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, in 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; Wollast, 1998; Zeh and Kieda, 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 enrich nitrite oxidizers and finally 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 Nitrosopumilus 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, Nitrospira, Nitrospira, fluorescence in situ hybridization, transmission electron microscopy, 16S rRNA

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Marine species have been found in four of the recognized nitrite-oxidizing bacterial genera (Nitrospina, Nitrospira, Nitrobacter, and Nitrooccus; Ward and Carlucci, 1983). For the genus Nitrospina (Alawi et al., 2007) no marine species are presently known. The recently described nitrite oxidizer Nitrolancetus hollandicus (Sorokin et al., 2012), which in contrast to the previously known protobacterial nitrite oxidizers belongs to the Chlortetrifi phylum, was isolated from a reactor treating sewage plant digester effluent and no data on its salt tolerance or environmental distribution is yet available.

Within the process of nitrification ammonia oxidation to nitrite is the rate-limiting step and nitrite rarely accumulates in the process of nitrification (Philips et al., 2002; Arp, 2009). This may explain why nitrite-oxidizing bacteria (NOB) are over-looked in marine environmental studies concerning nitrification. Recent findings in the Namibian oxygen minimum zone (OMZ) by Füssel et al. (2011), however, indicate that nitrite oxidation rates may even exceed ammonia oxidation rates. Marine Nitrospira species have been 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). Nitrospina species have been detected in both coastal and open ocean habitats (Suzuki et al., 2004; Delong et al., 2006; Raman 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 Nitrospina NOB may be natural nitrite-oxidizing partners of marine NOA (Mineur 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 Nitrospina and Nitrooccus NOB in equal abundance.

The elucidation of the coprophysiology 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 species and ultimately determining their key physiological traits is helpful because it will aid in designing directed environmental research. Knowledge of 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 cultured 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 INOCULUM 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°09′25″ N, 4°78′27″ E). An aliquot of 48 L North Sea water was filtered using a HF80S polyvinylidene 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, Schiedam, the Netherlands) sensors. Nitrite concentrations were determined offline in liquid samples withdrawn daily from the reactor using Merckquant test strips (Merck BV, Schiphof-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 480 μM NH₄Cl. To avoid nitrite toxicity, the reactor system was then switched to a continuous medium feeding system 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 NaN₂ 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 ml per day. To retain biomass, the reactor content was allowed to settle once for a week 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, 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 to prevent suboptimal nitrite oxidizer growth rates due to carbon limitation, the gas flow of 80 ml/min of air was supplemented to 80 ml/min of Ar:CO₂ (99%/1%) from 7.5 months onward. It has furthermore been reported that iron as well as phosphate may become limiting compounds for growth (van de Vossenberg et al., 2008) when a medium containing only natural sea salts is used to enrich marine microorganisms. Therefore, the influent was supplemented with 0.261 ml/L of 1 M KH₂PO₄ and 0.45 ml/L of a 5 g/L FeSO₄·7H₂O + 5 g/L ethylenediaminetetraacetic acid (EDTA) tetrade 3 solution from month eight onward. 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 Merckoquant® 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 of reagent A (10 g of sulfanilic acid in 1 L 1 M HCl) and 0.5 ml reagent B (1 g 1-N-naphtylethylenediamine dihydrochloride in 1 L distilled water) was incubated for 10 min at room temperature, and measurements were 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 mM 50 μl sample was mixed with 800 μl OPA reagent, incubated (20 min, room temperature, in the dark), and the extinction measured (420 nm). To measure in the range of 0.054–1000 μg (g/l OPA, incubated (20 min, room temperature, in the dark) and measured with a fluorescence spectrophotometer (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-hydroxy succinimide ester (FLUO5) labeled derivatives from Thermohybird (Ulm, Germany). To visualize Nitrosomonas AOB and Nitrospira NOB simultaneously, probes NEU 653 (FLUO5) and NTSPA 712 (Cy5) were used together with their respective competitors (competitor probes consisted of unlabeled oligonucleotides) in single hybridizations at a formaldehyde concentration of 35%. To detect Nitrospina sp. NOB, hybridizations were performed at 28% formaldehyde 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 (1%CTAB, 100 mM Tris, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M NaCl, pH 8), 50 μl lysozyme (10 mg/ml, 66200 U/mg) and 30 μl RNase A (10 mg/ml, ≥5000 U/mg). After addition of 50 μl of protease 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 analyses (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>EU8338</td>
<td>FISH</td>
<td>GCTGCCCTCCCGTAGGAGT</td>
<td>338</td>
<td>Most Bacteria</td>
<td>Amann et al. (1990)</td>
</tr>
<tr>
<td>EU8338 II</td>
<td>FISH</td>
<td>GACGCCACCCCGTAGGAGT</td>
<td>338</td>
<td>Most Planctomycetes</td>
<td>Daims et al. (1995)</td>
</tr>
<tr>
<td>EU8338 III</td>
<td>FISH</td>
<td>GCTGCCACCCCGTAGGAGT</td>
<td>338</td>
<td>Most Verrucomicrobia</td>
<td>Daims et al. (1998)</td>
</tr>
<tr>
<td>NEU653</td>
<td>FISH</td>
<td>CCCCTCTCTCCGACTCTA</td>
<td>653</td>
<td>Most halophilic and halotolerant</td>
<td>Wagner et al. (1995)</td>
</tr>
<tr>
<td>Competitor NEU653</td>
<td>FISH</td>
<td>TTCACCCCCCCTGGCG</td>
<td></td>
<td>Nitrosononas spp</td>
<td></td>
</tr>
<tr>
<td>NTSPA712</td>
<td>FISH</td>
<td>CGGCTCGACCCGGCCTGCC</td>
<td>712</td>
<td>Most members of the phylum</td>
<td>Daims et al. (2001)</td>
</tr>
<tr>
<td>Competitor NTSPA712</td>
<td>FISH</td>
<td>CGGCTCGACCCGGCCTGCC</td>
<td></td>
<td>Nitrospira</td>
<td></td>
</tr>
<tr>
<td>NTSPN693</td>
<td>FISH</td>
<td>TCCCAAATATACAAAGATTT</td>
<td>693</td>
<td>Nitrosopina gracilis</td>
<td>Juretschko (2000)</td>
</tr>
<tr>
<td>616F</td>
<td>PCR</td>
<td>AGAGTTTGATYMTGGCTCAG</td>
<td>8</td>
<td>Bacteria</td>
<td>Juretschko et al. (1998)</td>
</tr>
<tr>
<td>630R</td>
<td>PCR</td>
<td>CAKAAAGGAGGTGATCC</td>
<td>1529</td>
<td>Nitrospira</td>
<td>Juretschko et al. (1999)</td>
</tr>
<tr>
<td>NTSPA1158R</td>
<td>PCR</td>
<td>CCCGTTMTCCTGCGAATG</td>
<td>1158</td>
<td>Most Nitrospira</td>
<td>Maisner et al. (2006)</td>
</tr>
<tr>
<td>NSE87F</td>
<td>PCR</td>
<td>AGTGCGAAGGGTGAGGAATA</td>
<td>87</td>
<td>Most Nitrospira</td>
<td>This study</td>
</tr>
<tr>
<td>NSE1124R</td>
<td>PCR</td>
<td>TTCCTCCAGAGTCCGGGCATGA</td>
<td>1124</td>
<td>Most Nitrospira</td>
<td>This study</td>
</tr>
<tr>
<td>#601F</td>
<td>Sequencing</td>
<td>GTGCGAGCGCCGGCGGTT</td>
<td>479</td>
<td>Most bacteria</td>
<td>Ustunova et al. (2001)</td>
</tr>
<tr>
<td>M13F</td>
<td>Sequencing</td>
<td>GGAAGACGCGCGGCGAG</td>
<td></td>
<td>Region flanking plasmid cloning site</td>
<td>pGEMT easy vector –</td>
</tr>
<tr>
<td>M13R</td>
<td>Sequencing</td>
<td>CACGGAAACACGACTGTTGAGA</td>
<td></td>
<td>Region flanking plasmid cloning site</td>
<td>pGEMT easy vector –</td>
</tr>
</tbody>
</table>

*E. coli numbering.

GoTaq® 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 pGEM-T 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 (EcoR1, 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 ContigExpress program of the Vector NTI Suite 7.0 software package (Informax) was used to assemble full-length clone sequences. Cloned 16S rRNA gene sequences were compared with their closest relatives in the GenBank database by BLASTN searches. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). The Ribosomal Database Project (RDP) Classifier tool (RDP Naïve Bayesian rRNA classifier version 2.5, May 2012, RDP 16S rRNA training set 9) was used to evaluate the taxonomic position of sequences (Wang et al., 2007). Pairwise analyses to determine sequence identities were performed using the internet tool from the Georgetown University Medical Center.

16S rRNA gene sequences are available from GenBank under the 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 Nitrosospira sp. and Cb 18 (KC706459) “Candidatus Nitrospira salvia.” 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 GENOME 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 18.29 (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 × 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 RNA sequence of the enriched *Nitrospira*, was designed (primers NSE87F and NSE1124R). These primers were tested in PCR reactions (Ta: 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 marina* cells and plasmid DNAs containing partial (1073 nt) 16S rRNA gene sequences from *Nitrospira* sublineages I (Nitrospira defluvii-like, 2 plasmids), II (*N. marina*-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, 3 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, microbially ammonia oxidizers became active in the enrichment with ammonia as the substrate. Within 9 days, 500 μM NO<sub>2</sub> was produced from 500 μM NH<sub>4</sub><sup>+</sup>. 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 NH<sub>4</sub>Cl concentration to 3 mM (after 4.5 months of operation), NH<sub>4</sub><sup>+</sup> as well as NO<sub>2</sub><sup>-</sup> reactor concentrations remained zero indicating complete consumption of both nitrogen species and therefore an active co-culture of ammonia and nitrite oxidizers.
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 uncultified Planctomycetes, three to the genus Physiophaera within the Planctomycetes, two to uncultified α-proteobacteria, one to the genus Phaeobacter within the α-proteobacteria, and one to uncultuated Aaeriodiniumae 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 (FP514271, clone LE53). For another sequence (FL628323, clone NiaA0631, Schmidtova 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 Nitrospina gracilis strain 3/211 (FR865038). The

![Figure 1](image-url)
next taxonomically described match for the Nitrospira NOB clone sequence was 94% sequence identity with the 16S rRNA sequence of Nitrospira marina strain Nb-295 (X82559, Ehrich et al., 1995). The FISH analyses revealed that the biomass at the end of the ammonium-fed enrichment (Figure 2A) consisted mainly (approximately 80% of the total population) of bacteria hybridizing with probe NEU 653. This indicates dominance of halotolerant/halophilic Nitrosomonas-like AOB. In addition, around 10% of the bacterial population hybridized with probe NTSPA712 indicating the presence of Nitrospira-like NOB. No hybridization with probe NTSPN693 was observed which suggests that Nitrospira 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 of Nitrospira marina strain Nb-295 (X82559, Ehrich et al., 1995). The FISH analyses (Figure 3) 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 population) and decline in Nitrosomonas AOB (~5%), indicating that the population became dominated by Nitrospira NOB. The eight sequenced clones (AC1-8) obtained through PCR primers 16SF9I and NTPSNA1158R 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 taxonomically described species, the enriched North Sea Nitrospira is phylogenetically most related (94% identity) to Nitrospira marina strain Nb-295 (X82559, Figure 3). This analysis indicates that the enriched North Sea Nitrospira represents a new species for which the name “Candidatus Nitrospira salina” (“salina” = “salty”) is proposed. The closest relatives (>98.7% 16S rRNA gene sequence identity) of the enriched North Sea Nitrospira were bacteria from a biofilters of marine recirculating aquaculture systems (Figure 3, HMJ346625 and HQ686083). Sequence HMJ346625 is a clone sequence (clone SF_NOB_Cd08) derived directly from biofilter material (Brown et al., 2013). Sequence HQ686083, however, originated from an enrichment culture (M1 marine) derived from marine recirculation aquaculture system biofilter carrier material (Keuter et al., 2011). The nearly identical 16S rRNA gene sequences of the enriched North Sea Nitrospira and the marine aquaculture biofilter species indicate these are the same species.

The 16S rRNA gene sequence of clone AC6 is 99.9% identical to that of clone Ch18 based on pairwise analysis indicating that the Nitrospira NOB species represented by this clone was already present in the reactor prior to the switch to nitrite as a substrate.

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

The 434 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 single 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 Nitrosomonas AOB and Nitrospira NOB during the NOB enrichment.** In green: cell hybridizing with probe NEU653 (targeting most halophilic and halotolerant Nitrosomonas spp.). In red: cells hybridizing with probe NTSPA712 (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 species

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...
targeted PCR followed by cloning and sequencing indicated the ammonia as the substrate. The results from 16S rRNA gene sequence of "Candidatus Nitrospira salsa" (99.5% sequence identity to sequence NC_014355, Lücke et al., 2010) and clone P1_15 an insert resembling the 16S rRNA gene sequence of Nitrospira defluvii (98.7%) of "Candidatus Nitrospira salsa". Surprisingly, North Sea time series clone P3_7 contained an insert most resembling the 16S rRNA gene sequence of Nitrospira defluvii (99.5% sequence identity to sequence NC_014355, Lücke et al., 2010) and clone P1_15 an insert resembling the 16S rRNA gene sequence of Nitrospira moscovienensis (97% sequence identity to NR_029287, Eribet et al., 1995), which are Nitrospira species associated with freshwater environments.

DISCUSSION
BIOREACTOR CULTIVATION OF MARINE NORTH SEA NITRIFIERS

The bio reactor approach adopted to enrich North Sea nitriﬁers proved successful. Within 6 months a marine assemblage of AOB and NOB was obtained by means of cultivation with ammonia as the substrate. The results from 16S rRNA gene targeted PCR followed by cloning and sequencing indicated the presence of putative Nitrosomonas-like AOB (S/20 clones), Nitrospira-like NOB (1/20 clones), and Nitrosopina-like NOB (2/20 clones), representing novel species. The FISH analysis demonstrated the abundance of the Nitrosomonas-like AOB (80% of the total bacterial population) and Nitrospira-like NOB (18%) of the total bacterial population, but failed to detect Nitrospina cells. Based on these results Nitrosomonas AOB and Nitrospira NOB are assumed responsible for the observed conversion of 2 mmol of ammonium per liter per day. Switching to nitrate as the sole substrate resulted in a high enrichment (80% of the total population based on FISH analysis) of Nitrospira NOB within another 6 months. The Nitrospira 16S rRNA gene sequences obtained from this point in time proved identical to the sequence obtained from the AOB/NOB co-culture indicating the species originally present in the marine assemblage was successfully stimulated. Phylogenetic analysis showed the enriched North Sea Nitrospira represents a novel species (“Candidatus Nitrospira salina”) only distantly related (94% 16S rRNA gene sequence identity) to the next taxonomically described species Nitrospira marina. In the study by Keuter et al. (2011) the Nitrospira 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 the enriched North Sea Nitrospira may be catalyzed by microorganisms with even higher affinities for ammonia. Moreover, AOB have been shown to be outnumbered by ANA (based on 16S rRNA and amoA gene copy numbers) in time series of Dutch coastal North Sea water (Wuchter et al., 2006; Pitcher et al., 2011). The enriched North Sea Nitroso monas AOB therefore might exhibit a low abundance in coastal 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.

RELEVANCE OF THE ENRICHED NITRIFIERS IN DUTCH COASTAL NORTH SEA WATER

It has been reported by Pommerning-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 likewise adapted to relatively low substrate conditions. This thought is strengthened by the origin of clone sequence F628323 (clone NtA40631) which shares 99% sequence identity to the sequence of the enriched North Sea Nitrospira NOB. This clone sequence was retrieved from brackish water from an anoxic fjord Nitinat Lake (Schmidtova et al., 2009), for which an ammonium concentration between 20 and 200 μM was reported. Maximum ammonium concentrations in the coastal North Sea water from which the enrichment is derived have, however, been reported to range from 10 to 13 μM during the winter months (Pitcher et al., 2011) which implies aerobic ammonia oxidation will likely be catalyzed by microorganisms with even higher affinities for ammonia. Moreover, AOB have been shown to be outnumbered by ANA (based on 16S rRNA and amoA gene copy numbers) in time series of Dutch coastal North Sea water (Wuchter et al., 2006; Pitcher et al., 2011). The enriched North Sea Nitrosomonas AOB therefore might exhibit a low abundance in coastal 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.

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Nitrospina species have often been detected in marine environ-
ments (e.g., Mincer et al., 2007; Reman et al., 2010; Santoro et al.,
2010; Füssel et al., 2011). Based on our present study, we cannot
exclude that Nitrospina NOB may be present in greater abun-
dance or contributing to a greater extent to in situ nitrite oxidation. Cul-
tivation in a bioreactor set-up offers a higher degree of control
over environmental parameters (pH, substrate concentration,
product concentration) than more traditional batch cultivation.
Selection for a particular species due to the cultivation condi-
tions, 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
nitrite tolerances for different species of Nitrospira NOB ranging
from a low 1.5 mM for enrichment culture AaH1 derived from a
marine sponge to intermediate (6 mM) for Nitrosopumilus marina
to high (15–25 mM) for freshwater Nitrospira species. Based on this,
the present bioreactor cultivation would be expected to yield a
Nitrospira NOB associated with low levels of nitrite. Indeed the
closest relatives (based on 16S rRNA gene sequence analysis) of
the enriched North Sea Nitrospira originated from recirculation
aquaculture systems which were described as relatively low nitrite
environments (10–40 μM nitrite reported by Keurer et al., 2011;
nitrite below detection reported by Brown et al., 2013). Substrate
concentration may have contributed to the dominance of “Candida-
tatus Nitrospira salina” in the final enrichment instead of Nitrospira
marina-like or Nitrospira-like NOB.

Strikingly, the only pools (P3 and P4) from which “Candidatus
Nitrospira salina” sequences were derived were also the only pools
consisting of samples from outside the winter months (spring and
summer; Table 2). Nitrospira defluvii (enriched from wastewater
treatment sludge, Speck et al., 2006; Mincer et al., 2008; Lücker
et al., 2010) and Nitrospira moscoviensis (isolated from a partially
corroded area of an iron pipe of a heating system, Ehrich et al.,
1995) are commonly associated with freshwater environments.
The detection of clone sequences most related (99.5% to Nitra-
spira defluvii, 97% identity to Nitrospira moscoviensis, respectively)
to 16S rRNA gene sequences from these Nitrospira species may be
cauised by terrestrial input (e.g., riverine influx) at the sampling site.

OUTLOOK

Further research, e.g., selective inhibition experiments of AOA
versus AOB 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. Collect-
ion 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 Nitrospina 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 facilitates
further in-depth studies such as determination of phys-
ical constraints and comparison to other NOB species. Such
characterizations will increase our understanding of microbial
nitrogen cycling.

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