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# Molecular evidence for anaerobic ammonium–oxidizing (anammox) bacteria in continental shelf and slope sediments off northwest Africa

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*Abstract*

The presence and abundance of anaerobic ammonium–oxidizing (anammox) bacteria was investigated in continental shelf and slope sediments (300–3000 m water depth) off northwest Africa in a combined approach applying quantitative polymerase chain reaction (q-PCR) analysis of anammox-specific 16S rRNA genes and anammox-specific ladderane biomarker lipids. We used the presence of an intact ladderane monoether lipid with a phosphocholine (PC) headgroup as a direct indicator for living anammox bacteria and compared it with the abundance of ladderane core lipids derived from both living and dead bacterial biomass. All investigated sediments contained ladderane lipids, both intact and core lipids, in agreement with the presence of anammox-specific 16S rRNA gene copies, indicating that anammox occurs at all sites. Concentrations of ladderane core lipids in core top sediments varied between 0.3 and 97 ng $g^{-1}$ sediment, with the highest concentrations detected at the sites located on the shelf at shallower water depths between 300 and 500 m. In contrast, the $C_{20}$ [3]-ladderane monoether-PC lipid was most abundant in a core top sediment from 1500 m water depth. Both anammox-specific 16S rRNA gene copy numbers and the concentration of the $C_{20}$ [3]-ladderane monoether-PC lipid increased downcore in sediments located at greater water depths, showing highest concentrations of $1.2 \times 10^8$ copies $g^{-1}$ sediment and 30 pg $g^{-1}$ sediment, respectively, at the deepest station of 3000 m water depth. This suggests that the relative abundance of anammox bacteria is higher in sediments at intermediate to deep water depths where carbon mineralization rates are lower but where anammox is probably more important than denitrification.

Recent studies have indicated that anammox, the anaerobic ammonium oxidation to dinitrogen gas with nitrite as electron acceptor, is a key process in the global marine nitrogen cycle (Arrigo 2005; Hulth et al. 2005), constituting a novel route to remove fixed inorganic nitrogen, which was so far attributed to heterotrophic denitrification. This process has been detected in various environments, i.e., anoxic water columns (Dalsgaard et al. 2003; Kuypers et al. 2003), marine and estuarine sediments (Dalsgaard and Thamdrup 2002; Trimmer et al. 2003), freshwater lakes (Schubert et al. 2006), terrestrial hot springs (Jaeschke et al. 2009*b*), and polar marine sediments and sea ice (Rysgaard and Glud 2004*a*; Rysgaard et al. 2004). In the oxygen minimum zones (OMZ) off Namibia (Kuypers et al. 2005), Chile (Thamdrup et al. 2006), and Peru (Hamersley et al. 2007), anammox was shown to be the dominant $N_2$ production pathway, while denitrification was minor or even absent. In marine sediments the relative contribution of anammox to total $N_2$ production measured by $^{15}N$ isotope pairing ranged from a few percentage points to ca. 70% and was suggested to be correlated with organic matter mineralization rates in the sediment and water depth (Dalsgaard and Thamdrup 2002; Thamdrup and Dalsgaard 2002; Engström et al. 2005). Schmid et al. (2007) and Penton et al. (2006) found evidence for the presence of anammox bacteria in a wide range of anoxic ecosystems covering arctic, temperate, and tropical shelf sediments. Little, however, is known about the occurrence and distribution of anammox bacteria with sediment depth or along continental slopes or even in the deep sea, although recent studies by Engström et al. (2009), Glud et al. (2009), and Trimmer and Nicholls (2009), all based on $^{15}N$ isotope incubations, give different indications on the relative importance of anammox at greater water depths.

The anammox process is so far linked to one group of organisms forming a distinct phylogenetic group within the *Planctomycetes* (Strous et al. 1999; Schmid et al. 2007). The group of anammox bacteria is currently associated to at least four genera, "*Candidatus* Brocadia," "*Candidatus* Kuenenia," "*Candidatus* Anammoxoglobus," and "*Candidatus* Scalindua" (Kartal et al. 2007; Schmid et al. 2007; Van de Vossenberg et al. 2008). So far, available 16S rRNA gene sequences from marine environments were found to be closely related to "*Candidatus* Scalindua" sp. (Kuypers et al. 2005; Schubert et al. 2006; Schmid et al. 2007), while in terrestrial hot springs they were closely related to "*Candidatus* Brocadia" and "*Candidatus* Kuenenia" (Jaeschke et al. 2009*b*). Anammox bacteria contain a separated intracytoplasmic compartment called the anammoxosome, where anammox catabolism was shown to take place (Lindsay et al. 2001; van Niftrik et al. 2004, 2008). The membrane of this organelle consists of unusual ladderane lipids forming a dense barrier, which reduces the permeability of the membrane to small molecules, e.g., the toxic

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intermediate of the anammox reaction, hydrazine, which can easily permeate less dense bacterial membranes (Sinninghe Damsté et al. 2002). The ladderane core lipid contains three or five linearly concatenated cyclobutane rings either ester or ether bound to the glycerol backbone, which is unprecedented in nature.

Ladderane core lipids have been applied as biomarkers for anammox bacteria in the anoxic waters of the Black Sea (Kuypers et al. 2003) as well as in oxygen minimum zones (OMZs) off Namibia (Kuypers et al. 2005), Peru (Hamersley et al. 2007), and the Arabian Sea (Jaeschke et al. 2007). However, these lipids may not necessarily indicate the presence of active anammox bacteria but may also be derived from fossil biomass because ladderane core lipids do not occur as such in anammox cell membranes but are derived from intact ladderane lipids containing polar headgroups. Recently, Boumann et al. (2006) and Rattray et al. (2008) have shown that intact ladderane lipids comprise phosphocholine (PC), phosphoethanolamine (PE), or phosphoglycerol (PG) as the major headgroups attached to the glycerol backbone. Intact phospholipids are more soluble in water and much less resistant to enzymatic cleavage because of their ionic nature; therefore they decompose rapidly after cell death, i.e., by losing their polar headgroup (White et al. 1979; Harvey et al. 1986). Intact phospholipids are thought to be suitable biomarkers for the study of living microbial communities (Sturt et al. 2004). Indeed, Jaeschke et al. (2009*a*) showed that a specific $C_{20}$ [3]-ladderane monoether lipid with a PC headgroup is a potentially suitable indicator for active anammox bacteria in marine sediments. However, direct comparison of this new marker for the detection of living anammox bacteria with other molecular markers like real-time quantitative polymerase chain reaction (q-PCR) or fluorescence in situ hybridization has not yet been performed.

In this study, we investigated the presence and abundance of anammox bacteria in marine sediments off northwest Africa derived from a range of water depths varying between 300 and 3000 m using different molecular techniques. To distinguish between living anammox cells and their dead remnants, we compared abundances of an intact $C_{20}$ [3]-ladderane monoether-PC lipid vs. ladderane core lipids with sediment depth using different high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) techniques. Furthermore, q-PCR was used to quantify the amount of 16S rRNA gene copies of anammox bacteria in different environments (Schmid et al. 2005; Hamersley et al. 2007), and these data were compared with the lipid results.

## Methods

*Study sites and sampling*—The sediment cores analyzed in this study were collected during R/V *Meteor* cruise M65/1 from the continental shelf and slope off northwest Africa in June 2005 (Mulitza et al. 2006). This area is characterized by coastal upwelling of nutrient-rich deep waters to the euphotic zone resulting in high primary productivity. Elevated oxygen consumption related to relatively high rates of organic matter decomposition results in hypoxic conditions (low oxygen, 2.0–0.2 mL $L^{-1}$) at intermediate water depths between 300 and 700 m (Stramma et al. 2008). In total 14 sediment cores were retrieved off Senegal and Guinea (16.5°N–8.5°N) from water depths ranging between 300 and 3000 m (Table 1; Fig. 1). The sediments consisted mainly of homogenous greenish muds. All sediment cores were obtained by multicorers. Cores for pore water analysis were transferred to the cooling lab immediately after recovery and processed at a temperature of about 4°C. Cores for lipid and deoxyribonucleic acid (DNA) analysis were sectioned into 1-cm slices immediately after recovery and stored at −20°C until used for analysis.

*Ladderane lipid analysis*—Ladderane core lipids: Typically ca. 6–20 g of freeze-dried and homogenized sediment were ultrasonically extracted 5× using a dichloromethane (DCM)-methanol mixture (2 : 1 by volume). The extracts were combined and the bulk of the solvent was removed by rotary evaporation under vacuum, and the extract was further dried over a $Na_2SO_4$ column. An aliquot of the lipid extract was saponified with aqueous 1 mol $L^{-1}$ KOH in methanol for 2 h. Nonsaponifiable lipids (neutral lipids) were extracted out of the basic solution (pH > 13) using DCM. Fatty acids were obtained by acidifying the residue to pH 3 and subsequent extraction with DCM. The fatty acid fraction was methylated by adding diazomethane ($CH_2N_2$) to convert fatty acids into their corresponding fatty acid methyl esters (FAMEs). The excess $CH_2N_2$ was removed by evaporation. To remove very polar components, aliquots of the FAMEs were eluted with ethyl acetate over a small column filled with silica. Polyunsaturated fatty acids were removed by eluting the aliquots with ethyl acetate over a small $AgNO_3$ (5%) impregnated silica column, yielding a saturated fatty acid fraction. An aliquot of the neutral fraction was eluted with DCM : methanol (1 : 1 volume : volume [v : v]) over a small column filled with activated aluminum oxide. Both fatty acid and neutral fractions were dissolved in acetone and then filtered through a 0.45-$\mu$m, 4-mm diameter polytetrafluoroethylene filter.

These fractions were analyzed by HPLC coupled to positive ion atmospheric pressure chemical ionization MS/MS as described by Hopmans et al. (2006) with some modifications. Specifically, separation was achieved using a Zorbax Eclipse XDB-$C_8$ column (3.0 × 250 mm, 5 $\mu$m; Agilent) and a flow rate of 0.18 mL $min^{-1}$ MeOH. The source settings were vaporizer temperature 475°C, discharge current 2.5 $\mu$A, sheath gas ($N_2$) pressure 30 (arbitrary units), auxiliary gas ($N_2$) pressure 5 (arbitrary units), capillary temperature 350°C, source collision-induced dissociation (CID) −10 V. Argon pressure was maintained at 1.5 mTorr in the second quadrupole. Quantification of ladderane core lipids was done by using an external calibration curve using standards of isolated methylated ladderane fatty acids containing the [3]- and [5]-ladderane moieties (Fig. 2, I–III) and a monoether containing the [3]-ladderane moiety (Fig. 2, IV). A detection limit (defined by a signal to noise ratio of 3) of 30–35 pg injected on-column was achieved with this technique. The reproducibility of the concentrations based on duplicate measurements was between 3% and 8%.

Table 1. Characteristics of study sites off northwest Africa.

| Sta. GeoB | Date (2005) | Latitude (°N) | Longitude (°W) | WD (m) | TOC (%) |
|---|---|---|---|---|---|
| 9501 | 12 Jun | 16°50′38 | 16°43′96 | 330 | 2.6 |
| 9506 | 14 Jun | 15°36′50 | 18°20′99 | 2964 | 1.2 |
| 9508 | 15 Jun | 15°29′97 | 17°56′82 | 2385 | 2.0 |
| 9510 | 15 Jun | 15°25′00 | 17°39′23 | 1567 | 2.4 |
| 9512 | 16 Jun | 15°20′21 | 17°21′99 | 787 | 2.2 |
| 9513 | 16 Jun | 15°19′10 | 17°17′70 | 498 | 1.3 |
| 9520 | 19 Jun | 13°49′77 | 17°35′45 | 1102 | 1.8 |
| 9521 | 19 Jun | 13°50′87 | 17°29′43 | 522 | 2.3 |
| 9525 | 21 Jun | 12°38′40 | 17°52′75 | 2648 | 2.8 |
| 9526 | 21 Jun | 12°26′12 | 18°03′37 | 3223 | 2.6 |
| 9528 | 23 Jun | 09°09′96 | 17°39′84 | 3062 | 0.9 |
| 9529 | 24 Jun | 09°21′18 | 17°22′13 | 1234 | 1.1 |
| 9534 | 26 Jun | 08°54′04 | 14°56′14 | 493 | 4.2 |
| 9535 | 26 Jun | 08°52′53 | 14°57′64 | 666 | 4.3 |

TOC was analyzed on sediment core top samples. WD, water depth.

The index of ladderane lipids with five cyclobutane rings, an index for the relative chain length of ladderane lipids, was calculated according to Rattray (2008):

$$NL_5 = \frac{C_{20}[5]\text{ fatty acid}}{C_{18}[5]\text{ fatty acid} + C_{20}[5]\text{ fatty acid}} \quad (1)$$

The $NL_5$ was then converted to a temperature estimate using the equation of Rattray (2008):

$$NL_5 = 0.2 + \frac{0.7}{1 + e^{-\left(\frac{\text{Temp}-16.0}{1.6}\right)}} \quad (2)$$

The reproducibility of the $NL_5$ based on duplicate measurements was better than 0.005.

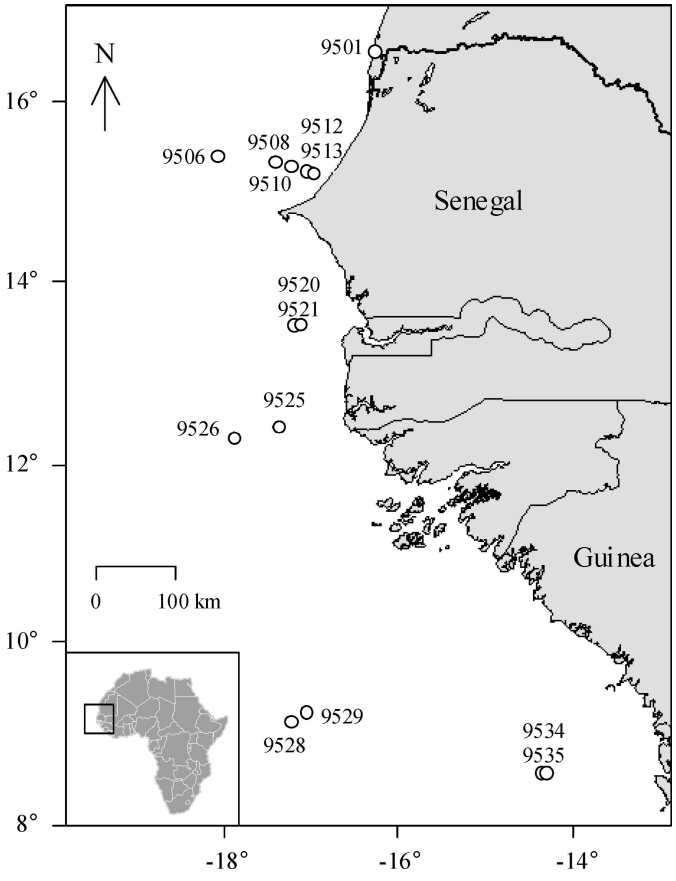


Fig. 1. Location of sediment cores investigated off northwest Africa.

$C_{20}$ [3]-ladderane monoether-PC lipid analysis: Sediment samples of 6–20 g were ultrasonically extracted according to the method of Bligh and Dyer (1959) with some modifications using a mixture of methanol, DCM, and phosphate buffer at pH 7.4 (2 : 1 : 0.8 v : v : v). After sonication for 10 min, further DCM and buffer were added to the mixture to achieve a final methanol : DCM : buffer ratio of 1 : 1 : 0.9 (v : v : v). The solution was centrifuged afterward to separate the DCM phase from the methanol : phosphate buffer. The DCM phase containing the lipids was kept, while the methanol : phosphate buffer phase was discarded. The samples were re-extracted three more times using the same procedure. The DCM fractions were combined, and the bulk of the solvent was removed by rotary evaporation under vacuum. An aliquot of the extract was dissolved in a DCM : methanol mixture (9 : 1, v : v) and filtered through a 0.45-$\mu$m, 4-mm diameter regenerated cellulose filter.

The $C_{20}$ [3]-monoether lipid containing a PC headgroup (Fig. 2, V) was analyzed by HPLC–electrospray ionization–MS/MS based on the method described by Boumann et al. (2006) with some modifications. Separation was achieved on a LiChrospher diol column (250 mm × 2.1 mm, 5-$\mu$m particles) maintained at 30°C. The following linear gradient was used with a flow rate of 0.2 mL $min^{-1}$: 90% A : 10% B to 70% A : 30% B over 10 min, maintained for 20 min, then to 35% A : 65% B in 15 min, maintained for 15 min, then back to 100% A for 20 min to re-equilibrate the column, where A = hexane : 2-propanol : formic acid : 14.8 mol $L^{-1}$ $NH_{3aq}$ ratios 79 : 20 : 0.12 : 0.04 (v : v : v : v) and B = 2-propanol : water : formic acid : 14.8 mol $L^{-1}$ $NH_{3aq}$ ratios 88 : 10 : 0.12 : 0.04 (v : v : v : v). Detection of the $C_{20}$ [3]-monoalkylether-PC was achieved by collision-induced dissociation selective reaction monitoring (SRM) of the transition from *m*/*z* (mass-to-charge ratio)

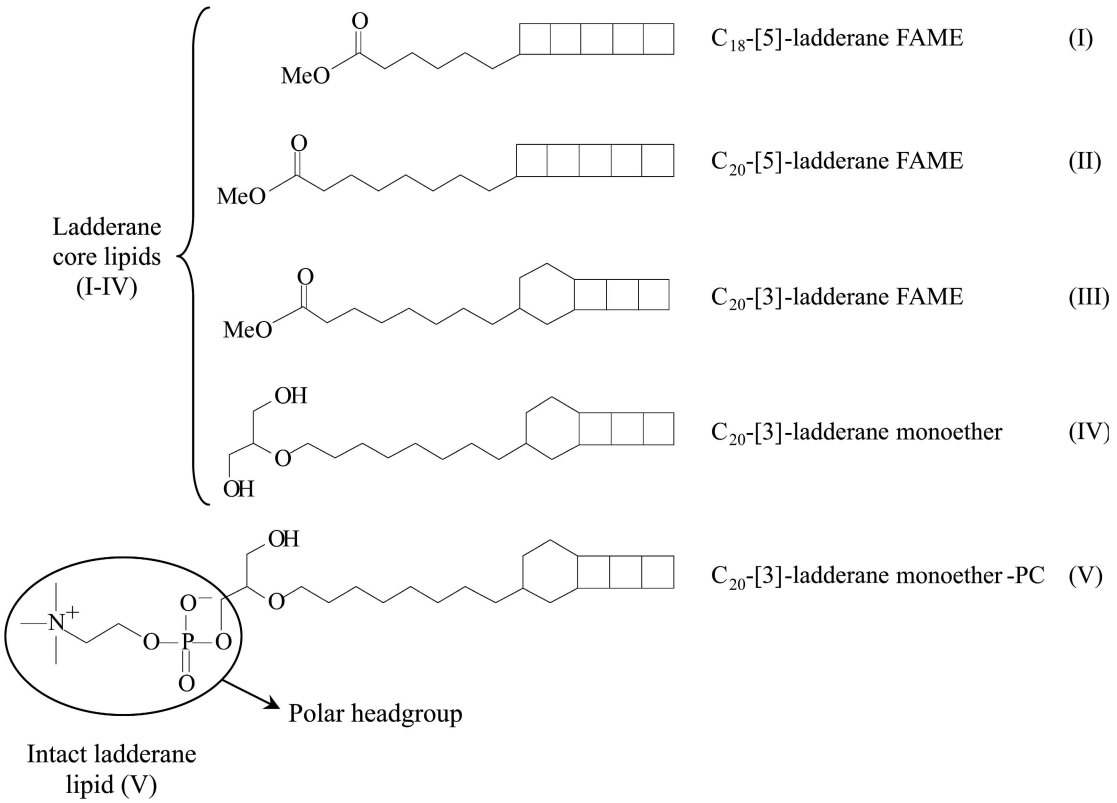


Fig. 2. Chemical structures of the ladderane lipids analyzed in this study; ladderane "core" lipids: (I) $C_{18}$ [5]-ladderane FAME, (II) $C_{20}$ [5]-ladderane FAME, (III) $C_{20}$ [3]-ladderane FAME, (IV) $C_{20}$ [3]-ladderane monoether, "intact" ladderane lipid: (V) $C_{20}$ [3]-ladderane monoether-PC. FAME, fatty acid methyl ester.

530 $[M+H]^+$ to *m/z* 184 (corresponding to the PC headgroup), with 1.5 mTorr argon as collision gas and 20 V collision energy. Quantification of the intact ladderane monoetherlipid was done by an external calibration curve of an isolated $C_{20}$ [3]-ladderane monoether-PC standard (43% purity). A detection limit of 10 pg injected on-column was achieved with this technique. The analytical reproducibility based on duplicate measurements was better than 12% (Jaeschke et al. 2009*a*).

*DNA extraction and q-PCR*—Total DNA from sediment cores GeoB 9501, 9506, 9510, and 9513 was extracted from ca. 0.25–0.33 g sediment sample using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories). An aliquot of the total DNA extract was subjected to agarose gel electrophoresis to determine DNA quality and quantity. All extracts contained mainly higher molecular weight DNA ($\sim$20 kbp fragments) at a concentration of $>10$ ng $\mu L^{-1}$.

Undiluted, as well as 5 and 25 times diluted (in order to check for inhibition) DNA extracts were subjected to quantitative PCR (q-PCR) analysis. Since inhibition was not observed, the data from the undiluted samples were used for q-PCR.

Real-time PCR amplification was performed using the 16S rRNA gene specific primer set Brod541F (5′-GAGCACGTAGGTGGGTTTGT-3′)–Brod1260R (5′-GGATTCGCTTCACCTCTCGG-3′) for detecting anammox bacteria in sediments according to Penton et al. (2006). Reaction mixtures (20 $\mu$L) contained 1 unit of Picomaxx™ high-fidelity DNA polymerase, 2 $\mu$L of 10× Picomaxx PCR buffer (both Stratagene), 0.25 mmol $L^{-1}$ each of deoxynucleotide triphosphate, 8 $\mu$g of bovine serum albumin, 0.25 $\mu$mol $L^{-1}$ of each primer, 50,000 times diluted SYBRgreen (a cyanine dye) (Molecular Probes), a final concentration of 10 nmol $L^{-1}$ fluorescein, 3 mmol $L^{-1}$ of $MgCl_2$, and ultrapure sterile water (Sigma). All reactions involved initial denaturing (4 min at 95°C), followed by 35–40 cycles including denaturing (30 s at 94°C), 40 s of primer annealing of 62°C, a fotomoment at 80°C for 25 s, and primer extension (60 s at 72°C). Real-time PCR was performed in triplet measurements per sample using an iCycler (Biorad). Calibration of the samples was performed by using a dilution series of the 719-bp purified PCR product, ranging from $10^7$ to $10^1$ copies per reaction, of a culture of "*Candidatus* Scalindua wagneri" were used as q-PCR standards. Real-time PCR was followed by melt curve analysis and gel electrophoresis to check for the presence of aspecific products such as primer dimers, which were not detected.

*Pore water and sediment chemistry*—Multicores were processed in a glove box under argon atmosphere at about 4°C. The sampling resolution was 0.25–1 cm. Pore water was extracted by pressure filtration using Teflon squeezers that were operated with argon at a pressure gradually increasing up to 4 bars. The pore water was retrieved through 0.2-$\mu$m cellulose acetate membrane filters. Ammo-

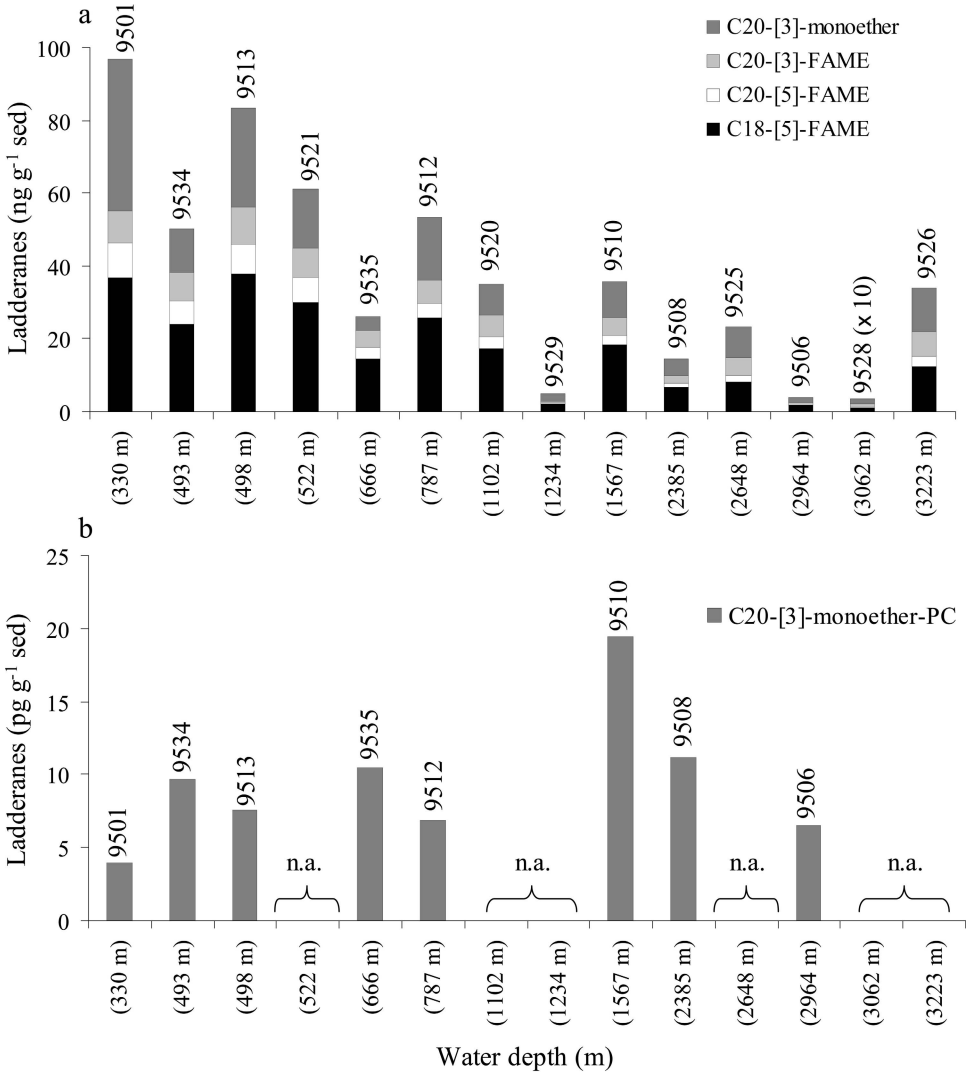


Fig. 3. (a) Concentrations and distribution of ladderane core lipids in surface sediments from different water depths off the shores of Senegal and Guinea, (b) concentration of the $C_{20}$ [3]-ladderane monoether-PC in selected surface sediments from different water depths off the shores of Senegal and Guinea.

nium and nitrate concentrations were determined on board immediately after sampling using a segmented flow autoanalyzer and standard colorimetric techniques.

For bulk organic carbon analyses, freeze-dried and powdered surface sediment (0–1 cm) samples were decalcified with dilute (2 mol $L^{-1}$) HCl, rinsed with demineralized water to remove $CaCl_2$, and freeze-dried again. Total organic carbon (TOC) was measured against a benzoic acid laboratory standard (C = 68.80%) on a Carlo Erba Flash elemental analyzer coupled to a Thermofinnigan Delta$^{plus}$ mass spectrometer. The reproducibility based on duplicate measurements was better than 0.2%.

## Results

*Ladderane core lipid analysis*—A set of 14 sediment core tops (0–1 cm) derived from different water depths (ca. 300–3000 m) off northwest Africa (Fig. 1) was analyzed for ladderane core lipids. In all samples ladderane core lipids were detected (Fig. 3a). Their relative distribution was similar in all samples, i.e., they were generally dominated by the $C_{18}$ [5]-ladderane FAME (I), followed by the $C_{20}$ [3]-ladderane monoether (IV), being 1.5–3.5 times lower in concentration, and the $C_{20}$ [3]- and $C_{20}$ [5]-ladderane FAMEs (II and III), both 2–8 times lower in concentration. Total ladderane lipid abundances varied between 0.3 and 97 ng $g^{-1}$ sediment (Fig. 3a).

In addition to the surface sediments, four sediment cores from the Mauritania mudbelt and along the continental slope off Senegal were analyzed to study the ladderane core lipid distribution with sediment depth (Table 2; Fig. 4). The highest summed concentrations of the ladderane core lipids of 139 ng $g^{-1}$ sediment were detected at the shallowest Sta. GeoB9501 (water depth 330 m) at 1.5 cm depth. At Sta. GeoB9513 (water depth 500 m) summed ladderane lipid concentrations are 70 ng $g^{-1}$ sediment at

Table 2. Concentration of individual ladderane core lipids in the sediment cores from four stations.

| Sta. GeoB | Depth (cm) | Concentration (ng $g^{-1}$ sediment) $C_{18}$ [5]-FA | $C_{20}$ [5]-FA | $C_{20}$ [3]-FA | $C_{20}$ [3]-monoether |
|---|---|---|---|---|---|
| 9501 | 0.5 | 37.2 | 10.4 | 5.7 | 41.7 |
| | 1.5 | 42.6 | 14.5 | 14.4 | 67.2 |
| | 2.5 | 32.6 | 10.7 | 6.4 | 52.3 |
| | 3.5 | 28.1 | 8.1 | 4.6 | 25.9 |
| | 4.5 | 27.4 | 8.4 | 5.1 | 54.9 |
| 9513 | 0.5 | 41.7 | 9.0 | 7.3 | 12.4 |
| | 1.5 | 32.2 | 6.7 | 5.3 | 33.9 |
| | 2.5 | 21.7 | 4.9 | 3.7 | 30.5 |
| | 3.5 | 8.0 | 1.8 | 1.4 | 36.9 |
| | 4.5 | 14.5 | 3.6 | 2.7 | 27.1 |
| 9510 | 0.5 | 19.2 | 2.5 | 3.5 | 5.4 |
| | 1.5 | 27.3 | 4.3 | 6.3 | 32.1 |
| | 2.5 | 26.2 | 3.4 | 4.5 | 30.8 |
| | 3.5 | 12.6 | 2.1 | 2.9 | 15.6 |
| | 4.5 | 7.5 | 1.1 | 1.5 | 11.1 |
| 9506 | 0.5 | 1.5 | 0.2 | 0.3 | 2.3 |
| | 1.5 | 5.3 | 0.8 | 1.1 | 5.6 |
| | 2.5 | 9.1 | 1.6 | 2.5 | 12.4 |
| | 3.5 | 16.8 | 2.6 | 4.4 | 15.4 |
| | 4.5 | 12.6 | 1.4 | 2.2 | 17.0 |

FA, fatty acid.

the surface, which then decreased farther downcore to values of 48 ng $g^{-1}$ sediment at 4.5 cm depth (Table 2; Fig. 4b). At Sta. GeoB9510 (water depth 1500 m) summed concentrations of ladderanes increased from 30 ng $g^{-1}$ sediment in the surface to 70 ng $g^{-1}$ at 1.5 cm depth and then decreased farther downcore to values of 21 ng $g^{-1}$ sediment at 4.5 cm depth (Fig. 4c). At the deepest station, Sta. GeoB9506 (water depth 3000 m), summed ladderane concentrations increased from about 4 ng $g^{-1}$ sediment in the surface to 39 ng $g^{-1}$ sediment at 3.5 cm depth (Table 2; Fig. 4d).

Anammox bacteria have been found to alter their membrane composition in response to temperature changes, i.e., an increase in the amount of shorter chain ladderane fatty acids relative to the amount of longer chain fatty acids at lower temperatures and vice versa (Rattray 2008). The $NL_5$ (*see* Methods section) has been proposed as a means to quantify this relative change. The $NL_5$ of the ladderane lipids in the surface sediments and the sediment cores was generally low, varying between 0.11 and 0.22 in surface sediments, suggesting they were synthesized at temperatures below 10°C (Rattray 2008). Hardly any variation in the $NL_5$ was observed with sediment depth, but $NL_5$ values were generally slightly smaller at greater water depth (Fig. 4).

*$C_{20}$ [3]-ladderane monoether-PC lipid analysis*—Eight core tops (Table 1) were analyzed for the $C_{20}$ [3]-ladderane monoether-PC lipid. This lipid was detected in all core tops, and its concentration varied between 4 and 19 pg $g^{-1}$ sediment (Fig. 3b). The concentration of the $C_{20}$ [3]-ladderane monoether-PC lipid was also determined in four sediment cores derived from the Mauritania mudbelt and along the continental slope off Senegal (Fig. 4). Concentrations ranged between <1 and 30 pg $g^{-1}$ sediment. Lowest concentrations of <10 pg $g^{-1}$ sediment were detected at Sta. GeoB9501 and GeoB9513 located at 330 and 500 m water depth, respectively (Fig. 4a,b). The two deeper sites, GeoB9510 and GeoB9506, located at 1500 and 3000 m water depth along the slope, revealed higher concentrations of the $C_{20}$ [3]-ladderane monoether-PC lipid, with maximum values of 20 and 30 pg $g^{-1}$ sediment, respectively (Fig. 4c,d).

*q-PCR analysis*—q-PCR analysis using a primer set selective for anammox bacteria was performed for the four sediment cores, which were also analyzed for ladderane lipids. The 16S rRNA gene copy numbers per gram of sediment ranged between $6.7 \times 10^6$ and $1.2 \times 10^8$ (Fig. 4). As observed with the $C_{20}$ [3]-ladderane monoether-PC lipid concentrations, the lowest 16S rRNA gene copy numbers were at the two shallow sites, GeoB9501 and GeoB9513 (Fig. 4a,b), while the two sites located at deeper water depths revealed 16S rRNA gene copy numbers that were about 1–1.5 orders of magnitude higher (Fig. 4c,d).

*Pore water chemistry*—Pore water ammonium and nitrate concentrations were analyzed for four sediment cores derived from the Mauritania mudbelt and along the continental slope off Senegal (Fig. 4). Nutrient concentrations in the pore water ranged between 4 and 58 $\mu$mol $L^{-1}$ for ammonium and between 1 and 36 $\mu$mol $L^{-1}$ for nitrate. Ammonium concentrations were highest at the shallowest Sta. GeoB9501 (58 $\mu$mol $L^{-1}$) and decreased at the sites farther downslope with increasing water depth, where concentrations were only 7–8 $\mu$mol $L^{-1}$ at the deepest site, while nitrate concentrations of 36 $\mu$mol $L^{-1}$ were highest at the deepest Sta. GeoB9506 (Fig. 4).

## Discussion

*Spatial distribution and origin of ladderane lipids*—The distribution of ladderane core lipids in surface sediments reveals a distinct pattern with, in general, higher abundances in surface sediments from shallower water depth and closer distance to the coast (Fig. 3a). In contrast, the concentrations of the $C_{20}$ [3]-ladderane monoether-PC lipid, which is a marker for living anammox bacteria

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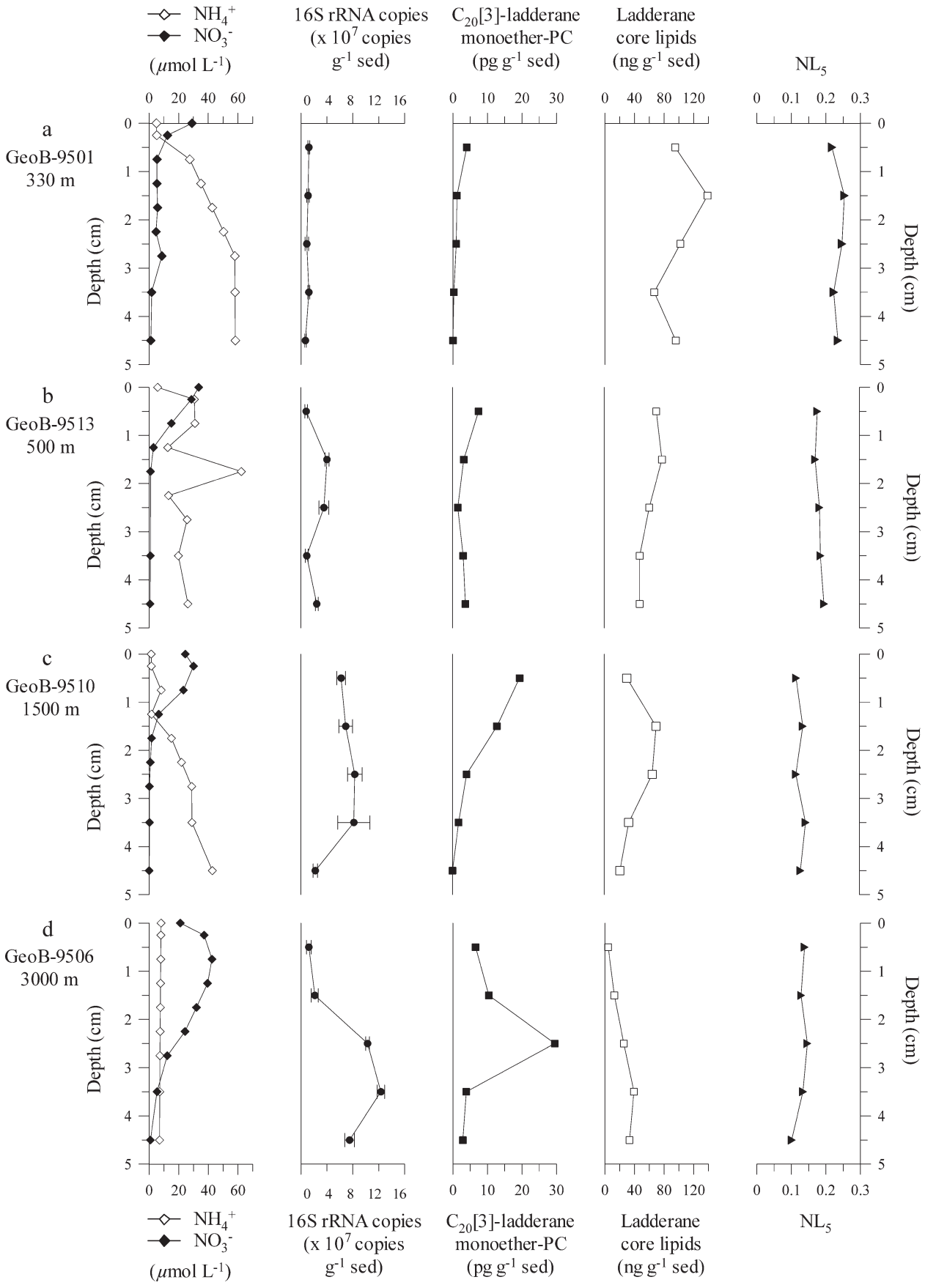
Fig. 4. Nutrient pore water profiles, 16S rRNA copy numbers, and distribution of anammox-specific ladderane membrane lipids in sediments from the mudbelt off Mauritania at (a) 330 m water depth, and (b–d) along the continental slope off Senegal at water depths of 500, 1500, and 3000 m, respectively. The first column shows pore water concentration profiles of $NH_4^+$ and $NO_3^-$. The second column shows the number of 16S rRNA gene copies of anammox bacteria. The third column shows the concentration of the $C_{20}$ [3]-ladderane monoether-PC. The fourth column shows the summed concentration of four different ladderane core lipids. The fifth column shows the $NL_5$, a temperature index based on the ratio of ladderane lipids with different chain length (*see* text). FAME, fatty acid methyl ester.


a
GeoB-9501
330 m
b
GeoB-9513
500 m
c
GeoB-9510
1500 m
d
GeoB-9506
3000 m
Depth (cm)
NH4+
NO3-
(μmol L-1)
16S rRNA copies
(x 10^7 copies
g-1 sed)
C20[3]-ladderane
monoether-PC
(pg g-1 sed)
Ladderane
core lipids
(ng g-1 sed)
NL5

(Jaeschke et al. 2009*a*), is highest in the surface sediment at a water depth of 1500 m, while it is lower at deeper and shallower water depths (Fig. 3b). The distribution of ladderane core lipids and the intact $C_{20}$ [3]-ladderane monoether-PC lipid in core top (0–1 cm) sediments thus show different patterns (Fig. 3). Especially at Sta. GeoB 9501, where highest concentrations of the core lipids were detected, the $C_{20}$ [3]-ladderane monoether-PC lipid is relatively low in concentration. This may indicate that some of the ladderane core lipids detected in the sediments may actually have originated from dead anammox bacterial biomass settling from the overlying OMZ. Ladderane lipids are known to be produced in OMZs off the shore of Peru, in the Arabian Sea, and in the Benguela upwelling area (Kuypers et al. 2005; Hamersley et al. 2007; Jaeschke et al. 2007), and ladderane core lipids have been found in settling particles in the Arabian Sea (Jaeschke et al. 2007). The OMZ off northwest Africa is less pronounced (hypoxic conditions) than these OMZs, although its intensity has increased over the past 50 yr (Stramma et al. 2008).

One way of evaluating the origin of ladderane lipids is to examine their relative distribution. Anammox bacteria have been found to adjust their membrane composition with temperature, i.e., an increase of the chain length of the ladderane fatty acids ($C_{20}$ [5]-ladderane FAME compared to the $C_{18}$ [5]-ladderane FAME) at higher growth temperature (Rattray 2008). The $NL_5$, which quantifies these changes, has low values, ranging from 0.11 to 0.22 in the surface sediments (Fig. 5), with slightly lower values at greater water depth. These values suggest that the ladderanes were synthesized at temperatures below 10°C (Rattray 2008), in line with CTD (conductivity-temperature-depth)-derived in situ bottom water temperatures of 2.5°C to 12°C. In contrast, ladderane lipids hypothetically produced in the overlying OMZ with temperatures of 14–15°C would likely possess slightly higher $NL_5$ values of ca. 0.3–0.4 (indicated by the dashed line in Fig. 5). The $NL_5$ values of the ladderane core lipids thus indicate that the ladderane lipids in the surface sediments are predominantly derived from anammox bacteria in the sediment. However, this does not fully exclude that part of the ladderane lipids are derived from anammox bacteria in the OMZ, nor that anammox may not be an important process in the OMZ. They potentially could be delivered by anoxic aggregates settling to the seafloor (Kuypers et al. 2005; Woebken et al. 2007). Part of the settling ladderane lipids might have been degraded during transport through the oxic water column, especially at greater water depths. Analysis of suspended particulate matter or sediment trap material is needed, though, to provide further insight into the possible occurrence of anammox bacteria in the oxygen-depleted water column off northwest Africa.

*Distribution of anammox bacteria within the sediment*—The depth profiles investigated at the three stations along the continental slope off Senegal show decreasing ladderane core lipid concentrations with increasing water depth (Fig. 4b–d). While Stas. GeoB9501 and GeoB9513 do not show much variation in ladderane concentration with sediment depth (Fig. 4a,b), it decreases at Sta. GeoB9510 below 2.5 cm depth (Fig. 4c), and at Sta. GeoB9506 it increases continuously with sediment depth (Fig. 4d). Apparent differences in the distribution of the core ladderane lipids vs. the intact ladderane lipid are evident in terms of total abundances and sediment depth. The concentration of the intact $C_{20}$ [3]-ladderane monoether lipid containing a PC headgroup is in general 3–4 orders of magnitude lower than the corresponding monoether core lipid. This large difference is partly because the core ladderane monoether also derives from intact ladderane monoether with PG and PE headgroups and ladderane ester–ethers and diethers with PC, PG, and PE headgroups (Boumann et al. 2006; Rattray et al. 2008), which we were not able to quantify with our current HPLC-MS/MS method. However, the major reason for this discrepancy is probably that the intact ladderane monoether-PC lipid represents the living anammox microbial biomass and, thus, the actual zone of living anammox bacteria and that these intact ladderanes are rapidly transformed into ladderane core lipids upon cell lysis (Jaeschke et al., 2009*a*). This explanation is partly supported by the 16S rRNA gene copy numbers of anammox bacteria in the sediment. They also reveal overall low values over the whole depth profile at the two shallow sites GeoB9501 and 9513, while highest 16S rRNA gene copy numbers were observed at the deepest site GeoB9506, increasing with sediment depth, similar to what is observed with the intact $C_{20}$ [3]-ladderane monoether lipid (Fig. 4). Ladderane core lipids have a much higher preservation potential and are actually the transformation products of the intact ladderane lipids. Therefore, they will accumulate below the depth where anammox bacteria are active, resulting in much higher concentrations compared to intact ladderanes. This likely explains the concentration profiles of the core

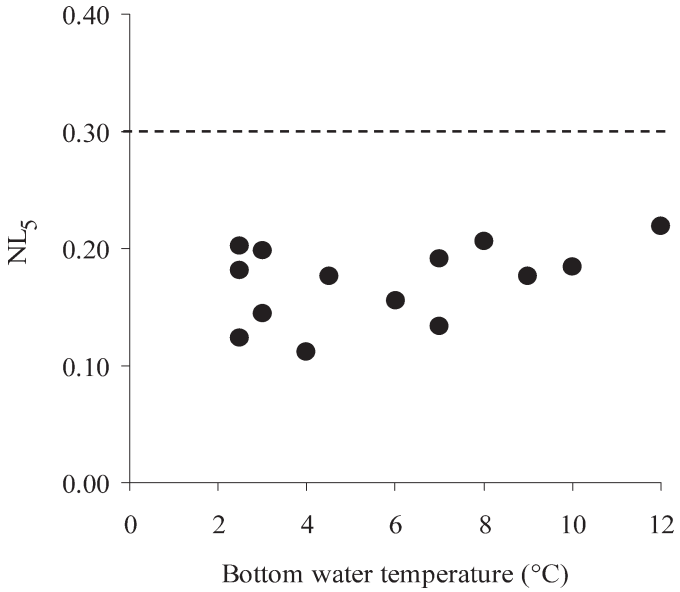



Fig. 5. $NL_5$ calculated for surface sediments in relation to bottom water temperatures. The dotted line indicates $NL_5$ values expected from ladderane lipids potentially synthesized in the overlying oxygen minimum zones.

ladderane lipids: relatively high at the surface in the cores GeoB-9501 and GeoB-9513 from the shallow sites, where highest abundances of anammox bacteria are at the surface, and relatively high at 3–5 cm depth in core GeoB-9506, where the level of highest anammox bacterial abundances is deeper, i.e., at 3.5 cm depth (Fig. 4). The ladderane core lipid concentration below the active zone of anammox bacteria remains relatively high or even increases due to the contribution of fossil lipids. This probably explains the different pattern of the core lipids compared to both the intact $C_{20}$ [3]-ladderane monoether-PC lipid and the 16S rRNA gene copy numbers.

*Distribution of anammox bacteria with water depth*—The concentration of the intact $C_{20}$ [3]-ladderane monoether-PC lipid and 16S rRNA gene copy numbers generally increase with increasing water depth, showing the opposite trend to the ladderane core lipids (Fig. 4). This is even more evident if we plot the $C_{20}$ [3]-ladderane monoether-PC lipid concentrations and 16S rRNA copy numbers averaged over the upper 5 cm of the sediment along the depth transect (Fig. 6a,b). This plot clearly reveals the increasing abundance of anammox bacteria in sediments at greater water depths. Ladderane core lipid concentrations along the same transect, however, reveal the opposite pattern (Fig. 6c), which may be due to the general better conditions for lipid preservation in the shallow sediments (lower oxygen penetration depth, less bioturbation).

From the 16S rRNA gene copy numbers and the intact $C_{20}$ [3]-ladderane monoether lipid data it is also apparent that with increasing water depth the zone of living anammox bacteria is extending deeper into the sediment (sites 9510 and 9506). This extension in sediment depth at which maximum levels of 16S rRNA gene copy numbers of anammox bacteria and intact $C_{20}$ [3]-ladderane monoether lipids are found with depth of the stations is probably linked with the penetration depth of nitrate, and probably oxygen as well, which also increases continuously with increasing water depth and is most distinct at the deepest Sta. GeoB9506 (Fig. 4d). This probably explains why in our larger set of surface sediments (i.e., 0–1 cm), concentrations of the intact ladderane lipid maximized in the sediments at a water depth of ca. 1500 m (Fig. 3b); in the deeper sediments the zone of highest anammox bacterial abundances is most likely below 1 cm depth.

Earlier studies by Dalsgaard and Thamdrup (2002) and Engström et al. (2005) showed that the relative importance of anammox (compared to denitrification) in coastal marine sediments is independent of pore water nitrate concentrations and is mainly influenced by organic matter mineralization rates in the sediment with a higher relative contribution of anammox in sediments receiving a lower flux of organic carbon. The concentration of the intact $C_{20}$ [3]-ladderane monoether-PC lipid (Fig. 7a) seems to be nonlinearly inversely related to pore water ammonium concentrations for the four sites along the slope profile. A similar relation can be found for 16S rRNA gene copy numbers of anammox bacteria, although less distinct (Fig. 7c). Engström et al. (2005) reported a similar relation between the relative contribution of anammox to total $N_2$

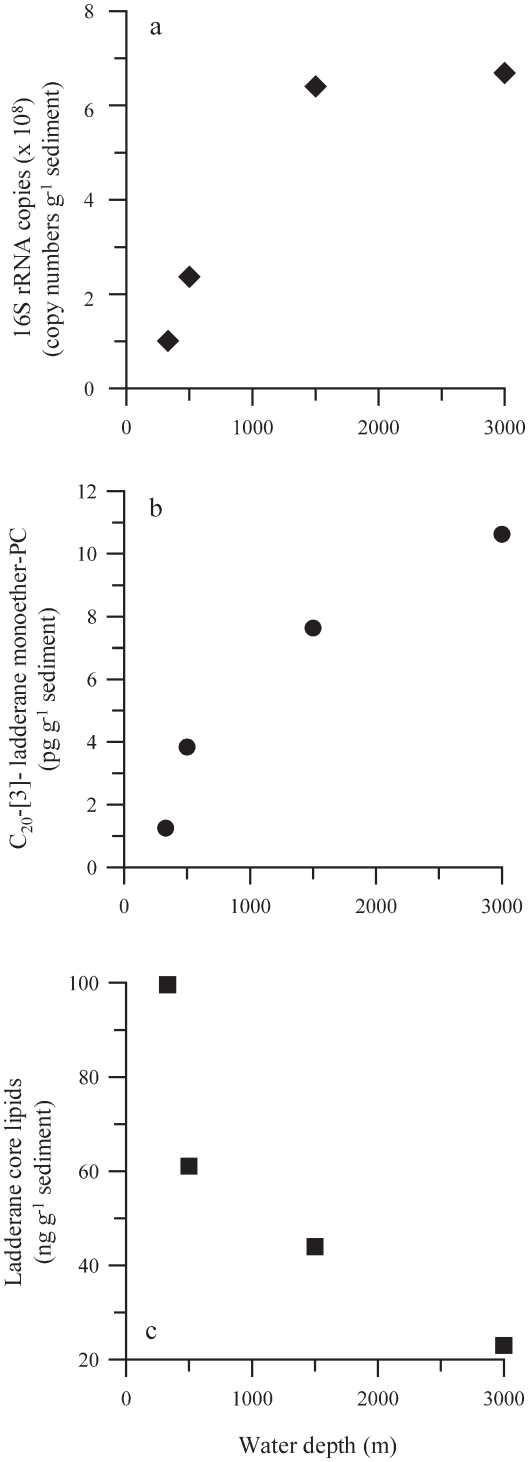



Fig. 6. Average concentrations for the upper 5 cm for different tracers for anammox bacteria in sediment cores as a function of water depth of the sediment cores. (a) 16S rRNA gene copy numbers, (b) $C_{20}$ [3]-ladderane monoether-PC, and (c) summed concentration of the four different ladderane core lipids (I–IV, Fig. 2).

production and pore water ammonium mineralization rates, where the relative importance of anammox was found to be highest at the least reactive site. The concentration of the intact $C_{20}$ [3]-ladderane monoether-

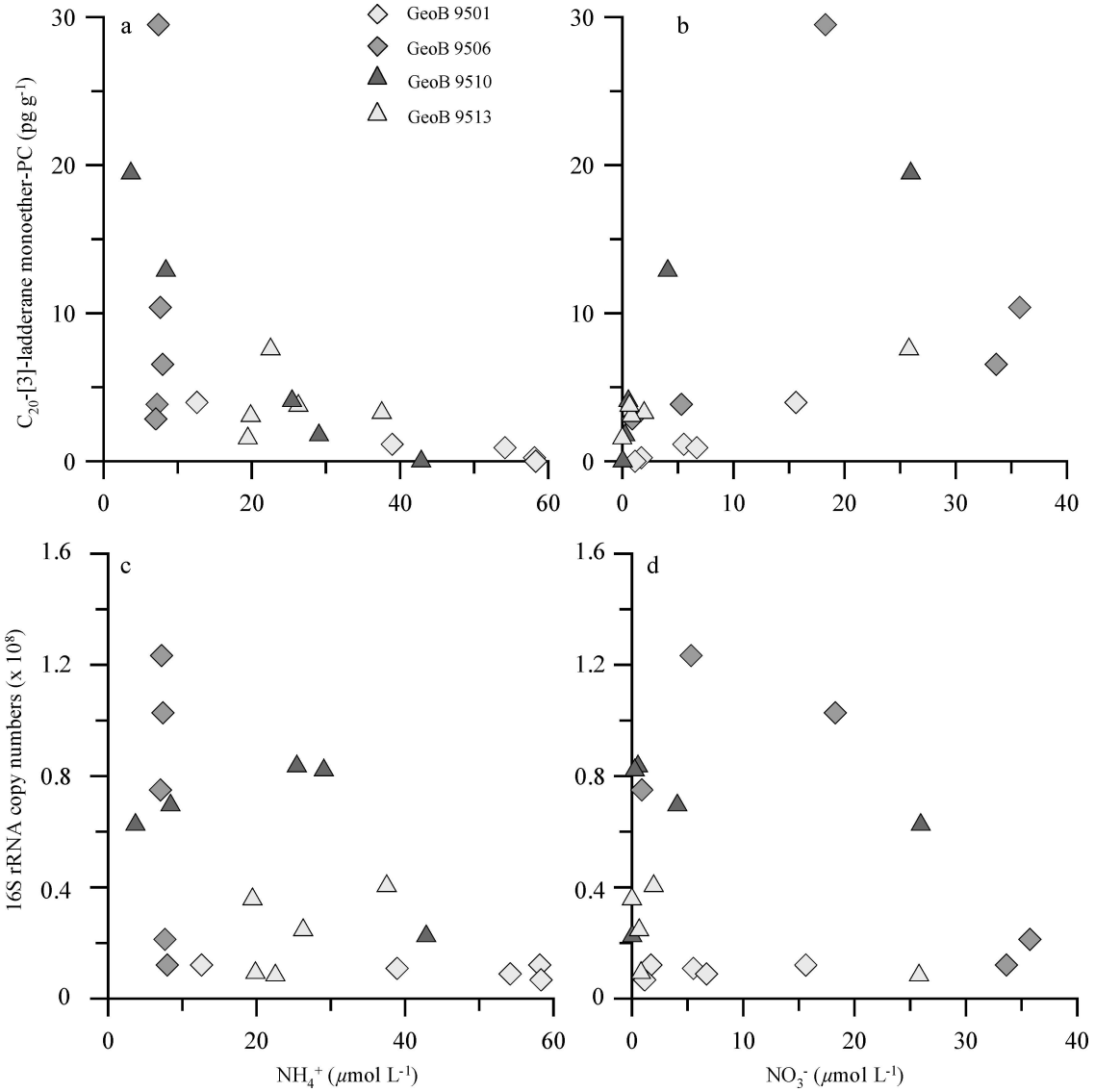



Fig. 7. (a, b) $C_{20}$ [3]-ladderane monoether-PC concentrations and (c, d) anammox-specific 16S rRNA copy numbers in relation to pore water $NH_4^+$ and $NO_3^-$ concentrations from sediment cores GeoB9501, 9506, 9510, and 9513, respectively.

PC lipid and the 16S rRNA gene copy numbers of anammox bacteria both seem not to be related to pore water nitrate concentrations (Fig. 7b,d). In fact, highest concentrations of the $C_{20}$ [3]-ladderane monoether-PC lipid and the 16S rRNA gene copy numbers are highest at low pore water ammonium and nitrate concentrations (Figs. 4, 7), which has also been observed for the water column in the Black Sea (Kuypers et al. 2003). Our data indicate that the relative importance of anammox in removing nitrogen from sediments seems to be highest at greater water depths where organic matter mineralization rates are lower and are in agreement with studies of coastal sediments by Thamdrup and Dalsgaard (2002). Recently, Trimmer and Nicholls (2009) measured $N_2$ production in intact sediment cores along a continental shelf to slope transect (50–2000 m water depth) in the North Atlantic, and their results show that, although total $N_2$ production was highest in shallow organic carbon-rich shelf sediments, the proportion of anammox relative to denitrification increased downslope, i.e., 33% on average on the shelf and reaching a maximum of 65% on the slope, indicating that anammox is increasingly important in deeper slope sediments where organic carbon concentrations and, therefore, overall $N_2$ production were lower. Their findings were in agreement with concentrations of ladderane lipids analyzed in sediments from the same sites (Jaeschke et al. 2009*a*). Our lipid and DNA data support these findings, extend them to even deeper sediments (ca. 3000 m) along the northwest African coast, and provide further evidence for the widespread occurrence of anammox and its effect on the marine nitrogen cycle.

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