DIPLOMARBEIT

Titel der Diplomarbeit

Ecophysiological investigation of nitrite-oxidizing bacteria of the genus *Nitrospira*

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A. Introduction

A.1 Nitrogen cycle

The nitrogen cycle (Fig. A.1) is one of the most significant and well-studied biochemical cycles, mostly due to the importance of nitrogen as component of nucleic acids, proteins and vitamins. Nitrogen and its different redox states can be found in all domains of life and various global areas (i.e. biosphere, atmosphere, lithosphere and hydrosphere) during the recycling of this element. In reference to that, prokaryotes play an essential role in this nitrogen cycle framed by five microbiological reactions: nitrogen fixation, nitrification, denitrification, anaerobic ammonium oxidation and mineralization (Bock *et al.* 2006). Some of these processes are exclusively catalyzed by prokaryotes, e.g. fixation of nitrogen and anaerobic ammonium oxidation (Hayatsu *et al.* 2008).

Fixation of nitrogen creates an interface between the nitrogen pool of the atmosphere and the biosphere as it converts chemically inert, gaseous dinitrogen (N_2) into bioavailable ammonia (NH_3). This is a reductive and ATP-dependent reaction that is performed exclusively by free-living and symbiotic prokaryotes belonging to various phyla, like *Actinobacteria*, *Bacteriodetes*, *Chloroflexi*, *Cyanobacteria*, *Eurarchaeota*, *Firmicutes*, *Spirochaetes* and *Proteobacteria* (Kneip *et al.* 2007; Berman-Frank *et al.* 2003). Nitrogen fixation is catalyzed by the nitrogenase enzyme complex, consisting of two separate enzymes – dinitrogenase and dinitrogenase reductase – which are inhibited by oxygen, even at conditions of very low oxygen concentration. The product of this reaction, ammonia, is then used for the synthesis of biomolecules, for instance proteins.

Ammonia remaining in the environment can be used as energy source under oxic conditions by converting ammonia to nitrite (NO_2^-) via hydroxylamine (NH_2OH). This oxidation is the first step of nitrification (for details see A.1.1), which is performed by two physiological groups of prokaryotes, ammonia oxidizers and nitrite oxidizers. Nitrite oxidizing bacteria (NOB) catalyze the second step of nitrification and convert nitrite to nitrate (NO_3^-). Nitrification is an aerobic process, but recently a way of anaerobic ammonium oxidation (anammox) was found (van de Graaf *et al.* 1995). Anammox bacteria belong to the phylum *Plantomycetes* and catalyze the transformation of ammonium to dinitrogen under anoxic

environmental conditions, with nitrite being an electron acceptor (Schmid *et al.* 2005). Another process that leads to the loss of nitrogen compounds in the biosphere is denitrification. Bacteria, archaea and fungi are capable of denitrification, which is a widespread dissimilatory reduction of nitrate to gaseous dinitrogen in anaerobic conditions (Cabello *et al.* 2004; Hayatsu *et al.* 2008).

Recently described processes, as a) hyperthermophilic N_2-fixing methane-producing archaea (Mehta *et al.* 2006), NO_2^- - oxidizing phototrophs (Griffin *et al.* 2007), NO_2^- - dependent anaerobic methane oxidation (Raghoebarsing *et al.* 2006), NO_3^- reduction to N_2 by foraminifera (Risgaard-Petersen *et al.* 2006), provide examples that science is still far away from full comprehension regarding the nitrogen cycle (Jetten 2008).

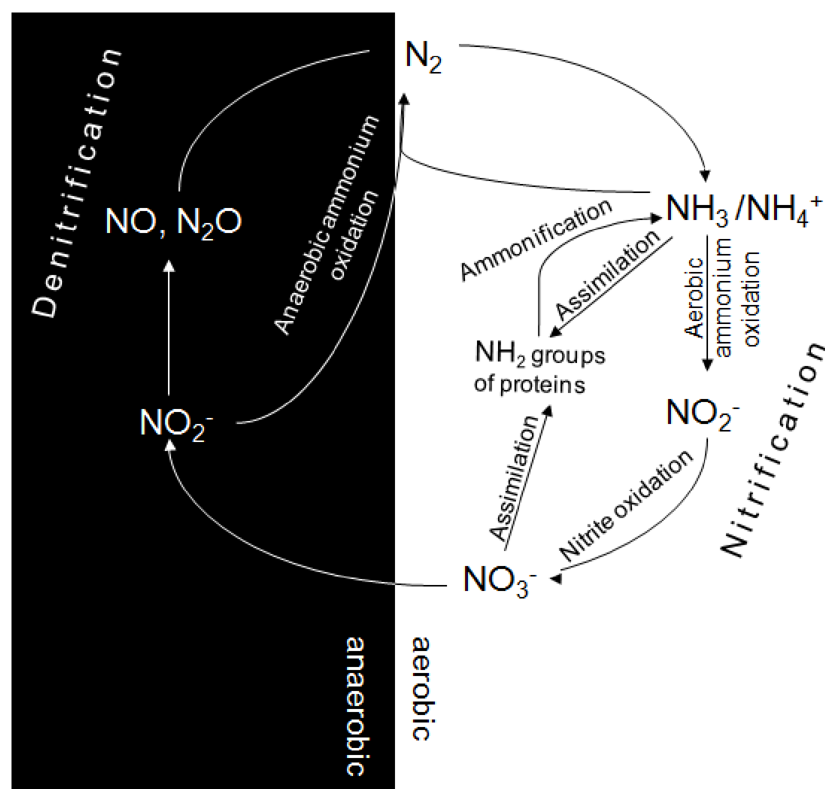


Figure A.1: The biogeochemical nitrogen cycle

A.1.1 Nitrification

A.1.1.1 Ammonia-oxidizing bacteria and archaea

Lithoautotrophic ammonia oxidizing bacteria (AOB), characterized by the prefix *Nitroso*-, belong to two phylogenetically distinct lineages within the β- and γ- subclasses of *Proteobacteria* (Koops *et al.* 2001). The genera *Nitrosospira*, *Nitrosolobus*, *Nitrosovibrio* and *Nitrosomonas* (including *Nitrosococcus mobilis*) belong to a monophyletic group within the *β-Proteobacteria*, whereas the genus *Nitrosococcus* is affiliated to the γ-subclass of *Proteobacteria* (Purkhold *et al.* 2000;Koops *et al.* 2001).

Most AOB have the potential to hydrolyze urea and use it as ammonia source (Koops *et al.* 2001). Ammonia is subsequently oxidized to intermediate hydroxylamine by the enzyme ammonia monooxygenase (AMO; Fig:A.2), which consists of 3 subunits, i.e. AmoA, AmoB, AmoC encoded by the genes *amoA*, *amoB*, *amoC* (Klotz *et al.* 1998). The subunit AmoA contains the active site of AMO and therefore *amoA* is used as a phylogenetic marker gene of AOB. The enzyme hydroxylamine oxidoreductase (HAO; Fig.A.2) catalyses the second reaction of ammonia oxidation. Hydroxylamine is oxidized to nitrite, which leads to the release of four electrons. Two of these electrons are required in the ammonia oxidation step catalyzed by AMO, the other two electrons are transfered to the respiratory chain for energy generation (Bock *et al.* 2006).

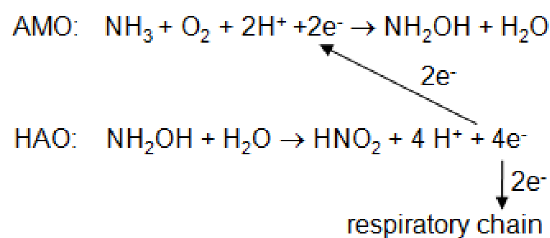


Figure A.2: Ammonia-oxidation of AOB catalyzed by AMO and HAO

Recently an *amoA*-related gene was discovered, together with a 16S rRNA gene of a crenarchaeota on a large genomic contig, which was isolated from a metagenomic soil DNA library (Treusch *et al.* 2005). The existence of ammonia-oxidizing archaea was finally proofed by the isolation of a marine chemolithoautotrophical crenarchaeota, that uses the conversion of ammonia to nitrite for gaining its energy (Könneke *et al.* 2005). Subsequent

studies found AOA in different habitats, like various soil types, marine water columns and marine sediments (Leininger *et al.* 2006 ; Francis *et al.* 2005).

A.1.1.2 Nitrite-oxidizing bacteria

The second step in the nitrification process, namely the oxidation of nitrite to nitrate, is catalyzed by nitrite-oxidizing bacteria (NOB). The lithotrophic NOB, characterized by the prefix *Nitro-*, are phylogenetically located within the α- (*Nitrobacter*), β- (*Nitrotoga*) γ- (*Nitrococcus*), δ- (*Nitrospina*) subclasses of *Proteobacteria* and the phylum *Nitrospira* (Bock *et al.* 2006, Alawi *et al.* 2007). The oxidation of nitrite to nitrate (equation A.1) is catalyzed by the membrane-associated enzyme nitrite oxidoreductase (Nxr) and the gained electrons are released and pass subsequently the respiratory chain (Bock *et al.* 2006, Alawi *et al.* 2007).

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

Equation A.1: Nitrite-oxidation of NOB catalyzed by Nxr

A.1.2 Genus *Nitrospira*

The genus *Nitrospira* represents a phylogenetic group of NOB within the phylum *Nitrospirae*. All known members of the genus *Nitrospira* are obligately chemolithotroph, they gain their energy by oxidizing of nitrite (Daims *et. al*, 2001). Former studies showed that some members of the genus *Nitrospira* can use organic carbon, like pyruvate (Daims *et al.* 2001).

Although a large diversity can be found within the genus *Nitrospira*, only three representatives of this group could be isolated successfully. The first isolated *Nitrospira* species is *Nitrospira marina*, which was enriched from water samples of the Atlantic Ocean (Watson *et al.* 1986). The second described species is *Nitrospira moscoviensis*, isolated from a sample from an iron pipeline of an urban heating system in Moscow, Russia (Ehrich *et al.* 1995). Additionally, two enrichments are available: "*Candidatus* Nitrospira defluvii", obtained from activated sludge samples and "*Candidatus* Nitrospira bockiana", enriched from deposits from a steel pipe of the heating system in Moscow, Russia (Spieck *et al.* 2006; Lebedeva *et al.* 2008).

The genus *Nitrospira* is divided into at least six sublineages (Fig.A.3). All 16S rRNA sequences in one sublineage are more than 94.9% similar, whereas the similarity of two sequences from different sublineages is below 94% (Watson *et al.* 1986; Daims *et al.* 2001). Sublineage I includes "*Candidatus* Nitrospira defluvii" and mainly sequences received from

wastewater treating plant (WWTP) samples. In comparison, sublineage II contains 16S rRNA sequences from many different habitats like soil, rhizosphere, WWTP, lake water and freshwater aquaria and also an isolated representative, *Nitrospira moscoviensis*. Sublineage III consists of sequences from Nullarbor caves, Australia, with no isolated or enriched member, whereas sublineage IV comprises *Nitrospira marina* and other sequences retrieved from sea water samples and marine sponges. Moreover, *Candidatus* Nitrospira bockiana represents sublineage V and an enrichment of thermophilic *Nitrospira* from a hot spring in the Baikal rift zone seems to form a sixth sublineage within this genus (Elena Lebedeva, manuscript in preparation). All these data show a high phylogenetical diversity within this genus as well as a wide spatial distribution of its members, as relatives of *Nitrospira* were found in various habitats: soil, ocean, sponges, freshwater, WWTP and recently in thermophilic habitats (Watson *et al.* 1986; Ehrich *et al.* 1995; Spieck *et al.* 2006; Daims *et al.* 2001; Lebedeva *et al.* 2008; Lebedeva *et al.* 2005; Foesel *et al.* 2008). Interestingly, the members of sublineage I, III, IV, V, VI seem to be adapted to a specific type of habitat, while sublineage II contains nitrite oxidizers that can be found in a wide range of different habitats (Daims *et al.* 2001).

A niche differentiation of members of *Nitrospira* sublineage I and II can be observed ,since these different groups of organisms can be found co-exististing in the same WWTP basin (Maixner *et al.* 2006). Recently, Maixner and colleagues (2006) showed that a putative niche differentiation of sublineage I and II populations of the genus *Nitrospira* can occur due to different affinities to nitrite. Members of the *Nitrospira* sublineage I were located in immediate vicinity to AOBs, where the nitrite concentration is higher – whereas the distribution of members of *Nitrospira* sublineage II did not showed such a relationship (Maixner *et al.* 2006).

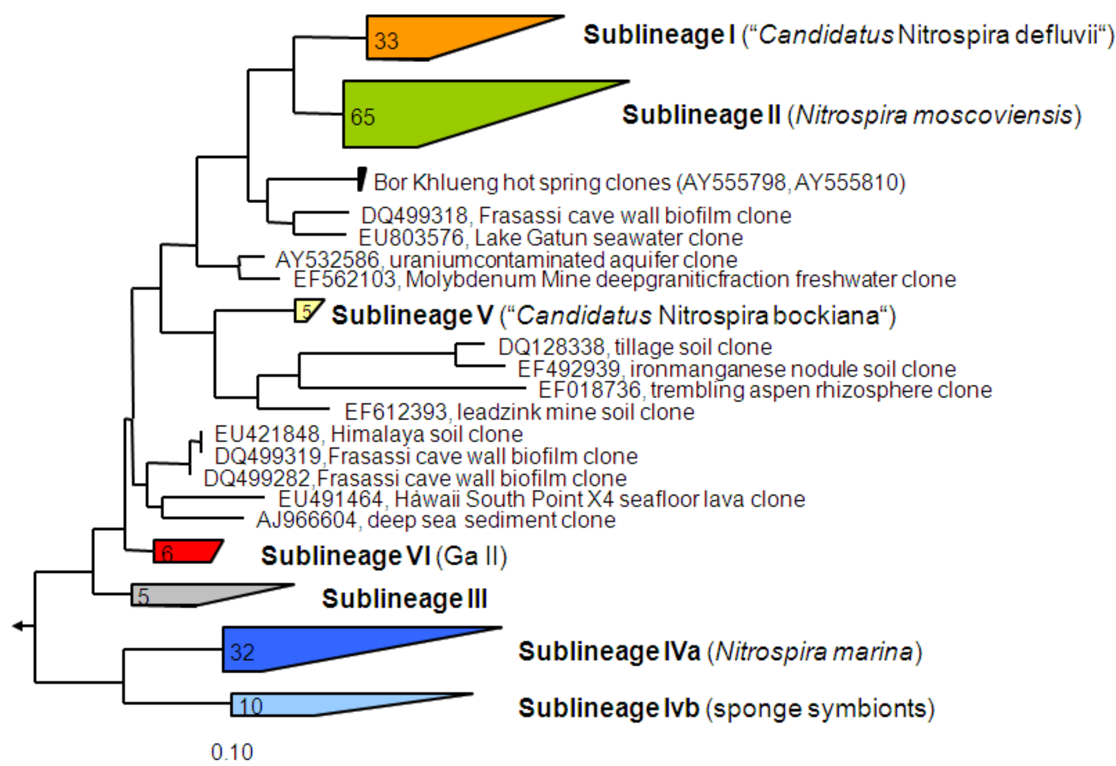


Figure A.3: Phylogenetic tree based on 16S rRNA gene sequences of selected *Nitrospira*-like bacteria and their clustering in different sublineages (I – VI), indicated by different colors. This image was kindly provided by Frank Maixner and was modified.

A.1.2.1 NOB in waste water treatment plants

Waste water treatments (WWTPs) are quite homogeneous ecosystems compared to natural habitats like soil, and for the ability to measure and control various physical and chemical parameters, combined with a high microbiological complexity, WWTPs are considered model-systems for research in microbial ecology (Daims *et al.* 2006).

For a long time *Nitrobacter*-like bacteria were considered representatives of dominant NOB in WWTPs, because they are easy to cultivate and therefore their abundance and their ecological importance were underestimated. Furthermore, the development, establishment and application of cultivation-independent techniques showed that other nitrite oxidizers play the key role in this environment. Several studies identified *Nitrospira*-related organisms as the most abundant and important NOB in WWTPs (Juretschko *et al.* 1998; Daims *et al.* 2001; Schramm *et al.* 1999; Daims *et al.* 2006).

Moreover, a niche differentiation of the two genera *Nitrobacter* and *Nitrospira* was observed (Schramm *et al.* 1999; Schramm *et al.* 2000). *Nitrobacter* is suggested to be a r-strategist, due to its lower affinity to substrate and oxygen and its higher growth rate compared to *Nitrospira*. In contrast *Nitrospira* seems to be adapted to low nitrite concentration with a higher oxygen affinity and might be a typical K-strategist(Schramm *et al.* 1999).

It could also be detected that members of AOB and NOB form separate, dense clusters in activated sludge and biofilms. Several studies showed a colocalization of members of these physiological groups (Juretschko *et al.* 1998; Schramm *et al.* 1998). Both partners do benefit from this colocalization, as AOB supply nitrite, the energy source of NOB, whereas NOB remove nitrite, which is toxic for AOB at high concentrations. Therefore, *Nitrospira* spp. coaggregate with *Nitrosococcus mobilis* in small microcolonies and members of the genus *Nitrospira* are often located in the immediate vicinity of AOB.

A.1.2.2 Nitrite oxidoreductase

The key enzyme of all known NOB is the membrane-associated enzyme nitrite oxidoreductase (Nxr). Nxr contains molybdenum and iron-sulfur clusters as cofactors and (Meincke *et al.* 1992), it belongs to the mononuclear molybdenum-cofactor-containing protein family. This protein family is divided into three classes, the sulfite oxidase family, the xanthine oxidase, and the dimethylsulfoxide (DMSO) reductase families (Hille 1996). All mononuclear molybdenum enzymes catalyze the transfer of one oxygen atom from water to a defined substrate by a two-electron redox reaction and vice versa (Kisker *et al.* 1997). Most DMSO family members, like Nxr of *Nitrobacter*, contain a molybdopterin guanosine dinucleotide as a cofactor of molybdenum (Kisker *et al.* 1997; Meincke *et al.* 1992; Hille 1996).

In *Nitrobacter*, the Nxr consists at least of the nitrite-oxidizing subunit α (115-130 kDa) and subunit β (65 kDa), which serves as an electron-channeling protein between subunit α and the respiratory chain (Spieck *et al.* 1996b). This intracellular enzyme is associated to the cytoplasmic and intracytoplasmic membranes, that can be found in *Nitrobacter* and *Nitrococcus* (Spieck *et al.* 1996a). Intracytoplasmic membranes are absent in *Nitrospira* and *Nitrospina*, whereas the periplasmic space seems to be enlarged in these genera. Nitrate can be reduced to nitrite by Nxr in *Nitrobacter hamburgensis* under anaerobic conditions (Kirstein *et al.* 1993).

Knowledge about the nitrite oxidation system of *Nitrospira* organisms is still limited, but recent studies showed that the Nxr of *Nitrospira moscoviensis* and *Nitrospira defluvii* consists at least of two subunits: the large α-subunit and the small β-subunit (Spieck et al. 1998; Lücker *et al.*, manuscript in preparation). The α-subunit and β-subunit of Nxr fulfill the same function in *Nitrospira* spp. as in *Nitrobacter*. NxrA of *Nitrospira defluvii* contains an N-terminal Fe/S binding center and molybdopterin cofactor-binding motifs, which is characteristical for the DMSO reductase family (Lücker *et al.*, manuscript in preparation). Moreover, a twin-arginine signal peptide can be found at the N-terminus of NxrA, which serves as a recognition site for the tat translocase system, that transport the folded protein into the periplasmic space. In comparison to NxrA, the electron transfer subunit, NxrB, lacks a signal peptide and therefore it is suspected that the tat-system translocates NxrA and NxrB both together as a folded protein complex to the periplasmic space.

Furthermore, several candidate genes for a third putative subunit could be identified recently within a genome study of *Nitrospira defluvii*, sequenced via an environmental genomic approach (Lücker *et al.*, manuscript in preparation). The γ-subunit is involved in the electron transport between the Fe/S complexes of NxrA and NxrB and the respiratory chain.

The β-subunit of Nxr was proven to be located in the periplasmic space by immunocytochemistry followed by electron microscopic in cells of *Nitrospira moscoviensis* (Fig.A.4) (Spieck *et al.* 1998). By oxidizing nitrite to nitrate, a proton motive force is generated to gain ATP via ATPase and so a possible accumulation of toxic nitrite in the cells can be avoided in the extracellular space.

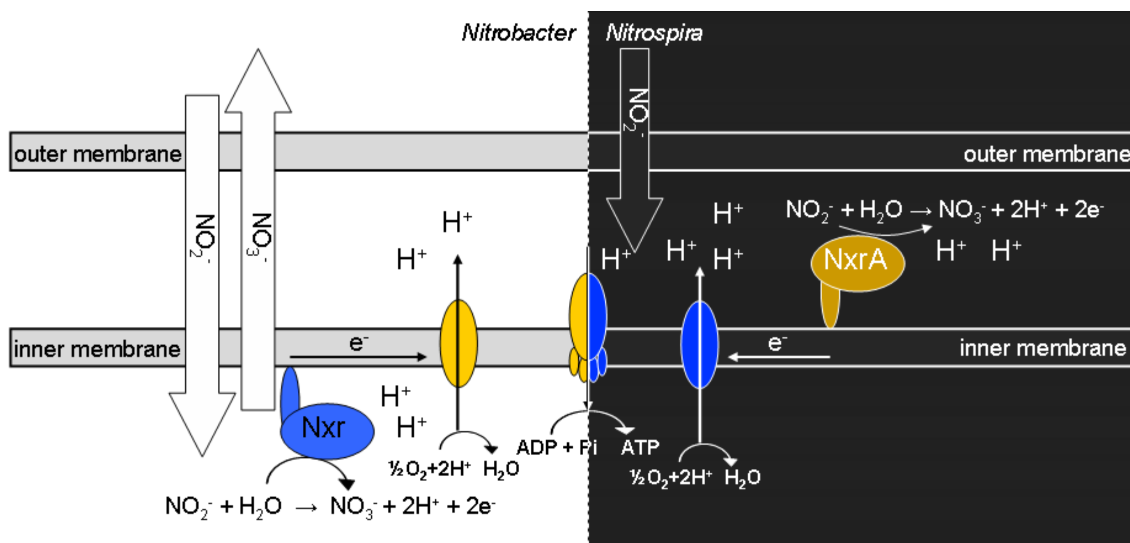


Figure A.4: Scheme of the nitrite oxidizing complex of the genera *Nitrobacter* and *Nitrospira* and the different location of Nxr within the cells. Here, arrangement and size of the subunits are drawn simplified.

Two copies of *nxrA*, encoding subunit α, and *nxrB*, encoding subunit β, were found in the recently sequenced genome of *Nitrobacter winogradskyi* Nb-255 (Starkenburg *et al.* 2006). It is remarkable that one set of copies forms an operon together with *nxrX*, which is probably involved in the folding process of Nxr, and other genes with possible accessory functions to Nxr. Other gene copies of each subunit are located elsewhere and independently in the genome.

In the genome of *Nitrospira defluvii nxrA* and *nrxB* genes are located in an operon (*nxrAB*), which is present twice on the chromosome (Fig.A.5) in contrast to *Nitrobacter*. Interestingly, the *nxrB* copies are identical on the amino acid level, whereas the *nxrA* copies differ from each other to 14.4% on the amino acid level (Maixner et al., manuscript in preparation).

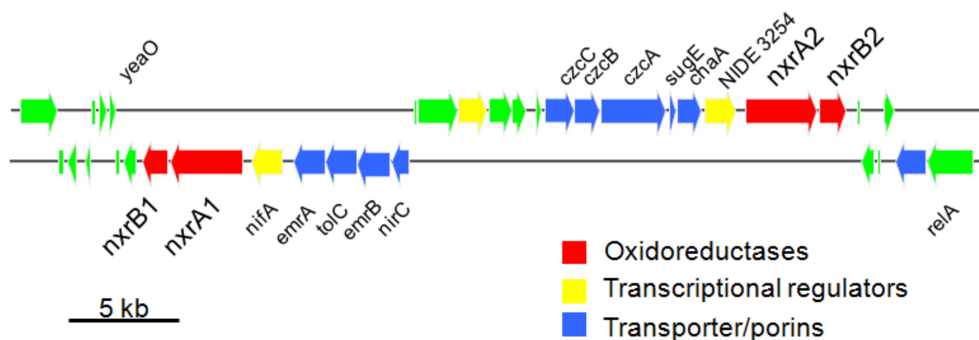


Figure A.5: Scheme of the genomic stretch of *N.defluvii* encoding the duplicated *nxrAB* operons; NIDE 3254: Sigma-54 dependent transcriptional regulator. This image was kindly provided by Frank Maixner and was modified.

For both genera, *Nitrospira* and *Nitrobacter* studies showed that subunits encoding genes of Nxr have the potential to be used as phylogenetic marker genes. Compared to the 16S rRNA genes *nxrA*, *nxrB* and *nxrX* show a higher sequence diversity and therefore a better phylogenetical resolution in the genus *Nitrobacter* (Maixner *et al.*, manuscript in preparation; Wertz *et al.* 2008; Vanparys *et al.* 2007, Poly *et al.* 2008).

A.2 Aims of this study

A.2.1 Characterization of the key enzyme nitrite oxidoreductase of *Nitrospira spp.*

A.2.1.1 Heterologous expression of the *nxrAB* operon and the *nxrA* gene of *Candidatus* Nitrospira defluvii

Although members of the genus *Nitrospira* represent an ecophysiologically significant group of NOBs, knowledge about their nitrite oxidizing system is still limited. In all known studies about Nxr of the genus *Nitrospira* the enzymes were isolated by heat treatment of membranes. In contrast this study uses a different approach, i.e. the heterologous expression of *nxrAB* and *nxrA*, which was chosen to characterize the Nxr of *Candidatus* Nitrospira defluvii. Heterologous expression is a tool to produce pure subunits, which then can be used to either investigate the activity of defined subunits, or to produce enough pure enzyme to characterize it by crystallographic methods. The aim of this study was the heterologous expression of the *nxrAB* operon as well as of the *nxrA* gene, isolated and amplified from an enrichment culture of *Candidatus* Nitrospira defluvii.

A.2.1.2 Investigation of the potential of the *nxrA* gene as a marker for gene expression and phylogeny

It could be shown that the β-subunit of Nxr can serve as a functional and phylogenetic marker for detection of representatives of the genus *Nitrospira* in environmental samples (Maixner, in prep.). In this study, the potential of the α-subunit-encoding gene as a phylogenetic marker for the genus *Nitrospira* was investigated. *NxrA*-specific primers were designed and established to analyze the occurrence and phylogeny of *Nitrospira* in different environments, like WWTP and soil. The second goal of this study was the analysis of gene expression of different *nxrA*

gene copies in the genomes of *Nitrospira* sublineage I organisms in the main WWTP of Vienna.

Furthermore, the occurrence of *Nitrospira* sublineage I in soil, detected by *nxrA* specific primers, was verified on 16S rRNA level and NOBs were enriched from a soil sample taken from the botanical garden of Vienna, where sublineage I organisms could be detected by *nxrA*- and 16S rRNA-based identification.

A.2.1.3 Micromanipulation of FISH-labelled *Nitrospira* sublineage II clusters in activated sludge flocs of different WWTPs and molecular analysis of the microbial floc community

In this study, a combination of both FISH and micromanipulation was used to identify different bacterial populations in single activated sludge flocs containing the target populations. The flocs were used for analysis of the bacterial composition in this micro-ecosystem, and to explore the potential of this technique for isolating single microcolonies of *Nitrospira* sublineage II.

The first aim of the study was to investigate bacterial biodiversity in a single activated sludge floc (harboring *Nitrospira* sublineage II) for comparison with the diversity of the whole bacterial community of an activated sludge detected in former studies (Juretschko *et al.* 1998). Putative differences could be a hint for new interaction partners of *Nitrospira* sublineage II. Former studies showed that colocalization of different physiological groups of organisms led to interactions between those partners, e.g. utilization of partly toxic waste products or secretion products, like soluble microbial products (Schramm *et al.* 1998). For instance, Kindaichi and colleagues (2004) showed an ecophysiological interaction between nitrifiers and heterotrophic bacteria in a carbon-limited autotrophic nitrifying biofilm via FISH-MAR. Thus, identification of a putative interaction partner of *Nitrospira* sublineage II organisms could give new insights into the bacterial foodchain within an activated sludge floc.

Additionally, the potential of this technique was tested to gain a single microcolony of *Nitrospira* sublineage II. Due to the strong adhesion characteristic of *Nitrospira* clusters compared to other bacteria in an activated sludge floc, mechanical shear techniques were used to isolate *Nitrospira* II population by minimizing the floc size.

For other methods – microfluidics, immunomagnetic capture, optical trapping and magneto-FISH – it could be shown that they offered the opportunity to separate and purify particular members of a diverse microbial community (Marcy *et al.* 2007; Ashkin 1997; Yeung *et al.* 2002; Kalyuzhnaya *et al.* 2006). The purified cells can then be used for genome sequencing.

Another field for this approach would be the isolation of living *Nitrospira* organisms directly from the activated sludge by mechanical shear techniques and further isolation by micromanipulation. After bead beating the chance to find *Nitrospira* cell cluster in intact flocs might be higher compared to other bacteria cells due to the strong adhesion characteristic of *Nitrospira*-like cells.

A.2.2 Analysis of the influence of low nitrite concentration and discontinuous aeration condition on *Nitrospira* sublineage I and II

Although one representative of *Nitrospira* sublineage II, *Nitrospira moscoviensis* could successfully be isolated over 25 years ago, an enrichment culture of sublineage II organisms out of activated sludge samples is still missing. The key of success for enrichment studies is a sound knowledge of the ecophysiology regarding the organism of interest.

Former studies showed that sublineage I organisms can outcompete sublineage II of *Nitrospira* by high nitrite concentrations, whereas sublineage II seems to be adopted to low nitrite concentrations. (Maixner *et al.* 2006) The concentration of oxygen seems be play a role in niche-differentiation of the two *Nitrospira* sublineages, indicated by the preference of members of sublineage II to be located in the middle of activated sludge flocs, where the oxygen content is lower than on the fringe of the floc (Schramm *et al.* 2000).

To obtain an enrichment culture of *Nitrospira* sublineage II, activated sludge from two different WWTPs was incubated at low nitrite concentrations. Furthermore, the influence of discontinuous aeration conditions were tested on the *Nitrospira* sublineage I and II populations. Fluorescence labeled probes and FISH were used to monitor changes in the biodiversity of incubated activated sludge samples.

B. Materials and methods

In this study, all used chemicals were of *p.a.* quality, if not stated otherwise. All buffers, media and solutions were produced using double distilled and filtered water ($H_2O_{bidist.}$) produced by a water purification facility (MQ Biocel, Millipore Corporation, Billerica, MA, USA) and were autoclaved in a watervapour-high-pressure autoclave (Varioclav 135S, H+P, Munich, Germany) for 20 min. at 121°C and 1.013×10^5 Pa pressure and stored at room temperature (RT), unless stated otherwise. Furthermore, chemicals, buffers and solutions unstable at high temperature were filtered sterile and if necessary, added after autoclaving. If not stated otherwise, all centrifugation steps were performed using a table-top centrifuge (Mikro 22R, Hettich, Tuttlingen, Germany) at RT.

B.1 Technical equipment

Table B.1: Technical equipment

Equipment	Company
Agarose gel electrophoresis apparature Sub-Cell GT	Bio-Rad Laboratories GmbH, Munich, Germany
Beadbeater Fast Prep FP 120	Savant Instruments Inc. Holbrook, NY
CCD camera AxioCam HRc	Carl Zeiss MicroImaging GmbH, Jena, Germany
Centrifuges:	
Mikro 20	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Rotina 35 R	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Galaxy Mini Centrifuge	VWR international, West Chester, PA, USA
Concentrator 5301	Eppendorf AG, Hamburg, Germany
Devices for gelelectrophoresis:	
Electrophoresis cell (Sub-Cell GT)	Bio-Rad Laboratories GmbH, Munich, Germany
Hoefer™ HE 33 - gel running tray (7x10cm)	Amersham Biosciences (SF) Corp., USA
Hoefer™ HE 33 Mini Horizontal submarine unit	Amersham Biosciences (SF) Corp., USA
Electrophoresis power supply (PowerPac Basic)	Bio-Rad Laboratories GmbH, Munich, Germany
Devices for SDS-PAGE analysis:	
Mini-PROTEAN Casting Stand Mini-PROTEAN Tetra Cell	Bio-Rad Laboratories GmbH, Munich, Germany

Electroporator Micro PulserTM	Bio-Rad Laboratories GmbH, Munich, Germany
Hybridisation oven UE-500	Memmert GmbH, Schwabach, Germany
icycler Thermal cycler	Bio-Rad Laboratories GmbH, Munich, Germany
Laminar flow hood, Safe 2010 Modell 1.2	Holten, Jouan Nordic, Allerod, Denmark
Magnetic stirrer RCT basic	IKA® Werke GmbH & Co. KG, Staufen, Germany
Microbiological incubator KB 115	Binder GmbH, Tuttlingen, Germany
Microscopes: Inverse microscope Axiovert 25 Epifluorescence microscope Axioplan 2 imaging Confocal laser scanning microscope LSM 510 Meta	Carl Zeiss MicroImaging GmbH, Jena, Germany Carl Zeiss MicroImaging GmbH, Jena, Germany Carl Zeiss MicroImaging GmbH, Jena, Germany
Magnetic stirrer: RCT basic	 IKA® Werke GmbH, Schwabach, Deutschland
Microwave MD6460	Microstar
Heatblock VWR Digital Heatblock	VWR international, West Chester, PA, USA
NanoDrop® ND-1000 UV/Vis spectrophotometer	NanoDrop Technologies Inc., Wilmington, DE, USA
pH meter inoLab pH Level 1	Wissenschaftlich technische Werkstätten (WTW) GmbH & Co. KG, Weilheim, Germany
Platform Shaker 2300	New Brunswick Co., Inc., Madison NJ, USA
Scales: OHAUS® Analytical Plus balance Sartorius BL 3100	 Ohaus Corporation, Pine Brook, NJ, USA Sartorius AG, Göttingen, Germany
Scanner Epson Expression 1680 Pro	Epson Deutschland GmbH, Meerbusch, Germany
Sonicator Bandelin Sonoplus HD2070	Bandelin electronic GmbH & Co. KG, Berlin, Germany
Sonotrode Bandelin Sonoplus UW 2070	Bandelin electronic GmbH & Co. KG, Berlin, Germany
TE77 semi-dry transfer unit	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Thermostatic circulator MultiTempTM III	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Transilluminator	Biostep GmbH, Jahnsdorf, Germany
Ultrasonic Cleaner SC100T	VWR International bvba/sprl, Leuven, Belgium
UV sterilizing PCR workstation	PeqLab Biotechnologie GmbH, Erlangen, Germany
Vortex Genie 2	Scientific Industries, New York, USA
Water baths:	

DC10 GFL® type 1004	Thermo Haake GmbH, Karlsruhe, Germany 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 Varioclav® 25 T H+P	H+P Labortechnik GmbH, Oberschleißheim, Germany H+P Labortechnik GmbH, Oberschleißheim, Germany

B.2 Expendable items

Table B.2: Expendable items

Expendable item	Company
Cover slips 24×50 mm	Paul Marienfeld, Bad Mergentheim, Germany
Eppendorf Reaktionsgefäße (ERT), various sizes	Eppendorf AG, Hamburg, Germany
Erlenmeyer-Kolben DURAN®, various sizes	Schott Glas, Mainz, Germany
Glascapillares (50 µl in 5.1 cm)	Idaho Technology Inc., Salt Lake City, UT, USA
Microseal „A“ Film	MJ Research, Waltham, MA, USA
Microseal® “B” film	Biorad, München, Germany
Mikrotiterplatte MicrosealTM 96,	V-Boden MJ Research, Waltham, MA, USA
MultiScreen 96-well plates	Millipore Corporation, Billerica, MA, USA
Petri dishes 94/16	Greiner Bio-one GmbH, Frickenhausen, Germany
Sampling vessels, 15mL 50 mL	Greiner Bio-One GmbH, Frickenhausen, Germany
slides, 10 Well	Paul Marienfeld, Bad Mergentheim, Germany
Electroporation cuvette (0.2 cm)	Bio-Rad Laboratories GmbH, Munich, Germany
slides, 76 x 26 mm	Carl Roth GmbH & Co., Karlsruhe, Germany
Tips, various volumes	Carl Roth GmbH & Co., Karlsruhe, Germany
PCR tubes (0.2 ml)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Pipette Tipps (various sizes)	Carl Roth GmbH Co. KG, Karlsruhe, Germany
Plastic cuvettes	Greiner Bio-One GmbH, Frickenhausen, Germany

Plastic pipettes (10 ml, 2 ml), single use, sterile	Barloworld Scientific Ltd., Staffordshire, UK
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
Syringe filter, single use, sterile, 1.20 µm pore size	Sartorius AG, Goettingen, Germany

B.3 Chemicals and enzymes

Table B.3: Chemicals

Chemical	Company
6x DNA Loading Dye	Fermentas Life Sciences Inc., Hannover, MD, USA
Acetic acid	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agar	Fluka Chemie AG, Buchs, Switzerland
Ammonium bicarbonate	Fluka Chemie AG, Buchs, Switzerland
Ammonium persulfate (APS)	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Ampicillin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Boric acid	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Brilliant Blue G-250	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Brilliant Blue R-250	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bromphenol blue	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Calcium chloride dihydrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Citifluor AF1	Agar Scientific Ltd., Stansted, UK
Coomassie brillant blue G-250	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Disodiumhydrogenphosphate dihydrate (Na2HPO4 x 2H20)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dithiothreitol (DTT)	Fluka Chemie AG, Buchs, Switzerland
Ethanol absolute	AustrAlco Österreichische Alkoholhandels GmbH, Spillern, Austria
Ethidium bromide (EtBr)	Fluka Chemie AG, Buchs, Switzerland

Ethylenediamine-tetraaceticacid (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ferrous ammonium sulfate hexahydrate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Ficoll® 400	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Formaldehyde (37% (w/w))	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Formamide (FA)	Fluka Chemie AG, Buchs, Switzerland
Glucose	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Glycerol (87% (w/v))	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Glycine	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Hydrochloric acid (HCl) (37% (w/w))	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Hydrogen Peroxid (H2O2), 30%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Imidazol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
IPTG (Isopropyl-ß-D-thiogalactopyranoside)	Fermentas Life Sciences Inc., Hannover, MD, USA
Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Kanamycin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Methanol	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Moviol 4-88	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
N,N,N´,N´-tetramethylethane-1,2-diamine (TEMED)	Fluka Chemie AG, Buchs, Switzerland
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Rotiphorese® NF-Acrylamide/ Bisacrylamide-solution 30 % (29:1)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Mallinckrodt Baker B.V., Deventer, Holland
Sodium dodecly sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

SYBR® Green I	Cambrex Bio Science, Rockland, Inc., Rockland, ME, USA
Tris	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Urea	USB, Corp., Cleveland, USA
X-Gal (5-brom-4-chlor-3-indolyl-β-D-galactopyranoside)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Xylencyanol	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Yeast extract	Oxoid Ltd., Hampshire, England

Table B.4: Enzymes

Enzyme	Company
Benzonase® Nuclease	Novagen, Darmstadt, Germany
Taq DNA Polymerase (5U/µl)	Fermentas Inc. Hannover, MD, USA
10 x Ex Taq polymerase-buffer	Fermentas Inc. Hannover, MD, USA
Buffer O	Fermentas Inc., Hannover, MD, USA
MspI (10 U/µl)	Fermentas Inc., Hannover, MD, USA
EcoRI (10 U/µl)	Fermentas Inc., Hannover, MD, USA
NdeI (10 U/µl)	Fermentas Inc., Hannover, MD, USA
Lysozyme human (≥ 100 U/µg)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Table B.5: Software

Software	URL	Reference
ARB software package	http://www.arb-home.de/	Ludwig *et al.* 2004
Basic Local Alignment Search Tool	http://www.ncbi.nlm.nih.gov/BLAST/	Altschul *et al.* 1990
probeBase	http://www.microbial-ecology.net/probebase/	Loy *et al.* 2003
probeCheck	http://www.microbial-ecology.net/probecheck/	Loy *et al.* 2008
Ribosomal Database Project	http://rdp.cme.msu.edu/	Cole *et al.* 2003
Clone Manager 5	-	Technelysium Pty Ltd
SignalP	http://www.cbs.dtu.dk/services/SignalP/	CBS Technical University of Denmark DTU
ChromasPro	http://www.technelysium.com.au/chromas.html	Scientific & Educational Software

B.4 Buffers, media and solutions

B.4.1 General buffers

PBS stock solution (Na_xPO_4) pH 7.2-7.4		
Solution 1	NaH_2PO_4	200mM (35.6g/L)
Solution2	Na_2HPO_4	200mM (27.6g/L)

pH of PBS stock solution was adjusted to 7.2-7.4 by adding solution 2 to solution 1.

1 x PBS pH 7.2-7.4	
NaCl	130 mM (7.6g/L)
PBS stock solution	10 mM (50 ml/L)
H_2O_{bidest}	ad 1000mL

3x PBS pH 7.2-7.4	
NaCl	390 mM (22.8g/L)
PBS stock solution	30 mM (150 ml/L)
H_2O_{bidest}	ad 1000mL

B.4.2 Buffers and solutions for agarose gel electrophoresis

All buffers and solutions for agarose gel electrophoresis were not autoclaved or sterile-filtrated.

10 x TBE pH 8.3-8.7	
Tris-HCl	162.0 g
EDTA	9.3 g
Boric acid	27.5 g
H_2O_{bidest}	ad 1000 mL

1 x TBE buffer	
10 x TBE	100 mL
H_2O_{bidest}	ad 1000 mL

50 x TAE pH 8.0	
Tris	2 M
Sodium acetate	500 mM
EDTA	50 mM

pH was adjusted to 8.0 with pure acetic acid.

1 x TAE	
50 x TAE	20 mL/L
H_2O_{bidest}	ad 1000 mL

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

DNA ladder
GeneRuler^{TM} 1kb (Fermentas Inc., Hannover, MD, USA)

Ethidium bromide stock solution
10 mg/ml ethidium bromide (EtBr) in H_2O_{bidest}

EtBr staining solution
EtBr stock solution diluted 1: 10.000 in H_2O_{bidest}

B.4.3 Buffers and solutions for isolation of plasmid DNA

P1 buffer	
Tris-HCl, pH 8.0	50 mM
EDTA	10mM
RNaseA	100µg/mL

NaOH/SDS solution (10mL stock)	
$H_2O_{bidest.}$	8mL
NaOH	1mL
10% SDS	1mL

Potassium acetate/acetate solution (10mL stock)	
KCl (5M)	6mL
$H_2O_{bidest.}$	2.85mL
Acetic acid (pure)	1.15mL

B.4.4 Buffers and solutions for Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

10% Ammoniumperoxodisulfit (APS)	
APS	10% w/v
$H_2O_{bidest.}$	ad 10mL

SDS PAGE lower buffer pH 8,8	
10% SDS	20mL
Tris	90.85g
$H_2O_{bidest.}$	ad 500mL

SDS PAGE upper buffer pH 6,8	
SDS	10g
Tris	30.2g
Glycin	114g
$H_2O_{bidest.}$	ad 1000mL

Coomassie staining solution	
Acetic acid	10% (v/v)
Methanol	4% (v/v)
Coomassie brilliant blue R250	0.25% (w/v)

Destaining solution	
Acetic acid	10% (v/v)

Protein ladder
PageRuler™ Prestained Protein ladder
(Fermentas Inc., Hannover, MD, USA)

4x SDS PAGE loading dye (Laemmli buffer)	
Tris/HCl pH= 6.8	200mM
Glycerol	40% (v/v)
SDS	8% (w/v)
Bromphenolblue	0.2% (w/v)
2-Mercaptoethanol	2.85% (v/v)
$H_2O_{bidest.}$	ad 100mL

B.4.5 Buffers and solutions for heterologous expression

All solutions for heterologous expression were filter-sterilized.

Sodium-Phosphate buffer

0.2M monobasic stock (500mL)	
$NaH_2PO_4*H_2O$	13.8g
$H_2O_{bidest.}$	ad 500mL

0.2M monobasic stock (500mL)	
$Na_2HPO_4*H_2O$	17.8g
$H_2O_{bidest.}$	ad 500mL

0.1M Na-Phosphate buffer pH 7.4	
$Na_2HPO_4*H_2O$	243mL
$NaH_2PO_4*H_2O$	ad until pH 7.4 is reached
H_2O_{bidest}	ad 600mL

Binding buffer pH 7.4	
0.1M Na-Phosphate buffer	200mL
NaCl	0.5M (58.44g/L)
Urea	8M (60,08g/L)
Imidazol	20mM (1.36g/L)
H_2O_{bidest}	ad 1000mL

B.4.6 Solutions for induction and selection

All solutions for induction and selection were filter-sterilized and stored at -20°C until further use.

X-gal solution	
X-gal	40 mg/mL

X-gal was dissolved in di-methylformamide (DMF) and stored in the dark at -20° C.

IPTG stock solution	
IPTG	1M

IPTG was dissolved in $H_2O_{bidest.}$

B.4.7 Antibiotics

The antibiotics were stored at -20°C and were added to medium to reach a final concentration of 100 µg/mL.

Ampicillin stock solution (Amp)	
Amp	100 mg/mL

Amp was dissolved in 50 % $EtOH_{abs}$.

Kanamycin stock solution (Kan)	
Kan	100 mg/mL

Kan was dissolved in $H_2O_{bidest.}$

B.4.8 Culture media for *Escherichia coli* (*E. coli*) strains

Luria-Bertani-medium (LB-medium) pH 7.0-7.5	
Tryptone	10.0 g/L
NaCl	5.0 g/L
Yeast extract	5.0 g/L
$H_2O_{bidest.}$	ad 1000 mL
For solid medium:	
agar	15 g/L

dYT-medium	
Tryptone	16.0 g/L
Yeast extract	10.0 g/L
NaCl	5.0 g/L
$H_2O_{bidest.}$	ad 1000 mL

SOC medium (Invitrogen Corporation,Carlsbad, CA, USA)	
Tryptone	2 % w/v
Yeast extract	0.5 % w/v
NaCl	10 mM
KCl	2.5 mM
$MgSO_4$	10 mM
$MgCl_2$	10 mM
Glucose	20 mM

B.4.9 Enrichment media of nitrifiers

Mineral salt medium for enrichment of nitrifiers from soil samples

(Bock *et al.* 1983)

Mineral salt medium with nitrite	
NaC1	0.5g
$CaCO_3$	0.003g
$MgSO_4x7H_2O$	0.05g
KH_2PO_4	0.15g
$(NH_4)_2Mo7O_{24}$-$4H_2O$	0.05mg
$FeSO_4x7H_2O$	0.15mg
$NaNO_2$	0.5g
$H_2O_{bidest-}$	ad 1000mL

Trace elements medium for enrichment of nitrifiers in a fed-batch experiment

(Maixner *et al.* 2006)

Trace elements medium with nitrite	
$NaNO_2$	448 mg
$NaHCO_3$	20 mg
KH_2PO_4	110 mg
$MgSO_4x7H_2O$	380 mg
$FeSO_4$ $x7H_2O$	7.2 mg
EDTA	10.3 mg
$CuSO_4x5H_2O$	0.5 mg
$ZnSO_4x7H_2O$	0.5 mg
$NaMoO_4x2H_2O$	0.5 mg
$H_2O_{bidest.}$	ad 1000ml

B.4.10 Solution for detection of nitrite consumption

Tüpfelreagenz	
ortho phosphoric acid	5mL
sulfanilamide	2g
H_2O_{bidest}	25mL

The components were mixed and 0.1g N-(1-Naphtyl-)ethylenediamine-dihydrochloride were added. Furthermore, the solution was filled up to its final volume of 50mL with H_2O_{bidest}. The solution was stored at 4°C in the dark.

B.5 RNA-based analyses

B.5.1 RNA isolation

RNA isolation is a method to isolate the whole RNA content (mRNA, tRNA, miRNA, siRNA) of cells in a sample. This opens the opportunity to study the gene expression of a defined sample. The expression of a gene of interest at the time point of sampling can be detected by reverse transcription PCR. During a reverse transcription PCR the isolated RNA is transcripted in complementary DNA (cDNA) by a virus enzyme, i.e. reverse transcriptase. The received cDNA can then be used as a template for amplifying a specific sequence via PCR and thus presence and absence of gene expression can be observed.

B.5.1.1 RNA isolation

After adding 1mL of Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) to 500µL activated sludge in a Lysing Matrix E tube the suspension was bead-beated for 45sec at speed 6.5. The lysate was incubated for 5min at room temperature (RT) and 0.2mL chloroform was added. After shaking the tubes by inverting for 1min the suspension was incubated for 2min at RT and centrifuged at 12,000 x g for 15min at 4°C. The colorless upper aqueous phase was transferred to a fresh tube. For precipitation of the RNA 0.5mL isopropyl alcohol was added to the aqueous phase and the samples were incubated at RT for 10min, followed by a centrifugation step (12,000 x g, 10min, 4°C). The RNA was washed with 1mL 75% ethanol and the suspension was centrifuged again (7,500 x g, 5min, 4°C). Finally, the pellet was air dried and the RNA was dissolved in 50 µl of RNAse-free $H_2O_{bidest./DEPC}$ by incubating for 10min at 50°C.

The purity of the RNA eluate and the presence of DNA contaminations respectively was tested by agarose gelelectrophoresis.

B.5.1.2 DNase digestion of isolated RNA

42.5µL RNA solution were mixed with 5µL 10x reaction buffer and 2.5µL DNase and incubated for 1h. After stopping the reaction by adding 5µl 25mM EDTA and incubation at 65°C for 10 min, the whole procedure from B.4.1.1, beginning with adding of 0,1mL chloroform was repeated with half of the volume of all solutions. After air-drying the RNA pellet the RNA was dissolved again in 50µL of RNAse-free $H_2O_{bidest./DEPC}$ by incubating for 10min at 50°C. 1µL of RNase inhibitor was added to avoid degradation of RNA and the sample was stored at -20°C until further usage.

B.5.2 cDNA synthesis by reverse transcription

The synthesis of cDNA was performed by using "RevertAid™ First Strand cDNA Synthesis Kit" (Fermentas, St. Leon-Rot, Germany) and was accomplished by following the manufacturers' manual. The enzyme RevertAid™ M-MuLV reverse transcriptase provided in this kit was isolated from Moloney Murine Leukemia Virus and has the ability to synthesize full-length cDNA up to13kb. Approximately 2µg RNA were used for reverse transcription by using gene specific reverse primer. The received cDNA was subsequently amplified by using the standard PCR protocol.

B.6 DNA-based analyses

B.6.1 DNA isolation

B.6.1.1 Genomic DNA isolation

Isolation of genomic DNA was performed by using PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., CA, USA) by following the manufacturers' manual. The last step of isolation of DNA with this kit includes the elution of DNA with 100µL $H_2O_{bidest.}$. The eluted DNA was then stored at -20°C until usage.

B.6.1.2 Plasmid DNA isolation (Miniprep)

Solutions

P1 buffer

NaOH/SDS solution

Potassium acetate/acetate solution

Procedure

The plasmid isolation is based on the principle of alkaline lysis, followed by organic precipitation of proteins and a terminal precipitation of DNA by isopropanol.

Overnight cultures of recombinant *E.coli* were harvested by centrifugation at 13,000 rpm for 1min. Afterwards the cell pellet was resuspended in 100µL P1 buffer and incubated at RT for 5min. For lysis of the cells 200µL NaOH/SDS solution were added and mixed with the cells by inverting the tubes several times. After incubation at 4°C for 5min all proteins were precipitated by adding 150µL potassium acetate/acetate solution. Additionally, the suspension was incubated for 5min at 4°C. To separate the DNA from all other cell components, like membrane fragments and precipitated proteins a centrifugation step were performed (13,000 rpm, 1min). The supernatant was transferred in a new ERT and 1 Vol. isopropanol was added, mixed and incubated for 10min at RT for precipitation of the plasmid DNA. After centrifugation for 1min at 13,000 rpm and discarding of the supernatant the DNA pellet was washed in 500µL ice-cold 70% ethanol, followed by a final centrifugation step (13,000 rpm, 1min). The supernatant was removed and the DNA pellet was dried on air. Finally, the DNA was dissolved in 50µL $H_2O_{bidest.}$ and all samples were stored at -20°C until further use.

B.6.1.3 Genomic DNA isolation by cooking the cell suspension

To isolate DNA from the enrichment of nitrite-oxidizing bacteria inoculated with dry grassland soil samples from the botanical garden of Vienna the cell suspension was cooked.

Procedure

1.5mL of the cell culture were centrifuged at 13,000 rpm for 8min. After discarding the supernatant the cell pellet was washed with 500µL $H_2O_{bidest.}$ and a centrifugation step (13,000rpm, 15min) was performed. Finally, the cells were eluated in 100µL $H_2O_{bidest.}$ and incubated at 95°C for 10min.

B.6.2 *In vitro* amplification of target DNA fragments by polymerase chain reaction

The polymerase chain reaction (PCR) is a technique to amplify DNA fragments with defined sequence by using a thermally stable DNA polymerase and cycles of denaturation of double strands, annealing of template and primer and elongation of the new strand. Since every new synthesized strand can be used as a template the amplification of DNA molecules is exponential during the PCR.

B.6.2.1 List of all used primers

Table B.6: List of all used primers

Primer	Sequence 5´-3´	Specificity	Ta [°C]	Product length	Reference
616V	AGA GTT TGA TYM TGG CTC	most *Bacteria*	56-54	1503bp	Juretschko *et al.*, 1998
1492R	GGY TAC CTT GTT ACG ACT T				Lane 1991
Arch21F	TTC CGG TTG ATC CYG CCG GA	most *Archaea*	56	1503bp	DeLong 1992
1492R	GGY TAC CTT GTT ACG ACT T				Lane 1991
616V	AGA GTT TGA TYM TGG CTC	*Nitrospira* cluster I	61	1437bp	Juretschko *et al.*, 1998
nt1431	TTG GCT TGG GCG ACT TC				Maixner *et al.* 2006
nxrBF706	AAG ACC TAY TTC AAC TGG TC	*nxrB Nitrobacter*/ *Nitrococcus*	56	724bp	Maixner, in preparation
nxrBR1431	CGC TCC ATC GGY GGA ACM AC				Maixner, in preparation
F19	TGG CAA CTG GGA CGG AAG ATG	*nxrB* Nitrospira	55-48	1239bp	Maixner, in preparation
R1237	GTA GAT CGG CTC TTC GAC CTG				Maixner, in preparation
nxrBF169	TAC ATG TGG TGG AAC A	*nxrB* genus *Nitrospira*	56	485 bp	Maixner, in preparation
nxrBR707	CGG TTC TGG TCR ATC A				Maixner, in preparation
Nxr3V	CGA GCG CAT ATG GAT AAA GTC CTC GCG	*nxrAB Nitrospira defluvii*	60	4877bp	this study
NxrR	TTA CGA GAA TTC CCC AGC CAG TTC ACG CGC TC				Maixner
NxrAR	TTA CGA GAA TTC CCC CGA CCT TGA TCT TGA TAT G	*nxrA Nitrospira defluvii*	62	3422bp	this study
F151	TGG GAR CGT GTG TAT CAC G	*nxrA*	47-61	663bp	this study
F151b	GT CAT CTC GAT CTC YTG CGT				this study
R831	CG TGC CAG GTR TAG TT				this study
F814	AAC TAY ACC TGG CAC GG	*nxrA*	53-61	1231bp	this study

R2062	TGG GAG CGC GTC TAT CAC G				this study
NxrA2L	GCC GTG CTC ATC GCG ACG	*nxrA*	62	272bp	this study
R831	CG TGC CAG GTR TAG TT				this study
NxrA1L	TTG ATC GTC ATC GGY ACT	*nxrA*	62	272bp	this study
R831	CG TGC CAG GTR TAG TT				this study

B.6.2.2 Amplification of target DNA fragments

Reagents

Reagents	Company
Magnesium chloride ($MgCl_2$)	(Fermentas Inc., Hannover, MD, USA)
Nucleotide mix (dNTP)	(Fermentas Inc., Hannover, MD, USA)
10 x Ex Taq polymerase buffer	(Fermentas Inc., Hannover, MD, USA)
Taq DNA polymerase	(Fermentas Inc., Hannover, MD, USA)
Bovine Serum Albumine (BSA)	(New England BioLabs Inc., Beverly, MA, USA)

Procedure

After cleaning the PCR hood (Ultraviolet Sterilizing PCR Workstation, Peqlab Biotechnology GmbH, Germany) with 3% H_2O_2 , the PCR hood was decontaminated by treating it and all opened pipette boxes with UV light for 15min. Simultaneously, all reagents except of the enzyme DNA-polymerase were thawed. After decontamination of the PCR hood all pipettes were wiped with 3% H_2O_2 and laid under the PCR hood. All following steps were performed under the PCR hood. The thawed reagents except of the DNA polymerase were vortexed briefly before they were added to master mix. After gentle mixing and aliquoting the master mixm, the thawed and mixed template was added to each approach, except the negative control. Positive controls were pipetted into the reaction mix outside of the PCR hood, to avoid cross-contaminations. Finally, the PCR was performed by application of an Icycler (Biorad, Munich, Germany) PCR cycler.

B.6.2.3 PCR reaction mix

Standard PCR reaction mix

Reagents	Volume (µL)	Final concentration	Stock concentration
$MgCl_2$	4	2mM	25mM
dNTP	5	2mM	0,2mM
Ex Taq polymerase buffer	5	1x	10x
Forward primer	1	1pmol/µL	50pmol/µL
Reverse Primer	1	1pmol/µL	50pmol/µL
Taq DNA polymerase	0.25	0.02 units/µL	5units/µL
Template DNA	1-3		
$H_2O_{bidest.}$.	ad 50		

PCR reaction mix for amplify DNA isolated from soil

Reagents	Volume (µL)	Final concentration	Stock concentration
$MgCl_2$	4	2mM	25mM
dNTP	5	2mM	0,2mM
Ex Taq polymerase buffer	5	1x	10x
Forward primer	1	1pmol/µL	50pmol/µL
Reverse Primer	1	1pmol/µL	50pmol/µL
BSA	0.25	0.05mg/mL	10mg/mL
Taq DNA polymerase	0.25	0.02 units/µL	5units/µL
Template DNA	1-3		
$H_2O_{bidest.}$	ad 50		

B.6.2.4 PCR programs

Standard PCR program

Table B.7: Standard PCR program

PCR-step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	35
Annealing[a]	46-65	30sec	
Elongation[b]	72	30sec - 100sec	
Final Elongation	72	10min	1

a) annealing temperature according to table B.6

b) elongation time depends on the size of the amplified DNA fragment. The rate of DNA synthesis by Taq DNA-polymerase is 1kp per 1min.

Table B.8: PCR program for amplification of *nxrB* fragment with F19/R1237 as primers

PCR-step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	35
Annealing	a	30sec	
Elongation	72	1min	
Final Elongation	72	10min	1

a) annealing temperature according to B.6

Table B.9: PCR program for amplification of 16sRNA genes with 21F/1492R 616V/1492R and 616V/Nt1431R as primers

PCR-step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	35
Annealing	a	30sec	
Elongation	72	1.5min	
Final Elongation	72	10min	1

a) annealing temperature according to B.6

Table B.10: PCR program for amplification of *nxrB* fragments of *Nitrospira* and *Nitrobacter* with 169F/707R and nxrBF706/nxrBR1431 as primers

PCR-step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	35
Annealing	a	30sec	
Elongation	72	30sec	
Final Elongation	72	10min	1

a) annealing temperature according to B.6

Table B.11: PCR program for amplification of *nxrA* fragments of *Nitrospira* with F814/R2062

PCR-step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	35
Annealing	a	30sec	
Elongation	72	2min	
Final Elongation	72	10min	1

a) annealing temperature according to B.6

Table B.12: PCR program for amplification of *nxrA* fragments of Nitrospira with F151mix/R831, NxrA1L/R831 and NxrA2L/R831 as primers

PCR-step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	35
Annealing	a	30sec	
Elongation	72	40sec	
Final Elongation	72	10min	1

a) annealing temperature according to B.6

B.6.2.5 High-fidelity PCR

High-fidelity PCR was performed by using high-fidelity reaction mix (Fermentas, St. Leon-Rot, Germany) by following the manufacturers' manual for a higher accuracy and a successful amplification of longer DNA fragments up to 10kp.

Table B.13: PCR program for amplification the *nxrAB* operon of *Nitrospira defluvii* with Nxr3V/NxrR as primers

PCR-step	Temp [°C]	Time	Number of cycles
Inital denaturation	95	3min	1
Denaturation	95	40sec	10
Annealing	60	40sec	
Elongation	68	6min	
Denaturation	95	40sec	25
Annealing	60	40sec	
Elongation	68	6min +10sec/cycle	
Final elongation	68	10min	1

Table B.14: PCR program for amplification the *nxrA* genes of *Nitrospira defluvii* with Nxr3V/NxrAR as primers

PCR-step	Temp [°C]	Time	Number of cycles
Inital denaturation	95	3min	1
Denaturation	95	40sec	10
Annealing	62	40sec	
Elongation	68	4min	
Denaturation	95	40sec	25
Annealing	62	40sec	
Elongation	68	4min +10sec/cycle	
Final elongation	68	10min	1

B.6.2.6 Gradient PCR

For evaluation of the optimal annealing temperature of new designed primer pairs it is necessary to perform a gradient PCR. This type of PCR program allows you to perform several PCR reactions in parallel under equal conditions except the annealing temperature, which differs in a defined range.

Table 15: Gradient PCR

PCR-step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	35
Annealing	a	30sec	
Elongation	72	30sec -100sec	
Final Elongation	72	10min	1

a) gradient of annealing temperatures ranged from 46°C-65°C

B.6.2.7 Addition of dATP-overhangs to PCR products

The existence of dATP-overhangs is essential for a successful insertion of the PCR product into Topo TA and Topo XL cloning® vectors. However, after longer storage of PCR products the adenosine-overhangs on both ends of the amplicons degrade and therefore the cloning efficiency is reduced.

Reagents	Volume (µL)	Final concentration	Stock concentration
$MgCl_2$	3	2,5mM	25mM
dATP	3	2,5µM	25µMl
Ex Taq polymerase buffer	3	1x	10x
Taq DNA polymerase	0.25	0.04units/µL	5units/µL
PCR product	20,75		

The thawed reagents were pipetted together and mixed gently. The addition of dATP-overhangs were performed at 72°C for 30min in Icycler (Biorad, Munich, Germany) PCR cycler.

B.6.3 Qualitative analysis of nucleic acids

B.6.3.1 Qualitative analysis and detection of DNA using agarose gelelectrophoresis

Horizontal agarose gelelectrophoresis is a tool for separation of DNA fragments by size. The size of the DNA fragment correlates with the migration distance of the negatively loaded phosphor backbone of the DNA in an electric field.

Solutions

1%-2% (w/v) agarose in 1xTBE buffer

loading dye

1kb DNA ladder

EtBr staining solution

Procedure

Depending on the size of the DNA fragments to be analyzed and type of analysis a 1%-2% (w/v) agarose gel was produced. Agarose was weighed, mixed with the corresponding Vol. of 1xTBE buffer and heated up in the microwave until agarose was melted completely. Subsequently, the solution was cooled to approximately 50°C and poured into a gel tray (Sub-Cell GT UV-Transparent Gel Tray (15x15cm), Biorad, Munich, Germany) with a comb to produce a defined number of pockets in the gel. After complete polymerization, the tray with gel was inserted into a gelelectrophoresis apparature (Sub-Cell GT, Biorad Muinch, Germany) and the comb was drawn out again. A 1:1 mixture of DNA sample and loading dye

was produced and pipetted into the pockets of the gel. A DNA ladder was applied on the outer pockets of the gel to determine the size of the DNA fragments of interest. Depending on the agarose concentration of the gel and type of analysis a separation of DNA fragments was done by a voltage 100V-130V for 50min and 80min. After separation the gel was put into the EtBr bath for staining for approximately 30min.

Nucleic acids visualization was done by placing the gel onto a transilluminator (Biostep GmbH, Jahnsdorf, Germany) emitting UV light (λ = 312 nm) and documented by digital photography using a gel documentation system (Biostep, Jahnsdorf, Germany).

B.6.3.2 Quantitative photometric analysis of nucleic acids

For photometric analysis of nucleic acids 1.5µL of DNA or RNA solution was pipetted onto the end of the fiber optic cable of a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). For determination of the concentration of nucleic acids the extinction at 260 nm with different extinction coefficients for RNA, double and single stranded DNA was used, following the Lambert Beer equation.

B.6.4 Cloning of gene amplificons

B.6.4.1 Classical cloning

Especially for heterologous expression the classical cloning approach is a necessary tool to provide insertion of specific DNA fragments into a pET system vector.

B.6.4.2 Double digestion of pET21b vector and insert

After amplification of the gene for subsequent heterologous expression with specific primers, which includes cutting sites of restriction enzymes, this DNA fragment and the pET21b vector were digested separately by two defined restriction enzymes (Fermentas Inc. Hannover, MD, USA).

Approach for pET21b vector	Approach for the gene of interest
2µL 10x reaction buffer O	2µL 10x reaction buffer O
1µL vector pET 21b (ca. 2µg)	1µL – 5µL DNA fragment (ca. 1µg)
0.5µL EcoRI restriction enzyme	0.5µL EcoRI restriction enzyme
0.5µL NdeI restriction enzyme	0.5µL NdeI restriction enzyme
ad 20µL $H_2O_{bidest.}$	ad 20µL $H_2O_{bidest.}$

These approaches were gently mixed and incubated at 37°C for 3h. Afterwards the solutions were incubated at 65°C for 20min to stop the digestion.

B.6.4.3 Ligation of pET21b vector and insert

Reaction mix for ligation:

Solution	Vol. [µL]
5x ligase reaction buffer	4µL
10mol ATP	1µL
digested vector DNA	2µL
digested insert DNA	3µL – 13µL
T4 DNA ligase (Invitrogen Corp., Carlsbad, CA, USA)	1µL
$H_2O_{bidest.}$	ad 20µL

After incubation at 4°C over night, a dialysis against $H_2O_{bidest.}$ was performed on nitrocellulose filters (0.025 µm, Ø 25 mm, MF Millipore, Millipore Corporation Billerica, MA, USA) for 20 min. This step stops the ligation reaction and removes the remaining salt from the solution.

B.6.4.4 Transformation of pET21b into electrocompetent cells by electroporation

B.6.4.4.1 Preparation of electrocompetent *E.coli* cells

750mL of dYT medium was inoculated with an over-night culture of the desired *E.coli* strain in a ratio of 1:1000 for preparation of electrocompetent cells. The culture was incubated at 37°C until the OD_{600} 0.5 to 0.7 was reached. All following steps were performed at 4°C with cooled solutions. The cells were stored at 4°C for 15min and harvested by centrifugation (5,000rpm, 15min, 4°C) in 50mL Greiner tubes. After washing the cells with ice-cold $H_2O_{bidest.}$ the resuspension was centrifuged (5,000rpm, 15min, 4°C) again. A second washing step was performed with 50mL 10% ice-cold glycerol, followed by centrifugation (5,000rpm, 15min, 4°C). After decantation of the supernatant the pellet was resuspended with approximately 1mL to 2mL 10% ice-cold glycerol, depending of the size of the cell pellet. This cell solution was then aliquoted a 100µL in screw tubes and stored at -80°C until further usage.

B.6.4.4.2 Transformation of electrocompetent cells by electroporation

1µL for retransformation of a vector or 5µL for transformation of a ligation solution were added to thawed electrocompetent cells and this solution was then gently stirred and pipetted into an ice-cold electroporation cuvette (0.2cm gap, Biorad, Munich, Germany). The cuvette was placed into the electroporator (MicroPulser, Biorad, Munich, Germany) and the transformation was performed at a voltage of 2.5 kV, capacity of 25 mF and resistance of 200 Ω for 4.5-5 msec.

After transformation, 250µL of SOC medium were added to the cells and the mixture was transfered into a screw cap. The solution was incubated at 37°C for 1h and subsequently 100µL, and 150µL of the cell suspension in case of new ligated vectors, were plated onto agar plates with antibiotic ampicillin. For retransformation 3µL and 10µL of the culture were used.

B.6.4.5 Cloning by using the TOPO TA and TOPO XL cloning kit

DNA received from environmental samples contains a mixture of various examples of the same gene. For successful sequencing it is necessary to isolate those different gene types. Cloning is the procedure for isolating and amplifying the single gene sequences and provides the possibility for successful Sanger sequencing.

B.6.4.6 Ligation

The TOPO TA and TOPO XL vectors are linear and contain single 3'thymidine overhangs, where the enzyme topoisomerase I is bound. This allows PCR products with dATP overhangs to ligate with the vector. The dATP overhang attacks the binding of the thymidine overhangs of vector with the enzyme topoisomerase I. This leads to the release of the enzyme, followed by the insertion of the PCR product and the circularization of the vector. The circularized vector can then be transformed into competent *E.coli* cells.

Procedure

Amplicons of the gene of interest were produced by PCR according to B6.2.2. PCR products which were stored in the fridge for more than one day were treated to add dATP overhangs (see B7.7)

A 2% agarose gel was made by dissolving low melting agarose (NuSieve® 3:1 Agarose low melting) in TAE buffer according to B6.3.1. After loading a DNA ladder into the outer pocket

of the polymerized gel the samples mixed with loading dye 1:1 were also pipetted into pockets and the gel was run at 100mA for 100min. Afterwards the gel was stained in SYBR® Green I staining solution for 30min. Nucleic acids visualization was performed by placing the gel onto a transilluminator (Biostep GmbH, Jahnsdorf, Germany) emitting UV light with a wavelength of 312 nm. The band with the predicted size was cut out by using a glass capillary and was blown out of the capillary with a yellow tip of a 200µL pipette in an ERT. Subsequently, the excised band was incubated at 70°C until the agarose was melted completely and 100µL $H_2O_{bidest.}$ were added to the melted solution and were mixed gently. The solution was incubated at 70°C for another 2min and 10µL were used for ligation.

Ligation mix for TOPO TA cloning kit

Solution	Vol [µL]	Company
PCR product	10	
TOPO TA vector	1.5	Invitrogen Corporation, Carlsbad,CA,USA
Salt solution	1.5	Invitrogen Corporation, Carlsbad,CA,USA

After ligation at room temperature for 20min the transformation was performed.

Ligation mix for TOPO XL cloning kit

Solution	Vol [µL]	Company
PCR product	10	
TOPO XL vector	1.5	Invitrogen Corporation, Carlsbad,CA,USA

After ligation at room temperature for 5min 1.5µL stop solution was added to the ligation mix and the transformation was performed.

B.6.4.7 Transformation

10µL (TOPO XL) or 13µL (TOPO TA) of ligation mix were added gently to thawed chemically competent *E.coli* TOP10 cells. After incubation of the transformation mixture for 30min at 4°C, a heat-shock for 30sec at 42°C was performed for transformation of vectors into the competent cells. Afterwards the reaction was incubated on ice immediately for 2min. 250µL of thawed and 20°C warm SOC medium was pipetted gently onto the transformed

cells. After incubation of the cell culture for 1h at 37°C horizontally on a shaker 100µL and 150µL of the suspension were plated onto LB-kan plates, where 40µL X-gal were plated out before for blue/white screening. The plates were incubated over night at 37°C. On the following day white colonies were picked and screened for right insertion.

B.6.5 Insert screening via PCR to identify positive clones

A PCR reaction mix was performed according to B.6.2.2. After picking *E.coli* colonies with a tooth stick, they were struck out on a master plate. The residual cells on the tooth stick were suspended in PCR reaction mix and the TOPO vector DNA inside the cells served as template for the PCR reaction. The primers bind to the flanking regions of the insert site on the vector and thus the insert independent of its sequence was amplified and the insert size could be estimated.

Reaction mix for insert screening PCR

Reagents	**Volume (µL)**	**Final concentration**	**Stock concentration**
$MgCl_2$	4	2mM	25mM
dNTP	5	2mM	0.2mM
Ex Taq polymerase-buffer	5	1x	10x
Forward primer	1	1pM/µL	50pM/µL
Reverse Primer	1	1pM/µL	50pM/µL
Taq DNA-polymerase	0.25	0.02 units/µL	5units/µL
$H_2O_{bidest.}$	ad 50		

Table B.16: List of primers for insert screening

Primer	**Sequence 5´-3´**	**Specificity**	**Ta [°C]**
M13F	GTA AAA CGA CGG CCA G	TOPO vector	60
M13R	CAG GAA ACA GCT ATG AC		
T7V	TAA TAC GAC TCA CTA TAG GG	pET21b(+)	56
T7R	GCT AGT TAT TGC TCA GCG G		

Table B.17: PCR program for insert screening

PCR step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	35
Annealing[a]	56-60	30sec	
Elongation[b]	72	30sec-4min	
Final Elongation	72	10min	1

a) annealing temperature according to B.16
b) elongation time depends on the size of the amplified DNA fragment. The rate of DNA synthesis by Taq DNA polymerase is 1kbp per 1min.

PCR products were analyzed via gelelectrophoresis according to Sec. B.6.3.1 and clones with the right insert length were used for further analyses.

B.6.6 Restriction fragment length polymorphism (RFLP)

RFLP is a method to determine the sequence diversity of a clone library without sequencing. A defined restriction enzyme cuts the gene of interest into fragments of different length. Due to point mutations a phylogenetic group gains or lacks restriction endonuclease cutting sites of this specific restriction enzyme, which leads to a different pattern of DNA fragments after agarose gelelectrophoresis. Consequently the diversity of a clone library can be estimated.

Table 18: List of all used restriction enzymes

Enzyme name	Cutting site	Buffer	Company
MspI	CC^GG	Tango	Fermentas Life Sciences Inc., Hannover, MD, USA

Procedure:

Solution	Vol [µL]	Final concentration	Stock concentration
Buffer Tango	1	1x	10x
$H_2O_{bidest.}$	3		
Enzyme MspI	1	1U/µL	10U/µL
PCR product	5		

All solutions are pipetted together and mixed gently. After incubation of the approach for 3h at 37°C the reaction is stopped by adding of 10µL loading dye which contains EDTA to inactivate the restriction enzyme. This mixture was then loaded onto a 2%-agarose gelelectrophroesis and run for 90min at 100V.

B.6.7 Sequencing

PCR products inserted into TOPO TA/TOPO XL vectors were sequenced by using the DNA Sequencer Applied Biosystems 3130 following the instruction manual. This technique is based on the chain-termination method, developed by Fredrick Sanger (Sanger *et al.* 1977). Therefore a PCR is performed with dNTPs as well as fluorescently labeled dideoxynucleotide triphosphates (ddNTPs), the four different ddNTPs are labelled with four different dyes in the reaction mix. Due to the lack of the 3'OH group of ddNTPS the elongation of a strand stops, when a ddNTP is incorporated. Statistically, an incorporation of a ddNTP occurs at every position of the sequence and therefore the sum of all fragments results in the whole sequence of the DNA molecule.

The synthesized fragments are separated electrophoretically and the colors of the ddNTPs are distinguished by a detector. Subsequently, the chromatogram of the detected colors can be used to determine the nucleotide sequence of the DNA molecule of interest.

Table B.19: List of the sequencing primers

Primer	Sequence 5´-3´	Ta [°C]
TopoSeq-F	AGC TTG GTA CCG AGC T	60
TopoSeq-R	GTA AAA CGA CGG CCA GT	

B.7 DNA sequence analyses

B.7.1 Proofreading of sequences by using chromasPro

The software program chromasPro (http://www.technelysium.com.au/chromas.html) is a tool used to illustrate the chromatograms achieved by Sanger sequencing, and to find and correct possible mistakes in the sequence generated automatically. Every sequence was proofread by using chromasPro.

B.7.2 Rapid sequence analyses by BLAST

BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) is an online tool used to compare sequences that result from Sanger sequencing with the NCBI database. By using BLAST, rapid taxonomic affiliation of the sequences of interest can be achieved.

The program compares the sequence of interest to the database to identify the most similar sequences in the dataset and ranks them according to their relevance.

B.7.3 Sequence analyses with the software program ARB

The software program ARB includes different tools for sequence database maintenance and sequence analyses, like sequence alignment, filter calculation, phylogenetic analyses and the possibility to design probes and primers.

B.7.3.1 Sequence alignment using the software program ARB

The proofread sequences achieved by Sanger sequencing were imported into an ARB database as a FASTA file. These sequences were then aligned automatically by use of the fast aligner tool of the editor. Afterwards the alignment was checked and if necessary corrected manually.

Protein-coding gene sequences, like *nxrA* sequences were additionally translated *in silico* into amino-acid sequences, which were then aligned again.

B.7.3.2 Phylogenetic analyses

To reconstruct the phylogeny of the investigated bacteria, the received 16S rRNA and functional protein sequences were calculated into phylogenetic trees using the software ARB (Ludwig *et al.* 2004). For calculation of phylogenetic trees conservation filters were used to exclude highly variable sequences positions and to cut off the primer sequences. Different calculation methods, like neighbor joining, maximum likelihood, maximum parsimony, treepuzzle and fitch were used to ensure a correct tree topology.

B.8 Fluorescence *in situ* hybridization (FISH)

FISH is a culture-independent method, which provides the possibility to identify the microbial community of complex environmental samples. Fluorescently labeled oligonucleotides are used to bind to specific regions of the ribosomal RNA (rRNA) and so it is possible to identify them at different taxonomic levels. Thus, the microbial diversity of an environment sample

can be estimated. FISH involves several steps, starting by fixation and immobilization of the cells, followed by hybridization of oligonucleotide probes to rRNA under stringent conditions. Finally, the detection of fluorescently labeled cells is performed by using epifluorescence microscopy.

B.8.1 Cell fixation

B.8.1.1 Cell fixation with paraformaldehyde (PFA)

Three volumes of 4% paraformaldehyde solution were gently mixed with one volume settled activated sludge and incubated at 4°C for 4h. To remove the 4% paraformaldehyde solution the sample was centrifuged at 13,000 rpm for 15min and after disposal of the supernatant the pellet was washed with 1xPBS. Subsequently, the sample was centrifuged again at 13,000 rpm for 15min. The pellet was then resuspended in one volume of 1xPBS and one volume of $ethanol_{abs.}$. All fixed samples were stored at -20°C.

B.8.1.2 Cell fixation with ethanol

One volume of $ethanol_{abs.}$ was added to one volume of settle activated sludge and fixed samples were stored at -20°C.

B.8.2 Fluorescence *in situ* hybridization (FISH)

B.8.2.1 List of all used 16S rRNA probes

Table B.20: List of all used 16S rRNA probes

Primer	Sequence 5' – 3'	Specificity	FA %	Reference
EUB I	GCT GCC TCC CGT AGG AGT	most *Bacteria*	0-50	Amann *et al.* 1990
EUB II	GCA GCC ACC CGT AGG TGT	*Planctomycetales*	0-50	Daims *et al.* 1999
EUB III	GCT GCC ACC CGT AGG TGT	*Verrucomicrobiales*	0-50	Daims *et al.* 1999
Ntspa-662	GGA ATT CCG CGC TCC TCT	genus *Nitrospira*	35	Daims *et al.* 2001
Ntspa-662 competitor	GGA ATT CCG CTC TCC TCT		35	Daims *et al.* 2001
Ntspa-1431	TTG GCT TGG GCG ACT TCA	sublineage I of the genus *Nitrospira*	35	Maixner *et al.* 2006

Ntspa-1151	TTC TCC TGG GCA GTC TCT CC	sublineage II of the genus *Nitrospira*	35-40	Maixner *et al.* 2006

B.8.2.2 Cell immobilization

One or two doses of 10µL of fixed activated sludge, depending on cell density of the respective sample, were pipetted onto a 10-wells slide and dried in the hybridization oven at 46°C for approximately 10min until all liquid parts were removed completely.

B.8.2.3 Dehydration of the sample

After immobilization of the cells on the slide, an ascending ethanol series was performed to dehydrogenize the sample. The slide was put successively into 50%, 80% and 96% ethanol solutions for 3 min per ethanol concentration.

B.8.2.4 In situ hybridization

Solutions

5M NaCl

1M Tris/HCl pH 8.0

0.5M EDTA pH 8.0

10% (w/v) SDS

Formamide (FA)

Hybridization buffer (46°C):

FA (conc.)	0	5	10	20	25	30	35	40	50	55	70
5M NaCl [µL]	180	180	180	180	180	180	180	180	180	180	180
1M Tris/HCl [µL]	20	20	20	20	20	20	20	20	20	20	20
MQ [µL]	800	750	700	600	550	500	350	400	300	250	100
FA [µL]	0	50	100	200	250	300	350	400	500	550	700
10% SDS [µL]	1	1	1	1	1	1	1	1	1	1	1

Washing buffer:

FA (conc.)	0	5	10	20	25	30	35	40	50	55
5M NaCl [mL]	9	6.3	4.5	2.15	1.49	1.02	0.7	0.46	0.18	0.1
1M Tris/HCl [mL]	1	1	1	1	1	1	1	1	1	1
0,5M EDTA [mL]	0	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MQ [mL]	ad 50mL	ad 50mL	ad 50mL	ad 50mL	ad 50mL	ad 50mL	ad 50mL	ad 50mL	ad 50mL	ad 50mL
10% SDS [mL]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05

9µL of hybridization buffer were pipetted onto the cells, 1µL of respective probe was added, then mixed with the pipette tip without scratching the cell layer. The remaining hybridization buffer was poured in a 50mL Greiner tube having a paper tissue inside, that served as a hybridization chamber. The slide was put into the hybridization chamber and was incubated at 46°C in the hybridization oven for 1.5-2h.

B.8.2.5 Washing of the hybridized samples

After hybridization, the slide was transferred into a 50mL Greiner tube with pre-warmed (48°C) washing buffer and incubated at 48°C for 10min in a waterbath. Afterwards, the slide was dipped into ice-cold $H_2O_{bidest.}$ for a few seconds and dried with compressed air. All slides were stored at -20°C in dark for further microscopic analysis.

B.8.3 Liquid fluorescence *in situ* hybridization

B.8.3.1 Dehydration of the sample

500µL of ethanol-fixated sample were centrifuged for 2min for 13,000rpm. After discarding the supernatant, an increasing ethanol series was performed to dehydrate the cells. Therefore, the sample was incubated for 3min with 500µL 80% ethanol, centrifuged for 2min and then the pellet was resuspended in 500µL 100% ethanol and incubated for 3min.

Afterwards, the ethanol series the cells were washed in 1xPBS.

B.8.3.2 In situ hybridization

For hybridisation, 200µL of hybridisation buffer with probe-dependent FA concentration was mixed with 10µL respective probe and added to the samples. This mixture was incubated for 2h at 46°C in the hybridisation oven.

B.8.3.3 Washing of the hybridized samples

For removal of the hybridisation buffer, the sample was centrifuged at 13,000rpm for 2min and the pellet was resuspended and washed with pre-warmed washing buffer for 10min at 48°C. After a final centrifugation step (13,000rpm, 2min) the approach was incorporated in 100µL 1xPBS. All samples were stored at 4°C for further use.

B.9 Protein-based analyses

B.9.1 Heterologous expression

B.9.1.1 List of primers for heterologous expression

Table B.21: List of all used primers for heterologous expression

Primer	Sequence 5' – 3'	Specificity	Tm	Comment
Nxr3V	CGA GCG CA^T ATG GAT AAA GTC CTC GCG	*Nitrospira defluvii nxrA/nxrAB* without signal peptide sequence	60-62	5‘ CA^T ATG 3‘ is the recognition site of the restriction enzyme NdeI
NxrR	TTA CGA G^AA TTC CCC AGC CAG TTC ACG CGC TC	*Nitrospira defluvii nxrAB* without signal peptide sequence	60	5' G^AA TTC 3' is the recognition site of the restriction enzyme EcoRI
NxrAR	TTA CGA G^AA TTC CCC CGA CCT TGA TCT TGA TAT G	*Nitrospira defluvii nxrA* without signal peptide sequence	62	5' G^AA TTC 3' is the recognition site of the restriction enzyme EcoRI

^Cutting site of the restriction enzyme

B.9.1.2 List of all used *E.coli* strains for heterologous expression

Table B.22: List of all used *E.coli* strains for heterologous expression

Strain	Company	Genotype	Reference
E.coli XL1 Blue	Stratagene	F'[Tn10, *pro*AB$^+$, *lac*I^q Δ(*lac*Z)M15], *end*A, *gyr*A96(nal^R), *thi*, *rec*A1, *rel*A1, *lac*, *gln*V44 *hsd*R17(r_K^- m_K^+)	Bullock 1987
E.coli BL21 (DE3)	Stratagene	F$^-$, *ompT*, *gal*, *dcm*, *hsd*S$_B$(r_B^- m_B^-), λ(DE3 [*lac*I lacUV5-T7])	Weiner M.P. 1994
E.coli C43 (DE3)	Avidis	F-, *omp*T, *lon*, *hsd*SB(r_B^-), *dcm*, *gal*, λ(DE3[*lac*I lacUV5-T7]) pLysS	Miroux *et al.* 1996
E.coli JM109 (DE3)	Promega	F' [*tra*D36, *pro*AB$^+$, *lac*I^q *lac*ZΔM15], *end*A, *thi*, *rel*A1, *sup*E44, *gyr*A96, *rec*A1, Δ(lac-proAB), *hsd*R17(r_K^- m_K^+), λ(DE3[*lac*I lacUV5-T7])	Yanisch-Perron *et al.* 1985
E.coli Tuner (DE3)	Novagen	F$^-$, *gal, omp*T, *dcm*, pLacI (Cam^R), *hsd*S$_B$ (r_B^- m_B^-), *lacY1*(DE3[*lac*I lacUV5-T7])	Khlebnikov *et al.* 2002

B.9.1.3 Expression of Nitrite oxidoreductase of *Nitrospira defluvii*

Procedure

After successful transformation of pET21b+ into an *E.coli* expression strain with the inserted gene of interest, the expression was tested. For expression of the gene of interest into one of the listed *E.coli* expression strains, a 5mL liquid LB-medium with ampicillin was inoculated with an *E.coli* colony and incubated over night at 37°C on a shaker. This overnight culture served as an inoculum for new cultures. 1/100 Vol. of overnight culture was mixed with 1 Vol. LB-medium with ampicillin. For good aeration, LB-medium should make up only 20% maximal of the total flask volume. After incubation for at 37°C 2h on a shaker, a defined amount of IPTG was added to one of the cultures for induction of the heterologous expression, while the other culture kept uninduced and served as a negative control. After another 4h to 6h (depending on the incubation temperature, which varied from 37°C to 19°C), the cells were harvested by centrifugation at 10,000rpm for 15min. After washing in 1xPBS an additional centrifugation step (10,000rpm, 15min) was performed, the supernatant was discarded and the cells were stored at -20°C for further use.

B.9.1.4 Detection of formation of inclusion bodies

Inclusion bodies are insoluble aggregates of non-native proteins and they represent one of the most common problems during heterologous expression. The inclusion bodies almost exclusively consist of the protein of interest which can constitute up to 30% of the total protein content of the cell. Yet, those inclusion bodies have a main disadvantage: Inside, the protein of interest is not folded properly and therefore an analysis to determine formation of inclusion bodies is essential for design of further experiments.

Solutions

Binding buffer

Procedure

The cell pellet was resuspended in 500µL to 1mL of binding buffer, this mixture was frozen and thawed again three times. After the last thaw step, lysozyme (1mg/µL) was added to suspension for enzymatic lysis of the cells and incubated for 1h at 37°C, followed by sonification (0.9sec pulse) of the cells at 60% for 5min on ice.

To differentiate between insolubility of the protein of interest and formation of inclusion bodies, the solution was split and one aliquot was mixed with 8M urea for denaturation of all proteins, followed by centrifugation for 30min at 13,000rpm at 4°C. A part of the pellet was resuspended in 100µL 1xPBS, then this solution and the supernatant were used for further analysis of a possible formation of inclusion bodies.

B.9.2 Separation of proteins by protein gel-electrophoresis

B.9.2.1 Protein denaturation

Solution

4x SDS PAGE loading dye

Procedure

For further analysis of the heterologous expressed proteins, the cells were resupended in 50µL 4xSDS PAGE loading dye. Afterwards, 1µL to 2µL benzonase nuclease (depending on the viscosity of the sample) were added and the suspension was incubated 1h at 4°C. Then the protein sample was cooked at 95°C for 5min and was immediately used for loading onto the SDS PAGE.

B.9.2.2 Sodium-dodecyl-sulfate-polyacrylamide gelelectrophoresis (SDS PAGE)

Solutions

10% Ammoniumperoxodisulfit (APS)

SDS PAGE lower buffer

SDS PAGE upper buffer

Coomassie staining solution

Destaining solution

Protein ladder

Procedure

The lower gel was filled into the gel casting chamber (Biorad, Munich, Germany) for polymerization with a layer of isopropanol on top. After polymerization, the isopropanol was removed and the upper gel was pipetted on the top of the lower gel. The comb was stuck into lower gel before polymerization. After complete polymerization, the gel was transferred into the gelelectrophoresis chamber (Mini Protean 3 Cell), which was filled with 1xSDS running buffer. The prepared samples were loaded into the slots of the gel and marker was added in the slots on both ends of the gel. A separation of denatured proteins took place by a voltage 80V for 90min. After separation the gel was put into the Coomassie staining solution for staining for approximately 1h. The gel was then put into the destaining solution to minimize the background. Afterwards, the gel was scanned by Epson scanner for digitalization.

B.10 Sampling at Waste water treatment plants

Activated-sludge samples from three different types of WWTPs were used for further analyses. The investigated plants differ remarkably in their design, source of sewage and their size. Main features of the different WWTPs are summarized in table.B.23.

Table B.23: Summary of the main features of the analyzed WWTPs

WWTP	Location	Wastewater type	Reactor type
main WWTP Vienna	Vienna	municipal and industrial	full-scale wwtp
WWTP University of veterinary medicine	Vienna	predominantly excrements from animals	primary clarifier
WWTP Ingolstadt	Bavaria	industrial	SBR with trickling filter

The activated-sludge samples from the main WWTP of Vienna and the WWTP of the University of veterinary medicine of Vienna were filled in a flask and stored at 4°C until transport, except the samples for the RNA isolation. These samples were pipetted in ERT and put in liquid nitrogen to avoid RNA degradation.

The activated-sludge samples from the WWTP of Ingolstadt were collected by our cooperation partner Mr.Herbert Kohler. For each sampling time point, 3 aliquots of settled activated sludge were filled in Greiner tubes for further analyses, like PFA and EtOH fixation for FISH and DNA-isolation.

B.10.1 Main WWTP of Vienna (HKA)

The main WWTP of Vienna in Simmering consists of mechanical, chemical and biological sewage purification steps. Nitrogen removal is performed in fifteen aeration tanks by simultaneous nitrification and denitrification. Furthermore, chemical phosphorous precipitation is accomplished by adding iron(III) sulfate into the aeration tanks.

The activated-sludge samples were collected in the aerobic zone of an aeration tank of the second biological purification stage. The aeration tanks are designed for simultaneous nitrification and denitrification with alternating aerobic and anaerobic zones. (Website of the main WWTP of Vienna: http://www.ebs.co.at/)

B.10.2 WWTP of the University of Veterinary Medicine of Vienna

The WWTP of the University of Veterinary Medicine of Vienna is the smallest WWTP investigated. It comprises mechanical and biological "pre-sewage purification" and consists of two aeration tanks. The effluent of this WWTP flows into the sewer system and finally to the main WWTP of Vienna.

The activated-sludge samples were taken from the aeration tank under aerated conditions.

B.10.3 WWTP of Ingolstadt (Zentralkläranlage Ingolstadt)

Similar to the main WWTP of Vienna, the WWTP of Ingolstadt combines mechanical, chemical and biological sewage-purification treatments. A major difference is the use of trickling filters with a pre-denitrification step. Also, further sludge treatment differs between these two WWTPs. The WWTP in Ingolstadt consists of three sludge digestors and three

centrifuges, where both the amount of organic matters and the water content are reduced. Finally, the sludge is dried and can be used for energy production.

The actived sludge was sampled in a tank by our cooperation partner Mr.Herbert Kohler, where nitrogen removal from the liquid form sludge dewatering takes place.

B.11 Fed-batch experiment for enrichment of *Nitrospira* sublineage II

For analysis of the influence of low nitrite concentration and discontinuous aeration conditions on *Nitrospira* sublineage I and II, an incubation experiment was set up in laboratory scale (Fig B.1).

B.11.1 Design of fed-batch experiment

Solutions

Trace elements medium for enrichment of nitrifiers in a fed batch experiment

Tüpfelreagenz

Procedure

Samples of activated sludge were incubated in parallel from two different WWTPs, "Zentralkläranlage Ingolstadt" (Germany) and the WWTP of the University of Veterinary Medicine of Vienna. For each WWTP, the activated sludge settled down, then the supernatant was disposed. Afterwards, buffered mineral medium for enrichment of nitrifiers was used for filling the sludge up to 700mL for each WWTP sample and then poured in two 1L flasks respectively. These flasks were kept in dark and at room temperature. Every hour, 0.5mL stock solution was pumped into all approaches to gain a final concentration of 0.1mg/L. Volume was reduced to 700mL every 2 weeks by decanting the supernatant after settling of the sludge. Additionally, for the first 60 days air was constantly pumped into all flasks. Then aeration conditions were changed to explore the effect of different aeration conditions during incubation. A procedure of interrupted aeration with an aeration interval of 10min air exposure, interrupted by 15min, was performed for 49 days. Every week, samples from all incubations were taken and used to perform PFA fixation and ethanol fixation of the cells to detect possible changes of the community by FISH analysis. Consumption of nitrite and

changes in pH value were checked weekly. When pH dropped under 7, 4mL of 875mM $NaHCO_3$ buffer were used to adjust the pH value to 7.7 to 7.5.

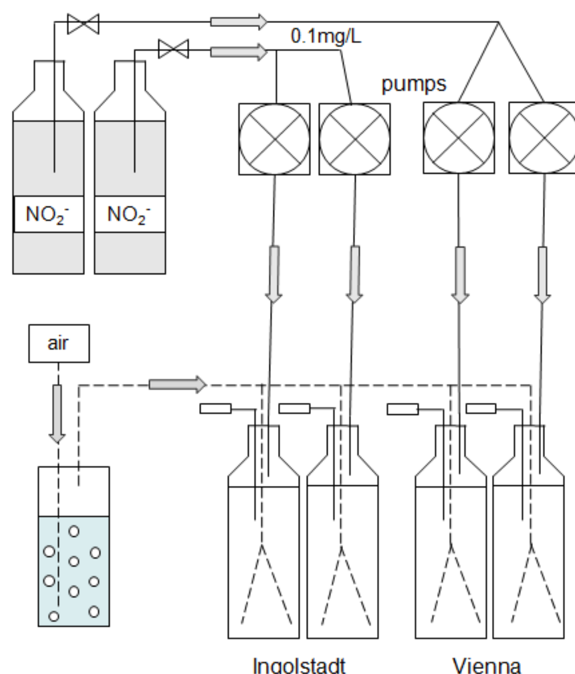


Figure B.1 : Schematic design of incubation of nitrite-oxidizing bacteria from activated-sludge samples

B.12 Enrichment of nitrite-oxidizing bacteria from a soil sample

Solution

Mineral salt medium for enrichment of nitrifiers from soil samples

Procedure

According to Bartosch *et al.* 2002, 1.5g of dry grassland soil were incubated in 4 300mL Erlenmeyer flaks in mineral salt medium with a nitrite concentration of 0.2g $NaNO_2$/L at 30°C in the dark. The consumption of nitrite was periodically tested with nitrite test stripes and 0.1mL of 5M $NaNO_2$ solution were added to regain a final concentration of 0.2g $NaNO_2$/L in case all nitrite was consumed. These steps were performed under a flame to avoid contamination of the enrichment culture.

Because the produced nitrate accumulated, two of the cultures were splitted and diluted to 1:1 and 1:10 respectively.

B.13 Micromanipulation of FISH-labeled cells

B.13.1 Sorting of activated sludge flocs by micromanipulation

Liquid FISH was performed with ethanol-fixed activated sludge samples and the cells were hybridized with the probe Ntspa1151 specific for members of the sublineage II of the genus *Nitrospira*.

After washing of the hybridized cells, the liquid FISH samples were diluted to 2-10fold depending on the concentration of activated sludge. Afterwards, approximately 70µL of the sample were pipetted onto a glass slide and single activated-sludge flocs were transferred in 30µL PCR $H_2O_{bidest.}$. Subsequently a PCR reaction was performed for amplification of the 16S rRNA genes of the bacteria inside the flocs.

Table B.24: PCR reaction for amplify all bacterial 16S rRNA genes inside the activated sludge flocs

PCR-step	Temp [°C]	Time	Number of cycles
Denaturation	95	5min	1
Denaturation	95	40sec	30-35[a]
Annealing	54	30sec	
Elongation	72	1.5min	
Final Elongation	72	10min	1

a) 30 cycles for amplification of 16S rRNA genes of Bacteria within the activated-sludge floc of the WWTP of the University of Veterinary Medicine of Vienna and 35 cycles when an activated-sludge floc of the WWTP of Ingolstadt was used as template.

C. Results

C.1 Heterologous expression of the *nxrAB* operon of *N. defluvii* in *E.coli*

For further characterization of the nitrite oxidoreductase, heterologous expression of the *nxrAB* operon of *Candidatus* Nitrospira defluvii was performed in *E.coli*. Table C.1 shows characteristic features of these two proteins, encoded by the *nxrAB* operon.

Table C.1: Characterization of the putative NxrA and NxrB proteins of *N.defluvii*

operon	DNA fragment (bp)	Protein (AA)	Protein (kDa)	Signal peptide prediction[a]
nxrAB	4877	NxrA: 1127	129	+
		NxrB: 430	50	-

[a] Signal peptide prediction by using the software SignalP 3.0

A primer pair Nxr3V/NxrR for amplifying the *nxrAB* operon of *Nitrospira defluvii* was designed, including the restriction enzymes cutting sites for the classical cloning approach and without the signal peptide of sequence of *nxrA* according to signal peptide prediction tool (SignalP 3.0).

To check for successful insertion of the potential *nxrAB* operon, the *nxrAB*-pET21b construct was transformed into *E.coli* XL1 blue by electroporation and screened according to Sec. B.6.5..

The expression of the inserted pET21b construct was tested by incubation of defined expression strains under different growth conditions and expression was induced with different concentrations of IPTG. After harvesting the cells, a SDS page was used for detection and visualization of heterologous expression. All results of heterologous expression in different *E.coli* strains and at different condition of growth and induction are summarized in table C.2. Figure C.1 shows the SDS page of *E.coli* tuner (DE3) samples, as an example for the highest expression level of the *nxrAB* operon.

Table C.2: Summary of all results of heterologous expression of *nxrAB* operon using different *E.coli* expression strains, various growth conditions and IPTG concentrations

Strain (*E.coli*)	**Temperature (°C)**	**Time of growth after IPTG (h)**	**IPTG**	**Expression**
JM109 (DE3)	37	4	1mM	----[a]
JM109 (DE3)	RT	4	1mM	----[c]
BL21 (DE3)	37	4	1mM	---+[c]
BL21 (DE3)	37	4	0.5mM	---+ ICB[b]
BL21 (DE3)	37	4	0.1mM	----[c]
BL21 (DE3)	37	6	0.5mM	---+[c]
BL21 (DE3)	RT	4	1mM	---+[c]
C43 (DE3)	37	4	0.2mM	---+[c]
C43 (DE3)	37	4	0.5mM	---+[c]
Tuner (DE3)	37	4	0.2mM	--++
Tuner (DE3)	37	4	0.5mM	--++[c]
Tuner (DE3)	RT	5	0.2mM	-+++
Tuner (DE3)	RT	12	0.2mM	-+++ ICB
Tuner (DE3)	**19**	**6**	**0.2mM**	**-+++ ICB**

a) Expression level categorized according to the amount of produced protein: maximal expression level: ++++; high expression level:-+++; average expression level: --++; low expression level: ---+; no expression: ----

b) formation of protein aggregations, protein in inclusion bodies (ICB)

c) formation of protein aggregations was not tested

bold: highest expression level observed in all performed expression experiments

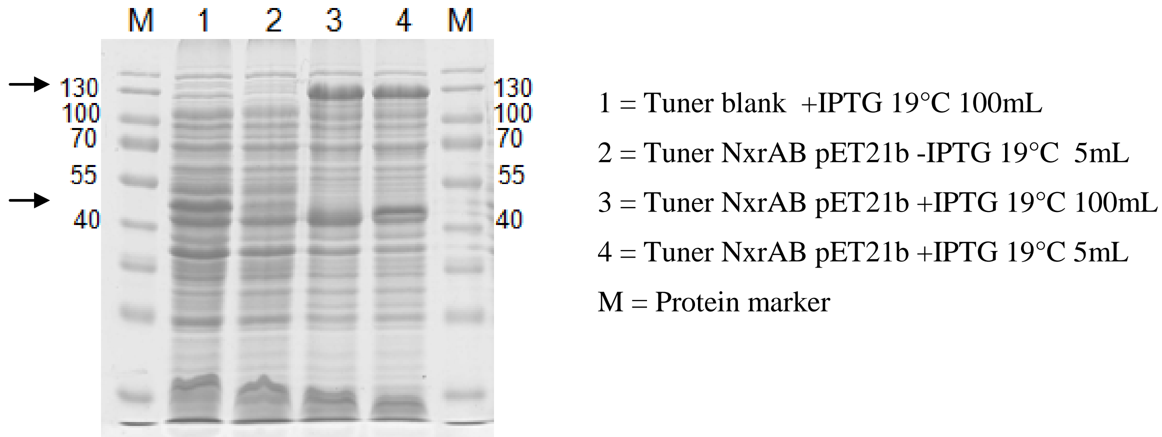


Figure C.1: 12% SDS page of *E.coli* tuner (DE3) with and without pET21b with *nxrAB* as insert; Tuner blank: *E.coli* tuner (DE3) without pET21b *nxrAB*; predicted size of NxrA: 129kDa and NxrB: 50kDa

Furthermore, solubility of the NxrA and NxrB protein and possible formation of inclusion bodies were tested by centrifugation and treatment with urea according to B.9.1.4. After lysis of the cell culture and centrifugation of the lysate, soluble proteins can be found in the supernatant, whereas insoluble proteins and inclusion bodies form part of the pellet. NxrA and NxrB could be detected in the pellet after centrifugation (fig.C.2, lane 4) indicating the formation of inclusion bodies. Furthermore, after treatment of the samples with 8M urea, a fraction of NxrA and NxrB became soluble (fig.C.2, lane 5), while the other part remained in the pellet forming protein aggregates (fig.C.2, lane 6).

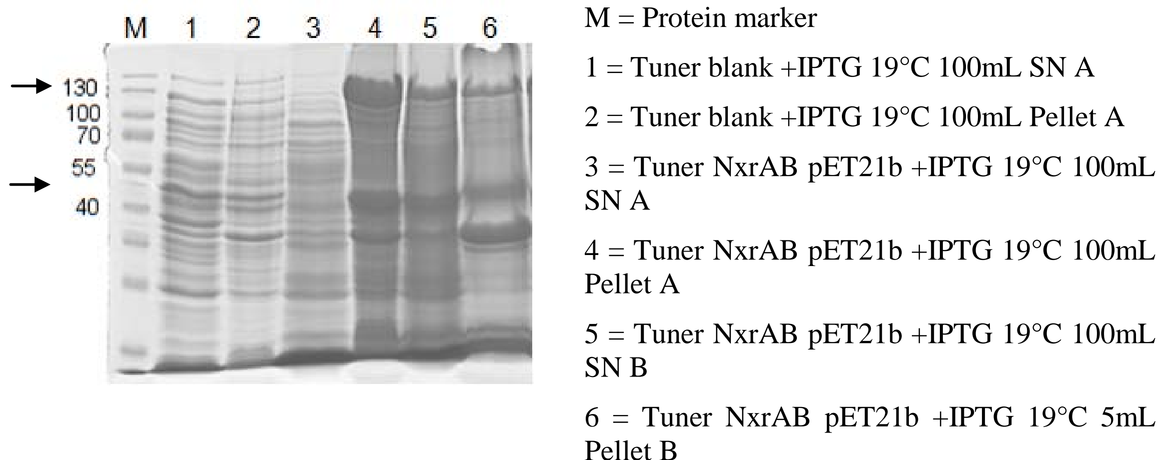


Figure C.2: 12% SDS page of *E.coli* tuner (DE3) with pET21b with *nxrAB* as insert; A: lysate in binding buffer B: lysate in binding buffer with 8M urea SN:supernatant; Tuner blank: *E.coli* tuner (DE3) without pET21b *nxrAB*; predicted size of NxrA: 129kDa and NxrB: 50kDa

C.2 Heterologous expression of the *nxrA* gene of *N.defluvii* in *E.coli*

Due to formation of inclusion bodies during the heterologous expression of *nxrAB* operon of *Candidatus* Nitrospira defluvii in *E.coli*, a heterologous expression experiment of the *nxrA* gene alone inserted into the expression vector pET21b(+) was performed. In table C.3 a summary of the significant data of *nxrA* and its gene product is listed.

Table C.3: Characterization of the putative NxrA protein of *N.defluvii*

Gene	DNA fragment (bp)	Protein (AA)	Protein (kDa)	Signal peptide prediction[a]
nxrA	3422	1127	129	+

[a] Signal peptide prediction by using the software SignalP 3.0

C.2.1 Amplification, cloning and insertion of *nxrA* into the expression vector

A primer pair (Nxr3V/NxrAR) for amplification of the *nxrA* gene of *Candidatus* Nitrospira defluvii was designed and the optimal annealing temperature of this primer pair was evaluated by gradient PCR (fig.C.3). An annealing temperature of 62.3°C was chosen, due to the occurrence of a band with the right size and the absence of any other band.

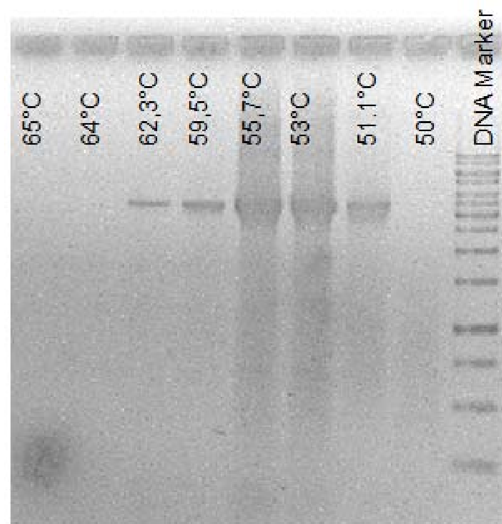


Figure C.3: 1%-agarose gelelectrophoresis of the gradient PCR for evaluation the optimal annealing temperature of the primer pair Nxr3V/NxrAR for amplification of the *nxrA* gene; predicted size of *nxrA* 3422bp

For checking the successful insertion of the potential *nxrA* gene, the *nxrA*-pET21b construct was transformed into *E.coli* XL1 blue by electroporation and screened according to Sec. B.6.5.

C.2.2 Heterologous expression in *E.coli*

According to section C.1.1, heterologous expression experiments of *nxrA* in different *E.coli* strains under diverse growth conditions were performed. Table C.4 shows a summary of all results of heterologous expression of *nxrA* gene using different *E.coli* strains and growth conditions. Additionally, the occurrence of a band with the predicted size of NxrA in sample with induced heterologous expression of *nxrA* could be observed (fig. C.4).

Table C.4: Summary of all results of heterologous expression of *nxrA* gene using different *E.coli* expression strains, various growth conditions and IPTG concentrations

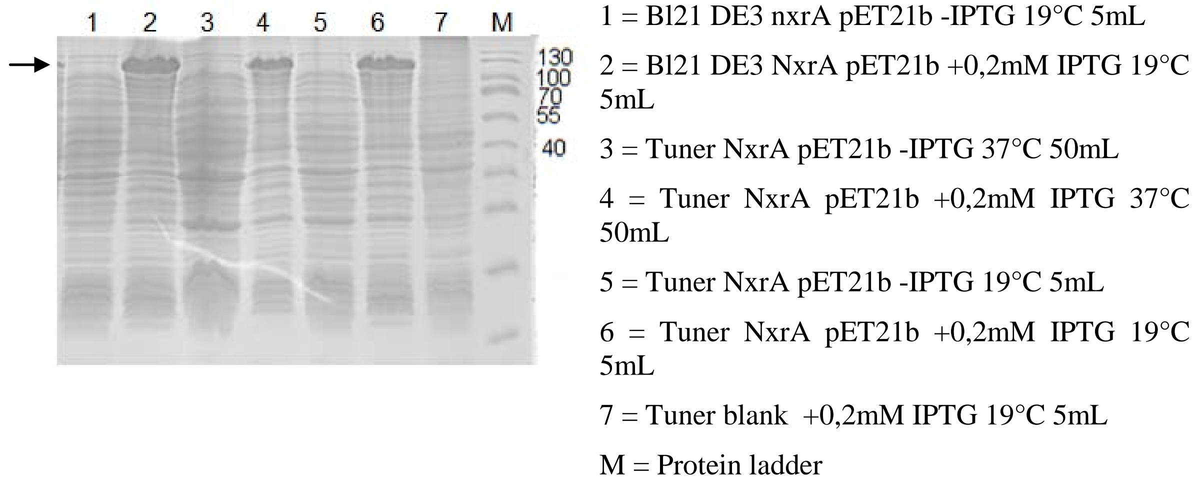
Strain (E.coli)	Temperature (°C)	Time of growth after IPTG (h)	IPTG	Expression
BL21 (DE3)	37	4	0,2mM	---+[a] ICB[b]
BL21 (DE3)	**19**	**6**	**0,2mM**	**-+++** [c]
Tuner (DE3)	37	4	0,2mM	--++ ICB
Tuner (DE3)	19	6	0,2mM	--++ [c]

a) expression level categorized according to the amount of produced protein: maximal expression level: ++++; high expression level:-+++; average expression level: --++; low expression level: ---+; no expression: ----

b) formation of protein aggregations, protein in inclusion bodies (ICB)

c) formation of protein aggregations was not tested

bold: highest expression level observed within all performed expression experiments



1 = Bl21 DE3 nxrA pET21b -IPTG 19°C 5mL

2 = Bl21 DE3 NxrA pET21b +0,2mM IPTG 19°C 5mL

3 = Tuner NxrA pET21b -IPTG 37°C 50mL

4 = Tuner NxrA pET21b +0,2mM IPTG 37°C 50mL

5 = Tuner NxrA pET21b -IPTG 19°C 5mL

6 = Tuner NxrA pET21b +0,2mM IPTG 19°C 5mL

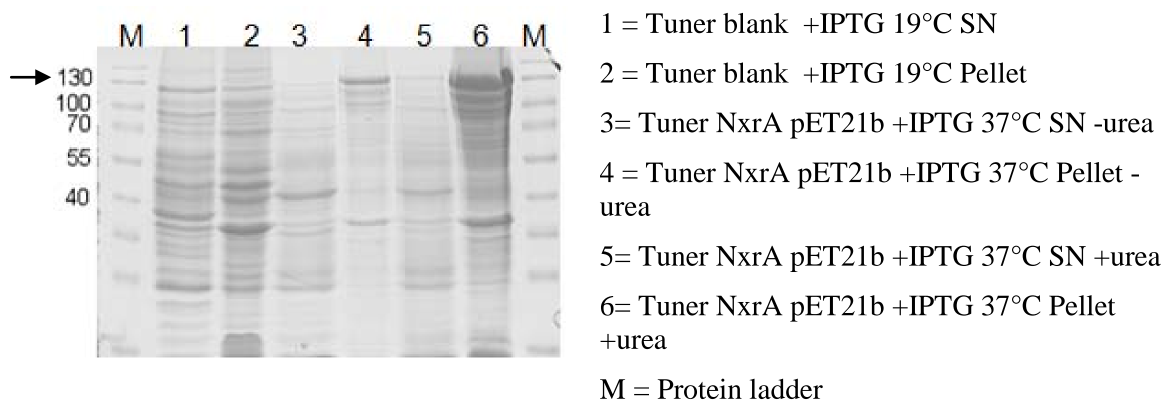
7 = Tuner blank +0,2mM IPTG 19°C 5mL

M = Protein ladder

Figure C.4: 12% SDS page of *E.coli* tuner (DE3) with and without pET21b with *nxrA* as insert; Tuner blank: *E.coli* tuner (DE3) without pET21b *nxrAB*; predicted size of NxrA: 129kDa

Formation of inclusion bodies was analyzed and confirmed by lysis, centrifugation of the lysate and treatment with 8M urea after harvesting of *E.coli* tuner (DE3) cell cultures (fig. C.5 lane 4). Even after treatment with 8M urea a large proportion of NxrA still was unsoluble and remained in the pellet after centrifugation (fig. C.5 lane 6)

The induction of heterologous expression of *nxrA* in *E.coli* BL21 (DE3) cells led also to protein-aggregation and formation of inclusion bodies (fig. C.6). After treatment of the samples with 8M urea a small fraction of NxrA became soluble (fig. C.6, lane 5), while the other part remained in the pellet as protein aggregates (fig. C.6, lane 6).



1 = Tuner blank +IPTG 19°C SN

2 = Tuner blank +IPTG 19°C Pellet

3= Tuner NxrA pET21b +IPTG 37°C SN -urea

4 = Tuner NxrA pET21b +IPTG 37°C Pellet -urea

5= Tuner NxrA pET21b +IPTG 37°C SN +urea

6= Tuner NxrA pET21b +IPTG 37°C Pellet +urea

M = Protein ladder

Figure C.5: 12% SDS page of *E.coli* tuner (DE3) with pET21b with *nxrA* as insert; -Urea: lysate in binding buffer +Urea: lysate in binding buffer with 8M urea SN:supernatant; Tuner blank: *E.coli* tuner (DE3) without pET21b *nxrA*; predicted size of NxrA: 129kDa

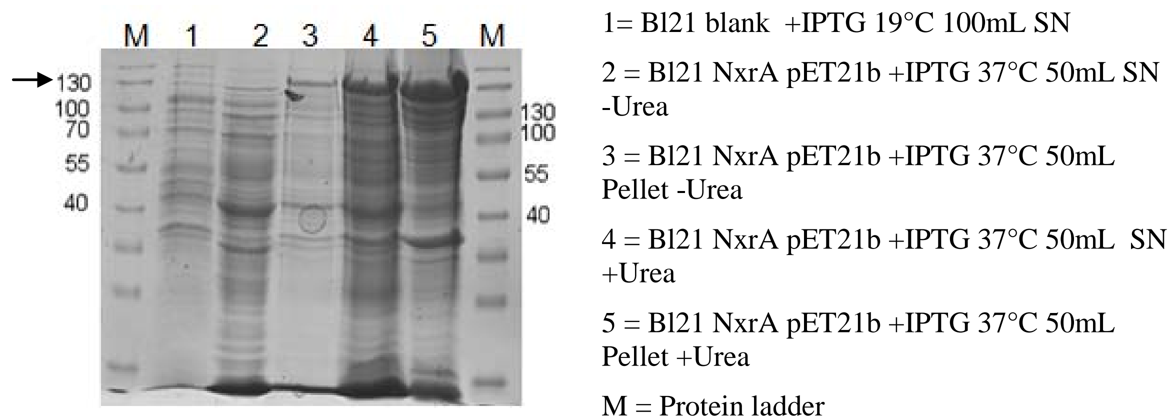


Figure C.6: 12% SDS page of *E.coli* BL21 (DE3) with pET21b with *nxrA* as insert; -Urea: lysate in binding buffer +Urea: lysate in binding buffer with 8M urea SN:supernatant; BL21 blank: *E.coli* BL21 (DE3) without pET21b *nxrA*; predicted size of NxrA: 129kDa

C.3 Investigation of different *nxrA* gene copies in the genome of *Nitrospira* spp.

C.3.1 Amplification of *nxrA* gene fragments from *Nitrospira* spp., cloning and RFLP analyses

To examine the potential of *nxrA* as a marker gene for expression analyses of different *nxrAB* operon copies in the genome of various *Nitrospira* spp. and as a putative phylogenetic marker gene of the genus *Nitrospira*, two primer pairs (F151mix/R831 and F814/R2062) specific for different parts of *nxrA* were designed. The 680bp-fragment, amplified with the primer pair F151mix/R831 includes the region, which encodes the Fe/S binding center of the active center of NxrA, whereas the primers F814 and R2062 are specific for the molybdenum lignand binding site of the protein and amplify a fragment with a size of 1248bp (Maixner *et al.*, manuscript in preparation).

A gradient PCR was performed to determine the optimal annealing temperature of the two different primer pairs according to Sec. B.6.2.6 (fig. C.7 and fig. C.8).

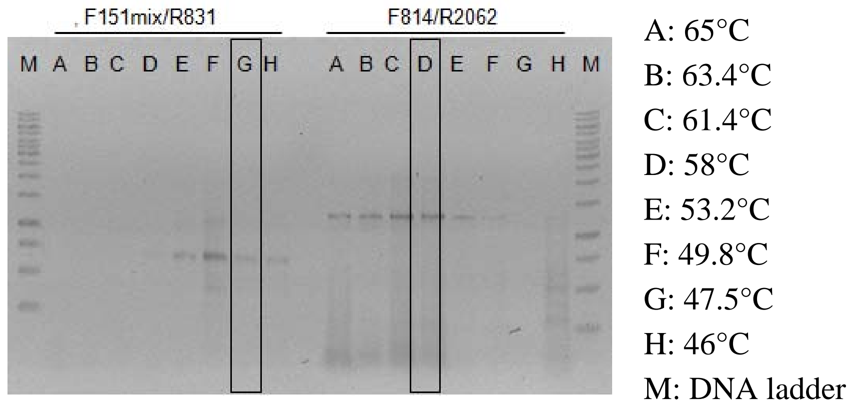


Figure C.7: 1% agarose gelelectrophoresis of the gradient PCR to determine the optimal annealing temperature of the primer pairs F151mix/R831 and F814/R2062; DNA: isolated from the main WWTP of Vienna; framed: optimal annealing temperature; predicted size: F151mix/R831: 663bp: F814/R2062: 1231bp

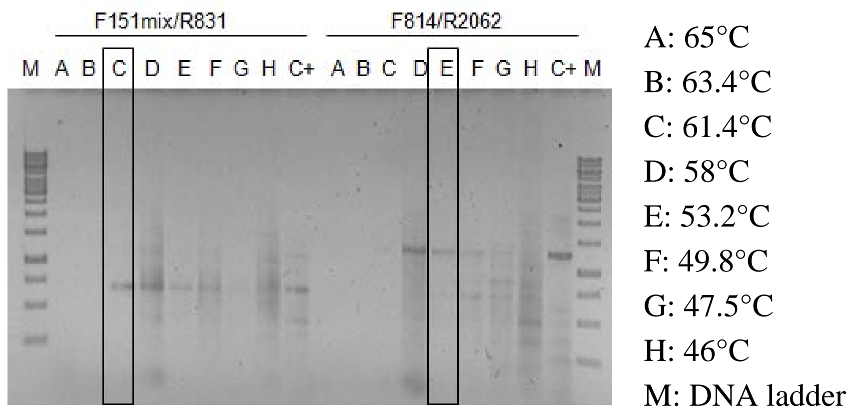


Figure C.8: 1% agarose gelelectrophoresis of the gradient PCR to determine the optimal annealing temperature of the primer pairs F151mix/R831 and F814/R2062; DNA: isolated from dry grassland of the botanical garden of Vienna; C+: positive control with DNA isolated from the main WWTP of Vienna; annealing temperature of F151mix/R831 positive control: 47.5°C; annealing temperature of F814/R2062 positive control: 58°C; framed: optimal annealing temperature; predicted size: F151mix/R831: 663bp: F814/R2062: 1231bp

Interestingly, the optimal annealing temperature seemed to be depend on the type of template DNA. The optimal annealing temperature ranged between 47.5°C and 61.4°C for the primers F151mix/R831 and between 58°C and 53.2°C for the primer pair F814/R2062.

After evaluation of the two primer pairs, *nxrA* fragments were amplified by PCR from different DNA samples, isolated from various habitats, like the main WWTP of Vienna, dry grassland soil from the botanical garden of Vienna and a pure culture of *Nitrospira moscoviensis*. Amplification of both fragments of *Nitrospira moscoviensis* was performed at 53.2°C, since a defined band occurred at this annealing temperature in every gradient PCR. The amplicons were cloned into the TOPO TA cloning vectors and the clones were screened for insertion by M13 PCR. Furthermore, RFLP was used to estimate the diversity of the *nxrA* DNA sequences. The results from the RFLP analysis are summarized in table C.5.

Table C.5: Results of the RFLP analysis of different template DNA amplified by two primer pairs to analyze the *nxrA* of various *Nitrospira* spp.

primer pair	DNA sample	number of RFLP patterns	number of unique RFLP patterns occurring only once	sequenced clones
F151mix/R831	WWTP Vienna	3	0	7
F151mix/R831	dry grassland soil	7	6	7
F151mix/R831	*N. moscoviensis*	5	0	9
F814/R2062	WWTP Vienna	4	2	6
F814/R2062	dry grassland soil	4	3	6
F814/R2062	*N. moscoviensis*	3	0	5

From two to three clones of each RFLP pattern the vector DNA was then isolated and the *nxrA* inserts were sequenced by the Sanger method.

C.3.2 Phylogeny of the *nxrA* gene fragments sequences

The received sequences were proofread by using of the software ChromasPro and by the use of the software ARB the nucleotide sequences were translated into amino acids and aligned. Furthermore, two different calculating-tree methods, neighbor joining and maximum likelihood were used to analyze the phylogeny of the different fragments on amino acid level. The short fragment was amplified with the primers F151mix/R831, which includes the Fe/S binding cluster (fig. C.7) and the long fragment was amplified with the primer pair F814/R2062, which encodes the molybdenum-lignand binding site of the NxrA protein (fig. C.8.).

Figure C.7: Neighbor joining tree of NxrA fragment F151mix/R831 (226aa) on amino acid level; brown font: sequences received from the main WWTP of Vienna; green font: sequences received from dry grassland soil from the botanical garden of Vienna; black font: sequences received from a pure culture of *Nitrospira moscoviensis*; blue font: sequences received from the genome of *Nitrospira defluvii*;

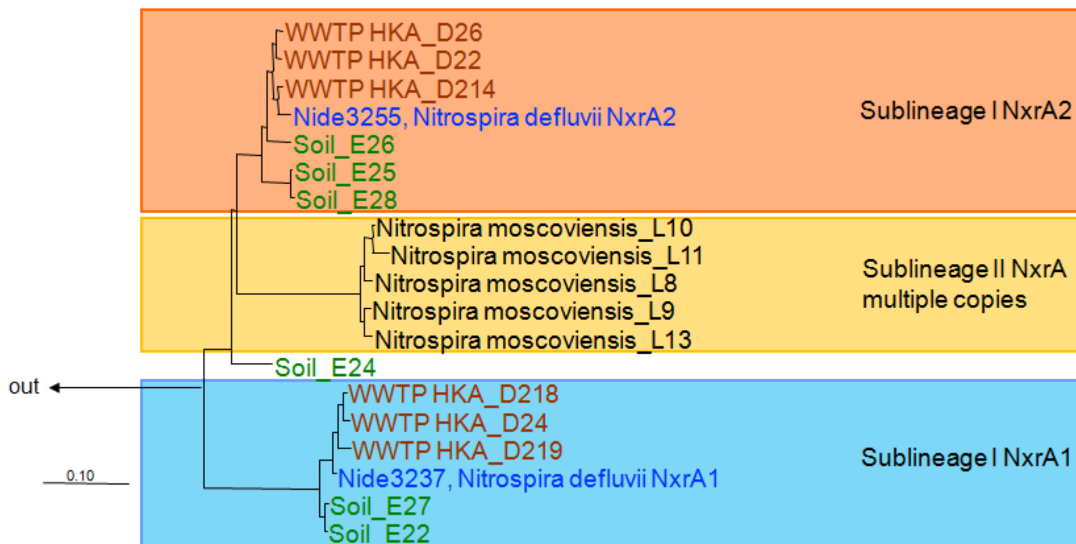


Figure C.8: Maximum likelihood tree of NxrA fragment F814/R2062 (422aa) on amino acid level; brown font: sequences received from the main WWTP of Vienna; green font: sequences received from dry grassland soil from the botanical garden of Vienna; black font: sequences received from a pure culture of *Nitrospira moscoviensis*; blue font: sequences received from the genome of *Nitrospira defluvii*;

Furthermore, distance matrixes were calculated for both *nxrA* fragment datasets on amino acid level. Table C.6 compares and sums up the most significant data from the distance matrixes calculated for the sequences received by using the primer pairs F151mix/R2062.

Table C.6: Summary and comparison of distance matrixes of the sequences amplified with the primer pairs F151mix/R831 and F814/R2062 on amino acid level

	Sequence differences in % on aa level	
	short fragment (F151mix/R831)	long fragment (F814/R2062)
sublineage I *nxrA1* cluster	0.5% - 3.3%	0.8% - 4.1%
sublineage I *nxrA2* cluster	0% - 7.6%	0.8% - 6.6%
comparison of *nxrA1* and *nxrA2* cluster of sublineage I	11.4% - 14.7%	13.1% - 19.8%
copies of *N.moscoviensis*	0.5% - 8,7%	1.8% - 3.3%
copies of *N.defluvii*	13.1%	13.1%

C.3.3 Expression analysis of the *nxrA* copies within the genome of *Nitrospira* spp. in the main WWTP of Vienna

C.3.3.1 Evaluation of two forward primers to differentiate between the different *nxrA* copy clusters

To differentiate between the two different *nxrA* copy clusters of *Nitrospira* sublineage I, which all sequences received from the main WWTPs of Vienna belong to, two new forward primers (NxrA1L and NxrA2L) were designed. The primer NxrAL1 is specific for the *nxrA1* cluster (in fig. C.7 in blue), whereas NxrAL2 covers all sequences within *nxrA2* cluster (in fig. C.7 in orange) of *Nitrospira* sublineage I. Both forwards primers were combined with 831R as reverse primer. The optimal annealing temperature was evaluated by a gradient PCR, which should lead to a length of amplicons of 272bp (fig. C.9) and was determined to be 62°C for both primer pairs.

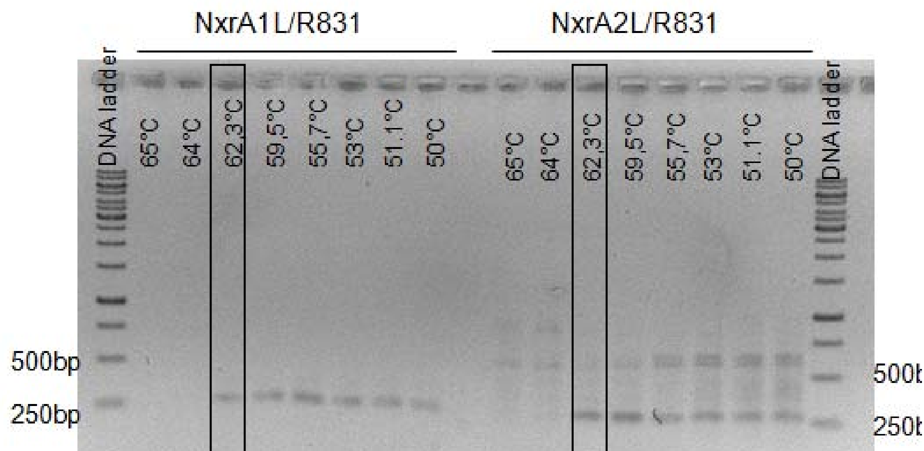


Figure C.9: 1%-agarose gelelectrophoresis of the gradient PCR to determine the optimal annealing temperature of the primer pairs nxrA1L/R831 and nxrA2L/R831 for separate amplification of both *nxrA* copy clusters of sublineage I of the genus *Nitrospira*; framed: optimal annealing temperature; predicted size: 272bp

C.3.3.2 RNA isolation from the main WWTP of Vienna, cDNA synthesis

RNA was isolated from samples of the main WWTP of Vienna to investigate the expression profile of *nxrA* at the sampling point in time. Fig. C.10 shows an agarose gelelectrophoresis picture of the isolated RNA duplicates to control purity and quality of the RNA samples.

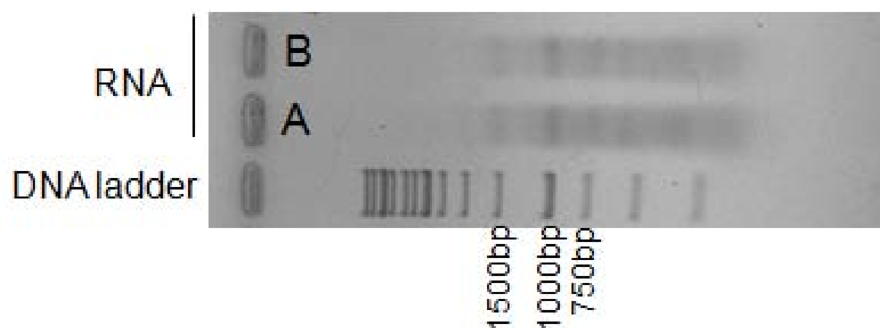


Figure C.10: 1%-agarose gelelectrophoresis picture of the isolated RNA from the main WWTP of Vienna; A and B are duplicates.

For reverse transcription of the isolated RNA the reverse primer R831 was used to synthesize cDNA from both *nxrA* copy clusters. Additionally, two PCR reactions with the two cluster specific primers were performed to detect possible differences in expression of these two *nxrA* gene copy clusters (fig. C. 11).

C.3.3.3 Analysis of the expression profile of two different *nxrAB* operon in the genome of *Nitrospira* sublineage I organisms

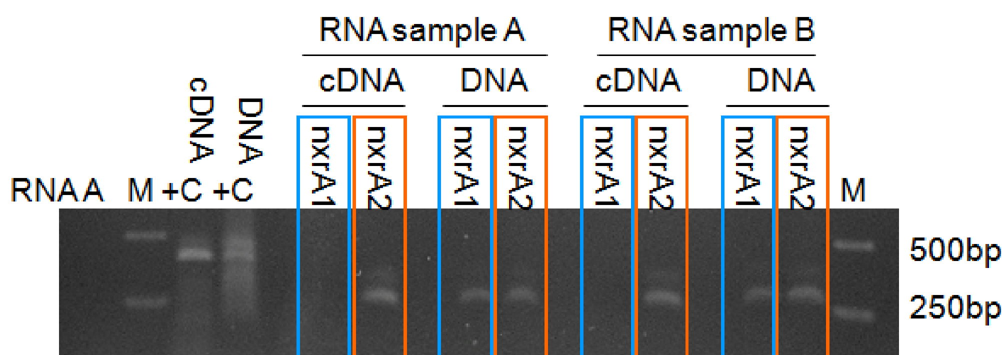


Figure C.11: 1%-agarose gelelectrophoresis picture of the PCR with different primer pairs and cDNA received from the main WWTP of Vienna as template; nxrA1: NxrA1L/R831 as primers for PCR; nxrA2: NxrA2L/R831 as primers for PCR; RNA A: RNA without reverse transcription from RNA sample A; M: DNA ladder; predicted size: 272bp

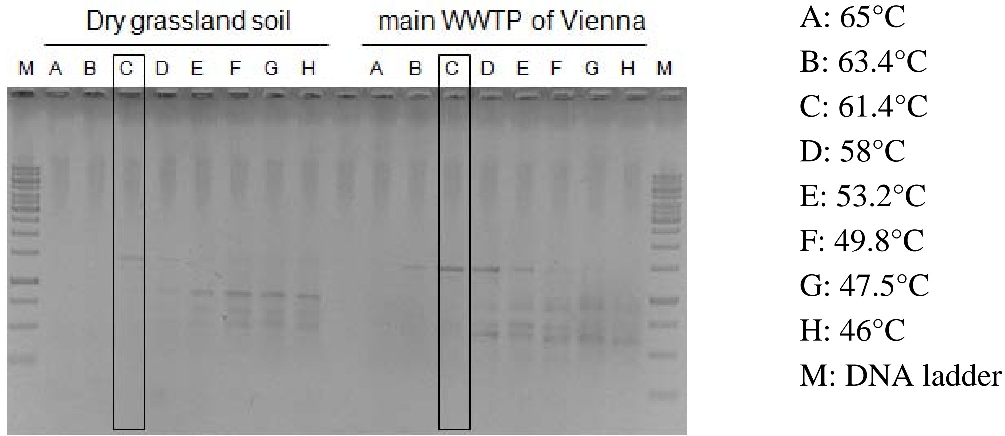
The PCR reaction with NxrAL1/R831 as primers specific for the *nxrA1* cluster of sublineage I did not lead to amplification (fig. C.11; framed in blue), so no RNA of this copy was transcribed at the sampling point in time, whereas a product of the PCR reaction with the primer pair NxrAL2/R831 was amplified (fig. C.11; framed in orange). There are two possible explanation for the absent of amplicons of *nxrA1*, i) there was no expression of the *nxrA1*-related genes at the moment of sampling or ii) due to the formation of secondary structures of the transcribed mRNA the primers could not bind.

C.4 Phylogenetic analysis of various soil samples on 16S rRNA level

C.4.1 Amplification of 16S rRNA genes from *Nitrospira* sublineage I, cloning and RFLP analyses

To verify the occurrence of *Nitrospira* sublineage I in dry grassland soil detected by *nxrA* as a putative new phylogenetic marker gene, a PCR reaction was performed to exclusively amplify *Nitrospira* sublineage I 16S rRNA genes with the primer pair 616V/nt1431R. The sublineage-I-specific primer nt1431 is usually used as a FISH probe, named Ntspa1431 (Maixner *et al.* 2006). Therefore the primer pair 616V/nt1431R had to be evaluated first by gradient PCR

with DNA isolated from dry grassland soil from the botanical garden of Vienna and the main WWTP of Vienna as template DNA (fig. C.12).



A: 65°C
B: 63.4°C
C: 61.4°C
D: 58°C
E: 53.2°C
F: 49.8°C
G: 47.5°C
H: 46°C
M: DNA ladder

Figure C.12: 1%-agarose gelelectrophoresis of the gradient PCR to determine the optimal annealing temperature of the primer pair 616V/nt1431R to amplify the 16S rRNA gene of sublineage I of the genus *Nitrospira*; framed: optimal annealing temperature

DNA isolated from dry grassland soil served as a template DNA for PCR with the annealing temperature of 61.4°C, as well as DNA extracted from a forest soil sample and an unfertilized garden soil sample (Table C.7).

Table C.7: Characterization of different soil samples for determination of *Nitrospira* sublineage I presence

Short name of soil sample	Description of soil sample	Presence of *Nitrospira* sublineage I
BG_soil	dry grassland soil from the Vienna botanical garden	+
KS_soil	unfertilized soil from a garden in Klagenfurt	+
WS_soil	soil from a mixed forest nearby Villach	-

The amplicons were cloned into TOPO TA vectors and the insertion of the right insert was controlled by M13 PCR. Due to low transformation and insertion efficiency, no RFLP analysis was performed, but the isolated vector DNA samples were sequenced directly.

C.4.2 Phylogeny of the 16S rRNA sequences of various soil samples

The received sequences were proofread and added to a database containing most known 16S rRNA sequences affiliated to the genus *Nitrospira*. The added sequences were aligned and a phylogenetic tree with some representatives from each sublineage of the genus *Nitrospira* was calculated (fig. C.12).

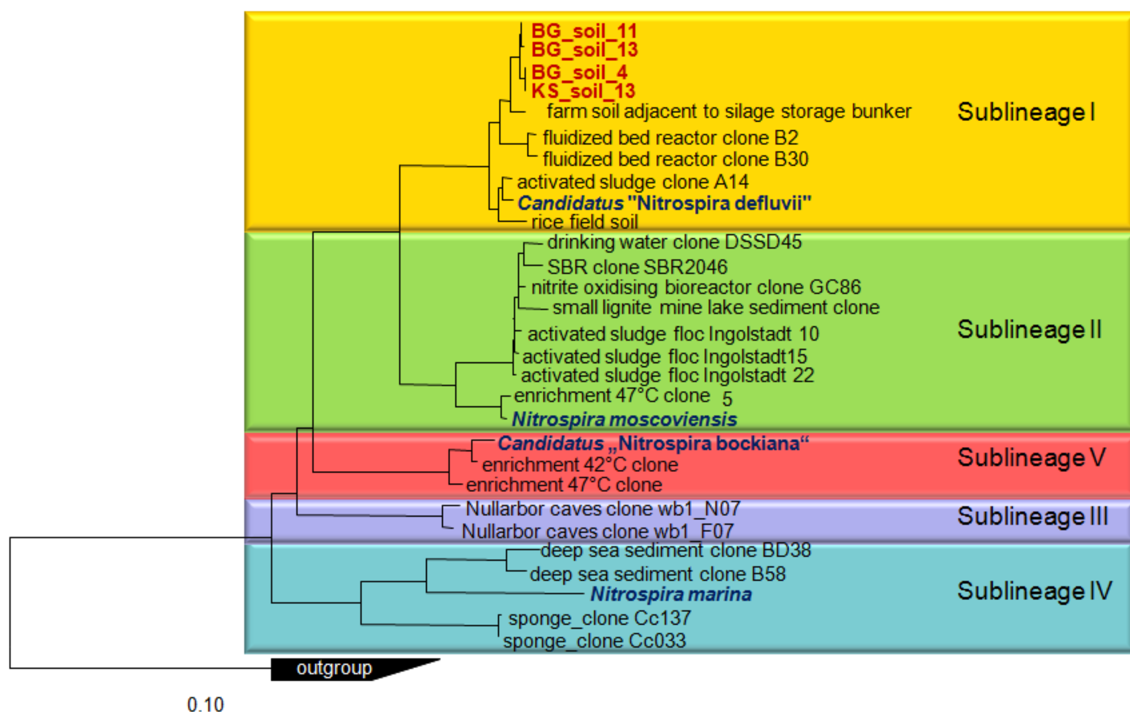


Figure C.12: Neighbor joining tree of the genus *Nitrospira* based upon 16S rRNA gene analysis; red font: sequences received from soil within this study; blue font: pure or enrichment cultures.

C.5 Enrichment of nitrite-oxidizing bacteria from dry grassland soil

1.5g of dry grassland soil of the Viennese botanical garden were incubated in 150mL mineral media (Sec. B.4.9) containing 0.2g natriumnitrite at 30°C in the dark to enrich nitrite-oxidizing bacteria, according to Sec. B.14. This environmental sample was used, due to the occurrence of sublineage I *Nitrospira*, detected by the cultivation-independent technique PCR according to Sec. C.4.

After the first consumption of nitrite, the period when nitrite had to be supplied became shorter and finally the cultures were fed with 0.1mL 5M $NaNO_2$ three times a week and white-brownish flocs were observed (table C.8).

Table C.8: Time interval for the first nitrite consumption (lag phase) of enrichment cultures growing on 0.2gNaNO2/L medium

Name of the culture	Lag phase (days)	Comments
A1_soil_BG	no consumption	-
A2_soil_BG	no consumption	-
B1_soil_BG	79	-
B2_soil_BG	57	this culture was used for further analyses

C.5.1 Identification of enriched nitrifying microorganisms

DNA was isolated by cooking cells according to B.6.1.3 from the culture B2_soil_BG and different PCR reactions with various primer pairs to identify the enriched nitrite-oxidizing organisms were performed (fig.C.13).

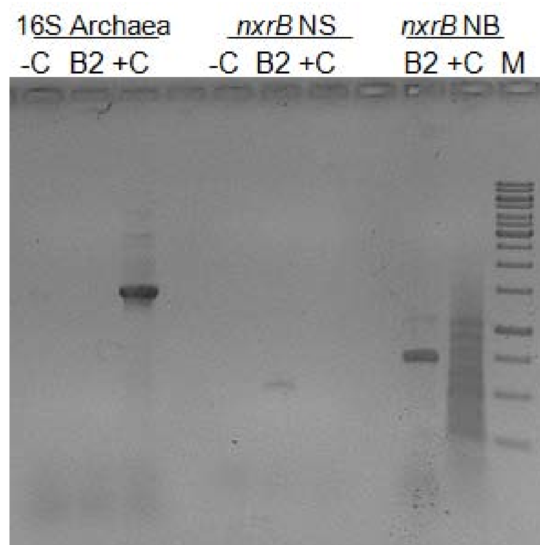


Figure C.13: 1%-agarose gelelectrophoresis of DNA isolated from B2_soil_BG as a template with different primer pairs to identify enriched nitrite-oxidizing organisms; M: DNA ladder; -C: negative control; +C: positive control; B2: B2_soil_BG as a template; 21F/1492R primer pair specific for 16S of archaea; 169F/707R primer pair specific for *nxrB* of the genus *Nitrospira*; 706F/1431R primer pair specific for *nxrB* of the genus *Nitrobacter*; predicted size: 21F/1492R: 1503bp; 169F/707R: 485bp; 706F/1431R: 724bp

The amplicons of *nxrB* of the genus *Nitrobacter* were cloned and three clones were sequenced. The sequences analyses via BLAST showed that the nitrite-oxidizing bacteria of the culture B2_soil_BG are affiliated to *Nitrobacter sp.* PJN1 and *Nitrobacter sp.* HTN8 (table C.9 and fig. C.14).

Table C.9: Summarization of the BLAST analysis results of *Nitrobacter* nxrB sequences received from the enrichment cultures

BLAST query	BLAST result		
Enrichment squence	**Accession number**	**Organism**	**Identity**
Soil_BG_1	AY508482	Nitrobacter sp. HTN8	95%
	AY508479	Nitrobacter sp. PJN1	93%
Soil_BG_2	AY508482	Nitrobacter sp. HTN8	95%
	AY508479	Nitrobacter sp. PJN1	95%
Soil_BG_8	AY508482	Nitrobacter sp. HTN8	95%
	AY508479	Nitrobacter sp. PJN1	95%

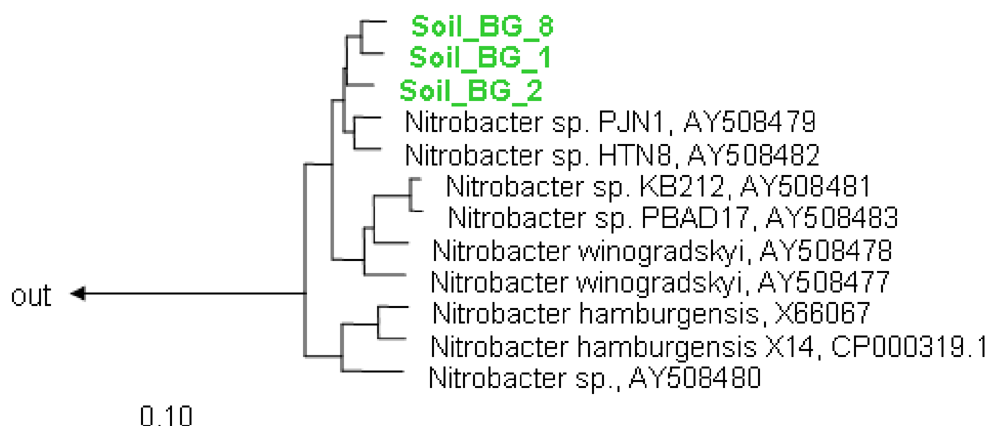


Figure C.14: Neighbor joining tree of nxrB sequences affiliated to the genus *Nitrobacter*; green: nxrB sequences received from the enrichment cultures

C.6 Micromanipulation of FISH-labeled *Nitrospira* sublineage II clusters in activated sludge flocs of different WWTPs and further analysis

C.6.1 Micromanipulation and PCR analysis of an activated sludge floc from the WWTP of the University of Veterinary Medicine of Vienna

Activated sludge from the WWTP of the University of Veterinary Medicine of Vienna was fixated in ethanol and used to perform a liquid FISH approach, according to B.8.3. Then a single activated-sludge floc was isolated by micromanipulation (see B.13) and a PCR was performed with 616V/1492R as primer pair to amplify most bacterial 16S rRNA genes for estimation of the total bacterial diversity within an activated sludge floc. After establishment of a clone library a RFLP analysis was performed and 27 clones were sequenced (table C.8)

C.6.2 Identification of the bacterial diversity within an activated sludge floc from the WWTP of the University of Veterinary Medicine of Vienna

The bacterial diversity was estimated by BLAST and was confirmed by adding the sequences to the SILVA database. Diversity is illustrated in fig. C.15. No sequence was affiliated to sublineage II of the genus *Nitrospira*, although the activated-sludge floc, which served as template, was sublineage II positive. Furthermore, eleven (in fig. C.15 with a bold frame) out of nineteen orders where the received sequences belong to, are known to harbor organisms found in WWTP. The only *Nitrospira* sequence found is affiliated to sublineage I.

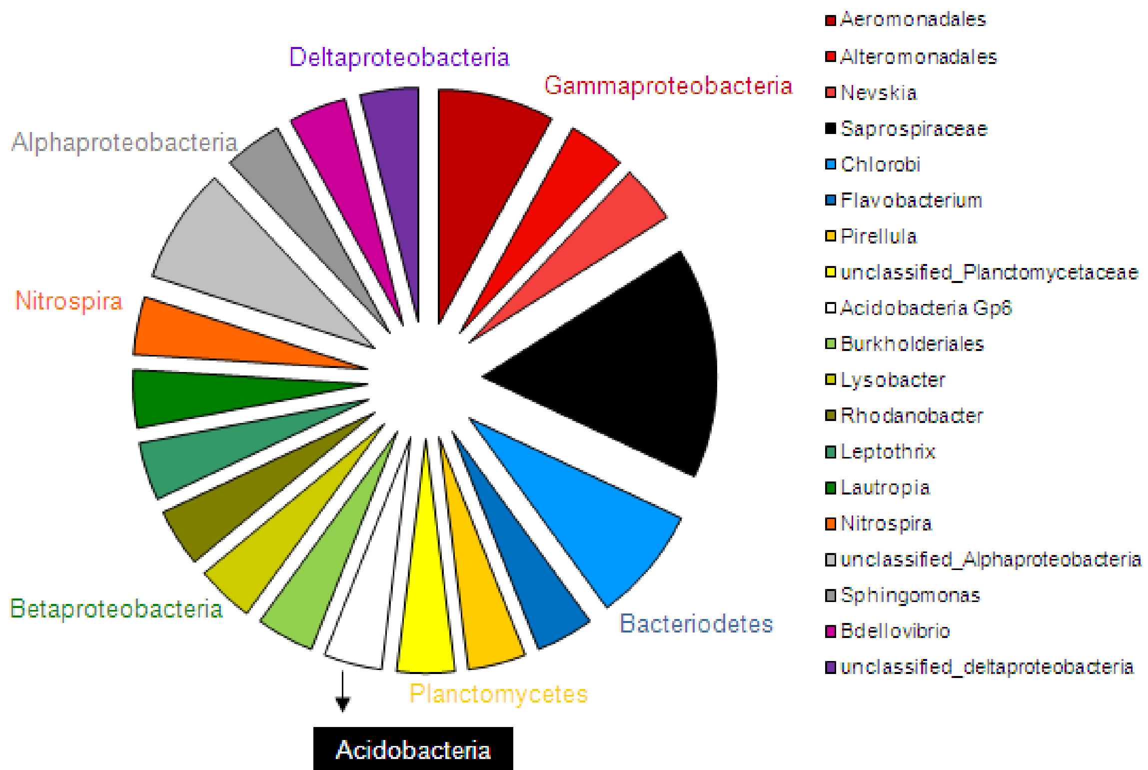


Figure C.15: Diagram of the bacterial diversity within the activated sludge floc from the WWTP of the University of Veterinary Medicine of Vienna; bold: organisms known to be present in WWTP

C.6.3 Micromanipulation and PCR analysis of an activated sludge floc from the WWTP of Ingolstadt

Additionally, another activated sludge floc from the WWTP of Ingolstadt was analyzed. For improvement of isolation of a microcolony of sublineage II of the genus *Nitrospira* a mechanical shear experiment was performed to minimize the size of the flocs by bead beating. An optimal bead beating setting for separation of the flocs was chosen as speed 5 for 45sec. In contrast to milder bead beating conditions the activated-sludge flocs treated under those settings ceased to show any adhesion effect to each other and could not aggregate to reform bigger flocs after separation. A huge number of single flocs was found, with different morphologies and a size range of 20-40µm was observed. Then a single activated sludge floc was isolated by micromanipulation (see B.13) and a PCR was performed with 616V/1492R as primer pair to amplify most bacterial 16S rRNA genes for estimation of the total bacterial diversity within an activated sludge floc. Although the amplification of the 16S rRNA genes

led to a very thin band at the agarose gelelectrophoresis, it was possible to analyze the diversity of the bacterial community of the activated-sludge floc by creating a clone library.

C.6.4 Identification of bacterial diversity in an activated sludge floc from the WWTP of Ingolstadt

After successful establishment of a clone library, defined clones were sequenced by the Sanger method. The results of the diversity analysis are shown in fig. C.16 and table C.10 compares the results of the two differentely treated activated sludge flocs.

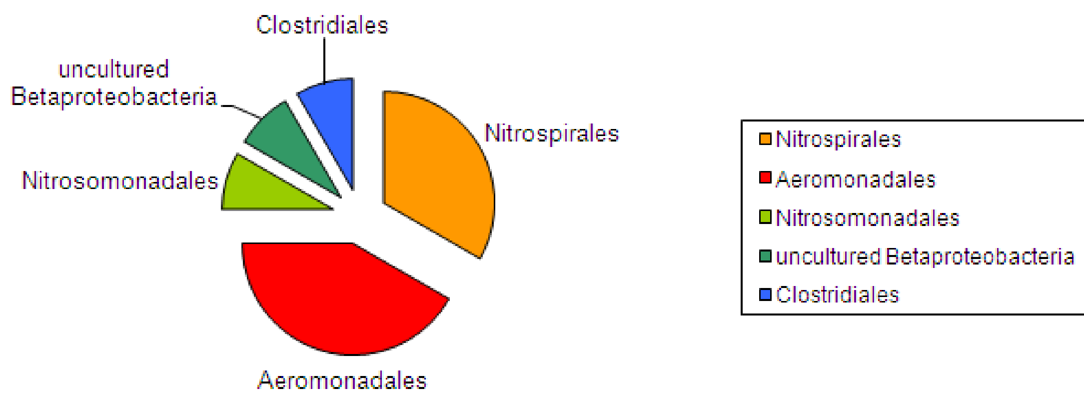


Figure C.16: Diagram of the bacterial diversity in the activated sludge floc from the WWTP of Ingolstadt; bold: organisms known to be present in WWTP

Table C.10: Comparison of two activated sludge flocs received from two different WWTP, the WWTP of the University of Veterinary Medicine of Vienna and the WWTP of Ingolstadt

Features	University of Veterinary Medicine WWTP	Ingolstadt WWTP
bead beated	no	yes
size	ca. 40µm	ca. 15µm
picked clones	50	43
RFLP pattern	30	7
sequences	27	12
different orders	19	5
Nitrospira sublineage II	0	4 (33% of all sequences)

C.7 Analysis of the influence of low nitrite concentration and discontinuous aeration conditions on *Nitrospira* sublineage I and II

C.7.1 Accomplishment of selective enrichment of nitrite-oxidizing bacteria

Activated sludge samples of two different WWTPs, “Zentralkläranlage Ingolstadt” (Germany) and the WWTP of the University of veterinary medicine of Vienna were incubated in parallel, according to B11.

Table C.11: Summary of the abundance of *Nitrospira* sublineage I and sublineage II in the inoculums of the incubation experiment with activated sludge of two different WWTPs

WWTP	presence of *Nitrospira* sublineage I	presence of *Nitrospira* sublineage II
University of Veterinary Medicine WWTP	++[a]	+++
Ingolstadt WWTP	+	++

a) classification of abundance of FISH-analyzed cell clusters ordered by the following categories: +++ high abundance; ++ medium abundance; + low abundance

Weekly samples were taken to perform PFA and ethanol fixation of the cells of all four approaches as well as a nitrite consumption check by a color reaction of the nitrite-testing solution (B.4.10) and the pH value via pH test strips. FISH, followed by microscopy, was used to determine changes in the abundance of *Nitrospira* sublineage I and sublineage II respectively. Interestingly, the pH value changed in all approaches only during the first month, whereas it remained at the same level during the remaining experiment. After the first 60 days, a permanent decrease of *Nitrospira* cell clusters in all four approaches was observed. Due to this loss of living *Nitrospira* cells, aeration was changed to discontinuous aeration with an interval of 10min air exposure, interrupted by 15min. After an additional 49 days the experiment was stopped because of a complete loss of *Nitrospira* biomass in the incubations of activated sludge of the WWTP of the Vienna University of Veterinary Medicine and an accumulation of nitrite was detected. Also nearly no cells of the genus *Nitrospira* could be observed in the incubations of activated sludge of the “Zentralkläranlage Ingolstadt” at the end of the experiment.

D. Discussion

D.1 Heterologous expression of the *nxrAB* operon and the *nxrA* gene of *N. defluvii* in *E.coli*

One aim of this study was the heterologous expression of the *nxrAB* operon and the *nxrA* gene of *Candidatus* Nitrospira defluvii to gain more information about the enzyme kinetic and enzymatic structure. However, the heterologous expression of the *nxAB* operon *Candidatus* Nitrospira defluvii in defined *E.coli* strains led to formation of inclusion bodies. The heterologous of the *nxrA* gene alone of Candidatus *Nitrospira defluvii* led also to the formation of inclusion bodies at 37°C with several expression strains. It remains to be determined whether the heterologous expression of *nxrA* in *E.coli* BL21 (DE3) at 19°C leads to a soluble protein, because this heterologous expression experiment resulted in the highest yield of NxrA.

Former studies showed that large multidomain proteins were often folded *in vivo* with the help of folding modulators, like molecular chaperons and folding catalysts (Baneyx *et al.* 2004). The failure of interaction with folding modulators can lead to formation of insoluble aggregates of overexpressed proteins.

Insoluble aggregates of non-native proteins, generally known as inclusion bodies, are a common problem during overexpression of recombinant proteins. These overexpressed proteins form 80% to 95% of all aggregated material in those inclusion bodies (Baneyx *et al.* 2004). Because of the purity of the aggregated protein and the resistance to protolysis in the inclusion bodies, they are used as a tool for heterologous expression of proteins which are toxic, as well as unstable in *E.coli*, and are easy to refold again (Baneyx *et al.* 2004; Baneyx 1999). The protocols available for protein purification and refolding applied in inclusion bodies are often expensive and time-consuming. Furthermore, there is no guarantee that large amounts of biologically active products can be obtained using *in vitro* refolding.

Various aspects had to be considered before performing the heterologous expression of the *nxrAB* operon as well as of the *nxrA* gene of *Candidatus* Nitrospira defluvii in *E.coli*, like the choice of expression vector and expression strains, conditions of growth and induction, etc. They are summarized in D.1.1 to D1.5.

D.1.1 Expression vector

The pET 21b(+) vector system was used while performing the expression studies. It was first described by Studier *et al.* 1990 and is one of the most often used expression systems for recombinant protein preparation. Expression regulation of the inserted protein of interest is based on the repressor system of the *lac* operon as well as on the T7 RNA polymerase of the enterobacteria phage 7. The pET 21b(+) vector includes a region encoding the *lac* repressor, LacI, a gene for ampicillin resistance and a hybrid T7 *lac* promoter – i.e. a T7 promoter, which is regulated by the downstream *lac* operator sequence. Additionally, a region encoding a HIS_6-tag can be found on the C-terminus of the gene of interest, inserted into pET21(+) vectors. HIS_6-tags are used as a tool for affinity purification, immunofluorescence and western blot analyses. The DE3 expression strains are lysogenized by a phage fragment, encoding the *lacI* gene and the T7 RNA polymerase, which is controlled by the IPTG inducible *lacUV5* promoter. LacI represses the expression of T7 RNA polymerase and T7 *lac* promoter of the plasmid until IPTG separates this binding and induces the expression of T7 RNA polymerase and the inserted gene of interest. The usage of pET 21b did not result in production of soluble NxrA and NxrB proteins. It is still an open question, whether the application of other vector-systems might lead to a positive result of the heterologous expression experiments.

D.1.2 Expression strains

Many different expression strains were used to obtain a successful expression of either NxrA and NxrB or NxrA only. Every *E.coli* host strain has special genetic characteristics possibly crucial for the production of recombinant proteins.

The highest yield of expressed NxrA and NxrB was achieved by the use of the pET21(b) expression vector in *E.coli* Tuner (DE3). The most significant difference between *E.coli* Tuner (DE3) and other expression strains is deletion of the lactose transporter (*lacYI*) in its genome (Khlebnikov *et al.* 2002). An active transport of lactose into the cells is suppressed because of this deletion, therefore IPTG is the only substance in the *E.coli* Tuner (DE3) cells that can regulate the release of *LacI* and activation of the heterologous expression respectively. So it is possible to regulate the induction of recombinant protein production – dependent on the concentration of IPTG – in a more sensitive way.

Compared to that, the use of *E.coli* BL21 (DE3) as a host strain led to the highest yield of produced NxrA only. This host strain is lysogenized by a fragment of the T7 phage and represents the origin of all other DE3 expression strains (Dubendorff *et al.* 1991; Studier *et al.* 1990).

The chances to gain a successful production of soluble NxrA and NxrB as well as NxrA only might increase as other expression strains are applied. The result of the expression experiment of *nxrAB* and *nxrA* is the formation of inclusion bodies. That can be seen as a hint that the gene product of *nxrAB* is not encoded by too many rarely used codons in *E.coli*. The problem apparently lies in the solubility and folding of the proteins respectively or in overproduction of the proteins, because of a too weak overexpression regulation.

A possible approach to remove these obstacles would bet he use other expression strains or different conditions of growth and induction.

The *E.coli* Tuner pLysS strain could be used to better regulate the overexpression. This *E.coli* strain has the same genetic characteristics like *E.coli* Tuner, but includes the pLysS plasmid, which carries a region encoding the T7 lysozyme, a natural inhibitor of T7 RNA polymerase (Khlebnikov *et al.* 2002; Studier 1991). So there is the chance to better control of overexpression of the gene of interest.

To increase the solubility of the recombinant proteins it is recommended to use the *E.coli* Origami B strain, which could improve the disulfide-bond formation in the cytoplasm and the correct folding of the protein respectively (Bessette *et al.* 1999)

D.1.3 Growth conditions

The choice of the optimal growth conditions seem to be as essential for a successful production of recombinant proteins as the choice of the vector system or host strain.

It is especially the growth temperature, often that is considered as a tool for minimizing the formation of inclusion bodies. Former studies showed that aggregation of recombinant proteins could be avoided by decreasing the cultivation temperature to slow down protein production(Sorensen *et al.* 2005b). In this study, cultivation at reduced growth temperature led to a higher visible expression, analyzed by SDS-page in both cases, NxrA and NxrB and NxrA alone.

Other growth factors, like the pH value and composition of the cultivation medium also increase solubility of expressed proteins. In some cases, the presence of a certain cofactor is required for the correct folding of the recombinant protein. The addition of defined cofactors or binding partners may minimize the formation of inclusion bodies and may lead to an increased solublility of proteins. (Sorensen *et al.* 2005b) It remains to be determined whether an addition of molybdopterin as cofactor of NxrA would increase the solubility of the protein.

D.1.4 Induction conditions

In this study, the IPTG concentrations ranged between 0.1mM and 1mM for expression of NxrA and NxrB and 0.2mM for NxrA. The best expression result was reached with an IPTG concentration of 0.2mM in *E.coli* Tuner (DE3). Although *E.coli* Tuner (DE3) is considered to be more sensitive to different IPTG concentrations, no differences in the expression of NxrA and NxrB induced with 0.2mM or 0.5mM IPTG could be observed. The expression level can be controlled by varying the inducer concentration (Sorensen *et al.* 2005a). A low expression level may lead to a higher yield of soluble proteins.

D.1.5 Other possible approaches to improve the solubility of the recombinant protein

One strategy to obtain a successful production of the protein of interest would be to alternate the position of the HIS_6-tag (Alam *et al.* 2002). In this study the HIS_6-tag was attached to the C-terminus of the recombinant proteins. A change of the attachment site from the C-terminus to the N-terminus of the HIS_6-tag could result in an expression of soluble recombinant proteins.

Another strategy would be the coexpression of chaperons being helpful in the proper *in vivo* folding of a recombinant protein. (Sorensen *et al.* 2005b) There are hints that a chaperon encoding gene exists in the genome of *Nitrobacter winogradskyi*, responsible for correct folding of the subunits of Nxr (Starkenburg *et al.* 2006). A similar gene could be found in the genome of *Candidatus* Nitrospira defluvii (Lücker *et al.*, manuscript in preparation). So the coexpression of a *Nitrospira defluvii* Nxr-specific chaperon would possibly improve the heterologous expression approach.

Recent studies showed the potential of solubilization and refolding of bacterial inclusion body proteins (Sorensen *et al.* 2005b; Singh *et al.* 2005; Willis *et al.* 2005; Vincentelli *et al.* 2004).

Purity and the high yield of expressed proteins are advantages of refolding inclusion body proteins. As most refolding protocols only refold a small amount of proteins and there is no guarantee that a received protein is still active, this uncertain outcome is a major drawback.

D.2 Investigation of different expression profiles of *nxrA* gene copies in the genome of *Nitrospira* spp.

Together with gene loss and horizontal gene transfer, gene duplication is an important evolutionary tool for asexual microorganisms to adapt to new environment conditions (Gevers *et al.* 2004; Riehle *et al.* 2001).

This study shows that *nxrA* exists in the genomes of various bacteria belonging to the genus *Nitrospira*. Investigation of different expression profiles of *nxrA* gene copies in the genome of *Nitrospira* sublineage I like bacteria

For *Candidatus* Nitrospira defluvii, a representative of sublineage I, it had been shown before via genome sequencing that two *nxrAB* operons exist in its genome (Lücker *et al.*, manuscript in preparation). The *nxrA* copies in the genome of *Candidatus* Nitrospira defluvii differ from each other by 14.4% on amino acid level and by 18.5% on DNA level, while the electron-channeling β-subunit-encoding copies are nearly identical. The difference between the subunits that interact with the substrate indicates different biochemical characteristics of the NxrA protein versions, like differences in the substrate-affinity or substrate-specificity. Former studies showed that various key enzymes of the nitrogen cycle, like *nifH* of *Azorhizobium caulinodans* or nirS of *Thauera mechernichensis* strain 27 are existent in the genome in duplicates (Iki *et al.* 2007; Etchebehere *et al.* 2005). Different transcription activities of these duplicated genes suggest functional differences between the paralogs.

In genome analyses of *Candidatus* Nitrospira defluvii, different regulation factors of transcription were found upstream the *nxrAB* operons (Lücker *et al.*, manuscript in preparation). All these findings indicate that the two *nxrAB* operons exhibit an ecophysiological “backup” for *Candidatus* Nitrospira defluvii to be able to react to changing environment conditions by expression of different *nxrA* paralogs (Gevers *et al.* 2004).

In this study, the exclusive expression of only one *nxrA* paralog in the genomes of *Nitrospira* sublineage I organisms could be observed in the main WWTP of Vienna. Possible

explanations of these findings are that i) the gene expression is regulated as a response to different external stimuli or ii) only one of the *nxrAB* paralogs is functional.

Maixner and colleagues (manuscript in preparation) observed that under starvation a certain amount of mRNA of *nxrB* is detectable in enrichment cultures of *N.defluvii*. It remains to be determined if just one of the *nxrAB* operons is expressed during starvation, because then one could assume that one *nxrAB* operon is expressed constitutively, while the transcription of the other operon copy is regulated by certain environmental conditions. In a recent study, it could be shown that two particulate methane monooxygenase (pMMO) isozymes with different methane oxidation kinetics are expressed differently in *Methylocystis* sp. strain SC2 (Baani *et al.* 2008). One pMMO isozyme was expressed constitutively, whereas the other type was expressed only at high-methane conditions. Furthermore, similar results were achieved in an expression analysis of two *nirS* copies in *Thauera mechernichensis* strain 27 (Etchebehere *et al.* 2005). Also in these denitrifying bacteria one gene copy was expressed constitutively, while the other *nirS* copy was positively regulated by nitrate.

Prospective incubation experiments with different nitrite concentrations and different substrates may be a key for better understanding of the ecophysiological role of the *nxrAB* duplicates.

D.2.1 Investigation of *nxrA* gene copies in the genome of *Nitrospira* sublineage II

The differences between the sequences received from the pure culture of *Nitrospira moscoviensis* ranged between 0.5%-8.7% for the short fragment (F151mix/R831) and 1.8%-3.3% for the long fragment (F814/R2062) on aa level. These results can be interpreted as a hint for the occurrence of multiple copies of *nxrA* in the genome of *Nitrospira moscoviensis*. It is still a open question whether multiple copies of *nxrA* indicate multiple copies of the whole *nxrAB* operon, like in *Candidatus* Nitrospira defluvii, or if they represent solo *nxrA* gene copies, as were found in the genome of *Nitrobacter winogradskyi* (Starkenburg *et al.* 2006).

Furthermore, it has to be tested in prospective studies if those multiple copies derive from the genome of a monoclonal culture or from a polyclonal culture.

Interestingly, the *nxrA* sequences received from the *Nitrospira moscoviensis* pure culture showed a higher similarity than the two *nxrA* copies within the genome of *Candidatus*

Nitrospira defluvii. It remains to be determined in prospective studies why there exist more *nxrA* copies in *Nitrospira moscoviensis* than in the genome of *Candidatus* Nitrospira defluvii. The answer to the question if the occurrence and ecological establishment of sublineage II organisms in various habitats are linked to a high number of *nxrA* copies in *Nitrospira moscoviensis*, would be another step to gain more insights into the ecophysiology of sublineage II of the genus *Nitrospira*.

D.3 Investigation of *nxrA* as a potential phylogenetic marker gene for the genus *Nitrospira*

Former studies showed that functional genes, involved in the nitrogen-cycle, like *nirK*, *amoA*, *amoC*, *nxrB*, *nxrX* could also serve as phylogenetic marker genes (Rotthauwe *et al.* 1997; Vanparys *et al.* 2007; Konneke *et al.* 2005). Especially for phylogenetically young groups it has been demonstrated that the phylogeny with functional genes leads to a fine-scale resolution of closely related populations, due to the higher diversity of functional gene sequences compared to 16S rRNA genes (Orso *et al.* 1994; Purkhold *et al.* 2000; Vanparys *et al.* 2007).

In this study, two different primer pairs were established to investigate the potential of *nxrA* as a potential phylogenetic marker gene for the genus *Nitrospira*. The amplicons of these two primer pairs differ in length as well in the binding region. The shorter *nxrA* fragment, amplified with the primer pair F151mix/R831, includes the region that encodes the Fe/S binding center of the active center of NxrA, whereas the primers F814 and R2062 are specific for the molybdenum lignand binding site of the protein and amplifies a longer fragment (Maixner *et al.*, manuscript in preparation).

Recently, a molecular approach for targeting a 322bp fragment of *nxrA* was developed to study the diversity of members of the genus *Nitrobacter* in different soil types (Poly *et al.* 2008; Wertz *et al.* 2008). The newly developed primers F1nor and R1nor that were used in these studies bind downstream of the region that encodes the molybdenum binding site.

D.3.1 Comparison of the *nxrA* phylogeny received from two different primer pairs

The phylogenetic trees received from the two *nxrA* datasets of the amplicons of the different primers are nearly identical. In both trees a clustering of sublineage I and sublineage II

sequences could be observed. Further sequences received from soil and WWTP clustered to different genomic *nxrA* copies of *Candidatus* Nitrospira defluvii. *Nitrospira moscoviensis* sequences also seem to build up a separate cluster containing multiple copies of *nxrA* in both trees (see fig. C.7 and fig. C.8).

Although both trees show a clustering of *Nitrospira* sublineage I and sublineage II sequences, the use of *nxrA* as phylogenetic maker gene might be problematic, due to the concurrent clustering of different *nxrA* copies in the genome of bacteria affiliated to the genus *Nitrospira*, compared to phylogenetic trees based on *nxrB* sequences (Maixner *et al.*, manuscript in preparation). A former study has already shown that the differences between multiple gene copies of *nxrA* affiliated to the genus *Nitrobacter* complicate the taxonomic interpretation of environmental samples (Poly *et al.* 2008).

Nevertheless, the comparison of the phylogenetic trees demonstrates that both datasets are incomplete. An indication of this incompleteness is that in the tree received from the shorter *nxrA* fragment, sequences from soil are affiliated to *Nitrospira moscoviensis*, whereas in the tree calculated with the dataset from the longer *nxrA* fragment, no clustering of soil sequences to *Nitrospira moscoviensis* could be observed. Also, the shorter *nxrA*-fragment dataset seems to be not complete due to the lack of soil sequences, which form a cluster with sublineage I *nxrA1*. This clustering could be observed in the phylogenetic tree calculated with the dataset from the longer *nxrA* fragment. Possible explorations of this observed incompleteness could lie either in a difference in coverage of the two studied primer pairs or in the too low number of sequenced clones.

Similar to the identical topology of the different phylogenetic trees of *nxrA* fragments, the comparison of the distance matrixes (table C.6) showed no significant differences between the similarities of the amplicons of the two primer pairs. As known from the analysis of the genome of *Candidatus* Nitrospira defluvii, the gene copies of the NxrA encoding gene *nxrA* differ from each other by 14.4% on amino acid level (Maixner *et al.*, manuscript in preparation). The parts of the *nxrA* gene, which is amplified by the primers F151mix and R831, as well as the region of the *nxrA* gene amplified by the primer pair F814/R2062, show a difference between the two *nxrA* copies in the genome of *Candidatus* Nitrospira defluvii of 13.1%. The high similarity between the two *nxrA* regions amplified by different primers pairs indicates that both primer pairs are equally suitable for analyses of the phylogeny of the genus

Nitrospira. However, the investigated fragments of the *nxrA* gene differ in their lengths. The longer region amplified by F814/R2062 compared to the shorter fragment amplified by F151mix/R831 might be better for phylogenetic analyses.

It still has to be examined whether *nxrA* can as a phylogenetic marker gene on the sublineage level by screening more *Nitrospira* pure cultures; because it also might be that the observed clustering does not reflect phylogeny, but functional similarity.

A hint that *nxrA* might have the potential to serve as a putative new phylogenetic marker gene is the detection of sublineage I affiliated bacteria in soil via the analysis of *nxrA* sequences and the confirmation of this finding on 16S rRNA level.

D.4 Detection of members of the sublineage I of the genus *Nitrospira* in soil

Soil represents a very heterogeneous ecosystem with a huge physio-chemical complexity and many micro-niches, where a very complex microbial community can be found in (Urich *et al.* 2008). Microorganisms in this environment are involved in the global cycles of carbon, sulfur and nitrogen and thus have an indispensable ecological function. Numbers of studies are dealing with nitrification in soils, due to its importance in agriculture and fertilization respectively (Avrahami *et al.* 2003, Bruns *et al.* 1999, Phillips *et al.* 2000, Fierer *et al.* 2009). More than the half of N-fertilizer is lost through leaching of nitrate, which is evoked by nitrifying organisms and dentrification processes (Freitag *et al.* 2005). However, most of the studies concerning nitrification in soil are focused on the oxidation of ammonia (Phillips *et al.* 2000; Webster *et al.* 2005; Avrahami *et al.* 2003). The discovery of AOA and their high abundance in soil led to a debate within the scientific community about their importance compared to their bacterial counterparts (Treusch *et al.* 2005; Leininger *et al.* 2006).

In contrast to AOA and AOB, knowledge about NOB in soil is still limited. Former studies showed the occurrence of *Nitrobacter*- and *Nitrospira*-like bacteria, mostly members of *Nitrospira* sublineage II in various soil types (Noll *et al.* 2005, Bartosch *et al.* 2002; Freitag *et al.* 2005;). Furthermore, in a recent study it was observed *Nitrospira*-like bacteria show a higher abundance in the rhizosphere of wild oat compared to the bulk soil (DeAngelis *et al.* 2009).

Although *Nitrospira* sublineage I was sporadically found in different soil types, like forest soil, corn field soil (Roesch *et al.* 2007) and fertilized grassland soil (Freitag *et al.* 2005),

sublineage I is still believed to represent a habitat-specific subcluster of the genus *Nitrospira* that is almost exclusively found in WWTPs.

In this study, representatives of *Nitrospira* sublineage I were found in two different soil types, in dry grassland soil from the botanical garden of Vienna and in unfertilized garden soil. The occurrence of sublineage I in soil was confirmed with *nxrA*, a putative new phylogenetic marker of the genus *Nitrospira* and on 16S rRNA level, with sublineage I specific primers.

Prospective studies are needed to investigate the abundance and the ecophysiological role of *Nitrospira* sublineage I in soil. Furthermore, expression analyses of *nxrA* copies in the genomes of *Nitrospira* sublineage I received from soil would be a further step to understand the role of the *nxrA* duplicates. Soil is more oligothrophic than WWTPs, so maybe, if the NxrA paralogs differ from each other in their substrate affinity a *nxrA* gene copy different from that in the WWTP would be expressed, but this hypothesis needs further investigations to be proved.

D.5 Micromanipulation of FISH-labeled *Nitrospira* sublineage II clusters in activated-sludge flocs of different WWTPs and further analyses

Activated-sludge flocs have a size of 40µm-125µm in average (Jorand *et al.* 1995; Nielsen 2002) and can be separated the one hand in the minor part (5%-20%) of bacterial cells, and on the other hand in extracellular polymeric substance (EPS), which is produced by the bacterial cells (Wilen *et al.* 2008). Nitrifying organisms are known to grow in dense cell aggregates that seem to form the fraction of the floc most resistant against deflocculation conditions. Former studies showed that different groups of microcolony-forming bacteria differ in their resistance against break-ups of their cell aggregates (Larsen *et al.* 2008; Klausen *et al.* 2004; Wilen *et al.* 2008). Members of the phyla *Nitrospira* and *Betaproteobacteria* form very strong microcolonies within activated-sludge flocs that remain almost intact under extreme physically shear conditions, due to strong adhesion characteristics of the cells (Klausen *et al.* 2004).

A recent study showed that the combination of FISH and micromanipulation can be used to gain more information on the bacterial composition of activated-sludge flocs (Thomsen *et al.* 2004). The focus of this study was the investigation of activated-sludge flocs, which harbored at least one *Nitrospira* sublineage II cell cluster. These flocs could be identified by using FISH and were successfully isolated by micromanipulation. Furthermore, the bacterial

diversity was determined by amplification of 16S rRNA genes by PCR and additional sequence analysis.

In a single activated-sludge floc from the WWTP of the University of Veterinary Medicine of Vienna with a size of approximately 40µm eight different bacterial phyla could be found. Although this high diversity could be detected in the microenviroment, no *Nitrospira* sublineage II sequence was received. Former studies showed that members of the genus *Nitrospira* were underestimated in PCR-based studies, compared to quantification with FISH (Juretschko *et al.* 2002) and microarray analyses (DeSantis *et al.* 2007). Furthermore, the same PCR-based underestimation could also be found for the physiological group AOB, where no known representatives could be detected in the sludge floc from the WWTP of the University of Veterinary Medicine of Vienna.

The occurrence of these groups of organisms in the mechanically-treated activated sludge floc from the WWTP of Ingolstadt was an indication for the biased lack of both *Nitrospira* sublinage II and AOB sequences in the Viennese WWTP sample, due to low cell disruption effectiveness of *Nitrospira* cells (Juretschko *et al.* 1998). It is of course difficult to compare activated sludge samples from two different WWTPs because the structure of the microbial community is strongly influenced by wastewater composition and design of the WWTP (Juretschko *et al.* 2002). Nevertheless, it is likely that a diversity reduction by minimizing the size of the activated sludge floc led to a reduction of the PCR bias for *Nitrospira* and AOB, which are often found in the close vicinity of *Nitrospira* (Schramm *et al.* 1998).

This study confirms results from former studies in terms of the stronger adhesion characteristic of *Nitrospira* cells and cells affiliated to the β-subclass of *Proteobacteria* (Larsen *et al.* 2008), due to the finding that all known AOB and NOB within the mechanical treated floc were affiliated with these phyla.

In this study, the diversity in the activated-sludge floc could be decreased via mechanical treatment by minmizing the size of the floc. This result shows the potential of mechanical treatment and micromanipulation to successfully isolate living *Nitrospira* organisms by using the strong adhesion properties of *Nitrospira* cells compared to other bacteria (Larsen *et al.* 2008).

Former studies already showed that by the use of micromanipulation, unknown bacteria could be successfully isolated out of very complex habitats, like soil (Ferrari *et al.* 2009; Dennis *et al.* 2008).

It is highly challenging to perform successful enrichment of *Nitrospira*-like bacteria from environment samples due to the occurrence of coexisting contaminants – *Nitrobacter*-like bacteria and heterotrophic microorganisms – that can overgrow a new *Nitrospira* enrichment culture very easily (Spieck *et al.* 2006). Members of the genus *Nitrobacter* are fast-growing organisms with lower affinity of nitrite compared to *Nitrospira*-like bacteria (Schramm *et al.* 1999; Kim *et al.* 2006) Therefore *Nitrobacter* can overgrow *Nitrospira* enrichment cultures in a medium with a higher nitrite concentration (Bartosch *et al.* 1999; Spieck *et al.* 2006). *Candidatus* Nitrospira defluvii was enriched by using dilution series and antibiotic to avoid the growth of *Nitrobacter*-like bacteria and heterotrophs, but dilution series are time and space consuming (Spieck *et al.* 2006).

By using micromanipulation combined with mechanical treatment the overgrowth would be also avoided, because *Nitrobacter* and *Nitrospira* cells could be separated by mechanical treatment due to the loose attachment to activated sludge flocs of *Alphaproteobacteria*, like *Nitrobacter* (Klausen *et al.* 2004; Larsen *et al.* 2008). Thus, a mechanically-treated activated-sludge floc could then serve as a putative *Nitrobacter*-free inoculum for further incubation of NOB.

Another field of application of the combination of FISH and micromanipulation with prior mechanical treatment lies in the isolation of *Nitrospira* cell cluster for genomic studies.

Isolation of single cells from environmental samples can be performed by several purification approaches, e.g. microfluidics, immunomagnetic capture, optical trapping and magneto-FISH (Marcy *et al.* 2007; Ashkin 1997; Yeung *et al.* 2002; Kalyuzhnaya *et al.* 2006). The choice of the appropriate isolation method depends on the target organisms, the characteristics of the environment as well as the throughput rate of the particular purification approach (Ishoey *et al.* 2008). Additionally, the combination of FISH and micromanipulation was used to detect and isolate target populations directly from various environmental samples in former studies (Lasken *et al.* 2005; Kvist *et al.* 2007). Subsequently, the genomic DNA can be amplified from a single isolated target cell by multiple displacement amplification (MDA) to achieve a sufficient amount of template DNA for single-cell genomic sequencing (Ishoey *et al.* 2008;

Lasken 2007; Dean *et al.* 2001; Hosono *et al.* 2003). Despite several disadvantages, like non-specific amplification, chimera formation and representation bias, MDA was successfully applied to allow single-cell genomic sequencing in former microbial ecology studies (Kvist *et al.* 2007; Mavingui *et al.* 2005; Mussmann *et al.* 2007; Binga *et al.* 2008). Furthermore, earlier studies showed that the use of multiple cells as template DNA decreases the representation bias (Bergen *et al.* 2005; Kalyuzhnaya *et al.* 2006; Binga *et al.* 2008) and that non-specific amplification, which is caused by contaminations could be reduced via micromanipulation by washing the template cells a few times (Ishoy *et al.* 2006; Kvist *et al.* 2007; Binga *et al.* 2008). Consequently, micromanipulation for isolation of a *Nitrospira* sublineage II cell cluster combined by MDA and 454 pyrosequencing could be a powerful tool to gain at least an extensive portion of the genome of bacteria belonging to *Nitrospira* sublineage II.

D.6 Analysis of the influence of low nitrite concentration and discontinuous aeration conditions on *Nitrospira* sublineage I and II

All living organisms have to deal with selective pressure and compete against other organisms for resources. Therefore different survival strategies evolved to successfully persist in a defined ecosystem. In ecology and also recently in the microbial ecology a model often used for different strategists is the concept of K-/r- selection (Andrews *et al.* 1986). This concept describes organisms with high growth rates and high substrate-turnover rates at high substrate concentrations as r-strategists, which dominate resource rich ecosystems. Conversely, organisms with low growth rates and high substrate affinity at low substrate concentrations can be characterized as K-strategists, which are adapted to stable and rather oligotrophic environments.

Former studies suggested that *Nitrospira* spp. represent typical K-strategists, whereas *Nitrobacter* spp. are r-strategists with a higher growth rate and lower affinities to nitrite and oxygen (Schramm *et al.* 1999; Downing *et al.* 2008; Kim *et al.* 2006; Nogueira *et al.* 2006; Blackburne *et al.* 2007; Sliekers *et al.* 2005).

Furthermore, in ecosystems, where members of *Nitrospira* sublineage I and sublineage II are present, a niche-differentiation due to different nitrite affinities can be observed. Former studies showed that sublineage I organisms can outcompete bacteria belonging to sublineage

II of the genus *Nitrospira* at high nitrite concentrations, whereas sublineage II like bacteria seems to be adopted to low nitrite concentrations (Maixner *et al.* 2006).

In this study, the influence of low nitrite concentration and discontinuous aeration conditions on *Nitrospira* sublineage I and II was analyzed with the goal to achieve an enriched culture of representatives of *Nitrospira* sublineage II. Therefore, two different activated-sludge samples were used as inoculum and were incubated in mineral medium with a low nitrite concentration. The WWTPs chosen for this enrichment experiment were known for their high content of bacteria belonging to *Nitrospira* sublineage II organisms in the activated sludge.

Although members of *Nitrospira* sublineage II were highly abundant in the activated sludge samples used as inoculum, a permanent decrease of biomass of both *Nitrospira* sublineage I and II was observed during the incubation in medium with low nitrite concentrations. These results contrast the findings of Maixner and colleagues (2006), who observed a relative increase of *Nitrospira* sublineage II compared to *Nitrospira* sublineage I at low nitrite concentrations. However, the designs of both incubation experiments were slightly different.

For example in the study of Maixner and colleagues (2006) 200mL Erlenmeyer flasks were used for incubation. Erlenmeyer flasks and a smaller volume seem to be better suitable for the initial phase of incubation of nitrifiers, because also Spieck and colleagues (Spieck *et al.* 2006) used Erlenmeyer flasks in the beginning of incubation for selective enrichment of *Candidatus* Nitrospira defluvii from activated sludge. Furthermore, the oxygen supply differed in both studies, due to the usage of porous stones at the end of the oxygen tube to minimize the size of the air bubbles in the incubated media from Maixner and colleagues (2006), whereas in this study the air tubes had just an open end, where larger air bubbles discarded. This aspect should not make any difference in the incubation design, since in WWTPs the aeration of the nitrification basins is very strong with a lot of large bubbles.

A possible explonation for the contrary results of the two studies (this study and Maixner *et al.* 2006) is the occurrence of different ecotypes in sublineage II. A recent showed that closely related members of the same genus represent different ecotypes. Coleman and colleagues (Coleman *et al.* 2007) discovered different *Prochlorococcus* populations that are adapted to high- and low-light conditions. A possible hint for the existence of various ecotypes in *Nitrospira* sublineage II, that are adapted differentially to high- and low- nitrite conditions, is

the presence of different sequence types belonging to *Nitrospira* sublineage II in fertilized soil compared to unfertilized soil (Freitag *et al.* 2005).

Since the incubated activated-sludge samples of this study and of Maixner and colleagues' (2006) were received from different WWTPs, the existence of sublineage II organisms being differentially adapted to various nitrite-, other N-compounds- and carbon-conditions cannot be excluded, due to the difference of the studied WWTPs in design and the composition of the sewage treated. The waste water treated in the WWTP of the University of Veterinary Medicine of Vienna consits predominantly of excrements from animals and therefore it is enriched in different carbon-compounds. It remains to be determined whether a *Nitrospira* sublineage II ecotype grows mixotrophically by using one of these carbon-compounds

The liquid from sluge dewatering form the WWTP of Ingolstadt that was also used as inoculum for the incubation experiments is highly enriched in ammonia. So in this habitat, the *Nitrospira* sublineage II organisms may be adapted to high nitrogen-conditions.

All these findings indicate a high ecophysiological diversity in sublineage II of the genus *Nitrospira* and further investigations are needed to determine this diversity in sublineage II and to gain more information about the ecophysiology of *Nitrospira* spp.

E. Summary

Nitrification is one of the key processes being a part of the biogeochemical nitrogen cycle and is performed by two physiological groups of prokaryote: ammonia oxidizers and nitrite oxidizers. The second step of nitrification, the oxidation of nitrite to nitrate, is catalyzed by nitrite-oxidizing bacteria (NOB). The oxidation of nitrite to nitrate is catalyzed by the membrane-associated enzyme nitrite oxidoreductase (Nxr), which consists at least two subunits, the large α-subunit and the small β-subunit, encoded by the *nxrAB* gene operon.

The focus of this study lies on the ecophysiological investigation of NOB of the genus *Nitrospira* in various environments. In the first part of this thesis the key enzyme of nitrite oxidation, Nxr, was investigated on protein-, as well as on DNA- and RNA-level. A heterologous expression of the *nxrAB* operon and the *nxrA* gene of *Candidatus* Nitrospira defluvii was performed to produce pure subunits, which then can be used to either investigate the activity of defined subunits, or to produce enough pure enzyme to characterize it by crystallographic methods. The heterologous expression of both the *nxrAB* operon and the *nxrA* gene led to the formation of inclusion bodies.

The potential of the *nxrA* gene that encodes the α-subunit of Nxr as a phylogenetic marker gene for the genus *Nitrospira* was tested. For that, two different primer pairs were designed to amplify fragments encoding the active center of NxrA. The amplicons of both primer pairs showed the same division into phylogenetic groups revealed by the 16S rRNA gene. Due to the usage of these *nxrA*-specific primer pairs the occurrence of *Nitrospira* sublineage I organisms, which was found mostly in WWTPs in various soil samples could be observed as well as a clustering of different *nxrA* copies in the genomes of *Nitrospira* sublineage I organisms. Expression analysis of the two copies of *nxrA* in the genomes of *Nitrospira* sublineage I organisms in the main WWTP of Vienna showed that only one of these gene duplicates was expressed during the sampling time point.

The second part of this thesis focuses on the application of FISH and micromanipulation to analyze bacterial diversity in the activated-sludge floc as a micro-environment, which habors *Nitrospira* sublineage II cellclusters. The comparison of the bacterial community in this microhabitat to the bacterial community of the whole WWTP showed no significant difference. Additionally, FISH-micromanipulation was used to isolate a cluster of *Nitrospira*

cells by minimizing the floc size which led to a decrease of diversity within. That procedure showed the potential of this method to isolate sublineage II organisms for genome sequencing directly from the activated-sludge floc.

Finally, in the last part of this thesis, activated sludge from two different WWTPs was incubated at low-nitrite concentrations and discontinuous aeration conditions to receive an enrichment of *Nitrospira* sublineage II. Although former studies showed that *Nitrospira* sublineage II seems to be better adopted to low-nitrite concentrations than sublineage I organisms, the incubation experiment in this study did not lead to a successful enrichment of members of *Nitrospira* sublineage II.

All these results show that prospective studies are needed to gain more insights into the ecophysiology of these slow-growing organisms to be able to optimize wastewater treatment processes.

F. Zusammenfassung

Nitrifikation stellt einen Schüsselprozess des globalen Stickstoffkreislaufes dar. Nitritoxidierende Bakterien (NOB) führen den zweiten Schritt der Nitrifikation, die Oxidation von Nitrit zu Nitrat durch, welcher von dem membranassozierten Enzym der NOB, der Nitritoxidoreduktase (Nxr) katalysiert wird. Die Nxr besteht aus mindestens zwei Untereinheiten, der nitritoxidierenden α-Unterheit und der kleineren β-Untereinheit, welche vom *nxrAB*-Operon kodiert werden.

Der Hauptaugenmerk dieser Studie lag auf der ökophysiologischen Untersuchung von NOB des Genus *Nitrospira* in unterschiedlichen Habitaten. Im ersten Teil dieser Studie wurde das Schüsselenzym der Nitritoxidation, die Nxr, auf unterschiedlichen Ebenen –auf RNA-, DNA- und Proteinebene – analysiert. Es wurde eine heterologe Expression des *nxrAB*-Operon, sowie des *nxrA* Genes des *Candidatus* Nitrospira defluvii durchgeführt, um weitere Einblicke in die Aktivität der einzelnen Untereinheiten der Nitrit oxidoreduktase zu erlangen. Allerdings führte die heterologe Expression des *nxrAB* operons, sowie die Expression des *nxrA*-Gens zur Bildung von Proteinaggregaten, sogenannten Inclusion Bodies.

Weiters wurde das Potential des *nxrA*-Gens, welches die Nxr α-Untereinheit kodiert, als Marker für die Phylogenie innerhalb des Genus *Nitrospira* untersucht. Zu diesem Zweck wurden zwei verschiedene Primer-Paare konstruiert, welche beide unterschiedliche Fragmente des aktiven Zentrums der NxrA amplifizieren. Die Amplifikate beider Primer zeigten die gleiche Unterteilung in einzelne phylogenetische Gruppen, ähnlich phylogenetischen Untersuchungen mit 16S rRNS Genen. Durch den Einsatz von *nxrA* als neues mögliches phylogenetisches Marker-Gen konnten Vertreter der *Nitrospira* sublineage I, die als typische Kläranlagenorganismen gelten, in Boden aufgefunden werden. Weiters konnte man ein Clustering von verschiedenen *nxrA*-Kopien innerhalb des Genomes von Nitrospira sublineage I Organismen beobachten. Expressionsanalysen der beiden *nxrA*-Duplikate innerhalb des Genoms von *Nitrospira* sublineage I aus der Hauptkläranlage Wien zeigten, dass zu dem Zeitpunkt der Probennahme nur ein *nxrA* Paralog exprimiert wurde.

Im zweiten Teil dieser Arbeit wurde die bakterielle Diversität innerhalb einer vereinzelten Klärschlammflocke mit Hilfe der Kombination zweier Methoden, FISH gekoppelt an Mikromanipulation, erforscht. Durch die Anwendung von FISH wurden Flocken identifiziert,

welche Zellcluster von *Nitrospira* sublineage II aufwiesen. Mittels Mikromanipulation wurde eine solche Flocke vereinzelt, um die bakterielle Diversität innerhalb dieses Mikrohabitats zu untersuchen. Der Vergleich der bakteriellen Diversität dieser vereinzelten Flocke mit jener der bakteriellen Gemeinschaft im gesamten Klärschlamm zeigte jedoch keine Unterschiede. Zusätzlich wurde das Potential der Kombination FISH-Mikromanipulation getestet, um einzelne Zellaggregate von *Nitrospira* sublineage II zu isolieren, welche mittels mechanischer Scherkräfte vom restlichen Flockenmaterial getrennt wurden. Die Reduzierung der bakteriellen Diversität innerhalb der verkleinerten Flocke zeigte, dass dieser Ansatz geeignet ist, um einzelne Zellcluster von *Nitrospira* sublineage II direkt aus dem Belebtschlamm zu isolieren.

Im letzten Teil dieser Arbeit wurde Klärschlamm von zwei verschiedenen Kläranlagen bei niedrigen Nitrit-Konzentrationen und diskontinuierlicher Belüftung inkubiert, um eine Anreicherung von *Nitrospira* sublineage II Organismen zu erreichen. Allerdings führte diese Inkubation zu keiner erfolgreichen Anreicherung von *Nitrospira* sublineage II.

Zusammengefasst zeigen die Ergebnisse dieser Arbeit die Notwendigkeit zukünftiger Untersuchungen, um weitere Einblicke in die Ökophysiologie dieser langsam wachsenden Bakteriengruppe zu erhalten, mit dem Ziel, Klärprozesse weiter optimieren zu können.

G. Appendix

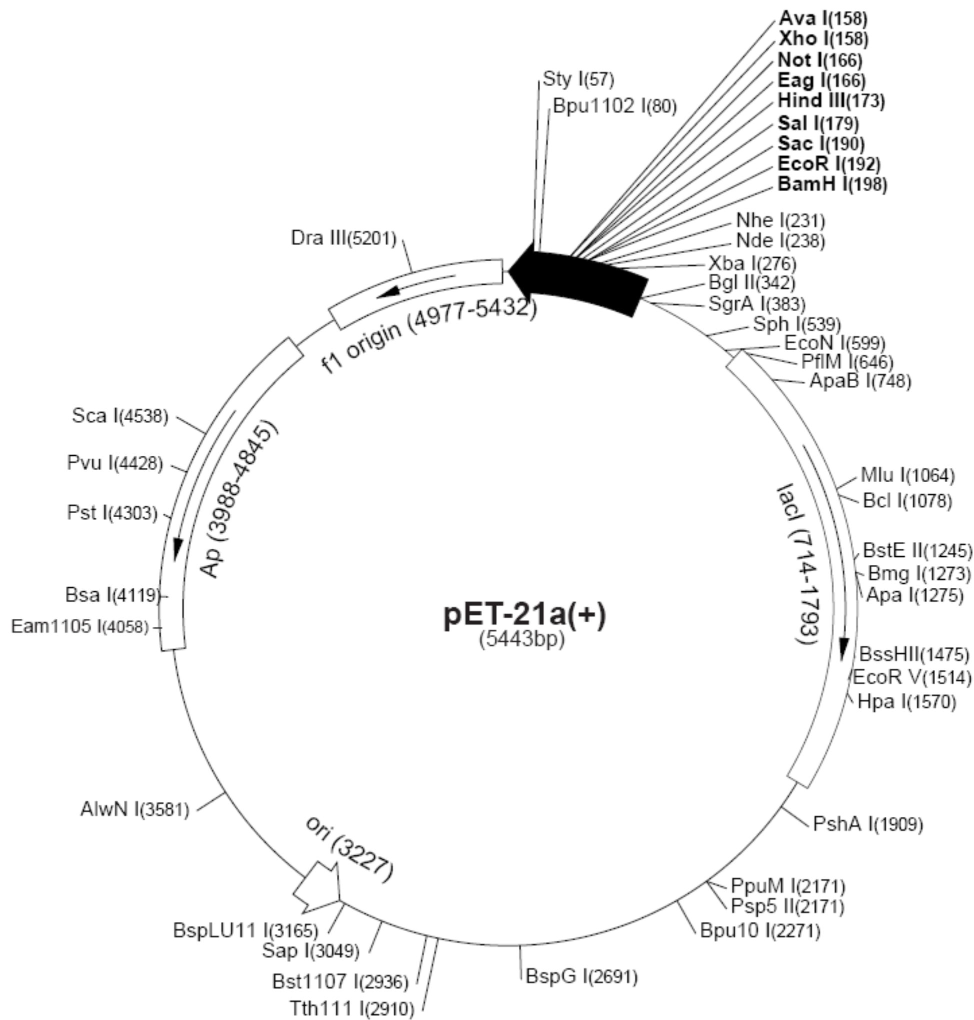
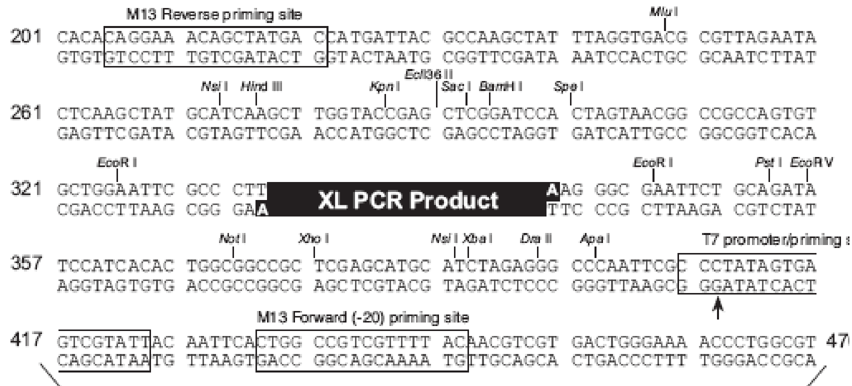
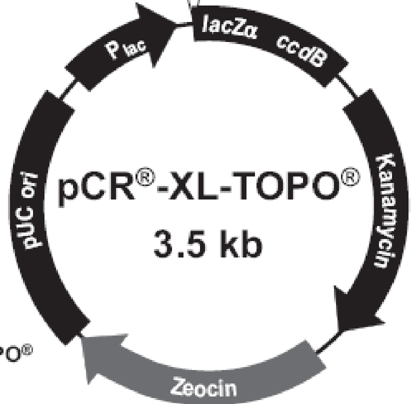


Figure: G.1: Map of the expression vector pET21a, which is identical to the map of pET21d(+)

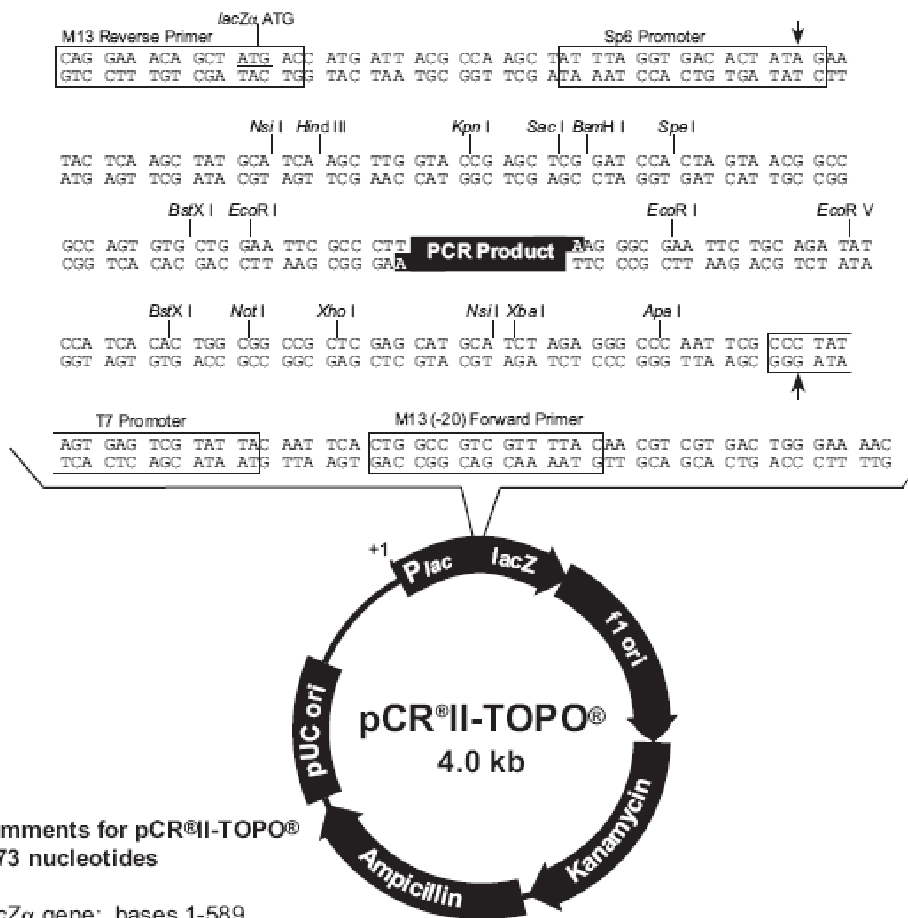




Comments for pCR®-XL-TOPO®
3519 nucleotides

Lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
Lac Za ORF: bases 217-576
Multiple Cloning Site: bases 248-399
TOPO® Cloning site: bases 336-337
T7 promoter priming site: bases 406-425
M13 Forward (-20) priming site: bases 433-448
Fusion joint: bases 577-585
*ccd*B lethal gene ORF: bases 586-888
Kanamycin resistance ORF: bases 1237-2031
Zeocin resistance ORF: bases 2238-2612
pUC origin: bases 2680-3393

Figure G.2: Map of the cloning vector TopoXL



Comments for pCR®II-TOPO®
3973 nucleotides

LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

Figure G.3: Map of the cloning vector pCR®II- TOPO of the TOPO TA cloning kit

G.1 Abbreviations

Table G.1: Table of abbreviations

α	alpha
β	beta
γ	gamma
λ	wavelength
µ	mikro (10-6)
°C	degree Celsius
%	percent
A	adenine
abs	absolute
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
APS	adenosine-5'-phosphosulfate
ARB	software package for phylogenetic analyses
bidist.	double distilled and filtered
BLAST	Basic Local Alignment Search Tool
Bp	base pair(s)
C	cytosine
c.d.	controlled disturbances
CLSM	Confocal Laser Scanning Microscope (or Microscopy)
Cy3	5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy3.18-derivative Nhydroxysuccimidester
Cy5	5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy5.18-derivative Nhydroxysuccimidester
DNA	desoxyribonucleic acid
dNTP	desoxy-nucleotide-tri-phosphate
E. coli	*Escherichia coli*
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 bomide
f	forward (used for labbeling of primers)
FA	formamide
Fig.	figure
FISH	fluorescence in situ hybridisation
FLUOS	5,(6)-carboxfluorescein-N-hydroxysuccimidester
g	gram(s)
G	guanine
h	hour(s)
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
k	kilo (103)
kan	kanamycin
KCl	potassium chloride
L	liter(s)
LB	Luria Bertani
m	milli (10-3)
M	molar
min	minute(s)
mol	mol
n	nano (10-9)
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NH_3	ammonia
NH_4^+	ammonium
N_2	dinitrogen
N_2O	nitrous oxide
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate

nxr, Nxr	nitrite oxidoreductase
NOB	nitrite-oxidizing bacteria
O2	molecular oxygen
o/n	overnight
p	pico (10-12)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
r	reverse (used for labelling of primers)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecyl sulphate
SBR	sequencing batch reactor
sec.	second(s)
sp.	species (singular)
spp.	species (plural)
Tab.	table
TAE	tris-actetate-EDTA
Taq	thermostable DNA-polymerase from *Thermus aquaticus*
TBE	tris-boric acid-EDTA
TEMED	N,N,N’,N’-tetra-methyl-ethylene-di-amine
Temp.	temperature
U	uracil
UV	ultraviolet
V	forward (used for labelling of primers)
Vol.	volume(s)
w/v	weight per volume
X-Gal	5-brom-4-chlor-3-indolyl-β-D-galactopyranoside

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