A novel marine nitrite-oxidizing *Nitrospira* species from Dutch coastal North Sea water

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**INTRODUCTION**

The ocean is the largest reservoir of fixed nitrogen on Earth containing about five times more fixed nitrogen than terrestrial systems (Gruber, 2008) which renders marine systems of major importance to global nitrogen cycling. Nitrogen, as the bioavailable forms of ammonium and nitrate, is one of the key nutrients in marine waters and may limit primary production especially in coastal systems (Downing, 1997; Wolster, 1998; Zeh and Kudela, 2011). Most of the fixed organic nitrogen in the ocean is converted to nitrate by remineralization consisting of ammonification and nitrification (Gruber, 2008). In the two-step process of nitrification, ammonia is oxidized first to nitrite by aerobic ammonia-oxidizing microorganisms and then to nitrate by aerobic nitrite-oxidizing microorganisms.

Marine microorganisms are important for the global nitrogen cycle, but marine nitrifiers, especially aerobic nitrite oxidizers, remain largely unexplored. To increase the number of cultured representatives of marine nitrite-oxidizing bacteria (NOB), a bioreactor cultivation approach was adopted to first enrich nitrifiers and ultimately nitrite oxidizers from Dutch coastal North Sea water. With solely ammonia as the substrate an active nitrifying community consisting of novel marine *Nitrosomonas* aerobic ammonia oxidizers (ammonia-oxidizing bacteria) and *Nitrospira* and *Nitrosospira* NOB was obtained which converted a maximum of 2 mmol of ammonia per liter per day. Switching the feed of the culture to nitrite as a sole substrate resulted in a *Nitrospira* NOB dominated community (approximately 80% of the total microbial community based on fluorescence in situ hybridization and metagenomic data) converting a maximum of 3 mmol of nitrite per liter per day. Phylogenetic analyses based on the 16S rRNA gene indicated that the *Nitrospira* enriched from the North Sea is a novel *Nitrospira* species with *Nitrospira marina* as the next taxonomically described relative (94% 16S rRNA sequence identity). Transmission electron microscopy analysis revealed a cell plan typical for *Nitrospira* species. The cytoplasm contained electron light particles that might represent glycogen storage. A large periplasmic space was present which was filled with electron dense particles. *Nitrospira*-targeted polymerase chain reaction analyses demonstrated the presence of the enriched *Nitrospira* species in a time series of North Sea genomic DNA samples. The availability of this new *Nitrospira* species enrichment culture facilitates further in-depth studies such as determination of physiological constraints, and comparison to other NOB species.

**Keywords:** marine nitrification, enrichment, *Nitrosomonas*, *Nitrospira*, fluorescence in situ hybridization, transmission electron microscopy, 16S rRNA

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Nitrospina species were isolated from a surface water sample of the Gulf of Maine (Watson et al., 1986) as well as from marine recirculation aquaculture system biofilters (Keuter et al., 2011; Brown et al., 2013). In addition they have been described as inhabitants of marine sponges (Hoffmann et al., 2009; Off et al., 2010). Nitrosopina species have been detected in both coastal and open ocean habitats (Suzuki et al., 2004; Delong et al., 2010; Berman et al., 2010) based on 16S rRNA gene sequences. Moreover, co-variation of archaeal amoA and 16S rRNA genes with Nitrospina-like 16S rRNA genes has been observed which suggests that Nitrosopina NOB may be natural nitrite-oxidizing partners of marine NOA (Miner et al., 2007; Santoro et al., 2010). Fluorescence in situ hybridization (FISH) analyses using probes targeting all nitrite-oxidizing genera known at that time by Füssel et al. (2011) on Namibian OMZ samples demonstrated the presence of only Nitrosopina and Nitrooccus NOB in equal abundance.

The elucidation of the ecophysiology of marine nitrite oxidizers in part is complicated by the difficulties in combining molecular data (e.g., presence and abundance of particular genes or species) with cultivation-derived parameters (e.g., proof of physiological capabilities, affinities, growth rates, salt tolerance). Increasing the availability of cultured strains and species, with cultivation-derived parameters (e.g., proof of physiological capabilities, affinities, growth rates, salt tolerance) and physiological constraints of different strains and species, for instance, enables making informed guesses about which particular strain or species inhabits a certain habitat. To increase the number of cultivated marine nitrite oxidizers, a bioreactor set-up was used in the present study to first enrich a marine assemblage of aerobic ammonia oxidizers and nitrite oxidizers and ultimately solely the nitrite oxidizers from North Sea coastal water. The microbial community composition was evaluated by FISH analyses and the phylogenetic position of the enriched aerobic ammonia oxidizers and nitrite oxidizers determined by 16S rRNA gene sequence [polymerase chain reaction (PCR) and metagenome data] based analyses. The cell plan of the enriched nitrite-oxidizing Nitrospina species was visualized with transmission electron microscopy (TEM) and, using a newly designed primer pair targeting Nitrospina species, its presence was detected in a time series (Wuchter et al., 2006; Pitcher et al., 2011) of high molecular weight DNA isolated from the same coastal sampling site.

**MATERIALS AND METHODS**

**INDICOLUM DESCRIPTION AND REACTOR SET-UP**

Water representative of Dutch coastal North Sea water (Pitcher et al., 2011) was collected in February 2007 at high tide at the jetty of the Royal Netherlands Institute for Sea Research situated on the island Texel (53°00′25″ N, 4°78′27″ E). An aliquot of 48 L North Sea water was filtered using a HF80S polysulfone capillary artificial Kidney/Hemofilter (Fresenius Medical Care Nederland BV, Nieuwkoop, the Netherlands). This resulted in 2 L of 24-fold concentrated biomass suspension and a cleared solution (filtrate) devoid of particles. The collected biomass suspension was incubated in a sterile glass and stainless steel reactor (adaptive, 2 L working volume). Heat-sterilized (20 min, 120°C, 15 kPa) filtrate supplemented with ammonium or nitrite (from 1 M sterile stocks of NH₄Cl and NaNO₂) was used as a medium. Oxygen and pH were monitored online using Applikon (Applikon Biotechnology BV, Scheider, the Netherlands) sensors. Nitrite concentrations were determined offline in liquid samples withdrawn daily from the reactor using Merckosquant test strips (Merck BV, Schiphol-Rijk, the Netherlands) and ammonium as well as nitrite concentration weekly using colorimetric methods (see below). The reactor was kept at a pH 7.8 with solutions of sterile 1 M NaHCO₃ and 0.6 M HCl, operated at room temperature 22 ± 2°C, stirred at 150 rpm, and supplied with an air flow of 80 ml/min.

**ENRICHMENT WITH AMMONIA AS THE SUBSTRATE**

The culture was amended with 300 μM NH₄Cl and incubated for 19 days as a batch and then for another 2 days after addition of 400 μM NH₄Cl. To avoid nitrite toxicity, the reactor system was thereafter switched to a continuous mode of operation using medium containing 750 μM NH₄⁺ at a dilution rate of 0.25 day⁻¹. When nitrite disappeared from the culture, indicating activity of nitrite oxidizers, the ammonium concentration was subsequently increased to 1.5 mM after 3 months, and further to 2, 3, and finally 10 mM after 4, 4.5, and 5.5 months, respectively. The reactor was switched to nitrite as the sole substrate after 7 months.

**ENRICHMENT WITH NITRITE AS THE SUBSTRATE**

To stimulate growth of the nitrite oxidizers, a batch mode of operation was adopted and 750 μM NaNO₂ provided as the substrate. Whenever nitrite was depleted, it was restored to 750 μM. In this manner, a total of 43 mmol of nitrite were supplied in the first month of operation with nitrite as the sole substrate. Wall growth was suspended and the biomass diluted fourfold by replacement of reactor content with medium respectively, 1 week and 1 month after the switch to nitrite. After 1 month, a fed-batch mode of operation was adopted by adding medium containing 30 mM NaNO₂ at a flow rate starting at 40 ml per day. The pump rate...
of the influent was increased manually in small (~10 ml day−1) steps whenever NO3− levels remained below 2 mg/L to a final rate of 100 mg per day. To retain biomass, the reactor content was allowed to settle once a week for at least 1 h after which clarified liquid was removed to maintain a maximum reactor volume of 2 L. Removal of wall growth and fourfold dilution of the biomass were performed as described above after 2 and 4 months. The influent nitrite concentration was raised to 20, 40, 60, 80 mM and finally 100 mM after 6, 9, 10, 10.5, and 11 months, respectively, by increasing the flow rate from 40 to 100 ml per day in 10 ml steps keeping NO3− levels below 2 mg/L. In order to prevent suboptimal nitrite oxidizer growth rates due to carbon limitation, the gas flow of 80 ml/min of air was supplemented with 10 ml/min Argon/CO2 (95%/5%) from 7.5 months onward.

Limitations of O2 may become limiting compounds for growth (van de Vossenberg et al., 2008) prior to medium replenishment resulting in an actual dilution rate of 0.05 day−1. This was operated for 12 months with nitrite as the sole substrate. The reactor was operated for 12 months with nitrite as the sole substrate. During the last month the biomass was no longer allowed to settle prior to medium replenishment resulting in an actual dilution rate of 0.05 day−1.

Chemical Analyses

To estimate nitrate concentrations liquid samples were measured directly using Merckomquet teststrips (range for nitrate 10–500 mg/L, nitrite 2–80 mg/L, Merck BV, Schiphol-Rijk, the Netherlands). At least once a week, 0.5 ml aliquots were centrifuged (5 min 10,000 × g) and the resulting supernatants used for more elaborate colorimetric analyses to monitor residual ammonium and nitrite concentrations. To measure nitrite, a colorimetric method adapted from Griess-Romijn-van Eck (1966) was used. A mixture of 50 μl with 0.5 ml of reagent A (10 g of sulfanilic acid in 1 L 1 M HCl) and 0.5 ml reagent B (1 g 1-N-naphthylethenediamine dihydrochloride in 1 L distilled water) was incubated for 10 min at room temperature, and measurements performed at 540 nm. Ammonium concentrations were determined using ortho-phthalaldehyde (OPA) reagent (Both, 1971; Taylor et al., 1974). The OPA reagent consisted of 8.54 g of OPA dissolved in 10 ml of absolute ethanol, with 50 μl of β-mercaptoethanol, and filled to 100 ml with sodium phosphate buffer (0.3 M pH 7.3). To measure ammonium concentrations between 0.25 and 5.0 μM 50 μl sample was mixed with 800 μl OPA reagent, incubated (20 min, room temperature, in the dark), and the extinction measured (410 nm). To measure in the range of 5–300 μM, 100 μl of sample was mixed with 2 ml OPA reagent containing only 0.054 g/100 ml OPA, incubated (20 min, room temperature, in the dark) and measured with a fluorospectrophotometer (excitation 411 nm, emission 482 nm, slit size 5 nm, 600 V).

Fluorescence in situ hybridization

Biomass was harvested from 20 ml reactor material by centrifugation (10 min 10,000 × g) and fixed for FISH analyses by addition of 4% w/v paraformaldehyde, incubating on ice (2 h), centrifuging (15 min 10,000 × g) and washing the resulting pellet with phosphate buffered saline (PBS, pH 7.2) and finally adding PBS and 100% EtOH (1:1) to reach a volume of 10% of the original sample. Fixed material was stored at −20°C until analysis. FISH analyses on fixed biomass from the start, after 1 and 6 months of the nitrite-fed period were performed as described by Amann et al. (1990), using 10 μl fixed material per hybridization. Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) mounting medium with DAPI (4,6-diamidino-2-phenylindole) was used to enhance the fluorescent signal and stain all DNA. Specifications and details of probes used in this study are presented in Table 1. Probes were purchased as Cy-3, Cy-5, and 5(6)-carboxyfluorescein- N-hydroxyuccinimide-ester (FLUOS) labeled derivatives from Thermohybird (Ulm, Germany). To visualize Nitrospira AOB and Nitrosira NOB simultaneously, probes NEU 653 (FLUOS) and NTSPA 712 (Cy3) were used together with their respective competitors (competitor probes consisted of unlabeled oligonucleotides) in single hybridizations at a formamide concentration of 35%. To detect Nitrospina sp. NOB, hybridizations were performed at 28% formamide concentration with probe NTSPN693. To stain all bacteria, a mixture of probes EUB338, EUB338 II, and EUB338 III was used for all hybridizations. Microscopic inspections were performed at a 1000-fold magnification. For image acquisition a Zeiss AxioPlan 2 epifluorescence microscope (Zeiss, Jena, Germany) was used with the standard software package (version 3.1). Abundance estimates of cells hybridizing with a particular probe were based on visual inspection of three randomly taken FISH microscopy pictures per hybridization.

Extraction high molecular weight DNA

Biomass was harvested from 20 ml reactor content by centrifugation (20 min, 2400 × g) after 3.5 months with ammonia, and after 6 months with nitrite as the sole substrate, respectively. Biomass was also harvested from 50 ml reactor content after 12 months with nitrite as the substrate. High molecular weight DNA was extracted using a cetyltrimethyl-ammoniumbromide (CTAB) and sodium dodecyl sulfate (SDS)-lysis-based method adapted from Zhou et al. (1996). Biomass was suspended and incubated for 30 min at 37°C in a mixture of 675 μl CTAB extraction buffer (1g/100 ml CTAB, 100 mM Tris, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, pH 8), 50 μl lysisyme (10 mg/ml, 66200 U/mg) and 30 μl Rnase A (10 mg/ml, ≥5000 U/mg). After addition of 50 μl of proteinase K (10 mg/ml, 20 U/mg) and incubation for 30 min at 37°C, the mixture was supplemented with 150 μl 10% SDS and incubated at 65°C for 2 h. DNA was recovered by phenol/chloroform extraction and isopropanol precipitation after which it was suspended in 40 μl ultrapure water (MilliQ, Millipore SA, Molsheim, France) and stored at 4°C until use.

PCR reactions, cloning, sequencing, and sequence analyses

Polymerase chain reaction reactions (30 cycles, followed by a final extension for 10 min at 72°C) were performed in a T gradient PCR apparatus (Whatman Biometra, Göttingen, Germany) using...
Table 1 | Oligonucleotide specifications.

<table>
<thead>
<tr>
<th>Name</th>
<th>Used for</th>
<th>Sequence (5′–3′)</th>
<th>Position</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338 FISH</td>
<td>FISH</td>
<td>GCTGCCCTCCCGTAGGAGT</td>
<td>338</td>
<td>Most Bacteria</td>
<td>Amann et al. (1990)</td>
</tr>
<tr>
<td>EUB338 II FISH</td>
<td>FISH</td>
<td>GCAGCCACCCGTAGGTGT</td>
<td>338</td>
<td>Most Planctomycetales</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>EUB338 III FISH</td>
<td>FISH</td>
<td>GCTGCCACCCGTAGGTGT</td>
<td>338</td>
<td>Most Verrucomicrobiales</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>NEU653 FISH</td>
<td></td>
<td>CCCCCTGCTCGACTCTA</td>
<td>653</td>
<td>Most halophilic and halotolerant</td>
<td>Wagner et al. (1995)</td>
</tr>
<tr>
<td>Competitor NEU653 FISH</td>
<td></td>
<td>TCCCACCCCCCCCTGCG</td>
<td></td>
<td>Nitrospira</td>
<td></td>
</tr>
<tr>
<td>NTSPA712 FISH</td>
<td></td>
<td>CCAGTTCCGCGCGGCGT</td>
<td>712</td>
<td>Most members of the phylum</td>
<td>Daims et al. (2001)</td>
</tr>
<tr>
<td>Competitor NTSPA712 FISH</td>
<td></td>
<td>GCAGCCACCCGTAGGTGT</td>
<td></td>
<td>Nitrospira</td>
<td></td>
</tr>
<tr>
<td>NTSP693 FISH</td>
<td>COMPETITOR</td>
<td>TCCCACCGCCGGCGGCGGCT</td>
<td>693</td>
<td>Most halophilic and halotolerant</td>
<td>Wagner et al. (1995)</td>
</tr>
<tr>
<td>616F PCR</td>
<td></td>
<td>AGAGTTTGATYMTGGCTCAG</td>
<td>8</td>
<td>Bacteria</td>
<td>Juretschko et al. (1998)</td>
</tr>
<tr>
<td>630R PCR</td>
<td></td>
<td>CATAAAAGGAGGTGATCC</td>
<td>1529</td>
<td>Bacteria</td>
<td>Juretschko et al. (1998)</td>
</tr>
<tr>
<td>NTSP1158R PCR</td>
<td></td>
<td>CCCGGTMTCTGGCAGGTA</td>
<td>1158</td>
<td>Most Nitrospira</td>
<td>Maisner et al. (2006)</td>
</tr>
<tr>
<td>NSE7F PCR</td>
<td></td>
<td>AGGTGCCAACGGGTGGAGAATA</td>
<td>87</td>
<td>Most Nitrospira</td>
<td>This study</td>
</tr>
<tr>
<td>NSE1124R PCR</td>
<td></td>
<td>TCTTCCAGGAGTCCGGCCATG</td>
<td>1124</td>
<td>Most Nitrospira</td>
<td>This study</td>
</tr>
<tr>
<td>610IIF Sequencing</td>
<td></td>
<td>GTGCCAGCAGGCGGCGG</td>
<td>479</td>
<td>Most bacteria</td>
<td>Udvardo et al. (2001)</td>
</tr>
<tr>
<td>M13F Sequencing</td>
<td></td>
<td>GAAACCACGCGGCG</td>
<td></td>
<td>pGEMT easy vector</td>
<td></td>
</tr>
<tr>
<td>M13R Sequencing</td>
<td></td>
<td>CAGGAACGCGCAGCG</td>
<td></td>
<td>pGEMT easy vector</td>
<td></td>
</tr>
</tbody>
</table>

*E. coli numbering.

GoToaq Green Master Mix (Promega Benelux BV, Leiden, the Netherlands). A PCR cycle consisted of, 1 min at 95 °C, 1 min at annealing temperature (Ta) and 1.5 min at 72 °C. For each 25 µl volume PCR reaction, 1 µl of 10-fold diluted high molecular weight DNA was used as the template. Resultant products were cloned using the pGEMT Easy vector cloning kit (Promega Benelux BV, Leiden, the Netherlands). Plasmid DNA was extracted using the GeneJet Plasmid Miniprep Kit (Fermentas GMBH, St. Leon-Rot, Germany). Clones were checked by restriction analysis of plasmid DNA (EcoRI, Fermentas GMBH, St. Leon-Rot, Germany). Sequencing (Sanger method) was performed at the division DNA diagnostics of the Human Genetics department of the University Medical Centre Nijmegen St Radboud. The Contig-Express program of the Vector NTI Suite 7.0 software package (InforMax) was used to assemble full-length clone sequences. General bacterial primers 616F and 630R (Ta: 56 °C) were used to amplify bacterial 16S rRNA gene sequences from DNA extracted after 5.5 months of operation with ammonia as the sole substrate. The resultant product was cloned, plasmids isolated and sequencing performed on 20 clones using sequencing primer 610IIF. For six clones the entire insert sequence was derived by additional sequencing reactions with primers M13F and M13R.

16S rRNA gene sequences are available from GenBank under the following accession numbers: KC706457-706479. For sequences sharing at least 99% sequence identity (see “Results”) a representative sequence has been submitted. For the North Sea nitrifier enrichment cultures clone Cb9 (KC706457) represents the Nitrosomonas sp., Cb12 (KC706458) the Nitrosopina sp. and Cb 18 (KC706459) "Candidatus Nitrospira sulfu." For the North Sea time
series sequences clone P3_4 represents the cluster of 15 Nitrospira sequences.

**METAGENOME SEQUENCING AND RECONSTRUCTION OF THE 16S rRNA GENE SEQUENCE OF THE DOMINANT NOB**

DNA extraction performed on 50 ml reactor biomass at the end of the incubation with nitrite as the sole substrate yielded 15 μg DNA based on spectrophotometric estimation using NanoDrop technology (Thermoscientific, USA). Eight microgram was subsequently used for pyrosequencing using the Roche 454 GS FLX Titanium sequencer (Roche, Switzerland) at the Department of Human Genetics Nijmegen at the Center for Molecular Life Sciences, Institute for Genetic and Metabolic Disease of the Radboud University Nijmegen Medical Center. To estimate Nitrospira abundance, all generated reads were mapped using CLC Bio Genomics Workbench (version 5.5.1) to a custom 16S rRNA gene sequence database which consisted of all unaligned sequences in release 10.28 (2,328,464 sequences) of the RDP 16S rRNA database (Cole et al., 2009), from which all sequences containing “uncultured” or “unidentified” in the description were removed. The resultant database (available upon request) contained 339,774 16S rRNA gene sequences. Through mapping, cutoff 90% identity over 90% of the read length) of the sequence reads on this database Nitrospira sp. 16S rRNA gene sequence reads were identified. The 16S rRNA gene sequence of the dominant Nitrospira NOB was reconstructed through a de novo assembly of those reads using the CLC genomics workbench.

**TRANSMISSION ELECTRON MICROSCOPY**

To investigate the cell morphology of the enriched NOB using TEM, biomass harvested from 100 ml reactor content (by centrifugation for 20 min., 2400 x g) after 8 months of operation with nitrite as the sole substrate was taken. Cryofixation was performed by high pressure freezing and was followed by freeze-substitution in acetone containing 2% osmium tetroxide, 0.2% uranyl acetate, and 1% water, embedding in Epon resin and sectioning using an ultramicrotome for TEM analysis. Sample preparation was performed as described previously by van Niftrik et al. (2008).

**DETECTION OF THE ENRICHED NITROSPIRA NOB IN COASTAL NORTH SEA WATER**

To verify that the enriched Nitrospira originated from the North Sea and was not a contaminant from our laboratory, high molecular weight DNA samples from a North Sea time series (Wuchter et al., 2006; Pitcher et al., 2011) were screened for the presence of Nitrospira by PCR analyses. A new primer pair perfectly matching the full-length 16S rRNA sequence of the enriched Nitrospira, was designed (primers NSE87F and NSE1124R). These primers were tested in PCR reactions (Tc: 80°C) using the DNA extracted from the enrichment after 6 months of operation with nitrite as the substrate as a template. In addition, test reactions were performed using high molecular weight DNA extracted from Nitrospira defluvii and Nitrospira marinae cells and plasmid DNAs containing partial (1073 nt) 16S rRNA gene sequences from Nitrospira sublineages 1 (Nitrospira defluvii-like, 2 plasmids), II (N. marinae-like, 2 plasmids), and IV (N. marina-like, 2 plasmids). To screen the North Sea time series, six pools were prepared from partial aliquots (3 μl of each sample) of the high molecular weight DNA samples from the time series (see Table 2 in the Results). Prior to amplification, 5 μl of each pool was purified by excision of DNA-containing bands from low-melting point agarose gel (Electron wide range, low melting agarose, VWR BDH Prolabo) after electrophoresis to remove substances possibly interfering with PCR amplification. PCR products were cloned, and for 25 clones (3–5 clones picked per pool) plasmid DNA was extracted and sequencing performed with primers M13F and M13R.

**RESULTS**

North Sea AOB and NOB enrichment with ammonia as the substrate

After a lag phase of 10 days, microbial ammonia oxidizers became active in the enrichment with ammonia as the substrate. Within 9 days, 500 μM NO3− was produced from 500 μM NH4+. A second aliquot of 400 μM ammonium induced further nitrite accumulation at a higher rate (400 μM within 2 days) which indicates growth of ammonia oxidizers. After adopting a continuous mode of operation (D = 0.25 per day) to avoid nitrite toxicity, all supplied ammonium (750 μM) was converted to nitrite in a 1:1 ratio up to 3 months of operation. Hereafter, the nitrite concentration dropped to zero within a 14 day period indicating a rapid increase in nitrite oxidizer activity. During the subsequent stepwise increase of the influent NH4Cl concentration to 3 mM (after 4.5 months of operation), NH4+ as well as NO2- reactor concentrations remained zero indicating complete consumption of both nitrogen species and therefore an active co-culture of ammonia and nitrite oxidizers.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>North Sea time series high molecular weight DNA samples.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample pool</td>
<td>Time series samples</td>
</tr>
<tr>
<td>P2</td>
<td>Jan-Feb-Mar 2004: 02-Jan, 14-Jan, 30-Jan, 09-Feb, 15-Feb, 16-Feb, 23-Feb, 01-Mar, 08-Mar, 15-Mar, 22-Mar, 29-Mar</td>
</tr>
<tr>
<td>P6</td>
<td>Jan 2005: 14-Jan, 25-Jan, 31-Jan</td>
</tr>
</tbody>
</table>

*Pools consisted of a mixture of 3 μl of each time series sample.*
A novel North Sea Nitrospira species

converting 0.75 mmol of nitrogen per liter per day. The raise to 10 mM NH₄Cl (after 5.5 months of operation) resulted in an ammonium and nitrite accumulation to final concentrations (at 6 months of operation) of 1.8 mM and 100 μM, respectively. During the last half month of operation therefore approximately 2 mmol of nitrogen were consumed per liter per day.

The PCR performed with general bacterial primers on DNA extracted from biomass after 5.5 months of operation with ammonium as the substrate yielded correct-sized inserts (1500 nt). The 20 clones picked for plasmid isolation yielded 20 partial (695–845 nt) 16S rRNA gene sequences of which six contained recognizable nitrifier 16S rRNA sequences based on BlastN searches of the National Center for Biotechnology Information (NCBI) database and taxonomic assignment using the Classifier tool of the RDP. Taxonomic assignment of the remaining sequences resulted in five sequences assigned to uncultured bacteria, two to unclassified Planctomycetes, three to the genus Physiophaera within the Planctomycetes, two to unclassified α-proteobacteria, one to the genus Phaeobacter within the α-proteobacteria, and one to unclassified Archaea within the Chloroflexi. Nearly full-length 16S rRNA gene sequences generated from the clones containing a recognizable nitrifier sequence resulted in three Nitrosomonas (AOB) sequences (clones Cb9, 10, and 15; >99% shared sequence identity), two Nitrospina (NOB) sequences (clones Cb12 and 16; 99.5% shared sequence identity), and one Nitrospira (NOB) sequence (clone Cb18). The phylogenetic position of the putative Nitrosomonas-like AOB is shown in Figure 1 which illustrates that the sequence from the enrichment culture is related to Nitrosomonas marina but does not cluster closely to any cultivated Nitrosomonas species. The closest match in the NCBI database (96% sequence identity) with a cultivated species was the 16S rRNA gene sequence of Nitrosomonas sp. NM51 (Purkhold et al., 2000). This implies that the enriched AOB may represent a previously uncultured Nitrosomonas species. The closest match in the NCBI database (99% sequence identity) was to an unpublished marine clone sequence (FJ514271, clone LX3). For another sequence (FJ628523, clone NitA08631, Schmidova et al., 2009) sharing 99% sequence identity to the sequence of the enriched North Sea AOB it was known that this sequence was retrieved from brackish water from the anoxic fjord Nitinat Lake, which is an environment with an ammonium concentration between 20 and 200 μM. The Nitrospina (NOB) sequences shared only 92% sequence identity to the 16S rRNA gene sequence of the cultivated species Nitropina gracilis strain 3/211 (FR865038). The

![FIGURE 1](https://example.com/figure1.png)
Within the first month of the nitrite-fed period (manual supply of *Nitrospira marina* sequence was 94% sequence identity with the 16S rRNA sequence probe NTSPN693 was observed which suggests that *Nitrospira* ant/halophilic ing with probe NEU 653. This indicates dominance of halotoler-
((approximately 80% of the total population) of bacteria hybridiz-
ning with probe NTSPN693 was observed which suggests that *Nitrosospira* NOB were a minority within the nitrifier community.

**NORTH SEA NOB ENRICHMENT WITH NITRITE AS THE SUBSTRATE**

Within the first month of the nitrite-fed period (manual supply nitrite to 0.5–1 mM final concentrations) an average nitrite con-
sumption rate of 1 mmol per liter per day was observed. Fourfold dilutions of the biomass, after 1 week and after 1 month, respec-
tively, did not result in any observed change in nitrite consumption rate. During the operation in a fed-batch mode (between 1 and 11 months of operation) with stepwise increasing influent nitrite concent-
ation (from 10 to 80 mM) followed by continuous oper-
ation (D = 0.05 per day; last month of operation) the nitrite consumption rate increased to a final value of 3 mmol per liter per day. The fourfold dilutions after 2 and 4 months of opera-
tion, again did not affect the observed nitrite consumption rate.

The FISH analyses (Figure 2) of biomass after 1 and 6 months of operation with nitrite as the substrate revealed an increase in *Nitrospira NOB* (to a final ~80% of the total bacterial popula-
tion) and decline in *Nitrosomonas NOB* (~4%), indicating that the population became dominated by *Nitrospira NOB*.

The eight sequenced clones (AC1-8) obtained through PCR with primers 616F0 and NTSPA1158R using DNA extracted after 6 months of operation, contained 99% identical inserts based on pairwise alignment. Therefore the fully sequenced insert of clone AC6 was used as a representative for phylogenetic analysis (Figure 3). When looking at 16S rRNA gene sequences of taxo-

The 454 sequencing run on DNA extracted from biomass at the end of the incubation with nitrite as the sole substrate (after 12 months) generated, after quality trimming, 1,216,565 sin-
gle reads with an average length of 405 nt. The mapping of all reads to the custom 16S rRNA gene sequence database resulted in 198 mapped reads, of which 147 mapped to *Nitrospira* sp. 16S rRNA gene sequences. This implies an abundance of *Nitrospira* sp. 16S rRNA genes within the total population of 74% which is in agreement with the 80% abundance estimated from the FISH analysis after 6 months of operation with nitrite as the substrate.

The 16S rRNA gene sequence of the dominant *Nitrospira* NOB reconstructed from the 147 *Nitrospira* sp. reads exhibited 99.9% sequence identity to the earlier obtained (clone Ch18 and Ac6) sequences resulting from PCR analysis, suggesting that the same species persisted as the dominant NOB within the reactor.

**TEM ANALYSIS OF THE ENRICHED NORTH SEA *Nitrospira* sp.**

The biomass was mainly situated in small aggregates in the culture. This was reflected in the electron microscopy pictures generated with the TEM analysis of the biomass, harvested from the enrichment after 8 months. These showed dense clumps of cells seemingly embedded in extracellular material (Figure 4A). Some typical morphological features of a representative cell are pointed out in Figure 4B. Most striking is the large periplasmic space containing many electron dense particles. In addition, large electron light particles are visible in the cytoplasm.

**FIGURE 2** Fluorescence microscopy pictures of the abundance of *Nitrospira NOB* and *Nitrosospira NOB* during the NOB enrichment. In green: cell hybridizing with probe Probe NOB (targeting most halophilic and halotolerant *Nitrospira NOB* spp.). In red: cells hybridizing with probe NTSP712 (targeting most members of the phylum *Nitrospira*). (A) Biomass after 1 month; (B) after 3 months; (C) after 6 months of operation with nitrite as the only substrate.
DETECTION OF THE ENRICHED Nitrospira NOB IN COASTAL NORTH SEA WATER

Nitrospira-targeted primer pair 616F/NTSP A1158R did not yield significant amplicons (data not shown) for DNA samples from the North Sea time series, and therefore primers NSE87F and NSE1124R were developed. This primer pair, designed to specifically target the enriched "Candidatus Nitrospira salsa", yielded correct-sized (1073 nt) amplicons with all tested templates (high molecular weight DNA from the North Sea enrichment, Nitrospira defluvii and N. moscoviensis as well as plasmid DNA from respectively sublineage I (Nitrospira defluvii-like), II (N. moscoviensis-like), and IV (N. marina-like). This demonstrates that this primer pair functions well for all tested Nitrospira species and does not specifically target the enriched North Sea species.

Screening of the six separate pools (see Table 2) from the North Sea time series of high molecular weight DNA samples with this primer pair resulted in 25 16S rRNA gene clone sequences of which 22 contained a Nitrospira sequence. Three (clones P3_4, P3_5, and P4_29) clones exhibited a sequence identity of...
presence of putative targeted PCR followed by cloning and sequencing indicated the ammonia as the substrate. The results from 16S rRNA gene of AOB and NOB was obtained by means of cultivation with fiers proved successful. Within 6 months a marine assemblage

The bioreactor approach adopted to enrich North Sea nitri-

BIOREACTOR CULTIVATION OF MARINE NORTH SEA NITRIFIERS

DISCUSSION

BIOREACTOR CULTIVATION OF MARINE NORTH SEA NITRIFIERS

The bioreactor approach adopted to enrich North Sea niti-

RELEVANCE OF THE ENRICHED NITRIFIERS IN DUTCH COASTAL NORTH SEA WATER

It has been reported by Pommerening-Röser et al. (1996) that affinity for ammonia varies among members of different lineages within the AOB genus Nitrosomonas but tends to be relatively similar within a specific lineage. The clustering of the enriched North Sea Nitrosomonas AOB 16S rRNA gene sequence with Nitrosomonas species commonly associated with low substrate environments (Figure 1) therefore suggests this species is like-

a novel species (“Candidatus Nitro-

Nitrospira marina. In the study by Keuter et al. (2011) the Nitrosira in marine enrichment M1, derived from a marine recirculation aquaculture system, is hypothesized to originate from North Sea water because the system was started and refreshed with North Sea water and its contribution to in situ nitrification may be minor. The retrieval of clone sequences (3/20) from the North Sea time series nearly identical to the 16S rRNA gene sequence of “Candidatus Nitrospira salina” proves that this species does occur in the North Sea. The higher abundance of clone sequences (17/22) forming a separate distinct cluster suggests that another Nitrospira species may actually be more abundant and potentially contribute more to in situ nitrification. Moreover, our data suggested that a minor portion of the nitrifier community may have consisted of Nitrospira NOB after 5.5 months of enrichment with ammonium.
Nitrospira species have often been detected in marine environments (e.g., Mincer et al., 2007; Raman et al., 2010; Santoro et al., 2010; Füssel et al., 2011). Based on our present study, we cannot exclude that Nitrospira NOB may be present in greater abundance or contributing to a greater extent in situ nitrite oxidation. Cultivation in a bio-reactor set-up offers a higher degree of control over environmental parameters (pH, T, substrate concentration, product concentration) than more traditional batch cultivation. Selection for a particular species due to the cultivation conditions, however, cannot be excluded completely. Our enrichment was performed with a maximum nitrite concentration of 750 μM (during the first month of operation). Off et al. (2010) reported was performed with a maximum nitrite concentration of 750 μM versus other Nitrospira sp. and Nitrospira sp.).

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OUTLOOK

Further research, e.g., selective inhibition experiments of AOA versus NOB activity (Van et al., 2012), may clarify the role of the enriched Nitrosomonas AOB species in Dutch coastal North Sea water nitrification. Bioreactor enrichments adopting more strin- gent substrate levels may result in marine microbial assemblages with a totally different species composition, which would be useful to compare and contrast to the one presently described. Collection of in situ abundance data (e.g., by quantitative PCR analyses) for different species of NOB (e.g., “ Candidatus Nitrospira salina”) versus other Nitrospira sp. and Nitrospira sp.) may help identify which NOB are of relevance to in situ nitrification. Seasonality in the abundance of this species may be corroborated by future reac- tor or laboratory enrichment experiments performed at different temperatures. Screening of a high resolution time series may aid in elucidating temporal changes in NOB community composition. The availability of the new Nitrospira species enrichments will facilitate further in-depth studies such as determination of phys- iological constraints and comparison to other NOB species. Such characterizations will increase our understanding of microbial nitrogen cycling.


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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