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„Expanding the Knowledge on Nitrifying Bacteria and Their Ecological Niches: From Cultures to the Environment and Back Again“

verfasst von

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Table of Contents

1 INTRODUCTION

1.1 The Nitrogen Cycle

Nitrogen (N) is an essential macronutrient for all organisms and viruses, as it is a major constituent of nucleic and amino acids (AA) – molecules, which allow for information storage and processing, as well as cell functioning. Because all natural systems are dependent on N, understanding of the global N-cycle is crucial. The N-cycle (see Figure 1.1 for a schematic overview with emphasis on biologically catalyzed reactions) comprises a variety of N-compounds with different redox states, ranging from the most reduced at -3 (ammonia/ammonium, NH_3/NH_4^+) to the most oxidized at +5 (nitrate, NO_3^-). The interconversion of these N-compounds by (micro-) organisms is usually tightly linked to other element cycles (e.g. carbon- and sulfur-cycle). This coupling can be either due to concomitant incorporation of these elements into biomass in certain ratios, or due to coupling of redox reactions involving different elements during respiration. The wide range of N-compound redox states and reduction potentials supports a plethora of pathways allowing for generation of energy and reducing equivalents, which are predominantly carried out by specialized bacteria and archaea (Simon & Klotz 2013).

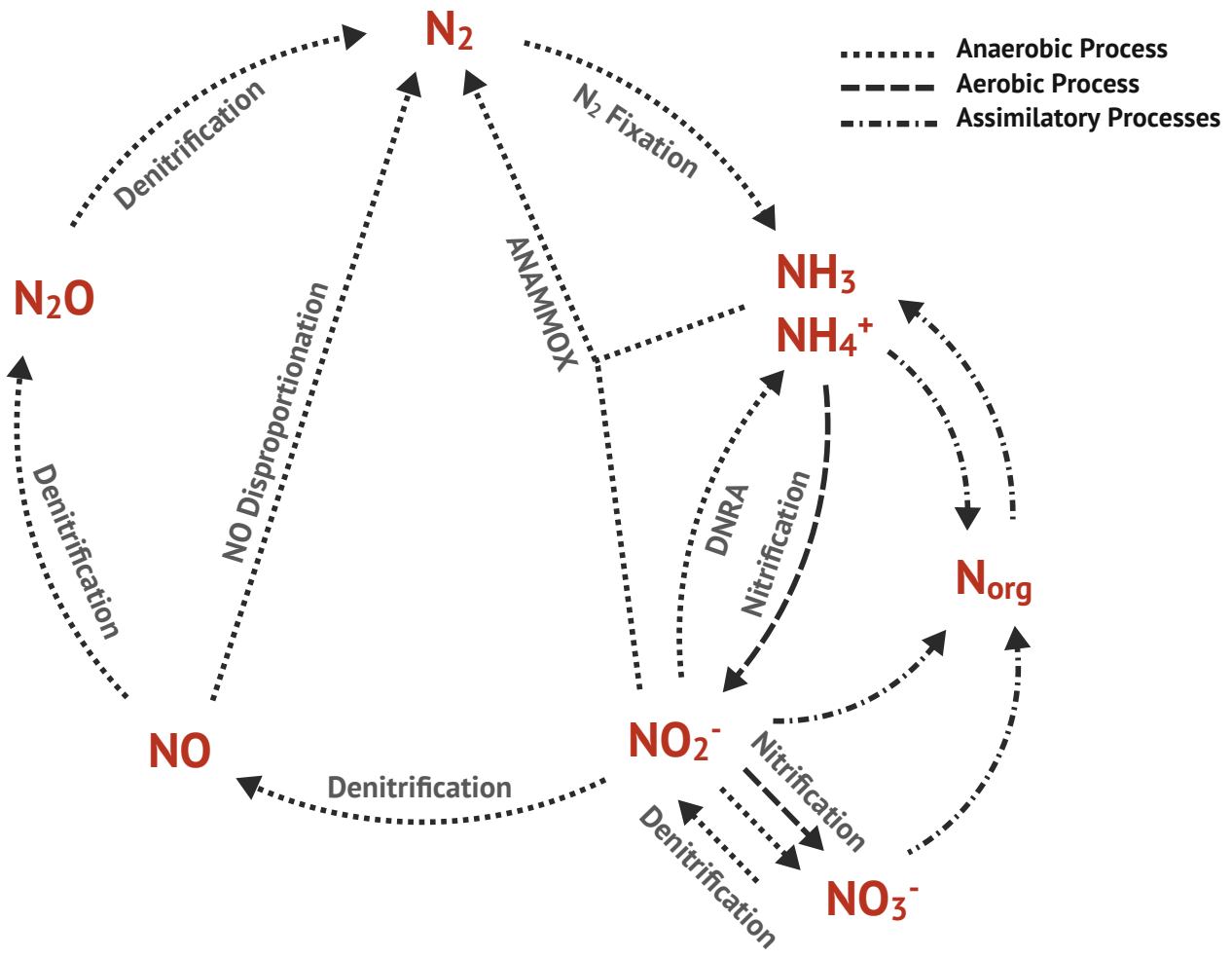

Figure 1.1 Schematic N-cycle with emphasis on biologically catalyzed processes. N-compounds are depicted in red, process names in grey. Arrowheads indicate direction of the reactions. Anaerobic processes are indicated by dotted lines, aerobic processes by dashed lines. Dashed/dotted lines indicate assimilatory processes. ANAMMOX: anaerobic ammonium oxidation. DNRA: dissimilatory nitrite or nitrate reduction to ammonium. Modified from Canfield et al. (2010).

1.1.1 Nitrogen Fixation

The largest reservoir of N is found as gaseous N_2, making up 78% of today's atmospheric composition. Due to its stable triple bond, N_2 is chemically inert and therefore not readily available to organisms for incorporation into cellular compounds. The energetically costly conversion of N_2 into more reactive and thereby bio-available forms of N (N_r), a process termed N-fixation, is biologically only carried out by specialized members of bacteria and archaea (e.g. Mehta & Baross 2006; Dekas et al. 2009; Canfield et al. 2010). Another natural, albeit not biological, N_r-input stems from atmospheric fixation of N_2 during lightning (Schumann & Huntrieser 2007). This however constitutes only a minor source of N_r compared to biological N-fixation (approximately 5 Tg N^*y^{-1} as compared to 250 Tg N^*y^{-1}, Schumann & Huntrieser 2007 and Gruber & Galloway 2008 respectively). In addition to natural inputs of N_r, human activities have drastically increased bio-available N. Anthropogenic inputs of N_r from combustion of fossil fuels, cultivation of legumes and, most importantly, the Haber-Bosch process result in almost as much available N_r as from all natural sources together (Canfield et al. 2010). The additional anthropogenic N_r has led to imbalances and acceleration of the natural N-cycle and to eutrophication of various ecosystems (Gruber & Galloway 2008; Martínez-Espinosa et al. 2011).

1.1.2 Nitrification

Biological N-fixation yields NH_3, which is subsequently protonated to NH_4^+ to a large proportion at acidic to moderately alkaline pH (pK_a 9.24). NH_4^+ can be readily assimilated into biomass by bacteria, archaea and Eukaryotes. Under aerobic conditions, ammonia-oxidizing microorganisms (AOM), which are found both among bacteria (AOB) and archaea (AOA), oxidize NH_3 to nitrite (NO_2^-) to gain energy. While the resulting NO_2^- is toxic to most organisms when it accumulates in the environment, NO_2^- can nonetheless be utilized for assimilatory, dissimilatory, and respiratory processes by specialized groups of organisms (Simon 2002; Simon & Klotz 2013, for a definition of assimilation, dissimilation, and respiration see Moreno-Vivián & Ferguson 1998). Under aerobic conditions, aerobic nitrite oxidizers, which are so far only known among bacteria (NOB), oxidize NO_2^- to NO_3^- to gain energy. The resulting NO_3^- can be readily assimilated as N-source by many organisms and is a common electron acceptor in absence of O_2. Due to the syntrophy between AOM and NOB (collectively termed nitrifiers), these guilds are often found in close spatial association, as NOB rely on NO_2^- produced by AOM, and AOM on removal of their metabolism's toxic end product, which has been shown to inhibit ammonia oxidizing activity in *Nitrosomonas europaea* (Stein & Arp 1998). Recently however, it has become more and more apparent that especially in marine systems, NOB may be decoupled from AOM, and instead obtain their substrate from anaerobic reduction of NO_3^- to NO_2^- by other organisms (e.g. Füssel et al. 2012; Beman et al. 2013). Both AOM and NOB generate ATP and reducing equivalents during oxidation of N-compounds, which are used for CO_2-fixation. Nonetheless, some AOM and NOB have been found to be able to grow facultatively or even obligatorily mixotrophically (e.g. Clark & Schmidt 1966; Bock 1976; Krümmel & Harms 1982; Daims et al. 2001; Spieck et al. 2014 and Qin et al. 2014 respectively). Additionally to chemolithotrophic NOB, anaerobic phototrophic bacteria have been recently described that use NO_2^- as electron donor for anoxygenic photosynthesis and thereby generate NO_3^- (Griffin et al. 2007).

1.1.3 Heterotrophic Nitrification

In addition to predominantly chemolithotrophic generation of NO_3^- by AOM and NOB, heterotrophic nitrifiers, which couple NH_3 oxidation to reduction of organic matter also produce NO_3^- (Hora & Iyengar 1960; Bock & Wagner 2006). However, the relative importance of this alternative NO_3^- generating process, which can be carried out by bacteria, archaea, fungi, and algae, is little studied (Bock & Wagner 2006).

1.1.4 Anaerobic Nitrite and Nitrate Utilizing Pathways

NO_2^- cannot only be utilized by NOB, but occupies a central position in the nitrogen cycle, as it can be used by five different guilds of (micro-) organisms for respiration. Under anoxic conditions, both NO_3^- and NO_2^- can be reduced to ammonium (dissimilatory nitrite or nitrate reduction to ammonium, DNRA) by either chemolithotrophic or fermenting bacteria (Simon 2002; Burgin & Hamilton 2007). Alternatively, NO_3^- and NO_2^- can be used as electron acceptors by denitrifiers, a highly diverse group of mostly heterotrophic organisms found among archaea, bacteria, some fungi and even foraminifera (Risgaard-Petersen et al. 2006; Francis et al. 2007). Denitrifiers catalyze a series of N-transformations from NO_3^- to NO_2^-, NO, N_2O and ultimately to N_2, where NO_2^-, NO, N_2O or N_2 are possible end products. Which of these processes – DNRA or denitrification – occurs, was found to depend on C/N and NO_3^-/NO_2^- ratios, as well as applied generation time in anoxic incubations of marine sandy sediments (Kraft et al. 2014). Another anaerobic pathway utilizing NO_2^- is anaerobic ammonium oxidation (ANAMMOX), carried out by specialized *Planctomycetes*. These organisms combine reduction of NO_2^- with oxidation of NH_4^+ to N_2 and thereby take a shortcut in the N-cycle (Mulder et al. 1995; Kartal et al. 2011). Another, only recently discovered link in the N-cycle is nitric oxide (NO) disproportionation to N_2 and O_2, carried out by *Methylomirabilis oxyfera*, which couples methane (CH_4) oxidation to NO_2^- reduction (Raghoebarsing et al. 2006; Ettwig et al. 2010).

The N-cycle is strongly dependent on specialized bacteria and archaea. Some N-interconversions can indeed exclusively be carried out by members of these groups of organisms. It is therefore essential to understand the physiology and constraints of these organisms to better grasp controls over N-availability in both natural and anthropogenic systems, N-fluxes and – as N_2O is a potent green house gas – their impact on climate change.

1.2 A Brief Overview of Aerobic Nitrite Oxidizing Bacteria

More than 120 years ago, Sergei Winogradsky was the first to conclusively show that nitrification is a chemolithotrophic process, and described the first NOB, a *Nitrobacter* strain (Dworkin 2012). Even though nitrification and NOB have been known for a long time, there is still limited knowledge on the physiology of NOB. This is mostly due to the limited number of cultivated NOB strains, which results from the difficulty to obtain axenic cultures of these organisms in the laboratory (e.g. Spieck & Lipski 2011). Indeed, until the discovery of the marine NOB genera *Nitrospina* (*Nitrospina gracilis*) and *Nitrococcus* (*Nitrococcus mobilis*) in 1971, the only NOB in culture belonged to the genus *Nitrobacter* (Watson & Waterbury 1971). *Nitrospira marina*, member of the fourth described NOB genus *Nitrospira*, was isolated in 1986 (Watson et al. 1986). Only after molecular methods such as FISH became widely used in the 1990s, efficient screening methods for enrichment cultures by other means than light microscopy became available and applicable. Screening methods in combination with the recognition that the NO_2^- concentration used during enrichment can have a significant effect on the NOB obtained (e.g. Bartosch et al. 1999; Spieck & Lipski 2011), have led to more described NOB isolates during the last 20 years. Today, six genera of NOB are known, which are scattered over the phylogenetic tree.

1.2.1 Genera Adapted to High Nitrite Concentrations

Nitrobacter is a diverse genus within *Alphaproteobacteria* that shows highly similar 16S rRNA gene sequences between strains and is therefore assumed to be evolutionarily young (Orso et al. 1994). Members of *Nitrobacter* are the best studied NOB, as they are most amenable to cultivation and have been known the longest. *Nitrobacter* strains are usually tolerant to high NO_2^- concentrations (e.g. Bartosch et al. 1999; Spieck & Lipski 2011) and are considered r-strategists (Schramm et al. 1999; Kim & Kim 2006; Nogueira & Melo 2006). *Nitrococcus*, which was so far only found in marine environments, where it can constitute a large proportion of detectable NOB (Füssel et al. 2012),

belongs to the *Gammaproteobacteria* (Teske et al. 1994). Of this genus, only one species is described so far (Watson & Waterbury 1971). *Nitrolancea hollandica* is the only described gram-positive NOB species, belongs to the phylum *Chloroflexi*, and was discovered only very recently (Sorokin et al. 2012 and 2014). Unlike *Nitrobacter* and *Nitrococcus*, *Nitrolancea* contains no complex intracytoplasmic membrane systems (Sorokin et al. 2012). Nonetheless, in all three genera, the key enzyme for NOB, nitrite oxidoreductase (NXR, catalyzing the reversible oxidation of NO_2^- to NO_3^-) is closely related (Sorokin et al. 2012) and was shown or predicted to be facing the cytoplasm (Sundermeyer-Klinger et al. 1984 and references therein; Spieck & Lipski 2011; Sorokin et al. 2012). Because NXR in these genera faces the cytoplasm, NO_2^- oxidation does not contribute to the proton motive force (PMF), as this reaction consumes H_2O and yields two protons on the "wrong", more electronegative side of the membrane (Lücker et al. 2013).

1.2.2 Genera Adapted to Low Nitrite Concentrations

Nitrospira, which belongs to the phylum *Nitrospirae* (Ehrich et al. 1995), is a widely distributed and highly diverse genus of NOB. *Nitrospira* occur both in marine and terrestrial ecosystems and comprise six known phylogenetic lineages (Daims et al. 2001; Lücker 2010). Only few *Nitrospira* pure cultures are described, which comprise only five of the six lineages (Watson et al. 1986; Ehrich et al. 1995; Lebedeva et al. 2008 and 2011; Keuter et al. 2011; Ushiki et al. 2013; Nowka et al. 2015). *Nitrospira* have been shown to be K-strategists in regard to substrate utilization (Schramm et al. 1999; Kim & Kim 2006; Nogueira & Melo 2006). *Nitrospina* is the only known genus of the phylum *Nitrospinae*, and has so far only been found in marine environments (Lücker et al. 2013), where it can constitute the most important NOB (e.g. Beman et al. 2013). So far, only two species of *Nitrospina* have been obtained in pure culture (Watson & Waterbury 1971; Spieck et al. 2014). *Nitrotoga* is an only recently discovered NOB genus, which belongs to *Betaproteobacteria*. Only two enrichment cultures of this NOB are available (Alawi et al. 2007 and 2009), which have been obtained from permafrost soil and activated sludge. Both enrichments are adapted to very low NO_2^- concentrations and low temperatures, which is currently extrapolated to be a feature of the entire genus. The NXR of *Nitrospira*, *Nitrospina* and *Nitrotoga* are related to a different cluster of the type II DMSO reductase family than NXR of *Nitrobacter*, *Nitrococcus* and *Nitrolancea* (Lücker et al. 2010, 2013, and unpublished data). Unlike in *Nitrobacter*, *Nitrococcus* and *Nitrolancea*, the NXR in *Nitrospira*, *Nitrospina* and *Nitrotoga* is anchored in the plasma membrane facing the periplasm (Spieck et al. 1998; Lücker et al. 2013; Nowka et al. 2014). This NXR-orientation makes the necessity for dedicated NO_2^- transporters obsolete, and additionally contributes to the generation of a PMF during NO_2^- oxidation (Lücker et al. 2013), which could be the reason for the overall slightly higher growth yield of *Nitrospira* strains as compared to *Nitrobacter* strains reported by Nowka et al. (2014).

It is not yet known why *Nitrobacter*, *Nitrococcus* and *Nitrolancea* have overall higher NO_2^- tolerances than *Nitrospira*, *Nitrospina* and *Nitrotoga*. However, it was suggested that the periplasmic orientation of NXR allows to both utilize lower NO_2^- concentrations and might be responsible for the inhibitory effects of high NO_2^- concentrations (Spieck & Lipski 2011). Whether the inhibitory effect of high NO_2^- concentrations in *Nitrospira*, *Nitrospina* and *Nitrotoga* is due to excess reducing power channeled into the respiratory chain from NO_2^- oxidation, and whether tolerance of high NO_2^- concentrations in *Nitrobacter*, *Nitrococcus* and *Nitrolancea* is due to relatively constant NO_2^- concentrations in the cytoplasm which are maintained by the activity of transporters remains to be determined.

1.2.3 Alternative Energy Generation Pathways in Nitrite Oxidizing Bacteria

Even though NOB are usually associated with oxidation of NO_2^- to NO_3^-, there are reports of alternative energy generation pathways. Several *Nitrobacter* strains have been found to grow better

mixotrophically (i.e. utilizing organics in addition to NO_2^-, O_2, and CO_2) and even entirely heterotrophically than autotrophically (Bock et al. 1983 and 1990). While no other NOB than *Nitrobacter* have been reported to grow heterotrophically, mixotrophy is a more widespread phenomenon. Growth of *Nitrospina watsonii, Nitrospira defluvii*, *Nitrospira marina,* and *Nitrospira marina Ecomares* was stimulated by concomitant incubation with NO_2^-, pyruvate and – except *N. defluvii* – yeast extract and peptone (Watson et al. 1986; Spieck et al. 2006; Keuter et al. 2011; Spieck et al. 2014). Also *Nitrospira* in activated sludge were shown to incorporate pyruvate under nitrifying conditions (Daims et al. 2001). Gruber-Dorninger et al. (2015) found that subpopulations of *Nitrospira* lineage I could utilize formate both under nitrifying conditions and as sole substrate. Additionally, *Nitrospira japonica* was able to utilize formate as sole substrate for energy generation and growth under aerobic conditions (Ushiki et al. 2013).

Under anoxic conditions, *Nitrobacter* strains were found to couple NO_3^- reduction to oxidation of organic compounds (e.g. Freitag et al. 1987), *Nitrolancea hollandica* could also couple NO_3^- reduction to oxidation of formate (Sorokin et al. 2012 and 2014), and *Nitrospira moscoviensis* was shown to denitrify NO_3^- to NO_2^- while oxidizing H_2 (Ehrich et al. 1995). Only recently, *Nitrospira moscoviensis* was found to be able to also couple H_2 oxidation to reduction of O_2, further expanding the physiological repertoire of this organism (Koch et al. 2014).

All these findings indicate that NOB might not only perform NO_2^- oxidation in the environment, but might be associated with other, quite unexpected substrate transformations.

1.2.4 Isolation of Nitrite Oxidizing Bacteria

Many different isolation methods have been employed for obtaining the existing NOB cultures. Mostly combinations of several methods were successful (e.g. Nowka et al. 2015). Dilution series were commonly employed, and are especially applicable for planktonic cells (e.g. Watson & Waterbury 1971; Ehrich et al. 1995). However, many *Nitrospira* grow in cauliflower-like microcolonies, which are often interspersed with contaminating cells and therefore need to be broken apart to obtain single cells before dilution series are performed (e.g. Nowka et al. 2015). Breaking apart *Nitrospira* microcolonies however is often difficult due to the strong cohesion among cells (e.g. Larsen et al. 2008). Another often employed method for separation of NOB from contaminating cells is Percoll density centrifugation, where different cell types are separated according to their density (e.g. Ehrich et al. 1995; Lebedeva et al. 2008; Nowka et al. 2015). Sometimes, antibiotics were added to enrichments of *Nitrospira* to reduce heterotrophic contaminants, as some *Nitrospira* strains show tolerance against low concentrations of beta-lactam antibiotics and acriflavine (Spieck et al. 2006; Nowka et al. 2015). Targeted cell separation of NOB from enrichment cultures have only recently been described, either by fluorescence activated cell sorting (Ushiki et al. 2013) or micromanipulation with tweezing lasers (Nowka et al. 2015). Most NOB – with the exception of some *Nitrobacter* strains (Bock et al. 1983; Sorokin et al. 1998) and *Nitrolancea hollandica* (Sorokin et al. 2012 and 2014) – do not grow on solid agar medium, which has been attributed to too high O_2 concentrations (Spieck & Lipski 2011). Recently, Nowka et al. (2015) reported that *Nitrospira defluvii* grew on agarose, but not agar plates, and *only* in presence of heterotrophic bacteria. This was contributed to the presence of inhibiting substances in commercially available agar, but not agarose, and a yet unknown interaction between heterotrophs and *N. defluvii*.

The fact that so few NOB are cultured, reflects their fastidious nature and the difficulty of obtaining pure cultures of these organisms. Many NOB grow very slowly, which likely results from the low energy gain from the oxidation of NO_2^- to NO_3^- (ΔG_0' = -77 kJ*mol^{-1} respectively, calculated from ΔE_0'

listed in Madigan et al. 2012). Additionally, especially *Nitrospira*, *Nitrospina* and *Nitrotoga* are easily inhibited by high NO_2^- concentrations, and therefore require continuous substrate addition. Both the slow growth and the continuous feeding mode that most NOB require, make cultures susceptible to contaminating organisms. Even though NOB grow autotrophically in mineral medium, organic substances, which are secreted by living NOB or made available by cell lysis, can – due to the commonly employed batch feeding mode – accumulate in the medium and thereby sustain growth of heterotrophic contaminants.

1.3 Nitrogen Cycling in Wastewater Treatment Plants

1.3.1 Operational Modes for Nitrogen Removal

Compared to natural water systems, wastewater is strongly enriched in N_r. The most abundant N-species in wastewater is NH_4^+, which mostly stems from breakdown of organic matter (Wagner & Loy 2002). Increased input of N_r into natural water bodies can lead to eutrophication, excessive algal blooms and respiration, which is followed by oxygen depletion. Therefore, N_r has to be removed from wastewater prior to discharge into natural systems by conversion into gaseous N-compounds. In most wastewater treatment plants (WWTP), N_r is removed biologically, usually via sequential nitrification and denitrification. Two strategies for separation of nitrification and denitrification are commonly employed in activated sludge – either spatially or temporally (see scheme in Figure 1.2 A and B respectively).

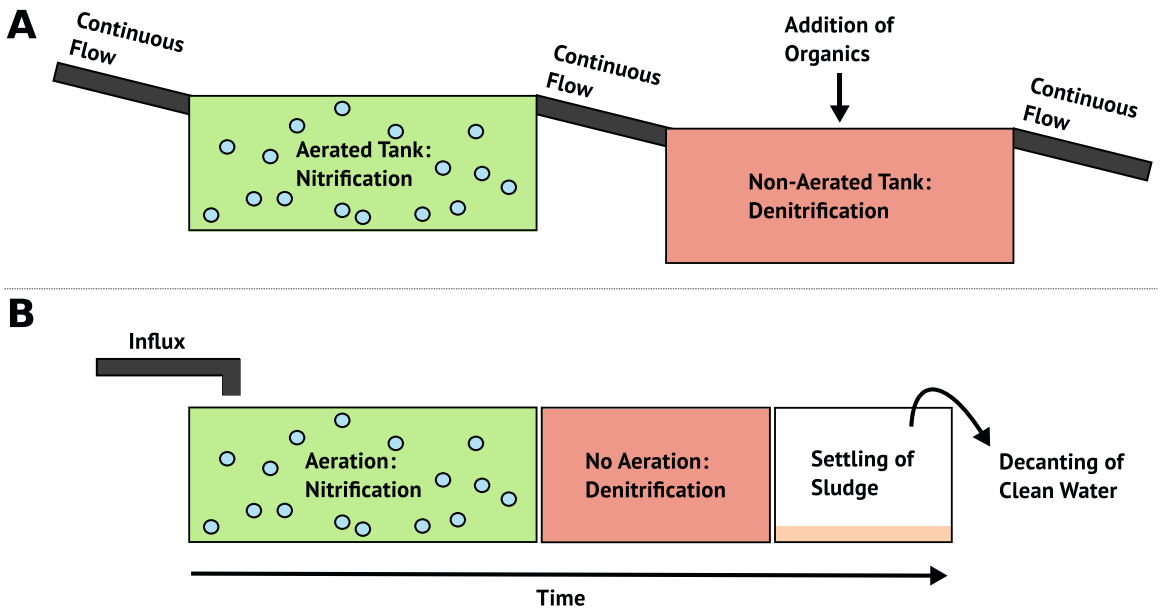

Figure 1.2 Schematic of separation possibilities of nitrification and denitrification. A: Spatial separation between aerated nitrification and non-aerated denitrification basins with continuous water flow. Note the necessity of adding organics as electron donor for denitrification, if it occurs downstream of nitrification. B: Temporal separation between nitrification and denitrification in a sequencing batch reactor by intermittent aeration and non-aeration. After settling of sludge, clean water is decanted.

In WWTP with spatial nitrification and denitrification separation, wastewater sequentially flows through two different basins, one aerated basin for nitrification and one non-aerated basin for denitrification. The sequence of these basins differs between WWTP setups. For setups with denitrification downstream of the nitrification basin, external electron donors (organics) have to be added (Figure 1.2, A), for nitrification downstream of the denitrification basin, water has to be

recycled for removal of NO_3^-. In sequencing batch reactors (SBR), a temporal separation between nitrification and denitrification is achieved, where aeration and non-aeration is alternated over time (Figure 1.2, B). Here, wastewater is pumped into a tank in batches and clean water is decanted after treatment.

In addition to full nitrification to NO_3^- followed by denitrification, partial nitrification to NO_2^-, followed by denitrification can be employed (e.g. Schmidt et al. 2003). As an alternative to conventional nitrification-denitrification, ANAMMOX can be employed for N-removal following partial nitrification to NO_2^- (e.g. Fux et al. 2002).

1.3.2 Nitrifiers in WWTP

Prior to the application of *in situ* molecular methods such as fluorescence *in situ* hybridization (FISH) to activated sludge samples, it was assumed that mainly *Nitrosomonas europaea* (AOB) and *Nitrobacter sp*. (NOB) were responsible for nitrification. In the 1990s, at the onset of FISH, it was recognized that the nitrifier community in activated sludge is much more complex.

In addition to the previously known AOB *Nitrosomonas europaea*, additional species of betaproteobacterial AOB are abundant and actively oxidizing ammonia in WWTP, mostly belonging to the genera of *Nitrosomonas* (including *"Nitrosococcus" mobilis*) and *Nitrosospira*. The gammaproteobacterial genus of *Nitrosococcus* occurs mostly in saline habitats and does not play a major role in nitrification in WWTP (Daims et al. 2009). The role of the recently discovered AOA (Könneke et al. 2005) for ammonia oxidation in WWTP is controversial. While there is some evidence for numerical dominance of *Thaumarchaea* carrying the key enzyme for ammonia oxidation, ammonia monooxygenase (more specifically the alpha-subunit *amoA*), over AOB (e.g. Kayee et al. 2011; Bai et al. 2012), so far, ammonia-oxidizing activity of these *amoA*-carrying *Thaumarchaea* was not shown (Limpiyakorn et al. 2013). The only published study investigating activity of *Thaumarchaea* in activated sludge showed that their population size could not be sustained by the measured ammonia oxidation rates, and that *Thaumarchaea* did not incorporate ^{14}C-bicarbonate under nitrifiying conditions (Mußmann et al. 2011).

Nitrobacter are indeed rarely found in large numbers in WWTPs by *in situ* techniques, while they can be readily isolated in the laboratory due to their comparably fast growth. Instead *Nitrospira spp*., which are more difficult to obtain in pure cultures, are usually the dominant NOB in WWTP both in numbers and activity (e.g. Juretschko et al. 1998; Schramm et al. 1998; Daims et al. 2001). In WWTP, *Nitrospira* lineage I, II and IV are found (Daims et al. 2009). In addition to *Nitrospira*, *Nitrotoga* was recently also found to constitute the major NOB group present in some WWTPs (Lücker et al. 2015). So far, *Nitrotoga* has been mostly associated with cold temperatures, as both described enrichment cultures were obtained at temperatures well below 20 °C (Alawi et al. 2007 and 2009), and because all WWTPs containing detectable *Nitrotoga* were operated at low temperatures (Lücker et al. 2015).

1.3.3 Wastewater Treatment Plants as Model Systems

The physical and chemical parameters of a WWTP are, unlike conditions in most other environments constantly monitored. This supplies the researcher with a large amount of information on the investigated system (Daims et al. 2006a). In addition to WWTPs being well-controlled systems, they harbor a huge diversity of microorganisms, which allows central ecological questions to be addressed, such as how predation or viral lysis affect communities, how organisms compete for common substrates, and how niche differentiation occurs within functional groups. Due to the constant replenishment of nutrients by fresh wastewater, communities in activated sludge are very active and

the cell density in activated sludge is extremely high. This makes activated sludge well suited for FISH-based techniques, which can be difficult to implement in environments such as soils, where cells are less abundant or active (Wagner et al. 2006). Given the constant removal of excess activated sludge from WWTPs, only actively growing organisms can stably remain in the system, whereas inactive cells and resting stages are flushed out. This results in constant loss of inadequately adapted strains from activated sludge, which is e.g. used for selection against NOB in partial nitrification setups (Schmidt et al. 2003). However, even though only well adapted organisms can persist in WWTPs, multiple similar groups of organisms are often found to coexist in WWTPs. Maixner et al. (2006) found that two lineages of *Nitrospira* (lineage I and II) coexisted in activated sludge, and showed that *Nitrospira* lineage I occurred closer to AOB and was adapted to higher NO_2^- concentrations than lineage II. But even within one *Nitrospira* lineage, different subpopulations were found to stably coexist over several years in activated sludge (Gruber-Dorninger et al. 2015). These subpopulations were shown to differ in substrate utilization patterns, preferred NO_2^- concentrations, and co-aggregation patterns with AOB populations. These findings imply that WWTPs are ideal systems to study adaptation, niche differentiation and evolution of microorganisms.

1.4 Theories on Diversity and Competition

Two central questions in ecology are what determines the observed community composition in natural systems and how the observed diversity is sustained.

1.4.1 Deterministic or Stochastic Community Assembly

Two main concepts have been proposed on the driving forces in community assembly – the deterministic theories, based on the niche-concept (e.g. Hutchinson 1957), and the stochastic, neutral theories, most prominently the unified neutral theory of biodiversity (Hubbell 2001).

Historically, ecologists tended to explain the observed community composition entirely by the sets of environmental factors that given species require to successfully establish and reproduce. Hutchinson was one of the first to formulate the concept of "niche" as an n-dimensional hypervolume, i.e. as a set of environmental parameters (consumable resources such as food, but also state factors such as temperature), which a given species needs to exist (Hutchinson 1957). Because of these defined requirements of species, similar environments are expected to select for similar sets of species, and species are expected to co-occur non-randomly. More diverse habitats (e.g. temperate brown soil as opposed to the euphotic water layer of a stratified lake) should be associated with more diverse species assemblages due to more available dimensions in niche space. Closely tied to the niche-concept is the concept of competitive exclusion. It postulates that "complete competitors cannot coexist" or, differently coined, "ecological differentiation is the necessary condition for coexistence" (Hardin 1960). If two or more species have exactly the same requirements and the system is in equilibrium state, the species, which has a slight advantage in fitness over the other (i.e. is more successful in reproduction) will survive, while the inferior species will go extinct, principles which can be traced back to Darwin's "The Origin of Species" (1859). Stable coexistence is only possible by ecological differences between species, e.g. different substrate spectra. However, competitive exclusion can impossibly be experimentally falsified, as exact congruency of niche-dimensions for two species cannot be established with any certainty (Hardin 1960).

During the last decades, alternative models explaining community assembly have become more popular, which assume purely stochastic processes to shape species distribution, with no regard to a specific species' ecological requirements or adaptations. The most prominent model, the "unified neutral theory of biodiversity" (henceforth referred to as neutral theory), was proposed in 2001 by S.

Hubbell. It assumes strict equality in fitness of individuals across all species within one trophic level (e.g. trees in tropical forests), i.e. every individual has the same probability of death, birth, and the same speciation and immigration rates – irrespective of species affiliation. Species are assumed to only differ in their abundance, both on local and regional scale, which thereby influences the combined probabilities of a species to expand or decline in a given environment. Furthermore, environments are assumed to be saturated with individuals – no new individual can establish unless another dies. A "gap" produced by death is immediately filled by birth of a new individual belonging to an already present species, by immigration of an individual from the regional species pool, or by evolution of a new species. Which of these scenarios takes place is determined by chance or probability. Even though the model is neutral (i.e. dependent on chance), patchiness between environments is created by dispersal limitation of individuals. Consequently, community similarity decreases with geographic distance and consistent co-occurrence of species is not expected (Hubbell 2001 and 2005).

The proposed neutral theory has sparked considerable controversy among ecologists, because species-specific traits are entirely ignored. This controversy has produced a large number of publications on how well neutral theory fits, and a lower number of studies on how well it predicts patterns in community assembly (e.g. Hubbell 2001; Thompson & Townsend 2006, for studies on microorganisms see e. g. Sloan et al. 2006; Woodcock et al. 2007; Dumbrell et al. 2010; Langenheder & Székely 2011). For microbial ecologists, neutral theory is intriguing, since much of the physiological diversity (or niche) of microorganisms occurring in nature is unknown. If species assembly could be explained by neutral processes alone, much of the riddle of bacterial diversity would be solved. However, there are several problems associated with simply applying Hubbell's neutral theory for bacteria and archaea. First, it assumes that they are overall dispersal limited and that the paradigm of "everything's everywhere, but the environment selects" (de Wit & Bouvier 2006 and references therein) can be neglected. More importantly however, neutral theory was developed to model distribution of one trophic level of organisms. Definition of trophic levels for bacteria and archaea is difficult, because exact physiology and substrate utilization patterns can vary between closely related strains, and is mostly unknown (Sloan et al. 2006). Nonetheless, a neutral species distribution model was shown to fit both samples of assumedly single trophic levels of bacteria (e.g. Sloan et al. 2006, for AOB and denitrifiers), and to predict and fit samples containing multiple trophic levels (e.g. Woodcock et al. 2007, for bacterial communities in water holes of beech trees). While neutral processes have been proposed to be important, many studies show that *both* stochastic and deterministic processes play a role in microbial community assembly and composition (e.g. Dumbrell et al. 2010 for fungi of one assumed trophic level and Langenheder & Székely 2011 for multiple trophic levels of bacteria), similar to *both* genetic drift and natural selection acting on genome evolution and speciation.

1.4.2 Factors Sustaining Diversity

Connected to the above-discussed factors determining community assembly, is how the observed diversity of organisms, which often (seemingly) share the same resources, is sustained. While in neutral theory, stochasticity, i.e. immigration and speciation sustain diversity (e.g. Sloan et al. 2006), in the following section, more deterministic factors sustaining diversity will be discussed.

Hutchinson (1961) was one of the first to explicitly address the question of how species utilizing the same resources can coexist as the "paradox of the plankton": How can the diversity observed in phytoplankton be sustained in a homogenous habitat like the well mixed surface waters, although all species require essentially the same nutrients? Hutchinson identified some possible mechanisms in

sustaining this paradoxical diversity, most importantly the temporal variability of a given environment, i.e. the absence of an equilibrium state (Hutchinson 1961). If conditions favoring one species over others change too fast for out-competition of the remaining species, several species can coexist for a longer time period. The later proposed "intermediate disturbance hypothesis" (Grime 1973), stating that at levels of intermediate disturbance, species diversity is highest, is essentially based on the same idea. Additional factors proposed to influence species diversity are niche partitioning, selective predation, parasitism or susceptibility to viruses, symbiosis, and immigration from a seed bank in the case of microorganisms – the latter corresponds to neutral theory immigration. Niche partitioning is thought to primarily stem from avoidance of competition between organisms (e.g. Peña et al. 2010). If organisms are competing for a common substrate, mutations allowing to use resources not yet used, or to partition a given resource at low or high concentrations (e.g. different NO_2^- preference by different lineages or sublineage-populations of NOB, Maixner et al. 2006; Gruber-Dorninger et al. 2015) will be favored by selection. Diversity by niche differentiation is thought to be sustained by both narrow and partly overlapping niches. Selective predation may play an important role for diversity of multicellular organisms, for bacteria and archaea however, this is likely of minor importance, as predation on microorganisms is mostly size- but not taxon-specific (Pernthaler 2005). Viruses however are often highly specific for certain species and have been suggested to act as a control-mechanism on species abundance, most prominently in the "killing the winner" model formulated by Thingstad (2000).This model proposes that the most active bacteria and archaea (which would be dominant in absence of viruses) are most diminished by specific viruses. Other groups (which are less competitive in the absence of viruses) invest more into defense strategies and can therefore co-occur. Although this model was initially developed for aquatic systems, viral abundance is high in many environments and could therefore have a strong impact on communities in other habitats as well. Brockhurst et al. (2006) could experimentally show with two *Pseudomonas* strains in a chemostat that selective viral susceptibility can promote coexistence.

1.5 Diversity Assessment of Bacteria and Archaea, Microdiversity and Single Cell Genomics

After the onset of cultivation-independent methods in microbiology it was recognized that the diversity of bacteria and archaea in the environment by far exceeds prior estimates, as only a tiny fraction of strains can be cultivated by commonly employed techniques. Today, high throughput sequencing of 16S rRNA gene amplicons is most frequently used to assess diversity of bacteria and archaea in natural samples. The obtained quality filtered sequences are usually grouped into operational taxonomic units (OTU) at essentially arbitrary similarity thresholds – for 16S rRNA gene amplicons a 97 % identity cutoff is commonly employed. This grouping of sequences is assumed to be necessary to minimize the effect of PCR and sequencing errors, which can cause considerable amounts of artifacts (Kunin et al. 2010). Additionally, grouping of sequences reduces the effect of multiple, non-identical rRNA gene operons per genome. OTUs obtained from 16S rRNA gene amplicons often contain multiple, closely related sequences, a phenomenon that has been termed molecular microdiversity (Moore et al. 1998). There is mounting evidence that this molecular microdiversity not only stems from artifacts and multiple rRNA gene copies per genome, but can also stem from real functional diversity, as functionally different strains have been shown to share 16S rRNA gene identities above 97 %, or even 100 %. For example, environmentally co-occurring, isolated *Prochlorococcus* strains with 16S rRNA gene sequence identities > 97 % were shown to be adapted to different light intensities (Moore et al. 1998) and closely related, naturally co-occurring *Nitrospira* subpopulations were shown to differ in their substrate utilization patterns (Gruber-Dorninger et al. 2015). When comparing multiple samples, e.g. across environmental gradients, the recently developed method of oligotyping can identify potentially more meaningful groups of sequences than arbitrary

OTU cutoffs can (Eren et al. 2013; Buttigieg & Ramette 2015). Oligotyping can aid in developing hypotheses on ecological differences between closely related organisms, which can then be confirmed experimentally e.g. by exposure of the environmental samples to different, strictly controlled conditions and assessment of activity patterns of the occurring oligotypes. So far however, oligotyping has mostly been used to show correlations of oligotype occurrence with certain environmental parameters, without follow-up studies verifying this assumed dependence. However, not only organisms with highly similar 16S rRNA gene sequences have been shown to be functionally different, but also strains with identical 16S rRNA genes. A drastic example are pathogenic strains of *Bacillus anthracis*, which have exactly the same 16S rRNA gene sequence as strains of the mostly non-pathogenic *Bacillus cereus* (Bavykin et al. 2004). Similarly, *Brevundimonas alba* strains with identical 16S rRNA genes show functional differences (Jaspers & Overmann 2004), as well as strains of *Salinibacter ruber* (Peña et al. 2010).

Most studies examining *phenotypic* functional differentiation between microdiverse organisms rely on cultivation of the investigated strains (e.g. Moore et al. 1998; Jaspers & Overmann 2004; Hahn & Pöckl 2005; Haverkamp et al. 2009; Peña et al. 2010; Antón et al. 2013). In contrast, investigation of potential differences between microdiverse organisms in the environment – with the exception of the study performed by Gruber-Dorninger et al. (2015) – mostly relies on assessment of *genotypic* differences.

Information on genomic differences of coexisting, closely related strains in environmental samples can only efficiently be obtained by single cell genomics (SCG), as metagenomics approaches usually result in assembly of consensus-genomes or -contigs and therefore cannot resolve intra-species diversity (e.g. Stepanauskas 2012; Fitzsimons et al. 2013). Additionally, SCG allows to obtain much more genomic information of rare organisms in the environment than metagenomics approaches do, and has for example been used for prediction of the physiology of members of the OP9/*Acribacteria* phylum, which lacks cultivated strains (Dodsworth et al. 2013).

SCG applications however still have some limitations. SCG relies on physical separation of target cells from the sample, which is usually achieved by fluorescence activated cell sorting or micromanipulation. Separation of single cells however can be laborious for non-planktonic communities, where cells grow in aggregates or biofilms (Rinke et al. 2014). Usually, SCG approaches are non-targeted (Clingenpeel et al. 2015), as few microorganisms can be identified based on specific morphology or pigmentation, and DNA of cells, which have undergone a typical FISH protocol, is less suitable for, and paraformaldehyde fixation entirely inhibits downstream workflow (Clingenpeel et al. 2014). Isolated cells are lysed to make their DNA accessible for random amplification by multiple displacement amplification (MDA, Dean et al. 2001), which is necessary to obtain sufficient amounts of DNA for genome sequencing. Lysis efficiency however strongly depends on the cell type and the lysis protocol used (e.g. Clingenpeel et al. 2015). In SCG, usually only partial genomes can be recovered due to uneven chromosome amplification in MDA reactions (Clingenpeel et al. 2014). Additionally, chromosomes of single cells can suffer breakage during cell lysis, resulting in fragmented genomic information. Several possibilities for obtaining more than one chromosome copy as starting material, and therefore more even overall amplification and higher recovery rates have been suggested. Dichosa et al. (2012) proposed stalling of cell division in growing cells for artificially obtaining a higher chromosome copy number per single cell, and Fitzsimons et al. (2013) employed cultivation of single cells in agarose micro-droplets for obtaining clonal colonies for MDA. However, both methods rely on growth of cells in the laboratory.

Nonetheless, even though SCG is far from providing the sequence of entire genomes, valuable insights into niche-differentiation, genome and species evolution have been gained from studies employing SCG. For example, coexistence of 100s of microdiverse wild *Prochlorococcus* strains has recently been characterized by SCG, and has been attributed to stable niche partitioning over millions of years between these groups, which share a large proportion of their genome, but additionally show distinct flexible gene inventories (Kashtan et al. 2014). In the future, SCG will hopefully trigger more testable hypotheses on differences between microdiverse organisms, further elucidating their phenotypic functional differentiation in the environment.

1.6 Aims of This Study

In this thesis, both cultivation-dependent and -independent techniques were employed and developed to expand the knowledge on physiology and ecological niches of nitrifying bacteria, with the main focus on NOB.

1.6.1 Assessment of the Denitrifying Potential in *Nitrospira moscoviensis*

The key enzyme of nitrite oxidizers, NXR, catalyzes both the oxidation of NO_2^- to NO_3^- and the reduction of NO_3^- to NO_2^-, thus carrying out the first step in denitrification. While the denitrifying potential of *Nitrobacter* has been well characterized (e.g. Freitag et al. 1987), so far, only one anecdotal report of NO_3^- reduction coupled to H_2 oxidation in *Nitrospira moscoviensis* was reported (Ehrich et al. 1995). Recently however, it has been shown that *Nitrospira moscoviensis* can also couple anaerobic formate oxidation to NO_3^- reduction (Hanna Koch, unpublished data).

In the present study, denitrification coupled to oxidation of simple organic substrates was assessed for a *N. moscoviensis* pure culture to gain further insight into potential alternative energy generation pathways of this nitrite oxidizer.

1.6.2 Workflow Assessment for Investigation of Active, Coexisting, Microdiverse Nitrifiers

Recently, Gruber-Dorninger et al. (2015) published a study on niche-partitioning in stably coexisting, closely-related *Nitrospira* in activated sludge. In that study, a combination of assessment of substrate utilization patterns and spatial co-aggregation of *Nitrospira* subpopulations with AOB was employed. While the techniques used successfully elucidated several phenotypic differences between coexisting, closely related *Nitrospira*, more hypotheses on physiological differences could be generated and more targeted experiments performed, if the genetic potential and differences of coexisting strains was known.

In the course of a project aiming at obtaining genome sequences of 100 coexisting nitrifiers by SCG, and thereby gaining information on their functional differentiation, genomic plasticity and evolution, a workflow was developed for the investigation of microdiverse nitrifiers from activated sludge (Tae Kwon Lee, unpublished data). Similar to the proof of principle study using pure cultures by Huang et al. (2009), the developed workflow relies on stable isotope labeling, detection of active cells by Raman spectroscopy and sorting by optical tweezing. Additionally, it exploits the intrinsic ability of nitrifiers to form clonal, structurally rigid microcolonies (Juretschko et al. 1998; Daims et al. 2001; Larsen et al. 2008), which allows for the investigation of these hard-to-maintain and slow growing organisms irrespective of growth, while obtaining multiple chromosome copies for MDA. Because of this rigid microcolony structure, activated sludge can be disrupted without strong effects on the integrity of nitrifier microcolonies (Larsen et al. 2008). Disrupted microflocs containing nitrifiers can be identified by their characteristic Raman spectra (Pätzold et al. 2008) and then sorted by optical tweezing. Sorted microflocs are subjected to MDA, PCR screening, and microfloc metagenome

sequencing. In addition to the identification of nitrifiers, Raman spectra allow to identify *active* microorganisms after incubation with stable isotopes, as incorporation of stable isotopes into biomass causes distinct peak shifts. ^{13}C and D_2O were used for labeling, which produce peak shifts from 1003 cm^{-1} to 967 cm^{-1} for ^{12}C- and ^{13}C-phenylalanine respectively (Huang et al. 2004 and 2007), and a D-C peak in the region of 2200 cm^{-1} from D_2O incorporation (Berry et al. 2015). In addition to obtaining the targeted nitrifier genomes, genomic information of cells adhering to nitrifier microcolonies is expected to yield insights into close symbiotic and functional interactions (Blainey 2013).

In the present study, the described workflow for the assessment of genomic plasticity and possible functional differentiation of microdiverse, coexisting, active nitrifiers in activated sludge from WWTP Klosterneuburg was further developed and evaluated, and MDA products from 24 sorted microflocs were sequenced in a pre-study.

1.6.3 Enrichment, Physiological Characterization and Isolation of Novel Nitrite Oxidizers

Although NOB carry out an essential step in the biological N-cycle, few cultured representatives are available to study those important organisms in more detail. The best-studied NOB are members of the genus *Nitrobacter*, which are easier to cultivate in the laboratory than most other NOB (e.g. Spieck & Lipski 2011). The onset of the use of molecular methods such as PCR and FISH has facilitated monitoring of enrichment cultures dramatically, leading to more enriched and isolated NOB strains during the last decades. However, there is still a huge lack of knowledge regarding this group of organisms, which is also highlighted by the recent discovery of two entirely new genera of NOB – *Nitrotoga*, a member of *Betaproteobacteria*, and *Nitrolancea*, of the phylum *Chloroflexi* (Alawi et al. 2007; Sorokin et al. 2012 and 2014).

In the present study, NOB were enriched from the municipal WWTP Klosterneuburg, resulting in the characterization of a novel, mesophilic member of *Nitrotoga*.

2 MATERIALS AND METHODS

2.1 Chemicals, Solutions, Media

All buffers, media and solutions were prepared with double distilled, sterile filtered (i.e. filtered through 0.2 µm pore size filters) water (ddH_2O, MQ Biocel, Millipore). Buffers, media and solutions were inactivated by autoclaving for 20 min at 121 °C and $1.013*10^5$ Pa.

2.1.1 Conventional Fluorescence *in situ* Hybridization

Table 2.1 DAPI/PBS Solution, 10 µg*ml^{-1}

DAPI (Roth, 6335.1)	100 µg
Ad 10 ml 1x PBS, sterile filtered	

Table 2.2 Ethylenediaminetetraacetic acid (EDTA), 0.5M, pH 8

$Na_2EDTA*2H_2O$ (Roth, 99%, 8043.2)	46.5 g
pH adjusted to 8 with NaOH (Roth, 99%, 6771.1) while stirring and heating, ad 250 ml ddH_2O, autoclaved	

Table 2.3 NaCl, 5M

NaCl (Roth, 99.5%, 3957.2)	146.1 g
Ad 500 ml ddH_2O, autoclaved	

Table 2.4 Phosphate buffered saline (PBS), 10x, 1x

	10x stock	1x
NaCl (Roth, 99.5%)	80 g	140 mM
KCl (Roth, 99%, P017.2)	2 g	2.7 mM
$Na_2HPO_4*2H_2O$ (Roth, 99.5% 4984.1)	18 g	6.5 mM
KH_2PO_4 (Sigma-Aldrich, 99%, P5655)	2.4 g	1.5 mM
pH adjusted to 7.4, ad 1 l ddH_2O, autoclaved		

Table 2.5 Sodium dodecyl sulfate (SDS), 10% (w/v)

SDS (Roth, 99%, 4360.2)	5 g
Ad 50 ml ddH_2O, sterile filtered	

Table 2.6 Tris*HCl, 1M, pH 8

Tris (Roth, 99.3%, AE15.3)	30.3 g
pH adjusted to 8 with smoking HCl (Roth, 4625.1), ad 250 ml ddH_2O, autoclaved	

Table 2.7 Additional chemicals and solutions used for conventional fluorescence *in situ* hybridization (FISH).

Citi Fluor AF1 (Agar Scientific Limited)
Ethanol denatured (AustrAlco)
Formalin (Roth, 37 % ROTIPURAN®, 4979.1)
Formamide (deionized, Roth, 99.5%, P040.1)
Probes (Biomers and Thermo Scientific)

2.1.2 Polymerase Chain Reaction

Table 2.8 Chemicals and solutions used for polymerase chain reaction (PCR).

Bovine Serum Albumin, 20 mg*ml^{-1} (BSA, Fermentas, B14)
ddH_2O (Fresenius water, Kabi)
dNTPs, 2 mM (Fermentas, R0242)
$MgCl_2$, 25 mM (Fermentas, EP0402)
Primer (Biomers and Thermo Scientific)
Taq buffer, 10x (KCl, Fermentas, EP0402)
Taq Polymerase recombinant, 5U*µl^{-1} (Fermentas, EP0402)

2.1.3 Agarose Gel Electrophoresis

Table 2.9 Tris/Borate/EDTA (TBE) buffer, 10x

	10x stock	1x
Tris (Roth, 99.3%, AE15.3)	108 g	89 mM
Boric acid (Roth, 99.8%, 6943.1)	55 g	89 mM
$Na_2EDTA*2H_2O$ (Roth, 99%, 8043.2)	7.4 g	2 mM
pH adjusted to 8.3 – 8.7, ad 1l ddH_2O		

Table 2.10 Additional solutions and chemicals for agarose gel electrophoresis.

GelRed Nucleic Acid Stain 10,000x in DMSO (Biotium, 41002)
GeneRuler 1 kb and 100 bp DNA Ladder (Thermo Fisher Scientific, SM0313)
LE Agarose (Biozym, 840004)
Loading Dye, 6x (Thermo Fisher Scientific, #R0611)

2.1.4 Measurement of Nitrite and Nitrate

Table 2.11 N-naphthylethylene diamine dichloride (NEDD), 0.1 %

NEDD (Roth, 98%, 4342.1)	250 mg
Ad 250 ml ddH_2O, NEDD bottle wrapped in aluminum foil due to light sensitivity, stored at 4 °C	

Table 2.12 Sulfanilamide solution, 2%

Sulfanilamide (Sigma-Aldrich, 99%, S9251-100G)	5 g
Ad 250 ml 3 M HCl, stored in the dark at 4 °C	

Table 2.13 Vanadium(III) chloride (VCl_3) solution

VCl_3 (Sigma-Aldrich, 97%, 208272-25G)	400 mg
1 M HCl	50 ml
VCl_3 was weighed into a tube in a fume hood. VCl_3 bottle wrapped in aluminum foil due to light sensitivity, stored at 4 °C	

Table 2.14 Additional chemicals for measurement of NO_2^- and NO_3^-.

$NaNO_2$ (Roth, 99.7%, 8604.1)
$NaNO_3$ (Roth, 99%, A136.1)

2.1.5 Capillary Electrophoresis

Table 2.15 NaOH, 10 mM, valeric acid, 2 mM

NaOH (Roth, 99%, 6771.1)	0.1 g
Valeric acid (Sigma-Aldrich, 99%, V-0125)	50 µl
Ad 250 ml ddH_2O, sterile filtered, stored at 4 °C in a glass bottle to avoid precipitation	

Table 2.16 Additional chemicals for capillary electrophoresis.

Na Acetate (Sigma-Aldrich, 99%, S8750-250G)
Na Citrate (Roth, 99%, 3580.1)
Na Formate (Fluka, 99%, 71539)
Na Fumarate (Merck, 99%, 8.20584.0500)
Na Pyruvate (Sigma-Aldrich, 99%, 15990)
Na Succinate (Sigma-Aldrich, 98%, 14160-100G)

2.1.6 Cultivation of Nitrite Oxidizing Bacteria

Table 2.17 Ampicillin stock, 1000x

Ampicillin (Sigma-Aldrich, A0166-5G)	100 mg
Ad 1 ml in 50% ethanol, sterile filtered, stored at -20°C	

Table 2.18 Mineral medium, 10x

	10x stock	1x medium
$CaCO_3$ (Roth, 99%, P012.2)	0.1 g	0.10 mM
NH_4Cl (Merck, p.a., 1.01145.0500)	0.1 g	0.19 mM
NaCl (Roth, 99.5%, 3957.2)	5 g	8.56 mM
$MgSO_4*7H_2O$ (Merck, p.a., 1.05886.0500)	0.5 g	0.20 mM
KH_2PO_4 (Sigma-Aldrich, 99%, P5655-500G)	1.5 g	1.10 mM
Ad 1 l ddH_2O, stored at 4 °C		

Table 2.19 Mineral medium, 1x

10x mineral medium stock	100 ml
pH adjusted to 8.4 – 8.5, ad 1 l ddH_2O, autoclaved	

Table 2.20 Selenite/tungstate trace element solution, 1000x

	1000x stock
NaOH (Roth, 99%, 6771.1)	0.5 g
$Na_2SeO_3.5\ H_2O$ (Fluka, 99%, 00163-25G)	3 mg
$Na_2WO_4.2\ H_2O$ (Sigma-Aldrich, 99%, 72070-100G)	4 mg
Salts dissolved in 900 ml ddH_2O, then ad 1 l ddH_2O, stored at 4°C	

Table 2.21 Trace element solution, 1000x

	1000x stock
$MnSO_4*H_2O$ (Roth, 98%, 4487.1)	34.4 mg
H_3BO_3 (Roth, 99.8%, 6943.1)	50 mg
$ZnCl_2$ (Riedel-de Haen, 98%, 31650)	70 mg
$Na_2MoO_4*2H_2O$ (Alfa Aesar, 98%, A19222)	72.6 mg
$CuCl_2*2H_2O$ (AnalaR NORMAPUR VWR, 99%, 23093.233)	20 mg
$NiCl_2*6H_2O$ (Riedel-de Haen, 97%, 13613)	24 mg
$CoCl_2*6H_2O$ (Sigma-Aldrich, C8661-25G)	80 mg
$FeSO_4*7H_2O$ (Roth, 99%, P015.1)	1000 mg
Components added sequentially from top, filled to 995 ml with ddH_2O, add 2.5 ml smoking HCl (Roth, 4625.1), then added $FeSO_4*7H_2O$, stored at 4°C	

Table 2.22 Additional chemicals and solutions for cultivation of nitrite oxidizing bacteria.

Baktopur (Sera)
Dimethyl sulfoxid (DMSO, Roth, 99.5%, A994.1)
Ethanol (Merck, molecular grade, 1.08543.0250)
Fe(III) Citrate (Sigma-Aldrich, 3522-50-7)
Isoleucine (Roth, 98.5%, 3922.2)
Leucine (Roth, 98.5%, 3984.1)
Liquid N_2 (Messner Group)
Na Acetate (Sigma-Aldrich, 99%, S8750-250G)
Na Citrate (Roth, 99%, 3580.1)
Na Formate (Fluka, 99%, 71539)
Na Fumarate (Merck, 99%, 8.20584.0500)
Na Pyruvate (Sigma-Aldrich, 99%, 15990)
Na Succinate (Sigma-Aldrich, 98%, 14160-100G)
$NaNO_2$ (Roth, 99.7%, 8604.1)
$NaNO_3$ (Roth, 99%, A136.1)
Noble Agar (Difco, 214220)
Oxalic acid (Sigma-Aldrich, 33506-1KG)
Plaque agarose (Biozym, 840100)
Sieve 3:1 Agarose (Biozym, 850091)
Valine (Roth, 98.5%, 4879.1)

2.1.7 Cultivation of *E. coli*

Table 2.23 Ampicillin stock, 1000x

Ampicillin (Sigma-Aldrich, A0166-5G)	100 mg
Ad 1 ml in 50% ethanol, sterile filtered, stored at -20°C	

Table 2.24 Lysogeny broth (LB) medium

NaCl (Roth, 99.5%, 3957.2)	5 g
Trypton (Oxoid, LP0042B)	10 g
Yeast Extract (Oxoid, LP0042B)	5 g
Agar (for solid medium, bacto, 214010)	20 g
Ad 1 l ddH_2O	

2.1.8 Restriction Digestion

Table 2.25 Enzymes and solutions used for restriction digestion.

ddH_2O (Fresenius water, Kabi)
MspI (Fermentas, ER0542)
Tango buffer (Fermentas, # BY5)

2.1.9 Solutions for Plasmid Extraction

Table 2.26 NaOH, 2N

NaOH (Roth, 99%, 6771.1)	1.6 g
NaOH pellets added to 20 ml ddH_2O	

Table 2.27 NaOH/SDS solution

2N NaOH	2 ml
10 % SDS (Roth, 99%, 4360.2)	2 ml
Ad 20 ml ddH_2O, stored at 4°C	

Table 2.28 P1 buffer

1 M Tris*HCl pH 8	1 ml
0.5 M EDTA	400 µl
RNAse A (Fermentas, EN0531)	20 mg
Ad 20 ml ddH_2O, stored at 4°C	

Table 2.29 K acetate/acetate solution

K acetate (Roth, 99%, T874.2)	5.8896 g
Acetic acid (Roth, 100%, 3738.2)	2.3 ml
Ad 20 ml ddH_2O, stored at 4°C	

2.1.10 Solutions for Percoll Gradient Centrifugation

Table 2.30 NaCl, 0.9%

NaCl (Roth, 99.5%, 3957.2)	0.9 g
Ad 100 ml ddH_2O, autoclaved	

Table 2.31 NaCl, 1.5 M

NaCl (Roth, 99.5%, 3957.2)	8.766 g
Ad 100 ml ddH_2O, autoclaved	

Table 2.32 Additional solutions used for Percoll density gradient centrifugation.

Percoll (autoclaved, Sigma-Aldrich, P4937-25mL)

2.1.11 Microautoradiography FISH

Table 2.33 Developer D19 solution

D19 (Kodak, 146 4593)	40 g
Ad 1 l ddH_2O, bottle wrapped in aluminum foil due to light sensitivity, stored at room temperature (RT)	

Table 2.34 Na-thiosulfate fixative solution

Na-Thiosulfate (Sigma-Aldrich, 99%, 217263-1KG)	30 g
Ad 1 l ddH_2O, stored at 4 °C	

Table 2.35 Additional chemicals and solutions used for microautoradiography FISH.

^{12}C-Sodium Bicarbonate (JT Baker, 100%, 0263)
^{14}C-Sodium Bicarbonate (500 µCi, Hanke Hartmann Analytic)
NTB autoradiography emulsion (Kodak, 889 5666), bottle wrapped in aluminum foil due to light sensitivity, stored at 4 °C

2.1.12 DNA Isolation and RNA Digestion

Table 2.36 Hexadecyltrimethyl ammonium bromide (CTAB)/NaCl Solution

NaCl (Roth, 99.5%, 3957.2)	4.1 g
CTAB (Roth, 98%, 9161.1)	10 g
Dissolved NaCl in 80 ml ddH_2O, slowly added CTAB while stirring and heating to 65°C until dissolved (3 h), then ad 100 ml ddH_2O, autoclaved	

Table 2.37 Lysozyme solution, 100 mg*ml^{-1}

Lysozyme (Sigma-Aldrich, 90%, L1667-1G)	100 mg
Ad 1 ml ddH_2O, sterile filtered, freshly prepared	

Table 2.38 PEG 8000 Solution, 30%

PEG 8000 (Sigma-Aldrich, 81268)	12 g
NaCl (Roth, 99.5%, 3957.2)	3.74 g
Ad 40 ml ddH_2O, autoclaved	

Table 2.39 Phosphate buffer, 120 mM

Na_2HPO_4*$2H_2O$ (Roth, 99.5% 4984.1)	10.045 g
NaH_2PO_4*H_2O (Roth, 98%, T878.1)	0.4912 g
pH adjusted to 8.0, ad 500 ml ddH_2O	

Table 2.40 Proteinase K solution, 10 mg*ml^{-1}

Proteinase K (Roche, 3115836001)	10 mg
Ad 1 ml ddH_2O, sterile filtered, stored at -20 °C	

Table 2.41 Tris/EDTA (TE) buffer

Tris (Roth, 99.3%, AE15.3)	1.2 g
EDTA (Roth, 99%, 8043.2)	0.37 g
pH adjusted to 8, ad 1 l ddH_2O, autoclaved	

Table 2.42 Tris/NaCl/SDS (TNS) buffer

Tris*HCl (Roth, 99%, 9090.1)	15.76 g
NaCl (Roth, 99.5%, 3957.2)	1.1688 g
SDS (Roth, 99%, 4360.2)	20 g
pH adjusted to 8.0, ad 200 ml ddH_2O, autoclaved	

Table 2.43 Additional chemicals, enzymes and solutions used for DNA isolation and RNA digestion.

Dry ice (Linde Gas)
Elution buffer (PowerSoil DNA Isolation Kit, MoBio, 12888-50)
Ethanol, 70% (Merck, molecular grade, 1.08543.0250)
Isopropanol (Roth, 99.5%, 9866.1)

NaCl, 5M (Roth, 99.5%, 3957.2)
RNase ONE™ Ribonuclease (Promega, M4261)
Roti-Chloroform/Isoamylalkohol (24/1 pH 8, Roth, X984.3)
Roti-Phenol (pH 8, Roth, 0038.2)
Roti-Phenol/Chloroform/Isoamylalkohol (25/24/1, pH 8, Roth, A156.2)
SDS, 10% (Roth, 99%, 4360.2)

2.1.13 Catalyzed Reporter Deposition FISH

Table 2.44 H_2O_2 solution, 100x

H_2O_2 (Sigma-Aldrich, 30%, H1009-5ML)	1 µl
Ad 300 µl 1x PBS	

Table 2.45 HCl, 0.01 M

37% HCl (Roth, 4625.1)	0.828 ml
Add HCl to 1 l ddH_2O	

Table 2.46 Hybridization buffer for CARD-FISH, 25% Formamide

5M NaCl	3.6 ml
1 M Tris*HCl	0.4 ml
ddH_2O	9.0 ml
Dextransulfate (Sigma-Aldrich, D8906-50G)	2.0 g
Formamide (deionized, Roth, 99.5%, P040.1)	5.0 ml
Blocking reagent	2.0 ml
10% SDS	40 µl
After dextransulfate addition, vortexed and heated to 50°C until dissolved, then cooled to RT and remaining ingredients added, aliquoted and stored at -20 °C.	

Table 2.47 Proteinase K solution, 15µg*ml^{-1}

Proteinase K (Roche, 3115836001)	10 mg
Ad 1 ml ddH_2O, then diluted 1:667 in 1x PBS, sterile filtered	

Table 2.48 Tyramide mixture for signal amplification

FITC tyramide stock	2 µl
100x H_2O_2 solution	10 µl
Ad 1 ml CARD-FISH amplification buffer	

Table 2.49 Additional chemicals and solutions used for catalyzed reporter deposition (CARD) FISH.

FITC Tyramides
H_2O_2, 30% (Sigma-Aldrich, H1009-5ML)
HPR probes *Thaumarchaea*, EUBmix (Biomers)
Methanol (Sigma-Aldrich, 99.6%, 179957-1L)

2.1.14 Incubations for Microdiversity Project

Table 2.50 PBS/Tween20 Solution

Tween20 (Sigma-Aldrich, P9416)	500 µl
Ad 100 ml 1x PBS, UV-treated and sterile filtered into UV-treated tubes	

Table 2.51 Additional chemicals and solutions for incubations.

^{12}C-Na bicarbonate (JT Baker, 100%, 0263)
^{13}C-Na bicarbonate (Cambridge Isotope Lab, 99%, CLM-441-5)
D_2O (Sigma-Aldrich, 99.9%, 151882-500G)
Nessler reagent (Sigma-Aldrich, CLM-441-5)

2.2 Consumables

Table 2.52 Consumables used in this study.

Consumable	Manufacturer
10-well microscope slides, black epoxy resin color mask	Marienfeld
50 ml tubes, PP, DNAse and RNAse free	Greiner
96 well plates, flat bottom, transparent	Greiner
Aluminum crimp seals	Sigma-Aldrich
Aluminum-spotted slides Al136	EMF Corporation
Cellulose acetate filter, 0.45 µm	IWAKI, Asahi Glass Co Ltd
ColiRollers plating beads	Merck Millipore
Eppendorf tubes, PP, various sizes	Biozym, Greiner, Eppendorf
Erlenmeyer flasks, various sizes	Roth
Glass bottles, screw-cap, various sizes	Schott
Glass capillaries, hollow rectangle	CM Scientific
Glass cover slips, 24x50 cm	Marienfeld
Lysing matrix tubes	MP Biomedicals
Microscope slides, cut edges, frosted end	Roth
Minisart filter, 1.2 µm	Sartorius stedium
Nalgene syringe filter, 0.2 µm	Thermo Fisher Scientific
Nitrite and nitrate test stripes	Merckoquant
Nunc plastic inoculation loops, single use	Thermo Fisher Scientific
Omnifix luer-lock syringes, sterile, various sizes	Braun
Parafilm	M
PCR film, adhesive	Eppendorf
PCR plates, 96 wells, skirted	Eppendorf
Petri dish, 90 mm diameter	Sterilin
Rezist gas filter, 0.2 µm, 7 bar	Whatman
Rubber stoppers, butyl, various sizes	GMT
Serological pipettes, sterile, single use, various sizes	VWR
Serum bottles, various sizes, Wheaton	Sigma-Aldrich
Sterican needles, sterile, various sizes	Roth
TCTP filter, 10 µm	Merck Millipore
Tips, various sizes, with and without filter	Biozym
Transfer tips, 15 µm diameter, 20° angle	Eppendorf
TSTP filter, 3 µm	Merck Millipore
Ultra-clear thinwall centrifuge tubes, 13.2 ml	Beckman coulter

2.3 Equipment

Table 2.53 Equipment used in this study.

Equipment	Manufacturer
Analytical Plus (analytical balance)	Ohaus
Axio Imager M1 and Axioplan 2 imaging (epifluorescence microscopes)	Zeiss
BL3100 and BL6100 (balance)	Sartorius
Centrifuge 5804R, FA 45-30-11 and F34-6-38 rotors	Eppendorf
Concentrator 5301	Eppendorf
DMI6000, TCS SP8 X, and CTR 6500 (inverted confocal laser scanning microscope)	Leica
Eppendorf Research Pipettes, various sizes	Eppendorf
Filtration tower, fritted glass base (2.5 cm), conical rubber stopper and aluminum clamps	Merck Millipore
Galaxy Mini Microcentrifuge C12XX	VWR
Hybridization oven	Memmert
ICS-3000, Ion Pac AG11 (4x50 mm), AS11 (4x250 mm) columns (ion chromatography system)	Dionex
Infinite M200 PRO multimode microplate reader	Tecan
LabRam HR Evolution (Raman microscope)	Horiba Jobin-Yvon
LaminAir Model 1.2 (laminar flow hood)	JOUAN Nordic A/S
Mikro 22R (microcentrifuge)	Hettich
MQ Biocel (water purification system)	Merck Millipore
MiniSpin plus (microcentrifuge)	Eppendorf
Muffle furnace	
NanoDrop ND-1000	Thermo Fisher Scientific
NanoDrop ND-3300	Thermo Fisher Scientific
Observer D1, Transfer Man NK2 (micromanipulation microscope)	Zeiss, Eppendorf
Optima L100 XP Ultracentrifuge, with SW 41 Ti rotor	Beckman Coulter
P/ACE MDQ (capillary electrophoresis system)	Beckman Coulter
ProfiLine pH 3110 (pH-meter)	WTW
RCT Basic (magnetic stirrer/hot plate)	IKA-Werke
Rotina 35R (microcentrifuge)	Hettich
Sono Puls HD2070, UW2070 (sonicator probe)	Bandelin
Sub-Cell GT (agarose gel electrophoresis system)	BioRad
Suction Flask (100 ml), glass	Schott
T100 PCR (thermocycler)	BioRad
UST-C30M-8R UV Transilluminator	Biostep
UV Sterilizing PCR Workstation	PEQLAB
Vacuum/Pressure Pump DOA-P730-BN	Pall
Vortex Genie 2	Scientific Industries
Water bath	GFL

2.4 Kits

Table 2.54 Kits used in this study.

Kit	Manufacturer
CEofix Anions 5 Kit	Analis
PowerSoil DNA Isolation Kit	Mobio
QIAquick PCR purification kit	Qiagen
Quant-iT PicoGreen dsDNA Assay Kit	Thermo Fisher Scientific
REPLI-g Single Cell Kit	Qiagen
TOPO TA Cloning Kit, pCR 4-TOPO Vector	Thermo Fisher Scientific

2.5 Probes for FISH

Table 2.55 Probes used for FISH.

Probe ID	Name	FA (%)	Sequence	Competitor	Specificity	Reference
Bde525	-	35	GAT CCC TCG TCT TAC CGC	-	Genus *Bdellovibrio*	Mahmoud et al. 2007
Bet42a	L-C-bProt-1027-a-A-17	35	GCC TTC CCA CTT CGT TT	GCC TTC CCA CAT CGT TT	*Betaproteobacteria*	Manz et al. 1992
Cl6a192	S-*-Nsm6a-0192-a-A-20	35	CTT TCG ATC CCC TAC TTT CC	CTT TCG ATC CCC TGC TTT CC	Cluster 6a, *Nitrosomonas oligotropha/ureae* cluster	Adamczyk et al. 2003
Eub338 I	S-D-Bact-0338-a-A-18	0-50	GCT GCC TCC CGT AGG AGT	If used by itself, Eub338 II and III	Most bacteria	Amann et al. 1990
Eub338 II	S-*-BactP-0338-a-A-18	0-50	GCA GCC ACC CGT AGG TGT	If used by itself, Eub338 I and III	*Planctomycetes*	Daims et al. 1999
Eub338 III	S-*-BactV-0338-a-A-18	0-50	GCT GCC ACC CGT AGG TGT	If used by itself, Eub338 I and II	*Verrucomicrobia*	Daims et al. 1999
Gam42a	L-C-gProt-1027-a-A-17	35	GCC TTC CCA CAT CGT TT	GCC TTC CCA CTT CGT TT	*Gammaproteo-bacteria*	Manz et al. 1992
Hsal723	-	35	GCC CAG TTA GCT GCC TAC GCC	-	*Micavibrio*-like bacteria	Dolinšek et al. 2013
Hsal866	-	35	CCC AGG CGG TGT GCT AAT CAC T	-	*Micavibrio*-like bacteria	Dolinšek et al. 2013
Ncom1025	-	35	CTC GAT TCC CTT TCG GGC A	-	Cluster 8 *Nitrosomonas communis*	Juretschko 2000
NEU	S-*-Nsm-0651-a-A-18	40	CCC CTC TGC TGC ACT CTA	TTC CAT CCC CCT CTG CCG	Most halophilic and halotolerant *Nitrosomonas spp.* (*Nitrosomonas halophila*, *eutropha*, and *europaea*, "*Nitrosococcus*" *mobilis*)	Wagner et al. 1995
Nit3	S-G-Nbac-1035-a-A-18	40	CCT GTG CTC CAT GCT CCG	CCT GTG CTC CAG GCT CCG	*Nitrobacter*	M. Wagner et al. 1996
Nmar830	-	35-40	GCC TAG TAA GGC CCA ACA	-	Cluster6b, *Nitrosomonas marina* cluster	Juretschko 2000
Nmv/Ncmob	S-S-Nmob-0174-a-A-18	35	TCC TCA GAG ACT ACG CGG	-	*"Nitrosococcus" mobilis* (*Nitrosomonas* lineage)	Pommerening-Röser, Rath, and Koops 1996
Nolimar712	-	40	GCC TTC GCC ATC GAT GTT CT	CGC CTT CGG CAC CGG TGT TCC	Cluster 6, *Nitrosomonas urea, oligotropha, marina, aestuarii*	Juretschko 2000
NonEUB	-	0-60	ACT CCT ACG GGA GGC AGC	-	Complementary to Eub338	Wallner, Amann, and Beisker 1993
Nscry1004	S-*-Nscry-1004-a-A-19	35	ACTCACCTCTCAG CGAGCT	-	*Nitrosomonas cryotolerans*	Juretschko 2000
Nso1225	S-F-bAOB-1224-a-A-20	35	CGC CAT TGT ATT ACG TGT GA	-	Betaproteo-bacterial AOB	Mobarry et al. 1996
Nso190	S-F-bAOB-0189-a-A-19	55	CGA TCC CCT GCT TTT CTC C	-	Betaproteo-bacterial AOB	Mobarry et al. 1996

Nsv443	-	30	CCG TGA CCG TTT CGT TCC G	-	*Nitrosospira spp.*, relatively unspecific	Mobarry et al. 1996
Ntlc439	S-G-Ntlc-439-a-A-18	40	TTG CTT CGT CCC CCA CAA	CAT CGT TTA CTG CTC GGA	*Nitrolancea hollandica*	Sorokin et al. 2012
Ntoga122	S-G-Ntoga-0122-a-A-19	35-40	TCC GGG TAC GTT CCG ATA T	TCW GGG TAC GTT CCG ATA T & TCY GGG TAC GTT CCG ATG T	Genus *Nitrotoga*	Lücker et al. 2015
Ntoga221/ FGall221b	S-F-Gall-0221-b-A-18f	30-35	TAT CGG CCG CTC CGA AAA	CAT CGG CCG CTC CGA AAG	Genus *Nitrotoga*	Lücker et al. 2015
Ntspa1151	S-*-Ntspa-1151-a-A-20	35	TTC TCC TGG GCA GTC TCT CC	-	*Nitrospira* lineage II, some lineage IV	Maixner et al. 2006
Ntspa1431	S-*-Ntspa-1431-a-A-18	35	TTG GCT TGG GCG ACT TCA	-	*Nitrospira* lineage I	Maixner et al. 2006
Ntspa662	S-G-Ntspa-662-a-A-18	35	GGA ATT CCG CGC TCC TCT	GGA ATT CCG CTC TCC TCT	Genus *Nitrospira*	Daims et al. 2001
Ntspa712	S-*-Ntspa-712-a-A-21	35-50	CGC CTT CGC CAC CGG CCT TCC	CGC CTT CGC CAC CGG TGT TCC	Phylum *Nitrospira*	Daims et al. 2001

2.5.1 Mixes of Probes

Table 2.56 Frequently used probe mixes.

Mix Name	Probes included
AOBmix	Cl6a192 + competitor, Ncom1025, NEU + competitor, Nso1225
EUBmix	Eub338 I, Eub338 II, Eub338 III
HSALmix	Hsal723, Hsal866
NTSPAmix	Ntspa662+ competitor, Ntspa712+ competitor

2.6 Primers for PCR

Table 2.57 Primers used for PCR. Primer pairs are shaded in the same color.

Primer ID	Name	Sequence	Annealing	Target	Length	Specificity	Reference
M13V		GTA AAA CGA CGG CCA G	60 °C	-	-	-	-
M13R		CAG GAA ACA GCT ATG AC					
8F	S-D-Bact-0008-b-S-20	AGA GTT TGA TYM TGG CTC	54 °C	16S rRNA	1486 bp	Most bacteria	Juretschko et al. 1998
1492R	S-*-Proka-1492-a-A-19	GGY TAC CTT GTT ACG ACT T					Loy et al. 2002
341F	S-D-Bact-0341-a-S-17	CCT ACG GGA GGC AGC AG	54 °C	16S rRNA	464 bp	Most bacteria	Herlemann et al. 2011
785R	S-D-Bact-0785-a-A-21	GACTACHVGGGTAT CTAATCC					
BamoF (141)	-	TGG GGR ATA ACG CAY CGA AAG	60 °C	16S rRNA	1084 bp	Betaproteo-bacterial AOB	McCaig et al. 1994
Nso1225R	S-F-bAOB-1224-a-A-20	CGC CAT TGT ATT ACG TGT GA					Mobarry et al. 1996
8F	S-D-Bact-0008-b-S-20	AGA GTT TGA TYM TGGCTC	54 °C	16S rRNA	1150 bp	*Nitrospira*	Juretschko et al. 1998
Ntspa1158R	-	CCC GTT MTC CTG GGC AGT					Maixner et al. 2006
Ntspa662F	S-G-Ntspa-662-a-A-18	AGAGGAGCGCGGAA TTCC	54 °C	16S rRNA	496 bp	*Nitrospira*	Daims et al. 2001
Ntspa1158R	-	CCC GTT MTC CTG GGC AGT					Maixner et al. 2006
amoA1F	-	GGG GTT TCT ACT GGT GGT	50 °C	*amoA*	490 bp	Betaproteo-bacterial AOB	Rotthauwe, Witzel, and Liesack 1997
amoA2R	-	CCC CTC TGC AAA GCC TTC TTC					
nxrB169F	-	TAC ATG TGG TGG AAC A	56 °C	*nxrB*	485 bp	*Nitrospira*	Pester et al. 2014
nxrB638R	-	CGG TTC TGG TCR ATC A					
nxrB706F	-	AAG ACC TAY TTC AAC TGG TC	56 °C	*nxrB*	724 bp	*Nitrobacter* and *Nitrococcus*	Frank Maixner, unpublished
nxrB1431R	-	CGC TCC ATC GGY GGA ACM AC					

2.7 Cultures and Activated Sludge Samples

Nitrospira moscoviensis (Ehrich et al. 1995) biomass was obtained from Hanna Koch and subsequently kept in aerobic batch culture.

Activated sludge was sampled at the full scale municipal WWTP Klosterneuburg (KNB) in October 2013, January, and April 2014 from basin "Schwachlastbelebung 1" (activated sludge basin, KNB1), "Schwachlastbelebung 2" (activated sludge basin, KNB2), and "Trübwasserspeicher" (basin for treatment of reject water, KNB3). WWTP Klosterneuburg treats municipal wastewater, which is relatively constant in its composition from winter to summer. In autumn however, influx wastewater differs from other seasons due to high wine mash content, as WWTP Klosterneuburg receives wastewater from winemakers in the region (Karl Weiss, personal communication). Since 2011, WWTP Klosterneuburg employs the so-called "Hybridverfahren", which consists of conventional physical wastewater treatment (removal of solids, oil and grease), and three consecutive sludge basins. Wastewater first enters an aerated basin ("Schwachlastbelebung 1") for initial removal of organics and concomitant nitrification, which is separated from a non-aerated basin for denitrification ("Hochlastbelebung") by a settling tank. A second intermittently aerated basin for nitrification/denitrification ("Schwachlastbelebung 2") follows afterwards. Both sludge and water is recycled between the basins.

2.8 Conventional Fluorescence *in situ* Hybridization Protocol

2.8.1 Chemicals and Solutions

For chemicals and solutions used for conventional fluorescence *in situ* hybridization (FISH), please refer to the following tables:

- DAPI/PBS solution: Table 2.1
- EDTA solution: Table 2.2
- NaCl solution: Table 2.3
- PBS buffer: Table 2.4
- SDS solution: Table 2.5
- Tris*HCl buffer: Table 2.6
- Probes used: Table 2.55 and Table 2.56
- Additional chemicals and solutions: Table 2.7

2.8.2 General Procedure for FISH

rRNA targeted FISH allows for detection of specific groups of (micro-) organisms in the environment by exploiting hybridization of fluorescently labeled complementary nucleic acids to an organism's ribosomal RNA. FISH was essentially carried out as described by Daims et al. (2005). Whenever possible, probes were used in a hierarchical manner, to confirm presence of specific populations with a second probe of similar or slightly broader specificity. Each new sample that was investigated by FISH, was hybridized with NonEUB probes labeled with all fluorophores used (FitC, Cy3, Cy5) to assess possible unspecific binding of DNA and/or fluorophores to sample-structures. For probes requiring competitors (Table 2.55), those were always included in FISH, however are not mentioned in the text for sake of easier reading.

2.8.2.1 Sample Fixation

For FISH targeting gram-negative cells, biomass was fixed with formalin in 1x PBS. Biomass was either fixed in 3 % formalin for 2 h at 4 °C, 3 % formalin for 30 min at room temperature (RT), or 2 % formalin at 4 °C over night (O/N). After fixation, cells were centrifuged (17,949 g, 10 min, 4 °C) and

washed with 1x PBS twice. Then, biomass was resuspended in ice-cold 50 % 1x PBS, 50 % ethanol (molecular grade). For FISH targeting gram-positive cells, no fixation was employed, but cell material was directly resuspended in ice-cold 50% 1x PBS, 50 % ethanol (molecular grade). This will henceforth be referred to as "ethanol fixation". Fixed biomass was always stored at -20 °C.

2.8.2.2 Agarose-Coating of Slides, Sample Immobilization and Dehydration

Agarose-coating of microscope slides was employed to avoid loss of biomass during washing steps. Slides were dipped into melted 0.2 % LE agarose in ddH_2O, dried with compressed air, and stored at RT. For immobilization of biomass, 3-10 µl of fixed sample were applied onto wells and dried at 46 °C. Immobilized biomass was dehydrated by an ethanol series (50 %, 80 % and 96 % denatured ethanol, 3 min each) and then let dry at RT.

2.8.2.3 Stringent Hybridization and Washing

Stringent hybridization conditions were ensured via formamide concentration in the hybridization buffer, to only allow hybridization of the used probe(s) to target organisms. Hybridization buffers were prepared according to Table 2.58. Onto each well, 10 µl of stringent hybridization buffer were applied and mixed with 1 µl of the respective probe working stock(s), yielding final probe concentrations of 0.5 pmol*µl^{-1}. Microscope slides were incubated in the dark at 46 °C for 1.5 h - O/N in tightly closed hybridization chambers (PP-tubes), containing tissue that was soaked in the remaining hybridization buffer.

Table 2.58 Composition of hybridization buffer for formamide concentrations between 0 % and 70 %. All volumes are given in µl. SDS was added last to avoid precipitation.

Formamide (%)	0	5	10	20	25	30	35	40	45	50	70
5M NaCl	180	180	180	180	180	180	180	180	180	180	180
1M Tris*HCl	20	20	20	20	20	20	20	20	20	20	20
ddH_2O	800	750	700	600	550	500	450	400	350	300	100
Formamide	0	50	100	200	250	300	350	400	450	500	700
10% (w/v) SDS	1	1	1	1	1	1	1	1	1	1	1

After hybridization, excess probe and formamide were removed by a washing step under stringent conditions ensured via NaCl concentration in the washing buffer, which was prepared according to Table 2.59, and pre-warmed to 48 °C. Microscope slides were transferred to washing buffer and incubated at 48 °C in the dark for 10 min. Then, slides were briefly dipped into ice-cold ddH_2O to remove traces of salts, dried with compressed air, and stored in the dark until analysis. For long-term storage, dry slides were stored at -20 °C.

Table 2.59 Composition of washing buffer for formamide concentrations between 0 % and 70 %. All volumes are given in µl unless otherwise specified.

Formamide (%)	0	5	10	20	25	30	35	40	45	50	70
5M NaCl	9,000	6,300	4,500	2,150	1,490	1,020	700	460	300	180	0
1M Tris*HCl	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
0.5M EDTA	0	0	0	500	500	500	500	500	500	500	500
ddH_2O ad	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml

When two or more probes, which required different formamide concentrations were used, double hybridizations were performed. Sample was hybridized with probes requiring higher formamide concentration first. After washing, a short ethanol series (1 min incubation each) was carried out prior to the second hybridization at lower formamide concentration with the respective probe(s) for 1.5 – 2 h.

2.8.2.4 DAPI staining

To visualize all cells, samples were stained with 4',6-diamidino-2-phenylindole (DAPI) after FISH. Per well, 15 µl of DAPI/PBS solution were added. Slides were incubated in the dark for 5 min, briefly dipped into ddH_2O to remove excess DAPI, and dried by compressed air.

2.8.2.5 Embedding of FISH-Slides

Slides were embedded in CitiFluor AF1 anti-fading mounting medium prior to analysis on epifluorescence or confocal laser scanning microscopes (CLSM) to avoid bleaching and to ensure optimal pH for probe excitation and emission.

2.9 Microscopy

For screening purposes, epifluorescence and bright field microscopy were employed on Zeiss Axio Imager M1 and Zeiss Axioplan 2 imaging microscopes. For image acquisition, a CLSM (Leica DMI6000, TCS SP8 X) was used, which was equipped with a white light laser and a UV laser for excitation of DAPI. Image acquisition was done using Leica LAS software, with a pinhole setting of 1 airy unit (detection of fluorescence from 1 µm in z-dimension). For quantification of specific populations and for analysis of colocalization of two populations, images (1024x1024 pixel, 400 Hz) were acquired at random positions distributed over the entire well. For activated sludge samples, 40 images per analysis from at least two different wells were acquired. For enrichment cultures, 20 images were acquired per analysis. Positions of image acquisition were pre-defined using the “Mark and Find” and “Adaptive Focus Control” tools implemented in the LAS software package.

2.10 Image Analysis with daime

For quantification of specific FISH-defined populations and colocalization analyses of two populations, daime 2.0 was used (Daims et al. 2006b). For analyses of activated sludge images, specific population signals were segmented using the “Edge Detection” algorithm. EUBmix or DAPI signals (reference space) were segmented using “RatsL”. For all segmentations, a minimum pixel size of 10 was defined. Autofluorescence or fluorescence due to unspecific binding of probes to matrix components visible in both population image series was removed automatically by exclusion of objects showing > 60% congruency. In addition, all images were manually checked for autofluorescence and unspecific fluorescence signals. Biomass fractions of specific populations relative to overall signals from EUBmix/DAPI were calculated using the “Biovolume fraction” tool. For colocalization analyses, two additional steps were employed. Pixels present in both specific populations (due to adjacent growth of microorganisms and slight overlap of fluorescence) create artificially enhanced colocalization and were therefore subtracted from both specific population channels. Lastly, specific population signals were added to the reference space, and pixel holes in the reference space were filled. Spatial arrangement tests between two specific populations were carried out using the “2D Inflate” algorithm against a reference space. For analysis of enrichment cultures, images were segmented with “RatsL” for all channels, with a pixel minimum of 10. Biovolume fraction was calculated as described above.

2.11 General Polymerase Chain Reaction Protocol

2.11.1 Chemicals and Solutions

For chemicals and solutions used for PCR, please refer to the following tables:

- Chemicals and solutions: Table 2.8
- Primers used: Table 2.57

2.11.2 Procedure for PCR

All handling of PCR components was done in a UV-sterilizing PCR workstation (Peqlab), using sterile filter tips. Non-DNA and non-enzymatic reagents were UV-treated prior to usage. Unless otherwise specified, PCR reactions contained 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 pmol*µl^{-1} of each primer, 1x Ex Taq Polymerase buffer (KCl) and 0.025 U*µl^{-1} Taq DNA Polymerase. For each PCR, a negative control containing ddH_2O instead of DNA template was included to rule out contamination of the used chemicals. When possible, positive controls were included for PCR, consisting of a plasmid carrying the target sequence, or DNA from a target organism. Unless otherwise specified, each PCR run consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing for 30 s at stringent temperatures depending on the used primer pair (Table 2.57), and elongation at 72 °C. At the end of all runs, a final elongation at 72 °C for 10 min was included.

2.12 Agarose Gel Electrophoresis

2.12.1 Chemicals and Solutions

For chemicals and solutions used for agarose gel electrophoresis, please refer to the following tables:

- TBE buffer: Table 2.9
- Additional chemicals and solutions: Table 2.10

2.12.2 Procedure for Agarose Gel Electrophoresis

PCR products, restriction digests and extracted genomic DNA were visualized by agarose gel electrophoresis. Gels were cast and run in SubCell GT Systems (BioRad) at 90 or 120 V for restriction digests/genomic DNA and PCR products respectively. Gels were prepared by melting 1.5 – 2 % (w/v) LE agarose in 1x TBE buffer in a microwave. Before loading, five volumes of DNA-containing sample were mixed with one volume 6x loading dye. For qualitative assessment of PCR products, gels were pre-stained by addition of 2 µl 10,000x GelRed per 100 ml gel-volume before casting of gels. Gels were post-stained in GelRed (3x GelRed in ddH_2O) for 30 min for restriction digests and genomic DNA to better visualize actual lengths of DNA fragments. For each agarose gel electrophoresis, GeneRuler 1 kb or 100 bp was included as a length standard.

2.13 Measurement of Nitrite and Nitrate

2.13.1 Chemicals and Solutions

For chemicals and solutions used for measurements of NO_2^- and NO_3^-, please refer to the following tables:

- N-naphthylethylene diamine dichloride (NEDD) solution: Table 2.11
- Sulfanilamide solution: Table 2.12
- VCl_3 solution: Table 2.13
- Additional chemicals and solutions: Table 2.14

2.13.2 Procedure for Griess-Based Measurements

All Griess-based methods rely on the formation of a colored azo-dye complex between NO_2^-, sulfanilamide and N-naphthylethylene diamine dichloride (NEDD). $NO_2^-$and NO_3^- standards were prepared by serial dilution in mineral medium, ranging from 50 µM for NO_2^- and 62.5 µM for NO_3^- to 0 µM (mineral medium only). Samples were diluted in mineral medium to maximum expected concentrations of 30 µM to avoid extrapolation from standards. Measurements were carried out in

transparent 96-well plates with flat bottom (Greiner). Both standards and samples were measured in technical duplicates. Absorbance was measured on an Infinite 200 PRO multimode microplate reader (Tecan).

2.13.2.1 Nitrite Measurement

NO_2^- was measured by a Griess reaction protocol based on Griess-Romijn van Eck (1966). For each new experiment, 2 % sulfanilamide and 0.1 % NEDD solutions were mixed in a 1:1 ratio, in sufficient amounts for all samples and standards to be measured. Per well, 50 µl diluted sample or standard were applied, and subsequently mixed with 50 µl sulfanilamide/NEDD mixture using a Multipette stream (Eppendorf). Absorbance was measured at 545 nm and sample concentration inferred from standards.

2.13.2.2 Nitrate Measurement

NO_3^- detection is based on the reduction of NO_3^- to NO_2^- and the subsequent measurement of NO_2^- by Griess reaction. Measurement of NO_3^- was adapted from Miranda et al. (2001) by inclusion of an NO_2^- standard to account for inaccuracies in NO_3^- determination in presence of high NO_2^-, similar to García-Robledo et al. (2014). As a reducing agent for NO_3^- reduction, vanadium(III) chloride (VCl_3) was used. 50 µl of diluted samples or standards (both NO_3^- and NO_2^-) were applied per well and mixed with 50 µl VCl_3 solution. Immediately after VCl_3 addition, 50 µl sulfanilamide/NEDD mixture (as described in 2.13.2.1) were added. Well plates were sealed with plastic foil and incubated at 37 °C in the dark for 30 – 45 min. Absorbance was measured at 540 nm. NO_3^- concentration was inferred by calculating absorbance stemming from NO_2^- in the sample (according to values obtained by protocol described in 2.13.2.1) in presence of VCl_3 from the NO_2^- standard included with all NO_3^- measurements. This theoretical absorbance stemming from sample-NO_2^- was then subtracted from the measured total sample absorbance in presence of VCl_3. Sample NO_3^- concentration was subsequently inferred from the "residual" absorbance from NO_3^- standards.

2.13.3 Procedure for Ion Chromatography

Ion chromatography (IC) measurements of NO_3^- and NO_2^- were performed by Ludwig Seidl on ICS-3000 (Dionex) equipped with Ion Pac AG11 (4x50 mm) and AS11 (4x250 mm) columns. As eluent, KOH ranging from 0.5 – 10 mM was employed on a run time of 30 min per sample. Analysis of chromatograms was done with Chromeleon Chromatography Data System Software. Combined NO_2^- and NO_3^- standards were prepared by serial dilution in mineral medium, ranging from 100 $mg*l^{-1}$ (2.17 mM NO_2^-, 1.61 mM NO_3^-) to 0 $mg*l^{-1}$ (mineral medium only). Both samples and standards were diluted 1:10 in ddH_2O for measurements. Concentrations of NO_2^- and NO_3^- in samples were inferred from the standard.

2.14 Measurement of Organic Anions by Capillary Electrophoresis

2.14.1 Chemicals and Solutions

For chemicals and solutions used for measurements of organic anions by capillary electrophoresis (CE), please refer to the following tables:

- NaOH/valeric acid solution: Table 2.15
- Additional chemicals and solutions: Table 2.16

2.14.2 Procedure for CE Measurements

CE measurements were carried out on a P/ACE MDQ Molecular Characterization System (Beckman Coulter) equipped with 32 Karat Software. CEofix Anions 5 Kit was used for measurement of organic

anions. Standards for analyzed compounds were prepared in mineral medium and serially diluted to concentrations ranging from 1 – 0 mM. Samples and standards were mixed in a ratio of 1:1 with 10 mM NaOH/2 mM valeric acid for charging (NaOH) and inclusion of an internal standard for normalization of peak areas (valeric acid). Both standards and samples were measured in duplicates. Peak areas of measured compounds were normalized to the peak area of valeric acid. Sample concentrations were then inferred from standards.

2.15 Statistical Analyses, Diagrams and Graphics

Data evaluation of NO_3^-, NO_2^- and CE measurements was done with Microsoft Excel. For statistical analyses and plotting of data, RStudio (R 3.0.2, RStudio 0.98.978, R Core Team 2013, including packages ggplot2, plyr, extrafont, reshape2, stringr) was used. Graphics were generated with Inkscape (0.48.5).

2.16 Cultivation and Investigation of *N. moscoviensis'* Denitrifying Potential

2.16.1 Chemicals and Solutions

For chemicals and solutions used for cultivation and investigation of *N. moscoviensis'* denitrifying potential, please refer to sections describing conventional FISH (2.8), Griess based NO_2^- and NO_3^- measurements (2.13), and CE measurements (2.14), as well as the following tables:

- Mineral medium: Table 2.18 and Table 2.19
- Trace element solutions: Table 2.20 and Table 2.21
- Additional chemicals and solutions for cultivation of NOB: Table 2.22

2.16.2 Aerobic Cultivation of *N. moscoviensis*

All manipulation of cultures was done next to a flame or in a laminar flow cabinet to ensure sterile conditions. Only sterile filter tips were used for handling of cultures. *N. moscoviensis* was cultivated in batch cultures in mineral medium (adapted from Ehrich et al. 1995) supplemented with 5 mM NO_2^- in Erlenmeyer flasks, closed with aluminum caps. Cultures were kept at 37 °C in the dark without shaking. Every five to ten days, NO_2^-concentration was assessed by nitrite test stripes. Upon depletion of substrate, sterile filtered $NaNO_2$ was added to a final concentration of 5 mM. When biomass became inactive due to accumulation of high concentrations of NO_3^- after prolonged batch feeding, biomass was harvested by centrifugation (8,228 g, 20 min, 25 °C), washed with NO_x-free mineral medium and again pelleted, until all traces of NO_x were removed. Washed biomass was then resuspended in fresh mineral medium and further cultivated. For transfers, cultures were diluted 1:30 – 1:50 in mineral medium containing 1 mM NO_2^-, and then further cultivated. Every one to two months, aliquots of all *N. moscoviensis* cultures were formalin-fixed as described in section 2.8.2.1. To screen for presence of contaminants, FISH with general bacterial probes (EUBmix) and a specific probe for *Nitrospira* lineage II (Ntspa1151) was performed. Additionally, all cells were stained with DAPI. When contaminant cells were observed (cells only stained by DAPI or only stained by DAPI and EUBmix probes but not by the specific *Nitrospira* probe), the respective cultures were killed.

2.16.3 Experiments With *N. moscoviensis* Under Anoxic Conditions

To test potential alternative pathways for energy generation in *N. moscoviensis* under anoxic conditions, oxidation of acetate, branched AA (equimolar mix of valine, leucine, and isoleucine), citrate, ethanol (molecular grade), formate, fumarate, pyruvate, and succinate coupled to NO_3^- reduction was investigated.

2.16.3.1 Preparation of Serum Bottles and Rubber Stoppers

Serum bottles were washed both with and without detergent to remove potentially inhibitory traces of salts and organics. From butyl rubber stoppers, potentially inhibitory substances were removed by autoclaving them in ddH_2O multiple times, followed by treatment with diluted oxalic acid for 24 h, and subsequent washing and autoclaving in ddH_2O. Dry, clean stoppers were autoclaved in beakers before setting up the experiments.

2.16.3.2 Preparation of Anoxic, Sterile Serum Bottles for Cultivation

Washed serum bottles were made anoxic by flushing with N_2 for 2 min, closing with cleaned butyl rubber stoppers and subsequently pulling out the N_2 flushing needle. Serum bottles were sealed with aluminum crimps and autoclaved.

2.16.3.3 Preparation of Anoxic Mineral Medium

Anoxic mineral medium was prepared according to Widdel & Bak (1992). Mineral medium was autoclaved in a custom-made Widdel-flask containing a magnetic stir bar. The flask was equipped with a gas inlet connected to a cotton filter, two peripheral valves for addition of liquids, and tubing for filling of bottles with anoxic medium. All manipulation (see Figure 2.1 for an overview of the setup) was done next to a flame to ensure sterile conditions.

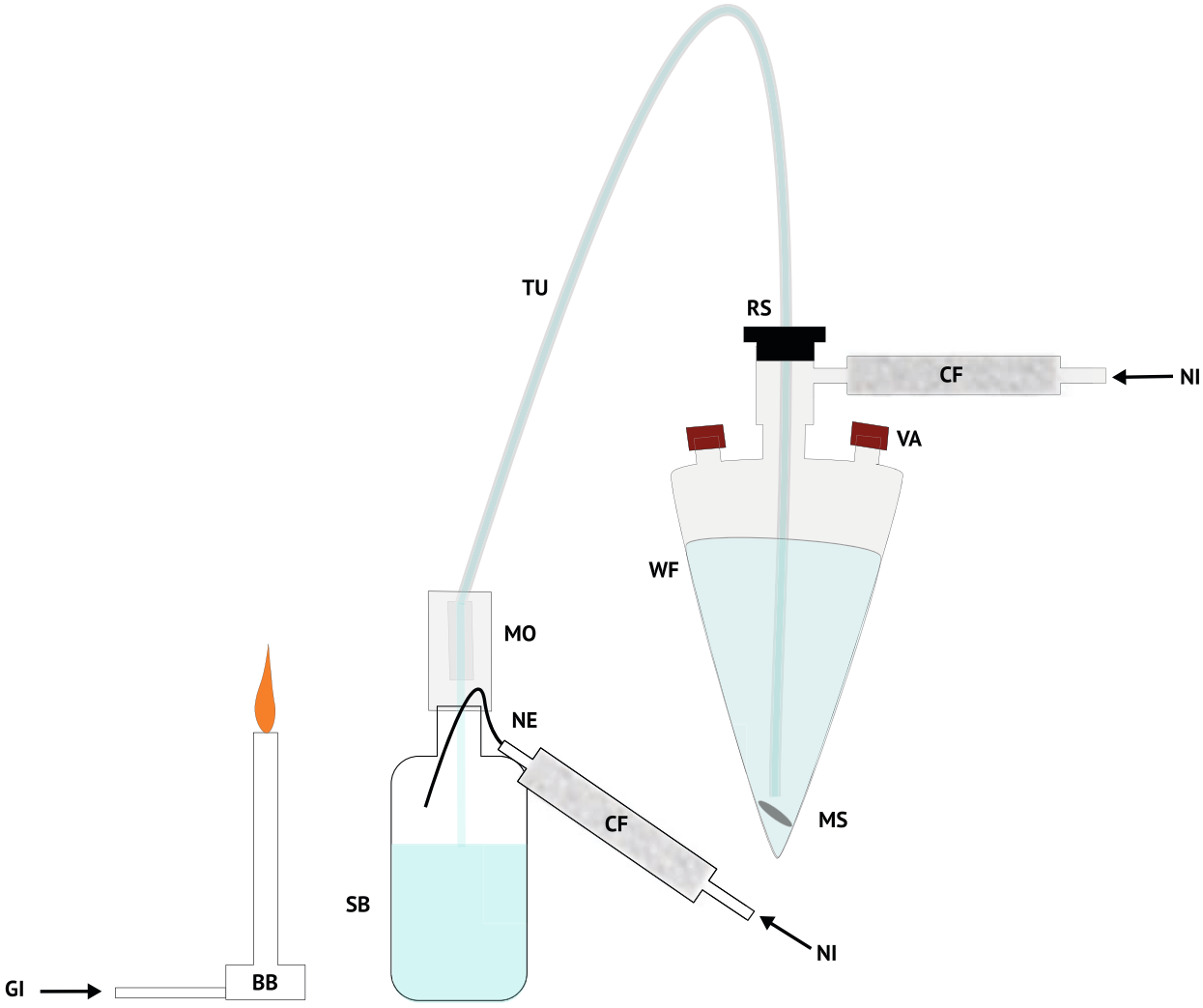

Figure 2.1 Schematic of Widdel-flask and filling procedure. BB: Bunsen burner, CF: Cotton filter, GI: Gas inlet, NE: Needle, NI: N_2-inlet, MO: Medium outlet, MS: Magnetic stir bar, RS: Rubber stopper, SB: Schott bottle, TU: Tubing, VA: Valve, WF: Widdel-Flask.

The flask headspace was flushed with N_2 for 5 min with untightened side valves, while stirring to remove residual O_2. Under N_2-pressure, the medium was then cooled to RT. Sterile filtered trace elements and selenite/tungstate trace elements were added via a side valve, while flushing with N_2. Sterile Schott bottles were flushed with N_2, and, while flushing, were filled with the anoxic medium. Medium bottles were closed with sterile butyl rubber stoppers, sealed with plastic screw caps with aperture and stored in the dark at RT.

2.16.3.4 Preparation of Anoxic Substrates

Substrate stock solutions were freshly prepared in mineral medium in serum bottles, in concentrations where 0.5 ml substrate addition to incubations resulted in approximately 1 mM final concentration. All used serum bottles were closed with butyl rubber stoppers and aluminum crimps. Non-volatile, heat stable substrate solutions (NO_3^-, succinate, fumarate, formate, acetate, and branched amino acids) were transferred to serum bottles containing magnetic stir bars and closed. Solutions were made anoxic by three cycles of vacuum and N_2-pressure application for 20 min and 10 min respectively and were subsequently autoclaved. For non-volatile, heat-labile substrates (NO_2^-, pyruvate, and citrate), closed serum bottles containing a magnetic stir bar were autoclaved. Substrate solutions were sterile filtered and injected by syringe and needle into sterile serum bottles. Under sterile conditions, solutions were made anoxic as described above. Volatile ethanol stock solution was prepared by injecting 70% ethanol (molecular grade) into closed serum bottles containing anoxic mineral medium. Afterwards, diluted ethanol solution was autoclaved. All substrate stocks were stored in the dark at 4 °C.

2.16.3.5 Assessment of Denitrification Potential

Two consecutive experiments were conducted to assess the denitrification potential of *N. moscoviensis* (see Table 2.60 for an overview of the experimental setup). For each experiment, control incubations testing for presence of O_2 (incubation with NO_2^- only), oxidation of internal storage compounds (incubation with NO_3^- only), and biomass activity (incubation with formate and NO_3^-) were included. Each substrate combination was tested in biological duplicates. All manipulation was done next to a flame to ensure sterile conditions. N_2-flushed, sterile syringes and needles were used for manipulation of anoxic liquids and gases. For injection into Schott bottle stoppers, 23 G needles (Braun, 4657667) were used, for serum bottle rubber stoppers, 27 G (Braun, 4657705) needles with smaller diameter to avoid formation of holes.

Table 2.60 Overview of the experimental setup for assessment of the denitrification potential of *N. moscoviensis*. Substrates were added to final concentrations of approximately 1 mM each.

Experiment	Liquid/Bottle Volume (ml)	Substrates Tested	Notes
1	12/30	Acetate + NO_3^-	
		Citrate + NO_3^-	
		Fumarate + NO_3^-	
		Pyruvate + NO_3^-	
		Succinate + NO_3^-	
		Formate + NO_3^-	Control for activity of cells
		NO_3^--only	Control for storage compounds
		NO_2^--only	Control for anoxic conditions
2	20/50	Branched AA + NO_3^-	
		Ethanol + NO_3^-	
		Formate + NO_3^-	Control for activity of cells
		NO_3^--only	Control for storage compounds
		NO_2^--only	Control for anoxic conditions

Sterile, anoxic medium was distributed to serum bottles. After injection of medium, equal volumes of headspace were withdrawn to avoid overpressure. Sterile, anoxic substrate stock(s) were added to bottles to final concentrations of approximately 1 mM each (Table 2.60). *N. moscoviensis* biomass was harvested (8,228 g, 20 min, 25 °C) and washed in fresh anoxic mineral medium until no NO_x was detectable on test stripes. Biomass was then resuspended in 2 ml anoxic medium and injected into a serum bottle containing anoxic medium to minimize O_2 contamination. Cell suspension was gently homogenized by inverting, and was stored on ice until inoculation. Per serum bottle, 1 ml of cell suspension was added. Remaining inoculum was formalin- and ethanol-fixed for FISH. After inoculation, CO_2/N_2 (20/80 %) gas mixture was added as carbon source (3 ml for experiment 1, and 6 ml for experiment 2). At each sampling time point, 0.5 ml liquid were withdrawn and stored on ice until centrifugation (20,817 g, 20 min, 4 °C). Supernatant (SN) was transferred to fresh tubes and stored at -20 °C until analysis of substrate concentrations. NO_2^- and NO_3^- concentrations were assessed by Griess-based methods as described in 2.13.2. Organic anion concentration (acetate, citrate, formate, fumarate, pyruvate, and succinate) was assessed by CE measurements as described in 2.14. Following the last time point, 2 ml of each incubation setup were centrifuged (20,817 g, 10 min, 4 °C) and biomass was formalin-fixed to screen for contaminants by FISH. Incubations were carried out for 147 (experiment 1) and 49 days (experiment 2) respectively.

2.17 Microdiversity of Nitrifying Bacteria

2.17.1 Chemicals and Solutions

For chemicals and solutions used for experiments in course of the microdiversity project, please refer to sections describing conventional FISH (2.1.1), PCR (2.1.2) and the following tables:

- H_2O_2 solution for CARD-FISH: Table 2.44
- HCl solution for CARD-FISH: Table 2.45
- Hybridization buffer for CARD-FISH: Table 2.46
- Proteinase K solution for CARD-FISH: Table 2.47
- Tyramide mixture for CARD-FISH: Table 2.48
- Additional chemicals and solutions for CARD-FISH: Table 2.49
- PBS/Tween 20 solution for microfloc-sorting: Table 2.50
- Additional chemicals and solutions for incubations: Table 2.51

2.17.2 Processing of Activated Sludge Samples

After sampling of activated sludge from WWTP Klosterneuburg, 50 ml per sampling basin were formalin- and ethanol-fixed as described in 2.8.2.1. Additionally, sludge was pelleted by centrifugation (10,000 g, 10 min, 4 °C) and stored at -20 °C for DNA extraction. Sludge SN was sterile filtered by subsequent filtration through 1.2 µm, 0.45 µm, and 0.2 µm filters. Unfixed sludge and sterile SN were stored at 4 °C until further processing. An overview over experiments carried out at different sampling time points is depicted in Table 2.61.

Table 2.61 Overview of experiments conducted at different sampling time points with sludge from WWTP Klosterneuburg, second nitrification basin (KNB2).

Sample	Experiments
October 2013	• FISH Screening • Sonication Optimization
January 2014	• FISH Screening • Sonication Optimization • Pre-study (incubation of sludge with heavy isotopes, Raman-activated microfloc sorting, and metagenome sequencing of 24 microflocs)
April 2014	• FISH Screening • Sonication Optimization • D_2O incubation test (assessment of minimum incubation time and D_2O concentration needed for nitrifier labeling)

2.17.3 FISH Screening, Nitrifier Quantification and Colocalization Assessment

Samples obtained from WWTP Klosterneuburg were screened by conventional FISH for presence of several functional groups. For AOB screening, probes Cl6a192, NEU, Ncom1025, Nsv443, Nmv/Ncmob, Nolimar712, Nscry1004, Nmar830, Nso190, and Nso1225 were used, for NOB screening, probes Ntspa1431, Ntspa1151, Ntspa451, Ntspa835, Ntspa662, Ntspa712, Nit3, Ntoga122, and Ntlc439. For Ntlc439 (targeting *Nitrolancea hollandica*), ethanol-fixed sample was used. Additionally, FISH screening for *Micavibrio*-like predators of *Nitrospira* (HSALmix) was employed.

Nitrifiers in samples from January and April 2014 were quantified and tested for colocalization by FISH as described in section 2.9 and 2.10. FISH for colocalization analyses was carried out with AOBmix, NTSPAmix and EUBmix.

2.17.4 Catalyzed Reporter Deposition FISH

To screen for presence of *Thaumarchaea* in non-incubated KNB samples and KNB2 samples incubated in presence of heavy isotopes from January 2014, CARD-FISH (Lebaron et al. 1997; Schönhuber et al. 1997) was employed, following a modified protocol from Laura Sauder and Jasmin Schwarz. This protocol employs a proteinase K instead of a lysozyme treatment for permeabilization of cells and includes a methanol/H_2O_2 inactivation step for endogenous peroxidases. Additionally, instead of TNT buffer for washing steps, 1xPBS was used.

For *Thaumarchaea* CARD-FISH, three formalin-fixed samples were used; activated sludge from KNB, *Thaumarchaea* enrichment from Laura Sauder (positive control for Thaum726 probe), as well as *E. coli* (positive control for CARD-FISH). Sludge from KNB was separately hybridized with *Thaumarchaea* probe Thaum726, EUBmix probes and NonEUB probe as combined control for unspecific binding and activity of endogenous peroxidases. *Thaumarchaea* enrichment was hybridized with Thaum726, and *E. coli* with EUBmix probes only.

In short, biomass was dehydrated by a conventional ethanol series and subsequently coated with a thin layer of 0.2 % LE agarose (in ddH_2O). Slides were let dry completely at RT. Permeabilization of cells was done by proteinase K digestion at RT for 10 min. Slides were then washed in ddH_2O for 1 min to remove excess proteinase K. Residual proteinase K was inactivated by incubation of the slide in 0.01 M HCl for 20 min. To remove HCl, slides were again washed in ddH_2O for 1 min. Endogenous peroxidases were inactivated by O/N incubation in 99% methanol and 0.3% H_2O_2. Subsequently, slides were washed twice in ddH_2O for 1 min, then rinsed in denatured ethanol and dried completely. 299 µl hybridization buffer containing 25% formamide were mixed with 1 µl HRP probe stock solution and 1 µl respective unlabeled competitor probes, giving final HRP probe concentrations of 0.17 ng*µl^{-1} each. 20 µl of hybridization buffer mixed with probes were applied per well. Slides were incubated in humid chambers containing tissue soaked in 25% formamide (in ddH_2O) at 46 °C for 3 h, and subsequently washed in regular washing buffer (Table 2.59) for 15 min at 48 °C. For signal amplification, slides were incubated in 1x PBS for 5 min. Then excess liquid was removed, while not drying biomass. Per well, 20 µl FITC tyramide mixture were added, slides were put into a humid chamber containing water-soaked tissue, and incubated at 46 °C for 30 min. Subsequently, slides were washed in the dark in 1x PBS for 15 min and twice in ddH_2O for 1 min each to remove excess tyramides. Slides were dehydrated by dipping into denatured ethanol, DAPI-stained and then stored at -20 °C until microscopic analysis.

2.17.5 Optimization of Sonication Conditions

Optimal sonication conditions for obtaining nitrifier microflocs were assessed for all activated sludge samples. For the October 2013 sample, only formalin-fixed sludge was available, else, unfixed activated sludge was used. Sludge was diluted 1:10 in 1x PBS to a final volume of 0.5 ml in 2 ml tubes. Hereafter, diluted sludge was sonicated for 5, 10, 20, 30, 40, 50 and 60 s, using 60% power and 1 cycle settings with a sonication probe, while cooling the sample on ice to minimize heating. Sonicated samples were then pelleted by centrifugation (17,949 g, 10 min, 4 °C) and either formalin-fixed (unfixed sample), or directly resuspended in 50% 1x PBS, 50% ethanol (formalin-fixed sample). Sonication efficiency was assessed by conventional FISH, using both general nitrifier probes (AOBmix and NTSPAmix) and specific AOB probes (Cl6a192 and NEU). Conditions yielding sufficient dissection

of sludge, floc diameters between 5 – 20 µm, and intact nitrifier microcolonies were then employed for dissection of sludge before Raman measurements and sorting of microflocs.

2.17.6 Raman-Activated Microfloc Sorting to Investigate Nitrifier Microdiversity: A Pre-Study

An overview of the entire workflow for the pre-study conducted in course of the microdiversity project is depicted in Figure 2.2. The pre-study was carried out to assess efficiency of sludge incubation, sonication optimization and FISH screening, Raman microscopy and sorting of microflocs, and MDA and subsequent PCR screening. Eventually, 24 MDA products were sequenced at the Joint Genome Institute (JGI) to obtain the metagenome of the respective sorted microfloc.

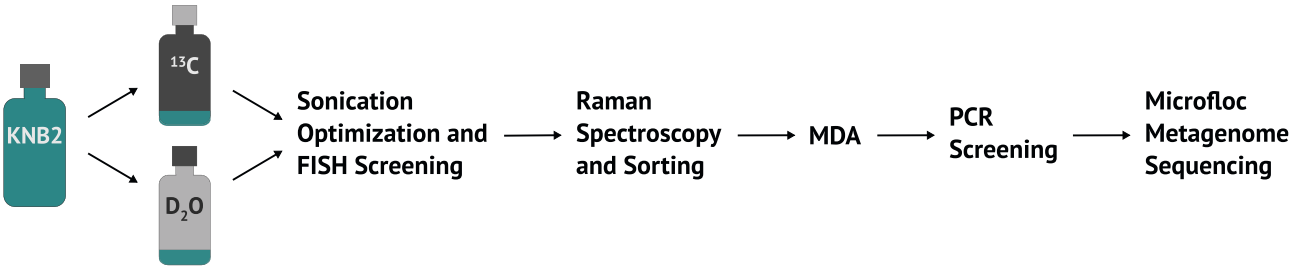

Figure 2.2 Workflow of the pre-study. Activated sludge from KNB2 was incubated for seven days either in presence of ^{13}C-bicarbonate or D_2O. After incubation, sonication conditions for optimal sludge dissection were assessed, FISH screening was conducted, and sonicated microflocs were sorted according to their Raman spectra. MDA was performed on sorted microflocs, and screening PCRs were carried out to assess presence of bacterial and nitrifier DNA. Based on those results, 24 MDA products were selected for metagenome sequencing of the respective sorted microfloc.

2.17.6.1 Incubation Conditions

Incubations were carried out by Tae Kwon Lee. Activated sludge from KNB2 was incubated in biological duplicates either in presence of D_2O or ^{13}C-bicarbonate. Sludge for ^{13}C incubations was pre-incubated at RT for three days without addition of any energy source to remove ^{12}C-organics. For D_2O incubations, sterile filtered SN was concentrated 2x in a vacuum concentrator and subsequently mixed 1:1 with 100% D_2O, yielding 50% D_2O in the incubations. Both D_2O and ^{13}C incubations were supplemented with 5 mM Na bicarbonate as C-source. For ^{13}C incubations, this was added as ^{13}C-, for D_2O incubations as ^{12}C-bicarbonate. Biomass was incubated shaking (90 rpm) at RT, in serum bottles wrapped in aluminum foil and closed with rubber stoppers. Every day, aliquots were withdrawn for formalin fixation, Raman, pH measurement and assessment of NH_4^+, NO_2^- and NO_3^-. Daily, pH was adjusted 8, and NH_4^+ to a final concentration of 1 mM. Biomass was incubated for seven days.

2.17.6.2 Sonication Optimization, FISH Screening, Nitrifier Quantification and Colocalization Testing

Nitrifier presence, abundance and colocalization between AOB and NOB were assessed after seven days of incubation as described in 2.17.3. Optimal sonication conditions for obtaining nitrifier microflocs for Raman sorting were assessed as described in 2.17.5. Sonicated microflocs were screened by FISH.

2.17.6.3 Raman Spectroscopy and Sorting of Microflocs

Raman spectroscopy and sorting of sonicated microflocs was performed by Tae Kwon Lee. In total, 80 microflocs were sorted with a tweezing laser in glass capillaries. 20 flocs were sorted in a targeted manner from biological replicate 1 of both ^{13}C and D_2O incubations. In the targeted sorting approach, flocs were only sorted if they showed both cytochrome (indicative of nitrifiers) and activity signatures (^{13}C-phenylalanine shift to 967 cm^{-1}, or D-C peak around 2200 cm^{-1}) from incorporation of either

^{13}C-bicarbonate or D_2O into biomass. Additionally, for each treatment, 20 flocs were sorted regardless of presence of cytochrome or activity signatures, to assess selective enrichment of nitrifiers by targeted, as compared to random sorting.

2.17.6.4 MDA and PCR Screening

MDA with REPLI-g kit was performed by Tae Kwon Lee. Sorted microflocs were lysed by a combination of bead beating with UV-treated micro-beads and subsequent alkaline lysis. MDA products were stored at -20 °C.

Aliquots of MDA products were diluted 1:100 in ddH_2O for PCR. 5 µl of diluted MDA product each were used as template for PCR runs. Screening PCRs were done with some modifications to the protocol described in 2.11 – final primer concentration was 1 pmol*µl^{-1} each and final polymerase concentration 0.04 U*µl^{-1}. Initial denaturation was done for 4 min, and denaturation for each cycle for 40 s. MDA products were screened with general bacterial primer sets (8F and 1431R, 341F and 785R) to assess microfloc recovery from the capillary, lysis, and MDA success. Additionally, PCR screens for presence of nitrifier DNA were carried out. AOB specific screening PCRs were carried out with primers targeting *amoA* (amoA1F, amoA2R) and the 16s rRNA gene (BamoF, Nso1225R). *Nitrospira* specific screening PCRs contained primers targeting *nxrB* (nxrB169F, nxrB638R) and two primer sets for 16S rRNA genes (8F and 1158R, 662F and 1158R). Each PCR was carried out at least twice, once by Tae Kwon Lee, once by Katharina Kitzinger. Based on the outcome of the PCR screen, 24 MDA products were selected for microfloc metagenome sequencing at the JGI.

2.17.7 D_2O Incubation Test

In order to test minimum incubation time and D_2O concentration necessary for detection of D_2O incorporation into nitrifier biomass, activated sludge from KNB2 (April 2014 sample) was incubated in duplicates in presence of 0, 25, 50 and 75% D_2O in sterile filtered sludge SN. The setup of the experiment was done as described in 2.17.6.1. For incubations, however, instead of tightly closed serum bottles, serum bottles were incubated closed by loose aluminum caps to allow for air exchange. Each day, NH_4^+ and pH were adjusted, and aliquots were removed for optimization of sludge disruption, FISH and Raman.

Various treatments were tested to determine optimal disruption of sludge. For sonication, additionally to the settings described above (2.17.5), 10% power for 60 s, and 30% power for 30 s and 50 s were tried. Additionally, 1:10 diluted sludge was disrupted by bead beating in lysing matrix A tubes with only the large ceramics ball retained for 5 m*s^{-1} and 4 m*s^{-1} for 30 s each. Disruption by shear force by pipetting up and down five times with 23 G, 26 G and 27 G (with 23 G having the largest, 27 G the smallest diameter) needles, and dissection of sludge by douncing in a 1.5 ml tube with a plastic douncer was also tested. Each dissection method was evaluated by FISH.

2.18 Enrichment, Characterization and Isolation of Novel Nitrite Oxidizing Bacteria

2.18.1 Chemicals and Solutions

Please refer to sections describing conventional FISH (2.8), PCR (2.11), agarose gel electrophoresis (2.12), and NO_2^- and NO_3^- measurements (2.13) for chemicals and solutions used for the respective methods.

For chemicals and solutions used for cultivation, please refer to the following tables:

- Ampicillin stock solution: Table 2.17
- Mineral medium: Table 2.18 and Table 2.19
- Trace element solutions: Table 2.20 and Table 2.21
- Additional chemicals and solutions for cultivation of NOB: Table 2.22

For chemicals and solutions used for transformation and isolation of plasmids, as well as restriction digestion, please refer to the following tables:

- Ampicillin stock solution: Table 2.23
- LB medium: Table 2.24
- NaOH solution: Table 2.26
- NaOH/SDS solution: Table 2.27
- P1 buffer: Table 2.28
- K acetate/acetate solution: Table 2.29
- Solutions and enzymes for restriction digestion: Table 2.25

For chemicals and solutions used for Percoll density gradient centrifugation, please refer to the following tables:

- NaCl solutions: Table 2.30 and Table 2.31
- Additional solutions for density gradient centrifugation: Table 2.32

For chemicals and solutions used for microautoradiography (MAR) FISH, please refer to the following tables:

- Developer solution: Table 2.33
- Fixative solution: Table 2.34
- Additional chemicals and solutions used for MAR-FISH: Table 2.35

For chemicals and solutions used for DNA isolation and RNA digestion, please refer to the following tables:

- CTAB/NaCl solution: Table 2.36
- Lysozyme solution: Table 2.37
- PEG 8000 solution: Table 2.38
- Phosphate buffer: Table 2.39
- Proteinase K solution: Table 2.40
- TE buffer: Table 2.41
- TNS buffer: Table 2.42
- Additional chemicals and solutions for DNA isolation and RNA digestion: Table 2.43

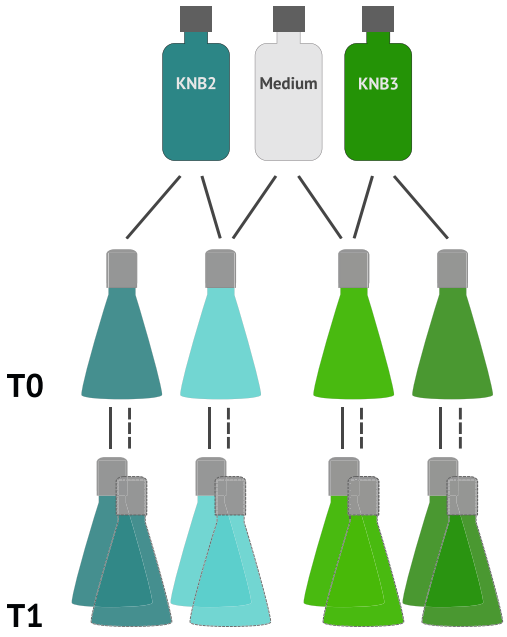

Figure 2.3 Overview of setup and first transfers of nitrite oxidizer enrichments from WWTP Klosterneuburg. Solid lines represent normal transfers, dashed lines represent bottleneck setups, where inoculum was filtered.

2.18.2 Initial Setup

Nitrite oxidizing bacteria were enriched from activated sludge from WWTP Klosterneuburg. Activated sludge taken from the second nitrification basin (KNB2) and the basin for treatment of reject water (KNB3) was diluted either 1:3,000 (KNB2) or 1:750 (KNB3) due to their different density. Incubations were prepared either in 150 ml mineral medium or in 150 ml sterile filtered sludge SN in 300 ml Erlenmeyer flasks loosely closed with aluminum caps (Figure 2.3). All manipulation of enrichment cultures was done next to a flame or in a laminar flow cabinet to ensure sterile conditions. Only sterile filter tips were used for handling of enrichment cultures. Biomass was initially incubated in presence of 1 mM NO_2^- in the dark at RT. Every two to three days, NO_2^- concentration was assessed by nitrite test stripes. Upon depletion of substrate, sterile filtered $NaNO_2$ was added to a final concentration of 1 mM.

2.18.3 Transfer and Cultivation Regime

Before each transfer, 1 ml aliquots of culture were centrifuged (17,949 g, 10 min, 4 °C), SN was discarded, and biomass formalin-fixed for screening FISH. FISH was initially performed with probes for *Nitrospira* lineage I (Ntspa1431), genus and phylum *Nitrospira* (Ntspa662 and Ntspa712 respectively) and general bacterial probes (EUBmix). Cultures showing high numbers of nitrite oxidizers were transferred; cultures overgrown with non-target cells were discontinued. At least two flasks of each cultivation line were kept at all times, in order to have backups, in case the newest transfers failed. Upon inhibition of cultures by accumulated high concentrations of NO_3^-, cultures were washed and resuspended in fresh mineral medium as described in 2.16.2.

3 weeks after initial inoculation, first transfers (Figure 2.3, T1) were made, where inoculum was diluted in Erlenmeyer flasks 1:200 in either mineral medium or in sludge SN diluted 1:2 in mineral medium for those incubations that were initially inoculated in sterile filtered sludge SN. Additionally to normal transfers, bottleneck transfers were done to select for planktonic nitrite oxidizers, where inoculum was filtered through a 1.2 µm filter and diluted 1:25.

From the second transfer round on, or the first washing (upon inhibition by accumulated NO_3^-), no sterile filtered sludge SN was included in any of the transfers – biomass was incubated in mineral medium only. Transfers were done as described for T1, with the exception being the use of 100 ml Schott bottles containing 40 ml mineral medium, instead of using Erlenmeyer flasks. For the bottleneck setup from KNB2 (mineral medium only incubation, T1), inverse-filtration transfers were done to select for small nitrite oxidizer microcolonies. The filtration tower was autoclaved before filtration, and was – after each filtration of biomass – disinfected by submersion in 70% denatured ethanol. Filters were also dipped into 70% ethanol and dried before filtration of cultures. Either 200 µl or 1 ml of inoculum were filtered onto either 3 µm or 10 µm pore size polycarbonate filters and subsequently washed with 15 ml mineral medium to remove planktonic cells. Filters were

incubated for 24 h in 40 ml mineral medium in 100 ml Schott bottles supplemented with 1 mM NO_2^-. Subsequently, filters were removed from incubations.

From that point on, two separate cultivation lines were kept – one cultivated in muffled (500 °C, 4 h) Schott bottles (Schott bottle enrichment, SBE) and one line in muffled Erlenmeyer flasks (Erlenmeyer flask enrichment, EFE). EFE cultures were transferred every two to three months in dilutions of 1:50 and fed with 1 – 3 mM NO_2^-. SBE cultures were transferred every three to eight weeks in dilutions of 1:200 and supplemented with 1 – 5 mM NO_2^-.

2.18.4 PCR, Cloning, and Sequencing of Amplified Genes

For PCR, cloning, and sequencing of amplified genes, 1 ml culture aliquots were removed from the two enrichment lines and biomass was pelleted (17,949 g, 4 °C, 10 min). SN was removed and cell pellets resuspended in 50 µl ddH_2O. Cells were lysed by five cycles of freezing and thawing (-80 °C and 70 °C, 5 min each). 5 µl of crude cell lysate were used as template for PCR. For both enrichment lines, primers specific for *Nitrospira* 16S rRNA genes (8F, Ntspa1158R) were used, for SBE general bacterial 16S rRNA gene targeting primers (8F, 1492R), primers targeting *nxrB* of *Nitrospira* (nxrB169F, nxrB638R), and primers for *nxrB* of *Nitrobacter* and *Nitrococcus* (nxrB706F, nxrB1431R) were used additionally. PCR products were visualized by agarose gel electrophoresis.

PCR products were purified using QIAquick PCR Purification Kit (Qiagen). Cloning and transformation was carried out with pCR4 TOPO-TA Cloning Kit as recommended by the manufacturer, with OneShot Top10 competent *E. coli* (Thermo Fisher Scientific). Ampicillin was used as selective agent. After O/N incubation at 37 °C, clones were picked for inoculation of a master plate and M13 colony PCR to confirm presence of an insert of the desired length. M13 PCR products were visualized by agarose gel electrophoresis. Clones, which showed inserts of the desired length were inoculated in selective LB medium O/N at 37 °C, and shaken (200 rpm). Additionally, M13 PCR products of clones carrying an insert were digested by restriction enzyme MspI to check for different restriction patterns, indicating different sequences of cloned PCR products. In PP-tubes, 5 µl PCR product were mixed with 1 µl 10x tango restriction buffer, 3.5 µl ddH_2O and 0.5 µl MspI. Tubes were incubated for 1 h at 37 °C. Afterwards, the reaction was stopped by addition of loading dye. Restriction patterns were analyzed by agarose gel electrophoresis, and for each pattern, one to two clones were selected for sequencing.

For plasmid extraction by alkaline lysis, O/N grown cells were transferred to PP-tubes and pelleted (20,817 g, 2 min), resuspended in 100 µl P1 buffer, and incubated at RT for 5 min for RNA digestion. Tubes were then supplemented with 200 µl of NaOH/SDS, incubated for 5 min on ice and repeatedly inverted until 150 µl K acetate/acetate solution were added. Tubes were incubated on ice for 5 min for precipitation of proteins. Precipitated protein was pelleted and DNA-containing SN was transferred to a clean PP-tube. Plasmid DNA was precipitated by mixing with one volume of isopropanol for 10 min at RT. Then, plasmid DNA was pelleted and washed with ice-cold 70% molecular grade ethanol. The plasmid DNA pellet was air dried and resuspended in 50 µl ddH_2O. DNA concentration was assessed by NanoDrop ND-1000. Plasmid solutions were diluted to approximately 80 $ng^*\mu l^{-1}$ and Sanger sequenced by Microsynth.

Obtained sequence base calls were manually curated, and low quality bases removed for further analysis. Primer and plasmid sequences were removed. When applicable, forward and reverse reads were aligned, assembled and exported as fasta files using Codon Code Aligner 5.0.1.

Curated sequences were compared by nucleotide megablast (Zhang et al. 2000) against the nucleotide collection database for a fast overview of sequence affiliation. Nitrite oxidizer sequences were then aligned using the SINA alignment tool (Pruesse et al. 2012) and the received alignment was loaded into Arb. Sequences were checked for chimera by manually chopping sequences in half and separately placing them into reference trees. When chopped sequences fell to different branches in the tree, the sequence was assumed to be chimeric. Non-chimeric sequences were placed in reference phylogenetic trees of nitrite oxidizers by Sebastian Lücker and Jasmin Schwarz.

2.18.5 Erlenmeyer Flask Enrichment

2.18.5.1 FISH Screening

FISH screening of EFE was done with the probe combination specified above (2.18.3), and also regularly checked with probes specific for *Nitrotoga* (Ntoga122, Ntoga221).

2.18.5.2 Micromanipulation

For EFE cultures, micromanipulation was employed to separate small, morphologically homogenous, putative nitrite oxidizer colonies from contaminating cells. A Zeiss Observer D1 microscope equipped with Eppendorf Transfer Man NK2 was used. For physical isolation of target cells, sterile Eppendorf Transfer Tips with a diameter of 15 µm and a 20° angle were used. A drop of diluted culture was spotted onto a petri dish, and one colony at a time was impaled on the capillary tip. Each colony was ejected into a sterile 100 µl drop of mineral medium containing 1 mM NO_2^-, transferred to a sterile 1.5 ml PP-tube, and incubated in the dark at RT. NO_2^- consumption was assessed every one to two weeks and upon depletion of substrate, NO_2^- was added to 1 mM. After consumption of 2 mM NO_2^-, the volume was adjusted to 400 µl. The culture was scaled up to 100 ml in Erlenmeyer flasks after oxidation of approximately 10 mM NO_2^- and was further cultivated as described above.

2.18.5.3 Antibiotic Treatment

To assess possible antibiotic resistances of target NOB, aliquots of EFE were supplemented with antibiotics. *Nitrospira defluvii* had previously been enriched and isolated by a combination of treatment with ampicillin and acriflavine (Nowka et al. 2015). Accordingly, cultures were supplemented with sterile filtered Sera baktopur (81 $g*l^{-1}$ butylglycol, 2.1 $g*l^{-1}$ acriflavine, 50 $mg*l^{-1}$ methylene blue) and ampicillin. Cultures were supplemented with final concentrations of 10 and 0.105 $mg*l^{-1}$ of acriflavine and 150 and 50 $mg*l^{-1}$ ampicillin. Additionally, a combination of 0.105 $mg*l^{-1}$ acriflavine and 50 $mg*l^{-1}$ ampicillin was tested.

2.18.6 Schott Bottle Enrichment

2.18.6.1 FISH Screening

Starting from the split of cultivation lines, FISH screening of SBE was done with probes specific for *Nitrotoga* (Ntoga122, Ntoga221), *Betaproteobacteria* (Bet42a) and EUBmix probes. Quantification of cells targeted by Ntoga122 was done as described in 2.9 and 2.10. In addition, SBE was regularly screened for presence of *Nitrospira* and *Nitrobacter* (Nit3).

2.18.6.2 Microautoradiography FISH

Microautoradiography FISH (MAR-FISH) was performed as described by Andreasen & Nielsen (1997) and Lee et al. (1999) to assess autotrophic lifestyle of NOB in SBE under nitrifying conditions. Starved SBE culture (no NO_2^- addition for three days, no NO_2^- measurable in culture SN) was used. All treatments were incubated in presence of 42.25 µM Na bicarbonate. For ^{14}C incubations, this amounted to a total activity of 10 µCi per vial. Where applicable, NO_2^- was added to a final concentration of 1 mM.

Four treatments were included in the experiment – first, living SBE incubated with ^{14}C and NO_2^-, second, living SBE incubated with ^{12}C and NO_2^- (cold control to assess NO_2^- oxidation), third, living SBE incubated with ^{14}C without NO_2^- (no substrate control to account for internal storage compounds), and lastly formalin-fixed SBE in sterile filtered culture SN incubated with ^{14}C and NO_2^- (dead control to assess unspecific binding of ^{14}C to biomass or matrix). 4 ml of culture per treatment were incubated in the dark at RT in serum bottles tightly closed with rubber stoppers. At the beginning of the experiment, 0.5 ml per treatment were removed, pelleted (20.817 g, 4 °C, 10 min), and SN transferred to a fresh tube. SN was then stored at -20 °C, to allow for assessment of the exact amount of radiolabel employed. After 6 h and 20 h incubation respectively, 1 ml per treatment was removed and formalin-fixed. SN was again stored at -20 °C. Throughout the experiment, NO_2^- concentration was followed in the cold control with test stripes to replenish NO_2^- for radiotracer incubations as necessary.

FISH was performed with formalin-fixed samples both from the 6 h and 20 h time point on agarose-coated cover slips. Wells were drawn with a fatty acid pen to avoid spreading of hybridization buffer. After FISH, samples were DAPI stained. Thus treated cover slips were coated with pre-warmed (43 °C), carefully mixed NTB autoradiography emulsion in the dark under infrared-light by slowly dipping cover slips into emulsion. Cover slips were then horizontally placed in a box with silica gel-pads, wrapped in aluminum foil and incubated at 4 °C for exposure. One and two weeks exposure time was tested. MAR signals were developed in the dark room by subsequently exposing cover slips to developer for 7 min at RT, stopping solution (ice-cold ddH_2O) for 1 min, fixative (ice-cold Na thiosulfate) for 2 min, and ice-cold ddH_2O for 1 min. Cover slips were then let dry in the dark at RT and subsequently stored at 4 °C until microscopic analysis on the CLSM. Prior to analysis, a droplet of CitiFluor was placed on each well. Images were recorded for all treatments, separately for fluorescence and bright field mode, as fluorescence signals and silver grains had different focal planes. Images of different focal planes were later merged.

2.18.6.3 Raman Spectroscopy

Raman spectra of formalin-fixed SBE sample were acquired to assess, if SBE cells show pronounced cytochrome signatures, which are indicative of nitrifiers. Raman spectroscopy was done on a confocal Raman microscope (LabRam HR Evolution, Horiba Jobin-Yvon) equipped with a 532 nm laser. LabSpec6 Software was used for spectra acquisition. Diluted sample was applied onto an aluminum-spotted slide, let dry and washed in ddH_2O to remove salt traces. Cells were measured using the 532 nm laser, with 300 $gr*mm^{-1}$ diffraction grating. Spectra were recorded in the range of 400 – 3,200 cm^{-1}.

2.18.6.4 Cryostock Preparation

Cryostocks of SBE were prepared analogous to Vekeman et al. (2013) and as implemented by Mario Pogoda. Biomass (50 – 100 ml culture) was harvested as described in 2.16.2, washed with fresh mineral medium, and concentrated in 3 – 5 ml medium. 20% dimethyl sulfoxide (DMSO) was prepared in mineral medium and sterile filtered. In screw-cap tubes, DMSO solution (0.6 ml) was distributed, the same volume of cell suspension added, carefully mixed by inverting and immediately flash-frozen in liquid N_2. Then, tubes were transferred to -80 °C freezers for long-term storage. For reactivation of cells, cryostocks were thawed in a water bath set to 25 °C, washed with fresh medium to remove traces of DMSO, resuspended in mineral medium supplemented with 1 mM NO_2^-, and transferred to Schott bottles for further cultivation (2.18.3).

2.18.6.5 Nitrite Tolerance Assessment

NO_2^- tolerance of SBE was assessed by incubation in presence of NO_2^- concentrations ranging from 1 - 30 mM. Mineral medium (40 ml) was distributed to 100 ml Schott bottles and supplemented with 1, 5, 10, 15, 20 or 30 mM NO_2^- in duplicates. 25 ml of dense SBE culture were washed as described in 2.16.2, resuspended in 3 ml mineral medium and kept on ice until inoculation. Per bottle, 200 µl of cell suspension were added. Remaining inoculum was formalin- and ethanol-fixed for FISH. At each sampling point, 200 µl of culture were removed, centrifuged (20,817 g, 10 min, 4 °C) and SN stored at -20 °C until analysis of NO_2^- and NO_3^- by Griess-based methods as described in 2.13.2. Biomass was incubated in the dark at RT for 46 days. After complete oxidation of NO_2^-, or at the end of the experiment, 2 ml per treatment were formalin-fixed for FISH screening.

NO_2^- oxidation rates for each treatment were inferred from the two consecutive sampling time points where NO_2^- depletion slopes were steepest. As absolute cell counts of *Nitrotoga* for each sampling point could not easily be obtained due to the presence of contaminating cells, rates were normalized to the amount of NO_2^- already consumed at the earlier of the two time points used for rate calculations. This was done to account for growth of *Nitrotoga* during incubation before maximum NO_2^- oxidation occurred.

2.18.6.6 Temperature Optimum Assessment

Temperature optimum of SBE was assessed by incubation at temperatures ranging from 4 – 46 °C. 24 h before the start of the experiment, Schott bottles were filled with 40 ml mineral medium each, and supplemented with 1 mM NO_2^-. Three bottles each were pre-incubated at the respective temperatures (4, 14, 20, 24, 28, 37, or 46 °C). The inoculation and sampling procedure was done as described in 2.18.6.5. Bottles were incubated in the dark at the respective temperatures and regularly sampled for 102 h, but kept at the respective temperatures for over three months to check for possible substrate depletion over longer time frames. NO_2^- and NO_3^- concentration in the SN was assessed both by Griess-based methods and by IC, as described in 2.13.2 and 2.13.3 respectively. Again, after complete oxidation of NO_2^-, 2 ml per treatment were formalin-fixed for FISH screening.

2.18.6.7 DNA Isolation for Genome Sequencing

To obtain genomic DNA from SBE for sequencing of the enrichment's metagenome, two different DNA isolation protocols were used to extract DNA from two different time points (June and October 2014). For all handling, sterile filter tips were used. During DNA extraction, reaction setups were only mixed by inverting, not by vortexing, to avoid DNA fragmentation. Tube contents were mixed after each addition of chemicals or solutions. All steps involving handling of phenol or chloroform were carried out in a fume hood. Approximately 50 ml of SBE culture were pelleted (8,228 g, 20 min, 25 °C) per DNA extraction per tube. Each protocol was carried out in three to four replicates per time point.

Both for June and October samples, DNA was extracted by enzymatic lysis (lysozyme and proteinase K treatment) according to "Bacterial genomic DNA isolation using CTAB" (JGI, version 3). The only modification was to carry out the phenol/chloroform/isoamylalcohol extraction before the chloroform/isoamylalcohol treatment. In short, cell pellets were resuspended in 740 µl TE buffer and transferred to 2 ml PP-tubes. 20 µl lysozyme solution were added, and setups were incubated for 5 min at RT. Then, 40 µl 10 % SDS were added. 8 µl proteinase K solution were added, and setups incubated for 1 h at 37 °C. After incubation, 100 µl 5M NaCl solution were added. Then, 100 µl of pre-warmed (65 °C) CTAB/NaCl solution were added, and tubes were incubated for 10 min at 65 °C. 0.5 ml phenol/chloroform/isoamyl alcohol (pH 8) were added, and tubes were centrifuged (20,817 g, 10 min, RT). The resulting upper aqueous phase was carefully transferred into a fresh 2 ml PP-tube, and mixed with 0.5 ml Chloroform/Isoamylalcohol (pH 8). Then, tubes were again centrifuged (20,817 g, 10 min,

RT) to remove traces of phenol from reactions. The resulting upper aqueous phase was again carefully transferred into a fresh 2 ml PP-tube. Subsequently, 0.6 volumes of Isopropanol were added and tubes were incubated for 30 min at RT to precipitate DNA, before pelleting DNA by centrifugation (20,817 g, 15 min, RT). SN was carefully discarded and the DNA pellet was washed with 0.8 ml of 70% ethanol. Tubes were again centrifuged (20,817 g, 10 min, RT) and ethanol carefully discarded. The DNA pellet was dried at RT until it became transparent, then, DNA was resuspended in 60 µl elution buffer from MoBio PowerSoil kit.

The second protocol employed for DNA extraction from October samples was done analogous to Roey Angel's protocol for "DNA and RNA extraction from soil" (Version 3). This method relies on mechanical lysis by bead beating in presence of phenol and precipitation of DNA by PEG8000. In short, biomass was resuspended in 375 µl of phosphate buffer and 125 µl of TNS buffer, and transferred to lysing matrix E tubes. Then, cell suspension was mixed with 500 µl of TE buffered phenol (pH 8). Bead beating was done for 30 s at 4.5 $m*s^{-1}$ while cooling with dry ice. Setups were centrifuged (20,817 g, 3 min, RT) and supernatant carefully transferred to a fresh 2 ml PP-tube. Subsequently, 800 µl phenol/chloroform/isoamyl alcohol (pH 8) were added to the supernatant, and tubes were again centrifuged (20,817 g, 3 min, RT). The resulting upper aqueous phase was carefully transferred into a fresh 2 ml PP-tube and mixed with one volume of chloroform/isoamyl alcohol (pH 8), before setups were centrifuged (20,817 g, 3 min, RT). Again, the resulting upper aqueous phase was carefully transferred into a fresh 2 ml PP-tube and subsequently mixed with 1 ml PEG8000 solution. Then, tubes were centrifuged (20,817 g, 60 min, 4 °C). SN was carefully discarded and the pellet washed in 1 ml of ice-cold 70 % ethanol. Tubes were again centrifuged (20,817 g, 20 min, 4 °C), and ethanol carefully removed. The DNA pellet was dried at RT until it became transparent, then, DNA was resuspended in 100 µl elution buffer from MoBio PowerSoil kit.

RNA digestion was done according to "RNase 1 Treatment" (JGI, version 1.2) for all extraction methods before sending DNA for sequencing, without DNA precipitation at the end. In short, 170 µl of DNA in elution buffer were gently mixed with 20 µl 10x RNase I buffer and 10 µl RNase I. Setups were incubated for 1 h at 37 °C, then RNase I was inactivated by heating to 70 °C for 15 min.

DNA concentration and quality was assessed by NanoDrop ND-1000, gel electrophoresis (2%) and Quant-iT PicoGreen on a NanoDrop ND-3300, following a downscaled protocol by Roey Angel. In short, 1x TE buffer was prepared with nuclease- and nucleic-acid-free ddH_2O, and PicoGreen was diluted 200x in 1x TE buffer. DNA standard was diluted to 10, 5 and 2.5 $ng*\mu l^{-1}$. Samples were diluted from values obtained from NanoDrop ND-1000 to fall within the standard range. Both samples and standards were diluted 1:10 in PicoGreen solution, mixed by vortexing, incubated for 5 min at RT, and measured in duplicates on NanoDrop ND-3300. DNA was shipped on dry ice to be sequenced in cooperation with Søren Karst, Mads Albertsen, and Per Halkjer Nielsen at University of Aalborg, Denmark.

2.18.6.8 Dilution to Extinction Setups

In order to obtain higher enrichments of target nitrite oxidizers, serial dilutions were prepared. Initially, various vial types were tested for activity and maintained high enrichment of target cells. Muffled cultivation glass tubes closed with aluminum caps, muffled glass vials with plastic screw-lids, 50 ml PP-Greiner-tubes and 2 ml PP-tubes were tested, containing either 5 ml (glass vials and Greiner tubes) or 0.5 ml (2 ml tubes) mineral medium supplemented with 1 mM NO_2^-.

Serial dilutions were done in PP-Greiner-tubes, in 1:10 dilution steps up to 10^{10} fold dilution. NO_2^- oxidation was checked weekly, and upon depletion, NO_2^- was added to a final concentration of 1 mM. The highest dilution that was still active was assessed by light microscopy, and, if only few contaminant cells were observed, served as an inoculum for both a batch culture and another serial dilution.

2.18.6.9 Percoll Density Gradient Centrifugation

Percoll density gradient centrifugation was carried out analogously to Ehrich et al. (1995) and Off (2012). NaCl solutions and Percoll were autoclaved separately prior to usage to avoid gelling of Percoll in presence of NaCl. 250 ml of SBE culture were pelleted, concentrated in 1 ml 0.9% NaCl and carefully mixed with 34.5 ml Percoll and 15 ml 1.5 M NaCl. 7 ml of cell suspension each were transferred to 13.2 ml plastic centrifugation tubes and centrifuged in a swing out rotor (SW 41 Ti) on a Beckman Coulter Optima L100 XP Ultracentrifuge (12,000 g, 4 °C, 2 h). After centrifugation, fractions of the resulting brownish cell band were removed in duplicates by three different methods. Cell fraction was removed either by puncturing the tube with a heated, sterile needle from the side, or by removing the target fraction either by needle or 200 µl pipette tip from the top opening by plunging through layers above the target band. All fractions were assessed by light microscopy for presence of target cells, and for each method, one fraction was serially diluted (1:10, up to 10^{11} fold dilution) as described above. Additionally, the remaining fractions were used as inoculum for a liquid batch culture in Schott bottles.

2.18.6.10 Filtration

To reduce the number of contaminant cells in SBE, various filtration strategies were employed. 5 ml of grown enrichment culture were filtered through a 0.45 µm filter and further incubated with 1 mM NO_2^- in 50 ml PP Greiner tubes. Inocula for transfers were filtered through 1.2 µm filters, subsequently pelleted by centrifugation (8,228 g, 20 min, 25 °C), and washed in mineral medium to remove potential organic residues from filtration. Cultures that became inhibited by accumulated NO_3^- were also filtered through 1.2 µm filters before washing with fresh medium and further incubation.

2.18.6.11 Antibiotic Treatment

As two *Nitrotoga* draft genomes encode genes for acriflavine resistance (Sebastian Lücker, unpublished data), cultures were supplemented with sterile filtered baktopur. Final concentrations of 21, 4.2, 2.1, 0.42, 0.21 and 0 $mg*l^{-1}$ acriflavine were tested in Greiner tubes.

2.18.6.12 Cultivation on Solid Media

Two strategies were employed to test growth on solid media (Figure 2.4). Either normal or phosphate-free (no KH_2PO_4) mineral medium was prepared with solidifying agent. 500x phosphate stock was sterile filtered and added after autoclaving to phosphate-free medium. Optimal pH of phosphate stock to give the final desired pH of 7.8 was assessed with autoclaved, phosphate-free liquid medium, which was supplemented with KH_2PO_4 stocks of pH 7.6, 7.8 and 8.0. Additionally, activity of SBE culture in those liquid media was tested.

Normal mineral medium for plating was supplemented with 1% noble agar or 1% sieve agarose before autoclaving. Phosphate-free mineral medium was supplemented with 1% noble agar, 1% sieve agarose or 1% plaque agarose before autoclaving. Before solidifying of autoclaved media, sterile filtered NO_2^- (final concentration 1 mM), trace element solution, and – for medium without KH_2PO_4 – 500x phosphate stock of pH 8 was added, and plates were poured. 5 – 10 µl of 1:100 to 1:1,000 diluted cultures were plated with ColiRollers beads. Plates were wrapped with Parafilm and incubated in the dark at RT. Grown colonies were picked with sterile pipette tips, resuspended in 1x PBS and cell morphology was investigated by light microscopy. Cells with promising morphologies were streaked out onto fresh plates of the same type. Colony PCR with general bacterial primers (8F, 1492R) was performed on grown colonies and directly sequenced. Sequences were analyzed as described in 2.18.4.

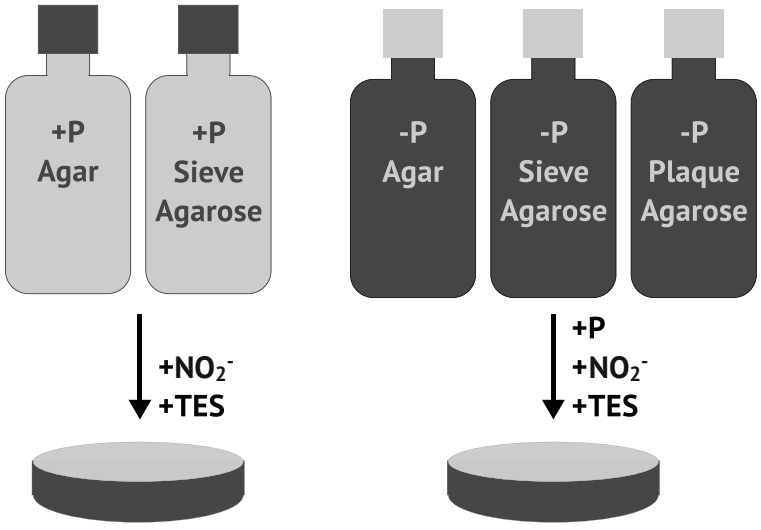

Figure 2.4 Overview of solid media trials. To the left: Normal media containing phosphate (+P). To the right: Media without phosphate addition (-P) before autoclaving.

To test whether colonies grown on "phosphate-free" solid medium supplemented with plaque agarose were growing due to phosphate addition after autoclaving or because of the agarose type used, normal solid mineral medium with phosphate added before autoclaving was also prepared with plaque agarose, and colonies grown on "phosphate-free" plaque agarose medium were streaked out on normal mineral medium plaque agarose plates.

3 RESULTS

3.1 Comparison of Nitrite and Nitrate Measurement Methods

3.1.1 Comparison of Conventional and Adapted Griess-Based Nitrate Measurement

An adapted protocol from Miranda et al. (2001) and García-Robledo et al. (2014) was developed during this study to overcome underestimation of NO_3^- in presence of high NO_2^- concentrations.

NO_2^- and NO_3^- of the same concentration were found to show different absorbance after incubation in presence of VCl_3, the reducing agent that catalyzes conversion of NO_3^- to NO_2^- in the sample. NO_3^- and NO_2^- standards incubated at identical conditions (same volumes of sample, VCl_3 and sulfanilamide/NEDD mixture, 30 min incubation at 37 °C) had different slopes (Figure 3.1), with steeper slopes for NO_3^- standards.

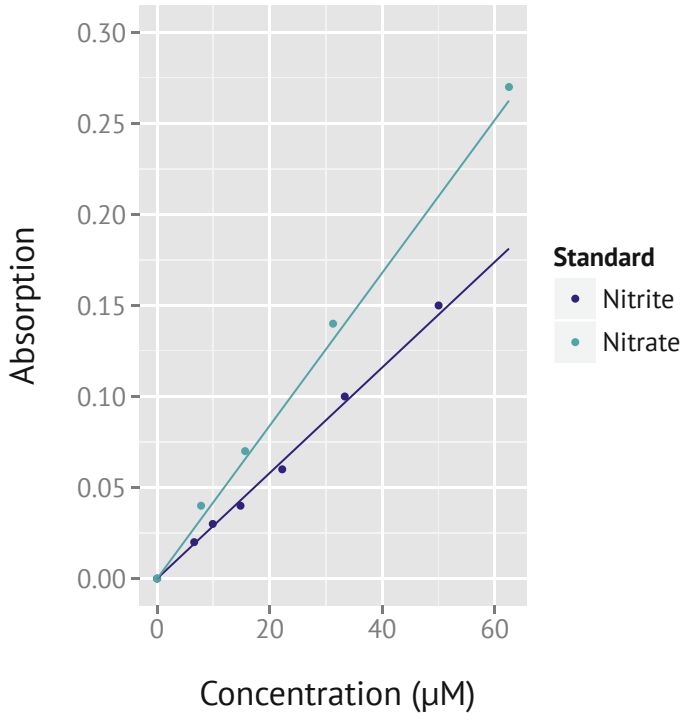

Figure 3.1 Standard curves for NO_2^- (dark blue) and NO_3^- (light blue) incubated under identical conditions in presence of VCl_3. Note the different slopes of the two standard curves.

In conventional NO_3^- assessment, concentrations of NO_x (NO_2^- obtained from the reduction of NO_3^- by VCl_3 and original NO_2^- in the sample) are inferred from the NO_3^- standard only. Subsequently, NO_2^- concentrations obtained from separate Griess-reactions are subtracted. Application of that protocol to measurements from temperature experiments with SBE produced negative NO_3^- values at high NO_2^- concentrations (Figure 3.2, middle graph panel).

The developed adapted protocol for NO_3^- determination minimized negative values for NO_3^- at high NO_2^- concentrations (Figure 3.2, right graph panel). This adapted protocol includes NO_2^- standards for each NO_3^- measurement. From separately assessed NO_2^- concentration in the sample by Griess

reaction, a theoretical absorbance stemming from NO_2^- in presence of VCl_3 was calculated for each sample by using the NO_2^- standard incubated with VCl_3. That theoretical absorbance stemming from sample-NO_2^- was then subtracted from measured NO_x absorbance. Residual absorbance was assumed to only stem from NO_3^- in the sample. NO_3^- concentrations were subsequently inferred from the NO_3^- standard.

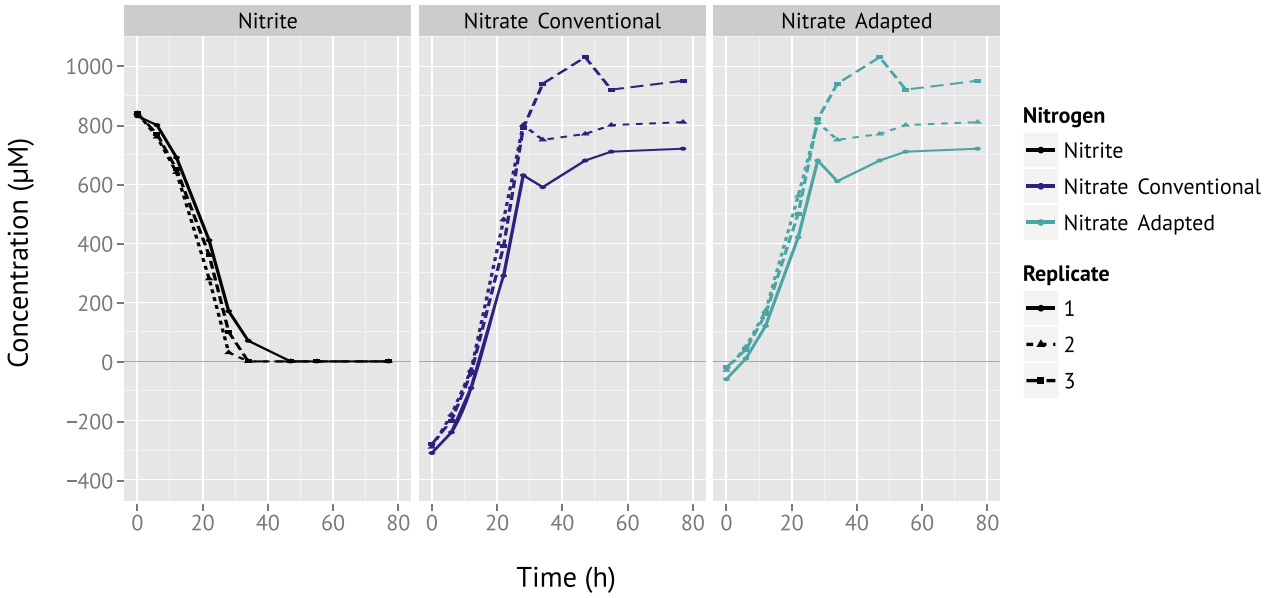

Figure 3.2 Measured NO_x content in 28°C incubations from temperature optimum experiment with SBE, as obtained by Griess-based measurements. On the x-axis, incubation time is depicted, on the y-axis, measured concentration of NO_x. Measured NO_2^- concentrations are depicted in the left graph panel in black for comparison. Dark blue values (middle graph panel) were obtained by using the conventional protocol for NO_3^- determination. Light blue values (right graph panel) were obtained from the developed adapted protocol for NO_3^- determination.

3.1.2 Comparison of Griess-Based Methods and Ion Chromatography

Most NO_x measurements during this thesis were carried out by Griess-based measurements, however, samples from the SBE temperature optimum experiment were additionally assessed by IC. Exemplary graphs of sample-NO_2^- and -NO_3^- measured both by Griess-based methods and IC are depicted in Figure 3.3. While obtained NO_2^- values are similar between methods, NO_3^- values differ, especially after almost complete NO_2^- depletion (28 h post incubation). In IC measurements, the detection limit of NO_2^- was 0.1 mM.

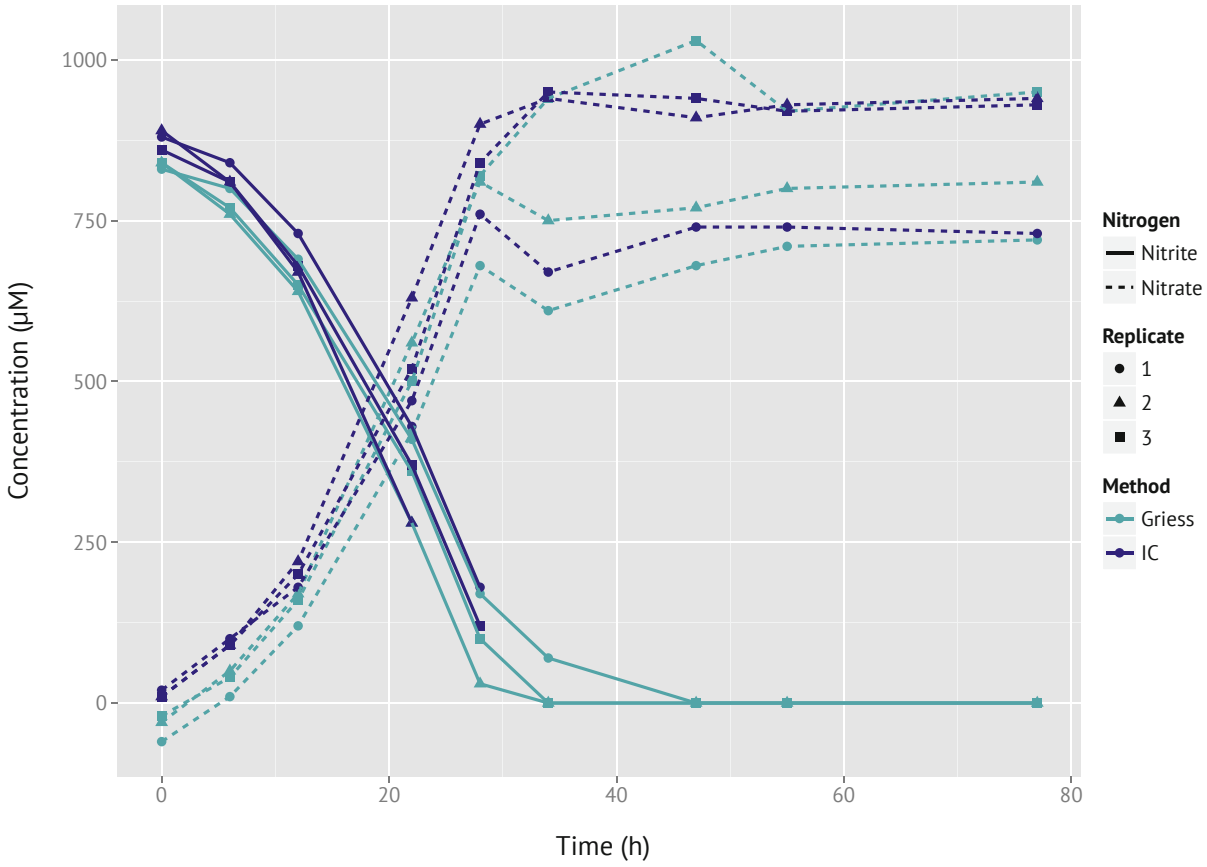

Figure 3.3 Comparison of NO_2^- and NO_3^- measurements by Griess-based methods (light blue) and IC (dark blue) for 28°C incubations during temperature optimum experiment. On the x-axis, incubation time is depicted, on the y-axis, measured concentration of NO_x. NO_2^- detection limit in IC measurements was 0.1 mM.

3.2 Investigation of the Denitrifying Potential of *N. moscoviensis*

In order to test alternative energy generation pathways in *N. moscoviensis* under anoxic conditions, biomass was incubated with NO_3^- and tricarboxylic acid (TCA) cycle precursors and intermediates (acetate, citrate, fumarate, pyruvate, and succinate; experiment 1), as well as branched AA and ethanol (experiment 2). In both experiments, *N. moscoviensis* was additionally incubated with only NO_3^- (control for internal storage compounds), only NO_2^- (control for anoxic conditions), and, as control for biomass activity, with formate and NO_3^-, which had been previously shown to be utilized under anoxic conditions (Hanna Koch, unpublished data).

N. moscoviensis readily oxidized formate while reducing NO_3^- to NO_2^- without observable lag-phase in both experiments (Figure 3.4). While formate oxidation and NO_3^- reduction initially occurred nearly stoichiometrically, substrate conversion was decoupled after five and seven days in experiment 1 and 2 respectively. While formate was completely consumed in both experiments, not all NO_3^- was reduced to NO_2^-, with approximately 100 µM residual NO_3^- in experiment 1, and 200 – 300 µM NO_3^- in experiment 2.

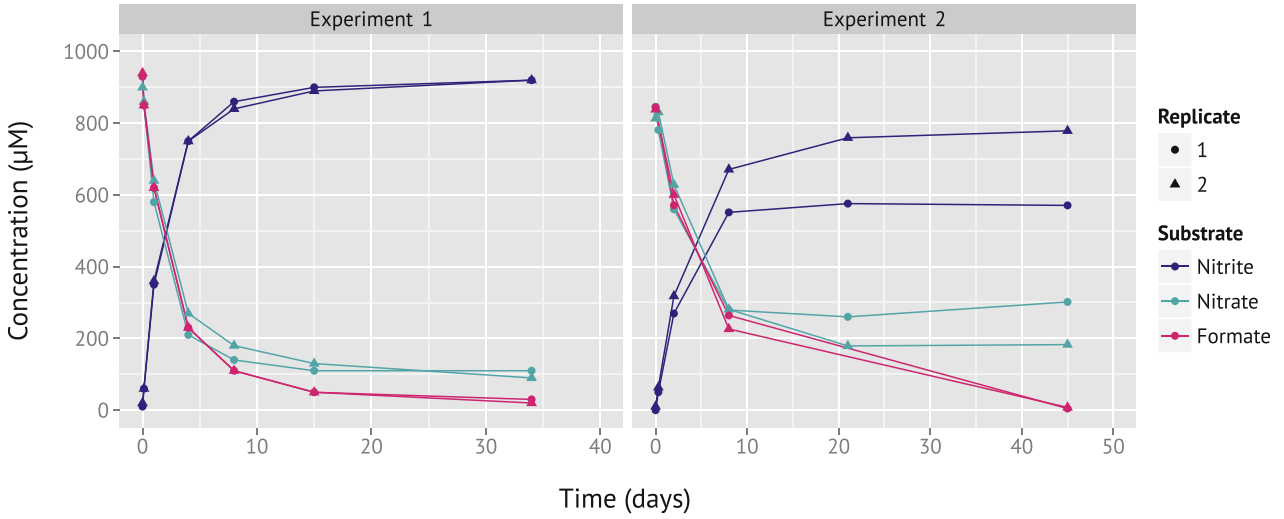

Figure 3.4 Anaerobic formate oxidation coupled to NO_3^- reduction in *N. moscoviensis* over 34 and 45 days (experiment 1 and experiment 2 respectively). Biomass was incubated in biological duplicates (replicate 1 and 2). Depicted data points represent means of Griess-based NO_x, and CE measurements of formate in technical duplicates.

While formate and NO_3^- were readily used, no substrate turnover was observed for any other substrate combination throughout the first experiment. NO_x measurements from the first 34 days of the first experiment are depicted in Figure 3.5. Organic anion concentrations as measured by CE did not change during the experiment either (data omitted from graph for better clarity).

In experiment 2, no NO_3^{--} reduction was observed apart from incubations containing formate and NO_3^-. However, NO_2^- concentrations in NO_2^--only controls slightly decreased over time (Figure 3.6).

No contaminants were observed by FISH in any sample of experiment 1 and 2, neither in inoculum, nor after the last time point taken. All observed cells were stained both by DAPI, EUBmix and Ntspa1151 probes.

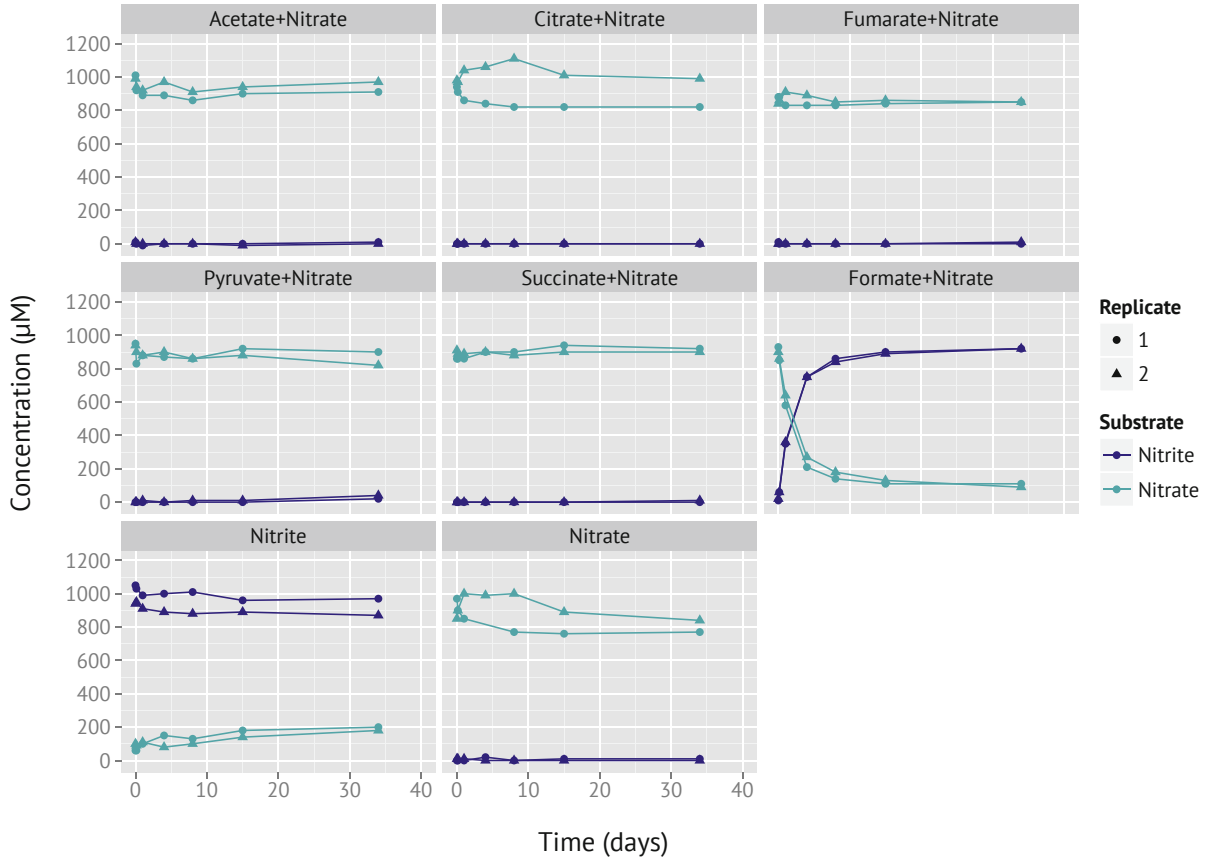

Figure 3.5 Experiment 1. Incubations of *N. moscoviensis* under anoxic conditions with TCA cycle precursors and intermediates and NO_3^- over 34 days in biological duplicates (replicate 1 and 2). On the x-axis, incubation time is depicted, on the y-axis, measured concentration of NO_x. Graph panels depict the different substrate combinations. Depicted data points represent means of NO_x concentrations from Griess-based measurements in technical duplicates. CE measurements of organic anions are omitted for clarity.

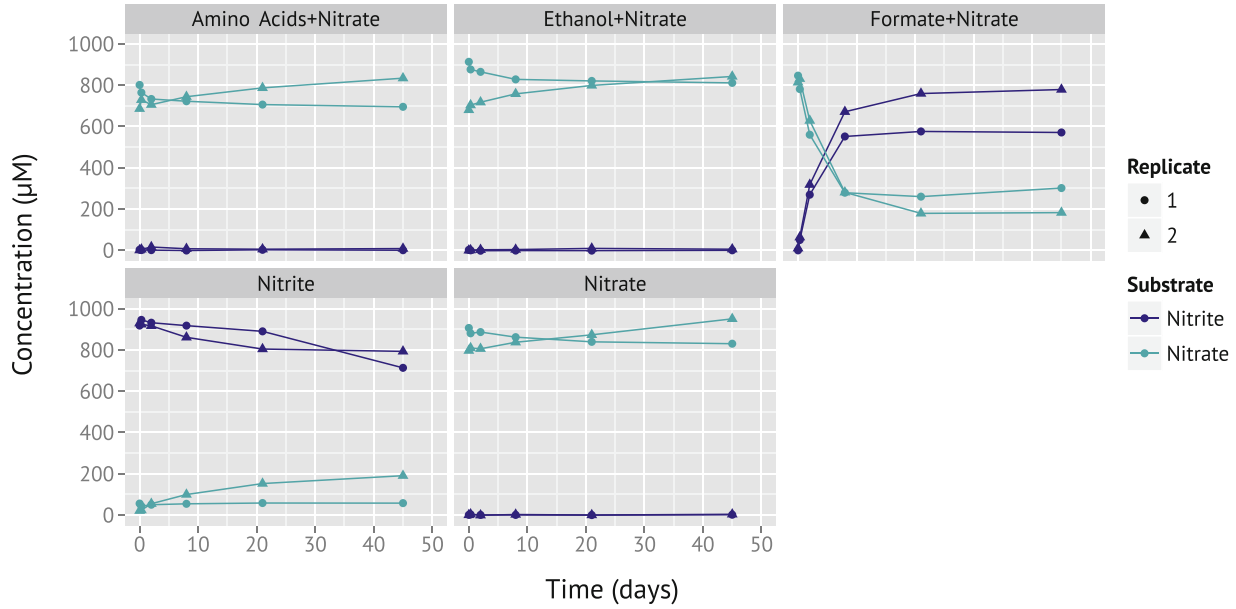

Figure 3.6 Experiment 2. Incubations of *N. moscoviensis* under anoxic conditions with branched AA and ethanol and NO_3^- over 34 days in biological duplicates (replicate 1 and 2). On the x-axis, incubation time is depicted, on the y-axis, measured concentration of NO_x. Graph panels depict the different substrate combinations. Depicted data points represent means of NO_x concentrations from Griess-based measurements in technical duplicates.

3.3 Microdiversity of Nitrifying Bacteria

3.3.1 FISH Screening

All samples obtained from WWTP Klosterneuburg (October 2013, January and April 2014) were screened by FISH for presence of nitrifier populations and *Micavibrio*-like predators of *Nitrospira*.

The same AOB populations, targeted by probes NEU, Cl6a192, and Ncom1025, were observed in all samples. However, these probe-defined AOB populations were not mutually exclusive, but a partial overlap between colonies stained by probes Cl6a192 and Ncom1025 was observed, where Ncom1025 hybridized exclusively to colonies also stained by Cl6a192. In addition to those colonies targeted by both Ncom1025 and Cl6a192, other colonies were stained only by the Cl6a192 probe. Therefore, three probe-defined AOB populations were detected, one targeted by probe NEU, one only by Cl6a192 and one both by Cl6a192 and Ncom1025 (Figure 3.7). Even though the AOB populations present were the same for all samples, dominant morphologies changed from near spherical AOB colonies in October 2013 to sparser, larger colonies in January and April 2014 (compare Figure 3.7 and Figure 3.9).

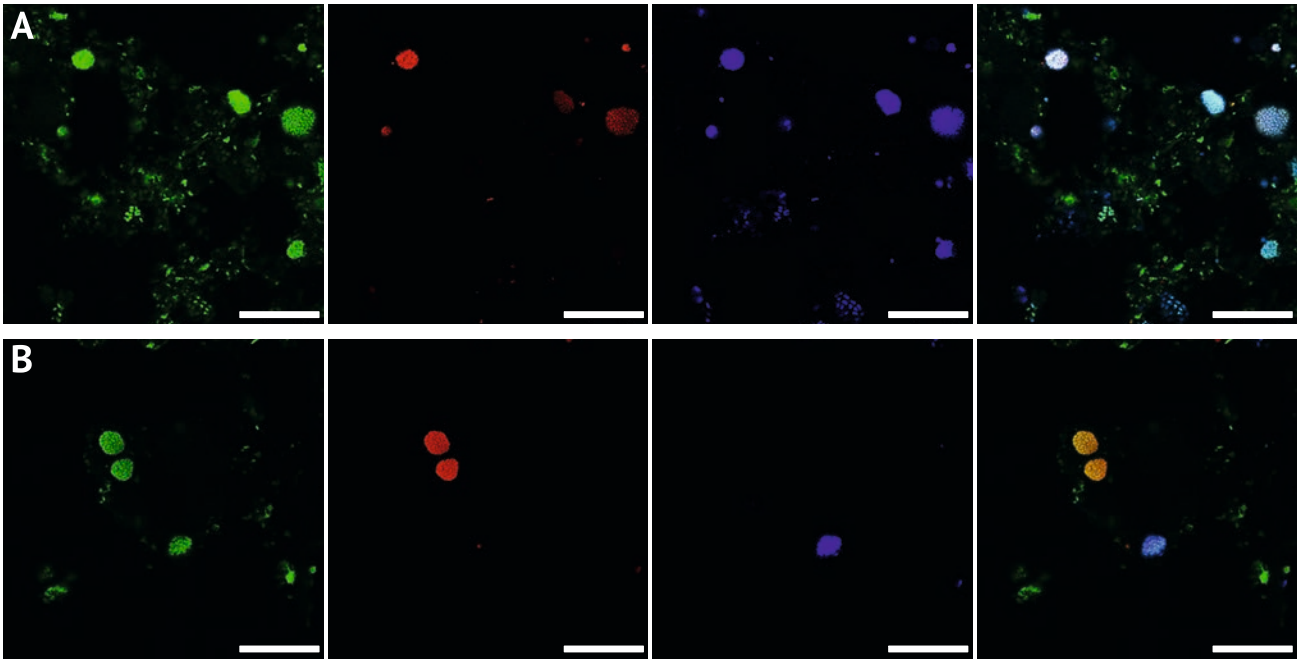

Figure 3.7 Representative images of AOB populations present in WWTP Klosterneuburg, October 2013 sample, KNB2. Single fluorescence channels are depicted in the first three images, overlay thereof in images to the right. A: Signal overlap of probe Ncom1025 and Cl6a192. Green depicts *Betaproteobacteria* (Bet42a), red shows cells targeted by Ncom1025, and blue shows cells targeted by Cl6a192. Note that not all cells with Cl6a192 signals also show Ncom1025 signals. B: AOB populations targeted by NEU and Cl6a192. Green depicts *Betaproteobacteria* (Bet42a), red depicts cells targeted by NEU, and blue depicts cells targeted by Cl6a192. Scale bars represent 25 µm.

In all samples, the only detectable *Nitrospira* population belonged to *Nitrospira* lineage I (targeted by probe Ntspa1431). No other nitrite oxidizers were observed by FISH. *Micavibrio*-like predators of *Nitrospira* (targeted by HSALmix) were present in all samples, and were always observed in close proximity to, or within, *Nitrospira* colonies (Figure 3.8).

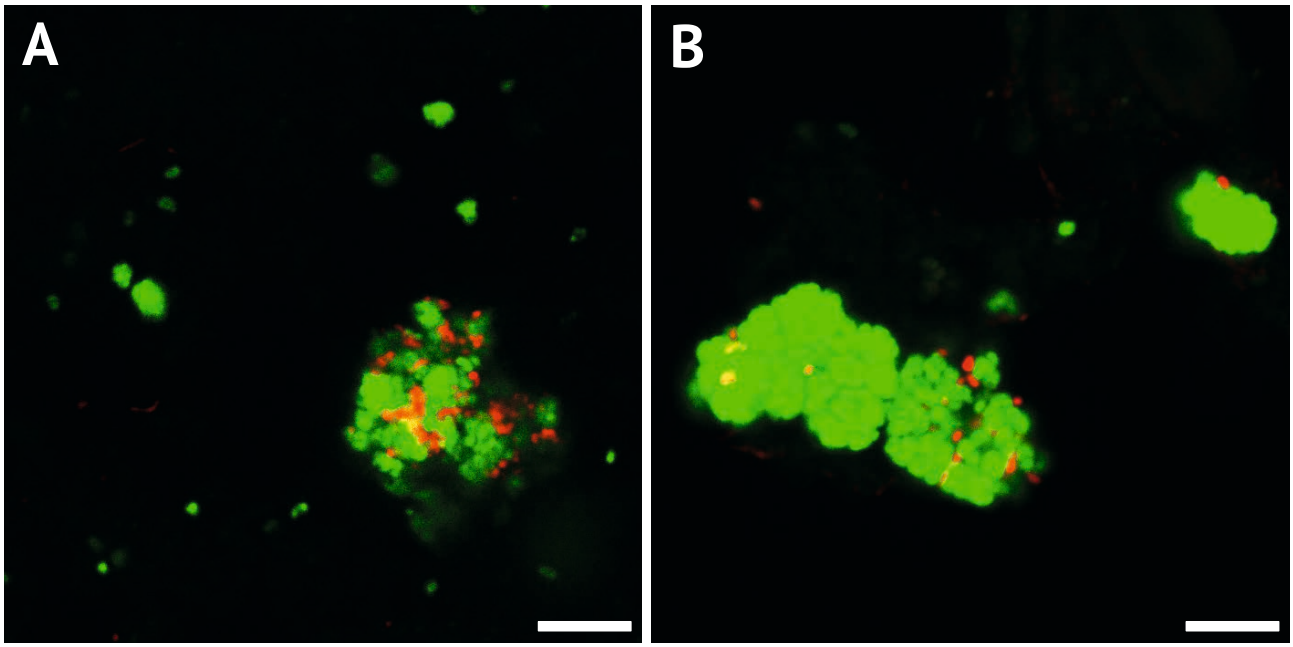

Figure 3.8 *Micavibrio* (red, HSALmix) and *Nitrospira* (green, NTSPAmix) in WWTP Klosterneuburg KNB2. A: October2013 sample. B: January 2014 sample.

No *Thaumarchaea* were observed in activated sludge samples from January 2014 by CARD-FISH, neither before nor after incubation with heavy isotopes.

3.3.2 Quantification and Colocalization of AOB and *Nitrospira*

Nitrifier biomass was quantified in samples from January 2014 for all three sampling basins. KNB2 contained the highest fraction of nitrifiers, were approximately 4 % and 5 % of the total bacterial biomass consisted of AOB and *Nitrospira* respectively (Table 3.1, exemplary image Figure 3.9). KNB1 and KNB3 contained similar amounts of AOB (2.7 % in KNB1 and 3.2 % in KNB3 respectively) and *Nitrospira* (4.0 % in KNB1 and 4.3 % in KNB3 respectively). In no basin, AOB and *Nitrospira* were found to co-localize in the acquired 2-dimensional images.

Table 3.1 Relative AOB and *Nitrospira* biovolume fractions of overall bacterial biomass (EUBmix) in WWTP Klosterneuburg samples from first activated sludge basin 1 (KNB1), nitrification/denitrification basin (KNB2), and basin for treatment of reject water (KNB3).

	KNB1	KNB2	KNB3
AOB	2.7 %	4.3 %	3.2 %
Nitrospira	4.0 %	5.1 %	4.3 %

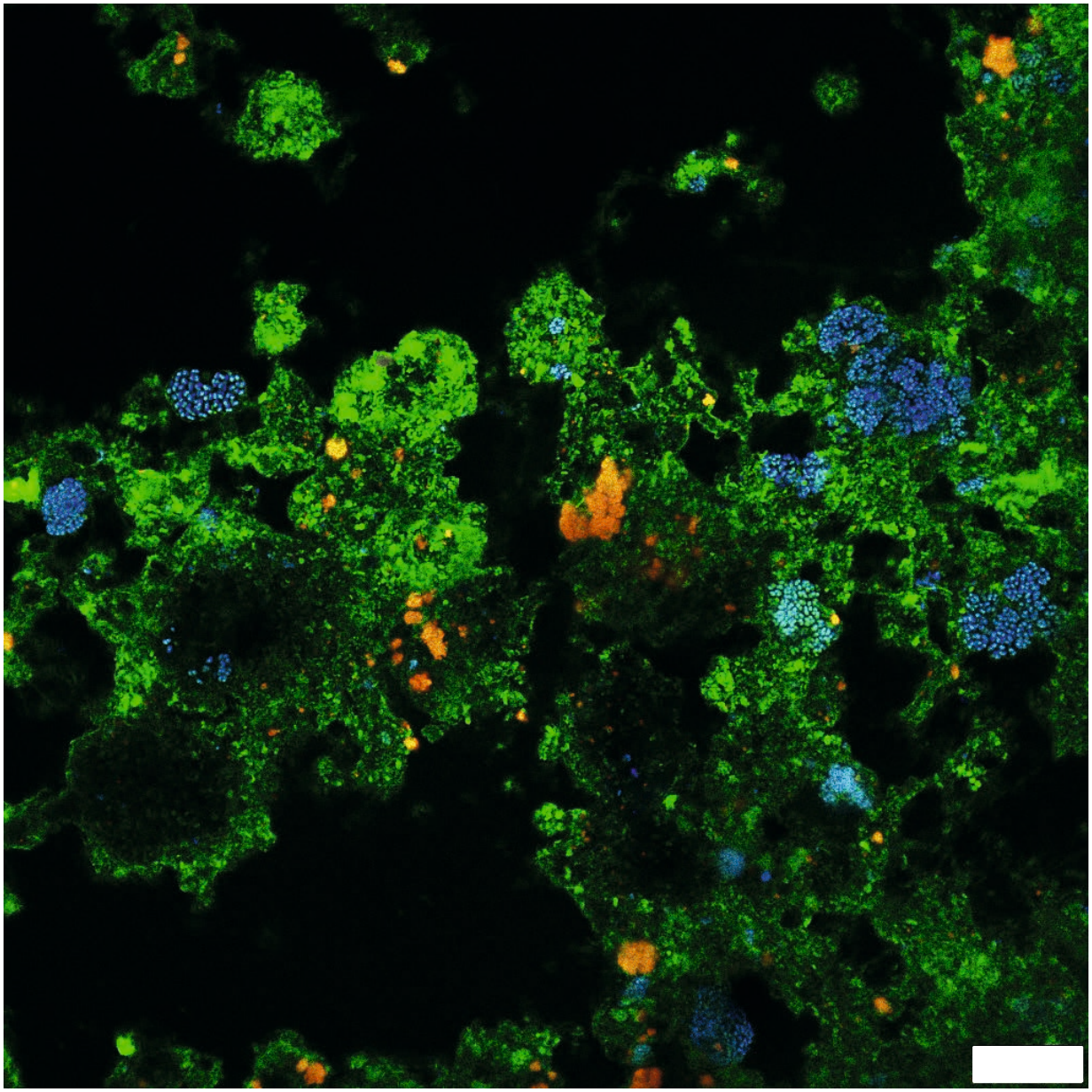

Figure 3.9 Exemplary image from images for quantification of AOB and *Nitrospira* in activated sludge from KNB2, January 2014 sample. Bacterial biomass targeted by EUBmix is depicted in green, AOB (AOBmix) are shown in blue, *Nitrospira* (NTSPAmix) in orange. Scale bar represents 25 µm.

3.3.3 Raman-Activated Microfloc Sorting to Investigate Nitrifier Microdiversity: A Pre-Study

As a pre-study, workflow efficiency for investigation of microdiverse nitrifiers was assessed. Sludge was incubated in presence of either ^{13}C-bicarbonate or D_2O, followed by FISH screening and sonication optimization. Aditionally, acquisition of Raman spectra, sorting of microflocs by optical tweezing, as well as MDA and PCR screening were assessed. Based on the outcome of the PCR screenings, 24 MDA products were selected for sequencing of the respective microfloc metagenome at JGI.

After seven days of incubation either in presence of ^{13}C-bicarbonate or D_2O, incubated sludge was screened for presence of AOB and NOB populations, and sonication conditions were optimized for obtaining nitrifier microflocs for Raman spectroscopy and sorting.

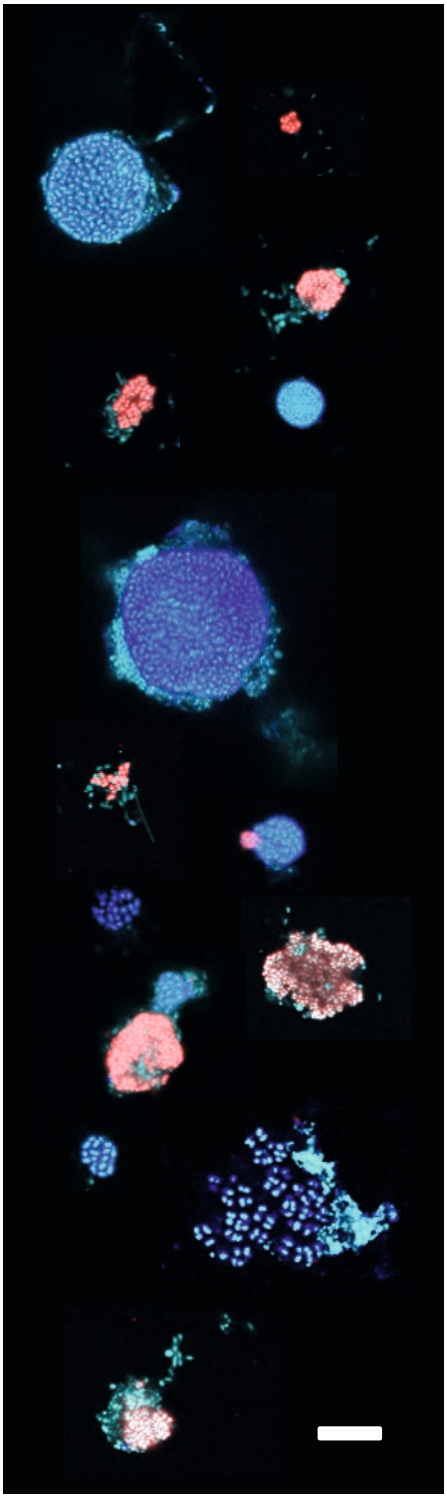

Figure 3.10 Image-collage of sonicated microflocs from KNB2, January 2014 sample. AOB are depicted in blue (AOBmix), *Nitrospira* in red (NTSPAmix), all cells (stained by DAPI) are depicted in cyan. Note that some microflocs contain both AOB and *Nitrospira* colonies. Scale bar represents 10 µm.

Under both incubation conditions, the same FISH-defined AOB and NOB populations were present as in sludge before incubation (native sludge). AOB and *Nitrospira* abundances, which were quantified for replicate 1 of both treatments, were similar between the two incubations and native sludge. The total nitrifier fraction ranged between 7 – 9 % (Table 3.2). No colocalization between AOB and *Nitrospira* in acquired 2-dimensional images was observed. While *Micavibrio*-like cells were frequently found within *Nitrospira* colonies in native sludge, *Micavibrio* was not detectable by FISH after seven days of incubation with ^{13}C-bicarbonate or D_2O.

Table 3.2 Relative AOB and *Nitrospira* biovolume fractions of overall bacterial biomass (EUBmix) in samples from native KNB2, and after seven days of incubation in presence of ^{13}C-bicarbonate or D_2O for biological replicate 1.

	Native KNB2	^{13}C Treatment	D_2O Treatment
AOB	4.3 %	4.4 %	3.3 %
Nitrospira	5.1 %	5.0 %	3.3 %

For both native and incubated sludge with ^{13}C-bicarbonate and D_2O, sonication results were found to be optimal at 30s, 60% power and 1 cycle settings. Under those conditions, sludge was sufficiently disrupted and most obtained microflocs had diameters of 5 – 20 µm. This size fraction contained mostly one single nitrifier colony, and sometimes both AOB and *Nitrospira* colonies. Two different AOB or two different *Nitrospira* colonies in one microfloc of this size were never observed (see Figure 3.10 for exemplary images of microflocs). There was no observable bias in presence of FISH-defined populations between native and incubated, or sonicated and non-sonicated sludge.

When sorting microflocs from ^{13}C-bicarbonate and D_2O treatments, it was noted by Tae Kwon Lee that under both incubation conditions as well as in native sludge, many flocs with cytochrome peaks also possessed a Raman peak at 967 cm^{-1}. The latter usually is used as activity marker in ^{13}C incubations due to phenylalanine peak shift from 1003 (^{12}C-phenylalanine) to 967 cm^{-1} (^{13}C-phenylalanine). In KNB2, however, putative nitrifiers possessed inherent peaks in that marker region. Therefore, it was not possible to differentiate between active and inactive cells in ^{13}C incubations. For each treatment, 20 microflocs were sorted randomly (whether or not the microfloc showed activity and nitrifier markers), and 20 microflocs were sorted in a targeted manner (only when they showed both nitrifier and activity signatures in their Raman spectra).

PCR screens to assess presence of bacterial DNA in MDA products were carried out with two general bacterial primer sets targeting the 16S rRNA gene, resulting in one long and one short amplicon. Additionally, PCR screens to assess nitrifier DNA presence were carried out with two primer sets targeting AOB 16S rRNA and *amoA* genes, and three primer sets targeting *Nitrospira*. For *Nitrospira* screens, two primer sets targeting the 16S rRNA gene were used, resulting in one long and one short amplicon, and one primer set was used that targeted *nxrB*.

PCR runs were found to only give consistent results, when template, i.e. diluted MDA product, was vortexed thoroughly and additionally mixed by pipetting prior to setting up PCR reactions. In Table 3.3, fractions of positive overall and nitrifier-specific PCR screens are listed according to treatment and sorting strategy. Overall success of obtaining MDA products containing bacterial DNA was similar between approaches ($\chi^2 = 2.05$, d.f. = 3, $p = 0.56$), with the highest efficiency in targeted sorting from D_2O treatment (80%, other approaches 60 – 65 %). No significant enrichment in nitrifier-positive screens in targeted (19/29) as compared to random sorting (11/25) was found ($\chi^2 = 2.52$, d.f. = 1, $p = 0.11$).

Table 3.3 Overview of MDA products containing bacterial DNA (counted as positive, when at least one PCR screen was successful), and fraction of AOB- and/or *Nitrospira*-positive PCR screens thereof. Success rates are listed according to treatment (^{13}C and D_2O) and sorting strategy (random and targeted).

Screening Outcome	^{13}C Treatment				D_2O Treatment			
	Random Sorting		Targeted Sorting		Random Sorting		Targeted Sorting	
Bacterial DNA present	12/20	60 %	13/20	65 %	13/20	65 %	16/20	80 %
AOB or *Nitrospira* present	4/12	33 %	7/13	54 %	7/13	54 %	12/16	75 %
AOB and *Nitrospira* present	1/12	8 %	1/13	8 %	2/13	15 %	6/16	38 %
***Only* AOB present**	2/12	17 %	1/13	8 %	1/13	8 %	2/16	13 %
***Only Nitrospira* present**	1/12	8 %	5/13	38 %	4/13	31 %	4/16	25 %

PCR screening results, as well as information obtained from Raman spectra for each sorted microfloc are summarized for all sorting strategies and incubation treatments in Figure 3.11.

Interestingly, short amplicons were highly significantly more often amplified than large amplicons of the same gene (general 16S rRNA gene: overall 51 positive screens, 49 short, 35 long amplicons obtained, $\chi^2 = 13.22$, d.f. = 1, $p < 0.001$; *Nitrospira* 16S rRNA gene: overall 18 positive screens, 16 short, five long amplicons obtained, $\chi^2 = 13.83$, d.f. = 1, $p < 0.001$). For general bacterial 16S rRNA gene amplicons, *only* small amplicons were obtained 16 times, while *only* large amplicons were obtained twice, for *Nitrospira* 16S rRNA gene amplicons, *only* long amplicons were obtained once, while *only* short amplicons were obtained from twelve MDA products.

For microflocs with cytochrome signatures, nitrifier screening PCRs were significantly more often positive, than for microflocs without cytochrome signatures (no cytochrome signature: five nitrifier-positive screens out of 17 MDA products containing bacterial DNA, with cytochrome signature: 25 nitrifier-positive screens out of 35 MDA products containing bacterial DNA, $\chi^2 = 6.868$, d.f. = 1, $p < 0.01$).

Based on PCR screening outcome and Raman signature, 24 MDA products were selected for microfloc metagenome sequencing at JGI (Figure 3.11). All ten MDA products with positive PCR screens for both AOB *and Nitrospira*, as well as four with *only* AOB-, and seven with *only Nitrospira*-positive screens were selected. Additionally, three MDA products with only general bacterial-positive PCR screens, but with cytochrome signatures in their respective microfloc Raman spectra were selected for sequencing.

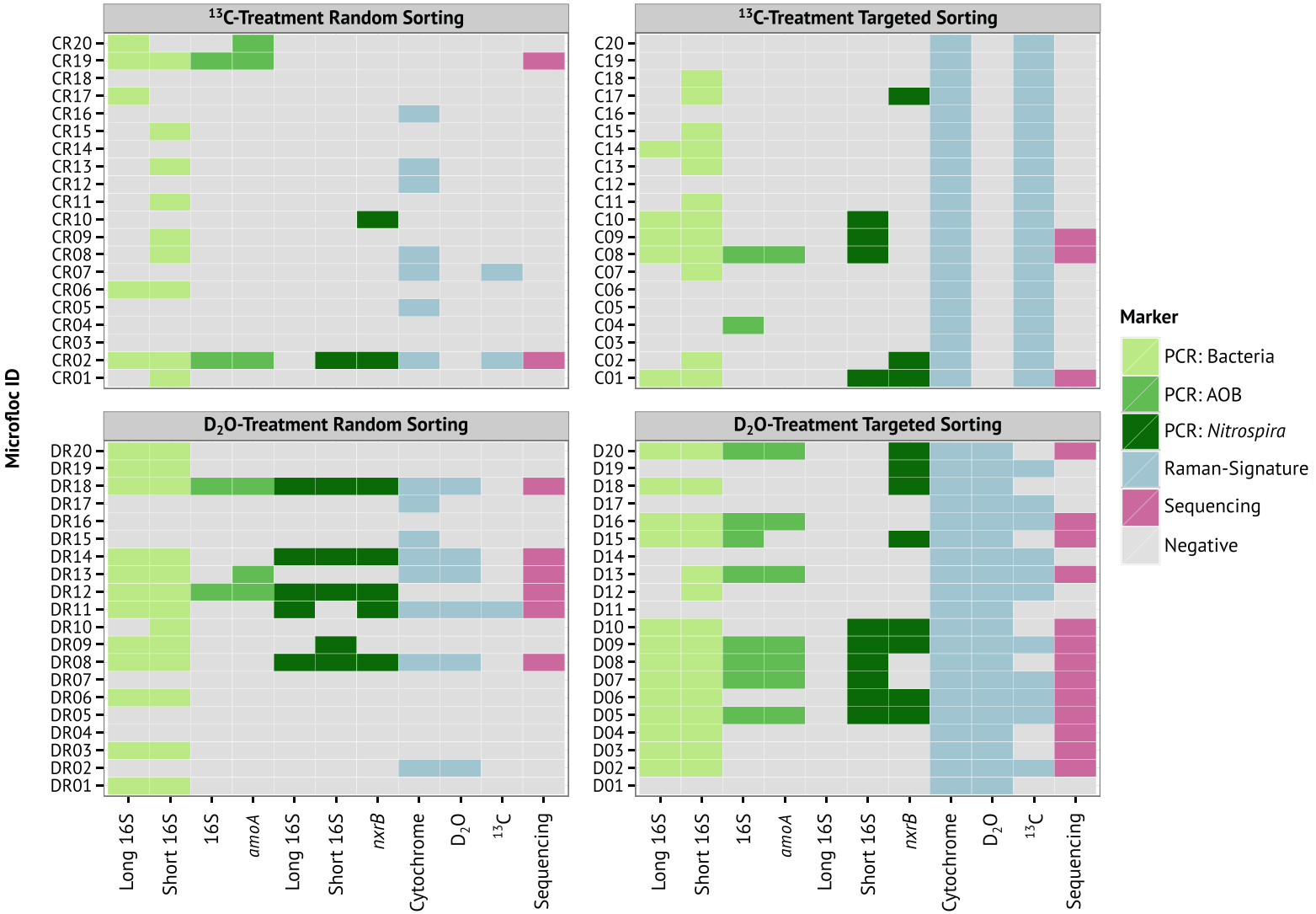

Figure 3.11 Overview of information obtained on sorted microflocs. Each row represents one microfloc; panels represent treatments (^{13}C and D_2O incubations) and sorting strategy (random or targeted). PCR screening results are colored in shades of green according to primer specificity (see legend). General bacterial amplicons are depicted in column 1 – 2, AOB-specific amplicons in column 3 – 4, and *Nitrospira*-specific amplicons in columns 5 – 7. Information obtained from microfloc Raman spectra is depicted in blue (column 8 – 10), and indication whether MDA products were selected for sequencing in column 11 (pink). Colored scales represent presence of PCR product, Raman signature or selection for sequencing. Grey fill represents absence thereof.

Preliminary sequencing results obtained from JGI are depicted in Figure 3.12. Three sequencing runs failed, and one (D06) contained only low sequencing depth. All other sequenced microfloc metagenomes contained either *Nitrospira*- or *Nitrosomonadales*-related sequences, as determined by contig megablast and classification to order level by JGI after decontamination procedures.

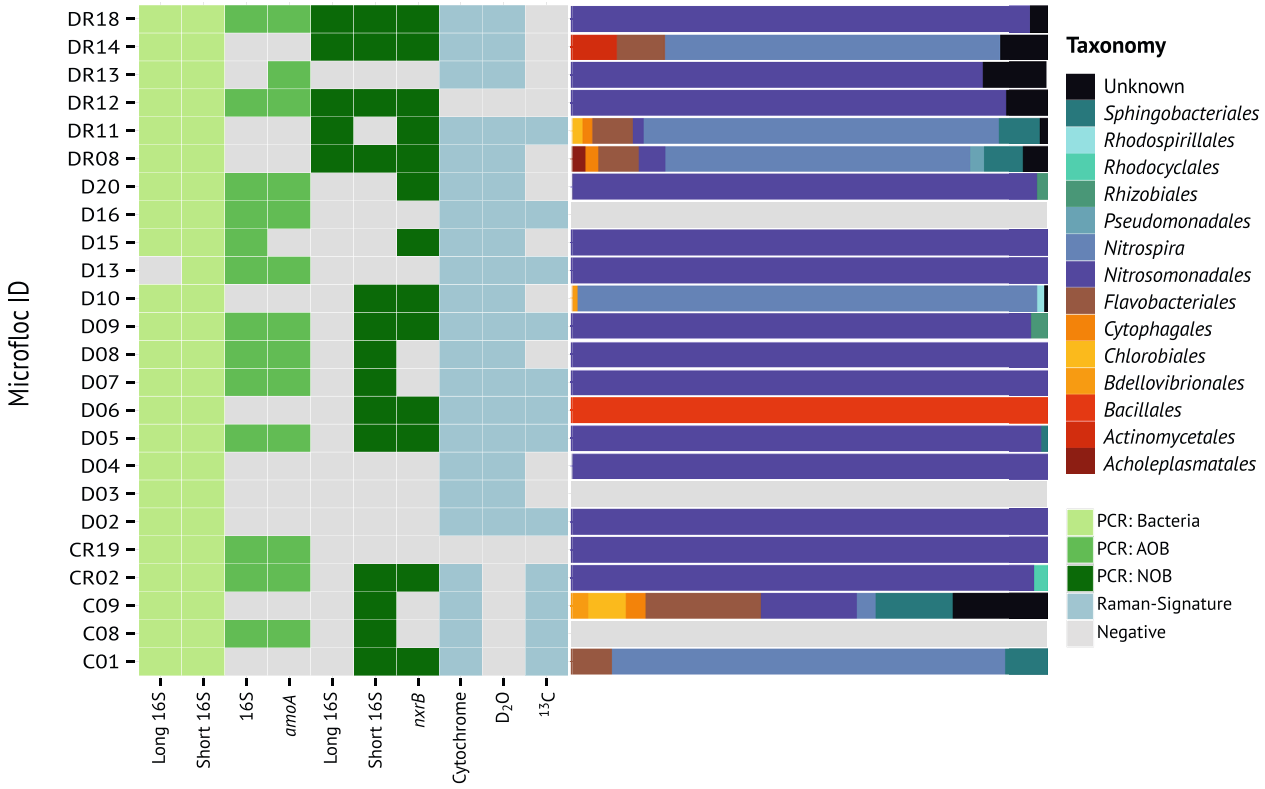

Figure 3.12 Preliminary sequencing results obtained from JGI. Contigs obtained from sequencing of MDA products were assigned taxonomy to order level by megablast. Taxonomy bars represent 100 % of contigs after decontamination procedures.

3.3.4 D_2O Incubation Test

To assess the minimum incubation time and minimal concentration of D_2O necessary for sufficient label incorporation into biomass to be seen in Raman spectra, activated sludge from KNB2 was incubated in presence of 0 %, 25 %, 50 % and 75 % D_2O in biological duplicates.

The same FISH-defined populations of nitrifiers as in native sludge were present throughout the incubation (Cl6a192/Ncom1025, NEU, and Ntspa1431). In contrast to the previous incubation (3.3.3), *Micavibrio*-like bacteria were observed within *Nitrospira* colonies throughout the incubation at all D_2O concentrations tested.

After seven days of incubation, slightly less *Nitrospira* were observed by quantitative FISH in 75 % D_2O treatments as compared to lower D_2O concentrations (4.8 – 5.4 % as compared to 5.2 – 8 %), for AOB abundance, no clear trend was seen (Table 3.4).

Table 3.4 Relative AOB and *Nitrospira* biovolume fractions of overall bacterial biomass (EUBmix) in samples from native KNB2 (April sample), and after seven days of incubation in presence of 0 %, 25 %, 50 %, and 75 % D_2O for replicates 1 and 2.

	Native KNB2	0 % D_2O		25 % D_2O		50 % D_2O		75 % D_2O	
		R1	R2	R1	R2	R1	R2	R1	R2
AOB	4.3 %	5.3 %	4.4 %	6.0 %	4.6 %	6.1 %	4.4 %	4.2 %	4.9 %
Nitrospira	4.9 %	8.0 %	7.2 %	6.9 %	6.0 %	6.6 %	5.2 %	4.8 %	5.4 %

Throughout the incubation time, sonication optimization was evaluated. However, the previously applied settings of 30s, 1 cycle, 60% power led to a selective loss of FISH signals from AOB population Cl6a192 and a strong reduction in *Nitrospira* colonies after sonication as compared to non-sonicated sludge. Although various sonication conditions, as well as other dissection methods (bead beating, application of shear force by suction through needles, and douncing) were tested, none were successful in retaining all FISH-defined populations.

Nonetheless, when sonicated sludge (30s, 1 cycle, 60% power) was introduced into capillaries and measured on the Raman microscope, cytochrome signatures were observed for many microflocs. D_2O incorporation into putative nitrifier biomass was first detectable after two days in 50 % D_2O incubations.

3.4 Enrichment, Characterization and Isolation of Novel Nitrite Oxidizing Bacteria

3.4.1 Initial Setup and Phylogeny of Enriched Nitrite Oxidizing Bacteria

Initial Erlenmeyer flask cultures inoculated with activated sludge from KNB2 and KNB3 depleted 1 mM NO_2^- within one week after inoculation. Thereafter, 1 mM NO_2^- was consumed within one to three days. No pronounced differences in activity were observed for incubations containing sterile filtered sludge SN and mineral medium, or between the source of inocula (KNB2 or KNB3). Initially, an increase in *Nitrospira* lineage I was observed by FISH in all incubations.

Upon changing of cultivation flasks from Erlenmeyer flasks to Schott bottles, which was done to minimize possible input of organics from lab air, *Nitrospira* abundance (as assessed by FISH) decreased in all SBE, and finally *Nitrospira* disappeared within two weeks. Instead, two other cell morphologies became dominant. The observed large oval-shaped bacteria were stained very brightly, while irregular bean-shaped bacteria gave weak signals with EUBmix probes (Figure 3.13). Even though *Nitrospira* was eventually not detectable any longer by FISH or PCR (*Nitrospira* specific 16S rRNA gene and *nxrB* targeting primers), NO_2^- was still actively oxidized to NO_3^-. Therefore, both SBE and EFE cultures were kept as parallel cultivation lines from that point on.

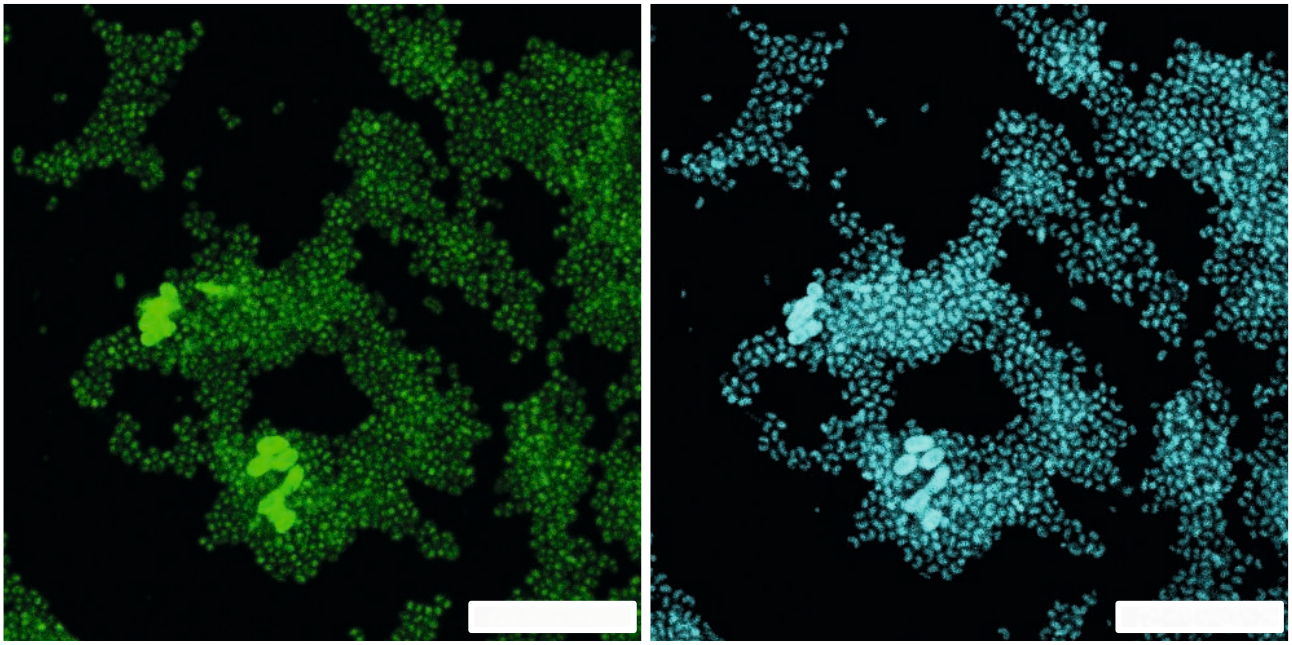

Figure 3.13 Exemplary image of the two observed morphologies in SBE, bacterial biomass (EUBmix) in green on the left, all cells (DAPI-stained) in cyan on the right side. Note the difference in fluorescence intensity between oval- and bean-shaped bacteria in the left image. Scale bars represent 10 µm.

In order to identify the unknown nitrite oxidizers in SBE, and to establish a phylogeny for *Nitrospira* lineage I in EFE, general bacterial (SBE) and *Nitrospira* specific 16S rRNA gene amplicons (EFE) were cloned, transformed into *E. coli* and sequenced. For EFE, *Nitrospira* lineage I sequences 99.5% – 99.8% identical to *N. defluvii* were obtained (see Table 9.1 and Table 9.2 for best BLAST hits and sequences).

Obtained general 16S rRNA gene sequences from SBE were affiliated with *Zoogloea oryzea* (*Betaproteobacteria*), *Brevundimonas sp.* (*Alphaproteobacteria*), *Sphingopyxis macrogoltabida*

(*Alphaproteobacteria*), and Candidatus *Nitrotoga arctica* (*Betaproteobacteria*). Best BLAST hits for *Nitrotoga*-affiliated and non-nitrite oxidizer sequences are listed in Table 9.3 and Table 9.5 respectively, *Nitrotoga*-affiliated sequences are given in Table 9.4. The most abundant restriction digest pattern of M13 PCR products (twelve out of 24) corresponded to *Nitrotoga*-, the second most abundant (five out of 24) to *Zoogloea oryzea*-affiliated sequences.

Chimera-checked sequences affiliated with *Nitrotoga* showed 98.2 % – 98.5 % identity to Ca. *N. arctica*, and inter-sequence identity of 99.6 % – 100 %, with six variants differing in single bases. All obtained *Nitrotoga sp.* sequences from SBE cultures fell into a clade of the genus *Nitrotoga* without described representatives (Figure 3.14).

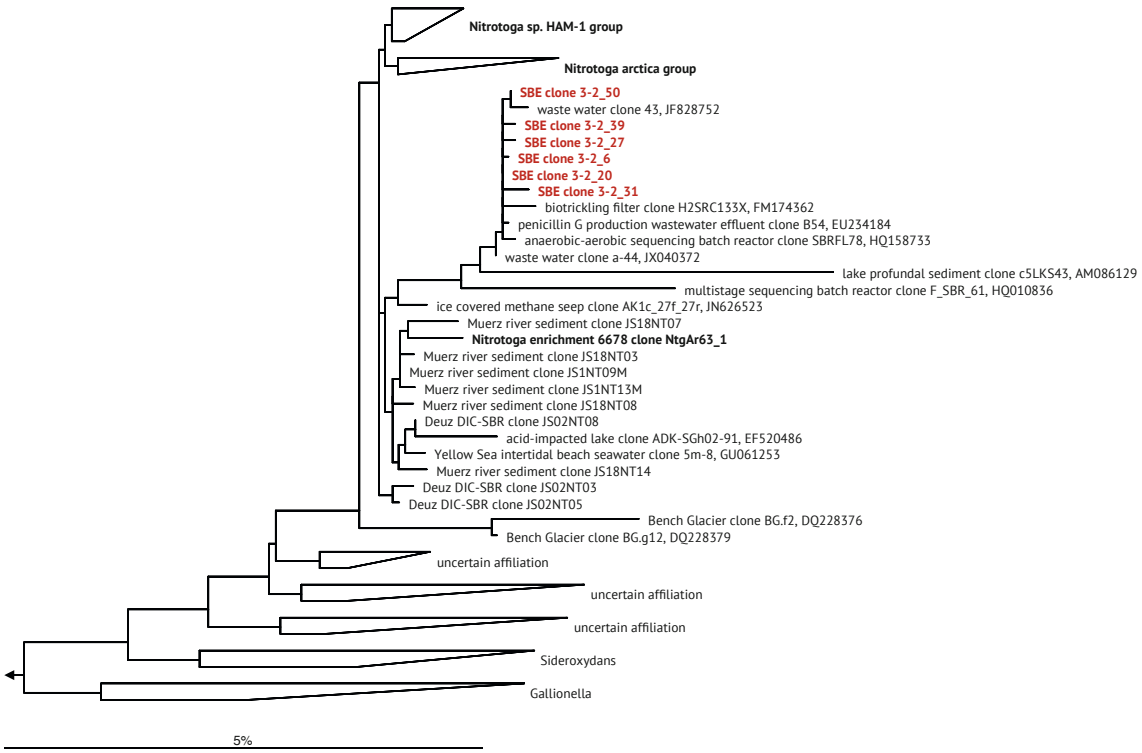

Figure 3.14 Phylogeny of *Nitrotoga*-affiliated sequences obtained in this study and previously known *Nitrotoga*- and *Gallionella*-affiliated sequences. Sequences obtained from SBE are depicted in bold red, other known enrichments of *Nitrotoga sp.* in bold black. PhyML tree, generated with a 50% *Gallionellaceae* conservation filter by courtesy of Sebastian Lücker.

3.4.2 *Nitrospira* Enrichments

3.4.2.1 Attempts to Obtain Higher Enrichment of *Nitrospira* Lineage I

One month after initial inoculation, *Nitrospira* accounted for 33 % of the total biomass (DAPI-stained) in EFE from KNB2, as determined by quantitative FISH (see Figure 3.15 for representative images). No substantially higher enrichment was obtained at any later time point (based on non-quantitative FISH). Instead, most transfers contained less *Nitrospira*. Varying the total amount of biomass transferred (1:2 – 1:200 dilutions) or the pH of the mineral medium used (between 7.6 – 8) did not result in consistent trends of higher or lower *Nitrospira* enrichment. *Nitrotoga* was observed once in EFE, however at low abundance.

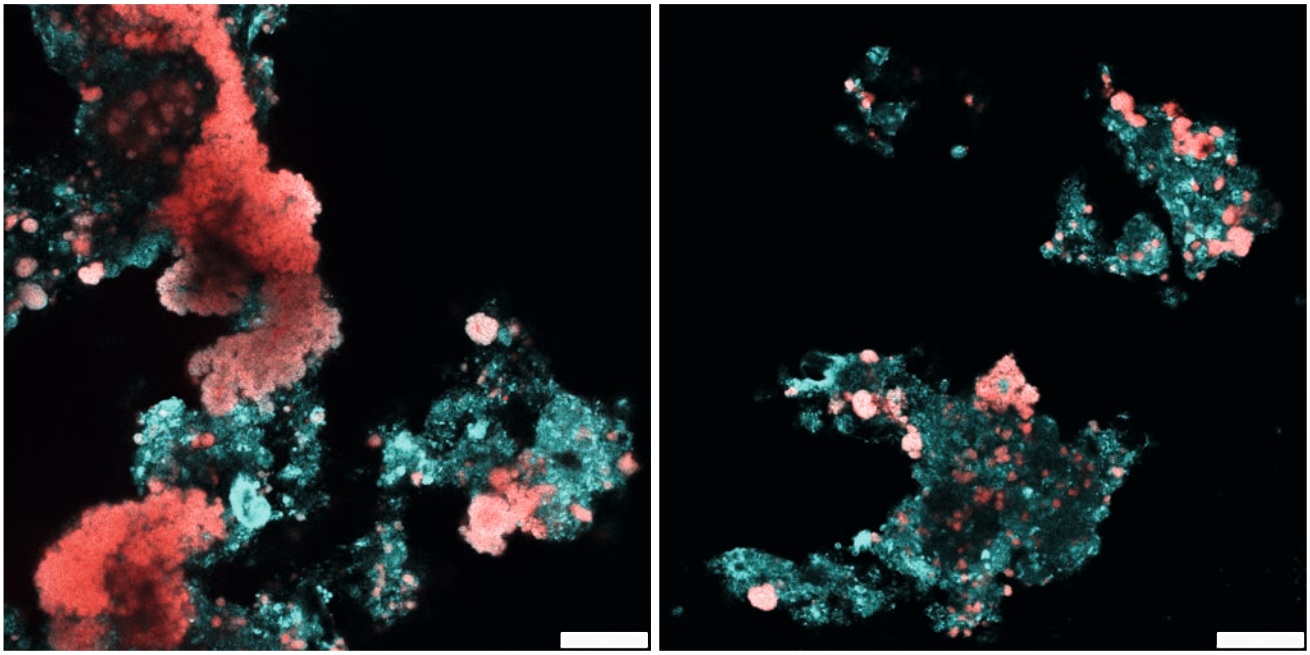

Figure 3.15 Representative images of EFE enrichment, one month after initial inoculation. Biomass was stained by DAPI (cyan), *Nitrospira* is depicted in light red (NTSPAmix). Scale bars represent 25 µm.

One culture containing small (20 – 30 µm in diameter) *Nitrospira* colonies without other detectable cells, was used for micromanipulation to isolate those colonies from other cells in the enrichment. While four out of five sorted colonies oxidized NO_2^- to NO_3^-, all were overgrown by other bacteria after further cultivation.

Antibiotics treatment with ampicillin and acriflavine did not result in higher *Nitrospira* enrichment. All cultures supplemented with ampicillin were rapidly overgrown by other bacteria, and while NO_2^- was depleted, no NO_3^- was detectable. Cultures supplemented with 10 mg*l^{-1}acriflavine did not utilize NO_2^- at all, while cultures supplemented with 0.105 mg*l^{-1} oxidized NO_2^- to NO_3^-, but contained only few *Nitrospira* after four weeks of incubation.

3.4.3 *Nitrotoga* Enrichments

After identification of the unknown nitrite oxidizer in SBE as *Nitrotoga sp.*, SBE were monitored by FISH using *Nitrotoga*-specific probes Ntoga122 and Ntoga221. *Nitrotoga*-specific probes exclusively stained all bean-shaped cells in SBE. Due to the characteristic shape of *Nitrotoga* in SBE (at least under the applied cultivation conditions), the degree of *Nitrotoga* enrichment was – in addition to FISH – also monitored by light microscopy. Approximately one month after switching from Erlenmeyer flasks to Schott bottles, *Nitrotoga* accounted for 80% of the bacterial biomass (targeted by EUBmix), as assessed by quantitative FISH. Throughout, *Nitrotoga* stayed similarly or higher enriched. The most dominant contaminating cell type was large oval-shaped *Betaproteobacteria* (stained by Bet42a probe).

While *Nitrotoga* was never observed to form colonies, but occurred as planktonic cells, other *Betaproteobacteria* grew both planktonic and in large flocs. Floc formation of contaminating cells especially occurred after prolonged batch cultivation.

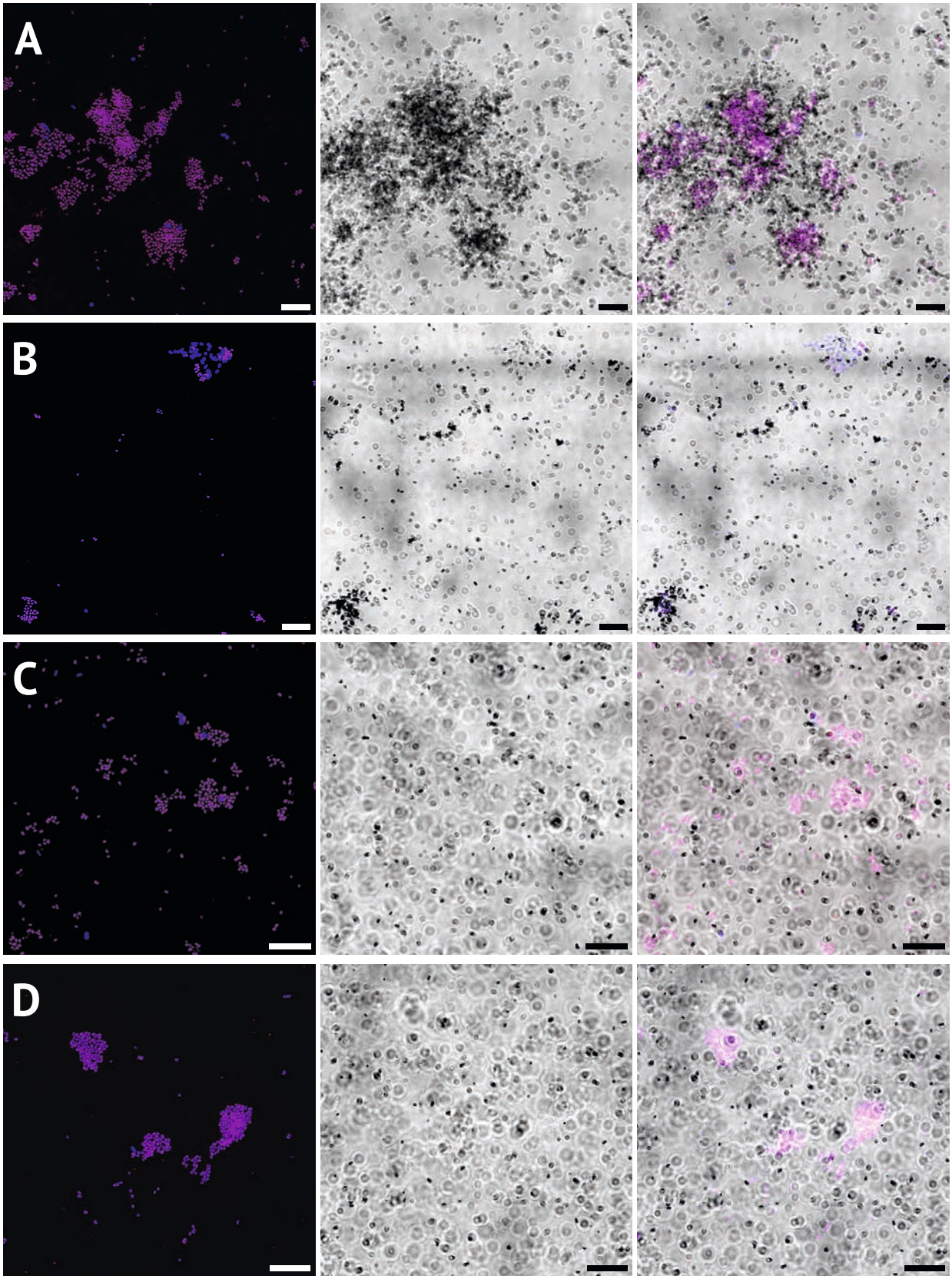

Figure 3.16 MAR-FISH images of SBE. Bacterial biomass targeted by EUBmix probes is depicted in blue, *Nitrotoga* is depicted in violet due to binding of both bacterial and *Nitrotoga*-specific probes (Ntoga122). Bright field images of silver grain formation are shown in black and white. A, B: SBE incubated with ^{14}C-bicarbonate and NO_2^-. C: No substrate control, SBE incubated with ^{14}C-bicarbonate but no NO_2^-. D: Dead control incubated with ^{14}C-bicarbonate and NO_2^-. Scale bars represent 10 µm.

3.4.3.1 MAR-FISH

In order to assess, whether nitrite oxidizers other than *Nitrotoga* were present in SBE, MAR-FISH was carried out under nitrifying conditions. Supplemented 1 mM NO_2^- was used up only at the end of the incubation. At both time points taken (6 h and 20 h), ^{14}C incorporation only co-occurred with biomass in incubations containing living SBE supplemented with ^{14}C-bicarbonate and NO_2^-. Silver grain formation was always observed on top of multiple (> 15 – 20) *Nitrotoga* cells. While silver grains often occurred on top of *Nitrotoga* interspersed with other cells (Figure 3.16, A), no silver grain formation was observed on top of clusters of non-*Nitrotoga* cells (Figure 3.16, B). In no-substrate and dead-control incubations (Figure 3.16, C and D respectively), some sparse silver grain formation occurred, but this was randomly distributed over the entire well, similar to slide surface outside of wells, and not associated with cells. Longer incubation time (20 h instead of 6 h) and longer exposure time (two weeks instead of one week) resulted in larger areas of silver grain formation, but no change in the observed activity pattern.

3.4.3.2 Raman Spectroscopy

To assess, whether *Nitrotoga* in SBE show cytochrome signatures, which are indicative of most nitrifiers, Raman spectra were acquired. Only cells of *Nitrotoga*-like morphology (bean-shaped cells) showed characteristic cytochrome signatures. Repeatedly measured Raman spectra of one *Nitrotoga*-like cell are depicted in Figure 3.17. Cytochrome intensities as well as peak intensity between 2200 – 2600 cm^{-1} decreased with increasing number of measurements of the same cell.

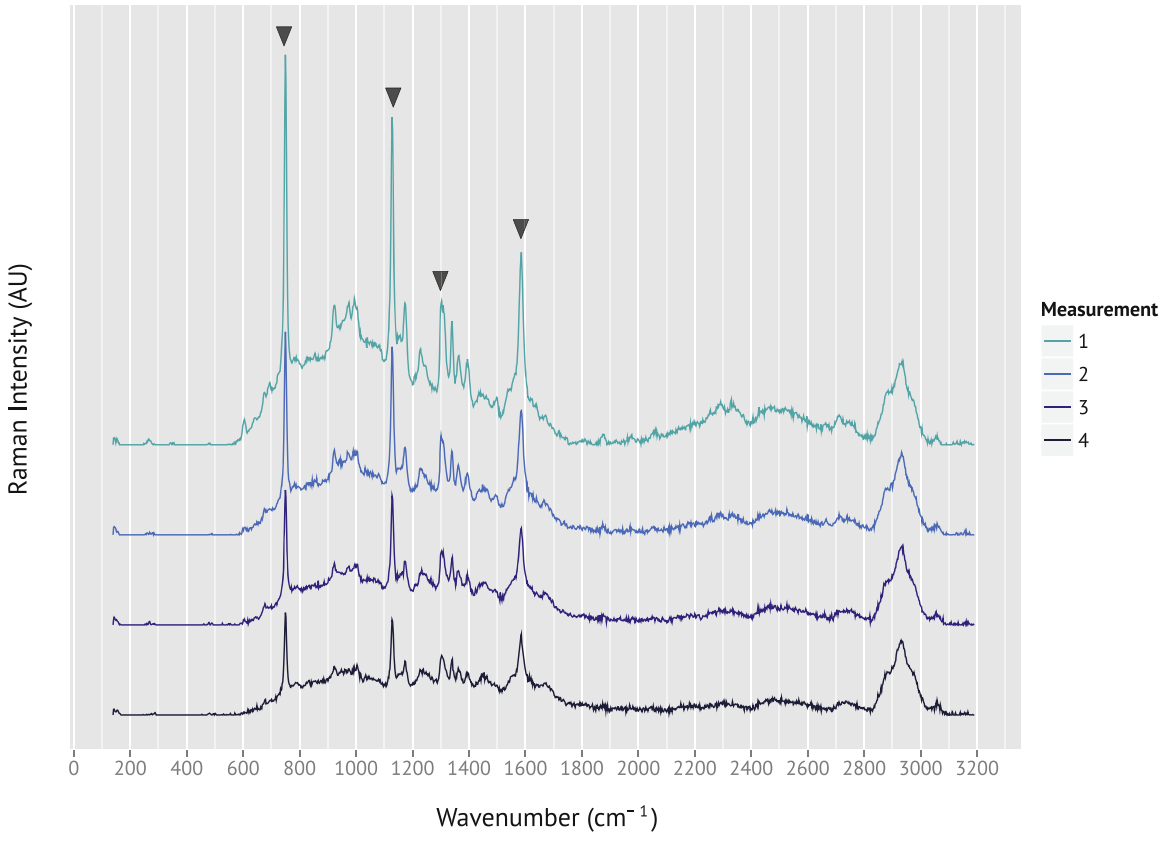

Figure 3.17 Raman spectra of one *Nitrotoga*-like cell. Spectra were baselined using the SNIP algorithm as implemented in scattr (David Berry, unpublished). Spectra were not normalized. Consecutive measurements are vertically stacked to better visualize decreasing cytochrome (grey arrows) intensities and peak intensity at 2200 – 2600 cm^{-1} with increasing number of measurements. On the x-axis, wavenumber is depicted, on the y-axis, Raman intensity.

3.4.3.3 Cryoconservation

A cryopreserved SBE culture was thawed two weeks and four months after preparation of cryostocks. *Nitrotoga* could be successfully revived and oxidized NO_2^- within two days after revival. The degree of enrichment of revived *Nitrotoga* was similar to the cultures used for cryostock preparation.

3.4.3.4 Physiological Characterization: Substrate Tolerance

Substrate tolerance of SBE was tested over a range of NO_2^- concentrations from 1 – 30 mM. Within 15 days, NO_2^- was depleted in treatments containing up to 10 mM (Figure 3.18), while treatments containing 15, 20, and 30 mM NO_2^- did not utilize NO_2^- during this time. The fastest depletion occurred at 1 mM (1.5 days), followed by 5 mM (approximately 2.5 days), and 10 mM NO_2^- (five and eleven days for replicate 1 and 2 respectively). SBE supplemented with 1, 5 or 10 mM NO_2^- showed high enrichment of *Nitrotoga* after complete oxidation of NO_2^-, with only few contaminants (*Betaproteobacteria)* present, as observed by FISH.

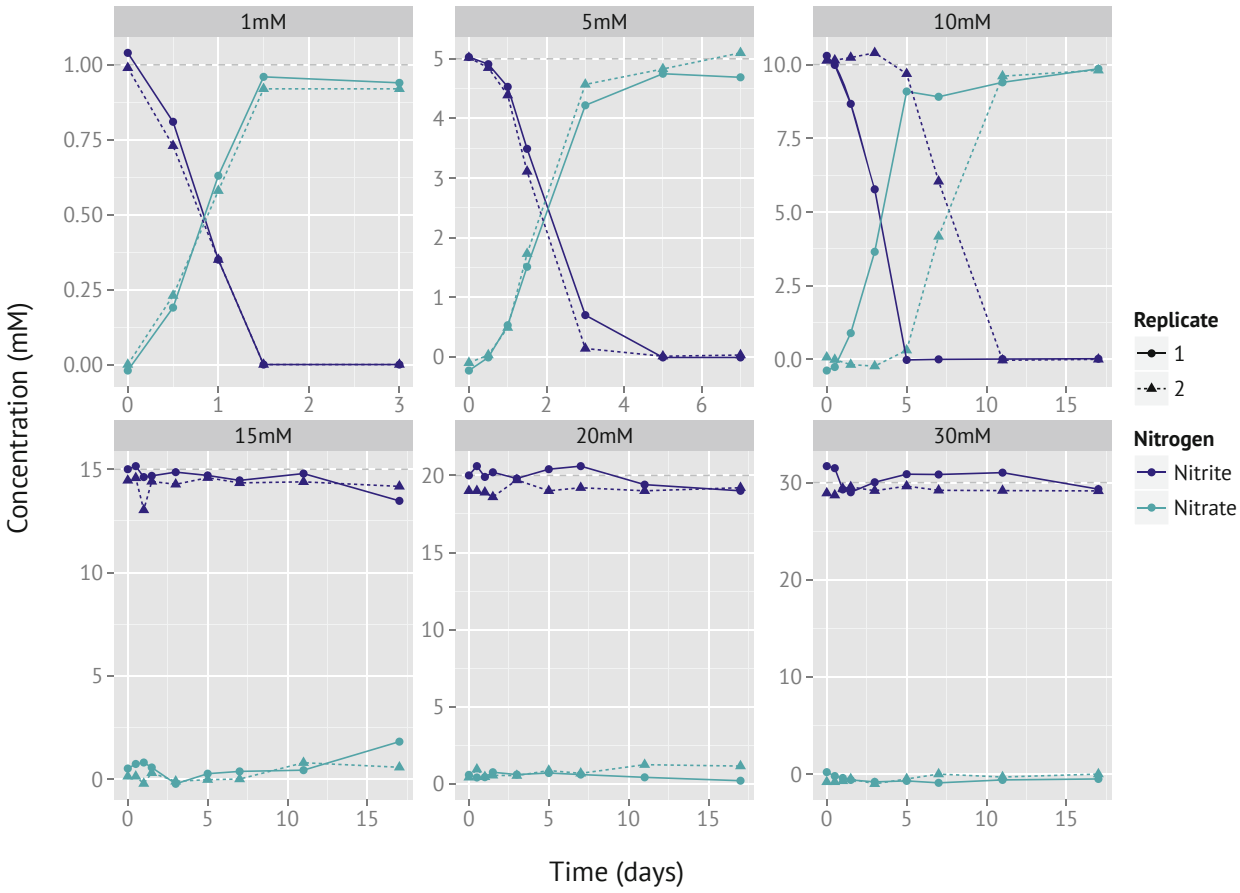

Figure 3.18 NO_2^- oxidation to NO_3^- during substrate tolerance experiment with SBE over 17 days of incubation in biological duplicates (replicate 1 and 2). On the x-axis, incubation time is depicted, on the y-axis, measured concentration of NO_x. Panels represent different NO_2^- amendments, with the added NO_2^- concentration indicated in the title and as dashed grey lines in the graphs. Note the different scaling of x- and y-axes for different NO_2^- amendments. Depicted data points represent means of NO_x concentrations from Griess-based measurements in technical duplicates.

While no NO_2^- depletion was observed within 17 days for NO_2^- concentrations higher than 10 mM, replicate 1 of the 15 mM treatment oxidized NO_2^- to NO_3^- within 23 days (data not shown), and was highly enriched in *Nitrotoga*. The only observable contaminants were again *Betaproteobacteria*.

Replicate 2 of the 15 mM, both replicates of the 20 mM and replicate 1 of the 30 mM treatment oxidized NO_2^- to NO_3^-, within 46 days. However, for these incubations, hardly any *Nitrotoga* were detectable by FISH after depletion of NO_2^-. Instead, cells stained by *Nitrobacter* probe Nit3 were abundant. Replicate 2 of the 30 mM treatment did not deplete NO_2^- in the course of the experiment, and contained very little biomass at the end of the experiment, with no detectable *Nitrobacter* cells.

3.4.3.5 Physiological Characterization: Temperature Optimum and Activity Range

Temperature optimum for NO_2^- oxidation of SBE was assessed by incubation in biological triplicates over a range from 4 °C to 46 °C, representing temperature optima of most described nitrite oxidizing bacteria. All bottles were supplemented with 1 mM NO_2^-.

NO_2^- oxidation occurred fastest at 28 °C (depletion after 34 h), followed by 24 °C (47 h), and 20 °C (77 h) incubations. SBE incubated at 14 °C and 37 °C started oxidizing, however did not deplete NO_2^- in the first 102 h of the experiment, where culture aliquots were regularly sampled. No NO_2^- depletion was observed for SBE incubated at 4 °C and 46 °C during this time (see Figure 3.19 for IC measurements of NO_x during the first 102 h).

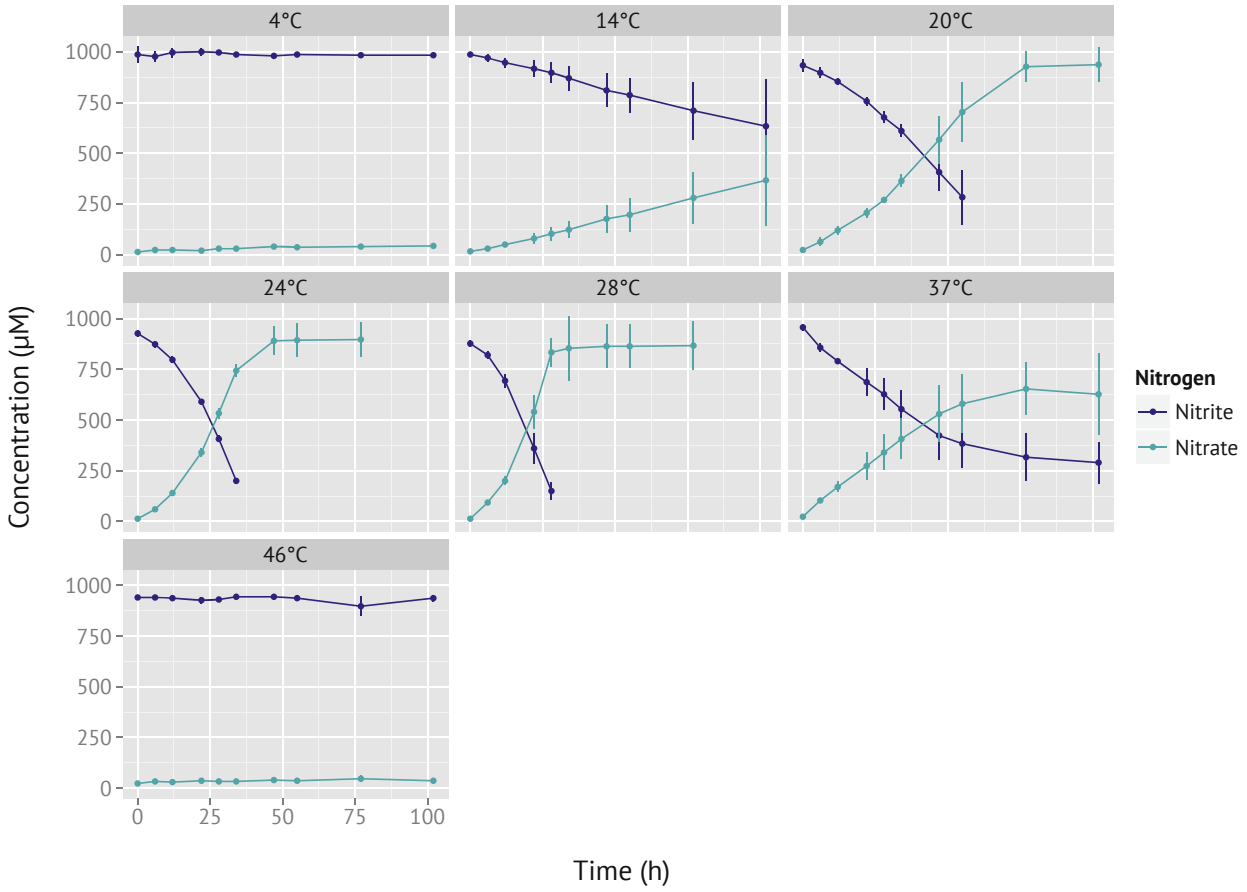

Figure 3.19 NO_2^- oxidation to NO_3^- during SBE incubations at temperatures from 4 °C to 46 °C for 102 h. On the x-axis, incubation time is depicted, on the y-axis, measured concentration of NO_x. Depicted NO_x concentrations represent means of biological triplicates, error bars indicate standard deviations. Graph panels represent different incubation temperatures, with the respective temperature indicated in the panel title. NO_x was measured by IC, detection limit of NO_2^- was 0.1 mM.

NO_2^- oxidation rates between 6 h and 22 h after incubation differed accordingly between incubation temperatures (Figure 3.20), with the significantly highest rate occurring at 28 °C (ANOVA, incubation temperature as factor, Tukey HSD test, $p < 0.01$).

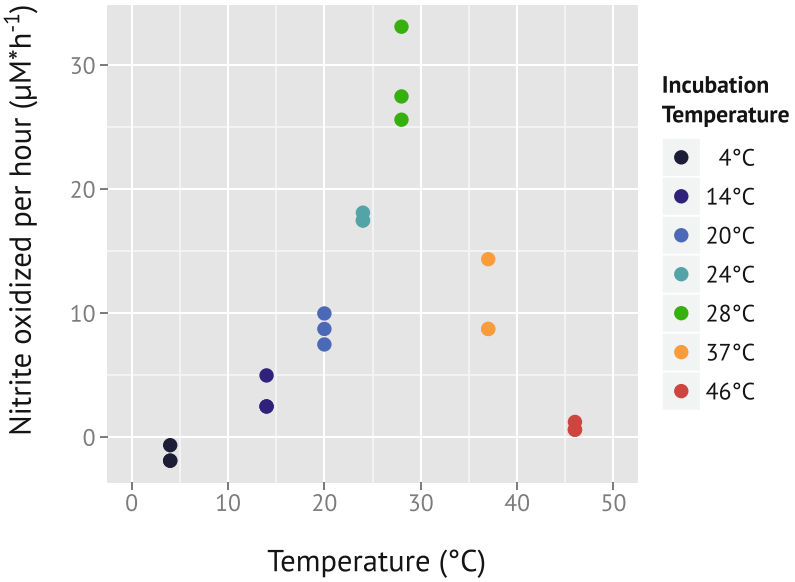

Figure 3.20 NO_2^- oxidation rates between 6 h and 22 h after inoculation at temperatures between 4 °C and 46 °C. Each dot represents one of biological triplicates. Rates were calculated from NO_2^- measurements by IC.

No strong differences between the degree of *Nitrotoga* enrichment at the different temperatures or between triplicates were observed by FISH. Prolonged cultivation did not result in higher enrichment at any temperature as compared to cultures kept at RT.

While NO_2^- was oxidized (albeit slowly) in 4 °C incubations within few weeks, no NO_2^- depletion was observed for SBE incubated at 46 °C over three months.

3.4.3.6 DNA Extraction

DNA from SBE was extracted from two time points by two different methods, one relying on enzymatic lysis and subsequent CTAB precipitation of non-nucleic acid, soluble cell material, and one using bead beating with phenol and precipitation of nucleic acids by PEG 8000. All extractions yielded sufficient amounts of genomic DNA for sequencing (minimum 2,000 ng). However, over 4x more DNA was obtained from mechanical lysis and precipitation of DNA with PEG8000 as compared to enzymatic lysis from the same amounts of biomass from the extractions carried out in October (Table 3.5).

Table 3.5 Overview over DNA yield from SBE at different time points and extraction methods. Identical amounts of biomass were used for the two different extraction methods from October SBE sample. DNA was quantified by PicoGreen after RNAse treatment.

Sample	Extraction Method	Total DNA (ng)
SBE June	Enzymatic lyis and CTAB	3,200
SBE October	Enzymatic lyis and CTAB	5,000
SBE October	Mechanical lysis and PEG8000	22,800

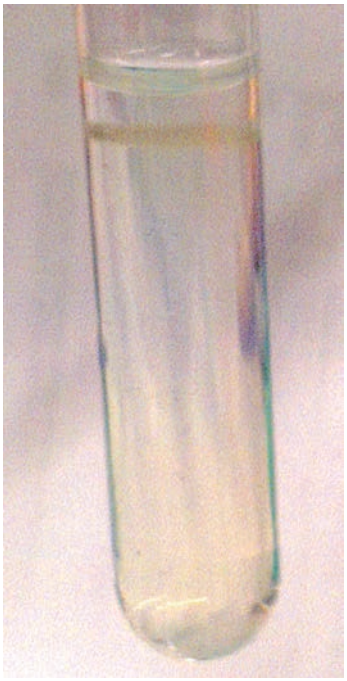

Figure 3.21 Centrifugation tube after Percoll density gradient centrifugation. Note the brown cell band near the top.

3.4.3.7 Attempts to Obtain an Axenic Culture

Various approaches were taken to obtain higher enrichment or an axenic culture of *Nitrotoga*. SBE culture was serially diluted, and Percoll density gradient centrifugation, various filtration strategies and supplementation of SBE with various concentrations of acriflavine were tested. Figure 3.22 summarizes all attempts made to obtain an axenic culture of *Nitrotoga*.

Dilution to extinction setups were active up to a 10^6 – 10^7-fold dilution within few weeks. However, these dilutions still contained non-*Nitrotoga*-like cells in similar amounts as batch cultures. Higher dilutions did not consume NO_2^- even after incubation for several months.

Percoll density gradient centrifugation resulted in a brown cell band (Figure 3.21), containing high amounts of *Nitrotoga* (as determined by light microscopy). However, the fraction also contained other cell morphologies, irrespective of the method used for removal of the target fraction from centrifugation tubes. No higher enrichment of *Nitrotoga* as compared to batch cultures could be obtained from serially diluted or further cultivated Percoll fractions.

0.45 µm filtered SBE did not consume added NO_2^- over incubation of several months. Filtration of the inoculum or entire batch culture through 1.2 µm filters temporarily reduced the amount of flocs, but did not result in loss of contaminant cells.

While SBE oxidized NO_2^- in all acriflavine treatments (21, 4.2, 2.1, 0.42, and 0.21 $mg*l^{-1}$), *Nitrotoga* were less enriched as compared to SBE without addition of acriflavine.

3.4.3.8 Isolation of *Nitrotoga* on Solid Medium

Growth of *Nitrotoga* on solid medium was tested both on conventional mineral medium autoclaved with noble agar or sieve agarose, and on mineral medium prepared without phosphate addition prior to autoclaving. Phosphate-free medium was supplemented with noble agar, sieve or plaque agarose. Sterile filtered phosphate stock was added after autoclaving.

On conventional mineral medium supplemented with agar or sieve agarose, formation of milky-white, initially circular, later amoeba-shaped colonies was observed within ten days. Light microscopic analysis revealed large oval-shaped cells, morphologically identical to the most frequent contaminating *Betaproteobacteria* in batch cultures of SBE. Colony PCR with a general primer set targeting bacterial 16S rRNA genes and direct sequencing of PCR products was carried out. Thus analyzed colonies were affiliated with *Zoogloea resiniphila* strain PIV-3C2y (99.8 % identity, accession nr. AJ505854.1). No additional colony formation was observed during two months of incubation.

To test activity of SBE in medium where phosphate was added after autoclaving, phosphate-free mineral medium was autoclaved without solidifying agents, subsequently supplemented with 500x phosphate stock of pH 7.6, 7.8 or 8.0, yielding pH of 7.4, 7.6, and 7.8 respectively, and inoculated with SBE. SBE was active fastest in medium supplemented with phosphate stock of pH 8.0. Therefore, phosphate-free medium was prepared with agar, sieve and plaque agarose and supplemented with

phosphate stock of pH 8.0 after autoclaving. After plating of SBE culture, plates were incubated for almost one month, before grown colonies were analyzed by light microscopy. Most analyzed colonies consisted of oval-shaped microorganisms or cocci growing in chains. However, one of the analyzed colonies growing on plaque agarose plates consisted of cells with *Nitrotoga*-like morphology, and was streaked out on fresh plates. Growing *Nitrotoga*-like colonies were circular, slightly yellowish and almost transparent. They grew slowly and had a diameter of 0.2 – 1 mm after one month of incubation. Colony PCR on grown colonies confirmed affiliation with *Nitrotoga* (100% identity to Uncultured bacterium clone a-44 16S ribosomal RNA gene, accession nr. JX040372.2, 98.4 % identity to Ca. *N. arctica*). Liquid batch cultures were inoculated from the same *Nitrotoga* colonies that were used for colony PCR. Those liquid cultures oxidized NO_2^- within six to eight days. No other cells than *Nitrotoga* were observed in liquid cultures inoculated from picked colonies by FISH.

To test, whether growth of *Nitrotoga* on solid medium was due to phosphate addition after autoclaving or due to the agarose type (plaque agarose) used, normal mineral medium containing phosphate was autoclaved with plaque agarose, and *Nitrotoga* colonies were streaked out on these plates. After one month of incubation, no colony formation was observed.

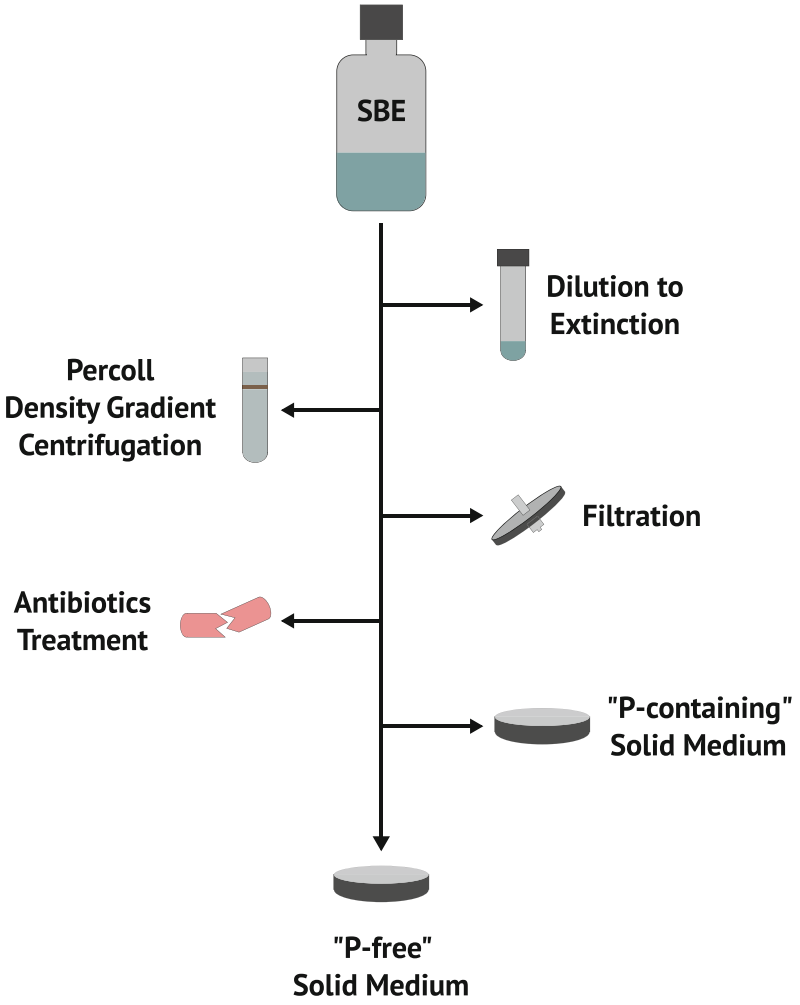

Figure 3.22 Overview over different approaches taken to obtain an axenic culture of *Nitrotoga*. "P-containing" solid medium refers to normal mineral medium supplemented with solidifying agent, "P-free" solid medium refers to mineral medium prepared without phosphate addition prior to autoclaving.

4 DISCUSSION

4.1 Comparison of Nitrite and Nitrate Measurement Methods

It had previously been observed that in Griess-based measurements of NO_3^-, NO_3^- concentrations are underestimated in presence of excess (> 3x) NO_2^- (Miranda et al. 2001; Beda & Nedospasov 2005; García-Robledo et al. 2014). While in most natural environments NO_3^- exceeds NO_2^- concentration, the opposite was the case for the experiments conducted in this thesis. Therefore, an adapted protocol for Griess-based NO_3^- measurement was developed, which is similar to a solution published by García-Robledo et al. (2014).

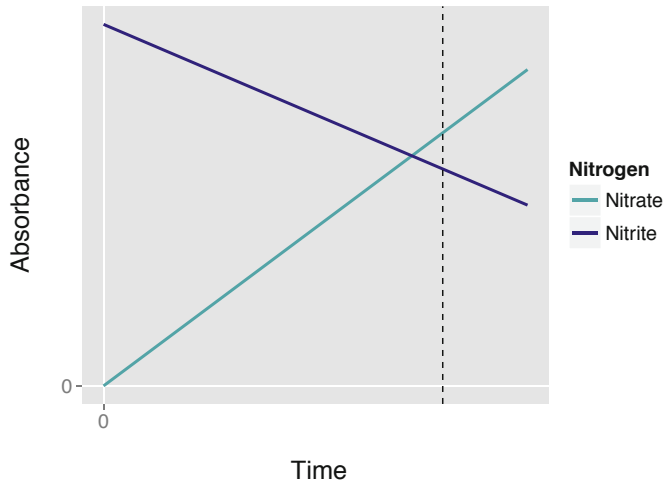

Figure 4.1 Schematic of absorbance behavior of equal concentrations of NO_2^- and NO_3^- respectively in presence of VCl_3 and Griess reagent. Dashed line represents time of absorbance measurement. Note that absorbance from NO_2^- decreases at a slower rate than absorbance from NO_3^- increases.

Both adaptations employ NO_2^- standards to account for differing absorbance of NO_2^- and NO_3^- in the sample in presence of VCl_3 (Figure 3.1). The different behavior of NO_3^- and NO_2^- likely stems from instability of the colored azo-dye complex in presence of VCl_3, and may additionally be influenced by the incubation temperature. Azo-dye from sample-NO_2^- is formed at the beginning of, and then gradually degrades during incubation. Azo-dye from reduced NO_3^- in the sample however is continually formed, as NO_3^- reduction is time dependent and VCl_3 does not reduce all NO_3^- in the sample during the employed incubation times. Degradation of azo-dye complex occurs at a slower rate than NO_3^- reduction (schematically shown in Figure 4.1 for equal concentrations of NO_2^- and NO_3^-).

Unlike García-Robledo et al. (2014), the method developed here does not employ sequential NO_2^- and NO_x (NO_3^-) measurement of the same sample aliquot on different plates, adapted chemical composition of VCl_3 solution, and incubation at 60 °C, but relies on the same procedure and chemicals as described by Miranda et al. (2001). A possible further development to reduce pipetting steps could be sequential measurement of NO_2^- and NO_x (NO_3^-) in the same plate. Samples and standards could be measured after addition of Griess reagent only (NO_2^- measurement), could then be mixed with VCl_3, incubated to allow for NO_3^- reduction, and again be measured in the same plate (NO_3^-/NO_x measurement). Applicability of this adaptation remains to be tested.

NO_2^- and NO_3^- measurements by IC are – unlike Griess-based measurements – independent of one another and additionally require fewer pipetting steps, which leads to lower cumulative measurement errors. However, IC measurements require more expensive equipment, measurements require more time than Griess-based measurements, and the NO_2^- detection limit in presence of Cl^- is high with the IC setup used in this study (0.1 mM in presence of 8.75 mM Cl^-). Use of different IC columns would likely permit a much lower detection limit of NO_2^-, making this method also suitable for experiments employing lower substrate concentrations.

4.2 Investigation of the Denitrifying Potential of *N. moscoviensis*

Although members of *Nitrospira*, and not *Nitrobacter*, represent the most important nitrite oxidizers in most known WWTP (Juretschko et al. 1998; Daims et al. 2001; Lücker et al. 2015) and are widely distributed in natural systems (Daims et al. 2001 and references therein), their physiology has attracted more attention only recently (e.g. Koch et al. 2014; Nowka et al. 2014).

In this study, the denitrifying potential of *N. moscoviensis*, which belongs to the ubiquitously distributed *Nitrospira* lineage II, was investigated to gain further insight into the physiology of this canonically nitrite oxidizing bacterium. For all tested compounds (acetate, branched AA, citrate, ethanol, fumarate, pyruvate, succinate), genes potentially allowing for internal utilization and – for some compounds – compound uptake are encoded in the recently sequenced *N. moscoviensis* genome (Hanna Koch, unpublished data). As unambiguous annotation of transporters can be difficult, and approximately 50 % (Hanna Koch, personal communication) of the predicted coding sequences in *N. moscoviensis* are as yet uncharacterized genes, selection of compounds to be tested was mainly based on indications for internal utilization.

4.2.1 Selection of Compounds to be Tested

While no specific transporters for TCA cycle intermediates and precursors (acetate, pyruvate, succinate, fumarate, citrate) were found in the genome, once inside the cell, they could directly be fed into the oxidative TCA cycle. Analogous to the respiratory chain proposed for *N. defluvii* (Lücker et al. 2010), electrons yielded from oxidation of these compounds could enter the respiratory chain of *N. moscoviensis* via succinate oxidation by succinate dehydrogenase (complex II). Additionally, electrons from NADH, which is generated in the oxidative TCA cycle, could enter the respiratory chain via complex I. Complex I translocates H^+ across the membrane, thereby contributing to the generation of a proton motive force (PMF). Subsequently, electrons could be transferred via complex II to the quinone pool. From there, electrons are likely directly transferred to NXR (Figure 4.2), as is typical for nitrate reductases (Simon & Klotz 2013). The interaction between NXR and the quinone pool might be facilitated by alternative NXR ɣ-subunits (Holger Daims, personal communication), which however remains to be elucidated.

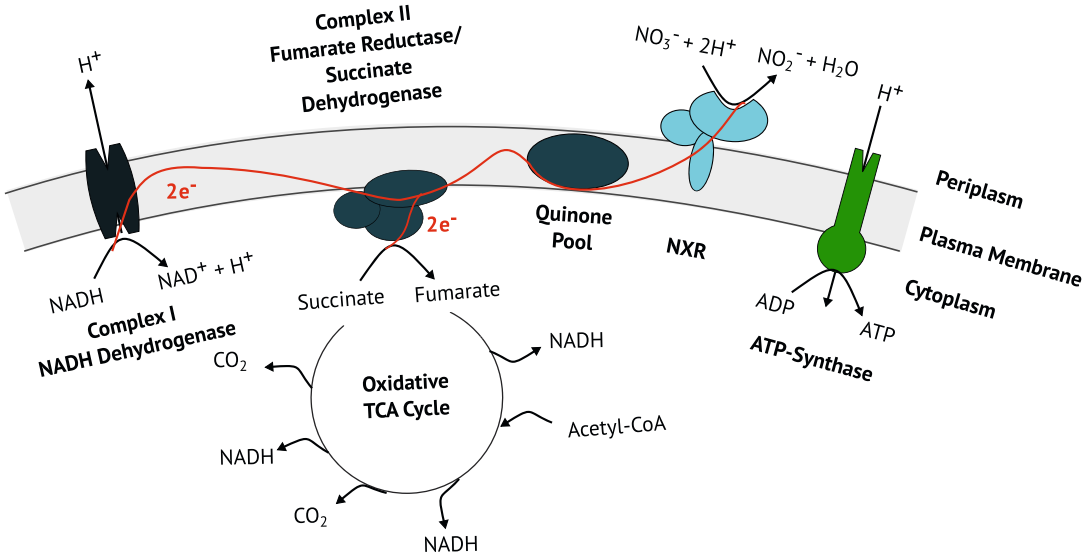

Figure 4.2 Schematic overview of possible entry into and electron flow within N. moscoviensis respiratory chain from TCA cycle to NO3- under anoxic conditions. Electrons from NADH generated in the oxidative TCA cycle could enter the respiratory chain at complex I. Electrons from succinate oxidation could enter via complex II, and be transferred to NO3- via quinone pool. Electron flow is depicted in red. Modified from Lücker et al. (2010).

Essentially the same pathway would be possible for branched AA, whose degradation product is acetyl-CoA, which again could be fed into the TCA cycle. *N. moscoviensis* encodes for at least a partial branched AA degradation pathway, and additionally – as well as *N. defluvii* (Lücker 2010) – for a branched AA transporter (Hanna Koch, unpublished data), indicating possible uptake of these AA from the environment.

Ethanol, as a membrane permeable compound, requires no dedicated transporter, and could be oxidized by the encoded cytoplasmic alcohol dehydrogenase, while concomitantly reducing NAD+ to NADH. From NADH, electrons could then be transferred to the membrane bound NADH dehydrogenase (complex I), fed into the quinone pool, and follow the electron transport chain described above.

4.2.2 Reasons for Lack of Utilization of These Compounds

The fact that none of the tested compounds (acetate, pyruvate, succinate, fumarate, citrate, branched AA, and ethanol) could be used for energy generation under the conditions employed can have several reasons.

First, the used *N. moscoviensis* biomass could have been inactive. This however was not the case, as biomass incubated with formate and NO_3^- readily depleted formate and reduced NO_3^- to NO_2^- (see discussion below).

Second, the reactions may not be energetically possible. The transfer of electrons from NADH to NO_3^- (for ethanol, but also for formate) theoretically yields more energy than NO_2^- oxidation (approximately ΔG_0'=-143 kJ*mol^{-1} and -77 kJ*mol^{-1} respectively, calculated from ΔE_0' listed in Madigan et al. 2012). Additionally, formate oxidation coupled to NO_3^- reduction readily occurs (see discussion below). Coupling of succinate oxidation to NO_3^- reduction (for TCA cycle intermediates and precursors) is energetically less favorable, but nonetheless yields almost the same energy as NO_2^- oxidation (approximately ΔG_0' = -75 kJ*mol^{-1} and -77 kJ*mol^{-1} respectively). Therefore, all compounds tested would likely yield sufficient energy to generate ATP and reducing equivalents. Nonetheless, mere comparisons of ΔG_0' do not reflect the actual energy yield from reactions *in-vivo* (e.g Caldwell et al. 2008). In this specific case, H^+ formation from NO_2^- oxidation may not equal the proton pumping activity of complex I, longer reverse electron transport necessary for regeneration of reducing equivalents under NO_2^- oxidizing conditions may diminish (Figure 4.3), and synthesis of additional enzymes needed for the respective pathways may further affect the actual energy yield.

Third, the respective compounds might not have been able to enter the cell due to lack of membrane permeability or expressed transporters. This scenario seems likely for the tested TCA cycle intermediates and precursors (acetate, pyruvate, succinate, fumarate, and citrate), as those compounds are central metabolites and thus, once localized in the cytoplasm, *N. moscoviensis* should have been able to use them. As all are charged molecules, they cannot easily diffuse through membranes, therefore, lack of utilization of these compounds likely is due to the lack of a transporter encoded in the genome, or a lack of expression thereof. Interestingly, some other *Nitrospira* strains are capable of pyruvate uptake. *N. defluvii, N. marina* and *N. marina Ecomares* have been shown to grow mixotrophically with pyruvate and NO_2^- (Watson et al. 1986; Spieck et al. 2006; Keuter et al. 2011). In a study performed by Daims et al. (2001), *Nitrospira* from activated sludge were also shown to aerobically incorporate ^{14}C-pyruvate in presence of NO_2^-, however, under anoxic conditions in presence of NO_3^-, no pyruvate incorporation was observed. This apparent discrepancy between

utilization of this central metabolite under oxic, but not anoxic conditions may reflect faster uptake of pyruvate by heterotrophic, e.g. denitrifying bacteria, as compared to *Nitrospira*.

Fourth, the tested compounds may have entered the cells, but could not be used for energy generation. Both branched AA and ethanol likely could enter the cytoplasm of *N. moscoviensis*, either via the encoded branched AA transporter (unless it was not expressed or is not functional, which cannot be excluded), or by diffusion respectively. The degradation pathway for valine, leucine and isoleucine in *N. moscoviensis* is largely present, however, according to KEGG metabolism maps, some canonical enzymes are missing. This incomplete degradation pathway may preclude utilization of branched AA for energy generation. Branched AA could however still be assimilated when energy is available from other pathways (e.g. NO_2^- oxidation). Possible uptake and assimilation under nitrifying conditions could be assessed by concomitant incubation with radiolabeled branched AA, followed by MAR-FISH. Such an experiment has previously been performed by Gruber-Dorninger et al. (2015), where valine incorporation into biomass of *Nitrospira* lineage I was examined by MAR-FISH in activated sludge. In those incubations however, no incorporation of ^{14}C-valine was observed. Lack of ethanol utilization by *N. moscoviensis* for energy generation may be due to substrate specificity of the encoded alcohol dehydrogenase for alcohols other than ethanol. One intriguing possibility could be oxidation of methanol by the encoded alcohol dehydrogenase in *N. moscoviensis*. This could enable yet another possible syntrophy (or detoxification mechanism) between AOB and NOB, as AOB can – although without growing – oxidize methane to methanol (Bock & Wagner 2006). Whether the alcohol dehydrogenase in *N. moscoviensis* can accept methanol however is purely speculative and its true substrate(s) remain to be determined.

4.2.3 Coupling of Formate Oxidation and Nitrate Reduction in *N. moscoviensis*

In control incubations with formate and NO_3^- to assess whether biomass was active, substrates were readily utilized. While it had previously been shown that *N. moscoviensis* is capable of formate oxidation both under aerobic and anoxic conditions, with O_2 and NO_3^- respectively as electron acceptor respectively (Hanna Koch, unpublished data), the outcome of the here conducted control incubations is interesting nonetheless.

Formate oxidation and NO_3^- reduction theoretically occur equimolarly – formate oxidation yields, and NO_3^- reduction requires two electrons. Electrons from formate are transferred to NADH by the cytoplasmic formate dehydrogenase, and are subsequently channeled into the respiratory chain at complex I (Figure 4.3, A). However, in both conducted experiments, while formate was consumed completely, NO_3^- was not depleted. The decoupling of formate oxidation and NO_3^- reduction observed after initial stoichiometric conversion (Figure 3.4) might be due to presence of O_2, which could serve as energetically more favorable electron acceptor than NO_3^-. However, as control incubations containing only NO_2^- did not oxidize NO_2^- (experiment 1), or only slightly after prolonged incubation (experiment 2), this scenario seems unlikely. Alternatively, decoupling of formate oxidation and NO_3^- reduction could be due to channeling of electrons from formate into the reductive TCA cycle for fixation of CO_2 (Figure 4.3, B). In this scenario, electrons from NADH would either directly be used for CO_2 fixation, or fed into the quinone pool via complex I, and transferred onto fumarate to regenerate succinate. The latter electron flow could even produce a (weak) PMF over the membrane due to the H^+ pumping activity of complex I, thereby allowing for ATP production.

The observed decoupling of NO_3^- reduction and formate oxidation suggests that *N. moscoviensis* can fix CO_2 and thus possibly grow under the applied conditions. Use of formate as sole substrate for energy generation and growth under oxic conditions has previously been shown for *Nitrospira japonica*

(lineage II) by cultivation in formate-containing medium (Ushiki et al. 2013). Additionally, under aerobic conditions without addition of any other energy source, *Nitrospira* lineage I in activated sludge incorporated ^{14}C-labeled formate into biomass (Gruber-Dorninger et al. 2015). Interestingly, formate utilization seems to be a widespread feature of NOB, as not only some *Nitrospira*, but also some *Nitrobacter* isolates and *Nitrolancea hollandica* can utilize this compound (Starkenburg et al. 2006; Sorokin et al. 2012 and 2014), which represents an energetically cheaper way to regenerate NADH than via reverse electron flow from NO_2^- oxidation (Figure 4.3, C). Additionally, formate utilization for energy generation should yield more ATP both aerobically and anaerobically than oxidation of NO_2^- (approximately ΔG_0' = -143 kJ*mol^{-1} and -220 kJ* mol^{-1} for anaerobic and aerobic formate oxidation respectively, and -77 kJ* mol^{-1} for NO_2^- oxidation). While comparisons of ΔG_0' do not necessarily represent energy yields *in-vivo*, biomass yield from formate oxidation under anoxic (with NO_3^- as terminal electron acceptor) and aerobic conditions, in comparison to yields from NO_2^- oxidation could give further insights into the actual efficiency of these alternative metabolic pathways.

Interestingly, although *N. moscoviensis* – just as *N. defluvii* (Lücker et al. 2010) – encodes for a nitrite reductase (NirK, Hanna Koch, unpublished data), catalyzing the conversion of NO_2^- to NO, this likely did not occur in large amounts during the conducted experiments, as NO_2^- was nearly stoichiometrically formed from NO_3^-. Large-scale respiratory (or dissimilatory) NO_2^- reduction to NO could also lead to accumulation of NO in toxic amounts due to the lack of a known NO-reductase encoded in the genome of *N. moscoviensis* (Hanna Koch, unpublished data). NirK-expression and/or activity may therefore be tightly regulated. Starkenburg et al. (2008) suggested that NirK-expression and NO-levels in *Nitrobacter* have a mostly regulatory function in diverting the direction of the electron flow from O_2 to NADH for CO_2 fixation under NO_2^- oxidizing conditions. While the respiratory chains in *Nitrospira* and *Nitrobacter* differ strongly – especially regarding the NXR and the terminal oxidase (Lücker et al. 2010), where the latter was suggested to be inhibited by NO in *Nitrobacter* (Starkenburg et al. (2008) – NirK-expression and NO-levels could also represent an important regulatory mechanism in *Nitrospira*. Interestingly, Courtens et al. (2015) recently found that NO_2^- oxidation in *Nitrospira*-dominated activated sludge was stronger inhibited than in *Nitrobacter*-dominated communities, which may indicate a different effect of NO on these two genera. Further elucidation of the intriguing role of NO in *Nitrospira* awaits investigation.

The ability to oxidize formate could be advantageous for *N. moscoviensis* in many ways. Not only could *N. moscoviensis* gain energy and possibly grow during periods of O_2 depletion by NO_3^- reduction coupled to formate oxidation, but formate is also a cheaper way to regenerate reducing equivalents than by reverse electron flow from NO_2^- oxidation. Additionally, it is also tempting to speculate about a potential syntrophy between aerobically and anaerobically growing *N. moscoviensis* populations. In soil particles or biofilms, where O_2 is rapidly depleted at the outer layers (Stewart and Franklin 2008 and references therein), cells in oxic layers could oxidize NO_2^- to NO_3^-, which could – in presence of formate e.g. supplied by fermentation– in turn be reduced to NO_2^- by cells growing in anoxic pockets. Such a substrate regeneration loop was experimentally shown for *Nitrobacter* strains grown in anoxic medium containing pyruvate, NO_3^- and NO_2^-. This setup contained an O_2-permeable tubing, which sustained an aerobic, NO_2^- oxidizing biofilm, while the anaerobically growing bulk-medium population of *Nitrobacter* reduced NO_3^- to NO_2^- and oxidized pyruvate (Freitag et al. 1987).

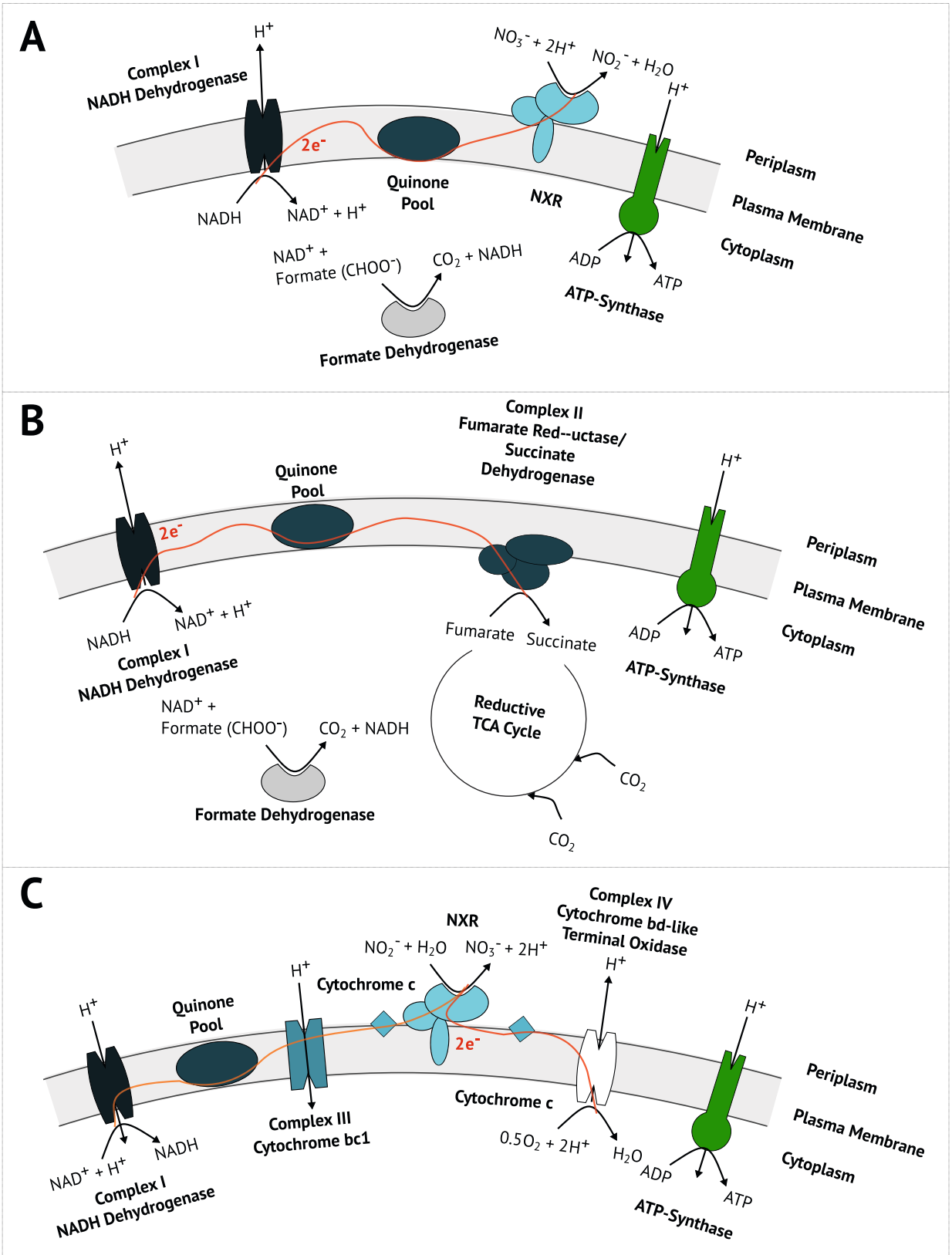

Figure 4.3 Schematic overview of entry into and electron flow within *N. moscoviensis'* respiratory chain. A: Anaerobic energy generation from formate and NO_3^-. Electrons from formate oxidation are transferred to NADH and channeled into the respiratory chain at complex I. B: CO_2 fixation. Electrons from formate oxidation could be used for CO_2 fixation via the reductive TCA cycle. C: Energy generating pathway and production of reducing agents under NO_2^- oxidizing conditions. Electron flow is depicted in red, reverse electron flow in orange. Modified from Lücker et al. (2010).

4.2.4 Summary

Recently, it has become more and more apparent that *Nitrospira* are not strictly dependent upon their canonical substrates NO_2^- and O_2. Indeed, *N. moscoviensis* can utilize alternative substrates for energy generation, both relying on nitrogen redox-reactions (coupling of formate oxidation to NO_3^- reduction) and utilizing substrates independent of nitrogen cycling, by aerobic oxidation of H_2, which was shown to also sustain growth (Koch et al. 2014). As these findings regard only one species, it is very well possible that this is only the tip of an iceberg of physiological versatility in *Nitrospira*.

4.3 Microdiversity of Nitrifying Bacteria

In order to investigate the efficiency and applicability of the workflow adapted by Tae Kwon Lee for sorting active nitrifier microflocs, a pre-study was conducted, where activated sludge from WWTP Klosterneuburg was incubated in presence of NH_4^+ and either ^{13}C-bicarbonate or D_2O for labeling of active microorganisms. Nitrifier abundance was quantified by FISH before and after incubation, and optimal disruption conditions were assessed. For each treatment, 20 microflocs were sorted randomly (no regard to nitrifier and activity Raman signatures) and 20 were sorted only if they showed both nitrifier and activity signatures. MDA was performed, and MDA products screened with several different primer sets. Based on the screening outcome, 24 MDA products were selected for microfloc metagenome sequencing.

4.3.1 The Study System: FISH-Defined Bacterial Populations in WWTP Klosterneuburg

In the study system chosen for the initial workflow assessment for investigation of nitrifier microdiversity, three AOB populations and one NOB population were detected by FISH. For the detected AOB populations, an overlapping hybridization pattern between Cl6a192 and Ncom1025 was found, which we had previously also observed in a different WWTP (Gruber-Dorninger et al. 2015). Those observations raise concerns regarding probe specificity and call for a re-evaluation of those frequently used AOB probes. Because of this lacking specificity, no certain phylogenetic affiliation of the observed AOB populations can be inferred by FISH. However, likely *Nitrosomonas* belonging to the *N. oligotropha/ureae* cluster (targeted by Cl6a192) and *N. eutropha/europaea/halophila* and *"Nitrosococcus" mobilis* cluster (targeted by NEU) were present in activated sludge from WWTP Klosterneuburg. The only detected NOB were members of *Nitrospira* lineage I (targeted by Ntspa1431). All nitrifier populations detected by FISH grew as microcolonies (Figure 3.9), making this study system suitable for the employed approach for the assessment of nitrifier microdiversity.

The overall abundance of nitrifiers in WWTP Klosterneuburg (approximately 10 % of bacterial biomass, as measured by quantitative FISH) was typical for activated sludge (Juretschko et al. 1998; Larsen et al. 2008; Lücker et al. 2015). No statistically significant colocalization was observed in 2-dimensional images of AOB and *Nitrospira*. One possible explanation for the lacking colocalization could be the resolution of AOB and *Nitrospira* probes used for those analyses. Previously, we could show for a different WWTP that multiple coexisting *Nitrospira* populations within one lineage show dramatically different colocalization patterns in regard to different AOB subpopulations (Gruber-Dorninger et al. 2015). Such an analysis however is not possible without rRNA gene sequence information and specific probes for the nitrifier populations present in the respective system. Therefore it remains to be tested, whether specific colocalization patterns between populations exist. Nonetheless, when focusing through sludge flocs, AOB and *Nitrospira* colonies were frequently observed close to each other in three dimensions, indicating a possible syntrophic interaction at least between some of the nitrifiers present.

Interestingly, *Micavibrio*-like bacteria occurred in all native (non-incubated) sludge samples investigated. Those bacteria have previously been suggested to be specific predators of *Nitrospira* lineage I (Dolinšek et al. 2013). Indeed, FISH signals from *Micavibrio*-like cells were exclusively found within, or in close proximity of *Nitrospira* colonies in activated sludge from WWTP Klosterneuburg, supporting a close interaction between those organisms (Figure 3.8). Because *Micavibrio*-like bacteria occur so tightly associated with *Nitrospira*, it is likely that genomic information on *Micavibrio*-like bacteria can be obtained from infected, sorted, and sequenced *Nitrospira* microcolonies, possibly yielding more information on this interesting microbial interaction.

4.3.2 Raman-Activated Microfloc Sorting to Investigate Nitrifier Microdiversity: A Pre-Study

4.3.2.1 Community Composition after Incubation

The community composition before and after incubation with heavy isotopes (^{13}C-bicarbonate and D_2O respectively) was almost identical to native sludge regarding the presence and abundance of nitrifier populations. However, no *Micavibrio*-like bacteria were detected by FISH after the 7-day incubation. Interestingly, in the second incubation experiment performed to determine minimum incubation time and D_2O concentration to observe label-incorporation in Raman spectra (see section 2.17.7 and 4.3.3), *Micavibrio* was still found after seven days of incubation. Therefore, it seems unlikely that loss of *Micavibrio* in this pre-study stems from isotope toxicity (Filiou et al. 2012 and references therein). The main difference between the two experiments was (apart from the sampling time) that in the second incubation, flasks were only loosely closed, allowing for air exchange, while flasks in the first incubation were closed airtight by rubber stoppers. This may have caused suboptimal O_2 availability in the first incubation and directly or indirectly resulted in loss of *Micavibrio*-like bacteria.

4.3.2.2 Nitrifier Representation in Sonicated Microflocs

FISH-defined nitrifier populations were detected in sonicated microflocs in similar ratios to each other as in non-sonicated sludge. This is in accordance with Larsen et al. (2008), who previously showed that in contrast to other bacteria, only a small fraction of nitrifier microcolonies disperse upon dissection with shear force. Nonetheless, as the resolution of the used FISH-probes was low, sonication bias against specific nitrifier strains cannot be excluded. Some of the FISH-screened microflocs contained both AOB and *Nitrospira* microcolonies, and almost all observed microflocs contained some non-nitrifier cells adhering to the respective nitrifier microcolonies (Figure 3.10). This allows, unlike for most other "SCG" approaches, direct information on potential interactions between nitrifiers and their surrounding cells based on their genome content (Blainey 2013). For example, repeatedly occurring non-nitrifiers in the "mini-metagenomes" from sorted microflocs could indicate a symbiosis, or could even represent so far unknown nitrifiers.

4.3.2.3 PCR Screening Success and Dependence on Amplicon Length

Screening PCRs of MDA products from sorted microflocs only gave consistent results between screening replicates, when template was vigorously mixed before setting up PCR reactions. This may be due to the hyperbranched structure of MDA products (e.g. Hutchison & Venter 2006), which could cause uneven distribution of DNA unless thoroughly mixed.

A possibly related observation is that short amplicons were significantly more readily amplified than long ones (49 and 35 successful PCRs targeting bacterial 16S rRNA gene for short and long amplicons respectively, and 16 and five for *Nitrospira* 16S rRNA gene). This might be due to inaccessibility of long stretches of DNA because of hyperbranched DNA strands. Alternatively, primer coverage might differ between short and long amplicons of the same gene and thus yield different amplification patterns. Observed inconsistencies between positive screening PCRs for functional marker genes (*amoA* or *nxrB*) and specific 16S rRNA genes (AOB and *Nitrospira* specific) may also occur for the same reason. Another cause could be uneven amplification of DNA during MDA reactions (e.g. Stepanauskas 2012), which should however be somewhat counteracted by the presence of multiple, presumably clonal chromosomes from nitrifier microcolonies as MDA templates (Dichosa et al. 2012; Fitzsimons et al. 2013). For the planned sorting, PCR screening and sequencing of 100 microflocs, it is therefore advisable to use at least two primer pairs per screening target (general bacterial DNA, AOB or *Nitrospira* DNA), targeting short amplicons to avoid false negatives due to branched structure of the MDA product or uneven amplification.

4.3.2.4 No Significant Enrichment of Nitrifiers Between Sorting Strategies, But Due to Presence of Cytochrome Signature

Interestingly, no significant enrichment in nitrifier-positive PCR screens was found between random and targeted sorting. While the number of sorted microflocs was small, and therefore, the power of the statistical test is relatively low that outcome nonetheless implies that the main advantage of sorting microflocs according to their Raman spectra is, to determine whether a given microfloc was active, and not to specifically target nitrifiers. Nonetheless, for MDA products from microflocs, which showed cytochrome signatures, nitrifier PCR screens were significantly more often positive than for MDA products from microflocs lacking cytochrome signatures. These observations imply that the main selection for nitrifiers already occurred during sonication, and sonication already enriched for nitrifiers in the specific size class that is suitable for microfloc sorting. This is in accordance with Larsen et al. (2008) who found that dissection of sludge specifically enriches for nitrifying microcolonies in the flocculated fraction.

4.3.2.5 Discrepancies Between Cytochrome Signature and Nitrifier Screening Outcome

In four cases, nitrifier-positive PCR results were obtained, although the Raman spectra of the sorted microflocs did not show cytochrome signatures. This could be due to the spatial resolution of Raman spectra as employed in this study. Spectra typically were acquired only from a small area of the total sorted microfloc, and, as the microflocs consist not only of nitrifiers, but several cell types (Figure 3.10), it is possible that the actual nitrifier was missed when acquiring Raman spectra. Alternatively, this could be due to DNA from lysed nitrifiers adhering to sorted microflocs.

In ten cases, PCR screens for microfloc MDA products were *only* positive for general bacterial screens, although the respective sorted microfloc did show pronounced cytochrome signatures. This could have several reasons. First, the used nitrifier-specific primers might not target the specific nitrifier strain present in the sorted microfloc. Second, non-nitrifier cells with pronounced cytochrome signatures might have wrongly been identified as putative nitrifiers based on their Raman spectra. While cytochrome signatures are typical for nitrifiers (Pätzold et al. 2008), some non-nitrifier species also show strong cytochrome signatures, although rarely in such intensity as observed for nitrifiers (Christoph Böhm, unpublished data). Third, nitrifiers present in the sorted microfloc could be more resistant to cell lysis than the adhering, “contaminating” bacteria. Lysis efficiency can generally be problematic for SCG, as the volume of lysis chemicals has to be minimal (Stepanauskas 2012), and nitrifiers are difficult to lyse, likely due to extracellular polymers (for AOB: Juretschko et al. 1998; Foesel et al. 2008, for NOB: Hanna Koch and Holger Daims, personal communication). Whether insufficient lysis of nitrifiers is an obstacle for amplification, could be assessed by trying different lysis methods (e.g. bead beating, freeze/thaw cycles, alkaline lysis, enzymatic lysis, and combinations thereof) and quantification of nitrifier DNA fraction as compared to overall DNA by quantitative PCR or deep sequencing of general bacterial 16S rRNA gene amplicons.

4.3.2.6 PCR Screening and Raman Signatures Compared to Contig Megablast

Overall, PCR screening results fit the initial microfloc metagenome sequencing results obtained from JGI. However, strangely, when PCR screens were positive for both AOB and *Nitrospira*, contigs were only assigned to *Nitrosomonadales* and never to both *Nitrosomonadales* and *Nitrospira*. While this may change once all sequences are investigated in more depth and assigned a more refined taxonomy, the dominance of *Nitrosomonadales*-related contigs is nonetheless striking. Interestingly, the diversity in sequenced MDA products containing *Nitrospira* is (on an order level) generally higher than for MDA products containing *Nitrosomonadales*. Whether this is an artifact of this fast, initial analysis, or whether there is a biological reason behind this observation (e.g. sequenced *Nitrospira* microcolonies generally containing more other cells) remains to be investigated.

In order to allow the finding of nitrifiers that are not detected by the primers used for PCR screening, three microfloc MDA products were sequenced that did not yield any AOB/*Nitrospira* positive PCR screens, but whose Raman spectra showed both cytochrome and activity signatures. While one sequencing run failed, for the remaining two microflocs contigs assigned to *Nitrosomonadales* were obtained. These could represent novel AOB within this order, which are not targeted by the used primer sets, or alternatively, the negative screening outcome with the used primers could be due to difficulties in amplification of branched MDA products and/or uneven amplification.

4.3.3 Influence of D_2O Concentration and Incubation Time on Microfloc Labeling

In the experiment designed to identify minimum incubation time and D_2O concentration necessary for observing D-C peaks in nitrifier Raman spectra, pronounced bias in nitrifier community composition after sonication was observed. Nitrifier microcolonies in the activated sludge sample from April 2014 seemed to be overall less stable than from other sampling time points.

No FISH signals from the AOB population targeted by probe Cl6a192 were observed after sonication, and abundance of *Nitrospira* as compared to undisrupted sludge was strongly decreased. This change in sonication resistance could be due to changed conditions in WWTP Klosterneuburg. While no change in operational mode was introduced, or changes in overall N_r-discharge were observed in WWTP Klosterneuburg at the time of sampling (Karl Weiss, personal communication), changes in climate or wastewater influx composition may not only affect microorganisms present (e.g. Günther et al. 2012), but could also affect floc rigidity. The variance in sludge and microcolony rigidity between samples from different time points shows the importance of thorough FISH-based screening before sorting of microflocs, as selective loss of populations after sonication would certainly diminish the chances of obtaining microdiverse nitrifiers.

Label incorporation into putative nitrifier microflocs was observed after two days of incubations with 50 % D_2O concentration. Abundance shifts of entire *Nitrospira* lineages, but also sublineage populations upon high or low substrate concentration had previously been observed (Maixner et al. 2006; Gruber-Dorninger et al. 2015). Therefore, short incubation times will likely allow for investigation of a close to "natural" community composition, as incubation for several days might induce shifts in strain abundances and eventually reduce the microdiversity present, as the investigated community adapts to the laboratory environment.

4.3.4 Summary and Outlook

Some aspects of the workflow still can be improved for the planned sorting and metagenome sequencing of 100 nitrifier microflocs. Based on the initial results of, and insights from the already conducted experiments, the following steps are suggested.

A thorough assessment of nitrifier diversity based on functional genes (*amoA* and *nxrB*) as well as specific 16S rRNA genes would help to identify a suitable WWTP for the planned large-scale experiment. Ideally, this WWTP should contain sufficient, but not too high nitrifier microdiversity on marker gene level, to obtain several closely related genomes for both AOB and NOB, while not undersampling, which could hamper understanding of the system. Selecting a system with moderate microdiversity on marker gene level is especially important, as identical marker gene sequences may hide genome-wide and functional microdiversity (Bavykin et al. 2004; Jaspers & Overmann 2004; Peña et al. 2010). Additionally, a thorough evaluation of different lysis protocols to quantitatively obtain nitrifier DNA might help to gain even more insight into microdiverse nitrifiers, due to better genome recovery from sequenced microfloc metagenomes.

After each sampling of activated sludge, nitrifier presence and microcolony stability has to be carefully assessed by FISH in order to not exclude certain populations from analysis due to loss during sonication.

Incubations should be continuously aerated to avoid O_2 depletion and therefore suboptimal conditions. Optimizing incubation conditions might allow for even faster labeling of the active target population, and therefore investigation of a system closer to *in situ* community composition.

An additional layer of biological information could be obtained by sorting *active* microflocs from differently incubated biomass, e.g. from incubations at low and high NH_4^+ concentration, or from incubations at different temperatures. Potential differences between the active nitrifiers sorted from different incubations may give additional information on, and allow for more detailed predictions of their physiology and respective ecological niches. Incubations should be carried out with D_2O and not ^{13}C-bicarbonate as labeling strategy, as putative nitrifiers can show inherent peaks in the region of ^{13}C-phenylalanine. For all treatments, incubations without D_2O addition, which are otherwise treated identically, should be included for comparison of Raman spectra to exclude the possibility of D-C like peaks occurring in unlabeled cells. Additionally, these incubations could serve to assess a possible isotope effect on nitrifiers and other microorganisms in D_2O incubations.

After incubation with D_2O, nitrifier presence and abundance should be re-assessed, and sonication again optimized. After sorting, lysis and MDA of microflocs, PCR screenings should be ideally carried out with primer sets for short amplicons. Since screening PCRs with nitrifier-specific functional and 16S rRNA genes sometimes gave inconsistent results, at least two different genes for AOB and NOB respectively should be included in screenings. Additionally, screening PCRs targeting *Micavibrio*-like bacteria could be included to specifically select *Nitrospira* microcolonies infected with *Micavibrio* for microfloc metagenome sequencing. Regarding the selection of MDA products for sequencing, those which show positive PCR screens both for AOB and NOB are expected to give most information on possible interactions between those syntrophic guilds, and are therefore among the most interesting. Nonetheless, as novel nitrifiers may not be targeted by the used primer sets, while showing cytochrome spectra, these may be discovered by sequencing of MDA products from microflocs with cytochrome signatures and only positive PCR screens for general bacterial 16S rRNA genes.

While much work remains to be done, initial results from the conducted pre-study to investigate workflow efficiency are promising. It remains to be seen, how well the sequences obtained can be binned (i.e. assigned to one organism), how much genomic information they contain (genome coverage and fragmentation), and how large the fraction of nitrifier DNA in raw sequencing reads is (Stepanauskas 2012; Blainey 2013). However, once all technical questions are resolved, this approach for investigation of a coherent functional group in a complex system will allow for addressing some of the most basic questions in ecology and microbiology in more detail:

Which functional differences lie beyond near identical marker genes?
How does microdiversity evolve and how is it sustained?
How do (micro-) organisms interact?
How does this interaction and adaptation to specific microniches affect their evolution?

4.4 Enrichment, Characterization and Isolation of Novel Nitrite Oxidizing Bacteria

Nitrite oxidizing bacteria were enriched from the municipal WWTP Klosterneuburg, which was also investigated in course of the microdiversity project. In two different cultivation lines, *Nitrospira* lineage I and *Nitrotoga* were enriched.

4.4.1 Selection for Different Nitrite Oxidizers: A "Bottle Effect"

Interestingly, two different NOB were enriched under almost identical conditions. The only difference between enrichments of *Nitrospira* and *Nitrotoga* was the incubation flask and culture volume used – once 100 ml in Erlenmeyer flasks (*Nitrospira* enrichment) and once 40 ml in Schott bottles (*Nitrotoga* enrichment). The type of cultivation flask was initially changed to exclude potential input of organics from laboratory air, and thereby reduce growth of heterotrophic contaminating cells.

Cultivation in Schott bottles consistently selected for *Nitrotoga* and against *Nitrospira*, even though *Nitrospira* lineage I were the only FISH-detectable NOB in the inoculum. It has previously been observed that cultivation conditions strongly affect the enriched community. Temperature was shown to be one major determinant in enrichment of *Nitrotoga*, *Nitrospira* or *Nitrobacter* from a municipal WWTP, under otherwise identical conditions (Alawi et al. 2009). NO_2^- concentration is also a known selective factor in enrichmen of NOB (Spieck & Lipski 2011). For AOB enrichments, Lebedeva et al. (2005) observed bottle-dependent growth, where AOB stably grew in tubes but not in flasks, which was attributed to O_2 availability. The opposite effect was observed by Spieck et al. (2006) for NOB, who noted that *Nitrospira defluvii* grew better in Erlenmeyer flasks than in glass tubes. This effect was also attributed to different gas diffusion between vial types, where the larger medium surface area in Erlenmeyer flasks was assumed to allow for higher O_2 concentration (Spieck et al. 2006). However, *Nitrospira* lineage I were later found to be more susceptible to high O_2 concentrations than *Nitrospira* lineage II (Park & Noguera 2008), and *N. defluvii* was shown to lack canonical reactive oxygen species (ROS) defense mechanisms (Lücker et al. 2010). Therefore, an unknown, other factor than O_2 availability may have promoted growth of *N. defluvii* in Erlenmeyer flasks as opposed to glass tubes.

In the present study, while temperature, NO_2^- concentration and medium composition were constant, the overall medium volume (100 ml and 40 ml) and surface-to-volume ratio between Erlenmeyer flasks and Schott bottles could have selected for different NOB. Reduced air exchange in Schott bottles closed with screw caps may have caused lower O_2 concentrations (due to respiration), as compared to Erlenmeyer flasks closed with loose caps. Continuous air exchange in Erlenmeyer cultures may have caused higher input of volatile organics from the surrounding laboratory air, thereby selecting directly or indirectly against *Nitrotoga* or for *Nitrospira* lineage I. Additionally, it cannot be excluded that the glass composition itself influenced the NOB community present.

4.4.2 Loss of *Nitrospira* Upon Prolonged Cultivation

Why the *Nitrospira* enrichment was not stable in its degree of enrichment cannot be answered with any certainty. It seems unlikely that the decrease in *Nitrospira* was due to a specific soluble inorganic nutrient, which had only been present in the WWTP inoculum, as washing of the entire culture and further cultivation did not cause a strong decrease in *Nitrospira* numbers. It is however possible that a specific mutualistic interaction partner was lost upon transfers, leading to a decrease of *Nitrospira*. Interestingly, initially, micromanipulated colonies from *Nitrospira* enrichments did oxidize NO_2^-, but were later overgrown by other microorganisms. Multiple washing steps of the sorted microcolony might help to remove accidentally co-sorted cells, however if those contaminating cells grow within *Nitrospira* colonies, which cannot be excluded, they likely cannot be removed by simple washing.

4.4.3 Enrichment, Characterization and Isolation of the Mesophilic Candidatus *Nitrotoga fabula*

Within one to two weeks after shifting the cultivation flasks from Erlenmeyer flasks to Schott bottles, *Nitrotoga* outcompeted *Nitrospira*. *Nitrotoga* present in Schott bottle enrichment was closest related to a *Nitrotoga* sequence obtained from a WWTP (accession nr. JX040372.2, Figure 3.14). While *Nitrotoga* can be the only detectable NOB in municipal WWTPs (Lücker et al. 2015), *Nitrotoga* likely was not of major importance in WWTP Klosterneuburg, as it could not be detected by FISH in any of the activated sludge samples collected between October 2013 and April 2014. Interestingly, all *Nitrotoga* 16S rRNA gene sequences obtained fell into a sublineage of the genus without any previous enrichment cultures available.

The most abundant contaminants in the *Nitrotoga* enrichments were other *Betaproteobacteria* (targeted by Bet42a probe). Those contaminants likely belonged to the genus *Zoogloea*, the most frequently obtained sequence (apart from *Nitrotoga*) and only other *Betaproteobacterium* (apart from *Nitrotoga*) in the clone library prepared from amplified 16S rRNA genes of the enrichment. Additionally, cells from *Zoogloea* colonies, which grew on agar, sieve and plaque agarose plates, had the same morphology as the betaproteobacterial contaminants observed by FISH.

4.4.3.1 MAR-FISH: *Nitrotoga* is the Most Important NOB of the Enrichment

In MAR-FISH, no cells except for *Nitrotoga* were associated with silver grain formation. Therefore, *Nitrotoga* certainly is the most important NOB present in the enrichment. Nonetheless, it cannot be excluded that other NOB were present in the enrichment. This is both because FISH has a detection limit of approximately 10^3 – 10^4 cells*ml^{-1} (Amann et al. 1995), and silver grain formation was only observed on top of several, but not single *Nitrotoga* cells. Single, sporadic cells of other NOB would likely not have been associated with silver grains and thus not detected.

4.4.3.2 Raman: *Nitrotoga* Show Characteristic Nitrifier Signatures

All Raman spectra from *Nitrotoga*-like cells had pronounced cytochrome signatures, which are indicative of nitrifiers (Pätzold et al. 2008), a characteristic that was exploited in the microdiversity project described above. As observed in previous studies, cytochrome intensity decreased with the number of measurements, likely due to photo-oxidation (Pätzold et al. 2008; Okotrub & Surovtsev 2014). Additionally, a peak in the region of 2200 cm^{-1} was observed during the first measurement of *Nitrotoga*-like cells, which disappeared after repeated measurements, likely due to bleaching. That observation shows the importance of including controls for Raman-based studies employing stable isotopes as activity markers, as D-C peaks from incorporation of D_2O into biomass also lie in this region (Berry et al. 2015). Without knowledge of Raman spectra of unlabeled cells, the observed bleachable peaks in Raman spectra of *Nitrotoga*-like cells at 2200 cm^{-1} might wrongly be interpreted as activity marker in such studies.

For the most abundant, oval-shaped contaminants of the enrichment, no cytochrome signatures were observed, which further supported the assumption that they were not NOB.

4.4.3.3 Substrate Tolerance of Enriched *Nitrotoga*

Nitrotoga enrichments utilized up to 10 mM NO_2^- within 15 days of incubation (Figure 3.18), and one replicate incubated with 15 mM depleted NO_2^- within 23 days. The observed differences in the time needed to deplete NO_2^- between replicates of the 10 mM and 15 mM NO_2^- incubations could be due to uneven ratios of *Nitrotoga* to contaminating cells. Contaminating cells in the inoculum were rare and occurred mostly as large flocs. Therefore, different total amounts of *Nitrotoga* may have been present between replicates.

SBE incubated with 15 mM, 20 mM and 30 mM, which utilized NO_2^- only after up to 46 days of incubation or not at all (replicate 2, 30 mM NO_2^-) were found to mainly contain *Nitrobacter* and hardly any *Nitrotoga*. This observation implies that some *Nitrobacter* cells were present in the inoculum. However, *Nitrobacter* was apparently not competitive enough to overgrow *Nitrotoga* at concentrations up to 10 mM NO_2^-. This is in agreement with reported *Nitrobacter* NO_2^- tolerances, which generally are higher than those of *Nitrospira*, *Nitrospina*, and *Nitrotoga* (Spieck & Lipski 2011). Possibly, *Nitrobacter* cells were very rare in the enrichment, explaining the long lag phase before NO_2^- was depleted in those incubations where *Nitrobacter* dominated. It seems unlikely that the observed NO_2^- depletion at concentrations of 1 mM – 15 mM within 23 days was due to activity of *Nitrobacter*, both because *Nitrobacter* was not detectable by FISH, and because consumption of NO_2^- started much earlier as compared to those incubations where *Nitrobacter* became dominant. Nonetheless, it cannot be excluded that *Nitrobacter* started utilizing NO_2^- at initial high concentrations (10 mM and 15 mM NO_2^-) and was later outcompeted by *Nitrotoga*, once NO_2^- concentrations were lower.

All SBE, which depleted up to 15 mM NO_2^- within 23 days, were highly enriched in *Nitrotoga*, which implies that the maximum NO_2^- tolerance of *Nitrotoga* lies at approximately 15 mM. This substrate tolerance is comparable to described strains of *Nitrospira* (summarized in Lebedeva et al. 2008; Off et al. 2010), however differs by one order of magnitude from the reported substrate tolerance of 1.2 mM NO_2^- of the only described enriched *Nitrotoga* strain, Ca. *N. arctica* (Alawi et al. 2007). Based on Ca. *N. arctica* and another enrichment of *Nitrotoga* at low NO_2^- concentration (0.3 mM) from a WWTP, the genus *Nitrotoga* has been proposed to be adapted to low NO_2^- concentrations (Alawi et al. 2007 and 2009). Recently however, Lücker et al. (2015) showed by MAR-FISH that *Nitrotoga* in activated sludge were still active in presence of 10 mM NO_2^-, challenging this view for the first time. The *Nitrotoga* enrichment that was obtained in the present study further corroborates that some strains can tolerate much higher NO_2^- concentrations than previously reported for *Nitrotoga* enrichments.

Determination of NO_2^- oxidation rates at different substrate concentrations and thereby inferring the NO_2^- optimum of the enrichment was not possible, as absolute cell counts or protein content (as proxy thereof) of *Nitrotoga* could not be obtained due to presence of contaminating cells. Normalization of NO_2^- oxidation rates to cell counts or protein content however is vital, as mere comparisons of NO_2^- depletion rates at the steepest part of the slope do not consider growth of cells during the incubation, and therefore overestimate rates at high substrate concentrations. This is depicted in Figure 4.4 for NO_2^- oxidation rates of 1 mM, 5 mM and 10 mM NO_2^-. In Figure 4.4, A, rates were directly inferred from the steepest part of the slope without normalization, indicating highest rates at approximately 5 mM, with almost equally high rates at 10 mM NO_2^-. In Figure 4.4 B, rates were normalized to the already consumed amount of NO_2^- at the steepest part of the slope (approximately 0.25 mM, 1 mM and 2.5 mM for incubations initially supplied with 1 mM, 5 mM and 10 mM respectively) indicating equally high rates at 1 mM and 5 mM NO_2^-, and much lower rates at 10 mM NO_2^-. While this normalization certainly does not accurately reflect cell numbers, as linear growth cannot be assumed, it does show the need for normalization in such experiments. Additionally, one has to account for the fact that at the steepest part of the slope, the NO_2^- concentration is already lower than the initial concentration (in this experiment rates were calculated at approximately 0.75 mM, 4 mM and 7.5 mM residual NO_2^- instead of the originally supplied 1 mM, 5 mM, and 10 mM NO_2^-). This further skews such comparisons, which are however frequently reported in the literature without reporting normalization procedures (e.g. Off et al. 2010; Off 2012; Ushiki et al. 2013; Spieck et al. 2014). One alternative to normalization by direct cell counting or protein content is determination of copy number of specific marker genes as done for enrichments of thermophilic *Nitrospira* by Edwards et al. (2013).

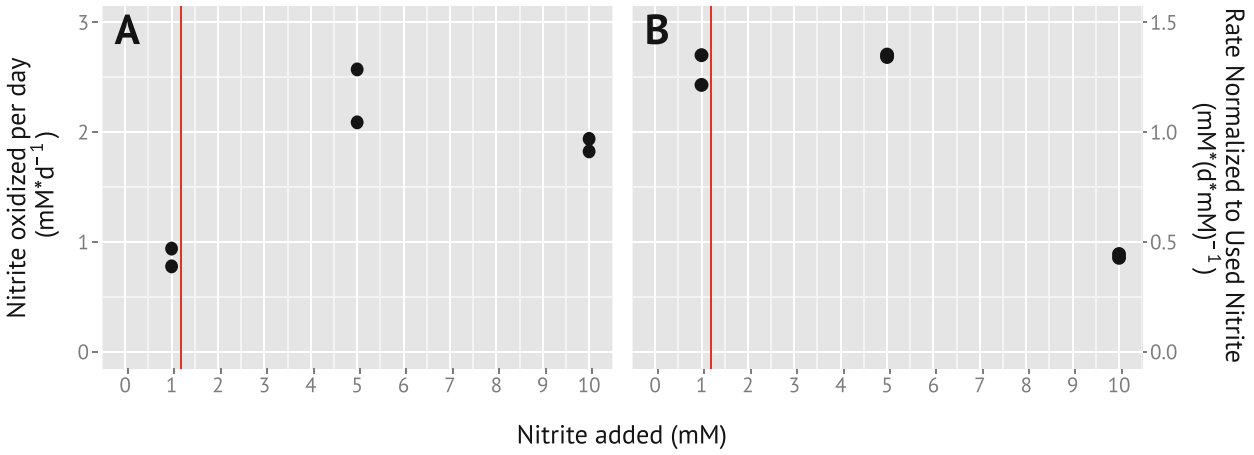

Figure 4.4 Comparison between NO_2^- oxidation rates from substrate tolerance tests with *Nitrotoga* enrichment. Rates were inferred from steepest point of the slopes. A: NO_2^- oxidation per day without normalization. B: NO_2^- oxidation per day normalized to total NO_2^- already consumed at the steepest slope. Red lines represent reported maximum NO_2^- tolerance of the previously described Ca. *N. arctica*.

Lebedeva et al. (2008) observed for an enrichment and pure culture of *Nitrospira bockiana* that NO_2^- tolerance can be influenced and altered by the presence of contaminating cells. Therefore, exact oxidation rates, NO_2^- optimum and tolerance range should be re-evaluated with a pure culture of *Nitrotoga*, where optimum and range are not affected by contaminating cells, and where absolute *Nitrotoga* cell counts or protein content can easily be included.

4.4.3.4 Temperature Optimum of Enriched *Nitrotoga*

Temperature optimum of the *Nitrotoga* enrichment was tested in presence of 1 mM NO_2^- at 4 °C, 14 °C, 20 °C, 24 °C, 28 °C, 37 °C, and 46 °C, spanning a temperature range containing optima of most described NOB (Table 4.1). During the time span investigated in detail, NO_2^- oxidation was observed between 14 °C and 37 °C. Upon prolonged incubation, NO_2^- was also depleted at 4 °C, but not at 46 °C. Oxidation rates were nearly symmetrically distributed across the temperatures tested, with the maximum NO_2^- oxidation rates at 28 °C (Figure 3.20). Optima around 28 °C are common for NOB (see Table 4.1 for some described NOB temperature ranges and optima). However, the genus *Nitrotoga* has so far been associated with low temperature optima, as both available enrichment cultures show adaptation to low temperatures (Alawi et al. 2007 and 2009). Even though *Nitrotoga* was found to be active at temperatures up to 27 °C in activated sludge, as investigated by MAR-FISH, *Nitrotoga* occurred exclusively in cold WWTP (Lücker et al. 2015). The present enrichment therefore is the first to not only tolerate mesophilic conditions, but to be adapted to higher temperatures.

Nonetheless, temperature ranges and optima may be influenced both by the NO_2^- concentration tested and by the presence of contaminating cells (Lebedeva et al. 2008). Therefore, temperature range and optimum should be re-evaluated with a pure culture of *Nitrotoga*.

Table 4.1 Overview of temperature optima and activity ranges in selected NOB.

Organism	Temperature Range	Temperature Optimum	Reference
Ca. *Nitrotoga arctica* enrichment (*Betaproteobacteria*)	4 – 22 °C	10 °C	Alawi et al. 2007
Nitrobacter alkalicus (*Alphaproteobacteria*)	?	25 – 28 °C	Sorokin et al. 1998
Nitrococcus mobilis (*Gammaproteobacteria*)	14 – 40 °C	25 – 30 °C	Watson & Waterbury 1971
Nitrolancea hollandica (*Chloroflexi*)	? – 46 (growth) / 63 C (activity)	40 °C	Sorokin et al. 2014
Nitrospina gracilis (Nitrospina)	14 – 40 °C	25 – 30 °C	Watson & Waterbury 1971
Nitrospira Aa01 enrichment (*Nitrospira* lineage IV)	17 – 30 °C	28 – 30 °C	Off et al. 2010
Nitrospira bockiana (*Nitrospira* lineage V)	28 – 44 °C	42 °C	Lebedeva et al. 2008
Nitrospira calida (*Nitrospira* lineage VI)	37 – 58 °C	46 – 52 °C	Lebedeva et al. 2011
Nitrospira defluvii (*Nitrospira* lineage I)	10 – 32 °C	32 °C	Off et al. 2010; Nowka et al. 2015
Nitrospira enrichments (*Nitrospira* lineage II and VI)	? – 60 °C	45 – 50 °C	Edwards et al. 2013
Nitrospira japonica (*Nitrospira* lineage II)	20 – 35 °C	31 °C	Ushiki et al. 2013
Nitrospira lenta (*Nitrospira* lineage II)	10 -32 °C	28 °C	Nowka et al. 2015
Nitrospira marina (*Nitrospira* lineage IV)	15 – 30 °C	25 – 30 °C	Watson et al. 1986
Nitrospira moscoviensis (*Nitrospira* lineage II)	33 – 40 °C	39 °C	Ehrich et al. 1995

4.4.3.5 Attempts to Obtain an Axenic Culture of *Nitrotoga*

The failure to obtain an axenic culture of *Nitrotoga* from dilution series, Percoll density gradient centrifugations (and subsequent dilution series), and filtration trials could be due to a lack of discrimination between *Nitrotoga* and contaminating cells by the used pore sizes and too high dilution increments. As approximately 80 – 90 % of all cells in the enrichment were *Nitrotoga*, 1:10 dilutions may by chance never have resulted in the isolation of only *Nitrotoga* cells. Dilution steps of 1:2 might have been more successful at high dilutions.

4.4.3.6 Growth of *Nitrotoga* on Solid Medium

Growth of *Nitrotoga* on solid medium was both tested on normal mineral medium (with phosphate addition before autoclaving) and on mineral medium without phosphate addition prior to autoclaving. This was done according to Tanaka et al. (2014), who found that solid medium contained high H_2O_2 levels when phosphate was autoclaved together with agar or agarose. H_2O_2 can inhibit growth of ROS-sensitive microorganisms on solid medium. While there was no knowledge available on presence of catalases and other detoxification systems for ROS in *Nitrotoga*, e.g. *Nitrospira defluvii* lacks such systems (Lücker et al. 2010), and is also sensitive to high concentrations of dissolved O_2 (Christiane Gruber-Dorninger and Hanna Koch, personal communication). *Nitrotoga* was enriched only in Schott bottles, where the cultivation medium was assumed to contain lower O_2 levels than in Erlenmeyer flasks. We therefore hypothesized that *Nitrotoga* might grow on solid medium, when phosphate was

added after autoclaving, and levels of ROS thereby minimized. After initial tests to ascertain that *Nitrotoga* can grow in medium where phosphate was added after autoclaving, enrichment culture was plated on three different solid media (noble agar, sieve and plaque agarose). Indeed, after one month of incubation on solid plaque agarose medium supplemented with 1 mM NO_2^-, a colony with cells of *Nitrotoga*-like morphology was detected and streaked out onto new plates of the same type. After another month, grown colonies served as inoculum for liquid mineral medium and template for colony PCR with general bacterial primers, which confirmed growth of *Nitrotoga* on plaque agarose plates. *Nitrotoga* was active and grew in liquid mineral medium that was inoculated from grown colonies, and liquid culture could again be plated and readily formed colonies on solid plaque agarose medium, where phosphate was added after autoclaving. *Nitrotoga* was however not able to form colonies within one month on plaque agarose plates, where phosphate was added before autoclaving. This indicates that growth of *Nitrotoga* on plates was indeed due to absence of H_2O_2, and not exclusively due to the specific agarose used (plaque agarose). Unlike agar and sieve agarose, medium prepared with plaque agarose did not result in slight brownish color, indicative of caramelized sugars after autoclaving. This implies that plaque agarose was least contaminated with organics of the tested solidifying agents.

The observations that unambiguous *Nitrotoga* sequences were obtained from sequencing of colony PCR amplicons that no floc formation occurred in liquid cultures inoculated from colonies, which had previously been due to betaproteobacterial contaminants, and that no other cells than *Nitrotoga* were detectable by FISH, tentatively suggest that an axenic culture of *Nitrotoga* was obtained. This would represent the first isolated member of the genus *Nitrotoga*. Whether this really is the case, however still needs to be more thoroughly examined.

The only pure culture NOB which could so far be grown on solid medium are *Nitrobacter* (Bock et al. 1983; Sorokin et al. 1998) and *Nitrolancea hollandica* (Sorokin et al. 2012 and 2014). *Nitrospira defluvii* grows on agarose plates, however only in presence of heterotrophic bacteria, never as pure *Nitrospira* colonies (Off 2012; Nowka et al. 2015). It is intriguing to speculate that indeed it could be formation of H_2O_2 in conventional solid medium preparation that has stalled growth of other NOB on plates so far, and that *N. defluvii* only grows in presence of other bacteria, because those possess ROS detoxification mechanisms, which locally reduce the H_2O_2 concentration. Whether this is the case, remains to be examined.

4.4.3.7 Candidatus *Nitrotoga fabula*

The *Nitrotoga* strain enriched (and possibly isolated) in this study belongs to a sublineage of *Nitrotoga* without cultured or enriched representatives, and shows a drastically different physiology as compared to previously described *Nitrotoga* enrichments (Alawi et al. 2007 and 2009). Additionally, the 16S rRNA gene identity to the closest enriched relative (Ca. *N. arctica*) is 98.5 %, which is (slightly) below the currently suggested threshold for species demarcation of 98.65 % (Kim et al. 2014). Therefore, a new species of this genus, Candidatus *Nitrotoga fabula* (fabula, latin, “small bean”, named after their morphology), is provisionally suggested.

Ca. *N. fabula* is the first described mesophilic member of the genus *Nitrotoga*. It grows planktonic, over a range from 4 °C – 37 °C, with a temperature optimum of 28 °C and tolerates up to 15 mM NO_2^-. Both temperature and substrate adaptation drastically differ from previous *Nitrotoga* enrichments and therefore imply that *Nitrotoga* may be competitive over a much broader range of environmental conditions than previously assumed. As the genome of Ca. *N. fabula* is currently being sequenced, more information on this novel NOB will soon be available, allowing for more targeted experiments.

Substrate tolerance of Ca. *N. fabula* is similar to many *Nitrospira* strains. Ca. *N. fabula* can therefore be assumed to be a K-strategist (e.g. Nowka et al. 2014). Nonetheless, Ca. *N. fabula* grows – in comparison to many *Nitrospira* – fast, and causes visible turbidity after oxidation of only few mM NO_2^-. The fast growth, and the ability of Ca. *N. fabula* to grow on solid medium may enable biochemical studies, which have been difficult to carry out with other NOB than *Nitrobacter* due to their fastidious nature. As *Nitrotoga* – like *Nitrospira* and *Nitrospina* (Lücker et al. 2010 and 2013) – possess a periplasmic NXR (Nowka et al. 2014), Ca. *N. fabula* may enable functional and structural investigation of the periplasmic version of this NOB marker enzyme.

However, *Nitrotoga* is also interesting from an evolutionary point of view. *Nitrotoga* are the only known NOB within the *Betaproteobacteria*, and could shed light on horizontal gene transfer of NXR and other genes allowing for NO_2^- oxidation. *Nitrotoga* is therefore an excellent model organism for studying the modularity of physiology in bacteria and archaea. Additionally, the genus *Nitrotoga* seems to be evolutionarily relatively young, as all known 16S rRNA gene sequences show high identities to one another (Figure 3.14). Nonetheless, Ca. *N. arctica* and Ca. *N. fabula*, which share a 16S rRNA gene identity of 98.5 %, are physiologically highly divergent. Therefore, *Nitrotoga* sequences, which have been found from various environments (Lücker et al. 2015 and references therein), could indicate rapid functional diversification within this young genus.

5 CONCLUSION

In this study, three projects, which all aimed at elucidating adaptations of nitrifying bacteria to their environment were carried out.

It was confirmed that *N. moscoviensis* can utilize formate and NO_3^- for energy generation under anoxic conditions, which may allow those bacteria to survive during periods of anoxia, or even enable a syntrophy between aerobically and anaerobically growing *N. moscoviensis* populations.

A method to obtain genomic information on microdiverse nitrifiers from activated sludge was further developed and assessed. It was shown by FISH that nitrifier microflocs could be obtained from activated sludge by sonication. Further, it was shown that the previously developed workflow of incubation of activated sludge in presence of heavy isotopes is feasible for D_2O but not for ^{13}C-bicarbonate in the investigated WWTP Klosterneuburg. Targeted Raman activated microfloc sorting did not significantly enrich for nitrifiers, as the main enrichment occurred during sonication, but microflocs with cytochrome signatures significantly more often contained nitrifiers than those without cytochromes. While some steps in the workflow can still be optimized, overall, the employed strategy seems well suited to obtain (at least partial) genomes from nitrifiers in activated sludge, which will allow for generation of hypothesis regarding niche differentiation, symbiosis and evolution of coexistence of microdiverse nitrifiers.

Last, a novel species of the genus *Nitrotoga*, tentatively named Candidatus *Nitrotoga fabula*, was enriched from activated sludge from WWTP Klosterneuburg, characterized, and possibly obtained in pure culture. This novel species belongs to a sublineage of *Nitrotoga* without described relatives and shows drastically different physiology as compared to previously described enrichments. It is the first described mesophilic species of the genus, indicating that the environmental niche of *Nitrotoga* is likely much wider than previously assumed. Additionally, Ca. *N. fabula* grows relatively fast in liquid medium, and is able to grow on plaque agarose plates where phosphate was added after autoclaving to avoid H_2O_2 formation during autoclaving of phosphate and agarose or agar (Tanaka et al. 2014). Growth of Ca. *N. fabula* on solid medium will simplify further research on this interesting species. This novel solid medium preparation strategy might also enable cultivation of other NOB on solid medium, which could fuel and simplify future research on this important functional group.

6 SUMMARY

Chemolithotrophic nitrification is a key process in the global nitrogen cycle, and is exclusively carried out by specialized microorganisms – namely ammonia oxidizing bacteria and archaea, and nitrite oxidizing bacteria. As these organisms are difficult to obtain and keep in axenic culture, only limited knowledge is available on their physiology. In the present study, both cultivation dependent and cultivation independent approaches were taken to gain further insight into physiology, evolution, and niche differentiation of these organisms, with the main focus on nitrite oxidizing bacteria.

First, the denitrification potential of an axenic culture of *Nitrospira moscoviensis*, a nitrite oxidizing bacterium, was assessed. Biomass was incubated under anoxic conditions in the presence of nitrate (NO_3^-) and either tricarboxylic acid cycle precursors or intermediates, branched amino acids, ethanol or formate. It was shown that of the tested compounds, *N. moscoviensis* can only utilize formate and NO_3^- for energy generation under anoxic conditions. This may allow *N. moscoviensis* to gain energy during periods of anoxia, or even enable a syntrophy between aerobically and anaerobically growing *N. moscoviensis* populations in the environment.

Second, a method to obtain genomes of microdiverse coexisting nitrifiers from activated sludge was further developed and evaluated in a pre-study to gain insights into mechanisms allowing for coexistence and niche differentiation. Activated sludge was incubated under nitrifying conditions in the presence of heavy isotopes (^{13}C or D_2O) to allow for detection of active microorganisms. Incubated sludge was dissected by sonication, and it was shown by FISH that this method is suitable for obtaining single nitrifier microcolonies within microflocs. Subsequently, Raman spectra were acquired for the obtained microflocs. Raman spectra allowed for detecting active nitrifiers based on the presence of cytochrome signatures, which are indicative of nitrifiers, and presence of activity marker signatures. It was shown that incubation is feasible with D_2O, but not with ^{13}C as activity marker, as some nitrifiers possessed inherent peaks in the ^{13}C marker region. Obtained microflocs were sorted by optical tweezing. Cells of sorted microflocs were lysed, their DNA amplified by multiple displacement amplification (MDA), and MDA products were screened by PCR for bacterial and nitrifier DNA. It was shown that targeted sorting of active nitrifiers did not significantly enrich for nitrifiers in comparison to random sorting. Nonetheless, microflocs whose Raman spectra contained cytochrome signatures significantly more often contained nitrifier DNA than those without. These observations imply that the main enrichment for nitrifiers already occurred during sonication, as nitrifiers grow in very rigid microcolonies, which are resistant to dissection. This also implies that the main advantage of using Raman spectroscopy is to identify active microorganisms. Lastly, 24 MDA products were selected for microfloc metagenome sequencing. While some steps in the workflow can still be optimized, overall the employed strategy seems well suited to obtain (at least partial) genomes from nitrifiers in activated sludge, which will allow for the generation of hypotheses regarding niche differentiation, symbiosis and evolution of coexistence among microdiverse nitrifiers.

Finally, a novel species of the genus *Nitrotoga*, tentatively named Candidatus *Nitrotoga fabula* (fabula, lat. “small bean”, named after the characteristic morphology of this species), was enriched from activated sludge from WWTP Klosterneuburg, characterized, and likely obtained in axenic culture. This novel species belongs to a sublineage of *Nitrotoga* without described relatives and shows drastically different physiology when compared to the two previously described enrichments, which were obtained at low substrate concentrations and temperatures. Ca. *N. fabula* is the first described mesophilic species of the genus, with a NO_2^- tolerance of approximately 15 mM and a temperature optimum of 28 °C, indicating that the environmental niche of *Nitrotoga* is likely much wider than

previously assumed. Additionally, a novel solid medium preparation was employed, which allowed cultivation of Ca. *N. fabula* on plates and will simplify further research on this evolutionarily highly interesting and hardly investigated nitrite oxidizers.

Taken together, the insights gained in this study have expanded the knowledge on nitrifying bacteria regarding substrate utilization of *N. moscoviensis* and physiological adaptations within the hardly investigated genus *Nitrotoga*. Additionally, the further development and evaluation of the workflow for studying microdiverse coexisting nitrifiers is expected to yield invaluable information on niche differentiation, symbiosis and evolution of these important organisms.

7 ZUSAMMENFASSUNG

Im globalen Stickstoffkreislauf ist chemolithotrophe Nitrifikation ein Schlüsselprozess, der ausschließlich von spezialisierten Mikroorganismen – nämlich von Ammoniak-oxidierenden Bakterien und Archeen sowie Nitrit-oxidierenden Bakterien – ausgeführt wird. Die Kultivierung und Kulturerhaltung von Ammoniak- und Nitritoxidierern sind äußerst schwierig, weshalb nur beschränktes Wissen über ihre Physiologie vorhanden ist. In dieser Studie wurden sowohl Anreicherungs- und Kultivierungstechniken als auch kultivierungsabhängige Methoden angewandt, um mehr Einblick in die Physiologie, Evolution und Nischendifferenzierung dieser wichtigen funktionellen Gruppe zu erhalten. Ein besonderes Augenmerk lag dabei auf Nitritoxidierern.

Zuerst wurde das Denitrifikationspotential einer Reinkultur des Nitritoxidierers *Nitrospira moscoviensis* weiter erforscht. *N. moscoviensis*-Biomasse wurde mit Nitrat (NO_3^-) und verschiedenen Intermediaten und Vorstufen des Zitronensäure-Zyklus sowie mit verzweigten Aminosäuren, Ethanol oder Formiat inkubiert. Von den getesteten Substraten konnte *N. moscoviensis* lediglich Formiat und NO_3^- zur Energiegewinnung nutzen. Die Verwendung dieser Substrate könnte *N. moscoviensis* eine alternative Energiegewinnungsmöglichkeit in Abwesenheit von Sauerstoff als terminalem Elektronenakzeptor eröffnen. Zusätzlich könnte dieser Stoffwechselweg auch eine Symbiose zwischen aerob und anaerob lebenden *N. moscoviensis*-Populationen in der Umwelt ermöglichen.

Im zweiten Teil der Studie wurde eine Methode zur Untersuchung von möglichen Unterschieden auf Genom-Ebene und der sich daraus ergebenden funktionellen Differenzierung zwischen mikrodiversen koexistierenden Nitrifikanten in Belebtschlamm weiterentwickelt und in einer Vorstudie bewertet. Belebtschlamm wurde hierfür unter nitrifizierenden Bedingungen mit schweren Isotopen (^{13}C und D_2O) inkubiert, um aktive Mikroorganismen zu markieren. Danach wurden die Belebtschlammflocken durch Sonifizieren in Mikroflocken zerkleinert, wobei gezeigt werden konnte, dass diese Methode geeignet ist, einzelne Nitrifikanten-Kolonien in Mikroflocken zu erhalten. Danach wurden Raman-Spektren von Mikroflocken aufgenommen. Raman-Spektroskopie ermöglicht die Identifizierung aktiver Nitrifikanten, weil diese charakteristische Zytochrom-Signaturen aufweisen und der Einbau schwerer Isotope in Biomasse in den Spektren sichtbar ist. Es konnte gezeigt werden, dass D_2O als Aktivitätsmarker gut geeignet ist, nicht jedoch ^{13}C, da manche Nitrifikanten in ihren Raman-Spektren Peaks in der Region des ^{13}C-Aktivitätsmarkers aufweisen. Ausgewählte Mikroflocken wurden mittels optischer Pinzette sortiert. Die so vereinzelten Mikroflocken wurden lysiert, ihre DNA mittels multiple displacement amplification (MDA) amplifiziert und MDA-Produkte durch PCR auf Anwesenheit von bakterieller und Nitrifikanten-DNA überprüft. Dadurch konnte gezeigt werden, dass gezieltes Sortieren von Nitrifikanten-Mikroflocken aufgrund ihres Raman-Spektrums im Vergleich zu zufälligem Sortieren nicht zu einer Anreicherung von Nitrifikanten-DNA in den erhaltenen MDA-Produkten führte. Nichtsdestotrotz enthielten MDA-Produkte von Mikroflocken mit Zytochrom-Signaturen signifikant häufiger Nitrifikanten-DNA als MDA-Produkte von Mikroflocken ohne Zytochrom-Signaturen. Diese Ergebnisse deuten darauf hin, dass die eigentliche Selektion auf Nitrifikanten bereits durch das Zerkleinern von Belebtschlammflocken erfolgte, da Nitrifikanten als sehr robuste Mikrokolonien wachsen. Des Weiteren bedeutet dies, dass der Hauptvorteil der Raman-Spektroskopie darin liegt, aktive Mikroorganismen zu identifizieren. Aufgrund der PCR-Screening-Ergebnisse wurden 24 MDA-Produkte ausgewählt, um die Metagenome der sortierten Mikroflocken zu sequenzieren. Obwohl noch einige Schritte im Workflow optimiert werden könnten, ist die hier angewandte Methode für die Analyse von Genomen mikrodiverser Nitrifikanten aus Belebtschlamm sehr gut geeignet. Die in dieser Studie gewonnenen Erkenntnisse werden in Zukunft das Erstellen und Überprüfen von Hypothesen

zur Nischendifferenzierung, Symbiose und Evolution von Koexistenz mikrodiverser Nitrifikanten in Belebtschlamm ermöglichen.

Im letzten Teil dieser Studie wurde eine neue *Nitrotoga*-Spezies aus Belebtschlamm angereichert, charakterisiert und höchstwahrscheinlich in Reinkultur erhalten. Diese neue Spezies wurde provisorisch Candidatus *Nitrotoga fabula* (fabula, lat. „kleine Bohne“, nach der charakteristischen Morphologie dieser Spezies) genannt. Diese neue Art gehört einer Untergruppe von *Nitrotoga* an, in der bisher keine beschriebenen Anreicherungen zu finden sind. Außerdem unterscheidet sie sich physiologisch deutlich von den zwei beschriebenen Anreicherungen, die beide bei niedrigen Temperaturen und Substratkonzentrationen angereichert wurden. Ca. *N. fabul*a ist mit einem Temperaturoptimum von 28 °C und einer Substrattoleranz von etwa 15 mM NO_2^- die erste beschriebene mesophile Spezies in der Gattung *Nitrotoga*. Dies impliziert, dass die ökologische Nische der Gattung *Nitrotoga* viel breiter ist als zuvor angenommen. Zusätzlich wurde eine neue Art der Festmedium-Zubereitung angewandt, welche die Kultivierung von Ca. *N. fabul*a auf Agarose-Platten ermöglichte. Diese Kultivierungsmethode wird in Zukunft die Forschung an diesen evolutiv höchst interessanten und kaum erforschten Nitritoxidierern erleichtern.

Zusammengefasst erweitern die Erkenntnisse, die in dieser Studie gewonnen wurden, das Wissen über mögliche Substrate von *Nitrospira moscoviensis* abseits von Nitritoxidation und die physiologischen Anpassungen der bisher kaum erforschten Gattung *Nitrotoga*. Außerdem wurde eine Methode zur Untersuchung von mikrodiversen koexistierenden Nitrifikanten in Belebtschlamm weiterentwickelt und beurteilt, die in Zukunft Erkenntnisse von unschätzbarem Wert bezüglich Nischendifferenzierung, Symbiose und Evolution dieser wichtigen Organismen ermöglichen wird.

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Anne and Jasmin – thank you for always helping out when I was stuck with one or the other problem, for lending me a hand when I had too much to do for my own 2, and for the many work- and life-related discussions and suggestions!
Chrissy – thank you for always lending me your ear and wits, and for making me feel so welcome at our shared bench!
Basti – thank you for all discussions, suggestions and input, as well as for being the first to expose me to quinone pools and respiratory chains.
Markus – thanks a lot for all your microscope, MAR-FISH and Raman support!
Michi and Steph – without you, this last year would have been half the fun and very likely a lot more frustrating, thank you for being there, for having coffee, beer, and Jausen-Pausen.
Bela – thank you for being such a nice coffee-break companion, for many discussions, and for being a great R "cheat-sheet".
Roey, Allen, Karin, Stephanie, Julia, Claus, Martin, Adrian, Flo – thank you for making this last year so great!
All other Domies – thank you for the nice working atmosphere!

Karo – thank you for being such a great friend. Studying biology (and generally living in Vienna) would not be the same without you. Also: thank you for getting rid of all those extra commas!

Alven – thank you for bearing with me no matter how excited or frustrated I am, for understanding all the long lab-evenings, for always being there and supporting me. And: thank you for correcting all the strange English sentences that found their way into this thesis!

And, last, I want to thank my mum and dad for always being there for me, for supporting me in whatever I am pursuing, for encouraging me whenever I need it, and for being the best parents I can imagine.

9 APPENDIX

9.1 Sequencing Results From Nitrite Oxidizer Enrichments

Table 9.1 Best BLAST hits and closest described relative of obtained *Nitrospira* 16S rRNA gene sequences from EFE culture. BLAST hits last accessed November 2014.

Clone ID	Sequence Length	Best BLAST Hit	Accession Nr.	Identity	Closest Described Hit	Accession Nr.	Identity
EFE-04	912 nt	Uncultured bacterium clone B112-43 16S ribosomal RNA gene, partial sequence	KF010765.1	99.89 %	Candidatus *Nitrospira defluvii* 16S ribosomal RNA, complete sequence	NR_074700.1	99.78 %
EFE-09	982 nt	Uncultured bacterium clone B112-43 16S ribosomal RNA gene, partial sequence	KF010765.1	99.90 %	Candidatus *Nitrospira defluvii* 16S ribosomal RNA, complete sequence	NR_074700.1	99.90 %
EFE-12	912 nt	Uncultured *Nitrospirae* bacterium partial 16S rRNA gene, clone C100B0W9	HE613584.1	99.78 %	Candidatus *Nitrospira defluvii* 16S ribosomal RNA, complete sequence	NR_074700.1	99.56 %
EFE-17	927 nt	Uncultured bacterium clone B112-43 16S ribosomal RNA gene, partial sequence	KF010765.1	99.78 %	Candidatus *Nitrospira defluvii* 16S ribosomal RNA, complete sequence	NR_074700.1	99.68 %
EFE-23	941 nt	Uncultured bacterium clone B112-43 16S ribosomal RNA gene, partial sequence	KF010765.1	99.89 %	Candidatus *Nitrospira defluvii* 16S ribosomal RNA, complete sequence	NR_074700.1	99.89 %

Table 9.2 *Nitrospira* 16S rRNA gene sequences obtained from EFE culture.

>EFE-04

AGAGTTTGATTCTGGCTCAGAACGAACGCTGGCGGCGCGCCTAATACATGCAAGTCGAGCGAGAAGGTGTAGCAATACACTTGTAAAGCGG
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CGGGTTCGGAGGGAAAGCCACACTGTGAGTGCGGCGCTCTTTGATGAGCTCATGTCCTATCAGCTTGTTGGTAGGGTAACGGCCTACCAAG
GCTTTGACGGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGCACTGCGACACGGGCCAGACTCCTACGGGAGGCAGCAGTAAGGAATA
TTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGGGGGATGAAGGTCTTCGGATTGTAAACCCCTTTCGGGAGGGAAGATGGAGC
GAGCAATCGTTCGGACGGTACCTCCAGAAGCAGCCACGGCCAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGTGGCAAGCGTTGTTCGG
ATTCACTGGGCGTACAGGGTGTGTAGGCGGTTTGGTAAGCCTTCTGTTAAAGCTTCGGGCCCAACCCGGAAAGCGCAGAGGGTACTGCCAG
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TGTCGGCGGGTTACCGCCGGTGCCGCAGCTAACGCATTAAGTATCCCGCCTGGGAAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGAC
GGG

> EFE-09

TCGGGTTCGGAGGGAAAGCCACACTGTGAGTGCGGCGCTCTTTGATGAGCTCATGTCCTATCAGCTTGTTGGTAGGGTAACGGCCTACCAA
GGCTTTGACGGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGCACTGCGACACGGGCCAGACTCCTACGGGAGGCAGCAGTAAGGAAT
ATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGGGGGATGAAGGTCTTCGGATTGTAAACCCCTTTCGGGAGGGAAGATGGAG
CGAGCAATCGTTCGGACGGTACCTCCAGAAGCAGCCACGGCCAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGTGGCAAGCGTTGTTCG

GATTCACTGGGCGTACAGGGTGTGTAGGCGGTTTGGTAAGCCTTCTGTTAAAGCTTCGGGCCCAACCCGGAAAGCGCAGAGGGTACTGCCA
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CGAGCGCAACCCCTGCTTTCAGTTGCTACCGGGTCATGCCGAGCACTCTGAAAGGACTGCCCAGGATAACGGG

>EFE-12

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CGGGTTCGGAGGGAAAGCCACACTGTGAGTGCGGCGCTCTTTGATGAGCTCATGTCCTATCAGCTTGTTGGTAGGGTAACGGCCTACCAAG
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GGG

>EFE-17

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CGGGTTCGGAGGGAAAGCCACACTGTGAGTGCGGCGCTCTTTGATGAGCTCATGTCCTATCAGCTTGTTGGTAGGGTAACGGCCTACCAAG
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TGTCGGCGGGTTACCGCCGGTGCCGCAGCTAACGCATTAAGTATCCCGCCTGGGAAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGAC
GGGGGGCCCGCACAAGCG

>EFE-23

TTGATGAGCTCATGTCCTATCAGCTTGTTGGTAGGGTAACGGCCTACCAAGGCTTTGACGGGTAGCTGGTCTGAGAGGACGATCAGCCACA
CTGGCACTGCGACACGGGCCAGACTCCTACGGGAGGCAGCAGTAAGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTG
GGGGATGAAGGTCTTCGGATTGTAAACCCCTTTCGGGAGGGAAGATGGAGCGAGCAATCGTTCGGACGGTACCTCCAGAAGCAGCCACGGC
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AAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACTATGGATACTAAGTGTCGGCGGGTTACCGCCGGTGCCGCAGCTAACGCATTAA
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CGCAACGTGAAGAACCTTACCCAGGTTGGACATGCACGTAGTAGAAAGGTGAAAGCCTGACGAGGTAGCAATACCAGCGTGCTCAGGTGCT
GCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGCTTTCAGTTGCTACCGGGTCATGCCG
AGCACTCTGAAAGGACTGCCCAGGATAACGGG

Table 9.3 Best BLAST hits and closest described relative of obtained *Nitrotoga* 16S rRNA gene sequences from SBE culture. BLAST hits last accessed November 2014.

Clone ID	Sequence Length	Best BLAST Hit	Accession Nr.	Identity	Closest Described Hit	Accession Nr.	Identity
SBE-06	1464 nt	Uncultured bacterium clone a-44 16S ribosomal RNA gene, partial sequence	JX040372.1	99.9 %	Candidatus *Nitrotoga arctica* clone 6680 16S ribosomal RNA gene, partial sequence	gb|DQ839562.1|	98.4 %
SBE-20	1464 nt	Uncultured bacterium clone a-44 16S ribosomal RNA gene, partial	JX040372.1	100.0 %	Candidatus *Nitrotoga arctica* clone 6680 16S ribosomal RNA gene, partial	DQ839562.1	98.5 %

		sequence			sequence		
SBE-27	1464 nt	Uncultured bacterium clone a-44 16S ribosomal RNA gene, partial sequence	JX040372.1	99.9 %	Candidatus *Nitrotoga arctica* clone 6680 16S ribosomal RNA gene, partial sequence	DQ839562.1	98.4 %
SBE-28	1465 nt	Uncultured bacterium clone a-44 16S ribosomal RNA gene, partial sequence	JX040372.1	99.9 %	Candidatus *Nitrotoga arctica* clone 6680 16S ribosomal RNA gene, partial sequence	DQ839562.1	98.4 %
SBE-31	1465 nt	Uncultured bacterium clone a-44 16S ribosomal RNA gene, partial sequence	JX040372.1	99.7 %	Candidatus *Nitrotoga arctica* clone 6680 16S ribosomal RNA gene, partial sequence	DQ839562.1	98.2 %
SBE-39	1464 nt	Uncultured bacterium clone a-44 16S ribosomal RNA gene, partial sequence	JX040372.1	99.9 %	Candidatus *Nitrotoga arctica* clone 6680 16S ribosomal RNA gene, partial sequence	DQ839562.1	98.4 %
SBE-49	1464 nt	Uncultured bacterium clone a-44 16S ribosomal RNA gene, partial sequence	JX040372.1	100 %	Candidatus *Nitrotoga arctica* clone 6680 16S ribosomal RNA gene, partial sequence	DQ839562.1	98.5 %
SBE-50	1464 nt	Uncultured bacterium clone a-44 16S ribosomal RNA gene, partial sequence	JX040372.1	99.9 %	Candidatus *Nitrotoga arctica* clone 6680 16S ribosomal RNA gene, partial sequence	DQ839562.1	98.4 %

Table 9.4 *Nitrotoga* 16S rRNA gene sequences obtained from SBE culture.

>SBE-06
AGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATA
TATCGGAACGTACCCGGAAATGGGGGATAACGCAGCGAAAGTTGCGCTAATACCGCATATGCCCTGAGGGGGAAAGCGGGGGATCCGCAAG
GACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGGGGTAAAGGCCTACCTAGGCGACGATCCGTAGCTGGTCTGAGAGG
ACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAGCGCATGGGTGAATAACCTGTGTGGATGACG
GTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAG
CGTGCGCAGGCGGTCTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAAGGCTAGAGTACGGCAGAG
GGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGTCGATACTGACGCT
CATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGGTGTTGGGGGGGGAGACTT
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CATTAATTGCCATCATTCAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC
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CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTG
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CTGGGGTG

>SBE-20
AGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATA

TATCGGAACGTACCCGGAAATGGGGGATAACGCAGCGAAAGTTGCGCTAATACCGCATATGCCCTGAGGGGGAAAGCGGGGGATCCGCAAG
GACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGGGGTAAAGGCCTACCTAGGCGACGATCCGTAGCTGGTCTGAGAGG
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CGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAAGGCTAGAGTACGGCAGAG
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CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTG
TACACACCGCCCGTCACACCATGGGAGCGGGCTCTACCAGAAGCAGCTAGCCTAACCGCAAGGAGGGCGGTTGCCACGGTAGGGTTCGTGA
CTGGGGTG

>SBE-27

AGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATA
TATCGGAACGTACCCGGAAATGGGGGATAACGCAGCGAAAGTTGCGCTAATACCGCATATGCCCTGAGGGGGAAAGCGGGGGATCCGCAAG
GACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGGGGTAAAGGCCTACCTAGGCGACGATCCGTAGCTGGTCTGAGAGG
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CTGGGGTG

>SBE-28

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CGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAAGGCTAGAGTACGGCAGAG
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>SBE-31

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GTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAG

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>SBE-39

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GACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGGGGTAAAGGCCTACCTAGGCGACGATCCGTAGCTGGTCTGAGAGG
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CGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAAGGCTAGAGTACGGCAGAG
GGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGTCGATACTGACGCT
CATGCACAAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTTCACGCCCTAAACGATGTCAACTAGGTGTTGGGGGGGGAGACTT
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GCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCCAGAGATGGCTTGGTGCCC
GAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
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TTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCTCACAAAGCCGATCGTAGT
CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTG
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CTGGGGTG

>SBE-49

AGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATA
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GACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGGGGTAAAGGCCTACCTAGGCGACGATCCGTAGCTGGTCTGAGAGG
ACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAGCGCATGGGTGAATAACCTGTGTGGATGACG
GTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAG
CGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAAGGCTAGAGTACGGCAGAG
GGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGTCGATACTGACGCT
CATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGGTGTTGGGGGGGGAGACTT
CCTTAGTACCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAA
GCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCCAGAGATGGCTTGGTGCCC
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CTGGGGTG

>SBE-50

AGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTGGCGAACGGGTGAGTAATA
TATCGGAACGTACCCGGAAATGGGGGATAACGCAGCGAAAGTTGCGCTAATACCGCATATGCCCTGAGGGGGAAAGCGGGGGATCCGCAAG
GACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGGGGTAAAGGCCTACCTAGGCGACGATCCGTAGCTGGTCTGAGAGG
ACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAGCGCATGGGTGAATAACCTGTGTGGATGACG
GTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAG
CGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAAGGCTAGAGTACGGCAGAG
GGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGTCGATACTGACGCT
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CCTTAGTACCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAA
GCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCCAGAGATGGCTTGGTGCCC

GAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
CATTAATTGCCATCATTCAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC
TTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCTCACAAGGCCGATCGTAGT
CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTG
TACACACCGCCCGTCACACCATGGGAGCGGGCTCTACCAGAAGCAGCTAGCCTAACCGCAAGGAGGGCGGTTGCCACGGTAGGGTTCGTGA
CTGGGGTG

Table 9.5 Best BLAST hits for 16S rRNA gene sequences of non-nitrite oxidizers from SBE cultures. BLAST hits last accessed November 2014.

Clone ID	Sequence Length	Best BLAST Hit	Accession Nr.	Identity	Closest Described Hit	Accession Nr.	Identity
SBE-01	990 nt	Uncultured bacterium clone eff65 16S ribosomal RNA gene, partial sequence	JN245751.1	96.9 %	*Zoogloea oryzae* gene for 16S rRNA, partial sequence, strain: A-4	AB201044.1	96.9 %
SBE-12	1024 nt	*Zoogloea oryzae* gene for 16S rRNA, partial sequence, strain: A-4	AB201044.1	100 %			
SBE-13	991 nt	Uncultured bacterium clone eff65 16S ribosomal RNA gene, partial sequence	JN245751.1	99.8 %	*Zoogloea oryzae* gene for 16S rRNA, partial sequence, strain: A-4	AB201044.1	99.8 %
SBE-14	1000 nt	Uncultured *Caulobacteraceae* bacterium clone 14 16S ribosomal RNA gene, partial sequence	HQ184340.1	99.6 %	*Brevundimonas sp.* R043 16S ribosomal RNA gene, partial sequence	KC252872.1	99.7 %
SBE-18	965 nt	Uncultured bacterium clone DR336 16S ribosomal RNA gene, partial sequence	JF429339.1	99.8 %	*Zoogloea oryzae* gene for 16S rRNA, partial sequence, strain: A-4	AB201044.1	99.5 %
SBE-32	1024 nt	*Zoogloea oryzae* gene for 16S rRNA, partial sequence, strain: A-4	AB201044.1	100 %			
SBE-34	1000 nt	*Pseudomonas aeruginosa* PA96 genome	CP007224.1	99.9 %			
SBE-47	1027 nt	Uncultured bacterium clone KSC2-79 16S ribosomal RNA gene, partial sequence	DQ532295.1	99.2 %	*Sphingopyxis macrogoltabida* gene for 16S rRNA, partial sequence, strain: BSN11	AB675371.1	98.7 %
SBE-48	322 nt	*Zoogloea oryzae* gene for 16S rRNA, partial sequence, strain: A-4	AB201044.1	100 %			

9.2 Abbreviations

Table 9.6 List of abbreviations used in this thesis. Commonly used chemical formulas are also listed.

Abbreviation	
AA	Amino acids
amoA	Ammonia monooxygenase gene, alpha-subunit
ANAMMOX	Anaerobic ammonium oxidizing bacteria
ANOVA	Analysis of variance
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
AOM	Ammonia Oxidizing Microorganisms (AOB and AOA)
ATP	Adenosine Triphosphate
BSA	Bovine serum albumin
Ca.	Candidatus species
CARD	Catalyzed reporter deposition
CE	Capillary electrophoresis
CH_4	Methane
Cl^-	Chloride
CLSM	Confocal laser scanning microscope
CTAB	Hexadecyltrimethyl ammonium bromide
D_2O	Deuterium oxide (deuterated water)
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNRA	Dissimilatory nitrite or nitrate reduction to ammonium
EDTA	Ethylenediaminetetraacetic acid
EFE	Erlenmeyer flask enrichment (*Nitrospira* enrichment)
FISH	Fluorescence *in situ* hybridization
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
IC	Ion chromatography
JGI	Joint Genome Institute
KNB	Klosterneuburg
KNB1	First nitrification basin WWTP Klosterneuburg
KNB2	Second nitrification basin WWTP Klosterneuburg
KNB3	Basin for treatment of reject water WWTP Klosterneuburg
LB	Lysogeny broth medium
MAR	Microautoradiography
MDA	Multiple displacement amplification
N	Nitrogen
N_2	Dinitrogen
N_2O	Nitrous oxide
NEDD	N-naphthylethylene diamine dichloride
NH_3	Ammonia
NH_4^+	Ammonium
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite oxidizing bacteria
NO_x	Nitrite and Nitrate
N_r	Reactive N-compounds
NXR	Nitrite oxidoreductase
nxrB	Nitrite oxidoreductase beta-subunit
O/N	Over night
O_2	Dioxygen

OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMF	Proton motive force
PP	Polypropylene
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acids
RT	Room temperature
SBE	Schott bottle enrichment (*Nitrotoga* enrichment)
SBR	Sequencing batch reactor
SCG	Single cell genomics
SDS	Sodium dodecylsulfate
SN	Supernatant
TBE	Tris/Borate/EDTA buffer
TCA	Tricarboxylic acid (citric acid)
TE	Tris/EDTA buffer
TNS	Tris/NaCl/SDS buffer
UV	Ultraviolet
VCl_3	Vanadium(III) chloride
WWTP	Waste water treatment plant

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11 CURRICULUM VITAE

PERSONAL DATA

- Born October 8, 1989 in St. Pölten
- Austrian citizenship

EDUCATION

- Master thesis on *Microdiversity and Physiology of Nitrifiers* in Ass. Prof. Holger Daims' group, *Department of Microbiology and Ecosystem Science,* University of Vienna, October 2013 to December 2014
- Erasmus exchange year at University of Bergen, Bergen, Norway, August 2012 to July 2013
- Master curriculum in *Molecular Microbiology* with focus on *Microbial Ecology* at University of Vienna since 2012
- Bachelor in *Biology* with focus on *Ecology* at University of Vienna, 2009 to 2012, passed with honors
- Matura degree in 2008, passed with honors
- Exchange semester *St. John's Preparatory School*, Minnesota (USA), January to May 2006
- High school *Stiftsgymnasiums Melk* with specialization in modern languages, 2004 to 2008
- Middle school *Stiftsgymnasium Melk*, 2000 to 2004
- Primary School *Englische Fräulein* in St. Pölten, 1996 to 2000

INTERNSHIPS AND WORK EXPERIENCE

- Tutor for a practical course on Fluorescence *in situ* hybridization at University of Vienna, summer term 2014
- Internship in Prof. Tron Frede Thingstad's group for *Marine Microbiology*, University of Bergen, *MINOS Mesocosm experiment to further elucidate the microbial loop concept*, Mai and June 2013
- Internship in Prof. Nils-Kåre Birkeland's group for *General Microbiology*, University of Bergen, *Heterologous expression and purification of sigma-factors from* Verrucomicrobium Methylacidiphilum kamtchatkense *Kam1*, October 2012 to Februar 2013
- Internship in Prof. Holger Daims' group at the *Division of Microbial Ecology*, University of Vienna, *Microdiversity of* Nitrospira *in activated sludge*, April to June 2012
- Tutor for an applied chemistry course for biologists at University of Vienna, winter term 2011 and summer term 2012
- Trainee with a DFG project in *Parque Nacional de Podocarpus*, Ecuador in Prof. Wolfgang Wilcke's group, *Aluminum tolerance of tropical tree saplings*, August to September 2011

SKILLS AND METHODS IN THE FIELD OF MICROBIOLOGY

- Extensive skills in fluorescence *in situ* hybridization (incl. MAR-FISH, DOPE-FISH, FISH on filters, FISH in liquid phase, CARD-FISH, Clone-FISH) and fluorescence microscopy (incl. laser scanning microscopy and laser-microdissection)
- Aerobic and anaerobic cultivation and enrichment of slow growing microorganisms
- PCR based experiments (incl. primer design and MDA)
- Raman spectroscopy of microorganisms
- Basic skills in flow cytometry
- Software package *ARB* for phylogenetic analyses

- *daime* software for spatial arrangement analyses of microbial communities and analyses of probe dissociation curves
- Basic skills in *R*

PUBLICATIONS

- Koch, H., Albertsen, M., Kitzinger, K., Herbold, C., Lücker, S., Spieck, E., Nielsen, P. H., Wagner, M., Daims, H., *In Preparation*. Nitrification by circular feeding: Versatile nitrite-oxidizing bacteria cleave urea in an extended interaction with ammonia oxidizers.
- Gruber-Dorninger, C., Pester, M., Kitzinger, K., Savio D.F., Loy, A., Rattei, T., Wagner, M. & Daims, H., 2015. Functionally relevant diversity of closely related *Nitrospira* in activated sludge. *The ISME JournaL,* 9(3).
- Daebeler, A., Schwarz, J., Kitzinger, K., Koch, H., Daims, H., 2014. *Haloalkaliphilic nitrite-oxidizing bacteria in Austrian soda lakes*. Talk at ISME Conference, Seoul.
- Lee, T. K., Kitzinger, K., Wozak, M., Palatinszky, M., Schmid, M., Berry, D., Albersten, M., Nielsen, P., Woyke, T., Daims, H., Wagner, M., 2014. *Raman-based microcolony genomics for studying the microevolution and ecology of nitrifiers*. Talk at ISME Conference, Seoul.

LANGUAGES

- German (mother tongue)
- English (fluent)
- Norwegian, Bokmål (good)
- French (intermediate)
- Spanish (intermediate)
- Swedish (beginner)
- Danish (beginner)
- Latin