RESEARCH ARTICLE

Diversity and spatio-temporal distribution of ammonia-oxidizing *Archaea* and *Bacteria* in sediments of the Westerschelde estuary

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Abstract

The diversity and spatio-temporal distribution of ammonia-oxidizing *Archaea* (AOA) and *Bacteria* (AOB) were investigated along a salinity gradient in sediments of the Westerschelde estuary. Sediment samples were collected from three sites with different salinities, and at six time points over the year. Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA and *amoA* gene fragments was used to identify the AOA and AOB present. Members of the AOA were mainly belonging to the *Crenarchaeota* Group 1, which were found at all sites, while members of the genus *Nitrosomonas*, which were abundant at the brackish sites, and of the genus *Nitrosospira*, which were present in early spring at the marine sites, were found to be the dominant AOB. Statistical analysis indicated that salinity and temperature were the main factors controlling the diversity and distribution of both AOA and AOB. Variability in net primary production rates was also correlated with species composition of both groups, but changes in the nitrite concentration only to the distribution of the AOA.

Introduction

Chemolithoautotrophic nitrifying bacteria are using the energy obtained from the oxidation of ammonia or nitrite for cellular functions, mainly carbon fixation and biomass production. The best-studied nitrifying organisms are the ammonia-oxidizing bacteria (AOB) that are involved in the first and rate-limiting step of the nitrification. Their detection has been mainly performed using the 16S rRNA and *amoA* (ammonia monooxygenase subunit A) gene specific to AOB. They are affiliated to the beta- and gamma subdivision of the *Proteobacteria* (Head *et al.*, 1993; Purkhold *et al.*, 2000). Because they are difficult to cultivate, little is known about their ecology and physiology (Urakawa *et al.*, 2006). Another group of organisms that has recently been demonstrated to perform ammonia oxidation are members of the *Crenarchaeota*. Indication of this physiological trait was mainly based on the detection of *amoA* genes in uncultivated archaeons (e.g. Venter *et al.*, 2004; Treusch *et al.*, 2005) and on the isolation of *Nitrosopumilus maritimus*, which could grow chemoautotrophically by oxidizing ammonia to nitrite (Konneke *et al.*, 2005). By performing nitrification, both ammonia-oxidizing *Archaea* (AOA) and AOB play an important role in the global nitrogen cycle, even to the extent that it could decrease the negative impact of eutrophication, when it is coupled to denitrification (Seitzinger, 1988). Because they could dominate the oxygen consumption and carbon fixation in ammonia-rich systems (Andersson *et al.*, 2006a), their processes are especially quantitatively important in the nitrogen and carbon budgets of estuaries (e.g. Soetaert & Hernan, 1995; Garnier *et al.*, 2001; Andersson *et al.*, 2006a).

Estuaries and their intertidal sediments experience large fluctuations in hydrological, morphological and chemical conditions; therefore, the nitrifyers that are present might experience large changes as well. Some species may be limited to a narrow habitat, while others may tolerate a much wider range of environmental conditions. For example, de Bie *et al.* (2001) showed that different communities of AOB appeared at different salinities in the water column of the Westerschelde estuary. In a recent study, Bernhard *et al.* (2007) showed the presence of functionally distinct communities of AOB along a salinity gradient in a New England estuary. Although there are no detailed studies on the dynamics of AOA in estuaries, Beman & Francis (2006) suggested that AOA exhibited consistent spatial

structuring. Besides their distribution along the gradients in the estuary, organisms in the sediment also show distinct seasonal variations, because of changes in e.g. temperature, irradiance, river discharge, etc. The effect of environmental factors might also be related with their way of living, i.e. free-living or attached. For example, it has been shown that if AOB are particle-attached, their residence time in the estuary would increase and concomitantly they have more time to develop their population size, however, they would be exposed more often to resuspension and settling (Brion *et al.*, 2000; Andersson *et al.*, 2006b).

So far, there are only a few publications describing the dynamics of AOB (e.g. de Bie *et al.*, 2001; Bollmann & Laanbroek, 2002) and very little is known about the factors controlling the diversity and distribution of AOA and AOB. In this paper, we have studied the diversity and spatio-temporal distribution of both AOA and AOB in sediments of the Westerschelde estuary. Samples from different sites along the salinity gradient were collected at different times of the year. Subsequently, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA and *amoA* gene fragments were used to determine the diversity and distribution of AOA and AOB. Statistical analysis was used to correlate these results with environmental parameters in order to identify those that determined the diversity and distribution of AOA and AOB.

Materials and methods

Sampling and chemical analysis

Three different intertidal sites in the Westerschelde estuary, Appelzak (A), Biezelingsche Ham (B), and Paulina Polder (P), with average salinities of 5.4‰, 17.6‰, and 20.4‰, respectively, were selected as study sites (Fig. 1). At each site a high (1) and mid-shore (2) station was sampled. The difference in emersion time between the high- and mid-shore stations ranged from 1.5 to 2.5 h. Because pilot experiments of samples from high- and mid-shore stations showed the same distribution patterns of microorganisms, only the results of the mid-shore stations were shown and discussed. Station positions were determined by Global Positioning System. Each location was sampled six times between February and September 2003. Sampling was done during low tide between 10 a.m. and 2 p.m. Information on the key physical and biological parameters at these sites can be found in Forster *et al.* (2006).

Surface sediments were collected with a contact corer (Ford & Honeywill, 2002). Mixed samples from five different replicates were used for DNA extraction, chlorophyll a (*chl a*), water content, and grain size analyses. Pore water was obtained by centrifugation (10 min at 700 ***g***). The supernatant was filtered over glass microfibre filters (Whatman grade GF/F) and frozen (− 20 °C).

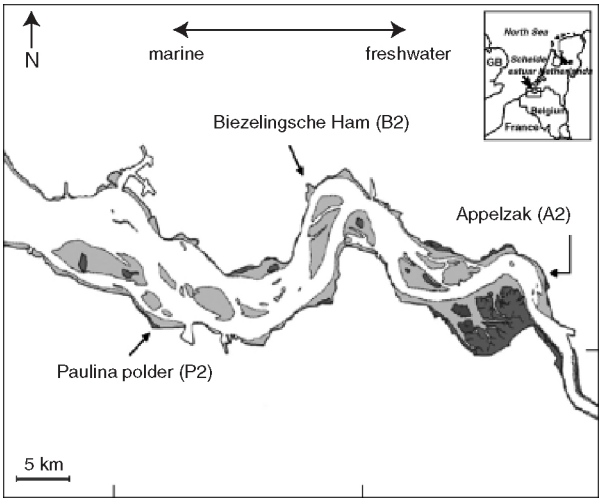

Fig. 1. Map of the sampling locations, Appelzak (a), Biezelingsche Ham (b) and Paulina Polder (P) in the Westerschelde estuary. The stations in each location are: middle shore A2 (51″23′ 00N; 004″14′ 19E), B2 (51″26′ 36N; 003″55′ 43E), and P2 (51″21′ 07N; 003″43′ 45E).

Sediment surface temperatures were measured with a digital thermometer during each sampling trip. The mean irradiance at all sites was recorded at hourly intervals using a Li-Cor Li-192 sensor (Li-Core Biosciences, Lincoln). Chl *a* was quantified using HPLC after acetone extraction (Rijstenbil, 2003). NO_2^-–N, NO_3^-–N, NH_4^+–N, and PO_4^{3-}–P were measured using standard colorimetric techniques (Grasshoff, 1976) on a Skalar SA 4000 segmented flow analyzer (Skalar Analytical B.V., Breda, the Netherlands). Salinity was measured using a titration method with SAC 80 (Radiometer, Copenhagen, Denmark). The percentage of water was determined by the loss of weight of the sample after 48 h of freeze drying. Total organic carbon (TOC) was measured with an elemental analyzer (Elementar, Vario EL). Sediment grain size and silt content of the sediment were determined by granulometric analysis using laser diffraction analyzer (Mastersizer 2000, Malvern Instruments Ltd, Worcestershire, UK). The information about the calculation of primary productivity from measured data can be found in detail in Forster *et al.* (2006).

DNA extraction and PCR amplification

DNA was extracted from 100 to 200 mg of the sediment sample using the Soil DNA Extraction Kit (Mo Bio Laboratories Inc., Carlsbad) combined with vortexing at maximum speed for 10 min. PCR was carried out in a Thermocycler (Biometra, Goettingen, Germany). In order to detect AOB, two different primer sets were used: (i) amoA-1F-GC (5′-GC-clamp- GGG GTT TCT ACT GGT GGT-3′)/amoA-2R-TC

(5′-CCC CTC TGC AAA GCC TTC TTC-3′) targeting 490 bp of the ORF of ammonia monooxygenase subunit A (*amoA*) gene (Rotthauwe *et al.*, 1997; Nicolaisen & Ramsing, 2002), and (ii) CTO189f-GC (5′-GC-clamp-GGA GRA AAG YAG GGG ATC G-3′)/CTO654r 5′-CTA GCY TTG TAG TTT CAA ACG C-3′) amplifying a 465-bp fragment of the 16S rRNA gene from *Proteobacteria* β-subgroup AOB (Kowalchuk *et al.*, 1997). AOA were detected using the primer set (i) amoA-23F (5′-ATG GTC TGG CTW AGA CG-3′)/amoA-616R (5′-GCC ATC CAT CTG TAT GTC CA3′), which targets the *amoA* gene of AOA (G. Nicol, pers. commun.). In addition, we used the primer set PARCH519F (5′-CAG CCG CCG CGG TAA-3′)/ARCH915R-GC (5′-GC-clamp- GTG CTC CCC CGC CAA TTC CT-3′) to target the 16S rRNA gene of *Archaea* (Coolen *et al.*, 2004), including those that might be involved in ammonia oxidation.

All amplification reactions (50 μL) contained 10–20 ng genomic DNA, determined with the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington), as template, each primer at a concentration of 0.5 μM and PCR buffer supplied with enzyme (2.5 U), dNTPs (200 μM) and $MgCl_2$ (1.5 mM). PCR conditions were similar to those described by Kowalchuk *et al.* (1997) Rotthauwe *et al.* (1997), and Coolen *et al.* (2004). The archaeal-*amoA* gene fragments were amplified using the following protocol: an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min.

DGGE and phylogenetic analysis

For DGGE, 6% polyacrylamide gels with a denaturant gradient of 30–70% for the bacterial *amoA* and 16S rRNA gene PCR products, of 25–65% for the archaeal 16S rRNA gene, and of 15–55% for the archaeal *amoA* gene fragments were used. Approximately 300 ng of PCR product, estimated by comparison with the amount of the molecular weight DNA marker, were applied to each well. Electrophoresis was run at a constant voltage of 100 V for 16 h at 60 °C. Subsequently, the gels were incubated for 30 min in a solution containing ethidium bromide (0.5 μg mL^{-1}), rinsed for 30 min in water, and photographed under UV illumination using the Gel Doc 1000 System (Bio-Rad Laboratories, Hercules).

Major bands were excised from the gel with a sterile scalpel, and incubated overnight at 4 °C in 50 μL nucleic acid-free water to extract the DNA from the gel slice. One or two microliters of the extract was used as template for reamplification. The PCR products were checked for single bands on DGGE and subsequently sequenced with one of the primers by a commercial company (BaseClear, Leiden, the Netherlands).

For phylogenetic analysis, known sequences of bacterial and archaeal *amoA* were obtained from GenBank. Alignments of *amoA* DNA sequences were done using the software program MEGA3 (Kumar *et al.*, 2004). Trees based on 16S rRNA gene sequences (*c.* 350 nucleotides for AOA and 420 nucleotides for AOB) were created with the software program ARB (Ludwig *et al.*, 2004) using the Neighbour Joining algorithm with automatic correction (i.e. Olsen or Felstenstein). Gaps were introduced to align the sequences, but excluded from the calculations.

DGGE profiles were analyzed using Phoretix 1D Pro (Nonlinear Dynamics, Newcastle upon Tyne, UK) in order to obtain a matrix containing the band percentage values of each sample as described by Massieux *et al.* (2004). The obtained data matrix was used for statistical analysis. In most cases the same amount of PCR product was loaded onto the gel. However, sometimes the results of different gels had to be used. To facilitate gel-to-gel comparison, the same samples were ran on the different gels. The detection limit of the bands was determined manually with the PHORETIX software according to the faintest band on the gel. An adjustment of the total band intensity for each lane was performed according to the lane with the lowest DNA concentration.

Statistical analysis

All data analyses on the DGGE profiles and environmental variables were performed using the software program PRIMER 6 (Clarke & Gorley, 2006). The similarity of DGGE profiles was determined using the Bray–Curtis index of similarity after square root transformation. A similarity matrix prepared for all sites was used to make a dendogram, using weighted-group average linkage in cluster analysis and nonmetric multidimensional scaling (MDS) plots. MDS is an ordination technique that represents the samples as points in a two-dimensional space. The relative distances between the points are in the same rank order as the relative similarities of the samples, i.e. points that are close together represents samples that are very similar in community composition.

The BIO-ENV procedure in the software package PRIMER 6 (Primer-E Ltd, Plymouth, UK) was used to relate main environmental factors to bacterial communities. This analysis consists of the similarity matrix obtained from DGGE profiles to the resulting matrix of Euclidean distances obtained after normalization of the environmental data. The analysis allowed us to obtain the variables with the greatest correlations (weighted Spearman rank correlations). PRIMER 6 (Primer-E Ltd) was also used to calculate species richness (total number of species: S) and the Shannon index (H′), (Magurran, 1988) from the DGGE values.

Nucleotide sequence accession numbers

The DNA sequences determined in this study were deposited in the GenBank database under the accession numbers EF551005–EF551039.

Results

Environmental parameters

Pore water salinity fluctuated between February and September 2003 at all three locations (2–17‰ at A2 (the mid-shore station of Appelzak), 14–25‰ at B2 (the mid-shore station at Biezelingsche Ham), and 19–29‰ at P2 (the mid-shore station of Paulina Polder) (Table 1). The salinity was the lowest in early spring and the highest in late summer at all stations. Similarly, the surface temperature peaked at 22 °C between July and September 2003, and was the lowest (4 °C) in February 2003 at all locations. The average tidal exposure was 12 h day^{-1}. Sediments were relatively homogeneous at all sites. They contained a large proportion of silty particles (average of 63%) and had an average median grain size of about 49 μm and high water content (average of 52%). The TOC, chl *a*, NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} peaked in spring, usually in April at A2 and B2, and in May–July at P2. The NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} ranged between 25 and 366 μM, 1–11, 48–443 and 7–115 μM, respectively. The nutrients showed a trend with an increase towards the estuary mouth. The chl *a* and TOC values were higher at A2 with an average value of 83 mg m^{-2} and 3%, respectively, than B2 and P2 with concentrations of 48–49 mg m^{-2} and 1%, respectively. The net primary productivity ranged between 159 and 356 mg C m^{-2} day at all sites which followed the trend of being the lowest in the early spring and the highest in the late summer (Table 1).

Diversity and distribution of AOA

DGGE analysis of DNA fragments obtained with primers targeting the 16S rRNA genes of *Archaea* showed between 7 and 15 bands (Fig. 2a). Six bands were excised from the DGGE profiles and sequenced. All sequences grouped with sequences of the marine *Crenarchaeota* Group 1 (Fig. 2b); bands 1, 2, and 6 belonged to group 1.1a, while bands 3, 4, and 5 were related to group 1.1b, which mainly consisted of sequences obtained from soil samples. Sequences of the different bands that were excised from the same position, but in different lanes gave the same sequence. Only dominant bands could be sequenced successfully, so other *Archaea* might also be present although in lower numbers.

DGGE analysis of *amoA* gene fragments obtained by PCR amplification with primers specific for archaeal *amoA* sequences, was used to identify *Archaea* that have the potential to oxidize ammonia. Ten bands, as indicated in Fig. 3a, were excised and sequenced. Bands at the same position in the gel, but from different lanes gave the same sequences. Figure 3b shows the phylogenetic affiliation; sequences obtained from bands 1, 2, 5, 6, 8, and 9 grouped

Table 1. Overview of the environmental parameters of the different sites in the Westerschelde estuary*

Sites†	Sampling time	Salinity (%)	T (°C)	Exposure time (h day^{-1})	Water content (%)	MGS (μm)	Silt content (%)	NH_4^+ (μM)	NO_2^- (μM)	NO_3^- (μM)	PO_4^- (μM)	TOC (%)	NPP (mg C m^{-2} day^{-1})	Chl a (mg m^{-2})
A2	Feb	2	4	12	47	50	62	25	3	296	10	1	−9	12
	Mar	5	10	14	70	41	70	51	1	143	21	3	−146	87
	Apr	11	10	10	75	24	87	322	8	199	64	4	−159	292
	May	12	16	13	58	39	70	159	5	48	42	1	113	63
	July	14	22	13	50	47	63	74	1	104	37	4	60	21
	Sept	17	20	13	58	44	68	52	1	48	18	2	136	24
B2	Feb	14	4	11	44	77	41	41	2	146	7	0	−2	4
	Mar	16	9	12	55	45	150	150	1	188	27	2	−14	72
	Apr	20	10	11	66	31	366	366	3	321	115	2	172	127
	May	23	16	11	51	29	185	185	11	104	23	2	37	34
	July	23	21	11	55	34	133	133	4	54	28	–	–	37
	Sept	25	21	12	48	56	100	100	2	160	15	1	114	22
P2	Feb	19	5	12	31	98	43	43	8	80	11	0	11	5
	Mar	18	9	14	39	53	175	175	1	86	32	1	−59	39
	Apr	23	10	12	36	76	311	311	2	429	38	0	130	83
	May	25	15	10	54	41	212	212	3	442	112	2	49	110
	July	28	20	12	50	36	233	233	11	83	56	1	103	34
	Sept	29	22	14	44	52	193	193	5	86	20	0	356	17

*Data are from Forster *et al.* (2006).

†A2, mid-shore station at Appelzak; B2, mid-shore station at Biezelingse Ham; P2, mid-shore station at Paulina Polder.

T, temperature; MGS, medium grain size; NPP, netto primary production.

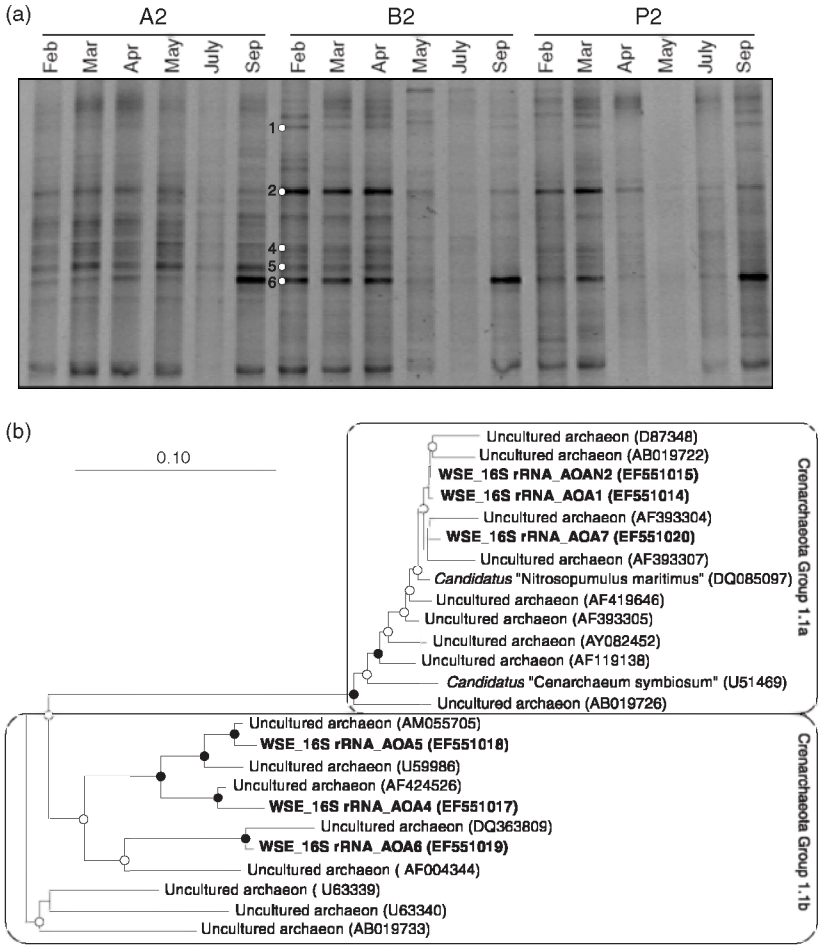

Fig. 2. (a) DGGE patterns of 16S rRNA gene fragments obtained after enzymatic amplification with primers specific for all *Archaea* and genomic DNA extracted from sediment samples collected from the mid shore of the sites A, B and P (see Fig. 1) between February and September 2003. (b) Neighbor-joining tree showing the phylogenetic affiliations of *Archaea* derived from the sequences of the excised DGGE bands shown in (a). The sequences obtained in this study are printed bold. Accession numbers are in parentheses. The bar represents 10% sequence difference. Black circles represent bootstrap values (1000 replicates) between 90% and 100%; open circles represent values from 50% to 90%. The sequences of *Sulfolobus acidocaldarius* (D14876), *Acidianus ambivalens* (X90484), *Pyrolobus fumari* (X99555), and *Thermofilum pendens* (X14835) were used as outgroup, but pruned from the tree.

into cluster A, while the sequences of the other bands grouped into cluster B (clusters names according to Beman & Francis, 2006). Bands from cluster A were related with the sequences of San Francisco Bay (SF), Elkhorn Slough (ES), and Huntington Beach (HB). Bands 3 and 4 were similar to the sequences obtained from the soils (e.g. Grass 2, German soil and Alpine 1), and band 7 in cluster B was identical to the sequence from Jasper Ridge soil (JS).

Statistical analysis of the DGGE data showed, at a level of 55% similarity, two clusters, i.e. one cluster consisting of all samples collected in February, March, and April (including the May samples at A2), and a second cluster consisting of samples collected in May, July, and September (Fig. 4a). At a level of 60% similarity, however, the A2 samples collected in early spring were clearly separated from the B2 and P2 samples. In the samples collected in May, July, and September, only those collected in September grouped separately. Salinity, temperature, net primary productivity and nitrite concentration were the environmental parameters that could best explain the clustering of these species at all sites ($\rho = 0.59$, $P < 0.01$). Figure 4b–e shows the relationship of AOA community composition with strongly correlated environmental data in a two-dimensional plot. For example, samples taken in early spring, when salinity or temperature was low, clustered together.

The number of DGGE bands, as an estimate of 'richness' (S), ranged between 7 and 15, while the Shannon–Weaver index H′ (an indicator of 'diversity') fluctuated between 1.83 and 2.64. Salinity and temperature were the factors best correlating with diversity.

Diversity and distribution of AOB

DGGE analysis of DNA fragments obtained with primers specific for the 16S rRNA gene of AOB showed between 5 and 10 bands. Nine bands were excised from the DGGE profiles, sequenced and used for phylogenetic analysis (Fig. 5). Sequences of bands excised from the upper part of the gel showed high similarity with sequences of members of the genus *Nitrosomonas*; bands 1 and 2, which were abundant at the brackish site of the estuary, were affiliated to members of the *N. oligotropha* and *N. urea* cluster. Band 4, that was present at all salinities, although most abundant at the brackish site, was affiliated to members of cluster *N. marina*

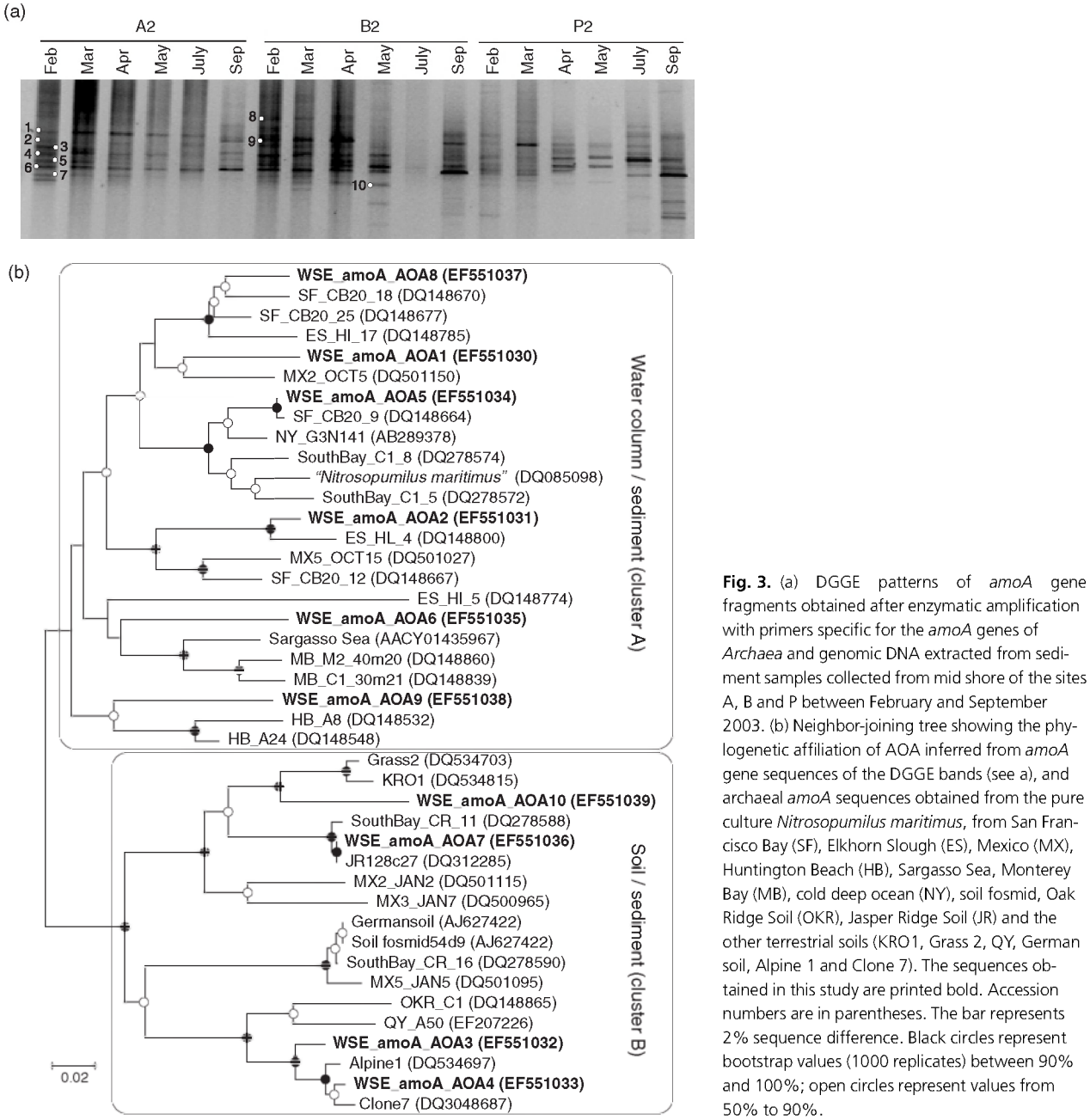

Fig. 3. (a) DGGE patterns of *amoA* gene fragments obtained after enzymatic amplification with primers specific for the *amoA* genes of *Archaea* and genomic DNA extracted from sediment samples collected from mid shore of the sites A, B and P between February and September 2003. (b) Neighbor-joining tree showing the phylogenetic affiliation of AOA inferred from *amoA* gene sequences of the DGGE bands (see a), and archaeal *amoA* sequences obtained from the pure culture *Nitrosopumilus maritimus*, from San Francisco Bay (SF), Elkhorn Slough (ES), Mexico (MX), Huntington Beach (HB), Sargasso Sea, Monterey Bay (MB), cold deep ocean (NY), soil fosmid, Oak Ridge Soil (OKR), Jasper Ridge Soil (JR) and the other terrestrial soils (KRO1, Grass 2, QY, German soil, Alpine 1 and Clone 7). The sequences obtained in this study are printed bold. Accession numbers are in parentheses. The bar represents 2% sequence difference. Black circles represent bootstrap values (1000 replicates) between 90% and 100%; open circles represent values from 50% to 90%.

like-bacteria, while bands 3, 5 and 6 grouped with *N. europae*/*N. mobilis*-like bacteria. Band 3 was most abundant in the summer at all sites, while bands 5 and 6 were only present as a small fraction of the community at all sites. Bands 7, 8 and 9, which appeared in early spring at high salinity, were closely related to uncultured *Nitrosospira*-like bacteria (Fig. 5b).

Statistical analysis indicated that at a level of 60% similarity, two clusters were formed, one cluster consisting of samples collected in the February, March, and April (except P2-April) and another cluster consisting of samples collected in May, June, and September (Fig. 6a). At a level of 70% similarity, however, the samples collected at A2 during February, March and April grouped separately from those collected at sites B2 and P2 in the same period. But the samples of A2 and B2 collected during May, July, and September all grouped together. The bands that determined this clustering were band 3, *Nitrosomonas halophila*, which started to be dominant especially in May, July and September (except at P2), and band 7, 8, 9, affiliated to members of the genus *Nitrosospira*, that were abundant at B2 and P2 during February, March and April. The variations in species

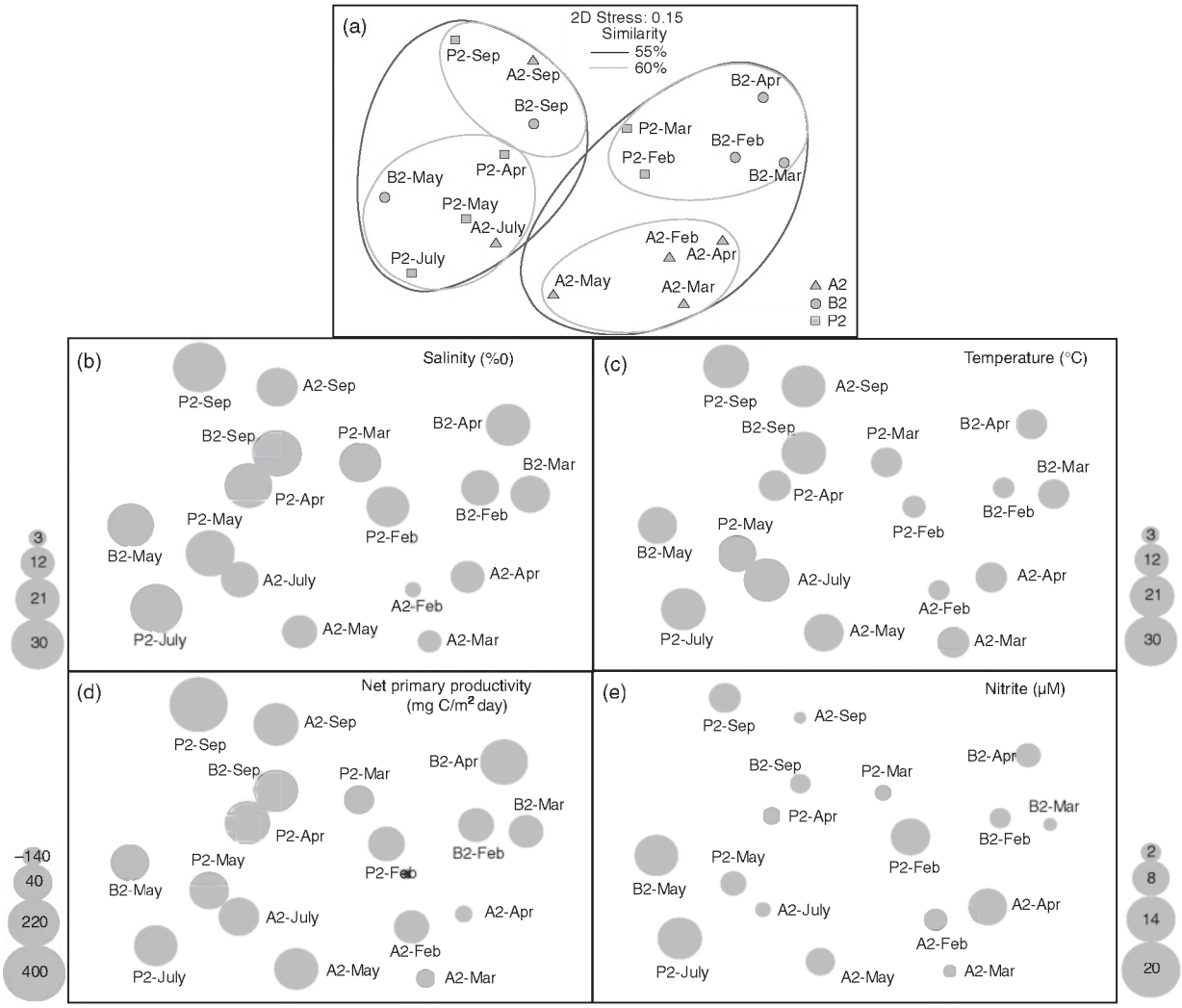

Fig. 4. Multi-Dimensional Scaling (MDS) ordination with cluster analysis (a), and with superimposed circles, which area is proportional to the selected environmental parameters (b–e) obtained from the DGGE gels of *amoA* gene fragments specific to AOA in the samples of A2, B2 and P2 between February and September 2003.

composition of AOB was significantly correlated and best explained by changes in salinity, temperature and net primary productivity ($\rho = 0.73$, $P < 0.01$) (Fig. 6b–d).

The number of the DGGE bands, as an indicator of richness (S), ranged from 5 to 10, and the diversity (H′) between 1.22 and 1.83. S and H′ were the highest at all sites in February, March, and April, when the salinity and temperature were relatively low.

Nine *amoA* gene fragments were excised from the gel representing all sites in March and April, and sequenced (Fig. 7a). Bands 1, 2, 3 and 4 were related to *Nitrosomonas nitrosa*, and bands 5, 6, 7, 8 and 9 were related to members of the genus *Nitrosospira* (Fig. 7b). From the profiles, it was obvious that the abundance of *N. nitrosa* was specific to the brackish site A2 in March and April, and of genus *Nitrosospira* to the marine site P2. Although *N. nitrosa* was present in B2 in March, still the dominant species was a member of genus *Nitrosospira* in April. The result indicated that salinity played a primary role in determining the species distribution in the estuary. The same DGGE pattern was observed throughout the six sampling times at A2. For B2 and P2, February samples showed the same pattern as the March and April samples. Amplification of *amoA* genes from samples B2 and P2 collected in May, June, and September failed.

Discussion

Diversity and distribution of AOA

Statistical analysis derived from *amoA* gene indicated that salinity, temperature, nitrite concentration, and net primary productivity rates produced major effects on the

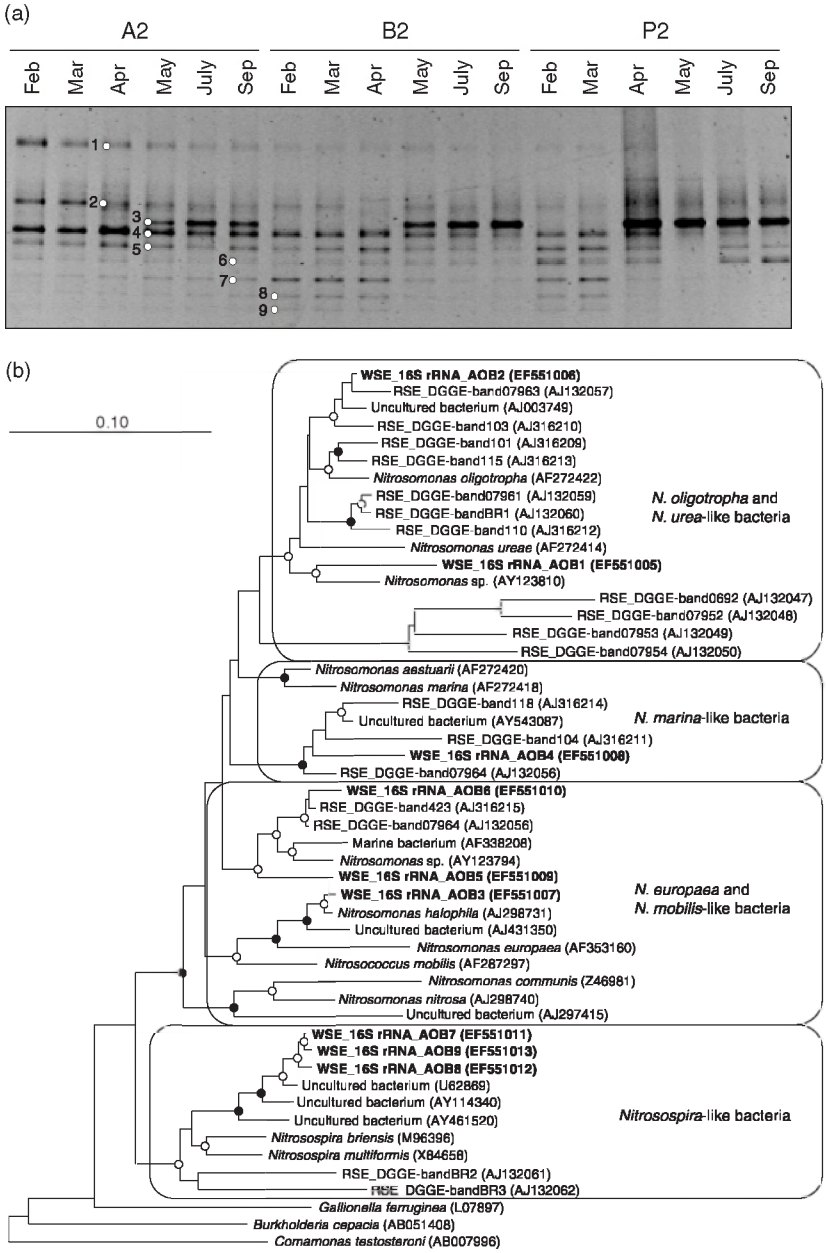

Fig. 5. (a) DGGE patterns of 16S rRNA gene fragments obtained after enzymatic amplification with primers specific for the 16S rRNA genes of all AOB, and the genomic DNA extracted from sediment samples collected from mid shore of sites A, B and P (see Fig. 1) between February and September 2003. (b) Neighbor-joining tree showing the phylogenetic affiliations of AOB inferred from sequences of the excised DGGE bands shown in (a). The sequences obtained in this study are printed bold. Accession numbers are in parentheses. The bar represents 10% sequence difference. Black circles represent bootstrap values (1000 replicates) between 90% and 100%; open circles represent values from 50% to 90%. The sequences of *Gallionella ferruginea, Burkholderia cepacia*, and *Comamonas testosteroni* were used as outgroup.

distribution of the AOA. It is obvious that variations in temperature separated the AOA communities from samples collected in February, March, and April, from those collected in May, July, and September. In early spring, the variations in salinity were very large to the extent that brackish sites were clustering differently than marine sites. The spatial variability of AOA has also been shown in the studies of Francis *et al.* (2005) and Beman & Francis (2006). However, in our study, there is another factor playing a role in separating the samples collected in September from other samples, since the variations in salinity and temperature were not big enough to cause any effect. The net primary productivity rates increased almost triple in September although chl *a* concentration continued to decrease after blooming in April. Interestingly, the marine *Crenarchaeota* in the water columns have been shown to increase after blooming and negatively correlated with chl *a* concentration (Murray *et al.*, 1998). Although it has been hypothesized by Konneke *et al.* (2005) that addition of organic matter and low concentrations of ammonium might limit the growth of marine *Crenarchaeota*, there is not enough evidence to prove this. In our study, nitrite has also been found as one of the parameters, which might cause differences in AOA distribution, however no effect of inorganic nitrogen species on the

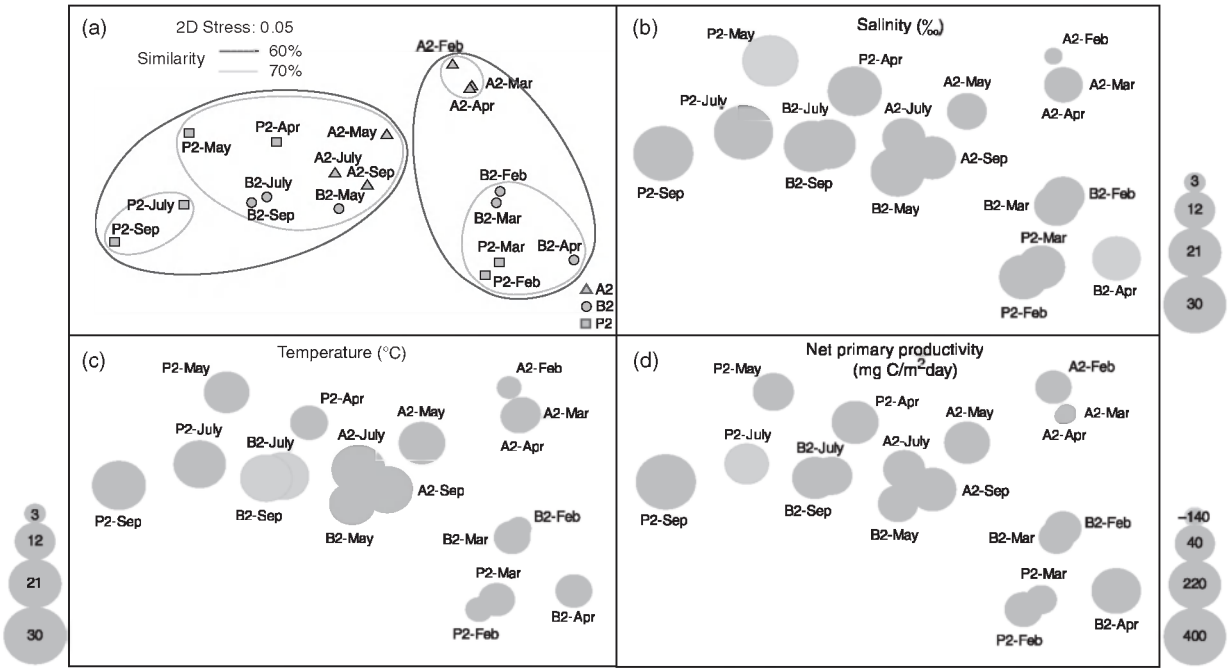

Fig. 6. Multi-Dimensional Scaling (MDS) ordination with cluster analysis (a) and with superimposed circles which area is proportional to selected environmental parameters (b–d), obtained from the data of DGGE gels of 16S rRNA gene products specific to AOB of the *Betaproteobacteria* in the samples of A2, B2 and P2 between February and September 2003.

AOB was observed. Similar to the study of Murray *et al.* (1999) in the Santa Barbara channel, Wuchter *et al.* (2006) showed in an incubation experiment with samples from the North Sea, that the abundance of marine *Crenarchaeota* correlated positively with nitrite, but, that the abundance of *Beta* and *Gammaproteobacteria* was not influenced. Although, a possible toxic effect of a high nitrite concentration on *Crenarchaeota* has also been observed (Wuchter *et al.*, 2006).

Diversity and distribution of AOB

Comparative sequence analysis of AOB gave nine different sequences with 16S rRNA gene analysis and only two types of AOB with the *amoA* analysis. The two types were *N. nitrosa*, specific to the brackish site, and one member of the genus *Nitrosospira*, abundant at the marine site. With the 16S rRNA gene analysis, three sequences grouped in the cluster of *N. europae*/*N. mobilis*-like bacteria including *N. nitrosa*. However, the distributional patterns of these sequences obtained via two primers were different. In addition, although there were three different *Nitrosospira* sequences with 16S rRNA gene analysis, the amplification with *amoA* primer gave five sequences, that were within one cluster in the phylogenetic tree. It might be related to an organism with five different operons. In the 16S rRNA gene analysis, the three different bands were also closely related in the phylogenetic tree. Interestingly, although *N. halophila* became abundant after April within the 16S rRNA gene analysis, the analysis of *amoA* gene did not detect this organism, which might be due to the limited specificity of the primers (Ivanova *et al.*, 2000).

While 16S rRNA gene analysis indicates the effect of temperature, salinity and net primary productivity, *amoA* results show only the effect of salinity on the distribution of AOB. Bernhard *et al.* (2005) showed a similar result with *amoA* analysis, whereby salinity was considered as the only stress factor, that selects a narrow range of best-adapted AOB. In a recent study, however, Bernhard *et al.* (2007) showed that salinity was an important factor, but not the primary factor in determining the distribution of AOB. Using 16S rRNA gene as a molecular marker, we found similar sequences of salinity- and temperature-tolerant AOB as was found by de Bie *et al.* (2001) and Bollmann & Laanbroek (2002).

There are only a few studies showing the impact of temperature on the community structure of AOB. Similar to our results, Avrahami *et al.* (2003) demonstrated that *amoA* sequences belonging to the *Nitrosospira* cluster were dominant at low temperature (4–10 °C) in incubated soil samples and disappeared completely at high temperature (30 °C). In the study of Coci *et al.* (2005) there was a change in community structure observed with 16S rRNA gene and a decrease in nitrification rate after 1 week of incubation of the sediment from the Westerschelde. They suggested that temperature might explain these changes. It is known that

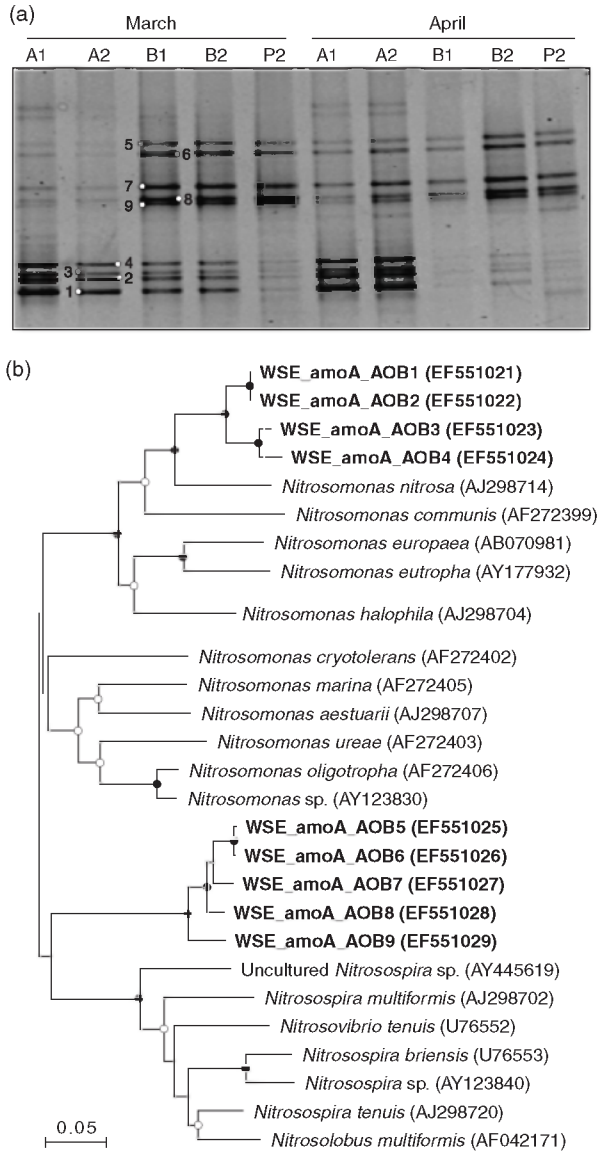

Fig. 7. (a) DGGE patterns of *amoA* gene fragments obtained after enzymatic amplification with primers specific for the *amoA* genes of AOB and genomic DNA extracted from sediment samples collected from the high- (1) and mid- shore (2) of sites A, B and P in March and April 2003. (b) Neighbor-joining tree showing the phylogenetic affiliation of AOB inferred from *amoA* gene sequences of the DGGE bands shown in (a). The sequences obtained in this study are printed in bold. Accession numbers are in parentheses. The bar represents 5% sequence difference. Black circles represent bootstrap values (1000 replicates) between 90% and 100%; open circles represent values from 50% to 90%. The sequence of *Nitrosococcus oceanus* (U96611) was used as outgroup, but pruned from the tree.

nitrification might vary with season, temperature, as well as with substrate level, oxygen, pH and salinity (e.g. Kemp & Dodds, 2002; Andersson *et al.*, 2006a). Although in this study there was no measurement of nitrification rates, the other parallel studies about the Westerschelde showed that, for example, a high nitrification rate was observed in the winter of 2003, since the decrease of the temperature could induce bacteria to increase their substrate needs for optimal growth (Andersson *et al.*, 2006b). These changes in the rate and growth might be reflected in the composition and diversity of the organisms as observed in our study.

Our study shows the coexistence of AOA and AOB in sediments of the Westerschelde estuary. These two groups of organisms might play an important role in the nitrification. However, the contribution of each group to the nitrification process is unknown and needs further study. Although there is no evidence of large numbers of nitrifying bacteria in marine waters, marine *Crenarchaota* constitute up to 20–30% of the total prokaryotic community (Karner *et al.*, 2001). A higher abundance of AOA in sediments, in comparison with AOB, have been suggested by Beman & Francis (2006) and Francis *et al.* (2005). In our study, spatial and temporal patterns of these nitrifying organisms and the main factors that influence their diversity and distribution were found to be similar in the sediments of Westerschelde estuary. While variability in salinity caused the spatial distribution, temperature was the primary seasonal factor affecting the diversity and distribution of the AOA and AOB. However, the correlation analysis of 16S rRNA and *amoA* gene profiles of both groups indicates that AOB show a more distinct and greater temporal and spatial distribution in community structure, which might be due to their response to temperature and salinity. The variability in the net primary productivity caused also changes in the distribution of both groups. Because environmental parameters covary, it is difficult to pinpoint only one environmental parameter responsible for the distribution of AOA and AOB. Because nitrite was also found as one of the parameters, causing differences in AOA distribution, but not in AOB, it might be a determining factor in the niche differentiation between AOA and AOB.

Because of the large number of samples, PCR-DGGE was chosen over other techniques to analyze the diversity of AOA and AOB. However, we are aware of the possible limitations of this technique (e.g. Cilia *et al.*, 1996; Kowalchuk *et al.*, 1997). Future studies on the niche differentiation of the nitrifying bacteria and archaea in the Westerschelde estuary should include other techniques, such as quantitative PCR, stable isotope probing and measurements of nitrifying activity.

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