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# ***NirS*-containing denitrifier communities in the water column and sediment of the Baltic Sea**

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## Abstract

The aim of this study was to compare structural differences in the denitrifying microbial communities along the environmental gradients observed in the water column and coastal sediments of the Baltic Sea. To link community structure and environmental gradients, denitrifier communities were analyzed by terminal restriction fragment length polymorphism (T-RFLP) based on *nirS* as a functional marker gene for denitrification. *NirS*-type denitrifier community composition was further evaluated by phylogenetic analysis of *nirS* sequences from clone libraries. T-RFLP analysis indicated some overlap but also major differences of communities from the water column and the sediment. Shifts in community composition along the biogeochemical gradients were observed only in the water column while denitrifier communities were rather uniform within the upper 30 mm of the sediment. Specific terminal restriction fragments (T-RFs) indicative for the sulfidic zone suggest the presence of nitrate-reducing and sulfide-oxidizing microorganisms that were previously shown to be important at the suboxic-sulfidic interface in the water column of the Baltic Sea. Phylogenetic analysis of *nirS* genes from the Baltic Sea and of sequences from marine habitats all over the world indicated distinct denitrifier communities that grouped mostly according to their habitat. We suggest that these subgroups of denitrifiers had developed after selection through several factors, i.e. their habitats (water column or sediment), impact by prevalent environmental conditions and isolation by large geographic distances between habitats.

## 1 Introduction

The Baltic Sea is one of the largest brackish water systems in the world (Meyer-Reil et al., 2000). After the last glaciation the Baltic Sea has been connected continuously with the Atlantic Ocean over the past 8000 years via the Danish straits (Sohlenius et al., 1996; Andr n et al., 2000a; Sohlenius et al., 2001). These straits provide the only

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salt water supply from the North Sea. Water exchange with the North Sea depends on wind-induced movements (Meyer-Reil et al., 2000) and mainly affects the upper layers of the water column while water exchange in the deeper layers is quite rare and occurs approximately once in a decade (Feistel et al., 2003a, b; Nausch et al., 2003). In the  
5 Gotland Deep, one of the deepest basins (250 m depth) in the central Baltic Sea, well-mixed surface water is separated from dense bottom waters by a halocline at about 70 m depth (Kotilainen et al., 2002). This barrier leads to frequent anoxic conditions in deeper waters and to accumulations of sulfide ( $S^{2-}$ ) that diffuses out of the sediment. Therefore, low oxygen ( $O_2$ ) and sulfidic conditions with concurrent availability of nitrate  
10 ( $NO_3^-$ ) at the suboxic-sulfidic interface can be found in the water column (Brettar and Rheinheimer, 1991; Hannig et al., 2006a).

Thus, conditions in the water column should be adequate for denitrification and it has been assumed that water column denitrification contributes significantly to the nitrogen budget of the Baltic Sea (Rönner and Sørensen, 1985). Significant denitrification  
15 activity has indeed been proven for the suboxic-sulfidic interface, indicating sulfide-driven chemoautotrophic denitrification (Brettar and Rheinheimer, 1991; Hannig et al., 2006b<sup>1</sup>).

Cultivation-independent approaches based on analysis of functional marker genes for denitrification revealed the lowest denitrifier diversity in the suboxic-sulfidic inter-  
20 face in the central Baltic (Hannig et al., 2006a). In contrast, cultivation-dependent approaches showed the highest diversity and density of heterotrophic denitrifying bacteria (Brettar et al., 2001) with a predominance of *Shewanella baltica* and *Pseudomonas* sp. (Brettar and Höfle, 1993; Höfle and Brettar, 1996) in that interface. Furthermore, an  $\epsilon$ -proteobacterium, related to *Thiomicrospira denitrificans* was identified as an impor-  
25 tant chemolithoautotrophic sulfide-oxidizer and nitrate-reducer (Labrenz et al., 2004; Höfle et al., 2005) and suggested to be predominantly responsible for autotrophic den-

<sup>1</sup>Hannig, M., Lavik, G., Kuypers, M. M. M., Woebken, D., Martens-Habbena, W., and Jürgens, K.: Shift from denitrification to anammox after inflow events in the central Baltic Sea, submitted, 2006b.

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itrification in the Gotland Deep (Brettar et al., 2006).

Heterotrophic and autotrophic denitrifiers reduce oxidized nitrogen compounds  $\text{NO}_3^-$  and nitrite ( $\text{NO}_2^-$ ) to the gaseous intermediates nitric oxide and nitrous oxide and to dinitrogen gas as the end product in a dissimilatory process. Denitrifying bacteria are phylogenetically widespread, thus a 16S rRNA gene-based approach is inappropriate to explore the communities of organisms belonging to this functional group. *NirK* and *nirS*, coding for copper and cytochrome *cd*<sub>1</sub>-containing nitrite reductase, respectively were used as functional marker genes to target denitrifiers in PCR-based approaches (Braker et al., 1998). Both enzymes are functionally equivalent and catalyse the reduction of  $\text{NO}_2^-$  to NO, which is the key reaction in denitrification (Zumft, 1997). These genes have been targeted to study denitrifier communities in a variety of habitats such as soils (Avrahami et al., 2002; Priemé et al., 2002), aquifers (Santoro et al., 2006) waste water treatment plants (Throbäck et al., 2004) and marine habitats (Braker et al., 2000; Liu et al., 2003; Jayakumar et al., 2004; Castro-González et al., 2005; Hannig et al., 2006a). Studies of these communities in marine habitats indicated that denitrifier diversity and community structure in the water column and sediments of the world's oceans were at least in part determined by environmental gradients of  $\text{O}_2$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{S}^{2-}$  (Liu et al., 2003; Castro-González et al., 2005; Hannig et al., 2006a).

Although data on denitrifier diversity in different aquatic and terrestrial habitats are rapidly accumulating, the principle differences in denitrifier composition between sediment and water column are not known. The Baltic Sea is an ideal system for this comparison of denitrifier community structure as gradients in  $\text{O}_2$ ,  $\text{NO}_3^-$  and  $\text{S}^{2-}$  occur both in the water column (e.g., in the central basins), at the scale of meters, and in coastal sediments (e.g. in the Bodden in the southern Baltic Sea), at scales of mm to cm. The Bodden at the “Nordreügensche Boddenkette” are formed by the accumulation of sand, which separates areas of shallow water from the outer Baltic Sea (Meyer-Reil et al., 2000). The water column in these shallow water areas is mixed thoroughly, therefore anoxic conditions occur only in the sediments. There is evidence that coastal sediments are very important in removal of riverborne nitrogen by denitrification (Voss

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et al., 2005). Denitrification rates have been measured in Baltic sediments (e.g. central Gulf of Finland) using the isotope pairing method (Tuominen et al., 1998). It revealed that the bulk of denitrification was coupled to  $\text{NO}_3^-$  production by nitrification. However, in the Bodden sediment only low amounts of  $\text{NO}_3^-$  were produced by nitrification at the aerobic-anaerobic interface resulting in low summertime denitrification rates in this area (Dahlke, 1990). Besides denitrification, also anaerobic ammonium oxidation (anammox) contributes to nitrogen loss in coastal sediments (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen et al., 2004).

Here, we studied communities of denitrifiers in the water column of the central Baltic Sea and in coastal sediments by applying the functional marker gene *nirS* amplified from community DNA. Shifts in *nirS*-type denitrifier communities along biogeochemical gradients were analysed by terminal restriction fragment length polymorphism analysis (T-RFLP) as a fingerprint technique. Additionally, *nirS* genes from the Bodden sediment were cloned, sequenced and included into a phylogenetic tree comprising all *nirS* sequences from marine habitats available from public databases. Analysis of these sequence data suggests that the heterogeneity of *nirS* genes is driven by the different ecological niches that the respective marine denitrifiers occupy. These niches seem to be defined by the habitat, water column or sediment, shaped by the prevalent environmental conditions, and can be isolated by large geographic distances.

## 2 Material and methods

### 2.1 Study area and sampling

Two different sites in the Baltic Sea were studied, namely Rassower Strom which is located in the “Nordrügensche Boddengewässer” (54°33′ N; 13°12′ E) for sediment sampling, and the Gotland Deep (240 m water depth) in the central Baltic Sea (Baltic Sea monitoring station 271; 57°19′ N, 20°03′ E) for water column sampling (Fig. 1). Water samples were collected at the Gotland Deep during a cruise in August 2004. Coastal

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sediment samples were taken in July 2004 at the Rassower Strom, a shallow water coastal inlet of the southern Baltic Sea (4 m water depth). Since the water column in these shallow water areas is continuously mixed by wind, anoxic conditions occur only in the sediments. Sampling at the Gotland Deep was performed on board of the R/V “A. v. Humboldt” with a rosette water sampler (5 l) mounted on a Seabird CTD-system measuring profiles of temperature, conductivity, O<sub>2</sub>, fluorescence and turbidity. For DNA extraction, seawater was filtered immediately after sampling through Durapore® filters (0.2 μm; Millipore, Billerica, MA, USA). The filters were snap-frozen in sterile reaction tubes in liquid nitrogen and stored at –20°C. Sediment samples were collected on board of the ship R/V “Prof. F. Gessner” with a multicorer (Black et al., 2002). Two subsets of samples were analyzed from the same core. First, subsamples from the top 3 cm according to the oxic layer (0 to 5 mm depth), the suboxic layer (5 to 12 mm depth), and the sulfidic layer (12 to 30 mm depth) were taken for PCR amplification and cloning of *nirS* genes. A second set of subsamples was obtained by slicing the sediment at 1, 2, 5, 7, and 10 cm depth. The corresponding layers (0 to 1, 1 to 2, 2 to 5, 5 to 7 and 9 to 10 cm) were homogenized and used for T-RFLP analysis of *nirS* genes. Measurements of chemical parameters and DNA extractions were performed within 1 h after sampling.

## 2.2 Determination of chemical parameters in samples from the water column and sediment from the Baltic Sea

Oxygen (O<sub>2</sub>), ammonium (NH<sub>4</sub><sup>+</sup>), sulfide (S<sup>2-</sup>) concentrations and pH were determined for samples from both sampling sites. In water samples, O<sub>2</sub> was measured by the Winkler method as described by Grasshoff (1983). Ammonium was determined in the water column with the modified indophenolblue method of Koroleff (1983), where NO<sub>3</sub><sup>-</sup> is reduced to NO<sub>2</sub><sup>-</sup> by a copper cadmium column and determined as NO<sub>2</sub><sup>-</sup> (Grasshoff, 1983). Sulfide was measured photometrically by the modified methylene blue method of Fonselius (1983).

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In the sediment samples  $O_2$  and  $S^{2-}$  concentrations and pH were measured with micro-sensors only in the upper 3 cm, whereas  $NO_3^-$  and  $NO_2^-$  were measured at all depths using an ion chromatograph (Sykam, Fürstfeldbruck, Germany). Microsensor-based measurements of  $O_2$ ,  $S^{2-}$ , and pH were performed to describe spatial small-scale variations in the sediments. Measurements of dissolved  $O_2$  were done with fiber-optic micro-sensors (type B2, tip diameter  $<50 \mu m$ ; PreSens GmbH, Regensburg, Germany) connected to an  $O_2$ -meter (Microx TX2; PreSens GmbH). Dissolved  $S^{2-}$  was measured with a potentiometric  $Ag/Ag_2S$  electrode (tip diameter of  $140 \mu m$ ; Revsbech et al., 1983) modified according to Cypionka (1986). The  $S^{2-}$ -sensor was connected to a mV-meter (3403 True RMS Voltmeter; Hewlett Packard, Böblingen, Germany). An ion-selective pH-mini-electrode (tip diameter of 1 mm; Toepffer Lab System PHM-146, Göppingen, Germany) was connected to a pH-meter. The micro-sensors were mounted together on a computer-controlled motorized micromanipulator (Märzhäuser GmbH, Wetzlar, Germany) and inserted stepwise into the sediment. Porewater for analyses of  $NO_3^-$  and  $NO_2^-$  was gained by centrifugation. Nitrate and  $NO_2^-$  were measured by ion chromatography (Sykam) at a wavelength of 220 nm.

### 2.3 Nucleic acid extraction from water and sediment samples

Nucleic acid extraction for all water samples from frozen filters was performed as described by Weinbauer et al. (2002). DNA and RNA were obtained by mechanical and chemical extraction using glass beads and sodium lauryl sarcosinate-phenol. Prior to PCR, DNA extracts were purified from RNA by incubation with RNase I (Roche Diagnostics, Mannheim, Germany) for 30 min at  $37^\circ C$ , and DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, ME, USA).

From sediment samples (0.5 g), DNA was extracted adding 0.5 g glass beads,  $900 \mu l$  extraction buffer (Kramer and Singleton, 1993) and  $100 \mu l$  acid-washed polyvinylpyrrolidone (PVPP; Sigma-Aldrich, Deisenhofen, Germany; Ogram et al.,



1998). First, cells were lysed by freezing (30 min,  $-70^{\circ}\text{C}$ ) and thawing ( $65^{\circ}\text{C}$ , 5 min) with concurrent shaking of the suspension at 1000 rpm (Thermomixer; Eppendorf, Hamburg, Germany). In addition, cells were disrupted by bead beating twice for 30 s at  $5.5\text{ m s}^{-1}$  (Fastprep Celldisrupter; Bio101, Carlsbad, CA, USA) and centrifuged at  $12\,000\times g$  at  $4^{\circ}\text{C}$  for 1 min. With the supernatant two extraction steps were performed: (1) with phenol-chloroform-isoamylalcohol [24:24:1, v/v/v (volume in volume in volume)] and (2) with chloroform-isoamylalcohol (24:1, v/v). Afterwards the DNA was concentrated by ethanol precipitation (2 volumes of 96% ethanol, 1/10 volume of sodium acetate, 30 min at  $-70^{\circ}\text{C}$ ) and centrifuged ( $16\,000\times g$ , 15 min,  $4^{\circ}\text{C}$ ). The pellet was suspended in  $50\ \mu\text{l}$  sterile *aqua bidest*. Humic acids were removed with PVPP columns (Ogram et al., 1998; Mendum et al., 1998) and the QIAquick Purification Kit (Qiagen, Hilden, Germany).

## 2.4 PCR amplification

Amplification of *nirS* genes was done with the primer pair nirS1F and nirS6R (MWG Biotech, Ebersberg, Germany) as described by Braker et al. (1998). *NirS* was amplified in a total volume of  $25\ \mu\text{l}$  containing  $1\ \mu\text{l}$  of environmental DNA,  $2.5\ \mu\text{l}$  of 400 ng BSA (Roche, Mannheim, Germany)  $\mu\text{l}^{-1}$ ,  $200\ \mu\text{M}$  of each desoxyribonucleoside triphosphate (Roche Applied Science, Mannheim, Germany) and 1.25 U Red AccuTaq DNA polymerase (Sigma, Taufkirchen, Germany). PCRs were performed in a 9700 thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) or in an iCycler iQ (Biorad Laboratories, Inc., Hercules, CA, USA) with conditions modified according to Braker et al. (2000) and Braker et al. (2001). "Touchdown"-PCR started with  $57^{\circ}\text{C}$  until it reached  $52.5^{\circ}\text{C}$  in the first ten cycles. Additional 25 cycles were performed at an annealing temperature of  $55^{\circ}\text{C}$ . For water samples the same program was used except that 26 cycles were performed with an annealing temperature of  $56^{\circ}\text{C}$ .

Aliquots of  $100\ \mu\text{l}$  PCR products were loaded on 1.5% [w/v (weight/volume)] agarose gels (SeaKem<sup>®</sup> LE agarose; Cambrex Bio Science, Rockland, ME, USA) and sepa-

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rated by electrophoresis (120 V, 45 min). Bands were visualised by UV excitation after staining with ethidium bromide ( $0.5 \text{ mg l}^{-1}$ ). Bands of the expected size were excised from the gel and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany).

## 5 2.5 Terminal restriction fragment length polymorphism (T-RFLP) analysis for sediment and water samples

For T-RFLP analysis the primer nirS1F was 5'-end labelled with 6-carboxyfluorescein (MWG Biotech, Ebersberg, Germany). The purified PCR products were digested with 0.3 U of the restriction enzyme *HhaI* [GCG'C] at 37°C over night according to the manufacturer's instructions (Promega, Madison, WI, USA). Digested products were cleaned with Autoseq G-50 columns (Amersham Biosciences, Chalfont St. Giles, UK) according to the manufacturer's instructions. Aliquots of the digest ( $2 \mu\text{l}$ ) were mixed with  $12 \mu\text{l}$  deionised formamide (Applera, Darmstadt, Germany) and  $0.2 \mu\text{l}$  of an internal DNA fragment length standard (X-Rhodamine MapMarker<sup>®</sup> 30–1000 bp; BioVentures, Murfreesboro, TN, USA). Terminal restriction fragments (T-RFs) were separated with an automated DNA sequencer (ABI 310, Applied Biosystems, Darmstadt, Germany). The length of fluorescently labelled T-RFs was determined by comparison with the internal standard using GeneScan 3.71 software (Applied Biosystems).

## 2.6 Analysis of terminal restriction fragments (T-RFs)

20 Peaks  $\geq 60$  units of fluorescence from different samples were normalized by an iterative standardization procedure (Dunbar et al., 2001). The relative abundance of T-RFs in percent was determined by calculating the ratio between the heights of a given peak and the normalized total peak height of each sample. Richness of each sample corresponds to the number of T-RFs. Commonly used diversity indices, namely Shannon diversity and Shannon evenness index, were calculated:

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Shannon diversity index  $H$ :

$$H = - \sum p_i \cdot \log_2 p_i \quad (1)$$

Shannon evenness index  $J$ :

$$J = \frac{H}{H_{\max}} = \frac{\sum p_i \cdot \log_2 p_i}{\sum \hat{p}_i \cdot \log_2 \hat{p}_i} \quad (2)$$

- 5 where  $p_i$  is the proportion (relative abundance) of T-RF  $i$  in the community and  $\hat{p}_i$  is the equally distributed relative abundance.

## 2.7 Cloning and sequencing

Unlabelled PCR products (50 ng) of the sediment sample from the upper three centime-  
ters (0 to 5 mm, 5 to 12 mm, and 12 to 30 mm depth) were cloned using the TA Cloning  
10 Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Clones were  
screened for inserts of the proper size using vector specific primers (T7, M13). The  
inserts were sequenced as described previously by Avrahami et al. (2002).

## 2.8 Phylogenetic analysis

*NirS* clones were aligned to sequences from the EMBL database with the  
15 ARB fast aligner feature. Phylogenetic analyses were performed with ARB  
(<http://www.arb-home.de>). Trees were reconstructed with the distance matrix-based  
neighbour joining method (ARB) and the overall topology was confirmed by trees  
calculated with PROTPARS and PROTML (PHYLP; Felsenstein, 1993). Nucleotide  
sequences retrieved from the Bodden sediments have been deposited in the EMBL  
20 database under accession numbers AM238454 to AM238510.

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### 3 Results

#### 3.1 Field measurements

In the water column of the Gotland Deep oxygen ( $O_2$ ) decreased rapidly below the halocline (at a depth of 70 m) from  $300 \mu\text{M}$  in the oxic layers to low concentrations ( $10$ – $50 \mu\text{M}$ ) in the suboxic zone (80 to 220 m) (Fig. 2a). Oxygen was below the detection limit ( $1 \mu\text{M}$ ) at a depth of 220 m and in the sulfidic zone (225 to 232.5 m). While nitrate ( $\text{NO}_3^-$ ) concentration was low in the oxic layers it increased below the halocline and highest concentrations ( $13 \mu\text{M}$ ) were measured at a depth of 220 m. Below this depth  $\text{NO}_3^-$  concentration declined rapidly. Nitrite ( $\text{NO}_2^-$ ) concentration showed two peaks at depths of 55 m and 225 m. In contrast, ammonium ( $\text{NH}_4^+$ ) was consistently low in the upper water column and increased below 225 m. Sulfide ( $\text{S}^{2-}$ ) could be detected at a depth of 230 m, reaching  $3.5 \mu\text{M}$  at 232.5 m.

In the coastal sediment sample  $O_2$  concentration decreased rapidly from  $300 \mu\text{M}$  to 0 within the top 2 millimetres (Fig. 2b). Nitrite was detected at two depths (0 to 2 mm and 2 to 5 mm depth) with concentrations of  $0.25 \mu\text{M}$  and  $0.1 \mu\text{M}$ , respectively (data not shown) and was below the detection limit below 5 mm depth. Nitrate concentrations in the overlying water were  $0.26 \mu\text{M}$  whereas  $\text{NO}_3^-$  in the sediment was below the detection limit in all layers. Sulfide started to accumulate in the sediment from a depth of 7 mm and reached its highest concentration ( $0.5 \text{ mM}$ ) at a depth of 30 mm.

#### 3.2 Denitrifier community profiles along biogeochemical gradients

The communities of *nirS*-type denitrifiers from water samples (Gotland Deep) and coastal sediments (southern Baltic Sea) along the biogeochemical gradients at both sampling sites were analysed by T-RFLP of *HhaI*-digested *nirS* amplicons. The restriction endonuclease *HhaI* showed the highest level of resolution compared to *TaqI* and *MspI* as indicated by the highest number of peaks observed (data not shown). In total, 22 different *nirS* T-RFs (terminal restriction fragments) were detected after hydrolysis

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with *Hhal* in samples from both sampling sites. Representative T-RFLP profiles for water column and sediment are shown in Fig. 3.

In the water column of the Gotland Deep, in total 16 different *nirS* T-RFs were found. Diversity (H, Shannon diversity) and evenness (E, Shannon evenness) levels were lowest (H=0.48; E=0.44) in the upper oxygenated zone and highest at depths from 100 to 200 m in the suboxic zone (H, 1.74 to 1.86; E, 0.84 to 0.90) (data not shown). In the suboxic zone changes in *nirS*-type denitrifier community structure were mainly based on changes in relative abundance of T-RFs. While the T-RF of 111 bp dominated the denitrifier community profile in the water column at 70 to 85 m, the relative abundance of the 36-bp T-RF increased with water depth and it became dominant at a depth of 200 m. A strong shift in the community structure occurred from 200 m (suboxic zone) to 225 m (start of sulfidic layers). In contrast to the suboxic zone, the 47-bp fragment was dominant in the sulfidic zone (225–232.5 m) (Fig. 3a). Some *nirS* T-RFs were specific for some layers of the water column at the Gotland Deep, i.e. those of 218, 295 and 380 bp. The majority of the T-RFs occurred at several depths but were unique to the water column, i.e. T-RFs of 45, 47, 118, 120, 275, 299, 341, and 537 bp.

The occurrence and relative abundance of *nirS* T-RFs in the coastal sediment profiles was more homogenous (H, 0.95 to 1.34; E, 0.66 to 0.76) than in the water column. However, the occurrence of the T-RFs of 346, 97 and 240 bp was restricted to the sediment layers of 0 to 1, 1 to 2 and 9 to 10 cm depth, respectively (Fig. 3b). In total, 11 different T-RFs were detected in five different sediment layers (0 to 10 cm). The T-RFs of 36 and 111 bp, which occurred also in several depths of the water column, were predominant at all sediment depths. Many T-RFs were detected exclusively in the sediment samples, i.e. the 72-, 97-, 240-, 346-, 347-, and 385-bp T-RF. Several T-RFs i.e. those of 36, 107, 111, 238, and 273 bp occurred both in samples from sediment and water column.

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### 3.3 Phylogenetic affiliation of *nirS* sequences from marine habitats

A total of 57 *nirS* clones from environmental DNA, which were obtained from the coastal Baltic Sea sediment sample (BSS = Baltic Sea sediment), were included into a phylogenetic tree (Fig. 4). *NirS* sequences from the Gotland Deep station had been gathered in 2003 (Hannig et al., 2006a) and were also considered (BS). Besides sequences from taxonomically described isolates, the overall tree also included all *nirS* sequences of unknown affiliation retrieved from other marine systems that were available in the database. These sequences were retrieved from the water column of the eastern South Pacific (ESP, Castro-González et al., 2005), the Arabian Sea (ASW, Jayakumar et al., 2003), and the Northern Baltic Sea (CBBS, Tuomainen et al., 2003) and sediments of the Pacific Northwest (PNW, Braker et al., 2000) and the eastern tropical North Pacific (ETNP, Liu et al., 2004).

Phylogenetic analysis revealed seven major clusters of marine *nirS* sequences (marine Clusters I–VII). The overall topology was supported by the parsimony and maximum likelihood algorithm and clusters were defined if sequences were consistently grouped together. Sequences from Baltic Sea sediment (BSS Clusters A to F) belonged to seven subclusters of *nirS* genes (Fig. 4). The majority of BSS sequences (29) belonged to BSS Clusters A to E and were placed in marine Cluster I. This cluster also included *nirS* genes from marine sediments (PNW Clusters III and IV; ETNP Cluster Ib and Ic) and water column (ASW Clusters XI and XII; ESP Cluster I) as well as sequences from the water column (BS) and from a cyanobacterial bloom (CBBS) from the Baltic Sea. An in-depth analysis of Cluster I-sequences showed that *nirS* sequences from the oxic (0 to 5 mm depth), anoxic (5 to 12 mm depth), and sulfidic zone (12 to 30 mm depth) of the Baltic Sea sediment were not clustered according to the prevalent environmental conditions in these zones (Fig. 5). In contrast, sequences from all three zones were found distributed in several shared subclusters within Cluster I.

In marine sequence Cluster II, BSS Cluster F grouped together with sequences from

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several clusters from the ASW (Clusters IV, VII, VIII, IX), the ESP (Clusters IIa, IIb, IIc, III), and the Baltic Sea (BS Cluster IV, V, one sequence each from the BS and the CBBS). Marine Cluster II also comprised sequences from denitrifying isolates (*Pseudomonas* spp., *Thauera mechernichensis*, *Azoarcus tolulyticus* and marine isolates affiliated with *Marinobacter* spp. and *Halomonas variabilis*).

Marine sequence Clusters III, IV, V, VI, and VII harboured no sequences from the Baltic Sea sediment but sequences from the water column of the Baltic Sea and from the other marine habitats. Cluster III contained sequences from the Baltic Sea (Cluster VIII) and the ESP (Cluster IV). Cluster IV contained *nirS* from the ASW (Clusters I, II and III), from the ETNP (Clusters Ia and Ib), and the Baltic Sea (Clusters VIa and VIb) and *nirS* from *Ralstonia eutropha*. Marine *nirS* sequences from the Baltic Sea (Clusters VIIa and VIIb) and the ETNP (Cluster Ia) were related to those from *Paracoccus denitrificans* and *Roseobacter denitrificans*. Most sequences found in Cluster VI originate from cultivated denitrifiers e.g. *Thauera* spp., *Azoarcus* spp. and *Pseudomonas stutzeri* and related marine isolates. Sequences from the ETNP (Cluster Ic) and the Baltic Sea (BS Cluster IX) were also grouped in this cluster. Marine Cluster VII consisted exclusively of *nirS* genes from clones from the Baltic Sea (Cluster X), the ASW (Cluster V) and the PNW (Cluster II).

## 4 Discussion

A number of studies to date have explored denitrifier communities from marine habitats but they all were from distinct geographic locations. Either from the water column or sediment, they were separated by large geographic distances and presumably influenced by distinct environmental conditions. In this study *nirS*-type denitrifier communities were explored from water column (Gotland Deep) and sediment samples (Rassower Strom, Bodden) of the Baltic Sea (Fig. 1). Despite comparably close geographic location (550 km distance) both locations differed in salinity (Bodden, 7 to 9 psu; Gotland Deep, 7 to 13 psu) and anthropogenic influence (Hübel et al., 1998). Common to

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both habitats are the strong gradients of biogeochemical parameters (Fig. 2).

Oxygenated surface water in the Gotland Deep is separated by a stable halocline from deeper waters at 60 to 70 m. This halocline prevents vertical mixing and results in long-lasting suboxic zones and sulfidic/anoxic conditions on top of the sediment.

5 However, recently even deep layers of the Gotland Deep were provided with oxygen ( $O_2$ ) because of several inflows of dense North Sea from summer 2002 till summer 2003 (Feistel et al., 2003a, b; Feistel et al., 2004). After disappearance of sulfide ( $S^{2-}$ ) in 2003, a newly developed redoxcline appeared during 2004 at a depth of 230 m where the concentration of nitrate ( $NO_3^-$ ) decreased and that of  $S^{2-}$  increased towards the sediment. In contrast, the sediment samples of the Bodden were anoxic below the first 10 2 mm. Sulfide appeared below a depth of 7 mm and accumulated at concentrations two orders of magnitude higher than in the water column. The accumulation of  $S^{2-}$  in the anoxic layers of both habitats suggests active sulfate reducing microbial communities.

#### 4.1 Denitrifier communities along vertical chemical gradients in the Baltic Sea water column and sediment

15 Terminal restriction fragment length polymorphism (T-RFLP) of *nirS* amplicons has been successfully used as a fingerprinting method to analyse denitrifier community structure (Braker et al., 2001; Castro-González et al., 2005; Hannig et al., 2006a). We focused on *nirS* as a functional marker gene to detect denitrifiers since amplification of 20 *nirK* was shown to fail occasionally for marine samples (Braker et al., 2000). Whether this reflects primer bias or is of ecological relevance remains unknown. Furthermore, the majority of studies on marine denitrifier communities had focused on *nirS*, thus making comparisons between Baltic and other marine systems based on this gene more reasonable.

25 T-RFLP analysis of *nirS* genes demonstrated partial overlaps between water and sediment communities but revealed also unexpected dissimilarities between communities despite comparable physico-chemical gradients in the water column and the sediment. While community profiles from the water column showed strong vertical

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shifts, particularly from suboxic to sulfidic conditions, this was not the case for the sediment community profiles (Fig. 3). Minor changes in *nirS*-type denitrifier, *Bacteria* and *Archaea* community composition despite strong small-scale biogeochemical gradients were also observed with depth in a sediment core collected at Puget Sound, WA (Braker et al., 2001). Sequence analysis of clones from the Bodden sediment core showed a comparably uniform community composition for the oxic, suboxic, and anoxic zone by grouping sequences in common subclusters (BSS Cluster A to F). This also agrees with *nirS* sequences retrieved from three layers of the Puget Sound sediment core clustering in a common Puget Sound cluster (Braker et al., 2000). In the Bodden, the low water level together with wind induced intermixture of the top sediment layers leads to a physical input of O<sub>2</sub> into the upper sediment. Furthermore, the sediment stratification and the distribution of porewater solutes (e.g. O<sub>2</sub>) can be influenced by bioturbation through feeding and locomotion activity of marine invertebrates. Thereby, O<sub>2</sub> is inserted into the anoxic sediment layers (Forster et al., 1995). After a bioturbation event, the observed chemical gradients, which are the result of differential metabolic activities of microbial communities in the sediment, are re-established much faster than gradients in bacterial community structure.

In contrast, the water column zonation of denitrifier communities is probably more stable stratified as each layer extends across a range of several meters. The highest level of diversity of cultivable denitrifying heterotrophic bacteria was found at the suboxic-sulfidic interface in the water column of the Gotland Deep (Brettar et al., 2001). This was not visible by T-RFLP analysis which revealed approximately similar numbers of T-RFs in the different layers. With a concurrent accumulation of NO<sub>3</sub><sup>-</sup> in the water column the community structure shifted with depth from 85 to 200 m depth (Figs. 2 and 3). However, the most pronounced shift occurred with the transition from the suboxic to the sulfidic zone. At the suboxic-sulfidic interface, with the frequent concurrence of NO<sub>3</sub><sup>-</sup> and H<sub>2</sub>S, autotrophic denitrification has been demonstrated (Brettar and Rheinheimer, 1991; Hannig et al., 2006b<sup>1</sup>). Our community profiles, e.g. the dominance of the 47-bp T-RF and the appearance of the 273-bp T-RF, indicate that specific planktonic

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denitrifiers are adapted to thrive under sulfidic conditions. Similar shifts in denitrifier community composition and the appearance of abundant T-RFs specific for the sulfidic zone were recently observed for the water column at one station in the vicinity of the Gotland Deep in August and October 2003 (Hannig et al., 2006a).

5 Two dominant T-RFs (36 and 111 bp) were found in all layers of the sediment and were identical to T-RFs dominating the oxygenated and the suboxic zones in the water column. The 273-bp T-RF seems to be indicative of denitrifier communities in sulfidic environments as it was also found in the respective zones in the water column. This T-RF may hint on a more general occurrence of organisms capable of autotrophic  
10 nitrate reduction and sulfide oxidation, such as *Thiomicrospira denitrificans*-like bacteria. These  $\epsilon$ -proteobacteria were identified as some of the dominant denitrifiers at the suboxic-sulfidic interface of the water column at the Gotland Deep (Labrenz et al., 2005; Brettar et al., 2006). Unfortunately, the *nirS* sequences of these organisms are not known so far and therefore this information was not available to be evaluated with  
15 our data.

#### 4.2 Phylogeny of *nirS* sequences from marine environments

Community profiles were studied by T-RFLP based on cleavage of *nirS* PCR products using a single restriction enzyme. This approach is suitable to analyse shifts in denitrifier communities along environmental gradients but may be limited due to the occurrence of identical T-RFs that correspond to sequences from different clusters within  
20 the *nirS* tree. A better resolution can be achieved by the use of different restriction enzymes (Osborn et al., 2006) or phylogenetic analysis of sequences from clones.

The phylogenetic tree (Fig. 4), containing most of the published *nirS* sequences from marine environments, showed that only few *nirS* sequences were similar to those from denitrifying isolates (e.g. in marine Cluster II: ASW Cluster VIII, 95.6 to 100% identical to *Pseudomonas aeruginosa*; ESP Cluster III, 93.7 to 96.9% identical to *Pseudomonas fluorescens*). Whether these genes were indeed derived from *Pseudomonas* spp. is unknown because a distribution of denitrification genes via horizontal gene  
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transfer among phylogenetically unrelated organisms is possible (Etchebehere and Tiedje, 2005). Therefore our results confirm that the marine environment is strongly dominated by diverse and novel *nirS*-type denitrifiers that are not cultured yet.

Most environmental *nirS* sequences grouped in separate subclusters according to the site from which they were obtained. Marine Cluster II consisted almost exclusively of water column sequences with four sequences from the Bodden sediment as the sister group. Furthermore, sequences of marine Cluster III were obtained from the water column of the Baltic Sea and the eastern South Pacific (ESP). The habitat, water column or sediment, obviously has a strong impact on denitrifier community composition. Nonetheless, there were also some interesting overlaps of denitrifier communities. Only within marine Cluster I, water column and sediment sequences grouped within the same subclusters (Fig. 5). Three of them harboured sequences from both sites of the Baltic Sea, covering sequences from all sediment depths but only from the oxygenated zone of the water column. This is not surprising since horizontal currents lead to constant water exchange within the upper layers of the Baltic. Further, within the shallow coastal areas a continuous exchange between sediments and overlying water occurs.

Interestingly, a group of sequences from all depths of the Bodden sediment, grouping in marine Cluster I, were closely related to *nirS* genes found in a sediment core from Puget Sound, WA. Sediments from both locations share similar features, i.e. denitrifier communities were stable with depth within the core, and electron acceptors O<sub>2</sub> and NO<sub>3</sub><sup>-</sup> were consumed within the first few millimetres. However, no S<sup>2-</sup> was detected at these depths in the core from Puget Sound (A. H. Devol, personal communication). Only little *nirS* sequence overlap in denitrifier communities was observed for sediments at Puget Sound and offshore Washington coast (Braker et al., 2000). This was attributed to the isolation of microbial communities by large distances and to the degree of degradation of the organic matter reaching the sediment. Distinct sediment characteristics at large geographic distances, differences in bottom topology at the metre scale and small scale (cm) differences due to meio- and macrofaunal abundance were shown to impact marine sediment denitrifier communities to an extent ranking in the

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order km>m>cm (Scala and Kerkhof, 2000). However, we conclude that in sediments with comparable environmental conditions similar *nirS*-type denitrifier communities can develop despite large geographic distances. Physico-chemical parameters (e.g. O<sub>2</sub>, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, PO<sub>4</sub><sup>-3</sup>, dissolved organic carbon, salinity) were shown to impact the composition and distribution of denitrifier communities (Liu et al., 2003; Taroncher-Oldenburg et al., 2003; Jayakumar et al., 2004; Castro-Gonzalés et al., 2005; Hannig et al., 2006a). The *nirS* tree (Fig. 4) reveals that similar environmental conditions (with respect to O<sub>2</sub>, NO<sub>3</sub><sup>-</sup>, and NO<sub>2</sub><sup>-</sup>) in the water column of the distantly located ESP and the ASW also seem to have triggered the development of similar communities (ESP Cluster I and ASW Cluster XI, within marine Cluster I).

In conclusion, from the data base obtained so far it seems that distinct marine *nirS*-type denitrifier communities occupy different ecological niches which are defined by the habitat, water column or sediment, shaped by the prevalent environmental conditions, and can be isolated by large geographic distances. *NirS* subgroups are mostly site-specific but overlap if communities are impacted by similar environmental conditions. It is evident that denitrifiers are widespread, occurring also in habitats in which denitrification is not possible, e.g. due to lack of NO<sub>3</sub><sup>-</sup>, presence of O<sub>2</sub> or high S<sup>2-</sup> concentrations. However, all the DNA-based studies focus on elucidating the genetic potential for denitrification but they do not reveal whether these communities are actively denitrifying or not. Most cultured denitrifiers grow preferentially as heterotrophs in the presence of O<sub>2</sub> but not as denitrifiers if possible. Other metabolic pathways must be active also in denitrifiers found deep in the sulfidic zone. Thus, the ability to denitrify is probably not the factor that exclusively selects for subgroups of denitrifier communities in the different habitats. We assume that the subclusters of closely related *nirS* sequences belong to the same bacterial taxa. These are adapted to a specific niche due to their set of genetic and phenotypic characters among which the capacity for denitrification is only one. Nothing is known yet about the phylogenetic affiliation and ecophysiology of the organisms from which the novel *nirS* sequence types from marine habitats were retrieved. Future studies should therefore aim to combine different approaches

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in order to gain further insights in the adaptation and regulation of denitrifying communities; for example, species-specific activity measurements and phylogenetic analysis, metagenomic libraries and cultivation-based studies.

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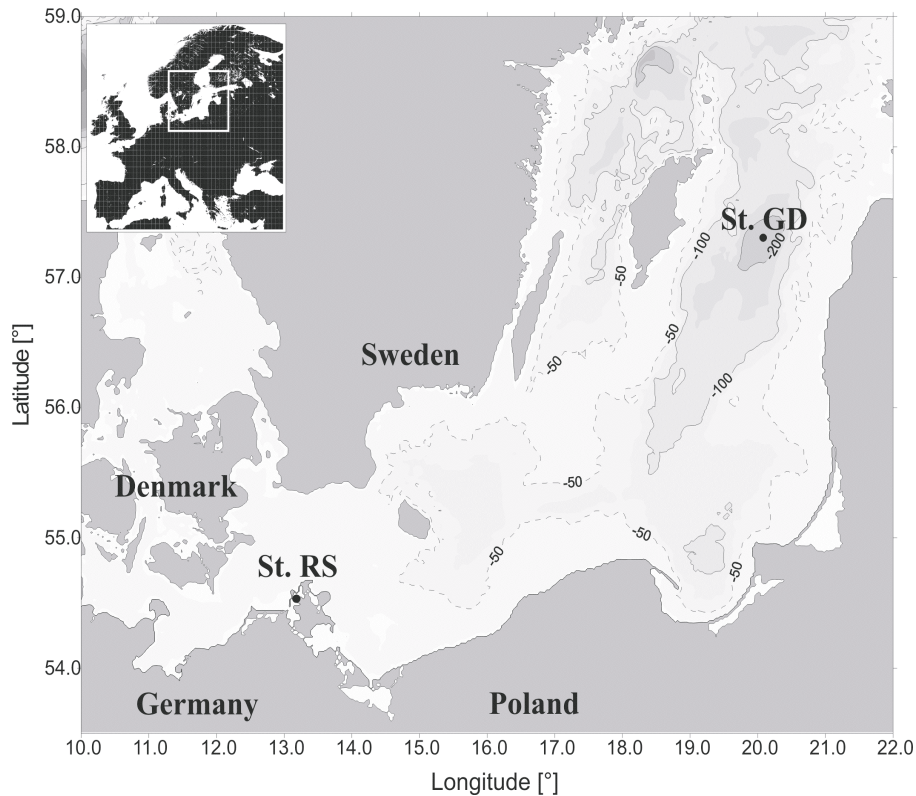
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**Fig. 1.** Sampling stations in the Baltic Sea. Station Rassower Strom (St. RS) in the Bodden next to the island of Rügen (Germany) and Gotland Deep (St. GD) in the central Baltic Sea (Map courtesy of Jan Donath, Baltic Sea Research Institute Warnemünde).

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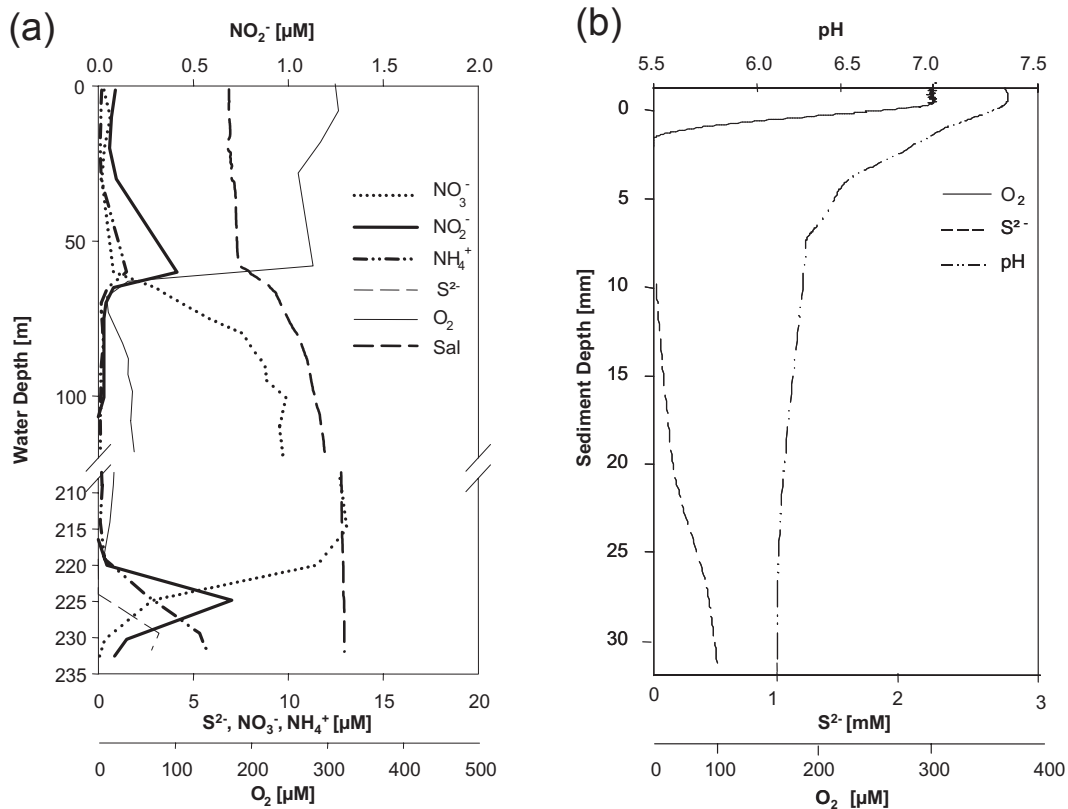
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**Fig. 2.** Physical and chemical parameters of sampling sites in the Baltic Sea; **(a)** water column in the Gotland Deep, **(b)** coastal sediment in the Bodden.

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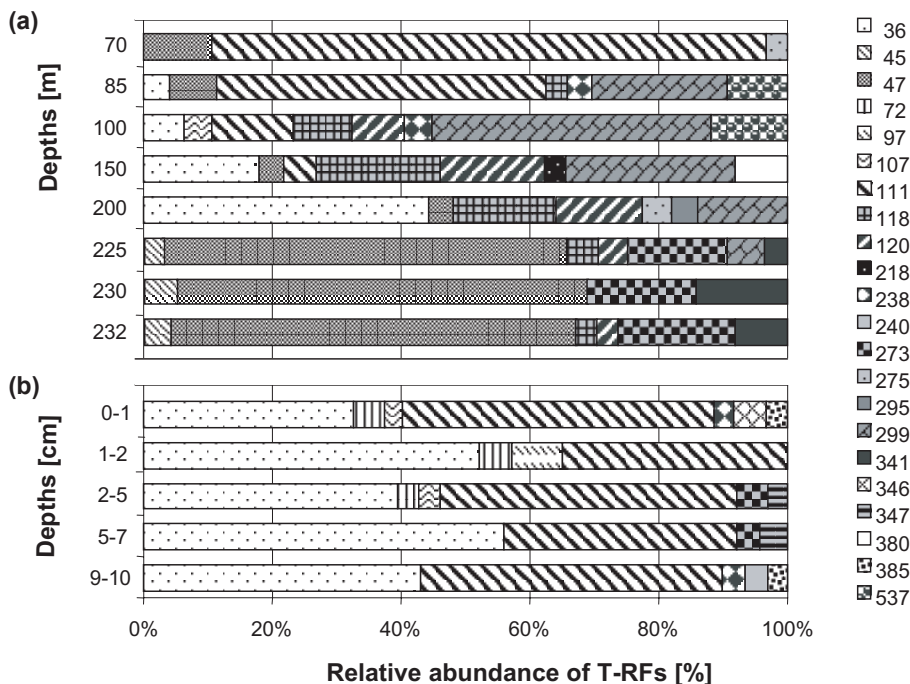
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**Fig. 3.** Relative abundance of terminal restriction fragments (T-RFs) of *nirS* amplicons. Numbers indicate the size of the fragment in base pairs after T-RFLP analysis. **(a)** samples from the water column at the Gotland Deep; **(b)**, samples from a sediment core taken in the Bodden.

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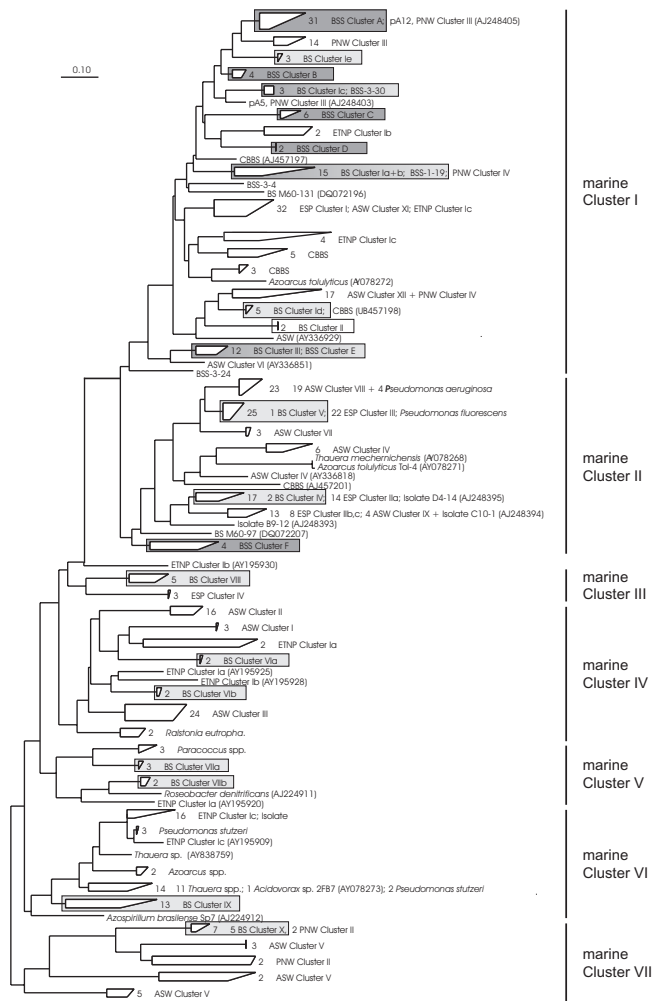


Fig. 4.

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**Fig. 4.** Phylogenetic tree of *nirS* sequences from all marine habitats. Tree was calculated by the neighbour joining method based on an alignment of 166 amino acids. Roman numbers indicate marine *nirS* sequence clusters. *NirS* sequences from sediment samples from the Baltic Sea were labelled with BSS. Clones were labelled with numbers to indicate from which depth of the sediment sequences were retrieved (1, 0 to 5 mm; 2, 5 to 12 mm; 3, 12 to 30 mm) and a second number for the respective clone. *NirS* clusters from Baltic Sea sediments were labelled with A to F. The number after the polygon indicates the number of clones of a given cluster. ASW, Arabian Sea water column; BS, Baltic Sea water column; CBBS, cyanobacterial bloom Baltic Sea; ESP, eastern South Pacific sediment; ETNP, eastern tropical North Pacific sediment; PNW, Pacific Northwest sediment.

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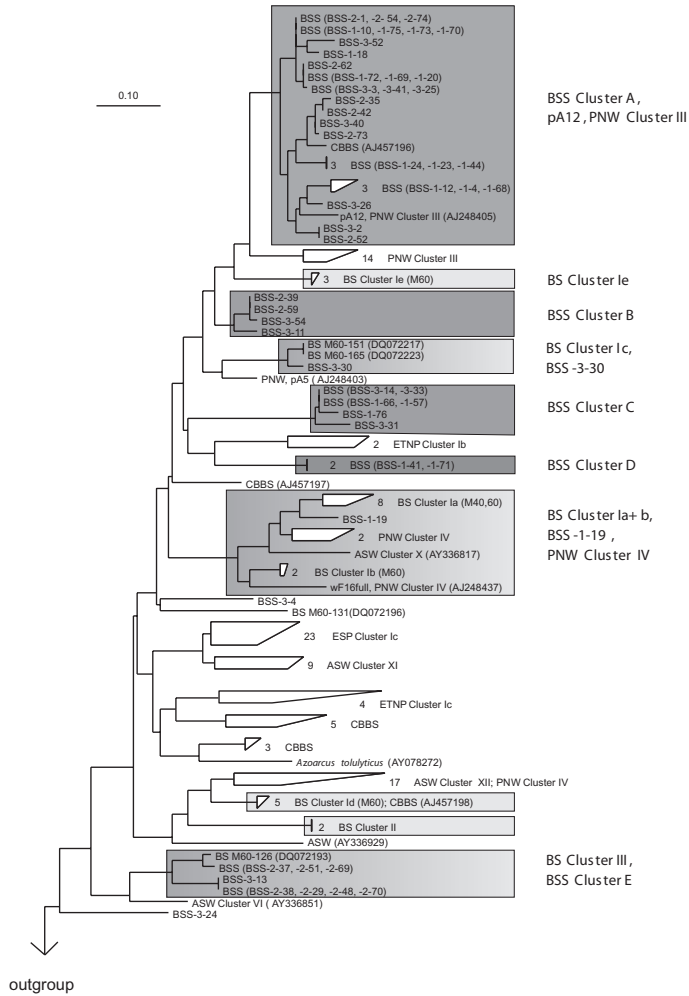


Fig. 5.

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**Fig. 5.** Phylogenetic tree of *nirS* sequences clustering in marine Cluster I. *NirS* sequences from sediment samples from the Baltic Sea were labelled with BSS. Clones were labelled with numbers to indicate from which depth of the sediment sequences were retrieved (1, 0 to 5 mm; 2, 5 to 12 mm; 3, 12 to 30 mm) and a second number for the respective clone. *NirS* clusters from Baltic Sea sediments were labelled with A to F. The number after the polygon indicates the number of clones of a given cluster. ASW, Arabian Sea water column; BS, Baltic Sea water column; CBBS, cyanobacterial bloom Baltic Sea; ESP, eastern South Pacific sediment; ETNP, eastern tropical North Pacific sediment; PNW, Pacific Northwest sediment.

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