# Activity and ecophysiology of nitrite-oxidizing bacteria in natural and engineered habitats

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Boris Nowka

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Jun. Prof. Dr. Mirjam Perner

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## List of abbreviations

| | |
|---|---|
| °C | degree Celsius |
| % | percentage |
| µl | microliter |
| µm | micrometer |
| AOA | ammonia-oxidizing archaea |
| AOB | ammonia-oxidizing bacteria |
| BCA | bicinchoninic acid |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pairs |
| CDS | coding sequence |
| cm | centimeter |
| DAPI | 4',6-diamidino-2-phenylindole |
| DGGE | denaturing gradient gel electrophoresis |
| DNA | deoxyribonucleic acid |
| DO | dissolved oxygen |
| EM | electron microscopy |
| EPS | extracellular polymeric substances |
| Fig. | figure |
| FISH | fluorescence *in situ* hybridization |
| fmol | femtomole |
| g | gram |
| h | hour |
| HPLC | high-performance liquid chromatography |
| l | liter |
| mM | millimolar |
| min | minute |
| nm | nanometer |
| NOB | nitrite-oxidizing bacteria |
| NXR | nitrite oxidoreductase |
| PCR | polymerase chain reaction |
| RAS | recirculation aquaculture system |

| | |
|---|---|
| RFLP | restriction fragment length polymorphism |
| rpm | revolutions per minute |
| rRNA | ribosomal ribonucleic acid |
| s | second |
| SEM | scanning electron microscope |
| SMP | soluble microbial products |
| Tab. | table |
| TEM | transmission electron microscope |
| v | volume |
| WWTP | wastewater treatment plant |

# Chapter I

## Introduction

### *The nitrogen cycle*

The nitrogen cycle (Fig. 1.1) is a key process for life on earth. In the atmosphere and in natural waters nitrogen (N) exists mainly as dinitrogen gas ($N_2$). The smaller fraction of the total N is fixed as ionic and organic forms, but these are the forms that are elementary for biogeochemical processes. In the biomass, nitrogen is a major component of proteins and nucleic acids. During the course of the nitrogen cycle the oxidation states of nitrogen occur in a range from –III (ammonia) to +V (nitrate). These different nitrogen molecules serve as electron donors or acceptors in biological transformations, which are almost exclusively catalyzed by microorganisms. Due to these biological transformations the fluxes of nitrogen are large and most nitrogen forms occur only transitionally.

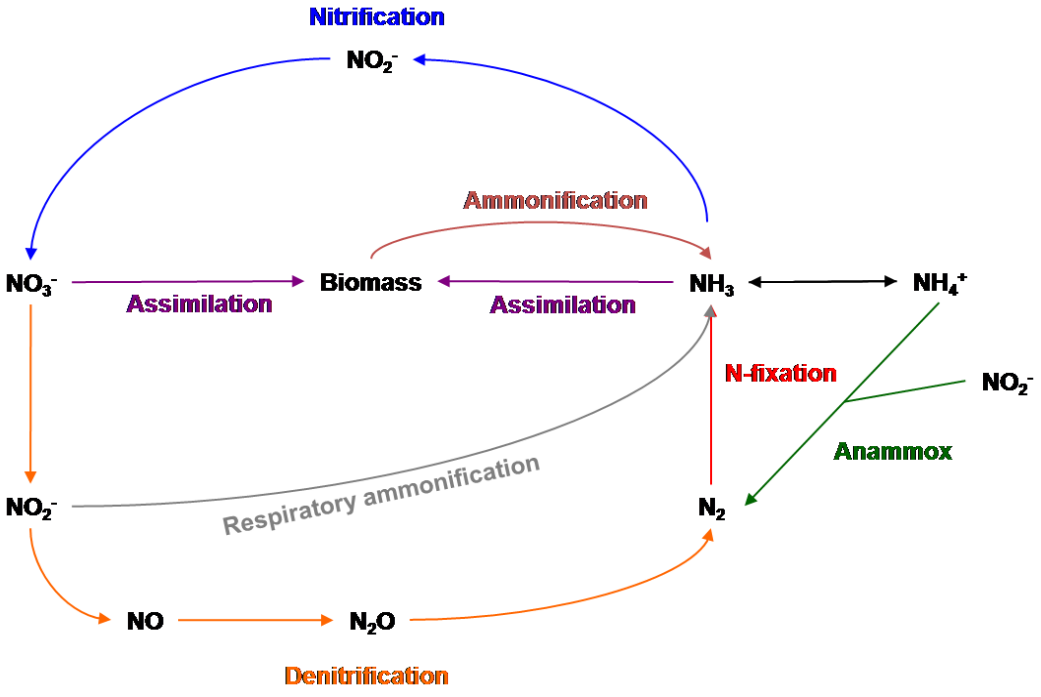


**Fig. 1.1:** Schematic illustration of the biogeochemical nitrogen cycle.

The input sources of fixed N are the biological and industrial nitrogen fixation and to a minor percentage natural processes like lightning. Nowadays, the anthropogenic creation of fixed nitrogen for fertilization use accounts for about the same quantities to terrestrial systems as biological nitrogen fixation by microorganisms (Galloway et al., 2004). The biological nitrogen fixation is catalyzed by archaea (Murray and Zinder, 1984) and bacteria, which reduce the inert dinitrogen gas to ammonia (Postgate, 1970). The fate of ammonia is to get either assimilated into biomass (Zehr and Ward, 2002) and later released by microbial ammonification (Burger and Jackson, 2003), or to get oxidized in two steps into nitrate by chemolithoautotrophic microorganisms in a process called nitrification (Prosser, 1989). Nitrate is assimilated into biomass by plants, fungi and bacteria (Lin and Stewart, 1997), or is used as respiratory substrate in oxygen-limited environments. In respiratory ammonification, nitrate is reduced via nitrite to ammonia (Simon, 2002), and in denitrification nitrate is reduced via a series of intermediates to dinitrogen (Zumft, 1997). A further dinitrogen generating process is the anaerobic ammonium oxidation (anammox), in which nitrite and ammonium are converted to dinitrogen gas (Kuypers et al., 2003).

### *Nitrification*

Nitrification is the process of the oxidation of ammonia to nitrate by two different functional groups of specialized chemolithoautotrophic microorganisms. The first step, the oxidation of ammonia to nitrite, is catalyzed by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). In a second step nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate. The activity of NOB prevents that nitrite accumulates to concentrations, which are toxic for most organisms in the environment (Philips et al., 2002). Further, it provides the oxidation product nitrate, a main nitrogen source for microbes and plants and a respiratory substrate in oxygen-limited environments.

Nitrification occurs in nearly every oxic environment, from rivers and lakes, aerated soils, ocean water and sediments, to biological wastewater treatment systems (WWTPs). At the oxic-anoxic interfaces of these ecosystems the nitrate generating process is coupled to anaerobic denitrification, in which the fixed nitrogen is converted to atmospheric dinitrogen. The bioavailable nitrogen inventory of the diverse ecosystems is regulated by the balance between inputs of ammonia from nitrogen fixation and the loss through denitrification.

### *Nitrification in wastewater treatment*

Besides the natural production of ammonia by degradation of organic matter, the anthropogenic input of fixed nitrogen has increased enormously over the last decades, especially through agricultural fertilization (Galloway et al., 2004). The benefit of an increased food production is attended by environmental problems like eutrophication of aquatic and terrestrial systems and a general acidification (Gruber and Galloway, 2008). Further, industrial and domestic sewage produces large amounts of anthropogenic ammonia. Thus, it is an important task of wastewater treatment plants to sufficiently remove nitrogen to prevent accumulation of toxic ammonia, nitrite and nitrate in receiving waters (Camargo and Alonso, 2006). Most WWTPs use an oxic nitrification stage and a subsequent anoxic denitrification stage. The different WWTPs use a variety of process flow sheets for nitrogen removal according to the characteristics of wastewater composition. Thus, the nitrification process underlies varying parameters like solids retention time (SRT), feeding and aeration patterns, and recycle ratio (Okabe et al., 2011). Due to these changing conditions, the microbial nitrification in WWTPs is sometimes difficult to maintain and occasionally breaks down. Nitrifying bacteria are slow-growing bacteria, which are sensitive to physical and chemical disturbances. Moreover, they are strongly affected by various environmental factors like temperature, pH, dissolved oxygen (DO) concentration, and wastewater composition with varying inhibitory substances (Daims and Wagner, 2010). In order to protect environmental health and to achieve the strict regulations on nitrogen discharge into receiving water bodies an efficient nitrogen removal is required, which is depending on the composition and functioning of microbial communities. An important issue is the interaction of the two functional groups which are specialized in either ammonia or nitrite oxidation. Often nitrite oxidation behaves more sensitively than ammonia oxidation and an unbalanced activity between AOB and NOB can result in nitrite accumulation to toxic concentrations (Knapp and Graham, 2007). Such an unbalanced activity was obtained in the presence of free ammonia at high pH, at high temperatures, and at low DO concentrations (Okabe et al., 2011), thus, these factors are important for establishing a stable nitrification process.

### *Ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA)*

The AOB are found in the *Betaproteobacteria* and *Gammaproteobacteria*. Currently, the genera *Nitrosomonas* and *Nitrosospira* comprise the betaproteobacterial AOB. Members of

both genera were found in WWTPs, where *Nitrosomonas* mostly constitutes the largest fraction of ammonia-oxidizing populations (Wagner and Loy, 2002) and *Nitrosospira* only prevails under adverse conditions such as low ammonium or DO concentrations (Li et al., 2007). The genera *Nitrosolobus* and *Nitrosovibrio* have been superseded by *Nitrosospira* due to high 16S rRNA similarities (Aakra et al., 2001). However, other molecular and morphological analyses support the separation into distinct genera (Ida et al., 2005). The only known genus within the *Gammaproteobacteria* is *Nitrosococcus*.

The discovery of AOA revealed a further group of nitrifying organisms, which are affiliated with the proposed phylum *Thaumarchaeota* (Brochier-Armanet et al., 2008; Spang et al., 2010). After the identification of ammonia monooxygenase (*amo*) genes on archaeal genome fragments (Treusch et al., 2005) and the first isolation of an ammonia-oxidizing archaeon (Könneke et al., 2005), an increasing number of representatives of the globally distributed AOA has been identified so far (Stahl and de la Torre, 2012). In wastewater treatment, AOA seem to have a minor role in nitrogen removal, although they were occasionally found WWTPs (Mußmann et al., 2011).

***Nitrite-oxidizing bacteria (NOB)***

NOB are widely distributed and have evolved a great diversity of populations adapted to most variable environments. Besides the richness of these bacteria in moderate habitats, NOB were also detected in extreme ecosystems like permafrost soils (Alawi et al., 2007), geothermal springs (Lebedeva et al., 2005), and alkaline soils and sediments (Sorokin et al., 1998). To date, six genera of aerobic chemolithoautotrophic NOB are known by phylogenetic classification (Fig. 1.2).

(i) The first described nitrite oxidizer belongs to the genus *Nitrobacter* within the *Alphaproteobacteria* (Winogradsky, 1892; Stackebrandt et al., 1988). Compared to most other NOB, members of this genus are easier to cultivate under increased nitrite concentrations. Since *Nitrobacter* are also distributed in a wide range of environments, most knowledge on NOB date back to the early isolates of four so far described *Nitrobacter* species. At first, *N. winogradskyi* (Winslow et al., 1917) was isolated from soils. The next species were the soil-derived *N. hamburgensis* (Bock et al., 1983) and *N. vulgaris* (Bock et al., 1990). Representatives of the latter nitrite oxidizer were also isolated from various environments like freshwaters, stones, and sewage (Bock et al., 1990). The most recent isolate originated from soda lake sediments and soda soil (Sorokin et al., 1998). Different molecular analyses

revealed that the aforementioned wide distribution is associated with a great diversity of *Nitrobacter* strains. Since the 16S rRNA gene sequences of *Nitrobacter* strains are highly similar (Orso et al., 1994; Freitag et al., 2005), other molecular strategies were used to clarify phylogenetic divergence. One approach illustrated the genomic heterogeneity by determining rRNA gene restriction patterns, DNA hybridization characteristics, and DNA base compositions (Navarro et al., 1992a). Other studies focused on the intergenic spacer region (IGS) as phylogenetic marker (Navarro et al., 1992b; Grundmann et al., 2000), and found a large microdiversity of *Nitrobacter* soil communities. Recent targets for analyzing the phylogenetic diversity of *Nitrobacter* were genes encoding subunits of the key enzyme of nitrite oxidation, the nitrite oxidoreductase (NXR) (Vanparys et al., 2007; Poly et al., 2007), offering a higher resolution than 16S rRNA to differentiate *Nitrobacter* strains.

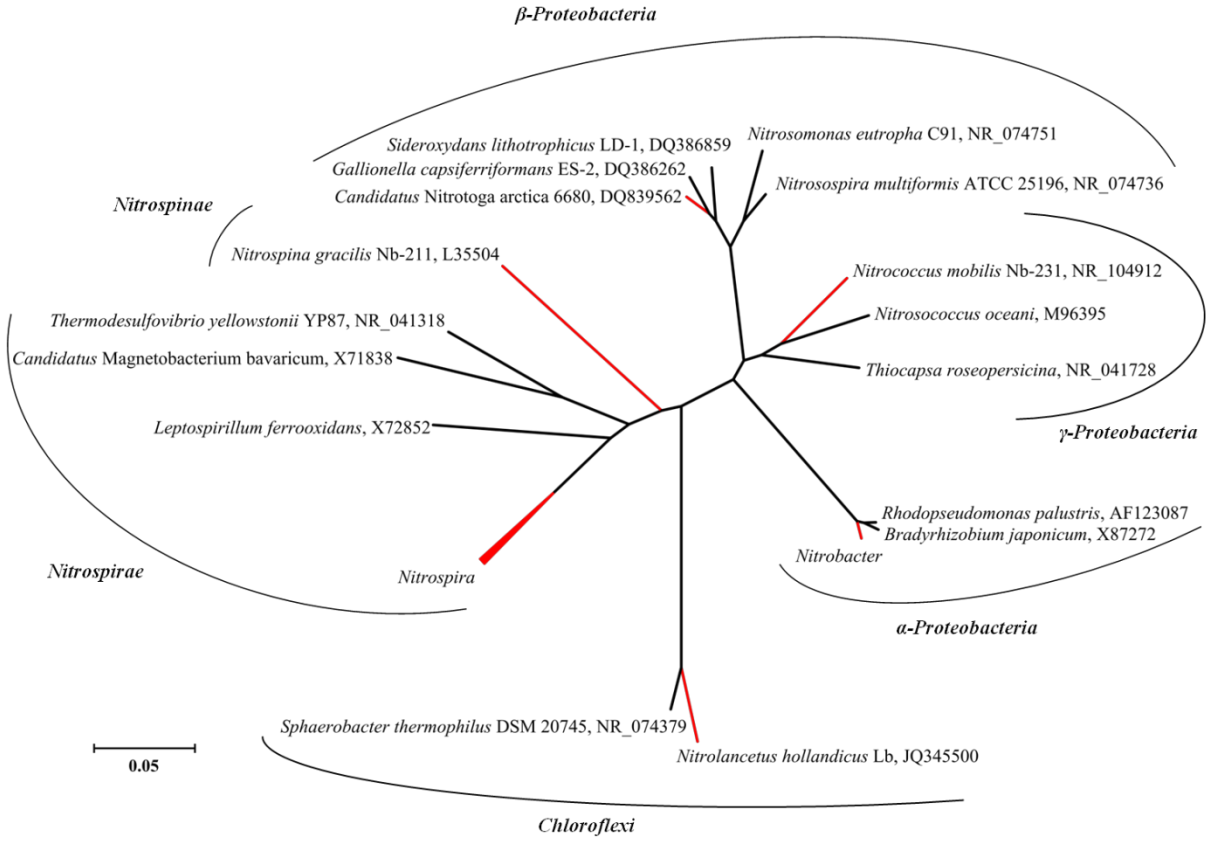


**Fig. 1.2:** 16S rRNA gene-based phylogeny reflecting the affiliations of nitrite-oxidizing bacteria. A neighbor-joining tree is shown. Nitrite-oxidizing bacteria are highlighted by red branches. Scale bar indicates 5% estimated sequence divergence.

(ii) Watson and Waterbury (1971) isolated one marine nitrite oxidizer from either of the two genera *Nitrococcus* and *Nitrospina* within the *Gammaproteobacteria* and *Nitrospinae*, respectively (Watson and Waterbury, 1971; Lücker et al., 2013). Strain *Nitrococcus mobilis* was derived from Pacific Ocean surface water, strain *Nitrospina gracilis* from Atlantic Ocean surface water. Recently, another *Nitrospina* species has been described (Spieck et al., 2014). *Nitrospina*-like bacteria were detected in several ocean water environments such as in oxygenated open ocean water (Fuchs et al., 2005; DeLong et al., 2006) and marine sediments, but also in anoxic marine sediments (Davis et al., 2009) and oxygen minimum zones (OMZs) (Labrenz et al., 2007; Fuchsman et al., 2011; Beman et al., 2013).

Since the isolation of *Nitrococcus mobilis* more than four decades ago (Watson and Waterbury, 1971), only few studies revealed the presence of members of this genus in ocean water, and non of these bacteria were identified in non-marine habitats yet. However, two studies identified *Nitrococcus*-like bacteria in OMZ waters (Ward et al., 1989; Füssel et al., 2012). Nitrite oxidation activities in these oxygen-limited settings were measured at high rates (Newell et al., 2011; Kalvelage et al., 2013; Beman et al., 2013), but no correlation to NOB abundance could be observed and sometimes substantial NOB populations were observed even without detectable nitrite oxidation activity (Füssel et al., 2012).

(iii) The genus *Nitrospira* within the *Nitrospirae* (Watson et al., 1986; Spieck and Bock, 2005) is regarded as the most diverse and widely distributed group of NOB. *N. marina* was the first described representative, isolated from ocean water (Watson et al., 1986). Several years later, *N. moscoviensis* was derived from urban heating water (Ehrich et al., 1995). The indicated wide distribution was further verified by a highly enriched culture of *Candidatus* N. defluvii from activated sludge of a WWTP (Spieck et al., 2006), followed by the two thermophilic pure cultures of *N. bockiana* (Lebedeva et al., 2008) and *N. calida* (Lebedeva et al., 2010) from geothermal springs, the marine isolate Ecomares 2.1 (Keuter et al., 2011), as well as the two strains ND1 and *N. japonica* (Fujitani et al., 2013; Ushiki et al., 2013) isolated from activated sludge. The long times between the first described *Nitrospira* species point out that members of this genus received initially less attention. However, after these nitrite oxidizers were found in nitrifying aquarium filters (Hovanec et al., 1998), lab scale reactors (Burrell et al., 1998), and WWTPs (Juretschko et al., 1998), it became obvious that *Nitrospira* are the most abundant NOB in these engineered systems. Further, the increasing number of 16S rRNA gene sequences indicated the wide distribution in the most varying environments (Daims et al., 2001). *Nitrospira* were detected in different soils (Dunbar et al., 1999; Bartosch et al., 2002; Freitag et al., 2005), sediments (Li et al., 1999; Altmann et al., 2003), freshwater

(Hovanec et al., 1998; Stein et al., 2001), subsurface fluids (Gihring et al., 2006; Swanner and Templeton, 2011), cave biofilms (Holmes et al., 2001; Chen et al., 2009), hot springs (Anitori et al., 2002; Kanokratana et al., 2004; Lebedeva et al., 2005), and marine sponges (Hoffmann et al., 2009; Off et al., 2010), costal water (Haaijer et al., 2013), and recirculation aquaculture systems (RAS) (Keuter et al., 2011; Brown et al., 2013). Analyses based on the currently available 16S rRNA gene sequences revealed six phylogenetic lineages within the genus *Nitrospira* (Daims et al., 2010), however, the increasing number of sequences with uncertain affiliation indicates a potential larger differentiation of *Nitrospira* phylogeny.

Lineage I mainly consists of *Nitrospira* sequences obtained from artificial ecosystems like WWTPs, and hitherto contains one enrichment culture of *Candidatus* N. defluvii (Spieck et al., 2006) and one pure culture of strain ND1 (Fujitani et al., 2013). Within the nitrifying community of engineered systems lineage I *Nitrospira* are considered to be the most abundant NOB (Juretschko et al., 1998; Okabe et al., 1999; Daims et al., 2001). Lineage II contains the two isolates *N. moscoviensis* from heating water (Ehrich et al., 1995) and *N. japonica* from a WWTP (Ushiki et al., 2013). Besides further members detected in activated sludge samples (Schramm et al., 1998; Maixner et al., 2006), sequences of this lineage were also retrieved from diverse habitats of soils and freshwater systems (Daims et al., 2010). In lineage III, only a few 16S rRNA sequences from Nullarbor cave system group together (Holmes et al., 2001), lacking cultivated representatives. Lineage IV, which contains the species *N. marina* (Watson et al., 1986), consists of marine *Nitrospira* including planktonic representatives from deep sea sampling sites and sediments, as well as sponge associated *Nitrospira* (Off et al., 2010). The most recently added *Nitrospira* lineages are lineage V, containing species *N. bockiana* from heating water (Lebedeva et al., 2008) and a few sequences obtained from soil, and lineage VI, which is formed by the moderate thermophilic strain *N. calida* (Lebedeva et al., 2010) and several sequences from hot springs.

(iv) All previously described nitrifying bacteria were mesophilic or thermophilic organisms. The first cold-adapted nitrite oxidizer was enriched by Alawi et al. (2007) from permafrost-affected soils of the Siberian Arctic, and named *Candidatus* Nitrotoga arctica. The newly discovered betaproteobacterium oxidized nitrite at a temperature range between 4°C and 22°C (Alawi et al., 2007). In addition to the enrichment of close related NOB from a wastewater treatment plant at temperatures of 10°C and 17°C (Alawi et al., 2009), 16S rRNA sequences related to *Nitrotoga*-like bacteria were detected in river biofilm (Brümmer et al., 2003), lake sediments (Schwarz et al., 2007), cave biofilm samples (Chen et al., 2009), nitrifying bioreactors (Karkman et al., 2011), and a drinking water filter (White et al., 2012).

The aforementioned enrichment of *Nitrotoga*-like bacteria from permafrost soil and activated sludge, and the diverse allocation of sequences with high similarity indicate that these NOB are more widely distributed in cold-affected environments than previously thought.

(v) The most recently discovered nitrite oxidizer is *Nitrolancetus hollandicus* (Sorokin et al., 2012), which belongs to the phylum of *Chloroflexi*. This organism was enriched and isolated from a laboratory-scale nitrifying bioreactor inoculated with sewage and was adapted to a broad temperature range of 25-63°C (Sorokin et al., 2012). Until now, no further nitrite oxidizer within the *Chloroflexi* has been detected.

### *Nitrite as an energy source*

NOB gain the energy required for growth from the oxidation of nitrite to nitrate. The key enzyme of this reaction is the nitrite oxidoreductase (NXR), which shuttles two electrons per oxidized $NO_2^-$ into the electron transport chain. The NXR is suggested to be a membrane-associated complex of three structural subunits (Sundermeyer-Klinger et al., 1984). The alpha subunit (NxrA) contains the substrate-binding site with a molybdopterin cofactor (Mo-co) (Meincke et al., 1992), and the beta subunit (NxrB) transfers the electrons from the NxrA to the membrane-integral gamma subunit (Kirstein and Bock, 1993), which is a membrane anchor of the holoenzyme and channels the electrons to the electron transport chain (Rothery et al., 2008). However, the exact compositions of the NXR complexes of the diverse NOB are structurally different and await further experimental clarification.

The NXR of *Nitrospira* is located on the outer site of the cell membrane, facing the periplasmic space (Spieck et al., 1998). Thus, nitrite oxidation occurs outside of the cell and no nitrite/nitrate transport is needed. The electron translocation through the cytoplasmic membrane and the consumption of protons in the cytoplasm during the reduction of $O_2$ creates a proton gradient for ATP production by a membrane-bound ATP synthase (Hooper and DiSpirito, 1985). In contrast, the NXR of *Nitrobacter* is located on the cytoplasmic site of the cell membrane and on the intracytoplasmic membranes (ICM) (Spieck et al., 1996). Therefore, a transport of nitrite and nitrate through the cytoplasmic membrane is needed (Starkenburg et al., 2006). The exact mechanism of energy conservation on the cytoplasmic side of the inner membrane awaits further investigation.

The process of nitrite oxidation is described by the following equation:

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

$2\ H^+ + 2\ e^- + ½\ O_2 \rightarrow H_2O$

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$NO_2^- + ½\ O_2 \rightarrow NO_3^- \qquad \Delta G_0' = -74.05\ kJ \cdot mol^{-1}$

For *Nitrobacter*, it was demonstrated that the NXR also catalyzes the reversible reaction, the reduction of nitrate under oxygen-limited conditions and with organic compounds as electron donors (Sundermeyer-Klinger et al., 1984; Freitag et al., 1987).

***Activity and ecophysiology of NOB***

With the recently discovered nitrifying bacteria of *Nitrotoga* and *Nitrolancetus*, six genera of NOB are known. Since these groups appear to be phylogenetically and physiologically most diverse, the question arises which factors provide niche specialization and differentiation. Previous studies especially addressed the competition of *Nitrobacter* and *Nitrospira* in activated sludge of WWTPs (Wagner et al., 2002), where the metabolic activity and growth parameters of nitrifiers are an important issue for the operational performance and stability. First investigations aimed at nitrite availability as a major factor for niche differentiation. Referring to the suggested ecological differentiation of microorganisms (Andrews and Harris, 1986), Schramm et al. (1999) proposed that *Nitrobacter* can be regarded as *r*-strategists with high maximum nitrite oxidation activity but low substrate affinity. In contrast, *Nitrospira* are regarded as *K*-strategists, which are characterized by low maximum activity but high substrate affinity. This might be one explanation why *Nitrobacter* achieve higher growth rates in laboratory cultures under high nitrite concentrations. Further, it indicates the advantage of *Nitrospira* under limited nitrite availability in wastewater treatment and in most natural environments. Several follow-up studies confirmed these contrary strategies between both genera (Kim and Kim, 2006; Nogueira and Melo, 2006; Blackburne et al., 2007). The high phylogenetic diversity within the genus *Nitrospira* implied also physiological differences within genera. Indeed, Maixner et al. (2006) could demonstrate that higher nitrite concentrations favored the growth of lineage I and selected against lineage II *Nitrospira*.

Beside the importance of nitrite availability several other factors determining the abundance and distribution of NOB were discussed, for example that niche separation occurs according to varying preferences for DO concentrations, due to a higher affinity for oxygen of *Nitrospira* compared to *Nitrobacter* (Okabe et al., 1999; Schramm et al., 1999; Downing and Nerenberg, 2008). Again, a varying selectivity was observed also within the genus *Nitrospira*, where community shifts of lineage I and II occurred due to differing DO concentrations (Park and Noguera, 2008).

Another ecological key parameter is the temperature. As described in the previous chapter, NOB have most diverse preferences for growth temperatures. A study suggested that variations of the seasonal temperature changed the community composition of nitrifying bacteria in several WWTPs (Siripong and Rittmann, 2007). Additionally, a cultivation-based approach studied the influence of temperature on the population structure of NOB (Alawi et al., 2009), revealing the advantageous growth of *Nitrotoga* under low temperatures.

For some NOB it was shown that organic compounds can stimulate growth (Steinmüller and Bock, 1976; Watson et al., 1986; Keuter et al., 2011), other studies observed no (Ehrich et al., 1995) or even inhibitory effects by organic substrates (Watson and Waterbury, 1971). Thus, organic compounds may select for different NOB and their capacity for growing mixotrophically.

So far, investigations on the selective effect of pH had received little attention. Until now, the only known alkaliphilic NOB is *Nitrobacter alkalicus* from soda lakes and soils (Sorokin et al., 1998). In low pH environments, evidence for nitrification was obtained especially in biofilms, indicating that nitrifiers had adapted to acidic conditions (Gieseke et al., 2006).

Secondary products from paper-bleaching, coke and steel processing industries like phenol, cyanide, and chlorate/chlorite can have strong inhibitory effects on nitrification in wastewater and other environments. A previous study suggested a resistance of *Nitrospira defluvii* against chlorite by expressing the enzyme chlorite dismutase (CLD) (Maixner et al., 2008). This enzyme converts chlorite ($ClO_2^-$) to chloride ($Cl^-$) and oxygen (Van Ginkel et al., 1996), thus indicating a protective function against chlorite, which occurs as byproduct of the chlorination of activated sludge. Furthermore, a cultivation-based approach and genome analysis have revealed that *N. defluvii* possesses genes coding for beta-lactam antibiotics (Spieck et al., 2006; Lücker et al., 2010).

### *Aim of the study*

The current knowledge of the ecophysiology and niche differentiation of NOB is mainly composed of characterizations of few representatives. However, in recent years several investigations have revealed an enormous phylogenetic diversity, especially within the genus *Nitrospira*. This leads to the consideration that single populations within the known six genera of NOB have their own distinct ecophysiological niches. To address this hypothesis, a main objective of this study was to isolate two *Nitrospira* strains from wastewater, which belong to main *Nitrospira* lineages I and II. Subsequently, these nitrite oxidizers should provide morphological and physiological insights. Furthermore, the study aims at determining the nitrite oxidation kinetics and growth parameters of the two new isolates in comparison to five other NOB from the genera *Nitrobacter*, *Nitrospira* and newly discovered *Nitrotoga*, which has been studied to only a limited extent.

### *Overview of chapters and work contributions*

The general introduction in **Chapter I** has provided background information on the current knowledge related to the topics of this study. It started with an overview of the processes in the nitrogen cycle and the ecological role of nitrification in the environment, especially in wastewater treatment. The next section focused on the diversity and abundance of the particular functional group of NOB, followed by the description of known factors providing niche differentiation. Finally, the mechanisms of nitrite oxidation and energy generation were described.

**Chapter II** deals with the diversity and characteristics of *Nitrospira* lineages I and II. Several directed approaches were used to isolate two strains from activated sludge. The isolated strain *Nitrospira defluvii* A17 and *Nitrospira* strain BS10 were subsequently investigated in morphological and physiological traits. Additionally, a variety of enrichments in *Nitrospira* lineages I and II from extreme and moderate natural ecosystems were phylogenetically analyzed.

This manuscript (in preparation) was conceived and written by Boris Nowka. B.N. performed the isolation of *Nitrospira* strain BS10, the physiological experiments and the morphological analyses. Sandra Off performed the isolation of *N. defluvii* A17 and the

extraction and cloning of the 16S rRNA gene sequences from *Nitrospira* enrichments. B.N. and S.O. performed physiological and morphological analyses of *N. defluvii*.

In **Chapter III**, the nitrite oxidation kinetics and growth parameters of members of the three most abundant genera of NOB in non-marine habitats were investigated. A highly sensitive microsensor respiration system was used to measure nitrite oxidation kinetics of six pure cultures – three of *Nitrobacter* and three of *Nitrospira* – and one enrichment culture of recently discovered *Nitrotoga*. Further, a new method for biofilm disruption was developed, enabling cell quantification of hitherto uncountable cell aggregations. The comparison of the different strains was completed by the determination of the specific growth characteristics.

This manuscript (in preparation) was conceived and written by Boris Nowka. B.N. performed all experiments and analyses in this study.

**Chapter IV** contains the summary and conclusion of the presented studies in English and German.

# Chapter II

## Natural distribution of *Nitrospira* lineage I and II and differentiation of two new isolates from activated sludge by morphological and physiological features

Boris Nowka[1], Sandra Off[1], Holger Daims[2], and Eva Spieck[1]

[1]Biocenter Klein Flottbek, Microbiology and Biotechnology, University of Hamburg, Ohnhorststr. 18, D-22609 Hamburg, Germany

[2]Department of Microbiology and Ecosystem Science, Division of Microbial Ecology, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria

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Keywords: Nitrification / *Nitrospira* / diversity / isolation / wastewater / physiology

**Abstract**

The second step of nitrification, the oxidation of nitrite to nitrate, is vital for the functioning of the nitrogen cycle in natural and engineered ecosystems. Nevertheless, our understanding of this process is still limited. Members of the genus *Nitrospira* are the most widely distributed and diverse known nitrite-oxidizing bacteria. During recent years, the diversity of *Nitrospira* 16S rRNA and functional gene sequences has increased remarkably, but only few representatives could be brought into laboratory cultures. In this study, the number of cultured *Nitrospira* was expanded by a variety of enrichments in lineages I and II, which were derived from extreme and moderate ecosystems such as permafrost-affected soils and freshwater. Further, two *Nitrospira* from activated sludge were isolated using novel approaches. Enriched lineage I *N. defluvii* was separated from concomitant heterotrophs by taking advantage of its resistance against ampicillin and acriflavine. A novel lineage II *Nitrospira*, named *N. lenta*, was initially enriched at 10°C in co-culture with *Nitrotoga* and finally purified by cell sorting. Both strains were characterized with regard to biofilm formation, tolerated levels of nitrite and nitrate, and temperature optima for growth. The new cultures will help to further explore physiological and genomic differences between *Nitrospira* from widespread lineages of this highly diverse genus.

## Introduction

The two-step process of nitrification is the microbially catalyzed oxidation of ammonia to nitrite and further to nitrate. The second step, nitrite oxidation, is performed by chemolithoautotrophic nitrite-oxidizing bacteria (NOB), a phylogenetically heterogeneous group (Teske et al., 1994; Ehrich et al., 1995; Lücker et al., 2013). Since their discovery in ocean water (Watson et al., 1986), NOB of the genus *Nitrospira* are considered as the main drivers of nitrite oxidation in a wide range of environments. Their distribution encompasses extreme environments like permafrost soils (Alawi et al., 2007) and hot springs (Lebedeva et al., 2005), moderate habitats in soils (Freitag et al., 2005) and sediments (Altmann et al., 2003) as well as sea- (Watson et al., 1986) and freshwater (Hovanec et al., 1998). Moreover, *Nitrospira* are the dominant NOB in most wastewater treatment plants (WWTPs) (Burrell et al., 1998; Juretschko et al., 1998; Daims et al., 2001). The genus *Nitrospira* forms a deeply branching lineage in the bacterial phylum *Nitrospirae* (Ehrich et al., 1995). Until now, *Nitrospira* has been divided into six phylogenetic lineages (Daims et al., 2001; Lebedeva et al., 2008; Lebedeva et al., 2010), of which lineages I and II have been detected in wastewater treatment facilities (Schramm et al., 1998; Daims et al., 2001; Lopez-Vazquez et al., 2013). Functional differences between these lineages include preferences for different concentrations of nitrite and dissolved oxygen (Maixner et al., 2006; Park and Noguera, 2008). In addition, Siripong and Rittmann (2007) concluded that the temperature may influence *Nitrospira* community composition.

Although *Nitrospira* are ubiquitously distributed, insights into their physiology are limited due to a small number of laboratory cultures and genomic sequences. As *Nitrospira* are notoriously recalcitrant to separation from heterotrophs, only the few members of *N. marina* (Watson et al., 1986; Keuter et al., 2011), *N. moscoviensis* (Ehrich et al., 1995), *N. bockiana* (Lebedeva et al., 2008), *N. calida* (Lebedeva et al., 2010), and *N. japonica* (Ushiki et al., 2013) have been isolated so far. Thus, much of the current knowledge about ecology and physiology of *Nitrospira* is based on studies performed with *in situ* techniques (Schramm et al., 1999; Daims et al., 2001; Altmann et al., 2003; Maixner et al., 2006) or enrichment cultures (Spieck et al., 2006; Alawi et al., 2009; Off et al., 2010).

The increasing anthropogenic nitrogen deposition has immense impact on the N-cycle, including the eutrophication of terrestrial and aquatic ecosystems and global acidification (Gruber and Galloway, 2008). Thus, the elimination of excess nitrogen from domestic and industrial wastewaters is crucial for environmental protection. Nitrification, including the activity of *Nitrospira*, is an essential step of this process and the lack of *Nitrospira* cultures

for advanced biological characterization is particularly unsatisfying in this context. Here we report on the isolation of two *Nitrospira* species from lineages I and II, which were derived from domestic activated sludge. This purification success was achieved by a combination of mechanical techniques for cell separation and chemical and antibiotic treatment for inhibition of heterotrophs. As a result, we present two different approaches for the directed isolation of *Nitrospira* and NOB in general.

## Materials and methods

### *Sampling sites*

*Nitrospira defluvii* (strain A17) and *Nitrospira* strain BS10 were separately isolated from enrichment cultures, which had been inoculated with activated sludge from the nitrification stage of the municipal WWTP in Köhlbrandhöft/Dradenau, Hamburg, Germany. The activated sludge samples were taken between November 1997 and December 1998 (*N. defluvii*) (Spieck et al., 2006) and in December 2006 (strain BS10) (Alawi et al., 2009). The Movile Cave in south-east Romania is an oxygen-limited freshwater ecosystem based on chemolithoautotrophy (Sarbu et al., 1996; Chen et al., 2009). Samples were taken in 2002 and 2003 from bacterial floating mats of a subsurface lake. Permafrost-affected soils in Samoylov Island, Lena Delta, Russia were sampled in 2004 (Alawi, 2007) from the active layer of a polygon transect (LD1-LD8) and a cliff (3301-3309). Samples of sandy to loamy soils were collected in African drylands in 2004 (Herpel, 2008): Namibian soils from Nama Karoo (Gellap East) (sample 74 without biocrust, sample 80 with biocrust) were collected in a depth of 0-5 cm. This region represents a shrub savanna with low annual rainfall. The second African sampling place was the desert ecoregion of Succulent Karoo in South Africa (Soebatsfontein), a desert region with the richest succulent flora on earth. In addition, samples were taken from a freshwater aquarium in 2011 and from Ebenheider sandstone in 2003 (Off, 2012).

### *Cultivation conditions*

The enrichment cultures from natural habitats were incubated at 17°C (permafrost soil), 22°C (Movile Cave), and 28°C (African soils; aquarium freshwater; sandstone). The enrichments from activated sludge containing *Nitrospira defluvii* (strain A17) or *Nitrospira*

strain BS10 were incubated at 28°C. The enrichments were performed as 0.15 to 3 l batch cultures in mineral medium with the following composition: 1000 ml distilled water, 0.02 g $NaNO_2^-$ as sole energy source, 0.007 g $CaCO_3$, 0.5 g NaCl, 0.05 g $MgSO_4$ x 7 $H_2O$, 0.15 g $KH_2PO_4$, 33.8 µg $MnSO_4$ x $H_2O$, 49.4 µg $H_3BO_3$, 43.1 µg $ZnSO_4$ x 7 $H_2O$, 37.1 µg $(NH_4)_6Mo_7O_{24}$, 973.0 µg $FeSO_4$ x 7 $H_2O$, 25.0 µg $CuSO_4$ x 5 $H_2O$. The pH was adjusted to 8.4 to 8.6 and changed to 7.4 to 7.6 two days after autoclaving. The cultures were started with inocula of 1% (v/v) and incubated in the dark. After the first detection of nitrite consumption, the cultures were stirred moderately (100 to 300 rpm) and nitrite was added frequently when consumed. The purity of the cultures (in terms of absence of heterotrophs) was tested repeatedly by incubating aliquots of the cultures in complex liquid (0.5 g $l^{-1}$ bactopeptone, 0.5 g $l^{-1}$ yeast extract, 0.5 g $l^{-1}$ meat extract, 0.584 g $l^{-1}$ NaCl in distilled water) or solid medium (2.5 g $l^{-1}$ meat extract, 2.5 g $l^{-1}$ casamino acids, 0.5 g $l^{-1}$ yeast extract, 1.0 g $l^{-1}$ $KH_2PO_4$, 0.5 g $l^{-1}$ NaCl, 15.0 g $l^{-1}$ agar in distilled water), and on mixotrophic solid medium (0.02 g $l^{-1}$ $NaNO_2^-$, 0.15 g $l^{-1}$ yeast extract, 0.15 g $l^{-1}$ bactopeptone, 0,055 g $l^{-1}$ sodium pyruvate, trace elements [see mineral medium], 15.0 g $l^{-1}$ agar in distilled water).

If not stated otherwise, subsequent experiments were performed with the obtained pure cultures of *N. defluvii* strain A17 and *Nitrospira* strain BS10.

*Enrichment and isolation*

Growth of *N. defluvii* was tested on solid medium plates, which were composed of mineral medium (see above) with 0.3 mM $NaNO_2^-$ as sole energy source and 13.0 g $l^{-1}$ agarose. Serial dilutions ($10^{-1}$ up to $10^{-8}$) were performed by stirring in glass tubes containing 5 ml mineral medium with 0.3 mM $NaNO_2^-$. To disrupt *Nitrospira* microcolonies into single cells, glass beads with a size of 1.7 to 2.0 mm were added and stirred for 10 s using a Vortex mixer at full speed. Nitrite-consuming cultures of the highest dilution were fed 3 to 4 times and then used as inocula for 150 ml cultures. The separation of *Nitrospira*-like bacteria from contaminants by Percoll density gradient centrifugation was performed as described by Ehrich and colleagues (1995). Ampicillin (50 µg $ml^{-1}$) was added to the enrichment of *N. defluvii* as described previously (Spieck et al., 2006). Cultures were further treated with the antiseptic acriflavinium chloride (acriflavine) in concentrations of 0.21 µg $ml^{-1}$ by using commercial sera baktopur® (sera GmbH, Heinsberg, Germany).

*Cell sorting*

Cells of the enrichment culture of *Nitrospira* strain BS10 were separated by an optical tweezers system (PALM MicroTweezers, Carl Zeiss Microscopy GmbH, Munich, Germany). The isolation was performed in a microscopy chamber (µ-Slide VI flat ibiTreat; ibidi GmbH, Martinsried, Germany) with two wells, which were connected by a capillary (30 µl volume). One well and the capillary contained 10 µl of sterile mineral medium, and the other well was filled with 10 µl of the enrichment culture of strain BS10. Single cells of the enrichment were visualized by bright-field microscopy at 1000x magnification (Axio Observer.Z1, Carl Zeiss, Jena, Germany), trapped by an infrared laser (wavelength of 1064 nm and 3 W output power), and moved through the capillary (approx. duration of 2-4 min) into fresh medium. The 10 µl inoculated medium was then transferred into a 1.5 ml tube containing mineral medium with 0.3 mM nitrite and incubated at 22°C.

*Chemical analyses*

Nitrite and nitrate concentrations were determined by high-performance liquid chromatography (HPLC) via ion-pair chromatography with a LiChrospher® RP-18 column (5 µm; 125 by 4 mm; Merck KGaA, Darmstadt, Germany) (Meincke et al., 1992) and UV detection in an automated system (HPLC-System LaChrom Elite®, VWR International GmbH, Darmstadt, Germany). Cell protein concentrations were measured by the bicinchoninic acid assay (BCA) (Smith et al., 1985) after cell lysis in 0.15 M NaOH and incubation at 90°C for 30 min.

*Electron microscopy*

For scanning electron microscopy (SEM) with a LEO 1525 electron microscope (Carl Zeiss, Jena, Germany) and for transmission electron microscopy (TEM) with a LEO 906E electron microscope (Carl Zeiss, Jena, Germany), pelletized cells were prepared as described previously (Keuter et al., 2011).

*Fluorescence* in situ *hybridization*

Aliquots of the *N. defluvii* isolate were fixed with paraformaldehyde (Amann et al., 1995). Fluorescence *in situ* hybridization (FISH) was performed as described elsewhere (Manz et al., 1992) with the 16S rRNA-targeted, Cy3-labeled probe S-G-Ntspa-0662-a-A-18, specific for the genus *Nitrospira* (Daims et al., 2001). Following FISH, the cells were stained with 4′,6-Diamidin-2′-phenylindol-dihydrochlorid (DAPI) and visually analyzed with an Axio ImagerM2 epifluorescence microscope (Carl Zeiss, Jena, Germany).

*Molecular and phylogenetic analyses*

DNA was extracted using the UltraClean Microbial DNA Isolation kit (MoBio Laboratories, Inc., USA) according to the manufacturer's instructions. Bacterial 16S rRNA genes were amplified by PCR with primer pair 27f/1492r (Lane, 1991) or primer pair 27f/Ntspa1158R (Lane, 1991; Maixner et al., 2006).

The PCR products were ligated into the pBluescript II SK+ vector cloning system (Stratagene, La Jolla, USA) and transformed into chemically competent cells according to the manufacturer's instructions. Prior to Sanger-sequencing, the plasmid primers T7 and T3 were used to re-amplify the cloned inserts. The 16S rRNA gene sequences were compared to sequences in public databases by Basic Local Alignment Search Tool (BLAST) searches (Altschul et al., 1990). Sequence alignment by ClustalW and neighbor-joining phylogenetic inference with the Tamura-Nei substitution model were performed using the MEGA software (Tamura et al., 2011). Bootstrap support for the phylogenetic tree was determined based on 1,000 iterations.

For specific PCR-amplification of 16S rRNA genes from *Nitrotoga*-like bacteria, the primer set NTG200F/NTG840R was used according to Alawi et al. (2007). For PCR-amplification of nitrite oxidoreductase subunit beta genes (*nxrB*) of *Nitrospira*, the primer set nxrB169f/nxrB638r was applied according to Pester et al. (2013). To test for the presence of *Nitrobacter*-like bacteria, primer set F1norA/R1norA targeting the gene of nitrite oxidoreductase subunit alpha (*nxrA*) of *Nitrobacter* was used as described elsewhere (Poly et al., 2007).

RFLP analysis was performed by colony PCR on 45 16S rRNA gene clones using the primers 27f and 1492r (Lane, 1991). For the subsequent endonuclease digestion, restriction

enzyme HapII (Fermentas, St. Leon-Rot, Germany) was used and unique RFLP patterns were identified after separation on a 3% agarose gel.

16S rRNA gene fragments for denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) were amplified by PCR with the primer pair GC-341f/907r (Lane, 1991) targeting most bacteria. DGGE with the PCR products was performed as described elsewhere (Alawi et al., 2007). The resulting bands were excised, re-amplified, and the partial 16S rDNA sequences were compared to sequences in public databases by BLAST (Altschul et al., 1990).

## Results

*Isolation of* Nitrospira defluvii *(lineage I)*

The starting enrichment culture for isolation consisted to 86% of *N. defluvii* and contained no other detectable NOB (Spieck et al., 2006). While the previous enrichment procedure had achieved a high abundance of *N. defluvii* relative to heterotrophic bacteria (Spieck et al., 2006), only a combination of various methods in this study allowed the final isolation of *N. defluvii* over a period of four years (Fig. 2.1).

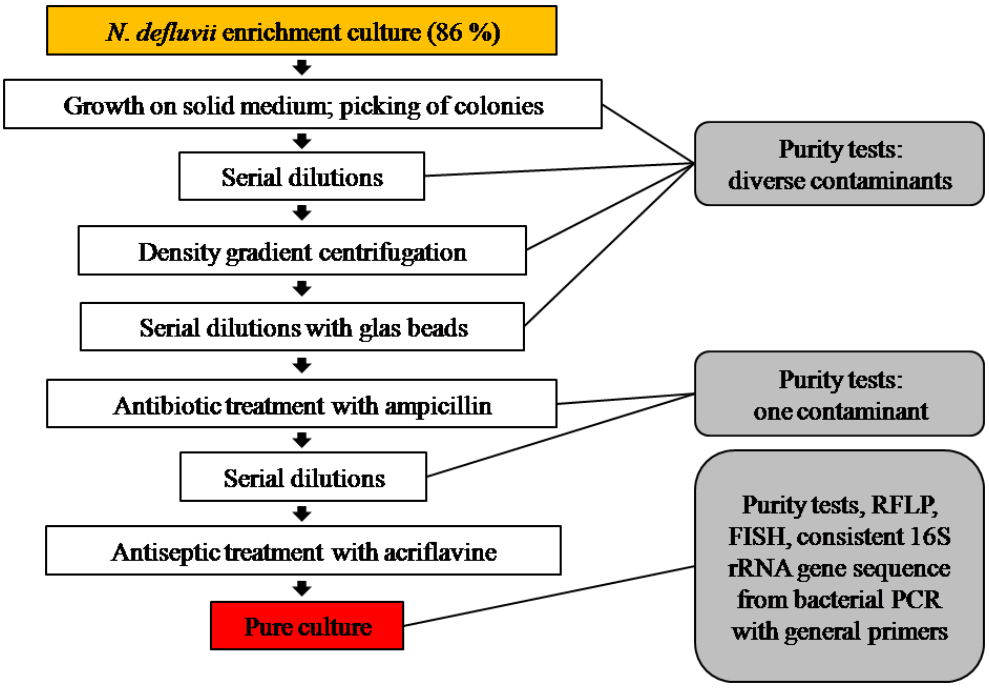


**Fig. 2.1:** Simplified flow chart of *Nitrospira defluvii* isolation procedure. All cultures were incubated at 28°C in mineral medium with 0.3 to 3 mM $NaNO_2$ as sole energy source. Transfer of cultures with inocula of one percent (v/v).

The first step of isolation was the successful cultivation of *N. defluvii* on solid mixotrophic medium with nitrite (Fig. 2.2). Notably, all previous attempts to cultivate *Nitrospira* on solid media had failed (unpublished data). On medium containing agarose instead of agar and a small amount of organic compounds, slow colony growth was observed. Within about two months, *N. defluvii* formed brownish, rough and convex colonies, which were identified later on in the enrichment in liquid mineral nitrite medium. During maturation, some of the *N. defluvii* colonies developed irregular extensions. Interestingly, colonies of *N. defluvii* were usually surrounded by colonies of heterotrophic bacteria and later attempts to purify *N. defluvii* on solid medium were not successful. Although subsequent inoculations did not result in pure cultures, an apparent decrease in the diversity of heterotrophs was observed after inoculation of complex solid media.

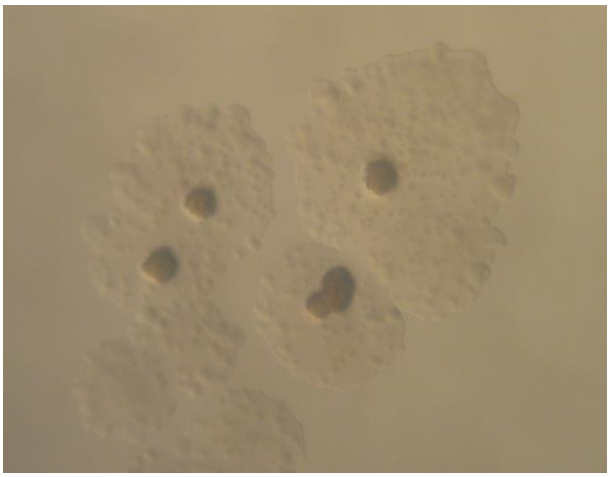

**Fig. 2.2:** Growth of *Nitrospira defluvii* enrichment culture on solid mixotrophic agarose medium (28°C). Within about two months *N. defluvii* formed brownish, rough and convex colonies, surrounded by heterotrophic microorganisms.

The purification procedure was continued by density gradient centrifugation, which separated a brownish band containing *Nitrospira* from brighter cell fractions of concomitant microorganisms. This procedure strongly increased the ratio of *N. defluvii* to other bacteria, but the presence of concomitant bacteria in the extracellular polymeric substances (EPS) was still observed by scanning electron microscopy (Fig. 2.3). Repeated serial dilutions were performed in glass tubes after extensive mixing of the cell inoculum with glass beads, which resulted in the disruption of the *Nitrospira* microcolonies and the detachment of heterotrophic microorganisms.

Consistent with our previous observation that *N. defluvii* is resistant to moderate concentrations (50 µg $ml^{-1}$) of ampicillin (Spieck et al., 2006), the ongoing treatment with this antibiotic resulted in the detection of only one remaining heterotrophic bacterium in the enrichment. As genome analysis had revealed a resistance gene against acriflavine (AcrA/B acriflavine resistance protein) (Lücker et al., 2010), this antiseptic was added repeatedly in concentrations of 0.21 µg $ml^{-1}$ to follow-up cultures over a period of several weeks with the aim to suppress the growth of the last detectable contaminant. Eventually this approach proved successful, and the purity of the *N. defluvii* culture was tested by inoculating liquid and solid complex media, where no growth of heterotrophic microorganisms was observed. RFLP analysis of 45 16S rRNA amplicons resulted in similar sequences with two different restriction patterns. Both corresponding cloned 16S rRNA genes were identical with *N. defluvii* strain A17. Additionally, only one sequence (belonging to *N. defluvii*) was obtained by direct sequencing of the 16S rRNA gene after PCR-amplification with primer set (27f/1492r) targeting most *Bacteria* (without cloning). Purity of the culture was also strongly supported by FISH, which revealed that all DAPI-detected cells hybridized to the *Nitrospira*-specific probe Ntspa662.

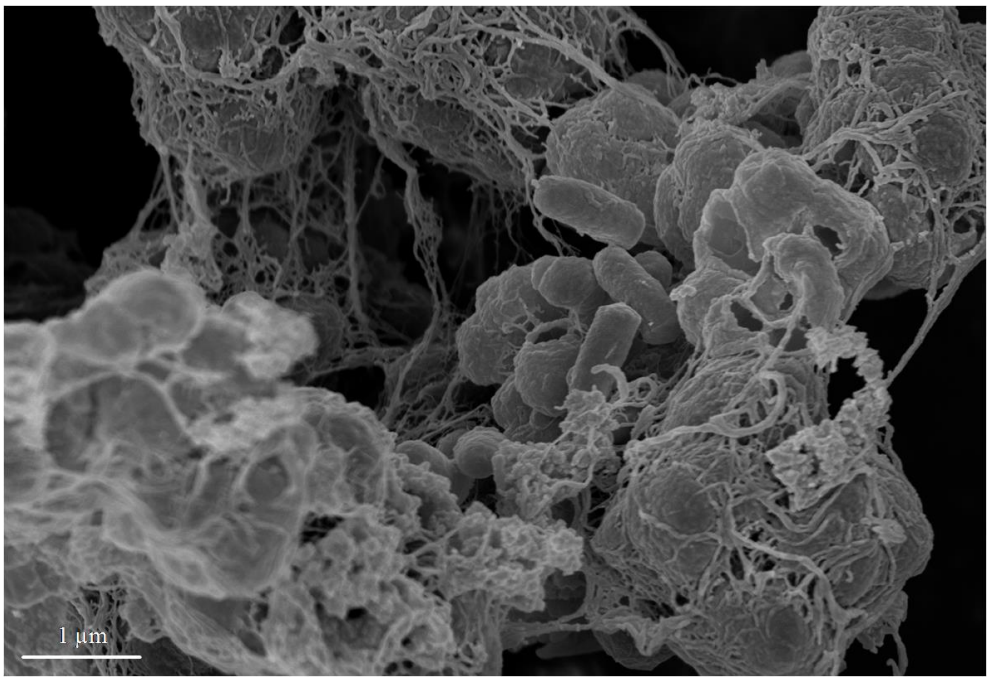


**Fig. 2.3:** SEM picture of *N. defluvii* enrichment culture. *N. defluvii* microcolonies embedded in an EPS network with attached rod-shaped concomitant bacteria. Magnification 37,400x.

*Isolation of* Nitrospira *strain BS10 (lineage II)*

A novel *Nitrospira* lineage II bacterium was derived from an activated sludge sample taken in December 2006 from the wastewater treatment plant Dradenau in Hamburg, Germany, which was also the sampling site of lineage I *N. defluvii* (Spieck et al., 2006; Alawi et al., 2009). With the initial goal to enrich *Nitrotoga*-like bacteria, a nitrite-oxidizing enrichment culture had been incubated at 10°C for about one year (Fig. 2.4).

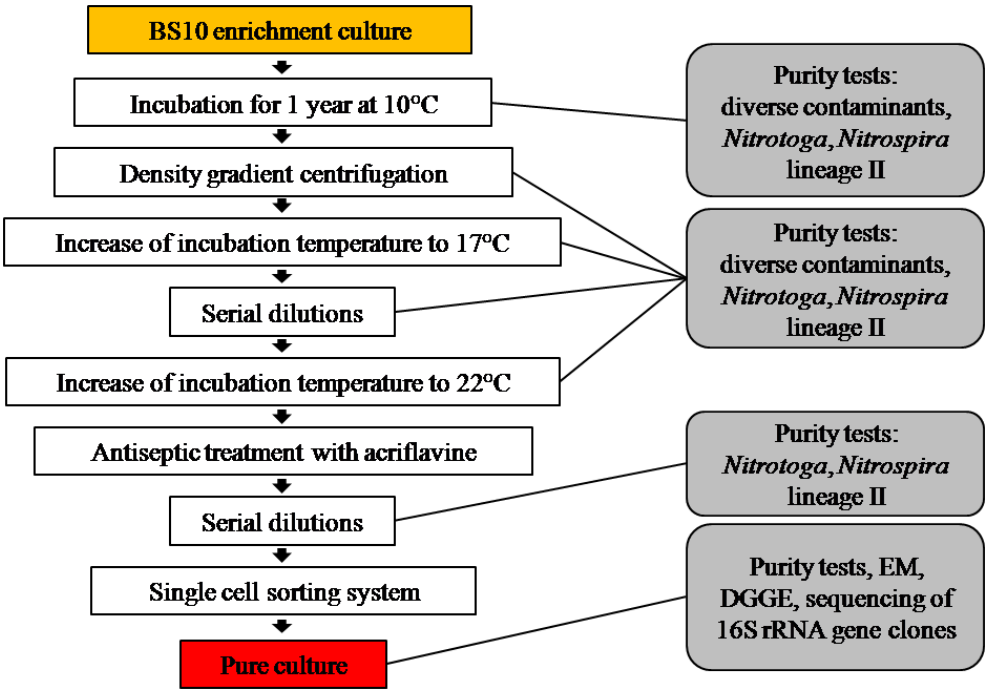


**Fig. 2.4:** Simplified flow chart of *Nitrospira* strain BS10 isolation procedure. All cultures were incubated at increasing temperatures in mineral medium with 0.3 to 3 mM $NaNO_2$ as sole energy source. Transfer of cultures with inocula of one percent (v/v).

PCR with genus-specific primers revealed the co-occurrence of *Nitrotoga*-like bacteria (not shown) and a *Nitrospira*-like bacterium related to *N. moscoviensis* (lineage II) in this enrichment (Pester et al., 2013). To isolate the novel *Nitrospira* lineage II representative, the culture was first concentrated by density gradient centrifugation, which apparently decreased the diversity of heterotrophs on complex solid media. Since *Nitrotoga* are regarded as NOB inhabiting low temperature environments (Alawi et al., 2007), a stepwise increase of the incubation temperature to 17°C and later to 22°C inhibited the growth of *Nitrotoga* and simultaneously enhanced proliferation of *Nitrospira*. Meanwhile, the culture was repeatedly fed with 0.3 mM nitrite, inoculated into fresh mineral medium for several times, and further

purified by serial dilutions. Similar to the isolation procedure of *N. defluvii* described above, the first key step towards the isolation of the novel *Nitrospira* strain was a treatment with acriflavine. After repeated acriflavine addition to several follow-up cultures and successive serial dilutions, no heterotrophic contaminants were found by inoculating liquid and solid complex media. Nevertheless, *Nitrotoga*-like bacteria were still detected by specific PCR with primers NTG200F/NTG840R (Alawi et al., 2007). The second key step was the application of a single cell sorting system (PALM MicroTweezers, Zeiss Munich), allowing the isolation of single cells for subsequent inoculation of pure cultures. By use of this micro tweezers system ten single cells of *Nitrospira* were separated and transferred into fresh nitrite medium. Six of these subcultures showed nitrite consumption after two weeks of incubation at 22°C. Their purity was confirmed visually by electron microscopy, by growth controls on liquid and solid complex media, and by DGGE analyses of genomic DNA with the universal bacterial primer set 341f-GC/907r (Lane, 1991). Two different DGGE bands occurred, which revealed identical sequences exclusively affiliated to *Nitrospira* lineage II. Additionally, cloning of twelve 16S rRNA genes PCR-amplified with primers 27f/1492r (Lane, 1991) and subsequent sequencing revealed one identical sequence of *Nitrospira* lineage II. The newly isolated organism is designated as *Nitrospira* strain BS10.

*Morphology and physiology of* Nitrospira *isolates*

According to earlier reports, *Nitrospira*-like bacteria modify their cell shapes in response to varying growth conditions (Spieck et al., 2006; Daims, 2013). Cells of *N. defluvii* are often found in large cell aggregates (Juretschko et al., 1998; Bartosch et al., 1999; Daims et al., 2001), consisting of small cell clusters organized as tetrads and embedded in EPS (Fig. 2.5 A). Under static conditions with 3 mM nitrite, *N. defluvii* cells formed loose aggregates with moderate EPS formation, but the amount of EPS increased in the presence of high nitrite concentrations (30 mM) or after addition of acriflavine as stress factors (not shown). The EPS consisted of floccular and filamentous structures that enclosed the cells almost completely but were interlaced with pores and voids. In case of a high cell density and nitrate accumulation, or after long periods of starvation, *N. defluvii* can occur as planktonic cells with a characteristic helical cell shape (Spieck et al., 2006). Cells of strain BS10 were helical-shaped and resembled cells of *N. moscoviensis* (Ehrich et al., 1995) (Fig. 2.5 B-F). In contrast to *N. defluvii*, their tendency to form cell aggregates was less pronounced and they occurred rather as planktonic cells. After repeated nitrite addition and nitrate accumulation, moderate to high

amounts of EPS were produced. Despite aggregation, the cells were separated by the surrounding EPS. Interestingly, in both *Nitrospira* strains (A17 and BS10) small extracellular membrane vesicles up to 100 nm in size were observed that seemed to be generated at the outer membrane and were also found attached to the inner EPS layer. Cells of both strains had an enlarged periplasmic space but lacked intracytoplasmic membranes and carboxysomes (Fig. 2.6).

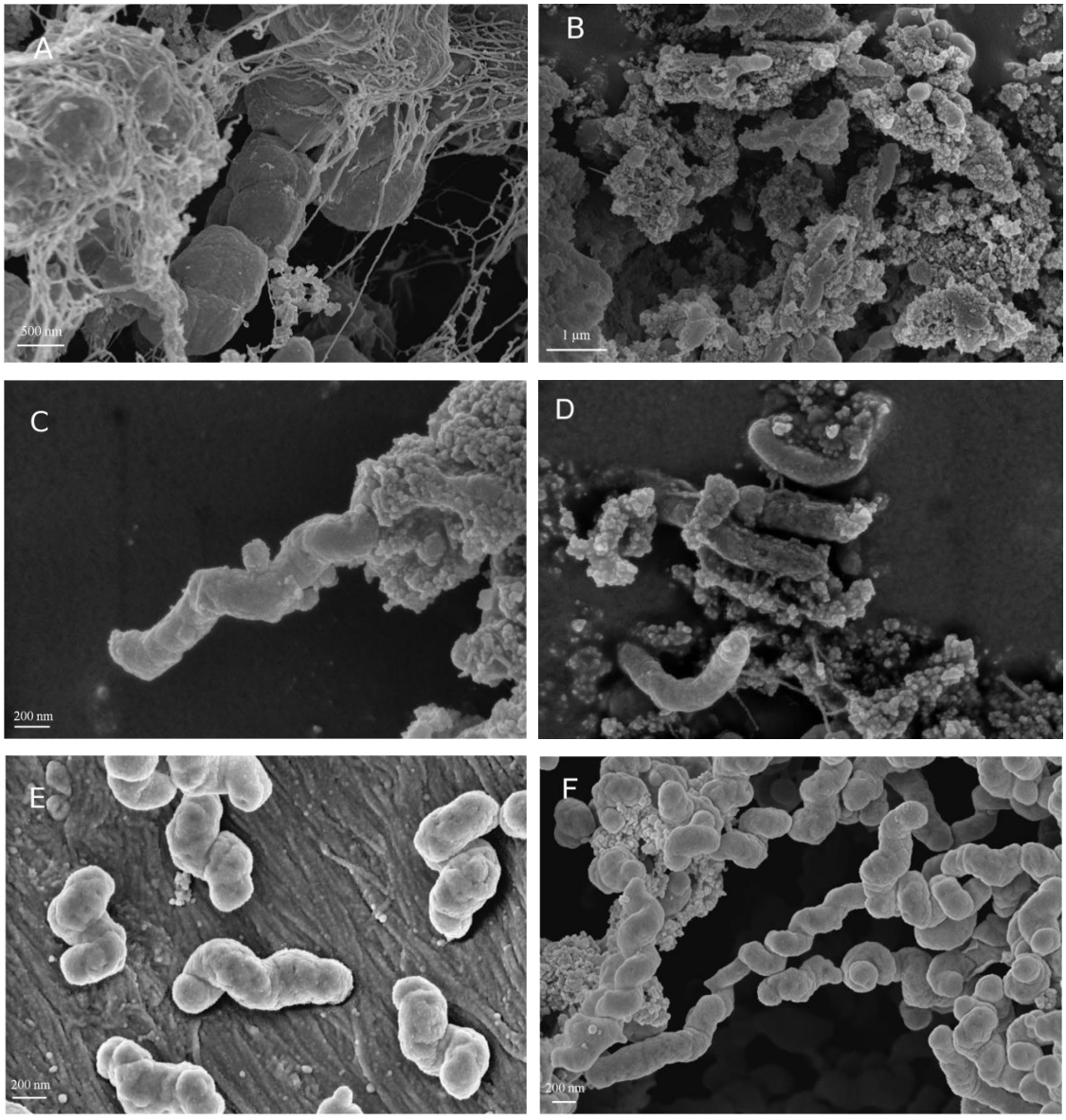


**Fig. 2.5:** SEM pictures of *Nitrospira* lineages I and II. Pictures with magnitudes from 2000-93,000x. Small microcolonies of *Nitrospira defluvii* (lineage I) with EPS filaments and tetrads as basic unit (A). *Nitrospira* strain BS10 (lineage II) cells embedded in EPS (B, C, D). Slightly aggregating (E) and planktonic (F) *Nitrospira moscoviensis* (lineage II) cells.

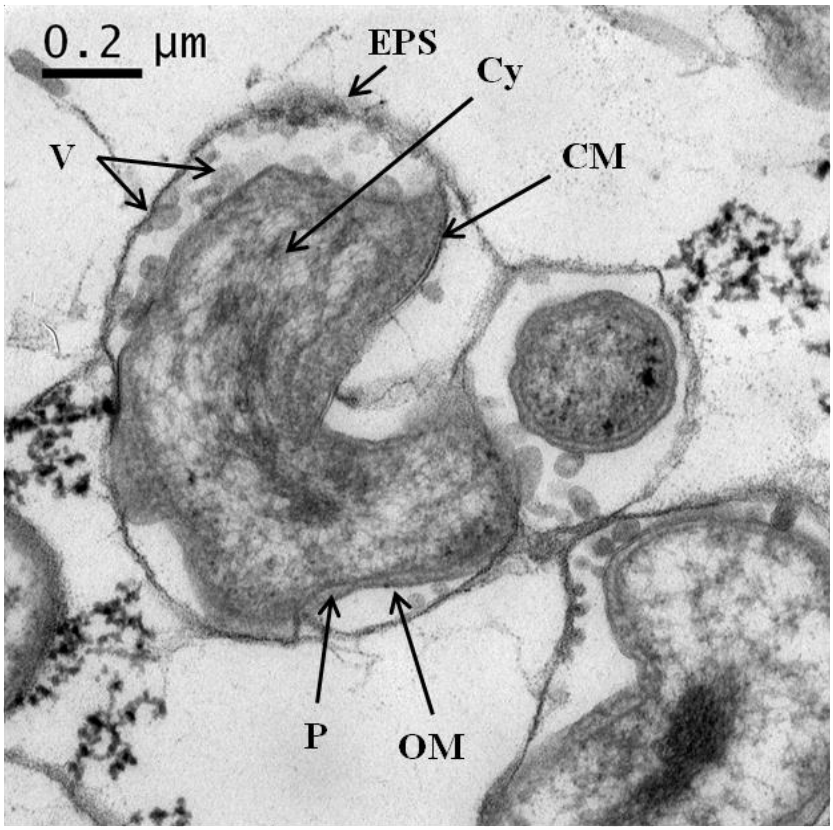


**Fig. 2.6:** TEM picture of *Nitrospira* strain BS10 microcolonies with thin layers (BS10) of EPS. The cells release membrane vesicles (V). Further cell components are the cytoplasmic membrane (CM), cytoplasm (Cy), periplasm (P), and outer membrane (OM).

Subsequently, key physiological properties of *N. defluvii* and strain BS10 were compared. The cultivation temperature of 28°C represented the growth optimum of both strains, and the temperature range (10 to 32°C) permitting growth of strain BS10 (Fig. 2.7 A) was similar to the temperature range of *N. defluvii* (Spieck and Lipski, 2011), but different to that of *N. moscoviensis* in the same phylogenetic lineage II (growth optimum at 39°C) (Ehrich et al., 1995). However, the two strains markedly differed in their tolerances against high nitrite concentrations. While *N. defluvii* grew at nitrite concentrations up to 30 mM, no growth of strain BS10 was observed above 2.5 mM nitrite (data not shown). Additionally, growth of strain BS10 was strongly inhibited in the presence of high concentrations of the substrate nitrite and product nitrate. For example, when the culture was started with 1 mM nitrate, the lag phase continued for approximately three weeks. With the tenfold concentration of 10 mM nitrate, the lag phase was extended up to eight weeks (Fig. 2.7 B). Nitrite-dependent growth curves are shown in Fig. 2.7 C and the different growth characteristics of both strains are exemplary compared in Fig. 2.7 D. Although *N. defluvii* had been supplied with a four-fold

higher nitrite concentration than strain BS10, *N. defluvii* consumed all nitrite ten days earlier than strain BS10.

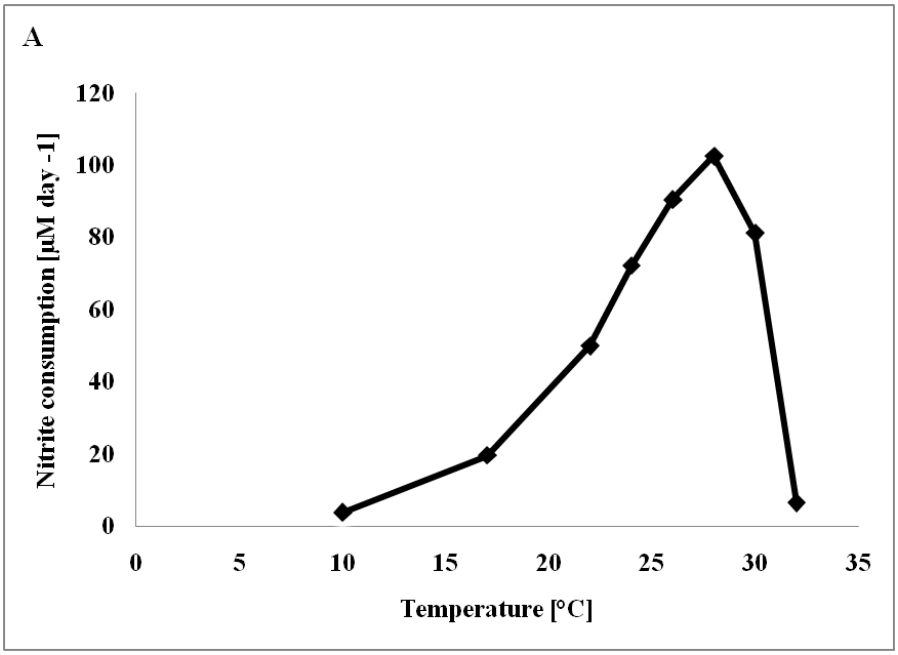


**Fig. 2.7 A:** Optimum growth temperature of the *Nitrospira* BS10 enrichment. Highest nitrite consumption rates measured with cultures of one percent inocula (v/v) in mineral medium with 0.3 mM $NaNO_2$ after 74 h of incubation at different temperatures.

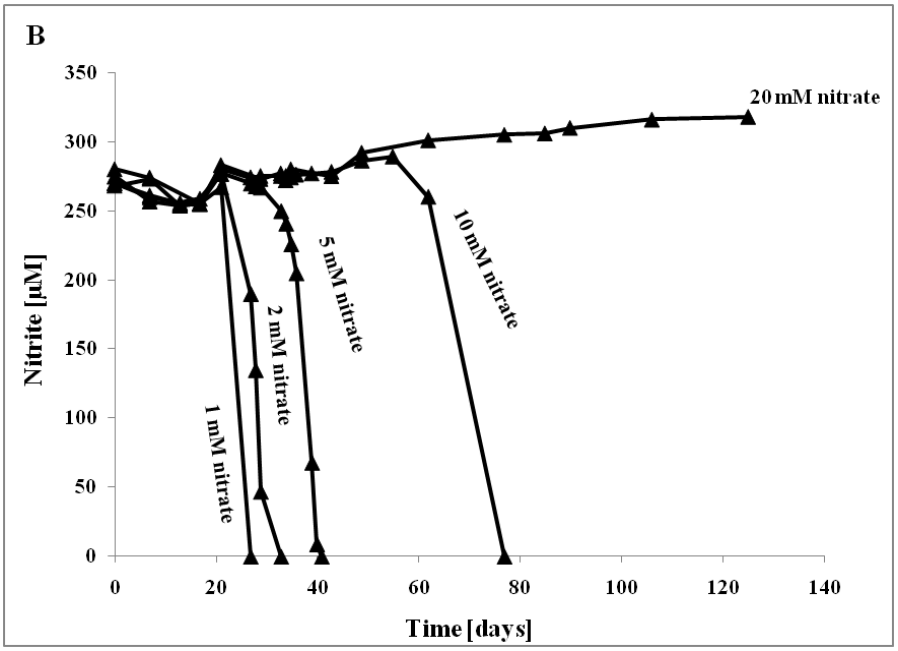


**Fig. 2.7 B:** Nitrate inhibition of *Nitrospira* strain BS10 cultures. The cultures were started with one percent inocula (v/v) and similar nitrite concentrations, but varying nitrate concentrations in mineral medium at 28°C. Hereby, the oxidation of nitrite was delayed with elevated nitrate concentrations, until 20 mM nitrate lead to total inhibition of activity.

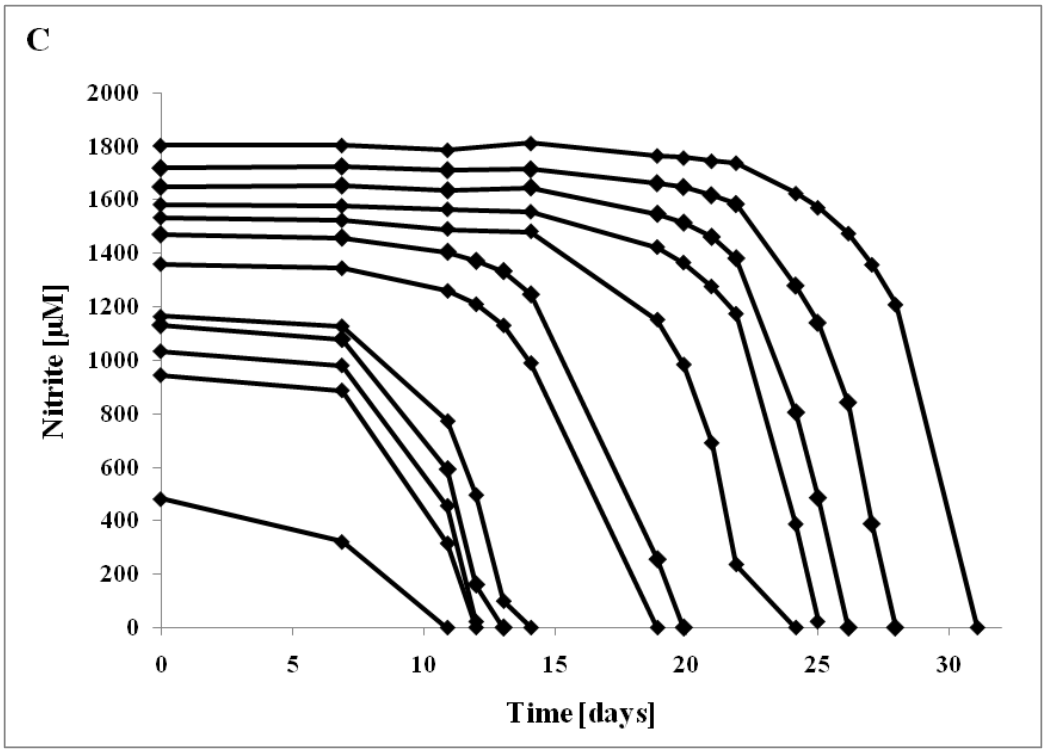


**Fig. 2.7 C:** Nitrite consumption of *Nitrospira* BS10 pure cultures. The cultures were started with one percent inocula (v/v) and different nitrite concentrations in mineral medium at 28°C. The duration of lag-phases increased in dependence on the initial nitrite concentration.

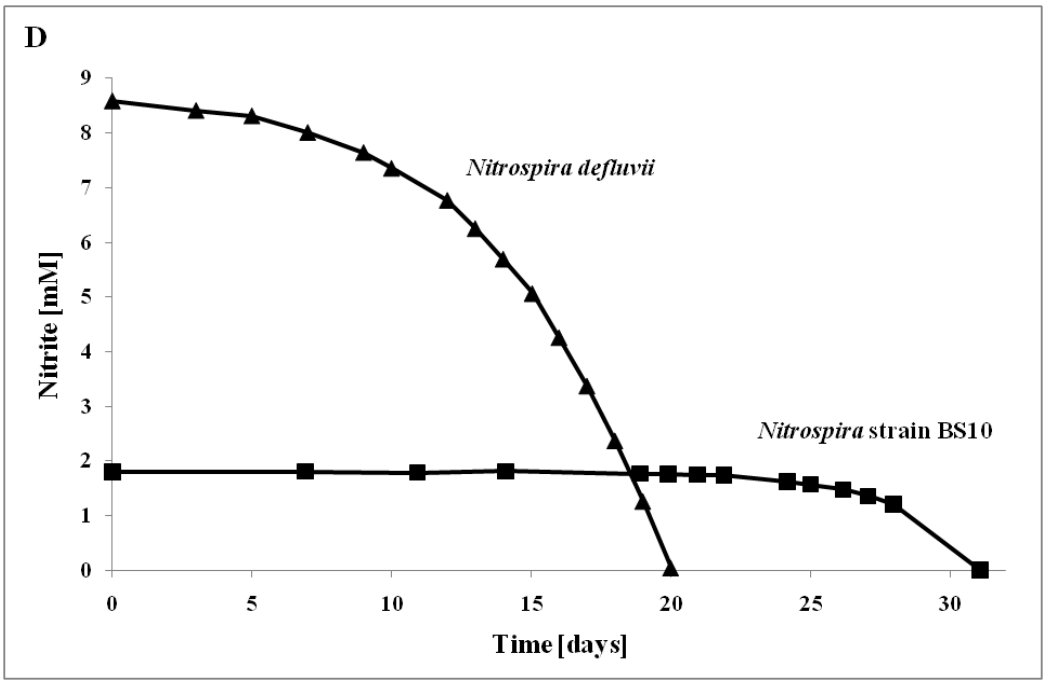


**Fig. 2.7 D:** Typical nitrite consumption curves of *N. defluvii* and strain BS10 pure cultures in mineral medium at 28°C. Before oxidizing 1.8 mM of nitrite strain BS10 demonstrated a *long-lasting* lag-phase of 25 days, whereas *N. defluvii* oxidized a fourfold amount of nitrite in 20 days with only a short lag-phase.

*Enrichment of* Nitrospira *from diverse habitats*

Besides activated sludge as eutrophic ecosystem, a variety of oligotrophic habitats like a freshwater aquarium were investigated. Further habitats were unique with regard to temperature (permafrost-affected soils in Siberia), water availability (Southern African drylands and sandstone) and oxygen content (Movile Cave in Romania). Cultivation was performed in standard mineral medium containing a low concentration of nitrite (0.3 mM) suitable for growth of *Nitrospira* and not promoting the enrichment of *Nitrobacter* (Bartosch et al., 1999). The incubation temperature of at least 17°C favored cells of *Nitrospira* in comparison to cold-adapted *Nitrotoga* (Alawi et al., 2007).

The phylogenetic tree of 16S rRNA gene sequences in Figure 2.8 shows that cultures with similar origin are not closely related, but are scattered in *Nitrospira* lineages I and II. In lineage I, the enrichment cultures Movile I3, freshwater aquarium and permafrost soil LD5 are closely related to *N. defluvii* (sequence similarities of 99.0%, 99.1% and 99.0%, respectively). The single sequence from the permafrost soil enrichment cultures 3301, 3305, and 3309 groups together with an environmental clone from arctic snow (FJ946576) and has a similarity of 98.4% to *N. defluvii*. The enrichment Namibia 74 shows a 16S rRNA gene sequence similarity of 97.9% to *N. defluvii* and affiliates with an environmental clone from dry steppe (JX255044). With a 16S rRNA gene sequence similarity of only 95.8% to *N. defluvii*, the second Namibian soil enrichment culture 80 is more distantly affiliated with the only cultured representative in lineage I. In lineage II, the 16S rRNA sequence of the here isolated *Nitrospira* strain BS10 is identical to an environmental sequence from a hypereutrophic lake (HQ827976) and most closely related to permafrost enrichment culture 3304 (similarity 99.6%). Otherwise, strain BS10 is only distantly related to the described species *N. moscoviensis* (similarity 96.1%) and the newly isolated *N. japonica* (similarity 96.1%), and therefore strain BS10 is designated as a new species. The most similar environmental sequence to permafrost enrichment culture LD3 originated from a ferromanganous micronodule (similarity 98.9%; AF293010). With a sequence similarity of 94.4% to strain BS10 and 94.9% to *N. moscoviensis*, permafrost enrichment culture LD3 is most distantly related to known cultures within lineage II. The sequence of enrichment culture South Africa 83 clusters together with a clone from a lava tube (similarity 98.6%; JF265912) and the sequence of enrichment culture LUA16 from natural sandstone with that of a paddy soil clone (similarity 98.4%; AJ617919). Their sequences have similarities of 97.8% and 97.1% to the next cultured representative of lineage II, *N. moscoviensis*, respectively.

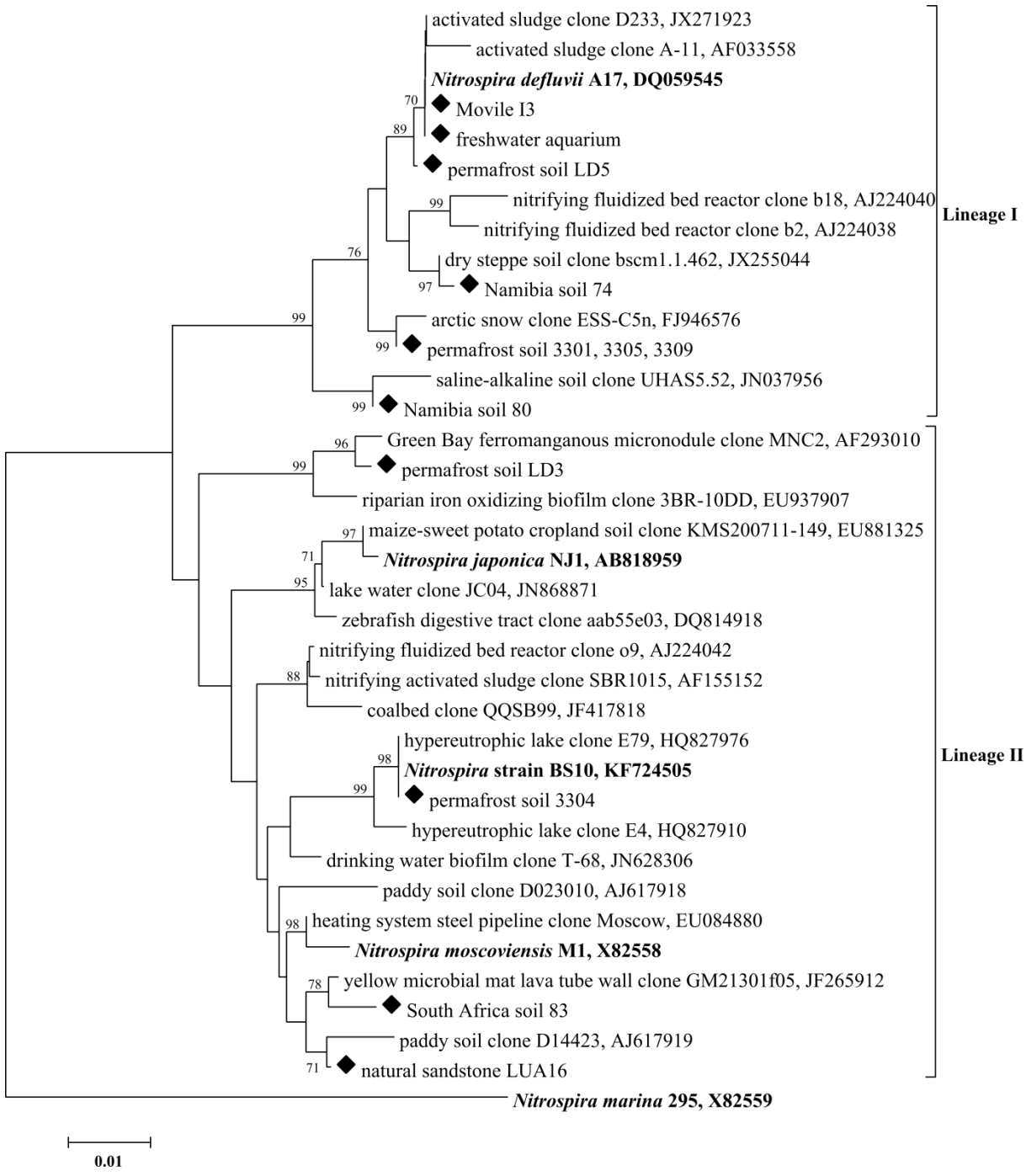


**Fig. 2.8:** 16S rRNA gene-based phylogeny of enriched and isolated members of the genus *Nitrospira*. A neighbor-joining tree is shown. Sequences of cultured representatives are printed in bold face. Sequences from organisms enriched in this study are marked by diamonds. The tree was constructed by using sequences of >830 bp. Nodes supported by bootstrap values >70% are indicated. Scale bar indicates 1% estimated sequence divergence.

## Discussion

### *Isolation of two* Nitrospira *strains of different lineages from a wastewater treatment plant*

*Nitrospira* species are known to be widely distributed (Daims et al., 2001), but knowledge about their morphological characteristics and physiology is mainly based on a small number of laboratory cultures and only one published genome sequence (Lücker et al., 2010). In WWTPs, the coexistence of several NOB including *Nitrospira* is assumed to cause stability (Daims et al., 2001), but not all representatives could be identified so far (Kruse et al., 2013). The major goal of this study was the isolation and characterization of two *Nitrospira* from a WWTP in order to search for differences affecting niche separation and to perform first biochemical experiments. Therefore, two independent procedures were implemented for isolating *Nitrospira defluvii* (lineage I) and *Nitrospira* strain BS10 (lineage II), the key NOB in activated sludge (Schramm et al., 1998; Daims et al., 2001; Maixner et al., 2006).

The standard isolation procedures in this study are based on cultivation in liquid autotrophic medium with nitrite as sole energy source, serial dilution techniques and density gradient centrifugation (Watson et al., 1986; Ehrich et al., 1995). However, both *Nitrospira* enrichments (*N. defluvii* and strain BS10) showed production of EPS, which embedded the cells tightly to microcolonies and due to the close co-localization of heterotrophic organisms and the slow growth rates of NOB (Prosser, 1989) these procedures were not sufficient for isolating *Nitrospira*. The strongest co-localization of heterotrophic bacteria occurred in the enrichment of *N. defluvii* (lineage I), which produced particularly high amounts of EPS. Here, the mixing of cell suspensions with sterile glass beads appeared to be a suitable strategy to further separate *Nitrospira* cells before inoculating serial dilutions.

Previous studies assumed that heterotrophic bacteria live in close vicinity to autotrophic nitrifiers, because they use excreted soluble microbial products (SMP) (Rittmann et al., 1994; Juretschko et al., 2002; Kindaichi et al., 2004). Since colony growth on solid mixotrophic medium succeeded with the enrichment culture of *N. defluvii*, but failed with pure cultures so far, *Nitrospira* might also benefit from the community, for example by utilizing metabolites and waste materials of the heterotrophs (Kindaichi et al., 2004). In contrast to NOB of the genus *Nitrobacter* (Sorokin et al., 1998), the gelling agent agarose was required for growth of *Nitrospira* on solid medium, which might be due to a higher purity with less inhibitory soluble constituents than found in agar (Johnson, 1995).

The selective enrichment of *N. defluvii* and strain BS10 was achieved by application of ampicillin for inhibiting growth of most heterotrophs. Beside the resistance against beta-

lactam antibiotics (Spieck et al., 2006), genomic analyses revealed an encoded acriflavine resistance protein (AcrA/B) in *N. defluvii* (Lücker et al., 2010). Acriflavine is a acridine dye used as antiseptic in aquarium water, which intercalates into DNA and inhibits protein biosynthesis (Lerman, 1964). Finally, the addition of acriflavine successfully inhibited the remaining heterotrophic bacteria in the enrichments of *N. defluvii* as well as strain BS10.

Since *Nitrospira* strain BS10 still coexisted with cells of *Nitrotoga*, a micro-tweezers single cell sorting system (Carl Zeiss, Munich, Germany) was used for the final isolation as described previously (Huber et al., 1995). Different to the recently introduced automated cell sorting system for separation of *Nitrospira* microcolonies (Fujitani et al., 2013; Ushiki et al., 2013), this micro-tweezers system allowed the manual separation of planktonic *Nitrospira* cells. Six of the ten subcultures of isolated cells remained active. This high separation efficiency illustrates the practical application and high potential of optical tweezers for the isolation of uncultured microorganisms and - at the same time - enables a fast isolation of single cells including underrepresented microorganisms.

*Characterization of lineage I and II* Nitrospira

Both isolated *Nitrospira* strains originated from the same WWTP (Dradenau, Hamburg) and previous studies revealed that both lineages can coexist in the same habitat, occupying distinct ecological niches, e.g. in terms of substrate or dissolved oxygen (DO) concentrations (Maixner et al., 2006; Park and Noguera, 2008). The substrate and product tolerances revealed in the present study consistently indicate such different adaption strategies, with *N. defluvii* (lineage I) appearing as more robust NOB compared to the fastidious strain BS10 (lineage II). The relatively low nitrite tolerance of strain BS10 compared to *N. defluvii* supports the hypothesis of Maixner et al. (2006) that high nitrite concentrations select for *Nitrospira* of lineage I and suppress lineage II.

During batch cultivation of NOB high nitrate concentrations can accumulate. First reports about nitrate inhibition of NOB were already given in the 1960s on *Nitrobacter* strains (Boon and Laudelout, 1962; Schön, 1965). Inhibiting effects of nitrate on *Nitrospira* were reported later on for lineage II *N. moscoviensis* (75 mM) (Ehrich et al., 1995), and marine lineage IV *Nitrospira* Ecomares 2.1 (80 mM) (Keuter et al., 2011). Again, with inhibitory effects at >35 mM nitrate, *N. defluvii* was less sensitive towards higher concentrations than strain BS10 with inhibition at >18 mM nitrate.

Besides the different adaptations to nitrogen concentrations, temperature has been revealed as further important factor for niche differentiation of NOB (Alawi et al., 2009). Although both investigated strains and all other so far investigated *Nitrospira* from activated sludge have their growth optimum around 28°C (Spieck et al., 2006; Fujitani et al., 2013; Ushiki et al., 2013), it was also reported that the diversity of *Nitrospira* in WWTPs was enhanced at lower temperatures (Siripong and Rittmann, 2007; Kruse et al., 2013). Accordingly, the initial enrichment at 10°C over a period of one year indicated that strain BS10 prevails at low temperatures, and might therefore have important impact on a robust maintenance of nitrification in low temperature wastewater treatment. Additionally, cultivation attempts performed at low temperature might be used as new strategy to enrich populations of *Nitrospira*, which are able to persist better in cold-affected environments than other populations of *Nitrospira*.

It is well known that especially *Nitrospira* lineage I reveal a strong biofilm production (Spieck et al., 2006; Daims, 2013). In this study, an increase in biofilm formation was detected as stress response (e.g. against nitrite and acriflavine), where EPS apparently function as protecting matrix (Flemming and Wingender, 2010). The existence of a cell to cell communication system has to be postulated to regulate planktonic versus aggregated life style of *Nitrospira* and biofilm density. Indeed, the observed vesicle release inside the EPS indicated a cell-cell exchange like the export of nucleic acids and extracellular enzymes (Flemming and Wingender, 2010) or for a cell-cell transmission mechanism of a quorum sensing signal (Mashburn and Whiteley, 2005; Remis et al., 2010) known for other Gram-negative bacteria. Since genome analyses gave no hints for a known quorum sensing system in *N. defluvii* (Lücker et al., 2010), the biochemical composition of these vesicles might be an interesting issue for studies on cell-cell interaction.

### *Distribution of* Nitrospira *lineage I and II in natural and artificial environments*

The diversity of publicly deposited *Nitrospira*-related 16S rRNA and *nxrB* gene sequences has increased enormously within recent years (Pester et al., 2013), but this genus is still represented by very few laboratory cultures from a small number of different habitats. Members of lineages I and II are the most frequently detected *Nitrospira* in non-marine environments. At the beginning of this study, cultures from these lineages were limited to one enrichment culture of *N. defluvii* (lineage I) (Spieck et al., 2006) and the pure culture of *N. moscoviensis* (lineage II) (Ehrich et al., 1995). Only recently, two more strains of *Nitrospira*

belonging to lineage I and II were isolated from activated sludge (Fujitani et al., 2013; Ushiki et al., 2013). Since the limited availability of cultures prevented to compare the physiological and genomic features between different lineages and within the same lineage, further moderate and extreme environments were sampled for inoculation of *Nitrospira*-specific enrichment cultures. In total, six additional enrichment cultures were obtained for *Nitrospira* lineage I and four cultures for *Nitrospira* lineage II (Fig. 3). These findings confirmed the known versatile distribution of representatives of lineage II (Daims et al., 2001; Freitag et al., 2005), and revealed that members of lineage I, which previously had been mostly found in artificial ecosystems like activated sludge, are also widespread in natural habitats like different soils, freshwater, and cave ecosystems.

It is remarkable that four distinct phylotypes of *Nitrospira* from permafrost soil were identified, which possibly can be explained by the existence of microniches. Since the samples were retrieved from different soil types of the active layer, like a cliff (3301, 3304, 3305, 3309), slope (LD5), and wall (LD3) of the polygon system, habitat specific conditions like water content or oxygen limitation might be linked to *Nitrospira* diversity. Especially the wall, slope and center of a polygon were diverse in terms of nitrogen availability and turnover (Sanders et al., 2008).

Interestingly, and in accordance with two enrichment cultures in this study, Pester et al. (2013) found that Namibian soils were hot spots of high *Nitrospira* diversity. Further, these authors presented a new cluster of *nxrB* sequences. Therefore, the affiliation of enrichment culture Namibia soil 80, which falls apart from the other 16S rRNA sequences in lineage I, needs further investigation.

Within lineage II, *Nitrospira* strain BS10 clearly differs from *N. moscoviensis* in temperature range and nitrite/nitrate tolerances (Ehrich et al., 1995) and from *N. japonica* in cell morphology (Ushiki et al., 2013). Together with the low similarities of the 16S rRNA genes of each 96.1%, which are well below the current species threshold of 98.7-99% (Stackebrandt and Ebers, 2006), these differences in physiology legitimate to designate strain BS10 as a new species.

*Description of* Nitrospira defluvii *sp. nov.*

De.flu.vi.i (L. gen. nom. of defluvium, named after the habitat where this organism was first isolated, wastewater)

Cells are Gram-negative short curved rods in microcolonies with a diameter of 0.5 to 1.2 µm or long spiral-shaped rods with 1 to 4 turns, 0.7 to 1.7 µm long, 0.2 to 0.4 µm wide. Aerobic chemolithoautotrophic nitrite oxidizer that uses carbon dioxide as sole carbon source. Optimum growth occurs at 28 to 32°C within a range between 10 to 34°C. The substrate optimum is 1.5 to 3 mM nitrite. Growth was observed up to a concentration of 30 mM nitrite. Cell growth is characterized by dense formation of flocs due to the strong production of extracellular polymeric substances.

Type strain *Nitrospira defluvii* A17 (Spieck et al., 2006) was isolated from activated sludge of the municipal wastewater treatment plant in Köhlbrandhöft/Dradenau, Hamburg, Germany. Nucleotide sequence data are available in the GenBank database under the accession number DQ059545.

*Description of* Nitrospira lenta *sp. nov.*

Lenta (L. fem. adj., named after the characteristic of being slow and tenacious)

Spiral-shaped rods with 1 to 3 turns, 1.0 to 2.3 µm long, 0.2 to 0.3 µm wide, non-motile, Gram-negative. Aerobic chemolithoautotrophic nitrite oxidizer that uses carbon dioxide as sole carbon source, optimum growth at 28°C within a range between 10 to 32°C. The substrate optimum is 0.3 to 1.2 mM nitrite. Growth inhibition occurs above 2.5 mM nitrite or 18 mM nitrate. Weak formation of flocs due to the production of extracellular polymeric substances is typical.

Type strain *Nitrospira lenta* BS10 was isolated from activated sludge of the municipal wastewater treatment plant in Köhlbrandhöft/Dradenau, Hamburg, Germany.

Nucleotide sequence data are available in the GenBank database under the accession number KF724505.

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# Chapter III

## Comparative oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as key factor for niche differentiation

Boris Nowka[1], Holger Daims[2], and Eva Spieck[1]

[1]Biocenter Klein Flottbek, Microbiology and Biotechnology, University of Hamburg, Ohnhorststr. 18, D-22609 Hamburg, Germany

[2]Department of Microbiology and Ecosystem Science, Division of Microbial Ecology, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria

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**Abstract**

*Nitrobacter*, *Nitrospira* and *Nitrotoga* are nitrite-oxidizing bacteria (NOB) commonly identified in many natural ecosystems and in wastewater treatment plants. Due to the limited number of available cultures, little is known about the physiological potential of growth and activity of NOB. Here we compare nitrite oxidation kinetics and growth properties of six pure cultures and one enrichment culture representing three genera of NOB. With half-saturation constants ($K_m$) between 9-27 µM nitrite, *Nitrospira* are adapted to live under significant substrate limitation. *Nitrobacter* showed a wide range of lower substrate affinities with $K_m$ values between 49-544 µM nitrite. However, the advantage of *Nitrobacter* is induced under excess nitrite conditions, sustaining high maximum specific activities ($V_{max}$) of 64-164 µmol nitrite per mg protein per h, contrary to lower activities of *Nitrospira* of 18-48 µmol nitrite per mg protein per h. For the first time, we report nitrite oxidation kinetics and growth parameters of an enrichment culture of *Candidatus* Nitrotoga arctica. The specific maximum activity ($V_{max}$= 26 µmol nitrite per mg protein per h) and substrate affinity ($K_m$= 58 µM nitrite) measured at low temperature of 17°C suggest that *Nitrotoga* could advantageously compete with other NOB in cold habitats. Together these findings support the hypothesis that NOB occupy different ecological niches according to the availability of nitrite. Further, in comparison with other studies the determined nitrite oxidation kinetics clearly discriminate between NOB derived from soil and those from activated sludge and freshwater systems.

## Introduction

Aerobic nitrite oxidation is the second part of nitrification, a key process in the global nitrogen cycle catalyzed by nitrite-oxidizing bacteria. These autotrophic, slow-growing bacteria use nitrite oxidation as their source of energy. Therefore, growth of NOB is directly linked to the availability of nitrite and the kinetics of its oxidation (Prosser, 1989). Nitrification occurs almost in every aquatic and terrestrial ecosystem, in natural as well as in artificial environments like wastewater treatment plants (WWTPs). Nitrite concentrations in these systems are most different, suggesting nitrite concentration as one major factor providing niche differentiation between NOB.

Known non-marine NOB belong to the genera *Nitrobacter* and *Nitrolancetus* within the *Alphaproteobacteria* and *Chloroflexi*, respectively (Winogradsky, 1892; Sorokin et al., 2012), *Nitrospira* within the *Nitrospirae* (Watson et al., 1986; Ehrich et al., 1995), and *Nitrotoga* within the *Betaproteobacteria* (Alawi et al., 2007). In general, NOB are regarded as widely distributed microorganisms. Though particular lineages may exist exclusively in certain ecosystems, phylogenetic analyses frequently reveal coexistence of different NOB genera in the same environmental samples (Burrell et al., 1998; Bartosch et al., 2002; Gieseke et al., 2003; Alawi et al., 2009; Attard et al., 2010). Additionally, subpopulations within the same genus can also coexist (Freitag et al., 2005; Maixner et al., 2006; Siripong and Rittmann, 2007; Park and Noguera, 2008), suggesting a spatial heterogeneity of different conditions in microenvironments. In an ecological context, these findings raise the question of factors providing niche differentiation and the functional differentiation between NOB and their sublineages. A well-studied hypothesis is that niche separation occurs due to varying preferences for dissolved oxygen (DO) concentrations, e.g. due to a high affinity for oxygen of *Nitrospira* (Okabe et al., 1999; Schramm et al., 2000; Gieseke et al., 2003; Sliekers et al., 2005; Downing and Nerenberg, 2008; Park and Noguera, 2008). A further advantage of some NOB is the capacity for growing mixotrophically (Steinmüller and Bock, 1976; Daims et al., 2001; Keuter et al., 2011). Further on, it was reported that temperature influences the population structure and diversity of NOB (Siripong and Rittmann, 2007; Alawi et al., 2009). However, the most obvious factor for niche differentiation is the utilization of the energy source nitrite. Due to their earlier discovery and availability in pure cultures (from the 1960s on) studies about nitrite oxidation kinetics of NOB were previously mainly performed with *Nitrobacter* cultures. The first estimations of oxidation kinetics of uncultured *Nitrospira* populations were done by microsensor measurements (Okabe et al., 1999; Schramm et al., 1999), and revealed high affinities for nitrite and thus suggesting a distinct differentiation of

substrate preferences. The two groups of ecological strategists (Andrews and Harris, 1986; Schramm et al., 1999) are the so-called *r*-strategists like *Nitrobacter* with high maximum nitrite oxidation activity and low substrate affinity, and the *K*-strategists like *Nitrospira*, which are characterized by low maximum activity but high substrate affinity, enabling these microorganisms to grow with low nitrite concentrations. Follow-up studies confirmed these contrary strategies (Wagner et al., 2002; Kim and Kim, 2006; Nogueira and Melo, 2006; Blackburne et al., 2007).

Intriguingly, the varying nitrite requirements of NOB are thought to be correlated with a different subcellular orientation and phylogenetic classification of two forms of the nitrite oxidoreductase (NXR), the key enzyme of nitrite oxidation (Spieck et al., 1998; Lücker et al., 2010). Members of *Nitrobacter*, *Nitrococcus* and *Nitrolancetus* have a cytoplasmically oriented form of NXR (Spieck and Bock, 2005; Sorokin et al., 2012). Instead, *Nitrospira* and *Nitrospina* contain phylogenetically distinct NXRs which are oriented towards the periplasmic space (Spieck et al., 1998; Lücker et al., 2010). These differences of the NXRs might result in a higher energy conservation of periplasmic NXR (Lücker et al., 2010).

In non-marine natural and engineered ecosystems, members of the genus *Nitrospira* represent the most dominant nitrite oxidizers in terms of diversity and population size (Hovanec et al., 1998; Juretschko et al., 1998; Daims et al., 2001; Altmann et al., 2003; Freitag et al., 2005; Lebedeva et al., 2005). However, the respective studies analyzed either uncultured *Nitrospira*-like bacteria by cultivation-independent approaches or they investigated heterogeneous enrichment cultures of not described species. Due to the lack of pure cultures, an exact attribution of oxidation kinetics to specific *Nitrospira* populations was uncertain until now. A further open question is the role of the newly discovered genus *Nitrotoga* (Alawi et al., 2007). After it was discovered in soil of the Siberian Arctic, members of this genus were quickly detected in diverse ecosystems, ranging from soils (Alawi et al., 2007; Sattin et al., 2009), biofilm (Chen et al., 2009), and freshwater (Percent et al., 2008; Li et al., 2011; Liu et al., 2012; White et al., 2012) to activated sludge (Alawi et al., 2009). Its preference for environments of low temperature and nitrite concentrations (Alawi et al., 2007; Alawi et al., 2009) makes *Nitrotoga* candidates for yet unrecognized important nitrite-oxidizers in oligotrophic ecosystems of cold regions.

Here we present a wide comparison of directly measured oxidation kinetics of members from known non-marine NOB by microsensor measurements, including the first pure culture results of lineage I and II *Nitrospira* from freshwater and activated sludge and the first analysis of an highly enriched *Nitrotoga* culture from permafrost soil. The results picture the

nitrite-dependent niche differentiation of NOB and give possible explanations for causes underlying their distribution in diverse ecosystems.

## Materials and methods

*Bacterial strains and culture conditions*

Seven different NOB species of three genera were investigated in this study. *Nitrobacter hamburgensis* X14 and *Nitrobacter winogradskyi* "Engel" were both isolated from soil (Sundermeyer and Bock, 1981; Bock et al., 1983), and *Nitrobacter vulgaris* $Ab_1$ from sewage (Bock et al., 1990). The isolates *Candidatus* Nitrospira defluvii A17 and *Candidatus* Nitrospira lenta BS10 were derived from activated sludge (Spieck et al., 2006; in prep.) and *Nitrospira moscoviensis* M-1 originated from a heating water system (Ehrich et al., 1995). *Candidatus* Nitrotoga arctica 6680 was highly enriched from permafrost soil (Alawi et al., 2007). In the following, the species with *Candidatus* status are designated as *N. defluvii*, *N. lenta* and enrichment *N. arctica*.

All *Nitrobacter* and *Nitrospira* were grown as batch cultures in 500 ml mineral medium with the following composition: 1 l distilled water, different concentrations of $NaNO_2$ as sole energy source, 0.007 g $CaCO_3$, 0.5 g NaCl, 0.05 g $MgSO_4$ x 7 $H_2O$, 0,15 g $KH_2PO_4$, 33.8 µg $MnSO_4$ x $H_2O$, 49.4 µg $H_3BO_3$, 43.1 µg $ZnSO_4$ x 7 $H_2O$, 37.1 µg $(NH_4)_6Mo_7O_{24}$, 973.0 µg $FeSO_4$ x 7 $H_2O$, 25.0 µg $CuSO_4$ x 5 $H_2O$. For the *Nitrotoga arctica* enrichment a modified trace element composition was used: 0.007 g $CaCO_3$, 0.5 g NaCl, 0.05 g $MgSO_4$ x 7 $H_2O$, 0,15 g $KH_2PO_4$, 100.0 µg $MnCl_2$ x 4 $H_2O$, 30.0 µg $H_3BO_3$, 144.0 µg $ZnSO_4$ x 7 $H_2O$, 36.0 µg $Na_2MoO_4$ x 2 $H_2O$, 2.1 mg $FeSO_4$ x 7 $H_2O$, 2.0 µg $CuCl_2$ x 2 $H_2O$, 190.0 µg $CoCl_2$ x 6 $H_2O$, 24.0 µg $NiCl_2$ x 6 $H_2O$. Before adding distilled water (ad 1 l), these supplements were dissolved in 12.5 ml HCl (25 %) (Widdel and Bak, 1992). The pH was adjusted to 8.4 – 8.6 and changed to 7.4 – 7.6 two days after autoclaving. The cultures were started with inocula of 1 % (v/v) and incubated in the dark at temperatures of 17°C (*N. arctica*), 28°C (all *Nitrobacter* strains, *N. defluvii* and *N. lenta*), and 37°C (*N. moscoviensis*). After the first detection of nitrite consumption, the *Nitrospira* and *Nitrobacter* cultures were stirred moderately (100 to 300 rpm). The enrichment *N. arctica* was cultivated without agitation.

*Chemical analyses*

Nitrite and nitrate concentrations were determined by high-performance liquid chromatography (HPLC) via ion-pair chromatography with a LiChrospher® RP-18 column (5 µm; 4x125 mm; Merck KGaA, Darmstadt, Germany) (Meincke et al., 1992) and UV detection in an automated system (HPLC-System LaChrom Elite®, VWR International GmbH, Darmstadt, Germany). Cell protein concentrations were measured by the BCA method (Smith et al., 1985) after cell lysis in 0.15 M NaOH and incubation at 90°C for 30 min.

*Disruption of* N. defluvii *aggregates and microcolonies*

Since *N. defluvii* forms extensive cell aggregates and microcolonies, we developed a method for disruption to enable single cell counting. At first, 1 ml of cell culture was shaken by the tool Mikro-Dismembrator S (Sartorius Stedim Biotech GmbH, Göttingen, Germany) for 40 min at a oscillation frequency of 1500 $^{1}$/min in a 3 ml PTFE-shaking flask with a chromium steel grinding ball (Ø 5 mm). Afterwards the cell culture was diluted in 10 ml $H_2O_{dest.}$ and sonicated for 10 min at 24 kHz frequency and 0.5 amplitude (UP200S Ultrasonic Processor, Hielscher Ultrasonics GmbH, Teltow, Germany). This procedure removed the bulk of extracellular polymeric substances (EPS) and resulted in single cells suitable for cell counting.

*Cell counting*

Aliquots of the cell cultures were diluted in 10 to 15 ml $H_2O_{dest.}$. Cell cultures were collected by filtration by Milli Pure Filter System (Sartorius GmbH, Göttingen, Germany) with membrane filters of 0.2 µm pore size (Merck Millipore, Billerica, USA). For cell counting, the filters were dried and pieces of the filters were subsequently stained with 0.01 mg ml$^{-1}$ DAPI (4',6-diamidino-2-phenylindole) for 3 min, immersed in $H_2O_{dest.}$ and 70 % ethanol for a few seconds. Cells were counted on dried filters by fluorescence microscopy (Axio Imager.M2, Carl Zeiss, Göttingen, Germany) and by use of a counting ocular (area of a square: 0.0004 mm$^2$ at 1000 magnification). Cell counts were calculated by the following equation: $cells_{overall}$ = (filter area (380 mm$^2$) / square area (0.0004 mm$^2$)) x (cell count per square). The cell count per square was generated from the average cell number of 30 squares.

*Activity measurements*

Activity measurements were performed with early-stationary phase cells in nitrite-limited mineral medium by the following procedure: 500 ml cultures of *N. defluvii* and the three strains of *Nitrobacter* were started with a nitrite concentration of 9 mM and *N. moscoviensis* with 5.7 mM. To avoid inhibition of cell growth, cultures of *N. lenta* and *N. arctica* were started with low nitrite concentrations of 0.27 mM and 0.66 mM, respectively, and replenished by these amounts when nitrite was consumed. Nitrite consumption and nitrate production were monitored frequently by HPLC. Between 12 h and 48 h after complete nitrite consumption (early-stationary phase) 50 ml aliquots of the cultures were filled into 100 ml flasks, stirred and incubated in thermo-constant rooms at corresponding temperatures until the start of the measurements (*N. arctica* at 17°C, all *Nitrobacter* strains, *N. defluvii* and *N. lenta* at 28°C, *N. moscoviensis* at 37°C). Nitrite-dependent oxygen consumption was measured in a micro-respiration system (Unisense AS, Denmark). This system consisted of an 1-channel oxygen sensor amplifier (OXY-Meter), a Clark-type oxygen microsensor (OX-MR; polarized for at least 48 h before use), a stirring system with glass coated magnets, 2-ml glass chambers with glass stoppers, a rack for 8 chambers, and the data acquisition software MicOx 3.0. The response time (90 %) of the oxygen microsensor was <15 s and the oxygen uptake of the microsensor was below 1 nM $d^{-1}$ (Gundersen et al., 1998). All measurements were carried out in a re-circulated water bath in thermo-constant rooms and 200 rpm stirring. Sub-samples of the early-stationary phase cells were filled into the 2-ml glass chambers, sealed with glass stoppers and immersed in the water bath. The micro-respiration sensor was inserted through a capillary hole inside the glass stopper. The measurements started with an initial equilibration of 15-30 min, nitrite was then added through a second capillary hole from stock solutions by a syringe.

*Calculation of oxidation kinetics and other properties*

Nitrite oxidation kinetics were estimated from multiple oxygen consumption rates at varying defined nitrite concentrations. Measurements of every nitrite-oxidizing strain were iterated with at least three different cultures. The amounts of consumed nitrite were calculated from oxygen consumption according to the ratio of nitrite oxidation to oxygen consumption of 1:0.5. By using the data analysis software SigmaPlot 12.0 (Systat Software GmbH, Erkrath, Germany) oxygen uptake rates were plotted according to total nitrite concentration and kinetic

characteristics were obtained by fitting a Michaelis-Menten kinetic with the following equation to the data:

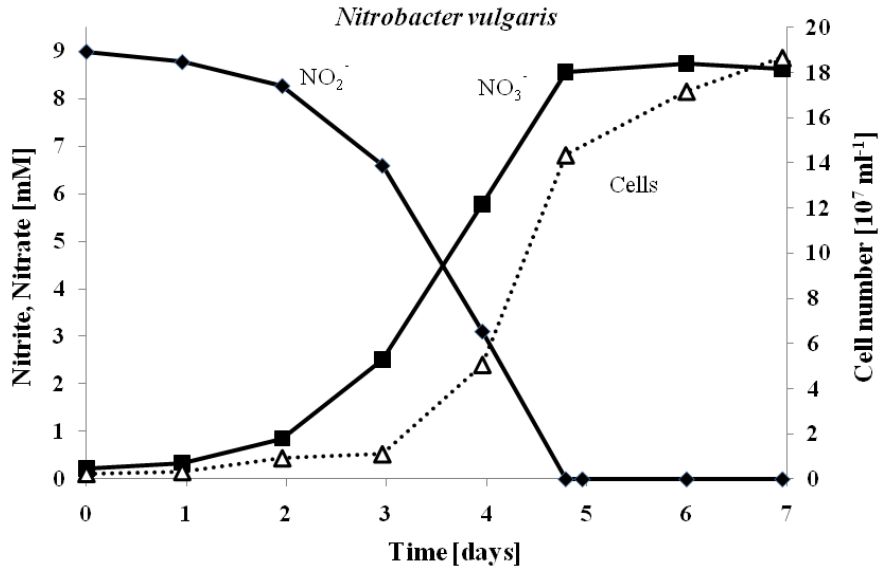
$V = (V_{max} \times [S]) / (K_m + [S])$

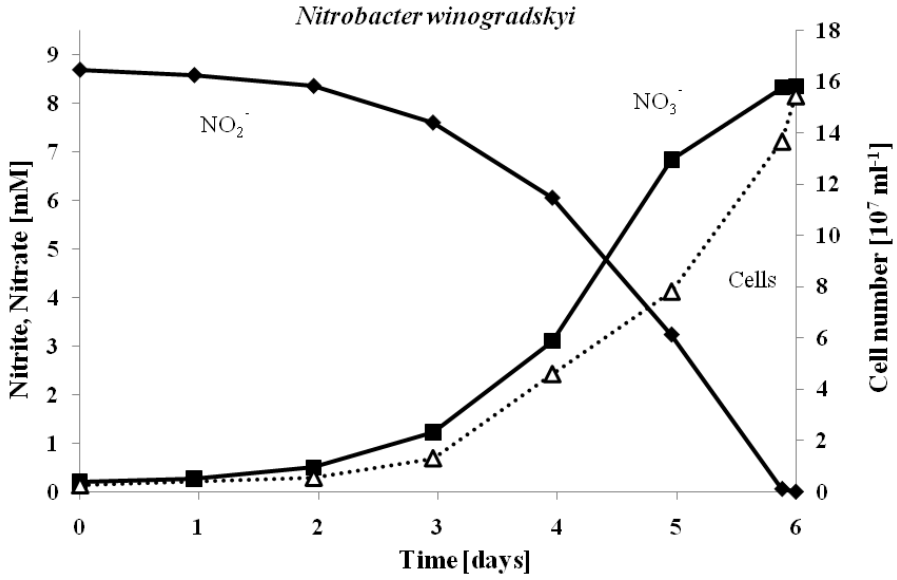
Here, V is activity, $V_{max}$ is maximum specific activity (µmol per mg protein per h), $K_m$ is half-saturation constant for nitrite oxidation (µM), and [S] is concentration of nitrite (µM). Nitrite uptake activity and cell growth were determined in 150 to 500 ml Erlenmeyer flasks of mineral medium with given nitrite concentrations at corresponding temperatures (described above) inoculated with 1 % (v/v) early-stationary phase cells. Sub-samples were withdrawn for determinations of nitrite and nitrate concentration (1 ml), cell counting (1 to 10 ml), and protein concentration (50 ml) as described above. Values of generation time, g (h), and cell activity, k (fmol $NO_2^-$ $cell^{-1}$ $h^{-1}$) were calculated from exponential growth phase as described (Belser and Schmidt, 1980). Protein growth yield, $Y_p$ (mg protein $mmol^{-1}$ nitrite), and cell growth yield, $Y_c$ (log of cells $mmol^{-1}$ nitrite), were calculated on the basis of cells or protein produced per mmol of nitrite oxidized.

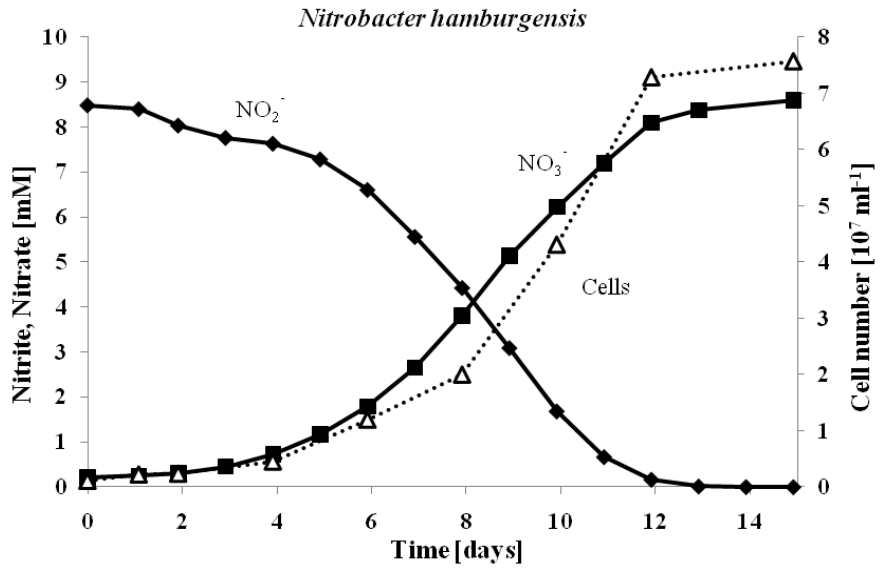
## Results

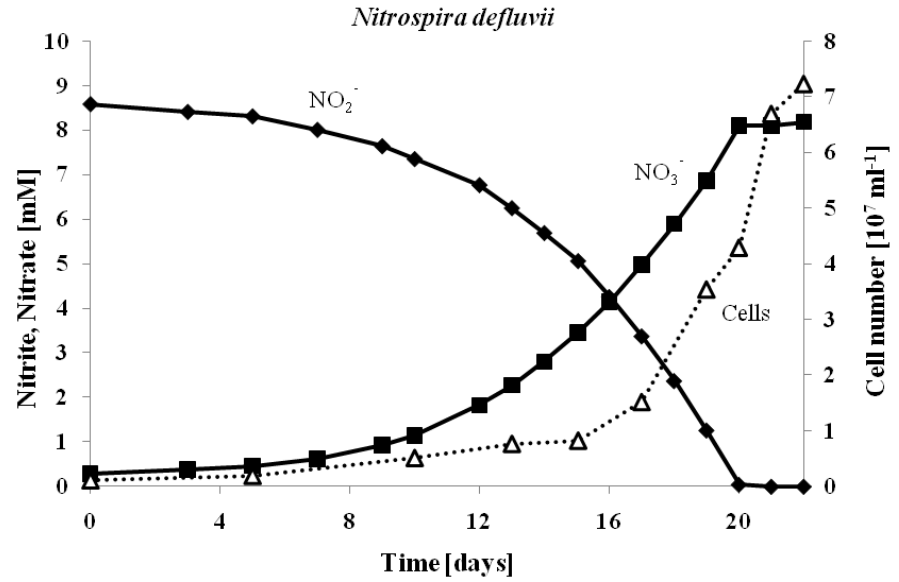
### *Growth of NOB in batch cultures*

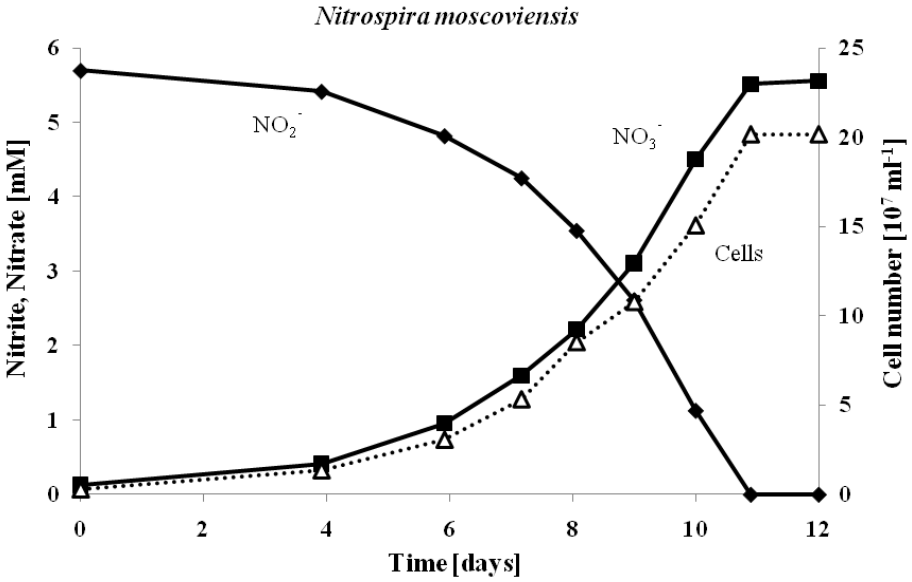
Fig. 3.1 summarizes cell growth, nitrite consumption and nitrate formation of NOB batch cultures in nitrite-limited mineral medium. Since lag-phases of *Nitrospira lenta* and the *Nitrotoga arctica* enrichment were strongly depending on the initial nitrite concentrations and growth of both was inhibited by high nitrite loads, substrate concentrations were reduced to 0.27 and 0.66 mM nitrite, respectively. In contrast, the *Nitrobacter* cultures and *Nitrospira defluvii* grew well with 9 mM nitrite, *N. moscoviensis* with 5.7 mM nitrite. For *Nitrobacter*, minimum generation times (g) of exponentially growing cells (Tab. 3.1) between 13 h (*N. vulgaris*) and 43 h (*N. hamburgensis*) were obtained. Generation times of the *Nitrospira* cultures ranged between 32 h (*N. moscoviensis*) and 37 h (*N. defluvii*, *N. lenta*), and the slowest growth was observed for *Nitrotoga arctica* (g= 44h).

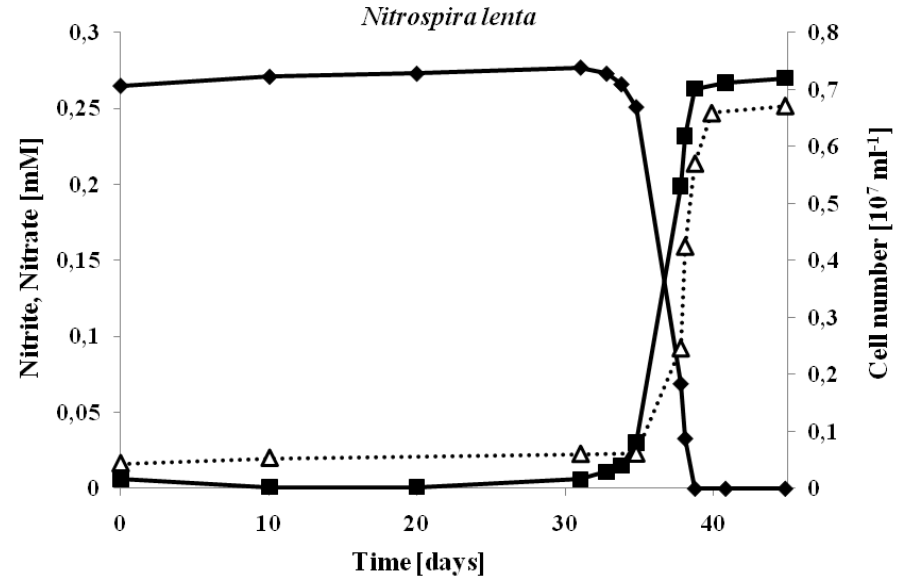

Nitrobacter vulgaris
NO2-
NO3-
Cells
Nitrite, Nitrate [mM]
Cell number [10^7 ml^-1]
Time [days]


Nitrobacter winogradskyi
NO2-
NO3-
Cells
Nitrite, Nitrate [mM]
Cell number [10^7 ml^-1]
Time [days]


Nitrobacter hamburgensis
NO2-
NO3-
Cells
Nitrite, Nitrate [mM]
Cell number [10^7 ml^-1]
Time [days]


Nitrospira defluvii
NO2-
NO3-
Cells
Nitrite, Nitrate [mM]
Cell number [10^7 ml^-1]
Time [days]


Nitrospira moscoviensis
NO2-
NO3-
Cells
Nitrite, Nitrate [mM]
Cell number [10^7 ml^-1]
Time [days]


Nitrospira lenta
Nitrite, Nitrate [mM]
Cell number [10^7 ml^-1]
Time [days]

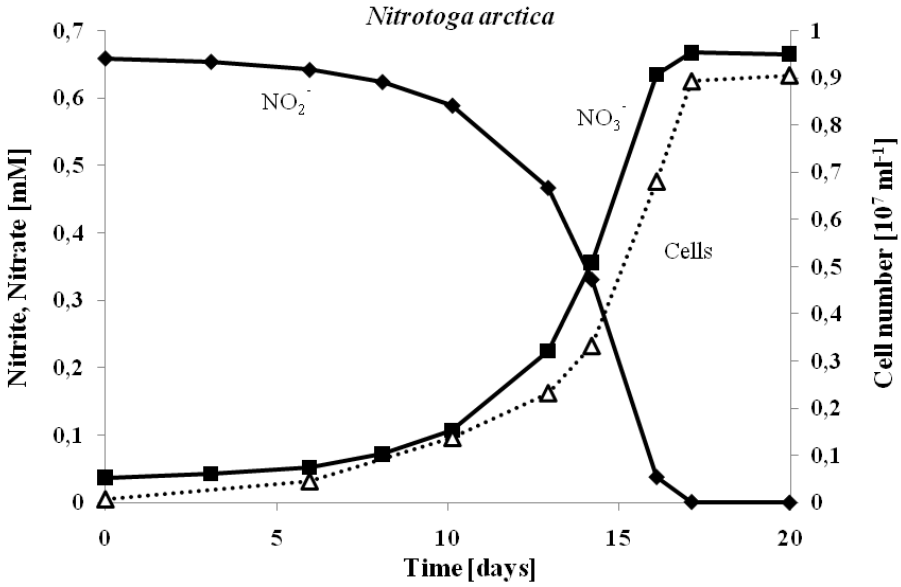



**Fig. 3.1:** Growth of NOB in nitrite-limited mineral medium batch cultures. *Nitrobacter vulgaris*, *Nitrobacter hamburgensis*, *Nitrobacter winogradskyi*, *Nitrospira defluvii*, and *Nitrospira lenta* were pure cultures incubated at 28°C. *Nitrospira moscoviensis* was incubated at 37°C and enrichment culture *Nitrotoga arctica* at 17°C. The cultures were started with one percent inocula (v/v) and initial provided nitrite was set to non-inhibiting concentrations. After complete depletion of nitrite, the cultures entered stationary phase.

*Cell activity and growth yields*

In batch cultures, cell activities (k) were calculated during exponential growth phases by simultaneous determination of cell counts and nitrite oxidation rates (Tab. 3.1). The results are consistent with $V_{max}$ values measured via oxygen consumption (see below). *N. vulgaris* showed the highest cell activity (13.1 fmol $NO_2^-$ per cell per h), followed by *N. hamburgensis* and *N. winogradskyi* (2.9 and 2.5 fmol $NO_2^-$ per cell h, respectively). Of the three studied *Nitrospira* strains *N. defluvii* attained highest cell activity (2.8 fmol $NO_2^-$ per cell per h), whereas *N. moscoviensis* had the lowest cell activity of all investigated cultures (0.6 fmol $NO_2^-$ per cell per h). *N. lenta* and the enrichment culture of *N. arctica* showed similar medium cell activities (2.1 and 2.0 fmol $NO_2^-$ per cell per h, respectively).

Further on, the nitrite-dependent growth yields of produced protein ($Y_p$) and cell concentrations ($Y_c$) are presented in Table 3.1. The results indicate that *Nitrospira* cultures have the highest yields of converting nitrite for protein synthesis. Among the *Nitrospira* cultures, *N. defluvii* showed the lowest and *N. moscoviensis* the highest protein yields. The calculated growth yields for protein of the *Nitrobacter* strains were slightly lower and the enrichment *N. arctica* revealed a similar protein yield. The yields of cells produced per nitrite

($Y_c$) were highest for the strains of *Nitrospira* lineage II, *N. moscoviensis* and *N. lenta*, whereas *N. defluvii* reached relatively low cell numbers. The further cell yields ranged between these values.

*Determination of oxidation kinetics*

The nitrite oxidation kinetics of maximum specific activity ($V_{max}$) and half-saturation constant ($K_m$) measured by microsensor-based oxygen consumption of six NOB strains and the *Nitrotoga* enrichment are presented in Fig. 3.2/3.3 and Tab. 3.1. The highest maximum oxidation activities were reached with members of the genus *Nitrobacter*. *N. vulgaris* from activated sludge showed by far the highest activity (164±9 µmol $NO_2^-$ per mg protein per h), followed by the soil-derived *N. winogradskyi* and *N. hamburgensis* (78±5 and 64±1 µmol $NO_2^-$ per mg protein per h). All studied *Nitrospira* strains reached lower maximum activities, though lineage I *N. defluvii* (48±2 µmol $NO_2^-$ per mg protein per h) isolated from activated sludge was able to attain nitrite consumption rates similar to those of *Nitrobacter* strains from soil. Both investigated lineage II *Nitrospira*, *N. moscoviensis* from heating water and *N. lenta* from activated sludge, showed less than half of this maximum activity (18±1 and 20±2 µmol $NO_2^-$ per mg protein per h, respectively). The enrichment of *Nitrotoga arctica* oxidized nitrite with a slightly higher maximum activity (26±3 µmol $NO_2^-$ per mg protein per h) than both lineage II *Nitrospira*.

The comparison of calculated half-saturation constants ($K_m$) also revealed a clear differentiation of nitrite oxidation affinities between the investigated NOB. The *Nitrospira* strains *N. defluvii* and *N. moscoviensis* (9±3 µM nitrite) were among the seven investigated NOB by far most affine towards nitrite. *N. lenta* revealed a three times higher value (27±11 µM nitrite). Of the three investigated *Nitrobacter* strains, *N. vulgaris* showed the best adaption to low nitrite concentrations (49±11 µM nitrite), though its affinity was still 1.8 to 5.4 times lower compared to *Nitrospira*. The lowest affinities were measured for *N. hamburgensis* and *N. winogradskyi* (544±55 and 309±92 µM nitrite, respectively). The nitrite affinity of the *N. arctica* enrichment culture (58±28 µM nitrite) was comparably to *N. vulgaris* in mid-range of the studied NOB.

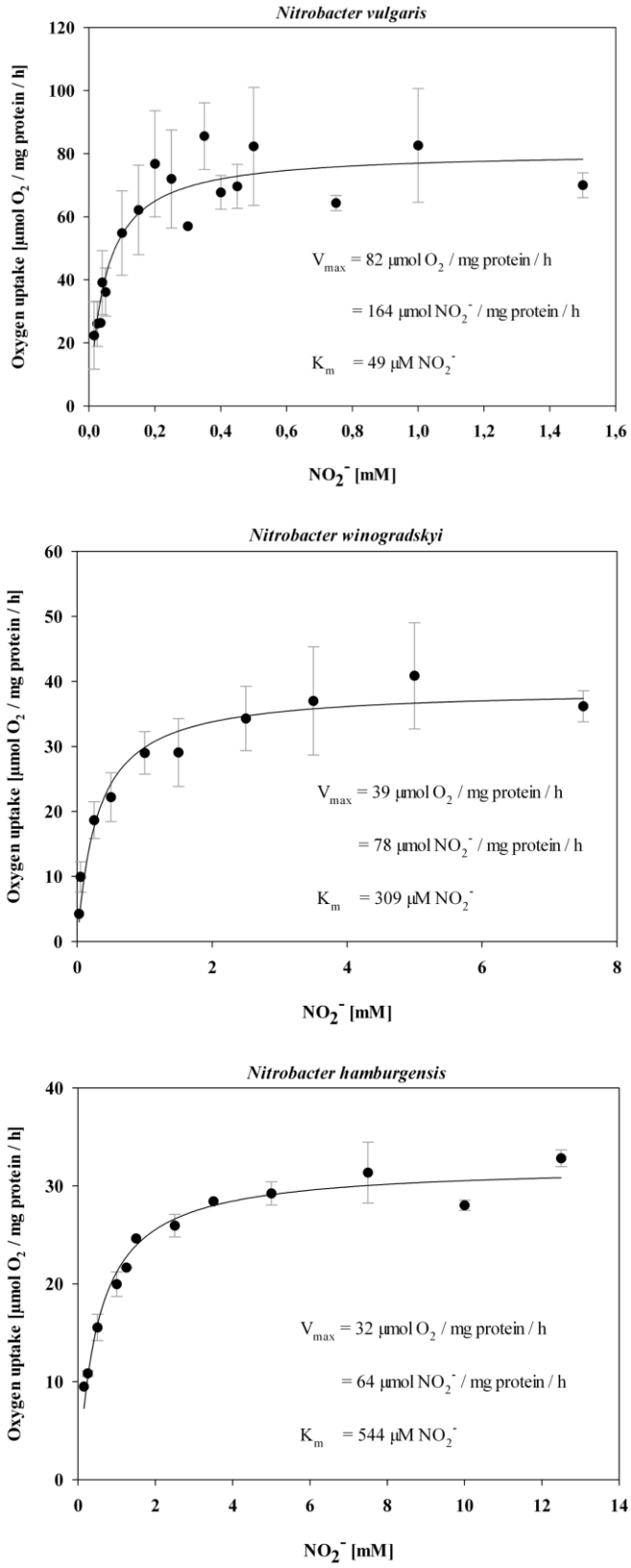



**Fig. 3.2 A:** Nitrite oxidation kinetics of *Nitrobacter*. Michaelis-Menten plots of oxygen uptake according to varying nitrite concentrations. Experiments were performed with cultures in early-stationary phase at 28°C. Value-points and standard deviations were calculated from threefold measurements. The kinetic parameters were calculated by fitting a Michaelis-Menten equation to the data. See experimental details and calculations in Materials and Methods.

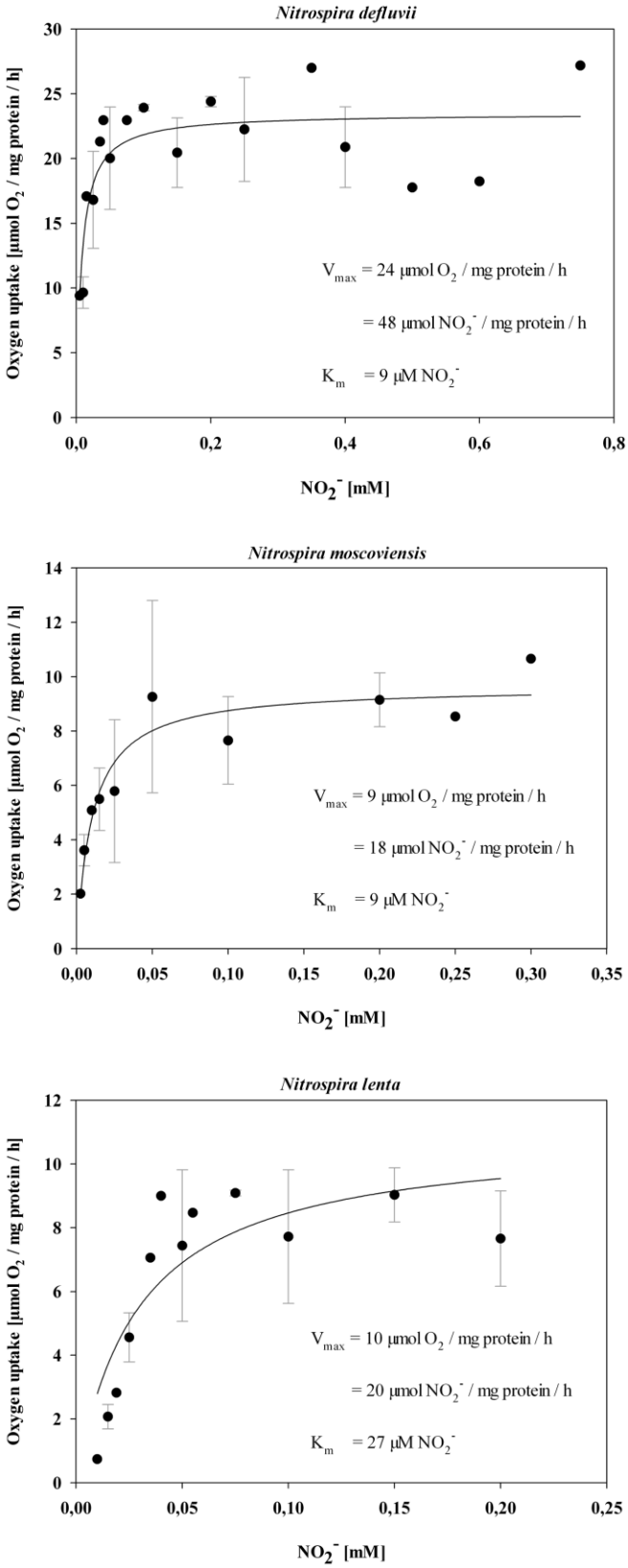



**Fig. 3.2 B:** Nitrite oxidation kinetics of *Nitrospira*. Michaelis-Menten plots of oxygen uptake according to varying nitrite concentrations. Experiments were performed with cultures in early-stationary phase at 28°C (*Nitrospira defluvii*, *Nitrospira lenta*), and 37°C (*Nitrospira moscoviensis*).Value-points and standard deviations were calculated from threefold measurements. The kinetic parameters were calculated by fitting a Michaelis-Menten equation to the data.

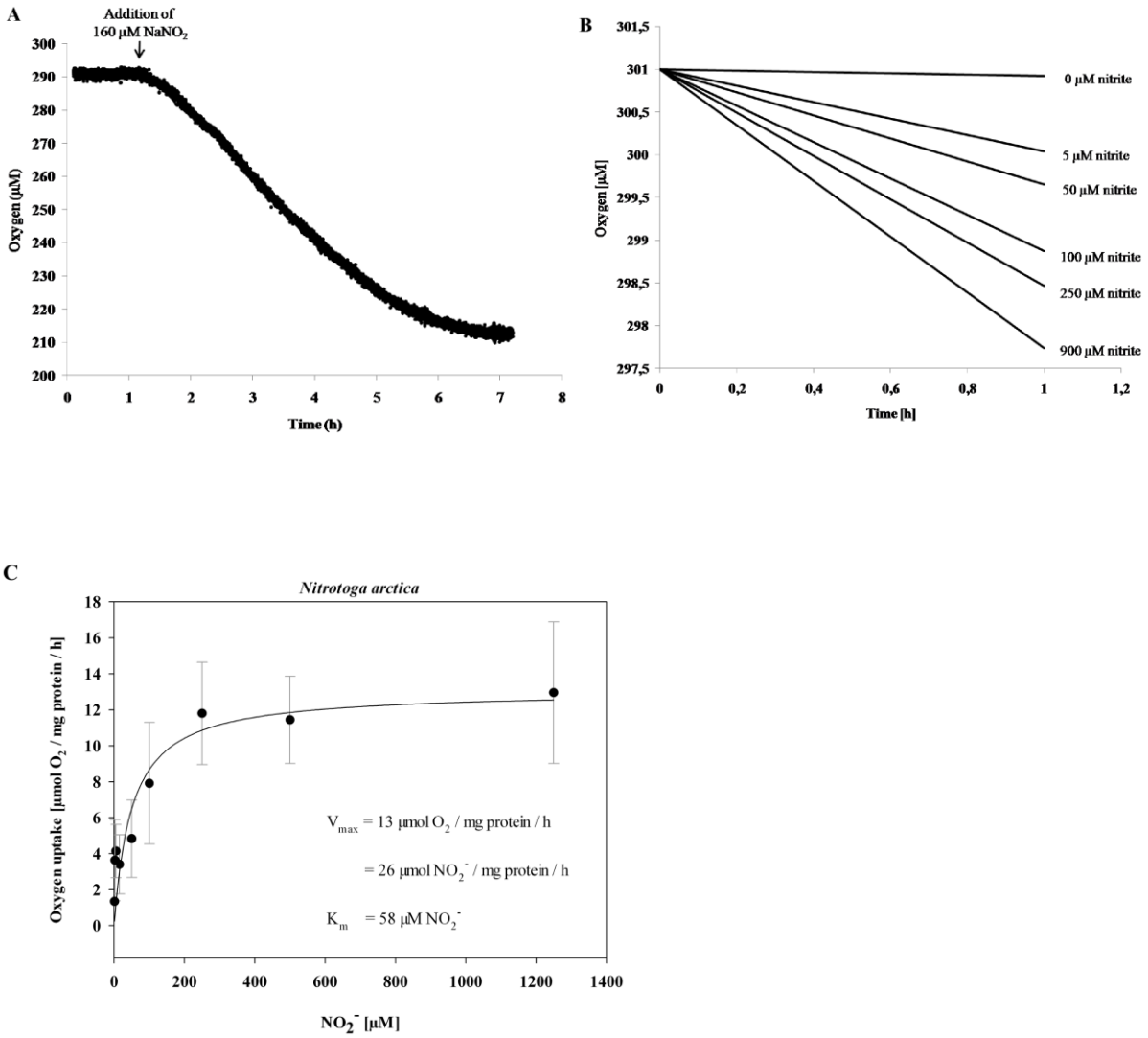



**Fig. 3.3:** Nitrite oxidation kinetics of *Nitrotoga arctica* enrichment culture. A. Nitrite-dependent oxygen uptake of early-stationary phase cells at 17°C. After equilibration of the cell aliquot and a stable oxygen concentration signal for ~1 h, the experiment was started by the addition of 160 µM $NaNO_2$. B. Nitrite-dependent oxygen uptake of early-stationary phase cells at 17°C with given nitrite concentrations. C. Michaelis-Menten plot of oxygen uptake according to nitrite concentration. The experiments were performed with early-stationary phase cells at 17°C. Values and standard deviations were calculated from threefold measurements. The kinetic parameters were calculated by fitting a Michaelis-Menten equation to the data. See experimental details and calculations in Materials and Methods.

## Discussion

Reported key growth parameters of pure NOB cultures like maximum specific activity ($V_{max}$), half-saturation constant for nitrite ($K_m$), and yield (Y) are mainly known for *Nitrobacter* (Prosser, 1989; Both et al., 1992; Laanbroek et al., 1994). The few known values of *Nitrospira* were all determined in enriched cultures (Schramm et al., 1999; Blackburne et al., 2007). Values of oxidation kinetics can largely fluctuate since kinetic constants are

affected by different growth conditions (Both et al., 1992; Laanbroek et al., 1994) and shift through different growth phases with changing substrate concentrations (Martens-Habbena and Stahl, 2011). Therefore, the aim of this study was to determine nitrite oxidation kinetics and growth parameters of non-marine NOB under standardized laboratory conditions, adjusting only strain specific temperatures and substrate preferences.

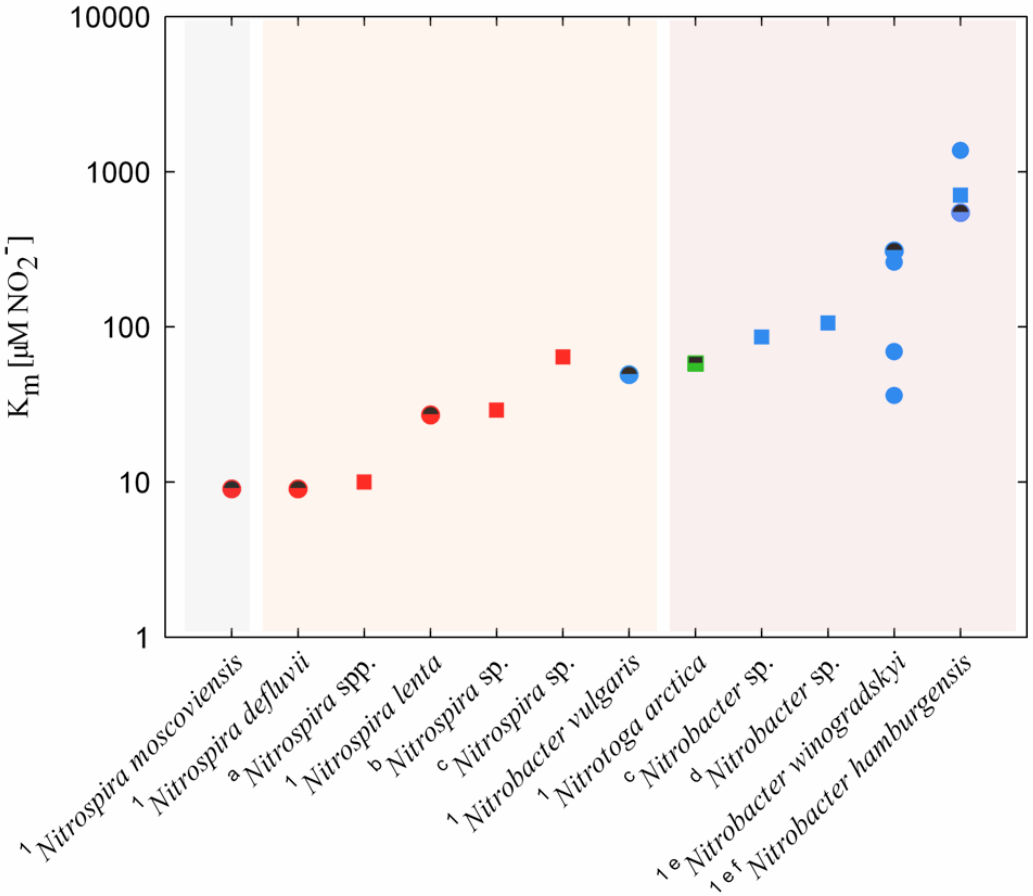



**Fig. 3.4:** Nitrite affinities of nitrite-oxidizing bacteria. Apparent $K_m$ of *Nitrospira* (red), *Nitrotoga arctica* (green), and *Nitrobacter* (blue). $K_m$ values are given for pure cultures (circles) and enrichment cultures (squares). Results from this study are marked by a black crescent. Environments are highlighted by the background colors grey (freshwater), orange (activated sludge) and bright red (soil). Values were obtained from the following references: [1]current study, [a]Schramm et al., 1999, [b]Maixner et al., 2006, [c]Blackburne et al., 2007, [d]Vadivelu et al., 2006, [e]Both et al., 1992, [f]Laanbroek et al., 1994.

*Nitrite oxidation kinetics*

Previous studies demonstrated that the availability of nitrite is an important factor for the competition of NOB, suggesting different nitrite oxidation kinetics for members of the genera *Nitrobacter* and *Nitrospira* (Schramm et al., 1999; Kim and Kim, 2006; Nogueira and Melo,

2006; Blackburne et al., 2007). Moreover, coexisting *Nitrospira* populations revealed distinct preferences for nitrite concentrations (Maixner et al., 2006). These findings indicate that substrate availability may account for the distribution of the different NOB in natural and artificial ecosystems. The comparison of $V_{max}$ and $K_m$ values for non-marine NOB strains in addition to results from literature strongly supports this assumption (Tab. 3.1 and Fig. 3.4), revealing specific adaptations for *Nitrospira*, *Nitrobacter*, *Nitrotoga* and their sublineages, and a distinct differentiation between populations from the different ecosystems.

Nitrospira

Members of the genus *Nitrospira* showed by far the highest affinities of all NOB investigated here. With the $K_m$ value of 9±3 µM nitrite, *N. defluvii* and *N. moscoviensis* have the lowest half-saturation constants determined for *Nitrospira* so far and a five times higher substrate affinity than *Nitrobacter vulgaris*, which has the highest affinity of all *Nitrobacter*. Interestingly, these *Nitrospira* strains belong to separate phylogenetical lineages I and II. Members of both lineages are widely distributed in natural habitats and were reported to be the main nitrite oxidizers in man-made ecosystems like WWTPs (Ehrich et al., 1995; Juretschko et al., 1998; Daims et al., 2001; Spieck et al., 2006; Siripong and Rittmann, 2007; Pester et al., 2013; Nowka et al., in prep.). The numerical dominance and the wide distribution of *Nitrospira* could be explained by their advantage of oxidizing nitrite with high affinity. Their $K_m$ values between 9 and 64 µM nitrite (Fig. 3.4) often resemble *in situ* conditions of ecosystems where these NOB were detected. In activated sludge of the WWTP where *N. defluvii* and *N. lenta* originated from, nitrite concentrations were measured between 7-8 µM in activated sludge samples (Kruse et al., 2013) and 7-96 µM in sewage influent (Hamburg Wasser, personal communication). *Nitrospira* have commonly been detected in oligotrophic ecosystems such as subsurface fluids (Swanner and Templeton, 2011) and freshwaters with nitrite in nanomolar concentrations and below detection limits, respectively (Martiny et al., 2005; Mußmann et al., 2013). These findings and the results of oxidation kinetics in this study support the suggested classification of *Nitrospira* as K-strategists with high affinities for the substrate nitrite (Schramm et al., 1999), hence, having an advantage in nature, where the nitrification intermediate nitrite hardly accumulates. Intriguingly, *Nitrospira* are not restricted to environments with low nitrite concentrations, but can also adapt to millimolar concentrations reported in subsurface fluids (Gihring et al., 2006) or sequencing batch reactors (Daims et al., 2001). *N. defluvii* even competes with the low affinity r-

strategists of *Nitrobacter*, which achieved 1.3-3.4 times higher maximum activities. Therefore, we propose that temporary peaks of high nitrite concentrations (e.g. in WWTPs) have low impact on *Nitrospira* dominance. However, continuously high nitrite loads were shown to result in advantageous growth of *Nitrobacter* (Bartosch et al., 1999; Nogueira and Melo, 2006).

Comparing the three studied *Nitrospira* species, lineage II *N. moscoviensis* and *N. lenta* revealed considerably lower oxidation activities than lineage I *N. defluvii*, which supports the hypothesis of Maixner et al. (2006) that lineage I outcompete lineage II *Nitrospira* under high nitrite concentrations. Interestingly, the $V_{max}$ of *N. defluvii* is much higher than that of *N. moscoviensis*, although the experimental temperature was 9°C lower, which suggests a high efficiency of *N. defluvii*. Despite relatively low maximum specific activity and cell specific activity of *N. moscoviensis* at a higher temperature of 37°C, this strain grows faster than the other *Nitrospira* strains. Since *N. moscoviensis* also revealed much higher yield values, one may speculate that this strain invests more energy in cell proliferation and growth. In difference, energy generated through nitrite oxidation may also be used for maintenance requirements proposed for *Nitrobacter* (Prosser, 1989; Vadivelu et al., 2006) and EPS production such as in *N. defluvii* cultures. Therefore, experimental studies are required to gain information about these issues in the genus *Nitrospira* and their different lineages.

In contrast to the maximum activities, the determined nitrite oxidation affinities allow no differentiation between phylogenetical lineages. *N. defluvii* (lineage I) and *N. moscoviensis* (lineage II) revealed the same high affinity, but they originated from different ecosystems (activated sludge vs. heating water). From the activated sludge derived strains, *N. lenta* (lineage II) revealed a three times lower nitrite affinity than *N. defluvii* (lineage I). The indicated advantage of *N. defluvii* under low and high nitrite concentrations raises the question if there are other factors providing the niche of *N. lenta* in wastewater treatment such as temperature, affinity to oxygen, availability of organics, and pH. These results are in agreement with the hypothesis that physiological properties are not identical between members of the same phylogenetic lineage and rather differ between individuals (Lebedeva et al., 2010).

**Tab. 3.1:** Kinetic constants and growth parameters of NOB cultures

| | *Nitrobacter vulgaris*[1] | *Nitrobacter hamburgensis*[1] | *Nitrobacter winogradskyi*[1] | *Nitrospira defluvii*[1] | *Nitrospira moscoviensis*[1] | *Nitrospira lenta*[1] | *Nitrotoga arctica*[2] |
|---|---|---|---|---|---|---|---|
| **Generation time, g (hours)** | **13**<br>12[a] | **43**<br>40[b], 63-84[e] | **26**<br>8-14[c], 41-87[e] | **37** | **32**<br>12[d] | **37** | **44** |
| **Maximum specific activity, $V_{max}$ (µmol $NO_2^-$ mg protein$^{-1}$ h$^{-1}$)** | **164±9** | **64±1** | **78±5** | **48±2** | **18±1** | **20±2** | **26±3** |
| **Saturation constant for activity, $K_m$ (µM $NO_2^-$)** | **49±11** | **544±55**<br>540-1370[e]<br>706-1240[3,f] | **309±92**<br>36-260[e]<br>170-1380[g] | **9±3** | **9±3** | **27±11** | **58±28** |
| **Maximum specific cell activity, k (fmol $NO_2^-$ cell$^{-1}$ h$^{-1}$)** | **13.1** | **2.9**<br>1.0-3.3[e]<br>3.7-7.4[3,f] | **2.5**<br>1.9-3.7[e]<br>12[h] | **2.8** | **0.6** | **2.1** | **2.0** |
| **Growth yield, $Y_p$ (mg protein mmol$^{-1}$ nitrite)** | **0.099** | **0.108** | **0.083** | **0.122** | **0.213**<br>0.120[d] | **0.142** | **0.102** |
| **Growth yield, $Y_c$ (log of cells mmol$^{-1}$ nitrite)** | **10.32** | **9.95** | **10.26** | **9.93** | **10.55** | **10.40** | **10.14** |

Values from the literature are printed in thin face: [a] Bock et al., 1990; [b] Bock et al., 1983; [c] Watson et al., 1989; d Ehrich et al., 1995; e Both et al., 1992; f Laanbroek et al., 1994; g Hunik et al., 1993; h Belser, 1979

[1] Pure culture, [2] Enrichment culture, [3] Mixed culture

Nitrobacter

While *Nitrobacter* species showed the highest maximum activities ($V_{max}$) of NOB, their affinities to oxidize nitrite are generally low. However, the $K_m$ values range from 36 to 1370 µM nitrite (Fig. 3.4), indicating a great diversity of *Nitrobacter* ecotypes, which agrees with the detected genetic heterogeneity within *Nitrobacter* communities revealed by several studies (Grundmann and Normand, 2000; Poly et al., 2007; Vanparys et al., 2007; Wertz et al., 2008). In this study, *N. vulgaris* derived from activated sludge revealed the highest nitrite affinity among the investigated *Nitrobacter*. For both soil-derived isolates *N. winogradskyi* and *N. hamburgensis* we determined 6.3-11.1 times lower nitrite affinities, which indicate that other environmental factors aside from nitrite concentration may account for *Nitrobacter* distribution. The potential of growing mixotrophically and chemoorganotrophically (Smith and Hoare, 1968; Bock, 1976; Steinmüller and Bock, 1976), and even anaerobically via nitrate reduction (Freitag et al., 1987; Ahlers et al., 1989), confers *Nitrobacter* a versatile metabolism. In accordance, the richness of *Nitrobacter* in soils was suggested to be related to the increase of organic matter content and high N availability (Poly et al., 2007; Ke et al., 2013; Attard et al., 2010). Although mixotrophic growth is also present in other NOB like *Nitrospira* (Watson et al., 1986; Daims et al., 2001; Keuter et al., 2011), the metabolic potential of *Nitrobacter* may be an important factor for competition despite disadvantageous oxidation kinetics under low nitrite concentrations of most environmental settings.

Nitrotoga

The nitrite oxidation kinetics of the enrichment *Nitrotoga arctica* from permafrost soil revealed that these bacteria are K-strategists like *Nitrospira*. The maximum specific activity was slightly higher than that of *N. moscoviensis* and *N. lenta*, but lower than the activity of the other NOB including the other soil derived *Nitrobacter*. However, *Nitrotoga arctica* can be classified between the lowest *Nitrospira* substrate affinities and the highest affinities of *Nitrobacter* (Fig. 3.4), and the $K_m$ value indicates a better adaption of *N. arctica* to low nitrite concentrations compared to the soil-inhabiting *Nitrobacter* strains. Additionally, these values were obtained at an incubation temperature of only 17°C in contrast to higher temperatures of 28°C and 37°C of the other cultures. This indicates that *Nitrotoga* are capable of prevailing in cold ecosystems. Accordingly, previous studies showed that *Nitrotoga* overgrew *Nitrospira* during long-term cultivation at 10°C (Alawi et al., 2009) and that the main nitrite oxidizer in

two nitrification bioreactors running at 5°C was identified as *Nitrotoga* (Karkman et al., 2011). Further, sequences with high similarity to *Nitrotoga*-like bacteria were detected in diverse habitats (Alawi et al., 2007; Schwarz et al., 2007; Chen et al., 2009; White et al., 2012), indicating that *Nitrotoga* is widely distributed in cold-affected environments.

**Acknowledgments**

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# Chapter IV


## Summary

The microbial process of nitrification prevents the accumulation of ammonia and nitrite in the environment, which are toxic for most organisms at high concentrations and can lead to eutrophication of freshwater and coastal marine ecosystems. In wastewater treatment systems (WWTPs), nitrification is the most crucial step for nitrogen removal. Although nitrite is generated ubiquitously by oxidation of ammonia under aerobic conditions, it hardly accumulates in most ecosystems. The conversion of nitrite to nitrate in the second step of nitrification is catalyzed by nitrite-oxidizing bacteria (NOB). Nitrite is an energy-poor substrate that requires highly specialized microorganisms. To date, the ability of aerobic nitrite oxidation is found in six genera of phylogenetically diverse NOB. However, most knowledge of their physiology and metabolism has been derived from studies of a limited number of pure cultures of *Nitrobacter*. Meanwhile, molecular techniques have revealed that members of the genus *Nitrospira* are often the dominant nitrite oxidizers in many habitats. Additionally, the new genus *Nitrotoga* was recently discovered in permafrost soil, which has been barely studied so far.

Currently, few pure cultures of *Nitrospira* and only a couple of *Nitrotoga* enrichments are available and details of their ecophysiology and niche differentiation are scarce. To overcome this limitation, the aim of this study was to bring two new *Nitrospira* species into pure culture and to gain new insights into their physiology and morphology. Subsequently, the nitrite oxidation kinetics and growth characteristics of members of the genera *Nitrospira, Nitrotoga,* and *Nitrobacter* were determined.

In the first part of this thesis, the successful isolation and characterization of the species *Nitrospira defluvii* (lineage I) and *Nitrospira lenta* (lineage II) is presented. Members of the phylogenetical *Nitrospira* lineages I and II have been frequently detected co-occurring in wastewater treatment. Similar physiological characteristics were the resistance against antiseptic acriflavine as well as the temperature ranges for growth with an optimum at 28°C. In contrast, the isolates differed in their tolerances against high nitrite and nitrate concentrations. *N. defluvii* appeared to be more robust with high tolerances, while *N. lenta* was more sensitive to high concentrations of the substrate and product of nitrite oxidation. Electron microscopic images revealed morphological features such as strong biofilm formation of aggregating *N. defluvii* cells as a response to high nitrite or acriflavine

concentrations. In contrast, the cells of *N. lenta* were mainly planktonic and excreted less extracellular polymeric substances (EPS). Consequently, the planktonic lifestyle of *N. lenta* allowed the separation of individual cells by a laser-based micro-tweezers system, which turned out to function as a fast and efficient isolation method for non-aggregating cells.

The phylogenetical investigations on *Nitrospira* enrichments from natural habitats revealed a wide distribution of lineage I and II representatives in extreme and moderate ecosystems such as drylands and freshwater. Since lineage I *Nitrospira* have mostly been found in engineered ecosystems such as WWTPs before, this uncovered wide distribution underlines their global importance in nitrification.

The availability of nitrite is suggested as a key factor providing niche differentiation of NOB. Previous studies have revealed that *Nitrobacter* and *Nitrospira* are adapted to different nitrite concentrations. But so far, nitrite oxidation kinetics of pure cultures are only known for strains of *Nitrobacter*. In the second part of this thesis, the nitrite oxidation kinetics and growth characteristics of three *Nitrospira* strains (including the new isolates), three *Nitrobacter* strains, and an enrichment culture of *Nitrotoga* were determined.

With the $K_m$ value of 9 µM nitrite, *N. defluvii* and *N. moscoviensis* showed by far the highest affinities for nitrite, suggesting members of *Nitrospira* as main nitrite oxidizers particularly in environments with low nitrite accumulation. The third investigated species of *Nitrospira*, *N. lenta*, had a lower affinity of 27 µM nitrite, which supports the hypothesis that members of *Nitrospira* occupy different microniches according to their preferred nitrite concentrations. The three *Nitrobacter* strains revealed lower nitrite affinities over a wide range of $K_m$ values between 49 (*N. vulgaris*) and 544 (*N. hamburgensis*) µM nitrite, which indicates that the genus *Nitrobacter* also contains diverse ecotypes. Further, their high maximum oxidation activities ($V_{max}$) of 64-164 µmol $NO_2^-$ per mg protein per h demonstrate that *Nitrobacter* prevails under elevated nitrite concentrations. The enrichment *Nitrotoga arctica* showed a medium nitrite affinity of 58 µM, but a relatively high maximum activity under a decreased temperature of 17°C with 26 µmol $NO_2^-$ per mg protein per h. Therefore, *Nitrotoga*-like bacteria might have notable impact on nitrification in cold-affected natural ecosystems such as soils, freshwater sediments, and biofilms, but also in engineered systems used in wastewater treatment. Together these findings provide a comprehensive overview of nitrite oxidation kinetics and growth characteristics of non-marine representatives of three NOB genera, which is important to understand their distribution in natural environments and engineered systems.

**Zusammenfassung**

Der Prozess der Nitrifikation verhindert die Anhäufung von Ammoniak und Nitrit, welche für die meisten Organismen in hohen Konzentrationen toxisch sind und zur Eutrophierung von Süßwasser- und küstennahen Meeresökosystemen führen können. Die Nitrifikation ist für die Stickstoffentfernung in Kläranlagen essentiell. Obwohl Nitrit durch die ubiquitäre Oxidation von Ammoniak unter aeroben Bedingungen generiert wird, akkumuliert es kaum in den meisten Ökosystemen. Der zweite Schritt der Nitrifikation, die Umsetzung von Nitrit zu Nitrat, wird durch Nitrit oxidierende Bakterien (NOB) katalysiert. Nitrit ist ein Substrat mit geringer Energie, das hochspezialisierte Mikroorganismen benötigt. Bisher ist die Fähigkeit der aeroben Nitritoxidation in sechs Gattungen phylogenetisch diverser NOB gefunden worden. Das meiste Wissen über die Physiologie und den Metabolismus stammt jedoch aus Untersuchungen an wenigen Reinkulturen von *Nitrobacter*. Mittlerweile haben molekulare Techniken aufgedeckt, dass Vertreter der Gattung *Nitrospira* in vielen Habitaten häufig die dominanten NOB sind. Desweiteren wurde vor kurzem die neue Gattung *Nitrotoga* in Permafrostboden entdeckt, die bisher kaum untersucht wurde.

Derzeit sind wenige Reinkulturen von *Nitrospira* und nur ein paar Anreicherungen von *Nitrotoga* vorhanden, zudem sind kaum Details über deren Ökophysiologie und Nischendifferenzierung bekannt. Aufgrund dieser Einschränkung war es das Ziel dieser Arbeit zwei neue Arten von *Nitrospira* in Reinkultur zu bringen und neue Erkenntnisse über deren Physiologie und Morphologie zu erlangen. Anschließend sollten die Kinetik der Nitritoxidation und die Wachstumseigenschaften der Gattungen *Nitrospira*, *Nitrotoga* und *Nitrobacter* bestimmt werden.

Im ersten Abschnitt dieser Dissertation wird die erfolgreiche Isolierung und Charakterisierung der zwei Arten *Nitrospira defluvii* (Abstammungslinie I) und *Nitrospira lenta* (Abstammungslinie II) dargestellt. Vertreter der phylogenetischen Abstammungslinien I und II von *Nitrospira* sind regelmäßig in Abwasser gemeinsam entdeckt worden. Gemeinsamkeiten in der Physiologie waren die Resistenz gegenüber dem Antiseptikum Acriflavin sowie der Temperaturbereich für das Wachstum (Optimum bei 28°C). Im Gegensatz dazu unterschieden sich die Isolate in deren Toleranz gegenüber hohen Nitrit- und Nitratkonzentrationen. *N. defluvii* war mit hohen Toleranzen robuster, während *N. lenta* sensitiver gegenüber hohen Konzentrationen des Substrates und des Produktes der Nitritoxidation war. Aufnahmen durch Rasterelektronenmikroskopie (REM) und Elektronenmikroskopie (EM) zeigten morphologische Eigenschaften, wie die starke

Biofilmbildung der aggregierenden Zellen von *N. defluvii* als Reaktion auf hohe Nitrit- oder Acriflavinkonzentrationen. Im Unterschied dazu kam *N. lenta* in planktonischer Zellform mit geringerer Ausscheidung extrazellulärer polymerer Substanzen (EPS) vor. Aufgrund des planktonischen Lebensstils von *N. lenta* war es möglich, die Trennung einzelner Zellen mit Hilfe eines laserbasierten Mikropinzetten-Systems durchzuführen, was sich als eine schnelle und effiziente Isolationsmethode für nicht-aggregierende Zellen herausstellte.
Die phylogenetischen Untersuchungen von *Nitrospira*-Anreicherungen aus natürlichen Habitaten deckten eine weite Verbreitung von Vertretern der Abstammungslinien I und II in extremen und moderaten Ökosystemen, wie durch Trockenheit betroffene Böden und Süßwasser, auf. Da *Nitrospira* der Abstammungslinie I bisher vornehmlich in technischen Systemen wie Kläranlagen gefunden wurden, unterstreicht diese bisher unbekannte weite Verbreitung deren globale Bedeutung in der Nitrifikation.

Die Verfügbarkeit von Nitrit wird als ein Schlüsselfaktor der Nischendifferenzierung von NOB angesehen. Vorherige Arbeiten haben aufgedeckt, dass *Nitrobacter* und *Nitrospira* an verschiedene Nitritkonzentrationen angepasst sind, jedoch ist die Kinetik der Nitritoxidation von Reinkulturen bisher nur für Stämme von *Nitrobacter* untersucht worden. Daher wurden in dieser Dissertation die Kinetik und die Wachstumseigenschaften von drei *Nitrospira*-Stämmen (unter anderem die neuen Isolate), drei *Nitrobacter*-Stämmen und einer *Nitrotoga*-Anreicherung bestimmt.

Mit einem $K_m$-Wert von 9 µM Nitrit zeigten *N. defluvii* und *N. moscoviensis* die bei weitem höchste Affinität für Nitrit, was Vertreter von *Nitrospira* als die wahrscheinlich vorherrschenden Nitritoxidierer, insbesondere in Umgebungen geringer Nitritakkumulation, macht. Die dritte untersuchte Art von *Nitrospira*, *N. lenta*, hatte mit 27 µM Nitrit eine geringere Affinität, was zu der Hypothese führt, dass Vertreter von *Nitrospira* verschiedene Mikronischen entsprechend ihrer präferierten Nitritkonzentration besetzen. Die drei *Nitrobacter*-Stämme zeigten mit den unterschiedlichen $K_m$-Werten zwischen 49 (*N. vulgaris*) und 544 (*N. hamburgensis*) µM Nitrit eine geringere Affinität zu Nitrit, was ebenfalls andeutet, dass diese Gattung diverse Ökotypen enthält. Als weiteres zeigten die hohen maximalen Oxidationsaktivitäten ($V_{max}$) von 64-164 µmol $NO_2^-$ / mg Protein / h, warum *Nitrobacter* bei erhöhten Nitritkonzentrationen vorherrscht. Die Anreicherung *Nitrotoga arctica* offenbarte mit 58 µM eine mittlere Affinität zu Nitrit, aber eine relativ hohe maximale Aktivität mit 26 µmol $NO_2^-$ / mg Protein / h, bei einer geringen Temperatur von 17°C. Daher könnten *Nitrotoga*-ähnliche Bakterien einen deutlichen Einfluss auf die Nitrifikation in

kältebetroffenen natürlichen Ökosystemen wie Erdböden, Süßwassersedimenten und Biofilmen, aber auch in technischen Systemen der Abwasseraufbereitung haben.

Zusammen verschaffen diese Ergebnisse einen umfassenden Überblick über die Kinetik der Nitritoxidation und die Wachstumseigenschaften nichtmariner Repräsentanten dreier Gattungen von NOB, was maßgeblich für das Verständnis über die Verbreitung in natürlichen Umgebungen und technischen Systemen ist.

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# Appendix

## List of publications

1. Boris Nowka, Sandra Off, Holger Daims, and Eva Spieck. Natural distribution and ecophysiology of *Nitrospira*: novel insights from laboratory cultures. (in preparation)

2. Boris Nowka, Holger Daims, and Eva Spieck. Comparative oxidation kinetics of nitrite-oxidizing bacteria: nitrite availability as key factor for niche differentiation. (in preparation)

3. Jessika Füssel and Boris Nowka, Sebastian Lücker, Phyllis Lam, Eva Spieck, Holger Daims, Philipp Hach, Sten Litman, and Marcel M. M. Kuypers. Metabolic versatility of a globally distributed nitrite oxidizer, *Nitrococcus* sp. (in preparation)

4. Sebastian Lücker, Boris Nowka, Thomas Rattei, Eva Spieck, and Holger Daims (2013). The genome of *Nitrospina gracilis* illuminates the metabolism and evolution of the major marine nitrite oxidizer. *Frontiers in Microbiology* 4: 27.

5. Michael Pester, Frank Maixner, David Berry, Thomas Rattei, Hanna Koch, Sebastian Lücker, Boris Nowka, Andreas Richter, Eva Spieck, Elena Lebedeva, Alexander Loy, Michael Wagner and Holger Daims (2013). *NxrB* encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing *Nitrospira*. *Environmental Microbiology*. doi: 10.1111/1462-2920.12300

6. Anja Spang, Anja Poehlein, Pierre Offre, Sabine Zumbrägel, Susanne Haider, Nicolas Rychlik, Boris Nowka, Christel Schmeisser, Elena Lebedeva, Thomas Rattei, Christoph Böhm, Markus Schmid, Alexander Galushko, Roland Hatzenpichler, Thomas Weinmaier, Rolf Daniel, Christa Schleper, Eva Spieck, Wolfgang Streit and Michael Wagner (2012). The genome of the ammonia-oxidizing *Candidatus* Nitrososphaera gargensis: insights into metabolic versatility and environmental adaptations. *Environmental Microbiology* 14: 3122–3145. doi: 10.1111/j.1462-2920.2012.02893.x

**Supplementary information**

>Nitrospira BS10_16S rRNA gene sequence

AGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCGCGCCTAATACATGCAAGT
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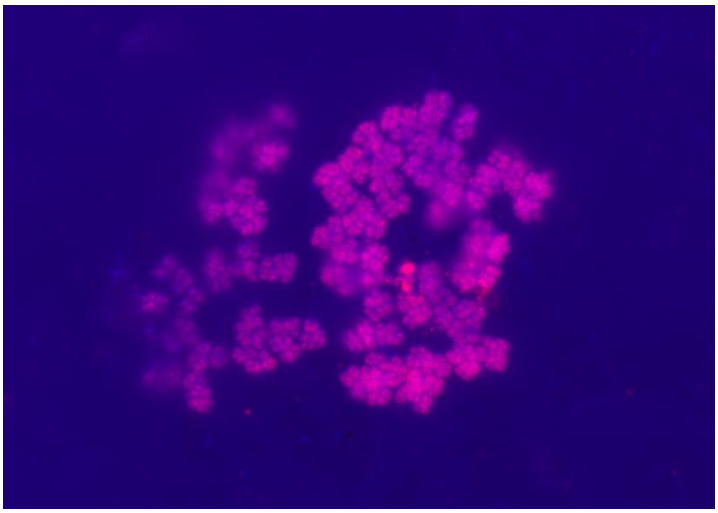

**Fig. S1:** Epifluorescence micrograph of *Nitrospira defluvii* cells hybridized with the *Nitrospirae*-specific probe Ntspa-712 (Cy3-labeled) and stained with DAPI.

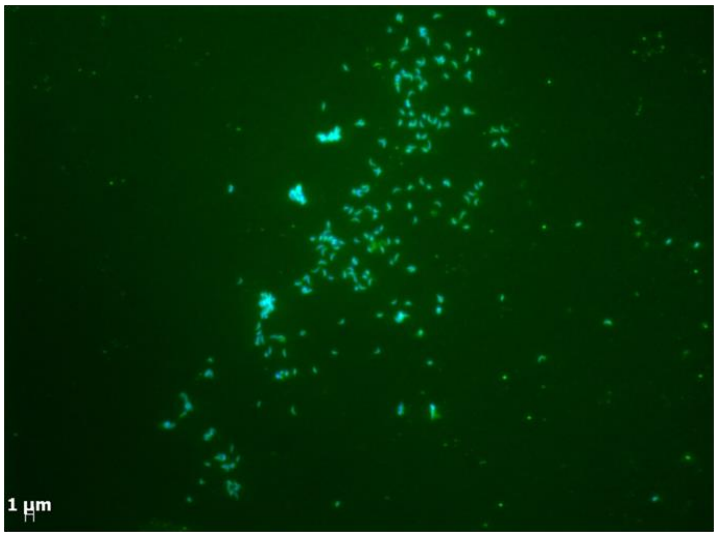


**Fig. S2:** Epifluorescence micrograph of *Nitrospira* strain BS10 cells hybridized with the *Nitrospira* sublineage II-specific probe Ntspa-1151 (FITC-labeled) and stained with DAPI.

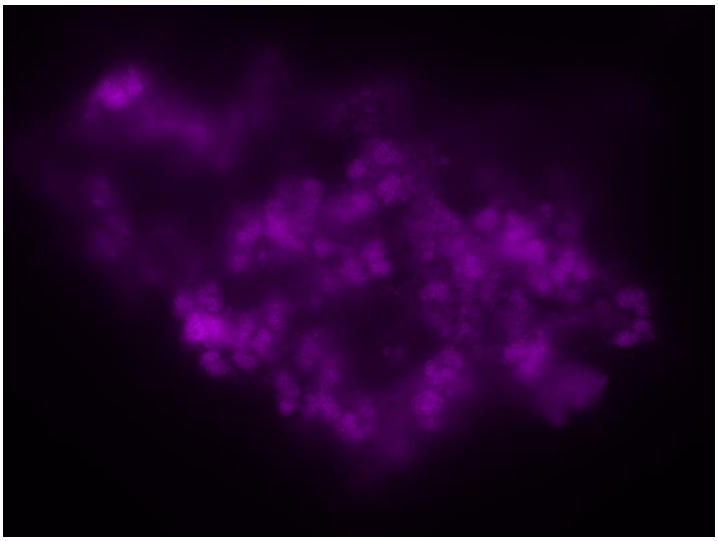

**Fig. S3:** Epifluorescence micrograph depicting aggregates of *Nitrotoga arctica* 6680 cells hybridized with the *Betaproteobacteria*-specific probe BET42a (Cy3-labeled) and stained with DAPI.

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