Characterization of *Candidatus* Nitrotoga and its Competitiveness in Co-Culture with *Nitrospira*

Dissertation

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ABSTRACT

Nitrification is a crucial microbial process during nitrogen removal in wastewater treatment plants (WWTPs) and recirculating aquaculture systems (RAS), where ammonia-oxidizing microbes (AOM) and nitrite oxidizing bacteria (NOB) prevent the accumulation of highly toxic ammonia and nitrite. NOB of the genus *Nitrospira* are usually detected in activated sludge or biofilters. However, *Nitrotoga* was recently reported as the main NOB in WWTPs, alone or in coexistence with *Nitrospira*. Their frequent detection in the same habitat raises the question of niche separation between these two genera.

The candidate genus *Nitrotoga* was first described as a cold-tolerant NOB with a distinct large periplasm. In contrast to *Nitrospira*-like NOB, only a few *Nitrotoga* members have been enriched, cultured, and characterized. Since their 16S rRNA gene sequences are nearly identical (> 99% similarity), assigning species within the genus based on sequence data alone is difficult. Four highly enriched *Nitrotoga* cultures sampled from different habitats were available for a comparative characterization, including physiology, phylogeny, and morphology. Based on results from these tests, cultures were selected for competition experiments with *Nitrospira* to determine factors enabling niche partitioning.

While they showed similar morphological features, each *Nitrotoga* culture was characterized by distinct physiological properties. *Nitrotoga arctica* (permafrost soil, active layer) and *Nitrotoga* BS (activated sludge) shared the same optimum temperature of 17°C, whereas *Nitrotoga* HW (biofilter RAS) and *Nitrotoga* 1052 (permafrost soil, bore core) grew best at 22°C. The cold adaption of the genus was confirmed by high activity down to 4°C but inhibition above 25 to 28°C. Regarding the pH, *Nitrotoga* tolerated alkalinity (about pH 8) better than acidity (about pH 6) in general. Nonetheless, *Ntg. arctica* and *Nitrotoga* HW had their optimum in the slightly acidic to neutral range, while *Nitrotoga* 1052 and *Nitrotoga* BS preferred slightly alkaline surroundings. The cultures were furthermore characterized by tolerating ammonium concentrations of 25 to 40 mM, and *Nitrotoga* BS was actually dependent on supplementation of ammonium. *Nitrotoga* were more easily affected by nitrate, and nitrite showed the most severe inhibitory effect at concentrations below those reported for most *Nitrospira*. The four *Nitrotoga* cultures

could also be distinguished by the gene encoding the nitrite oxidoreductase, the key enzyme for nitrite oxidation, with lower similarities than on 16S rRNA gene level.

Ntg. arctica and *Nitrotoga* BS were selected for co-cultivation experiments with *Nitrospira defluvii* in batch reactors operated at 17°C and different pH of either 6.4 or 7.4. Their abundance was evaluated by qPCR and FISH. *Nitrotoga* BS was clearly favored by its optimal growth conditions. It predominated over *Nsp. defluvii* at pH 7.4 with at least 94% relative abundance, but was suppressed at pH 6.4. In contrast, *Ntg. arctica* could not exploit its optimum at pH 6.4 and 17°C, but predominated at pH 7.4 as well, although it was not as abundant as *Nitrotoga* BS.

The positive influence of low temperature on *Nitrotoga* was also observed in a cold-freshwater RAS that was monitored from start-up over nearly two years. With a mean water temperature of 13°C, *Nitrotoga* found excellent conditions to colonize the biofilters, and was consistently detected together with *Nitrospira*. However, it was lost after disinfection with peracetic acid and hydrogen peroxide was implemented, whereas *Nitrospira* remained abundant in the biofilters.

These results underline the physiological and phylogenetic diversity within the genus *Nitrotoga*. They also demonstrate the positive selective effect of temperatures below 20°C on its competitiveness against *Nitrospira*, while pH plays a minor role. Together with recent publications, this work emphasizes the importance of *Nitrotoga* in engineered habitats and closes the knowledge gap in comparison to other NOB genera.

ZUSAMMENFASSUNG

Die Nitrifikation ist eine wichtige mikrobielle Reaktion bei der Stickstoffentfernung in Kläranlagen und Kreislaufanlagen für die Aquakultur, bei dem Ammoniak-oxidierende Mikroorganismen (AOM) und Nitrit-oxidierende Bakterien (NOB) die Anhäufung von hoch toxischem Ammoniak oder Nitrit verhindern. Für gewöhnlich dominieren NOB der Gattung *Nitrospira* in diesen Systemen. In letzter Zeit wurde jedoch *Nitrotoga* immer wieder als NOB in Kläranlagen gefunden, alleine oder in Koexistenz mit *Nitrospira*. Die häufige Beobachtung im selben Habitat wirft die Frage nach der Nischenbildung zwischen beiden NOB auf.

Die *Candidatus*-Gattung Nitrotoga wurde erstmals als Kälte-adaptiert mit auffällig vergrößertem Periplasma beschrieben. Im Gegensatz zu *Nitrospira*-ähnlichen NOB sind bisher nur wenige Vertreter von *Nitrotoga* angereichert, kulturviert und charakterisiert worden. Da ihre 16S rRNA Gensequenzen fast identisch sind (> 99% Übereinstimmung), ist die Zuordnung einzelner Arten innerhalb der Gattung auf Basis von Sequenzdaten schwierig. Vier hochangereicherte *Nitrotoga*-Kulturen unterschiedlicher Standorte wurden einer vergleichenden Charakterisierung unterzogen, die neben der Physiologie auch phylogenetische und morphologische Merkmale umfasste. Ausgehend von diesen Versuchen wurden Kulturen für Konkurrenzexperimente mit *Nitrospira* ausgewählt, um Faktoren für die Nischenbildung zwischen beiden NOB zu bestimmen.

Während die Kulturen eine einheitliche Morphologie besaßen, wiesen sie sehr unterschiedliche physiologische Eigenschaften auf. *Nitrotoga arctica* (Permafrostboden, Auftauhorizont) und *Nitrotoga* BS (Belebtschlamm) hatten ihr Temperaturoptimum bei 17°C, *Nitrotoga* HW (Kreislauf-Aquakultur) und *Nitrotoga* 1052 (Permafrostboden, Bohrkern) hingegen bei 22°C. Die Anpassung der Gattung an Kälte wurde durch ihre hohe Aktivität bei 4°C und ihre Hemmung ab 25 bis 28°C bestätigt. Im Hinblick auf den Einfluss des pH-Wertes tolerierte *Nitrotoga* leicht basische Bedingungen (etwa pH 8) insgesamt besser als saure (etwa pH 6). Dennoch hatten *Ntg. arctica* und *Nitrotoga* HW ihr Optimum im leicht sauren bis neutralen Bereich, während *Nitrotoga* 1052 und *Nitrotoga* BS eine leicht basische Umgebung bevorzugten. Charakteristisch war die Toleranz aller Kulturen gegenüber Ammoniumkonzentrationen zwischen 25 und 40 mM. *Nitrotoga* BS war darüber hinaus auf die Zugabe von Ammonium angewiesen. Hingegen reagierte *Nitrotoga*

empfindlicher auf erhöhte Konzentrationen an Nitrat und noch deutlicher auf Nitrit, mit maximal tolerierten Konzentrationen untern denen, die für die meisten *Nitrospira* ermittelt wurden. Darüber hinaus konnten die Kulturen mittels der Gensequenz für die Nitrite-Oxidoreduktase, das Schlüsselenzym der Nitrit-Oxidation, unterschieden werden, mit deutlich geringeren Übereinstimmungen als für das 16S rRNA Gen.

Ntg. arctica und *Nitrotoga* BS wurden in Co-Kultivierungsversuche mit *Nitrospira defluvii* eingesetzt. Die Versuche fanden in Batch-Bioreaktoren bei 17°C und pH 6,4 oder 7,4 statt. Die NOB-Häufigkeit wurde per qPCR und FISH ermittelt. *Nitrotoga* BS konnte von optimalen Wachstumsbedingungen bei pH 7,4 profitieren und dominierte mit mindestens 94% relativer Häufigkeit über *Nsp. defluvii*, wurde hingegen bei pH 6,4 verdrängt. Im Gegensatz dazu nutzte *Ntg. arctica* seine bevorzugten Bedingungen bei pH 6,4 und 17°C nicht, sondern war ebenfalls bei einem pH von 7,4 häufiger als *Nsp. defluvii*, wenn auch nicht so deutlich wie *Nitrotoga* BS.

Der positive Einfluss niedriger Temperatur auf *Nitrotoga* wurde ebenfalls in einer Kaltwasser-Kreislauf-Aquakulturanlage beobachtet, die seit der Starphase für zwei Jahre überwacht wurde. Bei durchschnittlichen 13°C Wassertemperatur fand *Nitrotoga* ideale Bedingungen zur Besiedlung der Biofilter und wurde durchgängig zusammen mit *Nitrospira* nachgewiesen. Jedoch wurde er nach der Desinfektion mit Peressigsäure und Wasserstoffperoxid aus dem Prozess gedrängt, während *Nitrospira* in den Biofiltern verblieb.

Diese Ergebnisse unterstreichen die physiologische und phylogenetische Vielfalt der Gattung *Nitrotoga*. Sie belegen den positiven Einfluss von Temperaturen unter 20°C auf ihre Konkurrenzfähigkeit gegenüber *Nitrospira*, wohingegen der pH-Wert eine untergeordnete Rolle spielt. Zusammen mit jüngsten Veröffentlichungen hebt diese Arbeit die Bedeutung von *Nitrotoga* in technisierten Habitaten hervor und erweitert die Erkenntnisse verglichen mit anderen NOB-Gattungen.

LIST OF PUBLICATIONS

Simone Wegen, Boris Nowka, Eva Spieck. Low Temperature and Neutral pH Define *Candidatus* Nitrotoga BS as a Competitive Nitrite-Oxidizer in Co-Culture with *Nitrospira defluvii*. (in preparation)

Contribution: SW designed research, conducted physiological tests with *Nitrotoga* BS and co-cultivation experiments, and performed data analysis; BN contributed nitrite oxidation kinetics. SW and ES drafted the manuscript.

Jennifer Hüpeden, Simone Wegen, Sandra Off, Sebastian Lücker, Yvonne Bedarf, Holger Daims, Carsten Kühn, Eva Spieck (2016). Relative Abundance of *Nitrotoga* in a Biofilter of a Cold-Freshwater Aquaculture Plant Appears To Be Stimulated by Slightly Acidic pH. *Applied and Environmental Microbiology.* 82, 1838–1845. doi 10.1128/AEM.03163-15

Contribution: SW designed and performed experiments with *Nitrotoga* sp. HW29, maintained the culture, and assisted in proof-reading and editing of the manuscript.

Simone Wegen, Jennifer Hüpeden, Carsten Kühn, Gregor Schmidt, Eva Spieck (2014). Comparing the nitrifying communities of two different freshwater recirculating aquaculture systems connected by the water flow. Poster presentation, Microbiology and Infection – 4th Joint Congress of DGHM and VAAM, October 2014

Simone Wegen, Sebastian Lücker, Carsten Kühn, Eva Spieck (2015). *Nitrotoga* - a main nitrite oxidizer in low temperature environments. Poster presentation, 6th Congress of European Microbiologists, June 2015

Simone Wegen, Eva Spieck (2017). Circum-neutral pH and low temperature define *Candidatus* Nitrotoga spp. as competitive nitrite oxidizer. Poster presentation, Microbiology and Infection 2017 – 5th Joint Conference of DGHM & VAAM, March 2017

Simone Wegen, Boris Nowka, Eva Spieck (2017). *Candidatus* Nitrotoga spp. competes with *Nitrospira defluvii* at low temperature in bioreactor experiments. Poster presentation, 5th International Conference on Nitrification and Related Processes (ICoN5), July 2017

ABBREVIATIONS

%	percentage
°C	degree Celsius
ΔG_0`	Gibbs free energy
Σ	total
AMO / *amoA*	ammonia monooxygenase / gene encoding the alpha subunit of AMO
Anammox	anaerobic ammonium oxidation
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
AOM	ammonia-oxidizing microbes
Aqua dist	distilled water
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
bp	base pairs
Comammox	complete ammonia oxidation (to nitrate)
Cy3/Cy5	cyanine dyes
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethyl sulfoxide
dO	dissolved oxygen
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia*, for example
EPS	extracellular polymeric substances
et al.	*et alii*, and others
EtOH	ethanol
F/R	forward/reverse orientation of primers
FA	formamide
FISH	fluorescence in-situ hybridization
FITC	fluorescein isothiocyanate
H_2O_2	hydrogen peroxide
h	hour(s)
HAO	hydroxylamine oxidoreductase
HDPE	high-density poly-ethylene
HGT	horizontal gene transfer
HPLC	high-performance liquid chromatography
ICM	intracytoplasmic membrane(s)
in-situ	on site
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilo bases
K_m	mean saturation constant for activity [mM nitrite]
L	liter
LAXI	medium containing LB, Ampicilin, X-Gal, and IPTG
LB	lysogeny broth
M	molar [mol/L]

min	minute(s)
mol	amount of substance
N	nitrogen
NAR	nitrate reductase
Nb.	*Nitrobacter* sp.
NCBI	National Center for Biotechnology Information
NH_3-N	ammonia derived nitrogen
NOB	nitrite-oxidizing bacteria
NirBC	assimilatory nitrite reductase
Nso.	*Nitrosomonas* sp.
Nsp.	*Nitrospira* sp.
NTC	non-template control
Ntg.	*Nitrotoga* sp.
NXR / *nxrB*	nitrite oxidoreductase / gene encoding the beta subunit of NXR
OPA	ortho-phtaldialdehyde
OTU(s)	operational taxonomic unit(s)
PAA	peracetic acid
PCR-H_2O	sterile distilled water treated with Diethyl pyrocarbonate for molecular biological methods
PBS	phosphate-buffered saline
pc	piece(s)
PCR / qPCR	polymerase chain reaction / quantitative PCR
pers. comm.	personal communication
PFA	para-formaldehyde
RAS	recirculation aquaculture system
RFU	relative fluorescence unit
RNA	ribonucleic acid
SD	standard deviation
sec	second(s)
SEM	scanning electron microscopy
SER	selenate reductase
sp.	species
t	tons
TAE	tris-acetate-EDTA buffer
TAN	total ammonia nitrogen
TEM	transmission electron microscopy
TNN	total nitrite nitrogen
Tris	tris(hydroxymethyl)aminomethane
rpm	revolutions per minute
U	enzyme unit
UP-H_2O	ultra-pure water
v/v	volume to volume
V_{max}	mean maximum specific activity [mM nitrite/mg protein · h]
w/v	weight to volume
WWTP(s)	wastewater treatment plant(s)
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CONTENTS

1 INTRODUCTION

1.1 THE NITROGEN CIRCLE AND NITRIFICATION

Nitrogen is among the most essential elements on Earth. It constitutes the majority of the atmosphere as N_2 gas and builds up biochemical molecules together with carbon, oxygen, and hydrogen, usually in its reduced form as an amino group (R-NH_2). Nitrogen conversion in the environment is best described as a ramified circle encompassing various biogeochemical reactions (Figure 1) that allow the fixation of otherwise inert N_2 into biomass and its release back into the atmosphere through decay of organic matter. Most steps in this circle are exclusively performed by microorganisms that use nitrogen compounds as electron donors or acceptors.

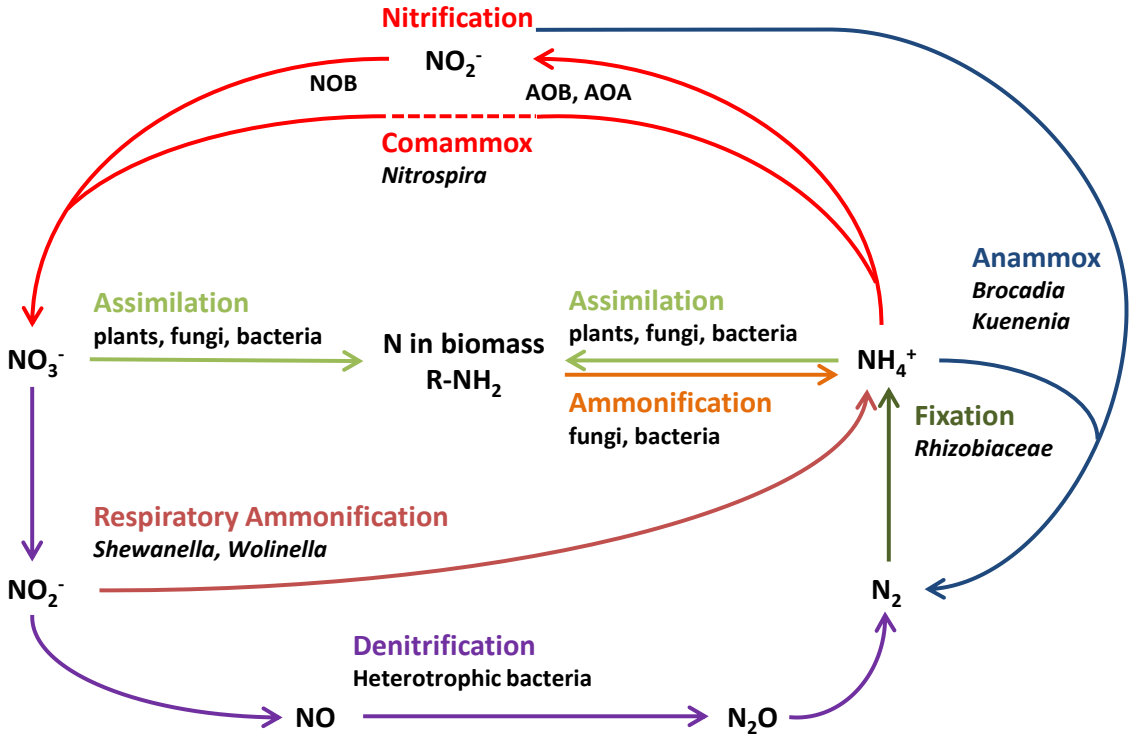

Figure 1: Schematic nitrogen cycle with crucial reactions and participating organisms (exemplary). Anammox = anaerobic ammonium oxidation; AOB/AOA = ammonia-oxidizing bacteria/archaea; Comammox = complete ammonia oxidation (via nitrite to nitrate); NOB = nitrite-oxidizing bacteria. The dashed red line at Comammox indicates oxidation of ammonia via nitrite to nitrate in one organism. To simplify matters, ammonia is shown in its ionized form (ammonium).

Although most nitrogen states are immediately processed further, accumulation of single compounds is possible and often causes severe problems where it occurs. Especially

ammonia (NH_3) and nitrite (NO_2^-) are toxins that impair in particular aquatic animals at relatively low concentrations.[1] Although less harmful, elevated nitrate levels (NO_3^-) damage aquatic life as well.[2] Furthermore, nitrite and nitrate are threatening to human health.[3,4] Nitrogen oxide and dioxide (NO, NO_2) promote acidification of aquatic ecosystems, and a general increase of N fluxes into water bodies facilitates eutrophication.[5] Last but not least, nitrous oxide (N_2O) is a potent greenhouse gas much more severe than CO_2.[6] Human activity by e.g. fertilization is connected to an escalating release of nitrogen into the environment.[7,8] Thus, balancing the nitrogen circle poses an important challenge to secure life on Earth by avoiding pollution of soil and water ecosystems and by limiting global warming.

As part of the nitrogen cycle (Figure 1), nitrification comprises the oxidation of ammonia (NH_3) to nitrite (NO_2^-) and further to nitrate (NO_3^-). It is a crucial process to prevent accumulation of the two first mentioned highly toxic N compounds by their conversion into a less harmful state. Nitrification is widespread in nature. As an oxygen dependent reaction, it occurs in aerated soils, sediments, limnic and marine aquatic environments above and at the transition to the anoxic zone.[9] Furthermore, it is of high importance in technical applications connected to water purification. Consequently, it is one of the most frequently studied microbial conversion in environmental engineering.[10]

1.2 NITRIFYING MICROORGANISM

Nitrification is carried out by distinct groups of microorganisms. Ammonia-oxidizing bacteria (AOB) or archaea (AOA) perform the first step, the oxidation of ammonia to nitrite. The second oxidation step to nitrate is achieved by nitrite-oxidizing bacteria (NOB). Furthermore, bacteria performing complete nitrification from ammonia to nitrate via nitrite (Comammox) in one organism were recently discovered. Since nitrification is oxygen-dependent, the involved microorganisms need oxygen as well. Additionally, bacteria performing anaerobic ammonium oxidation (Anammox) branch off nitrite from the nitrification process to generate N_2 by oxidizing ammonium. All these microbes are chemolithoautotrophs and utilize the energy derived from oxidation of ammonia or nitrite for CO_2 fixation to build up complex organic compounds. Initial discovery and description of AOB and NOB date back to the end of the 19th century, when Winogradsky

first enriched and cultivated these microbes.[11,12] Based on their common feature as nitrifying bacteria they were at first classified as one family, the *Nitrobacteriaceae*.[13,14] However, with the development and arising possibilities of sequence-based techniques,[15,16] it became apparent that nitrifying bacteria are not monophyletic but rather widely distributed over different bacterial phyla and classes (Figure 2). Recent discoveries of further chemolithoautotrophic microbes affiliated with nitrification enhance complexity of these fastidious organisms.

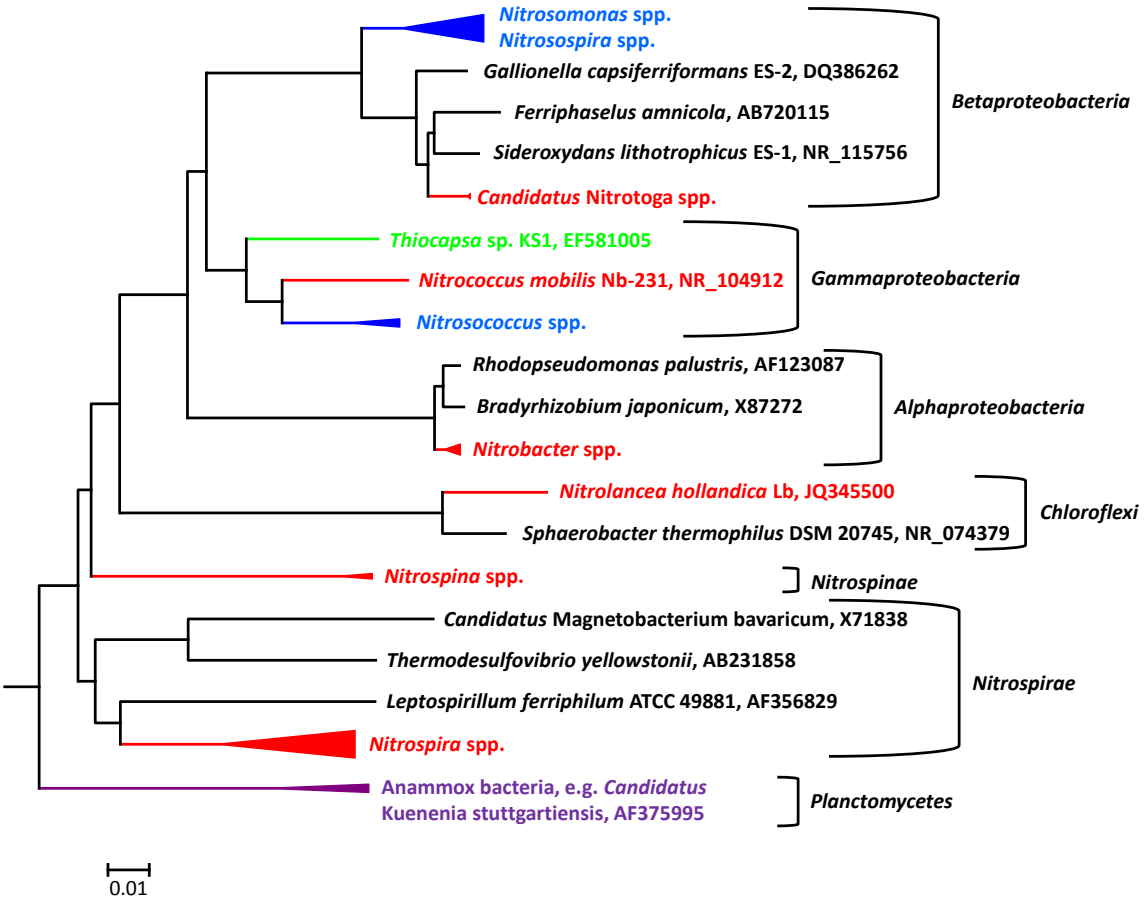

Figure 2: Phylogenetic tree illustrating the affiliation of to date known nitrifying bacteria based on their 16S rRNA gene sequence compared with further bacteria. NOB (red); AOB (blue); phototrophic nitrite oxidizer (green); Anammox bacteria (purple); AOA were used as outgroup (not shown). Brackets link phyla or classes. Scale bar indicates 1% sequence divergence.

1.2.1 Ammonia-Oxidizing Bacteria and Archaea (AOB/AOA)

Most AOB are members of the *Betaproteobacteria* and form a monophyletic group based on 16S rRNA and *amoA* gene sequences.[17–20] *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus mobilis* cluster into six lineages[21] with subdivisions suggested for

Nitrosospira.[20,22] Only two AOB species of the marine genus *Nitrosococcus* belong to the *Gammaproteobacteria*.[23–25]

AOB colonize soils,[18,26] limnic and marine aquatic habitats,[18,27–29] as well as technical applications related to sewage treatment like WWTPs[30–32] and RAS.[33–35] Ammonia oxidation is split in two parts: hydroxylamine (NH_2OH) is produced as an intermediate by the membrane associated ammonia monooxygenase (AMO) and further oxidized to nitric acid by the periplasmic hydroxylamine oxidoreductase (HAO). Thus, surroundings of AOB are subject to acidification

$NH_3 + O_2 + 2\ H^+ + 2e^-$	$\rightarrow NH_2OH + H_2O$		(AMO)
$NH_2OH + ½\ O_2$	$\rightarrow HNO_2 + 2\ H^+ + 2e^-$		(HAO)
$NH_3 + 1½\ O_2$	$\rightarrow HNO_2 + H_2O$	$\Delta\ G_0` = -275$ kJ/mol	($\Sigma 1$)

The last step generates two electrons that are channeled into the respiratory chain.[36,37] Some AOB possess urease that enables the use of urea (CH_4N_2O) as source for ammonia and CO_2[38–40] especially in acidic environments.[41,42] Based on the low energy gain ($\Delta\ G_0`$) from ammonia oxidation (eq. $\Sigma 1$), AOB have a slow growth rate with doubling times about half a day and more.[43]

Genes and enzymes for partial denitrification were found in most AOB[44,45] which allow them to perform nitrifier-denitrification, an incomplete denitrification with N_2O as the end product.[46–48] Although this process is carried out under aerobic conditions in contrast to heterotrophic denitrification, it is enhanced at low oxygen levels[46,48] and even allows cell growth.[49,50]

Discovery of AOA was initiated by identification of *amo* genes on archaeal genome fragments,[51] and first isolates were classified as *Crenarchaeota*.[52] Meanwhile, their existence lead to the assignment of the new phylum *Thaumarchaeota*,[53] and five phylogenetic AOA clusters were identified based on *amoA* gene sequences so far.[54] They are considered to be the globally most abundant archaea and colonize natural as well as engineered habitats where they can constitute the majority of ammonia oxidizers.[33,55–58] In some marine environments they are the only AOM and interact with *Nitrospina*-like NOB to complete nitrification.[59] AOA produce N_2O probably similar to nitrifier-

denitrification in AOB[60] which can ensure their survival at the oxicline. Consequently, they might be the main source for N_2O production in the ocean.[61]

1.2.2 ANAMMOX BACTERIA

In contrast to classical nitrifiers, bacteria capable of Anammox thrive under anoxic conditions and so far cluster exclusively into the *Planctomycetes* phylum. They were initially discussed based on theoretical thermodynamic calculations,[62] and first hints of their occurrence arose in denitrifying reactors.[63] These microbes use nitrite as electron acceptor to oxidize ammonium under formation of N_2 gas.[64,65] The generated energy is used for carbon fixation.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O \qquad \Delta G_0` = -357\ kJ/mol$$

Nitrogen is directly released into the atmosphere in an alternate way compared to nitrification and denitrification. Consequently, Anammox bacteria gained importance in WWTPs, since the especially toxic compounds nitrite and ammonia are directly removed without releasing harmful intermediates, and loss of fixed nitrogen is promoted. They are distributed globally in freshwater and marine ecosystems,[66,67] in WWTPs,[68,69] and RAS.[70] Anammox activity is indicated by nitrate production that is needed as reducing equivalent for CO_2 fixation.[71] Together with NOB, they are the only microbes contributing to the global nitrate pool. It is formed by nitrite oxidation via a nitrite oxidoreductase (NXR), the same enzyme that is crucial for NOB to meet their energy requirements.

1.2.3 NITRITE-OXIDIZING BACTERIA (NOB)

As is the case for AOB, discovery of NOB dates back to Winogradsky.[11,12] They are widely distributed over the phylogenetic tree (Figure 2) and their occurrence in natural and technical habitats is just as versatile.

The genera *Nitrobacter*[11,72] and *Nitrococcus*[73,74] belong to the *Alpha*- and *Gammaproteobacteria*, respectively. *Nitrobacter* species were isolated from soils,[75] freshwater,[75] sewage,[75] as well as acidic,[76] alkaline[77] and marine[78] environments. This genus is the most readily enriched, so it represented the best known NOB for a long time. In contrast, *Nitrobacter* are much less abundant in natural and engineered habitats compared to other NOB. *Nitrococcus mobilis* was isolated from Pacific ocean water,[73] and

further uncultivated members of this genus were found in oxygen minimum zone waters.[79,80] So far, they seem to thrive in marine environments only.

The genera *Nitrospira*[81] and *Nitrospina*[82,83] are assigned to and name givers of two separate bacterial phyla, the *Nitrospirae* and *Nitrospinae*. Based on 16S rRNA gene analysis, at least six distinct phylogenetic lineages can be assigned within the genus *Nitrospira*,[84] with an increasing number of sequences missing certain classification. Lineage I is represented by *Candidatus* Nitrospira defluvii[85,86] and lineage II by *Nitrospira lenta* BS10,[86] *Nitrospira japonica*,[87] and *Nitrospira moscoviensis*.[81] Members of these two lineages are often found in engineered habitats.[84,88] Lineage III lacks isolates so far but consists of cloned sequences e.g. from the Nullarbor cave.[89] Marine *Nitrospira* cluster in lineage IV with *Nitrospira marina*[90] and a sponge associated enrichment[91] as cultured representatives. Lineages V and VI contain isolates of *Candidatus* Nitrospira bockiana[92] and *Nitrospira calida*,[93] respectively. *Nitrospira* are considered to be the most abundant NOB in divers habitats including WWTPs,[30,94,95] RAS,[96] and other engineered systems. Additionally, they colonize diverse soils,[97] limnic and marine environments,[97] and moderately thermophilic NOB are also found among them.[93,98] *Nitrospina*-like NOB cluster into three clades, including the newly suggested *Candidatus* Nitromaritima that differs significantly from *Nitrospina gracilis*, the type strain of this genus.[99] So far, they were isolated and detected in marine habitats only.[59,73,80,83]

The just recently described *Nitrolancea hollandica* belongs to the phylum *Chloroflexi* and was isolated from a nitrifying bioreactor. It is thermotolerant and the only Gram-positive NOB to date.[100,101]

In addition, anoxic photosynthetic microorganisms that utilize reduced nitrogen compounds as electron donor were predicted.[62,102] Two such species were recently isolated from activated sludge and identified as *Rhodopseudomonas* (*Alphaproteobacteria*) and *Thiocapsa* (*Gammaproteobacteria*).[103,104] They are, however, not part of the oxygen-dependent nitrifying bacteria described above.

NOB obtain their energy by oxidizing nitrite to nitrate. The key enzyme mediating this reaction, nitrite oxidoreductase (NXR), is bound to the cytoplasmic membrane and consists of three subunits. The location of the substrate-binding unit NxrA and the electron-channeling unit NxrB differs between NOB. For *Nitrobacter*,[105,106] *Nitrococcus*,[105]

and *Nitrolancea*,[100] it faces the cytoplasm. The NXR of these genera is closely related to enzymes for respiratory nitrate reduction (NAR), especially from *E. coli* and other denitrifying bacteria. For *Nitrospira*[107,108] and *Nitrospina*[82] it is directed into the periplasmic space. Their NXR cluster together with that of *Candidatus* Kuenenia stuttgartiensis, an Anammox bacteria.[99,100,108] Although both types evolved independently, they belong to the type II group of dimethyl sulfoxide (DMSO) reductase-like molybdopterin-binding enzymes.[82,100,108,109] Orientation of the NXR might determine the affinity and sensitivity of an NOB to nitrite.[97,110] The actual nitrite oxidation is oxygen-independent as the oxygen atom for nitrate is obtained from water (eq. 1). The two released electrons are then transferred to oxygen under water formation (eq. 2).

$NO_2^- + H_2O$	$\rightarrow NO_3^- + 2\ H^+ + 2e^-$	$\Delta\ G_0` = +83$ kJ/mol	(1)
$½\ O_2 + 2\ H^+ + 2e^-$	$\rightarrow H_2O$	$\Delta\ G_0` = -157$ kJ/ mol	(2)
$NO_2^- + 1½\ O_2$	$\rightarrow NO_3^-$	$\Delta\ G_0` = -74$ kJ/mol	($\Sigma 2$)

Nitrite oxidation generates two electrons that are channeled into the respiratory chain. Since the energy gain of the overall reaction is low (eq. $\Sigma 2$), growth rates of NOB are even below those of AOB with hours to several days of doubling time.[75,110] Similar to *amoA* genes in AOA[54] or AOB,[20] *nxr* sequences can be used as phylogenetic marker for NOB in addition to 16S rRNA gene analysis.[111,112]

Apart from nitrite oxidation, NOB exhibit several alternative metabolisms and interactions with other microbes. Addition of simple organic compounds can support *Nitrobacter* and *Nitrospira*,[75,84,90] and exclusively heterotrophic growth is possible for some *Nitrobacter*.[113] Since NOB as well as AOB fix CO_2, they count as primary producers and support heterotrophs with organic matter.[114–116] In return, NOB profit from heterotrophic bacteria probably by taking up released cofactors or by lesser toxicity due to their nitrate reducing activity.[83] The extent of this interaction is still unknown but complicates isolation of NOB, as enrichments often grow better on plates or in liquid culture than pure cultures.[86]

Similar to certain AOB, some NOB possess urease. In an overall process called reciprocal feeding they can provide ammonia to urease-negative AOB in ammonia-deprived surrounding and in return get supplied with nitrite by the AOB.[117] Reciprocal feeding based on cyanate ($[OCN]^-$) was demonstrated as well,[118] since NOB are cyanase-positive and convert it to NH_3 and CO_2.[82,106,108]

Genes encoding hydrogenases were found in *Nitrospina gracilis*,[82] *Nitrolancea hollandica*,[100] and *Nitrospira moscoviensis.*[119] Growth and CO_2 fixation of the latter on H_2 and O_2 as sole electron donor and acceptor was confirmed[119] which indicates the Knallgas metabolism as an alternate autotrophic lifestyle for NOB. Their versatility is complemented by denitrification under anaerobic conditions, similar to nitrifier-denitrification in AOB, as was demonstrated for *Nitrobacter*,[120–122] where the nitrate reduction step to nitrite is probably performed by the NXR.[78,123]

Among the versatile *Nitrospira*, the first Comammox bacteria were discovered that perform complete ammonia oxidation to nitrate via nitrite.[124,125] Their occurrence was predicted based on thermodynamic considerations, since the higher energy gain gives a competitive advantages compared to microorganisms performing either ammonia or nitrite oxidation.[126]

$$NH_3 + 2\ O_2 \rightarrow NO_3^- + H_2O + H^+ \qquad \Delta G_0` = -349\ kJ/mol$$

Comammox *Nitrospira* cluster into *Nitrospira*-lineage II, based on 16S rRNA as well as *nxr* gene sequences. Their AMO, however, is affiliated with an unusual particulate methane monooxygenases (PMO)[124,125] originating from *Crenothrix polyspora*.[127] Thus, Comammox microbes might be mistakenly assigned as methanotrophes based on their *amo* sequence, since both enzymes are closely related.[127,128]

1.3 *CANDIDATUS* NITROTOGA

The cold-adapted *Candidatus* Nitrotoga arctica was enriched from permafrost-affected soils of the Siberian Arctic and first described by Alawi *et al.* in 2007.[129] Its 16S rRNA gene sequence (GenBank accession nr. DQ839562) clusters together with uncultivated environmental samples and *Gallionella ferruginea* as next taxonomically described relative. Thus, *Nitrotoga* is to date the only NOB assigned to the *Betaproteobacteria* (Figure 2). The particulate and wide periplasmic space is its most striking feature and name-giver of this candidate genus.[129]

A further enrichment originates from activated sludge of the WWTP in Hamburg-Dradenau. Again, it was separated from other NOB by incubation at lower temperatures of 10°C and 17°C. Its 16S rRNA gene sequence (*Nitrotoga* HAM-1, GenBank accession nr.

FJ263061) is 99.5% identical to that of *Ntg. arctica*, and it also possesses the characteristic wide periplasm.[130]

Two further *Nitrotoga* enrichments were recently described. *Nitrotoga* HW29 was sampled from biofilm material of a biofilter from a cold-freshwater RAS in Germany. Successful separation from *Nitrospira* was achieved at colder temperatures and by cultivation in acidic medium.[34] *Nitrotoga* AM1 originates from coastal sand in an eelgrass zone in Japan. Its optimum temperature was 16°C with nitrite oxidation up to 29°C. Its 16S rRNA gene is 99.6% identical to that of *Ntg. arctica*.[131]

Similar to *Nitrospira*, *Nitrotoga* are slow growing NOB with generation times between 44 and 54 h (*Ntg. arctica* and *Nitrotoga* AM1, respectively).[110,131] Little is known about their physiology due to the restricted number of enrichments in contrast to *Nitrobacter* or *Nitrospira*. The most striking difference is its adaption to lower temperature, whereas most NOB have growth optima in the mesophilic range.

Since its discovery, a great number of habitats turned out to be colonized by *Nitrotoga*. A screening of activated sludge from 13 WWTPs in Denmark based on 16S rRNA gene amplicon sequencing revealed that the NOB community was dominated by *Nitrotoga* in a few cases.[132] Lücker *et al.*[88] obtained similar results for 20 WWTPs, mainly located in Germany, investigated by fluorescence *in-situ* hybridization (FISH), and they reported that two plants were inhabited exclusively by *Nitrotoga*-like NOB. In both cases, temperatures were below 20°C. The actual *in-situ* nitrifying activity of *Nitrotoga* coupled to CO_2 fixation was demonstrated by incubating activated sludge.[88] These results challenge the assumption that *Nitrospira* is the most dominant NOB in WWTPs, and they broaden the possibilities for nitrification in technical applications at colder temperatures. Apart from WWTPs, *Nitrotoga*-like NOB were also detected in nitrogen-removing reactors for inorganic mine water at lower temperatures,[133] in a brackish RAS though they were not functionally relevant there,[134] and in a cold-freshwater RAS.[34,135] Furthermore, they were abundant in flow reactors located in the Äspö Hard Rock Laboratory below the Baltic sea,[136,137] inhabited freshly developed biofilms on reverse osmosis membranes fed with treated freshwater,[138] and colonized active filters for the treatment of drinking water.[139] Natural habitats with *Nitrotoga*-like NOB comprise cave systems like the Movile Cave in

Romania,[140] but also the subglacial lake Whillans in West Antarctica,[141,142] periglacial soils in Peru,[143] and the tidal reach of the Yangtze River, China.[144]

1.4 APPLIED NITRIFICATION

1.4.1 WASTEWATER TREATMENT PLANTS (WWTPS)

Wastewater treatment plants (WWTPs) combine nitrification and denitrification to clean sewage of N-loads before releasing it as drinking water into the environment. Different set-ups can be applied to ensure biological N-removal. Most WWTPs rely on aerated, activated sludge that ensures oxygen input for nitrification. Anoxic denitrification to generate N_2 gas is subsequently or intermittently performed. The need for organic carbon in the latter step is met by either adding simple organic compounds or untreated wastewater.[145–147]

Members of the genera *Nitrosomonas* and *Nitrospira* lineage I and II are the most common detected AOB and NOB in WWTPs, respectively.[30,84,94,95,101] Recently, *Candidatus* Nitrotoga was shown to be of considerate importance for nitrite oxidation in some WWTPs as well.[88,132] In contrast, members of the genus *Nitrobacter* are mostly of no relevance in these processes.[30] Apart from AOB and NOB, bacteria capable of Anammox open up an alternative way for N-removal at lower costs than traditional activated sludge processes.[69,148] Since nitrifying microbes are slow growing and react strongly to a change of environmental factors like pH, temperature, dissolved oxygen (dO), or inhibiting substances, they are easily washed out of processes. This often causes nitrification to be instable or fail.[149] NOB prove more sensitive than AOB, resulting in an accumulation of nitrite to toxic levels.[150,151]

1.4.2 RECIRCULATING AQUACULTURE SYSTEMS (RAS)

Construction of recirculating aquaculture systems (RAS) is gaining more and more importance to counter overfishing and to prevent interactions of fish rearing with the environment.[152,153] Excess feed and fish feces heavily load the rearing water with ammonium (ionized ammonia, NH_4^+) and free ammonia (NH_3), united as total ammonia nitrogen (TAN). To prevent loss in cultivated organisms due to TAN toxicity, to comply with environmental standards, and to decrease costs by re-using the process water,

biofiltration units are installed for water treatment[152,154] that employ a similar pattern as WWTPs. They are mostly operated as fixed, trickling, or moving-bed reactors. The latter contain carrier elements on which a biofilm with nitrifying and heterotrophic bacteria develops. To remove nitrate, an anoxic process can be added for denitrification. Since the content of organic carbon in the process water is low, external carbon needs to be supplied for a stable denitrifying reaction.[155]

Again, members of *Nitrosomonas* and *Nitrospira* are the most commonly detected AOB and NOB in RAS biofiltration,[156,157] but AOA and Comammox *Nitrospira*[158] were reported as well as, occasionally, members of the genus *Nitrobacter*.[159] Biofilters of marine RAS differ from their freshwater counterparts with regard to the nitrifying community. They contain AOA more often[33,160] and are in general inhabited by marine or salt-dependent AOB and NOB like *Nitrosococcus mobilis* and *Nsp. marina*.[35,96,134,161] As is the case for WWTPs, Anammox bacteria[70,156] as well as autotrophic or heterotrophic denitrifyers[157,162] were detected that enhance the potential for complete N-removal in RAS biofilters.

1.5 NICHE DIFFERENTIATION AMONG NOB

Niche differentiation or separation describes the approach of multiple species to coexist in a given habitat. If two species compete for the same niche, one will inevitably be driven out.[163] Knowledge about preferences of NOB for certain environmental conditions are of importance especially in the context of process stability in WWTPs or RAS.

A key factor for success of NOB is their affinity for nitrite and their maximum nitrite oxidation activity which can be linked to the growth rate. Kinetic measurements of different non-marine NOB led to their differentiation as *K*- or *r*-strategists.[164,165] *K*-strategists like *Nitrospira* express a high affinity for nitrite that allows them to grow under substrate limitation. These conditions are widespread in WWTPS or RAS. However, their maximum activity is low compared to *Nitrobacter*[110,165] or *Nitrolancea*. These *r*-strategists compensate their weak substrate affinity by higher maximum activities that enable fast growth at elevated or excess substrate concentrations.[99,109, 163,164] This explains the convenient enrichment of *Nitrobacter* in laboratory cultures at elevated nitrite levels, and why they are rarely reported in engineered habitats related to water purification. Nevertheless, several species coexist dependent on different substrate affinity even

within the *Nitrospira* genus, because sublineage I *Nitrospira* prefer higher nitrite contents than members of sublineage II.[168]

The adaption to low levels of nitrite in *Nitrospira* is coupled to a preference of microaerophilic growth.[95,169] They also show higher affinities for oxygen than *Nitrobacter*,[170] and genomic studies revealed the absence of common genes encoding for enzymes that ensure protection against reactive oxygen species in *Nsp. defluvii*.[108] Similar to the influence of nitrite, community shifts within a *Nitrospira* population were observed based on favorable dO concentrations.[171]

NOB differ in terms of optimum growth pH and temperatures that consequently influence niche separation. It was shown that the nitrifying community in WWTPs changes with seasonal temperature shifts without a loss of function.[32] Nitrification might occur from acidic to alkaline conditions, ranging from adaption to acidic pH in biofilms[172] to the isolation of an alkaliphilic *Nitrobacter*.[77] Nevertheless, nitrification in wastewater treatment proved most successful at mesophilic temperatures about 30°C and slightly alkaline conditions about pH 8.[173] With the observed coexistence of *Nitrotoga* and *Nitrospira* in WWTPs[88,132] and a cold-freshwater RAS[34] it is of interest to know what niches *Nitrotoga* can occupy without being suppressed by *Nitrospira*.

Based on two measured enrichments, *Nitrotoga* express a medium to high affinity for nitrite in the range of *Nitrobacter vulgaris* and *Nitrospira lenta*.[110,131] Further *Nitrobacter* species show much lesser affinities, whereas *Nitrospira* species are better adapted to low substrate concentrations.[110] Nevertheless, *Nitrotoga* was successfully enriched at lower nitrite contents.[129,130] The slow growth rate of *Nitrotoga* combined with their maximum activity comparable to *Nitrospira* species indicate that *Nitrotoga* is a *K*-strategist.[110] It can consequently colonize habitats with low nitrite levels, similar to *Nitrospira*.

The most notable difference between *Nitrotoga* compared to other NOB is their adaption to temperatures below 20°C. Thus, they might successfully occupy niches with this condition. This assumption is supported by habitats at which *Nitrotoga* was detected so far and was also demonstrated by Alawi and colleagues.[130] *Nitrotoga* is relevant in operating WWTPs with temperatures below 20°C as was revealed in screenings by Saunders *et al.*[132] and Lücker *et al.*[88] recently.

The influence of pH on the niche differentiation of *Nitrotoga* was so far not researched in depth. The successful separation of *Nitrotoga* HW29 from *Nitrospira*[34] and the enrichment of *Ntg. arctica* from acidic permafrost soil[129,174] actually indicate a positive effect of lower pH on the competitiveness of *Nitrotoga*.

1.6 AIM OF THIS STUDY AND OVERVIEW

Since only a few enrichments were characterized before, and above mentioned *in-situ* data are by default site-specific, the overall knowledge about this NOB genus is fragmentary. As important as *in-situ* analysis are to evaluate the versatility of bacterial communities in a habitat, highly enriched or even pure cultures of microorganisms are equally crucial to understand and assess their characteristics and behavior under changing external conditions. Therefore, this thesis focused on laboratory physiological experiments with NOB enrichment cultures based on their nitrite oxidizing ability, as it is directly linked to cell growth.[110,175]

To close the knowledge gap concerning *Nitrotoga*, one aspect of this thesis was the characterization and comparison of different *Nitrotoga* enrichments in hindsight to key physiological parameters, their phylogeny, and their morphology. Physiological experiments comprised the determination of temperature and pH optima, the influence of ammonium, nitrite, and nitrate, as well as inhibition by hydrogen peroxide (H_2O_2). Their phylogenetic relationship was analyzed using 16S rRNA and *nxrB* gene sequences. Each culture was furthermore examined by transmission and scanning electron microscopy to specify the appearance of this NOB and verify former results.

Furthermore, it is still unclear which conditions, apart from low temperature, favor *Nitrotoga* over other NOB. Thus, their competitiveness was investigated when faced with *Nitrospira*. *Nitrotoga* BS was co-cultured with *Nsp. defluvii* under changing pH at low temperature. This combination was of special interest, since both NOB were isolated from the same WWTP. For comparison, *Ntg. arctica* was also subjected to competition experiments with *Nsp. defluvii*. To determine NOB contents, qPCR had to be implemented for *Nitrotoga* and *Nsp. defluvii*.

In the third part of this thesis, a RAS was surveilled over two years with emphasis on the nitrifying potential and the nitrifying community in the biofiltration unit. The facility was

reconstructed and restarted in November 2013, and its monitoring was of eminent interest for process stability. *Nitrotoga* was detected in the biofilters before[34,135] and after reconstruction (this study), therefore this survey can help to broaden the knowledge concerning *Nitrotoga* in wastewater treatment.

2 MATERIAL AND METHODS

2.1 CULTURES AND CULTIVATION CONDITIONS

2.1.1 CULTURES

The following NOB were investigated in this thesis (Table 1). All *Nitrotoga* cultures are enrichments, *Nsp. defluvii* is a pure culture.

Table 1: Investigated NOB and basic information. Underlined names are used throughout this thesis.

Culture	Isolation Site	Cultivation Temperature
Candidatus Nitrotoga arctica 6680[129,176]	Sample 6680 from permafrost soil, active layer, 0 - 5 cm depths, Samoylov Island, Russia (N 72°22', E 126°28')	17°C
Nitrotoga BS = subculture of *Nitrotoga* HAM-1[130]	Activated sludge, WWTP Hamburg-Dradenau, Germany	17°C
Nitrotoga HW = *Nitrotoga* HW29[34]	Cold-freshwater RAS, Mecklenburg-West Pomerania, Germany	17°C
Nitrotoga 1052[176]	Sample 1052 from permafrost soil, bore core 90 cm depth, Kurungnakh Island, Russia (N 72°20', E 126°17')	22°C
Candidatus Nitrospira defluvii A17[85,86]	Activated sludge, WWTP Hamburg-Dradenau, Germany	28°C

2.1.2 GENERAL CULTIVATION PROCEDURES AND STANDARD MEDIUM COMPOSITION

Cultivation was performed in 150 ml autotrophic NOB medium in 300 ml Erlenmeyer flasks. Cultures were incubated at their standard cultivation temperature (Table 1) in the dark without shaking. To obtain cell cultures of high density, about 5 mM nitrite had to be consumed. It was stepwise supplied after complete consumption of previously added nitrite to avoid inhibition. Fresh cultures were inoculated with 1% preculture in 0.3 mM nitrite as initial starting concentration. If needed, ammonium was added to promote growth.

Autotrophic NOB medium (Table 2) was prepared after Bock[177] and supplied with trace elements by Widdel and Bak.[178] The pH was adjusted to 7.4 prior to autoclaving. Ultra-pure water (UP-H_2O) was generated with a Purelab flex by ELGA LabWater (Veolia Water Technologies GmbH, Celle, Germany).

Table 2: Autotrophic NOB medium.

NOB medium, 0.3 mM nitrite	**Σ 1 L**
10x NOB stock solution (A)	100 ml
Trace elements (B)	1 ml
2 M nitrite stock solution (C)	0.15 ml
UP-H2O	900 ml

(C) 2 M nitrite stock solution	**Σ 50 ml**
$NaNO_2$	6.9 g
UP-H_2O	50 ml

(A) 10x NOB stock solution	**Σ 1 L**
$CaCO_3$	0.07 g
NaCl	5.00 g
$MgSO_4$ x 7 H_2O	0.50 g
KH_2PO_4	1.50 g
UP-H_2O	1000 ml

(B) Trace elements	**Σ 1 L**
$FeSO_4$ x 7 H_2O	1.050 g
H_3BO_3	0.015 g
$MnCl_2$ x 4 H_2O	0.050 g
$CoCl_2$ x 6 H_2O	0.080 g
$NiCl_2$ x 6 H_2O	0.012 g
$CuCl_2$ x 2 H_2O	0.001 g
$ZnSO_4$ x 7 H_2O	0.072 g
Na_2MoO_4 x 2 H_2O	0.018 g
Na_2-EDTA	3.600 g
UP-H_2O	1000 ml
Adjust pH to 6 to dissolve components	

Autotrophic AOB medium (Table 3) was prepared after Krümmel and Harms.[179] Adjusting the pH was not necessary since the medium was buffered with $CaCO_3$.

Table 3: Autotrophic AOB medium.

AOB medium, 0.5 mM nitrite	**Σ 1 L**
10x AOB stock solution (A)	100 ml
Trace elements AOB (B)	1 ml
2 M ammonium stock solution (C)	0.25 ml
$CaCO_3$	5 g
UP-H_2O	900 ml

(C) 2 M ammonium stock solution	**Σ 50 ml**
NH_4Cl	5.35 g
UP-H_2O	50 ml

(A) 10x AOB stock solution	**Σ 1 L**
KH_2PO_4	0.544 g
KCl	0.744 g
$MgSO_4$ x 7 H_2O	0.493 g
NaCl	5.840 g
UP-H_2O	1000 ml

(B) Trace elements AOB	**Σ 1 L**
$MnSO_4$ x 4 H_2O	0.045 g
H_3BO_3	0.049 g
$ZnSO_4$ x 7 H_2O	0.043 g
$(NH_4)6Mo_7O_{24}$ x 4 H_2O	0.037 g
$FeSO_4$ x 7 H_2O	0.973 g
$CuSO_4$ x 5 H_2O	0.025 g
0.01 M HCl in UP-H_2O	1000 ml

Cultures were regularly checked for contamination by heterotrophic microorganisms on solid complex medium modified after Steinmüller and Bock[180] (Table 4). The pH was adjusted to 7.3 before adding agar and autoclaving. Plates were incubated between 17 and 28°C for three weeks.

Table 4: Modified purity agar.

Purity agar, modified	**Σ 1 L**
Agar Agar	15.0 g
NaCl	0.5 g
KH_2PO_4	1.0 g
Meat extract	5.0 g
Casein hydrolysate	5.0 g
Yeast extract	1.0 g
Add Aqua dist	1000 ml

Media were autoclaved at 121°C and 1 bar excess pressure for 20 min and subsequently stored at 4°C.

2.1.3 CELL HARVESTING

Cells were harvested by centrifugation at 10°C and 4,000 rpm for 30 min (Variofuge 3.0R, Heraeus Sepatech GmbH, Osterode, Germany) or 13,000 rpm for 15 min (Centrifuge 5414R, Eppendorf AG, Hamburg, Germany) until a visible pellet formed. The pellet was washed in 0.9% (w/v) NaCl and processed further depending on the following procedures.

2.2 MOLECULAR BIOLOGICAL METHODS

2.2.1 DNA ISOLATION

DNA was isolated from cell pellets with the Ultra Clean Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer's instruction and stored at -20°C for further applications.

2.2.2 POLYMERASE CHAIN REACTION (PCR)

PCR was based on the 16S rRNA gene or the *nxrB* gene. A standard master mix with Dream Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), a general PCR program, and all used primers are given in Table 5, Table 6, and Table 9. BSA (Thermo Fisher Scientific, Waltham, MA, USA) was added to circumvent PCR inhibition. The

reactions were run on a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany).

Table 5: Master mix for PCR with Dream Taq polymerase.

Component	Volume [µl] for 1 reaction of 25 µl	Final concentration
PCR-H_2O	17.75	
10x Dream Taq Green Buffer	2.50	1x
dNTPs (2 mM each)	2.50	0.2 mM each
Primer F (50 pmol/µl)	0.25	0.5 pmol/µl
Primer R (50 pmol/µl)	0.25	0.5 pmol/µl
Dream Taq polymerase (500 U/µl)	0.25	5 U/µl
BSA (20 µg/µl)	0.50	0.4 µg/µl
DNA	1.00	

Table 6: Program for PCR with Dream Taq polymerase.

Step	Temperature [°C]	Time [sec]	Cycles/others
1	95	240	Initial denaturation
2	95	35	Denaturation
3	See Table 9	45/30	Annealing 16S/*nxrB*
4	72	45/30	Elongation 16S/*nxrB* Repeat from step 2 for 30/35 times for 16S/*nxrB*
5	72	480/360	Final elongation 16S/*nxrB*
6	12	∞	Hold

If proof-reading activity was necessary, PCR was performed with Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). PCR reaction and program are listed in Table 7 and Table 8 (manufacturer's recommendation).

Table 7: Master mix for PCR with Phusion High-Fidelity polymerase.

Component	Volume [µl] for 1 reaction of 50 µl	Final concentration
PCR-H_2O	36.5	
Buffer 5x HF	10.0	1x
dNTPs (10 mM each)	1.0	0.2 mM each
Primer F (50 pmol/µl)	0.5	0.5 pmol/µl
Primer R (50 pmol/µl)	0.5	0.5 pmol/µl
Phusion High-Fidelity polymerase (2U/µl)	0.5	0.02 U/µl
DNA	1.0	

Table 8: PCR program for Phusion High-Fidelity polymerase.

Step	Temperature [°C]	Time [sec]	Cycles/others
1	98	30	Initial denaturation
2	98	10	Denaturation
3	See Table 9	30/10/45	Annealing 16S/*nxrB*/Cloning
4	72	30	Elongation Repeat from step 2 for 30/35 times for 16S and cloning/*nxrB*
5	72	300	Final elongation
6	12	∞	Hold

Table 9: List of primers and probes used in this study. Annealing temperature for Dream Taq polymerase; * annealing temperature for Phusion High-Fidelity polymerase; # primers for qPCR; c = unlabeled competitor; FA = optimal formamide concentration for FISH; ~ preparative PCR for Illumina MiSeq and 454 Pyrosequencing was performed elsewhere (MR DNA, Shallowater, TX, USA).

Primer	5'-3' sequence	Annealing temperature [°C]	Target sequence	Fragment size [kb]	References
27F	AGA GTT TGA TCM TGG CTC AG	55, 54* (for both F primers with 1492R)	16S rRNA gene, Bacteria	1.5 (with 1492R)	[181]
517F	CCA GCA GCY GCG GTA AN			1.0 (with 1492 R)	[181]
1492R	TAC GGY TAC CTT GTT ACG ACT T				[181]
515F~	GTG CCA GCM GCC GCG GTA A	Illumina MiSeq and 454 Pyrosequencing~	16S rRNA gene, Bacteria	0.3	[182] [182]
806R~	GGA CTA CHV GGG TWT CTA AT				
Nsp1158R	CCC GTT MTC CTG GGC AGT	58 (with 27F)	16 S rRNA gene, genus *Nitrospira*	1.1 (with 27F)	[168] [176]
Nsp60-kurzF	CGG GTG AGG AAT ACA TGG	64		0.6	[183]
Nsp662R	GGA ATT CCG CGC TCC TCT				
Nsp-nxrB169F	TAC ATG TGG TGG AAC A	56	*nxrB* gene, genus *Nitrospira*	0.5	[111] [111]
Nsp-nxrB638R	CGG TTC TGG TCR ATC A				
Ntg124F	ATC GGA ACG TAC CCG GAA A	65 (for both F primer with Ntg840R)	16S rRNA gene, genus *Nitrotoga*	0.7 (with Ntg840R)	[88] [129] [129]
Ntg200F	CTC GCG TTT TCG GAG CGG			0.6 (with Ntg840R)	
Ntg840R	CTA AGG AAG TCT CCT CCC				
Ntg-nxrBF	GAA ACS ATA TTC TGG AAT	56, 51*	*nxrB* gene, genus *Nitrotoga*	0.4	Lücker, unpublished Lücker, unpublished
Ntg-nxrBR	CGG GAC GCA TCA ATC A				
Nit3R	CCT GTG CTC CAT GCT CCG	60 (with 27F)	16S rRNA gene, genus *Nitrobacter*	1.0 (with 27F)	[184] from FISH probe NIT3 [185] from FISH probe Nb1000 and modified[176] [186] and modified[176]
Nb1000gF	CCA TGA CCG GTC GCA G	58		0.4	
Deg2R	GGT TTT TTG AGA TTT GCT AGG GG				
Ntg8-2F	TAA GCC CGG GGA TTT CAC AT	59#	qPCR for *Nitrotoga*	0.16	This study
Ntg8-2R	ATA CCC TGT GTG GAT GAC GG				
Nsp15F	ATT GCT ACC TCG TCA GGC TT	59#	qPCR for *Nsp. defluvii*	0.17	This study
Nsp15R	CGC ATT AAG TAT CCC GCC TG				

Table 9 continued.

Primer	5'-3' sequence	Annealing temperature [°C]	Target sequence	Fragment size [kb]	References
Nso190F	GGA GAA AAG CAG GGG ATC G	59	16S rRNA gene, Ammonia-oxidizing *Betaproteobacteria*	1.0	185
NitBR	TTA CGT GTG AAG CCC TAC CCA				27
amoA-1F	GGG GTT TCT ACT GGT GGT	60	*amoA* gene, *Betaproteobacteria*	0.5	128
amaA-2R	CCC CTC KGS AAA GCC TTC TTC				128
M13-20F	TTG TAA AAC GAC GGC CAG TG	61*	Plasmid specific primers	Depending on insert	
M13R	GGA AAC AGC TAT GAC CAT GA				
SP6	ATT TAG GTG ACA CTA TAG	50	Plasmid specific primers	Depending on insert	
T7	TAA ACG ACT CAC TAT AGG G				
Probe	**5'-3' sequence**	**FA [%]**	**Target**	**5'-labled**	**References**
Ntg122	TCC GGG TAC GTT CCG ATA T	40	Genus *Nitrotoga*	Cy3 or Cy5	88
Ntg122c1	TCW GGG TAC GTT CCG ATA T		-	-	88
Ntg122c2	TCY GGG TAC GTT CCG ATG T		-	-	88
Nsp712	GGA ATT CCG CGC TCC TCT	35	*Nitrospirae* phylum	Cy5 or FITC	84
Nsp712c	GGA ATT CCG CTC TCC TCT		-	-	84
Nsp662	CGC CTT CGC CAC CGG CCT TCC	50	Genus *Nitrospira*	Cy5 or FITC	84
Nsp662c	CGC CTT CGC CAC CGG TGT TCC		-	-	84
Nso190	CGA TCC CCT GCT TTT CTC C	55	Ammonia-oxidizing *Betaproteobacteria*	Cy3	185
Nso1225	CGC CAT TGT ATT ACG TGT GA	35		Cy3	185

2.2.3 GEL ELECTROPHORESIS

PCR products were evaluated on an 1.7% (w/v) agarose gel in 1x TAE buffer. Electrophoresis was performed on a Compact XS/S gel chamber (Biometra GmbH, Göttingen, Germany) for 30 min at 120 Volt. 100 bp+ or 1 kb ladder (both Thermo Fisher Scientific, Waltham, MA, USA) were added as size standard. DNA bands were visualized on a GelDocXR+ Molecular Imager with accompanying software ImageLab v5.0 (Bio-Rad Laboratories GmbH, Munich, Germany) after ethidium bromide staining.

2.2.4 SEQUENCING

Sequencing was performed at Eurofins Genomics GmbH (Ebersberg, Germany). PCR products or excised DNA bands from gel electrophoresis were cleaned up with the GenepHlow Gel/PCR kit (DFH300; Geneaid Biotech Ltd., Taiwan) according to manufacturer's instructions, mixed with the corresponding primer, and shipped for further processing. Sequences were evaluated with BLASTn[187] against the nr database at NCBI. 16S rRNA gene sequences were checked for chimeras using DECIPHER.[188]

2.2.5 NEXT GENERATION SEQUENCING

Genomic DNA of *Nitrotoga* cultures was send to MR DNA (Shallowater, TX, USA) for amplicon sequencing of the 16S rRNA gene with primers 515F and 806R.[182] *Ntg. arctica* and *Nitrotoga* HAM-1, the preculture of *Nitrotoga* BS, were analyzed by 454 Pyrosequencing, *Nitrotoga* BS, *Nitrotoga* HW, and *Nitrotoga* 1052 by Illumina MiSeq, each at a sequencing depth of 3000 reads. Chimeras were removed and denoised sequences grouped into operational taxonomic units (OTU) by MR DNA. Taxonomic classification of OTUs was performed by MR DNA using BLASTn against databases of GreenGenes, RDPII, and NCBI.

2.2.6 TA-CLONING

Cloning of PCR products was performed with the pGEM-T Easy Vector System (Promega Corporation, Madison, WI, USA). Ligation into the vector was performed after manufacturer's instructions.

PCR products derived from Phusion High-Fidelity polymerase are not suited for direct TA-cloning, since blunt-ends are created due to its proof-reading activity. To circumvent this

restriction, 0.5 µl Dream Taq polymerase was added per 50 µl sample after PCR with Phusion High-Fidelity polymerase and incubated at 72°C for 10 min to add an A overhang.

Transformation was performed in *E. coli* DH5α. 50 µl of briefly thawed cells were incubated with 2 µl ligation reaction mix for 20 min on ice, followed by a heat shock for 45 sec at 42°C and further incubation for 5 min on ice. 450 µl of LB medium[189] (Table 11) were added to the cells and incubated at 37°C for 1.5 h on a shaker. The transformation mix was transferred to LAXI plates (Table 11) and incubated over night at 37°C. Positive clones were selected by blue-white-screening.

Positive clones were incubated in 18 µl PCR-H_2O for 10 min at 96°C. 32 µl master mix with either Dream Taq or Phusion High-Fidelity polymerase, SP6/T7 or M13 F/R primers, were added for PCR (Table 10). Products were checked on an 1.7% agarose gel and those of correct size were sequenced. Chimeras were removed using DECIPHER.[188]

Table 10: PCR program for cloned inserts amplified with Dream Taq polymerase. For PCR with Phusion High-Fidelity polymerase see Table 8.

Step	Temperature [°C]	Time [sec]	Cycles/others
1	96	240	Initial denaturation
2	96	50	Denaturation
3	See Table 9	50	Annealing
4	72	120	Elongation Repeat from step 2 for 30 times
5	72	480	Final elongation
6	12	∞	Hold

Table 11: LB medium and LAXI plates. * added after autoclaving.

Component	LB medium Σ 500 ml	LAXI (plates) Σ 500 ml
Tryptone	5 g	5 g
Yeast extract	2.5 g	2.5 g
NaCl	5 g	5 g
Agar	-	7.5 g
Ampicillin (50 mg/ml)*	-	0.5 ml → 50 mg/L
X-Gal (20 mg/ml)*	-	0.625 ml → 50 mg/L
IPTG (0.1 M)*	-	1 ml → 0.2 mM
Add UP-H_2O	500 ml	500 ml

The pH was adjusted to 7, for LAXI before adding agar. Media were autoclaved at 120°C and 1 bar excess pressure for 20 min. Ampicillin, X-Gal, and IPTG were added after autoclaving, immediately before pouring plates when LAXI medium was below 60°C. Plates and liquid media were stored at 4°C.

2.2.7 QUANTITATIVE PCR (QPCR) FOR *NITROTOGA* AND *NSP. DEFLUVII*

Quantification of specific DNA fragments can be achieved by measuring fluorescence signals during PCR e.g. from SYBR Green used in this study. It emits an intense fluorescence when bound to double stranded DNA but not in the presence of single DNA strands or RNA. The signal intensity increases with an increase of PCR product. To actually quantify samples, a standard plasmid needs to be constructed with the target sequence inserted.

For construction of standard plasmids, 16S rRNA gene sequences were amplified and cloned into the pGEM-T Easy Vector System (Promega Corporation, Madison, WI, USA) as described above. For *Nsp. defluvii*, PCR products for cloning were generated with the semi-specific primer pair 27F/Nsp1158R, for *Ntg. arctica* with the specific primer pair Ntg124F/Ntg840R (Table 9). For each NOB, eight positive clones were selected, the inserts amplified with primers SP6/T7 (Table 9) and sequenced. One positive clone per NOB was than incubated in 10 ml LB medium with Ampicillin on a shaker at 37°C over night. Plasmids were isolated with the High Speed Plasmid Mini kit (PD300, Geneaid Biotech, Ltd, Taiwan) according to manufacturer's instruction. To adjust the copy numbers of each plasmid per µl for the standards, DNA contents (ng/µl) were measured on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the molecular weight (g/mol) of the plasmid plus insert was determined. The molecular weight was calculated with an online calculator at *http://www.encorbio.com/protocols/Nuc-MW.htm*. Copy numbers of plasmids were adjusted to 10^9 copies/µl for each NOB and these stock solutions were stored at -20°C. A working solution with 10^8 copies/µl was prepared to further dilute plasmids for calibration from 10^7 down to 10^2 copies/µl. The calibration was measured with every qPCR run to obtain a correlation curve and for quality control of the overall reaction as recommended by the MYQE guidelines.[190]

Furthermore, primers for qPCR had to be developed based on the sequences inserted into the plasmids. The aim was to obtain fragments between 150 and 250 bp in size with primers not binding to the counter NOB. The primers (Table 9) were designed using Primer-BLAST[191] and synthesized at Eurofins Genomics GmbH (Ebersberg, Germany). They were checked for correct amplification in an initial qPCR reaction with diluted standard

plasmids, the target NOB, the non-template NOB, and a negative control containing water.

Reactions were prepared with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instruction (Table 12). Triplicates per sample were run on a Bio-Rad CFX96 C1000 Touch Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany). Copy numbers were calculated by the accompanying software Bio-Rad CFX Manager 3.1 based on the regression curve of standard plasmid's amplification. The PCR program with added melt curve analysis is given in Table 13. Copy numbers for *Nitrotoga* were divided by two, since it possesses two copies of the 16S rRNA gene (S. Lücker, personal communication).

Table 12: Master mix for qPCR with SYBR Green.

Component	Volume [µl] 1 reaction of 16 µl 15 µl applied in qPCR	Final concentration
Maxima SYBR Green qPCR Master Mix (2x)	8.0	1x
PCR-H_2O	4.6	
Primer F (50 pmol/µl)	0.1	0.3 pmol/µl
Primer R (50 pmol/µl)	0.1	0.3 pmol/µl
DNA	3.2	

Table 13: qPCR program with melt curve analysis.

Step	Temperature [°C]	Time [sec]	Cycles/others
1	95	30	Initial denaturation
2	95	10	Denaturation
3	59	20	Annealing
4	72	30	Elongation Plate Read at every cycle Repeat from step 2 for 44 times
5	95	60	Dissociation before melt curve
6	65 → 95	10 per step	Melt curve analysis steps of 0.5°C; Plate Read at every step

Regression curves, coefficients of determination (r^2), and efficiency were calculated based on the standard plasmids. The regression curve was used as calibration to quantify samples; r^2 rates the fit of the data compared to the curve and should be above 0.990. The efficiency of the reaction should be 100 ± 10%, indicating that the DNA doubles with every PCR cycle. The slope of the curve should be about 3.3 = ln (10)/ln (2), also indicating a doubling of DNA during each cycle, equal to a tenfold DNA increase every 3.3 cycles.

Melt curves were generated for every sample after the last PCR cycle as quality control. The temperature was increased in defined steps while simultaneously measuring the fluorescence signal. When the double stranded DNA dissociated, a sharp decrease in fluorescence occurred. This signal change appeared in a narrow temperature range and peaks at the same temperature for all samples containing a PCR product of the same sequence. Multiple peaks in one sample or several peaks in the overall batch indicated primer dimers or amplification of unwanted fragments.

2.2.8 PFA-FIXATION OF GRAM-NEGATIVE CELLS FOR FISH

The protocol for fixation was modified from Amann and colleagues.[192] Fresh cell pellets were suspended in 20 to 50 µl 0.9% (w/v) NaCl, depending on pellet size, and threefold volume of 4% (w/v) para-formaldehyde (PFA) in phosphate-buffered saline (PBS). After fixation on ice for one hour, the pellet was washed twice in PBS buffer and suspended in 20 to 50 µl PBS buffer, depending on size, and the same volume of ice cold EtOH. The fixation was stored at -20°C until hybridization.

2.2.9 FLUORESCENCE *IN-SITU* HYBRIDIZATION (FISH)

The hybridization protocol was modified after Manz and colleagues.[193] Probes were synthesized at Eurofins Genomics GmbH (Ebersberg, Germany), prepared as 50 ng/µl stock solutions, and kept at -20°C in the dark. Before FISH, working solutions with 5 ng/µl probe and competitor(s) were prepared in hybridization buffer (Table 14).

A PFA-fixed sample of activated sludge from the WWTP in Hamburg-Dradenau was used as positive control, since it contained microcolonies of *Nitrospira*, *Nitrosomonas*, and *Nitrotoga*.

For hybridization, fixed cell material was transferred to a well of a diagnostic slide, dried at room temperature, and stepwise dehydrated in 50%, 80%, and 96% (v/v) EtOH for 3 min each. Samples were covered with 9 µl probe working solution and transferred to a humidity chamber with hybridization buffer (Table 14). After 2 h of hybridization at 46°C, the slides were washed for 20 min at 48°C in washing buffer (Table 15) and subsequently rinsed with cold distilled water. After drying at room temperature, samples were counterstained with DAPI (4',6'-diamidino-2-phenylindole) and mounted in Citifluor AF1 (Science Services GmbH, Munich, Germany).

Fluorescence was detected on an Axio ImagerM2 epifluorescence microscope equipped with the following Zeiss filter sets: 38HE for FITC, 43HE for Cy3, 49 for DAPI, and 50 for Cy5. Photos were taken with an AxioCamMRm and processed with the AxioVision40 v4.8.2.0 software (all Carl Zeiss Microscopy GmbH, Jena, Germany).

Table 14: Hybridization buffer for different FA concentrations.

Component [µl]	35% FA	40% FA	50% FA	55% FA
5 M NaCl	360	360	360	360
1 M Tris HCL, pH 7.2	40	40	40	40
Formamide (FA)	700	800	1000	1100
Aqua dist	900	800	600	500
10% SDS	2	2	2	2

Table 15: Washing buffer for different FA concentrations.

Component [µl]	35% FA	40% FA	50% FA	55% FA
5 M NaCl	700	460	180	100
1 M Tris HCL, pH 7.2	1000	1000	1000	1000
Aqua dist	Add 50 ml			
10% SDS	50	50	50	50
0.5 M EDTA pH 8.0	500	500	500	500

2.3 ELECTRON MICROSCOPY

To obtain high cell amounts necessary for electron microscopic methods, 10 ml of an active *Nitrotoga* culture were transferred into 500 ml autotrophic NOB medium with 0.5 mM nitrite. Substrate was stepwise supplied until 10 mM nitrite were consumed. Cultures were incubated at the standard cultivation temperature (Table 1) in the dark without agitation.

2.3.1 TRANSMISSION ELECTRON MICROSCOPY (TEM)

For TEM, cell pellets were suspended in 2.5% (v/v) glutaraldehyde in PBS, fixed for 30 min at room temperature, and additionally 1 h on ice. The cells were then washed three times in 75 mM cacodylate buffer. After an overnight incubation in 2% (w/v) osmium tetroxide, the fixation was embedded in Spurr resin.[194,195] Ultrathin sections of this resin were stained and contrasted with uranyl acetate and lead citrate as described elsewhere.[196,197] Micrographs were recorded with a charge-coupled device camera model 794 on a LEO 906E electron microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.3.2 SCANNING ELECTRON MICROSCOPY (SEM)

For SEM, cell pellets were fixed in 1% PFA (w/v) in PBS and 0.25% (v/v) glutaraldehyde in PBS for 20 min each. Dehydration was performed by increasing ethanol concentration for 10 min each: 30%, 50%, 70%, 90%, and 96% (v/v), followed by 2 times of 20 min in 100% EtOH, and a final dehydration step over night in 100% EtOH. The cells were than critical point dried with a Balzers CPD 030 and sputter-coated with a SCD 050 (both Bal-Tec, Schalksmühle, Germany). Micrographs were taken on a LEO 1525 electron microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.4 CHEMICAL ANALYSES

2.4.1 HPLC FOR QUANTIFICATION OF NITRITE AND NITRATE

Nitrite and nitrate were quantified by high-performance liquid chromatography (HPLC) with a modified method for ion pair chromatography.[198,199] A LiChrospher 100 RP-18 endcapped column (5 µm, LiChroCART 125 x 4 mm; Merck KGaA, Darmstadt, Germany) was used for separation. Peaks were recognized by their retention time in an automated system (Hitachi LaChrom Elite, VWR International GmbH, Darmstadt, Germany) with UV detection at 225 nm. The system was operated isocratic at a flow of 1 ml/min. The eluent was composed of 1.16 g TBAHS in 1 L UP-H_2O with 10% (v/v) HPLC-grade methanol, and adjusted to pH 6.4 with NaOH.

Data were acquired and processed with the software EZChrom Elite 3.3.2 (VWR International GmbH, Darmstadt, Germany) and quantification was based on peak area. The method was calibrated with a solution of nitrite and nitrate between 10 and 2000 µM.

Samples for HPLC were centrifuged, 500 µl transferred to HPCL glass vials (Fisher Scientific GmbH, Schwerte, Germany) and 25 µl injected. If organic or salt contents were expected, samples were diluted with UP-H_2O to prevent column deterioration and occurrence of peaks that might hinder quantification.

2.4.2 SPOT-TEST FOR NITRITE

The Griess-Ilosvay spot-test[200] was used to quickly check cultures and physiological tests for nitrite consumption. The solution (Table 16) formed a pink color in the presence of nitrite. Sulfanilamide was dissolved in *ortho*-phosphoric acid before adding further components. The solution was stored at 4°C in a lightproof bottle.

Table 16: Griess-Ilosvay solution.

Component	**Σ 100 ml**
Sulfanilamide	4 g
Ortho-phosphoric acid (85%)	10 ml
N-(1-naphthyl)ethylenediamine dihydrochloride	0.2 g
UP-H_2O	Add 100 ml

2.4.3 AMMONIUM QUICK TEST AND AMMONIUM QUANTIFICATION

A semi-quantitative ammonium test was carried out with Quantofix Ammonium (Macherey-Nagel GmbH & Co. KG, Düren, Germany) modified from manufacturer's instructions: 500 µl sample and one drop of NaOH solution were used.

Ammonium quantification was performed with a modified *Ortho*-phtalaldehyde (OPA) assay.[201,202] 950 µl OPA-reagent (Table 17A) and 50 µl sample were directly mixed in a cuvette (polysterene, Sarstedt AG & Co KG, Nümbrecht, Germany) and incubated in the dark at room temperature for 1 h to allow complete derivatization. Absorption of the reaction product was measured on an UV spectrophotometer (Jenway 6300, Bibby Scientific Ltd., Staffordshire, UK) at 420 nm. A stock solution with 10 mM NH_4Cl was prepared for calibration. The OPA-reagent was stored at 4°C in a lightproof bottle.

Table 17: OPA-reagent (A) and phosphate buffer (B).

(A) OPA-reagent	**Σ 100 ml**
Ortho-phtalaldehyde (OPA)	540 mg
Ethanol, HPLC-grade	10 ml
β-mercaptoethanol	50 µl
Phosphate buffer, 0.2 mM pH 7.3	Add 100 ml

(B) Phosphate buffer, 0.2 mM pH 7.3	
KH_2PO_4 in UP-H2O (0.2 mM)	ca. ¼
K_2HPO_4 in UP-H2O (0.2 mM)	ca. ¾
Both solutions were mixed to adjust pH to 7.3	

2.5 PHYLOGENY

Phylogenetic analysis of *Nitrotoga* was based on cloned fragments of the 16S rRNA and *nxrB* genes, amplified with primers 27F/1492R and Ntg-nxrBF/Ntg-nxrBR, respectively. Cloned inserts were amplified with M13 primers. PCR was performed with Phusion High-Fidelity DNA polymerase for proof-reading. 10 to 20 clones per batch were picked. 16S rRNA gene products were sequenced with 3 different primers: M13-20F and M13R to get the full sequence and additionally 517F for verification (Table 9). *NxrB* products were sequenced with Ntg-NxrBF primer. Sequences were merged and aligned by MUSCLE.[203] Neighbor-joining trees[204] were generated by the Maximum Composite Likelihood method[205] and 500 bootstrap repetitions[206] with MEGA7 (v. 7.0.21).[207] Nodes supported by bootstrap values above 50% are indicated.

2.6 PHYSIOLOGICAL CHARACTERIZATION

2.6.1 pH AND TEMPERATURE OPTIMA, AMMONIUM INFLUENCE

Experiments to evaluate pH and temperature optima as well as ammonium influence were carried out in triplicates in 100 ml Erlenmeyer flasks containing 50 ml autotrophic NOB medium with 1 mM nitrite. Flasks were inoculated with 1% preculture and incubated in the dark without shaking.

For *Nitrotoga* BS and *Nitrotoga* 1052, media for pH and temperature experiments were supplemented with 0.1 mM ammonium.

For pH tests, medium was sterile filtrated after adjusting the pH between 5.5 and 8.5. The pH was measured immediately after inoculation and this value taken as starting point. Cultivation took place at the temperatures given in Table 1.

Temperature optima were evaluated between 4 and 32°C.

The ammonium influence test was performed at the temperatures given in Table 1, except for *Nitrotoga* HW that was incubated at 22°C. Ammonium concentrations between 0 and 10 mM were applied.

Samples were taken regularly to measure nitrite and nitrate by HPLC. Appendix I contains nitrite oxidation curves for each parameter. Activities were calculated per sample during

exponential phase between 800 to 0 µM nitrite and then averaged. The highest activity per experiment and culture was defined as 100% to generate optimum curves for pH, temperature, and ammonium influence.

2.6.2 AMMONIUM, NITRITE, NITRATE, AND H_2O_2 INHIBITION

Experiments were carried out in glass test tubes with 10 ml autotrophic NOB medium in duplicates, except for H_2O_2 in triplicates. They were inoculated with 1% preculture and incubated in the dark without shaking. Tests were performed at the temperatures given in Table 1, except for *Nitrotoga* HW that was tested at 22°C. The spot-test was performed daily to evaluate time until nitrite was completely consumed.

The ammonium inhibition experiment was started with 1 mM nitrite and 0 to 40 mM ammonium. Free ammonia concentrations were determined by calculators at http://home.eng.iastate.edu/~jea/w3-research/free-ammonia/nh3.html and http://www.hbuehrer.ch/Rechner/Ammonia.html, based on TAN concentrations, pH, and temperature as describes elsewhere.[208,209]

The nitrite inhibition test was started with 1 to 20 mM nitrite and 0.1 mM ammonium.

The nitrate inhibition experiment was started with 1 mM nitrite, 0.1 mM ammonium, and 0 to 20 mM nitrate.

The test to evaluate H_2O_2 inhibition was started with 1 mM nitrite, 0.1 mM ammonium, and H_2O_2 levels between 0 and 0.1% (v/v). It was performed with *Nsp. defluvii* as well.

2.7 CO-CULTIVATION EXPERIMENTS

For co-cultivation experiments, two separate autoclavable MiniBio Reactor systems with a net volume of 500 ml were used (Applikon Biotechnology B.V., Delft, The Netherlands). The bundle included the software *my*-Control v1.1 to set-up and control the process, as well as the software BioXpert Lite v1.12 to measure process parameters online via a LAN-connected PC and to add off-line obtained data.

All experiments employed the same conditions (Table 18). Reactor runs were repeated three times with either *Nitrotoga* BS or *Ntg. arctica*, both combined with *Nsp. defluvii*.

Table 18: Standard process for co-cultivation experiments.

Total volume:	**350 ml**
Medium	NOB (Table 2)
Substrate	1 mM $NaNO_2$
Supplements	0.1 mM NH_4Cl
Temperature	17°C
pH	6.4 or 7.4
Stirrer	10 rpm
Aeration	none
Inoculation	1% = 3.5 ml per NOB

Electrodes for pH were calibrated between pH 4.0 and 7.0 before autoclaving. Electrodes for dissolved oxygen (dO) were calibrated at 100% oxygen saturation after autoclaving and subsequent equilibration over night at process temperature.

Stirring was kept low at 10 rpm and aeration was forgone, since fresh inoculated NOB are sensitive to shearing and oxidative stress. However, the slow stirring allowed enough uptake of oxygen during exponential nitrite oxidation, so that no oxygen limitation occurred. Ammonium was supplemented to support ammonium dependent *Nitrotoga*.

For nitrite and nitrate determination by HPLC and for photometrical ammonium quantification, 2 ml samples were taken daily with a sterile 12 cm cannula via a septum. Nitrite oxidation activity was calculated during complete exponential nitrite consumption, starting when about 10% initial nitrite was oxidized. At the end of each experiment when nitrite was completely converted to nitrate, cells were harvested in two 50 ml tubes: one for DNA isolation and subsequent qPCR, the other for fixation in 4% PFA (w/v) for FISH.

To evaluate NOB distribution, qPCR was conducted as described above with three technical replicates per reactor run. Relative abundance of *Nitrotoga* and *Nsp. defluvii* was calculated from copy number per ml. To confirm qPCR results, FISH was performed with PFA-fixed samples as described above. Cy3-labeled probe Ntg122 for *Nitrotoga* and Cy5-labeled probes Nsp662 and Nsp712 for *Nitrospira* as well as unlabeled competitor probes for both NOB were applied in one hybridization step with 40% FA and corresponding wash buffer. To evaluate NOB contents before inoculation, 100 µl of the precultures were incubated at 96°C for 10 min and subsequently, qPCR was conducted as described above. Obtained copy numbers were divided by 100 to account for 1% inoculation.

2.8 MONITORING OF A RECIRCULATION AQUACULTURE SYSTEM (RAS)

2.8.1 OVERVIEW RAS IN HOHEN WANGELIN

The monitored cold-freshwater RAS located in Hohen Wangelin (Mecklenburg-West Pomerania, Germany) was used to cultivate rainbow trout (*Oncorhynchus mykiss*). It was re-started in November 2013 after a complete overhaul, including the construction of a major biofilter unit in addition to two already existing smaller biofilters (left and right). A specialty in this process was the re-use of formerly applied biocarriers in the two smaller biofilters. The carriers were disinfected, dried, and stored after the previous run.[34]

The facility consisted of two rearing basins, three moving-bed biofilters, and further equipment for water purification (drum filter, sludge retention) and conditioning (intern circulation with mixing, CO2 degassing, and water influx). It was supplied with freshwater from a nearby well that was mixed with purified water from the biofilters and process water from the rearing tanks (Figure 3). Before entering intern circulation or biofiltration, rough particles were mechanically removed from the process water. The biofiltration was carried out stepwise with water channeled into the major biofilter first and then entering the smaller biofilters. Each biofilter contained biocarriers (GEA 2H Water Technologies GmbH, Wettringen, Germany) made of high-density poly-ethylene (HDPE) on which the nitrifying biofilm developed. Purified water was directed from biofiltration to intern circulation or mechanical cleaning. The rearing tanks were supplied with a mix of purified and freshwater (intern circulation).

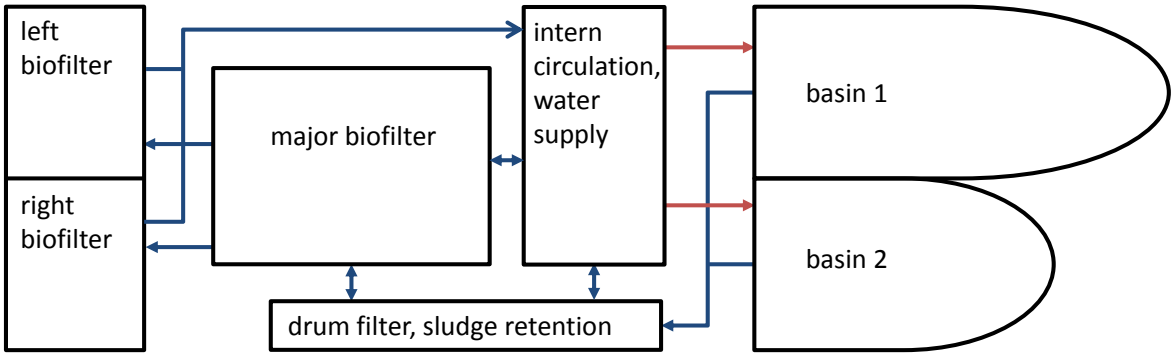

Figure 3: Overview of the rainbow trout RAS in Hohen Wangelin (simplified). Arrows indicate water flow; red arrows indicate influx into fish basins.

2.8.2 ACTIVITY TESTS

The potential nitrifying activity was checked regularly over two years from November 2013 to October 2015 with a focus on the start-up phase and disinfection steps with Wofasteril based on peracetic acid (PAA) and H_2O_2 (KESLA HYGIENE AG, Bitterfeld-Wolfen, Germany). For the major biofilter, eight biocarriers of type 2H-BCN 012 KLL were placed in 50 ml medium in a 300 ml Erlenmeyer flask. For the left and right biofilter a mixture of two 2H-BCN 012 KLL and eight 2H-BCN 009 was applied. Activities were tested separately for NOB and AOB in autotrophic NOB and AOB media (Table 2 and Table 3) with 1 mM substrate in duplicates per biofilter. The flasks were incubated in the dark on a round shaker at 120 rpm at 17°C. Samples were taken at least every hour, and nitrite and nitrate levels were determined by HPLC.

For NOB, the activity as consumed total nitrite nitrogen (TNN) per hour and per m^3 biofilter (g/hm^3) was calculated based on nitrite consumption in NOB medium. For AOB, the activity as consumed total ammonia nitrogen (TAN) per hour and per m^3 biofilter (g/hm^3) was calculated based on nitrite and nitrate formation in AOB medium. The NOB in the biofilm on the biocarriers immediately oxidized nitrite to nitrate, and consequently, no nitrite accumulated to calculate AOB activity from. Since the stoichiometry of ammonia conversion over nitrite to nitrate is 1:1:1 and the AOB were the rate-limiting factor, as the NOB could not produce nitrate faster than the AOB produced nitrite, the latter two N-compounds were summed up.

2.8.3 POPULATION ANALYSIS

Different methods were used to determine AOB and NOB in the nitrifying biofilm on the biocarriers.

For DNA isolation and PFA-fixation, biofilm material was harvested. At least 10 biocarriers were transferred to 50 ml tubes with 20 ml 0.9% (w/v) NaCl and glass beads. The tubes were shaken vigorously for 1 h at 4°C. The supernatant with detached biofilm was transferred to 2 ml tubes and centrifuged. More supernatant was added until a visible pellet formed. Two pellets were necessary for one biofilter: one pellet was PFA-fixated for FISH; DNA was isolated from the second pellet with the PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer's instructions.

FISH was performed with specific probes (Table 9) for *Nitrotoga*, *Nitrospira*, and ammonia-oxidizing *Betaproteobacteria* as described above.

PCR was performed with species specific or semi-specific primer sets for the 16S rRNA gene of *Nitrotoga, Nitrospira*, *Nitrobacter*, and ammonia-oxidizing *Betaproteobacteria*, or primers for the *nxrB* of *Nitrospira*, and the *amoA* of betaproteobacterial ammonia-oxidizers (Table 9). Reactions contained BSA to circumvent PCR inhibition by biofilm components. PCR products were sequenced after checking for correct band size on a 1.7% (w/v) agarose gel.

For TEM, biofilm material was scratched from carriers with a scalpel, directly transferred into 0.9% (w/v) NaCl, and pelletized. Samples for TEM were prepared as described above.

2.8.4 PHYSIOLOGICAL TESTS WITH BIOCARRIERS

To assess the influence of pH, temperature, ammonium, and nitrate on the short-term performance of the nitrifying biofilm, modified activity tests were conducted. They were performed with three biocarriers of type 2H-BCN 012 KLL of the major biofilter in 25 ml medium in 100 ml Erlenmeyer flasks in duplicates in the dark on a shaker at 120 rpm. Media for AOB and NOB were prepared as mentioned before, depending on the investigated parameter. Temperature was kept at 17°C, except for the temperature test that was performed at 10 and 17°C. Activities were calculated as described above, except for AOB during the nitrate inhibition tests, where it was calculated from ammonium consumption measured by the OPA-assay.

3 RESULTS

3.1 PURITY OF *NITROTOGA* CULTURES

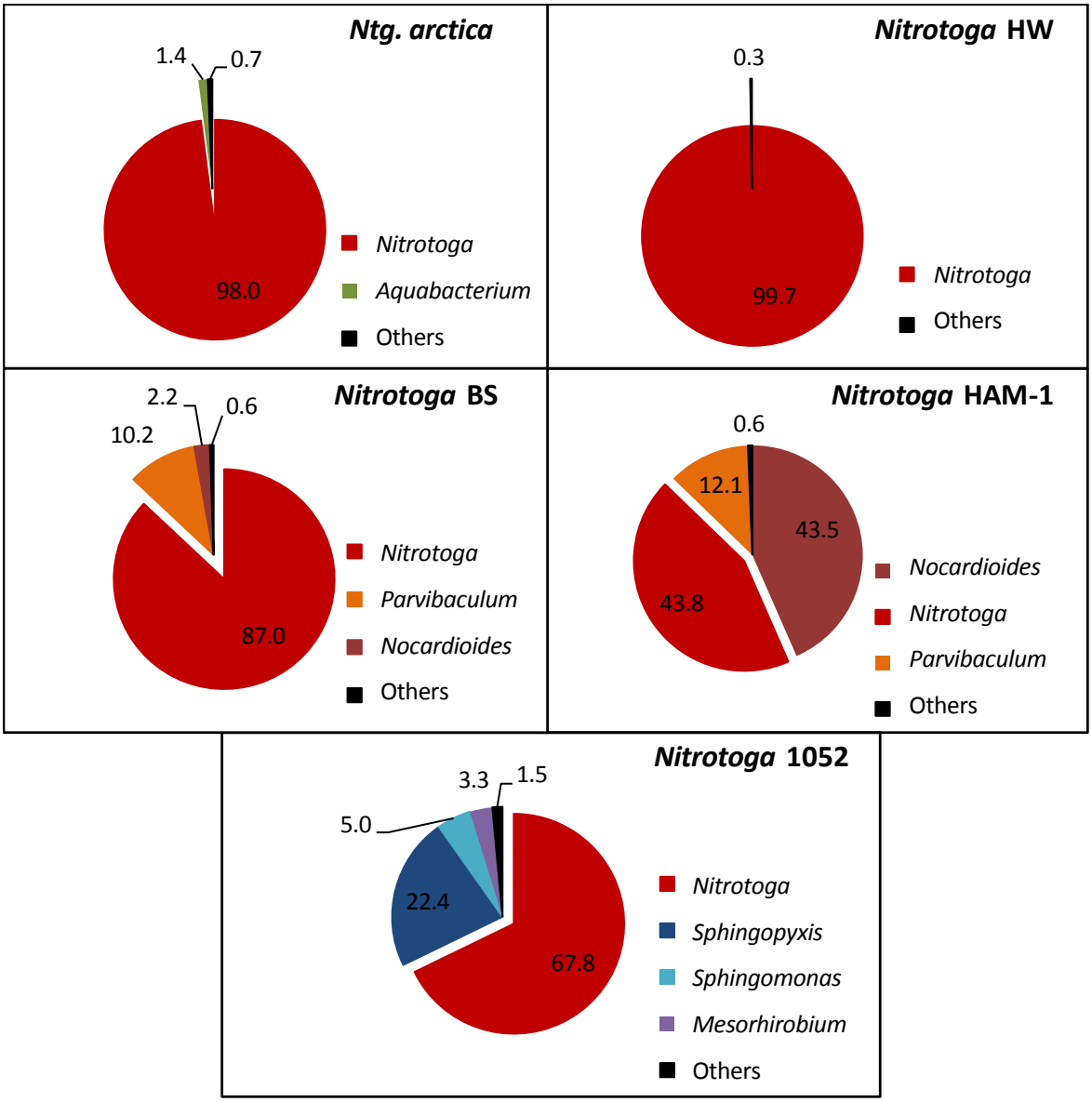

Figure 4: Purity of *Nitrotoga* cultures determined by 16S rRNA gene amplicon sequencing. *Ntg. arctica* and *Nitrotoga* HAM-1: 454 Pyrosequencing; all other cultures: Illumina MiSeq. *Nitrotoga* HAM-1 was maintained by Boris Nowka. Numbers indicate the percentage of OTUs assigned to the respective bacterial genera given in the legends.

Ntg. arctica and *Nitrotoga* HW did no longer contain any cultivable heterotrophic bacteria on solid complex medium. Near purity was revealed for these two cultures by 16S rRNA gene amplicon sequencing (Figure 4) with 98 and more than 99% of OTUs assigned to *Nitrotoga*. *Nitrotoga* BS was higher enriched than its preculture *Nitrotoga* HAM-1 (87%

compared to about 44%). Both enrichments contained cultivable heterotrophs, and similar bacteria were identified during amplicon sequencing: *Parvibaculum* belonging to the *Alphaproteobacteria* and *Nocardioides* of the *Actinobacteria* phylum. Two members of the latter genus were isolated from *Nitrotoga* BS that were closely related to *N. ganghwensis* (JF505976) and *N. hwasunensis* (JX841082) (this work, Bachelor thesis A. Schwabauer). About 68% OTUs of *Nitrotoga* 1052 were assigned to *Nitrotoga*. It coexisted with Sphingomonadaceae, members of the *Alphaproteobacteria* phylum. Next described relatives of accompanying bacteria in *Nitrotoga* cultures based on OTUs from 16S rRNA gene amplicon sequencing are listed in Table 19.

Table 19: Next described relatives of co-cultured bacteria in *Nitrotoga* enrichments based on OTUs obtained during 16S rRNA gene amplicon sequencing. A range of similarities indicates different OTUs with the same next described relative.

Next described relatives	Similarity [%]	Accession nr.	Taxonomy
Ntg. arctica			
Aquabacterium sp. Aqua2	95.6	AF089858.1	*Betaproteobacteria*, Burkholderiales, *Aquabacterium*
***Nitrotoga* BS**			
Nocardioides ganghwensis strain VN2013-65	99.3	KX449292.1	*Actinobacteria*, Propionibacteriales, Nocardioidaceae, *Nocardioides*
Parvibaculum sp. MBNA2	96.0	FN430653.1	*Alphaproteobacteria*, Rhizobiales, Rhodobiaceae, *Parvibaculum*
***Nitrotoga* HAM-1**			
Nocardioides ganghwensis strain VN2013-65	99.7	KX449292.1	*Actinobacteria*, Propionibacteriales, Nocardioidaceae, *Nocardioides*
Parvibaculum sp. MBNA2	94.9 - 97.0	FN430653.1	*Alphaproteobacteria*, Rhizobiales, Rhodobiaceae, *Parvibaculum*
***Nitrotoga* 1052**			
Sphingopyxis chilensis strain BBCC2226	97.5 - 98.2	KY787171.1	*Alphaproteobacteria*, Sphingomonadales, Sphingomonadaceae, *Sphingopyxis*
Novosphingobium sp. strain LYH5	99.0	KY126353.1	*Alphaproteobacteria*, Sphingomonadales, Sphingomonadaceae, *Novosphingobium*
Sphingomonas echinoides strain IIL-Asp29	99.6	KX380919.1	*Alphaproteobacteria*, Sphingomonadales, Sphingomonadaceae, *Sphingomonas*
Mesorhizobium sp. Z121_1	96.5	KF295447.1	*Alphaproteobacteria*, Rhizobiales, Phyllobacteriaceae, *Mesorhizobium*
Mesorhizobium sp. Cag14	98.1	FN546870.1	

3.2 PHYSIOLOGICAL CHARACTERIZATION

3.2.1 PH OPTIMA

When evaluating the pH optimum, the cultures showed distinct behaviors (Figure 5). They had in common that no activity was observed at slightly acidic conditions below pH 6.1 (*Nitrotoga* HW) to 6.7 (*Nitrotoga* BS, *Nitrotoga* 1052). Nevertheless, *Ntg. arctica* and

Nitrotoga HW were best adapted to weak aciditys: *Ntg. arctica* had the lowest optimum of all four enrichments at pH 6.4 and *Nitrotoga* HW maintained high activity at pH 6.1 (80%). Altogether, the latter culture was least sensitive to changing pH with a broad optimum from 6.8 to 7.2 and about 80% activity at more alkaline conditions. In contrast, *Ntg. arctica* was delayed above its optimum pH (40% activity). *Nitrotoga* 1052 and *Nitrotoga* BS both grew best at approximately pH 7.3 and were most sensitive to slight acidity. While *Nitrotoga* 1052 tolerated alkaline surroundings above pH 8 quite well, activity of *Nitrotoga* BS decreased to below 10% under this condition.

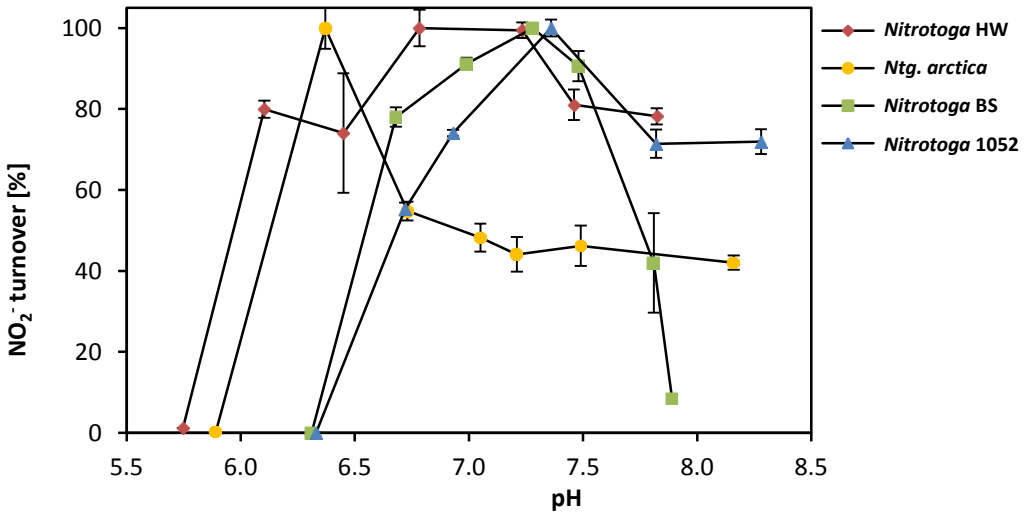

Figure 5: pH optima for *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 3. Symbols partially overlapped; SD partially too small to see.

3.2.2 TEMPERATURE OPTIMA

Two different temperature optima were observed for the four investigated enrichments (Figure 6). Overall, cold adaption of *Nitrotoga* was confirmed by activity between 20% and 60% at 4°C compared to the optimum and by growth inhibition at temperatures above 25°C to 28°C. *Ntg. arctica* and *Nitrotoga* BS grew best at 17°C and showed high activities at 4°C and 10°C as well (60% and 80%, respectively). In contrast, *Nitrotoga* HW and *Nitrotoga* 1052 proliferated best at 22°C, but they were delayed with less than 50% activity at temperatures below 10°C. However, these two cultures had a broader temperature range up to 25°C.

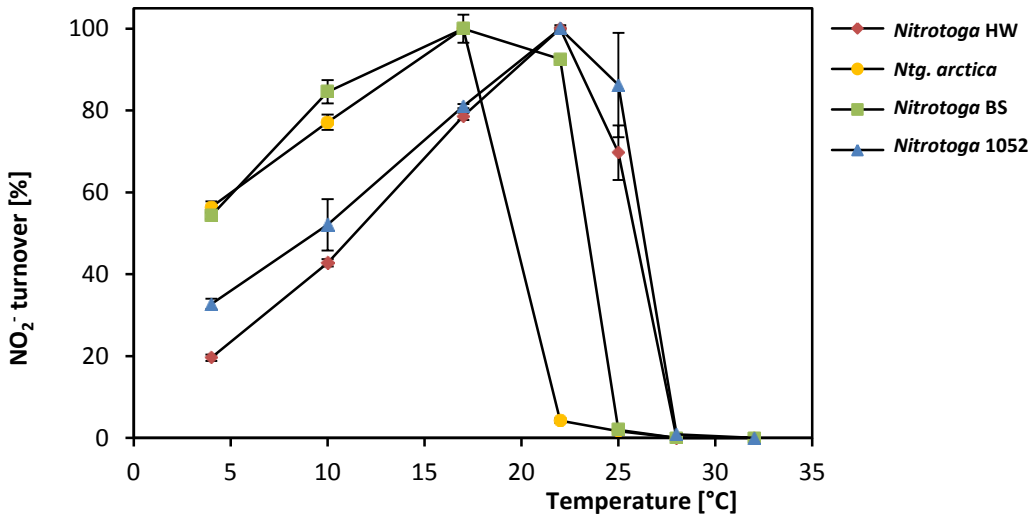

Figure 6: Temperature optima for *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 3. Symbols partially overlapped; SD partially too small to see.

3.2.3 AMMONIUM INFLUENCE

Since reliable cultivation of *Nitrotoga* BS could only be ensured by adding ammonium to the medium, while *Ntg. arctica*, *Nitrotoga* HW, and *Nitrotoga* 1052 were cultivated without its supplementation, the influence of this component was tested on all four enrichments (Figure 7). For an ammonium-deprived preculture of *Nitrotoga* BS, addition of ammonium had a visible positive effect on growth performance, whereas it was considerably slower without ammonium. In contrast, *Nitrotoga* BS was not influenced whether ammonium was added or not in a similar experiment if the preculture was ammonium-supplemented (results not shown). Addition of up to 10 mM ammonium did not influence nitrite oxidation in *Ntg. arctica*, *Nitrotoga* HW, or *Nitrotoga* 1052 compared to samples without supplementation.

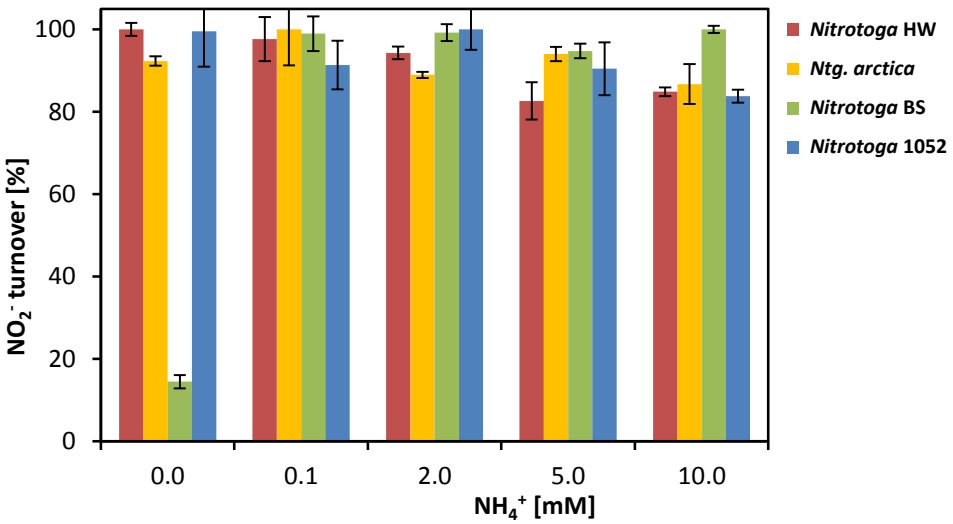

Figure 7: Influence of ammonium on growth of *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 3.

3.2.4 INHIBITION BY AMMONIUM

An inhibition test with ammonium was conducted, since the cultures showed no negative growth tendencies during the ammonium influence test (see above). It became apparent that all four enrichments shared a high tolerance (Figure 8). *Ntg. arctica* and *Nitrotoga* HW were active in the presence of 30 mM and 40 mM ammonium, respectively, without severe effects on nitrite oxidation compared to lower ammonium concentrations. In contrast, *Nitrotoga* BS and *Nitrotoga* 1052 were delayed at higher ammonium contents, but nevertheless grew at 30 mM and 25 mM ammonium, respectively.

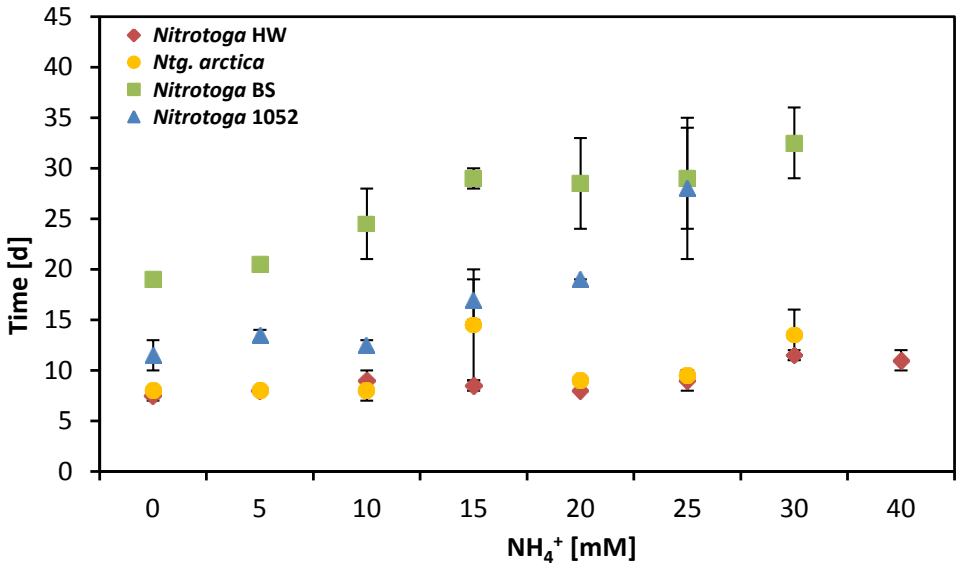

Figure 8: Inhibition of *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052 by ammonium. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 2. Symbols partially overlapped; SD partially too small to see.

3.2.5 INHIBITION BY NITRITE

Elevated nitrite concentrations had a strong inhibiting effect on the growth of *Nitrotoga* (Figure 9). The highest tolerance was demonstrated by *Nitrotoga* HW with 10 mM, whereas the other three cultures did not grow above 4 mM nitrite. Additionally, complete oxidation of nitrite lasted considerably longer at the highest tolerated concentration.

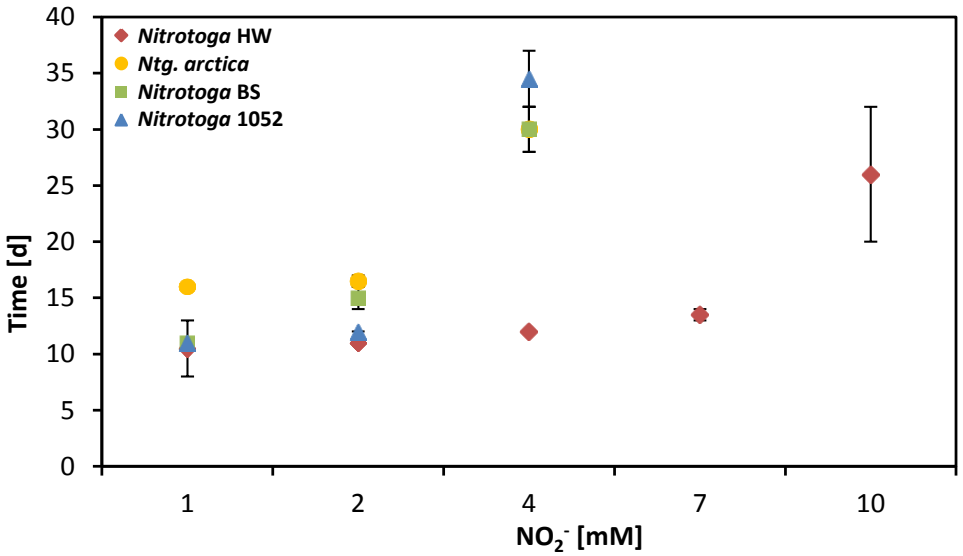

Figure 9: Inhibition of *Nitrotoga* HW, *Nitrotoga arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052 by nitrite. Mean and SD calculated from n = 2. Symbols partially overlapped; SD partially too small to see.

3.2.6 INHIBITION BY NITRATE

Elevated nitrate concentrations (Figure 10) had a weaker effect on growth of *Nitrotoga* compared to nitrite but affected the enrichments more than addition of ammonium. *Nitrotoga* BS was by far the most tolerant culture with fast nitrite oxidation in the presence of up to 20 mM nitrate. The remaining cultures preserved at lower concentrations of 10 and 15 mM nitrate, but needed considerably longer to completely oxidize 1 mM nitrite under these conditions.

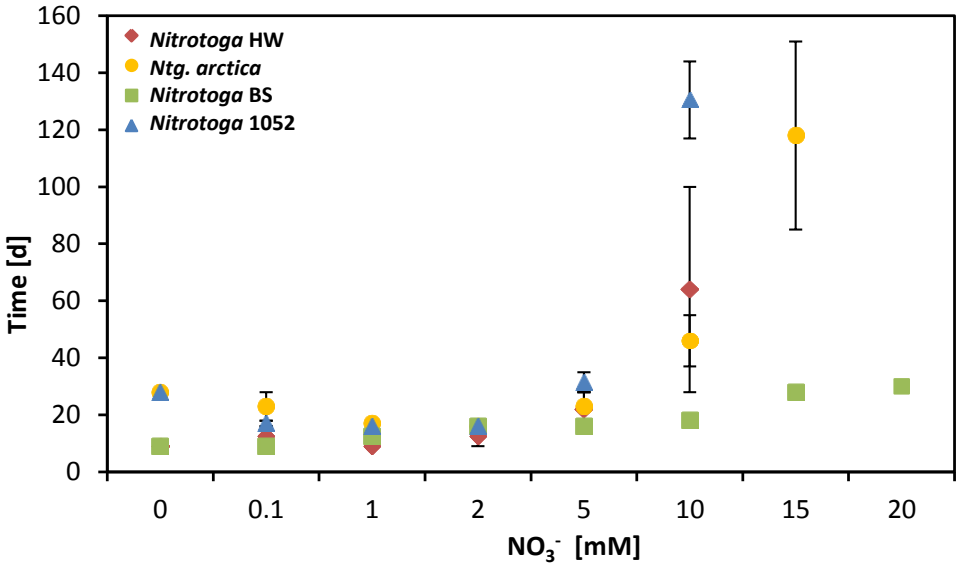

Figure 10: Inhibition of *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052 by nitrate. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 2. Symbols partially overlapped; SD partially too small to see.

3.2.7 INHIBITION BY H_2O_2

Inhibition of *Nitrotoga* by H_2O_2 was evaluated because a disinfection step with PAA and H_2O_2 in the investigated RAS (see below) led to a suppression of *Nitrotoga* in the process. An influence on *Nsp. defluvii* was surveyed as well, since similar *Nitrospira* were found in the RAS that were further detected after disinfection started. Approximately 10^{-4}% H_2O_2 were applied as final concentration in the RAS process water. No inhibition was observed up to 0.01% H_2O_2 in the NOB cultures tested, and only a minor delay in nitrite oxidation occurred at 0.1% H_2O_2, except for *Nitrotoga* 1052 (Figure 11) that was not impaired at all.

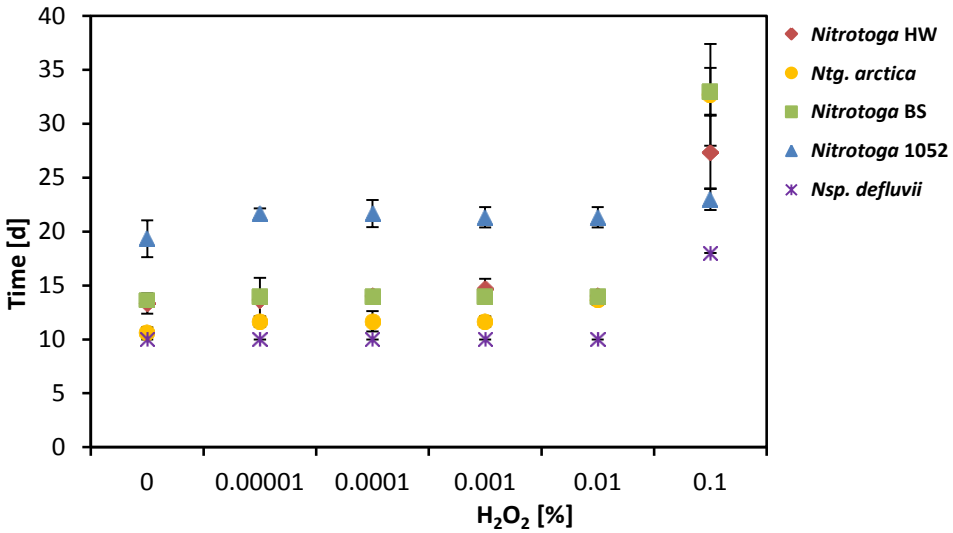

Figure 11: Inhibition of *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, *Nitrotoga* 1052, and *Nsp. defluvii* by H_2O_2. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 3. Symbols partially overlapped; SD partially too small to see.

3.3 PHYLOGENY – RELATIONSHIP OF DIFFERENT *NITROTOGA* CULTURES

3.3.1 16S RRNA GENE SEQUENCE

Based on 16S rRNA genes, all investigated *Nitrotoga* cultures were closely related with less than 1% sequence difference (Figure 12, Table 20), and all clones obtained from the same enrichment clustered together. *Ntg. arctica* and *Nitrotoga* 1052 were the most similar cultures; *Nitrotoga* HW and *Nitrotoga* BS were the most distant. The next described cultivated relatives are iron-oxidizing bacteria: *Sideroxydans lithotrophicus* strain ES-1 (GenBank: NR_115756) and *Gallionella capsiferriformans* strain ES-2 (GenBank: DQ386262). Like *Nitrotoga*, they belong to the Gallionallaceae family within the order Nitrosomonadales, class *Betaproteobacteria*.

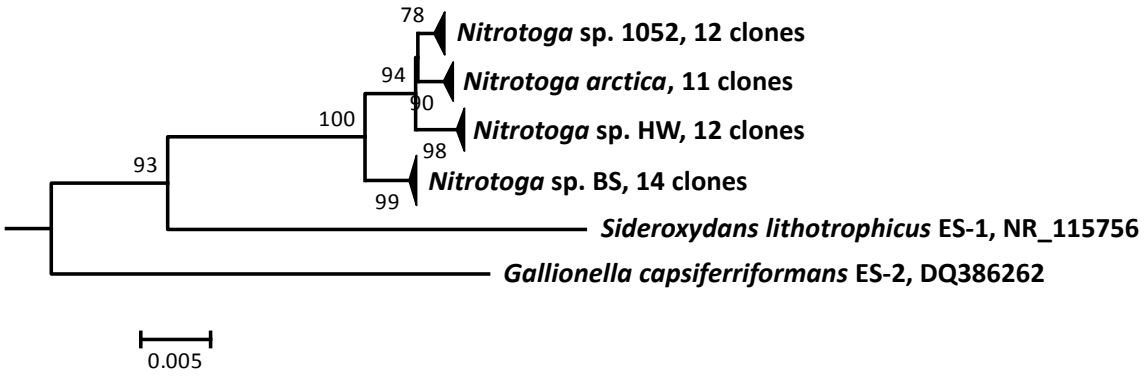

Figure 12: Neighbor-joining tree of cloned 16S rRNA gene sequences of investigated *Nitrotoga* cultures, with described next relatives for comparison. *Nitrosomonas europaea* ATCC 25978 (GenBank: HE862405) was used as outgroup. Scale bar indicates 0.5% sequence divergence.

Table 20: Overview of 16S rRNA gene sequence similarities based on data from Figure 12.

Similarity [%]	*Nitrotoga* 1052	*Nitrotoga* BS	*Nitrotoga* HW	*S. litho-trophicus* ES-1	*G. capsiferri-formans ES-2*
Ntg. arctica	99.7	99.1	99.5	95.0	94.1
	***Nitrotoga* 1052**	99.2	99.6	95.1	94.1
		***Nitrotoga* BS**	99.0	95.3	94.3
			***Nitrotoga* HW**	95.1	94.0

Sequences of the following cultures were submitted to NCBI during previous studies: *Ntg. arctica* (*Candidatus* Nitrotoga arctica clone 6680 16S ribosomal RNA gene, partial sequence; GenBank: DQ839562.1),[129] a preculture of *Nitrotoga* BS (*Candidatus* Nitrotoga sp. enrichment culture clone HAM-1 16S ribosomal RNA gene, partial sequence; GenBank: FJ263061.1),[130] and *Nitrotoga* HW (Uncultured *Candidatus* Nitrotoga clone HW29 16S ribosomal RNA gene, partial sequence; GenBank: KT778545.1).[34] These database

sequences of *Ntg. arctica* and *Nitrotoga* HW29 cluster together with the respective clones of *Ntg. arctica* and *Nitrotoga* HW obtained in this study. In contrast, sequences of *Nitrotoga* BS clones did not match the sequence of its preculture *Nitrotoga* HAM-1. Sequences of all 16S rRNA gene clones are listed in Appendix II. A detailed neighbor-joining tree of the most distant clones per culture and further *Nitrotoga*-affiliated sequences obtained from NCBI are shown in Appendix II 1. The sequence of *Nitrotoga* BS clone 15 was submitted to NCBI under GenBank accession number MF555727 (Wegen *et al.*, in preparation).

3.3.2 *nxrB* Gene Sequence

Based on the sequence of the *nxrB* gene, the cultures were more distant related (Figure 13, Table 21) than on 16S rRNA gene level. Again, cloned sequences of the same enrichment clustered together, and *Ntg. arctica* and *Nitrotoga* 1052 had the most similar sequences, while *Nitrotoga* HW and *Nitrotoga* BS were most distant. The most similar gene found at NCBI encodes for a putative selenate reductase of *Candidatus* Acetothermus autotrophicum (large contig sequence, contig 3, GenBank: AP011802.1) with a query coverage of about 95% but only about 66% sequence similarity. The most similar NOB-related sequences were *nxrB* sections in the genomes of *Nsp. defluvii* and *Nsp. moscoviensis* strain NSP M-1 (GenBank: FP929003.1 and CP011801.1, respectively) with a low query coverage below 30% but higher sequence similarity of about 80 to 83%.

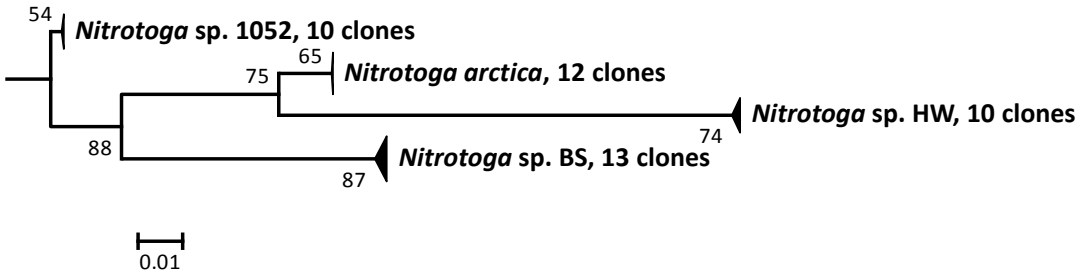

Figure 13: Neighbor-joining tree of cloned *nxrB* gene sequences of investigated *Nitrotoga* cultures. Cloned sequences without similarities to *nxrB* were used as outgroup. Scale bar indicates 1% sequence divergence.

Table 21: Overview of *nxrB* gene sequence similarities based on data from Figure 13.

Similarity [%]	*Nitrotoga* 1052	*Nitrotoga* BS	*Nitrotoga* HW
Ntg. arctica	93.5	89.6	88.6
	***Nitrotoga* 1052**	92.5	84.5
		***Nitrotoga* BS**	81.6

Compared to *nxrB* of other nitrifying bacteria, those of *Nitrotoga* form a distinct group. Nonetheless, they are more closely related to periplasm-oriented *nxrB* e.g. from *Nitrospira* or Anammox bacteria, than to those facing the cytoplasm, e.g. from *Nitrobacter* (Figure 14). To date, no stand-alone sequences for the *nxrB* of *Nitrotoga* were submitted to NCBI. Sequences of cloned *nxrB* are listed in Appendix II. A detailed *nxrB* tree is given in Appendix II 2.

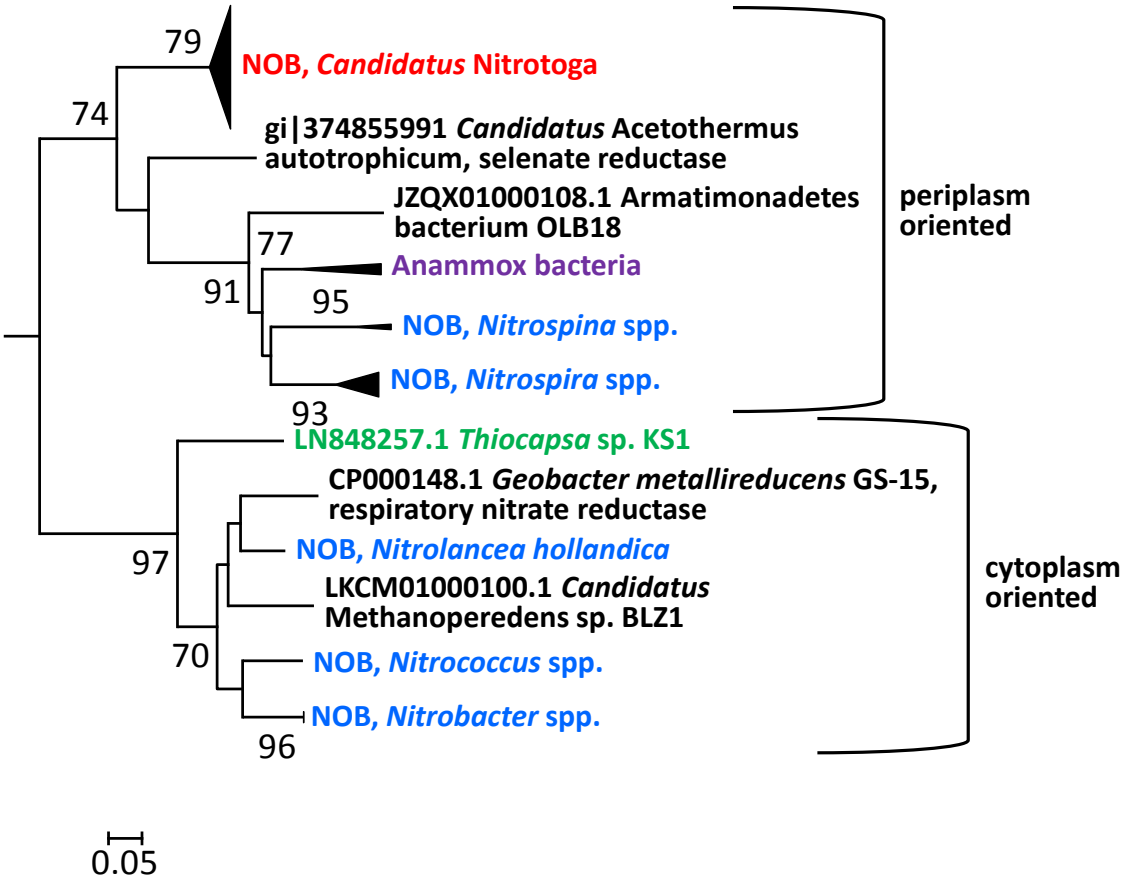

Figure 14: Neighbor-joining tree of cloned *nxrB* gene sequences of investigated *Nitrotoga* cultures (red) compared to *nxrB* gene sequences of different NOB (blue), Anammox bacteria (purple), a phototrophic nitrite oxidizing *Thiocapsa sp.* (green), and related sequences of other bacteria (black). Scale bar indicates 5% sequence divergence. The dimethylsulfide dehydrogenase beta subunit of *Pseudomonas litoralis* strain 2SM5 (GenBank: LT629748.1) was used as outgroup.

Furthermore, primers for *Nitrotoga nxrB* generated sequences that did not cluster within the actual *nxrB* sequences (used as outgroup in Figure 13). These sequences and further details about their similarity are listed in Appendix II.

3.4 MORPHOLOGY – ELECTRON MICROSCOPY

In TEM micrographs, the wide irregular shaped periplasm was the most striking feature of all *Nitrotoga* cultures investigated, hence why this NOB is named "Nitrotoga". The periplasm was easily distinguishable by its uniform shading and fine particular appearance compared to the cells. They were much more rich in contrast and appeared roughly granulated by storage materiel like glycogen. Due to their orientation in ultrathin sections, cells appeared as short, thick, sometimes curved rods, while others seemed to be coccoid as can be seen with two cells of *Nitrotoga* HW in Figure 15A. Paired cells of *Ntg. arctica* are visualized in Figure 15B. They were still connected by their periplasm probably as a result of incomplete cell division. Several of these conjoined cells were also observed in *Nitrotoga* HW and *Nitrotoga* 1052. An overview of a microcolony of *Nitrotoga* BS is given in Figure 16. In natural or engineered habitats like soil, RAS, or WWTPs, *Nitrotoga*-like bacteria clustered in similar loose aggregates that are less dense than microcolonies of *Nitrosomonas* or *Nitrospira*. All four investigated enrichments of *Nitrotoga* showed the same morphological features. More SEM micrographs are depicted in Appendix III.

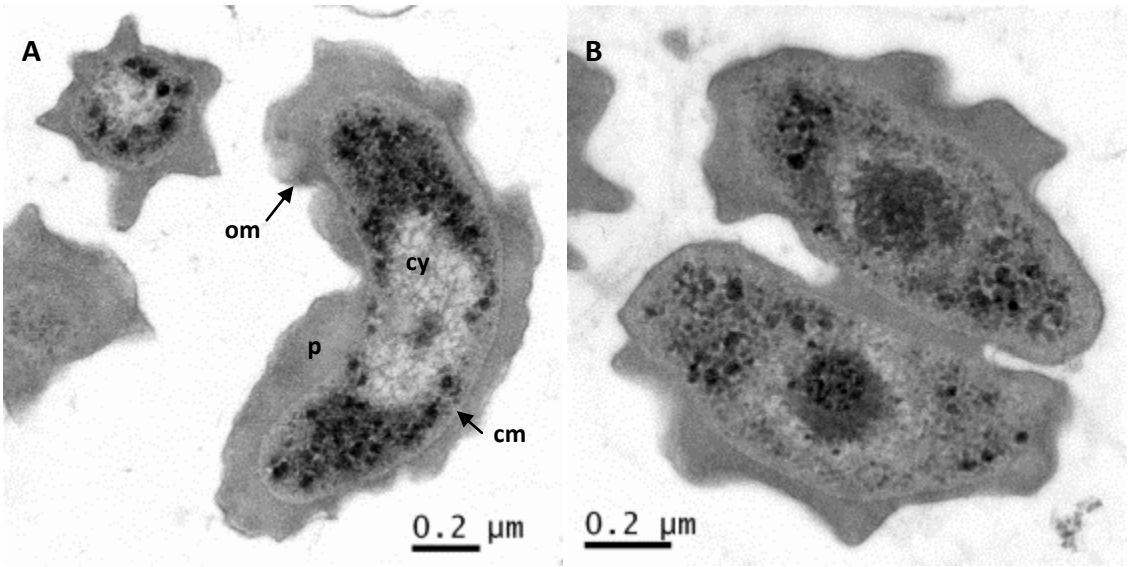

Figure 15: Detailed TEM micrographs of *Nitrotoga* HW and *Ntg. arctica*. **A:** Coccoid and rod shaped cells of *Nitrotoga* HW. The coccoid appearance resulted from orientation of the cell in ultrathin sections. **B:** Paired cells of *Ntg. arctica* connected by the periplasm probably after cell division. Cm = cytoplasmic membrane; cy = cytoplasm; om = outer membrane; p = periplasm.

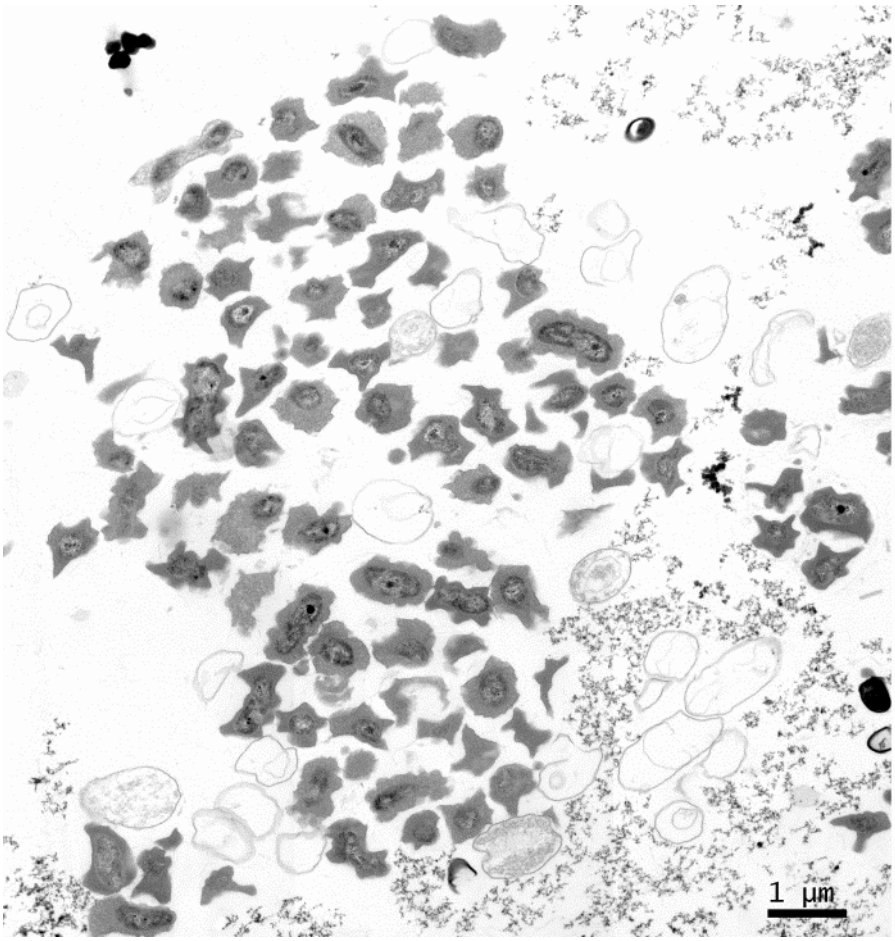

Figure 16: TEM overview of a microcolony of *Nitrotoga* BS that also contained empty cell envelopes. Cells appeared as short, thick, sometimes curved rods, or even as cocci, depending on orientation in ultrathin sections.

In SEM micrographs, all investigated *Nitrotoga* enrichments showed the same features. They appeared as irregular, more coccoid cells, with budding structures as part of the irregular and wide periplasmic space, as can be seen in an overview of *Ntg. arctica* (Figure 17) and a detailed picture of *Nitrotoga* HW (Figure 18). In general, samples were hard to observe since cells were covered by particles that might originate from EPS, and potential contaminations could not be definitely visualized. Some cells were too wrinkled to evaluate their actual appearance, which was probably caused by fixation when the wide periplasm collapsed during dehydration. Further SEM micrographs of all investigated *Nitrotoga* enrichments are shown in Appendix III. The appearance of *Nitrotoga* cultures investigated in this study differs from *Nitrotoga* AM1 that is more homogeneously rod-shaped.[131]

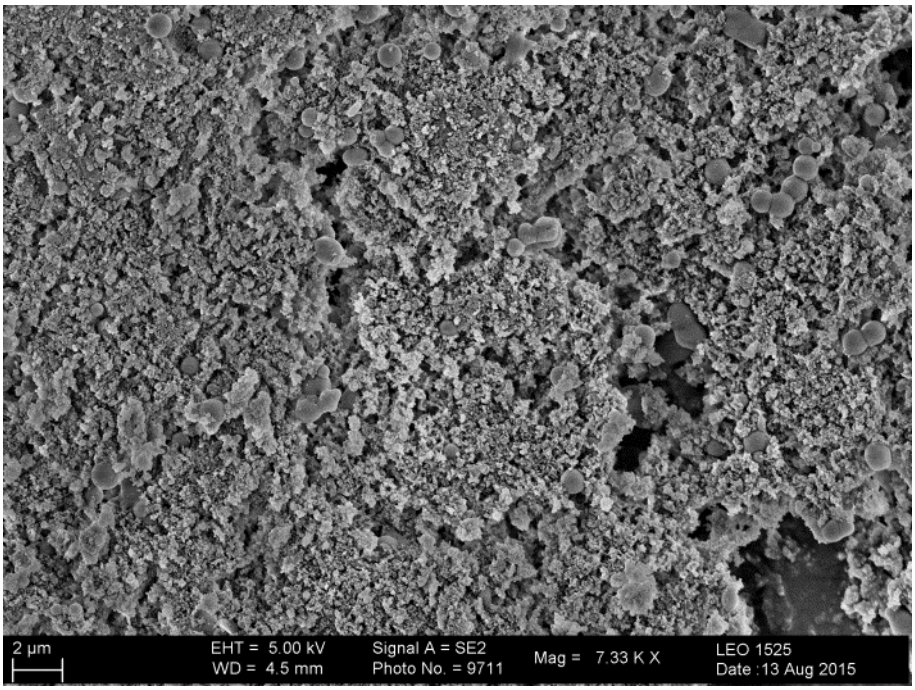

Figure 17: SEM overview of *Ntg. arctica*. For the most part, samples were covered by particles but occasionally, cells were visible amidst or above these particles. They were of irregular shape and appeared more coccoid than in TEM micrographs.

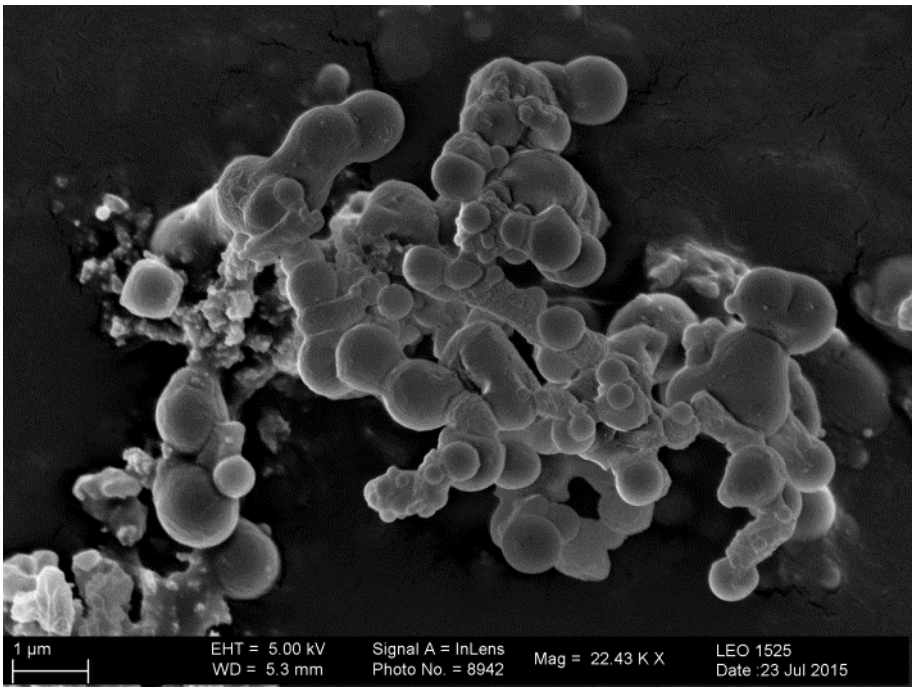

Figure 18: Detailed SEM picture of *Nitrotoga* HW. The cells were of irregular, coccoid shape with small buds. This shape is in accordance with the wide and irregular periplasm seen in TEM micrographs. Cell envelopes seemed to be wrinkled occasionally, probably due to dehydration during sample preparation.

3.5 qPCR – IMPLEMENTATION FOR *NITROTOGA* AND *NSP. DEFLUVII*

The aim qPCR is the quantification of specific DNA fragments in a sample. To this end, a standard plasmid had to be prepared with the target sequence cloned inside. Specific primers were designed based on the same sequence. Since *Nsp. defluvii* and *Ntg. arctica* had to be compared in co-cultivation experiments, they were chosen as source for DNA standards of the 16S rRNA gene.

Several positive clones were picked per NOB, PCR products of correct size (Figure 19) were sequenced, and plasmids were isolated from clones *Nitrotoga*-8 and *Nitrospira*-15 that both had 100% sequence similarity to *Ntg. arctica* DQ839562.1 and *Nsp. defluvii* NR_074700.1, respectively. Based on these plasmids, primer pairs Ntg8-2F/R and Nsp15F/R (Table 9) were designed. The amplified products had a size of about 160 and 170 bp for *Nitrotoga* and *Nsp. defluvii*, respectively.

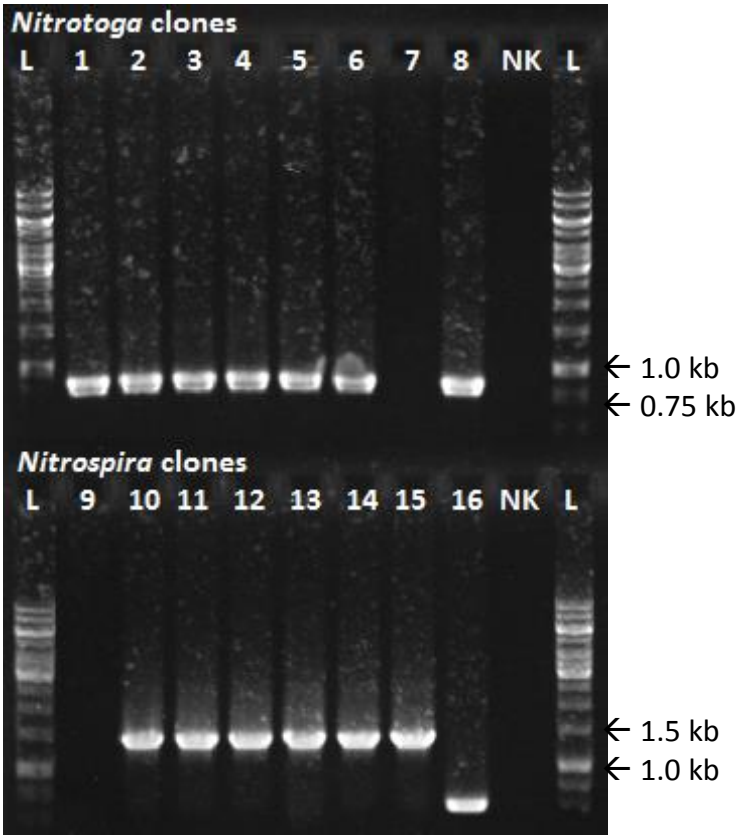

Figure 19: *Nitrotoga* clones 1 to 8 and *Nsp. defluvii* clones 9 to 15. L = Ladder 1 kb, NK = negative control without DNA. Inserts were amplified using SP6/T7 primer. Correct DNA-bands for *Nitrotoga* are located between 0.75 and 1 kb and for *Nsp. defluvii* between 1 and 1.5 kb. No plasmid in Ntg7 and Nsp9, wrong insert in Nsp16.

Test reactions were performed for both NOB to verify the designed primers and to estimate the quality of the overall reaction.

Exemplary reaction for *Nsp. defluvii* (Figure 20)

The reaction was performed with plasmids containing the 16S rRNA gene fragment from *Nsp. defluvii*, ten-fold diluted from 10^7 down to 10^2 copies/µl. Furthermore, a negative control without DNA, a non-template control (NTC) containing genomic DNA from *Ntg. arctica*, and a positive control containing genomic DNA of *Nsp. defluvii* were tested in one qPCR run. A similar exemplary qPCR run for standards of *Ntg. arctica* is shown in Appendix IV 9.

The upper left picture (Figure 20A) shows the amplification signals for plasmids ranging from 10^7 copies/µl (far left curves) down to 10^2 copies/µl (far right curves). The fluorescence of SYBR Green, measured as relative fluorescence units (RFU), is correlated to the cycle number. The lower horizontal line at 0 RFU indicates the baseline. Signals from NTC and negative control should not exceed this line. The fluorescence increases exponentially during amplification, eventually crossing the threshold for quantification Cq (upper horizontal line at about 300 RFU). This threshold cycle is correlated to the copy number per µl and used for calibration and quantification. Signals go into stationary phase when amplification stops due to shortage of reaction compounds.

The upper right picture (Figure 20B) contains the standard curve generated from signals of standard plasmids. Efficiency (E), correlation coefficient (r^2), and slope are indicated. These values are used for quality control of the reaction (MIQE guidelines[190]).

The lower left picture (Figure 20C) shows the melt curve for each sample. At lower temperatures, the signal of SYBR Green is intense, since the DNA is double stranded. At the melting point when the double strand dissociates completely, a sharp decline in signal is observed. The horizontal lines are signals of NTC and negative controls that contain no PCR product and consequently show no change in fluorescence.

Data from the melt curves are processed to generate melt peaks, given in the lower right picture (Figure 20D). The rate of signal change (d(RFU)/dT) is correlated to the temperature. The peaks top at the same temperature for all samples, indicating a clean PCR product with no contaminations. Again, the lower horizontal line contains NTC and

negative controls. Samples with contaminations or primer dimers would show a defined peak at lower temperatures as well. This was not observed during reactions in this study.

The designed primer for *Nitrotoga* and *Nitrospira* yielded good results for standard plasmids. There were no reactions with the NTC and the negative control. Genomic DNA of the targeted NOB was successfully recognized. Melt curve analysis confirmed the correct template in all reactions.

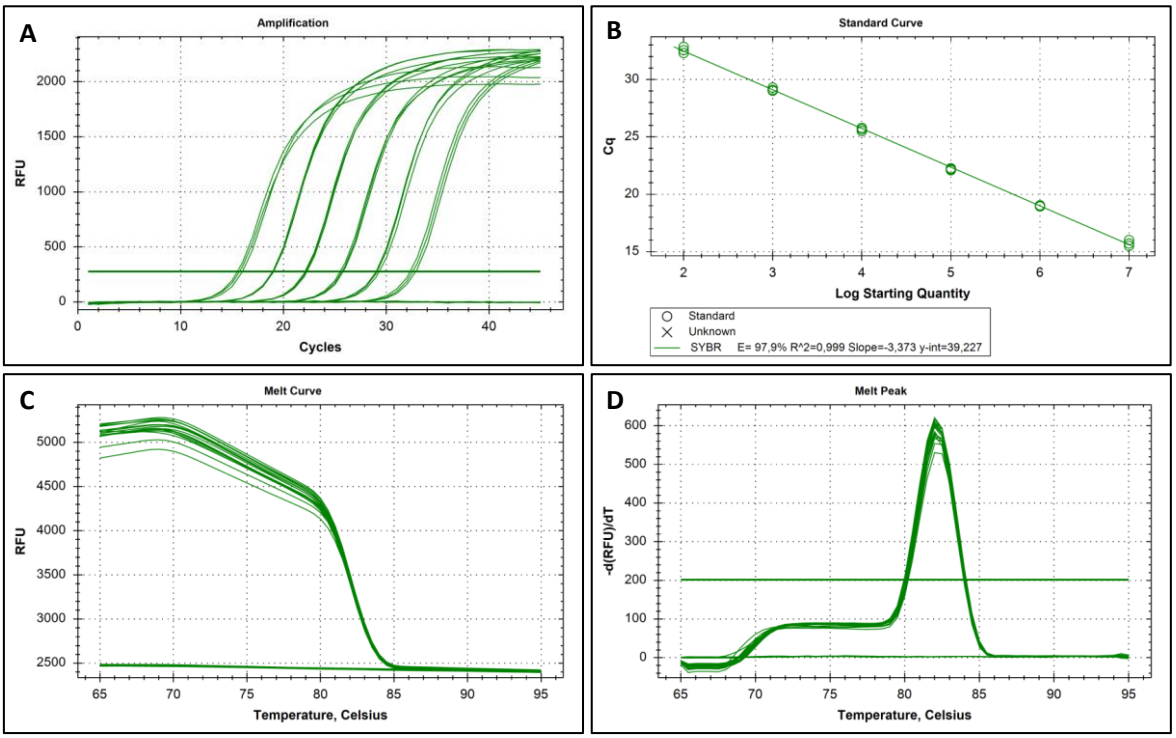

Figure 20: Exemplary qPCR run with standards for *Nsp. defluvii*, pictures were exported from software. **A:** Amplification curves for each standard and controls. Samples with identical dilution cluster together at the same reaction cycle, since they contain the same amount of DNA template. The positive control was excluded from the diagram to simplify matters. **B:** Regression curve calculated from standards. The box under the curve contains efficiency (E) of the reaction, regression coefficient (r^2 = R^2), and the slope. **C:** Melt curves of all samples after the last qPCR cycle. **D:** Melt peak of all samples derived from corresponding melt curves.

3.6 CO-CULTIVATION EXPERIMENTS – *NITROTOGA* VS. *NITROSPIRA*

3.6.1 GENERAL RESULTS

The aim of co-cultivation experiments was to gain further insights into niche differentiation between *Nitrotoga* and *Nitrospira* at given pH and temperature conditions. *Nsp. defluvii* was combined with either *Ntg. arctica* or *Nitrotoga* BS in three independent processes. Bioreactors were operated at 17°C and either pH 6.4 or 7.4 to

cover optimum pH for *Ntg. arctica*, *Nitrotoga* BS, and *Nsp. defluvii*. The medium contained 1 mM substrate and 0.1 mM ammonium to avoid inhibition of *Nitrotoga* BS.

Some results were consistent in all experiments and did not depend on NOB combination. Only minor pH shifts were observed during cultivation (± 0.2). Nitrite was stoichiometrically oxidized to nitrate and ammonium was not consumed in detectable amounts (not shown). No oxygen limitation occurred: dO levels followed nitrite consumption but increased to near saturation after its oxidation (Appendix IV). Most noticeable, nitrite oxidation was faster at pH 7.4 compared to 6.4, including shorter lag-phases. Correspondingly, nitrite turnover during exponential phase was faster at pH 7.4 for both NOB combinations. Obtained results and data for each run are summarized in Table 22 (*Nitrotoga* BS and *Nsp. defluvii*) and in Table 23 (*Ntg. arctica* and *Nsp. defluvii*).

Table 22: Process parameters and results for co-cultivation of *Nitrotoga* BS and *Nsp. defluvii*. * pH before inoculation; # until 1 mM nitrite was completely oxidized. Relative abundance of NOB calculated from qPCR results. Absolute values are listed in Appendix IV 7.

Run	pH*	pH start	pH end	Nitrite turnover [µm NO_2^-/d]	Duration [d]#	Relative abundance			
						Ntg. BS [%]		*Nsp. defluvii* [%]	
						Start	End	Start	End
A	**6.4**	6.42	6.28	120	22	65.4	**13.4**	34.6	**86.6**
	7.4	7.40	7.31	267	9		**94.9**		**5.1**
B	**6.4**	6.48	6.48	182	18	47.7	**15.4**	52.3	**84.6**
	7.4	7.34	7.35	332	7		**95.4**		**4.6**
C	**6.4**	6.41	6.57	164	17	12.7	**61.8**	87.3	**38.2**
	7.4	7.41	7.46	265	10		**94.3**		**5.7**

Table 23: Process parameters and results for co-cultivation of *Ntg. arctica* and *Nsp. defluvii*. *pH before inoculation; # until 1 mM nitrite was completely oxidized. Relative abundance of NOB calculated from qPCR results. Absolute values are listed in Appendix IV 8.

Run	pH*	pH start	pH end	Nitrite turnover [µm NO_2^-/d]	Duration [d]#	Relative abundance			
						Ntg. arctica [%]		*Nsp. defluvii* [%]	
						Start	End	Start	End
A	**6.4**	6.43	6.47	65	20	36.4	**69.0**	63.6	**31.0**
	7.4	7.33	7.23	120	13		**60.8**		**39.2**
B	**6.4**	6.43	6.55	89	37	18.1	**18.2**	81.9	**81.8**
	7.4	7.41	7.17	186	17		**55.2**		**44.8**
C	**6.4**	6.43	6.56	105	13	28.6	**30.3**	71.4	**69.7**
	7.4	7.40	7.32	148	9		**78.4**		**21.6**

3.6.2 *NITROTOGA* BS VS. *NSP. DEFLUVII*

Nitrotoga BS clearly dominated all three processes at pH 7.4 with at least over 94% of NOB content and it increased during each repeat compared to the relative abundance at inoculation (Table 22, Figure 22). Apart from run B, absolute copy numbers of *Nitrotoga* BS were higher at pH 7.4 than at pH 6.4. Correspondingly, *Nsp. defluvii* reached lower copy numbers at pH 7.4 than at pH 6.4. It constituted the NOB majority under more acidic conditions, except for repeat C (Figure 21). FISH micrographs confirmed the NOB distribution for each run (Figure 22). *Nsp. defluvii* formed characteristic microcolonies, whereas *Nitrotoga* BS occurred in more loose aggregates or as single cells.

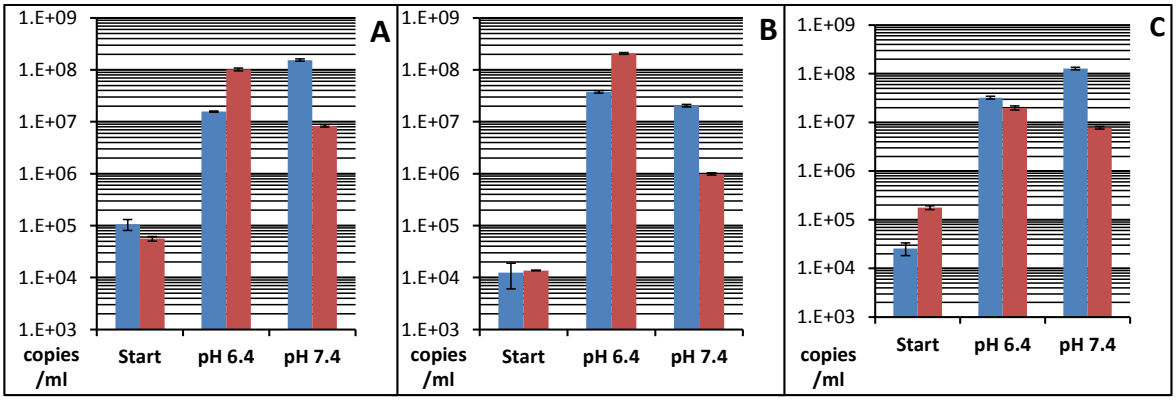

Figure 21: Copy numbers at process start and after co-cultivation for *Nitrotoga* BS (blue) and *Nsp. defluvii* (red). Three independent repeats are indicated by **A**, **B**, and **C**.

Nitrite and nitrate levels as well as dO of each process are summarized in Appendix IV 1-3. Copy numbers are listed in Appendix IV 7. Initial copy numbers per ml varied between 10^4 and 10^5 but never exceeded a factor of ten between *Nitrotoga* and *Nitrospira*. After co-cultivation, 10^7 to 10^8 copies per ml were reached except for *Nsp. defluvii* at pH 7.4 in process B with about 10^6 copies per ml.

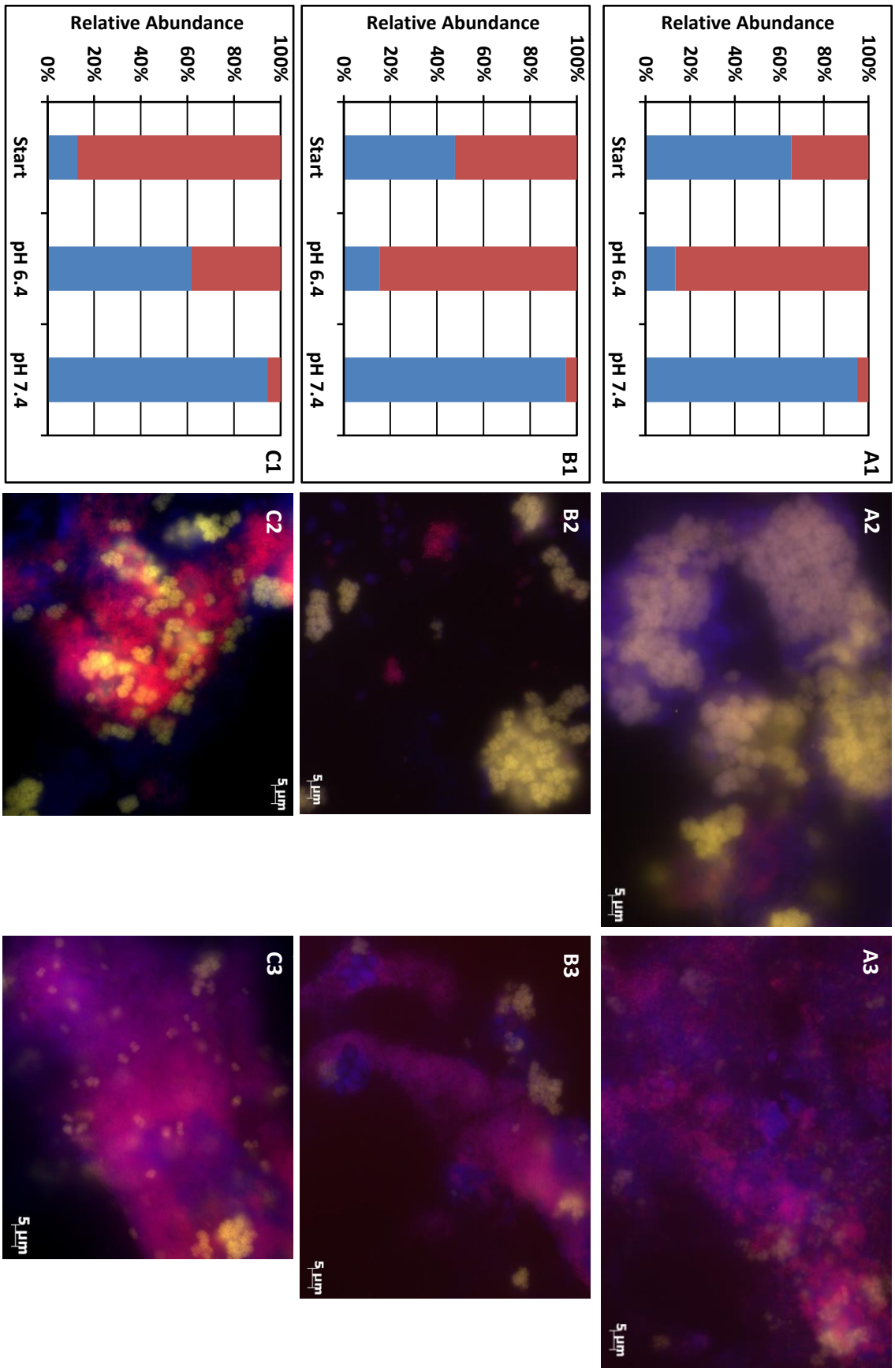

Figure 22: Co-cultivation of *Nitrotoga* BS and *Nsp. defluvii*. Three independent repeats are indicated by **A**, **B**, and **C**. **1:** Distribution of *Nitrotoga* BS (blue) and *Nsp. defluvii* (red) before and after complete oxidation of 1 mM nitrite under both pH conditions calculated by qPCR. **2:** Exemplary FISH micrographs for pH 6.4 and **3:** for pH 7.4. *Nitrotoga* BS = Cy3, magenta; *Nsp. defluvii* = Cy5, yellow; all cells = DAPI, blue.

3.6.3 *NTG. ARCTICA* VS. *NSP. DEFLUVII*

When co-culturing these two NOB, *Ntg. arctica* dominated at pH 7.4 as well. It increased under this condition compared to the inoculum and reached higher copy numbers than at pH 6.4. Except for run A, copy numbers of *Nsp. defluvii* were higher under acidic conditions (Table 23, Figure 23). Run A was also the only instance at which *Nsp. defluvii* decreased at pH 6.4, whereas the other two slightly acidic processes showed no shift in relative NOB abundance compared to the inoculum. FISH micrographs confirmed the NOB distribution for each run (Figure 24). Again, *Nsp. defluvii* was distinguishable from *Ntg. arctica* by formation of dense microcolonies.

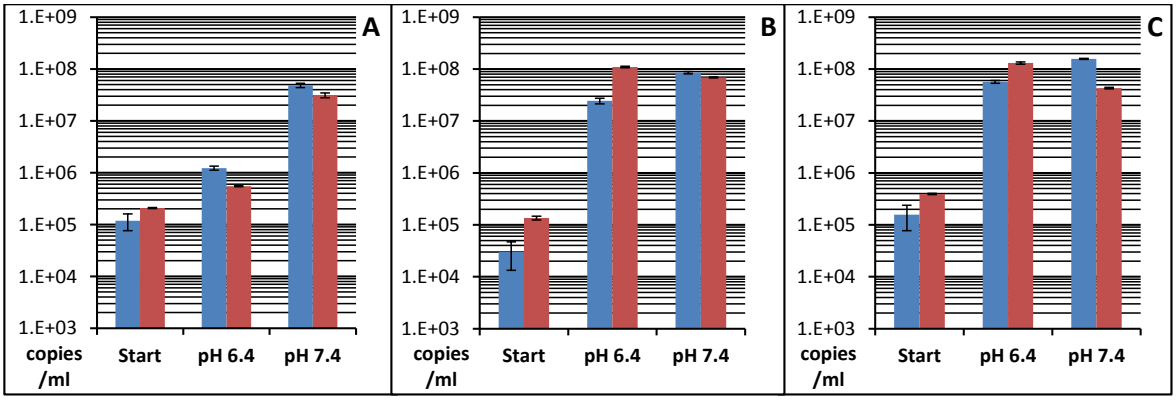

Figure 23: Copy numbers at process start and after co-cultivation for *Ntg. arctica* (blue) and *Nsp. defluvii* (red). Three independent repeats are indicated by **A**, **B**, and **C**.

Nitrite and nitrate levels as well as dO of each process are summarized in Appendix IV 4-6. The copy numbers are listed in Appendix IV 8. Initial copy numbers per ml fluctuated about 10^5, but never exceeded a factor of ten between *Nitrotoga* and *Nitrospira*. After cultivation, copies per ml increased up to between 10^7 to 10^8 except for run A at pH 6.4 with lower cell contents of approximately 10^6 copies per ml.

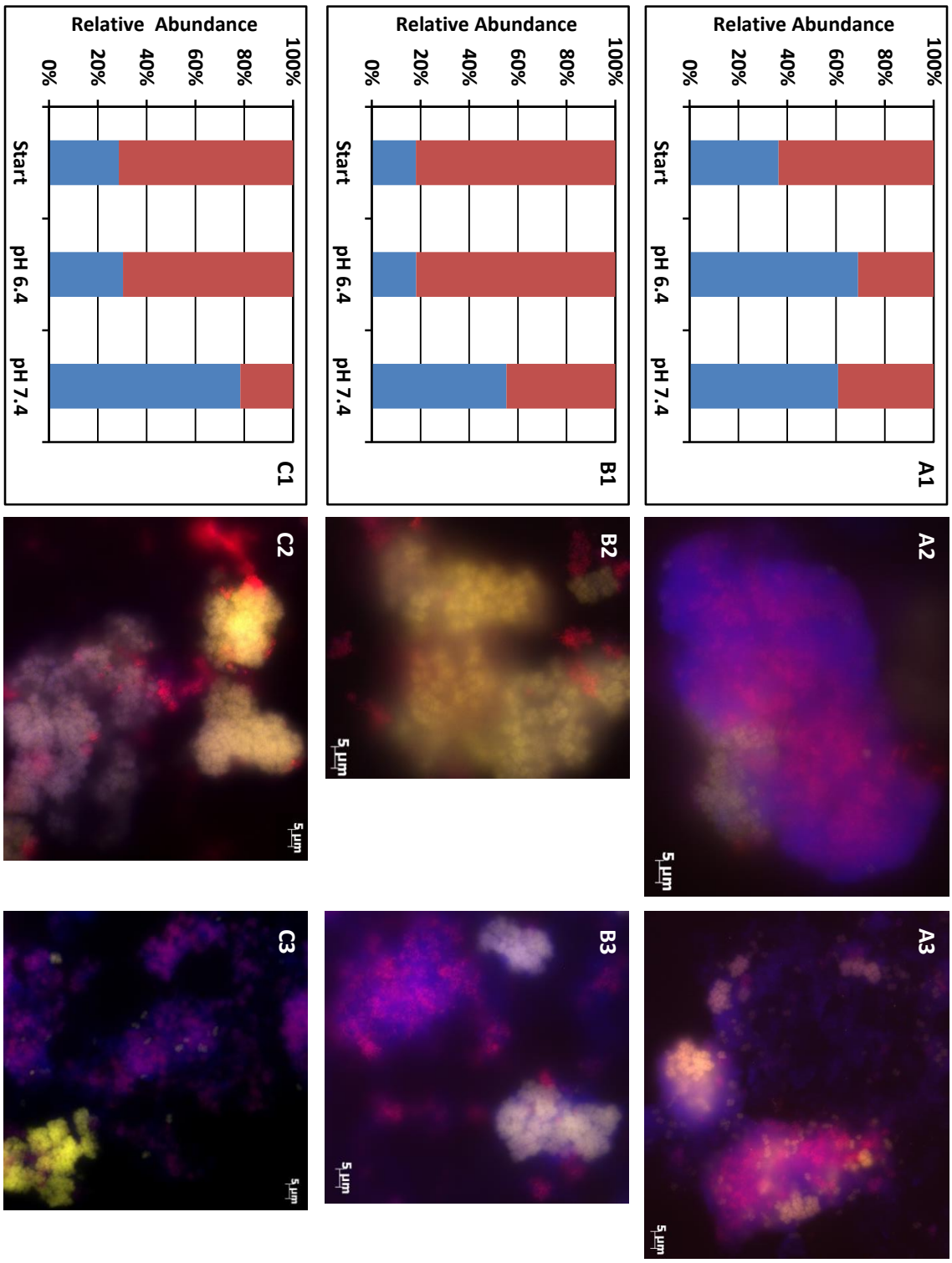

Figure 24: Co-cultivation of *Ntg. arctica* and *Nsp. defluvii*. Three independent repeats are indicated by **A**, **B**, and **C**. **1:** Distribution of *Ntg. arctica* (blue) and *Nsp. defluvii* (red) before and after complete oxidation of 1 mM nitrite under both pH conditions calculated by qPCR. **2:** Exemplary FISH micrographs for pH 6.4 and **3:** for pH 7.4. *Ntg. arctica* = Cy3, magenta; *Nsp. defluvii* = Cy5, yellow; all cells = DAPI, blue.

3.7 MONITORING NITRIFICATION IN RAS

The investigated RAS was re-started after a complete overhaul and addition of a new biofilter unit (major biofilter). Since monitoring of the previous process in the same facility revealed *Nitrotoga* as an important NOB in the biofiltration process,[34] the reconstruction was a good opportunity to observe the establishment of the NOB community, with a focus on *Nitrotoga*, and the course of nitrifying activity. Furthermore, recycled biocarriers from the previous run were used in the two smaller biofilters to potentially facilitate biofilm formation on the material. In the course of the process, disinfection with Wofasteril based on PAA and H_2O_2 was established, and its influence on nitrification was evaluated. Averaged water parameters over two years process duration are summarized in Table 24. The progress of these parameters it depicted in Figure 25.

Table 24: Water parameters and stocking in the rainbow trout RAS, November 04th 2013 to October 27th 2015.

Parameter	Mean ± SD	Min	Max
Temperature [°C]	13.2 ± 1.6	10.4	17.1
pH value	6.8 ± 0.01	6.8	7.0
Ammonium	0.55 ± 0.73	0.0	5.9
Nitrite [mg/l]	0.56 ± 0.30	0.0	2.2
Nitrate [mg/l]	73.0 ± 31.8	12.6	169.0
Fish [t]	12.6 ± 5.8	0.3	25.7
Fish [pc * 1000]	77.4 ± 24.9	2.5	123.2
Feed [kg/d]	127.4 ± 59.9	0.0	245.0

The amount of cultivated fish increased to its maximum of about 25 t (July and August 2014) but was reduced afterwards. The feed per day followed this course and was occasionally limited to counter high levels of ammonium or nitrate. Especially during the first two months of operation, elevated ammonium levels were observed, peaking at about 6 mg/L at the end of December 2013. This was prevented by addition of the major biofilter in January 2014, and overall ammonium contents stayed below 1 mg/L afterwards. The highest nitrite and nitrate concentrations were observed during times of maximum fish stocking in July and August 2014. Generally, nitrate levels exceeded ammonium and nitrite concentrations by far. Temperature and pH were stable over two years of rainbow trout rearing.

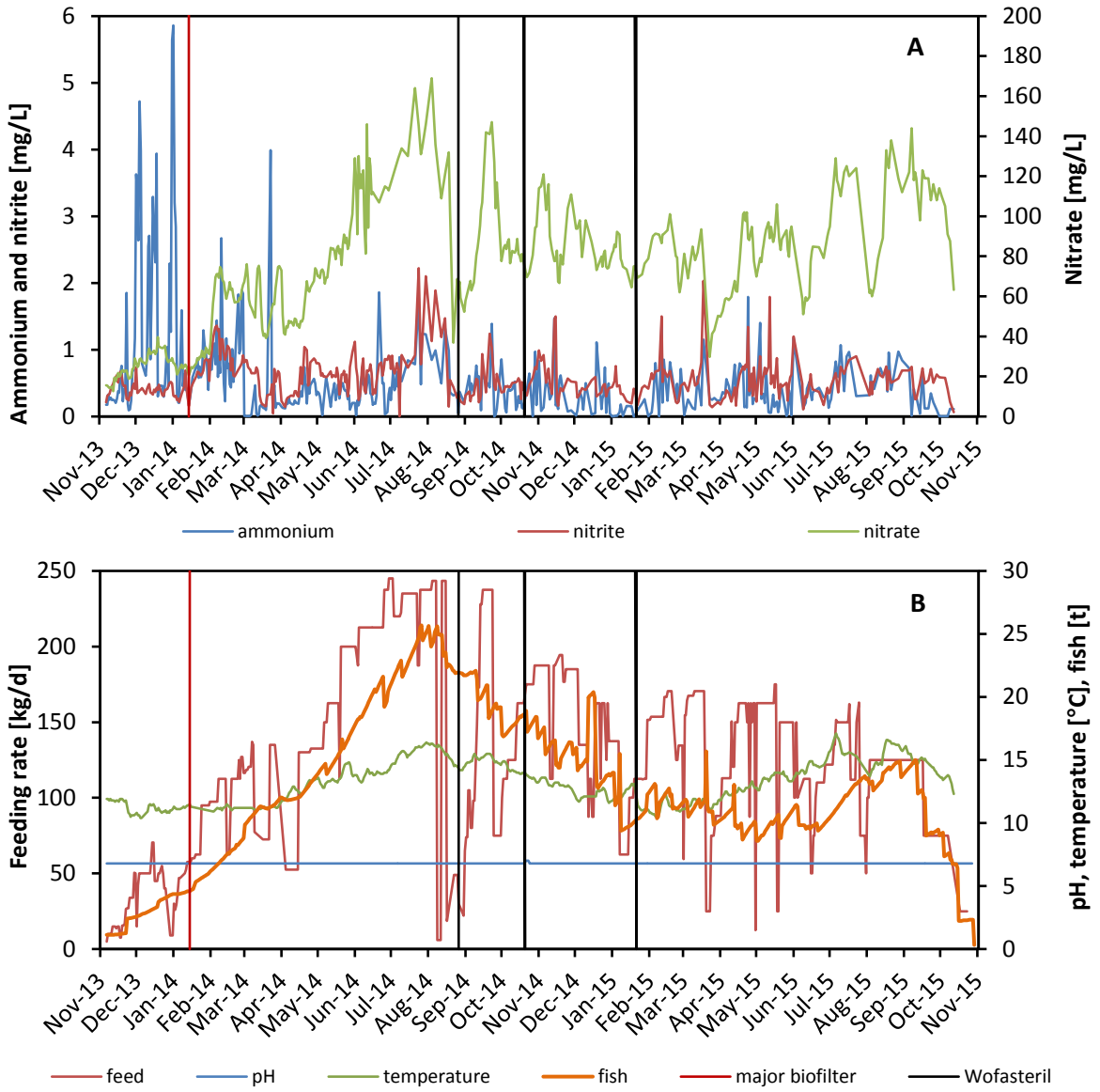

Figure 25: Concentration of ammonium, nitrite, and nitrate (**A**), as well as temperature, pH, feeding rate, and fish stocking (**B**) during two years of rainbow trout cultivation in the RAS at Hohen Wangelin. The red vertical line indicates the addition of the major biofilter for water purification; the black lines illustrate disinfection with Wofasteril.

3.7.1 NITRIFYING ACTIVITY IN BIOFILTERS AND INFLUENCE OF DISINFECTION

A stable nitrifying potential developed shortly after the new process started (Figure 26). In general, NOB showed higher activities than AOB but were more sensitive to Wofasteril with a visible decrease of nitrite turnover shortly after disinfection steps. The breakdown of NOB activity at the end of August 2014 was caused by higher amounts of Wofasteril that cut the turnover in half compared to previous measurements in June 2014. AOB were not that severely affected. The application of reduced amounts of disinfectant had no negative influence on the overall nitrifying performance. NOB activities remained

stable or increased after Wofasteril input. Regarding the overall process, AOB and NOB reached average activities about 11.8 ± 1.3 g/hm³ and 16.4 ± 1.8 g/hm³, respectively. After about two month, the major biofilter reached similar or higher turnover rates compared to the smaller biofilters left and right. All obtained activities are listed in Appendix V 1

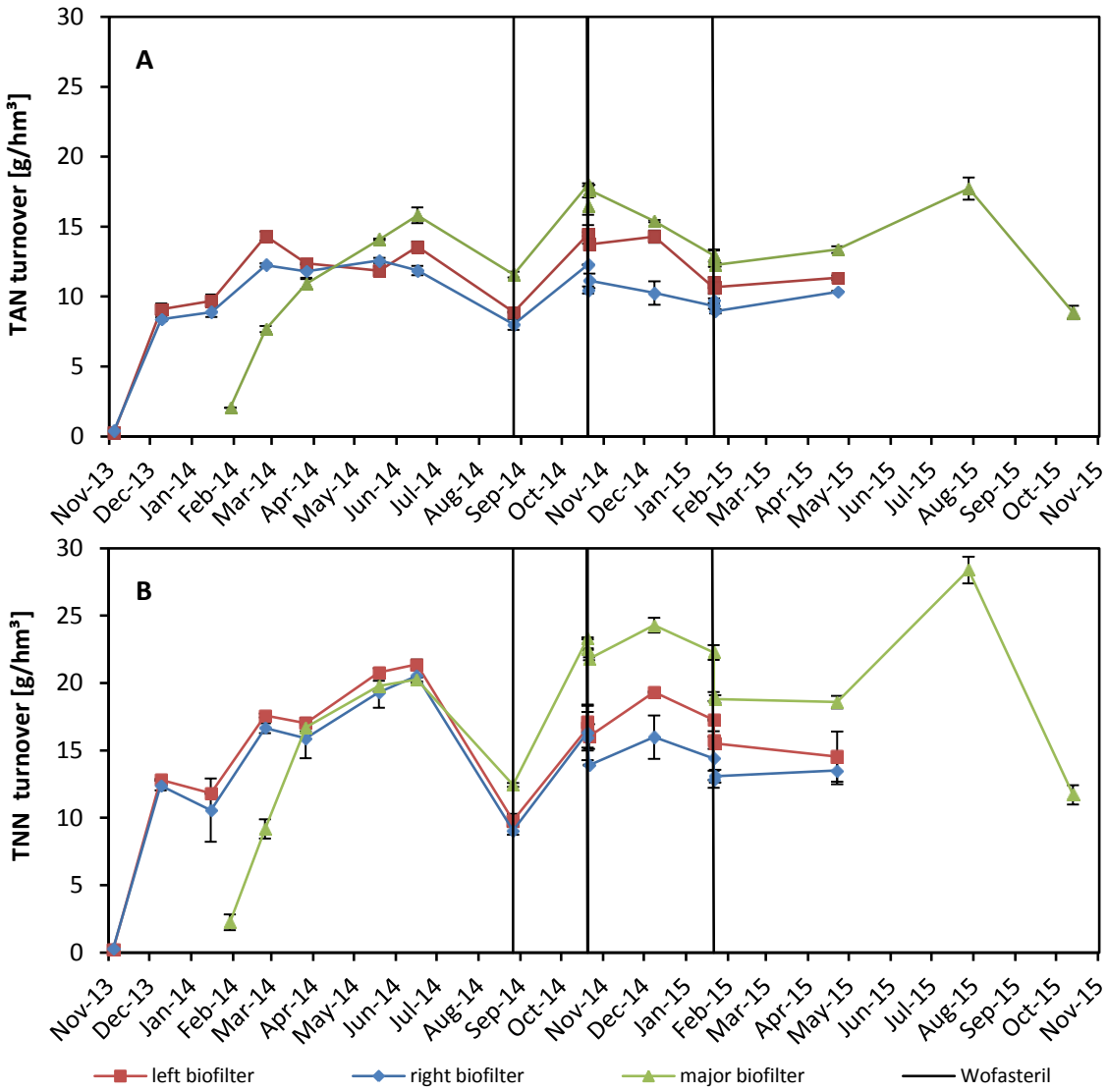

Figure 26: Potential nitrifying activity in the three biofilter modules (left, right, major) of the rainbow trout RAS. Vertical black lines indicate disinfection treatment with Wofasteril. **A:** Activity of the AOB calculated as consumed TAN in g per h and m³ biofilter. **B:** Activity of the NOB calculated as consumed TNN in g per h and m³ biofilter. The major biofilter was connected in January 2014. The left and right biofilters were disconnected from the process in May 2015 due to decreased fish stocking. Negative controls without biocarriers showed no nitrite consumption (results not shown). Mean and SD calculated from n = 2. SD partially too small to see.

3.7.2 NITRIFYING COMMUNITY

The nitrifying community was embedded in a thick brown biofilm colonizing the biocarriers (Figure 27). Population was analyzed by different methods after harvesting the biofilm.

Figure 27: Biocarriers from the biofilter of the rainbow trout RAS colonized by a thick biofilm, sampled in October 2014. **A:** left biofilter; **B:** right biofilter; both with types BCN 012 KLL (big black carrier) and BCN 009 (smaller black and white carriers); **C:** major biofilter with type BCN 012 KLL. The biofilm is visible as a brown structure within the compartments of the carriers. Bar = 1 cm.

By PCR with specific or semi-specific primer pairs for the 16S rRNA gene, *Nitrospira* and members of the Nitrosomonadaceae were always detected. Results were confirmed by PCR with specific primer pairs for *nxrB* of *Nitrospira* and betaproteobacterial *amoA*. Sequence-based discrimination between *Nitrosomonas* and *Nitrosospira* was not possible. *Nitrotoga* was only detectable before disinfection was implemented in August 2014. PCR products of *Nitrobacter* usually resulted in non-analyzable sequences. Results of community evaluation by PCR are summarized in Table 25. Next described relatives of selected sequences are listed in Appendix V 2.

By FISH, *Nitrotoga*, *Nitrospira*, and *Nitrosomonas* were found in exemplary samples from May 2014 (Figure 28). *Nitrotoga* was no longer detectable after disinfection was implemented (results not shown).

Table 25: Detected AOB and NOB by PCR. + positive reaction; - no reaction; +- weak reaction; * disinfection step with Wofasteril; n.d. not determined.

Sampling date	Nitrosomona-daceae	β-proteobac-terial *amoA*	*Nitrotoga*	*Nitrospira*	*Nitrospira nxrB*
04-Nov-13	+	+	+	+	-
09-Dec-13	+	+	+-	+	+
15+29-Jan-14	+	+	+	+	+
24-Feb-14	+	n.d.	+	+	n.d.
26-Mar-14	+	+	+-	+	+
19-May-14	+	+	+	+	+
16-Jun-14	+	+	+	+	+
26-Aug-14*	+	+	+-	+	+
20-Oct-14*	+	+	-	+	+
08-Dec-14	+	n.d.	-	+	n.d.
21-Jan-15*	+	+	-	+	+
22-Apr-15	+	+	-	+	+
28-Jul-15	+	+	-	+	+
13-Oct-15	+	+	-	+	+

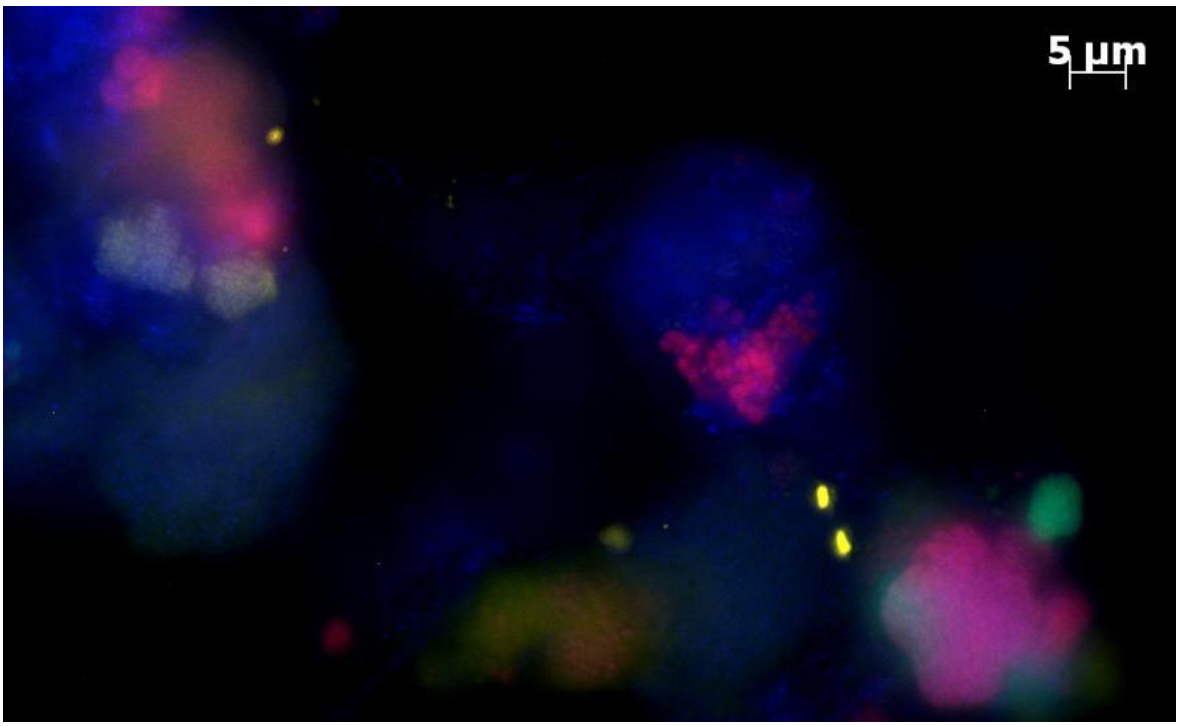

Figure 28: FISH of the major biofilter sampled in May 2014. *Nitrotoga*, *Nitrospira*, and *Nitrosomonas* coexisted in the biofilm. *Nitrosomonas* = Cy3, magenta; *Nitrotoga* = Cy5, yellow; *Nitrospira* = FITC, green; all cells = DAPI, blue. The yellow signal in the lower center of the picture is not *Nitrotoga* but background fluorescence of the sample while recording the FITC signal.

In TEM micrographs of fixed biofilm from May 2014 (Figure 29), loose aggregates of *Nitrotoga* were found together with microcolonies of *Nitrospira* and *Nitrosomonas*. However, samples dating from April 2015 no longer contained *Nitrotoga*-like cells. *Nitrospira* and *Nitrosomonas* were still present (Figure 30) together with microcolonies of *Nitrosospira*-like bacteria (Figure 31).

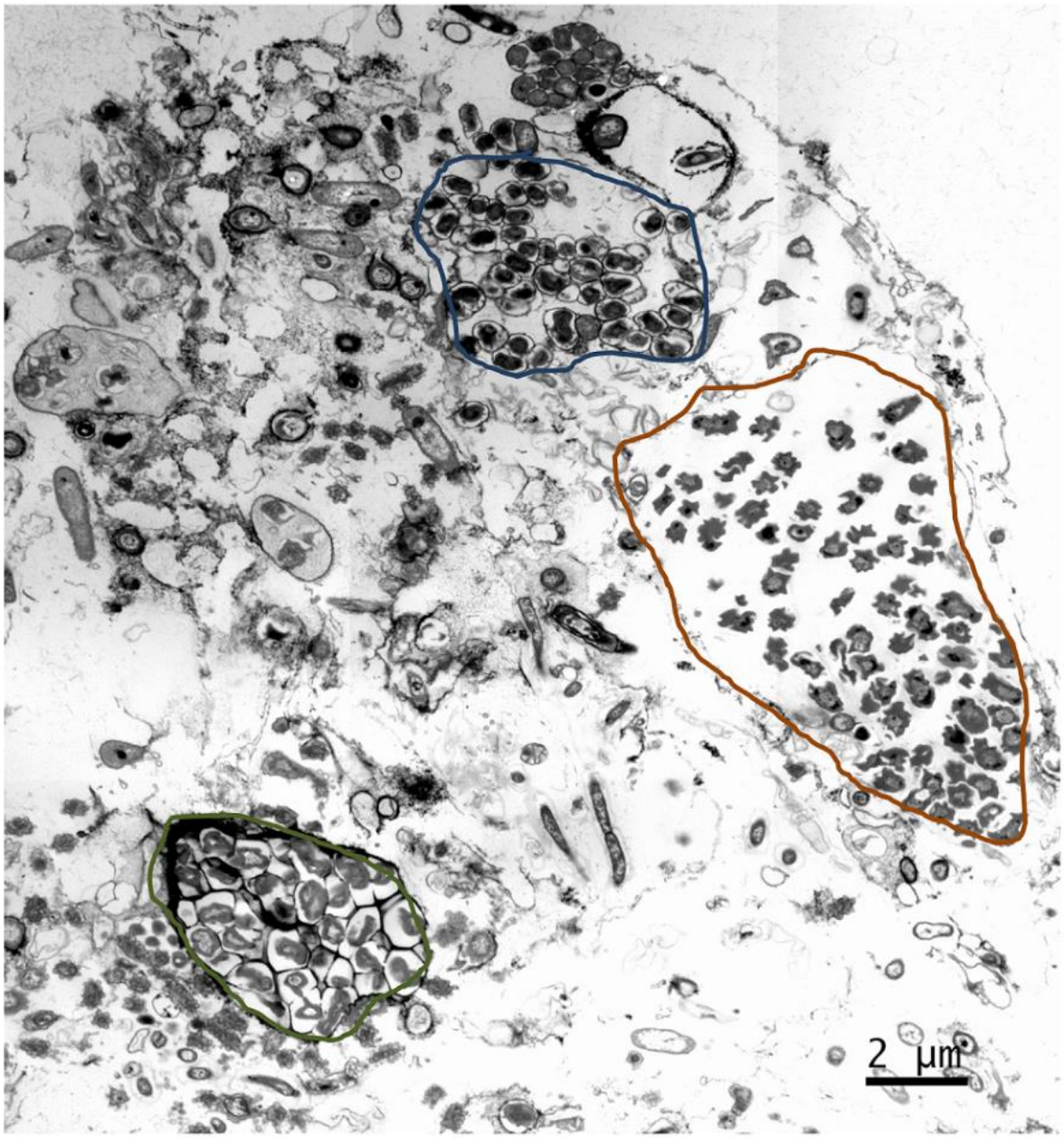

Figure 29: TEM micrograph of fixed biofilm from the major biofilter of the rainbow trout RAS sampled in May 2014. Microcolonies of *Nitrotoga* are marked in brown (center right), *Nitrospira* in blue (center top), and *Nitrosomonas* in green (lower left corner).

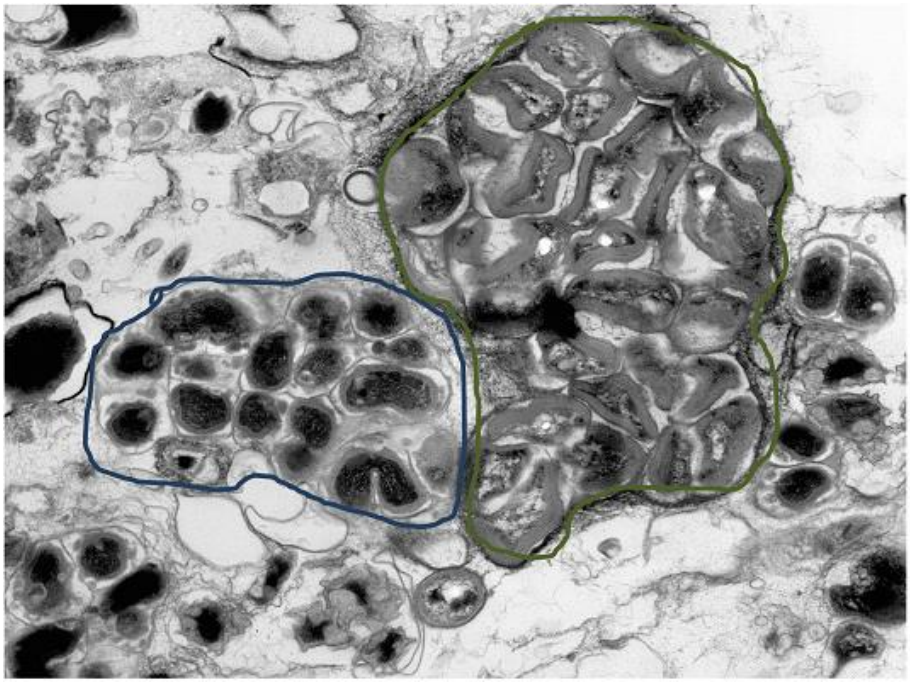

Figure 30: TEM micrograph of fixed biofilm from the major biofilter of the rainbow trout RAS sampled in April 2015. Microcolonies of *Nitrospira* and *Nitrosomonas* are marked in blue and green, respectively.

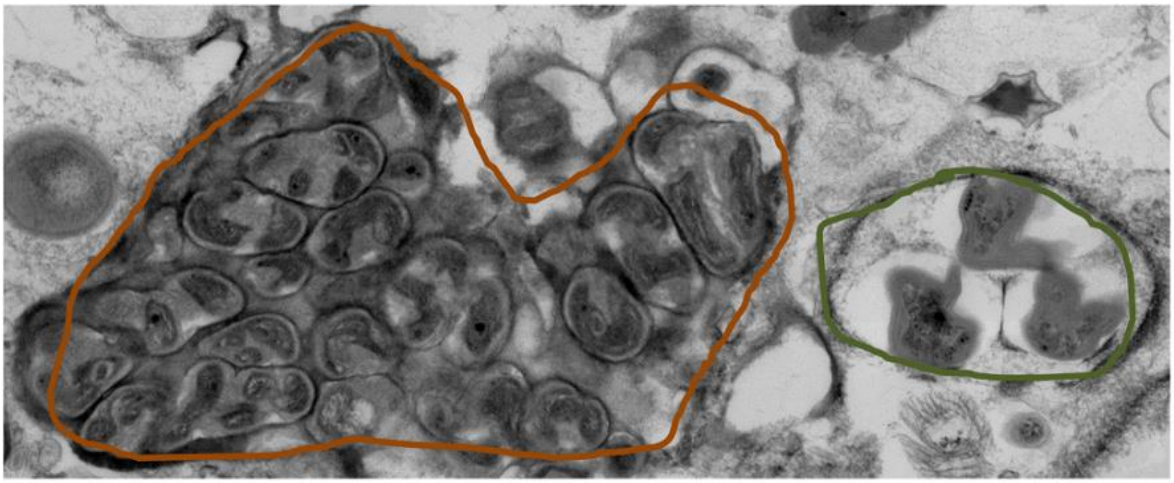

Figure 31: TEM micrograph of fixed biofilm from the major biofilter of the rainbow trout RAS sampled in April 2015. Microcolonies of *Nitrosospira*-like cells and *Nitrosomonas* are marked in orange and green, respectively.

Concluding, AOB and NOB represented by *Nitrosomonas* and *Nitrospira* were consistently present during the process, ensuring complete nitrification. There was a notable change after the implementation of disinfection with Wofasteril in August 2014. *Nitrotoga* was suppressed and no longer detectable, whereas it was abundant before. In exchange, a considerably amount of *Nitrosospira*-like cells emerged in TEM micrographs that were, however, not distinguishable from *Nitrosomonas* by molecular methods.

3.7.3 NITRIFICATION IN RAS UNDER CHANGING PHYSIOLOGICAL CONDITIONS

The influence of temperature and pH on potential nitrite oxidizing activity in the rainbow trout RAS was tested with biocarriers sampled from the major biofilter in June 2014. Two tests ranging from pH 6.3 to 8.1 were conducted at 10 and 17°C. Activity was lower at 10°C with a mean nitrite turnover of 16.8 ± 1.7 g/hm³ compared to 26 ± 3.9 g/hm³ at 17°C and an averaged ratio of 1.5 between 17 and 10°C. However, pH had no visible influence. Activity stayed nearly constant, especially at 10°C. At 17°C, maximum activities were reached at pH 7.2, 7.4, and 7.8.

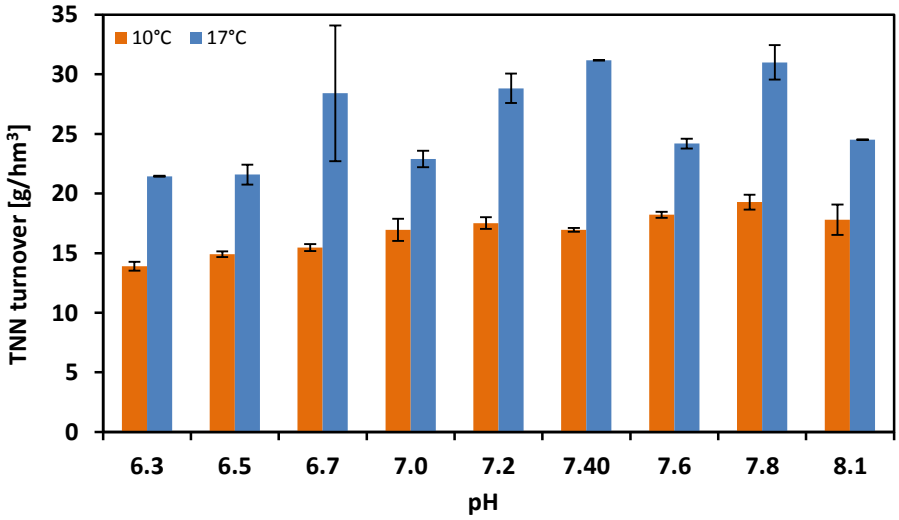

Figure 32: Influence of pH on potential nitrite oxidation in the rainbow trout RAS determined at 10 and 17°C. Negative controls without biocarriers showed no activity (results not shown). Mean and SD calculated from n = 2.

This test confirmed the higher nitrifying potential at 17°C that was also calculated from a previous test performed at 10 and 17°C with all three biofilters, sampled in February 2014 (Table 26). The major biofilter was just connected to the RAS one month before, thus its absolute activities were lower compared to the other biofilters.

Table 26: Turnover of ammonium and nitrite at 10 and 17°C and ratio of nitrifying potential at 17°C compared to 10°C.

	Biofilter	10°C	17°C	Ratio 17°C/10°C
TAN turnover [g/hm³]	Left	10.7 ± 0.1	14.3 ± 0.3	1.34
	Right	8.5 ± 0.4	12.3 ± 0.1	1.45
	Major	4.3 ± 0.2	7.7 ± 0.2	1.79
TNN turnover [g/hm³]	Left	16.2 ± 0.4	17.6 ± 0.1	1.09
	Right	13.4 ± 0.2	16.7 ± 0.4	1.25
	Major	6.9 ± 0.1	9.2 ± 0.7	1.33

Nitrate had a visible effect on the activity of NOB in the biofilm (Figure 33). Nitrite turnover decreased with increasing nitrate concentration, and maximum activity occurred without nitrate addition. In contrast, the AOB were not influenced by nitrate with comparable activities over the whole evaluated nitrate range. Biocarriers for this test were sampled from the major biofilter in June 2014.

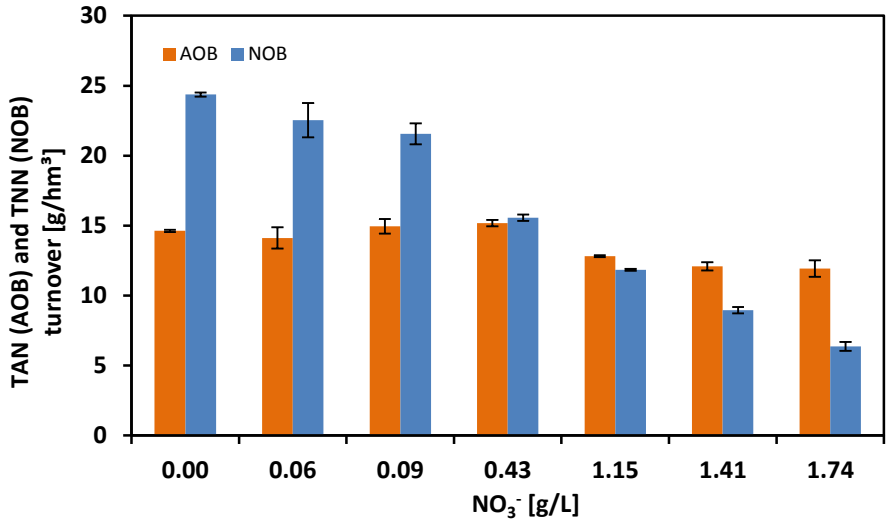

Figure 33: Influence of initial nitrate concentrations on the potential nitrifying activity of AOB and NOB. Negative controls without biocarriers showed no activity (results not shown). Mean and SD calculated from n = 2.

The influence of different ammonium concentrations was only tested for the AOB (Figure 34). Potential activity increased with increasing ammonium up to 25 mg/L and stabilized at higher concentrations. Biocarriers for this test were taken from the major biofilter in October 2014.

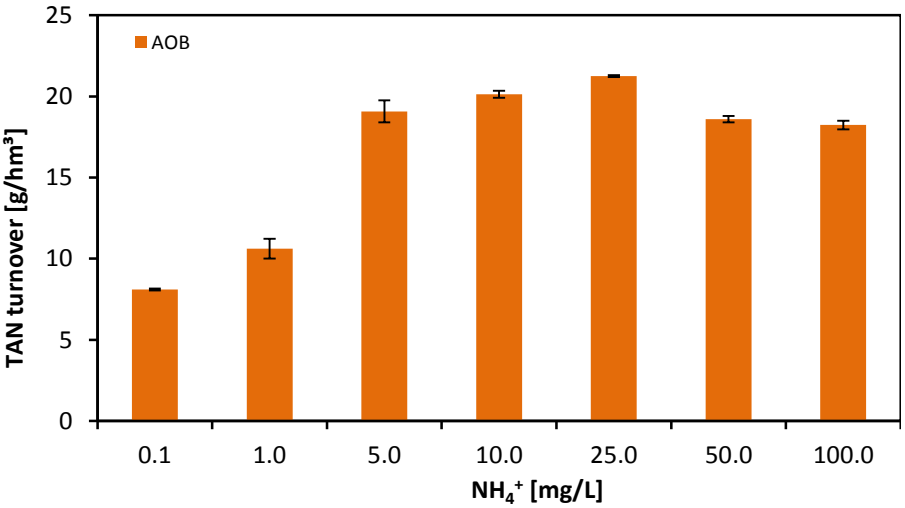

Figure 34: Influence of initial ammonium concentrations on the potential nitrifying activity of AOB. Negative controls without biocarriers showed no activity (results not shown). Mean and SD calculated from n = 2.

4 DISCUSSION

4.1 CULTIVATION OF *NITROTOGA*

4.1.1 OBSERVATIONS DURING CULTIVATION

Cultivation of established and acquisition of new *Nitrotoga* enrichments basically relies on prolonged incubation at temperatures below 20°C.[34,129–131] In this study, all *Nitrotoga* cultures were kept at 17°C, only *Nitrotoga* 1052 was maintained at 22°C (Table 1). The exception was based on the fact that *Nitrotoga* 1052 grew best under this condition and that no further NOB were detected in the culture (pers. comm. E. Spieck). This approach was confirmed by determining 22°C as the optimal growth temperature for *Nitrotoga* 1052 in this study (Table 27). Variation of pH can support the enrichment, as was observed for *Nitrotoga* HW,[34] but was no longer necessary during long-term cultivation. All investigated enrichments were successfully maintained at a pH about 7.4, although *Ntg. arctica* and *Nitrotoga* HW favored lower pH conditions (Table 27).

Nitrotoga grows well in standard autotrophic mineral NOB medium described above (Table 2) but nevertheless, supplementation of further components might be necessary. This was demonstrated for *Nitrotoga* BS that depended on the addition of ammonium (this study, Bachelor thesis A. Schwabauer). However, growth of *Nitrotoga* in autotrophic AOB media (Table 3) with 0.5 mM ammonium as sole energy source was not possible. Under the applied culturing conditions, ammonium was not consumed and consequently no nitrite or nitrate formed.

Similar to *Nitrospir*a, *Nitrotoga* tends to aggregate in culture[85,195] at standard culturing conditions. This behavior was observed in all investigated enrichments except for *Nitrotoga* 1052. In contrast to non-aggregating NOB like *Nitrobacter*,[195] *Nitrotoga* 1052 did not reach cell densities visible to the naked eye. These observations were in accordance with *Nitrotoga* 1052 being the most challenging enrichment if high cell densities were needed. Cell aggregates and formation of microcolonies can enhance the tolerance towards adverse conditions in the environment,[86,210] and the lack of aggregates in *Nitrotoga* 1052 might explain why this culture was more sensitive to N-compounds than the other three enrichments (Table 27).

4.1.2 HETEROTROPHIC BACTERIA IN *NITROTOGA* CULTURES

Each *Nitrotoga* culture investigated in this study harbored distinct concomitant bacteria. Like other NOB, *Nitrotoga* counts as a primary producer and can sustain a community of heterotrophs. It was also shown that this community is quite stable during subcultivation, since *Nitrotoga* BS and its preculture *Nitrotoga* HAM-1 contained the same heterotrophic genera: *Nocardioides* and *Parvibaculum* that are both members of the *Actinobacteria* and *Alphaproteobacteria*, respectively (Figure 4). Two different *Nocardioides* originating from *Nitrotoga* BS were cultured on complex medium, and the closest relative of one was reported to be capable of nitrate reduction as are several strains of this genus.[211] Contamination of NOB cultures with *Nocardioides* was reported for *Nsp. bockiana* as well.[92] The further detected genus *Parvibaculum* was so far not described as nitrate reducer, but some species can metabolize alkanes.[212–214]

In *Nitrotoga* 1052, the main concomitant heterotrophs were members of the alphaproteobacterial Sphingomonadaceae family. These bacteria were detected in biofilms of faucets[215] and systems for drinking water treatment,[139] biofilters of a freshwater RAS,[158] and rapid sand filters,[216] similar environments inhabited by *Nitrotoga* as well. They also coexisted with *Nitrotoga* and *Nitrospira* in the Movile cave.[140] Sphingomonadacea can degrade organic compounds, some are phototrophic, but a general role in nitrogen cycling was not reported.[217] However, they initiate biofilm formation by forming a monolayer of cells and EPS onto which further bacteria can attach.[138] Additionally, the alphaproteobacterial *Mesorhizobium* grew in co-culture with *Nitrotoga* 1052. Members of this genus inhabit nitrifying biofilms[218] e.g. in biofilters of aquaculture systems,[219] and they take part in the nitrogen cycle by N-fixation in plant nodules.[220,221] Additionally, *Mesorhizobium* were reported to share similarities with NOB species like enzymes for nitrite assimilation in *Nb. winogradskyi* Nb-255,[106] hydrogenases in *Nsp. moscoviensis*,[119] or carbon-monoxide dehydrogenase in *Nb. hamburgensis* X14.[78] Among others, *Mesorhizobium* cluster into the order Rhizobiales together with *Nitrobacter*.

Although *Nitrotoga* HW and *Ntg. arctica* did no longer contain cultivable heterotrophs, 16S rRNA gene amplicon sequencing revealed remaining microbes. No dominance of a single genus was found in *Nitrotoga* HW, while the majority of further OTUs in *Ntg.*

arctica were assigned to *Aquabacterium* that is also a member of the *Betaproteobacteria*. The initial described species originated from a drinking water system and could use nitrate as an alternate electron acceptor apart from oxygen.[222] This might explain their coexistence, as *Ntg. arctica* can supply *Aquabacterium* with nitrate. However, nitrate loss was not detected in experiments with *Ntg. arctica*.

As was show for nearly pure *Ntg. arctica* and *Nitrotoga* HW, concomitant bacteria are not essential to enable growth, though they could be beneficial by reducing nitrate or by scavenging other inhibitory substances. Maintaining axenic cultures is a frequently reported issue for unculturable bacteria, because they depend on concomitant microbes, miss supplements, or are inhibited by nutrient rich media.[223,224] Nevertheless, growth of *Nitrotoga* in pure cultures should not be problematic.

4.2 DIVERSITY WITHIN THE *CANDIDATUS* GENUS NITROTOGA

Since only a few enrichments are available, physiological and morphological properties of *Nitrotoga* remained understudied so far. Furthermore, obtained 16S rRNA gene sequences hinted at a very close relationship between different enrichments and clones from diverse habitats. The genus is described as cold-adapted considering the enrichment success at colder temperatures[129,130] and habitats in which it was discovered. It might further tolerate moderate acidity, since *Ntg. arctica* was enriched from acidic permafrost soils,[129,174] and the since successful enrichment of *Nitrotoga* HW and separation from *Nitrospira* was supported by lower pH of 5.7.[34] The cells share a uniform morphology based on description of *Ntg. arctica,*[129] *Nitrotoga* HAM-1,[130] and those found in a cold-freshwater RAS.[34] This study is to date the first that focuses on a comparative characterization of different *Nitrotoga* cultures obtained from a variety of habitats to further elucidate physiological, morphological, and phylogenetic properties of this genus.

4.2.1 PHYSIOLOGY

The four investigated *Nitrotoga* cultures proved physiologically far more diverse than was anticipated at the start of this thesis (Table 27). However, they were enrichments, and even though *Ntg. arctica* and *Nitrotoga* HW contained no cultivable heterotrophs, an influence of concomitant bacteria cannot be categorically excluded. In *Nitrotoga* BS, the heterotrophs might actually be responsible for the culture's tolerance towards nitrate,

since they were described as nitrate reducers. Nonetheless, no detectable loss of nitrate was observed during experiments.

Table 27: Overview of physiological parameters and culture relevant information of all four investigated *Nitrotoga* enrichments. Data in parenthesis indicate maximum values for temperature and minimum values for pH. Purity was evaluated based on 16S rRNA gene amplicon sequencing via Illumina MiSeq, except for * via 454 Pyrosequencing.

Culture	***Ntg. arctica***	***Nitrotoga* 1052**	***Nitrotoga* BS**	***Nitrotoga* HW**
Origin	Permafrost soil, active layer	Permafrost soil, bore core	Activated sludge	Biofilter RAS
Purity [%]	98*	68	87	99
Cultivable heterotrophs	None	Yes	Yes	None
Supplements	None	None	Ammonium	None
Temperature optimum [°C]	17 (22)	22 (25)	17 (22)	22 (25)
pH optimum	6.4 (6.4)	7.4 (6.7)	7.3 (6.7)	6.8-7.2 (6.1)
Tolerance				
Ammonium [mM]	30	25	30	40
Nitrite [mM]	4	4	4	10
Nitrate [mM]	15	10	20	15

Temperature and pH

The cold adaption of *Nitrotoga* was confirmed by optimal growth temperatures of 17°C (*Ntg. arctica*, *Nitrotoga* BS) and 22°C (*Nitrotoga* HW, *Nitrotoga* 1052). None of the four enrichments grew at 28°C or above, but their nitrite oxidizing activities at 4°C ranged from 20 to 60%. In this aspect, *Nitrotoga* differs from other NOB genera that mostly grow best in the mesophilic range e.g. *Nitrospira*[85,225] and *Nitrobacter*.[75,177] Some NOB are thermotolerant like *Nitrolancea hollandica* with activity between 25 and 63°C[100] or moderately thermophilic as certain *Nitrospira*,[93,98,226] but they are all less active at temperatures about 10°C. The adaption of *Nitrotoga* to lower temperature and its sensitivity to warmer conditions was also supported by an early enrichment obtained from a bioreactor from colleagues in Seattle. The initial enrichment was kept at 22°C and contained *Nitrotoga*, *Nitrospira*, and *Nitrobacter*, as was evaluated by sequencing specific 16S rRNA gene fragments. Simultaneous inoculated subcultures lost *Nitrotoga* when incubated at 28°C, whereas it remained at 17 and 22°C. Nevertheless, the enrichment *Nitrotoga* AM1 oxidized nitrite up to temperatures of 29°C, though its optimum temperature was 16°C,[131] and *Nitrotoga* showing *in-situ* activity at 27°C were recently observed in activated sludge.[88]

Each *Nitrotoga* culture showed distinct characteristics at changing pH conditions (Figure 5, Table 27), but most of them preferred pH values that resembled the ones prevailing at their sampling sites. *Nitrotoga* HW grew best at pH 6.8 to 7.2 in correspondence to the condition in the RAS it was isolated from,[34] and *Ntg. arctica* favored a pH of 6.4 comparable to the acidic soil it originated from.[129,174,176] *Nitrotoga* BS (this study) and *Nsp. defluvii*[34] had nearly the same pH optimum of 7.3 to 7.4 which is noteworthy since they were isolated from the same WWTP. The activated sludge as initial inoculum had a similar pH value.[85,86] *Nitrotoga* 1052 was the only culture with a different optimum pH of 7.3 compared to pH 5.5 at its sampling site, which was surprising since it proved quite sensitive to moderate acidity.

Thus, members of the genus *Nitrotoga* are not obligatorily adapted to acidic conditions. Some, like *Nitrotoga* BS and *Nitrotoga* 1052, ceased growth under moderate acidic conditions but favored neutral or slightly alkaline pH, while *Nitrotoga* HW was the most acid tolerant culture with about 80% activity at a minimum tolerated pH of 6.1. At first glance, this did not correspond to the successful separation of *Nitrotoga* HW from *Nitrospira* in acidic medium at pH 5.7. However, cultivation was prolonged to nine month under these conditions[34] to allow suboptimal, slow growth that was not monitored during physiological tests in this study. While some experiments demonstrated that *Nitrospira*, and not *Nitrobacter*, is the dominant NOB at a pH about 4,[172,227] other revealed that *Nitrospira* cultures appear more sensitive concerning acidity than *Nitrobacter*.[228] This sensitivity might explain the separation success of *Nitrotoga* HW from *Nitrospira* by prolonged incubation.[34] The only cultured acidophilic NOB known to date, *Nitrobacter* IOacid with optimal activity at pH 5.5, was isolated from acidic soil.[76] This corresponds to *Ntg. arctica* that was likewise enriched from acidic soil and showed the lowest pH optimum in this study. Nonetheless, most described NOB cultures of either *Nitrospira*, *Nitrobacter*, and *Nitrolancea* preferred neutral to slightly alkaline pH.[34,73,75,81,90,101,177] This might result from an optimal pH of 8 for nitrite oxidation via NXR that was, however, only determined for *Nb. winogradski*.[229] Nitrate reduction to nitrite via NXR was favored at pH 6.[229] This was also supported by former studies that revealed a rather narrow optimal pH for nitrite oxidation in *Nb. winogradskyi*.[230]

Influence of ammonium

Nitrotoga cultures differed in their needs for supplements. It was commonly observed that *Nitrotoga* BS grew worse under prolonged subcultivations in our laboratory. Initial investigations whether its growth could be supported by supplements hinted at a positive effect of ammonium (Bachelor thesis A. Schwabauer) with the additional advantage that the medium remains autotrophic. These first findings were verified during this study. Ammonium deficient cultures of *Nitrotoga* BS grew much better in ammonium supplemented medium when subcultured, compared to medium without ammonium (Figure 7). This did not occur, however, when the preculture was fed with ammonium not long before subcultivation. In this case, nitrite oxidation was comparable with or without ammonium. It is possible that *Nitrotoga* BS cannot utilize nitrite as nitrogen source for biosynthesis. Several NOB like *Nsp. defluvii*,[108(SI Results)] *Nitrospina gracilis*,[82] and *Nitrobacter*[78,106] possess assimilatory nitrite reductase for the reduction of nitrite to ammonium. In contrast, *Nitrolancea hollandica* lacks genes for this purpose and consequently could only be cultured when supplemented with ammonium.[100,101] This might be the case for *Nitrotoga* BS as well. Since it was isolated from a WWTP with sufficient ammonium loading, it might have lost this function through adaptive gene loss.[231] All sequenced NOB contain ammonium transporters as do most microbes to fulfill their nitrogen requirements,[232] and *Nitrotoga* BS is probably dependent on external ammonium. A positive effect of ammonium was also demonstrated for the recently described *Nitrotoga* AM1.[131] The authors discussed that enhanced growth resulted from energy conversion by down-regulating the expression for assimilatory nitrite reductase (NirBC) as was observed in *Nb. winogradsky*.[233] In contrast, *Nitrotoga* HW was cultured without addition of ammonium, although it was isolated from a RAS where this substance was abundant as well. A positive effect of ammonium was furthermore not observed in enrichments of *Ntg. arctica*, *Nitrotoga* HW, and *Nitrotoga* 1052 during experiments in this thesis. These ambiguous results impede a final conclusion on the dependence of *Nitrotoga* on ammonium as long as no genomic information is available for the investigated cultures.

In addition to the ammonium dependence of *Nitrotoga* BS, all investigated *Nitrotoga* enrichments shared a tolerance towards ammonium about 25 to 40 mM. This was also

demonstrated by *Nitrotoga* AM1 (30 to 40 mM).[131] The effect on other NOB appears to be culture dependent: *Nb. winogradskyi* was inhibited by comparable ammonium concentration of 35 mM[233] in pure culture, whereas *Nitrolancea hollandica* tolerated at least 200 mM ammonium.[100,101] *Nsp.* Ecomares grew in the presence of 50 to 80 mM ammonium and it was postulated that its resistance is linked to periodically high ammonium levels in the marine RAS it was isolated from.[96] This fits with the highest ammonium tolerance observed in *Nitrotoga* HW that was likewise enriched from a RAS. Nonetheless, the ammonium levels tolerated by *Nitrotoga* and most other NOB are considerably lower than concentrations that AOB can withstand. Their resistance is culture-dependent as well and determines niche separation.[234] Generally, most *Nitrosomonas* species can thrive in several 100 mM,[21,40] whereas *Nitrosospira* appear to favor lower ammonium contents about 200 mM at maximum.[21,235]

Actually, it is assumed that ammonium is not the inhibiting N-compound, but free ammonia (NH_3), since it can easily diffuse through membranes. Its concentration cannot be measured directly but has to be calculated based on TAN levels, pH, and temperature as described elsewhere.[208,209] In general, AOB and AOA tolerate free ammonia better than NOB[208,236,237] which is consistent with their higher ammonium resistance. Nevertheless, reports about the behavior of NOB towards free ammonia are quite controversial. *Nitrospira* appear to be less tolerant than *Nitrobacter*,[228] although other studies found the same concentration of 10 mg/L NH_3-N inhibitory for both NOB genera.[238,239] *Nsp.* Ecomares isolated from a marine RAS withstood similar levels,[96] while two *Nitrospira* strains from a WWTP were affected by much lower concentrations of about 0.9 and 4.3 mg/L NH_3-N.[240] *Nitrotoga*-like NOB appear to be moderately tolerant compared to other NOB with growth in about 3.3 to 6.4 mg/L NH_3-N observed in this study (Table 28) and 1.3 mg/L NH_3-N reported for *Nitrotoga* AM1.[131] The highest tolerated free ammonia concentration was exhibited by *Nitrotoga* HW probably in correspondence to its origin from a RAS, similar to *Nsp.* Ecomares.[96]

Table 28: Free ammonia concentrations tolerated by *Nitrotoga*.

Culture	pH, temperature	Ammonium [mM NH_4Cl]	Free ammonia [mg NH_3-N/L]
Nitrotoga HW	7.4, 22°C	40	6.4
Ntg. arctica	7.4, 17°C	30	3.3
Nitrotoga BS	7.4, 17°C	30	3.3
Nitrotoga 1052	7.4, 22°C	25	4.0
Nitrotoga AM1[131]	8.1, 19°C	30	1.3

Inhibition by nitrite, nitrate, and H_2O_2

The investigated *Nitrotoga* cultures differed in hindsight to inhibition by nitrite. Most noteworthy, the initial described enrichment of *Ntg. arctica* by Alawi *et al.* (2007) tolerated up to 1.2 mM nitrite,[129] while 4 mM were observed in this study. It remained unclear, however, whether the increased tolerance was acquired during prolonged cultivation, or if it was attributed to a higher fitness of the culture used in this study compared to the one tested by Alawi and colleagues. Furthermore, *Nitrotoga* from activated sludge showed *in-situ* activity in the presence of 10 mM nitrite[88] which corresponds to the maximum tolerated substrate concentration determined for *Nitrotoga* HW in this thesis. The various NOB genera preserve at different nitrite levels. *Nitrolancea hollandica* was characterized as resistant towards 75 mM nitrite,[100,101] followed by *Nitrobacter* species that tolerated 10 to 45 mM nitrite,[241] whereas *Nitrospira* appeared to be generally more sensitive.[242] Nevertheless, there is a high diversity within the genus *Nitrospira* concerning nitrite inhibition: *Nsp. lenta* ceased growth at concentrations above 2.5 mM, while *Nsp. defluvii* tolerated about 30 mM nitrite.[86] Further *Nitrospira* cultures could thrive in maximum substrate concentrations ranging from 6 to 30 mM.[81,90,92,243] Thus, *Nitrotoga* appears to be the most sensitive NOB so far with 4 to 10 mM nitrite tolerated at maximum. The nitrite resistance of NOB might be linked to higher substrate affinity. High K_m values were measured for *Nitrolancea* and *Nitrobacter*, and they tolerated higher nitrite concentrations compared to *Nitrospira* and *Nitrotoga*.[100,110] The sensitivity of the latter two NOB genera towards nitrite underlines their time-consuming enrichment at low substrate levels.

As was the case for ammonium and nitrite, *Nitrotoga* appear to be more sensitive to nitrate than other NOB. The least affected culture, *Nitrotoga* BS, did thrive in concentrations of 20 mM nitrate. However, its high tolerance might be attributed to

concomitant heterotrophs that are capable of nitrate reduction. Different *Nitrospira* species were inhibited above 18 to 80 mM nitrate[81,86,96] and product inhibition was observed for pure cultures of *Nb. winogradskyi*[244] as well. The latter NOB tolerated up to 128 mM nitrate,[245] and non-competitive inhibition was reasoned as a possible mechanism.[230]

A disinfection step based on PAA and H_2O_2 was carried out in the RAS investigated in this study, and *Nitrotoga* was no longer detectable in the biofilters after its implementation (Table 25). However, the four *Nitrotoga* cultures were not negatively affected by up to 0.1% H_2O_2 in laboratory tests (Figure 11). Consequently, it was most likely not the main reason why *Nitrotoga* was eliminated from the biofilters of the investigated RAS. So far, it is not known which modes of protection *Nitrotoga* possess against oxidative stress. *Nsp. defluvii* is catalase-negative but was likewise not influenced by H_2O_2 under the experimental conditions, probably because it has several other enzymes that might function as H_2O_2 scavengers.[108(Table S2 and SI Results)]

4.2.2 MORPHOLOGY

All investigated cultures shared the same morphology as was revealed in SEM and TEM micrographs. The cells appeared as slightly curved, short rods with a characteristic wide periplasmic space. In ultrathin sections, paired cells sharing one periplasm were occasionally observed probably due to incomplete cell division. The cells revealed a bubble-like structure in SEM micrographs but were difficult to visualize by this method. They were covered by a lot of particles potentially due to sample preparation. The recently described enrichment *Nitrotoga* AM1 formed more uniform, longer rods, and its morphology in SEM micrographs differed considerably from the appearance of *Nitrotoga* cultures observed in this study.[131]

Other NOB genera can be easily distinguished from *Nitrotoga* in electron micrographs based on their ultrastructure. *Nitrospira* cells are pleomorphic short rods or spirilla that release membrane vesicles and are associated as dense microcolonies surrounded by thick layers of EPS.[34,81,85–87,91,130,246] However, they were also observed as single planktonic cells.[81,86,93] *Nitrotoga* forms microcolonies under the applied culturing conditions and in natural or engineered habitats as well.[34,130]. However, their microcolonies appear looser than those of *Nitrospira*, and the surrounding EPS is less dense.[34,130] *Nitrobacter*-like NOB

appear as short pleomorphic rods with intracytoplasmic membranes (ICM) located at one cell pole.[75,113,177,246] Cells of the most recently described NOB genus *Nitrolancea* are quite large, short lancet-shaped rods that lack ICM.[100,101]

Since the energy yield generated by nitrite oxidation is low, NOB need high amounts of their key enzyme. In *K*-strategists like *Nitrospira*[107] and *Nitrotoga*, the NXR is located in the periplasmic space and probably responsible for the fine granulated structure of *Nitrotoga*'s extended periplasm (pers. comm. E. Spieck), while it is anchored in the ICM facing the cytoplasm in *r*-strategist NOB like *Nitrobacter*.[123]

4.2.3 PHYLOGENY

Based on the 16S rRNA gene, all four enrichments were closely related with at most 1% sequence divergence between *Nitrotoga* BS and *Nitrotoga* HW. This value corresponds to a clone library of uncultured *Nitrotoga* from WWTPs derived by Lücker and colleagues with sequence similarities over 98%.[88] The probability of microdiversity within the genus was addressed in their study as well and can be supported by the four here investigated cultures. Although *Nitrotoga* have two *rrn* operons (based on the genome of *Ntg. arctica*, pers. comm. S. Lücker), cloned rRNA gene sequences obtained from the same enrichment clustered together and did not intersperse with those of others (Figure 12). This shows that sequences were more similar within one culture than between different cultures. The possession of several 16S rRNA gene copies is shared by the closest relatives of *Nitrotoga*. *Sideroxydans lithotrophicus* strain ES-1 (Accession nr. NR115756) and *Gallionella capsiferriformans* strain ES-2 (Accession nr. DQ386262) contain two and three *rrn* operons, respectively (rrnDB 5.1, accessed 13.03.2017).[247]

A better resolution, with sequence divergence of 6.5 to 18.4% between *Nitrotoga* cultures, was obtained by sequencing a part of their *nxrB* gene. However, primers for *Nitrotoga nxrB* did not work on environmental samples and hindered a more detailed examination of habitats containing *Nitrotoga*-like NOB. The primers were designed based on the *nxrB* sequence of *Ntg. arctica* with the aim of amplifying a gene section similar to that obtained in *Nitrospira* by their respective *nxrB* primers (pers. comm. S. Lücker). Since *Nitrotoga nxrB* sequences proved quite divergent, the non-compatibility of the corresponding primers might be explained, as well as recurrent unspecific DNA bands during *nxrB* PCR in other cultures apart from *Ntg. arctica* and in environmental sampled.

Nonetheless, about 20% sequence divergence was also found in *nxrB* sequences of e.g. *Nitrospira* lineage I[111] which probably indicates a higher conserved sequence region at their primer binding sites.

The NXR of *Nitrotoga* forms a separate class within the known NXR (Figure 14) far from either the periplasm-oriented *Nitrospira*-type or cytoplasm-oriented *Nitrobacter*-type. This hints at a different evolutionary ancestor for NXR in *Nitrotoga*. Just recently, a NXR was discovered in *Thiocapsa* KS1 that forms a new cluster within the cytoplasm-oriented NXR.[104] Horizontal gene transfer (HGT) was hypothesized based on members within the *Thiocapsa* genus devoid of NXR or nitrite-oxidizing ability in general, but the donor remained unknown.[104] So far, *Nitrotoga* is exclusively associated with nitrite oxidation, but the lack of a close relative on genus-level with the same property does not exclude HGT. However, it is equally possible that the NXR of *Nitrotoga* evolved independently, since the DMSO reductase family is characterized by a high degree of diversity.[248] The most similar sequence found at NCBI codes for a putative selenate reductase (SER) in *Acetothermus autotrophicum* (GenBank: AP011802.1) that also belongs to the type II DMSO reductases. A connection of SER to NOB, apart from its reference in phylogenetic trees, was already reported for *Nitrospina gracilis*, where the NXR gamma subunit shares the solubility in the periplasm with SER.[82] The localization of alpha and gamma NXR subunits was so far not evaluated in *Nitrotoga*, but a solubility of the complete NXR complex might explain the extremely wide periplasm of *Nitrotoga*.

Nevertheless, the *nxrB* sequences of *Nitrotoga* were closer related to those of Anammox bacteria or NOB possessing a periplasm-oriented NXR than to those facing the cytoplasm. This suggests that the NXR of *Nitrotoga* is indeed facing in this direction and corresponds to *Nitrotoga* being a *K*-strategist.[110] Labeling experiments with monoclonal antibodies recognizing the beta-subunit of the NXR[105,246,249] in *Nitrotoga* enrichments support this suggestion even further (pers. comm. E. Spieck).

In terms of phylogenetic discrimination, *Nitrotoga* can be compared to *Nitrobacter* or the Nitrosomonadaceae. Different *Nitrobacter* species are very closely related at the 16S rRNA gene level as well, and the additional analysis of the *nxrB* sequence allowed a much better species differentiation for this NOB.[112] The same applies for the betaproteobacterial ammonia-oxidizers *Nitrosomonas* and *Nitrosospira* that could

likewise be hardly discriminated based on 16S rRNA gene sequence, whereas the *amoA* as marker gene facilitated the classification of different clusters.[20,22,128] In contrast, *Nitrospira* can be distinguished based on their 16S rRNA gene sequence alone. They cluster into different lineages that were divided by a sequence divergence below 94%,[84] and these lineages are also supported by the *nxrB* gene as phylogenetic marker.[111] This underlines the evolutionary distance between the betaproteobacterial *Nitrotoga* and *Nitrospira*, since the latter is a member of the deep-branching and consequently very old phylum *Nitrospirae*.

4.2.4 HIGH DIVERSITY OF *NITROTOGA* EMPHASIZES SEVERAL SPECIES WITHIN THE GENUS

The investigated *Nitrotoga* cultures proved far more diverse during this study than first anticipated. Based on 16S rRNA gene sequences they appear to belong to one species. The maximum sequence divergence of barely 1% is just overlapping with the currently accepted value of 99% sequence similarity proposed by Stackebrandt and Ebers,[250] above which further tests are recommended to define a culture as genomic independent. Before, a divergence of even 97% was mandatory.[251] The similar morphology underlines the resemblance of all investigated cultures which is common for members of the same genus. Several species could be easily assigned when applying *nxrB* as a marker gene. A cut-off of 95% sequence similarity was suggested for *Nitrospira*,[111] and the maximum similarity observed for *nxrB* of *Nitrotoga* in this study was 93.5% between *Ntg. arctica* and *Nitrotoga* 1052. The different physiological properties provide even more evidence for several *Nitrotoga* species, and the diverse isolation sites of the investigated cultures support this further: permafrost soil (*Ntg. arctica*[129] and *Nitrotoga* 1052[176]), activated sludge (*Nitrotoga* BS[130]), and biofilm from a RAS (*Nitrotoga* HW[34]). Especially the differences between the two spatial most closely sampled cultures, *Ntg. arctica* and *Nitrotoga* 1052, is a strong evidence for multiple species. Both enrichments differ in pH and temperature optimum as well as their sensitivity towards ammonium and nitrate. Their behavior mirrors the different soil depths they were isolated from. *Ntg. arctica* was enriched from the upper active layer that undergoes freezing and thawing periods.[129,174] In contrast, *Nitrotoga* 1052 was sampled from 90 cm depth where the soil is permanently frozen.[176] It might still be adapted to conditions that prevailed before it was conserved. This could explain the higher temperature and pH optima of *Nitrotoga* 1052, as well as

the decreased activity at 4 to 10°C compared to *Ntg. arctica*, and the deviating pH optima compared to the soil pH. Reports of *Nitrotoga*-like NOB from different habitats that tolerate mesophilic temperatures above 25°C[88,131] expand the probability of even more unknown members within this genus.

4.3 *NITROTOGA* COMPETES SUCCESSFULLY WITH *NITROSPIRA*

Nitrotoga and *Nitrospira* colonize similar habitats together,[88,130,132] but niche differentiation between both NOB is still unknown. To uncover conditions that favor one NOB over the other, co-cultivation experiments were conducted that aimed at temperature and pH as crucial parameters. Batch reactors were inoculated with *Nsp. defluvii* and either *Ntg. arctica* or *Nitrotoga* BS. Since *Nitrotoga* is found in colder environments, the temperature was kept at 17°C, the optimum growth temperature for *Ntg. arctica* and *Nitrotoga* BS. Two different pH conditions (6.4 and 7.4) were applied to investigate a possible adaption of *Nitrotoga* to slightly acidic pH values as was reported before[34] and to cover optimum pH of the three cultured NOB. After several process repeats, it became apparent that while individual pH and temperature optima can work in favor of *Nitrotoga*, these optima allowed no general conclusion about the performance of *Nitrotoga* in co-culture with *Nitrospira*.

In experiments with *Nitrotoga* BS and *Nsp. defluvii* (Figure 22), the former was considerable more competitive at pH 7.4 which can be explained by the combined positive effect of optimum pH and temperature. Both NOB favor similar pH conditions, but *Nsp. defluvii* achieved only 50% nitrite oxidation activity at 17°C,[34,this study] while this is the optimum growth temperature for *Nitrotoga* BS. Reverse results were obtained at pH 6.4, where *Nsp. defluvii* was more competitive despite the low temperature. In individual experiments, *Nitrotoga* BS was inhibited in this pH range (Figure 5), whereas *Nsp. defluvii* maintained 70% nitrite oxidizing activity under this condition.[34]

When *Ntg. arctica* and *Nsp. defluvii* were combined (Figure 24), the former was more successful at pH 7.4 as well. With abundances below 80%, *Ntg. arctica* was not as competitive against *Nsp. defluvii* as *Nitrotoga* BS under the same conditions. Furthermore, this result did not match with the optimum growth parameters for *Ntg. arctica* (Figure 5) that rather forecasted a predominance at pH 6.4 and 17°C. However,

unlike *Nitrotoga* BS, it was less affected by acidic surroundings, as its relative abundance did not decline at pH 6.4.

The experimental approach of co-cultivation applied in this study had some drawbacks. First of all, experiments were set-up as batch tests with 1 mM nitrite substrate that did not allow the formation of sufficient biomass. Thus, absolute nitrite turnover was compared between parallel started batch experiments at pH 6.4 and 7.4 and was not normalized to cell counts or protein content. This hindered a more thoroughly evaluation of activities between both pH conditions, although nitrite turnover was consistently lower at pH 6.4 compared to pH 7.4. As mentioned above, *Nitrotoga* BS was not a pure culture but coexisted with heterotrophic bacteria. Among them, putative nitrate reducers were found that might have influenced competition experiments. However, nitrite conversion to nitrate was stoichiometric without detectable loss of nitrate. Furthermore, *Nsp. defluvii* would likewise benefit from nitrate reduction, since it was co-cultured with concomitant bacteria of *Nitrotoga* BS and additionally, NOB coexist with several further bacteria in natural or engineered environments.

Success of *Nitrotoga* is obviously dependent on further factors apart from optimal pH and temperature. A combination of both can be advantageous as was the case for *Nitrotoga* BS (this study). This was also observed for *Nitrotoga* HW that was sampled from a RAS and separated from *Nitrospira* by adjusting the pH to acidic conditions.[34] However, this applies not to all *Nitrotoga* cultures as was revealed by *Ntg. arctica*, since it could not outcompete *Nsp. defluvii* under the combined effect of favored pH and temperature conditions. In a monitoring of different WWTPs in Denmark and Germany, not every WWTP was inhabited by *Nitrotoga* despite favorable temperatures, while *Nitrospira* prevailed in all systems.[88,132] In a survey by Lücker and colleagues, *Nitrotoga* did not thrive in WWTPs that treated animal rendering waste.[88] The RAS monitored during this study lost *Nitrotoga* after disinfection with PAA and H_2O_2. While H_2O_2 had no negative effect on *Nitrotoga* cultures in laboratory tests (this study), an inhibition by PAA or by an increased concentration of organic substances from lysed microorganisms could not be excluded, because NOB are often sensitive towards more complex organics.[252–254] Nonetheless, *Nitrospira* sufficed for nitrite oxidation in the RAS. It tolerated the disinfection and probably occupied the niche that *Nitrotoga* vacated.

To date, only one further study addressed the competition of *Nitrotoga* and *Nitrospira* in laboratory experiments, but it focused on the influence of changing nitrite availability. Kinnunen *et al.* found *Nitrotoga* to be more abundant at higher substrate contents, while lower nitrite levels selected for *Nitrospira*.[255] Their experimental set-up featured substrate concentrations close to K_m values of *Nitrospira* and *Nitrotoga*[110] which was reflected in their results.[255] Since substrate was oversaturated during competition experiments in this study, a comparison cannot be drawn. Furthermore, Kinnunen and colleagues applied a long-term cultivation approach and a microbial community from a natural habitat in their experiments[255] in contrast to short-term batch processes and defined cultures used in this study.

While inhibiting substances, pH, and substrate availability are essential for niche separation, low temperature is still the most decisive parameter enabling successful cultivation of *Nitrotoga* and its separation from other NOB. All cultures investigated during this thesis as well as the most recently described *Nitrotoga* AM1 were initially enriched between 10 and 17°C.[34,129–131,176]

4.4 NITRIFICATION IN A COLD-FRESHWATER RAS

Nitrification is essential in RAS biofilters to secure the health of cultivated animals by converting ammonia and nitrite to less toxic nitrate.[2,5] The biofilter contribute significantly to investment costs and amount to about 0.2 to 1.1 € per kg produced fish per year.[154,256] Consequently, a tight monitoring of nitrogen removal is indispensable to secure process stability and sustainability. Especially during the start-up phase of a RAS, ammonium or nitrite peaks occur since the nitrifying biofilm is still developing. This leads to a loss of reared organisms, increased water exchange, and elevated nitrogen levels discharged into the environment.[257–259] To accelerate the development of sufficient nitrifying activity, start-up enhancers can be introduced into the system.[258,260–262]

In the cold-freshwater rainbow trout RAS in Hohen Wangelin monitored from November 2013 to October 2015, the two smaller biofilters were filled with a mix of re-used and fresh biocarriers. The surface of the re-used biocarriers was already conditioned by biofilm formation during the previous process (monitored from July 2010 to September 2011).[34] This was supposed to reduce the period of unstable nitrification, since

physiochemical interactions[263] were no longer crucial to colonize the biocarriers, and the biofilm formation[264] could start immediately.

4.4.1 POTENTIAL NITRIFYING ACTIVITY IN HOHEN WANGELIN

At process start, only the two small biofilters with re-used biocarriers were connected. The nitrifying activity developed fast within one month post-start (November to December 2013, Figure 26), and the turnover observed for NOB was higher than for AOB from the start. The major biofilter needed two months to reach activities comparable to those of the two smaller units, and NOB were faster than AOB as well. In contrast to the results presented here, AOB were reported as more active than NOB in other RAS during start-up phases, while the latter usually achieved higher activities in established processes.[243,259,261] Elevated activities of NOB prevents the accumulation of nitrite in the system, and might result from a higher abundance of NOB compared to AOB in RAS.[96,134,156,161] When the two smaller biofilters were disconnected from the system (May 2015, Figure 26) due to lower fish stocking, a particularly sharp increase of NOB activity was observed in the major biofilter probably in response to an increased N-loading. The decrease in October 2015 corresponds to even lower feeding and fish density, and the rainbow trout rearing was eventually stopped during the same month.

Ammonium peaks were observed especially during the first three months which is typical for new started processes (Figure 25).[259] However, they ceased after the major biofilter was put into operation, and occasionally measured elevated ammonium or nitrate levels were later on countered by a short-term reduced feeding rate. The RAS in Hohen Wangelin was characterized by a low temperature and a controlled pH of 6.8, first of all to ensure best conditions for the fish, but also to keep the amount of free ammonia below toxic levels as was described previously,[256] since it increases with increasing temperature and pH. The highest measured ammonium level of about 6 mg/L corresponds to a free ammonia concentration of hardly 10 µg/L, while much higher concentrations of 0.16 to 1.1 mg/L free ammonia were evaluated to be lethal for 50% of a rainbow trout population within 96 hours.[265] Average acute toxicity for several freshwater and marine species were about 200 times higher than those measured in Hohen Wangelin.[265,266]

From August 2014 onward, disinfection with Wofasteril based on PAA and HP was implemented, and the first treatment resulted in a drastic decrease of nitrifying activity.

The NOB proved more sensitive than the AOB which was observed in further RAS during similar treatments as well.[267] Nonetheless, the nitrifying potential reached former levels within a month, and follow-up disinfection events with lower dosage of PAA and HP had only short-term influence on TAN and TNN turnover (Figure 26). PAA is a strong disinfectant and works synergistic with HP.[268] These compounds have the advantage of fast and easy degradation to oxygen and water and thus do not burden the environment via discharged water or increase the carbon to nitrogen ratio in the system. Furthermore, they are harmless for fish, and failures in biofiltration were not observed with correct application.[267,269]

It is important to notice that the potential nitrifying activity was determined at 17°C, whereas the RAS was operated at about 13°C mean water temperature. This implies lower activities *in-situ* that might compromise the process. Nonetheless, AOB and NOB were able to convert 1 mM substrate in these activity test, which was 30 (ammonium) to 80 times (nitrite) above the concentrations measured in the process water on average. This highlights the potential of the nitrifying biofilm to cope with higher nitrogen loads than were detected in Hohen Wangelin.

4.4.2 THE NITRIFYING COMMUNITY IN HOHEN WANGELIN

Generally, the most common nitrifiers reported in RAS biofiltration are *Nitrosomonas* (AOB) and *Nitrospira* (NOB)[156,157] with species specifically adapted to freshwater, brackish, or marine conditions.[34,96,259,270] This was also the case in Hohen Wangelin. The biofilters were consistently colonized with *Nitrosomonas*-like AOB and *Nitrospira*-like NOB, as was revealed by genus-specific PCR, FISH, and TEM. The next described relative with regard to *Nitrospira*-like NOB in Hohen Wangelin was *Nsp. defluvii* based on 16S rRNA and *nxrB* gene sequences obtained in this study and also by amplicon sequencing using Illumina MiSeq for samples from April 2015 (pers. comm. J. Hüpeden).[270] Furthermore, colonization with *Nitrotoga* was confirmed by PCR, FISH, and TEM. Temperatures of 10 to 17°C and a pH of 6.8 in the process water provided suitable conditions for this genus. The highly enriched *Nitrotoga* HW29 (investigated as *Nitrotoga* HW in this study) was obtained by long-term cultivation from the Hohen Wangelin system.[34] *Nitrotoga*-like NOB were also discovered in a marine RAS[259] as well as during the previous process in the Hohen Wangelin freshwater RAS.[34,135]

However, the disinfection with PAA and HP led to a change in the nitrifying community as of August 2014. *Nitrotoga* was no longer detectable, but since HP had no negative effect on *Nitrotoga* cultures in individually experiments (this study), its suppression was probably based on PAA or other unknown factors connected to disinfection. The possible release of organic matter from lysed microbes might have affected *Nitrotoga* more than *Nitrospira*, since the latter was continuously detected. While AOB and NOB can utilize simple organic molecules like formate or pyruvate,[241,271] they are often inhibited by more complex substances.[252–254] An increase of organic carbon results in a higher carbon to nitrogen ratio in the rearing waters that was reported to reduce the nitrifying activity in aquaculture systems, since it supports heterotrophic bacteria.[219]

Nitrosospira-like AOB emerged in the biofilm after disinfection started as was revealed by TEM. However, the shift from exclusively *Nitrosomonas* to a coexistence with *Nitrosospira*-like AOB could not be unambiguously confirmed by FISH or PCR, since applied probes and primers targeted both AOB genera. Amplicon sequencing via Illumina MiSeq of samples from April 2015 revealed a predominance of *Nitrosospira*-related *amoA* genes,[270] while *Nitrosomonas*-like AOB prevailed based on 16S rRNA genes (pers. comm. J. Hüpeden). The coexistence of both AOB was reported in a WWTP before, where *Nitrosospira* dominated during winter.[32] Furthermore, a psychrotolerant *Nitrosospira* was described recently,[235] which supports its detection in colder habitats like the RAS monitored in this study.

Nonetheless, disinfection and a shift in community did not impair the potential nitrifying activity. The role of *Nitrotoga* was probably taken by *Nitrospira*-like NOB, since *Nitrobacter* was not detected. Further studies demonstrated that the nitrifying community composed of *Nitrosomonas*-like AOB and *Nitrospira*-like NOB stays stable and maintains sufficient activity during disinfection with PAA and HP.[267]

4.4.3 SHORT-TERM STABILITY OF NITRIFICATION AT PHYSIOLOGICAL CHANGES

Physiological tests conducted with biocarriers revealed an overall stable short-term nitrifying activity in the biofilters of the Hohen Wangelin RAS. TAN and TNN removal were faster at 17°C compared to 10°C (Figure 32, Table 26), with a ratio of about 1.6 for TAN turnover and 1.3 for TNN turnover at 17 °C compared to 10°C. This is in accordance with more efficient nitrification in soils or WWTPs at more mesophilic temperature

conditions.[272–274] Since the RAS was operated at a mean water temperature of about 13°C, actual potential activity would amount to values between those calculated at 10 and 17°C.

No severe influence on activity was observed in a pH range from 6.3 to 8.1 (Figure 32). However, during the previous process at Hohen Wangelin, a distinct optimum was observed at pH 7.1, and activity decreased to about 50% at pH 6.[34] Since nitrification is strongly pH dependent and was reported to fail at pH 6.5,[275,276] the activity of AOB and NOB on the biocarriers was probably ensured by their localization in a dense biofilm that protected them from unfavorable conditions.[277–279]

Since nitrate accumulated in the process water, its influence on AOB and NOB performance was evaluated in activity tests with different initial nitrate concentrations (Figure 33). The AOB activity was not affected by up to 430 mg/L nitrate and decreased only minimally above this concentration. Insensitivity towards nitrate was also observed in other RAS[243,270] and in pure cultures of *Nitrosomonas*.[280,281] In contrast, NOB activity was reduced with increasing nitrate concentration, but mean nitrate values measured during the process (about 73 mg/L nitrate) still allowed 90% activity compared to TNN turnover without nitrate addition. At nitrate concentration above 1 mg/L, AOB were more active than NOB. Thus, nitrate accumulation can result in a reversal of the otherwise consistently observed ratio of higher nitrite oxidation over ammonia oxidation and consequently might lead to an accumulation of nitrite. It was reported that AOB and NOB respond less sensitive to nitrate than to ammonium and nitrite in general.[208] However, some NOB cultures were inhibited by concentrations similar to those applied in the nitrate influence experiment,[81,86,this study] while others tolerated much higher levels.[96] This underlines the difficulty of transferring results from pure or enrichment cultures to actual wastewater processes. Since reduced NOB activity with increasing nitrate levels was revealed in further RAS as well,[270] nitrate should be tightly monitored and removed from the process water either by water exchange or a denitrification unit.

The potential activity of AOB depended on the concentration of ammonium present as was revealed in laboratory tests. An optimum curve was observed with maximum activity at 25 mg/L ammonium, and TAN turnover decreased above and below this value (Figure 34). This underlines a much higher ammonium oxidation capacity of the biofilm in relation

to what was sufficient for a mean ammonium load of 0.55 mg/L in the RAS. Activities were lower at *in-situ* prevailing concentrations compared to those obtained with 18 mg/L ammonium (about 1 mM) during the standard activity tests. A similar influence of ammonium on TAN turnover was observed in biofilms of freshwater, brackish, and marine RAS, where optimum curves for AOB activities were obtained at higher ammonium concentrations than measured in the systems.[270]

The biofiltration process in the Hohen Wangelin RAS revealed a short-term stability of nitrification when challenged with increasing ammonium and nitrate levels. Since the tested concentrations were far above the highest measured concentration of nitrate (170 mg/L) and ammonium (6 mg/L), but still permitted fast nitrogen turnover, a short-term overload of these two N compounds should not lead to a lasting disturbance of the nitrifying biofilm. In addition, a quite high nitrite tolerance for NOB in biofilms from RAS biofilters, far above those measured *in-situ*, was revealed elsewhere[270] and supports the short-term resistance towards inorganic nitrogen observed in this study. This might again be explained by protection of AOB and NOB in a dense biofilm[277–279] or, in case of AOB tolerance towards nitrate, simply by an insensitivity of the AMO concerning this compound.

4.5 *NITROTOGA* IS RELEVANT FOR WASTEWATER PROCESSING

Recent studies underline the importance of *Nitrotoga* in engineered habitats connected to wastewater treatment where it coexists with *Nitrospira* or even dominates the NOB community.[34,88,132] Its competitiveness was demonstrated in laboratory experiments based on temperature and pH (this study) or substrate concentration,[255] but temperature seems to be the most decisive factor for success of *Nitrotoga*.[34,129,130,133,this study] Nitrification often slows down at low temperatures. A loss of 70 to 90% activity at 5 to 10°C compared to mesophilic conditions was reported before.[272–274,282] This is especially problematic in WWTPs during winter periods, since a longer sludge retention time is necessary for complete nitrification.[175] Colonization with *Nitrotoga* could counter these effects since they can maintain high activities under these conditions. However, seasonal shifts in NOB population in municipal WWTPs were so far not investigated in detail, while

for AOB, it was shown that *Nitrosospira* was more abundant than *Nitrosomonas* during winter.[32]

It was postulated before that *Nitrotoga* might be better adapted to slightly acidic pH than other NOB, since this was demonstrated for cultures of *Nitrotoga* HW[34,this study] and *Ntg. arctica* (this study). In general, impaired nitrification under acidic conditions is observed in wastewater treatment[283] probably due to inhibition of nitrifiers by free nitrous acid (HNO_2).[208,284] Other studies reported a fail of nitrification at a pH about 6.5.[275,276] In contrast to problems in sewage treatment, nitrification was possible in acidic soils[41] as well as in enrichment cultures with cell aggregates of *Nitrosospira* and *Nitrobacter*.[210] *Nitrotoga* could widen the pH range for nitrification. However, experiments during this study revealed that *Nitrotoga* is not universally adapted to more acidic conditions but rather to the predominant pH at the site the cultures were sampled from.

Compared to other NOB, *Nitrotoga* appeared to be more sensitive towards inorganic nitrogen (this study). Toxicity of ammonia, nitrite, and nitrate is mainly addressed in the context of aquaculturing, since their accumulation was regularly reported in these facilities, and because aquatic animals are especially vulnerable to these compounds.[1,2,5,285,286] Nevertheless, the *Nitrotoga* cultures investigated in this study were adapted to conditions in response to their isolation site. *Nitrotoga* HW, sampled from a cold-freshwater RAS, had the highest tolerance towards ammonium which corresponds to ammonium peaks that can occur in these systems. Further studies revealed that free ammonia drives niche separation among *Nitrospira* in WWTPs[240] and that nitrifying communities can adapt to increased concentrations of this compound.[279] *Nitrotoga* could undergo similar adaption and colonize WWTPs successfully, since ammonium and nitrite levels are rather low in these habitats. Apart from WWTPs,[88,130,132,this study] *Nitrotoga* was found in a freshwater and a marine RAS[34,135,243,259,this study] as well as in N-removing reactors for mine waters.[133] Due to a functional redundancy with widespread *Nitrospira*, but a slightly different physiology, *Nitrotoga* can ensure a more stable wastewater processing, since a versatile community is a key for robust nitrification.

4.6 OUTLOOK

During this thesis, the diversity of *Nitrotoga* enrichments became apparent. They exhibited distinct physiological properties that support the classification of several species within the genus. Contrasting their high similarity on 16S rRNA gene level, application of the *nxrB* gene as phylogenetic marker permits a better characterization of *Nitrotoga*-like NOB as is the case for other NOB.[111,112] Nonetheless, primers need to be improved or newly developed to circumvent false positive PCR products and to extent their use to genomic DNA isolated from environmental samples. The genomes of *Nitrotoga* cultures still await publication. These greatly missing data will reveal further details about the lifestyle of this NOB and again, the NXR is of high interest in this context. Results obtained in this study hint at a different evolutionary origin of NXR in *Nitrotoga* as it is separated from *nxrB* sequences of other NOB in neighbor-joining phylogenetic trees. Furthermore, enzyme isolation and characterization are necessary for a better assessment of its actual nitrifying capability in terms of substrate specificity or inhibition.

Nitrotoga was detected in diverse natural and engineered habitats before which challenges the assumption that *Nitrospira* is the most dominant NOB in sewage treatment. The fact that *Nitrotoga* can successfully colonize WWTPs[88,130,132] and RAS[34,135,243,259,this study] emphasizes its importance in wastewater purification and the necessity to include this NOB in models for process engineering. To this end, pure cultures originating from these habitats need to be analyzed in-depth concerning their kinetics for growth and nitrite oxidation.

The co-cultivation experiments performed in this study allowed an insight into the competitiveness of *Nitrotoga* against *Nitrospira* in dependence of temperature and pH value. However, only 17°C was tested so far as it represents the optimum growth temperature for *Nitrotoga* BS and *Ntg. arctica* but was simultaneously sufficient for growth of *Nsp. defluvii*. To better access niche differentiation between those two NOB genera, further experiments at 22°C are planned. While more mesophilic temperatures of 25 to 30°C pose the problem that *Nitrotoga* ceased growth under these conditions, 17 to 22°C reflect the temperature range observed in WWTPs that were colonized with *Nitrotoga*.[88,130,132] Additionally, competition experiments with *Nsp. lenta* are planned

under similar conditions, as this NOB was isolated from the same WWTP as *Nitrotoga* BS and *Nsp. defluvii*. Furthermore, it represents a lineage II *Nitrospira* in contrast to the lineage I *Nsp. defluvii* investigated so far.

To date, all cultures of *Nitrotoga* are exclusively non-marine. A potentially halophilic *Nitrotoga*-like NOB (clone AM0) was lost during enrichment of *Nitrotoga* AM1 by Ishii and colleagues.[131] However, the genus was already detected in biofilters of a marine RAS[243,259] which indeed suggests the existence of marine *Nitrotoga*. Together with the fact that some specimen were described to tolerate more mesophilic conditions,[88,131] the discovery of even more *Nitrotoga*-like NOB in habitats that where so far not considered to be colonized by this genus is possible.

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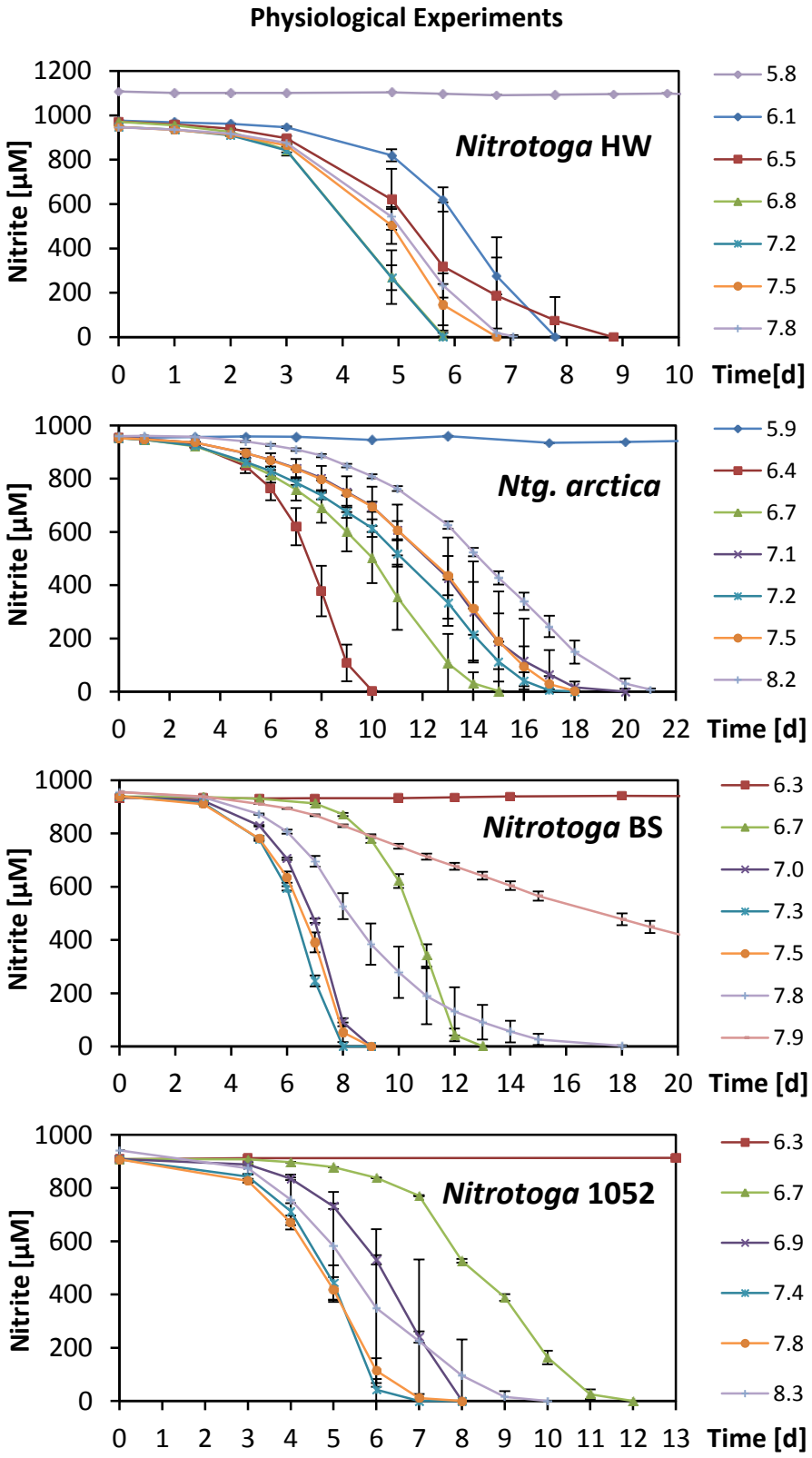
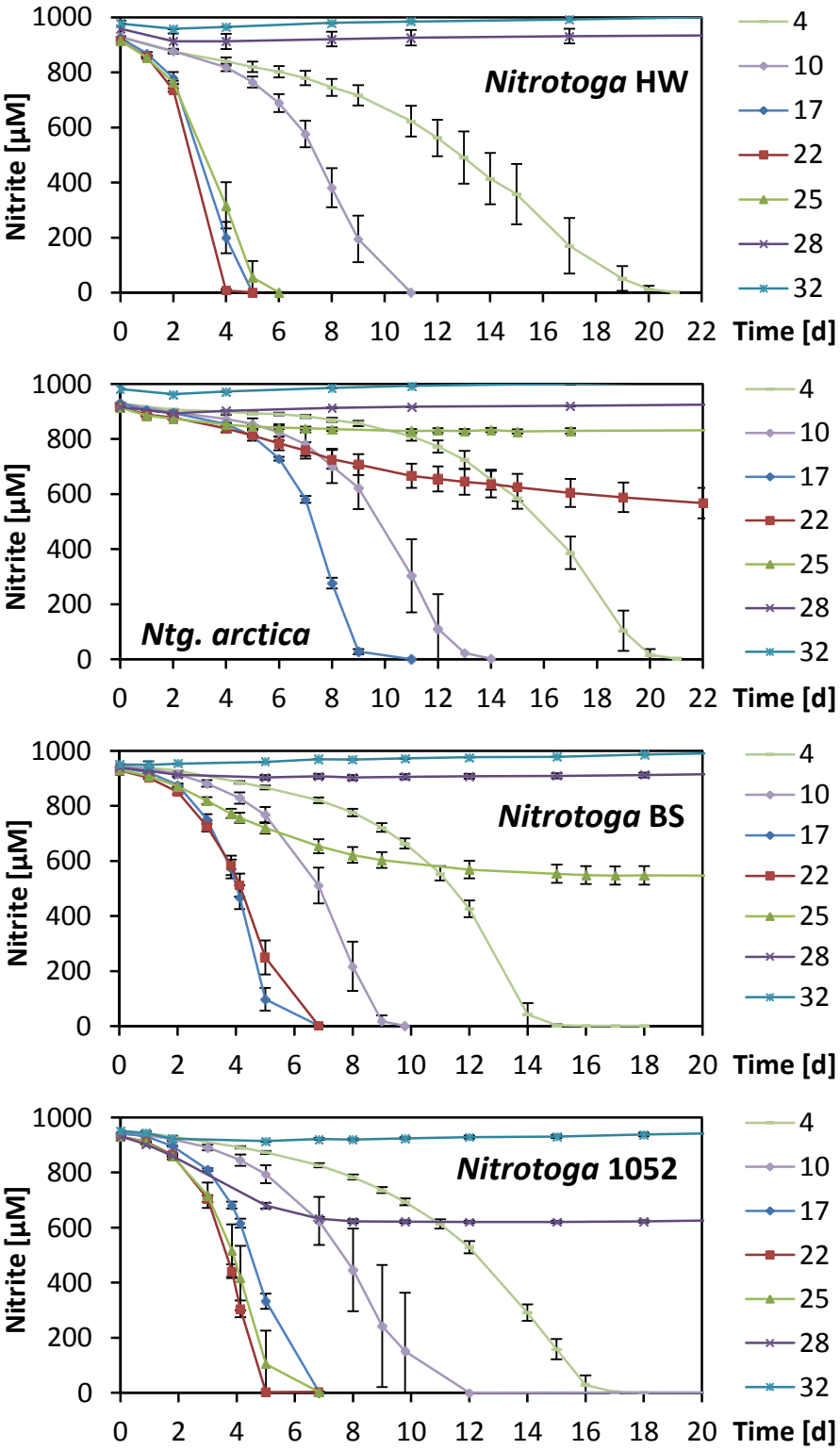
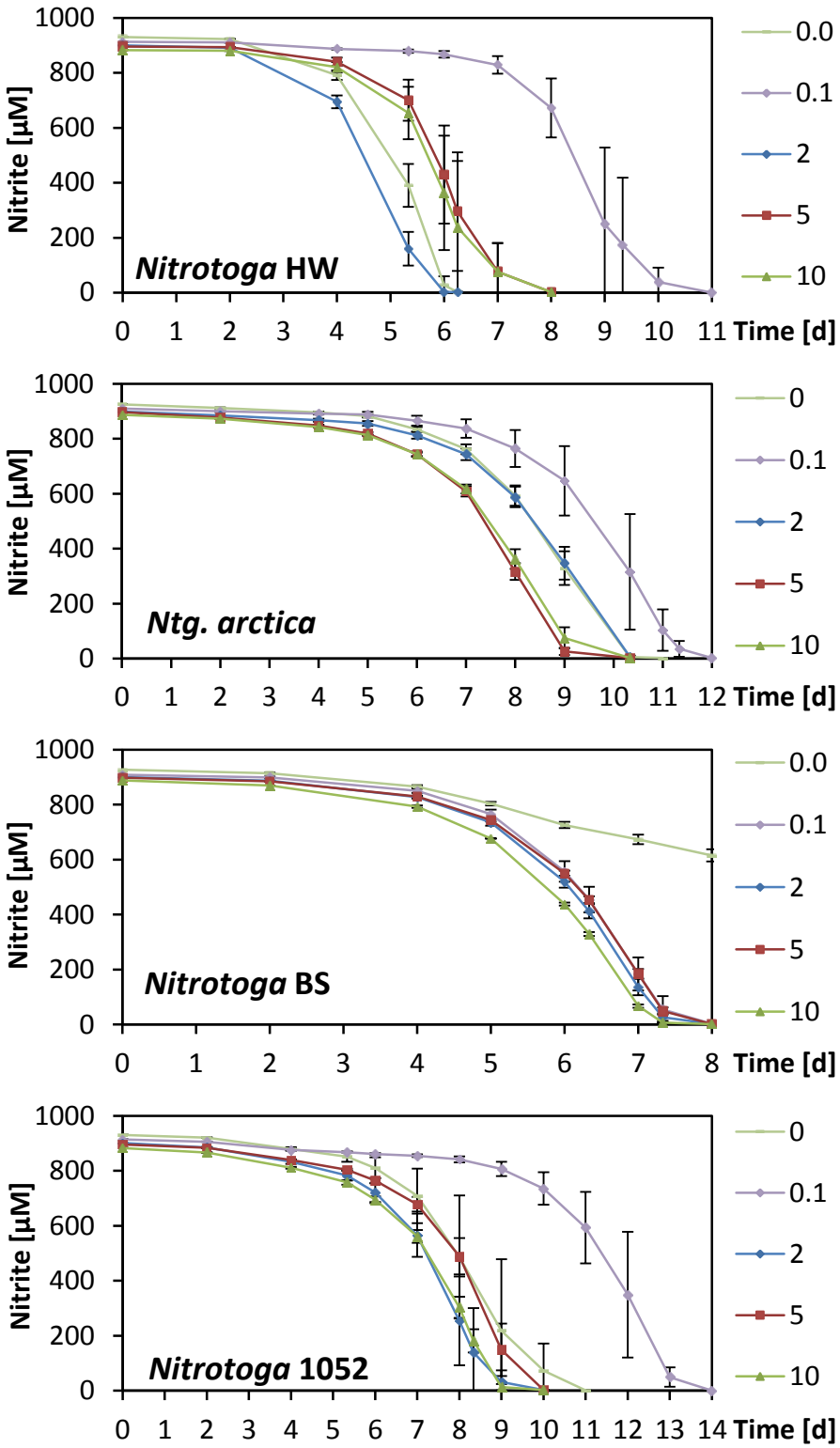
APPENDIX I



Appendix I 1: Influence of different pH values on growth of *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 3. Activity was calculated from nitrite values between approx. 800 and 0 µM.



Appendix I 2: Influence of temperatures from 4 to 32°C on growth of *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 3. Activity was calculated from nitrite values between approx. 800 and 0 µM.



Appendix I 3: Influence of 0 to 10 mM ammonium on growth of *Nitrotoga* HW, *Ntg. arctica*, *Nitrotoga* BS, and *Nitrotoga* 1052. Substrate concentration: 1 mM nitrite. Mean and SD calculated from n = 3. *Nitrotoga* BS finished oxidation of 1 mM nitrite at 0 mM ammonium within 32 days. Activity was calculated from nitrite values between approx. 800 and 0 µM.

APPENDIX II

Cloned Sequences and Further Phylogenetic Trees

The same sequences were shared by different clones (indicated by numbers).

16S rRNA gene sequences:

> Ntg BS clone 15, clone 24, clone 26

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAG
TGGCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGG
GAAAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGA
TCCGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACA
ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACAAGTT
AATACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGC
AAGGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCC
CCTGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTA
GGTGTTGGGGGAGGAGACTTCCTTAGTACCGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGA
ATTGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTG
CCAGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATG
ACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCCAAT
CTCAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGT
GAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGT
TGCCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAACC

>Ntg BS clone 16, clone 21, clone 23, clone 25, clone 28

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAG
TGGCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGG
GAAAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGA
TCCGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACA
ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACAAGTT
AATACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGC
AAGGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCC
CCTGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTA
GGTGTTGGGGGAGGAGACTTCCTTAGTACCGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGA
ATTGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTG
CCAGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATG
ACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCCAAT
CTCAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGT
GAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGT
TGCCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAGCC

>Ntg BS clone 17, clone 18, clone 19, clone 20, clone 22, clone 27

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAG
TGGCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGG
GAAAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGA
TCCGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACA
ATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACAAGTT
AATACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGC
AAGGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCC
CCTGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTA
GGTGTTGGGGGAGGAGACTTCCTTAGTACCGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGA
ATTGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTG
CCAGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATG
ACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCCAAT
CTCAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGT
GAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGT
TGCCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAACC

>Ntg HW clone 1, clone 3, clone 7, clone 8

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGTAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATG
GGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCATGGGTTAAT
ACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGG
AATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAAG
GCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCT
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GACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCCA
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AAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAA
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CACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAACC

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AGAGTTTGATCCTGGCTCAGATTGAANGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGTAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCATGGGTTAA
TACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
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AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
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>Ntg HW clone 5, clone 10, clone 11, clone 12

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
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AAGCGGGGGATTCGTAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCATGGGTTAA
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GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
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AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
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>Ntg HW clone 6, clone 9

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGTAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCATGGGTTAA
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ACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
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AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGTAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGAAAGCCTGATCCAGCCATGCCGCGNGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCATGGGTTAA
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AGAGTTTGATCATGGCTCAGATTGAASGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCTTGGAGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTAAGGGGGA
AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
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AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCTTGGAGGAGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTAAGGGGGA
AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
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AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCTTGGNGGCGAGTG
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AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
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AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCTTGGTGGCGAGTG
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AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
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GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCACATATGCCCTAAGGGGGA
AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
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TACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
TGTTGGGGGAGGAGACTTCCTTAGTACCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCT
CAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGTTG
CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAACC

>Ntg arctica clone 22, clone 23

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCTTGGAGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTAAGGGGGA
AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
TGTTGGGGGAGGAGACTTCCTTAGTACCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCT
CAGAAAGCCGATCATAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGTTG
CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAACC

>Ntg arctica clone 24

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCTTGGAGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTAAGGGGGA
AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
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TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
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CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAGCC

>Ntg arctica clone 27

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCTTGGAGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTAAGGGGGA
AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TACCCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
TGTTGGGGGAGGAGACTTCCTTAGTACCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
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ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGTTG
CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAACC

>Ntg arctica clone 28

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCTTGGNGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTAAGGGGGA
AAGCGGGGGATTCGTAAGAACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
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GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
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TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
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TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
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CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAACC

>Ntg 1052 clone 2, clone 31

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGCAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TAACCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
TGTTGGGGGAGGAGACTTCCTTAGTACCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCT
CAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGTTG
CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAGCC

>Ntg 1052 clone 7, clone 14

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGCAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TAACCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
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TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
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CAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGTTG
CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAGCC

>Ntg 1052 clone 8

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGCAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TAACCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
TGTTGGGGGAGGAGACTTCCTTAGTACCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCACAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCT
CAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGTTG
CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAGCC

>Ntg 1052 clone 11, clone 12, clone 37, clone 40, clone 47

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGCAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TAACCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
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TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
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>Ntg 1052 clone 13

AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGCAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TAACCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTATGGTGCGAGCGTTAGTCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
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AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCT
CAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGTTG
CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAGCC

>Ntg 1052 clone 42
AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGGGCAACCCTGGTGGCGAGTG
GCGAACGGGTGAGTAATATATCGGAACGTACCCGGAAATGGGGGATAACGTAGCGAAAGTTACGCTAATACCGCATATGCCCTGAGGGGGA
AAGCGGGGGATTCGCAAGGACCTCGCGTTTTCGGAGCGGCCGATATCGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATC
CGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGCCGGAAAGAAAACGCACGGGTTAA
TAACCTGTGTGGATGACGGTACCGGAAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGTGACTGCAA
GGCTAGAGTACGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCC
TGGGTCGATACTGACGGTCATGCACGAAAGCGTGGGGAGCAACCAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGG
TGTTGGGGGAGGAGACTTCCTTAGTACCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGCCAGGAACTTGCC
AGAGATGGCTTGGTGCCCGAAAGGGAACCTGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTCATTAATTGCCATCATTTAGTTGGGCACTTTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGTCGGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCTAATCT
CAGAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGA
ATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTTCTACCAGAAGCAGCTAGCCTAACCGCAAGGGGGGCGGTTG
CCACGGTAGGGTTCGTGACTGGGGTGAAGTCGTAACAAGGTAACC

nxrB gene sequences:

>Ntg BS 16, clone 19, clone 25, clone 28, clone 29, clone 32, clone 33, clone 34, clone 35, clone 36
GAAACGATATTCTGGAACAACGTCGAATCGAAGCCTTATGGTTTCTATCCGCTTGCCTGGGATGTGAAGGTTCTGGCCCTGCTGGGAGAGC
AAGCGCAACCGTGGTCAGGCAACAAGTACAATGGGACGACGATCTTTGAGGATCTGGGCATGAACCAGCGGCTCAAGGGTTATCTGCCTGA
TGAAATGGACTATGCGCATCCGAACCTTGGTGAAGATGAATGTCTTAAGATCTTGGATGGTGAAGGCGATTATATTAAGGGTCCAACTCAT
AAAAACTGGGGTTTCTTCTTCCCACGGATTTGTAATCACTGCACATTCCCAGGCTGCTTGGCAGCTTGCCCAAGAAAGGCGATCTACAAGC
GGCAAGAAGACGGTATCGTTTTGATTGATGCGTCCCG
>Ntg BS clone 23
GAAACCATATTCTGGAATAACGTCGAATCGAAGCCTTATGGTTTCTATCCGCTTGCCTGGGATGTGAAGGTTCTGGCCCTGCTGGGAGAGC
AAGCGCAACCGTGGTCAGGCAACAAGTACAATGGGACGACGATCTTTGAGGATCTGGGCATGAACCAGCGGCTCAAGGGTTATCTGCCTGA
TGAAATGGACTATGCGCATCCGAACCTTGGTGAAGATGAATGTCTTAAGATCTTGGATGGTGAAGGCGATTATATTAAGGGTCCAACTCAT
AAAAACTGGGGTTTCTTCTTCCCACGGATTTGTAATCACTGCACATTCCCAGGCTGCTTGGCAGCTTGCCCAAGAAAGGCGATCTACAAGC
GGCAAGAAGACGGTATCGTTTTGATTGATGCGTCCCG
>Ntg BS clone 30, clone 31
GAAACGATATTCTGGAACAACGTCGAATCGAAGCCTTATGGTTTCTATCCGCTTGCCTGGGATGTGAAGGTTCTGGCCCTGCTGGGAGAGC
AAGCGCAACCGTGGTCAGGCAACAAGTACAATGGGACGACGATCTTTGAGGATCTGGGCATGAACCAGCGGCTCAAGGGTTATCTGCCTGA
TGAAATGGACTATGCGCATCCRAACCTTGGTGAAGATGAATGTCTTAAGATCTTGGATGGTGAAGGCGATTATATTAAGGGTCCAACTCAT
AAAAACTGGGGTTTCTTCTTCCCACGGATTTGTAATCACTGCACATTCCCAGGCTGCTTGGCAGCTTGCCCAAGAAAGGCGATCTACAAGC
GGCAAGAAGACGGTATCGTTTTGATTGATGCGTCCCG
>Ntg HW clone 1, clone 3, clone 4, clone 5 clone 8, clone 9, clone 10, clone 11, clone 12
GAAACCATATTCTGGAATAACGTGGAATCGAAGCCTTATGGATTCTATCCGTTGGCCTGGGATGTGAAGGTTCTGGCTCTTTTGGGCGAAC
AGGCACAACCGTGGTCGGGTAACAAGTACAACGGCACGACGATCTTTGAAGACCTGGGCATGAACCAGCGGATCAAGGGCTATCTGCCGGA
TGAAATGGACTATGCGCATCCGAACCTGGGCGAAGACRAGTGCCTGAAGATTCTGGATGGCGAAGGGGACTATATCAAGGGCCCGACGCAC
AAGAACTGGGGTTTCTTCTTCCCGCGGATATGCAATCACTGTACCTTCCCTGGGTGCCTGGCTGCCTGCCCGAGAAAAGCGATCTACAAGC
GTCAAGAAGACGGAATCGTGCTGATTGATGCGTCCCG
>Ntg HW clone 2
GAAACGATATTCTGGAATAACGTGGAATCGAAGCCTTATGGATTCTATCCGTTGGCCTGGGATGTGAAGGTTCTGGCTCTTTTGGGCGAAC
AGGCACAACCGTGGTCGGGTAACAAGTACAACGGCACGACGATCTTTGAAGACCTGGGCATGAACCAGCGGATCAAGGGCTATCTGCCGGA
TGAAATGGACTATGCGCATCCGAACCTGGGCGAAGACGAGTGCCTGAAGATTCTGGATGGCGAAGGGGACTATATCAAGGGCCCGACGCAC
AAGAACTGGGGTTTCTTCTTCCCGCGGATATGCAATCACTGTACCTTCCCTGGGTGCCTGGCTGCCTGCCCGAGAAAAGCGATCTACAAGC
GTCAAGAAGACGGAATCGTGCTGATTGATGCGTCCCG
>Ntg arctica clone 15
GAAACGATATTCTGGAATAACGTGGAATCGAAGCCTTATGGATTCTATCCGCTTGCTTGGGATGTGAAGGTTTTGGCCCTCCTGGGAGAAC
AGGCGCAACCCTGGTCAGGCAACAAATACAGCGGCACGACGATCTTTGAAGACCTGGGCATGAATCAACGGATCAAGGGTTATCTGCCCGA
TGAAATGGACTATGCGCATCCGAACCTTGGGGAAGATGAGTGCCTTAAAATCCTGGATGGTGAAGGGGATTATATCAAGGGTCCGACGCAT
AAGAACTGGGGTTTCTTCTTCCCGCGGATATGTAACCACTGTACCTTCCCCGGTTGTTTGGCAGCGTGCCCGAGAAAGGCGATCTACAAGC
GGCAAGAAGATGGGATCGTTTTGATTGATGCGTCCCG
>Ntg arctica clone 16, clone 17, clone 18, clone 19, clone 21, clone 22, clone 25, clone 26, clone 27, clone 28
GAAACGATATTCTGGAATAACGTGGAATCGAAGCCTTATGGATTCTATCCGCTTGCTTGGGATGTGAAGGTTTTGGCCCTCCTGGGAGAAC
AGGCGCAACCCTGGTCAGGCAACAAATACAACGGCACGACGATCTTTGAAGACCTGGGCATGAATCAACGGATCAAGGGTTATCTGCCCGA
TGAAATGGACTATGCGCATCCGAACCTTGGGGAAGATGAGTGCCTTAAAATCCTGGATGGTGAAGGGGATTATATCAAGGGTCCGACGCAT
AAGAACTGGGGTTTCTTCTTCCCGCGGATATGTAACCACTGTACCTTCCCCGGTTGTTTGGCAGCGTGCCCGAGAAAGGCGATCTACAAGC
GGCAAGAAGATGGGATCGTTTTGATTGATGCGTCCCG
>Ntg 1052 clone 1, clone 2, clone 3, clone 5, clone 8, clone 10, clone 13,
GAAACGATATTCTGGAATAACGTGGAATCGAAGCCTTATGGATTCTATCCCCTTGCTTGGGATGTGAAGGTTTTGGCACTGCTGGGAGAAC
AAGCGCAACCGTGGTCAGGCAACAAGTACAACGGCACGACGATCTTTGAAGATCTGGGCATGAATCAGCGGATCAAGGGTTATCTGCCTGA
TGAAATGGACTATGCGCATCCGAACCTTGGGGAAGATGAATGCCTTAAGATTCTGGATGGTGAAGGGGATTATATCAAGGGCCCTACTCAC
AAGAACTGGGGTTTCTTCTTCCCACGGATTTGCAATCACTGCACCTTCCCAGGTTGCTTGGCAGCGTGCCCAAGAAAGGCGATCTACAAGA
GGCAAGAAGACGGGATCGTTTTGATTGATGCGTCCCG

>Ntg 1052 clone 9, clone 15, clone 16
GAAACGATATTCTGGAATAACGTGGAATCGAAGCCTTATGGATTCTATCCCCTTGCTTGGGATGTGAAGGTTTTGGCACTGCTGGGAGAAC
AAGCGCAACCGTGGTCAGGCAACAAGTACAACGGCACGACGATCTTTGAAGATCTGGGCATGAATCAGCGGATCAAGGGTTATCTGCCTGA
TGAAATGGACTATGCGCATCCGAACCTTGGGGAAGATGAATGCCTTAAGATTCTGGATGGTGAAGGGGATTATATCAAGGGCCCTACTCAC
AAGAACTGGGGTTTCTTCTTCCCACGGATTTGCAATCACTGCACCTTCCCAGGTTGCTTGGCAGCGTGCCCAAGAAAGGCGATCTACAAGA
GGCAAGAAGACGGGATCGTTTTAATTGATGCGTCCCG

Cloned sequences generated with *Nitrotoga nxrB* primers that are not *nxrB* gene sequences:

>Ntg BS clone 24
GAAACGATATTCTGGAATCTCCTGGCTGGCAATGTCGCGATACCAGGGCTCGGTCATCTTGCGCTTCGCGGGCAGGTTGAGCCATAGCTGA
AAGCCTTCCATCACGCCGTCTTTTTGCTCGGGGATTTCAGAATGAATTATGCCGCGCCCAGCGGTCATCCACTGCACACCGCCGTTTTCCA
GCAGCCCCTCATGTCCTGCGTTATCACGATGCCGCATCCGTCCTGACAGCATATAGGTGACCGTCTCGAAACCACGATGCGGGTGATCCGG
GAAACCCGCGATGTAGTCATCCGGATTATCGCTGCCGAATGCATCCAGCATCAAAAATGGATCAAGGCGCTGCTGCAGCTTGCCAGTAAGC
ACGCGGGTCAGCTTCACCCCCGCGCCGTCCGAGGTGGCAACGCCTTCGATGATGCGGTCGACACCGCGTGATTGATGCGTCCCG
>Ntg 1052 clone 4
CGGGACGCATCAATCGGACAAGGCATGAAGCTGGCAAAGGACTTGGGCAATCTGCCGGCCAACATATGCACCCCCAGCTATCTGGCGCAAC
AAGCAGTGGCTTTAGCCAAGGCGCACAAATCACTCAAAGTTACCGTGCTGGAAGAAAAGGATATGCAAAAGCTTGGCATGGGTTCGCTATT
GTCTGTGACCCGAGGCAGCGATGAACCCGCCAAGCTCATTACTCTGGAGTATCGCGGTGCCGATAAGAAACAAAAACCCATCGTACTGGTC
GGTAAAGGCATAACTTTTGACACTGGCGGCATCTCGCTCAAACCGGGTGCCGAGATGGATGAAATGAAATACGACATGTGCGGTGCAGCCA
GCGTGCTAGGTACGATGCAAGCCATTGCTGAAATTGGACTCAAGCTAAATGTAGTCGGCATTATTCCCACTTGCGAGAACATGCCTAGTGG
TTCCGCCACCAAACCGGGCGACATCGTCACCAGCATGTCCGGGCAGACCATCGAAATTTTGAATACCGATGCGGAAGGCCGTCTCATCCTG
TGCGATGCACTTACTTATGCGGCCAAGTTTGAGCCAACCTCGGTAATCGACATAGCCACCCTTACCGGCGCCTGCGTCATCGCATTAGGCC
ATGTTGCCAGCGGGCTTTATAGCAATCAGGATACCCTGGCACAGGAACTGCTGGCAGCAGGCGAGCAAACATATGACCGGGCTTGGCACAT
GCC
>Ntg 1052 clone 6, clone 14
GAAACGATATTCTGGAATCTCCTGGCTTGCGATGTCGCGATACCAAGGCTCGGCCATCTTACGCTTCGCAGGCAGGTTGAGCCACAGCTGA
AAACCTTCCATCACGCCATCTTTTTGCTCGGGAATTTCAGAATGAATTATGCCGCGTCCAGCAGTCATCCACTGAACGTCACCATGTTTCA
GCAGCCCCTCGTGTCCTGCATTATCTCGATGCCGCATACGTCCGGACAGCATATAGGTAACCGTCTCGAAACCACGATGCGGGTGATCCGG
GAAACCCGCGATGTAGTCGTCCGGGTTGTCGCTGCCGAATGCATCCAGCATTAAAAATGGATCAAGGCGGTGCTGCAGCTTGCCCGTAAGC
ACGCGGGTCAGTTTCACGCCTGCGCCATCCGAGGTGGCAACACCTTCGATGATGCGGTCGACACCGCGCGATTGGTGCAGCATTGTTTGAT
TGATGCGTCCCG
>Ntg 1052 clone 17
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TAGTCAGGCTATTGAGCAAATTGCTCAGCGTTATGCCATGCACTTGCCATGGACTATCCGTTTTCTGTTGCGCGGGATAGGCGCGATGCGC
CGCAGCGGGGCAAATCTGGTGAGCTACCTGCTGTTCGAAAAGAATTTTTGTCGTGCGATGATCGAGCTGGGATATCAGGATGCACTGAAAC
AGAAGCAAGAAATCCTGGCTTTTATAAATCAGCGCGGGAAAGCGGCGGAAAGCCTGCCACAATGAAAGCTGCCAGTACGGGTGCAAGTATG
GGCACGCCCCACCCATGCACCACGATGAAGCCGCTTACTTTCGCCCTGCTGCGCCTGTTGGTGGACGGAAAATTCCATTCCGGGGAAATGC
TGGCGCAGCGTTTGGGCATATCGCGCGCCAGCGTGAGCAATGCCTTGCATGGGGTAGATGGCTATGGCTTGGCTTTATACAGCGTGCCCGG
CCGTGGCTATTGCCTGAGCAATCCGCCGCAATGGCTTGATGCTGCACTTATTGCTCGCCATCTAGGTGAGCAGGCGGGACAATTCCAGATT
GAANTTTTCGACAGCTTGCCCTCCAGCAATACTCTGCTGTTGCAGCGCGCAGCGCAAGGCGCACCCAGCGGCAGCGTGCTGGTGGTGGAAC
TGCAAAGCGGCGGACGTGGGCGATTGGGACGGTCATGGCATTCCGGCGTGGGCAACGCGCTTACGTTCTCGCTGTTGTGGCGCTTTGAACT
TGATTTGTCGGCGCTGTCCGGCTTGAGCTTGGCAGTT
>Ntg 1052 clone 18
GAAACGATATTCTGGAATCTCCTGGCTTGMGATGTCGCGATACCAAGGCTCGGCCATCTTACGCTTCGCAGGCAGGTTGAGCCACAGCTGA
AAACCTTCCATCACGCCATCTTTTTGCTCGGGAATTTCAGAATGAATTATGCCGCGTCCAGCAGTCATCCAYTGAACGTCACCATGTTTCA
GCAGCCCCTCGTGTCCTGCATTATCTCGATGCCGCATACGTCCGGACAGCATATAGGTAACCGTCTCGAAACCACGATGCGGGTGATCCGG
GAAACCCGCGATGTAGTCGTCCGGGTTGTCGCTGCCGAATGCATCCAGCATTAAAAATGGATCAAGGCGGTGCTGCAGCTTGCCCGTAAGC
ACGCGGGTCAGTTTCACGCCTGCGCCATCCGAGGTGGCAACACCTTCGATGATGCGGTCGACACCGCGCGATTGGTGCAGCATTGTTTGAT
TGATGCGTCCCG
>Ntg 1052 clone 19
GAAACGATATTCTGGAATCTCCTGGCTTGCGATGTCGCGATACCAAGGCTCGGCCATCTTACGCTTCGCAGGCAGGTTGAGCCACAGCTGA
AAACCTTCCATCACGCCATCTTTTTGCTCGGGAATTTCAGAATGAATTATGCCGCGTCCAGCAGTCATCCACTGAACGTCACCATGTTTCA
GCAGCCCCTCGTGTCCTGCATTATCTCGATGCCGCATACGTCCGGACAGCATATAGGTAACCGTCTCGAAACCACGATGCGGGTGATCCGG
GAAACCCGCGATGTAGTCGTCCGGGTTGTCGCTGCCGAATGCATCCAGCATTAAAAATGGATCAAGGCGGTGCTGCAGCTTGCCCGTAAGC
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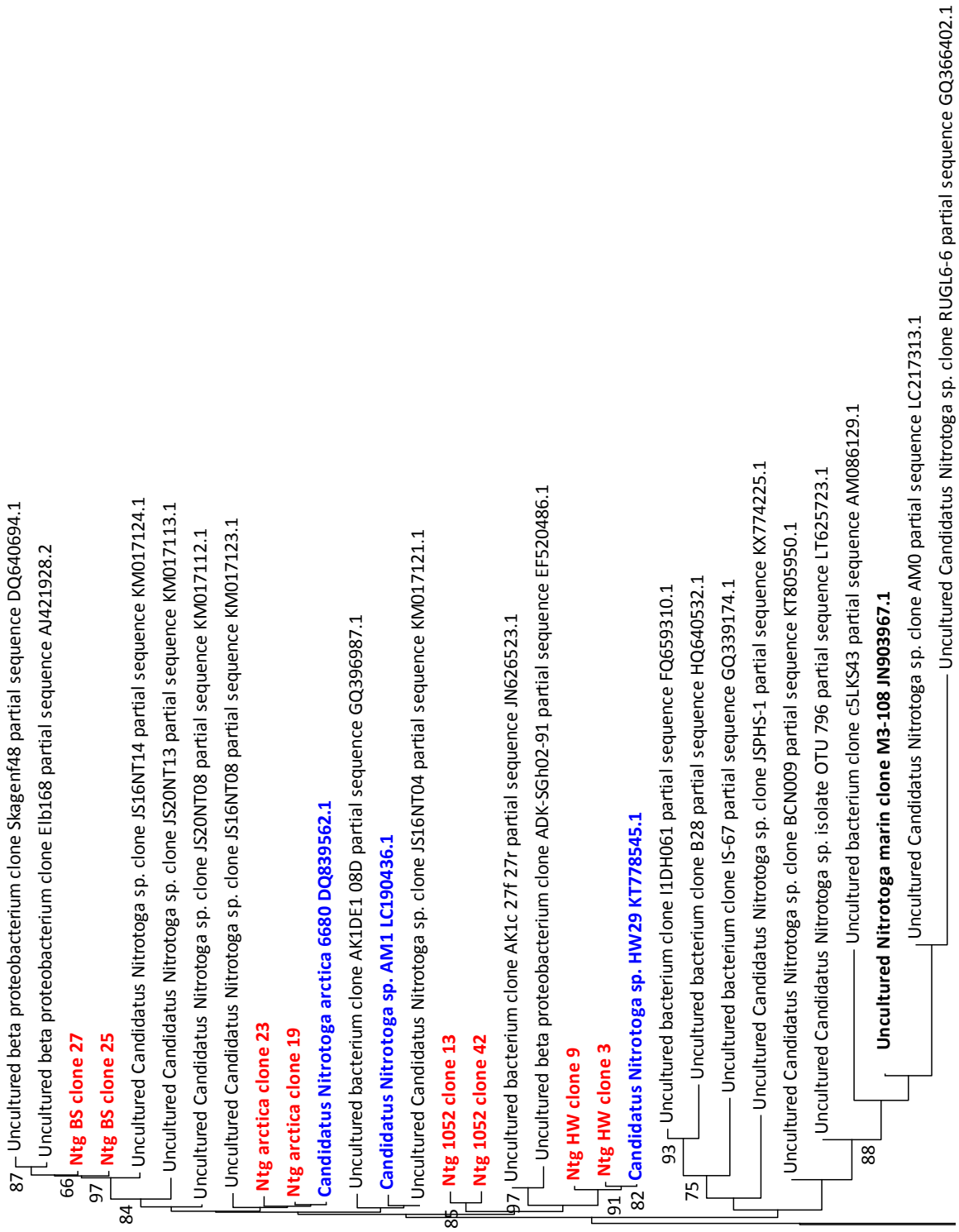
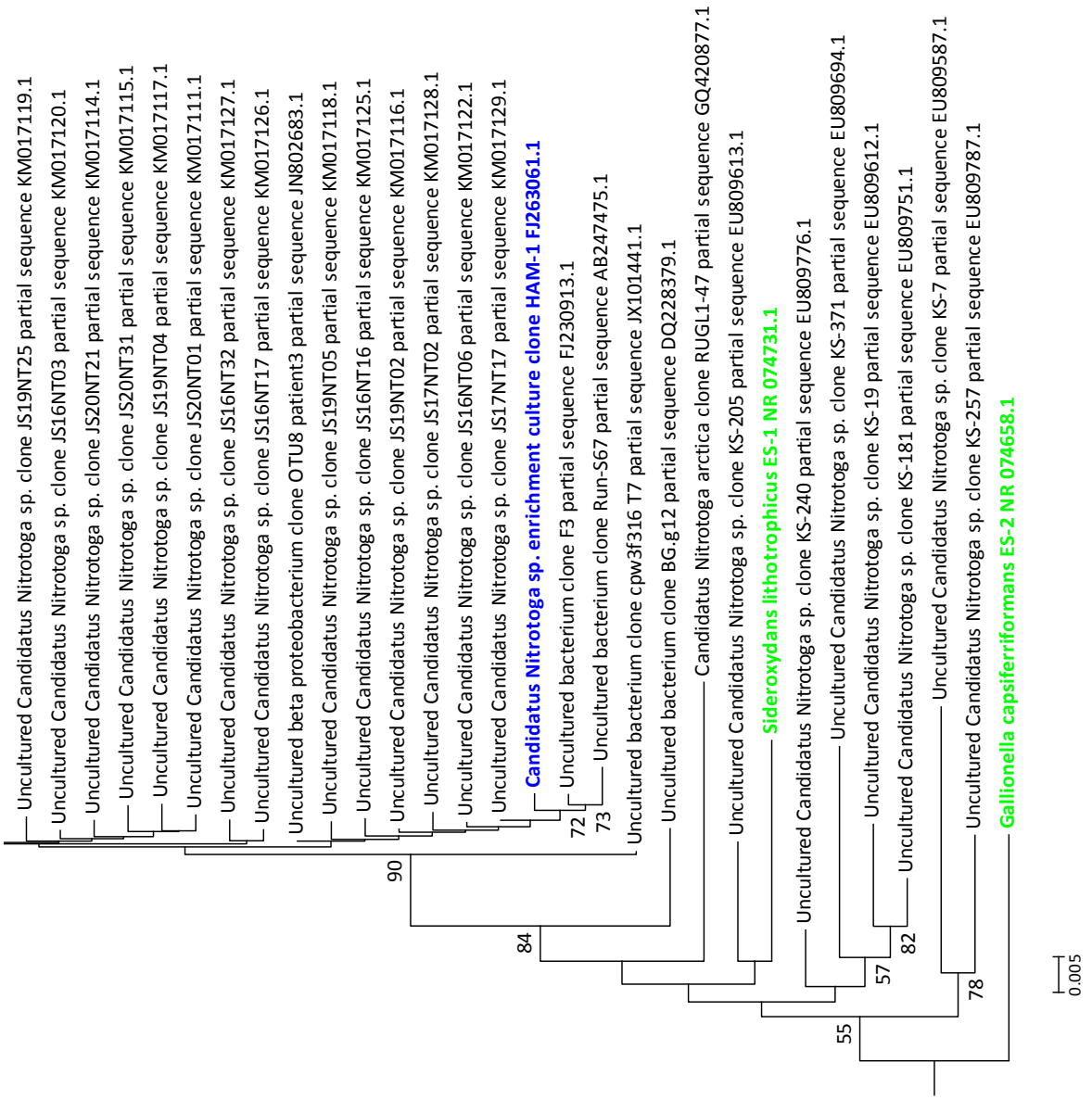
TGATGCGTCCCG



Appendix II 1: Detailed neighbor-joining tree of cloned 16S rRNA gene sequences of the most distant clones from all four investigated *Nitrotoga* cultures (red) compared to sequences deposited at NCBI, among them cultured *Nitrotoga* (blue), a marine *Nitrotoga* clone (**black**), and further cloned sequences designated as *Nitrotoga*, as well as the next described relatives (green). Scale bar indicates 0.5% sequence divergence. *Nitrosomonas europaea* ATCC 25978 (Accession nr. HE862405) was used as outgroup.



Appendix II 1 continued.

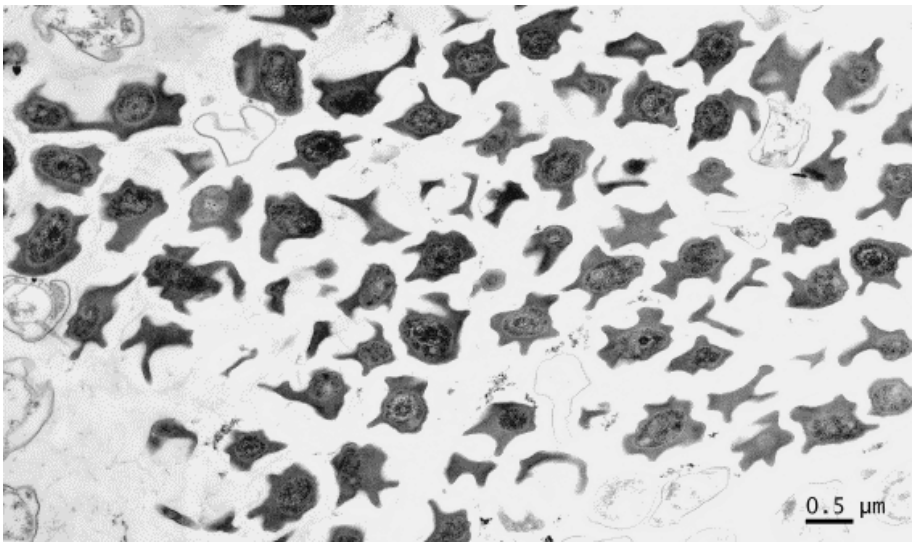
Appendix II 2: Detailed neighbor-joining tree of cloned *nxrB* gene sequences of all four investigated *Nitrotoga* cultures (red) compared to *nxrB* of different NOB (blue), Anammox bacteria (purple), a phototrophic nitrite oxidizing *Thiocapsa sp.* (green), and related sequences of other bacteria (black). Scale bar indicates 5% sequence divergence. The dimethylsulfide dehydrogenase beta subunit of *Pseudomonas litoralis* strain 2SM5 (Accession nr. LT629748.1) was used as outgroup.

Appendix II 3: Similarities of non-*nxrB* sequences cloned from *Nitrotoga* cultures. * discontiguous mega-blast was used, sequences of all other clones were evaluated by the megablast algorithm.

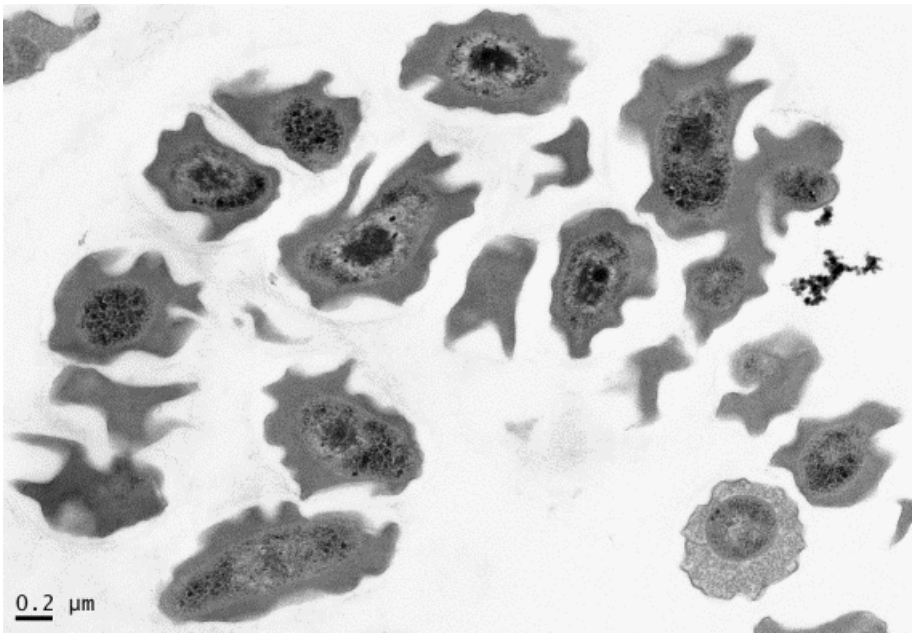
Clone nr.	Organism	Accession nr.	Query coverage [%]	Similarity [%]	Putative protein
***Nitrotoga* BS 24**	*Xanthomonas albilineans* GPE PC73, complete genome	FP565176.1	58	74	putative pirin-related protein protein_id="CBA16554.1
***Nitrotoga* 1052 4**	*Gallionella capsiferriformans* ES-2, complete genome	CP002159.1	92	75	PFAM: peptidase M17 leucyl aminopeptidase domain protein
	Sideroxydans lithotrophicus ES-1, complete genome	CP001965.1	83	78	PFAM: peptidase M17 leucyl aminopeptidase domain protein
***Nitrotoga* 1052 6, 18, 19**	*Janthinobacterium* sp. LM6, complete genome	CP019510.1	77-78	74-75	hypothetical protein, WP_010397537.1
Nitrotoga* 1052 17	*Sideroxydans lithotrophicus* ES-1, complete genome	CP001965.1	92	65	PFAM: Patatin PF01734; PFAM: biotin/lipoate A/B protein ligase; Helix-turn-helix, type 11 domain protein; biotin protein ligase domain protein" TIGR00121

APPENDIX III

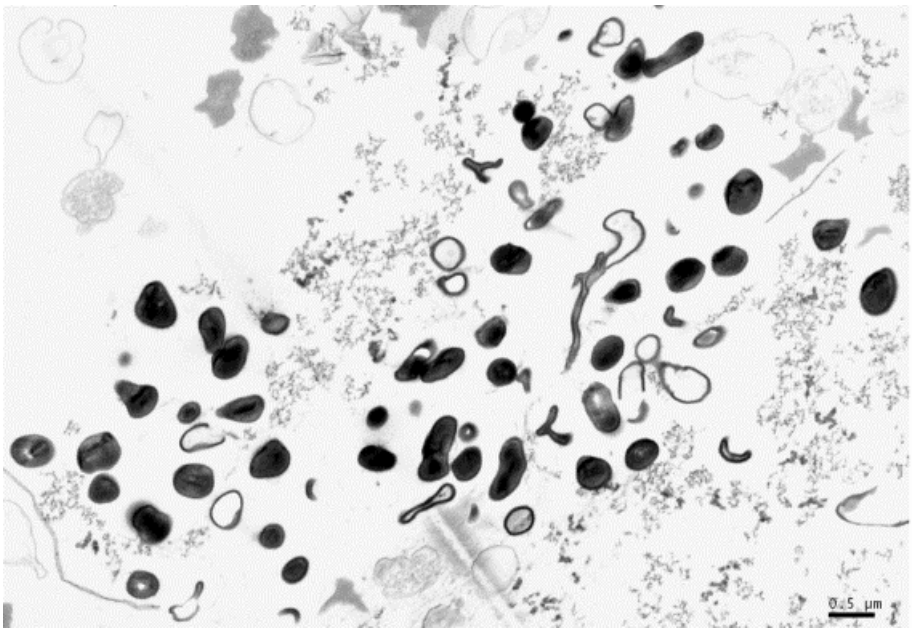
TEM and SEM Micrographs of *Nitrotoga*



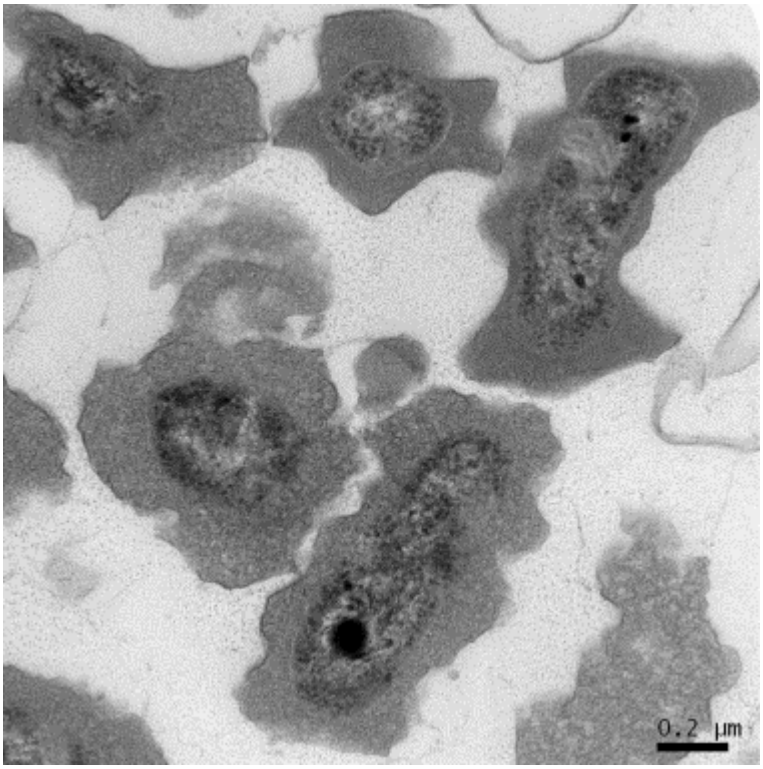
Appendix III 1: TEM overview of a *Ntg. arctica* microcolony. Cells appear as short rods or as cocci. The wide and irregular periplasm is clearly visible.



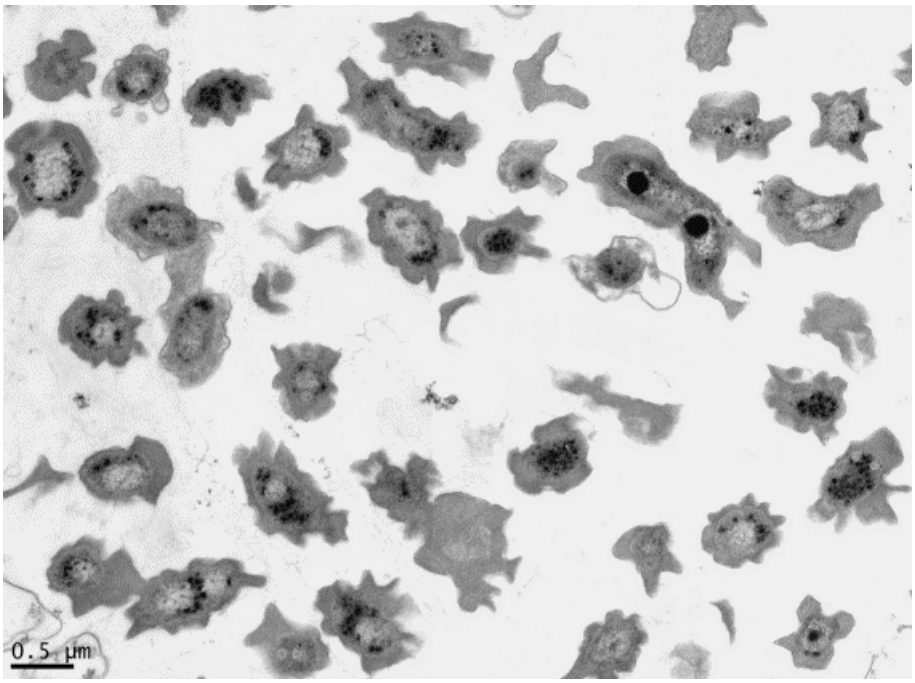
Appendix III 2: Detailed TEM picture of *Ntg. arctica* with three cells in one periplasm (upper right corner).



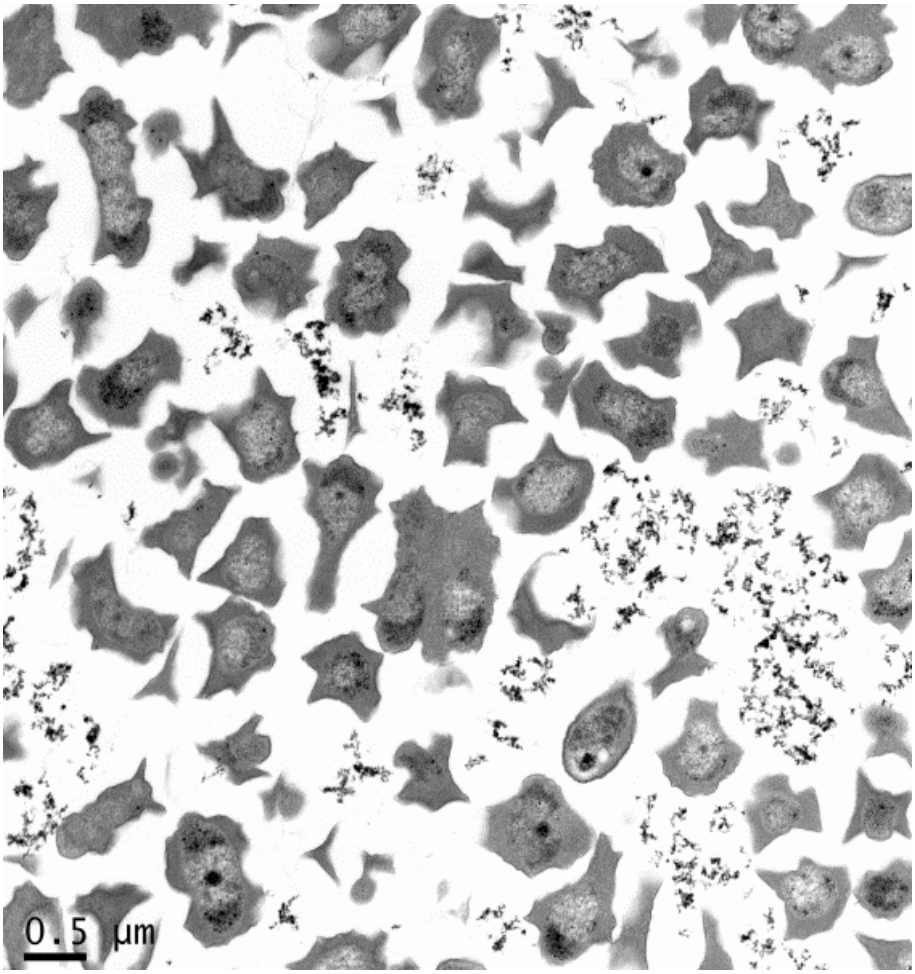
Appendix III 3: TEM overview of heavily contrasted contaminations in *Nitrotoga* BS. Some *Nitrotoga* cells are visible in the upper section of the picture.



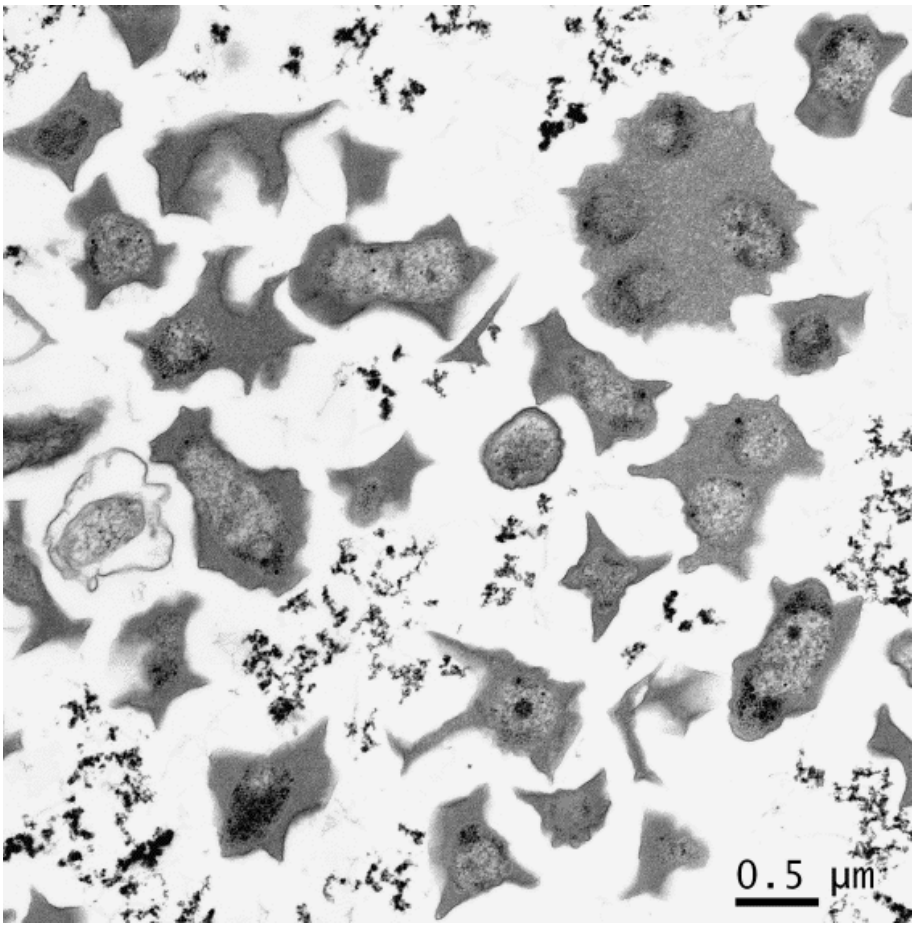
Appendix III 4: Detailed TEM micrograph of *Nitrotoga* BS.



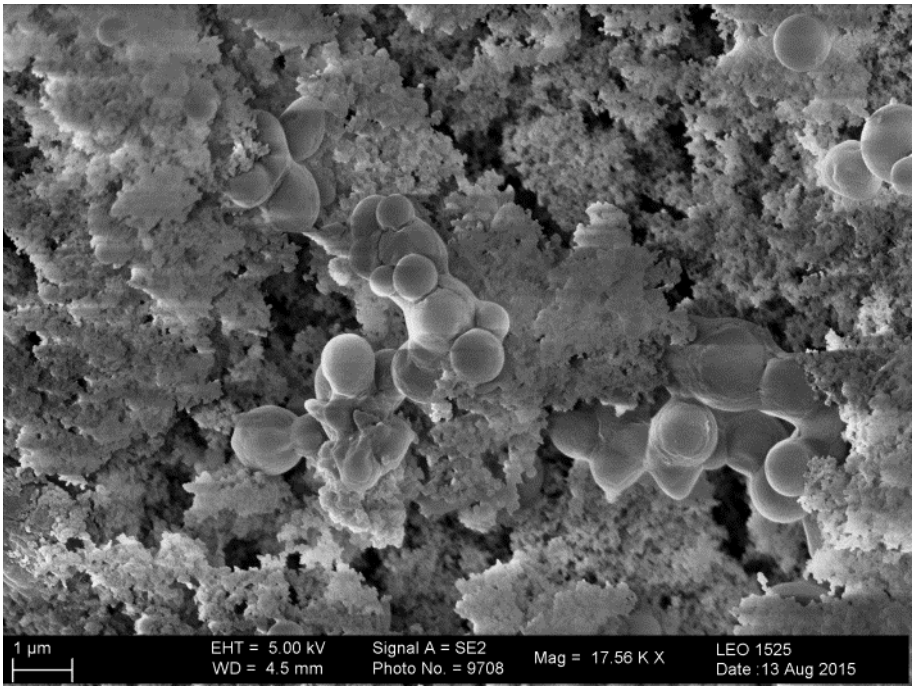
Appendix III 5: TEM overview of a *Nitrotoga* HW microcolony, containing several conjoined cells inhabiting one periplasm. The very long rod in the upper right corner might be dividing, due to the two dark centers and the constricted periplasm.



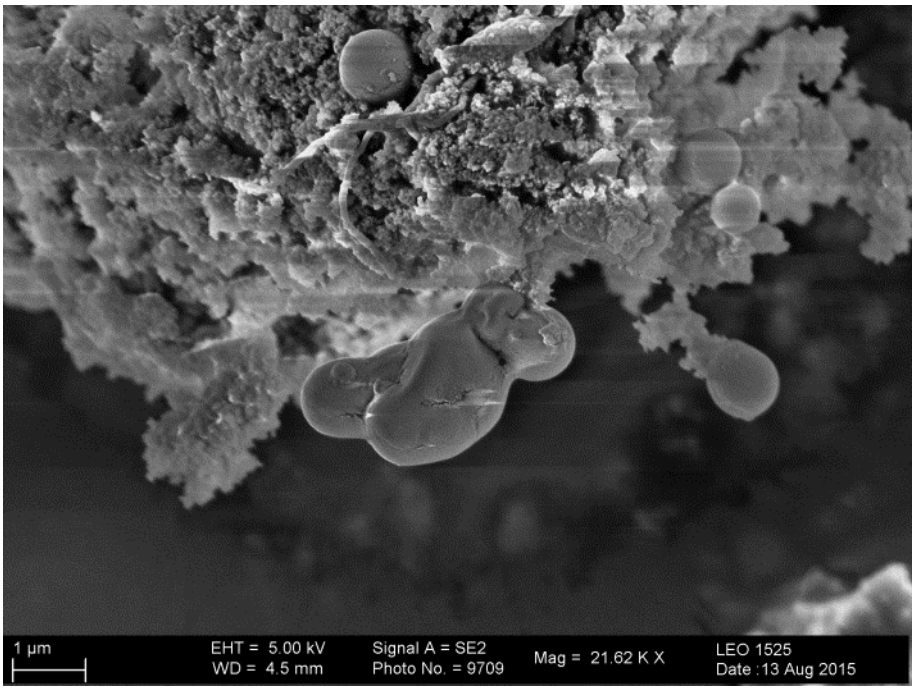
Appendix III 6: TEM overview of a *Nitrotoga* 1052 microcolony with paired cells in one periplasm.



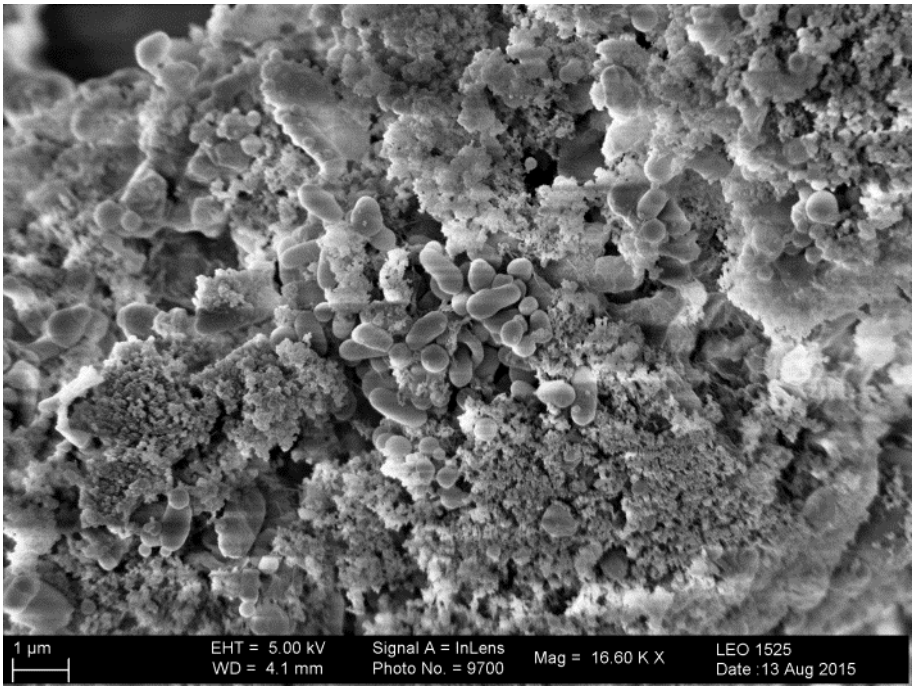
Appendix III 7: TEM micrograph of *Nitrotoga* 1052 with four apparently coccoid cells sharing one periplasm.



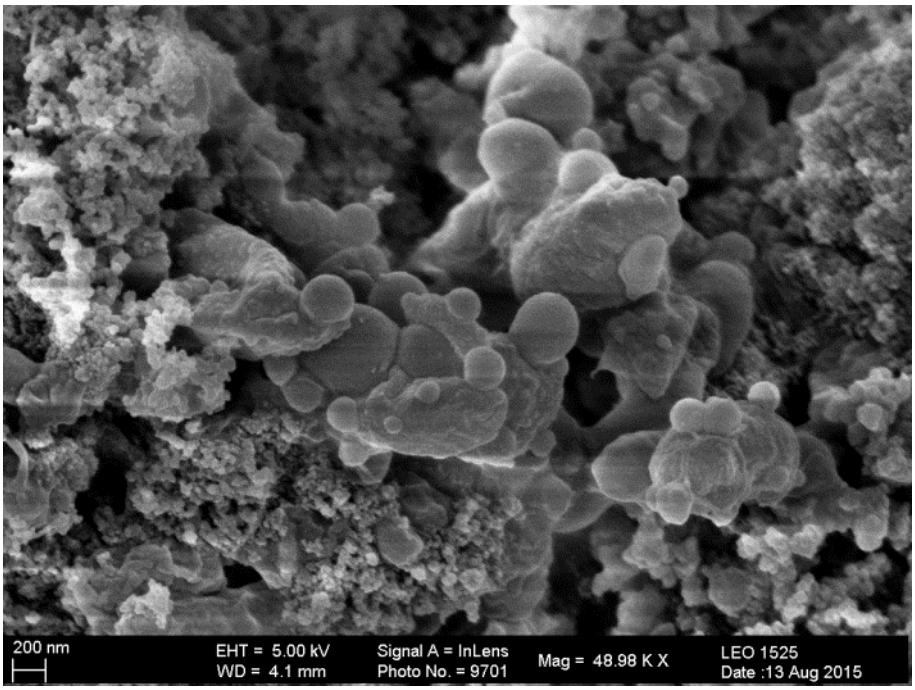
Appendix III 8: Detailed SEM picture of *Ntg. arctica* cells that appear as budding, irregular cocci on top of EPS particles. Shadow-like stripes occurred during image recording due to charging effects under the electron beam.



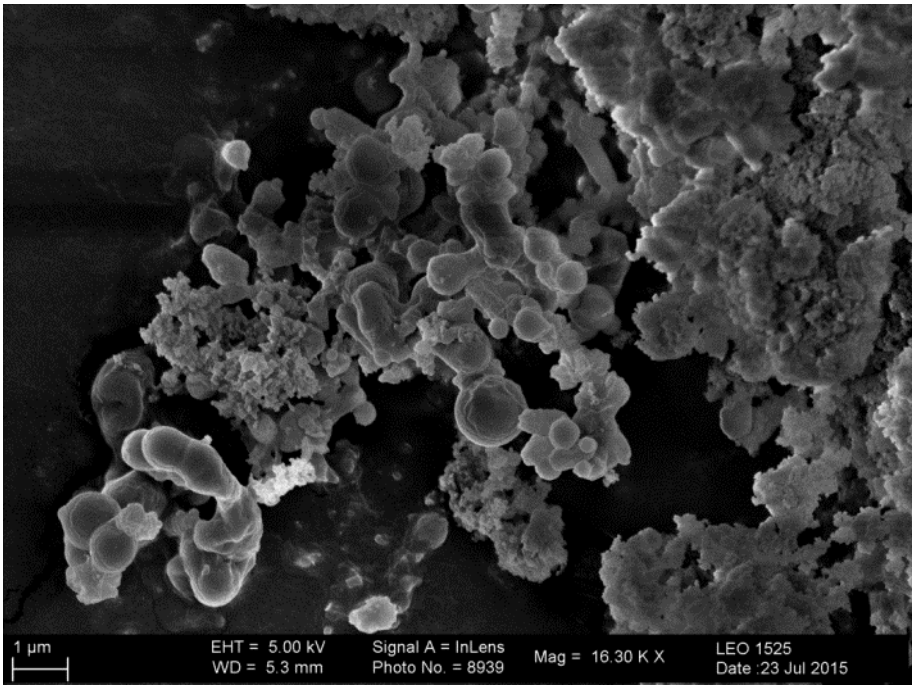
Appendix III 9: Detailed SEM micrograph of *Ntg. arctica*. The upper central part contains a spiraling structure, probably flagella or pili.



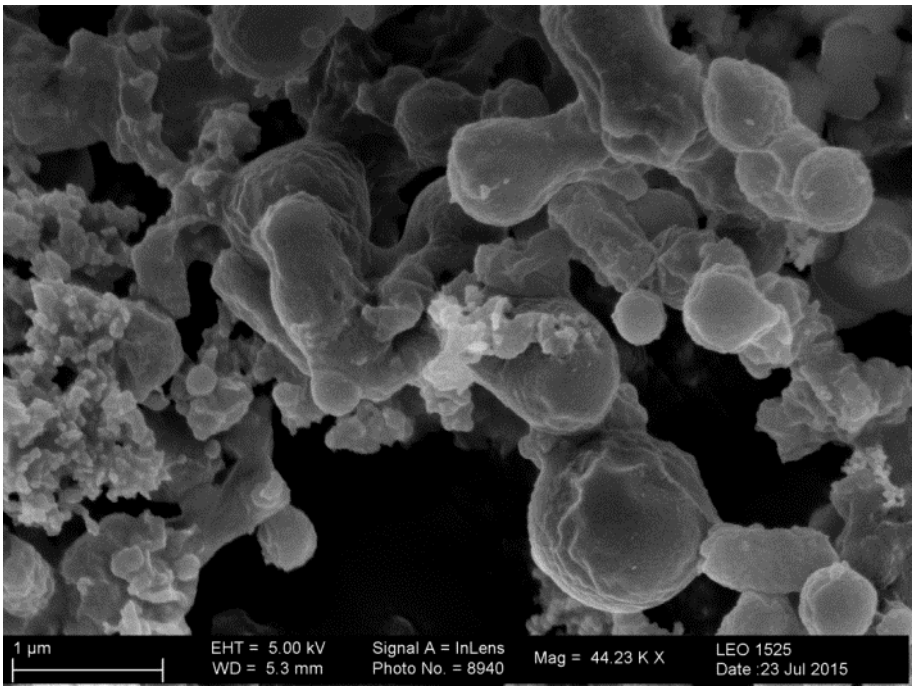
Appendix III 10: SEM overview of *Nitrotoga* BS, containing a possible contamination in the center, since these cells appear much more uniform than *Nitrotoga* cells.



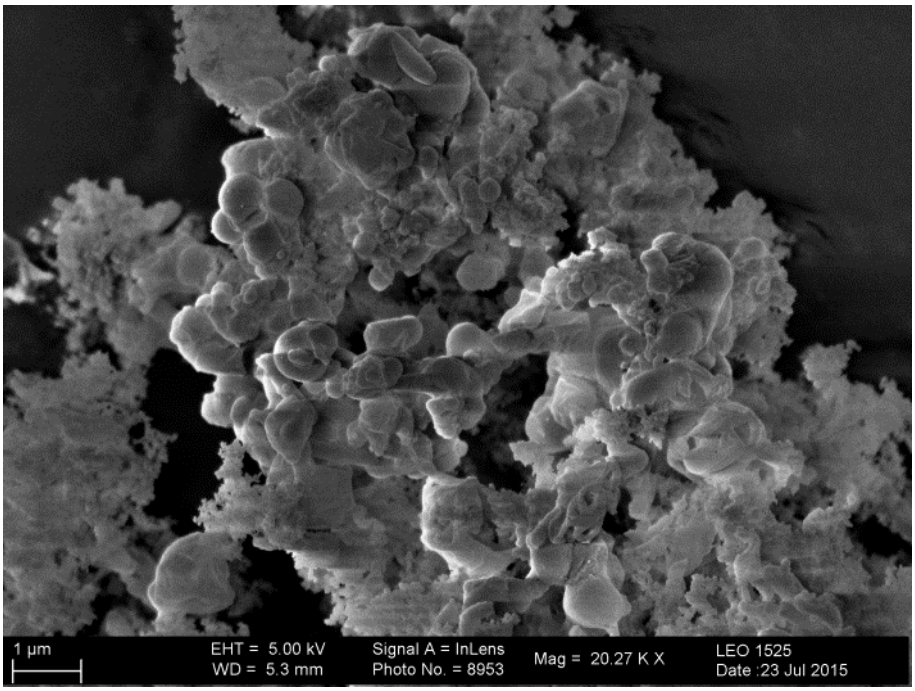
Appendix III 11: Detailed SEM picture of *Nitrotoga* BS, again showing irregular, coccoid cells with budding structures as was observed in other *Nitrotoga* cultures.



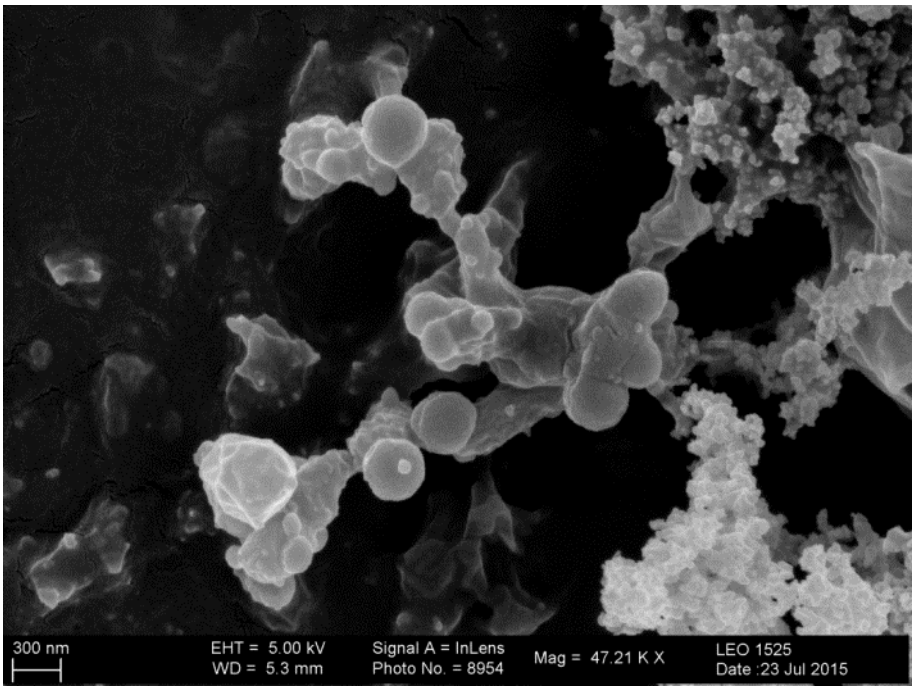
Appendix III 12: SEM micrograph of *Nitrotoga* HW.



Appendix III 13: Detailed SEM picture of *Nitrotoga* HW with wrinkled cell surface.

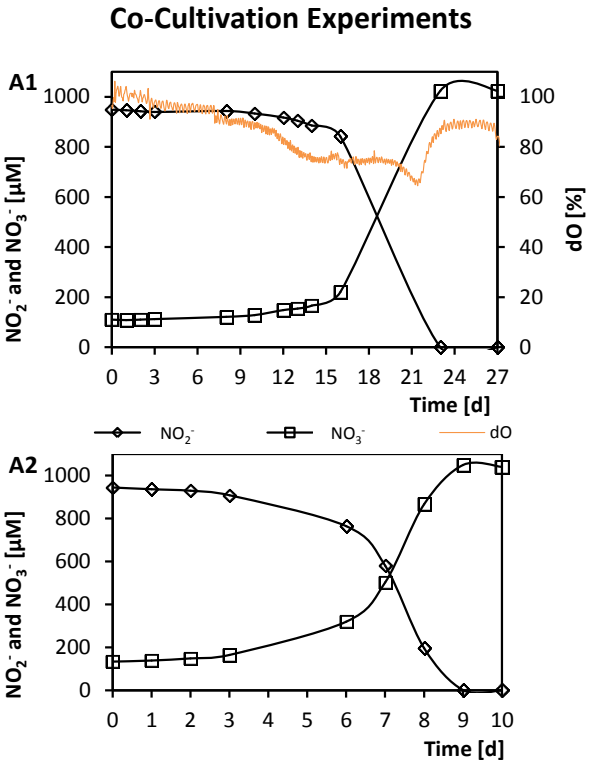


Appendix III 14: SEM micrograph of *Nitrotoga* 1052, depicting wrinkled, irregular cells.

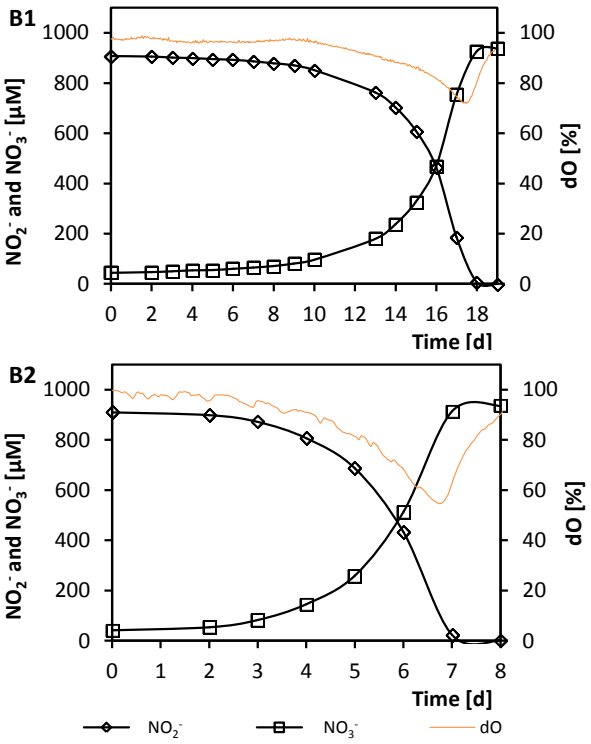


Appendix III 15: SEM micrograph of *Nitrotoga* 1052 with wrinkled cells.

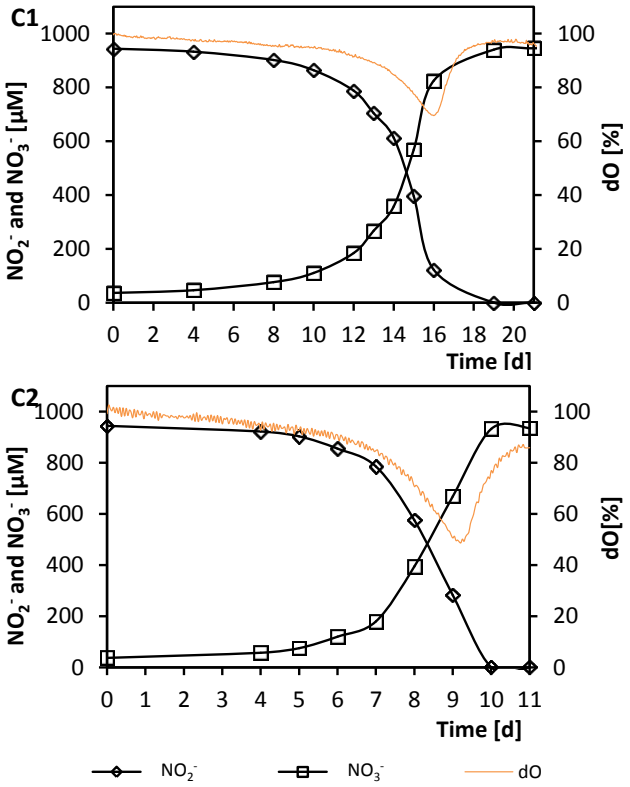
APPENDIX IV



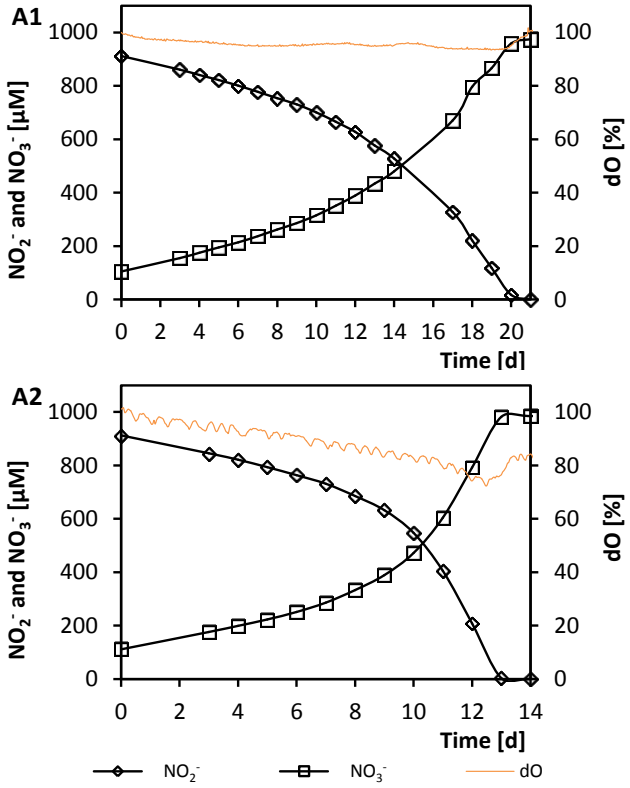
Appendix IV 1: Co-cultivation of *Nitrotoga* BS and *Nsp. defluvii*, run **A**. Nitrite, nitrate, and dO for pH 6.4 **(1)** and pH 7.4 **(2)**. Note different lengths of time axes for both processes. dO was not available during the process at pH 7.4 **(2)**.



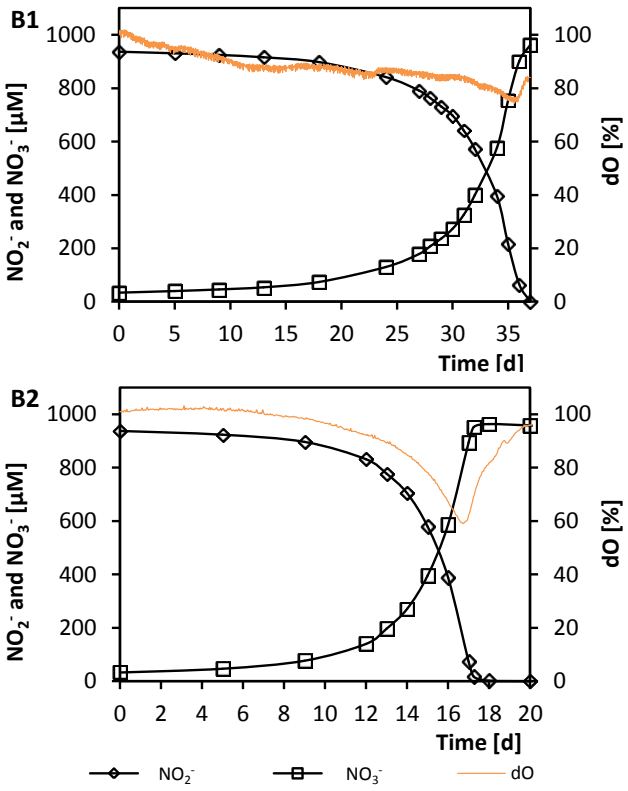
Appendix IV 2: Co-cultivation of *Nitrotoga* BS and *Nsp. defluvii*, run **B**. Nitrite, nitrate, and dO for pH 6.4 **(1)** and pH 7.4 **(2)**. Note different lengths of time axes for both processes.



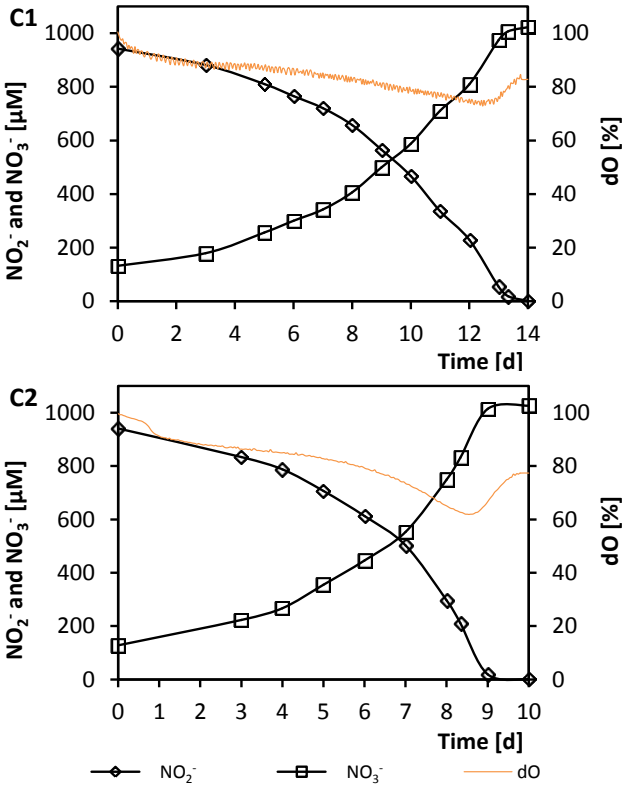
Appendix IV 3: Co-cultivation of *Nitrotoga* BS and *Nsp. defluvii*, run **C**. Nitrite, nitrate, and dO for pH 6.4 **(1)** and pH 7.4 **(2)**. Note different lengths of time axes for both processes.



Appendix IV 4: Co-cultivation of *Ntg. arctica* and *Nsp. defluvii*, run **A**. Nitrite, nitrate, and dO for pH 6.4 **(1)** and pH 7.4 **(2)**. Note different lengths of time axes for both processes.



Appendix IV 5: Co-cultivation of *Ntg. arctica* and *Nsp. defluvii*, run **A**. Nitrite, nitrate, and dO for pH 6.4 **(1)** and pH 7.4 **(2)**. Note different lengths of time axes for both processes.



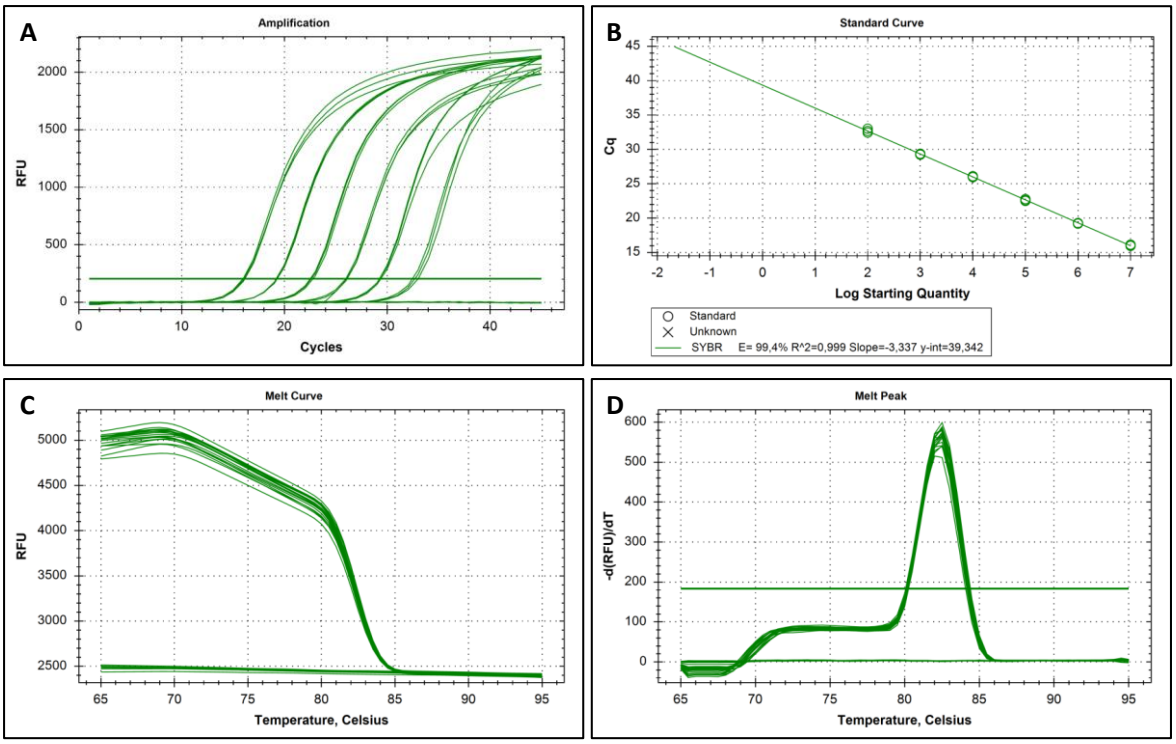
Appendix IV 6: Co-cultivation of *Ntg. arctica* and *Nsp. defluvii*, run **A**. Nitrite, nitrate, and dO for pH 6.4 **(1)** and pH 7.4 **(2)**. Note different lengths of time axes for both processes.

Appendix IV 7: Summarized qPCR results before inoculation and for reactor processes with *Nitrotoga* BS and *Nsp. defluvii*, given as copies per ml and relative abundance. Mean and SD calculated from technical triplicates.

Run	pH	*Nitrotoga* BS		*Nsp. defluvii*	
		[copies/ml]	[%]	[copies/ml]	[%]
	Start	$1.06*10^5 \pm 2.51*10^4$	65.4	$5.61*10^4 \pm 5.79*10^3$	34.6
A	**6.4**	$1.57*10^7 \pm 4.66*10^5$	13.4	$1.02*10^8 \pm 6.03*10^6$	86.6
	7.4	$1.54*10^8 \pm 7.81*10^6$	94.9	$8.34*10^6 \pm 4.11*10^5$	5.1
	Start	$1.25*10^4 \pm 6.43*10^3$	47.7	$1.37*10^4 \pm 2.50*10^2$	52.3
B	**6.4**	$3.77*10^7 \pm 2.13*10^6$	15.4	$2.08*10^8 \pm 8.64*10^6$	84.6
	7.4	$2.05*10^7 \pm 1.14*10^6$	95.4	$9.89*10^5 \pm 5.04*10^4$	4.6
	Start	$2.58*10^4 \pm 7.51*10^3$	12.7	$1.77*10^5 \pm 1.54*10^4$	87.3
C	**6.4**	$3.23*10^7 \pm 2.03*10^6$	61.8	$1.99*10^7 \pm 1.97*10^6$	38.2
	7.4	$1.28*10^8 \pm 8.01*10^6$	94.3	$7.80*10^6 \pm 4.53*10^5$	5.7

Appendix IV 8: Summarized qPCR results before inoculation and for reactor processes with *Ntg. arctica* and *Nsp. defluvii*, given as copies per ml and relative abundance. Mean and SD calculated from technical triplicates.

Run	pH	*Ntg. arctica*		*Nsp. defluvii*	
		[copies/ml]	[%]	[copies/ml]	[%]
	Start	$1.19*10^5 \pm 4.21*10^4$	36.4	$2.07*10^5 \pm 4.54*10^3$	63.6
A	**6.4**	$1.22*10^6 \pm 1.13*10^5$	69.0	$5.50*10^5 \pm 1.40*10^4$	31.0
	7.4	$4.85*10^7 \pm 4.77*10^6$	60.8	$3.13*10^7 \pm 3.38*10^6$	39.2
	Start	$3.01*10^4 \pm 1.68*10^4$	18.1	$1.36*10^5 \pm 1.17*10^4$	81.9
B	**6.4**	$2.44*10^7 \pm 3.05*10^6$	18.2	$1.10*10^8 \pm 3.57*10^6$	81.8
	7.4	$8.52*10^7 \pm 4.52*10^6$	55.2	$6.90*10^7 \pm 1.78*10^6$	44.8
	Start	$1.57*10^5 \pm 8.02*10^4$	28.6	$3.93*10^5 \pm 1.23*10^4$	71.4
C	**6.4**	$5.68*10^7 \pm 3.57*10^6$	30.3	$1.31*10^8 \pm 6.58*10^6$	69.7
	7.4	$1.57*10^8 \pm 3.36*10^6$	78.4	$4.32*10^7 \pm 1.45*10^6$	21.6



Appendix IV 9: Exemplary qPCR run with standards for *Ntg. arctica*, pictures were taken from software. **A:** Amplification curves for each standard and controls. Samples with identical dilution cluster together at the same reaction cycle, since they contain the same amount of DNA template. The positive control was excluded from the diagram to simplify matters. **B:** Regression curve calculated from standards. The box under the curve contains efficiency (E) of the reaction with standards, regression coefficient (r^2 = R^2), and the slope. **C:** Melt curves of all samples after the last qPCR cycle. **D:** Melt peak of all samples derived from corresponding melt curves.

APPENDIX V

Nitrification in RAS

Appendix V 1: Nitrifying activity in the three biofilters (left, right, major) of the rainbow trout RAS. Activities of AOB and NOB were calculated from TAN and TNN turnover in g per h and m³ biofilter, respectively. The RAS was stocked with fish in November 2013, rearing stopped in October 2015. The biofilters left and right were disconnected earlier due to decreased fish stocking. * disinfection step with Wofasteril; n.c. biofilter not connected; n.d. not determined.

Sampling date	AOB: TAN [g/hm³]			NOB: TNN [g/hm³]		
	Left biofilter	Right biofilter	Major biofilter	Left biofilter	Right biofilter	Major biofilter
04-Nov-13	0.3 ± 0.0	0.4 ± 0.0	n.c.	0.2 ± 0.0	0.3 ± 0.0	n.c.
09-Dec-13	9.1 ± 0.4	8.4 ± 0.1	n.c.	12.8 ± 0.1	12.4 ± 0.4	n.c.
15-Jan-14	9.71 ± 0.5	8.9 ± 0.3	n.c.	11.8 ± 0.1	10.6 ± 2.3	n.c.
29-Jan-14	n.d.	n.d.	2.1 ± 0.0	n.d.	n.d.	2.2 ± 0.6
24-Feb-14	14.3 ± 0.3	12.2 ± 0.1	7.7 ± 0.2	17.6 ± 0.1	16.7 ± 0.4	9.2 ± 0.7
26-Mar-14	12.4 ± 0.1	11.8 ± 0.5	10.9 ± 0.4	17.0 ± 0.1	15.9 ± 1.5	16.7 ± 0.3
19-May-14	11.9 ± 0.3	12.6 ± 0.2	14.1 ± 0.1	20.8 ± 0.3	19.3 ± 1.2	19.8 ± 0.4
16-Jun-14	13.6 ± 0.4	11.9 ± 0.3	15.8 ± 0.6	21.4 ± 0.4	20.5 ±0.5	20.3 ± 0.2
26-Aug-14*	8.9 ± 0.1	8.0 ± 0.4	11.6 ± 0.2	9.8 ± 0.5	9.0 ± 0.3	12.4 ± 0.1
20-Oct-14	14.4 ± 0.7	12.3 ± 0.0	18.0 ± 0.1	16.7 ± 1.7	16.3 ± 2.1	23.3 ± 0.1
20-Oct-14*	14.5 ± 0.1	10.5 ± 0.3	16.5 ± 0.6	17.1 ± 0.7	15.9 ± 0.7	22.5 ± 0.1
21-Oct-14	13.7 ± 0.4	11.1 ± 0.5	17.6 ± 0.4	16.0 ± 0.9	13.9 ± 0.0	21.8 ± 0.1
08-Dec-14	14.3 ± 0.4	10.3 ± 0.8	15.4 ± 0.1	19.3 ± 0.1	16.0 ± 1.6	24.3 ± 0.6
21-Jan-15	10.6 ± 0.4	9.3 ± 0.2	12.9 ± 0.4	17.3 ± 0.1	14.4 ± 1.6	22.3 ± 0.6
21-Jan-15*	11.0 ± 0.4	9.7 ± 0.2	12.7 ± 0.6	15.8 ± 0.7	12.9 ± 0.6	19.0 ± 0.3
22-Jan-15	10.7 ± 0.2	8.9 ± 0.2	12.3 ± 0.1	15.5 ± 0.1	13.1 ± 0.5	18.8 ± 0.3
22-Apr-15	11.3 ± 0.2	10.3 ± 0.1	13.4 ± 0.2	14.5 ± 1.9	13.5 ± 1.0	18.6 ±0.5
28-Jul-15	n.c.	n.c.	17.7 ± 0.8	n.c.	n.c.	28.4 ± 1.0
13-Oct-15	n.c.	n.c.	8.9 ± 0.5	n.c.	n.c.	11.7 ± 0.7
Mean activity	**12.0 ± 2.0**	**10.4 ± 1.5**	**13.0 ± 4.2**	**16.2 ± 3.1**	**14.7 ± 3.0**	**18.2 ± 6.5**
Overall mean		**11.8 ± 1.3**			**16.4 ± 1.8**	

Appendix V 2: Most similar sequences found at NCBI for exemplary sequenced PCR products from biofilm of the rainbow trout RAS at different time-points. * disinfection step with Wofasteril.

Sampling date	Biofilter	Target	Similarity [%]	Accession nr.	Organism
04. Nov 13	left	*nxrB Nitrospira* spp.	85%	KC884877.1	Uncultured Nitrospira sp. clone HKA-G9 nitrite oxidoreductase beta subunit (nxrB) gene, partial cds
			84%	FP929003.1	Candidatus Nitrospira defluvii chromosome, complete genome
09-Dec-13	right	*nxrB Nitrospira* spp.	90%	KC884877.1	Uncultured Nitrospira sp. clone HKA-G9 nitrite oxidoreductase beta subunit (nxrB) gene, partial cds
			88%	FP929003.1	Candidatus Nitrospira defluvii chromosome, complete genome
24. Feb 14	left	16S *Nitrosomonas* spp.	97%	JQ936545.1	Uncultured Nitrosomonas sp. clone DSL_Nmon25 16S ribosomal RNA gene, partial sequence
			97%	NR_074736.1	Nitrosospira multiformis strain ATCC 25196 16S ribosomal RNA gene, complete sequence
			97%	NR_104816.1	Nitrosovibrio tenuis strain Nv1 16S ribosomal RNA gene, complete sequence
		16S *Nitrobacter* spp.	Sequence not analyzable		
		16S *Nitrospira* spp.	94%	NR_074700.1	Candidatus Nitrospira defluvii 16S ribosomal RNA, complete sequence
		16S *Nitrotoga* spp.	99%	FJ263061.1	Candidatus Nitrotoga sp. enrichment culture clone HAM-1 16S ribosomal RNA gene, partial sequence
			99%	DQ839562.1	Candidatus Nitrotoga arctica clone 6680 16S ribosomal RNA gene, partial sequence
19-May-14	major	16S	99%	DQ839562.1	Candidatus Nitrotoga arctica clone 6680 16S ribosomal RNA gene, partial sequence
		Nitrotoga spp.	99%	FJ263061.1	Candidatus Nitrotoga sp. enrichment culture clone HAM-1 16S ribosomal RNA gene, partial sequence
		16S *Nitrobacter* spp.	Sequence not analyzable		
16. Jun 14	major	*nxrB Nitrospira* spp	90%	FP929003.1	Candidatus Nitrospira defluvii chromosome, complete genome
			84%	KC884861.1	Candidatus Nitrospira bockiana clone 17 nitrite oxidoreductase beta subunit (nxrB) gene,partial cds
		16S *Nitrospira* spp.	93%	NR_074700.1	Candidatus Nitrospira defluvii 16S ribosomal RNA, complete sequence
			93%	KF724505.1	Nitrospira lenta strain BS10 16S ribosomal RNA gene, partial sequence
		16S *Nitrosomonas* spp.	97%	AY856079.1	Nitrosospira sp. En13 16S ribosomal RNA gene, partial sequence
			97%	NR_104816.1	Nitrosovibrio tenuis strain Nv1 16S ribosomal RNA gene, complete sequence

Sampling date	Biofilter	Target	Similarity [%]	Accession nr.	Organism
26-Aug-14*	major	16S *Nitrospira* spp.	99%	NR_074700.1	Candidatus Nitrospira defluvii 16S ribosomal RNA, complete sequence
			99%	FP929003.1	Candidatus Nitrospira defluvii chromosome, complete genome
		16S *Nitrobacter* spp.	98%	JN688938.1	Mesorhizobium sp. RBC8 16S ribosomal RNA gene, partial sequence
			90%	KF618622.1	Nitrobacter vulgaris strain NBW3 16S ribosomal RNA gene, partial sequence
			90%	NR_074324.1	Nitrobacter winogradskyi strain Nb-255 16S ribosomal RNA gene, complete sequence
20-Oct-14*	right	16S *Nitrospira* spp.	94%	NR_074700.1	Candidatus Nitrospira defluvii 16S ribosomal RNA, complete sequence
			94%	FP929003.1	Candidatus Nitrospira defluvii chromosome, complete genome
		16S *Nitrosomonas* spp.	97%	AY856079.1	Nitrosospira sp. En13 16S ribosomal RNA gene, partial sequence
			97%	NR_104816.1	Nitrosovibrio tenuis strain Nv1 16S ribosomal RNA gene, complete sequence
08-Dec-14	major	*amoA Nitrosomonas* spp.	80%	AF016003.1	Nitrosospira sp. NpAV ammonia monooxygenase operon copy 2; ammonia monooxygenase 2 subunits C (amoC2), A (amoA2
			82%	AJ388585.1	Nitrosomonas urea amoA gene Nm10, clone pNU.2
		16S *Nitrobacter* spp.	95%	AB741445.1	Mesorhizobium amorphae gene for 16S rRNA, partial sequence, strain: GMC432
		16S *Nitrosomonas* spp.	96%	KC477402.1	Nitrosospira sp. APG3 16S ribosomal RNA gene, partial sequence
			96%	EF175095.1	Nitrosospira sp. EnWyke2 16S ribosomal RNA gene, partial sequence
22. Apr 15	right	*nxrB Nitrospira* spp.	91%	AB846883.1	Uncultured bacterium nxrB gene for nitrite oxidoreductase beta subunit, partial cds,, clone: nsxB-41
			89%	FP929003.1	Candidatus Nitrospira defluvii chromosome, complete genome
28. Jul 15	left	16S *Nitrospira* spp.	98%	KF724505.1	Nitrospira sp. BS10 16S ribosomal RNA gene, partial sequence
			98%	NR_029287.1	Nitrospira moscoviensis strain NSP M-1 16S ribosomal RNA gene, partial sequence
	right	16S *Nitrosomonas* spp.	98%	AY856079.1	Nitrosospira sp. En13 16S ribosomal RNA gene, partial sequence
			97%	NR_104816.1	Nitrosovibrio tenuis strain Nv1 16S ribosomal RNA gene, complete sequence

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DECLARATION ON OATH

I hereby declare in lieu of oath that I have written the present dissertation on my own and have not used any other resources and aids than the ones referred to in this thesis.

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Language Certification

As a native English speaker and certified translator I certify that the dissertation "Characterization of Candidatus Nitrotoga and its Competitiveness in Co-Culture with Nitrospira" by Simone Wegen is written in clear and correct English.

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