universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Cyanate utilization by and cryopreservation of *Nitrospira moscoviensis*"

verfasst von / submitted by

Mario Pogoda, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2015 / Vienna 2015

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 830
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Mikrobiologie, Mikrobielle Ökologie und Immunbiologie
Betreut von / Supervisor:	Prof. Dr. Dr. h.c Michael Wagner

1. Introduction:..... 4

1.1. Nitrogen cycle..... 4

1.2. Nitrite oxidizing bacteria..... 6

1.3. The genus *Nitrospira*..... 6

1.3.1. *Nitrospira moscoviensis*..... 8

1.4. Cyanate, a toxic nitrogen source..... 9

1.4.1. Appearance of cyanate in nature..... 9

1.4.2. Cyanate metabolism and the role of cyanase..... 11

1.5. Aims of this study..... 12

1.5.1. Exploring the capability of cyanate utilization by NOB..... 12

1.5.2. Cryopreservation of *Nitrospira moscoviensis*..... 12

2. Material and Methods:..... 13

2.1. Equipment and consumables..... 13

2.2. Chemicals..... 15

2.3. Kits..... 16

2.4. Buffers and solutions..... 17

2.5. 16S rRNA targeted oligonucleotide probes used for FISH..... 18

2.6. Software..... 18

2.7. Strains and standard growth conditions..... 18

2.8. Cultivation and maintaining..... 19

2.9. Fluorescence in situ hybridization..... 19

2.9.1. PFA-Fixation..... 19

2.9.2. Hybridization, probes and monitoring..... 20

2.10. Experimental procedure:..... 20

2.10.1. Preparation of flasks and rubber stoppers..... 20

2.10.2. Media and chemical components..... 21

2.10.3. Harvesting and washing *Nitrospira* cultures..... 21

2.10.4. Preparation of *Nitrospira* cells for dead-control..... 21

2.10.5. Sampling and chemical analysis:..... 21

2.10.6. pH measurement, OD assessment and protein analyses..... 22

2.11. Experiments..... 23

2.11.1. Exploring possible effects of cyanate and ammonia on the activity of *N. moscoviensis*..... 23

2.11.3. Chemical degradation of cyanate in the presence of nitrite..... 25

2.11.4. Degradation of cyanate to ammonium by *Nitrospira moscoviensis* (high cell density experiment)..... 26

2.11.5. Cryopreservation of *Nitrospira moscoviensis*..... 28

3. Results 30

3.1. Exploring possible effects of different cyanate and ammonium concentrations on the activity of *Nitrospira moscoviensis* 31

3.1.1. Effect of ammonium on the nitrite oxidation of *N. moscoviensis* 31

3.1.2. Effect of cyanate on the nitrite oxidation of *N. moscoviensis* 32

3.2. Degradation of cyanate to ammonium by *Nitrospira moscoviensis* (low cell density experiment) 33

3.2.1. Experimental set up influences the stability of pH 33

3.2.2. Degradation of cyanate by *Nitrospira moscoviensis* 33

3.3. Degradation of cyanate to ammonium by *Nitrospira moscoviensis* (high cell density experiment) 37

3.3.1. Increased cell density boost nitrite consumption 37

3.3.2. Cyanate degradation and ammonium formation by *Nitrospira moscoviensis* 39

3.4. Chemical degradation of cyanate in the presence of nitrite 40

3.4.1. Ammonium released in abiotic incubations with cyanate and nitrite 40

3.5. Cryopreservation and revival of *Nitrospira moscoviensis* 42

3.5.1. Recovering of activity after cryopreservation 42

4. Discussion 44

4.1. Effects of different cyanate and ammonium concentrations on the activity of *Nitrospira moscoviensis* 45

4.2. Degradation of cyanate to ammonium by *Nitrospira moscoviensis* 46

4.3. Cryopreservation and revival of *Nitrospira moscoviensis* 48

5. Summary 50

6. Zusammenfassung: 52

7. Abbreviations: 54

8. References: 56

9. Acknowledegements: 62

10. Curriculum vitae: 63

1. Introduction:

1.1. Nitrogen cycle

The huge diversity of microorganisms on our planet is crucial for the existence of all kinds of life. Many chemical processes which microorganisms carry out, such as recycling of key nutrients or degrading of organic matter are essential for the support and maintenance of life on earth (Madigan *et al*., 2008). Nitrogen is one of the most abundant elements on earth and is essential for the synthesis of nucleic acids, proteins and other relevant chemical compounds (Canfield *et al*., 2010). The nitrogen cycle represents a network of chemical transformations, by which nitrogen is converted into various redox states, ranging from -3 in ammonia (NH_3) up to +5 in nitrate (NO_3^-) (Fig.1) (Bock and Wagner, 2006). Various microorganisms are able to transform nitrogen compounds through oxidative or reductive processes. These processes are dependent on the metabolic capabilities of the microorganisms (Bock and Wagner, 2006; Madigan *et al*., 2008) and can be distinguished in several pathways.

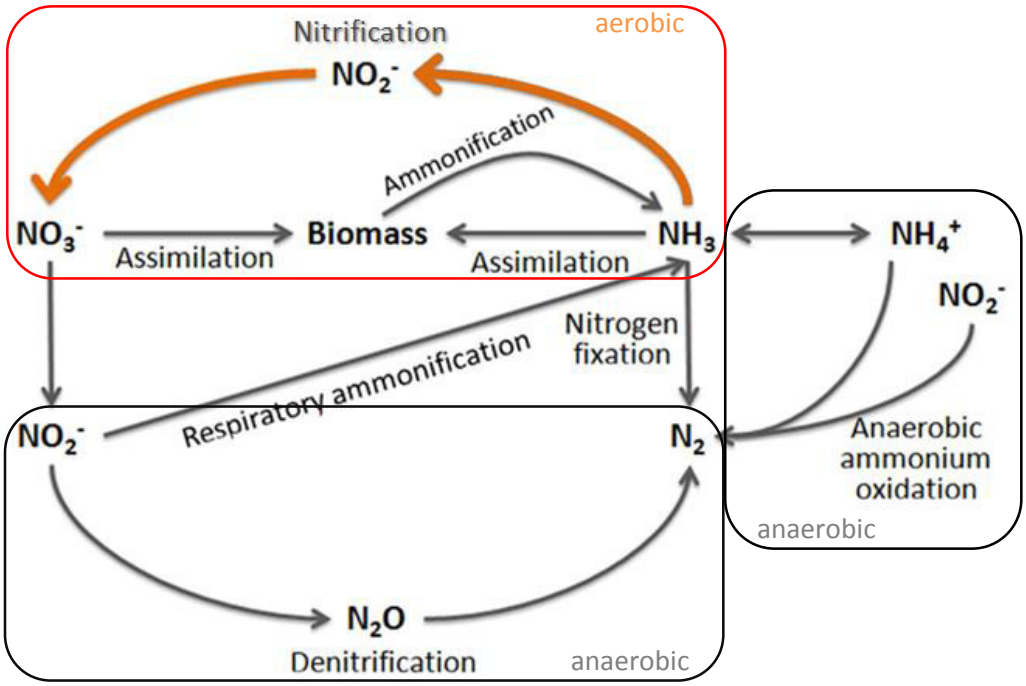

Fig.1: Schematic overview of the nitrogen cycle. The orange line shows the pathway of nitrification. Figure modified from Lücker (2013).

The first step in the biogeochemical nitrogen cycle is nitrogen fixation, which is performed exclusively by bacteria and archaea (Madigan *et al*., 2008). During nitrogen fixation, a large enzyme complex called nitrogenase catalyzes the breakage of the triple bond between the two nitrogen atoms and NH_3 is formed, which can be used as nitrogen source by other organisms (Bothe *et al*., 2007; Madigan *et al*., 2008).

Another natural source of NH_3 is decomposition, also known as ammonification. During this process, nitrogen from nitrated organic compounds (such as proteins or nucleotides) is released into the environment by heterotrophic microorganisms (Benbi and Richter, 2002) and later converted into inorganic NH_3 (White and Reddy, 2009).

The conversion of ammonia (NH_3) via nitrite (NO_2^-) into nitrate (NO_3^-) is called nitrification which is a key pathway within the nitrogen cycle (Prosser, 1989). Although this process requires oxygen, microorganisms involved in nitrification can also colonize hypoxic habitats (Lücker *et al*., 2013). Two distinct groups of microorganisms, ammonia oxidizing bacteria and archaea (called ammonia oxidizing microorganisms, AOM) catalyze the first step of nitrification by oxidizing ammonia to nitrite (Könneke *et al*., 2005; Madigan *et al*., 2008). The first part of this two-step process, the oxidation of ammonia to hydroxylamine is dependent on two electrons, which are received from the oxidation of hydroxylamine to nitrite (Bock and Wagner, 2006).

Nitrite oxidizing bacteria (NOB) perform the second step of nitrification by converting nitrite to nitrate (Bock and Wagner, 2006; Madigan *et al*., 2008). The coexistence between nitrite oxidizers and ammonia oxidizers leads to a mutualistic relationship (Stein and Arp, 1998; Okabe *et al*., 1999) with benefits for both groups. NOB are able to use the nitrite, produced by AOM, to gain their energy (Madigan *et al*., 2008), whereas the removal of nitrite, which is toxic for AOM at higher concentrations, is beneficial to the AOM (Stein and Arp, 1998).

Denitrification is a reductive process, which involves the conversion of nitrate to nitrogen via several reaction intermediates under anoxic conditions. First, nitrate is converted into nitrite, which is further reduced to nitric oxide (NO) and subsequently nitrous oxide (N_2O) is formed by reduction of NO. The final step of denitrification is the reduction of N_2O to nitrogen (N_2) (Bothe *et al*., 2007). Several prokaryotes, including heterotrophic bacteria (Carlson and Ingraham, 1983), and chemolithoautotrophic microorganisms (Baalsrud and Baalsrud, 1954), as well as eukaryotic organisms (Shoun and Tanimoto, 1991) can catalyze this process.

Anaerobic ammonium oxidation (anammox) is another oxidative process within the nitrogen cycle, where ammonium (NH_4^+) is oxidized under anoxic conditions (using nitrite as an electron acceptor)

into nitrogen (N_2) by a specialized group of bacteria (Mulder *et al*., 1995; Strous *et al*., 2006; Kuenen, 2008; Kartal *et al*., 2011). This so called "anammox" bacteria have been found in different habitats, Including freshwater, marine ecosystems and wastewater treatment plants (WWTPs) (Dalsgaard *et al*., 2005; Jetten *et al*., 2005).

1.2. Nitrite oxidizing bacteria

Nitrite oxidizing bacteria (NOB) perform the second step of nitrification by converting NO_2^- to NO_3^- (Bock and Wagner, 2006). These chemolithoautotrophic organisms use nitrite as their energy source and carbon dioxide (CO_2^-) as a carbon source. The oxidation of nitrite to nitrate (Equation 1) is catalyzed by the membrane-associated enzyme nitrite oxidoreductase (Nxr). The gained electrons are released and subsequently enter the respiratory chain (Bock *et al.,* 2006; Alawi *et al.,* 2007). Nxr can be found in different areas of the cell membrane. Nxr is located on the inner cell membrane of the intracellular membrane (ICM) in *Nitrobacter* and *Nitrococcus* (Meincke *et al*., 1992), whereas Nxr of *Nitrospira* is located in the periplasmic space (Spieck *et al*., 1998; Ehrich *et al*., 1995; Lücker et *al*., 2010).

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

Equation 1: Nitrite-oxidation of NOB into nitrate. This process is catalyzed by Nxr.

NOB can be found in nearly all terrestrial and aquatic environments and play a key role in wastewater treatment. NOB are phylogenetically diverse and belong to different subclasses of the *Proteobacteria* (*Alpha*, *Beta* and *Gamma*) and to the phyla *Chloroflexi*, *Nitrospirae* and *Nitrospinae*.

Over decades, only few NOB species could be isolated und cultivated, mainly because of the difficulties to cultivate and maintain these organisms under laboratory conditions (Sorokin *et al*., 2012; Alawi *et al*., 2007; Elbanna, 2012).

1.3. The genus *Nitrospira*

The genus *Nitrospira* belongs to the functional group of NOB within the phylum *Nitrospirae*. Hitherto, all known *Nitrospira* are chemolithotrophs, which gain their energy by oxidizing nitrite. All *Nitrospira*

are autotrophic, but some are also able to use organic carbon sources like pyruvate (Daims *et al.,* 2001). Members of the genus *Nitrospira* are barely studied and only few enrichment cultures are available, although they are among the most diverse and widespread nitrifiers in natural ecosystems and biological wastewater treatment plants (Lücker *et al*., 2010). Today, a big diversity with at least six different phylogenetic lineages could be detected within the genus *Nitrospira* (Fig.2). Cultivation-independent analyses of *Nitrospira* in sewage of wastewater treatment plants indicate their importance for nitrogen removal and processing of wastewater, whereas over decades it has been postulated that *Nitrobacter* is the key player in nitrification in this habitat due to cultivation bias (Juretschko *et al.,* 1998; Daims *et al.,* 2001; Schramm *et al.,* 1999; Daims *et al.,* 2006).

Most of the known members of the genus *Nitrospira* are uncultured (Burrell *et al.,* 1998; Juretschko *et al.,* 1998; Daims *et al.,* 2001; Freitag *et al*., 2005; Off *et al*., 2010).

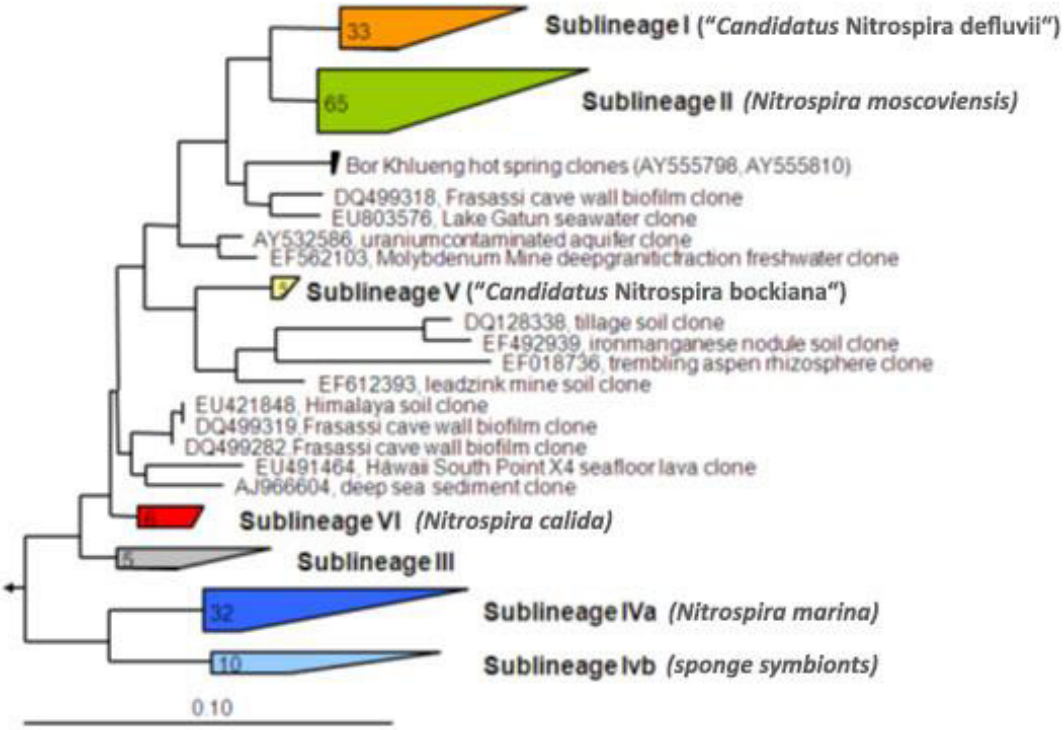

Fig.2: Phylogenetic tree based on 16S rRNA gene sequences of selected *Nitrospira* like bacteria and their clustering in different sublineages (I – VI), indicated by different colors (Koch 2009, Ecophysiological investigation of nitrite-oxidizing bacteria of the genus *Nitrospira.* Diploma thesis.)

Analyzes of all 16S rRNA gene sequences currently available show more than 94.9 % similarity in one sublineage. However, the similarity of two sequences from different sublineages is below 94% (Watson *et al.,* 1986; Daims *et al.,* 2001). Sequences of each sublineage can be found in different environments all over the world. Most sequences from sublineage I and the isolate "Candidatus *Nitrospira defluvii*" were obtained from WWTP. 16S rRNA gene sequences from sublineage II have been found in different habitats like oil, rhizosphere, WWTPs, lake water and freshwater aquaria. The isolates *Nitrospira moscoviensis* and *Nitrospira lenta* belong to this sublineage (Ehrich *et al.,* 1995; Nowka *et al*., 2014). Sublinage III contains only few sequences from the Nullarbor caves, Australia, without any enrichments or isolates. *Nitrospira marina* and other sequences retrieved from the sea and marine sponges, belong to sublineage IV. Sublineage V consists of *Candidatus Nitrospira bockiana. Nitrospira calida,* an enrichment from a hot spring in the Baikal rift zone forms the sixth and last sublineage within this genus (Lebedeva *et al.,* 2011). Beside sublineage II, which contains NOB that can be found in a wide range of other environments, all the other members of sublineage I, III, IV, V, VI seem to be adapted to a specific type of habitat (Daims *et al.,* 2001).

Some NOB are able to use organic compounds in addition to NO_2 and CO_2^-, including Candidatus *Nitrospira defluvii* and *Nitrospira marina*, which can grow mixotrophically (Watson *et al*., 1986; Spieck *et al*., 2006; Lücker *et al*., 2010; Daims *et al.,* 2001; Gruber-Dorninger *et al.,* 2014). *Nitrospira defluvii* is also able to assimilate formate without the presence of nitrite (Gruber-Dorninger *et al*., 2014).

1.3.1. *Nitrospira moscoviensis*

N. moscoviensis belongs to the widespread *Nitrospira* lineage II. It is a gram-negative, non-motile, non-marine, obligate lithoautotrophic nitrite-oxidizer, which was isolated from an enrichment culture initiated with a sample from a partially corroded area of an iron pipe of a heating system in Moscow, Russia (Ehrich *et al*., 1995). This bacterium has helical- to vibroid-shaped cells and is approximately 0.9–2.2 µm X 0.2–0.4 µm long. *N. moscoviensis* possesses an enlarged periplasmic space and lacks intracytoplasmic membranes and carboxysomes. The optimal growth conditions are at 39°C and pH 7.6–8.0 in a mineral medium with nitrite as sole energy source and carbon dioxide as sole carbon source. Incubation with more than 15 mM nitrite or 75 mM nitrate operates inhibitory. The doubling time at optimal condition is around 12 hours. The key enzyme for NO_2^- oxidation is the nitrite oxidoreductase (NXR), which is an iron-sulfur molybdoprotein located on the inner cell membrane of the periplasmic space (Spieck *et al*., 1998; Ehrich *et al*., 1995). The reaction catalyzed by this NXR is reversible, so that the enzyme can also reduce NO_3^- with electrons derived from organic compounds

(Lücker *et al*., 2010) and hydrogen (Ehrich *et al.,* 1995). The cytochromes of the cytoplasmic membrane belongs to the b- and c-type and the G+C content of DNA is around 56.9 ± 0.4 mol%.

As mentioned earlier, members of the genus *Nitrospira* can use organic compounds (Spieck *et al*., 1998; Daims *et al.,* 2001; Gruber-Dorninger *et al.,* 2014). Ehrich and colleagues indicated that organic substrate inhibits the activity of *N. moscoviensis* (Ehrich *et al.,* 1995). However, it is unknown if *N. moscoviensis* is able to use organic substrates in other conditions as carbon sources (mixotrophy) or for energy generation (organotrophy). Genomic analyses of *N. defluvii* and *N. moscoviensis* revealed that the genomes of this NOB encode pathways for the catabolic degradation and for the assimilation of acetate, pyruvate and formate (Lücker *et al.,* 2010; Koch, in preparation). New investigations also indicate that *N. moscoviensis* are able to utilize hydrogen (H_2) as an alternative energy source for aerobic respiration and can grow on hydrogen without any nitrite source (Koch *et al*., 2014). NOB are exposed to a plethora of potentially toxic substances in sewage. Because of these toxins, *N. moscoviensis,* like *N. defluvii*, possesses several multidrug efflux systems and transporters for heavy metals, organic solvents, and antimicrobials (Lücker *et al*., 2010). Genes for cyanate and arsenic resistance were found as well (Lücker *et al*., 2010).

1.4. Cyanate, a toxic nitrogen source

1.4.1. Appearance of cyanate in nature

Cyanate (CNO^-) is a reduced nitrogen compound, which can be found in aquatic and terrestrial ecosystems. In nature, cyanate is formed by spontaneous dissociation of urea in aqueous solution (Drinhuber and Schutz, 1948; Kamennaya *et al.,* 2008) or by photo-oxidation of cyanide (Raybuck *et al*., 1992). Despite the low concentrations (nM range) in marine waters, cyanate is a common N-source for cyanobacteria (Kamennaya *et al.,* 2008; Widner *et al.,* 2013; Kamennaya *et al.,* 2013). Cyanate is also common in wastewaters of cokes and steel industries (Zhang *et al*., 1998; Kim *et al*., 2006) and high amounts of cyanate can lead to problems in affected ecosystems (Bonaventura and Johnson, 1997; Kimochi *et al*., 1998).

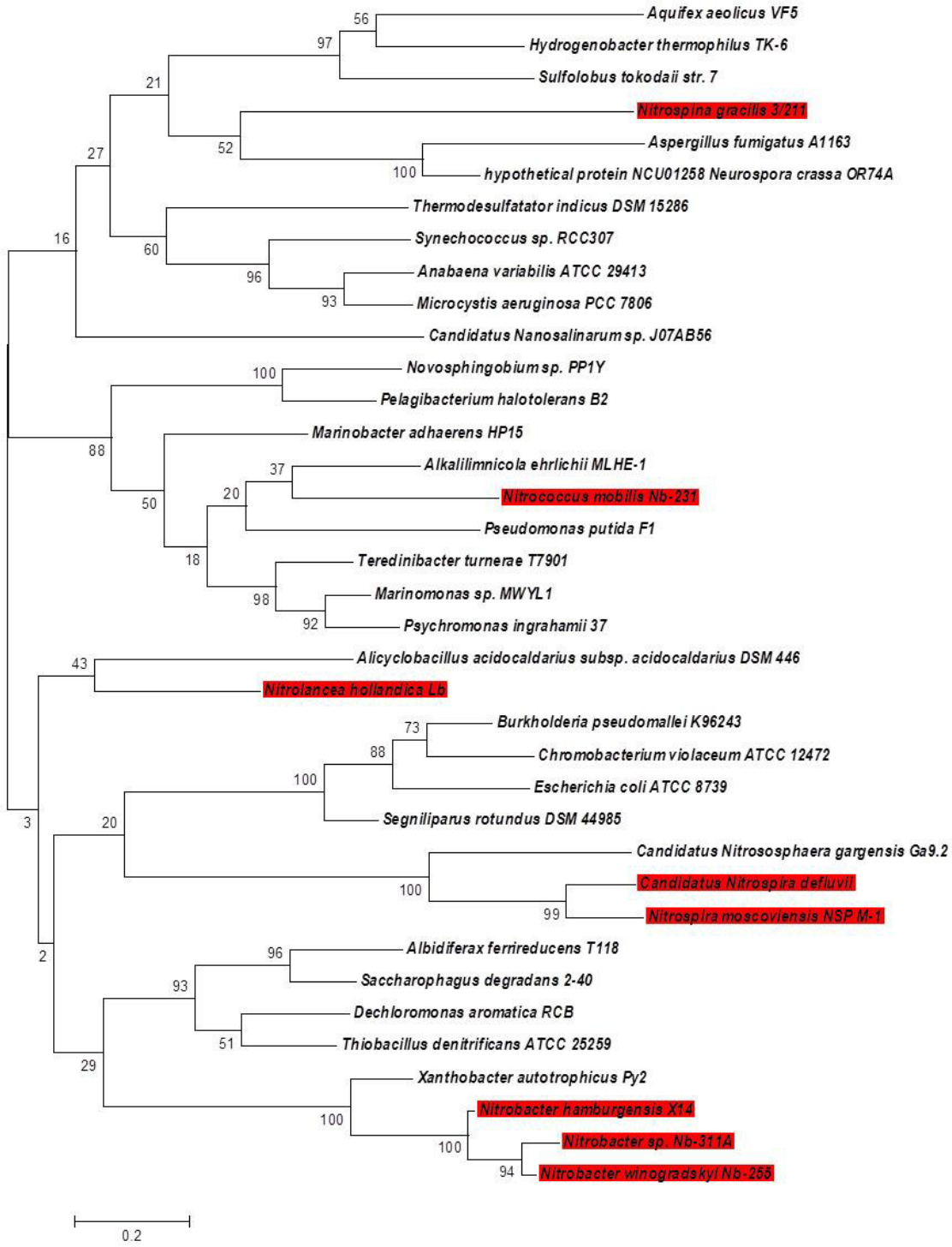

Fig.3: Maximum likelihood phylogenetic tree, showing amino acid sequences of cyanase from 37 organisms. Cyanase of NOB are shown in red. The depicted tree was constructed by using MEGA 6, version 6.0.5 (Tamura *et al.,* 2013). The scale bar represents 0.2 substitutions per site.

Despite the toxicity of cyanate, several studies indicate that cyanate might be an important nitrogen source to support the growth of aquatic microbes and, thus, may play a role in aquatic nitrogen cycling (Kamennaya and Post, 2013; Widner *et al*., 2013).

The utilization of cyanate by the cyanobacterial community is well-described and several other bacteria might be also able to use cyanate as a nitrogen source (Suzuki *et al*., 1996; Scanlan *et al*., 2009; Maeda and Omata, 2009). However, despite the successful usage of activated sludge processes for treating various industrial wastewaters, high concentration of cyanate and other toxic compounds severely inhibit the biological activity of activated sludge and other microbial communities (Amor *et al*., 2005; Kim *et al*., 2008; Kumar *et al*., 2003; Liu *et al*., 2005). Further investigations are necessary for better understanding the mechanisms by which high cyanate concentrations affect nitrification.

1.4.2. Cyanate metabolism and the role of cyanase

Cyanate is converted into ammonia (NH_3) and carbon dioxide (CO_2) in a bicarbonate-dependent reaction by the enzyme cyanase (EC 4.2.1.104), also known as cyanate lyase or cyanate hydratase (Johnson *et al.,* 1987). Structural analyses revealed that the cyanase of *Escherichia coli*, the first discovered cyanase (Taussig *et al.,* 1960), is a homodecamer of 17-kda subunit with three amino- acids as catalytic residues that bind cyanate and bicarbonate (Walsh *et al*., 2000). The catalysis mechanism of cyanase involves HCO_3^- as substrate and leads to the formation of a putative dianion intermediate, a combination of HCO_3^- and OCN^-, followed by a decarboxylation, which produces the first CO_2 molecule and carbamate. The adjacent spontaneous decarboxylation of CO_2 and carbamate leads to the production of a second CO_2 and ammonia (Anderson, 1980; Kozliak *et al*., 1995).

Genome analyses and BLAST searches of cyanase from *Escherichia coli* revealed gene homologues to the genes encoding cyanase in other *Proteobacteria*, cyanobacteria, plants and fungi (Fig.3) (Guilloton *et al*., 2002). Several studies on cyanate metabolism among different bacteria discovered that cyanases have diverse physiological functions, ranging from cyanate detoxification and production of NH_3 as an alternative N-source (Dorr and Knowles, 1989; Guilloton and Karst, 1987; Miller and Espie, 1994). In addition, CO_2 produced by cyanate degradation can be used for carbon fixation in photosynthetic cyanobacteria (Anderson and Little 1985; Luque-Almagro *et al*., 2008; Miller and Espie, 1994; Suzuki *et al*., 1996; Walsh *et al*., 2000).

Cyanate interacts with nucleophilic groups in proteins, which explain its toxic effect on organisms (Stark, 1965). Therefore, cyanases might have an important function in detoxification by lowering cyanate cell concentration in cyanate-rich environments (Guilloton and Karst, 1987).

1.5. Aims of this study

1.5.1. Exploring the capability of cyanate utilization by NOB

The main aim of this study was to examine the potential of *N. moscoviensis* to convert cyanate to ammonia and carbon dioxide. Interestingly, all sequenced NOB genomes encode a cyanase (Fig. 3). This enzyme might be important for detoxification, on the other hand cyanate might also be used as a nitrogen source, or to provide NH_3 to AOM, which lack cyanases. This ammonia, provided by NOB, could be an energy source for AOM which then oxidize it to nitrate, serving again as electron donor to the NOB.

Among all known AOM, only *Nitrososphaera gargensis*, an ammonia oxidizing archaea (AOA), is known to possess a cyanase. Interestingly, the cyanase of *N. defluvii* and *N. moscoviensis* are closely related to the one of *N. gargensis* (Fig.3). This finding suggests that respective genes could have been acquired by lateral gene transfer and *N. gargensis* has exchanged these genes with nitrifiers sharing the same niche (Spang *et al.,* 2012).

1.5.2. Cryopreservation of *Nitrospira moscoviensis*

High quality and stable long-term cryopreservation of bacteria are essential for long-term storage of these organisms. This is especially true for NOB, which are known to be difficult to cultivate and to maintain in the laboratory. Therefore, it is important to establish a good working protocol to cryopreserve NOB. The work of Vekeman and colleagues indicates several methods to cryopreserve different NOB species with great vitality after several months (Vekeman *et al.,* 2013). The aim of this study was to test different cryopreservation protocols for long-term storage of *N. moscoviensis*. For this purpose, pure cultures of *N. moscoviensis* were cryopreserved according to different protocols of Vekeman for two months to obtain a backup for further experiments (Vekeman *et al.,* 2013).

2. Material and Methods:

2.1. Equipment and consumables

Equipment	Company
AXIO-Imager M1 microscope	Zeiss, Oberkochen, Germany
CamSpec M107 Spectrophotometer	Spectronic Camspec Ltd, Garforth, UK
Centrifuges: **Rotina 35R** **Centrifuge 5804R** **Mini Spin** **Mini Star silverline**	 Andreas Hettingen GmbH & Co. KG, Tuttlingen, Germany Eppendorf AG, Hamburg, Germany Eppendorf AG, Hamburg, Germany VWR® International GmbH, PA, USA
Cover glasses 24 x 50 mm	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Digital Thermoblock **Mixing Block heating**	VWR® International GmbH, PA, USA Biozym scientific GmbH, Hessisch Oldendorf, Germany
Disposable syringe (1 ml, 3 ml, 5, ml, 30 ml)	B. Braun Melsungen AG, Melsungen, Germany
Disposable needles (100 sterican)	B. Braun Melsungen AG, Melsungen, Germany
Eppendorf reaction tubes (ERT)	Eppendorf AG, Hamburg, Germany
Erlenmeyer flasks different sizes	VWR® International GmbH, PA, USA
Galaxy mini centrifuge	VWR® International GmbH, PA, USA
Hybridization oven	Memmert GmbH + Co.KG, Germany
inoLab pH Level 1 -meter	WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Magnetic stirrer: **ArgoLab M2-D Pro Digital** **RCT Basic IKAMAG®**	 Giorgio Bormac S.r.l. Via della Meccanica, Carpi, Italy IKA Werke, Staufen, Germany
Merckoquant® Nitrite test strips: **Range: 2-80mg l^{-1}** **0.1-3 g l^{-1}**	Merck chemicals, Darmstadt, Germany
Merckoquant® Nitrate test strips **Range: 0-500 mg l^{-1}**	Merck chemicals, Darmstadt, Germany

Microscope slides, 10 reaction wells	Marienfeld Laboratory Glassware GmbH & Co. KG, Lauda-Königshofen, Germany
Pipette tips 2.5 µl	Biozym scientific GmbH, Hessisch Oldendorf, Germany
Pipette tips 1-200 µl	Lactan GmbH & Co KG, Graz, Austria
Pipette tips 100-1000 µl	Biozym scientific GmbH, Hessisch Oldendorf, Germany
Pipettes Serological (2 ml, 10ml, 25 ml)	VWR® International GmbH, PA, USA
Pipettes: Eppendorf research **0.2-2.5 µl ; 2-20 µl ; 20-200 µl ; 100-1000µl** **Multipipette stream**	 Eppendorf AG, Hamburg, Germany Eppendorf AG, Hamburg, Germany
Plastic Cuvettes	Greiner Bio-One GmbH, Frickenhausen, Germany
PS-Microplate, 96 wells, flat bottom, sterile	Greiner Bio-One GmbH, Frickenhausen, Germany
Reactions vessels 15 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Reactions vessels 50 ml (blue caps)	Greiner Bio-One GmbH, Frickenhausen, Germany
Reactions vessels 50 ml (green caps)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Rubber stoppers, 4.5 cm	Glasgerätebau Ochs Laborfachhandel e.K., Bovenden, Germany
Syringe Filter 0.45 µm **Syringe Filter 0.2 µm**	Thermo Fisher scientific, New York, USA Thermo Fisher scientific, New York, USA
Tecan reader Infinite 200	Tecan Group Ltd., Männedorf, Switzerland
Thermo Haake P5 water bath **Thermo Haake DC 10 water bath**	Thermo Fisher scientific, New York, USA Thermo Fisher scientific, New York, USA

Water bath GFL®	GFL®, Burgwedel, Germany

2.2. Chemicals

Chemicals	Company
Ammonium chloride (NH_4Cl_2) ≥ 99.8%	Merck chemicals, Darmstadt, Germany
Anthranilic acid ≥ 98%	Sigma-Aldrich, St. Louis, MO, USA
Bicarbonate ($NaCHO_3$)	Baker, Deventer, Holland
Boric acid >99.8% p.a., ACS, ISO	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Calcium chloride ($CaCl_2$) ≥ 99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Calcium carbonate (CaCO3) ≥ 99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Citifluor AF1, **Glycerol/PBS solution**	Agar Scientific Ltd., Stansted, UK
Di-chlorisocyanuric acid sodiumsalt dehydrate ≥ 98%	Fluka Chemie AG, Buchs, Switzerland
Cobalt (II) chloride hexahydrate ($CoCl_2$ $6H_2O$)	Sigma-Aldrich, St. Louis, MO, USA
Copper (II) sulfate ($CuSO_4$) ≥ 99%	Sigma-Aldrich, St. Louis, MO, USA
Dimethyl sulfoxid (DMSO)	Fluka Chemie AG, Buchs, Switzerland
96% Ethanol	96% Ethanol, denatured Inhouse
Ethylenediamine tetra acetic acid **disodium salt dihydrate >99% p.a.,** **ACS (Na_2EDTA dihydrate)**	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Formaldehyde (37% (w/w))	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Formamide (FA)	Fluka Chemie AG,Buchs, Switzerland
Iron (II) sulfate heptahydrate (Fe_2SO_4 $7H_2O$) ≥ 99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Magnesium sulfate monohydrate ($MgSO_4$) ≥ 99,5%	Merck chemicals, Darmstadt, Germany
Manganese (II) sulfate monohydrate ($MnSO_4H_2O$)≥ 98%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
N-(1-Naphtyl)-ethylenediamine 2HCl ≥ 98%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Nessler's reagent	Fluka Chemie AG, Buchs, Switzerland
Nickel (II) chloride hexahydrate ($NiCl_27H_2O$) ≥ 97%	Riedel-de Haën AG, Seelze, Germany
37% Paraformaldehyde	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
O-phosphoric acid ≥ 85%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium cyanate (KOCN) ≥ 96%	Sigma-Aldrich, St. Louis, MO, USA
Potassium phosphate monobasic (KH_2PO_4) ≥ 99%	Sigma-Aldrich, St. Louis, MO, USA
Sodium chloride (NaCl) ≥ 99.5%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hydroxide (NaOH) ≥ 99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium hydrogen carbonate ($NaHCO_3$)≥ 99.7%	Merck chemicals, Darmstadt, Germany
Sodium molybdate dihydrate (Na_2MoO_4 $2H_2O$) ≥ 99.5%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium nitrite ($NaNO_2^-$) ≥ 98.7%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium nitroprusside dehydrate ($C_5FeN_6Na_2O$ $2H_2O$) ≥ 99%	Fluka Chemie AG, Buchs, Switzerland
Sodium dihydrogen phosphate dihydrate ($NaH_2PO_42H_2O$)≥ 98%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_42H_2O$)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium salicylate ≥ 99.5%	Sigma-Aldrich, St. Louis, MO, USA
Sulfanilamide ≥ 98%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
tris(hydroxymethyl)-aminomethane	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Zinc chloride ($ZnCl_2$) ≥ 98%	Riedel-de Haën AG, Seelze, Germany

2.3. Kits

Used kits	Company
Pierce® BCA protein assay kit	Thermo Scientific, Waltham, MA, USA

2.4. Buffers and solutions

PBS stock solution	
Na_2HPO_4-Dihydrate [0.2 M]	35.6 g
NaH_2PO_4-Dihydrate [0.2M]	31.20 g
PBS (1 x)	
NaCl [130 mM]	7.6 g
Na_2PO_4 (PBS-Stock-solution) [10 mM]	50ml l^{-1}
MQ	ad 1000 ml
pH 7.2-7.4	
Formaldehyde (4%)	
Formalin (37 %)	21,6 ml
MQ	178,4 ml
EDTA [0.5 M]	
EDTA	186 g
MQ	ad 1000 ml
To adjust pH to 8, crystalline NaOH was added	
NaCl [5 M]	
NaCl	292.2 g
MQ	ad 1000 ml
Tris / HCl [1 M]	
Tris	30.3 g
MQ	ad 250 ml
pH was adjusted to 8 with fuming HCl.	
SDS [w/v 10 %]	
SDS	5 g
MQ	ad 50 ml
NaOH [2 N]	
NaOH	4 g
MQ	ad 50 ml

2.5. 16S rRNA targeted oligonucleotide probes used for FISH

Probe	Sequence 5´-3´	Binding position (*E. coli* 16SrRNA nomenclature)	Target	FA %	Reference
General Probes					
EUB338-I	GCT GCC TCC CGT AGG AGT	338–355	most *Bacteria*	0-50	(Amann *et al.*, 1990)
EUB338-II	GCA GCC ACC CGT AGG TGT	338–355	*Planctomycetales*	0-50	(Daims *et al.*, 1999)
EUB338-III	GCT GCC ACC CGT AGG TGT	338–355	*Verrucomicrobiales*	0-50	(Daims *et al.*, 1999)
Probes for *Nitrospira moscoviensis*					
Ntspa1151	TTC TCC TGG GCA GTC TCT CC	1151 - 1170	Sublineage II of the genus *Nitrospira*	35-40	(Maixner *et al.*, 2006)

2.6. Software

Software	URL	References
Adobe Illustrator CS6	http://www.adobe.com/	Adobe Systems Incorporated, San José, CA, USA
Mega 6	http://www.megasoftware.net/	Tamura *et al.*, 2013
ProbeBase	http://www.microbial-ecology.net/probebase/	Loy *et al.*, 2003
Origin® 9.1	http://www.originlab.de/	OriginLab Corporation, Northampton, MA, USA

2.7. Strains and standard growth conditions

The nitrite-oxidizing bacterium *N. moscoviensis* was grown in a mineral NOB medium containing (per 1 liter): 10 mg $CaCO_3$; 500 mg NaCl; $MgSO_4x7H_20$; 150 mg KH_2PO_4; 10 mg NH_4Cl, as well as 1 ml sterilized NOB-specific trace elements solution (NOB-TES) added after autoclaving. NOB-TES contains (per 1 liter): 34,4 mg $MnSO_4x1H_2O$; 50 mg H_3BO_3; 70 mg $ZnCl_2$, 72,6 mg $Na_2MoO_4x2H_2O$; 20 mg $CuCl_2x2H_2O$;

24 mg $NiCL_2x6H_2O$, 80 mg $CoCL_2x6H_2O$; 1g $FeSO_4x7H_2O$. All salts, except $FeSO_4x7H_2O$ were dissolved in 997,6 ml MQ water and 2,5 ml of 37% (smoking) HCL was added before dissolving $FeSO_4x7H_2O$ salt. The pH was adjusted to 8.6 before autoclaving and dropped to 7.6 after autoclaving.

For all experimental incubations, media components were the same, except that no NH_4Cl was added for incubations with cyanate or ammonium (NOB medium without NH_4^+).

2.8. Cultivation and maintaining

Both media types (with and without NH_4^+) can be used for growing *N. moscoviensis*, but cultures grew better with the standard NOB media, containing NH_4Cl. All cells were grown in 150, 300 and 1000 ml Erlenmeyer flasks at 37°C for several months in the dark and were fed with 2 mM natrium nitrite solution (final concentration) twice a week. For inoculation, 10 ml of culture were transferred into freshly prepared bottles with NOB media. After reaching the nitrate inhibition range (approximately 75 mM) cultures were centrifuged (9289 g, 15 min, room temperature) and washed twice with fresh NOB media. Nitrite and nitrate concentrations were checked by colorimetric nitrate and nitrite stripes (*Merck* KGaA, Darmstadt, Germany) and the purity of the culture was checked each month by using FISH and microscopy. Therefore, 2 ml of each culture were used for PFA-fixation. During the whole time, nearly all cultivations showed no contaminations. Contaminated cultures were killed by autoclaving.

2.9. Fluorescence in situ hybridization

2.9.1. PFA-Fixation

All PFA fixations were done according to a standardized protocol (Daims *et al.,* 2005). First, 2 ml of sample were taken and centrifuged at 19721 g for 10 min. After discarding the supernatant the pellet was resuspended in 1x PBS and incubated with 4% PFA (3:1; PFA:PBS) for 2 hours at 4°C and centrifuged again with the same parameter as described above. After discarding the supernatant, the pellet was washed with 1 ml PBS, the cell suspension was pelleted by centrifugation again and the resulting cell pellet was resuspended in one volume 1 x PBS and one volume 96% ethanol. Fixed samples were stored at -20°C for long time periods.

2.9.2. Hybridization, probes and monitoring

All hybridizations were performed with PFA fixed biomass according to the standard Fluorescence in situ hybridization (FISH) protocol (Daims *et al.,* 2005).

For hybridization, 5-10 µl PFA fixed biomass of the pure culture of *N. moscoviensis* were transferred on a 10 well slide. The samples were dried at 46°C and dehydrated by an increasing alcohol series (50%, 80% and 96%) each step for 3 min. Hybridization buffer and washing buffer were prepared for the optimal stringency of the probe. Fixed samples were treated with 10 µl hybridization buffer and 1 µl of each probe and incubated in a hybridization chamber (50 ml Greiner Reactions vials with a piece of tissue soaked with the hybridization buffer) for 2 hours at 46°C in dark. After hybridization, all slides were washed in warmed-up washing buffer for 10 min at 48°C in dark. At last, all slides were washed twice with ice-cold MQ water for few seconds and dried by compressed air.

Probes (the lists of all used 16S rRNA targeted oligonucleotide probes see 2.5) were used either single or double labeled at a working concentration of 5 pmol μl^{-1} for Indocarbocyanine (Cy3) and 8 pmol $\mu l^{-1}$for Fluorescein (Fluos) labeled probes. EUB338I, EUB338II and EUB338III were used in combination (EUB338mix) to detect most Bacteria (Daims *et al*., 1999), including *N. moscoviensis* and possible contaminants of pure cultures, as well as Ntspa1151 to detect *N. moscoviensis* specifically. The optimal formamide concentration was at 35% for all probes. In addition, DAPI staining was done after hybridization.

To prevent fading of fluorescence dyes, all slides were covered with Citifluor (AF2). Cells were observed with an AXIO-Imager M1 microscope (Zeiss, Oberkochen, Germany).

2.10. Experimental procedure:

2.10.1. Preparation of flasks and rubber stoppers

All incubations were done in 100 ml Schott bottles (Schott AG, Mainz, Germany), which were washed twice with 6 M HCL solution to remove all possibly contaminating components. All rubber stoppers (Ochs, Bovenden, Germany) were autoclaved several times in fresh MQ water to remove all components, leaking out of the rubber stoppers.

2.10.2. Media and chemical components

The mineral NOB medium was prepared and pH adjusted as described above (2.7). No NH_4Cl was added into the medium to investigate of possible ammonia production from cyanate. Medium was pre-warmed to 25°C to avoid stress for the cells.

All chemical solutions were prepared freshly at the day of the experiment. 20 ml of 100 mM KOCN and $NaNO_2$ was prepared and sterile filtered with a 0.2 µm GTBP filter (Millipore, Billerica, MA, USA). 100 mM $NaHCO_3$ solution was prepared and autoclaved the day before.

2.10.3. Harvesting and washing *Nitrospira* cultures

All washing steps were done under sterile conditions to avoid contaminations. Cultures were transferred into 50 ml reaction tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and centrifuged for 15 min at 9289 g at 25°C. The supernatant was discarded and the cell pellet was dissolved in fresh, warm NOB medium. Cells were pelleted by centrifugation and resuspended with fresh media again. Nitrite and nitrate concentrations were checked by using colorimetric stripes (*Merck* KGaA, Darmstadt, Germany). These steps were repeated until no nitrite or nitrate could be detected. Finally, the cell pellets were dissolved with 20 ml NOB media and stored on ice before the cells were further processed.

2.10.4. Preparation of *Nitrospira* cells for dead-control

To inactivate *Nitrospira* cells, washed cells were fixed with 4 % PFA according to the protocol described above (Daims *et al.,* 2005).

2.10.5. Sampling and chemical analysis:

2.10.5.1. Sampling

All samples were taken by disposable syringes and needles (B. Braun Melsungen AG, Germany). Amounts and time points of sampling varied among experiments. For each experiment, all samples were spun down for 10 min at 19721 g and approximately 1 ml of supernatant was stored at -20°C.

2.10.5.2. Chemical analysis

2.10.5.2.1. Detection of ammonium, nitrite and cyanate

Release of ammonium was analyzed by spectrophotometry via the sodium dichloroisocyanuric acid method (modified version from Kandeler and Gerber, 1988). Nitrite concentrations were measured by spectrophotometry using the sulfanilamide N-1-naphtylethylenedamine dihydrochloride (NEED) reagent method (Stickland and Parsons, 1972). Cyanate was measured after derivatization with 2-aminobenzoic acid to quinazoline-2,4-dione (Guilloton and Karst, 1985).

All spectrophotometric analyses were performed in 96-well plates with the Infinite 200 Pro spectrophotometer (Tecan Group AG, Männedorf, Switzerland). Quick checks of nitrite levels were done via colorimetric nitrite stripes (*Merck* KGaA, Darmstadt, Germany).

2.10.6. pH measurement, OD assessment and protein analyses

2.10.6.1. pH measurement

At the beginning and at the end of each experiments the pH of the medium was measured with the inoLab pH-level 1 meter (WTW Wissenschaftliche-Technische Werkstätten GmbH, Weilheim, Germany).

2.10.6.2. OD assessment

OD assessment and protein analyses were done at the beginning and at the end of each experiment to determine the amount and density of cells in all biotic incubations. 500 µl sample were transferred into plastic cuvettes (Greiner Bio-One GmbH, Frickenhausen, Germany) and OD was measured at OD_{578nm} and OD_{436nm}.

2.10.6.3. Protein analyses

All protein analyses were performed with the Pierce BCA protein assay kit (Thermo scientific). This is a detergent-compatible formulation based on bicinchoninic acid (BCA) for colorimetric detection and quantitation of total protein. This method combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) using a unique reagent containing bicinchoninic acid (Smith *et al.,* 1985). The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion and exhibits a strong absorbance at 562 nm, which in nearly linear with increasing protein

concentration over a broad working range (20-2000 µg/ml). All analyses were done via the CAM SPEC M107 spectral photometer (Spectronic Camspec Ltd, Garforth, UK).

2.11. Experiments

2.11.1. Exploring possible effects of cyanate and ammonia on the activity of *N. moscoviensis*

2.11.1.1. Incubation with ammonium

All Schott bottles were prepared as described in 2.10.1. In each Schott bottle, 100 ml NOB medium prepared with 1 mM $NaNO_2$ and different ammonium concentrations (0, 1, 2, 3, 5 mM = endconcentration) were added. 150 ml of *N. moscoviensis* culture was washed twice with fresh NOB medium to remove all remaining nitrite and nitrate. Washed cells were evenly distributed into all prepared Schott bottles and were incubated without shaking at 37°C in the dark for 96 hours. All incubations were done in duplicates (Tab.1). Sampling and chemical analyses were performed as described in 2.10.5.

Tab.1: Schematic overview of all incubation conditions which were used for the experiment. Each substrate was adjusted to get the final concentration as described. All incubations were performed in duplicates. All ammonium inhibition treatments were incubated for 96 hours.

Incubations	Ammonium [mM]	Nitrite [1 mM]	Culture	Time [h]
A (2x)	0	+	+	96
B(2x)	1	+	+	96
C (2x)	2	+	+	96
D (2x)	3	+	+	96
E (2x)	5	+	+	96

2.11.1.2. Incubation with cyanate

All Schott bottles were prepared as described in 2.10.1. In each Schott bottle, 100 ml standard NOB medium prepared with 1 mM $NaNO_2$ and different cyanate concentrations (0, 1, 2, 3, 5 mM = endconcentration) were added. Additionally, 1 mM $NaHCO_3$ was added to all incubation to sustain chemical activity of cyanase. 150 ml of *N. moscoviensis* culture was washed twice with fresh NOB

medium to remove all remaining nitrite and nitrate. Washed cells were evenly distributed in all prepared Schott bottles and were incubated without shaking at 37°C in the dark for 96 hours. All incubations were done in duplicates. Samples were taken in short time intervals (Tab.2) to get a detailed overview of all chemical processes and the effects of cyanate on *Nitrospira*. Sampling (Tab.3) and chemical analyses were performed as described in 2.10.5.

Tab.2: Schematic overview of all sample time points for all cyanate inhibition groups. Sample amounts were taken as mentioned above and performed as described in 2.10.5. Approx. 500 µl were used for pH analyses from the first and last sampling timepoint.

Day 0	Day 1	Day 2
0 h (1500 µl)	24 h (1000 µl)	48 h (1000 µl)
4 h (200 µl)	28 h (200 µl)	52 h (200 µl)
8 h (200 µl)	32 h (200 µl)	56 h (200 µl)
12 h (1000 µl)	36 h (1000 µl)	60 h (1500 µl)

Tab.3: Schematic overview of all incubation conditions which were used for the experiment. Each substrate was adjusted to get the final concentration as described. All incubations were performed in duplicates. All cyanate inhibition treatments were incubated for 60 hours.

Incubations	Cyanate [mM]	Nitrite [1 mM]	Bicarbonate [1 mM]	Culture	Time [h]
A (2x)	0	+	+	+	60
B (2x)	1	+	+	+	60
C (2x)	2	+	+	+	60
D (2x)	3	+	+	+	60
E (2x)	5	+	+	+	60
F (2x)	0	+	+	-	60

2.11.2. Degradation of cyanate to ammonium by *Nitrospira moscoviensis* (low cell density experiment)

All Schott bottles were prepared as described in 2.10.1. Mineral NOB medium (without NH_4^+ source) and 20 ml of 100 mM $NaNO_2$ and KOCN solutions were prepared freshly. The medium was autoclaved and sterile-filtrated TES was added. For each Schott bottle, 100 ml NOB media was prepared with 1 mM $NaHCO_3$. Three different incubation groups were set up to investigate cyanate degradation. The first with 1mM KOCN and 1 mM $NaNO_2$; the second with 1 mM KOCN only and the third with 1 mM $NaNO_2$ only to control for activity. In addition, abiotic incubations were performed by either adding 1 mM KOCN and 1 mM $NaNO_2$, or only 1 mM cyanate to the NOB medium. 250 ml *N. moscoviensis* culture were washed twice with fresh, warm NOB medium to remove all remaining nitrite and nitrate. Washed cells were evenly distributed in all prepared Schott bottles, closed by rubber stoppers and incubated without shaking at 37°C in dark for 120 hours. All incubations were done in triplicates (Tab.4). Sampling and chemical analyses were performed as described in 2.10.5.

Tab.4: Schematic overview of all incubation conditions. Each substrate was added to get the final concentration as described. All incubations were performed in triplicates. All groups were incubated for 120 hours.

Incubations	Nitrite (1mM)	Cyanate (1mM)	Bicarbonate (1 mM)	Culture	Time [h]
A (3x)	+	-	+	+	120
B (3x)	+	+	+	+	120
C (3x)	-	+	+	+	120
D (3x)	+	+	+	-	120
E (3x)	-	+	+	-	120

2.11.3. Chemical degradation of cyanate in the presence of nitrite

Two Schott bottles were prepared as described in 2.10.1. In each Schott bottle, 100 ml NOB medium was added and samples (1 ml) were taken. Next, 1 mM $NaNO_2$ was added to the first bottle and 1 mM KOCN to the second bottle with 100 ml NOB medium. Samples were taken again. For the last step, 1 mM KOCN was added to the first bottle and 1 mM $NaNO_2$ to the second bottle to get both substrates in the end in each Schott flask. 1 ml samples were taken for this last step too. Both bottles were stored

without shaking at 37°C in dark for 120 hours (Tab. 5). Sampling and chemical analyses were performed as described in 2.10.5.

Tab.5: Schematic overview of all incubations which were used for the experiment. Each substrate was adjusted to get the final concentration as described. At the end of Step 2, all incubations contained both substrates. All incubations were done without cells and incubated for 120 hours.

Incubations	Cyanate [1 mM]	Nitrite [1 mM]	Time [h]
Bottle A (Step 1)	**-**	**+**	**120**
Bottle B (Step 1)	**+**	-	**120**
Bottle A (Step 2)	**+**	-	**120**
Bottle B (Step 2)	**-**	**+**	**120**

2.11.4. Degradation of cyanate to ammonium by *Nitrospira moscoviensis* (high cell density experiment)

All Schott bottles were prepared as described in 2.10.1. Approximately 1600 ml *N. moscoviensis* culture were washed twice with fresh, warm NOB medium to remove all remaining nitrite and nitrate. Washed cultures were transferred to fresh media with 500 µM cyanate for 48 hours. Later, pretreated *N. moscoviensis* cultures were washed twice to remove all remaining nitrite and cyanate. Mineral NOB medium (without any NH_4^+ sources) and 20 ml of 100 mM $NaNO_2$ and KOCN solutions were prepared freshly. The medium was autoclaved and sterile-filtrated TES was added. For each Schott bottle, 50 ml NOB media were prepared with 1.5 mM $NaHCO_3$. All incubations were performed in the same way as described in 2.11.2. Washed cells were evenly distributed in all prepared Schott bottles, closed by rubber stoppers and incubated without shaking at 37°C in dark for 31-96 hours.

All incubations were performed in triplicates (Tab.6). Sampling (Tab.7) and chemical analyses were performed as described in 2.10.5. After 96 hours, remaining biomass from the biotic incubation with cyanate-only were washed and treated with nitrite for one week and prepared for the dead control incubation. To inactivate *N. moscoviensis* cells, washed cells were fixed with 4 % PFA as described in 2.10.4. Protein analyses were performed as described in 2.10.6.3.

The fixed cells were treated as all biotic incubations with 1 mM KOCN only, closed by rubber stoppers and stored without shaking at 37°C in dark for 96 hours. Sampling was performed with the same time points as described below (Tab.7)

Tab.6: Schematic overview of all incubations which were used for the experiment. Each substrate was adjusted to get the final concentration as described. All incubations were performed in triplicates. All cyanate / nitrite treatments were incubated for 31 hours, all cyanate-only treatments for 96 hours. Dead control was performed later, to avoid reduction of the biomass for the first step.

Incubations	Cyanate [1 mM]	Nitrite [1 mM]	Bicarbonate [1.5 mM]	Culture	Time [h]
A (3x)	+	-	+	+	31
B(3x)	+	+	+	+	31
C (3x)	-	+	+	+	96
D (3x)	+	+	+	-	31
E (3x)	-	+	+	-	96
F (3X)	-	+	+	dead	96

Tab.7: Chronology of sampling during the analysis of cyanate degradation by *N. moscoviensis*. (Blue) All abiotic/biotic incubations with cyanate and nitrite for 31 hours. (Red) All abiotic/biotic incubations with cyanate-only for 96 hours.

Day 0	Day 1	Day 2	Day 3	Day 4
0 h (1.5 ml)	20 h (1.0 ml)			
1.5 h (1.0 ml)	24 h (1.0 ml)			
3 h (1.0 ml)	31 h (1.0 ml)			
6 h (1.0 ml)				
9 h (1.0 ml)				
12 h (1.0 ml)				Cyanate + Nitrite
Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**
0 h (1.5 ml)	20 h (1.0 ml)	48 h (1.5 ml)	72 h (1.0 ml)	96 h (1.5 ml)
1.5 h (1.0 ml)	24 h (1.0 ml)			
3 h (1.0 ml)	31 h (1.0 ml)			
6 h (1.0 ml)				
9 h (1.0 ml)				
12 h (1.0 ml)				Cyanate-only

2.11.5. Cryopreservation of *Nitrospira moscoviensis*

N. moscoviensis cells were grown with the standard mineral medium over few months to reach high cell density as described in 2.8. After reaching high cell density, biomass was concentrated via centrifugation (9289 g, 15 min, 25°C) and washed twice in fresh medium. Nitrite and nitrate concentration were checked after each washing step with colorimetric nitrite/nitrate stripes (*Merck* KGA, Darmstadt, Germany). Samples were taken for FISH analyses.

Three different preservation media were tested according to the recently published protocol (Vekeman *et al.,* 2013). DMSO was used as cryoprotective agent (1, 10%) in combination with two growth media (1/10 TSB and NOB). 1/10 TSB and NOB media were prepared and autoclaved before. 2% and 20% DMSO solutions were prepared and sterile filtrated briefly before the culture was added. Cells were collected to a final volume of 800 µl and transferred to cryotubes with 800 µl of each preservation media to a final volume of 1.6 ml (Tab.8). All samples were frozen in liquid nitrogen (-196°C) and subjected to long-term cryopreservation at -80°C. Each preservation condition was done in triplicates. The cryopreserved *N. moscoviensis* cultures were resuscitated after a preservation period of nine weeks. OD and protein measuring was performed as described in 2.10.6.

Tab.8: Overview of the best cryopreservation conditions for *N. moscoviensis*.

Incubations	Strain	Pre-preservation growth medium	Preservation medium	CPA
A (2x)	***N. moscoviensis***	**Standard medium (NOB)**	**1/10 TSB**	**10% DMSO**
B(2x)	***N. moscoviensis***	**Standard medium (NOB)**	**NOB**	**1% DMSO**
C (2x)	***N. moscoviensis***	**Standard medium (NOB)**	**NOB**	**10% DMSO**

For reactivation, all tubes with biomass were thawed quickly at 37°C in a water bath and immediately transferred into fresh 2ml tubes and centrifuged for 15 min at 8500 rpm at 4°C to minimize the toxic effect of DMSO. All thawed cultures were washed twice with an equal volume of fresh NOB media (without nitrite) to ensure the removal of DMSO. The cultures were incubated into 50 ml fresh NOB media with 500 µM $NaNO_2$ (final concentration). All cultures were incubated at 37°C without shaking in the dark. Nitrite concentration was monitored with colorimetric nitrite stripes and nitrite (1 mM)

was refed, if necessary. Once a week, 500 µl samples were taken from each bottle to analyze the $OD_{578-436\ nm}$ by spectralphotometry. 500 µl NOB media was added again to maintain the volume over the whole time.

3. Results

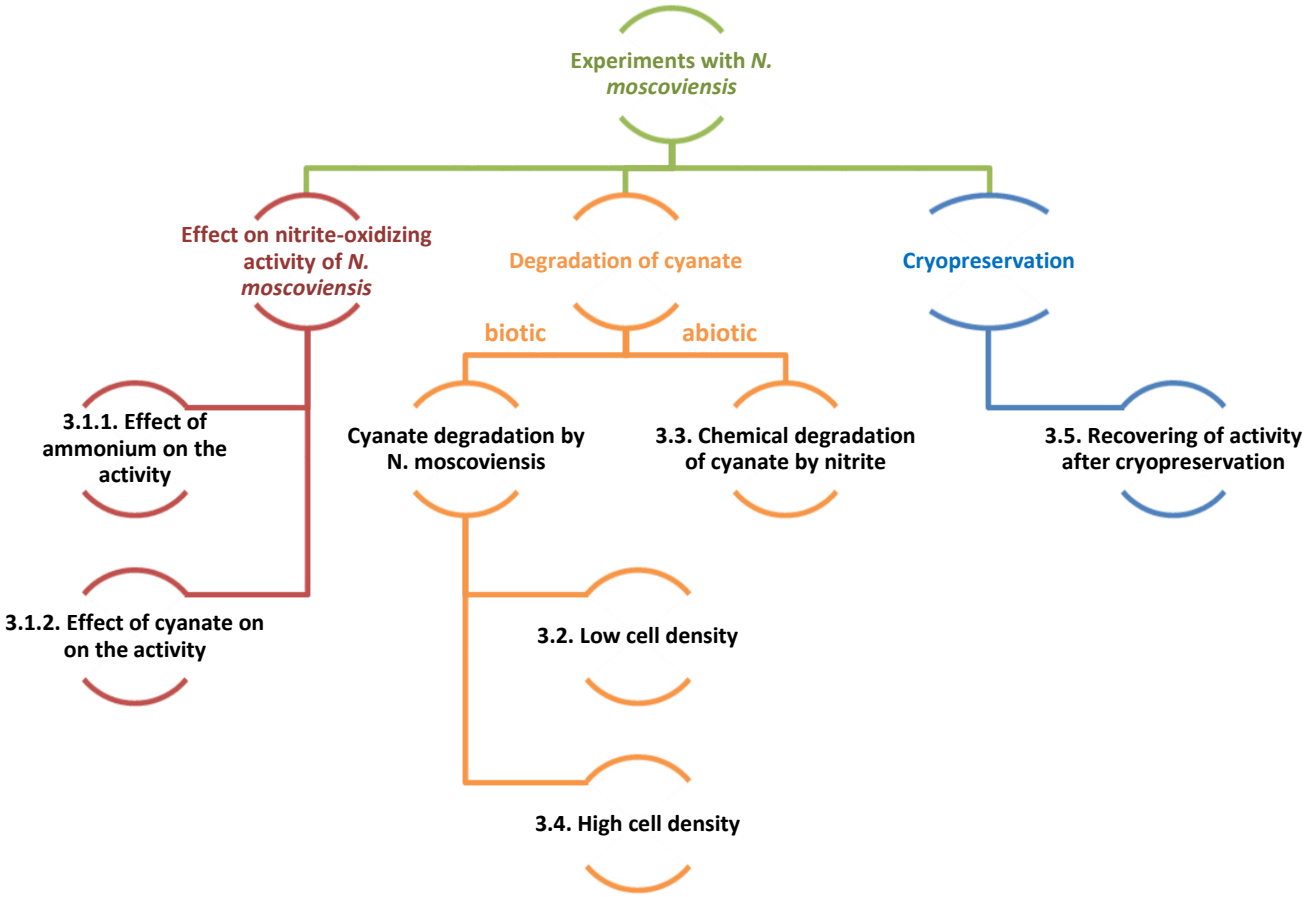

Fig.4: Flowchart of all presented experiments on *N. moscoviensis*. All chemical and physiological analyses with different cyanate and ammonium concentrations on *N. moscoviensis* are shown in **red**. All physiological experiments on biological decay of cyanate by cyanase of *N. moscoviensis* are shown in **orange**. Cryopreservation and revival of *N. moscoviensis* are shown in **blue**.

3.1. Exploring possible effects of different cyanate and ammonium concentrations on the activity of *Nitrospira moscoviensis*

In these two experiments, the potential toxicity of cyanate and ammonium was examined by using nitrite oxidation as a proxy for activity. Therefore, *N. moscoviensis* was incubated with 1mM nitrite and ammonium or cyanate concentrations ranging from 1 to 5 mM. Control incubations with nitrite only were performed to compare the activity of the cells over the whole time period. Samples for chemical analyses were taken every day and stored as described in 2.10.5. For all cyanate incubations, additional time points were taken as mentioned in 2.11.1.2.

3.1.1. Effect of ammonium on the nitrite oxidation of *N. moscoviensis*

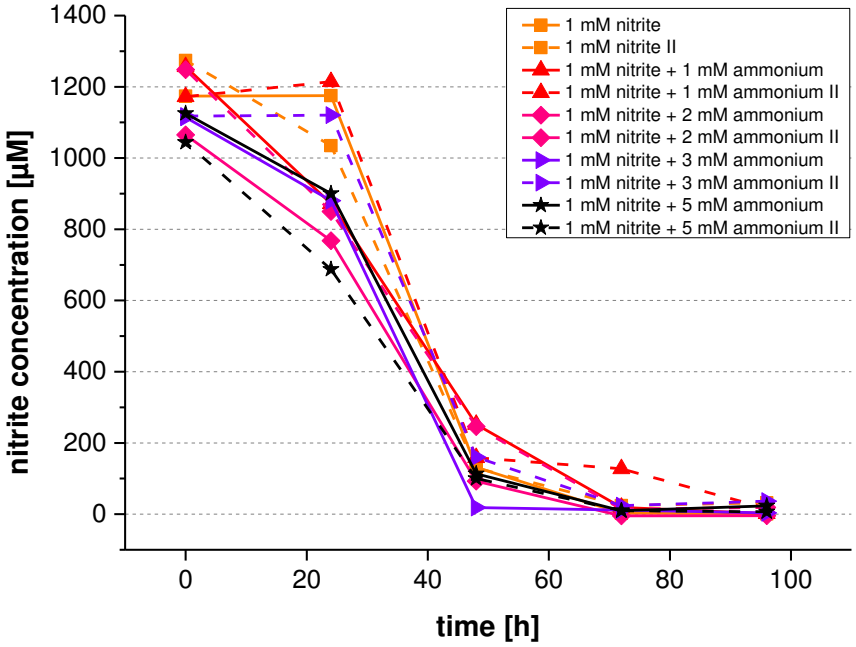

Fig.5: Nitrite concentrations in incubations with increasing ammonium concentrations and 1 mM nitrite. Control incubation (orange) contained only 1 mM nitrite. Incubation with increasing ammonium concentrations (1 mM – 5 mM) and 1 mM nitrite. All incubations were done in duplicates.

After 96 hours, all incubations with all applied ammonium concentrations consumed 1 mM nitrite. No significant differences between highest and lowest concentration could be observed (Fig.5). No inhibitory effect of ammonium in a concentration range of 1 -5 mM on nitrite oxidation by *N. moscoviensis* was detected.

3.1.2. Effect of cyanate on the nitrite oxidation of *N. moscoviensis*

For all incubations with cyanate, every day several samples for nitrite measurements were collected (Fig.6). The control incubation without cyanate consumed 1 mM nitrite in less than 24 hours, whereas the nitrite oxidation rate decreased with increasing concentrations of cyanate.

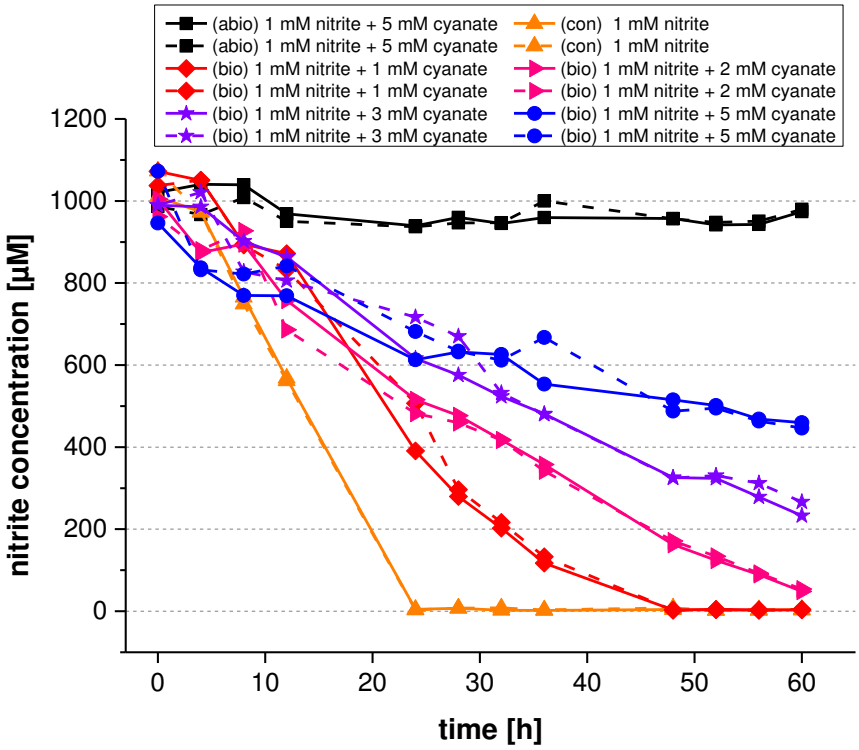

Fig.6: Nitrite concentrations in the incubations with increasing cyanate concentrations and 1 mM nitrite. Control incubation (**orange**) contained only 1 mM nitrite. Abiotic (**black**) incubation contained 1 mM nitrite and 5 mM cyanate. All biotic incubations were treated with 1mM nitrite, 1 mM bicarbonate and increasing cyanate concentrations (1 mM – 5 mM). All incubations were performed in duplicates.

Only biotic incubations with 1 and 2 mM cyanate were able to consume all nitrite within 60 hours. In the presence of 5 mM cyanate, approximately 50% of the nitrite was consumed by *N. moscoviensis* within 60 h. Nevertheless, despite the high cyanate concentrations, nitrite oxidation could be detected in all incubations. The abiotic incubations did not show any decrease of nitrite.

3.2. Degradation of cyanate to ammonium by *Nitrospira moscoviensis* (low cell density experiment)

An experiment with abiotic and biotic incubations at low biomass was performed to test the appropriate experimental setup for the main, high biomass cyanate-consumption experiment. *N. moscoviensis* was incubated with cyanate to investigate cyanate degradation by cyanase. Three different incubations were performed. Cultures were treated with cyanate and nitrite to investigate the nitrite oxidation activity of the cells in the presence of cyanate. Additionally, cultures were treated with cyanate-only to explore the degradation of cyanate by *N. moscoviensis*. Control incubation with nitrite were performed to compare the activity of the cells over the whole time period. Because of the chemical instability of cyanate and decay into ammonium, abiotic controls were performed. Samples for chemical analyses were taken every day and measurements were performed as described in 2.10.5.

3.2.1. Experimental set up influences the stability of pH

Over the whole time period, an increase of pH in all incubations with cyanate could be detected, while the pH of all control incubations containing only nitrite remained constant. No significant difference between abiotic and biotic incubations could be detected. (Fig.7 A-B).

3.2.2. Degradation of cyanate by *Nitrospira moscoviensis*

3.2.2.1. Analyses of nitrite consumption

In all biotic incubations with nitrite or cyanate and nitrite, consumption of nitrite could be detected over the whole time period (Fig. 8). The control incubation without cyanate depleted 1 mM nitrite after 24 hours. In comparison, all incubations with cyanate and nitrite had approximately 180 µM nitrite left after 24 hours. After 48 hours no nitrite could be detected in all biotic incubations with and without cyanate. At time point 54 hours, additional 1 mM nitrite was added in all biotic incubations. Again, all control incubations, as well as all incubations with cyanate depleted nitrite (1 mM) after 24 hours. The nitrite concentration in all abiotic incubations with cyanate and nitrite remained constant over 120 hours.

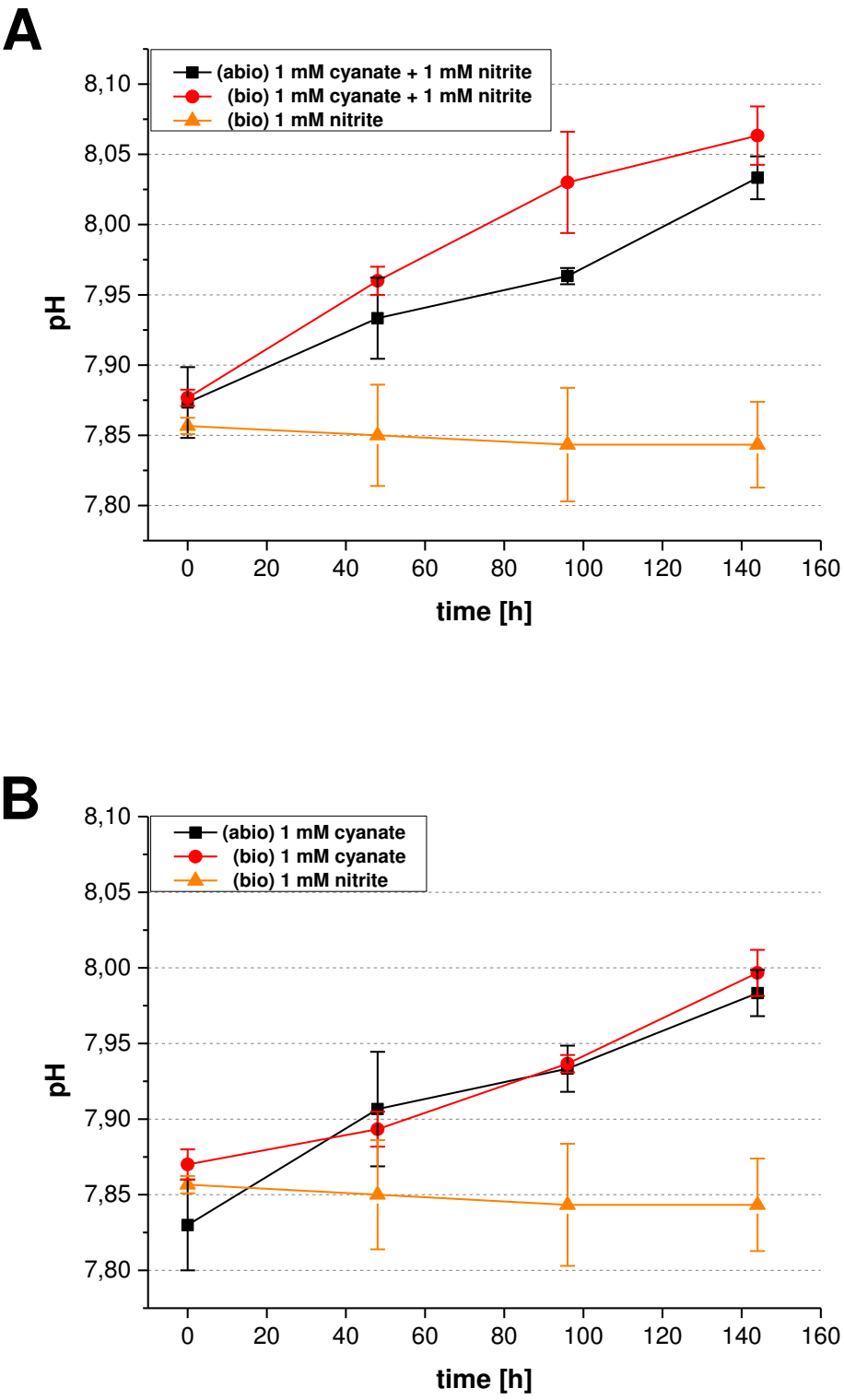

Fig.7: pH analyses of all biotic and abiotic incubations for 144 hours (**A-B**): (**A**) Abiotic (**black**) and biotic (**red**) incubation with cyanate and nitrite in NOB-media. Control (**orange**) incubation with nitrite-only. (**B**) Abiotic (**black**) and biotic (**red**) incubation with cyanate-only in NOB-media. Control (**orange**) incubation with nitrite-only. All incubations were done in triplicates.

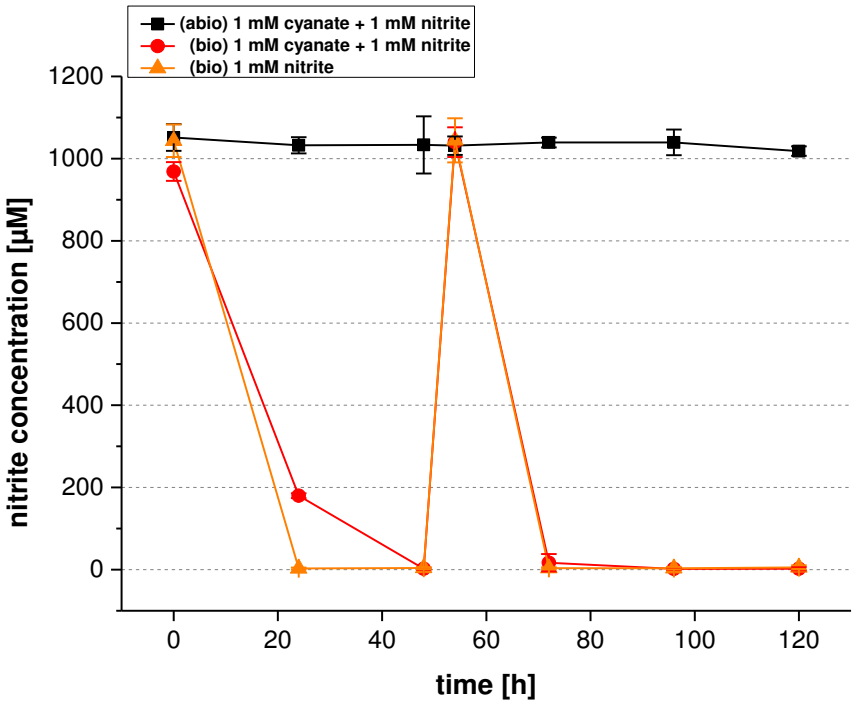

Fig.8: Concentration of nitrite after 120 hours. Abiotic (**black**) and biotic (**red**) incubations with 1 mM nitrite and 1 mM cyanate. All control incubations (**orange**) contained 1 mM nitrite-only and were re-added with 1 mM nitrite after 54 hours, as well as all biotic incubation with nitrite and cyanate (**red**). All incubations were performed in triplicates.

3.2.2.2. Analyses of ammonium released by cyanate degradation

Analyses of ammonium in all biotic incubations with cyanate indicated an increase of ammonium during the whole experiment (Fig.9 A-B). Adding both substrates (nitrite and cyanate) together resulted in higher ammonium formation in comparison to the incubations with cyanate-only. No significant differences between abiotic and biotic incubations could be detected in incubations with cyanate and nitrite or cyanate-only. Nevertheless, higher ammonium concentration at the beginning could be detected in all incubations with both substrates (Fig.9 A).

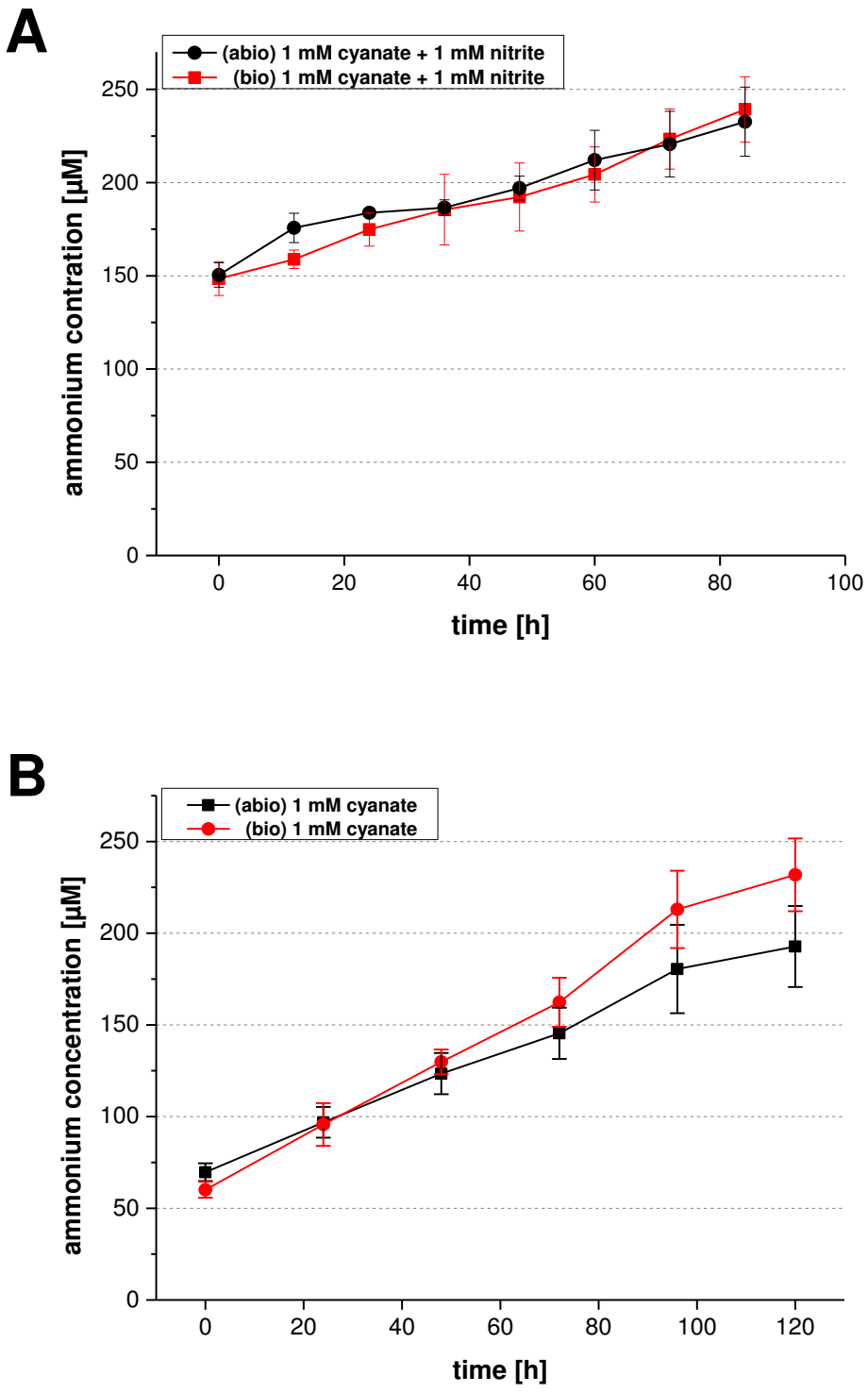

Fig.9: Concentration of ammonium (**A-B**). (**A**) Abiotic (**black**) and biotic (**red**) degradation incubations with 1 mM cyanate and 1 mM nitrite. (**B**) Abiotic (**black**) and biotic (**red**) degradation incubations with 1 mM cyanate-only. All incubations were done in triplicates.

3.3. Degradation of cyanate to ammonium by *Nitrospira moscoviensis* (high cell density experiment)

In the preceding low cell density experiment, no biological cyanate degradation by *N. moscoviensis* could be observed (Fig.9 A-B). To boost the biological activity and the presumptive degradation of cyanate by cyanase, more biomass of *N. moscoviensis* was used in the second experiment. The experimental setup was the same as described in 3.2. In addition, cells were treated with 0.5 mM cyanate for 48 hours before the start of the incubation, to possibly induce the expression of cyanase. All incubations with cyanate and nitrite were performed for 31 hours, while all treatments with cyanate-only were performed for 96 hours. Furthermore, dead controls with similar amounts of *N. moscoviensis* biomass and 1 mM cyanate were performed at the end of this experiment.

3.3.1. Increased cell density boost nitrite consumption

Analyses of protein concentrations of all biotic incubations indicated high and similar amounts of protein in all three high cell density incubations (Tab.9). The control incubation with nitrite-only (36 µg/ml) had the highest protein concentration, when compared to the incubations with cyanate (31 µg/ml for cyanate + nitrite and 27 µg/ml for cyanate-only). The use of high biomass boosted the nitrite consumption in all incubations (Fig.10 A-B). In the control incubation 1 mM nitrite was oxidized in less than 4 hours while incubations with 1 mM nitrite and 1 mM cyanate needed less than 6 hours to degrade their nitrite stock. Despite the high activity, a modest inhibition resulting from cyanate could be detected again. Due to the high activity rates (especially the control group), all additional feeding was performed with 2 mM instead of 1 mM nitrite. From the beginning, the abiotic incubations had (729 µM) less nitrite as the biotic ones (1051 µM). This difference in nitrite concentration was also found in all re-additions of nitrite (Fig.10 A-B).

Tab.9: Protein concentrations of all biotic incubations at time point 0. All incubations were done in triplicates. Protein analyses were performed as described in 2.10.6.3

Incubations	Biomass concentration [µg/ml]	Standard deviation
(Biotic) Cyanate + Nitrite	**31,25**	**± 5,54**
(Biotic) Cyanate-only	**27,64**	**± 5,66**
(Control) Nitrite-only	**36,10**	**± 3,94**

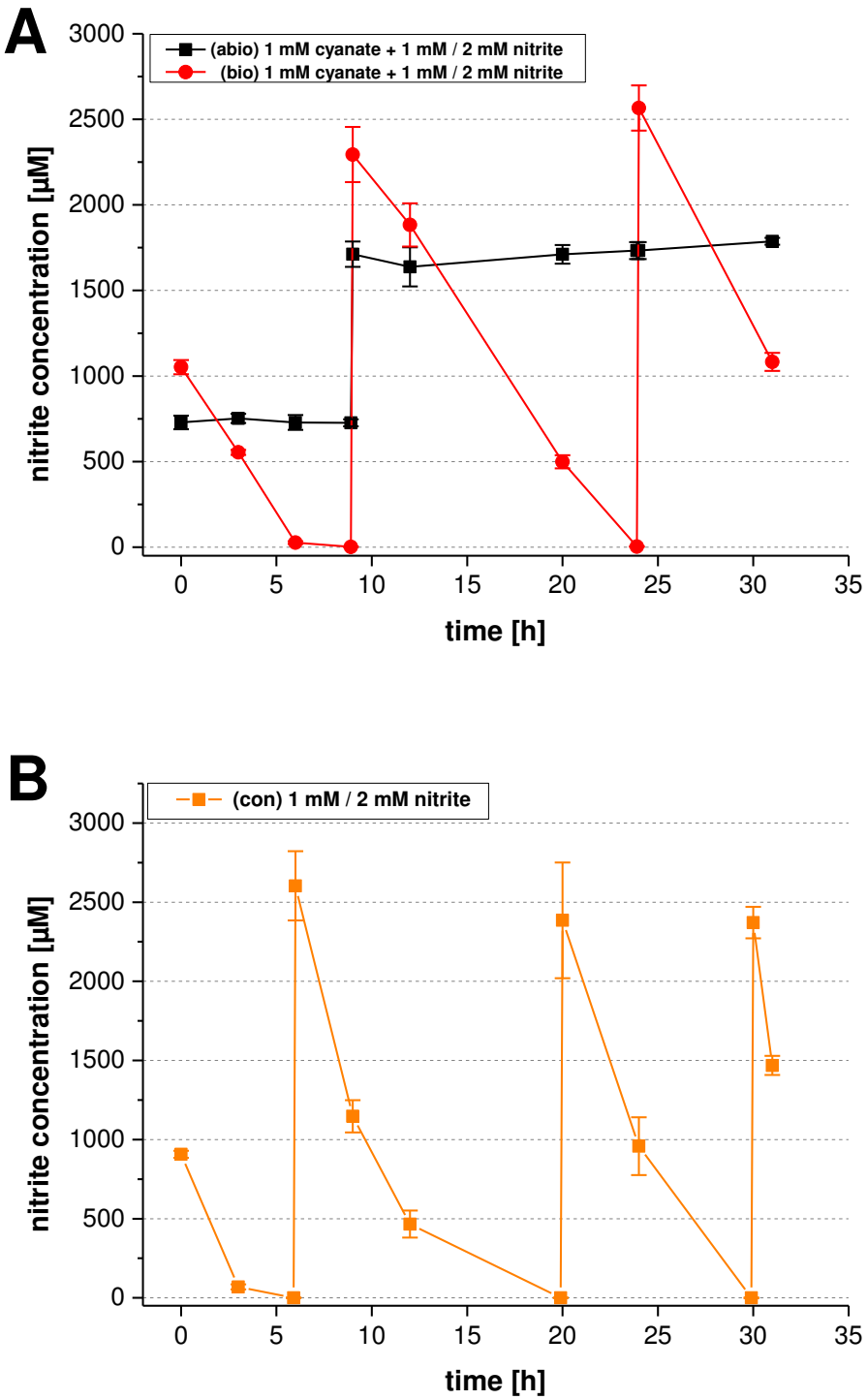

Fig.10: Concentration of nitrite after 31 hours incubation. (**A-B**) (**A**) Abiotic (**black**) and biotic (**red**) incubations with 1 mM nitrite and 1 mM cyanate. Additional 2 mM nitrite was added to all biotic incubations after 9 hours and 24 hours. 1 mM nitrite was added to all abiotic incubations after 9 hours. (**B**) Biotic control (**orange**) with 1mM nitrite. Additional 2 mM nitrite was added after 6 hours, 24 hours and 30 hours. All incubations were done in triplicates.

3.3.2. Cyanate degradation and ammonium formation by *Nitrospira moscoviensis*

Ammonium analyses of all abiotic and biotic incubations indicate the acceleration of cyanate decay by the presence of nitrite (Fig.11). Addition of nitrite at the beginning and later during the experiment (two times 2 mM at 9 and 24 hours in all biotic, and one time 1 mM at 9 hours in all abiotic incubations) led to spontaneous ammonium formation. Those shifts induced by nitrite make it hard to distinguish between chemical cyanate decay of the abiotic control and of the degradation induced by *N. moscoviensis* (Fig.11).

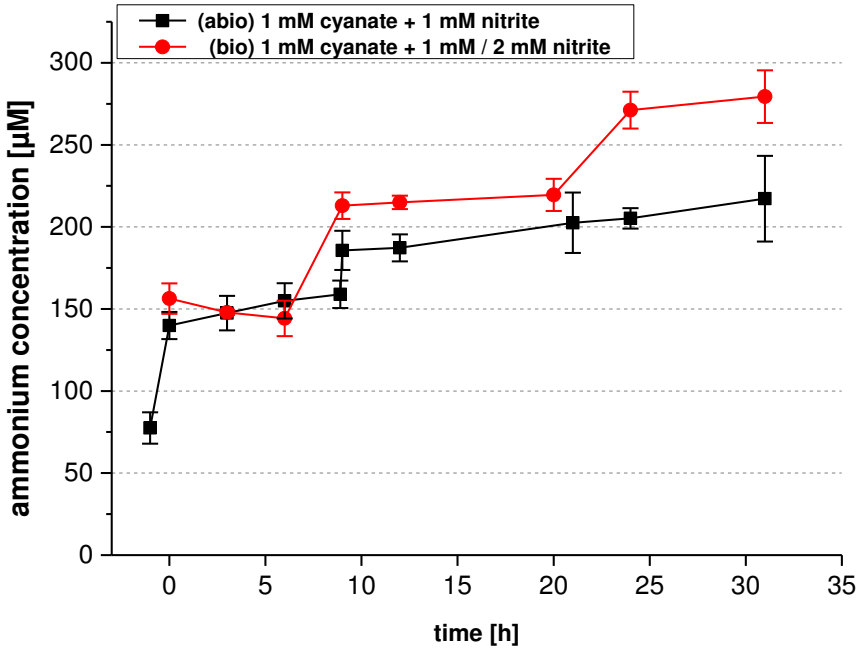

Fig.11: Concentration analyses of ammonium after 31 hours. Abiotic (**black**) and biotic (**red**) incubations with 1 mM cyanate and 1 mM nitrite. Sample (-1) was taken before nitrite was added. Additional 2 mM nitrite was added to all biotic incubations after 9 hours and 24 hours and 1 mM nitrite to all abiotic incubations after 9 hours. All incubations were done in triplicates.

Chemical analyses of ammonium in all abiotic and biotic incubations with only cyanate indicated the same rate of ammonium formation after 24 hours (Fig.12). However, at the end of the experiment (96 hours), a significant difference between abiotic and biotic incubation with cyanate could be detected. The additional dead control had equal concentrations as all abiotic incubations. These results were confirmed by cyanate analyses which showed reductions of cyanate in the same range as ammonium was released.

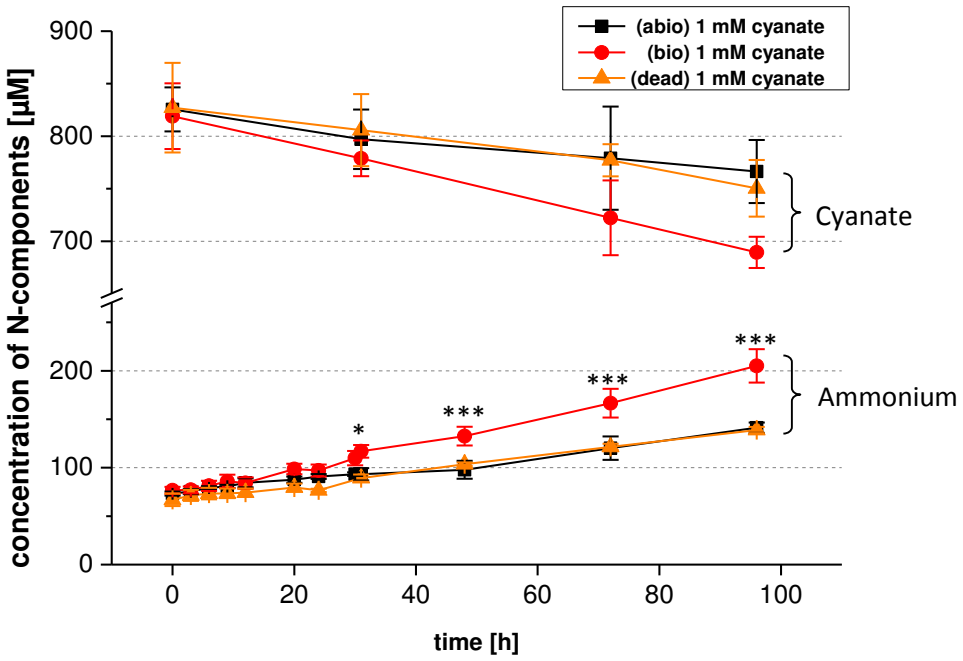

Fig.12: Concentration analyses of ammonia and cyanate after 96 hours (**A**): (**A**) Abiotic (**black**), biotic (**red**) and dead control (**orange**) incubations with 1 mM cyanate-only. Ammonium values are shown in the lower part, cyanate in the upper part of the diagram. Asterisks indicate statistical significance at P values of <0.05 (*) and <0.001 (***) between *N. moscoviensis* and dead biomass (Palatinszky *et al*., 2015). The significance was tested by using a two-way analysis of variance (ANOVA) including a Tukey's honest significant difference (HSD) test (Palatinszky *et al*., 2015).

3.4. Chemical degradation of cyanate in the presence of nitrite

In this experiment, the influence of nitrite on the chemical degradation of cyanate was explored in more detail. Therefore, an abiotic incubation with NOB medium was performed with the same concentrations of cyanate and nitrite as in biotic experiments described above. This experiment was performed in three steps. For each step, samples were taken for further analyses. First, normal NOB media without any substrate was prepared in two bottles. Second, in each bottle one substrate was added and at last, the 2nd substrate was added. Bottles with both substrates were incubates for several days and samples were taken every day for chemical analyses and stored as described in 2.10.6.

3.4.1. Ammonium released in abiotic incubations with cyanate and nitrite

At the beginning of the experiment approximately 150 µM ammonia could be detected in both bottles. This is the expected NH_4^+ concentration of standard NOB medium with NH_4^+ as N-source (Fig.13). Thereafter, to both

bottles one substrate with 1 mM concentration (Bottle A = nitrite; Bottle B = cyanate) was added. At time point 0, the second substrate were added (Bottle A = cyanate; Bottle B = nitrite). This addition resulted in an ammonium increase of approx. 150 µM in both bottles. Continuous ammonium formation could be detected till the end of the experiment in both bottles.

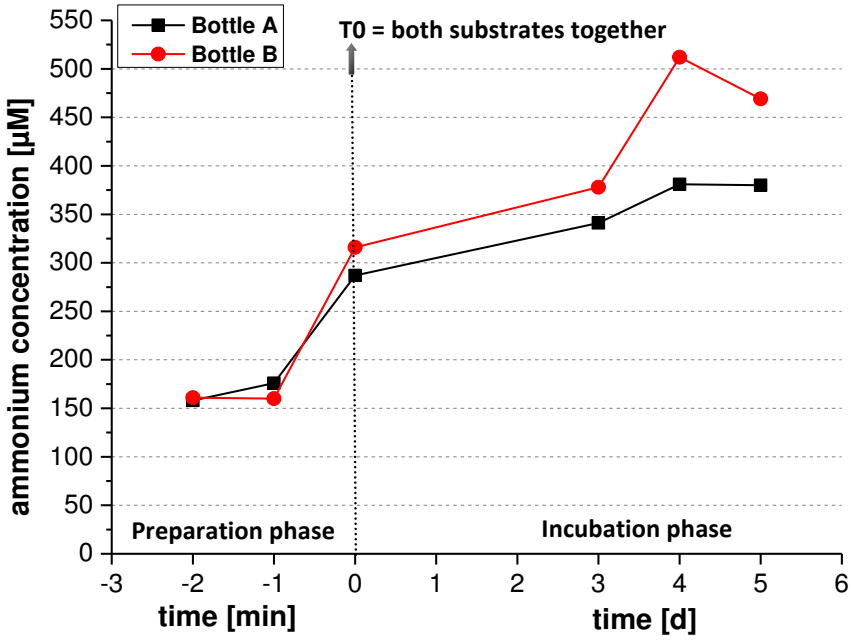

Fig.13: Ammonia measurements of the incubations with cyanate and nitrite, performed to explore the influence of nitrite on the chemical degradation of cyanate. The vertical dashed line split the graph in to time lines (left = minutes; right = days). After time point 0, both bottles had been stored and sampled for 120 hours.

3.5. Cryopreservation and revival of *Nitrospira moscoviensis*

For this experiment, different cryopreservation conditions were tested and cyro-stocks of a dense *N. moscoviensis* culture were performed as described in (Vekeman *et al.,* 2013). The dense culture was monitored by FISH to control the purity and condition of the cells. The culture was washed several times and was incubated with three different cryopreservation media as described in 2.11.5. All tubes were frozen in liquid nitrogen (-196°) and stored for 2 months at -80°C. Reactivation and the following incubation were performed as described in 2.11.5. To observe cell growth over time, fresh nitrite (1 mM) was re-added and samples were taken once a week to investigate the $OD_{578\text{-}436\ nm}$ as described in 2.10.6.2.

3.5.1. Recovering of activity after cryopreservation

After 3 days of thawing of the cryopreserved culture, 0.5 mM nitrite in all three groups was consumed, which indicates the activity of the strains (data not shown). Every week, 1 mM nitrite was re-added when nitrite was depleted to raise density of the culture. Higher nitrite concentration was avoided, to minimize stress for the cultures. After 7 days all three treated strains showed an increase of cells, which flatted significant after 27 days (Fig.14 A-B). At day 15, a highest $OD_{578\text{-}436\ nm}$ could be detected in both incubations which were treated with 1/10 TSB and 10% DSMO, while all other strains (NOB media + 1-10% DMSO) had lower $OD_{578\text{-}436\ nm}$ values. From day 27 to day 35 the optical density in all incubations was constant although nitrite was still consumed in a high rate. Except the light increase of $OD_{578\text{-}436\ nm}$ in the incubation with the cryopreserved cells, which were treated with 1/10 TSB and 10% DMSO at days 15 compared to the other incubations, all cryopreservation methods showed suitable activity recovery and comparable growth.

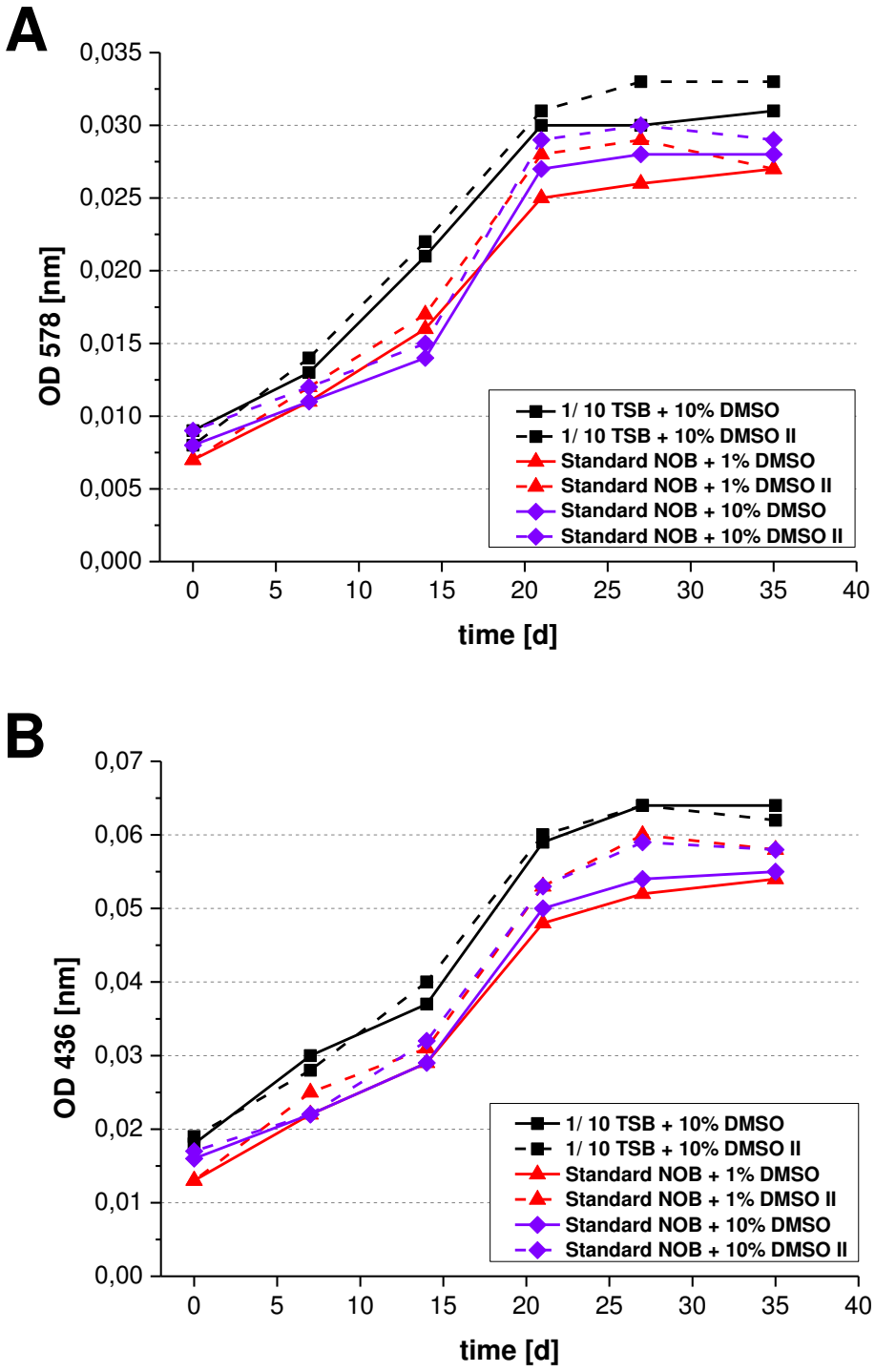

Fig.14: Analyses of the OD $_{578}$ and OD $_{436}$of reactivated *N. moscoviensis* cultures after 2 month at -80°C. All incubations were treated with 1 mM nitrite to maintain activity. (**A-B**) (**A**) Cryo-stocks with 1/10 TSB + 10% DMSO (**black**), Standard media + 1% DMSO (**red**) and Standard media + 1% DMSO (**purple**) measured at OD $_{578nm}$. (**B**) Cryo-stocks with 1/10 TSB + 10% DMSO (**black**), Standard media + 1% DMSO (**red**) and Standard media + 1% DMSO (**purple**) measured at OD $_{436nm}$. All incubations were done in duplicates.

4. Discussion

The increasing amount of toxic pollutants in wastewater leads to large problems for natural and engineered ecosystems (Bonaventura and Johnson, 1997; Kimochi *et al*., 1998). NOB are exposed to a plethora of potential toxic substances in sewage and it is known that high concentration of toxic pollutants severely inhibits the biological activity of activated sludge (Amor *et al.,* 2005; Liu *et al.,* 2005). However, the biochemical processes and degradation mechanisms of these substrates by microbes in the active sludge of wastewater treatment plants are barley studied. The genome of *N. defluvii*, a close relative of *N. moscoviensis* encodes several multidrug efflux systems and transporters for heavy metals, organic solvents, and antimicrobials (Lücker *et al.,* 2010), as well as addition genes for cyanate and arsenic resistance (Lücker *et al.,* 2010). Despite the fact that cyanases are common in all three domains of life and the metabolism of cyanate in *Escherichia coli* is well studied, it is still unclear why all NOB genomes encode cyanase and what use it might have for them.

The main target of this study was to examine the potential of *N. moscoviensis* to convert cell toxic cyanate (Stark, 1965) to ammonia and carbon dioxide. As mentioned before, all known NOB (*N. moscoviensis* included) encode a cyanase gene (Fig. 3). Among all AOM, only *Nitrososphaera gargensis* (AOA) encodes a cyanate hydratase.

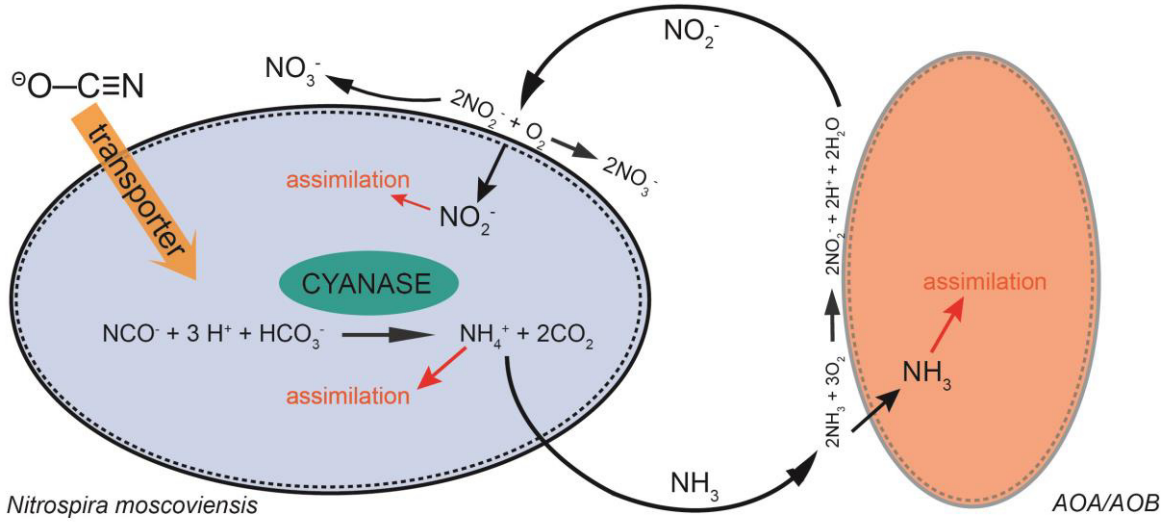

Fig.15: Schematic overview of the possible reutilization cycle of cyanate and chemical interactions between *N. moscoviensis* and ammonia-oxidizing microorganisms. Cyanate is taken up from the extracellular environment by cyanate transporter and degraded by the cyanase, in a bicarbonate including reaction, to ammonium and carbondioxide. Additional ammonia can be assimilated by AOAs/AOBs or oxidizer to nitrite, which again can be used by the NOBs.

The lack of cyanase in other AOM lead to the hypotheses that the ammonium which is generated by NOB through cyanate degradation could be used as energy source for co-existing AOM (Fig.15). On the other hand, AOM oxidize ammonium to nitrite, which can be further oxidized by NOB, resulting in a reciprocal feeding relationship. Despite the low cyanate concentrations in nature (Drinhuber and Schutz, 1948; Kamennaya *et al.,* 2008; Kamennaya *et al.,* 2013; Widner *et al*., 2013), cyanate is common in various aquatic ecosystems and can serve as an important energy and nitrogen source. So far, concentration of cyanate were not quantified in terrestrial ecosystems. With the help of new analytic methods, low concentrations of cyanate (nm range) are now detectable and might reveal new information about cyanate in thus far not investigated ecosystems.

4.1. Effects of different cyanate and ammonium concentrations on the activity of *Nitrospira moscoviensis*

The capability of *N. moscoviensis* to cope with a range of ammonium and cyanate concentrations was investigated. In several experiments *N. moscoviensis* was treated with increasing concentrations of ammonium or cyanate, and their effect on the nitrite oxidation rate was monitored.

The nitrite oxidation rate of *Nitrospira* was similar for all used ammonium concentrations tested here (1-5 mM) (Fig.5). 1 mM nitrite was depleted in all incubations after 72 hours. The inhibition resistance of *Nitrospira* to relatively high ammonium concentrations (up to 5 mM) is not surprising, since a co-existence of AOM and NOB is commonly reported. Analyses from Kim and colleagues confirm that several AOMs and a *Nitrospira* lineage II member coexist in sewage (Kim *et al.* 2011). Further analyses from Keuter and colleagues indicated that *Nitrospira ecomares* (similar to *Nitrospira moscoviensis*) could resist even higher ammonium concentrations (> 50 mM), too (Keuter *et al.,* 2011).

Next, *N. moscoviensis* was incubated with increasing cyanate concentration (see 3.1.2). In less than 24 hours, control incubations without cyanate depleted 1 mM nitrite and confirm the activity of used cells. While cyanate concentration of 1 mM induced a light decrease in nitrite oxidation activity (Fig.6), incubations with 2 mM or more cyanate significantly inhibited the metabolic activity of *N. moscoviensis*. Interestingly, even the highest applied cyanate concentration (5 mM) did not lead to a total inhibition of nitrite oxidation by *N. moscoviensis*. Experiments on *Nitrobacter* revealed that cyanate is a powerful inhibitor of nitrite oxidation at normal oxygen tensions (Butt and Lees, 1964). The presence of metabolic activity, indicated by nitrite oxidation, under these high cyanate concentrations suggests a high cyanate resistance of *N. moscoviensis*. Similar to this finding, a recent study revealed that a *Nitrospira* lineage II population in sewage of coke wastewater resists high concentrations of cyanate over several months (Kim *et al.,* 2011).

4.2. Degradation of cyanate to ammonium by *Nitrospira moscoviensis*

The aim of these experiments was to investigate the biological degradation of the cell toxic substrate cyanate by the enzyme cyanase of *N. moscoviensis*. For this, two experiments with different amounts of cells were incubated with cyanate and nitrite for several days. The release of ammonium by cyanate degradation was analyzed.

All incubations with cyanate showed an increase of pH during the incubations, while pH of all control incubations with nitrite were constant and stable (Fig.7 A-B). Using rubber stoppers instead of normal screw caps revealed less fluctuation of pH (data not shown), but still an increase of pH in the cyanate incubations was detected. This increase of pH in all cyanate treated incubations was due to release of ammonium by the degradation of cyanate, which did not significantly influence the nitrite oxidation activity of *N. moscoviensis* during these short-term incubations. Nevertheless, these pH increases (over pH 8) might inhibit the cell activity on a long term incubation, which has already been reported by Ehrich and colleges (Ehrich *et al.,* 1995). Therefore, modification of NOB medium with better buffer systems might be necessary for long-term incubations with cyanate.

The activity of cyanate fed and control inoculations, fed nitrite only, was checked by nitrite consumption. Depletion of nitrite could be detected in all biotic incubations (low and high cell density) during the whole experiment, while the nitrite concentration in all abiotic incubations was constant (Fig.8 and Fig.10). The amount of biomass affected nitrite oxidation rates strongly. With increased cell density, *N. moscoviensis* was able to degrade 1 mM nitrite in less than 5 hours (Fig.10 B). However, cyanate induced a slight reduction of the metabolic activity in *N. moscoviensis* independent of cell density in incubations (Fig.8 and Fig.10 A), which could be shown in 3.1.2.

Analyses of ammonium from the low cell density experiment revealed a constant, but low formation of ammonium by chemical cyanate degradation (Fig.9). Treatments with cyanate-only revealed a similar slope but less ammonium formation at the beginning in comparison to all incubations with cyanate and nitrite. Despite this differences of ammonium formation, the slope was similar and constant in both incubations. It appears that this interaction between cyanate and nitrite catalyzed chemical cyanate degradation into ammonium, which could be shown in 3.3 and 3.4 too (Fig.11, Fig.13). Despite this increase of ammonium at the beginning in comparison to all incubations with cyanate only, no significant differences between a chemical or biological degradation of cyanate could be detected in this experiment.

To circumvent the problem of differentiation between biotic and abiotic cyanate degradation we faced in the low biomass experiments, a higher concentration of biomass was used to accelerate and increase the biotic degradation of cyanate to ammonium by *N. moscoviensis* (3.3). This experiment was performed with the same

substrate concentrations as described before (see 3.2). In comparison to the low cell density experiment, sodium bicarbonate concentration was slightly increased (1.5 mM instead of 1 mM) to increase buffering capacity.

Again, the presence of nitrite enhanced ammonium release by abiotic cyanate degradation, as already observed in low biomass experiments (see 3.2.). Re-addition of 2 mM nitrite significantly increases the chemical degradation of cyanate (Fig.11). Despite the use of higher cell density, again no significant differences in cyanate degradation between abiotic and biotic experiments fed with cyanate and nitrite was observed after 31 hours. Re-addition of nitrite also aggravated the compromise between chemical and a possible enzymatic degradation of cyanate. Furthermore, cyanate concentrations could not be determined in all experiments fed with cyanate and nitrite. It appears that the use of high nitrite concentrations might affect the derivatization of 2-aminobenzoic acid to quinazoline-2,4-dione. To avoid these issues, other assays for cyanate measurement should be tested in future.

As discussed before, adding both substrates together increased ammonium release into the media. This chemical interactions between cyanate and nitrite is still unclear. According to current knowledge, cyanate itself is an instable chemical and decomposes slowly to ammonium and carbon dioxide. Other factors like pH, temperature or the concentration of cyanate itself, catalyzes the chemical degradation, too (Palatinszky *et al.,* 2015). Nevertheless, this spontaneous release of ammonium makes it hard to distinguish between chemical and biological cyanate degradation.

Still, chemical analyses of all high biomass cyanate-only incubations, revealed an increase of ammonium formation in biotic incubations, when compared to abiotic controls (medium only and dead control) after 48 hours, which became more significant after 96 hours (Fig.12). To confirm that the ammonium formed originates from cyanate degradation, cyanate concentrations were determined, too. The increase of ammonium concentration (Fig.12) was confirmed by cyanate decrease and supports our findings that *N. moscoviensis* can degrade cyanate and release ammonium into the medium (Fig. 12). These findings confirm biological cyanate degradation in all biotic cyanate only incubations. Despite the use of high concentrations of biomass, only a small difference between chemical and biological cyanate degradation by *N. moscoviensis* could be detected after 31 hours. All incubations with cyanate and nitrite were treated only for 31 hours, while all incubations with cyanate only were treated for 96 hours. It could be possible, that an increased incubation time with both substrates might also show a more significant biological degradation of cyanate. It is clear that a modification of the medium is necessary to confirm this hypothesis. The fact that nitrite catalyzes cyanate degradation immediately, as well as re-addition of nitrite aggravated significant a comparison between chemical and biological degradation of cyanate. Despite this results, biological cyanate degradation with the presence of nitrite cannot be excluded.

In this study, cyanate degradation of *N. moscoviensis* was shown for the first time. The only protein of *N. moscoviensis* known to catalyze this reaction is the cyanase. Further analyses, like transcriptomics or proteomics

would help to understand the role of cyanase in the *N. moscoviensis* metabolism and to detect additional proteins and processes involved in the cyanate degradation. Further analyses of cyanases on several species confirmed their diverse physiological functions, including cyanate detoxification and production of NH_3 as an alternative N-source (Dorr and Knowles, 1989; Guilloton and Karst, 1987; Miller and Espie, 1994).

The capability of *N. moscoviensis* to degrade cyanate and release ammonium to the environment, provides strong support for the above proposed cyanate-based circular feeding hypothesis. To confirm this hypothesis, the next step would be to assemble *N. moscoviensis* with the approximately same amount of an AOB e.g. *Nitrosomonas europeae* and supply this consortium with cyanate as sole nitrogen and energy source. Interestingly, Kim and colleges indicated that AOBs like *Nitrosomonas* could survive high cyanate treatments for several months (Kim *et al.,* 2011). We were already able to show this circular feeding recently with *Nitrospira moscoviensis* and *Nitrosomonas nitrosa* as a model organism (Palatinszky *et al.,* 2015).

Further analyses and experiments are urgent to reveal more insights into the role of NOB in cyanate degradation in the environment, as well as the role of cyanases in the metabolism of NOB, including cyanate as possible indirect energy source by feeding AOM, which lack cyanases.

4.3. Cryopreservation and revival of *Nitrospira moscoviensis*

High quality and stable long-term cryopreservation of bacteria are essential to maintain cells for further experiments. The aim of this study was to find a good long-term cryopreservation protocol for *N. moscoviensis*. Several protocols are already published and verified as good methods for many different species (Vekeman *et al.,* 2013; Spieck and Bock *et al*., 2006; Hoefman *et al*., 2013). Until now, no cryopreservation protocol for the pure culture of *N. moscoviensis* has been established. Vekeman and colleges developed a protocol for long-term cryopreservation on different nitrite-oxidizing bacteria (Vekeman *et al.,* 2013). NOB are slow growing organisms and are hard to maintain in lab conditions. For this, a universal and functional cryopreservation protocol for NOB is important. Here, *N. moscoviensis* cultures were treated with 3 different cryopreservation media and with different DMSO concentrations, which is a cryoprotectant agent (CPA) as described above (Tab.7). Nowadays, dimethyl sulfoxide (DMSO) is more commonly used as a cryoprotectant agent then glycerol (Hoefman *et al.,* 2012).

The activity of *N. moscoviensis* cultures revived after cryopreservation was confirmed by weekly observation of nitrite consumption, which was constant over the whole experiment. All three cryopreservation showed activity after several weeks and confirmed effective cryoprotection by DMSO (Fig.14 A-B). Similar to the obtained results of Vekeman and colleagues, 10% DMSO with ten-fold diluted trypticase soy broth showed slightly better activity recovery then other testes cryopreservation conditions (Vekeman *et al.,* 2013). Growth was monitored by

spectroscopy (OD_{578} and OD_{436}) and indicated normal and constant increases until 21 days after revival, which flattened significantly only after 35 days. This finding indicates the successful cryopreservation by using all three methods and a good protocol for further cryopreservations on *N. moscoviensis*. Although carbon rich media like TSB have some advantages for reactivation compared to standard medium, TSB is not a common medium for NOB cultivation and using TSB for cryopreservation instead of the common standard medium increases the chance for contaminations. To minimize contaminations, cryopreservation with standard medium should be preferred.

5. Summary

Nitrogen is a common element in the universe and necessary for all known forms of life on Earth. The nitrogen cycle is a key process, by which nitrogen is converted in various chemical forms by biological processes. The human activities and the production of wastewater and production of ammonia by the Haber-Bosch process have radically changed the global nitrogen cycle and show the urgency to avoid a breakdown of these systems. High concentrations of several pollutions affect several biological processes. Interactions of various microbes are able to degrade these toxic pollutions into harmless products, which can be used again by other species. This coexistence and their integration indicate the importance for the natural ecosystem. Not all biochemical processes are known so far and suggest the urgency for better knowledge of this complex system.

One of the key processes in the nitrogen cycle is nitrification, describing the sequential oxidation of ammonia via nitrite to nitrate. Nitrite oxidizing bacteria (NOB) catalyze for the second step of nitrification and occur in a wide range of aquatic and terrestrial ecosystems. Genome analyses in all sequenced NOB genomes confirm the presence of the *cynS* gene, which encodes the enzyme cyanase. This enzyme catalyzes the degradation of the cell toxic cyanate to ammonia and carbon dioxide. Cyanase can be used from cyanate detoxification or for production of NH_3 as an alternative N-source. Degradation of cyanate into ammonia can be used as a common energy source for ammonia oxidizing microorganisms (AOM) which live in a close vicinity to nitrite oxidizer. The biological degradation of ammonia leads to releasing of nitrite, which is the common energy source for NOB. Until now, very little is known about the cyanate metabolism in NOB. Therefore, various experiments were performed by incubating *N. moscoviensis*, a NOB belonging to the widely distributed genus *Nitrospira*, with cyanate to investigate the potential biological degradation of cyanate to ammonia and carbon dioxide by this organism.

The results indicate that *N. moscoviensis* can withstand high concentrations (up to 5 mM) of cyanate with less effect on their metabolic activity. Also higher concentrations of ammonium (> 5 mM) did not show any effect on the biological activity of *N. moscoviensis*. Ammonium release by cyanate degradation increased the pH constantly because of the weak buffered media. Re-adding of nitrite accelerates the chemical degradation of cyanate into ammonium, which could be indicated by the results. To maintain pH stability, rubber stoppers were used. Nevertheless, a better buffer system would be important for long-term experiments. All control incubations (nitrite-only) with less or high cell density indicated high oxidation rate and confirms cell activity. All treatments with 1 mM cyanate show a slight reduction of the metabolic activity, which is due to the presence of cyanate. No significant enhancement of ammonium formation by cyanate degradation could be exhibit in all the biotic treatments with cyanate and nitrite after 31 hours, even by using high cell density (31 µg/ml). In contrast to the incubation with cyanate and nitrite, the incubations with high cell density and with cyanate alone confirm a

significant difference between biotic and abiotic ammonium formation after 31 hours, which becomes more significant after 96 hours. This finding was confirmed by cyanate measurements and refers to biological degradation of cyanate to ammonium by cyanase. These results indicate degradation of cyanate by NOB for the first time and present interesting insights of the physiology of this bacteria.

In addition to the incubation experiments, different cryopreservation protocols for the *N. moscoviensis* were tested. All cryopreserved cells were stored for two months at -80°C. The results indicate the all two cryopreservation media (1/10 TSB and mineral NOB media), as well as DMSO concentrations (1 % and 10 %), which served as a cryoprotectant agent (CPA), show good activity recovery after two months. Spectrophotometric analyses of OD_{578} and OD_{436} confirm similar growth rate with all media combinations. The continuous growth ranged from day 0 to day 21 and descended to stable density from 21 days to 35 days. These findings confirm the successful cryopreservation of the slow growing NOB *N. moscoviensis* for several months and relieve the labor-intensive work on this bacteria.

6. Zusammenfassung:

Stickstoff ist ein weit verbreitetes Element im Universum und essentiell für alle bekannten Lebensformen auf unserem Planet. Der Stickstoffkreislauf spielt eine wichtige Rolle, wobei Stickstoff durch verschiedenste chemische Reaktionen in unterschiedlichsten Verbindungen eingebunden wird. Die Nitrifikation ist ein bedeutender Teilprozess des biogeochemischen Stickstoffkreislaufes und der biologischen Abwasseraufbereitung. Der Mensch hat durch die ständige Produktion von Abwasser und vor allem durch die Produktion von Stickstoffquellen durch das Haber-Bosch-Verfahren den globalen Stickstoffkreislauf drastisch verändert. Schadstoffe in hoher Konzentration beeinflussen die biologischen Prozesse der zusammenwirkenden Mikroflora. Diese Auswirkungen auf das globale Ökosystem zeigen, wie wichtig die Aufrechterhaltung seiner lebenswichtigen Funktionen ist. Die Interaktion von unterschiedlichsten Mikroorganismen können solche, zum Teil toxischen Schadstoffe in harmlose Produkte umwandeln, die für andere Organismen verwertbar sind. Diese Integration und Koexistenz zwischen unterschiedlichsten Spezies deutet auf ihre Wichtigkeit für das natürliche Ökosystem hin. Heutzutage sind nicht alle biochemischen Abläufe bekannt und weisen dadurch auf die Notwendigkeit für weitere Untersuchungen dieser komplexen Prozesse hin.

Der Hauptprozess der Nitrifikation ist die biologische Oxidation von Ammonium über Nitrit zu Nitrat, die von einer großen mikrobiellen Lebensgemeinschaft beeinflusst wird. Nitrit-oxidierende Bakterien (NOB), wichtig für den zweiten Schritt in der Nitrifikation, kommen in fast allen aquatischen und terrestrischen Ökosystemen vor. Obwohl NOB phylogenetisch eine sehr diverse Gruppe darstellt, haben Untersuchungen des Erbgutes von unterschiedlichen NOB gezeigt, dass diese alle ein *cynS* Gen besitzen. CynS kodiert für das Enzym Cyanase, welches den biochemischen Abbau des zelltoxischen Zyanat in Ammonium und Kohlendioxid durchführt. Die Verwendung von Cyanase ist vielseitig und reicht von Zyanat-Entgiftung der Zelle bis zu Freisetzung von NH_3, welches als weitere Stickstoffquelle herangezogen werden kann. Freisetzung von Ammonium könnte als Energiequelle für ammonium-oxidierende Mikroorganismen (AOM) dienen, die eng mit Nitritoxidierer zusammenleben. Der Abbau von Ammonium zu Nitrit wiederum könnte als Energiequelle für NOB fungieren. Über die Funktion des Enzyms im Metabolismus von NOB ist bis heute wenig bekannt. Weitere Untersuchungen sind diesbezüglich notwendig. Deshalb wurden physiologische Untersuchungen an *N. moscoviensis*, einem Vertreter der Nitritoxidierer, durchgeführt, um den biochemischen Zerfall von Zyanat zu Ammonium und Kohlendioxid durch das Enzym Cyanase zu untersuchen.

Die Untersuchungen mit *N. moscoviensis* bestätigen die Resistenz gegenüber hohen Zyanatkonzentrationen (bis zu 5 mM), trotz leichter Abnahme der Zellaktivität, gemessen durch den Nitrit-Abbau während der Inkubationen.

Auch eine Erhöhung der Ammoniumkonzentration (>5 mM) zeigte keinen deutlichen Effekt der biochemischen Aktivität von *N. moscoviensis*. Die Freisetzung von Ammonium durch den Zerfall von Zyanat erhöhte den ursprünglichen pH-Wert. Die Resultate bestätigten auch, dass die zusätzliche Zugabe von Nitrit den chemischen Zyanat-Zerfall nochmals deutlich beschleunigt. Durch die Verwendung von Gummistopfen anstelle der herkömmlichen Schraubverschlüsse wurde die pH-Stabilität gewährleistet. Nichtsdestotrotz ist eine Verbesserung des Puffersystems für Langzeitstudien notwendig. Alle Kontrollinkubationen (nur Nitrit) zeigten bei geringerer oder hoher Zugabe von Zellen eine hohe Oxidationsrate auf. Alle Inkubationen mit Zyanat (1 mM) weisen auf eine leichte Reduktion der Oxidationtsrate hin, welches auf der Wirkung von Zyanat selbst beruht. Keine signifikanten Unterschiede bezüglich der Ammoniumfreisetzung konnte bei allen Inkubationen mit Nitrit und Zyanat nach 31 Stunden erzielt werden. Auch Inkubationen mit hoher Zelldichte (31 µg/ml) konnten keine Unterschiede bei der Ammoniumfreisetzung bewirken.

Im Vergleich zu den Inkubationen mit Zyanat und Nitrit zeigten die Inkubationen mit nur Zyanat nach 31 Stunden eine deutliche Veränderung der Ammoniumfreisetzung, die nach 96 Stunden noch signifikanter wurde. Dieser Zyanat-Zerfall mit anschließender Freisetzung von Ammonium wurde auch durch chemische Analysen bestätigt und deutet auf einen biochemischen Abbau durch das Enzym hin. Dieses Resultat bestätigt zum ersten Mal den Zerfall von Zyanat durch einen Nitritoxidierer und liefert interessante Einblicke in die Physiologie von *N. moscoviensis.*

Zusätzlich zu den Inkubationsexperimenten wurden verschiedene Kryokonservierungsmethoden für eine Reinkultur von *N. moscoviensis* durchgeführt. Alle kryokonservierten Zellen wurden für 2 Monate bei -80°C gelagert. Die Resultate der Reaktivierung deuten darauf hin, dass beide Mediumtypen (1/10 TSB und NOB Medium), sowie beide DMSO Konzentrationen (1%-10%), die als Kryoschutzmittel wirkten, für eine gute Reaktivierungsaktivität geeignet sind. Spektroskopische Untersuchungen bei einer Optischen Dichte von OD_{578} und OD_{436} bestätigten eine ähnliche Wachstumsrate bei allen verwendeten Kombinationen. Eine kontinuierliche Wachstumsrate konnte vom Anfang bis zum Tag 21 festgestellt werden, die danach vom Tag 21 bis zum Tag 35 abflacht und konstant blieb. Dieses Resultat zeigte die erfolgreiche Kyrokonservierung des langsam wachsenden Bakteriums *N. moscoviensis* über mehrere Monate und erleichtert somit die schwere Aufrechterhaltung der Kulturen unter Laborbedingungen.

7. Abbreviations:

Anammox	Anaerobic ammonium oxidation
AF2	Citifluor
AOA	Ammonia oxidizing archaea
AOM	Ammonia oxidizing microorganisms
BCA	Bicinchoninic acid
CNO^-	Cyanate
CO_2	Carbon dioxide
CPA	Cryoprotectant agent
Cy3	Indocarbocyanine
DAPI	4',6-Diamidin-2-phenylindol
FA	Formamide
Fluos	Fluorescein
FISH	Fluorescence *in situ* hybridization
HCO_3^-	Bicarbonate
ICM	Intracellular membrane
KOCN	Potassium cyanate
N_2	Nitrogen
N_2O	Nitrous oxide
$NaHCO_3$	Natriumbicarbonate
NED	N-1-naphtylethylenedamine dihydrochloride
NH_3	Ammonia
NH_4^+	Ammonium
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite oxidizing bacteria
NXR	Nitrite oxidoreductase
PBS	Phosphate buffered saline

SCN	Thiocyanate
TES	Trace elements solution
TSB	Tryptone Soya Broth
WWTPS	Wastewater treatment plants

8. References:

Alawi, M., Lipski, A., Sanders, T., Pfeiffer, E.M., and Spieck, E. (2007). Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. ISME J *1*, 256–264.

Anderson, P.M. (1980). Purification and properties of the inducible enzyme cyanase. Biochemistry *19*, 2882-2888.

Anderson, P.M., Little, R.M. (1985). Kinetic properties of cyanase. Fed. Proc. *44*, 1053.

Amor, L., Eiroa, M., Kennes, C., Veiga, M.C. (2005). Phenol biodegradation and its effect on the nitrification process. Water Res. *39*, 2915-2920.

Baalsrud, K., Baalsrud, K.S. (1954). Studies on thiobacillus denitrificans. Archiv Für Mikrobiologie *20*, 34–62.

Benbi, D., Richter, J. (2002). A critical review of some approaches to modelling nitrogen mineralization. Biology and Fertility of Soils *35*, 168–183.

Bonaventura, C., and Johnson, F.M. (1997). Healthy environments for healthy people: bioremediation today and tomorrow. Environ. Health Perspect. *105* Suppl 1, 5–20.

Bothe, H., Ferguson, S., and Newton, W.E. (2007). Biology of the nitrogen cycle; 1st edition; Elsevier B.V., Oxford, UK; ISBN 978-0-444-53108-7 (Elsevier).

Bock, E., and Wagner, M. (2006). Oxidation of Inorganic Nitrogen Compounds as an Energy Source, Vol. 2. In The Prokaryotes, M.D.P. Dr, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, eds. (Springer New York), pp. 457–495.

Burrell, P.C., Keller, J., Blackall, L.L. (1998). Microbiology of a nitrite-oxidizing bioreactor. Appl Environ Microbiol *64*, 1878–1883.

Butt, W.D., Lees, H. (1964). The biochemistry of the nitrifying organisms. 8. The effects of oxygen tension, nitrite concentration, and cyanate concentration on nitrite oxidation by Nitrobacter. Can J Biochem. *42*, 1217-24.

Canfield, D.E., Glazer, A.N., and Falkowski, P.G. (2010). The Evolution and Future of Earth's Nitrogen Cycle. Science *330*, 192–196.

Carlson, C.A., Ingraham, J.L. (1983). Comparison of denitrification by Pseudomonas stutzeri, Pseudomonas aeruginosa, and Paracoccus denitrificans. Appl Environ Microbiol *45*, 1247–1253.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. Syst.Appl. Microbiol. *22*, 434–444.

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.H., Wagner M. (2001). In situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants. Appl Environ Microbiol *67*, 5273–5284.

Daims, H., Stoecker, K., and Wagner, M. (2005). Fluorescence in situ hybridization for the detection of prokaryotes. In Molecular Microbial Ecology, A.M. Osborne, and C.J. Smith, eds. (Taylor & Francis Group), pp. 213–223.

Daims, H., M.W. Taylor and M. Wagner (2006). Wastewater treatment: A model system for microbial ecology. Trends Biotechnol *24* (11): 483-9

Dalsgaard, T., Thamdrup, B., and Canfield, D.E. (2005). Anaerobic ammonium oxidation (anammox) in the marine environment. Research in Microbiology *156*, 457– 464.

Dirnhuber, P., Schutz, F. (1948). The isomeric transformation of urea into ammomium cyanate in aqueous solutions. Biochem. J. *42*, 628-632.

Dorr, P.K., and Knowles, C.J. (1989). Cyanide oxygenase and cyanase activities of *Pseudomonas fluorescens* NCIMB 11764. FEMS Microbiol *60*,289–294.

Ehrich, S., Behrens, D., Lebedeva, E., Ludwig, W., Bock, E. (1995). A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, *Nitrospira moscoviensis sp*. nov. and its phylogenetic relationship. Arch Microbiol *164*, 16–23.

Elbanna, K. (2012). A new simple method for the enumeration of nitrifying bacteria indifferent environments. Plant Soil Environ. *58*, 49–53.

Freitag, T.E., Chang, L., Clegg, C.D., Prosser, J.I. (2005). Influence of inorganic nitrogen management regime on the diversity of nitrite-oxidizing bacteria in agricultural grassland soils. Appl Environ Microbiol *71*, 8323–8334.

Gruber-Dorninger, C., Pester, M., Kitzinger, K., Savio, D.F., Loy, A., Rattei, T., Wagner, M., Daims, H. (2014). Functionally relevant diversity of closely related Nitrospira in activated sludge. ISME J. doi: 10.1038

Guilloton, M. and Karst, F. A (1985). Spectrophotometric Determination of Cyanate Using Reaction with 2 - Aminobenzoic Acid. Anal Biochem *149*, 291-295, doi: Doi 10.1016/0003-2697(85)90572-X.

Guilloton, M., Karst, F. (1986). Cyanate Specifically Inhibits Arginine Biosynthesis in *Escherichia coli* K12: a Case of By-product Inhibition. Journal of General Microbiology *133*, 655-665.

Guilloton, M., Karst, F. (1987). Isolation and characterization of *Escherichia coli* mutants lacking inducible cyanase. J. Gen. Microbiol. *133*, 645-653.

Guilloton, M., Espie, G.S., Anderson, P.M. (2002). What is the role of cyanase in plants? In: Goyal, A., Metha, A., Lodha, M.L. (Eds.), Reviews in Plant Biochemistry and Biotechnolgy. The Society for Plant Biochemistry and Biotechnolgy, New Delhi, India, pp. 57-79.

Hoefman, S., Van Hoorde, K., Boon, N., Vandamme, P., De Vos, P., Heylen, K. (2012). Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria, Plos One, 7 e34196.

Hoefman, S., Pommerening-Roser, A., Samyn, E., De Vos, P., Heylen, K. (2013). Efficient cryopreservation protocol enables accessibility of a broad range of ammonia-oxidizing bacteria for the scientific community. Res Microbial, *164*, 288-92.

Heylen, K., Ettwig, K., Hu, Z., Jetten, M., Kartal, B. (2012). Rapid and simple cryopreservation of anaerobic ammonium-oxidizing bacteria. Appl Environ Microbiol. *78*, 3010-3.

Johnson, W.V., Anderson, P.M. (1987). Bicarbonate is a recycling substrate for cyanase. J. Biol. Chem. *262*, 9021-9025.

Jetten, M.S.M., Cirpus, I., Kartal, B., Van Niftrik, L., Van de Pas-Schoonen, K.T., Sliekers, O., Haaijer, S., Van der Star, W., Schmid, M., Van de Vossenberg, J., *et al.* (2005). 1994–2004: 10 years of research on the anaerobic oxidation of ammonium. *33* (Pt 1), 119–123.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Roesser, A., Koops, H.P., *et al.* (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. Appl Environ Microbiol *64*, 3042–3051.

Kartal, B., Maalcke, W.J., de Almeida, N.M., Cirpus, I., Gloerich, J., Geerts, W., Op den Camp, H.J.M., Harhangi, H.R., Janssen-Megens, E.M., Francoijs, K.-J., *et al.* (2011). Molecular mechanism of anaerobic ammonium oxidation. Nature *479*, 127–130.

Kamennaya, N. A., Cherinhovsky, M. and Post, A.F. (2008). The cyanate utilization capacity of marine unicellular cyanobacteria. Limnol Oceanogr *53*, 2485–2494.

Kamennaya, N.A., and Post, A.F (2013). Distribution and expression of the cyanate acquisition potential among cyanobacterial populations in oligotrophic marine waters. Limnol Oceanogr *58*, 1959–1971

Kandeler, E. and Gerber, H. (1988). Short - Term Assay of Soil Urease Activity Using Colorimetric Determination of Ammonium. Biol Fert Soils *6*, 68-72.

Keuter, S., Kruse, M., Lipski, A., Spieck, E. (2011). Relevance of Nitrospira for nitrite oxidation in a marine recirculation aquaculture system and physiological features of a Nitrospira marina-like isolate. Environ Microbiol. *9*, 2536-47.

Kim, Y.M., Park, D., Lee, D.S., and Park, J.M. (2006). Instability of biological nitrogen removal in a cokes wastewater treatment facility during summer. Journal of Hazardous Materials *141*, 27-32.

Kim, Y.M., Park, D., Jeon, C.O., Lee, D.S., Park, J.M. (2008). Effect of HRT on the biological pre-denitrification process for the simultaneous removal of toxic pollutions from coke wastewater. Bioresource Technol. *99*, 8824-8832.

Kim, Y.M., Park, D., Jung, K.A., Lee, D.S., Park, J.M. (2009). Sudden failure of biological nitrogen and carbon removal in the full-scale pre-denitrification process treating cokes wastewater. Bioresource Technol. *100*, 4340-4347.

Kim, Y.M., Cho, H.U., Lee, D.S., Park, C., Park, D., Park, J.M. (2011).Response of nitrifying bacterial communities to the increased thiocyanate concentration in pre-denitrification process. Bioresource Technol. *102*, 913-922.

Kimochi, Y., Inamori Y., Mizuochi M., Xu K.-Q., and Matsumura M. (1998). Nitrogen Removal and N2O Emission in a Full-Scale Domestic Wastewater Treatment Plant with Intermittent Aeration. Journal of Fermentation and Bioengineering *86*, 202–206.

Koch, H., Galushko, A., Albertsen, M., Schintlmeister, A., Gruber-Dorninger, C., Lücker, S., Pelletier, E., Le Paslier, D., Spieck, E., Richter, A., Nielsen, P.H., Wagner, M., Daims, H. (2014). Growth of nitrite-oxidizing bacteria by aerobic hydrogen oxidation. Science. *345* (6200):1052-4

Kozliak, E.I., Fuchs, J.A., Guilloton, M.B., Anderson, P.M. (1995). Role of bicarbonate/CO_2 in the inhibition of *Escherichia coli* growth by cyanate. J.Bacteriol. *177*, 3213-3219.

Könneke, M., Bernhard, A.E., Torre, J.R. de la, Walker, C.B., Waterbury, J.B., and Stahl, D.A. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature *437*, 543–546.

Kuenen, J.G. (2008). Anammox bacteria: from discovery to application. Nat. Rev. Microbiol. *6*, 320–326.

Kumar, M.S., Vaidya, A.N., Shivaraman, N., Bal, A.S. (2003). Performance evaluation of a full-scale coke oven waste water treatment plant in an integrated steel plant. Indian J. Environ. Health *45*, 29-38.

Lebedeva, E.V., Off, S., Zumbrägel, S., Kruse, M., Shagzhina, A., Lücker, S., Maixner, F., Lipski, A., Daims, H., Spieck, E. (2011). Isolation and characterization of a moderately thermophilic nitrite-oxidizing bacterium from a geothermal spring. FEMS Microbiol Ecol. *75*, 195-204.

Liu, Y.Q., Tay, J.H., Ivanov, V., Moy, B.Y.P., Yu, L., Tay, S.T.L. (2005). Influence of phenol on nitrification by microbial granules. Process Biochem. *40*, 3285-3289.

Luque-Almagro, V.M., Huertas, M.-J., Saez, L.P., Luque-Romero, M.M., Moreno-Vivian, C., Castillo, F., Roldan, M.D., Blasco, R. (2008). Characterization of the *Pseudomonas pseudoalcaligenes* CECT5344 cyanase, an enzyme that is not essential for cyanide assimilation. Appl. Environ. Microbiol. *74*, 6280-6288.

Lücker, S. (2010). Exploring the ecology and genomics of globally important nitrite oxidizing bacteria. phd. Uni wien; AC08447806.

Lücker, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B., Rattei, T.,Damsté, J.S.S., Spieck, E., Paslier, D.L., *et al*. (2010). A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. PNAS *107*, 13479–13484.

Lücker, S., Nowka, B., Rattei, T., Spieck, E., and Daims, H. (2013). The Genome of Nitrospina gracilis Illuminates the Metabolism and Evolution of the Major Marine Nitrite Oxidizer. Front Microbiol *4*:27.

Maeda, S., Omata, T. (2009). Nitrite transport activity of the ABC-type cyanate transporter of the cyanobacterium *Synechococcus elongatus*. J Bacteriol. *191*, 3265-72.

Madigan, M.T., Martinko, J.M., Dunlap, P.V., and Clark, D.P. (2008). Brock Biology of Microorganisms, 12th edition. (Prentice-Hall, Upper Saddle River, NJ.).

Maixner, F. *et al.* (2006). Nitrite concentration influences the population structure of Nitrospira – like bacteria. Environ Microbiol *8*, 1487-1495, doi: DOI 10.1111/j.1462-2920.2006.01033.x419.

Meincke, M., Bock, E., Kastrau, D., Kroneck, P.M.H. (1992). Nitrite oxidoreductase from *Nitrobacter hamburgensis*: Redox centers and their catalytic role. Arch Microbiol *158*, 127–131.

Miller, A.G., Espie, G.S. (1994). Photosynthetic metabolism of cyanate by the cyanobacterium *Synechococcus* UTEX-625. Arch. Microbiol. *162*, 151-157.

Mulder, A., van de Graaf, A.A., Robertson, L.A., and Kuenen, J.G. (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiology Ecology *16*, 177–183.

Nowka, B., Off, S., Daims, H. and Spieck, E. (2014). Improved isolation strategies allowed the phenotypic differentiation of two *Nitrospira* strains from widespread phylogenetic lineages. FEMS Microbiol Ecol. DOI: http://dx.doi.org/10.1093/femsec/fiu031

Off, S., Alawi, M., Spieck, E. (2010). Enrichment and physiological characterization of a novel Nitrospira like bacterium obtained from a marine sponge. Appl Environ Microbiol *76*, 4640–4646.

Okabe, S., Satoh, H., and Watanabe, Y. (1999). In Situ Analysis of Nitrifying Biofilms as Determined by In Situ Hybridization and the Use of Microelectrodes. Appl. Environ. Microbiol. *65*, 3182–3191.

Palatinszky, M., Herbold, C., Jehmlich, N., Pogoda, M., Han, P., von Bergen, M., Lagkouvardos, I., Karst, S.M., Galushko, A., Koch, H., Berry, D., Daims, H. and Wagner, M. (2015). Cyanate as an energy source for nitrifiers. Nature. 524 (7563):105-8. doi: 10.1038/nature14856.

Prosser, J.I. (1989). Autotrophic nitrification in bacteria. Adv. Microb. Physiol. *30*, 125–181.

Qian, D., Jiang, L., Lu, L., Wie, C., Li, Y. (2011). Biochemical and structural properties of cyanases from Arabidopsis thaliana and Oryza sativa. PLoS One. 31; 6(3):e18300.

Raybuck, S.A (1992). Microbes and microbal enzymes for cyanide degradation. Biodegradation *3*, 3-18.

Scanlan, D.J., Ostrowski, M., Mazard, S., Dufresne, A., Garczarek, L., Hess, W.R., Post, A.F., Hagemann, M., Paulsen, I., Partensky, F. (2009). Ecological genomics of marine picocyanobacteria. Microbiol Mol Biol Rev. *73*, 249-99.

Schramm, A., de Beer, D., van den Heuvel, J. C., Ottengraf, S., Amann, R. (1999). Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: Quantification by in situ hybridization and the use of microsensors. Appl Environ Microb *65*, 3690-3696.

Smith, P.K, Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal Biochem. *150*, 76-85.

Shoun, H., Tanimoto, T. (1991). Denitrification by the fungus Fusarium oxysporum and involvement of cytochrome P-450 in the respiratory nitrite reduction. J. Biol. Chem. *266*, 11078–11082.

Sorokin, D.Y., Lücker, S., Vejmelkova, D., Kostrikina, N.A., Kleerebezem, R., Rijpstra, W.I.C., Damsté, J.S.S., Paslier, D.L., Muyzer, G., Wagner, M., et al. (2012). Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum Chloroflexi. The ISME Journal *6*:12, 2245–2256.

Spang, A., Poehlein, A., Offre, P., Zumbrägel, S., Haider, S. *et al* (2012). The genome of the ammonia-oxidizing Candidatus Nitrososphaera gargensis: insights into metabolic versatility and environmental adaptations. Environ Microbiol. *14*, 3122-45.

Spieck, E., Ehrich, S., Aamand, J., Bock, E. (1998). Isolation and immunocytochemical location of the nitrite-oxidizing system in *Nitrospira moscoviensis*. Arch Microbiol *169*, 225–230.

Spieck, E., and Bock, E. (2006). The lithoautotrophic Nitrite-Oxidizing Bacteria. In Bergey's Manual of Systematic Bacteriology, 2nd Edn., eds G. Garrity, D.J. Brenner, N.R. Krieg, and J. T. Staley (Springer New York), pp. 149-153.

Spieck, E., Hartwig, C., McCormack, I., Maixner, F., Wagner, M., Lipski, A., and Daims, H. (2006). Selective enrichment and molecular characterization of a previously uncultured Nitrospira-like bacterium from activated sludge. Environ. Microbiol. *8*, 405–415.

Stark, G.R. (1965). Reactions of cyanate with functional groups of proteins. III. Reactions with amino and carboxyl groups. Biochemistry *4*, 1030-1036.

Stein, L.Y., Arp, D.J. (1998). Loss of Ammonia Monooxygenase Activity in Nitrosomonas europaea upon Exposure to Nitrite. Appl. Environ. Microbiol. *64*, 4098– 4102.

Stickland, J. D. H., Parsons, T.R. (1072). A Practical Handbook of Seawater Analysis. Fisheries Research Board of Canada, Ottawa.

Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W., Horn, M., Daims, H., Bartol-Mavel, D., Wincker, P., *et al.* (2006). Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature *440*, 790– 794.

Suzuki, I., Sugiyama, T., Omata, T. (1996). Regulation by cyanate of the genes involved in carbon and nitrogen assimilation in the cyanobacterium *Synechococcus sp*. strain PCC 7942. J. Bacteriol. *178*, 2688-2694.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution *30*, 2725-2729.

Taussig, A. (1960). The synthesis of the induced enzyme, "cyanase", in E.coli. Biochem. Biophys. Acta. *44*, 510-519.

Vekeman, B., Hoefman, S., De Vos, P., Spieck, E., Heylen, K. (2013). A generally applicable cryopreservation method for nitrite-oxidizing bacteria. Syst Appl Microbiol. 36, 579-584.

Walsh, M.A., Otwinowski, Z., Perrakis, A., Anderson, P.M., Joachimiak, A. (2000). Structure of cyanase reveals that a noval dimeric and decameric arrangement of subunits is required for formation of the enzyme activity site. Structure *8*, 505-514.

Watson, S.W., Bock, E., Valois, V.W., Waterbury, J.B. and Schlosser, U. (1986). *Nitrospira marina* gen. Nov. Sp. Nov.: A chemolithotrophic nitrite-oxidizing bacterium. Archives of Microbiology *144*, 1-7.

Widner, B., Mulholland, M.R., and Mopper, K. (2013). Chromatographic Determination of nanomolar cyanate concentrations in Estuarine and Sea Waters by Precolumn Fluorescence Derivatization. Anal Chem *85*, 6661-6.

White, J.R., and Reddy, K.R. (2009). Biogeochemical Dynamics I: Nitrogen Cycling in Wetlands, Vol. 2. In Maltby E., Barker T. (ed.). Wetlands Handbook. 1st. Edition., (John Wiley & Sons), pp. 213–220.

Zhang, M., Tay, J.H., Qian, Y., Gu, X.S. (1998). Coke plant wastewater treatment by fixed biofilm system for COD and NH_3-N removal. Water Res. *32*, 519-527.

9. Acknowledegements:

Zum Abschluss möchte ich mich bei all denen bedanken, die für die Umsetzung dieser Arbeit mitgewirkt haben:

Einen großen Dank an Michael Wagner und Holger Daims für die Möglichkeit meine Arbeit hier zu schreiben und Teil dieses Departments zu sein.

Meiner Betreuerin Hanna Koch für all ihre Hingabe und Unterstützung über die gesamte Zeit und für die extra Motivation in schwierigen Zeiten.

An Alexander Galushko, Márton Palatinszky, Sebastian Lücker und Tae Kwon Lee für die vielen Hinweise in der Praxis und Theorie, sowie für die Hilfe bei vielen Präsentationen.

Petra Pjevac für ihre Hilfe und Wissen bei der Erstellung dieser Masterarbeit.

Martina Grill, unsere tolle TA die mich mit allem versorgt hat, was man im Labor benötigt.

Ein großes Dankeschön auch an Albert, Alexander, Allen, Anne, Bela, Brigitte, Claus, Daryl, Esther, Florian M., Florian S., Florian W., Felicitas, Gabriele, Jasmin, Karin, Katharina, Ken, Markus, Martin, Michaela, Orest, Penny, Roy, Stefanie, Stephanie und Stephan für alle die tolle Zeit im Labor und eure Hilfe, für die vielen Lacher die wir hatten, den Abenden im Gemeinschaftraum oder auf der Terrasse. Ich werde diese Zeit mit euch nie vergessen.

Danke auch an allen anderen DOMIES, die ich nicht erwähnt habe.

Zur guter Letzt möchte ich mich noch bei meiner ganzen Familie bedanken. Meinen Eltern, die mich über all die Jahre mit aller Kraft unterstützt haben. Meiner Lebensgefährtin Judith, für all Ihre Unterstützung und den Ansporn, die sie mir jeden Tag gibt. Meinen beiden Schwestern, Katharina und Agnes für all Ihren Rückhalt während meines Studiums. Und meinen Freunden, die mich seit Jahren durchs Leben begleiten.

10. Curriculum vitae:

Contact information

Name:	Mario Pogoda
Address:	Hartlgasse 19/15a
	1200 Wien
	Tel.: +436506419053
	Mail: mario.pogoda@gmx.at

Personal information

Date of birth:	17.12.1985
Place of birth:	Friesach/Austria
Sex:	male
Material status:	unmarried

Education

2000-2004	High school; BORG Auer v. Welsbach
1996-2000	Secondary school; Neumarkt in der Steiermark
1992-1996	Elementary school; Neumarkt in der Steiermark

University education

2010-2015	Master Molecular Microbiology, Microbial Ecology and Immunbiology
	University Vienna
2005-2010	Bachelor; Biomedicine and Biotechnology
	Veterinary Medicine University Vienna

Work experience

2.2015 -	Technical assistant (TA) at DOME (Division of Microbial Ecology)

Technical Skills

General laboratory skills	DNA isolation and purfication
Polymerase chain reaction (PCR)	Microscopy (CLSM, Eppifluorescence)
Cultivation skills (normal/modified Eukaryotic cell lines, Bacteria, Parasites, Viruses)	Fluorescenc-in-situ-hybridization (FISH)
Gel electrophoresis	Anatomy skills (laboratory animals)
Chemical and spectroscopic analyses	Bacterial cloning

Publications:

Palatinszky, M., Herbold, C., Jehmlich, N., Pogoda, M., Han, P., von Bergen, M., Lagkouvardos, I., Karst, S.M., Galushko, A., Koch, H., Berry, D., Daims, H. and Wagner, M. (2015). Cyanate as an energy source for nitrifiers. Nature. 524 (7563):105-8. doi: 10.1038/nature14856.