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; Zehr 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. The microbial mediators of nitrification have intrigued scientists ever since the hallmark publication by Winogradsky (1890) in which not only the ability of nitrifying organisms to withdraw energy from mineral substances was reported but it was also concluded that these microorganisms assimilate carbon from carbon dioxide. At present, after 120 years of research efforts, many nitrifying microorganisms are available in culture, but marine species are underrepresented. For instance, when evaluating the phylogeny of β-proteobacterial ammonia-oxidizing bacteria (AOB) Aakra et al. (2001) examined no less than 38 isolates of which only five had a marine origin. In the review by Koops and Pomerening-Röser (2001) on the distribution and ecophysiology of nitrifying bacteria the phylogenetic relationship of 19 cultured AOB species is shown and for only five of those species a preference for a marine habitat is indicated. In marine ecosystems ammonia-oxidizing archaea (AOA) species have been shown to outnumber their bacterial counterparts based on direct cell counts and gene (16S rRNA and amoA) copy numbers (Francis et al., 2005; Wuchter et al., 2006; Mincer et al., 2007). Currently, however, the only two cultured AOA species with a marine or estuarine origin are *Nitrospumilus* and *Nitrospira* species. 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.

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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 NaN₂O₃ provided as the substrate. Whenever nitrite was depleted, it was restored to 750 μM. In this manner, a total of 43 mmol of nitrite and 12 mmol of nitrate was required for complete ammonia oxidation to nitrate in the first 1.5 months of continuous operation. The reactor was switched to nitrite as the sole substrate, and 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.
of the influent was increased manually in small (≈ 10 ml day\(^{-1}\)) steps whenever NO\(^{3-}\) 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 per week. After a 1 h period 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 original sample. Fixed material was stored at \(\approx 20^\circ\) C until analysis. FISH analyses on fixed biomass from the start, after 1 and 6 months. The nitrite-fed period were performed as described by Amann et al. (1990), using 10 \(\mu\) l fixed material per hybridization. Vectorshield (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 Cy-6-carboxyfluorescein-N-hydroxysuccinimide-ester (FLUOS) labeled derivatives from Thermobialt (Ulm, Germany). To visualize Nitrosomonas AOB and Nitrospira NOB simultaneously, probes NEU 653 (FLUOS) and NTSPA 712 (Cy5) 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 20% 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 Axiosplan 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 \(\times \) 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 10 min at room temperature, and measurements performed at 540 nm. Ammonium concentrations were determined using ortho-phenaldialdehyde (OPA) reagent (Both, 1971; Taylor et al., 1974). The OPA reagent consisted of 8.54 \(\mu\) g of OPA dissolved in 10 ml of absolute ethanol, with 50 \(\mu\) l of \(\beta\)-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 \(\mu\) M 50 \(\mu\) l sample mixture was mixed with 800 \(\mu\) l OPA reagent, incubated (20 min, room temperature, in the dark), and the extinction measured (430 nm). To measure the range of 5–300 ppm, 100 \(\mu\) l sample mixture was mixed with 2 ml OPA reagent containing only 0.024 \(\mu\) g/100 ml 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 \(\times \) g) and fixed for FISH analyses by addition of 4% w/v paraformaldehyde, incubating on ice (2 h), centrifuging (15 min 10,000 \(\times \) 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 \(\approx 20^\circ\) C until analysis. FISH analyses on fixed biomass from the start, after 1 and 6 months. The nitrite-fed period were performed as described by Amann et al. (1990), using 10 \(\mu\) l fixed material per hybridization. Vectorshield (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. Specfications and details of probes used in this study are presented in Table 1. Probes were purchased as Cy-3, Cy-5, and Cy-6-carboxyfluorescein-N-hydroxysuccinimide-ester (FLUOS) labeled derivatives from Thermobialt (Ulm, Germany). To visualize Nitrosomonas AOB and Nitrospira NOB simultaneously, probes NEU 653 (FLUOS) and NTSPA 712 (Cy5) 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 20% 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 Axiosplan 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.

**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>EUB338 FISH</td>
<td></td>
<td>GCCTGCGCGCCGGTAGGAGT</td>
<td>338</td>
<td>Most Bacteria</td>
<td>Amann et al. (1990)</td>
</tr>
<tr>
<td>EUB338 II FISH</td>
<td></td>
<td>GCAGCCACCGCGCGTAGGAGT</td>
<td>338</td>
<td>Most Planctomycetes</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>EUB338 III FISH</td>
<td></td>
<td>GCTGCCACCCGTAGGAGT</td>
<td>338</td>
<td>Most Verrucomicrobiales</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>NEU653 FISH</td>
<td></td>
<td>CCCTTCTCTCGACCTCTA</td>
<td>653</td>
<td>Most heliophilic and halotolerant</td>
<td>Wagner et al. (1995)</td>
</tr>
<tr>
<td>Competitor NEU653 FISH</td>
<td></td>
<td>TCCATCCCCCTCTGCGG</td>
<td></td>
<td>Nitrosonomas spp.</td>
<td></td>
</tr>
<tr>
<td>NTSPA712 FISH</td>
<td></td>
<td>CGGGCTGACCGCGGCTCTCC</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>GCTGCCACCCGTAGGAGT</td>
<td></td>
<td>Nitrospirae</td>
<td></td>
</tr>
<tr>
<td>NTSPN683 FISH</td>
<td></td>
<td>GCCCTGAACAGCGCGCGTTT</td>
<td>683</td>
<td>Nitrospira gracilis</td>
<td>Juretschko (2000)</td>
</tr>
<tr>
<td>616F PCR</td>
<td></td>
<td>AAGGTGTTAGMTTGGCTCA</td>
<td>8</td>
<td>Bacteria</td>
<td>Juretschko et al. (1998)</td>
</tr>
<tr>
<td>NTSPA1158R PCR</td>
<td></td>
<td>CCCGTGAMTCGCGCGCTA</td>
<td>1158</td>
<td>Most Nitrospira praevalent</td>
<td>Mainserv et al. (2006)</td>
</tr>
<tr>
<td>NSE97F PCR</td>
<td></td>
<td>AGTGGCCGGCGCGGCTACAT</td>
<td>87</td>
<td>Most Nitrospira</td>
<td>This study</td>
</tr>
<tr>
<td>NSE1124R PCR</td>
<td></td>
<td>CGCCGAGCGGCGGCTA</td>
<td>1124</td>
<td>Most Nitrospira</td>
<td>This study</td>
</tr>
<tr>
<td>#809F Sequencing</td>
<td></td>
<td>GTCCACAGCCCGCGGT</td>
<td>479</td>
<td>Most bacteria</td>
<td>Udano et al. (2001)</td>
</tr>
<tr>
<td>M13F Sequencing</td>
<td></td>
<td>GTAAACGACGGCCGAG</td>
<td>Region flanking cloning site</td>
<td>pGEM vector easy vector</td>
<td>–</td>
</tr>
<tr>
<td>M13R Sequencing</td>
<td></td>
<td>CAGGAAACAGGCGGCGA</td>
<td>Region flanking cloning site</td>
<td>pGEM vector easy vector</td>
<td>–</td>
</tr>
</tbody>
</table>

*E. coli numbering.

GoTag® 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 pET-T Easy vector system (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 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 Naive 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 sequence analyses of the enrichment with ammonia as the substrate

General bacterial primers 616F and 630R (Ta 56°C, all primer details are listed in Table 1) were used to amplify bacterial 16S rRNA gene sequences from DNA extracted after 3.5 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 sequence analyses of the enrichment with nitrite as the substrate

After 6 months of operation with nitrite as the substrate, Nitrospira-targeted PCR (primer pair 616F and NTSPA1158R, Ta: 56°C) was performed on extracted DNA and the resulting product cloned. Eight clones (clones AC1-8) were randomly picked and sequenced with primer M13F. Additional sequencing was performed with primer M13R to obtain the full insert sequence of clone AC6.

Accession numbers

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 nitifier enrichment cultures clone Cb9 (KC706457) represents the Nitrosonomas sp., Cb12 (KC706458) the Nitrosopira sp. and Cb 18 (KC706459) “Candidatus Nitrospira saufa.” For the North Sea time...
Table 2 | North Sea time series high molecular weight DNA samples.

<table>
<thead>
<tr>
<th>Sample pool*</th>
<th>Time series samples</th>
</tr>
</thead>
<tbody>
<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.

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**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 NO\textsubscript{2}\textsuperscript{−} was produced from 500 μM NH\textsubscript{3}\textsuperscript{+}. 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\textsubscript{4}Cl concentration to 3 mM (after 4.5 months of operation), NH\textsubscript{3}\textsuperscript{+} as well as NO\textsubscript{2}\textsuperscript{−} 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 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 Phycisphaera within the Planctomycetes, two to unclassified α-proteobacteria, one to the genus Phaeobacter within the α-proteobacteria, and one to unclassified Anaerolineae within the Chloroflexi. Nearly full-length 16S rRNA gene sequences generated from the clones containing a recognizable nitrifier sequence resulted in three Nitrosonomas (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 Nitrosonomas-like AOB is shown in Figure 1 which illustrates that the sequence from the enrichment culture is related to Nitrosonomas marina but does not cluster closely to any cultivated Nitrosonomas species. The closest match in the NCBI database (96% sequence identity) with a cultivated species was the 16S rRNA gene sequence of Nitrosonomas sp. NM51 (Parkhiole et al., 2000). This implies that the enriched AOB may represent a previously uncultured Nitrosonomas species. The closest match in the NCBI database (99% sequence identity) was to an unpublished marine clone sequence (FJ514271, clone LXE3). For another sequence (FJ628523, clone NitA0631, 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 Nitrospina gracilis strain 3/211 (FR865838). The Nitrospina (NOB) sequences shared only 92% sequence identity to the 16S rRNA gene sequence of the cultivated species Nitrospina gracilis strain 3/211 (FR865838). The

![Figure 1](image-url)
Within the first month of the nitrite-fed period (manual supply of Nitrospira marina strain Nb-295 (X82539, 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 nitrite to 0.5–1 mM final concentrations) an average nitrite consumption rate of 1 mmol per liter per day was observed. Fourfold dilutions of the biomass, after 1 week and after 1 month, respectively, 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 concentration (from 10 to 80 mM) followed by continuous operation (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 operation, 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 population) and decline in Nitrosomonas AOB (∼7%), 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 taxonomically described species, the enriched North Sea Nitrospira is phylogenetically most related (94% identity) to Nitrospira marine strain Nb-295 (X82539, Figure 3). This analysis indicates that the enriched North Sea Nitrospira represents a new species for which the name “Candidatus Nitrospira salina” (“salsa” = “salty”) is proposed. The closest relatives (>98.7% 16S rRNA gene sequence identity) of the enriched North Sea Nitrospira were bacteria from a biofilter of marine recirculating aquaculture systems (Figure 3, HM345625 and HQ686083). Sequence HM345625 is a clone sequence (clone SF_NOB_C0B) 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 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 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.
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 salinii", 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...
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The bioreactor 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 fresh seawater 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 (1722) 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.
Nitrospina species have often been detected in marine environments (e.g., Mincer et al., 2007; Reman et al., 2010; Santoro et al., 2010; Pfeifer et al., 2011). Based on our present study, we cannot exclude that Nitrospina NOB may be present in greater abundance or contributing to a greater extent to in situ nitrite oxidation. Cultivation in bioreactor 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 nitrite tolerances for different species of Nitrospira NOB ranging from a low 1.5 mM for enrichment culture Aa01 derived from a marine sponge to intermediate (6 mM) for Nitrosopina marina to high (15–25 mM) for freshwater Nitrospira species. Based on this, the present bioreactor cultivation would be expected to yield a Nitrospina 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 Keuter et al., 2011; nitrite below detection reported by Brown et al., 2013). Substrate concentration may have contributed to the dominance of “Candidateatus Nitrospira salina” in the final enrichment instead of Nitrosopina marina-like or Nitrospina-like NOB.

Strikingly, the only pools (P3 and P4) from which “Candidateatus 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; Maixner 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., 1997) are commonly associated with freshwater environments. The detection of clone sequences most related (99.5% to Nitrospira defluvii, 97% identity to Nitrospira moscoviensis, respectively) to 16S rRNA gene sequences from these Nitrospira species may be caused by terrestrial input (e.g., riverine influx) at the sampling site.

OUTLOOK

Further research, e.g., selective inhibition experiments of AOA versus AOB activity (Yan et al., 2012), may clarify the role of the enriched Nitrosomonas AOB species in Dutch coastal North Sea water nitrification. Bioreactor enrichments adopting more stringent 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., “Candidateatus 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 reactor 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 physiological constraints and comparison to other NOB species. Such characterizations will increase our understanding of microbial nitrogen cycling.

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