures, enrichment cultures and environmental samples was established. Phylogenetic analysis showed that 16S rRNA gene and *nxrB* based phylogeny of *Nitrospira*-like bacteria is congruent to a high degree, without any evidence for HGT. Therefore, *nxrB* is suggested to be a suitable functional marker for the phylogenetic analysis of *Nitrospira*-like bacteria.

Molecular techniques nowadays not only provide great insight into the constitution of microbial communities in an environment but also allow statements about their activities. Nevertheless, the actual isolation of microorganisms, to gain detailed insight into the physiological capabilities and regulation mechanisms of organisms, remains as important as ever. Therefore here, investigating two different environments, not only a small window into the nitrifying community in an acidic forest soil, but also a first identification of several NOB and probably a new lineage of AOA was presented. Furthermore, members of a new sublineage of the previously identified thermophilic *Nitrospira*-like bacteria, growing at a temperature of 46°C, were shown to be active under nitrifying conditions.
Altogether these results are shed further light on the phylogenetic and physiological diversity of nitrifying microorganisms.

F. ZUSAMMENFASSUNG

Bakterien und Archaeen sind die hauptverantwortlichen Organismen für Nitrifikation, einem Schlüsselprozess im globalen Stickstoffkreislauf und können in fast allen Habitaten der Welt gefunden werden können. In dieser Studie wurden die nitrifizierenden Mikroorganismen zweier sehr unterschiedliche Habitate untersucht. Zum einen handelte es sich um Anreicherungen, die von heißen Quellen des Baikalsees (Russland) und Kamchatka (Russland) stammen, zum anderen um Boden- und Laubstreuproben (Bodenhorizonte L und F) eines temperaten, sauren Waldstandortes.

Die Ammoniak- und Nitrit-oxidierenden Mikroorganismen, der aus heißen Quellen stammenden, bei 46°C wachsenden Anreicherungen, wurden mittels Amplifizierung und Klonierung diverser molekularer Marker (16S rDNA, *AmoA*, *NxrB*) untersucht. Daraufhin wurden für diese Mikroorganismen spezifische Sonden entwickelt und die Mikroorganismen mittels FISH detektiert. Nitrit-oxidierende Mikroorganismen aus Anreicherung 8 wurden zusätzlich mittels Immunofluoreszenz-Technik auf die aktive Expression des, für Nitrit Oxidation verantwortlichen Enzyms, Nitritoxidoreduktase (NXR) hin untersucht und mittels Fluoreszenz *In Situ* Hybridisierung in Kombination mit Mikroautoradiographie auch funktionell getestet.

Durch Amplifizierung und Klonierung von 16SrDNA und *amoA* Genen, konnten die Ammoniak-oxidizerenden Mikroorganismen als zum Phylum der *Crenarchaeota* zugehörig identifiziert werden. Phylogenetische Analyse der Sequenzen zeigte, dass die Organismen in eine Gruppe unkultivierter Crenarchaeoten fallen, die zwischen der so genannten „marine group I.1a“ und der „SAGMGC-1 group“ liegt. Diese Gruppe von Crenarchaeoten konnte zuvor noch nicht mit Ammoniak-Oxidation in Verbindung gebracht werden. Die Nitrit-oxidizerenden Mikroorganismen konnten als *Nitrospira*-ähnliche Bakterien identifiziert werden, die aktiv das Enzym Nitritoxidoreduktase exprimierten und bei einer Temperatur von 43°C mit Nitrit als alleiniger Energiequelle aktiv Stoffwechsel betrieben.

Die nitrifizierenden Mikroorganismen eines temperaten sauren Waldstandortes wurden ebenfalls mittels Amplifizierung und Klonierung phylogenetischer und funktioneller Markergene charakterisiert. Im Gegensatz zu anderen Studien war es nicht möglich Gene bekannter Nitrifikanten aus den Laubstreuhorizonten zu amplifizieren. Dieses Ergebnis deutet darauf hin dass die Nitrifikation in diesen Horizonten möglicherweise hauptsächlich von heterotrophen Nitrifizierern durchgeführt wird. Für Horizont A war es hingegen möglich Gene aller terrestrisch vorkommenden Nitrifizierer zu detektieren. Für Ammoniak-

oxidizerende Archaeen und *Nitrospira*-ähnlichen Bakterien wurde außerdem auch Diversität innerhalb der phylogenetischen Gruppen festgestellt, was auf eine hohe Diversität nitrifizierender Mikroorganismen in diesem Bodenökosystem hinweist. Es darf natürlich trotzdem nicht vergessen werden dass alle Daten aus Klonbibliotheken stammen, weshalb keinerlei Aussagen über Häufigkeit, Aktivität oder Beitrag zum Stickstoffkreislauf getroffen werden können.

Für die Erforschung *Nitrospira*-ähnlicher Bakterien, wurde das *nxrB* Gen, das die β-Untereinheit der Nitritoxidoreduktase kodiert, hinsichtlich der Eignung als zusätzlicher molekularer Marker evaluiert. Es wurde eine Datenbank klonierter und sequenzierter *nxrB* Gene von verfügbaren Kulturen, Anreicherungen und aus Umweltproben erstellt. Phylogenetische Analyse der Sequenzen zeigte, dass die 16SrDNA und *nxrB* basierte Phylogenie *Nitrospira*-ähnlicher Bakterien zu einem hohen Grad kongruent ist und es keinerlei Hinweise auf lateralen Gen Transfer gibt. Dementsprechend könnte *nxrB* ein passender funktioneller Marker für die phylogenetische Analyse *Nitrospira*-ähnlicher Bakterien sein.

G. LIST OF ABBREVATIONS

16S rRNA	small subunit of rRNA
ε	molar extinction coefficient
λ	wavelength
µ	mikro (10-6)
#	number
°C	degree Celsius
%	percent
A	adenine; ampere
abs	absolute
Amo	ammonium monooxygenase
amoA	genes coding for subunits A, B and C of Amo
Amp	ampicillin
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
AOO	ammonia-oxidizing organisms
ARB	software package for phylogenetic analyses (from lat. *arbor*, "tree")
bidist	double-distilled and filtered
BLAST	basic local alignment search tool
bp	base pair(s)
c	centi (10-2)
C	cytosine
CARD	catalyzed reporter deposition
cDNA	complementary (*i.e.* reverse transcribed) DNA
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 (1,66018×10-24 g)
DAPI	4'-6'-di-amidino-2-phenylindole
DEPC	di-ethyl-pyrocarbonate
dist	plainly distilled

DGGE	denaturing gradient gel electrophoresis
DMF	N,N-di-methylformamide
DMSO	di-methylsulfoxid
DNA	desoxyribonuleic acid
dNTP	desoxy-nucleotide-tri-phosphate
dsrAB	genes coding for the subunits A and B of the the dissimilatory sulfite reductase
e^-	electron
E.	*Escherichia*
EDTA	ethylene-di-amine-tetra-acetic acid
e.g.	*exempli gratia* (lat., “example given”)
ERT	Eppendorf reaction tube
et al.	*et alteri* (lat., “and others”)
EtBr	ethidium bromide
etc.	et cetera (lat., “and so forth”)
EtOH	ethanol
F	forward (used for labeling of primers); Farat
FA	formamide
Fig.	figure
FISH	fluorescence *in situ* hybridization
Fluos	5,(6)-carboxyfluorescein-N-hydroxysuccimidester
g	gram(s)
G	guanine
h	hour(s)
H^+	Proton
Hao	hydroxylamine oxidoreductase
H_2O	water
H_2O_2	hydrogen peroxide
HB	hybridization buffer
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
i.e.	*id est* (lat., "that is")
IUPAC	International union of pure and applied chemistry

k	kilo (10^3)
K	guanine or thymine
Kan	kanamycin
KBL	kilobase-ladder (DNA length standard)
KCl	potassium acetate
KL	Klausenleopoldsdorf, Austria (study site)
l	liter(s)
lacZ	gene coding for β-galactosidase
lacZα	α-subunit of *lacZ*
LB	Luria Bertani
m	milli (10^{-3}); meter(s)
M	molar; adenine or cytosine
MAR	microautoradiography
Max.	maximum
MICDIF	MICrobial DIversity and ecosystem Functioning
min	minute(s)
ML	maximum likelihood
MP	maximum parsimony
n	nano (10-9)
N	adenine, thymine, guanine or cytosine
N.	*Nitrospira* or *Nitrosomonas*
NaCl	sodium chloride
NaOH	sodium hydroxide
Nar	nitrat reductase
narG/H	genes coding for the G or H subunit of Nar
NCBI	National Center for Biotechnology Information
n.d.	not determined
NH_2OH	hydroxlamine
NH_3	ammonia
nirK/S	genes coding for the K or S subunit of the nitrite reductase
HKA	"Hauptkläranlage", Main waste water treatment plant Vienna
NJ	neighbour joining
NO_2^-	nitrite
NO_3^-	nitrate

NOB	nitrite-oxidizing bacteria
nt	nucleotide(s)
Nxr	nitrite oxidoreductase
nxrA/B	genes coding for the A or B subunit of Nxr
O_2	molecular oxygen
ON	over night
OD	optical density, meassured at a wavelength of x nm
p.a.	*pro analyticum* (lat., "for analysis"), grade of purity
Pa	Pascal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PHYLIP	phylogeny interference package (software package for phylogenetic analyses)
pMMO	particulate methane monooxygenase
R	reverse (used for labeling of primers); adenine or guanine; resistance (against an antibiotic)
RDP	ribosomal database project
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal RNA
RT	room temperature; reverse transcription
S	cytosine or guanine
SAGMCG	South African gold mine crenarchaeotic group
SDS	sodium dodecyl sulfate
sec	second(s)
Sec.	section
SOC	Super Optimal broth with Catabolite repression (containing glucose)
sp.	species (singular)
ssp.	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
Temp.	temperature
ThAOA	thermophilic ammonia-oxidizing archaea
T_{inc}	incubation temperature
T_{opt}	optimal growth temperature
Tris	Trishydroxymethylaminomethan
TSA	tyramide signal amplification
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
WB	washing buffer
WWTP	waste water treatment plant
X-Gal	5-brom-4-chlor-3-indolyl-β-D-galactopyranoside

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I. APPENDIX

Table I. 1. BLAST hits of gene fragments amplified with general 16S rRNA gene primers from the thermal enrichment cultures (Section C. 1.2.).

enrichment	clone	best BLAST hit[a]	coverage	similarity	comment
Enrichment culture A	BA1	Uncultured *Nitrospira* sp. gene for 16S rRNA, partial sequence clone: HAuD-UB28	99%	**95%**	
	BA2	Uncultured bacterium clone TBb-29 16S ribosomal RNA gene, partial sequence	99%	**98%**	closest identified clone: uncultured *Nitrospira* clone (94% similarity)
	BA3	no sequence			
	BA4	Uncultured *Nitrospira* sp. gene for 16S rRNA, partial sequence clone: HAuD-UB28	100%	**96%**	
	BA5	Uncultured bacterium clone TBb-29 16S ribosomal RNA gene, partial sequence	100%	**97%**	
	BA6	Uncultured bacterium clone CV22 16S ribosomal RNA gene, partial sequence	100%	**96%**	
Enrichment culture C_a	BCa1	Uncultured β-proteobacterium clone RHG28c 16S ribosomal RNA gene, partial sequence	98%	**99%**	
	BCa2	Uncultured β-proteobacterium clone RHG28c 16S ribosomal RNA gene, partial sequence	99%	**99%**	clone found in thermal enrichment cultures by Hatzenpichler et al. (2008)
	BCa3	Uncultured bacterium clone TBb-29 16S ribosomal RNA gene, partial sequence	99%	**99%**	
	BCa4	chaos sequence			
	BCa5	Uncultured *Nitrospira* sp. gene for 16S rRNA, partial sequence clone: HAuD-UB28	100%	**96%**	
Enrichment culture 4	4-1	Uncultured bacterium partial 16S rRNA gene, clone CVCloAm2Ph140	99%	**97%**	closest identified clone: *Acidobacterium* sp.
	4-12	*Meiothermus rosaceus* 16S ribosomal RNA gene, partial sequence	100%	**99%**	
	4-17	Uncultured β-proteobacterium clone RHG28c 16S ribosomal RNA gene, partial sequence	100%	**98%**	clone found in thermal enrichment cultures by Hatzenpichler et al. (2008)
	B4-1	Uncultured bacterium clone 1-13 16S ribosomal RNA gene, partial sequence	100%	**98%**	closest identified clone: uncultured β-proteobacterium
	B4-2	*Caldimonas* sp. Han85 partial 16S rRNA gene, strain Han85	99%	**99%**	
	B4-3	Uncultured bacterium clone BS-B54 16S ribosomal RNA gene, partial sequence	100%	**95%**	closest identified clone: uncultured *Actinobacterium* sp.
	B4-4	*Caldimonas* sp. Han85 partial 16S rRNA gene, strain Han85	100%	**99%**	
	B4-5	Uncultured bacterium clone XJ118 16S ribosomal RNA gene, partial sequence	100%	**98%**	closest identified clone: uncultured green sulfur bacterium
	B4-6	Uncultured β-proteobacterium clone RHG28c 16S ribosomal RNA gene, partial sequence	97%	**96%**	clone found in thermal enrichment cultures by Hatzenpichler et al. (2008)
	B4-7	Uncultured β-proteobacterium clone RHG28c 16S ribosomal RNA gene, partial sequence	97%	**96%**	clone found in thermal enrichment cultures by Hatzenpichler et al. (2008)
	B4-9	*Caldimonas* sp. Han85 partial 16S rRNA gene, strain Han85	99%	**98%**	
	B4-10	Uncultured β-proteobacterium clone RHG28c 16S ribosomal RNA gene, partial sequence	98%	**96%**	clone found in thermal enrichment cultures by Hatzenpichler et al. (2008)
	B4-11	Uncultured γ-proteobacterium clone GSST58 16S ribosomal RNA gene, partial sequence	100%	**99%**	
	B4-12	*Caldimonas taiwanensis* strain On1 16S ribosomal RNA gene, partial sequence	98%	**99%**	
Enrichment culture 8	B1	Uncultured bacterium clone CV22 16S ribosomal RNA gene, partial sequence	100%	**95%**	closest identified clone: uncultured *Nitrospira* sp.
	B2	Uncultured bacterium clone CV22 16S ribosomal RNA gene, partial sequence	100%	**88%**	closest identified clone: uncultured *Nitrospira* sp.

	B3	Uncultured bacterium clone 1-13 16S ribosomal RNA gene, partial sequence	100%	**98%**	closest identified clone: uncultured β-proteobacterium
	B4	*Anoxybacillus voinovskiensis* gene for 6S ribosomal RNA, partial sequence	100%	**99%**	
	B5	*Anoxybacillus amylolyticus* 16S rRNA gene, type strain MR3C	99%	**98%**	
	B6	*Thermus* sp. R55-10 16S ribosomal RNA gene, partial sequence	99%	**99%**	
	B8	bad sequence			

[a] best BLAST hit for the clone partially sequenced with the Topo-Seq R primer.

Table I. 2. (Average) Similarity matrix of *Nirospira*-like 16S rRNA gene sequences retrieved from enrichment cultures A, C_a and 8 to other representatives and clones affiliated with the genus *Nitrospira*.

	Nde	Nmo	Nbo	NulC	Nmar	Spo	A/Ca	enr 8	8 sub II
"*Candidatus* Nitrospira defluvii" (Nde) (DQ059545)	100	94,2	90,1	90,8	88,6	88,7	92,0	92,2	92,2
Nitrospira moscoviensis (Nmo) (X82558)		100	90,5	91,2	89,2	89,2	93,3	92,9	98,3
"*Candidatus* Nitrospira bockiana" (Nbo) (EU084879)			100	90,3	88,3	89	93,3	93,6	89,8
Nullarbor caves clone wb1 (NulC) (AF317764)				100	90,9	89,8	93,4	94	89,7
Nitrospira marina (Nmar) (X82559)					100	92,3	90,6	90,9	86,3
Sponge clone (Spo) (EF076129)						100	90,6	90,4	87,4
A/C_a clones (A/C_a)[a]							100	96,1	-
Enrichment culture 8 cloneB1 (enr 8)[b]								100	92,6
Enrichment culture 8 sublineage II clones (8 sub II)[c]									100

[a] average similarity of all clones >1400 bp [b] average similarity of all clones (N1, N11) [c] only one clone >1400 bp was available

Table I. 3. Table showing the primer combinations used in attempts to amplify *nxrB* from various samples. "+"…PCR product was obtained, "-"…no PCR product was obtained, empty fields denote not determined combinations.

organism/sample	F14/R1239	F19/R1237	F14/R1237	F19/R1239	F916/R1239	F196/R1237	$T_{opt.}$[a]
Nitrospira marina		+	(+)				52.3
sponge DNA	-	-				-	x
Klausenleopoldsdorf soil			+			(+)	68
Rothwald forest soil						(+)	x
Enrichent culture 4/8		+					58
Enrichment culture A/Ca	-	+				(+)	58
Nitrospira bockiana						(+)	x
Nitrospira moscoviensis		+				(+)	all T (48-68)
Nitrospira defluvii		+					all T (48-68)
VetMed DNA		-	+	(+)			59.9
HKA-clone					+		59.3
BerylSpring DNA		+					59.9
DNA HKA						(+)	x

- …no PCR product was obtained; + …PCR product was obtained; x …$T_{opt.}$ was nto determined
[a] optimal annealing temperature; referring to the primers yielding the positive result not in brackets

Table I.4. Similarity matrix of *nxrB* sequences retrieved from enrichment cultures C_a and 8 to other representatives and clones affiliated with the genus *Nitrospira*.

	KuSt	NbMo	NbHa	Nde	Nmo	Nbo	Nma	BsII	Rwf	SBR	Klf	e8X10	e8X8	eCa1	eCa5	eCa7	av 8:Ca
Kuenenia stuttgartiensis genome (KuSt) fragment (CT573072)	100	40,7	40,4	60	61,3	61	61,8	60	59	62,4	60	61,4	61,7	60,2	59,8	60,2	
Nitrococcus mobilis Nb-231 (NbMo) (NZ_AAOF01000001)		100	75,9	46	45,1	46	43,5	46	45	44	45	45,9	45,9	45,5	45,6	45,5	
Nitrobacter hamburgensis X14 (NbHa) (X66067)			100	44	46,2	46	42,3	46	45	42,4	44	45,7	45,7	45,6	45,9	45,6	
"*Candidatus* Nitrospira defluvii" (Nde)				100	87,6	87	81,8	87	88	83,2	90	85,3	85,5	87,2	87,3	87,2	
Nitrospira moscoviensis (Nmo)					100	90	82,6	90	89	84,5	89	88,7	88,9	89,5	90,5	89,5	
"*Candidatus* Nitrospira bockiana" (Nbo)						100	84,4	91	87	84,6	88	90,7	90,9	90,7	91,6	90,7	
Nitrospira marina (Nma)							100	84	81	86,9	81	85,2	85,4	84,3	84,6	84,3	
Berry spring clone 11II (BsII)								100	87	84,2	88	89,2	89,6	100	98,6	100	
Rothwald forest clone 11 (Rwf)									100	83	92	84,6	84,8	87,4	88,1	87,4	
marine SBR enrichment clone H11 (SBR)										100	83	86	86,4	84,2	84	84,2	
acidic forest soil clone 5 (Klf)											100	84,9	85	87,7	87,9	87,7	
Enrichment culture 8 clone X10 (e8X10)												100	99,7	89,2	89,6	89,2	
enrichment culture 8 clone X8 (e8X8)													100	89,6	89,8	89,6	
Enrichment culture C_a clone 1 (eCa1)														100	98,6	100	
enrichmentculture C_a clone 5 (eCa5)															100	98,6	
Enrichment culture C_a clone 7 (eCa7)																100	
average similarity of clones from enrichment cultures 8 and C_a (av 8:Ca)																	89,5

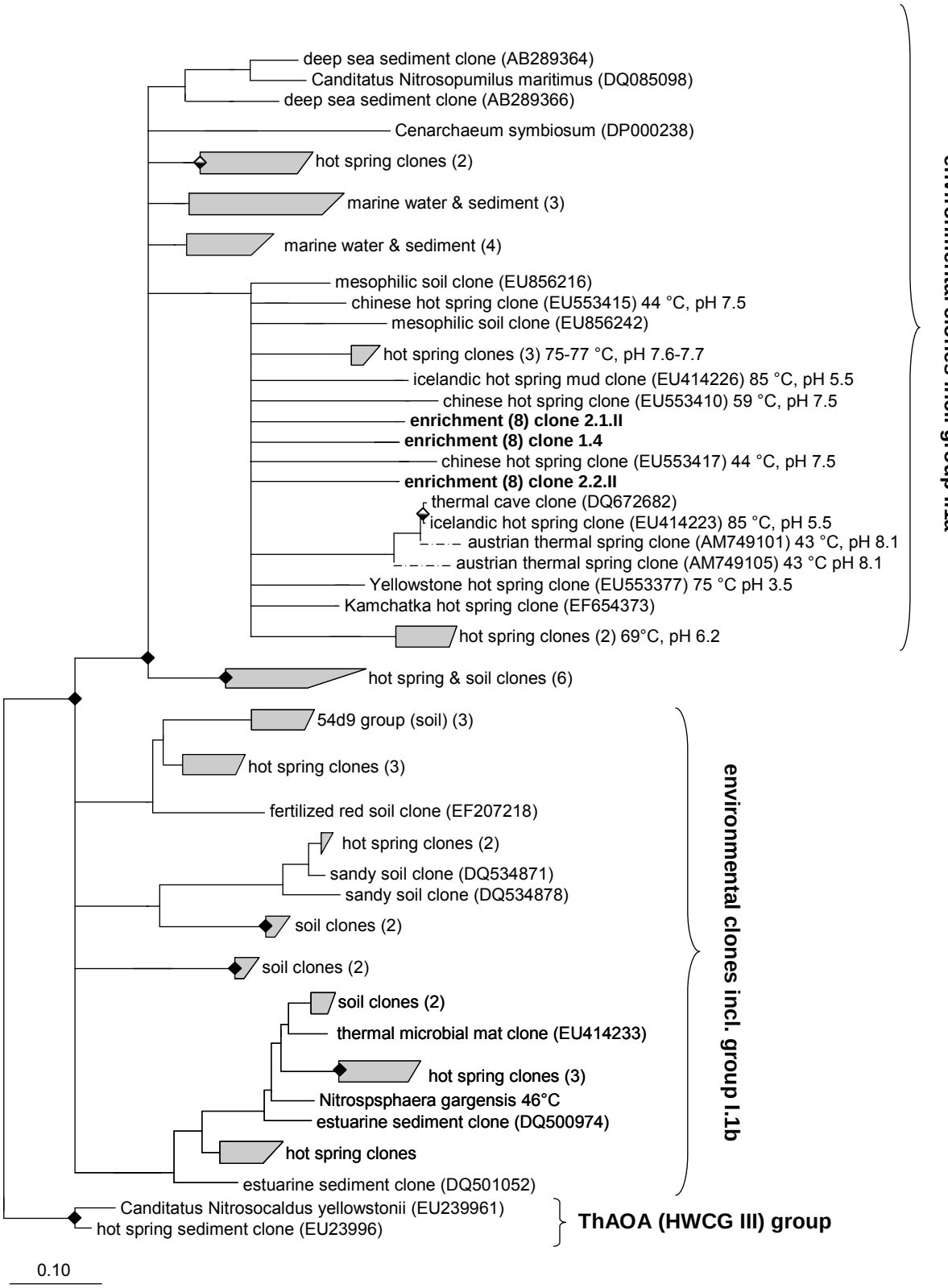

Figure I. 1. Unrooted consensus tree showing the position of crenarchaeal *amoA* gene fragments amplified from hot spring enrichment culture 8 and acidic forest soil (in bold). Evolutionary distance (Fitch) tree and MP tree topologies were considered for consensus. Phylogenetic calculations were done with sequences >169 aa positions, shorter sequences were added afterwards and are indicated by dashed lines. Nodes with filled squares and half filled squares indicate bootstrap support >90% and >70%, respectively. Accession numbers are written in brackets. Stars indicate *amoA* sequences that can be related to the 16S rRNA gene of *Crenarchaeota*. The scale bar represents 5% estimated nucleotide sequence difference.

Table I.5. Summary of amplified gene fragments, picked clones, RFLP patterns and retreived sequences from hot springs in Kamchatka (enrichment cultures 4 and 8) and at Lake Baikal (enrichment cultures A and C_a)

sample	amplified fragment	primer pair[a]	fragment size (bp)	# clones picked	insert in the right size	different RFLPs	# clones sequenced	# retreived sequences	
Enrichment culture 8	archaeal 16S	21F/1492R	1438	37	31	19/9/1/1[c]	9/6/1/1[d]	17	(9/6/1/1)[e]
	archaeal 16S	21F/958R	912	24	23	17/2/2/1/1	3/2/1/1/1	2	(2/-/-/-/-)
	archaeal *AmoA*	ArchAmoAF/R	453	33	31	30/1	7/1	31	
	16S Nitrospira	616V/712R	721	11	10	8/2	5/2	7	
	NxrB Nitrospira	F19/R1237	1238-1245	11	4	3/1	3/1	3	(2/1)
Enrichment culture 4	16S Nitrospira	616V/712R	721	11	11	11	5	5	
	NxrB Nitrospira	F19/R1237	1238-1245	no PCR product obtained					
enrichment cultureA	16S Nitrospira	616V/1158R	1153	9	9	-[b]	5	5	
	NxrB Nitrospira	F19/R1237	1238-1245	no clones					
Enrichment culture C_a	16S Nitrospira	616V/1158R	1153	9	9	-[b]	5	5	
	NxrB Nitrospira	F19/R1237	1238-1245	8	7	-	7	7	

[a] For detailed description of these primers see section B.7.4.
[b] RFLP didnt work
[c] e.g. please read: 19, 9 ant two single clones, resprecitvely, showed an unique pattern
[d] e.g. please read: 9 clones of the first RFLP pattern were sequenced, 6 clones of the second pattern, etc.
[e] e.g. please read: for 9 clones of the first pattern sequences were retrieved, for 6 clones of the second pattern, etc.

Table I.6. Summary of amplified gene fragments, picked clones, RFLP patterns and retrieved sequences.

amplified fragment	Primer pair[a]	fragment size (bp)	# clones picked	insert in the right size	different RFLPs	# clones sequenced	# retrieved sequences	
bacterial *AmoA*	AmoA1F/AmoA2R	453	19	11	-	11	8	
archaeal 16S	21F/1492R	1438	33	28	17/1[b]	6/1[b]	6	(6/-)[b]
archaeal 16S	21F/958R	912	33	25	21/2/1/1	3/2/1/1	4	(3/-/1/-)
archaeal *AmoA*	ArchAmoAF/R	634	12	6	-	3	2	
16S Nitrospira	616V/1158R	1165	23	16	5/4/2/2/1/1/1	4/1/1/1/1/1/1	8	(4/1/1/1/1/-/-)
NxrB Nitrospira	F14/R1237	1244	24	15	4/2/2/1/1/1/1/1/1	3/2/2/1/1/1/1/1/1	13	('3/2/2/1/1/1/1/1/1)
NxrB Nitrobacter	NxrBF706/NxrB1431	725	24	20	12/1/1/1	3/1/1/1	3	(3/-/-/-)

[a] For detailed description of these primers see section B.7.4.
[b] for interpretation of these results please refer to table I.3.

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