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MASTERARBEIT

Titel der Masterarbeit

„Exploring the distribution and activity of novel nitrite oxidizers in their natural and environmental habitats“

Verfasserin

Mag. Jasmin Schwarz

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„Die Neugier ist immer an
erster Stelle eines Problems,
das gelöst werden will.“
Galileo Galilei

1. Introduction

1.1. Nitrogen cycle

Microorganisms existed on our planet for billions of years before plants and animals appeared (Madigan *et al.*, 2008). They are essential for the support and maintenance of life on Earth because they carry out many chemical processes such as recycling of key nutrients or degrading of organic matter (Madigan *et al.*, 2008). Nitrogen is one of the most abundant elements on earth and is essential for the synthesis of nucleic acids and proteins (Canfield *et al.*, 2010). Within the nitrogen cycle (Figure 1), nitrogen exists stably at a wide range of redox states, ranging from -3 in ammonia (NH_3) to +5 in nitrate (NO_3^-) (Bock and Wagner, 2006). The transformations of nitrogen compounds by oxidative or reductive processes are dependent on the activity of different microorganisms (Bock and Wagner, 2006; Madigan *et al.*, 2008). The processes within the nitrogen cycle can be distinguished in dissimilatory pathways, where nitrogen compounds are used to gain energy (respiration) (Bothe *et al.*, 2007) or to dissipate an excess of reducing power (Moreno-Vivian *et al.*, 1999) and assimilatory pathways, where NH_3 or NO_3^- acting as the source of N atoms to build up N containing molecules (Bothe *et al.*, 2007). The most stable form of nitrogen is dinitrogen (N_2), which is an extremely inert molecule (Madigan *et al.*, 2008). The biological process which makes N_2 available to organisms is nitrogen fixation (Bothe *et al.*, 2007; Madigan *et al.*, 2008), which can be performed only by prokaryotes (Madigan *et al.*, 2008). These organisms are able to brake the triple bond between the two N atoms to form NH_3, which is catalyzed by a large enzyme complex called nitrogenase (Madigan *et al.*, 2008). The nitrogen from nitrated organic compounds (such as proteins or nucleotides) is released into the environment during decomposition (ammonification) (Benbi and Richter, 2002). This process is carried out by heterotrophic microorganisms (Benbi and Richter, 2002), resulting in the conversion to inorganic NH_3 (White and Reddy, 2009).

Nitrification is the oxidative pathway within the nitrogen cycle in which NH_3, the most reduced form, is converted to NO_3^-, the most oxidized form (Prosser, 1989). Microorganisms involved in this processes are aerobic organisms (Prosser, 1989), but can also colonize hypoxic habitats (Lücker *et al.*, 2013). The oxidation of NH_3 to NO_3^- via nitrite (NO_2^-) is carried out by two distinct groups of microorganisms (Prosser, 1989). Ammonia oxidizing bacteria and archaea (ammonia oxidizing prokaryotes, AOP) catalyze the first step by oxidizing ammonia to nitrite (Könneke *et al.*, 2005; Madigan *et al.*, 2008). Two of four electrons, received from oxidation of hydroxylamine to NO_2^- are needed for the first part of this reaction, the oxidation of ammonia to hydroxylamine (Figure 2A) (Bock and Wagner, 2006). Nitrification is subsequently completed by nitrite oxidizing bacteria (NOB), which convert nitrite to nitrate (Figure 2B) and delivers two electrons (Bock and Wagner, 2006; Madigan *et al.*, 2008) . Both guilds of organisms benefit from each other due to a mutualistic relationship (Stein and Arp, 1998; Okabe *et al.*, 1999), in which NOB use the nitrite produced by AOP to gain energy (Madigan *et al.*, 2008) and simultaneously remove the for AOP toxic nitrite (Stein and Arp, 1998).

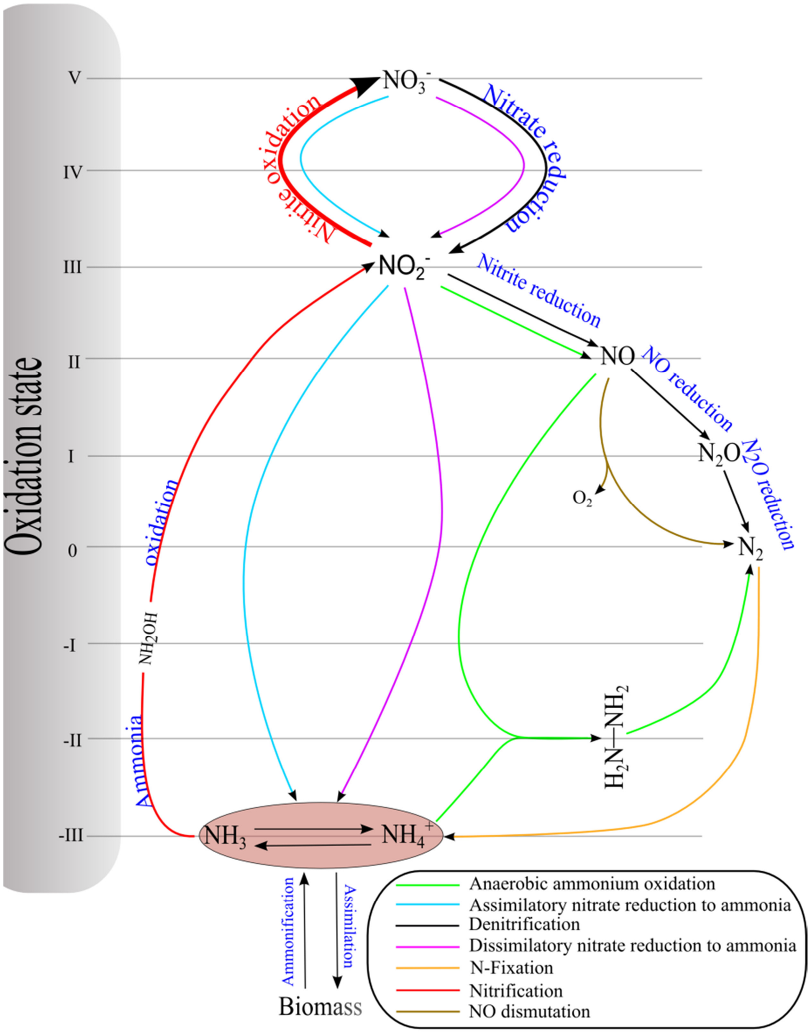


Figure 1 Nitrogen cycle (modified from Canfield *et al.*, 2010). Grey box: oxidation states of nitrogen in the respective nitrogen compound. Anaerobic ammonium oxidation (Anammox) is shown as suggested by Strous *et al.* and Kartal *et al.* (2006 and 2011). The oxidation of nitrite to nitrate carried out by NOB is indicated by the bold red arrow.

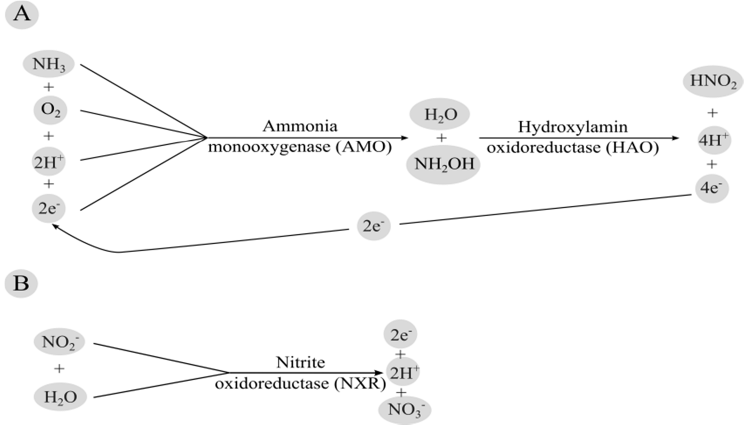


Figure 2 Redox reactions involved in nitrification. A Ammonia oxidation carried out by AOP, converts ammonia to nitrite. B Nitrite oxidation, carried out by NOB, converts nitrite to nitrate.

Denitrification is a reductive process involving the conversion of NO_3^- to NO_2^-, NO_2^- to nitric oxide (NO), NO to nitrous oxide (N_2O), and N_2O to N_2 (Bothe *et al.*, 2007). Various prokaryotes, mainly heterotrophic bacteria (Carlson and Ingraham, 1983), but also chemolithoautotrophic microorganisms (Baalsrud and Baalsrud, 1954) as well as eukaryotic organisms (Shoun and Tanimoto, 1991) are involved in this process. Another component of the nitrogen cycle is the anaerobic ammonium oxidation (Anammox, Figure 1). This pathway was detected in the 1980’s by A. Mulder (1992) and has been shown as a process mediated by microbes (Mulder *et al.*, 1995) in which NH_4^+ is oxidized by using NO_2^- as electron acceptor under anoxic conditions, leading to formation of N_2 (Strous *et al.*, 2006; Kuenen, 2008; Kartal *et al.,* 2011). Anammox organisms are phylogenetically affiliated to the order *Planctomycetales* (Jetten *et al.*, 2005). They can be found in freshwater and marine ecosystems as well as wastewater treatment plants (WWTPs) (Dalsgaard *et al.*, 2005; Jetten *et al.*, 2005).

The treatment of wastewater is important and has become essential because of sewage produced by an increasing number of humans, which would cause enormous problems if it is released untreated in natural water systems (Bonaventura and Johnson, 1997; Kimochi *et al.*, 1998). The consequences of discharging nitrogen to the environment will be oxygen depletion and eutrophication of aquatic ecosystems, intoxication of aquatic life and the contamination of groundwater by nitrate, which affects public health (Halling-Sørensen and Jorgensen, 1993). In WWTPs pollutants are removed by imitation of the natural self-purification capacity of aquatic environments, carried out by microbial communities (Amann *et al.*, 1998). Consequently, nitrification and denitrification processes play a major role not only in the environment but also in wastewater treatment (Juretschko *et al.*, 1998). The microbial nitrogen transformation process in wastewater treatment is fundamentally the same as in other environments such as soil, marine and freshwater habitats (Law *et al.*, 2012). However, WWTPs are engineered systems and there are differences to the environmental nitrification/denitrification processes such as higher concentrations of nitrogen (Law *et al.*, 2012). Therefore, an understanding of the processes involved in wastewater treatment and the knowledge about interaction of the communities, which contribute to removal of pollutants, are important to avoid a breakdown of this system (Wagner and Loy, 2002).

1.2. Nitrite oxidizers

NOB are chemolithoautotrophic organisms, using NO_2^- and carbon dioxide as the sole source of energy and carbon, respectively (Madigan *et al.*, 2008). Since the electron donor (NO_2^-) is more electropositive than the NAD^+/NADH couple, reducing power is obtained by reverse electron transport requiring energy from proton motive force (Madigan *et al.*, 2008). Some NOB are able to grow mixotrophically such as *Candidatus* Nitrospira defluvii (Spieck and Bock, 2006; Spieck *et al.*, 2006), which benefits from this lifestyle, using organic compounds from sewage in addition to NO_2^- and CO_2 (Lücker *et al.*, 2010).

The first NOB was already discovered 1892 by Sergei Winogradsky (Winogradsky, 1892). However, since 1892 the number of newly detected NOB was limited (Sorokin *et al.*, 2012) mainly because of difficulties to cultivate these organisms (e.g. Prosser, 1989; Alawi *et al.*, 2007; Elbanna, 2012; Sorokin *et al.*, 2012). Newly detected NOB were mainly phylogenetically affiliated to well-established groups of the *Alpha*-, *Gamma*- and *Deltaproteobacteria* and to the phylum *Nitrospirae* (Teske *et al.*, 1994; Ehrich *et al.*, 1995). Exceptions are the description of '*Candidatus* Nitrotoga arctica' by Alawi and coworkers (2007), *Nitrolancetus hollandicus* by Sorokin and colleagues (2012) as well as *Nitrospina*, which was already discovered in 1971 (Watson and Waterbury, 1971), but was later transferred from the class *Deltaproteobacteria* to the newly established bacterial phylum *Nitrospinae* (Lücker *et al.*, 2013).

This study focused on the distribution and activity of the newly detected nitrite oxidizing microorganisms '*Candidatus* Nitrotoga arctica' (Alawi *et al.*, 2007) and

Nitrolancetus hollandicus (Sorokin *et al.*, 2012), belonging to phylogenetic groups which were not thought to contain any NOB.

1.2.1. '*Candidatus* Nitrotoga arctica'

In 2007 a novel nitrite oxidizer was isolated from permafrost-affected soils of the Siberian Arctic and was preliminary classified as '*Candidatus* Nitrotoga arctica' (Alawi *et al.*, 2007). This organism is adapted to low temperatures and is phylogenetically affiliated to the *Betaproteobacteria*, representing the first NOB in this phylogenetic group (Alawi *et al.*, 2007). '*Candidatus* Nitrotoga arctica' forms coccoid to short rod-shaped cells (0.4–0.7 × 1.0 µm), which exhibit an extraordinary wide periplasmic space and contain no intracytoplasmic membranes or carboxysomes (Alawi *et al.*, 2007). The growth range of this organism was shown to be between 4°C and 22°C with an optimum at 10°C (Alawi *et al.*, 2007). Furthermore, they are adapted to low nitrite concentrations with an tolerance limit of 1.2 mM (Alawi *et al.*, 2007). The most closely related taxonomically described organism is *Gallionella ferruginea* (L07897) (Alawi *et al.*, 2007). *Gallionella* are iron oxidizing bacteria, which are characterized by the secretion of a twisted extracellular stalk, consisting of precipitated ferric iron oxide (Garrity *et al.*, 2005; Hallbeck and Pedersen, 2005). Originally, *Gallionella* was the only genus in the family *Gallionellaceae* of the order *Nitrosomonadales* (Hallbeck *et al.*, 1993; Garrity *et al.*, 2005; Hallbeck and Pedersen, 2005). But in 2007, it was suggested to transfer *Gallionellaceae* from the order *Nitrosomonadales* to the new order *Gallionellales*, which contains now two genera, *Gallionella* and *Sideroxydans* (Weiss *et al.*, 2007).

In addition to the isolation of '*Candidatus* Nitrotoga arctica' from permafrost-affected soils of the Siberian arctic, *Nitrotoga*-like bacteria were detected in WWTPs (Alawi *et al.*, 2009). The incubation of activated sludge from a WWTP in Hamburg at different temperatures revealed the existence of three distinct groups of nitrite oxidizers (Alawi *et al.*, 2009). Two of them encompass the already known genera *Nitrobacter* and *Nitrospira* and the third organism was shown to be affiliated to the newly detected '*Candidatus* Nitrotoga arctica' (Alawi *et al.*, 2009). The populations were shown to be not simultaneously active in WWTPs, but to be influenced by the temperature and availability of substrates (Alawi *et al.*, 2009). Similar observations were already made with nitrifying populations in other WWTPs (Avrahami and Conrad, 2003; Maixner *et* *al*., 2006; Siripong and Rittmann, 2007; Alawi *et al*., 2009). The study by Alawi and colleagues (2009) showed that the complexity of populations of NOB in WWTPs could be higher than previous expected. However, the importance and function of *Nitrotoga*-like bacteria in WWTPs are not known. Therefore, the aim of this study was to determine the nitrite oxidizing activity of *Nitrotoga* like bacteria in activated sludge from selected WWTPs at different temperature and nitrite concentrations by MAR-FISH analysis. Additionally the relative abundance and co-localization pattern of these organisms were determined in sludge of three WWTPs by digital image analysis. To determine the distribution of *Nitrotoga*-like bacteria in the environment activated sludge from selected WWTPs and sediment samples from lakes as well as river systems were screened via PCR and FISH. Additionally, in-depth phylogenetic analysis were performed to show the relationship of *Nitrotoga*-like 16S rRNA sequences obtained in this study to representatives of the genus *Nitrotoga*, *Gallionella,* and *Sideroxydans* publicly available in the NCBI Database.

1.2.2. *Nitrolancetus hollandicus*

In 2012, a new nitrite oxidizer was detected in a laboratory-scale bioreactor that is affiliated with the deep branching phylum *Chloroflexi* (green non-sulfur bacteria) (Sorokin *et al*., 2012). This phylum is a highly divergent group of bacteria that include anoxygenic photoautotrophs, aerobic chemoheterotrophs, thermophilic organisms and anaerobic organisms that obtain energy by reductive dehalogenation of organic chlorinated compounds (Gupta *et al*., 2013). However, chemolithoautotrophic nitrifying bacteria were not known in this phylum so far (Sorokin *et al*., 2012). The predominant morphotype within *Chloroflexi* is filamentous (Björnsson *et al*., 2002). They can be found in WWTPs (Kragelund *et al*., 2007), where they occur in relatively low numbers but are also thought to cause bulking incidents (Kragelund *et al*., 2007). However, this phylum includes also non-filamentous microbes, such as *Sphaerobacter thermophilus* and *Thermomicrobium roseum* (Hugenholtz *et al*., 2004), which are the most closely related organisms to *N. hollandicus* based on 16S rRNA phylogeny (Sorokin *et al*., 2012).

Nitrolancetus hollandicus is an aerobic, chemolithoautotrophic, lancet shaped (1 to 1.2 × 2 to 4 µm) organism that uses nitrite and CO_2 as sole energy and carbon source, respectively (Sorokin *et al*., 2012). *Nitrolancetus hollandicus* is not able to assimilate nitrogen from nitrite and requires ammonium for growth (Sorokin *et al*., 2012). Similar to '*Candidatus* Nitrotoga arctica' or *Nitrospira,* no intracytoplasmic membrane systems can be found (Sorokin *et al*., 2012). Like all other NOB, *Nitrolancetus* needs the membrane bound nitrite oxidoreductase (NXR) enzyme complex for nitrite oxidation (Tanaka *et al*., 1983; Sundermeyer-Klinger *et al*., 1984). The NXR consists of the *α* subunit (NxrA), which is thought to contain the catalytic side, the *β* subunit (NxrB), which is thought to channels the electrons from the *α* subunit to the *γ* subunit or directly to cytochrome *c* (Cyt*C*) (Sundermeyer-Klinger *et al*., 1984; Meincke *et al*., 1992; Kirstein and Bock, 1993) and the *γ* subunit (NxrC), which is suggested to function as a membrane anchor and channels the electrons from the *β* subunit to CytC (Rothery *et al*., 2008; Lücker *et al*., 2010). The genome of *Nitrolancetus hollandicus* contains four highly similar *nxrA* genes, three of which form a gene cluster and the fourth is suggested to be located in the *nxr* operon, containing the genes for the *β* (*nxrB*) and *γ* (*nxrC*) subunits of the NXR as well as the genes for CytC (*cytC*) and the *δ* subunit of the dissimilatory nitrate reductase (*narJ*) (Sorokin *et al*., 2012).

To obtain the complete sequences of all four *nxrA* genes of *Nitrolancetus hollandicus* a PCR was performed with specific primer sets. Furthermore, a PCR was performed to confirm the location of one of these copies within the NXR operon, using primer sets binding at the 3'end of the upstream *cytC* gene (encoding the electron carrier CytC) and at the 5'end of the downstream *nxrB* gene (encoding the *β*-subunit of the NXR). To identify the habitat of *Nitrolancetus hollandicus*, various environmental samples were screened by fluorescence in situ hybridization (FISH) and PCR.

2. Material and methods

2.1. Software

Software	URL	Reference
ARB	http://www.arb-home.de/	Ludwig *et al*., 2004
Basic Local Alignment Search Tool	http://www.ncbi.nlm.nih.gov/BLAST/	Altschul *et al*., 1990
ChromasPro	http://www.technelysium.com.au/chromas.html	Scientific & Educational Software
Daime	www.microbial-ecology.net/daime/	Daims *et al*. 2006
i-control^{TM}-Microplate Reader Software	http://www.tecan.com/i-control	Tecan Group Ltd., Männedorf, Switzerland
mathFISH	http://mathfish.cee.wisc.edu/index.html	University of Wisconsin-Madison, USA
OligoAnalyzer 3.1	http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx	IDT Integrated DNA Technologies, Iowa, USA
ProbeBase	http://www.microbial-ecology.net/probebase/	Loy *et al*., 2003
probeCheck	http://www.microbial-ecology.net/probecheck/	Loy *et al*., 2008
Ribosomal Database Project	http://rdp.cme.msu.edu/	(Maidak *et al*., 1996)
SINA	http://www.arb-silva.de/aligner/	Pruesse *et al*., 2012

2.2. Equipment and consumables

Equipment	Company
Analytical Plus balance	Ohaus, Nänikon, Switzerland
Bead beater	(BSP) Biosepc products, Bartlesville, Oklahoma, USA
CamSpec M107 Spectrophotometer	Spectronic Camspec Ltd, Garforth, UK
Centrifuges: Rotina 35R Mikro 22R Mikro 20R	 Andreas Hettingen GmbH & Co. KG, Tuttlingen, Germany Andreas Hettingen GmbH & Co. KG, Tuttlingen, Germany Andreas Hettingen GmbH & Co. KG, Tuttlingen, Germany
CLSM – confocal laser scanning microscope: LSM 512 META Leica LSM SP8	 Carl Zeiss AG, Jena, Germany ©Leica Microsystem, Wetzlar, Germany
Cover glasses 24 x 50 mm	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Digital Thermoblock	VWR International GmbH, PA, USA
Disposable cannulae (0.45 x 25 mm)	Lactan GmbH & Co KG, Graz, Austria
Disposable syringe (1 ml)	VWR® International GmbH, PA, USA
Dose-it 803	Integra Biosciences, Zizers, Switzerland
Eppendorf reaction tubes (ERT)	Eppendorf AG, Hamburg, Germany
Erlenmeyer flasks different sizes	VWR® International GmbH, PA, USA
Galaxy mini centrifuge	VWR® International GmbH, PA, USA
Haake Thermostat SC100	Thermo Fisher scientific, New York, USA
Hybridization oven	Memmert GmbH + Co.KG, Germany
Lysis Matrix Tubes A	QBIOgene, USA
Lysis Matrix Tubes E	QBIOgene, USA
Magnetic stirrer: Vioromag Maxi RCT Basic IKAMAG®	 Thermo Fisher scientific, New York, USA IKA Werke, Staufen, Germany

Merckoquant® Nitrite test strips: Range: 2-80mg l^{-1} 0.1-3 g l^{-1}	Merck chemicals, Darmstadt, Germany
Merckoquant® Nitrate test strips Range: 0-500 mg l^{-1}	Merck chemicals, Darmstadt, Germany
Micro haematocrit capillary tubes 75 mm Internal diameter: 1.5 mm external diameter: 1.55 mm not heparinized	Brand GmbH + Co.KG, Germany
Microscope slides, 10 reaction wells	Marienfeld Laboratory Glassware GmbH & Co. KG, Lauda-Königshofen, Germany
Milli-Q Biocel System Ultrapure Water (MQ)	Merck Millipore, Darmstadt, Germany
Nanodrop 1000 Spectrophotometer	Thermo Fisher scientific, New York, USA
Orbital (Platform) shaker innova 2300	New Brunswick scientific, Enfield, USA
Parafilm	Bemis Company Inc., Wisconsin, USA
PCR Cycler: Biorad T100^{TM} Biorad I-Cycler Eppendorf Mastercycler Gradient	BIO RAD Laboratories, Inc., UK BIO RAD Laboratories Inc., UK Eppendorf AG, Germany
Pipette tips 1-200 µl 0.1-10 µl	Lactan GmbH & Co KG, Graz, Austria
Pipette tips 1000 µl	Biozym scientific GmbH, Hessisch Oldendorf, Germany
Pipettes: Pipetman classic^{TM} Eppendorf research	Gilson$^{®}$, Middleton, USA Eppendorf AG, Germany
Quartz Cuvettes	Greiner, Frickenhausen, Germany
Reactions vessels 15 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Reactions vessels 50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Sanyo Incubator	Thermo Fisher scientific, New York, USA
SARTORIUS BL 6100 Max 6100 g	DWS, Data Weighing Systems, Inc., Illinois, USA
Scalpel	Lactan, Lancashire, UK
Screw cap reaction vessels 2 ml	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Stuart^{TM} Scientific roller mixer SRT1	Sigma-Aldrich, Missoui, USA
Syringe Filter 0.45 µm	Iwaki® cell biology, UK
Tecan reader Infinite 200	Tecan Group Ltd., Männedorf, Switzerland
Water bath	GFL$^{®}$, Burgwedel, Germany

2.3. Chemicals

Chemicals	Company
Acrylamide for molecular biology, ≥ 99% (HPLC)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Agar	Fluka Chemie AG, Buchs, Switzerland
Ammonium persulfate (APS)	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Ampicillin [100 mg ml^{-1}]	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Betain [5M]	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Bicarbonate ($NaCHO_3$)	J.T. – Baker, Deventer, Holland
[^{14}C]bicarbonate (^{14}C-$NaCHO_3$)	Hanke (Hartmann Analytic)
Boric acid >99.8% p.a., ACS, ISO	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Citifluor AF1, Glycerol/PBS solution	Agar Scientific Ltd., Stansted, UK

Chloroform/Isoamyl alcohol (24:1)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
CTAB (hexadecyltrimethyl ammonium bromide) > 98%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dimethyl sulfoxid (DMSO)	Fluka Chemie AG, Buchs Switzerland
96% Ethanol (96% Ph.Eur., extra pure)	Carl Roth GmbH & Co. KG, Karlsruhe Germany
96% Ethanol, denatured	Inhouse
Ethidium bromide [10 mg ml^{-1}]	Fluka Chemie AG, Buchs, Switzerland
Ethylenediamine tetra acetic acid disodium salt dihydrate >99% p.a., ACS (Na_2EDTA dihydrate)	Carl Rtoh GmbH & Co. KG, Karlsruhe, Germany
Formaldehyde (37% (w/w))	Carl Roth GmbH & Co. KG, Karlsruhe Germany
Formamide (FA)	Fluka Chemie AG,Buchs, Switzerland
Glycerol 99.5% p.a. waterfree	Carl Rtoh GmbH & Co. KG, Karlsruhe, Germany
Isopropanol (2-Propanol)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Kanamycin [100 mg ml^{-1}]	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Kodak D19 developer	Kodak, New York, USA
LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
LM-1	GE Healthcare, formerly Amersham Biosciences
Nessler's reagent	Fluka Chemie AG, Buchs Switzerland
NTB	Kodak, Rochester, New York
Sodium-Acetate-trihydrate > 99%, ACS, ISO	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium Nitrite (Na-NO_2^-) ≥ 98.7%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodiumthiosulphate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Tris PUFFERAN®, Buffer Grade	Carl Roth GmbH & Co. KG, Karlsruhe Germany
Phenol:Chloroform:Isoamyl Alcohol 25:24:1	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
37% Paraformaldehyde	Carl Roth GmbH & Co. KG, Karlsruhe Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4\,2H_2O$)	Carl Roth GmbH & Co. KG, Karlsruhe Germany
Di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4\,2H_2O$)	Carl Roth GmbH & Co. KG, Karlsruhe Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
SOC-Medium	Fermentas Inc. Hannover, MD, USA
Sybr® Green: 10.000 x concentration in DMSO	Cambrex Bio Science, Rockland, Inc., Rockland, ME, USA
Quant-iT™Picogreen®dsDNA Reagent and Kits	Invitrogen Corporation, Carlsbad, CA, USA
Tetramethyl Ammonium Chloride (TMAC)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)	Fluka Chemie AG, Buchs Switzerland
Tris	Carl Roth GmbH & Co. KG, Karlsruhe Germany
Yeast extract	Oxoid Ltd., Hampshire, England
X-Gal (5-brom-4-chlor-3-indolyl-ß-D-galactopyranoside)	Carl Roth GmbH & Co. KG, Karlsruhe Germany

2.4. Kits

Used Kits	Company
PCR-purification Kit	QIAGEN, Hilden, Deutschland
QIAquick Gel Extraction	QIAGEN, Hilden, Deutschland
DNeasy® Blood and Tissue Kit	QIAGEN, Hilden, Deutschland
Power soil DNA Isolation Kit	MO-BIO Laboratiories, Inc., Carlsbad, CA, USA
Topo® TA cloning Kit	Invitrogen Corporation, Carlsbad, CA, USA
TOPO® XL PCR cloning Kit	Invitrogen Corporation, Carlsbad, CA, USA

2.5. Enzymes

Enzymes and plasmids	Company
Taq DNA polymerase [5U μl^{-1}]	Fermentas, St. Leon-Rot, Germany
10 x Ex Taq polymerase-buffer	Fermentas, St. Leon-Rot, Germany
High Fidelity Taq	Fermentas, St. Leon-Rot, Germany
Phusion® High-Fidelity DNA polymerase Taq	New England BioLabs® GmbH
Ex Taq DNA Polymerase	Takara Bio Inc., Japan
aluI [10U μl^{-1}]	Fermentas, St. Leon-Rot, Germany
mspI [10U μl^{-1}]	Fermentas, St. Leon-Rot, Germany
ecoRI [10U μl^{-1}]	Fermentas, St. Leon-Rot, Germany
Lysozyme chicken (≥ 100U μg^{-1})	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Proteinase K	QIAGEN, Hilden, Deutschland
pCR™4-TOPO® 3956 bp	Invitrogen Corporation, Carlsbad, CA, USA
pCR® II-TOPO® 4000 bp	Invitrogen Corporation, Carlsbad, CA, USA
pCR-XL-TOPO® 3500 bp	Invitrogen Corporation, Carlsbad, CA, USA

2.6. GeneRuler

GeneRuler	Range	Company
1 kb DNA Ladder	250–10.000 bp	Fermentas, St. Leon-Rot, Germany
100 bp Plus DNA Ladder	100–3000 bp	Fermentas, St. Leon-Rot, Germany
MassRuler™ High range DNA ladder	1500–10.000	Fermentas, St. Leon-Rot, Germany

2.7. Buffer, media and solutions

2.7.1. General buffers and solutions

2.7.1.1. TE buffer (10 x)

Tris-HCl [1 M]	100 ml
EDTA [500 mM]	20 ml
MQ	880 ml

2.7.1.2.TE buffer (1 x)

10 x TBE	100 ml
MQ	ad 1000 ml

2.7.2. Buffer used for gel electrophoresis

2.7.2.1.TAE buffer (50 x)

Tris base [2 M]	242.28 g
Sodium acetate trihydrate [0.5 M]	68.04 g
N$_2$-EDTA dehydrate [0.05 M]	18.61 g
MQ	ad 1000 ml

2.7.2.2.TAE buffer (1 x)

TAE (50 x)	20 ml
MQ	ad 1000 ml

2.7.2.3.TBE buffer (10 x)

Tris base [0.9 M]	107.8 g
Boric acid [0.9 M]	55.0 g
Na$_2$-EDTA dihydrate	7.4 g
MQ	ad 1000

2.7.2.4.TBE buffer (1 x)

TBE (10 x)	100 ml
MQ	ad 1000 ml

2.7.3. Solutions used for selection

2.7.3.1. Ampicillin

Ampicillin stock solution [100 mg ml^{-1}] diluted in 50% EtOH

Ampicillin was added to media to a final concentration of [100 µg ml^{-1}]

2.7.3.2. Kanamycin

Kanamycin stock solution [100 mg ml^{-1}] diluted in MQ.

Kanamycin was added to media to a final concentration of [100 µg ml $^{-1}$]

2.7.3.3. Ethydium bromide

Ethydium bromide stock solution [10 mg ml^{-1}]	100 µl
MQ	1000 ml

2.7.3.4. Sybrgreen

Sybr® green stock solution	10 µl
TAE (1 x)	100 ml

2.7.3.5.X-Gal

X-Gal stock solution is diluted in di-methylformamide to a final concentration of 40 mg ml^{-1} and filter sterilized with 0.22 µm filters. X-Gal is stored at −20°C in the dark and was used for blue white screening necessary to identify clones with plasmids.

2.7.4. Solutions used for isolation of plasmid-DNA

2.7.4.1. Tris / HCl [1 M]

Tris	30.3 g
MQ	ad 250 ml

pH was adjusted to 8 with fuming HCl.

2.7.4.2. SDS [w/v 10 %]

SDS	5 g
MQ	ad 50 ml

2.7.4.3. NaOH [2 N]

NaOH	4 g
MQ	50 ml

2.7.4.4. Potassium acetate [5 M]

Potassium acetate	98 g
MQ	ad 200 ml

2.7.4.5. Buffer P1

Tris-HCl [1 M], pH 8	1 ml
EDTA [5 M], pH 8	400 µl
RNase A	100 µl ml^{-1}
MQ	20 ml

2.7.4.6. NaOH / SDS solution

NaOH [2 N]	20 ml
SDS [10 % w/v]	20 ml
MQ	160 ml

2.7.4.7. Potassium acetate / Acetate solution

Potassium acetate [5 M]	120 ml
Glacial acetic acid	23 ml
MQ	57 ml

2.7.5. Buffer and solutions used for fixation

2.7.5.1. Paraformaldehyde

A 4 % PFA working solution was prepared from a 37% PFA stock solution.

2.7.5.2. PBS stock solution

Na_2HPO_4-Dihydrate [0.2 M]	35.6 g
NaH_2PO_4-Dihydrate [0.2M]	31.20 g

NaH_2PO_4 was used for adjusting the pH-value of one liter Na_2HPO_4 to 7.2-7.4.

2.7.5.3. PBS (1 x)

NaCl [130 mM]	7.6 g
$NaxPO_4$ (PBS-Stock-solution) [10 mM]	50ml l^{-1}
MQ	ad 1000 ml

pH 7.2-7.4

2.7.6. Buffer and solutions used for hybridization

2.7.6.1. EDTA [0.5 M]

EDTA	186 g
MQ	ad 1000 ml

To adjust pH to 8, crystalline NaOH was added

2.7.6.2. NaCl [5 M]

NaCl	292.2 g
MQ	ad 1000 ml

2.7.6.3. SDS [10 % w/v]
See: 2.6.4.2 Solutions used for isolation of plasmid DNA

2.7.6.4. Tris/HCl [1 M]
See: 2.6.4.1 Solutions used for isolation of plasmid DNA

2.7.6.5. Formamide [100 %]

End concentration of formamide depends on required stringency.

2.8. Microorganisms

Species	Origin
Nitrolancetus hollandicus pure culture	Sorokin et al., 2012
Nitrolancetus hollandicus PFA fixed pure culture	Sorokin et al., 2012
Sharon 14.09.2009 mixed culture	Sorokin et al., 2012
Nitrotoga PFA fixed cultures	Kindly provided by Dr. Eva Spieck, Microbiology & Biotechnology Biocenter Klein Flottbek

2.9. Microorganisms used for cloning

Species	Strain	Type of cells	Genotype	T_{opt} [°C]	Growth media
E. coli	TOP10	Chemical competent	F^- mcrA Δ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) end A1 nupG	37	LB
E. coli	DH5α™-T1®	Chemical competent	F^- Φ80lacZΔM15Δ(lacZyA-argF)U169 recA1 endA1 hsdR17(r_k^-,m_k^+) phoA supE44 thi-1 gyrA96 relA1 tonA (confers resistance to phage T1)	37	LB

2.10. Primer used for sequencing

Primer (conc: 10 pmol μl^{-1})	Sequence 5'–3'	T_A [°C]	References
TopoF	AGC TTG GTA CCG AGC T	60	-
TopoR	TCT AGA TGC ATG CTC GA	60	-
M13F	GTA AAA CGA CGG CCA G	-	-
M13R	CAG GAA ACA GCT ATG AC	-	-
Nlho_NxrA0038F	GCC AGT GGG AAG AGT TCT ATA	60	Sorokin *et al.*, 2012

Nlho_NxrA3595R	GCC ACG TGC GTG TCC CGS GT	60	Sorokin *et al.*, 2012
Nlho_NxrA0658F	GAT GTC TGC GAG AGT GCC GAC	60	Sorokin *et al.*, 2012
Nlho_NxrA3070R	GTG ATC CAT GTA GTG ATG CTG	60	Sorokin *et al.*, 2012
Nlho_NxrA1294F	AAG TAC CTG GAG ACG AGC GAA	60	Sorokin *et al.*, 2012
Nlho_NxrA2381R	CTT GAA GAT CTC RTA GTC GG	60	Sorokin *et al.*, 2012
Nlho_NxrA1387F	GAC TAC CCG AAG AGC TAC GAC	60	Sorokin *et al.*, 2012
Nlho_NxrA2335R	CGC CGC GCC CAG CGG RTG GAT	60	Sorokin *et al.*, 2012
NxrB 168F	TAC ATG TGG TGG AAC A	60	Maixner, 2009
NxrB 638R	CGG TTC TGG TCR ATC A	60	Maixner, 2009

2.11. List of 16S rRNA targeted oligonucleotide probes used for FISH

Probe	**Sequence 5‘—3‘**	**Binding position (*E. coli* 16S rRNA nomenclature)**	**Target**	**FA %**	**Ref.**
Probes specific for *Nitrotoga*					
Ntoga122	TCC GGG TAC GTT CCG ATA T	122—140	*Candidatus* Nitrotoga arctica	40	Lücker, 2010
c1Ntoga122	TCW GGG TAC GTT CCG ATA T	122—140	-	-	Lücker, 2010
c2Ntoga122	TCY GGG TAC GTT CCG ATG T	122—140	-	-	Lücker, 2010
FGall178	TCC CCC TYA GGG CAT ATG	178—195	*Gallionellaceae*	30	Lücker, 2010
cFGall178	TCC CCC TYA GGG CKT ATG	178—195	-	-	Lücker, 2010
Ntoga1428	GCT AGC TGC TTC TGG T	1428—1443	*Candidatus* Nitrotoga arctica	25	This study[1]
cNtoga1428	GCT AAC TGC TTC TGG T	1428—1443	-	-	This study[2]
Ntoga221 (FGall221b)	TAT CGG CCG CTC CGA AAA	221—238	-	-	Lücker, 2010
Probes specific for AOBs					
Cluster6a192	CTT TCG ATC CCC TAC TTT CC	192—211	*Nitrosomonas oligotropha* lineage	35	Adamczyk *et al.*, 2003
cCluster6a192	CTT TCG ATC CCC TGC TTT CC	192—211	*Nitrosomonas eutropha* lineage	-	Adamczyk *et al.*, 2003
NSO1225	CGC CAT TGT ATT ACG TGT GA	1225—1244	*Betaproteobacterial* ammonia-oxidizing bacteria	35	Mobarry *et al.*, 1996

NEU	CCC CTC TGC TGC ACT CTA	653–670	Halophile and halotolerant members of genus *Nitrosomonas* including *N. mobilis*	40	Wagner *et al.*, 1995
cNEU (CTE)	TTC CAT CCC CCT CTG CCG	659–676	-	-	Wagner *et al.*, 1995
Probes specific for *Nitrospira*					
Ntspa662	GGA ATT CCG CGC TCC TCT	662–679	Genus *Nitrospira*	35	Daims *et al.*, 2001
cNtspa662	GGA ATT CCG CTC TCC TCT	662–679	-	-	Daims *et al.*, 2001
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	712–732	Genus *Nitrospira*	50	Daims *et al.*, 2001
cNtspa712	CGC CTT CGC CAC CGG TGT TCC	712–732	-	-	Daims *et al.*, 2001
Probes used for detection of *Nitrolancetus*					
Cfx1223	CCA TTG TAG CGT GTG TGT MG	1223–1242	phylum *Chloroflexi (green non sulfur bacteria)*	35	(Björnsson *et al.*, 2002)
GNSB-941	AAA CCA CACGCT CCG CT	941–957	Phylum Chloroflexi (green non sulfur bacteria)	35[4]	(Gich *et al.*, 2001)
Ntlc804	CAG CGT TTA CTG CTC GGA	804–821	*Nitrolancetus*	20	Sorokin *et al.*, 2012
Ntlc804c1	CAG CGT TTA CTG CGC GGA	804–821	*Nitrolancetus*	-	Sorokin *et al.*, 2012
Ntlc804c2	CAT CGT TTA CTG CTC GGA	804–821	*Nitrolancetus*	-	Sorokin *et al.*, 2012
Ntlc804c3	CAG CGT TTA CTG CTA GGA	804–821	*Nitrolancetus*	-	Sorokin *et al.*, 2012
Fsph811	TAG TCC ACA KCG TTT ACT	811–828	Family *Sphaerobacteraceae*	-	This study[2]
Ntlc439	TTG CTT CGT CCC CCA CAA	439–456	*Nitrolancetus*	40	Sorokin *et al.*, 2012
cNtlc439	TTG CTT CGT CCC CWA CAA	439–456	Competitor for Ntlc439	-	Sorokin *et al.*, 2012
General probes					
Bet42a	GCC TTC CCA CTT CGT TT	1027–1043 (23S rRNA)	*Betaproteobacteria*	35	(Manz *et al.*, 1992)
Gam42a[3]	GCC TTC CCA CAT CGT TT	1027–1043 (23S rRNA)	*Gammaproteobacteria*	-	(Manz *et al.*, 1992)
EUB338I	GCT GCC TCC CGT AGG AGT	338–355	most *Bacteria*	0-50	(Amann *et al.*, 1990)
EUB338II	GCA GCC ACC CGT AGG TGT	338–355	*Planctomycetales*	0-50	(Daims *et al.*, 1999)
EUB338III	GCT GCC ACC CGT AGG TGT	338–355	*Verrucomicrobiales*	0-50	(Daims *et al.*, 1999)
NONEUB338	ACT CCT ACG GGA GGC AGC	-	Control probe; complementary to EUB338	-	Wallner *et al.*, 1993

[1] Probedesign by Jasmin Schwarz

[2] Probedesign by Sebastian Lücker

[3] used as competitor for Bet42a.

2.12. List of used primer sets

Primer (conc: 50pmol µl^{-1})	Sequence 5'-3'	T_A [°C]	Comments	Ref.
General Primer sets				
616V (8f)	AGA GTT TGA TYM TGG CTC AG	62	General forward primer; screening	Juretschko *et al.*, 1998
1492R	GGY TAC CTT GTT ACG ACT T	62	General reverse primer; screening and cloning	McAllister *et al.*, 2011
M13F	GTA AAA CGA CGG CCA G	60	Determination of product size	Instruction Manual, TOPO®TA PCR Cloning Kit, Version U (10. April 2006)
M13R	CAG GAA ACA GCT ATG AC	60	Determination of product size	Instruction Manual, TOPO®TA PCR Cloning Kit, Version U (10. April 2006)
Primer sets specific for '*Candidatus* Nitrotoga arctica'				
Ntoga122F	ATA TCG GAA CGT ACC CGG A	62[1]/ 65[2]	Screening and Cloning	Lücker, 2010
Ntoga221F	TTT TCG GAG CGG CCG ATA	64	Screening (in combination with Ntoga1422r)	Lücker, 2010
Ntoga124F	ATC GGA ACG TAC CCG GAA A	63	Screening and Cloning (in combination with 1462bR)	This study[3]
Ntoga122bF	ATA TCG GAA CGT ACC CGG AAA	-	Screening and Cloning (in combination with 1462aR)	This study[3]
Ntoga215F	CTC RCG TTT TCG GAG CGG	63	Screening and Cloning (in combination with 1462bR	(Alawi *et al.*, 2007)[4]
Ntoga1422R	GCT GCT TCT GGT AGA ACC	65	Screening and Cloning	Lücker, 2010
Ntoga1462aR	CAC GAA CCC TAC CGT GGC AAC	-	Screening and Cloning	This study[3]
Ntoga1462bR	CGA ACC CTA CCG TGG CAA C	63	Screening and Cloning	This study[3]
Primer sets specific for 16S and *nxrA* of *Nitrolancetus*				
Ntlc188F	CAA GGC CGA TCA AGC AAA	63	Screening	(Sorokin *et al.*, 2012)
Ntlc1136R	TCT GGC TAG ACA TCC TCG	63	Screening	(Sorokin *et al.*, 2012)
Fsph811F	AGT AAA CGC TGT GGA CTA	62	Screening (used together with 1492R)	This study[5]

Nlho_NxrA00271F_NdeI	GAC TGA CAT ATG CGC CAG TGG GAA GAG TTC TAT A	-	With restriction site for NdeI Not used	This study[5]
Nlho_NxrA3600R_BamHI	CTG TCA GGA TCC TCG CAC YGC CAC GTG CGT GTC C	-	With restriction site for BamHI Not used	This study[5]
Nlho_NxrA0038F	GCC AGT GGG AAG AGT TCT ATA	68	Cloning	Sorokin *et al.*, 2012
Nlho_NxrA3595R	GCC ACG TGC GTG TCC CGS GT	68	Cloning	Sorokin *et al.*, 2012
Nlho_NxrA0031F	AAG GCA CGC CAG TGG GAA GAG TTC TA	60	Cloning, screening	Sorokin *et al.*, 2012
Nlho_NxrA3598R	TTT CGC ACY GCC ACG TGC GTG TCC CG	60	Cloning, screening	Sorokin *et al.*, 2012
Nlho_3566834F (cytcF)	TGG CGG AAC CGC GCA AAC GGC GTA	60	Used for testing; Binds to the 3' site of *cytC* gene	Sorokin *et al.*, 2012
Nlho_3567614R (nxrbR)	GGT GCC CGG CTT CGT CTC CAC GTT	60	Used for testing; Binds to the 5' site of *nxrB* gene	Sorokin *et al.*, 2012
Ntlc_NxrB11F	GAG CGC AAG TCT CGA TGA C	70	Used for screening of different environmental samples	This study[5]
Ntlc_NxrB735R	GAG ACG TGG ATA GCA GAG A	70	Used for screening of different environmental samples	This study[5]
Ntlc_NxrB21F	CTC GAT GAC TTC CAC CTT	68	Used for screening of different environmental samples	This study[2]
Ntlc_NxrB709R	TCT CGG ACT TAC CCG TCG C	68	Used for screening of different environmental samples	This study[5]
NbG_NxrB100F	AAR GGC ACC GAG TAC TG	64	Binds to *nxrB*-gene of group *Nitrobacter*; used for screening	This study[5]
NbG_NxrB694R	CGM CCA GTT GAA RTA GGT	64	Binds to *nxrB*-gene of group *Nitrobacter; used for screening*	This study[5]
NxrA2F	CAT TAC CCG CTC CAC CCA CGG G	68	Screening for chimera formation, C-terminal fragment of *nxrA2*	(Sorokin *et al.*, 2012)
NxrA2R	GTG CGT GTC CCG CGT CAG AAT T	68	Screening for chimera formation N-terminal fragment of *nxrA2*	(Sorokin *et al.*, 2012)
NxrA3F	TAC CCG CTC CAC CCA TGG AGT A	68	Screening for chimera formation C-terminal fragment of *nxrA3*	(Sorokin *et al.*, 2012)
NxrA3R	ATC GGT CCC CAG TAG TTC CAT G	68	Screening for chimera formation N-terminal fragment of *nxrA3*	(Sorokin *et al.*, 2012)
NxrA4F	TAC CCG CTC CAC TCA CGG AGT G	68	Screening for chimera formation C-terminal fragment of *nxrA4*	(Sorokin *et al.*, 2012)
NxrA4R	TCC CCA GTA GTT GAA CGC GTA G	68	Screening for chimera formation N-terminal fragment of *nxrA4*	(Sorokin *et al.*, 2012)

Primer sets specific for nxrB of *Nitrospina*				
Nspn_nxrA3218F	CGGACAGTCTGTTCCATA	52	Used as positive control	Lücker *et al*., 2013
Nspn_nxrC68R	GACCAGAAAGGATCGGTC	52	Used as positive control	Lücker *et al*., 2013
Nspn_nxrA1_3064F	CACTCTTGCTGGACGTCA	52	Cloning of nxrB1	Lücker *et al*., 2013
Nspn_nxrC1_111R	CATATCCACAACCACGTG	52	Cloning of nxrB1	Lücker *et al*., 2013
Nspn_A2_3064F	CATTCAGCATGGCAGAGC	52	Cloning of nxrB2	Lücker *et al*., 2013
Nspn_C2_111R	CAAATCGATCACCACTCC	52	Cloning of nxrB2	Lücker *et al*., 2013
Primer sets specific for the Genus Nitrospira				
nxrB19F	TGG CAA CTG GGA CGG AAG ATG	48	Amplification and cloning of *nxrB* of *Nitrospira sp. Ecomares* 2.1. and *Nitrospira Calida*	Maixner, 2009
nxrB1237R	GTA GAT CGG CTC TTC GAC CTG	48	Amplification and cloning of *nxrB* of *Nitrospira sp. Ecomares* 2.1. and *Nitrospira Calida*	Maixner, 2009
Ntspa1158R	CCC GTT MTC CTG GGC AGT	56	screening for *Nitrospira nxrB*	Maixner *et al*., 2006

1 T_A if used together with 1492R
2 T_A if used together with Ntoga1422r
3 Primer design by Jasmin Schwarz
4 modified by Sebastian Lücker
5 Primer design by Sebastian Lücker

2.13. Fluorescence in situ hybridization, quantification, and co-localization analysis

2.13.1. Fixation

2.13.1.1. PFA-Fixation

Fixation was done according to a standard protocol described elsewhere (Daims *et al*., 2005) with some modifications. For overnight fixation, one volume of 4% PFA and one volume of sludge was mixed and incubated at 4°C. To stop the fixation the samples were centrifuged at 14,000 rpm for 5 minutes. The pellet was washed with 1 x PBS twice, pelleted by centrifugation and resuspended in one volume 1 x PBS and one volume 96% ethanol. Fixed samples were stored at –20°C.

2.13.1.2. EtOH-Fixation

One volume of activated sludge sample was mixed with one volume of 96% ethanol. Fixed samples were stored at −20°C

2.13.2. Hybridization

Hybridization was performed with PFA fixed samples in accordance to the standard FISH protocol (Daims *et al.*, 2005). Depending on the sample, appropriate amounts were applicated on a well of a 10 well slide (Table 1).

Table 1 Amounts of sample applicated on wells

Culture	Amount [µl]
Pure culture	2–5
WWTP biomass	10
WWTP biomass with low cell density	2 x 10 or 10 + 5
WWTP biomass for MAR-FISH	15

For hybridization of *Nitrolancetus hollandicus* cells PFA-fixed pure culture and PFA-fixed samples, originated from a laboratory-scale bioreactor, were used (Sorokin *et al.*, 2012).

Depending on stringency required by probe, the preparation of hybridization- (HB) and washing buffer (WB) was performed with the appropriate formamide and NaCl concentration, respectively (Table 2). If the appropriate formamide concentrations of hierarchically used probes differed more than 5%, a serial hybridization was performed. For this approach, a separate hybridization was performed for each probe according to the standard FISH protocol (Daims *et al.*, 2005), starting with the highest formamide concentration.

Table 2 Pipetting scheme for preparation of washing and hybridization buffer

Washing buffer														
Fa (conc.) [%]	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**
5M NaCl [µl]	9000	6300	4500	3180	2150	1490	1020	700	460	300	180	100	40	0
1M Tris/ HCl [µl]	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
0.5 M EDTA [µl]	0	0	0	500	500	500	500	500	500	500	500	500	500	500
MQ [ml]	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50	ad 50
10% SDS [µl]	50	50	50	50	50	50	50	50	50	50	50	50	50	50

Hybridization buffer														
Fa (conc.) [%]	**0**	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**
5M NaCl [µl]	180	180	180	180	180	180	180	180	180	180	180	180	180	180
1M Tris/HCl [µl]	20	20	20	20	20	20	20	20	20	20	20	20	20	20
MQ [µl]	800	750	700	650	600	550	500	450	400	450	400	35	200	150
FA [µl]	0	50	100	150	200	250	300	350	400	450	500	550	600	650
10% SDS [µl]	1	1	1	1	1	1	1	1	1	1	1	1	1	1

2.13.3. Probes

Probes (for list of all used 16S rRNA targeted oligonucleotide probes see 2.11) were used either single or double labeled at a working concentration of 5 pmol µl^{-1} for Indocarbocyanine (Cy3) or Indodicarbocyanine (Cy5) labeled probes and 8 pmol µl^{-1} for Fluorescein (Fluos) labeled probes. To avoid false positive signals an appropriate set of hierarchically specific probes were used according to the multiple probe concept (Ludwig *et al.*, 1998). EUB338I, EUB338II and EUB338III were used in combination (EUB338mix) to detect most *Bacteria* (Daims *et al.*, 1999). Cluster6a, NEU and NSO1225 were used in combination (AOBmix) to cover the *Nitrosomonas oligotropha* lineage, halotolerant members of the genus *Nitrosomonas* and betaproteobacterial members of AOB. Cfx1223 and GNSB-941 were used in combination (Cfxmix) to cover the phylum *Chloroflexi*. The probe NONEUB338 was used to determine nonspecific binding of probes to bacterial cells.

2.13.4. Evaluation of the correct formamide concentration

To evaluate the optimal formamide concentration for novel probes a formamide series was performed. For that purpose activated sludge samples or pure cultures were hybridized with formamide concentrations ranging from 0 to 65% according to a standard protocol described elsewhere (Manz *et al*., 1992; Daims *et al*., 2005).

2.13.5. Mounting and imaging

Slides were covered with Citifluor (AF2) to prevent bleaching of fluorescence dyes. Fluorescence dyes were excited with laser light at specific wavelengths (Table 3). The emitted fluorescence light was detected and photographed with a confocal laser scanning microscope (CLSM). The LSM 5 (Zeiss) is equipped with an argon laser for excitation of Fluos and with two helium-neon-lasers for excitation of Cy3 and Cy5. The LSM SP5 (Leica) is equipped with a white light laser for excitation of Fluos, Cy3 and Cy5 as well as an UV laser for detection of 4'-6- diamidino-2-phenylindole (Dapi).

Table 3 Absorption and emission maximum of used fluorescent dyes

Fluorescent dye	Absorption maximum [nm]	Emission maximum [nm]
FLUOS	492	520
Cy3	550	570
Cy5	650	670

http://www.jacksonimmuno.com/technical/f-cy3-5.asp (accessed: 2013-06-19)

2.14. Abundance, co-localization, and activity of *Nitrotoga*-like bacteria

2.14.1. Quantification

For quantification of *Nitrotoga*-like bacteria in activated sludge samples from WWTP Deuz, Bad Zwischenahn and Langenzenn, probes binding to *Nitrotoga* 16S rRNA were used in combination with probes binding to most *Bacteria* (EUB338mix) or to *Betaproteobacteria* (Bet42a). The probe-bound fluorophores were excited and visualized in the appropriate channel of the CLSM. For each sample, fifty images were captured on randomly chosen positions. The optimal focal plane was determined in the channel of the general probe (EUB338mix or Bet42a). Analysis of the images for relative quantification of *Nitrotoga* in sludge samples was performed with the software *daime* (Daims *et al.*, 2006).

2.14.2. Co-localization

To obtain images for co-localization analysis, FISH was performed using probes specific for *Nitrotoga*-like bacteria, *Nitrospira*-like bacteria, and AOB. Afterwards, forty images (image stack) were captured with the CLSM. Each image consisted of three channels, containing the fluorescence signals of all three populations. The focusing of the biomass was performed in one of the three channels of the nitrifying populations *Nitrotoga* (Ntoga image series), *Nitrospira* (Nspira image series), or AOB (AOB image series), resulting in three image series consisting of forty images each. Subsequently, the co-localization patterns of *Nitrotoga* versus AOB, AOB versus *Nitrotoga*, *Nitrospira* versus AOB were analyzed with the software *daime* (Daims *et al.*, 2006). To show complementary effects of the two nitrite oxidizing populations, image stacks of the AOB image series, Nspira image series and Ntoga image series were combined resulting in a stack consisting of 120 images. Afterwards, the channels containing the fluorescence signals of the *Nitrotoga* and *Nitrospira* specific probes were combined by using the software *daime*. Subsequently, the co-localization pattern of NOB versus AOB was calculated by the same software.

For calculation of the co-localization pattern, the software scans the image pixel by pixel in the x and y dimensions and places dipoles between the populations describing a semicircle of the radius r (Daims *et al.*, 2006). All dipoles that touch a second different population will be recorded as a hit, whereas dipoles that hit no or the same population are recorded as a miss (Daims *et al.*, 2006). The probability (P(r)) that a dipole hits a population with the length r is calculated by dividing the hits by the sum of hits and misses (Daims *et al.*, 2006). P(r) is dependent on population densities (D_1 and D_2) (Daims *et al.*, 2006). Since the densities of the populations in the images can differ a comparison of the spatial relationships between different pairs of populations is difficult (Daims *et al.*, 2006). Therefore, P(r) is normalized by the products of the population densities D_1 and D_2, resulting in g(r) (Daims *et al.*, 2006). In case of a random distribution of two populations, the probability that a dipole hits both populations depends solely on the density of the two populations in the image (Daims *et al.*, 2006). Pair correlation values of one indicate random distribution of both populations, values below one indicate repulsion or displacement and values greater than one indicate co-localization of both organisms (Daims *et al.*, 2006). Because the diameter of most flocs is 50 to 70 µm and nitrifiers appear only within flocs, significant co-localization patterns can be observed until a maximum distance of 50 to 70 µm (Daims *et al.*, 2006).

2.14.3. Microautoradiography-Fluorescence in situ Hybridization

Microautoradiography-Fluorescence in situ Hybridization (MAR-FISH) was performed according to a protocol by Lee *et al.* and Nielsen *et al.* (Lee *et al.*, 1999; Nielsen *et al.*, 2003), using activated sludge samples from WWTP Bad Zwischenahn and WWTP Deuz. Activated sludge was stored at 4°C until use. Prior to incubation start, NH_3 and NO_2^- concentrations were measured in order to confirm that all endogenous NH_3 and NO_2^- were used up. For NO_2^- measurements, 10 µl of sludge supernatant were pipetted on a NO_2^--test strip (Merck chemicals). For measuring of NH_3 20 µl of supernatant were mixed with 20 µl of Neßlers reagent (Fluka). Yellow coloring of the mixture indicates presence of NH_3. If NO_2^- and NH_3 were still measurable, sludge was incubated at 4°C with slow agitation until nitrite and NH_3 were used up. For MAR-FISH incubation, 20 ml of activated sludge were diluted in a ratio of 1:5 with filter sterilized supernatant from the same WWTP and 5 ml thereof were aliquoted in 100 ml culture glass bottles, which were closed with rubber stoppers. For dead controls, 5 ml of PFA fixed sludge, diluted in a ratio of 1:5 with filter sterilized supernatant from the same WWTP, were transferred to 100 ml glass bottles. All experiments were performed in duplicates. Subsequently, the respective nitrite concentrations as well as 40 µl of [^{14}C]bicarbonate were added to each sample. Afterwards, 100 µl of each incubation flask were transferred to PCR tubes. For following the NO_2^- consumption during incubation, an additional replicate of the MAR incubation series was prepared containing [^{12}C]bicarbonate (cold incubation series) instead of [^{14}C]bicarbonate (hot incubation series). The incubation of cold and hot incubation series were performed at the respective temperature or respective NO_2^- concentration for 6 hours. During incubation, the NO_2^- concentration was measured every hour by pipetting 10 µl from cold incubation series on NO_2^--test strips. When used up, nitrite was replenished through the rubber stopper, using a 1 ml syringe and 0.45 x 24 needles (one drop ≈ 6 µl), in the cold and associated hot samples. After 6 hours of incubation 2 ml of mixed sample were taken from hot samples and dead controls. From there, 100 µl were pipetted in PCR-tubes for later scintillation measurements and the remaining biomass was centrifuged at

14,000 rpm for 10 minutes. The yielded supernatant was transferred to 2 ml ERTs and stored at −20°C for later NO_2^- measurements. The pelletized biomass was diluted in 1x PBS, mixed with 1 volume 4% PFA and, incubated overnight at 4°C for fixation. For end-fixation the samples were centrifuged at 14,000 rpm for 5 minutes. Pellets were washed with 1 x PBS twice, harvested by centrifugation and re-suspended with 1 volume 1 x PBS and 1 volume of 96 % Ethanol. Fixed samples were stored at −20°C. An overview of incubations, NO_2^- concentrations and temperatures are shown in Tables 4 and 5.

Table 4 Nitrite concentration series

Sample	Nitrite concentration [mM]
N1.1/N1.2	0
N2.1/N2.2	0.1
N3.1/N3.2	0.5
N4.1/N4.2	1
N5.1/N5.2	5
N6.1/N6.2	10
D1/D2	0.5

Incubation was performed at a constant temperature of 14°C.

Table 5 Temperature incubation series

Sample	Temperature [°C]
T1.1/T1.2	4
T2.1/T2.2	10
T3.1/T3.2	14
T4.1/T4.2	20
T5.1/T5.2	27
D1/D2	14

Incubation was performed at a constant nitrite concentration of 0.5 mM.

2.14.3.1. Imaging of fluorescence and microautoradiography signals

To minimize flock size, 100 µl of PFA fixed sludge sample were diluted with 400 µl 1 x PBS and pipetted in lysing matrix A tubes, containing only a ¼ ceramic bead. The lysis matrix tubes were shaken in a horizontal position for 10 minutes. Afterwards, the sludge was centrifuged at 10,000 rpm for 5 minutes and washed twice with 1 x PBS to remove abrasions that might cause autofluorescence. For hybridization, 15 µl of the incubated sludge were pipetted on cover glasses (high precision, 1.5H), on which three to four circles were made with a fatty acid pen to avoid dispersing of sample and hybridization buffer. The hybridization was performed as described elsewhere (Daims *et al.*, 2005) with two exceptions: 20 µl of hybridization buffer and 2 µl of each probe were used.

For visualization of MAR-signals, the hybridized samples were coated with pre-warmed LM1 (Amersham, GE Healthcare) or NTB (KODAK), put into a box and wrapped with aluminum foil. Since preliminary tests showed that prolonged incubation did not increase the number of MAR positive cells, all slides were incubated for one week at 4°C. For developing, slides were immersed in D19 developer for seven minutes and placed into ice-cold water for one minute to stop the reaction. For fixation, slides were dipped into ice-cold sodium-thiosulfate for four minutes and afterwards put into ice cold water for one minute (Table 6). Finally, slides were air-dried and embedded in Citifluor (AF2) for detecting and photographing the fluorescence and MAR signals with the CLSM.

Table 6 Reagents needed for developing of MAR-FISH slides

Solution	Concentration	Mix for 400 ml
Developer: Kodak D19	40 g l^{-1}	16 g
Stopping solution: double distilled water (ice cold)	-	-
Fixative: Sodiumthiosulfate (Sigma-Aldrich GmbH, Germany – ice cold)	30% w/v	120 g

2.15. DNA isolation, screening, cloning, and sequencing

2.15.1. DNA isolation

For DNA extraction from activated sludge and liquid manure samples (Table 7) a phenol:Chisam DNA isolation was performed. Therefore, 0.1 g of sludge were transferred to lysis matrix E tubes. After that, 500 µl AE-buffer, 50 µl 25% SDS and 600 µl phenol:chloroform:isoamyl alcohol (25:24:1) were added. For cell disruption, tubes were shaken twice for 15 seconds at 4.5 m s^{-1} in a bead beater. Afterwards the tubes were cooled down to avoid breakage during centrifugation. After centrifugation at 10,000 rpm for 10 minutes (4°C), the upper phase was transferred to a new 1.5 ml ERT and mixed with 600 µl chloroform:isoamyl alcohol (24:1). This mixture was inverted several times and centrifuged at 10,000 rpm for 10 minutes (4°C). Again the upper aqueous phase was transferred to a new 1.5 ml ERT and mixed with 0.6 volumes sodium acetate to increase salt concentration as well as 0.6 volumes of ice cold isopropanol for DNA precipitation. The ERT was inverted again several times and DNA was precipitated at –20°C for 1 hour.

To isolate DNA from soil and river sediments (Table 7), the Powersoil® DNA isolation Kit (MO BIO) was used according to the instruction manual (MO BIO Laboratories). Isolation of *Nitrolancetus hollandicus* genomic DNA from *Nitrolancetus hollandicus* pure culture was performed by using the CTAB DNA isolation protocol (JGI, http://my.jgi.doe.gov/general/index.html; DNA Isolation Bacterial CTAB Protocol; accessed: 2011-06-22) with two modifications: 25 µl of *Nitrolancetus hollandicus* pure culture cells were used for DNA isolation and the phenol:chloroform:isoamyl alcohol extraction was performed before the chloroform:isoamyl alcohol step to avoid carryover of phenol (Sorokin *et al*., 2012).

Table 7 Sampling points and sampling dates

Sample	**Sampling point**	**Date**	**Sampling done by**	**Stored at [°C]**	**Used for**	**Screened (cloned)**
Altmannstein SBR	WWTP Altmannstein, Germany	2007	S. Lücker	-20	screening and cloning	*Nitrotoga*
Ampfing SBR	WWTP Ampfing, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Bad Zwischenahn DIC-SBR	WWTP Bad Zwischenahn, Germany	2007	S. Lücker	-20	screening and cloning, quantification and spatial distribution analysis	*Nitrotoga*
Bad Zwischenahn DIC-SBR	WWTP Bad Zwischenahn, Germany	2012	S. Lücker	-20	MAR-FISH, cloning	*Nitrotoga*
Bruchmühlen DIC-SBR	WWTP Bruchmühlen, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Deuz DIC-SBR	WWTP Deuz, Germany	2007	S. Lücker	-20	screening and cloning, quantification and spatial distribution analysis	*Nitrotoga*
Deuz DIC-SBR	WWTP Deuz, Germany	2012	S. Lücker	-20	MAR-FISH	*Nitrotoga*
Hettstedt DIC-SBR	WWTP Hettstedt, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Huntlosen DIC-SBR	WWTP Huntslosen, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Ingolstadt SBR	WWTP Ingolstadt, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Kraftisried SSASB	WWTP Kraftisried, Germany	2007	S. Lücker	-20	screening, cloning	*Nitrotoga*
Langenzenn SBR	WWTP Langenzenn, Germany	2007	S. Lücker	-20	screening, cloning, quantification and spatial distribution analysis	*Nitrotoga*

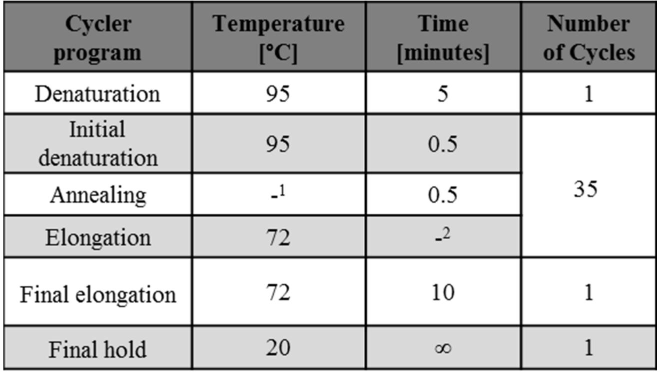
Lyss Ara fixed bed reactor	WWTP Lyss, Switzerland	2007	S. Lücker	-20	screening, cloning	*Nitrotoga*
Lyss TBA GZM membrane filtration	WWTP Lyss, animal rendering, Switzerland	2007	S. Lücker	-20	screening, cloning	*Nitrotoga*
Oberding fixed bed reactor	WWTP Oberding, Germany	2007	S. Lücker	-20	screening, cloning	*Nitrotoga*
Plattling TSASB	WWTP Plattling, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Radeburg DIC-SBR	WWTP Radeburg, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Seefeld SBR	WWTP Seefeld, Germany	2007	S. Lücker	-20	screening, cloning	*Nitrotoga*
Spenge DIC-SBR	WWTP Spenge, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Waldsassen SBR	WWTP Waldsassen, Germany	2007	S. Lücker	-20	screening, cloning	*Nitrotoga*
Weissthal DIC-SBR	WWTP Weissthal, Germany	2007	S. Lücker	-20	screening	*Nitrotoga*
Schwarza, river-sediment	Grafenbach, Lower Austria	2011	J. Schwarz	-20	screening	*Nitrotoga*
Pitten, river-sediment	Edlitz, Lower Austria	2011	J. Schwarz	-20	screening	*Nitrotoga*
Saubach, river-sediment	Pottschach, Lower Austria	2011	J. Schwarz	-20	screening	*Nitrotoga*
Mürz, river-sediment	Dietlergraben, Styria	2011	J. Schwarz	-20	screening, cloning	*Nitrotoga*
Feistritz, river-sediment	Feistritz am Wechsel, Styria	2011	J. Schwarz	-20	screening	*Nitrotoga*
Field, soil	Köttlach, Lower Austria	2011	J. Schwarz	-20	screening	*Nitrolancetus*
Terrace, soil	Althanstraße 14, Vienna	2011	J. Schwarz	-20	screening	*Nitrolancetus*
Liquid manure, cattle	Landwirtschaftliche Fachschule, Lower Austria, Warth	2011	J. Schwarz	-20	screening	*Nitrolancetus*
Farm, liquid manure, cattle	Ternitz, Lower Austria	2011	J. Schwarz	-20	screening	*Nitrolancetus*
Farm, liquid manure, swine	Styria	2011	S. Lücker	-20	screening	*Nitrolancetus*
Dutch drainage ditch sediments	Oorjipolder, Nijmegen, The Netherlands	2011	S. Lücker	-20	screening	*Nitrolancetus* *Nitrotoga*
Compost (upper side, bottom side, center)	Pottschach, Lower Austria	2011	J. Schwarz	-20	screening	*Nitrolancetus* *Nitrotoga*

Schilfgürtel left side, right side	Neusiedlersee, Burgenland	2004	S. Lücker	-20	screening	*Nitrolancetus* and *Nitrotoga*
Bergwerksee (small, big)	Langau bei Geras, NÖ	2004	S. Lücker	-20	screening	*Nitrolancetus* and *Nitrotoga*
Herrensee	Waren, Müritz, Germany	2004	S. Lücker	-20	screening	*Nitrolancetus* and *Nitrotoga*
Wörtenlacke sediment	Neusiedlersee, Burgenland	2004	S. Lücker	-20	screening	*Nitrolancetus* and *Nitrotoga*
Fettwiese	Melk, NÖ	2004	K. Hace	-20	screening	*Nitrolancetus* and *Nitrotoga*
Unterer Stinker	Neusiedlersee, Burgenland	2004	S. Lücker	-20	screening	*Nitrolancetus* and *Nitrotoga*
Fischteich Hessendorf	Langau bei Geras, Lower Austria	2004	S. Lücker	-20	screening	*Nitrolancetus* and *Nitrotoga*

2.15.2. Evaluation of annealing temperature

The correct annealing temperature for new primer sets was evaluated by performing a gradient PCR (Table 8). The temperature range was chosen according to the in silico melting temperature obtained with the online software tool OligoAnalyzer 3.1. (IDT Integrated DNA Technologies, Inc). The two-step gradient PCR protocol (Table 9) was used for evaluation of the annealing temperature of the *nxrA* primer set, which is a time-saving method, combining the annealing and the elongation step (Lopez and Prezioso, 2001).

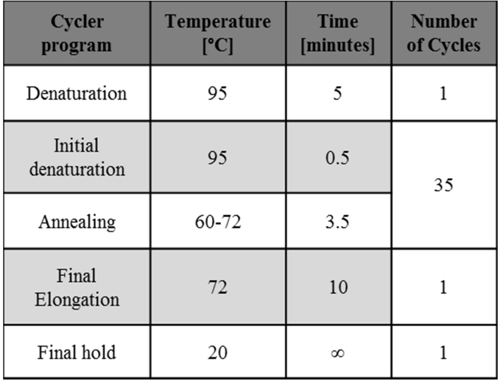
Table 8 Cycler program for gradient PCR



Cycler program	Temperature [°C]	Time [minutes]	Number of Cycles
Denaturation	95	5	1
Initial denaturation	95	0.5	35
Annealing	-[1]	0.5	
Elongation	72	-[2]	
Final elongation	72	10	1
Final hold	20	∞	1

1 for annealing temperature see 2.12. List of used primer sets;
2 depends on the length of the amplicon

Table 9 Cycler program for two-step gradient PCR



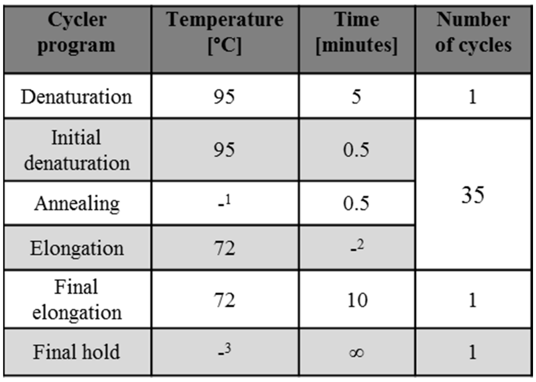
Cycler program	Temperature [°C]	Time [minutes]	Number of Cycles
Denaturation	95	5	1
Initial denaturation	95	0.5	35
Annealing	60-72	3.5	
Final Elongation	72	10	1
Final hold	20	∞	1

2.15.3. Screening and Cloning

2.15.3.1. Screening

For screening of environmental and activated sludge samples (Table 7) a PCR reaction (Table 10) was performed with primer sets specific for *Nitrolancetus hollandicus* as well as *Nitrotoga*-like 16S rRNA genes and primer sets specific for *nxrB* genes of group *Nitrobacter* as well as *nxrA* genes of *Nitrolancetus hollandicus* (for details see 2.12. List of used Primer sets). The obtained PCR products were analyzed with Gel electrophoresis (1% gel; 120 volt; 60 minutes). If amplicons of correct size were obtained a TOPO TA cloning reaction was performed.

Table 10 Cycler program for screening and cloning



Cycler program	Temperature [°C]	Time [minutes]	Number of cycles
Denaturation	95	5	1
Initial denaturation	95	0.5	35
Annealing	-[1]	0.5	
Elongation	72	-[2]	
Final elongation	72	10	1
Final hold	-[3]	∞	1

[1] for annealing temperature see 2.12. List of used primer sets;
[2] depends on the length of the amplicon
[3] 20°C for screening; 4°C for cloning

2.15.3.2. Cloning and sequencing of 16S rRNA and *nxrB* genes

After PCR purification (Quiagen), PCR products were cloned into the pCR® II-TOPO® or pCR™4-TOPO® Vector and subsequently transformed into one shot® TOP10 chemical competent cells according to the TOPO TA cloning protocol (Invitrogen, 2012). For a short-term backup, 32 clones were picked from LB-plates and transferred to an ampicillin [100 mg ml^{-1}] containing master plate, which was incubated at 37°C overnight. To screen for insert positive clones a M13-PCR was performed and positive clones were identified with gel electrophoresis (1% gel; 120 volt; 60 minutes). Clones containing correct inserts were digested with restriction enzymes to obtain the restriction fragment length polymorphism (RFLP) pattern. Therefore, 5 µl of the PCR product were mixed with 1 µl of the restriction enzymes mspI or aluI and 1 µl of Tango™buffer. After incubation for 3 hours at 37°C the digested products were loaded on a 2.5% gel (90 minutes; 100 volt) to identify the clones for Sanger-sequencing.

The clones were picked from the master plate and transferred to 5 ml of LB-liquid media containing 7 µl of ampicillin [100 mg ml^{-1}]. Subsequently, 700 µl of the overnight grown culture were mixed with 300 µl of 50% Glycerol for long term storage at −80°C. The remaining culture was harvested by centrifugation in a 2 ml ERT and the plasmid was isolated by the classic plasmid preparation protocol. For plasmid preparation the pellet was re-suspended in 100 µl P1 buffer and incubated for 5 minutes at room temperature. Subsequently, 200 µl of NaOH/SDS solution were added, the tubes were inverted several times and incubated for 5 minutes on ice to lyse the cells. During the incubation step, tubes were inverted again. For precipitation of proteins 150 µl of potassium/acetate solution were added and mixed by inverting. After incubation for 5 minutes on ice the precipitated proteins were pelletized by centrifugation for 1 minute at 13,000 rpm. The supernatant was transferred to 1.5 ml tubes and mixed with 1 volume of ice-cold isopropanol (2-propanol) and incubated for 10 minutes at room temperature for precipitation. DNA was pelletized by centrifugation for 1 minute at 13,000 rpm and washed 1 time with 500 µl 70% ethanol. After centrifugation, the pellet was air dried and dissolved in 50 µl double distilled water or 10 mM Tris.

Sanger-sequencing was performed with Topo or M13 primer sets. The obtained sequences were proofread with the software Chromas (Technelysium Pty Ltd) and corrected if required. To search for the closest relatives, the sequences were BLASTed (NCBI) (Altschul *et al.*, 1990). Proofed 16S rRNA genes were assembled with Chromas (Technelysium Pty Ltd). Afterwards, the 16S rRNA gene sequences were aligned using the SINA alignment tool (Pruesse *et al.*, 2012) and imported into ARB. The alignment was refined and phylogenetic trees were calculated by using the Silva database (SSURef_106_SILVA_NR_99.arb) in ARB (Ludwig *et al.*, 2004).

2.15.3.3. Cloning and sequencing of *Nitrolancetus hollandicus nxrA* genes

The four *nxrA* genes (*nxrA1*, *nxrA2*, *nxrA3* and, *nxrA4*) of *Nitrolancetus hollandicus* were amplified, using the newly designed primers NlHo_NxrA0038F and NlHo_NxrA3595R. To obtain high accurate sequences the high fidelity Taq polymerase (Fermentas) was used for amplification. The cycler Program for PCR is shown in Table 11. The products were excelled with micro haematocrit capillary tubes or by using a scalpel. The excelled gel were dissolved in 50 µl TE at 70°C for 10 minutes or extracted with the QIAquick Gel purification Kit according to QIAquick Gel purification Kit protocol (Quiagen). The cloning reaction was performed with the TOPO® XL PCR cloning kit using the pCR-XL-TOPO® 3.5 kb vector according to the TOPO® XL PCR cloning protocol (Invitrogen, 2012). Preparation of master plates for short time storage, identification of clones for sequencing and preparing of glycerol stocks for long time storage were performed as described previously in this work.

Table 11 Cycler program for cloning of *nxrA* genes

Cycler program	Temperature [°C]	Time [minutes]	Number of cycles
Denaturation	95	5	1
Initial denaturation	95	0.5	35
Annealing	-[1]	0.5	
Elongation	70	3.5	
Final elongation	70	10	1
Final hold	4	∞	1

[1] for annealing temperature see 2.12. List of used primer sets

Sanger-sequencing was performed with TopoF and TopoR primers as well as internal sequencing primers (Nlho_NxrA0658F, Nlho_NxrA1294F, Nlho_NxrA2335R and, Nlho_NxrA3070R) to obtain the full length sequence of the *nxrA* copies. The obtained sequences were BLASTed (NCBI) (Altschul *et al.*, 1990) to confirm the affiliation to the family of DMSO reductase type II enzymes. Subsequently, sequences were proofread, corrected and combined using the software Chromas (Technelysium Pty Ltd). Further phylogenetic analyses of *Nitrolancetus hollandicus nxrA* and calculation of phylogenetic trees were performed by S. Lücker. Newly designed primers (nxrA2F, nxrA3F, nxrA4F, nxrA2R, nxrA3R, and nxrA3R) were used for chimera screening of the obtained nxrA sequences, which had been formed during amplification due to the high similarity of the *Nitrolancetus hollandicus nxrA* (90.3%–95.8%) (Sorokin *et al.*, 2012). The cycler Program for PCR is shown in Table 11.

2.15.4. Calculation of phylogenetic trees

First, the phylogenetic affiliation of *Nitrotoga*-like 16S rRNA gene sequences obtained in this study with the genus *Gallionellaceae* within the class of *Betaproteobacteria* was confirmed. Therefore, the sequences were added in a 16S rRNA gene based tree containing the closest relatives of *Nitrotoga* and members of the families *Rhodocyclaceae* and *Comamonadaceae*. Subsequently, neighbor joining, maximum likelihood and maximum parsimony trees were calculated with full sequences of *Nitrotoga*-like 16S rRNA genes, obtained in this study, and with members of the *Betaproteobacteria*, which are closest related to the *Nitrotoga*-like 16S rRNA sequences. The tree was rooted by using an outgroup consisting of 9 members of the family *Rhodocyclaceae*. Further sequences were included by using the ARB parsimony interactive tool without changing the overall tree topology. Subsequently, a consensus tree was constructed based on maximum likelihood and maximum parsimony trees.

2.15.5. Design of new primer sets and probes specific for the genus *Nitrotoga*

The design of new primer sets and probes specific for the genus *Nitrotoga* was performed with the software ARB (Ludwig *et al.*, 2004). Therefore, *Nitrotoga*-like 16S rRNA sequences obtained in this study, members closest related to *Nitrotoga*-like bacteria and members of the family *Rhodocyclaceae* and *Comamonadaceae* were loaded into the ARB editor tool. Suitable regions for primer and probe binding were chosen manually and the specifity of the obtained primer and probe sequences were verified with the Ribosomal Database Project tool Probe match (Cole *et al.*, 2003; Cole *et al.*, 2009). Additionally, the obtained primers were analyzed with the software OligoAnalyzer, regarding GC content, melting temperature, and the possibility of hairpin, self-dimer as well as hetero-dimer formation (IDT Integrated DNA Technologies).

2.15.6. Confirmation of the genomic localization of *Nitrolancetus hollandicus nxrA1*

To verify the localization of *nxrA1* within the *nxr* operon a primer set (cytcF/nxrbR) was generated, binding to the 3'end of *cytC* and the 5'end of the *nxrB* gene (Figure 3). To confirm that the product, obtained by amplification with the cytcF/nxrbR primers, contained the expected *nxrA*, a nested PCR was performed using the Nlho-nxrA0038F and Nlho_nxrA3595R primers. The obtained amplicons were cloned (see 2.16.4.2) and Sanger-sequenced. Afterwards, the sequences were BLASTed (NCBI) (Altschul *et al.*, 1990) to confirm the phylogenetic affiliation to the family of DMSO reductase type II enzymes.

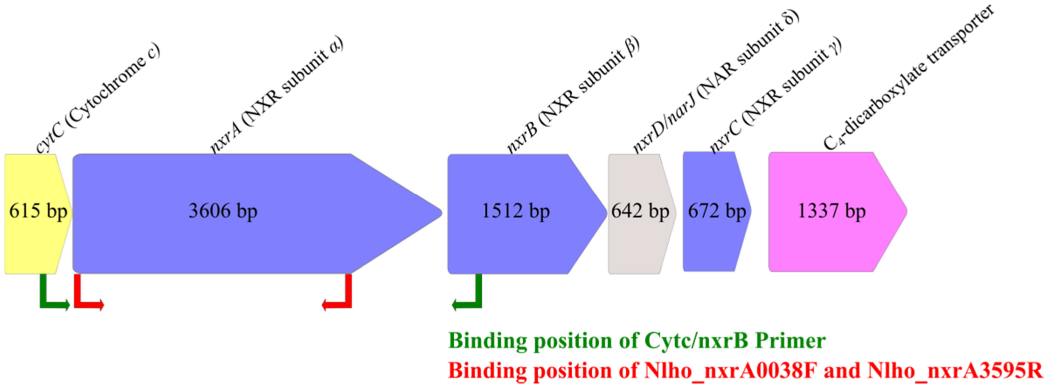


Figure 3 The conserved *nxr* region of the *Nitrolancetus hollandicus* genome with binding positions of Nlho_nxrA0038F/Nlho_nxrA3595R and cytc/nxrB-primers; to confirm the location of *nxrA1* upstream of *nxrB* and downstream of *cytC*, the following primer combinations were used: cytcF/nxrbR; cytcF/Nlho_nxrA3595R; Nlho_nxrA0038F/nxrbR; to obtain sequences of all four *nxrA* copies, one set of primer, which binds on the beginning and the end of *nxrA* genes (Nlho_NxrA0038F/Nlho_NxrA3595R), was used.

3. Results

3.1. Distribution, abundance, co-localization and phylogeny of *Nitrotoga*-like bacteria

Activated sludge samples from nineteen WWTPs and several environmental samples were screened for the presence of *Nitrotoga*-like bacteria via PCR using primer sets specific for the genus *Nitrotoga*. Additionally, the same activated sludge samples were screened for presence of *Nitrotoga*-like bacteria by FISH. Furthermore, the relative abundance and co-localization pattern was analyzed in three WWTPs.

PCR screening revealed the presence of *Nitrotoga*-like bacteria in eleven out of nineteen WWTPs, five river systems, one lake sediment as well as in a sediment sample from a dutch drainage ditch (Oorjipolder) (Table 12 and Table 13). Furthermore, *Nitrotoga*-like bacteria were detected in activated sludge samples from seven WWTPs using FISH (Table 13). Phylogenetic analysis of sequences obtained from seven WWTPs and one river system confirmed the affiliation of these sequences to the genus *Nitrotoga*. All sequences showed a high similarity of 98% and 99% to each other. However, phylogenetic analysis revealed two phylogenetic groups that are supported by three treeing methods, suggesting the existence of a diversity within the genus *Nitrotoga* (Figure 4).

Table 12 Detection of *Nitrotoga*-like bacteria in selected sediment samples

Environmental samples (sediments)	Detection of Nitrotoga
Feistritz	+
Bergwerksee big/small	–
Compost	–
Fettwiese	–
Fischteich Hessendorf (Landau)	–
Herrensee Sediment	–
Mürz	+
Oorjipolder	+
Östliche Wörtenlacke	–
Pitten	+
Saubach	+
Schilfgürtel right/left	+
Schwarza	+
Unterer Stinker	–

Used primer: Ntoga122F and Ntoga1422R as well as Ntoga124F and Ntoga1462bR (Schwarza, Mürz). No FISH was performed with those samples.

Table 13 Detection of *Nitrotoga*-like bacteria in selected activated sludge samples

WWTP	Reactor type	Type of treated sewage	Detection of Nitrotoga		*Nitrospira* sublineage	Temperature [°C]	Influent [mg l^{-1}]	Effluent [mg l^{-1}]			Sampling date 2007
			PCR	**FISH**			NH_4^+	NH_4^+	NO_2^-	NO_3^-	
Altmannstein	SBR	M	+	$+^1$	I + II	7	54.7	9.18	0.48	0.72	March 24
Ampfing	SBR	M + slaughter and dairy waste	-	$-^1$	II	13	nd	0.1	0.04	3.25	March 26
Bad Zwischenahn	DIC-SBR	M	+	+	-	16	60	0.25	0.15	6.5	May 23
Bruchmühlen	DIC-SBR	M	+	$+^1$	I	15	36	0.53	0.09	4.53	May 22
Deuz	DIC-SBR	M	+	+	I + II	13	nd	0.33	0.09	3.46	May 21
Hettstedt	DIC-SBR	M + external activated sludge	-	$-^1$	I + II	15	56	12.35	0.24	3	May 24
Huntlosen	DIC-SBR	M	-	$-^1$	I + II	17	68	0.13	0.03	2.2	May 23
Ingolstadt	SBR	activated sludge drainage	-	$-^1$	I + II	27	856	0.3	< 0.1	20.4	May 09
Kraftisried	single-stage activated sludge basin	A	-	$-^1$	I + II	7	397.5	35.3	6.2	17.4	January 29
Langenzenn	SBR	M	+	+	-	9	21.25	7.96	0.42	3.1	March 14
Lyss (ARA)	fixed bed reactor	M	+	$-^1$	I	12	20	1	0.1	18	January 29
Lyss (GZM)	membrane filtration plant	A	-	$-^1$	I + II	30	700	< 1	< 0.5	14	January 29
Oberding	fixed bed reactor	A	-	$-^1$	I + II	26	450	< 1	< 0.5	4	January 29
Plattling	two-stage activated sludge basin	A	-	$-^1$	I + II	30	750	1	< 0.5	3	January 29
Radeburg	DIC-SBR	M	+	-	I	14	nd	0	0.05	3.3	May 24
Rosenheim	SBR	M	$-^1$	$-^1$	I + II	36	970	nd	nd	nd	May 30
Seefeld	SBR	M	+	$-^1$	I	nd	8.32	1.59	nd	1.73	March 28
Spenge	DIC-SBR	M	+	$+^1$	I	14	24	< 0.2	0.05	1.38	May 22
Waldsassen	SBR	M + I	+	$+^1$	I	9	18.5	< 0.1	nd	3.45	March 27
Weisstal	DIC-SBR	M	+	$-^1$	I + II	nd	nd	0	0.02	4.4	May 21

Activated sludge from WWTPs screened positive for *Nitrotoga*-like bacteria. Detection of *Nitrospira* lineages I and II are also provided. Additional information about the type of sewage, reactor type, temperature and influent ammonia as well as effluent ammonia, nitrite and nitrate concentration are shown. A Animal rendering. I Industrial. M Municipal. Lücker (2010), modified. 1 Lücker (2010)

The abundance and co-localization pattern were analyzed in activated sludge from WWTPs Bad Zwischenahn and Langenzenn, where *Nitrotoga* is the only known NOB as well as in activated sludge from WWTP Deuz, where *Nitrotoga* coexists with *Nitrospira*-like bacteria. The abundance of *Nitrotoga*-like bacteria in activated sludge from WWTP Deuz was below 1% relative to all *Bacteria* and 3.5% relative to *Betaproteobacteria* (Table 14). However, pair correlation analysis in activated sludge from the same WWTP revealed a significant co-localization of *Nitrotoga* and AOB at 10 µm (Figure 5A). A similar pattern was obtained when the AOB image series was analyzed, but indicates a second possible pair correlation at 20 µm (Figure 5B). Spatial distribution analysis of *Nitrospira* and AOB revealed a significant pair correlation at 10 µm and 25 µm, which suggest a different co-localization pattern of two in WWTP Deuz existing *Nitrospira* lineages with AOB (Figure 5C), which is supported by earlier studies (Maixner *et al.*, 2006). Furthermore, the combined channels of the two nitrite oxidizing populations revealed a spatial distribution up to a distance of 35 µm. Additionally, both guilds of organisms are present at different distances, which indicates a niche adaptation of *Nitrotoga*-like bacteria and *Nitrospira*-like bacteria in activated sludge from WWTP Deuz (Figure 5D).

Similar to WWTP Deuz, the abundance of *Nitrotoga* in activated sludge from WWTP Langenzenn was below 1% relative to all *Bacteria* (Table 14). Spatial distribution analysis of the Ntoga image series showed two maxima between 0 and 10 µm (Figure 6A). Therefore, a pair correlation of the two functional groups could be assumed. However, the pair correlation is not significant, which is indicated by the +95% and –95% confidence limits. The pair correlation curve at distances larger than 15 µm suggests a random distribution of *Nitrotoga*-like bacteria and AOB. Analysis of the AOB images series showed displacement between *Nitrotoga*-like bacteria and AOB at distances below 35 µm (Figure 6B). Quantification of *Nitrotoga*-like bacteria in activated sludge from WWTP Bad Zwischenahn revealed an abundance of 1.4% relative to all *Bacteria* (Table 14), which is supported by earlier analysis (Lücker, 2010; data not shown). Interestingly, similar to Langenzenn no other known NOB is present in this sludge, but spatial distribution analysis revealed a co-localization between *Nitrotoga* and AOB (Lücker, 2010; data not shown).

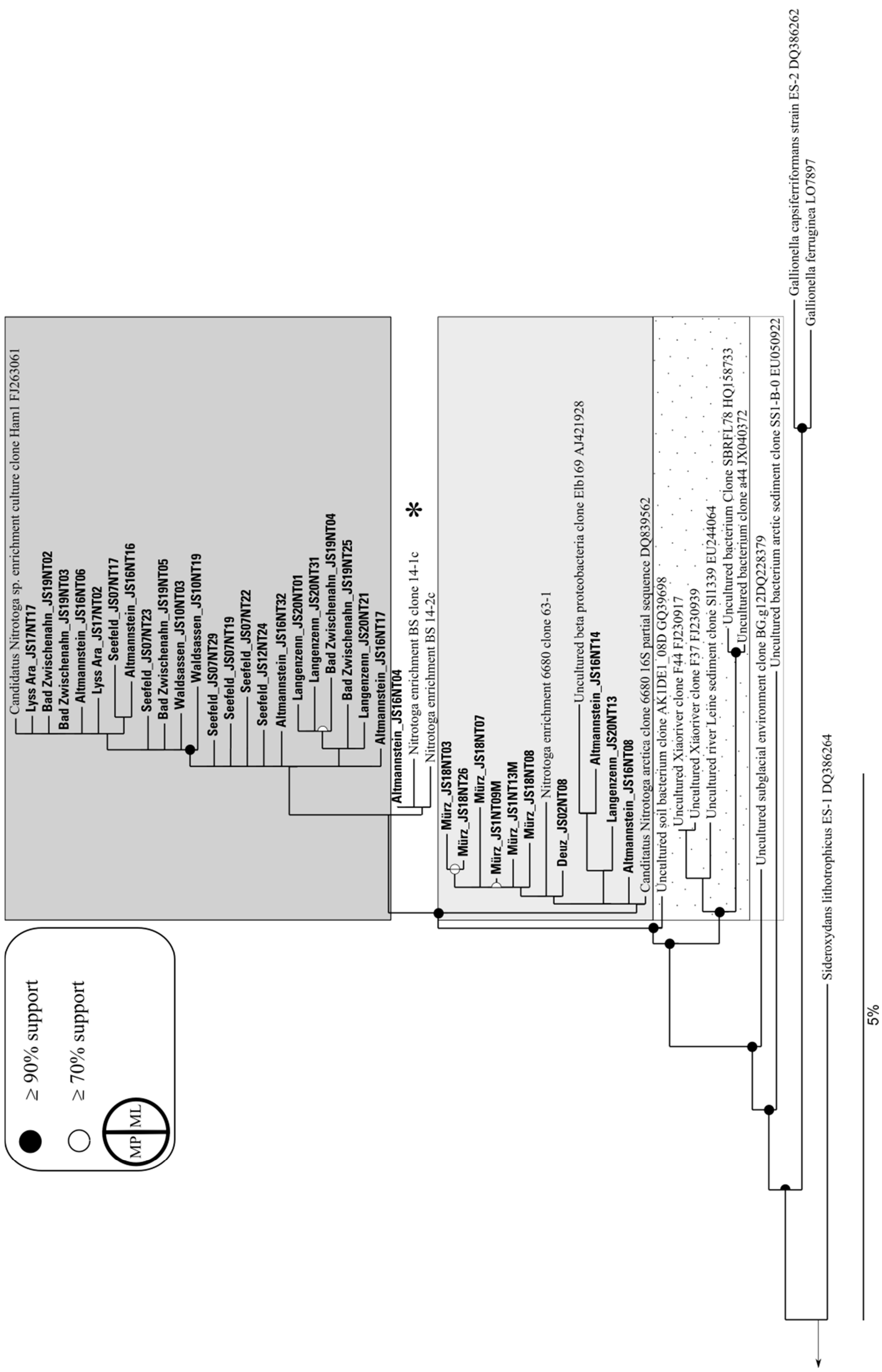


Figure 4 Phylogenetic analysis shows the affiliation of *Nitrotoga*-like 16S rRNA gene sequences obtained in this study to *Candidatus* Nitrotoga arctica, members of *Nitrotoga*-like bacteria and *Gallionellaceae* publicly available on the NCBI Database. The consensus tree is based on maximum parsimony and maximum likelihood calculations. Pie charts indicate the statistical support of nodes based on bootstrap analysis. Calculations of bootstrap values are based on 100 iterations. Additionally, a neighbor joining analysis was performed with jukes cantor correction and bootstrap analysis using 1000 iterations. Asterisk denotes sequences that have a different phylogenetic position in the neighbor joining tree. Sequences obtained in this study are indicated in bold face. The suggested groups of *Nitrotoga*-like bacteria are highlighted with the dark grey and light grey box. The dotted box shows 16S rRNA gene sequences whose affiliation to *Nitrotoga* is not confirmed. The white box shows 16S rRNA gene sequences of uncertain affiliation. Representatives of the family *Rhodocyclaceae* were used as outgroup. Scale bar represents 5% estimated sequence divergence. ML, maximum likelihood; MP, maximum parsimony.

Additionally to the quantification of *Nitrotoga*-like bacteria using the probe specific for the genus *Nitrotoga*, the relative abundance was determined by using the probe specific for the family *Gallionellaceae* (FGall178), which revealed similar results for all WWTPs, excluding the presence of *Nitrotoga*-like bacteria that are not targeted of the probe Ntoga122 (Table 14). Additional information about amplification and cloning of *Nitrotoga*-like 16S rRNA genes are provided in Supplementary Text S1 and S2.

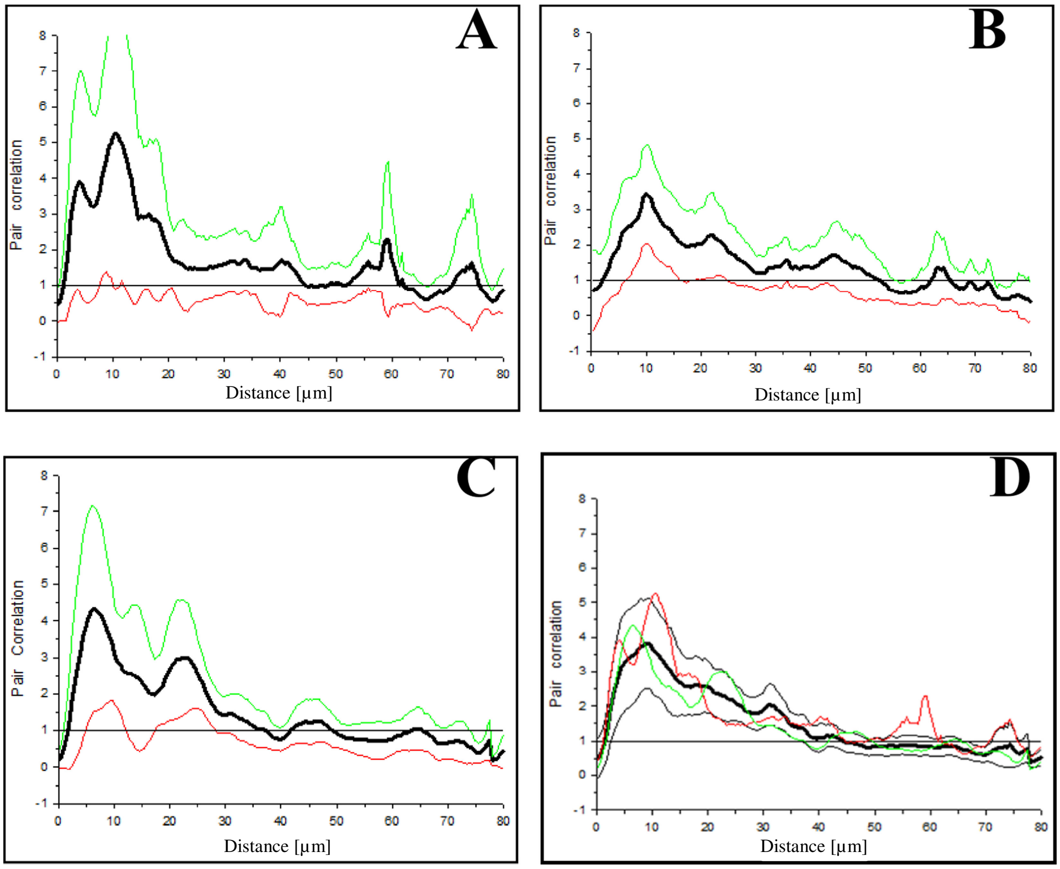


Figure 5 Colocalization patterns of NOB (*Nitrotoga* and *Nitrospira*) and AOB in activated sludge from WWTP Deuz. Red and green lines: positive and negative 95% confidence limits. Black line: pair correlation of two populations. The horizontal line marks a pair correlation of 1, which indicates a random distribution of the two populations. Values higher than 1 indicate a colocalization between two populations and values lower than 1 indicate repulsion or displacement.
A, B: Pair correlation analysis between *Nitrotoga*-like bacteria and AOB calculated with Ntoga image series (A) and calculated with AOB image series (B). C: Pair correlation between *Nitrospira*-like bacteria and AOB performed with Nspira image series. D: Colocalization analysis between NOB and AOB. Bold black line: pair correlation of the two functional groups. Thin black lines: positive and negative 95% confidence limits. For comparison, the pair correlation curves of the Ntoga image series and the Nspira image series (compare A and C) are indicated by the thin red and green lines, respectively.

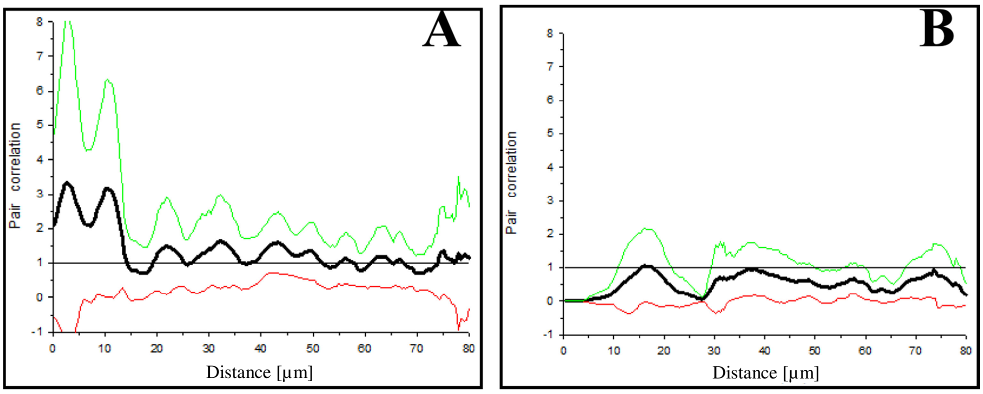


Figure 6 Co-localization analyses of *Nitrotoga*-like bacteria in sludge samples from WWTP Langenzenn relative to AOB. Green and red lines: positive and negative 95% confidence limits, respectively. Black line: pair correlation of two populations. A: Spatial distribution pattern of *Nitrotoga*-like bacteria versus AOB analyzed with the Ntoga image series. B: Spatial distribution pattern of *Nitrotoga*-like bacteria relative to AOB analyzed with the AOB image series.

Table 14 Relative abundance of *Nitrotoga*-like bacteria in selected activated sludge samples

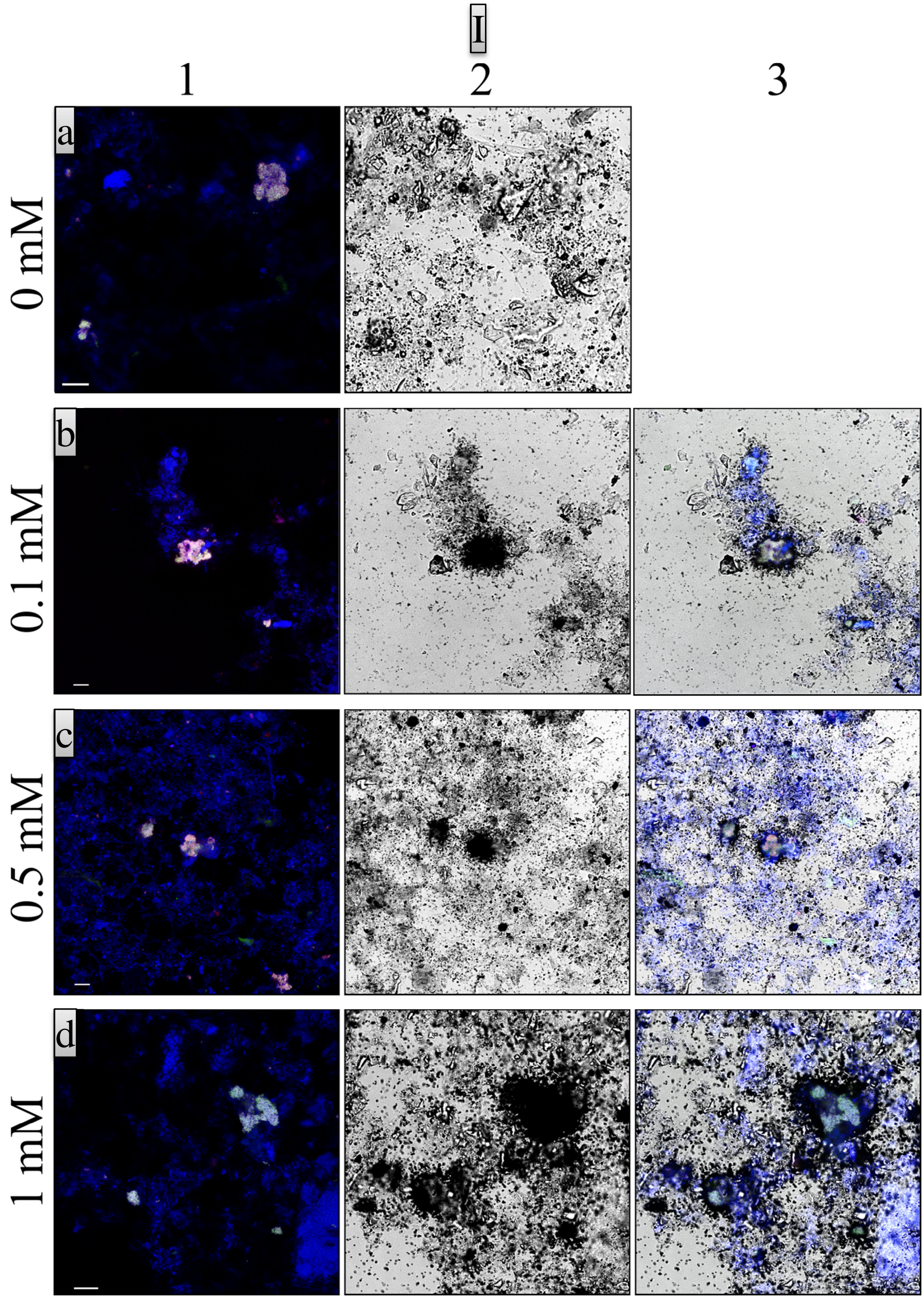
WWTP	Specific probe	General probes	Volume fraction [%]
Deuz-DIC-SBR	Ntoga122	EUBmix	0.4
	FGall178	EUBmix	0.5
	Ntoga122	Bet42a	3.5
Langenzenn-SBR	Ntoga122	EUBmix	0.2
	FGall178	EUBmix	0.5
Bad Zwischenahn-DIC-SBR	Ntoga122	EUBmix	1.4
	FGall178	EUBmix	1.6

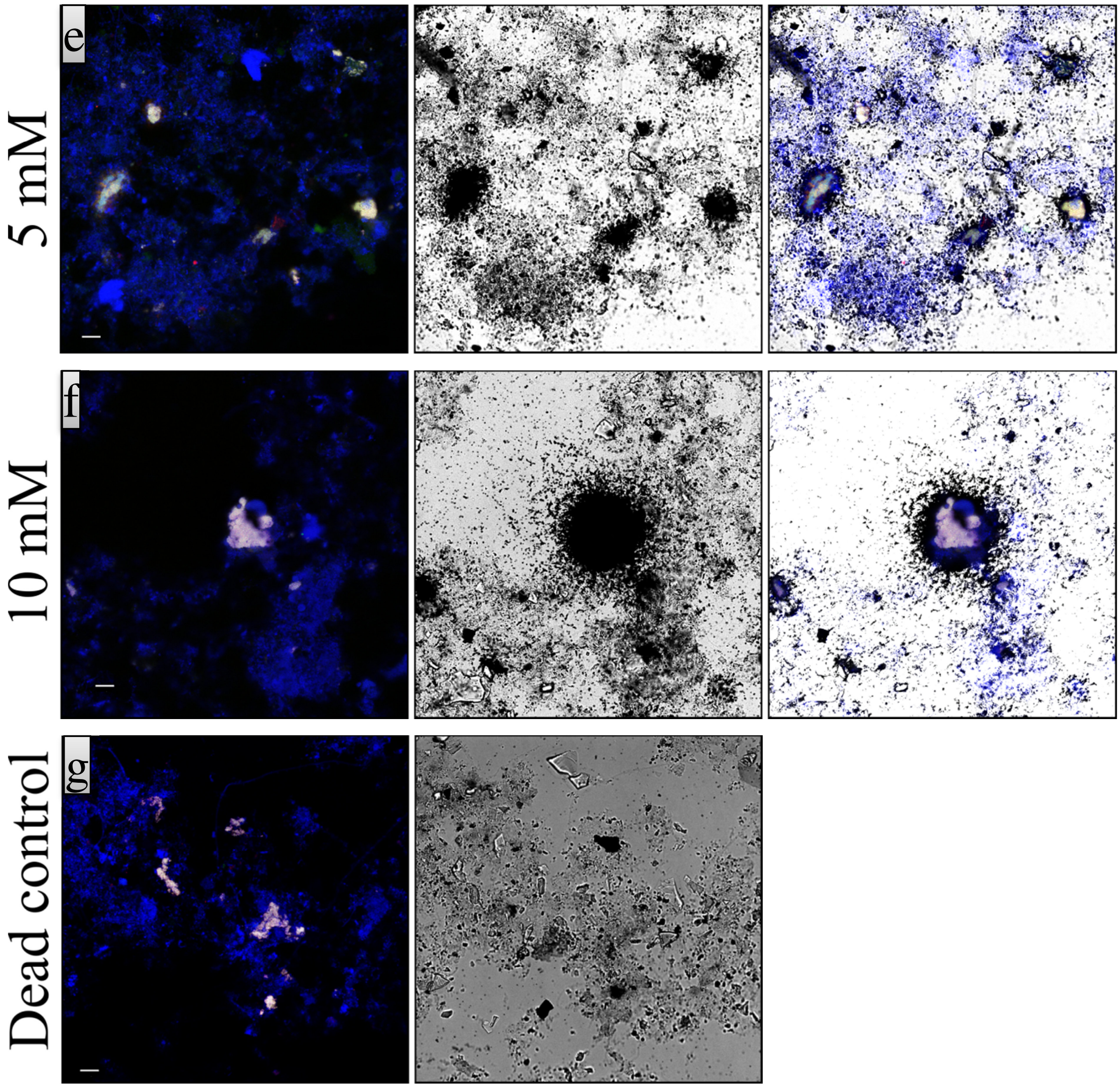
3.2. Functional analysis of *Nitrotoga*-like bacteria

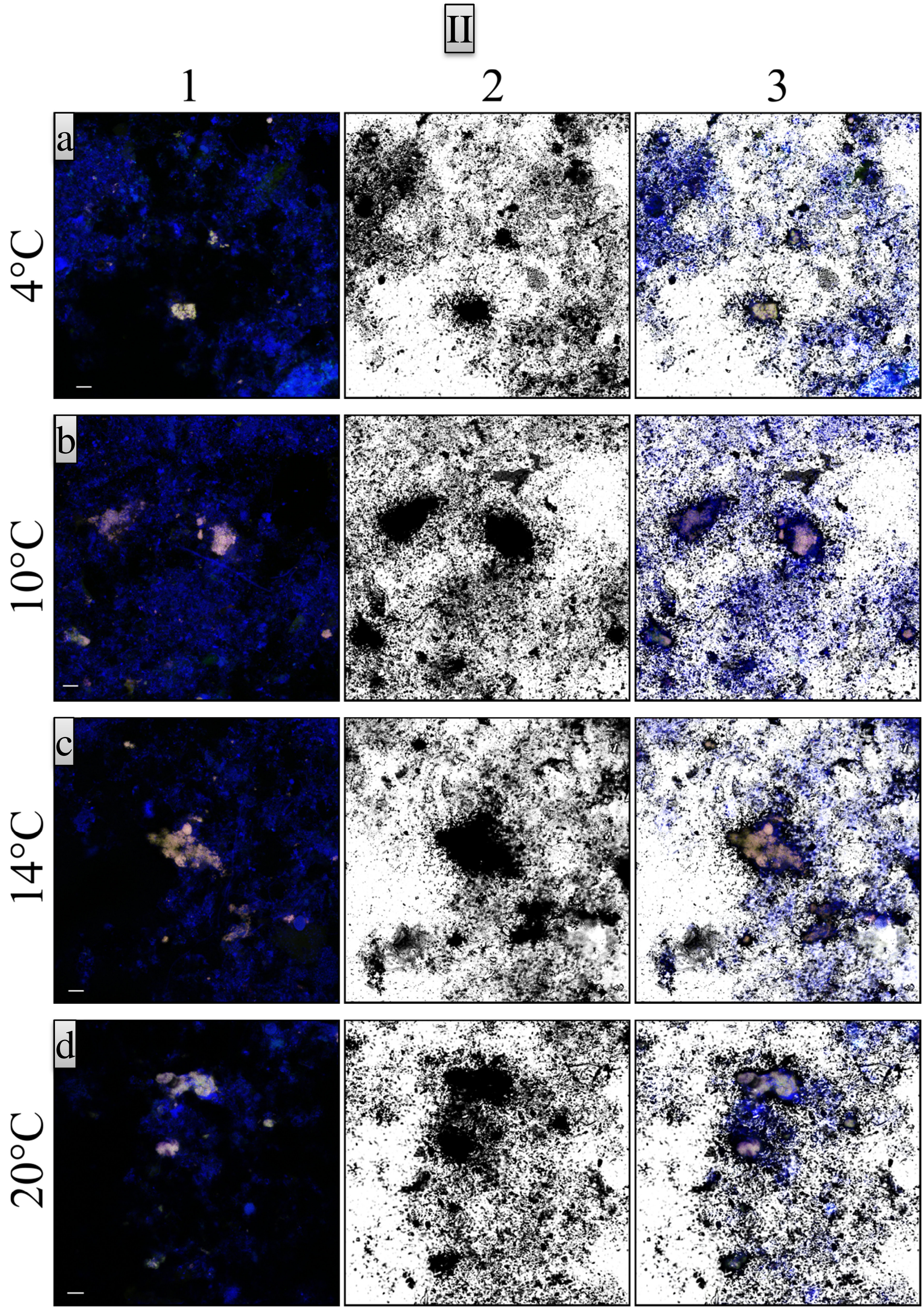
The NO_2^- oxidizing activity of *Nitrotoga*-like bacteria was verified in activated sludge samples from WWTPs Deuz and Bad Zwischenahn. Therefore, two incubation series with 6 different NO_2^- concentrations and with 5 different temperature conditions were performed. Prior to incubation start, the absence of endogenous NO_2^- and NH_3 was verified by measuring with NO_2^- test strips and Neßlers reagent, respectively. NO_2^- concentrations were monitored during the incubation with NO_2^- test strips and NO_2^- was replenished when necessary (Supplementary Figure S2). No consumption of NO_2^- was measured in the incubations with 5 mM and 10 mM NO_2^- (Supplementary Figure S2). The reason might be the lack of sensitivity of the used high range NO_2^- test strips (0.1–3 g l^{-1}) for small changes in NO_2^- concentrations. To ensure the absence of other known NOB in activated sludge of Bad Zwischenahn, FISH and PCR were performed, using probes and primer sets specific for *Nitrospira*-like and *Nitrobacter*-like bacteria (data not shown).

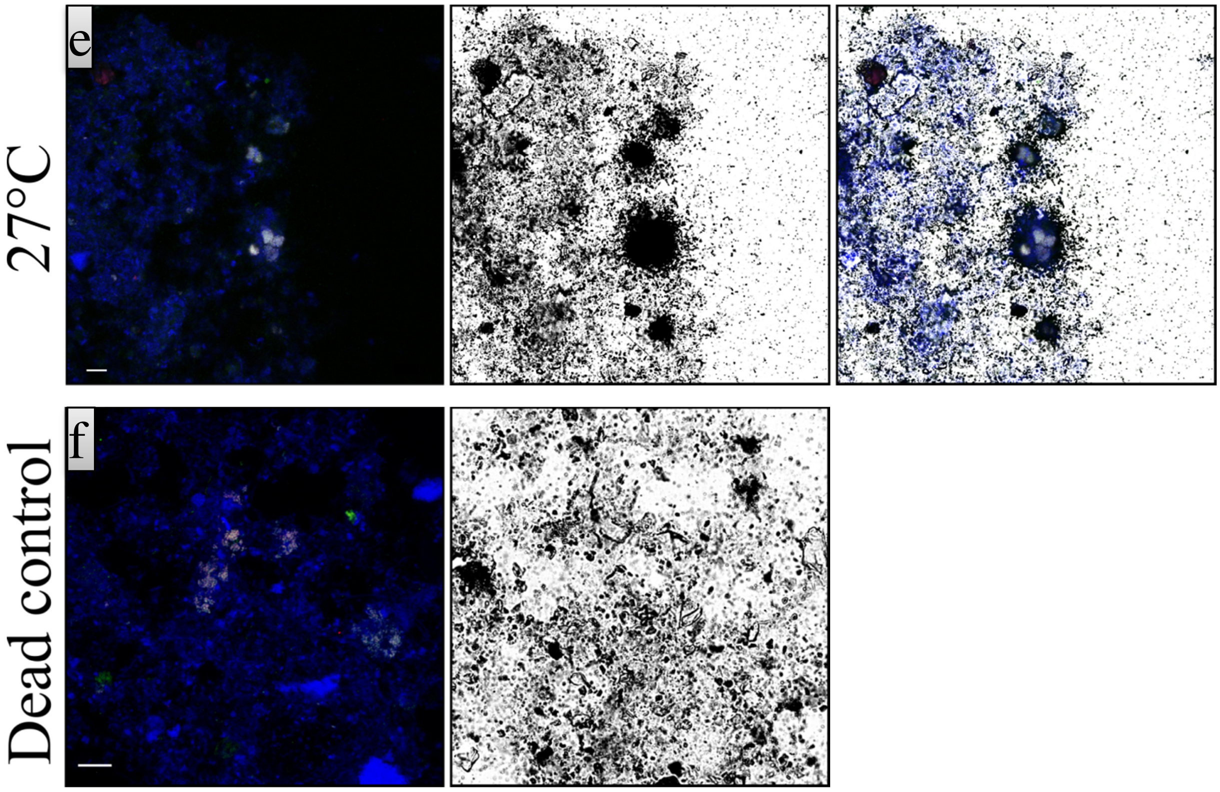
Temperature incubation was performed for 6 hours at 4°C, 10°C, 14°C, 20°C as well as 27°C with a NO_2^- concentration of 0.5 mM. NO_2^- incubation was conducted for 6 hours with 0 mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM as well as 10 mM NO_2^- in each incubation flask at a temperature of 14°C. Additionally, a control consisting of PFA fixed sludge (dead control) was incubated with 0.5 mM NO_2^- at a temperature of 14°C. No MAR signals were observed in dead controls, which confirmed that all formed silver grains resulted from biological reactions and not from unspecific binding of $[^{14}C]$bicarbonate to the cell surface or interaction between specimen and emulsion (chemography) (Figures 7I g, 7II f and Figures 8I g, 8II f). Furthermore, incubations without NO_2^- showed no formation of silver grains, which indicates the dependency of bicarbonate uptake on NO_2^- consumption (Figures 7I a, 7II a and Figures 8I a, 8II a). In activated sludge of WWTP Bad Zwischenahn CO_2-fixing activity of *Nitrotoga* could be detected at all NO_2^- concentrations and temperature conditions (Figure 7I b–f and 7II a–e) and only a small fraction of *Nitrotoga* cell aggregates showed no silver grain formation. Cluster which were MAR negative were either inactive or too small to distinguish their corresponding MAR signal against the background.

Since the abundance of *Nitrotoga* in activated sludge of WWTP Deuz was very low, it was difficult to find appropriate cell clusters. Nonetheless, *Nitrotoga* cluster that were detected, showed formation of silver grains, which indicates CO_2-fixation activity with NO_2^- as electron donor (Figures 8I b–f and 8II a–e). Unfortunately no *Nitrotoga* cluster was detected in activated sludge incubated with an NO_2^- concentration of 10 mM. In contrast to *Nitrotoga*, the second nitrite oxidizing organism present in activated sludge from WWTP Deuz was more abundant. MAR positive *Nitrospira* cell cluster could be detected at all nitrite concentrations and temperature conditions (Figures 8 I b–f and II a–e).

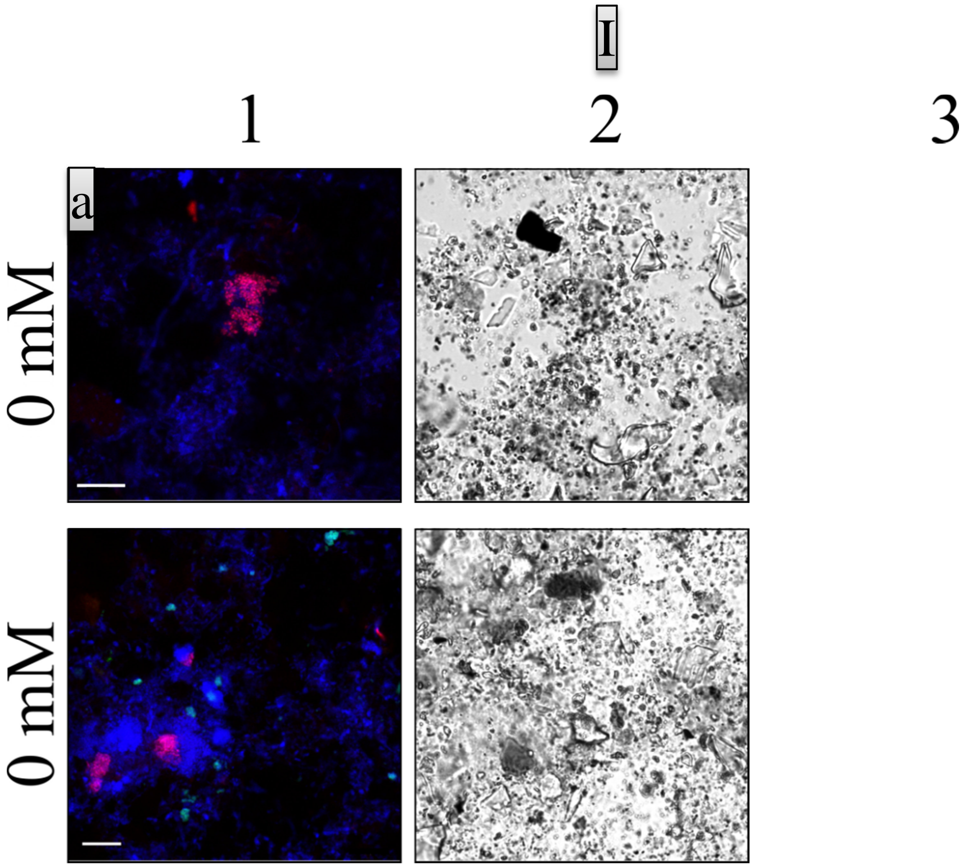

I
1
2
3
a
b
c
d
0 mM
0.1 mM
0.5 mM
1 mM

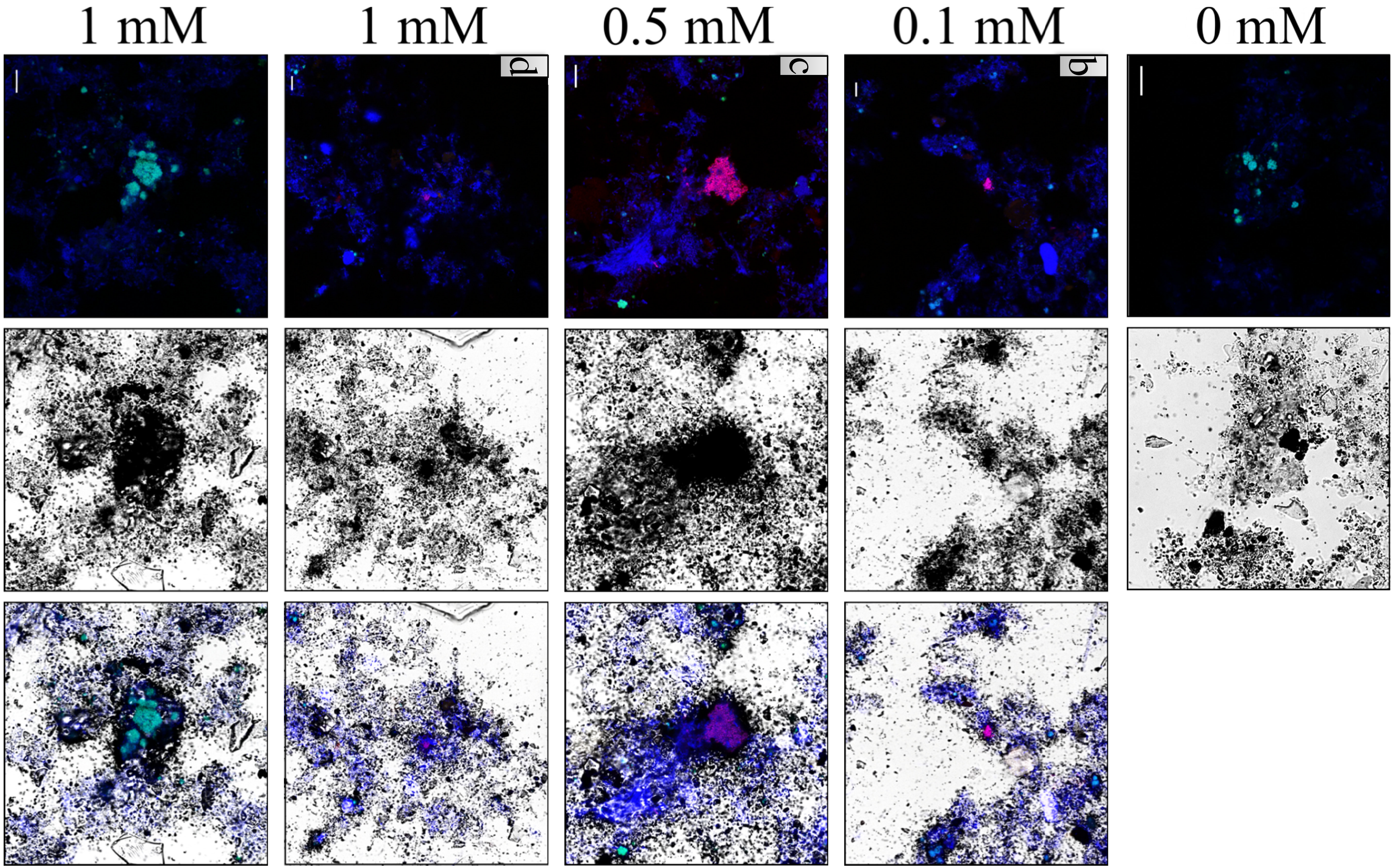

5 mM
e
10 mM
f
Dead control
g

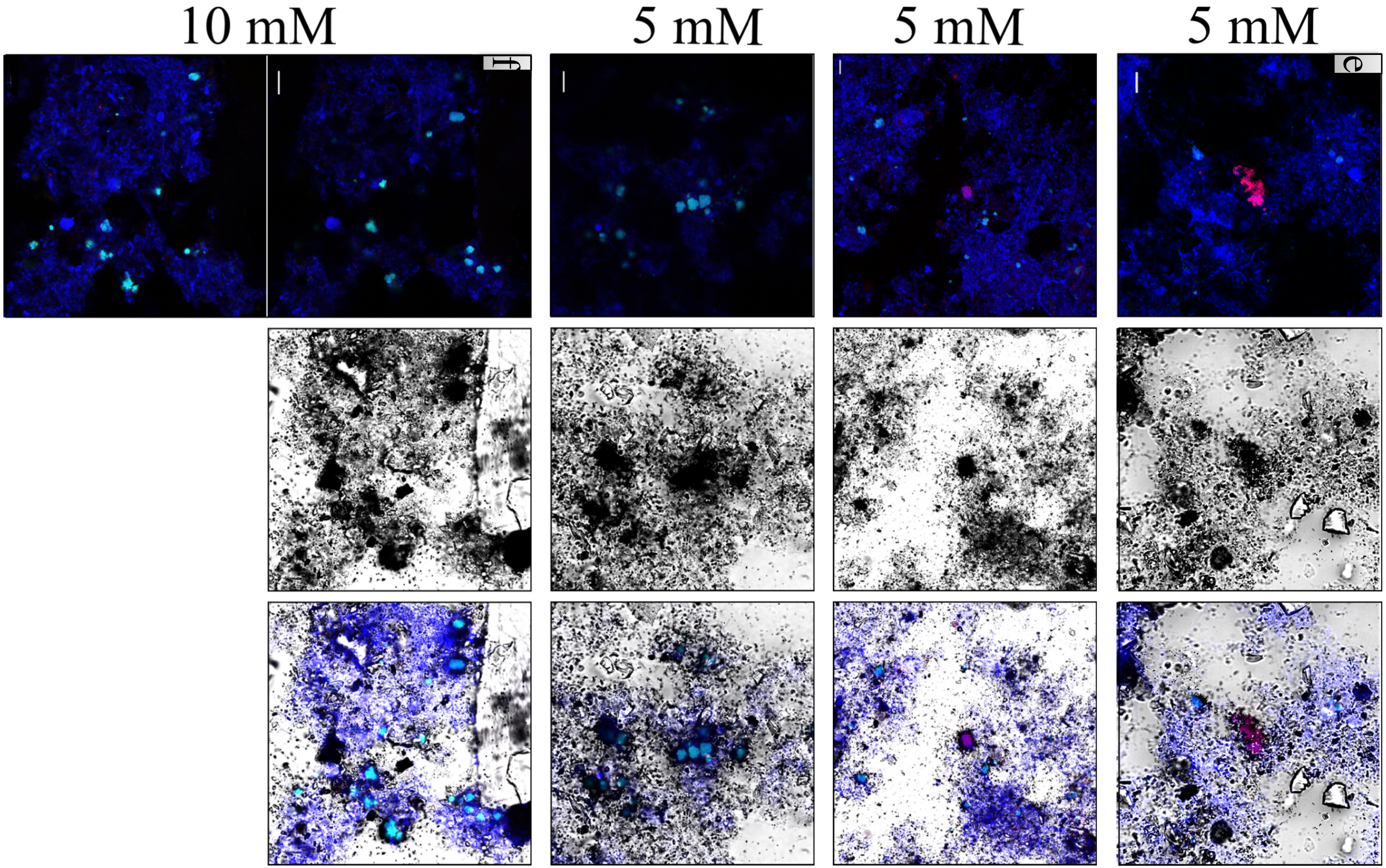

II
1
2
3
a
4°C
b
10°C
c
14°C
d
20°C



Figures 7I and 7II MAR-FISH images of activated sludge from WWTP Bad Zwischenahn. I, II: MAR-FISH images of temperature and nitrite incubation series after 6 hours of incubation; 1 FISH images: Ntoga122 (red) and Ntoga221 (green) targeting *Nitrotoga* cell clusters, EUB338mix (blue) targeting most *Bacteria*. *Nitrotoga* clusters appear in white due to simultaneously binding of probes Ntoga122, Ntoga221 as well as EUB338mix; other *Bacteria* appear in blue; 2 microautoradiographic images of the microscopic field as in panel 1; 3 overlay of images of panels 1 and 2; Scale bars indicate 10 µm.




1 mM
1 mM
0.5 mM
0.1 mM
0 mM
d
c
b


10 mM
5 mM
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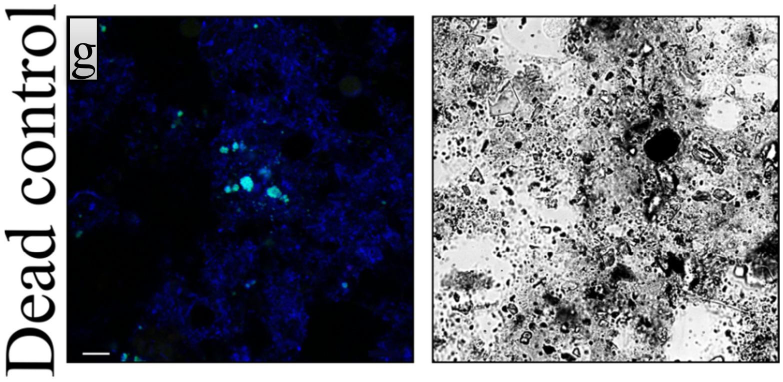
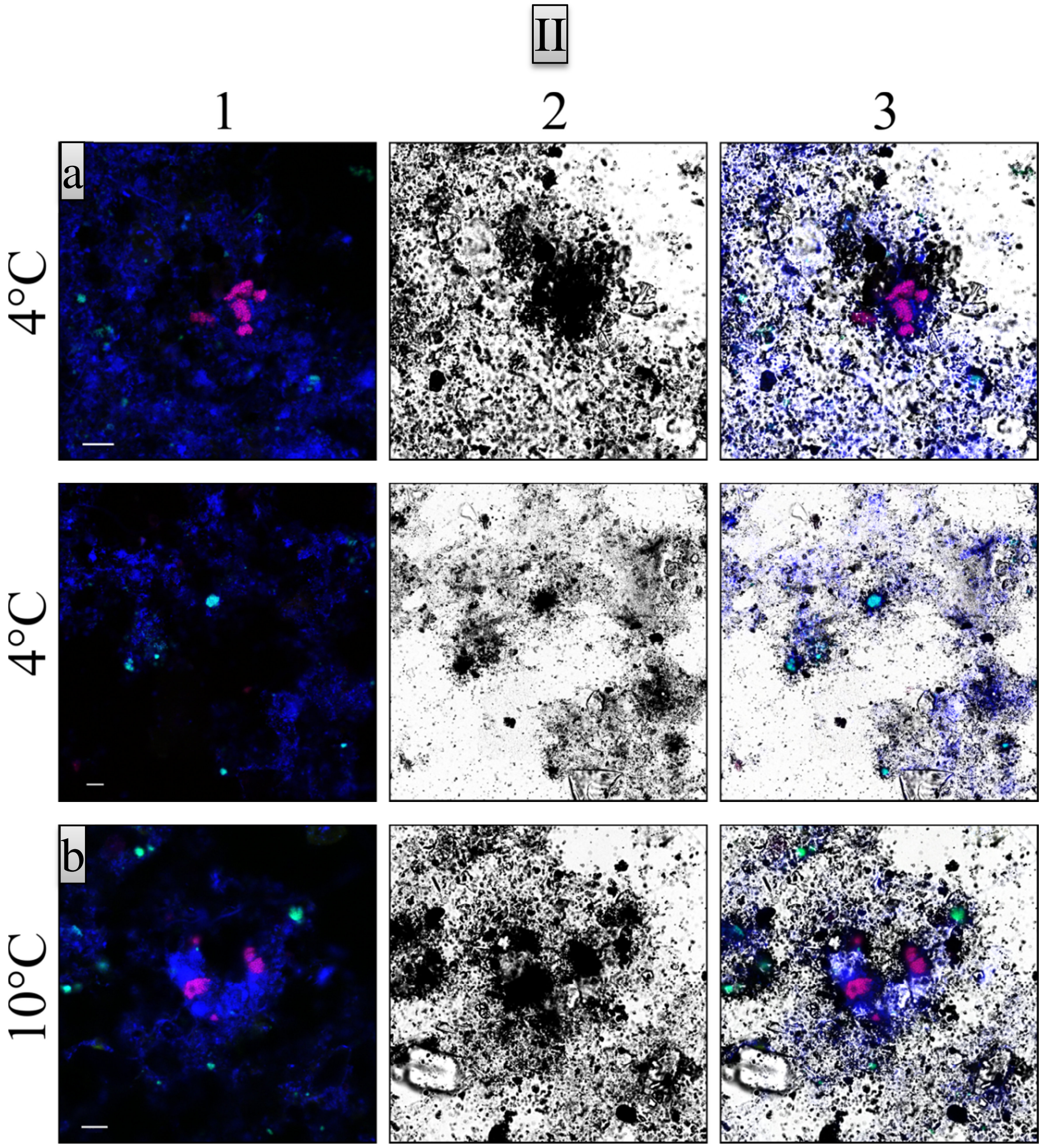
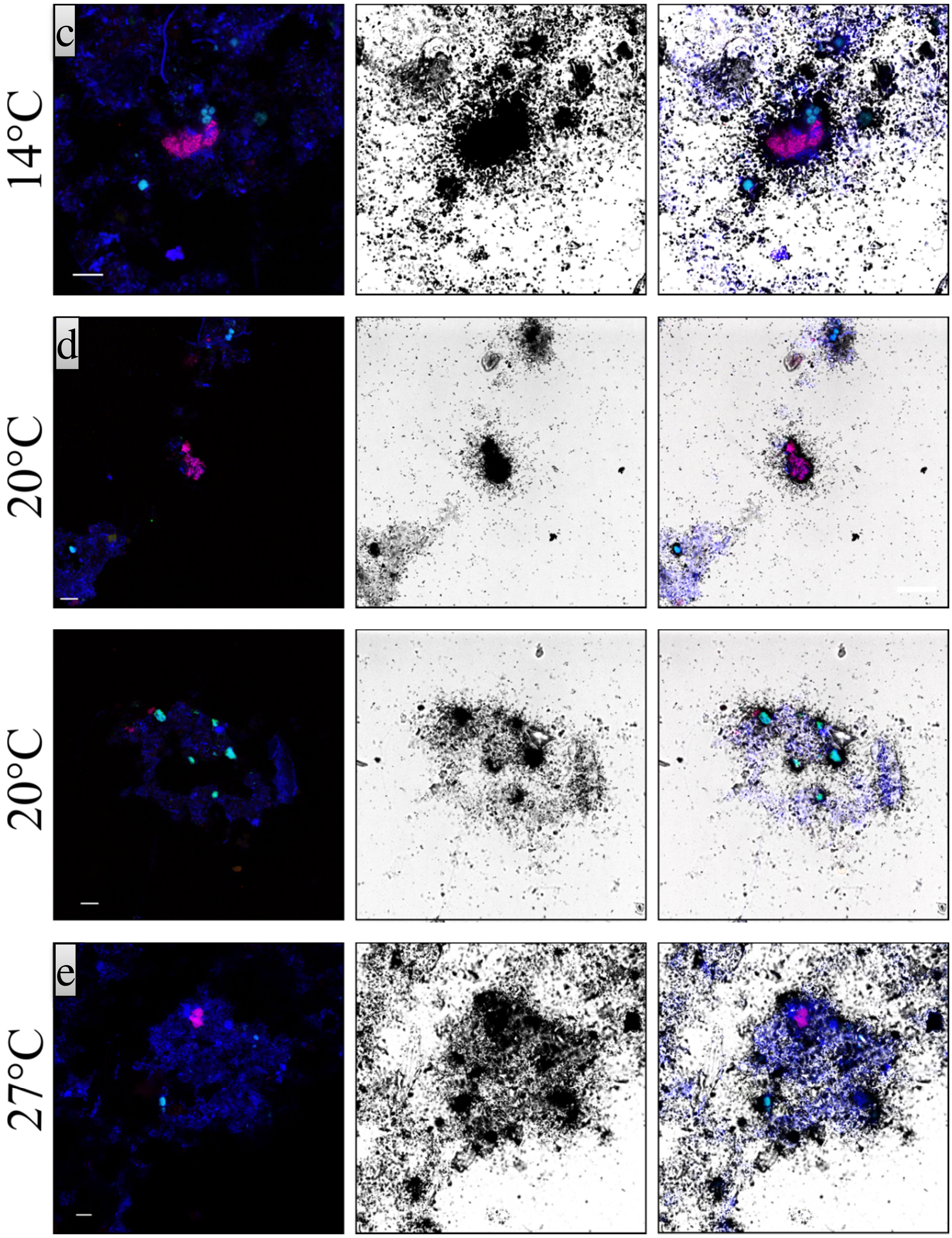
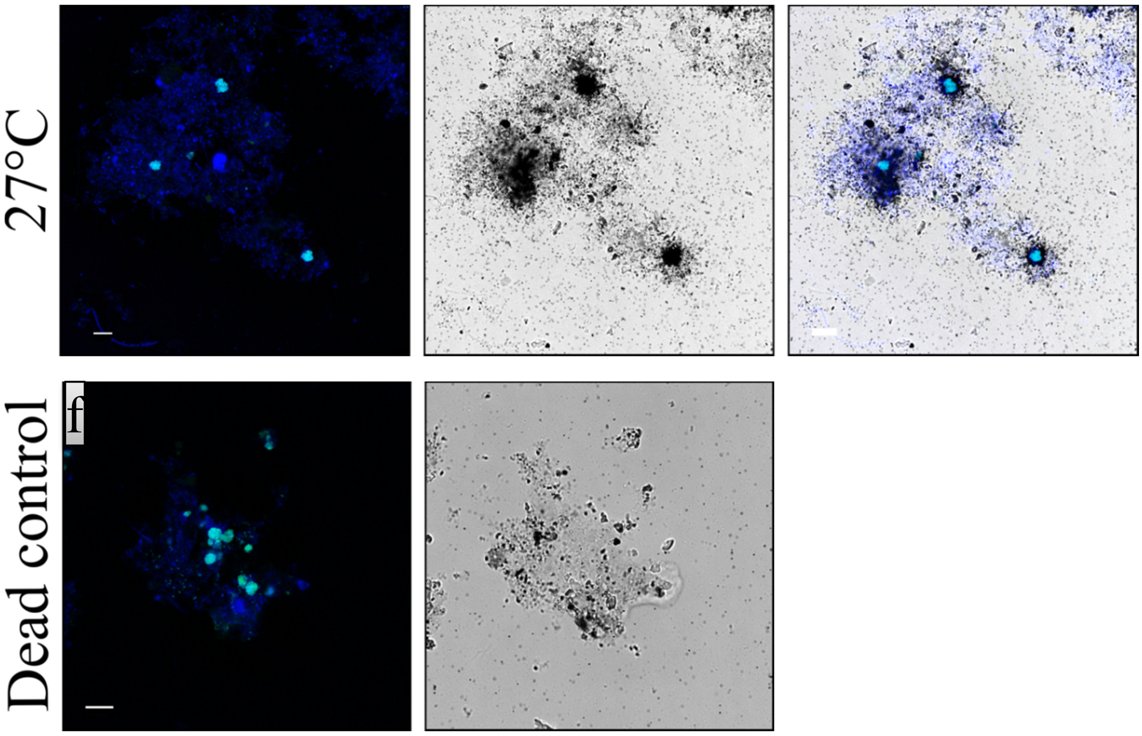
5 mM
f
e


Dead control
g


II
1
2
3
a
4°C
4°C
b
10°C


c
14°C
d
20°C
20°C
e
27°C



Figures 8I and 8II MAR-FISH of activated sludge from WWTP Deuz. I, II: MAR-FISH images of nitrite and temperature incubation series; 1 FISH images: Nspira662 (green) targeting *Nitrospira* cell clusters; Ntoga122 (red) targeting *Nitrotoga* cells and EUB338mix (blue) targeting most *Bacteria*; Clusters of *Nitrospira* appear in turquoise due to simultaneously binding of probes Nspira662 and EUB338mix; Clusters of *Nitrotoga* appear in magenta due to simultaneously binding of probes Ntoga122 and EUB338mix; Other *Bacteria* appear in blue*;* 2 microautoradiographic images of the same microscopic field as in panel 1; 3 overlay of images of panels 1 and 2; Scale bars indicate 10 µm.

3.4. *Nitrolancetus hollandicus*

3.4.1. *Nitrolancetus hollandicus nxrA* genes

Sequencing of the genomic DNA of *Nitrolancetus hollandicus* with high throughput techniques revealed the presence of four highly identically (90.3%–95.8%) *nxrA* genes (Sorokin *et al.*, 2012). One *nxrA* copy is suggested to be located directly upstream of the *nxrB* gene (*nxrA1*) (Sorokin *et al.*, 2012). The other three copies (*nxrA2*, *nxrA*3 and, *nxrA4*) form a cluster and are located in a different region of the genome (Sorokin *et al.*, 2012). Since the sequences of all *nxrA* genes were incomplete a set of primer (Nlho_nxrA038F and Nlho_nxrA3595R) were designed that targets all *nxrA* copies. Amplification with the Nlho_nxrA0038F and Nlho_nxrA3595R primer set revealed a product of the correct size of approximately 3.5 kb (Figure 9), which were cloned and Sanger-sequenced. Information about the 2000 bp and 500 bp products are provided in Supplementary Text S3. Further phylogenetic analysis of *Nitrolancetus hollandicus nxrA* genes were carried out by S. Lücker and revealed a relationship to NXR of

Nitrobacter spp. and *Nitrococcus mobilis* within the family of DMSO reductase type II enzymes (Sorokin *et al*., 2012).

The high similarity of the *nxrA* genes led to formation of chimeric sequences during PCR. To identify the correct sequence, primer sets specific for *nxrA1*, *nxrA2* and *nxrA3* (nxrA2F/R, nxrA3F/R, and nxrA4F/R) were used for PCR in different combinations. Amplicons were obtained with the primer combinations nxrA2F/nxrA2R, nxrA3F/nxrA3R and nxrA4F/nxrA4R (Figure 10). These results identified the chimeric sequences and confirmed the correct assembly of the sequence reads, obtained by high throughput sequencing. Further information about amplification and cloning of *Nitrolancetus nxrA* genes are provided in Supplementary Text S3 and S4.

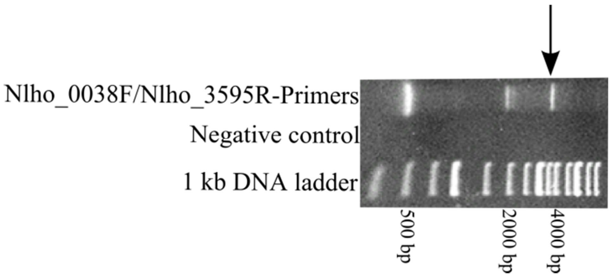


Figure 9 Gel electrophoresis image of the PCR product obtained by using the Nlho_nxrA0038F and Nlho_nxrA3595R primer. The arrow indicates the 3.5 kb amplicon.

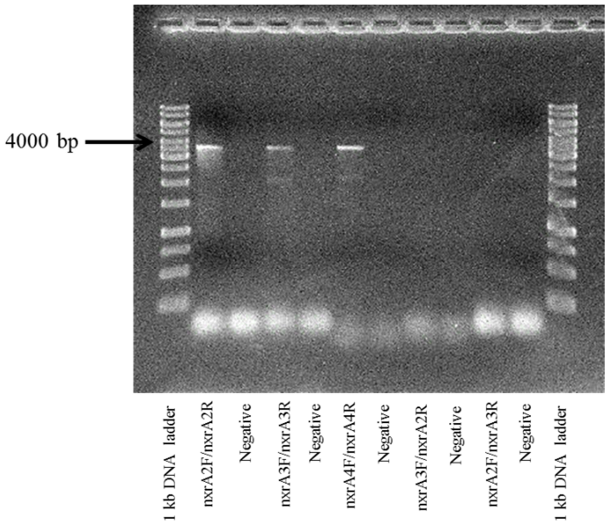


Figure 10 Gel electrophoresis image of the PCR products obtained with different combinations of primers nxrA2F, nxrA3F, nxrA4F, nxrA2R, nxrA3R, and nxrA4R. Not all of the used primer combinations are shown here. 5% DMSO was added to mastermix.

The genomic localization of *nxrA1* was confirmed with primers binding to the 3' end of the cytochrome *c* and to the 5' end of the *nxrB* genes. The used primer combinations (Nlho_nxrA0038F/nxrbR; cytcF/Nlho_nxrA3595R; cytcF/nxrbR) yielded amplicons of the expected size (Figure 11) which confirmed the localization of *nxrA* in the *nxr* operon. Furthermore, a nested PCR was performed with these amplicons, using the Nlho_nxrA0038F and Nlho_nxrA3595R primers. Subsequently, the obtained PCR products were cloned and Sanger-sequenced.

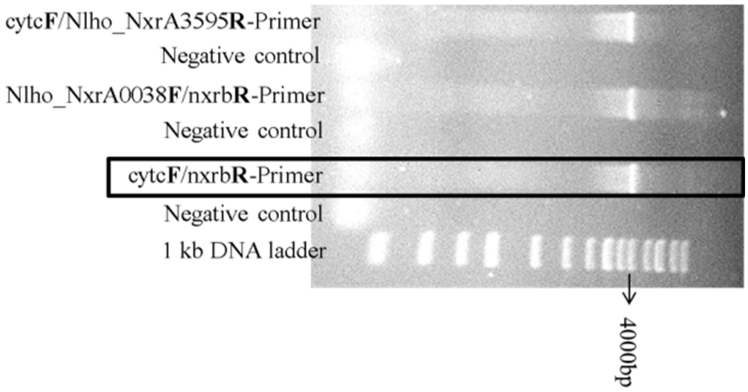


Figure 11 Gel electrophoresis image of the PCR products obtained by using the primer combinations cytcF/nxrbR, Nlho_nxrA038F/nxrbR, cytcF/Nlho_nxrA3595R. Black box indicates the PCR product obtained by using the cytcF/nxrbR primer set.

3.4.2. Hunting for '*Nitrolancetus hollandicus*': Screening of environmental samples

Nitrolancetus hollandicus is a thermotolerant NOB (temperature optimum for growth: 40°C), which tolerates high NO_2^- concentrations (up to 75 mM) and needs NH_3 for growth (Sorokin *et al.*, 2012). Therefore, environmental samples were chosen which might provide optimal conditions for this organism. Samples were screened via PCR using primers specific for 16S rRNA, *nxrB*, as well as *nxrA* genes. Additionally, FISH was performed with PFA fixed samples, using a general probe (EUB338mix) and probes specific for *Nitrolancetus hollandicus* and the phylum *Chloroflexi*. However, only FISH with activated sludge from WWTP Rosenheim revealed cell clusters, which were targeted by all three probes, suggesting the presence of a close relative of *Nitrolancetus hollandicus* (Figure 12). Probe adsorption to cell or particle surfaces was excluded by hybridization of the same activated sludge sample with NONEUB338 (data not shown). This result should be confirmed by PCR, using primers specific for *Nitrolancetus hollandicus* 16S rRNA genes. Furthermore, the phylogenetic affiliation of the obtained amplicon to *Nitrolancetus hollandicus* 16S rRNA must be confirmed by cloning and sequencing.

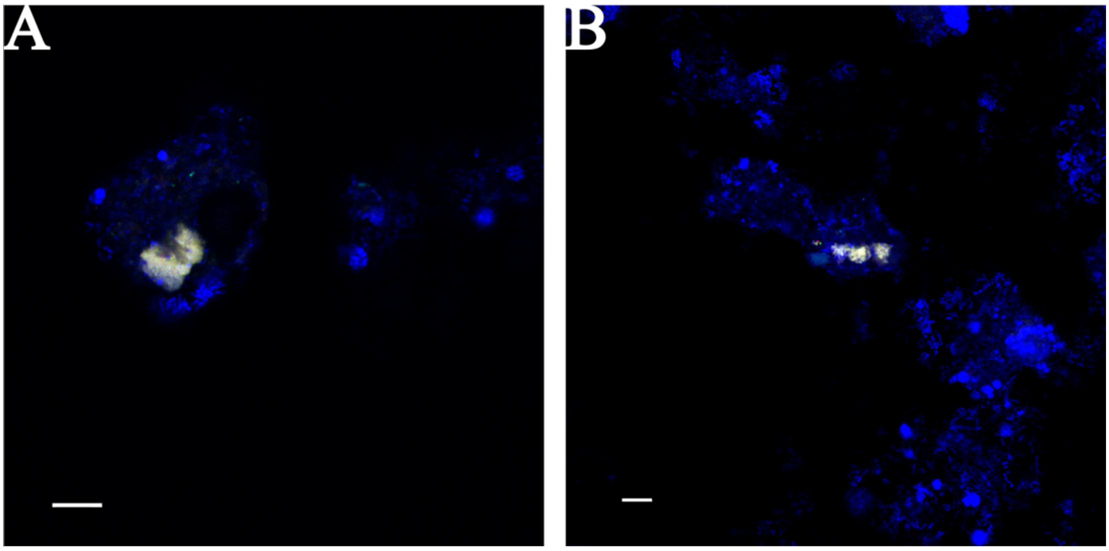


Figure 12 A and B: CLSM images of PFA-fixed sludge from WWTP Rosenheim; EUB338mix in blue targeting most *Bacteria*, Ntlc439 in red targeting *Nitrolancetus hollandicus*, Cfxmix in green targeting the phylum *Chloroflexi*; Clusters of *Nitrolancetus* appear in white due to simultaneously binding of Ntlc439, EUB338mix and Cfxmix; other bacteria appear in blue. Scalebars indicate 10 µm.

Additionally, the *'Nitrolancetus hollandicus'* 16S rRNA gene sequence was BLASTed against the NCBI Nucleotide Database (Altschul *et al.*, 1990) to increase the chance to detect the natural habitat of '*Nitrolancetus hollandicus*'. Indeed, in one case a sequence very similar to '*Nitrolancetus hollandicus*' 16S rRNA gene could be found. This sequence was submitted to NCBI Database (accession no: AB744381) by a Japanese team who screened soil samples to determine the changing of bacterial community during biological soil disinfestation (Mowlick, S. and Ueki, A. (2010). Analysis of bacterial community during biological soil disinfestations. Unpublished).

4. Discussion

4.1. ‘*Candidatus* Nitrotoga arctica’

4.1.1.Distribution and abundance of *Nitrotoga*-like bacteria

In 2007 ‘*Candidatus* Nitrotoga arctica’ was isolated from permafrost-affected soils of the Siberian Arctic (Alawi *et al*., 2007). Since then, close relatives of this organism were detected in activated sludge (Alawi *et al.,* 2009) or even in biological active filters for treatment of drinking water (Bereschenko *et al*., 2010; White *et al*., 2012). Intriguingly, further investigations performed in this study revealed the presence of *Nitrotoga*-like bacteria in a surprisingly high number of activated sludge and environmental samples, suggesting a wide distribution of these organisms even in moderate climate zones (Table 15).

Table 15 Activated sludge and environmental samples screened positive for *Nitrotoga*-like bacteria

Sample	**Sample-type**	**Temperature [°C]**	**Influent [mg l^{-1}]**	**Effluent [mg l^{-1}]**		
			NH_4^+	**NH_4^+**	**NO_2^-**	**NO_3^-**
Altmannstein SBR	Municipal activated sludge	7	54.7	9.18	0.48	0.72
Bad Zwischenahn DIC-SBR	Municipal activated sludge	16	60	0.25	0.15	6.5
Bruchmühlen DIC-SBR	Municipal activated sludge	15	36	0.53	0.09	4.53
Deuz DIC-SBR	Municipal activated sludge	13	nd	0.33	0.09	3.46
Langenzenn SBR	Municipal activated sludge	9	21.5	7.96	0.42	3.1
Lyss Ara Fixed bed reactor	Municipal activated sludge	12	20	1	0.1	18
Radeburg DIC-SBR	Municipal activated sludge	14	nd	0	0.05	3.3
Seefeld SBR	Municipal activated sludge	nd	8.32	1.59	nd	1.73
Spenge DIC-SBR	Municipal activated sludge	14	24	<0.2	0.05	1.38
Waldsassen SBR	municipal and industrial activated sludge	9	18.5	<0.1	nd	3.45
Weissthal DIC-SBR	Municipal activated sludge	nd	nd	0	0.02	4.4
Feistritz	River sediment	nd	nd	nd	nd	nd
Mürz	River sediment	nd	nd	nd	nd	nd
Oorjipolder	Durch drainage ditch sediment	nd	nd	nd	nd	nd
Pitten	River sediment	nd	nd	nd	nd	nd
Saubach	Stream sediment	nd	nd	nd	nd	nd
Schilfgürtel left/right	Lake sediment	nd	nd	nd	nd	nd
Schwarza	River sediment	nd	nd	nd	nd	nd

Additional information for WWTPs: temperature and influent as well as effluent concentration of NO_2^-, NO_3^- and, NH_3. Except of WWTP Bad Zwischenahn and WWTP Langenzenn, *Nitrospira*-like bacteria are present in all WWTPs. (Lücker, 2010). nd not determined.

Phylogenetic analysis of the *Nitrotoga*-like 16S rRNA gene sequences obtained in this study revealed a high similarity (98%–99%) to each other and to publicly available *Nitrotoga*-like organisms, suggesting a low diversity within the genus *Nitrotoga*. However, that is based on investigations of 16S rRNA gene sequences with a sometimes questionable resolution (Janda and Abott, 2007). It is possible that different species have high 16S rRNA gene similarity but exhibit a DNA hybridization (relative binding ratio) lower than 60–70% and therefore a high genetic diversity (Ullman and McCarthy, 1973; Fox *et al*., 1992; Janda and Abbott, 2007; Richter and Rosselló-Móra, 2009), which would indicate that they represent individual species (Stackebrandt and Goebel, 1994). Since DNA-DNA hybridization has a lot of disadvantages (Gevers *et al*., 2005), a strategy to solve the resolution limit of 16S rRNA gene sequences might be metagenomic analysis (Handelsman, 2004), calculation of the average nucleotide identity (Konstantinidis and Tiedje, 2005) or sequencing and analyzing of the bacterial pan-genome (Medini *et al*., 2005; Tettelin *et al*., 2005; Bentley, 2009). Additionally, Raman spectroscopy, which is already a capable approach for identification of pathogenic microorganisms in medical diagnostics (Harz *et al*., 2005; Popp *et al*., 2011), might be also applicable in microbial ecology. However, the high similarity between the 16S rRNA gene sequences might also reflect that *Nitrotoga* is a relatively young genus, which has already been shown for the genus *Nitrobacter* (Orso *et al*., 1994). Despite *Nitrobacter* strains have been isolated from various habitats, 16S rRNA gene-based phylogenetic analysis of all *Nitrobacter* species showed 16S rRNA gene similarities above 99% to each other and 98% similarity to the genera *Bradyrhizobium* and *Rhodopseudomonas*, suggesting that *Nitrobacter* evolved recently from a *Rhodopseudomonas*-like photosynthetic, nitrogen-fixing ancestor (Orso *et al*., 1994; Lücker, 2010). Surprisingly, a consensus tree, containing some of the publicly available 16S rRNA gene sequences related to '*Candidatus* Nitrotoga arctica' and sequences obtained in this study, unveiled two groups, which are supported by all treeing methods, suggesting the existence of a diversity within the genus *Nitrotoga*, which might imply the adaption to different environmental conditions. A similar pattern was already shown for subpopulations, co-occurring within *Nitrospira* sublineages I and II, showing high 16S rRNA gene sequence similarity to each other (Dorninger *et al*., manuscript in preparation). Interestingly, the diversity within *Nitrotoga*-like bacteria might be also supported by co-localization analysis in activated sludge from WWTP Deuz, which unveiled two significant peaks at 10 µm and 20 µm. A similar pattern has already been shown in a previous study, suggesting a pair correlation of *Nitrospira* lineage I and II with AOB at different distances because of adaptations to different nitrite concentrations (Maixner *et al.*, 2006). However, the co-localization pattern was only obtained by analyzing the AOB image series, whereas the spatial arrangement analysis of the Ntoga image series suggests a co-localization only at a distance of 10 µm. Additionally, the phylogenetic tree contains only one *Nitrotoga*-like 16S rRNA gene sequence obtained from WWTP Deuz. Therefore, the existence of these groups within the genus *Nitrotoga* has still to be verified by performing FISH and PCR, using group-specific probes and primers, respectively.

The wide distribution of *Nitrotoga* in the environment raised the question regarding the abundance of these organisms especially in WWTPs. In this context it is also important to analyze the co-localization pattern of *Nitrotoga*-like bacteria and AOP. Since both groups benefit from each other in a mutualistic relationship (Stein and Arp, 1998), co-localization has frequently been reported to occur between AOB and NOB *(*Schramm *et al.*, 1999; Maixner *et al.*, 2006). The abundance and spatial distribution pattern of *Nitrotoga*-like bacteria was analyzed in activated sludge samples from three WWTPs (Deuz, Langenzenn and, Bad Zwischenahn). In activated sludge from Langenzenn and Deuz the abundance of *Nitrotoga*-like bacteria was quite low (<1%). Interestingly, in activated sludge from WWTP Langenzenn, *Nitrotoga* seems to be the only known NOB. However, co-localization analyses suggested random distribution of *Nitrotoga* and AOB and revealed even displacement, when the AOB image series was analyzed. These quite unusual results might be explained by the presence of a so far undetected NOB that outcompete *Nitrotoga*-like bacteria from the vicinity of AOBs or by the presence of so far unknown AOB, which groups together with *Nitrotoga*-like bacteria. Because only probes have been used that target already known NOB and AOB, it is possible that unknown organisms have been overlooked. Furthermore, *Nitrotoga*-like bacteria might exhibit a different lifestyle, which makes a co-localization with AOB not necessary. In contrast to WWTP Langenzenn, the spatial distribution analyses in activated sludge from WWTP Deuz revealed a positive pair correlation between *Nitrotoga* and AOB. Additionally, the activated sludge harbors two lineages of *Nitrospira* with a relative abundance of 14% (Müller, 2008), showing significant co-localization with AOB. Both organisms need nitrite to gain energy and are therefore competitors for the same source of nutrition, where *Nitrospira*-like bacteria might be better adapted to the conditions in WWTP Deuz than *Nitrotoga*-like bacteria. The results indicate that *Nitrotoga*-like bacteria might have found their niche in activated sludge from WWTP Deuz, which is indicated by the co-localization pattern suggesting a positive relationship with AOB. Additionally, it was shown that the population is stable over a long time period. Activated sludge from WWTP Deuz was sampled at two different time points. In 2007, *Nitrotoga* was detected by PCR and FISH. Five years later the amount of *Nitrotoga* cells was lower but still detectable and nitrite oxidizing activity could be demonstrated (Figure 13). Similar to Langenzenn, in activated sludge from WWTP Bad Zwischenahn *Nitrotoga*-like bacteria are the only known NOB. However, spatial distribution analyses indicated co-localization of *Nitrotoga*-like bacteria and AOB (Lücker, 2010). In addition, the abundance was higher than in the two other two analyzed WWTPs (1.4%), which should be enough to maintain the nitrite oxidizing capacity in this sludge. Indeed, activated sludge from the DIC-SBR WWTP Huntlosen (Oldenburg, Germany) harbors only one nitrite oxidizing population (*Nitrospira*-like bacteria), which are present in a similar relative abundance of 1.3% (Müller, 2008).

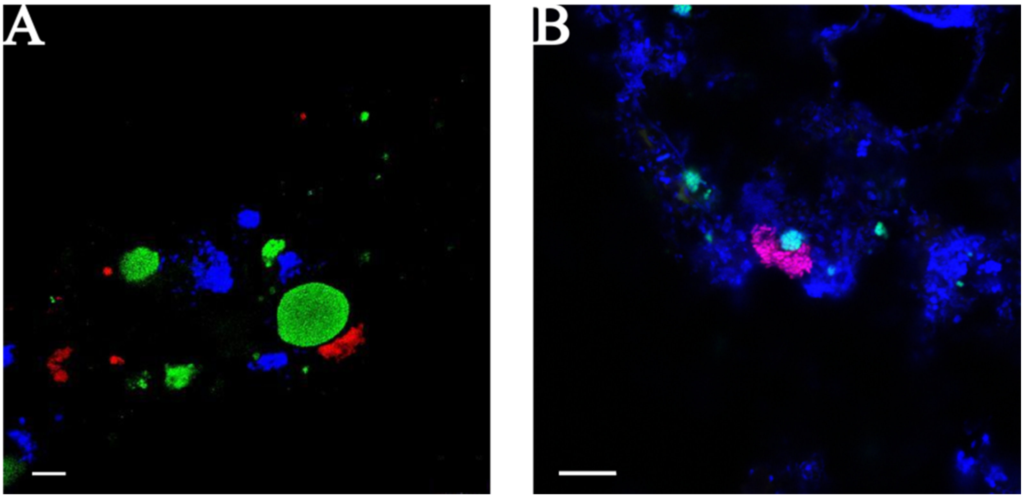


Figure 13 FISH images of fixed sludge from WWTP Deuz. A: Sampling date 2007; Ntoga122 targeting the genus *Nitrotoga* (red); AOBmix targeting members of AOB (green) and Ntspa662 targeting *Nitrospira* (blue); B: sampling date 2012; Ntoga122 targeting the genus *Nitrotoga* (red); Ntspa662 targeting *Nitrospira* (green); EUB338mix targeting most *bacteria* (blue). *Nitrotoga*-like bacteria occurs in magenta due to simultaneous binding of probes Ntoga122 and EUB338mix. *Nitrospira*-like bacteria appears turquoise due to simultaneous binding of probes Ntspa662 and EUB338mix. Other bacteria are shown in blue. In WWTP sludge of 2012 abundance of *Nitrotoga* was lower but still existent. Scalebars indicate 10 µm.

4.1.2. Activity of '*Nitrotoga*-like bacteria' in wastewater treatment plants

The nitrite oxidizing ability of *Nitrotoga* was so far only investigated by measuring the stoichiometrically oxidation of NO_2^- to NO_3^- during incubation of enrichments (Alawi *et al.*, 2007). Therefore, it was important to link the substrate uptake (NO_2^-) to the phylogenetic identity of the NO_2^- oxidizing organisms (Okabe *et al.*, 2004). Alawi and colleagues (2009) performed experiments with activated sludge samples from the conventional operated WWTP Köhlbrandthöft/Dradenau Hamburg, harboring the NOB populations *Nitrospira*, *Nitrobacter* and *Nitrotoga* (Alawi *et al.*, 2009). They incubated the activated sludge samples at 10°C, 17°C and 28°C with a NO_2^- concentration of 0.3 mM (Alawi *et al.*, 2009), whereby *Nitrotoga*-like cells could only be found in enrichments grown at 10°C and 17°C (Alawi *et al.*, 2009). In contrast to those results, in this study NO_2^- oxidizing activity of *Nitrotoga* in activated sludge from WWTP Bad Zwischenahn has been detected not only at the expected, but also at higher temperature (27°C) and NO_2^- concentrations (5 mM and 10 mM). Interestingly, the fraction of MAR positive cell clusters were substantially higher in all incubation conditions of both incubation series. Similar results were obtained when the NO_2^- oxidizing activity of *Nitrotoga* was analyzed in activated sludge from WWTP Deuz. However, screening of various WWTPs revealed the presence of *Nitrotoga*-like bacteria only in such WWTPs, which are operated at high temperatures, high influent NH_3 and high effluent NO_2^- concentrations (Table 15). Since the incubations were performed for 6 hours, it is conceivable that *Nitrotoga*-like bacteria are able to oxidize NO_2^- only for short time periods, whereas at longer time periods *Nitrotoga* might be outcompeted by other NOB populations. Such a switch in the population structure, which might be caused by different temperatures or substrate concentrations, has already been shown in other studies (Maixner *et al.*, 2006 and Alawi *et al.*, 2009). Since Bad Zwischenahn and Deuz are both differential integrated cycle strategy-sequencing batch reactors (DIC-SBR), one explanation for activity of *Nitrotoga*-like bacteria at high NO_2^- concentrations might be adaptions of these organisms to variable NO_2^- concentrations in such WWTPs. DIC-SBR is a biological wastewater treatment method based on the SBR process (SH+E Group, 2012). In SBR systems the treatment of activated sludge do not happen in separated tanks, but consists of timed sequences which include fill, react, settle, decant and, idle (U.S. EPA, 1992). DIC-SB reactors have two internal phases, whereby two wastewater fractions with different C/N load are generated (SH+E Group, 2012). The first load contains less carbon and more nitrogen (low C/N load) and the second load consists of a high carbon as well as low a nitrogen concentration (high C/N load) (SH+E Group, 2012). The loading of the reactor happens at different cycle times (SH+E Group, 2012), which suggests that in DIC-SBR systems microorganisms might have to deal with faster fluctuating nutrient concentrations than in conventionally operated WWTPs or in the natural environment. The activity of *Nitrotoga*-like bacteria at high temperatures might be an adaption to extreme temperature variations, since these organisms are closely related to '*Candidatus* Nitrotoga arctica'. The latter was isolated from permafrost affected soils, which are characterized by extreme temperature fluctuations of +25°C to -45°C (Wagner *et al.*, 2005; Alawi *et al.*, 2007).

Another explanation might be methodological problems during incubation leading to [^{14}C]bicarbonate uptake in the time period between bicarbonate was added and the flocs in the incubation flasks reached the appropriate temperature or NO_2^- concentration. However, the volumes of incubated sludge samples were small (5 ml), suggesting a fast diffusion of NO_2^- through the flocs. Additionally, 5 ml of sludge would have reached the appropriate incubation temperature in a very short time. In both cases it seems very unlikely that *Nitrotoga* cells would be able to incorporate enough [^{14}C]bicarbonate for MAR detection in this short time period.

The results for *Nitrospira*, the second nitrite oxidizing population in activated sludge from WWTP Deuz, were quite similar. They incorporated [^{14}C]bicarbonate at all temperatures and nitrite concentrations. However, since two lineages of *Nitrospira* exist in activated sludge from the WWTP Deuz (Müller, 2008), which are adapted to different nitrite concentrations (Maixner *et al.*, 2006) and *Nitrospira* are able to grow at a broad temperature range (Alawi *et al.*, 2009), the observed activity is not unusual.

4.2. Nitrolancetus hollandicus

4.2.1.*Nitrolancetus hollandicus nxrA* genes

Nitrite oxidoreductase (NXR) is the enzyme responsible for nitrite oxidation, and consists of a *α* subunit, *β* subunit and, *γ* subunit (Sundermeyer-Klinger *et al.*, 1984; Meincke *et al.*, 1992; Kirstein and Bock, 1993; Madigan *et al.*, 2008). The *α* subunit, which is oriented towards the cytoplasmic side of the cytoplasmic membrane in *Nitrolancetus*, contains the catalytic center and the *β* subunit channels the electrons to the γ subunit or the components of the respiratory chain (Sundermeyer-Klinger *et al.*, 1984; Meincke *et al.*, 1992; Kirstein and Bock, 1993). The γ subunit is suggested to function as a membrane anchor, which is probably involved in electron transport (Rothery *et al.*, 2008; Lücker *et al.*, 2010).

The *Nitrolancetus* genome harbors four copies of *nxrA* genes (Sorokin *et al.,* 2012). It is not uncommon that NOB harbor several paralogs of genes coding for subunits of the NXR. *Nitrobacter* and *Nitrospira* for example harbors more than one *nxrA* and *nxrB* genes (Starkenburg *et al.*, 2008; Lücker *et al.*, 2010). In *Nitrolancetus*, one *nxrA* gene likely forms a functional unit with the adjacent *nxrB* and *nxrC* genes, which is comparable to the conserved *nxr* region of *Nitrobacter* and *Nitrococcus* (Sorokin *et al.*, 2012). Three *nxrA* genes are clustered in a different genomic region containing also a two-response regulator (Sorokin *et al.*, 2012). Since regulatory systems are shown to enable bacteria to respond to environmental signals (Krell *et al.*, 2010) it might be possible that the response regulator in *Nitrolancetus* is responsible for controlling the expression of the *nxrA* genes according to environmental changes.

The sequences of all *nxrA* genes of *Nitrolancetus hollandicus* were obtained by cloning and Sanger-sequencing. Phylogenetic analysis of the NXR based on the α subunit revealed a high relationship to the NXR of *Nitrobacter* and *Nitrococcus* as well as to dissimilatory nitrate reductases (NARs) (Sorokin *et al.*, 2012) but is distinguishable from the NXR of *Nitrospira* which is related to the anammox organism '*Candidatus* Kuenenia stuttgartiensis' NXR (Lücker *et al.*, 2010). Interestingly, a NXR-like enzyme, similar to the NXR of *Nitrobacter*, *Nitrococcus* and *Nitrolancetus*, was found in the anaerobic, nitrite dependent methane oxidizer '*Candidatus* Methylomirabilis oxyfera', which belongs to the distinct phylum NC10, where the enzyme might function reversibly and reduce nitrate to nitrite (Ettwig *et al.*, 2010). Since these NXR enzymes are highly similar, it is suggested that a horizontal gene transfer established the cytoplasmically oriented NXR in ancestors of these bacteria (Sorokin *et al.*, 2012). Hence, the NXR enzymes are not restricted to NOB and occur in different bacteria involved in the N-cycle, which is an example for the exchange and functional adaption of genes during the evolution of these organisms (Klotz and Stein, 2008).

4.2.2.Hunting for *Nitrolancetus hollandicus*

Nitrolancetus hollandicus was discovered in a laboratory-scale bioreactor (Figure 14), operated at 35°C with a high NH_4^+ load of 428 mM to investigate AOB populations in partial nitrification lab-scale bioreactors (Vejmelkova *et al.*, 2012). The inoculum for the lab-scale bioreactor originated from a full-scale nitritation bioreactor (SHARON process) operated in the WWTP Rotterdam Dokhaven (Mulder *et al.*, 2001 and Vejmelkova *et al.*, 2012).

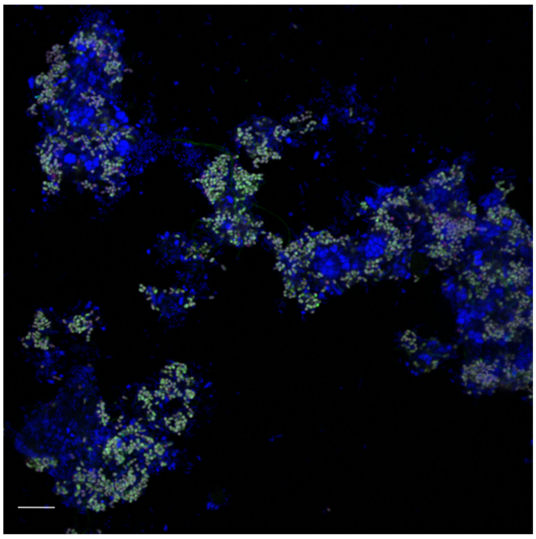


Figure 14 FISH image of *Nitrolancetus hollandicus* cells originated from the laboratory-scale bioreactor. EUB338mix (blue) targeting most *Bacteria*. Ntlc439 (red) targeting *Nitrolancetus hollandicus* cells and Cfxmix (green) targeting the phylum *Chloroflexi*. *Nitrolancetus hollandicus* cells appear in white due to simultaneously binding of probes Ntlc439, Cfxmix and EUB338mix. Other *Bacteria* appears in blue. Scalebar indicates 10 µm.

The temperature optimum of *Nitrolancetus* for growth is 40°C (Sorokin *et al.*, 2012). Additionally, this organism needs high NO_2^- levels to gain energy and NH_4^+ to assimilate nitrogen for growth (Sorokin *et al.*, 2012). Hence, the occurrence of *Nitrolancetus hollandicus* in most WWTPs is very unlikely. Therefore, for identification of the natural habitat of *Nitrolancetus hollandicus*, samples were chosen that are suggested to provide optimal conditions for this organism. For instance in compost or dung, the temperature, NO_2^- and the NH_4^+ concentration should be relatively high. And indeed, relatives of *Nitrolancetus hollandicus*, belonging to the thermophilic heterophrophic bacteria containing order *Sphaerobacterales*, have been detected in compost (Partanen *et al.*, 2010; Sorokin *et al.*, 2012). However, only close relatives of *Nitrolancetus hollandicus* have been found in an SHARON reactor in Korea (Sorokin *et al.*, 2012). In this study, screening of environmental samples revealed only a short *Nitrolancetus*-like 16S rRNA gene sequence (AB744381) originated from polluted soil

(Analysis of bacterial community during biological soil disinfestations; 2012; unpublished) and rare FISH signals, detected in an activated sludge sample from WWTP Rosenheim. The reason for the low detection rate of *Nitrolancetus* could be the occurrence in relatively small numbers in the environment because of the lack of appropriate conditions (Pedrós-Alió, 2006; Pedrós-Alió, 2012). Indeed, *Nitrolancetus hollandicus* could not be detected in the sludge used as inoculum for the laboratory-scale bioreactor but was enriched after incubation with high NH_4^+ concentrations and at high temperature (35°C) (Sorokin *et al*., 2012). At least for marine environments it is already known that only few species are very abundant and most microorganisms are rare, whereby 99% of this rare phylotypes were always rare and never become abundant (Galand *et al*., 2009). For these organisms conventional molecular methods are not sufficient to detect them and only high-throughput in-depth sequencing methods allow identification of these rare species (Pedrós-Alió, 2012). To detect *Nitrolancetus hollandicus* in its natural environment it might therefore be necessary to screen a high number of samples with high-throughput sequencing methods.

5. Summary

For a long time nitrite oxidizing bacteria (NOB) were thought to be restricted to four different bacterial phyla. However, after isolation of a cold-adapted NOB, '*Candidatus* Nitrotoga arctica' (*Betaproteobacteria*), in the Siberian permafrost and *Nitrolancetus hollandicus* (*Chloroflexi*) in a laboratory-scale bioreactor, it was realized that NOB are more diverse than expected. Since the knowledge about these organisms is limited, this study was focused on the investigation of these newly detected NOB. Interestingly, *Nitrotoga*-like bacteria were isolated from numerous activated sludge samples as well as lake and river sediments located in temperate climatic regions, which suggests wide-spread presence of *Nitrotoga* in the environment. The presence of this organism in wastewater treatment plants (WWTPs) raised the question about their abundance, function and co-localization pattern in such systems, since WWTPs are of fundamental importance for removal of sewage produced by humans. Furthermore, the knowledge about the interaction between communities, involved in removal of pollutants, is important to avoid a breakdown of these systems. Therefore, the abundance and spatial distribution pattern of *Nitrotoga*-like bacteria was analyzed in activated sludge samples from three WWTPs (Deuz, Langenzenn and, Bad Zwischenahn). Quantification revealed only low abundance (<1%) in activated sludge from WWTPs Deuz and Langenzenn but higher abundance (1.4%) in activated sludge from WWTP Bad Zwischenahn. In WWTP Deuz *Nitrotoga* co-occurs with a second nitrite oxidizing population (*Nitrospira*). FISH and digital image analysis of both groups unveiled a co-localization with ammonium oxidizing populations. Interestingly, these analyses revealed additionally a different distribution pattern for both NOB, suggesting a niche differentiation of these populations. In activated sludge from Bad Zwischenahn and Langenzenn, *Nitrotoga* is the only known nitrite oxidizing organism. The spatial distribution pattern of *Nitrotoga* in activated sludge from Bad Zwischenahn revealed a co-localization between *Nitrotoga*-like bacteria and ammonia oxidizing bacteria (AOB). Interestingly, the same analysis revealed displacement of *Nitrotoga*-like bacteria in activated sludge from WWTP Langenzenn, which might indicate the presents of an unknown NOB or AOB. Furthermore, *Nitrotoga*-like bacteria might exhibit a different lifestyle, which makes a co-localization with AOB not necessary. Since a nitrite oxidizing activity of *Nitrotoga* was so far only investigated by measuring the stoichiometrically oxidation of nitrite to nitrate during incubation of enrichments,

MAR-FISH analyses were performed to link the nitrite oxidizing activity to the phylogenetic identity of the nitrite oxidizing organisms. Interestingly, the nitrite oxidizing activity was not only shown at expected but also at high temperature (27°C) and nitrite concentrations (5 mM, 10 mM). However, *Nitrotoga*-like bacteria were only detected in such WWTPs, which are operated at low temperature (10-17°C) and NO_2^- concentrations (0.3 mM). Therefore, it might be suggested that *Nitrotoga* is able to survive higher temperature and nitrite concentration only for short incubation times but is outcompeted by other NOB during long incubation periods. Interestingly, 16S rRNA based in-depth phylogenetic analysis revealed the presence of a diversity within the genus *Nitrotoga*. Additionally, 16S rRNA gene sequences showed a very high similarity (98-99%) to each other, suggesting that *Nitrotoga* represents a very young genus. The results of this study suggests, that *Nitrotoga*-like bacteria found an ecological niche in natural and engineered habitats and might even be an important nitrite oxidizer in such WWTPs that provide optimal temperature and nitrite concentrations.

Nitrolancetus hollandicus is a thermotolerant (optimum at 40°C) NOB, which shows tolerance to high nitrite and ammonia concentrations. This organism has been detected in a laboratory-scale bioreactor, which was operated at 35°C with a high ammonia load of up to 428 mM. Additionally, a close relative of this organism has been detected in a SHARON reactor in Korea. Furthermore, phylogenetic analysis revealed the affiliation of *Nitrolancetus* to the class *Thermomicrobia* within the phylum *Chloroflexi*, in which this organism represents the first NOB. Interestingly, *Nitrolancetus hollandicus* was not detected in activated sludge originated from a full-scale nitritation bioreactor (SHARON process) operating in WWTP Rotterdam Dokhaven, which was used as inoculum for the laboratory-scale bioreactor. Therefore, for identification of natural habitats, various samples from engineered and natural habitats, which might provide optimal conditions, were screened by using different methods. However, only rare positive FISH signals in one wastewater treatment plant and a short 16S rRNA gene sequence submitted to NCBI Database by a Japanese team could be detected, suggesting a rare occurrence of this organism in the environment. Sequencing of the genomic DNA of *Nitrolancetus hollandicus* with high throughput techniques revealed the presence of four highly identically (90.3%-95.8%) *nxrA* genes. Since all *nxrA* gene sequences were incomplete one set of primer (Nlho_nxrA0038F/Nlho_nxrA3595R) was designed that targets all *nxrA* copies. Cloning and sequencing of these *nxrA* genes indicated a phylogenetic relationship to NXR of *Nitrobacter* spp. and *Nitrococcus mobilis* within the family of DMSO reductase type II enzymes. Interestingly, the NXR of these organisms is related to an NXR-like enzyme, detected in the anaerobic, nitrite dependent methane oxidizer '*Candidatus* Methylomirabilis oxyfera', suggesting that horizontal gene transfer established the NXR in ancestors of these bacteria. Furthermore, one *nxrA* gene copy was suggested to be located within the *nxr* operon which was confirmed via PCR.

6. Zusammenfassung

Lange Zeit wurde vermutet das Nitritoxidierer nur in vier verschiedenen bakteriellen Phyla zu finden sind. Aber nach der Entdeckung des an Kälte adaptierten Nitritoxidierers, ‚*Candidatus* Nitrotoga arctica' (*Betaproteobacteria*), im Permafrostboden Sibiriens und *Nitrolancetus hollandicus* (*Chloroflexi*) in einem Laborreaktor wurde erkannt, dass Nitritoxidierer diverser sind als bisher angenommen. Allerdings ist das Wissen über diese Mikroorganismen sehr limitiert, daher war das Ziel dieser Studie diese Wissenslücke zu verkleinern. Interessanterweise wurden bei einer Untersuchung verschiedenster Umweltproben, *Nitrotoga*-ähnliche Bakterien auch in zahlreichen Kläranlagen, See beziehungsweise Fluss Sedimenten entdeckt, was eine weite Verbreitung dieser Organismen in der Umwelt bedeuten kann. Diese Tatsache wirft auch die Frage über die Abundanz und die Funktion von *Nitrotoga* in der Umwelt im Allgemeinen und in Kläranlagen im Speziellen auf. Besonders in Kläranlagen ist es wichtig die ablaufenden Prozesse und die Interaktionen der in der Reinigung des Abwassers involvierten Organismen zu verstehen um ein zusammenbrechen des Systems zu verhindern. Es wurden die Abundanz und die Interaktion von *Nitrotoga* mit Ammoniak oxidierenden Organismen im Schlamm aus den Kläranlagen Bad Zwischenahn, Deuz und Langenzenn untersucht. Des Weiteren wurde die Funktion dieser Organismen im Schlamm der Kläranlagen Deuz und Bad Zwischenahn nachgewiesen. In zwei dieser Kläranlagen (Langenzenn und Bad Zwischenahn) stellt *Nitrotoga* den einzigen Nitrit oxidierenden Mikroorganismus dar. FISH und digitale Bildanalysen deuteten auf eine Kolokalisation zwischen *Nitrotoga*-ähnlichen Bakterien und Ammoniak oxidierende Mikroorganismen in den Belebtschlämmen der Kläranlagen Bad Zwischenahn und Deuz hin. Im Belebtschlamm der Kläranlage Langenzenn konnte sogar eine Verdrängung von *Nitrotoga* festgestellt werden, was zur Annahme führte, dass sich in diesem Schlamm noch unbekannte Nitrit oxidierende Bakterien oder sogar unbekannte Ammoniak oxidierende Organismen befinden. Des Weiteren könnten *Nitrotoga*-ähnliche Bakterien einen anderen Lebensstil aufweisen der sie von Ammoniak oxidierenden Organismen unabhängig macht. Die Abundanzen von *Nitrotoga*-ähnlichen Bakterien waren in den Belebtschlämmen der Kläranlagen Deuz und Langenzenn sehr gering (<1%). Allerdings koexistiert im Belebtschlamm der Kläranlage Deuz Nitrotoga mit zwei Gruppen von Nitrospira, wobei die Kolokalisationsanalysen unterschiedliche Verteilungsmuster für beide Organismen ergaben, was auf eine Nischendifferenzierung hindeutet. Im Belebtschlamm von Bad Zwischenahn ergab die Quantifizierung von *Nitrotoga*-ähnlichen Organismen eine Abundanz von 1.4% die allerdings hoch genug sein kann, um für eine ausreichende Entfernung des Nitrits zu sorgen. MAR-FISH Analysen wurden in den Belebtschlämmen aus Bad Zwischenahn und Deuz bei verschiedenen Temperaturen und Nitrit Konzentrationen durchgeführt. Ziel war es, eine Nitrit oxidierende Aktivität dieser Organismen in den Schlammproben beider Kläranlagen zu bestätigen, da die Fähigkeit des Wachstums mit Nitrit und Kohlenstoff als ausschließlicher Energie- und Kohlenstoffquelle bisher nur indirekt an Anreicherungskulturen beobachtet wurde. Interessanterweise wurde eine Einlagerung von [^{14}C]Bikarbonat nicht nur bei den erwarteten Temperaturen (4°C, 10°C, 14°C und 20°C) und Nitrit Konzentrationen (0,1 mM, 0,5 mM und 1 mM), sondern auch bei höheren Temperaturen (27°C) und Nitrit Konzentrationen (5 mM und 10 mM) beobachtet. Dieses Ergebnis deutet darauf hin, dass *Nitrotoga*-ähnliche Bakterien dazu fähig sind, kurze Zeit auch bei höheren Temperaturen und Nitrit Konzentrationen aktiv zu sein. Interessanterweise, ergaben auf 16S rRNS basierte phylogenetische Analysen Hinweise auf eine Diversität innerhalb des Genus. Zusätzlich zeigten die 16S rRNS Gen Sequenzen eine hohe Ähnlichkeit (98-99%) zueinander, was darauf hindeutet, dass es sich bei *Nitrotoga* noch um ein junges Genus handelt. Die Ergebnisse dieser Arbeit weisen darauf hin, dass *Nitrotoga*-ähnliche Bakterien eine ökologische Nische in natürlichen und künstlichen Habitaten gefunden haben und sogar wichtige Nitritoxidierer in jenen Kläranlagen sein könnten, die für diesen Organismus optimale Temperaturen und Nitritkonzentrationen bieten.

Nitrolancetus hollandicus ist ein thermotoleranter Nitritoxidierer, der eine Toleranz gegenüber hohen Nitrit (bis zu 75 mM) und Ammonium Konzentrationen (über 200 mM) aufweist. Dieser Organismus wurde in einem Laborreaktor entdeckt, der bei hohen Temperaturen (35°C) betrieben wurde und hohe Konzentration an Ammonium (428 mM) aufwies. Ein wenig später wurden nahe Verwandte dieses Organismus in einem SHARON Reaktor in Korea entdeckt. Interessanterweise offenbarten phylogenetische Analysen eine Zugehörigkeit dieses Organismus zur Klasse *Thermomicrobia* innerhalb des Phylums *Chloroflexi*, in welchen *Nitrolancetus* den ersten Nitritoxidierer darstellt. Zur Identifizierung des natürlichen Habitats dieses Organismus wurden verschiedene Proben untersucht die optimale Bedingungen (5-20 mM Nitrit und 4-200 mM Ammonium) für *Nitrolancetus* darstellen könnten. Mittels

Verwendung unterschiedlicher Methoden wurden allerdings nur einzelne FISH Signale im Belebtschlamm einer Kläranlage entdeckt. Zusätzlich wurde mittels BLAST (NCBI) eine kurze 16S rRNS Gen Sequenz, die eine hohe Ähnlichkeit zur *Nitrolancetus* 16S rRNS Gen Sequenz besitzt, in der NCBI Datenbank gefunden. Obwohl in zahlreichen Umweltproben nach *Nitrolancetus*-ähnlichen Bakterien gesucht wurde, wurden keine weiteren Hinweise auf das Habitat dieses Organismus gefunden, was auf ein seltenes Vorkommen von *Nitrolancetus* in der Umwelt hindeutet. Die Sequenzierung der genomischen DNS von *Nitrolancetus hollandicus* mittels Hochdurchsatz-Sequenzierungstechnologien ergaben die Präsenz von vier hoch ähnlichen (90,3-95,8%) *nxrA* Genen. Da alle vier *nxrA* Gene noch nicht vollständig sequenziert waren, wurden spezifische Primer entwickelt, die an diese Gene binden sollten. Klonierung und Sequenzierung der *nxrA* Gene von *Nitrolancetus hollandicus* offenbarten eine phylogenetische Verwandtschaft zu NXR von *Nitrobacter spp.* und *Nitrococcus mobilis* innerhalb der DMSO Reduktase Typ II Enzym Familie. Des Weiteren gab es Hinweise, dass einer dieser Kopien innerhalb des *nxr* Operons lokalisiert ist, was mittels einer spezifischen PCR überprüft und bewiesen wurde.

7. Supplementary material

7.1. Supplementary figures

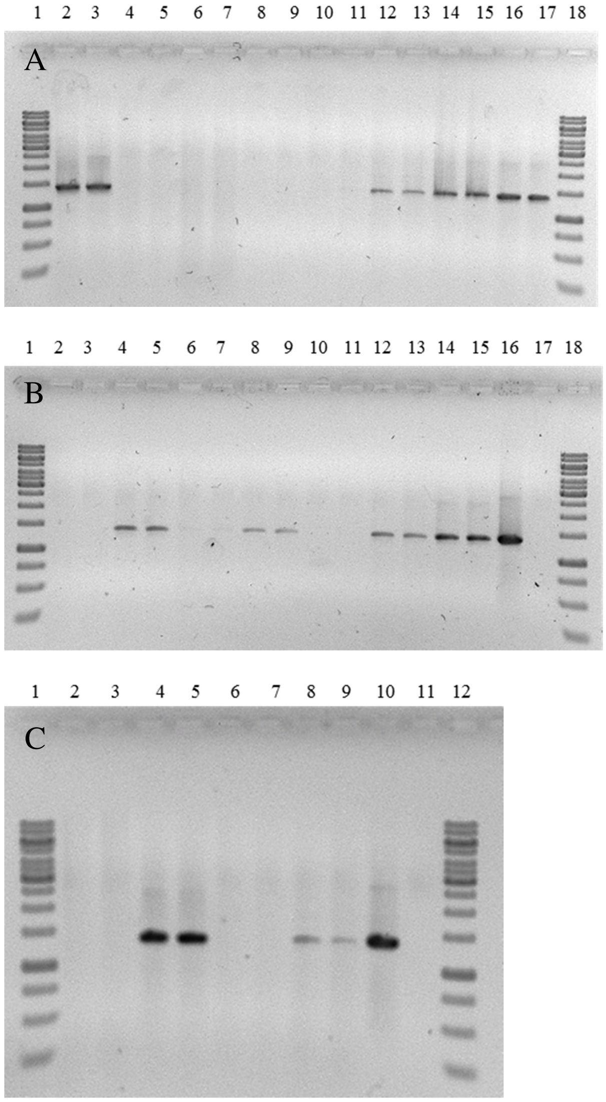
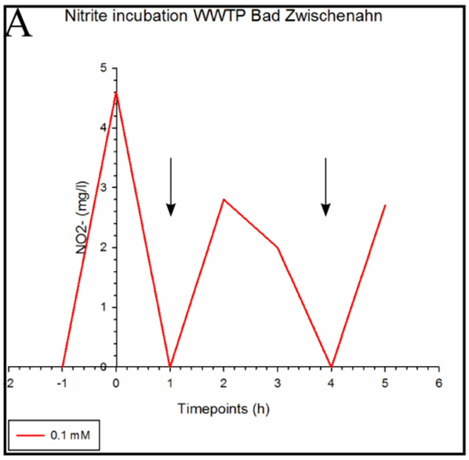


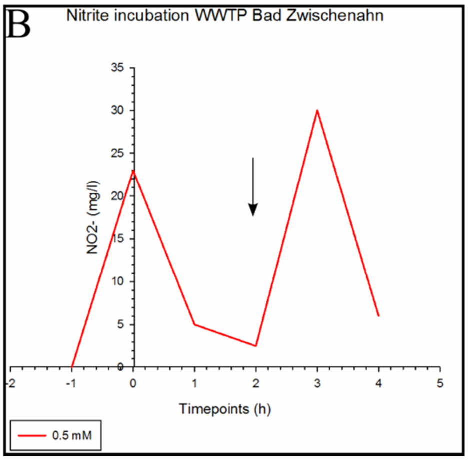
Figure S1 Gel electrophoresis of *Nitrotoga*-like 16S rRNA genes from different WWTPs; Used primer for amplification: Ntoga124F/Ntoga1462bR; Amplification of each template was carried out in duplicates; positive control: Plasmid Ntoga 66-63-1

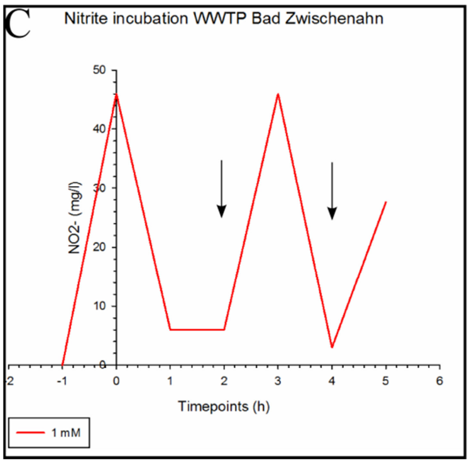
A: Lanes 1,18: 1 kb DNA ladder (Fermentas); Lanes 2,3: WWTP Spenge; Lanes 4,5: WWTP Plattling; Lanes 6,7: WWTP Ampfing; Lanes 8,9: WWTP Kraftisried; Lanes 10,11: WWTP Radeburg; Lanes 12,13: WWTP Deuz; Lanes 14,15: WWTP Seefeld; Lanes 16,17: WWTP Bruchmühlen;

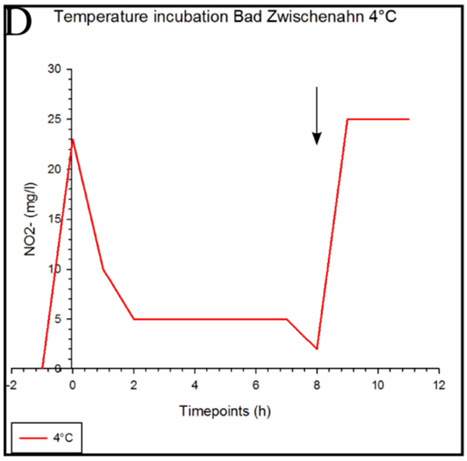
B: Lanes 1,18: 1 kb DNA ladder (Fermentas); Lanes 2,3: WWTP Oberding; Lanes 4,5: WWTP Altmannstein; Lanes 6,7: WWTP Ingolstadt; Lanes 8.9: WWTP Langenzenn; Lanes 10,11: WWTP Lyss GZM; Lanes 12,13: WWTP Lyss ARA; Lanes 14,15: WWTP Waldsassen; Lane 16: positive control; Lane 17 negative control;

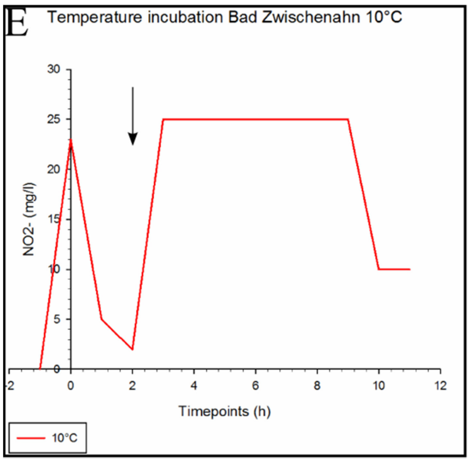
C: Lanes 1,12: 1 kb DNA ladder (Fermentas); Lanes 2,3: WWTP Hettstett; Lanes 4,5: WWTP Bad Zwischenahn; Lanes 6,7: WWTP Huntlosen; Lanes 8,9: WWTP Weissthal; Lane 10: positive control; Lane 12: negative control;

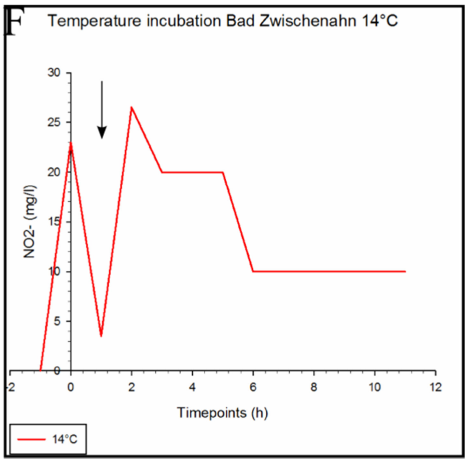

A
Nitrite incubation WWTP Bad Zwischenahn
NO2- (mg/l)
0
1
2
3
4
5
2
-1
0
1
2
3
4
5
6
Timepoints (h)
0.1 mM

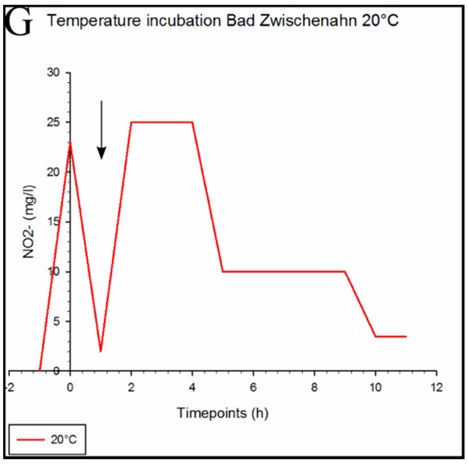

B
Nitrite incubation WWTP Bad Zwischenahn
NO2- (mg/l)
0
5
10
15
20
25
30
35
-2
-1
0
1
2
3
4
5
Timepoints (h)
0.5 mM

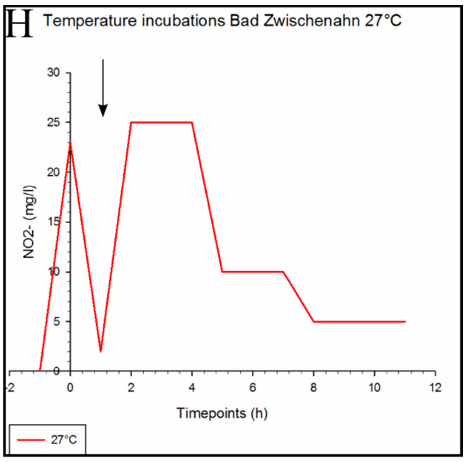

C
Nitrite incubation WWTP Bad Zwischenahn
NO2- (mg/l)
0
10
20
30
40
50
2
-1
0
1
2
3
4
5
6
Timepoints (h)
1 mM


D
Temperature incubation Bad Zwischenahn 4°C
NO2- (mg/l)
0
5
10
15
20
25
30
-2
0
2
4
6
8
10
12
Timepoints (h)
4°C


E
Temperature incubation Bad Zwischenahn 10°C
NO2- (mg/l)
0
5
10
15
20
25
30
2
0
2
4
6
8
10
12
Timepoints (h)
10°C


F
Temperature incubation Bad Zwischenahn 14°C
NO2- (mg/l)
0
5
10
15
20
25
30
-2
0
2
4
6
8
10
12
Timepoints (h)
14°C


G
Temperature incubation Bad Zwischenahn 20°C
NO2- (mg/l)
0
5
10
15
20
25
30
2
0
2
4
6
8
10
12
Timepoints (h)
20°C


H
Temperature incubations Bad Zwischenahn 27°C
NO2- (mg/l)
0
5
10
15
20
25
30
-2
0
2
4
6
8
10
12
Timepoints (h)
27°C

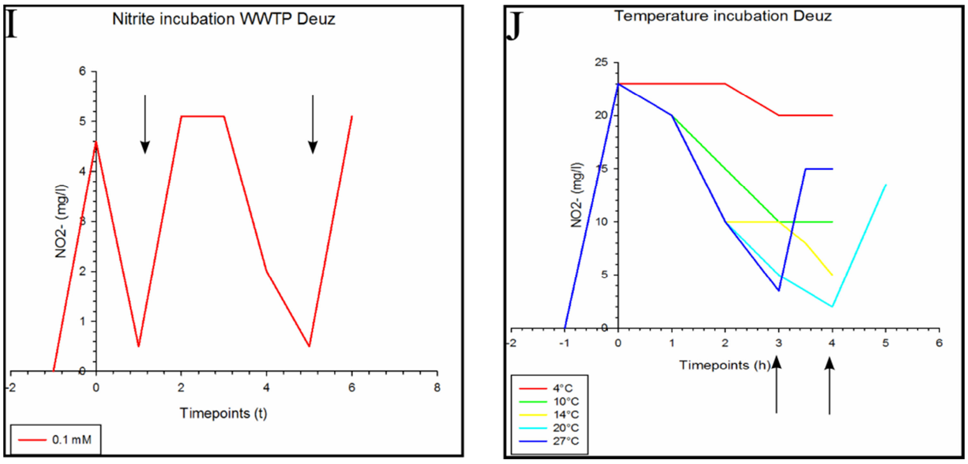


Figure S2 Nitrite feeding schemes. Shown is the nitrite concentration at certain timepoints. Addition of nitrite is indicated by black arrows. Measuring of nitrite was done with nitrite test strips.

A-C: Nitrite feeding scheme for nitrite incubations of activated sludge from WWTP Bad Zwischenahn; A nitrite feeding scheme for incubation with 0.1 mM nitrite; B nitrite feeding scheme for incubation with 0.5 mM nitrite; C nitrite feeding scheme for incubation with 1 mM nitrite; no consumption of nitrite was measured during incubation with 5 mM and 10 mM nitrite.

D-H: Nitrite feeding scheme for temperature incubations of activated sludge from WWTP Bad Zwischenahn; D nitrite feeding scheme for incubation at 4°C; E nitrite feeding scheme for incubation at 10°C; F nitrite feeding scheme for incubation at 14°C; G nitrite feeding scheme for incubation at 20°C; H nitrite feeding scheme for incubation at 27°C.

I-J: Nitrite feeding schemes for MAR-FISH incubations with activated sludge from WWTP Deuz; I nitrite feeding scheme for incubation with 0.1 mM; no nitrite feeding scheme is shown for 0.5 mM and 1 mM, because no replenishing of nitrite was necessary; no consumption of nitrite was measured during incubation with 5 mM and 10 mM nitrite; J Nitrite feeding schemes for temperature incubation series; red line incubation at 4°C; green line incubation at 10°C; yellow line incubation at 14°C; turquoise line incubation at 20°C; dark blue line incubation at 27°C.

Figure S3 Schematic illustration shows the binding of the Ntoga122F primer to a non-target sequence (*Dechlorosoma suillum*). The additional base in the non-target sequence leads to formation of a loop around the missing base (McIlroy *et al.*, 2011).

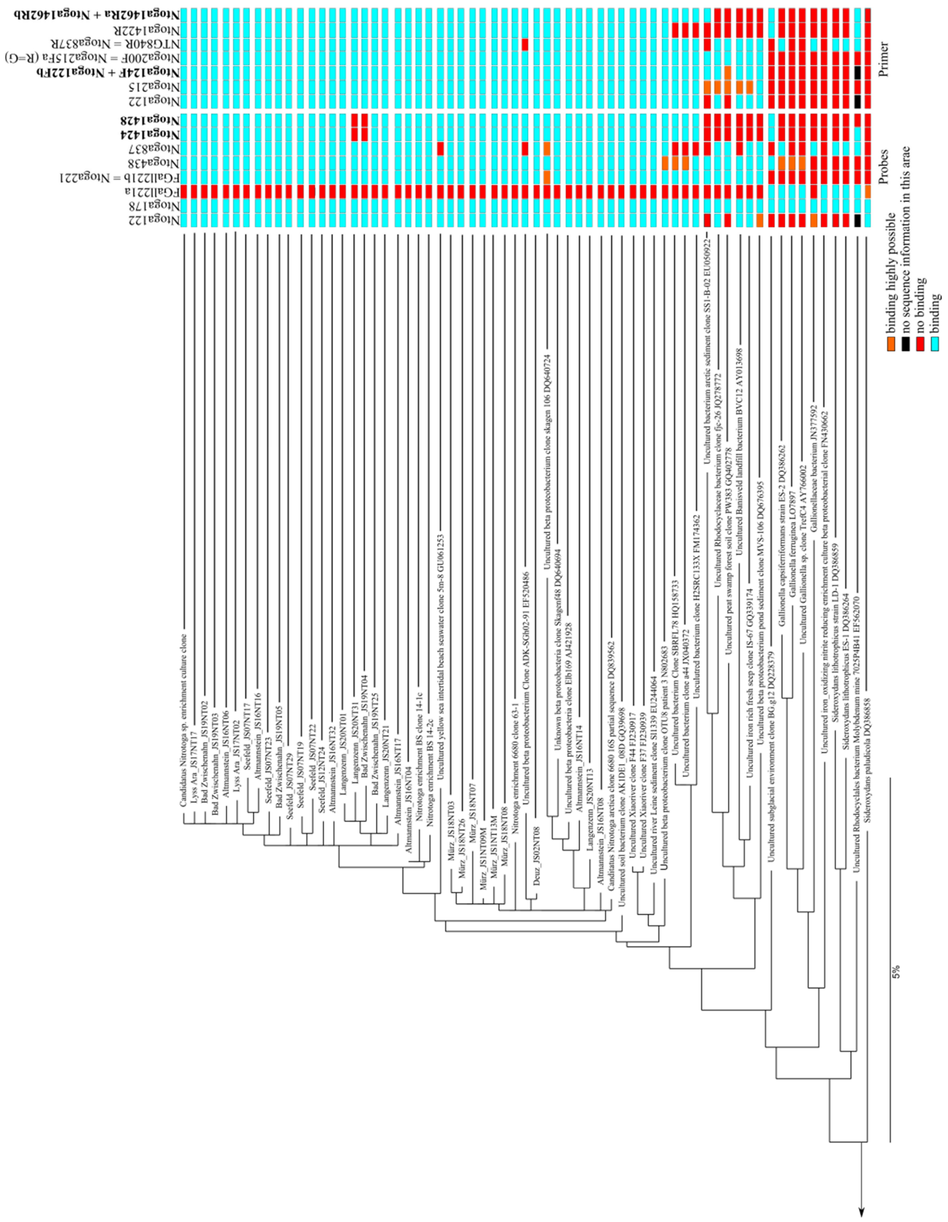


Figure S4 Coverage scheme of probes and primers based on in silico sequence comparison. Maximum likelihood tree containing some of the sequenced *Nitrotoga*-like 16S rRNA gene sequences obtained in this study and sequences of the genera *Nitrotoga*, *Gallionella* and, *Sideroxydans*, which are publicly available on the NCBI Database. New primers and probes, designed in this study, are indicated in bold.

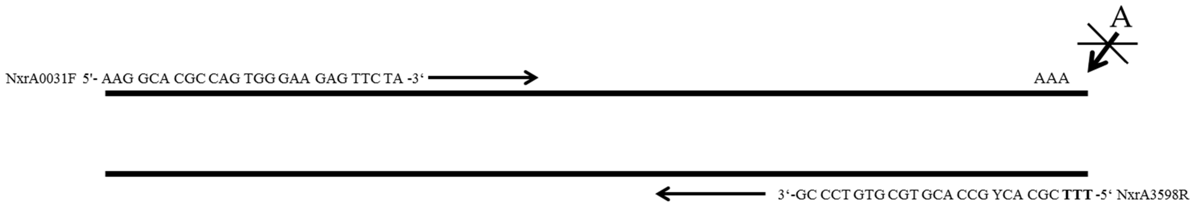


Figure S5 Forward and reverse primer sets used for *nxrA* amplification of *Nitrolancetus hollandicus*. The reverse primer (containing a 5’T) and the forward primer bind to their template and start to amplify this part of sequence. This leads to amplification of sequences that start with 5’A and ends with 3’A. The Taq polymerase is inefficient in adding a non-template A to an 3’A, which leads to problems during the cloning reaction (Brownstein *et al*., 1996). To use the primer for cloning, it was necessary to design new primer so that they contain 5’C or G instead of T.

7.2. Supplementary tables

Table S1 Influences of DNA extraction methods and used primers on detection of *Nitrotoga*-like bacteria in activated sludge from different WWTPs

WWTP	Reactor type	Type of treated sewage	Detection of Nitrotoga						*Nitrospira* sublineage	Temperature [°C]	Influent [mg l^{-1}]	Effluent [mg l^{-1}]			Sampling date 2007
			PCR[1]	**FISH[1]**	**PCR[2]**	**FISH[2]**	**PCR[3]**	**FISH[3]**			NH_4^+	NH_4^+	NO_2^-	NO_3^-	
Altmannstein	SBR	M	**-**	**+**	**+**	**nd**	**+**	**nd**	I + II	7	54.7	9.18	0.48	0.72	March 24
Ampfing	SBR	M + slaughter and dairy waste	**-**	**-**	**-**	**nd**	**-**	**nd**	II	13	nd	0.1	0.04	3.25	March 26
Bad Zwischenahn	DIC-SBR	M	**+**	**+**	**+**	**+**	**+**	**+**	-	16	60	0.25	0.15	6.5	May 23
Bruchmühlen	DIC-SBR	M	**+**	**+**	**+ (weak)**	**nd**	**+**	**nd**	I	15	36	0.53	0.09	4.53	May 22
Deuz	DIC-SBR	M	**+**	**+**	**-**	**+**	**+**	**+**	I + II	13	nd	0.33	0.09	3.46	May 21
Hettstedt	DIC-SBR	M + external activated sludge	**+**	**-**	**-**	**nd**	**-**	**nd**	I + II	15	56	12.35	0.24	3	May 24
Huntlosen	DIC-SBR	M	**-**	**-**	**-**	**nd**	**-**	**nd**	I + II	17	68	0.13	0.03	2.2	May 23
Ingolstadt	SBR	activated sludge drainage	**+**	**-**	**-**	**nd**	**-**	**nd**	I + II	27	856	0.3	< 0.1	20.4	May 09
Kraftisried	single-stage activated sludge basin	A	**+**	**-**	**+**	**nd**	**-**	**nd**	I + II	7	397.5	35.3	6.2	17.4	January 29
Langenzenn	SBR	M	**+**	**+**	**-**	**+**	**+**	**+**	-	9	21.25	7.96	0.42	3.1	March 14
Lyss (ARA)	fixed bed reactor	M	**-**	**-**	**-**	**nd**	**+**	**nd**	I	12	20	1	0.1	18	January 29
Lyss (GZM)	membrane filtration plant	A	**+**	**-**	**-**	**nd**	**-**	**nd**	I + II	30	700	< 1	< 0.5	14	January 29
Oberding	fixed bed reactor	A	**+**	**-**	**-**	**nd**	**-**	**nd**	I + II	26	450	< 1	< 0.5	4	January 29
Plattling	two-stage activated sludge basin	A	**nd**	**-**	**-**	**nd**	**-**	**nd**	I + II	30	750	1	< 0.5	3	January 29
Radeburg	DIC-SBR	M	**nd**	**+**	**-**	**-**	**+[4]**	**-**	I	14	nd	0	0.05	3.3	May 24
Rosenheim	SBR	M	**-**	**-**	**nd**	**nd**	**nd**	**nd**	I + II	36	970	nd	nd	nd	May 30
Seefeld	SBR	M	**+**	**-**	**+**	**nd**	**+**	**nd**	I	nd	8.32	1.59	nd	1.73	March 28
Spenge	DIC-SBR	M	**+**	**+**	**+**	**nd**	**+**	**nd**	I	14	24	< 0.2	0.05	1.38	May 22
Waldsassen	SBR	M + I	**+**	**+**	**+**	**nd**	**+**	**nd**	I	9	18.5	< 0.1	nd	3.45	March 27
Weisstal	DIC-SBR	M	**-**	**-**	**-**	**nd**	**+**	**nd**	I + II	nd	nd	0	0.02	4.4	May 21

Detection of *Nitrospira* lineages I and II are also provided. Additional information about the type of sewage, reactor type, temperature and influent ammonia as well as effluent ammonia, nitrite and nitrate concentration are shown.

1 isolation via Powersoil® DNA isolation Kit (MO BIO Laboratories); Primer sets: Ntoga122F and Ntoga1422R; BSA concentration 0.1 µg μl^{-1}(Müller, 2008);

2 Phenol/Chloroform DNA extraction; Primer sets: Ntoga122F/Ntoga1422R; BSA concentration: 0.1 µg μl^{-1};

3 Phenol/Chloroform DNA extraction; Primer sets: Ntoga124F and Ntoga1462bR; BSA concentration: 0.5 µg μl^{-1};

4 Positive after new DNA isolation;

M municipal; A animal rendering; I industrial; modified from Lücker (2010)

Table S2 Used $MgCl_2$ concentrations and there effect on amplification

$MgCl_2$ concentration [mM]	Effect on amplification
1	–
1.5	–
2	–
2.5	–
3	o
3.5	o
4	o

- negative effect, o no effect, + positive effect

Table S3 Tested PCR additives

PCR-additive	Concentration	Effect on amplification
DMSO	5%	+
TMAC	20 mM	–
TMAC	50 mM	–
TMAC DMSO	50 mM 5%	–
BSA	0.5 µg µl^{-1}	–
Glycerol BSA	5% 0.5 µg µl^{-1}	–
Glycerol DMSO	5% 5%	–
Glycerol BSA DMSO	5% 0.5 µg µl^{-1} 5%	–
TMAC BSA Glycerol	50 mM 0.5 µg µl^{-1} 5%	–

- negative effect, o no effect, + positive effect

7.3. Supplementary text

S1 Amplification and cloning of *Nitrotoga*-like bacteria 16S rRNA genes

Because the screening of the activated sludge sample from WWTP Altmannstein (Lücker, 2010) revealed FISH signals with probes specific for *Nitrotoga* but showed negative PCR results when using primer sets specific for *Nitrotoga*-like 16S rRNA genes (Ntoga122F/Ntoga1422R), it was assumed that the used primer set excludes certain *Nitrotoga*-like species. Therefore, the specific forward primer (Ntoga122F) was used in combination with the general reverse primer (1492R). However, this led to amplification of 16S rRNA genes belonging to the family *Comamonadaceae* and *Rhodocyclaceae*. Therefore, specific primer sets (Ntoga122F/Ntoga1422R) were used again. Interestingly, cloning and sequencing of the amplicon obtained with Ntoga122F and Ntoga1422R primers revealed still the presence of 16S rRNA gene sequences belonging to members of the family *Rhodocyclaceae*, whereas members of *Comamonadaceae* were not targeted anymore. Attempts for optimizing PCR conditions by increasing the annealing temperature or testing different magnesium concentrations did not improve the specifity of these primers (data not shown). Analyzing of both primers with ARB (Ludwig *et al.*, 2004) unveiled an inefficient mismatch at the 5'end of the reverse primer and a deletion mismatch at the 3' end of the forward primer. Interestingly, the mismatch at the 3'end of the forward primer was inefficient, since the polymerase was able to elongate the forward primer. A similar problem was already described for FISH probes by McIlroy and colleagues (2011). They described the binding of probes, containing a singly deletion mismatch to their non-targets, whereby the additional base in the non-target sequence leads to formation of a loop around the missing base (McIlroy *et al.*, 2011). A similar process might lead to binding of the forward primer to its non-target (Supplementary Figure S3). Since the mismatch of both primers seems to be inefficient, an amplification of non-target sequences might be possible. Consequently, new primer sets were designed by using the software ARB (Ludwig *et al.*, 2004). The coverage of the target group of the new primer pair is similar to the old primers (Supplementary Figure S4).

S2 Inconsistency of PCR amplification

DNA extraction from WWTP sludge and liquid manure samples was first performed with the PowerSoil® DNA isolation Kit (MO BIO), but yielded only low amounts of DNA. Consequently, a phenol:Chisam DNA-Isolation was performed, which increased the amount of DNA but also of co-extracted inhibitory substances, leading to false negative results because of inconsistent amplification of *Nitrotoga*-like 16S rRNA genes. Activated sludge and liquid manure contain a lot of PCR inhibitors, which are known to inhibit amplification (Opel *et al.*, 2010). Therefore, different PCR additives were tested to get reproducible PCR results (data not shown). The most common inhibitors which are known in environmental samples are huminic acids. Inhibition caused by huminic acids can be reduced by adding bovine serum albumin (BSA) to

PCR reactions (Schrader *et al.*, 2012). The recommended final concentration of BSA is 0.1 to 0.8 µg μl^{-1}, whereas the used BSA concentration was 0.1 µg μl^{-1}. To find the appropriate BSA concentration a BSA gradient was performed starting at 0.2 µg μl^{-1} and ending at 0.8 µg μl^{-1} final concentrations, which revealed an optimal BSA concentration of 0.5 µg μl^{-1}, resulting in a consistent amplification of *Nitrotoga*-like 16S rRNA genes (Figure S1).

S3 Amplification of *Nitrolancetus nxrA* genes

To obtain the sequences of all four *nxrA* genes a PCR was performed using the Nlho_NxrA0031F and Nlho_NxrA3598R primer set. Different polymerases and PCR additives were tested due to inconsistency in amplification of the *nxrA* genes (Supplementary tables S2 and S3). Best results were obtained with 5% DMSO (final concentration). Additionally, only polymerases with proof-reading activity were used to obtain high quality sequences. Both tested polymerases, Phusion® High-fidelity DNA polymerase (New England Biolabs®) and High Fidelity Taq polymerase (Fermentas), showed similar results. Therefore, for further amplification of the *nxrA* genes only the High fidelity Taq polymerase (Fermentas) was used, since this polymerase adds a poly-A tail to each sequence, making the cloning procedure easier.

Additionally, the amplification of the *nxrA* gene yielded also wrong sized amplicons (2000 bp, 500 bp). Therefore, different $MgCl_2$ concentrations were tested to avoid unspecific binding of the primer pair. However, tested $MgCl_2$ concentrations did not avoid mispriming, but seemed even to inhibit PCR amplification when using concentrations lower than 3 mM. Interestingly, analyzing of the used primer set with ARB (Ludwig *et al.*, 2004), revealed additional binding positions within the *nxrA* sequence. This could be verified for the 2000 bp amplicon by cloning and sequencing.

S4 Cloning of *Nitrolancetus nxrA* genes

Amplicons obtained with the Nlho_NxrA0031F and Nlho_NxrA3598R primers were cloned into pCR® XL-TOPO® 3.5 kb -Vector and subsequently transformed into DH5α^{TM}-T1 chemical competent cells according to the TOPO® XL PCR cloning protocol (Invitrogen 2012). But due to low cloning efficiency, no sequences of *nxrA* genes were obtained. Similar problems were already described by Brownstein and colleagues (1996) when they used primer containing an 5’ end T, leading to formation of sequences ending with a 3’ end A (Supplementary Figure S 5), whereby the Taq polymerase is inefficient in adding a non-template A to an 3’end A (Brownstein *et al.*, 1996). Indeed, the reverse primer Nlho_NxrA3585R used for amplification in this study contained a 5’ end T. Consequently, the reverse primer was shifted a few bases to overcome this problem.

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10. Curriculum vitae

Curriculum Vitae - Mag. Jasmin Schwarz - PART I	
Personal details	
Name:	Schwarz
Given Name:	Jasmin Stefanie
Academic degree:	Mag. Rer. Nat.
Nationality:	Austrian
Languages:	German (native speaker) English (fluent)
Education	
1982 - 1990:	Elementary and Secundary school
1990 - 1991:	HTBLVA-Mödling (Nachrichtentechnik)
1991 - 1996:	HTBLVA-Wien V (Textilbetriebstechnik und EDV)
14. July 1996:	A-level-degree
University education:	
1997 - 2004:	Ernährungswissenschaften (nutrition sciences) Degree thesis under tutorship of Prof. Jürgen König finished with Magistra (masters degree)
since 2008:	Molecular microbiology and immunology (Uni Wien) (A066/830)
Work experiences	
2008 - 2011:	Kompetenzzentrum für Gesundheitsförderung und Diätethik
February 2007 - September 2008:	Embers Consulting - Call center agent
30.07.2001 - 31. 08. 2001:	Rehabilitationszentrum Alland
19.08. 2000 - 13. 09. 2000:	Rehabilitationszentrum Grossgmain
02.08.1999 - 27. 08. 1999:	Rehabilitationszentrum Hochegg
31.07.1995 - 02.09.1995:	Fa. Merkur
02.08.1993 - 29.08.1993:	Fa. Hyuck - Austria GmbH
Laboratory experience:	
Molekulare Mikrobiologie (Prof. Busse)	
Molekularbiologische Laborarbeiten (Prof. Witte)	
Fluoreszenz in situ Hybridisierung (Prof. Wagner, Prof. Horn, Prof. Daims)	
Wasser-, Lebensmittel- und Betriebshygiene (Prof. Manafi)	
EDV und Biometrie (Prof. Weilguni)	
Histologie und Zytologie (Prof. Viehberger)	
Biochemisches Praktikum (Prof. Laggner)	
Chemische Übungen (Prof. Vierhapper)	
Mikroskopie pflanzlicher Lebensmittel (Prof. Till)	
Lebensmittelchemische Übungen (Prof. Lorbeer)	
Ernährungsphysiologie (Prof. König)	
since 2011: laboratory work at the department of microbial ecology (Prof. Wagner, Prof. Daims)	

Curriculum Vitae - Mag. Jasmin Schwarz - PART II

Skills

Computerknowledge
Excell
Words
Access

Additional courses:

English-courses (University of vienna)
First aid - course (Rotes Kreuz)
Rhetoric-course (Volkshochschule Wr. Neustadt)

Collaboration

Contribution on following books written by Sven-David Müller:
"Kochbuch für Fitness-Sportler und Bodybilder"
"Die 50 gesündesten und die 50 gefährlichsten Lebensmittel
"Süßstoffe"

Publications

Lopez-Vazquez C. M., Kubare M., Saroj D.P., Chikamba C., **Schwarz J.**, Daims H. and Brdjanovic D. (2013).
Thermophilic biological nitrogen removal in industrial wastewater treatment. Appl. Microbiol Biotechnol.
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Acknowledged contributions to following scientific works

Sorokin DY, Lücker S, Vejmelkova D, Kostrikina NA, Kleerebezem R, Rijpstra WI, Damsté JS, Le Paslier D,
Muyzer G, Wagner M, van Loosdrecht MC, Daims H. (2012)
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Front Microbiol. 4:27. doi: 10.3389/fmicb.2013.00027.

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