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Bacterial influence on amino acid enantiomerization in a coastal marine sediment

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Abstract

The contribution of D-isomers of aspartate (Asp), glutamate (Glu), alanine (Ala), and serine (Ser) to the total concentrations of dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA), and total hydrolyzable amino acids (THAA) was studied in a <2,300-yr-old coastal sediment. Concentrations of the different amino acid pools were typically 5 (DFAA), 100 (DCAA), and 20,000 nmol cm^{-3} (THAA), but variable amounts occurred, especially in the surface layer. D-amino acids were identified in all three amino acid fractions. The highest concentration of D-isomers occurred in the THAA fraction at a depth of 10 cm, where the maximum bacterial density has been observed (Jørgensen et al. 1990; Mar. Ecol. Prog. Ser. **59:** 39–54). This coincidence suggests that the D-amino acids originated in peptidoglycan of bacterial cell walls. The proportion of D-Asp, D-Glu, D-Ala, and D-Ser increased through the pools of DFAA and DCAA to THAA, suggesting a selective removal of L-amino acids to D-amino acids. The percentage of the four D-amino acids in the THAA pool increased with sediment depth to at least 4.3 m (~2,150 yr). This most likely indicated that peptidoglycan THAA were retained for a longer period of time in a particulate form compared to other proteinaceous material. The contribution of THAA from peptidoglycan to the THAA pool was estimated to range from >9% at the surface to at least 18% at 4.3 m deep, depending on the assumptions used in the calculation. The distribution of amino acid isomers in Aarhus Bay sediment demonstrated that bacterial peptidoglycan contributed to the pool of organic nitrogen (the four analyzed D-amino acids made up about 3% of particulate organic nitrogen [PON]). Although the peptidoglycan concentration increased with sediment depth, our observations do not indicate a long-term accumulation of peptidoglycan-derived amino acids.

All amino acids except glycine (Gly) may occur as either L- or D-stereoisomers. L-amino acids are most abundant in nature because they are constituents of all living material and, hence, are in most organic detritus. The formation of D-amino acids is primarily restricted to production of bacterial peptidoglycan and aging of amino acid in organic matter (the L-form can racemize to the D form). Eubacterial cells are surrounded by rigid walls, which are composed of peptidoglycan as the main building block (Schleifer and Kandler 1972). Peptidoglycan consists of alternate β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid strands cross-linked by short peptides. D-alanine (D-Ala) and D-glutamate (D-Glu) are two of the fundamental peptide components (Schleifer and Kandler 1972). In addition to a thick peptidoglycan cell wall in Gram-positive (G^+), relative to Gram-negative (G^-) bacteria, G^+ bacteria often have unique peptide interbridges in the peptide cross-linking. These interbridges are variable among G^+ subgroups and, therefore, taxonomically useful (Prescott et al. 1996). In addition to L-amino acids, the peptide interbridges may include D-amino acids other than D-Ala and D-Glu (e.g., D-aspartate [D-Asp] and D-leucine; Brückner et al. 1994).

Bacterial populations in marine environments typically have a dominance of G^- to G^+ bacteria at both oxic (Hagström et al. 2000) and anoxic conditions (Moriarty and Hayward 1982). This means that most D-amino acids in marine environments probably are derived from G^- cell walls; thus, the ability of G^- peptidoglycan to degrade is of major importance to the preservation of D-amino acids in these environments.

The structure of eubacterial peptidoglycan is believed to make the cell walls more resistant to degradation than the remaining part of the cell (McCarthy et al. 1998). This is supported by the presence of empty cell sacs in marine sediments, constituting 4–29% of the number of intact bacterial cells (Moriarty and Hayward 1982). Furthermore, peptidoglycan likely resides for several thousand years in the ocean because it is thought to account for a major and refractory part of the 4,000–6,000-yr-old high molecular weight dissolved organic matter (DOM) in the ocean (McCarthy et al. 1998). In addition to peptidoglycan, some cell membrane proteins of G^- bacteria appear to resist biological degradation and may contribute to the pool of refractive DOM (Tanoue et al. 1995)

A substantial fraction of amino acids in sediments occurs in a combined form (e.g., as peptides and proteins) and are often characterized as total hydrolyzable amino acids

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(THAA). THAA may account for a substantial portion (>60%) of the particulate organic nitrogen (PON) in marine sediments (Henrichs and Farrington 1987; Cowie et al. 1992; Dauwe and Middelburg 1998; Lomstein et al. 1998). A fraction of the THAA pool consists of bacterial cell walls in the form of living cells and as dead cell wall fragments. Because active bacteria can occur at even very large sediment depths (up to 250 m; Bottrell et al. 2000), bacteria may be dominant contributors of THAA at such sediment depths because most biological activity probably is restricted to prokaryotic organisms.

Biological activity and diagenetic processes modify the composition of THAA in marine sediments. Some amino acids are preferentially preserved or produced (e.g., Gly and β-alanine [β-Ala]), whereas other amino acids are depleted (e.g., lysine [Lys] and Glu; Degens et al. 1964; Burdige and Martens 1988; Cowie et al. 1992). A fraction of THAA in marine sediments consists of D-enantiomers. Pollock and Kvenvolden (1978) found that D-Ala, D-Asp, and D-Glu in surface sediment off California, USA, constituted ~8, 7, and 2% of the total amount of hydrolyzed Ala, Asp, and Glu, respectively. They suggested that these D-amino acids mainly originated from bacteria. A minor proportion of sediment amino acids are dissolved free amino acids (DFAA; Henrichs et al. 1984; Burdige and Martens 1990; Lomstein et al. 1998) and dissolved combined amino acids (DCAA; Lomstein et al. 1998). The DFAA pool in marine sediments can be highly dynamic and can be cycled several times per day (Christensen and Blackburn 1980; Sugai and Henrichs 1992; Lomstein et al. 1998).

The degree of racemization of hydrolyzable amino acids in protozoa, invertebrates, and skeletons of vertebrates has been used for chronological study (references in review by Schroeder and Bada 1976). The method is based on the fact that amino acids change their configuration from the natural L- to the D-form during aging, although at a low rate. The time for conversion of L-amino acids to an equilibrium mixture of L- and D-amino acids is estimated to be $\sim 10^4$ to $\sim 10^6$ yr at 0°C (Bada 1982). Amino acid enantiomerization has been studied in intact sediments (Bada et al. 1970; Kvenvolden and Peterson 1970) and in foraminiferal shells (protozoa) isolated from marine sediments (Schroeder and Bada 1976 and references therein). Geochronology of sediments is based on known rates of racemization and dependence of variables of the physical–chemical environment, such as temperature and water content (Schroeder and Bada 1976). Because of the low rate of racemization, D-amino acids in marine sediments have been suggested to originate from bacteria rather than from racemization (Pollock and Kvenvolden 1978). Lee and Bada (1977) and McCarthy et al. (1998) also explained the enantiomeric ratios of dissolved amino acids in seawater by the occurrence of marine bacteria and remnants of bacterial peptidoglycan.

The aim of the present study was to investigate the relative importance of D-amino acids in pore water and in the particulate organic matter pool of a coastal marine sediment. We hypothesize that peptidoglycan and, thus, bacterial cell walls are important components of organic matter in marine sediments. Furthermore, it was anticipated that mineralization processes (e.g., aerobic respiration, sulfate reduction, and methanogenesis) could affect the degree to which cell wall amino acids were mineralized or preserved. A 4.6-m-long sediment core, covering an age of 2,300 yr, was sectioned and analyzed for content of sulfate (SO_4^{2-}), methane (CH_4), chloride (Cl^-), particulate organic carbon (POC), and PON and for composition of stereoisomers of the DFAA, DCAA, and THAA pools.

Materials and methods

Study area—A sediment core was collected in June 1999 at Sta. 6 in Aarhus Bay, Denmark, (56°09′10″N, 10°19′20″E). Aarhus Bay is a 315-km^2 semienclosed embayment on the east coast of Jutland, connected to the Kattegat. The sediment is composed of 20% fine to very fine sand, 22% silt and 55% clay (Pejrup et al. 1996). A 4.6-m-long sediment piston core, 7 cm diameter, was collected. The core was cut into 3 major sections on board ship and brought to the laboratory. In the laboratory, cores were divided longitudinally, and 50-cm^3 subsamples from 29 different depth zones of the core were taken by disposable syringes with the tips removed. The age of the 4.6-m core was estimated to be a maximum of 2,300 yr, because at the same station, Pejrup et al. (1996) estimated the sediment 40 cm deep to be 200 yr old by Pb-210 dating. This corresponds to a sedimentation rate of 2 mm yr^{-1}. Water depth was 16 m, and bottom water temperature at the time of sampling was 4°C.

Sediment analysis—For analysis of SO_4^{2-} and Cl^- in the pore water, sediment samples were centrifuged at 4,416 × g, after which the supernatants were collected. Zinc acetate (20%, wt/wt) was added to preserve the pore water and to stop microbial activity. Concentrations of SO_4^{2-} and Cl^- were measured by ion chromatography according to Thomsen et al. (2001). Cl^- salinity in the upper sediment section (11 cm deep) was assumed to reflect salinity in the overlying seawater and was used as a reference for the deeper sediment layers. CH_4 was extracted from the sediment by adding 10 ml NaOH to 10 cm^3 of sediment in a 100-ml sealed glass flask. The sediment–NaOH mixture was thoroughly shaken, and CH_4 samples were withdrawn from the gas phase. Gas samples were analyzed by gas chromatography using flame ionization detection (Packard model 438A) after separation of methane on a Poropack Q-column (Hansen et al. 1998). Sediment density was measured gravimetrically on every third depth section on 50-cm^3 sediment samples. The water content was measured as weight loss of 10 cm^3 fresh sediment dried at 105°C for 24 h.

Intact sediment samples were stored frozen for later analysis of POC, total N (TN), DFAA, DCAA, and THAA. Content of POC and TN was determined on dried, ground, and H_2SO_3-treated sediment in a Carlo Erba NA-1500 CHN analyzer. The TN concentrations include inorganic and organic N. Inorganic N was not measured in the present sediment core, but NH_4^+ concentrations of 0.04–0.56 μM cm^{-3} have previously been found in the upper 2 m of sediment at Sta. 6 (Jørgensen et al. 1990). Occurrence of NO_3^- is restricted to the surface layer because negligible concentrations (<1 nM cm^{-3}) typically have been found below 5 mm at the station (Lomstein and Blackburn 1992). Compared to the

amount of TN of about 125 μM cm^{-3} in the sediment (indicated as PON in Fig. 1B), inorganic N presumably made up <0.5% of TN. When considering the analytical error in measuring TN (~3%), the obtained TN concentrations most likely are not different from PON and hereafter will be referred to as PON.

Porewater for DFAA and DCAA analysis was extracted from the sediment by centrifugation at 1,000 × g, followed by filtration of the supernatants through a 0.2-μm pore size membrane filter. DCAA pore-water samples (100 μl in 1.5-ml glass vials) were oven-dried at 60°C and transferred to Teflon reaction vessels before microwave hydrolysis (Jørgensen and Jensen 1997). The concentration of DCAA was obtained by subtracting the concentration of DFAA from the total dissolved concentration. Sediment for analysis of THAA was dried at 60°C and ground in a mortar, after which 20–30 mg of sediment was added to 1.5-ml glass vials and hydrolyzed by the microwave method. The hydrolyzed samples were redissolved in 1.25 M borate buffer adjusted to pH 9.5 (for DCAA) and pH 12 (for THAA), followed by filtration through a 13-mm diameter, 0.2-μm pore size filter.

Amino acids were quantified by high-performance liquid chromatography (HPLC) and fluorescence detection using two methods. Total amounts of DFAA, DCAA, and THAA were detected as fluorescent primary amines after an *o*-phthaldialdehyde (OPA) derivatization according to Lindroth and Mopper (1979) and Jørgensen et al. (1993). In addition to protein amino acids (except for Pro and Cys), the analysis included ornithine (Orn), α-aminobutyric acid (α-ABA) and γ-aminobutyric acid (γ-ABA). D- and L-isomers of Asp, Glu, serine (Ser), and Ala were measured by the method of Mopper and Furton (1991), with the exception that *N*-isobutyryl-L-cysteine (IBC) was used as a chiral agent (Brückner et al. 1994). The HPLC columns were 3.9 by 150 mm (Nova-Pak C18 steel column, Waters Associates) for the OPA method and 4.6 by 250 mm (Discovery C18 steel column, Supelco) for the IBC method.

D- *and* L-*amino acids in pure cultures*—Three bacteria strains (AT, B, and BJ) were analyzed for content of L- and D-isomers of Asp, Glu, Ser, and Ala. The bacterial cultures were originally isolated from the water column of Roskilde Fjord, Denmark, by L. Frette. Based on 16S RNA gene sequencing, the strains have tentatively been identified to the following genera: *Erythrobacter* (AT, a small, rod-shaped, G$^-$ bacteria), *Pseudomonas* (B, a mobile, long, rod-shaped, G$^-$ bacteria), and *Bacillus* (BJ, a large, mobile, rod-shaped, G$^+$ bacteria) (Frette and Johnsen unpubl. data).

The strains were grown in autoclaved Roskilde Fjord water enriched with ZoBell media. The cells were harvested in the early phase of stationary growth by centrifugation at 6,000 × g at 4°C for 10 min. The cells were washed three times in 0.9% NaCl. The cells were hydrolyzed and analyzed for L- and D-isomers of Asp, Glu, Ser, and Ala as described above. Samples for bacterial counts were fixed with 2% formaldehyde (final concentration), and stored refrigerated until staining with 4′,6-diamidino-2-phenylindole (DAPI) according to Porter and Feig (1980). The bacteria were counted at ×1,000 magnification by epifluorescence microscopy (Zeiss Axioplan). In addition to the three bacterial

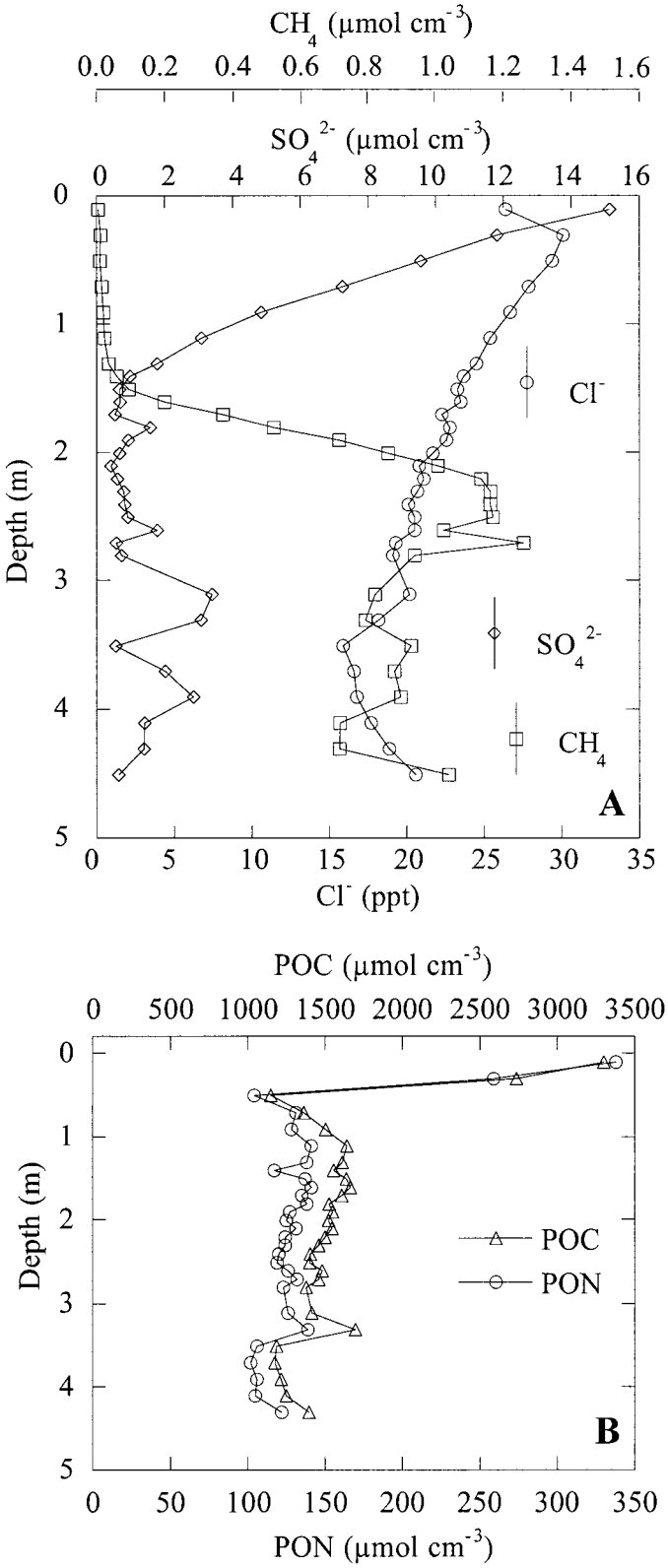


Fig. 1. Concentrations of (A) CH_4, SO_4^{2-}, Cl^-, and (B) POC and PON in the sediment. The Cl^- concentration in the upper sediment layer was assumed to be equivalent to the salinity of the seawater.

strains, peptidoglycan from the G^+ *Staphylococcus aureus* (Sigma-Aldrich) was hydrolyzed and analyzed for the four above-mentioned L- and D-isomers as described for sediment samples.

Results

Chemical sediment profiles—The sediment density was 1.30 g cm^{-3} throughout the core, whereas the porosity increased slightly with depth from 0.81 ml cm^{-3} at 0.1 m to 0.83 ml cm^{-3} at 4.6 m. The concentration of sulfate decreased from 15.1 μmol cm^{-3} (18.6 mM) in the upper sediment to <1 μmol cm^{-3} below 1.5 m (Fig. 1A). Some elevated concentrations of SO_4^{2-} (up to 3.4 μmol cm^{-3}) were measured between 3.0 and 4.6 m deep. The chloride concentration varied with depth, decreasing from 30.1‰ at 0.3 m to 15.9‰ at 3.5 m, below which it increased to 20.6‰ at 4.6 m (Fig. 1A). The concentration of CH_4 was < 0.04 μmol cm^{-3} in the upper 1.3 m, but it increased steeply to 1.13 μmol cm^{-3} at 2.2 m deep. From 2.2 to 4.5 m, CH_4 varied between 0.72 and 1.23 μmol cm^{-3} (Fig. 1A).

Particulate organic carbon and nitrogen—The amount of both POC and PON declined sharply with depth from 3,300 and 340 μmol cm^{-3}, respectively, at a depth of 0.1 m to 1,150 and 105 μmol cm^{-3}, respectively, at a depth of 0.5 m (Fig. 1B). Between depths of 0.5 and 1.0 m, there was a minor increase, below which the concentrations remained rather constant between 1,400 and 1,700 μmol cm^{-3} (POC) and 115 and 140 μmol cm^{-3} (PON). At the bottom of the core, lower concentrations of 1,175 to 1,250 μmol cm^{-3} (POC) and 102 to 106 μmol cm^{-3} (PON) were measured. The C : N ratio (POC : PON) was 11.56 $\pm$ 0.64 on average and did not demonstrate trends with depth, except for lower ratios of 9.76 and 10.58 at 0.1 and 0.3 m, respectively (data not shown).

Total amino acid pools—The concentration of DFAA decreased sharply from 25.3 nmol cm^{-3} at 0.1 m to 2.0–6.1 nmol cm^{-3} at 0.3–1.3 m deep (Fig. 2A). Lower concentrations occurred from 1.3 to 2.7 m (1.7–2.7 nmol cm^{-3}), but increased concentrations were found below 3.1 m (4.9–6.0 nmol cm^{-3}). The dominant DFAA were the protein amino acids Asp, Glu, Ser, and Ala, which together accounted for a 41 to 68 mole percentage of the DFAA pool.

In contrast to DFAA, the amount of DCAA increased with depth from 55 to 83 nmol cm^{-3} in the upper 0.5 m to 105 to 150 nmol cm^{-3} in deeper layers (Fig. 2B). Asp, Glu, Ser, Ala, and Gly accounted for 66–80% of the 19 identified DCAA, which included the three nonprotein amino acids Orn, α-ABA, and γ-ABA. Gly alone made up 14–18% of the DCAA pool.

The THAA pool decreased gradually from 58 μmol cm^{-3} at 0.1 m deep to 8.4 μmol cm^{-3} at 4.3 m deep (Fig. 2C). Glycine was the dominant THAA and increased in molar importance from 19% at 0.6 m, to 29% of all THAA at 4.3 m deep. Next to Gly, the major amino acids were Asp, Ala, Glu, and Ser, which accounted for 41 to 52% of the THAA pool. In contrast to Gly, the molar percentage of Asp, Glu, and Ser declined with increased sediment depth. THAA-N made up from 14.0 to 26.7% of the PON pool between 0.1 and 0.9 m, below which the contribution of THAA-N decreased to 8.7% at depths of 4.3 m (Fig. 2D).

The molar fraction of the nonprotein amino acid Orn increased from 0.3 to 1.8% between 0.1 and 1.7 m, whereas α-ABA and γ-ABA increased from top to bottom of the core and made up from 0 to 0.2% and from 0.1 to 0.6%, respectively, of the THAA pool (data not shown).

Occurrence of L- *and* D-*enantiomers*—Depth profiles of L-Asp, L-Glu, L-Ser, and L-Ala were similar to those of DFAA (Figs. 2A, 3A–D). The local DFAA maximum at 3 to 4 m deep was most pronounced for L-Ser (0.95 nmol cm^{-3}, Fig. 3C). Concentrations of the individual D-DFAA were always lower than their respective L-configurations. D-Glu, D-Ser, and D-Ala peaked at 0.1 m deep with concentrations of 0.46, 0.12, and 0.11 nmol cm^{-3}, respectively. Below 0.5 m, the concentration of each of the four D-amino acids was <0.09 nmol cm^{-3}.

In the DCAA pool, D- and L-isomers of Asp, Glu, Ser and Ala increased with depth as was observed for all DCAA (Figs. 2B, 4A–D). The concentration of the four L-DCAA increased about three times between 0.1 m (0.4 m for L-Glu) and 4.3 m; the concentration of L-Glu was higher at 0.1 than at 0.4 m. The order of importance of the D-DCAA were D-Asp $>$ D-Ala $>$ D-Glu $>$ D-Ser, with concentrations of 1.1 to 4.9, 0.72 to 2.7, 0.51 to 2.1, and 0.07 to 0.78 nmol cm^{-3}, respectively (Figs. 4A–D).

Profiles of the four L-THAA amino acids Asp, Glu, Ser, and Ala resembled the depth distribution of the total THAA pool (Figs. 2C, 5A–D). The L-amino acids decreased with depth from maximum concentrations of 9.7 (Asp), 6.1–6.4 (Ala and Glu), and 4.3 μmol cm^{-3} (Ser) at 0.1 m to <2.0 (Ala and Asp) and 0.5 μmol cm^{-3} (Glu and Ser) at 4.3 m. The concentration of all four D-THAA declined at depths from 0.1 to 0.4 m. Between 0.4 and 3 m, the concentrations were variable, but below 3 m they all decreased. In most depths, the D-THAA concentrations were less than 0.15 (Ser), 0.3 (Glu), 0.5 (Ala), and 1.5 μmol cm^{-3} (Asp).

Relative proportion of D-*amino acids*—In the DFAA pool, the proportion of D-Asp and D-Ala exceeded that of D-Glu and D-Ser, except at 0.1 m (Fig. 6A). The proportion is the percent D-amino acids according to the equation: (D-amino acid $\times$ 100)/(D-amino acid + L-amino acid). The proportion of D-Asp peaked between 0.9 and 2.0 m (up to 18%), whereas D-Ala constituted up to 10% between 1.5 and 2.7 m, but large variations occurred. Minimum values were 5.2% (D-Asp) and 3.9% (D-Ala) at 0.1 m. In contrast, the proportions of D-Ser and D-Glu had maxima at 0.1 m (9.0 and 6.2%, respectively). Deeper in the sediment, D-Ser and D-Glu varied between 1.8 and 6.5%, and 1.3 and 2.5%, respectively.

Among the DCAA, the proportion of D-Asp, D-Glu, D-Ser, and D-Ala always exceeded their respective contributions in the DFAA pool, except for a local minimum of D-Ser of 1.5% at 0.1 m (Figs. 6A,B). The four D-DCAA varied between 13.7 and 23.8% (D-Asp), 9.0 and 16.6% (D-Glu), 5.8 and 9.7% (D-Ser), and 11.5 and 15.4% (D-Ala) throughout the sediment core, with the lowest proportions between 1.5 and 2.7 m.

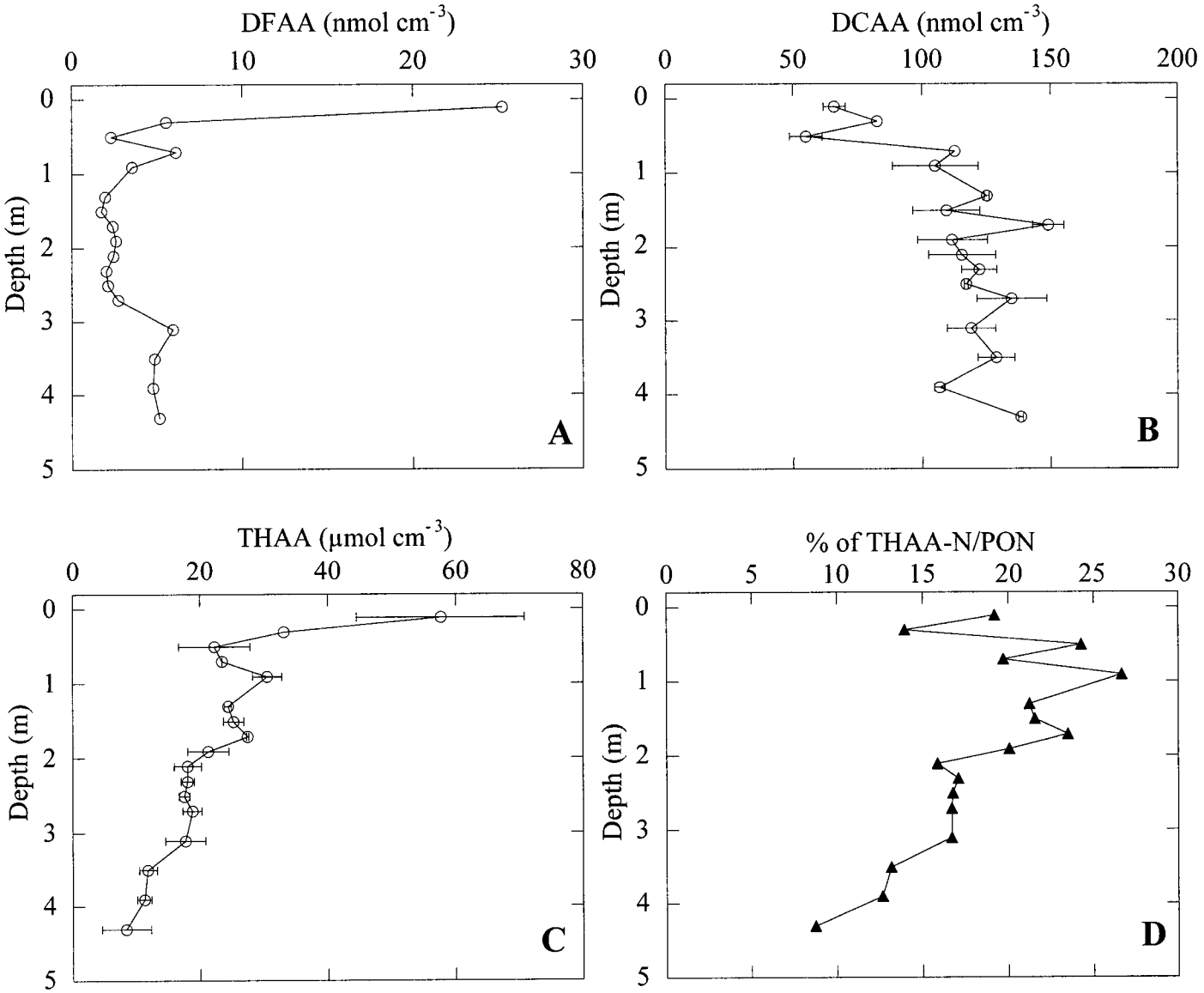


Fig. 2. Concentrations of (A) DFAA, (B) DCAA, (C) THAA, and (D) percent THAA-N versus PON. Mean values ± SD (n = 3) shown for A, B, and C.

The D-isomers of the THAA all increased from surface to bottom, with proportions of 13.7 to 30.2% (D-Asp), 8.9 to 15.2% (D-Ala), 8.1 to 13.6% (D-Glu), and 3.7 to 19.5% (D-Ser) between 0.1 and 4.6 m (Fig. 6C).

L- and D-amino acids in different bacteria—In order to relate occurrence of D- and L-amino acids in the sediment to the D- and L-amino acid content of bacterial cells, amino acids in whole-cell hydrolysates of the G^+ bacteria (BJ) and the two G^- strains (AT and B) and a commercial peptidoglycan were analyzed. The bacterial hydrolysates included both free and combined amino acids of the bacteria. We assumed all D-amino acids to originate in peptidoglycan; however, some D-amino acids may have been present in the bacterial cytoplasm because bacterial enzymes are known to produce free D-amino acids (Asano and Lübbehüsen 2000).

The G^+ BJ bacterium had a lower proportion of D-Asp and D-Ser than the G^- AT and B, whereas the proportions of D-Glu and D-Ala were similar in the three bacteria (Table 1). Peptidoglycan of the G^+ *Staphylococcus aureus* had a higher relative content of D-Asp, D-Glu, D-Ser, and D-Ala than the whole-cell hydrolysates of BJ, AT, and B.

For a comparison of D-amino acids in the three bacteria and in peptidoglycan, the amino acid content was normalized to D-Ala. In all three cultured bacteria, D-Asp and D-Glu were 1.1- to 1.9-fold more abundant than D-Ala, whereas the content of D-Ser was ~10 times lower than that of D-Ala (Table 2). The content of D-Ser in the B strain was close to the analytical detection limit. Peptidoglycan of *S. aureus* had a content of D-amino acids in the following order: D-Glu > D-Ala > D-Asp > D-Ser. Determination of the cell-specific concentration of D-Ala in strain AT and BJ demonstrated that the G^+ bacterium had a 1.7-fold higher content of D-Ala (6.6 amol cell^{-1}) than the G^- bacterium (3.8 amol cell^{-1}) (Table 2).

Table 1. Relative content of D-Asp, D-Glu, D-Ser, and D-Ala in whole cells of two G^- (AT and B) and one G^+ bacterium (BJ). Mean values ± 1 SD ($n = 3$) shown. Peptidoglycan was obtained from the G^+ *S. aureus.*

Bacteria	Relative content (%)			
	D-Asp	D-Glu	D-Ser	D-Ala
AT (G^-)	8.1 ± 1.2	11.2 ± 0.9	1.3 ± 0.4	6.9 ± 0.2
B (G^-)	7.7 ± 0.9	8.5 ± 0.3	0.8	5.1 ± 0.2
BJ (G^+)	5.9 ± 0.3	9.4 ± 0.8	0.5 ± 0.1	5.9 ± 0.4
Peptidoglycan (G^+)	22.5	31.9	2.5	24.6

Discussion

Sediment characteristics—The profiles of sulfate and methane indicate that the Aarhus Bay sediment core could be divided into three distinct zones: (1) a declining SO_4^{2-} (from 15 to 1.0 μmol cm^{-3}) and low CH_4 ($<$0.1 μmol cm^{-3}) zone in the upper 1.5 m, (2) a low SO_4^{2-} ($<$1.8 μmol cm^{-3}) and a high CH_4 (0.2–1.3 μmol cm^{-3}) zone between 1.5 and 3.0 m, and (3) a partly elevated SO_4^{2-} (0.6–3.4 μmol cm^{-3}) and high CH_4 zone below 3.0 m (Fig. 1A).

The declining sulfate concentration in zone 1 supports that microbial sulfate reduction was the dominant metabolic process for carbon oxidation in the sediment, as observed by Jørgensen et al. (1990) at the same location. The accumulation of CH_4 in zone 2 indicates a high activity of methanogenic bacteria. The decrease of CH_4 below 3 m probably was caused by anoxic oxidation of methane, coupled with sulfate reduction (Thomsen et al. 2001). The appearance of an increasing concentration of Cl^- (below 3.5 m) and CH_4 (below 4.3 m) in the sediment was unexpected. These changes appeared not to be caused by sampling artifacts (e.g., flushing of the sediment core with seawater) because this would have removed CH_4. Presumably, there was an in situ intrusion of seawater into the sediment from an unknown source. The slight increase of POC and PON at the bottom of the core (Fig. 1B) may indicate local changes of the sediment environment, possibly caused by intrusion of seawater, but to which extent this influenced the bacterial activity and the observed amino acid profiles, is unknown.

The division of sediment into three zones was mirrored in the occurrence of all DFAA, which declined steeply in zone 1, remained rather constant in zone 2, but increased in zone 3. A similar trend was observed for L-DFAA Glu, Ser, and Ala and in the proportion of the respective D-DFAA. In contrast, a three-zone pattern was not obvious for the concentrations of POC, PON, and THAA, which all decreased significantly in the upper 0.5 m and then subsequently slowly declined with depth. DCAA had an opposite pattern, as the concentrations doubled between 0.1 and 1.0 m, below which they remained rather constant.

Content of POC, PON, THAA, DCAA, and DFAA—The fraction of PON identified as THAA-N (an increase from 14 to 24% between 0.1 and 0.7 m, followed by a decrease to 9% at 4.3 m; Fig. 2D) is low compared to other studies. In sediment (1–6 cm) from the same location, Pedersen et al. (1999) previously found that THAA-N made up 46–51% of the total N. Similarly, in the upper 80 cm of Saanich Inlet (B.C., Canada) sediment, Cowie et al. (1992) found that 44.5–33.9% of the total N was THAA-N, and at seven stations in the North Sea, THAA-N contributed 25–60% to the total N (Dauwe and Middelburg 1998).

Despite THAA constituting a relatively low proportion of PON in the Aarhus Bay sediment, concentrations, depth profiles, and relative composition of THAA, DCAA, and DFAA were comparable to other coastal marine sediments. In the anoxic sediment of Cape Lookout Bight, North Carolina, USA, concentrations of DFAA were highest near the sediment–water interface (typically 20–60 μM compared to ~31 μM in the present study) and decreased asymptotically to 2–5 μM below 20 cm ($<$7 μM in the present study) (Burdige and Martens 1990). The concentration of DCAA of 66 nmol cm^{-3} at 11 cm deep in our study falls within concentrations previously measured in the upper ~5 cm of sediment of a shallow Danish Cove (Knebel Vig: 35–75 nmol N cm^{-3}; Lomstein et al. 1998). THAA in marine sediments have been

Table 2. Cell-specific concentrations of D-Ala of the AT and BJ species. The content of D-Asp, D-Glu, and D-Ser relative to D-Ala (mol/mol) in the AT, B, and BJ species and in the peptidoglycan of *S. aureus* is indicated as mean values ± 1 SD ($n = 3$). Except for the peptidoglycan of *S. aureus*, whole cells were used for all analyses. nd, no data.

Bacteria	D-Ala (amol cell^{-1})	Concentration		
		D-Asp/D-Ala	D-Glu/D-Ala	D-Ser/D-Ala
AT (G^-)	3.8	1.24 ± 0.12	1.55 ± 0.16	0.11 ± 0.03
B (G^-)	nd	1.50 ± 0.02	1.82 ± 0.13	0.07
BJ (G^+)	6.6	1.14 ± 0.16	1.91 ± 0.15	0.06 ± 0.01
Peptidoglycan (G^+)	nd	0.72	1.36	0.03

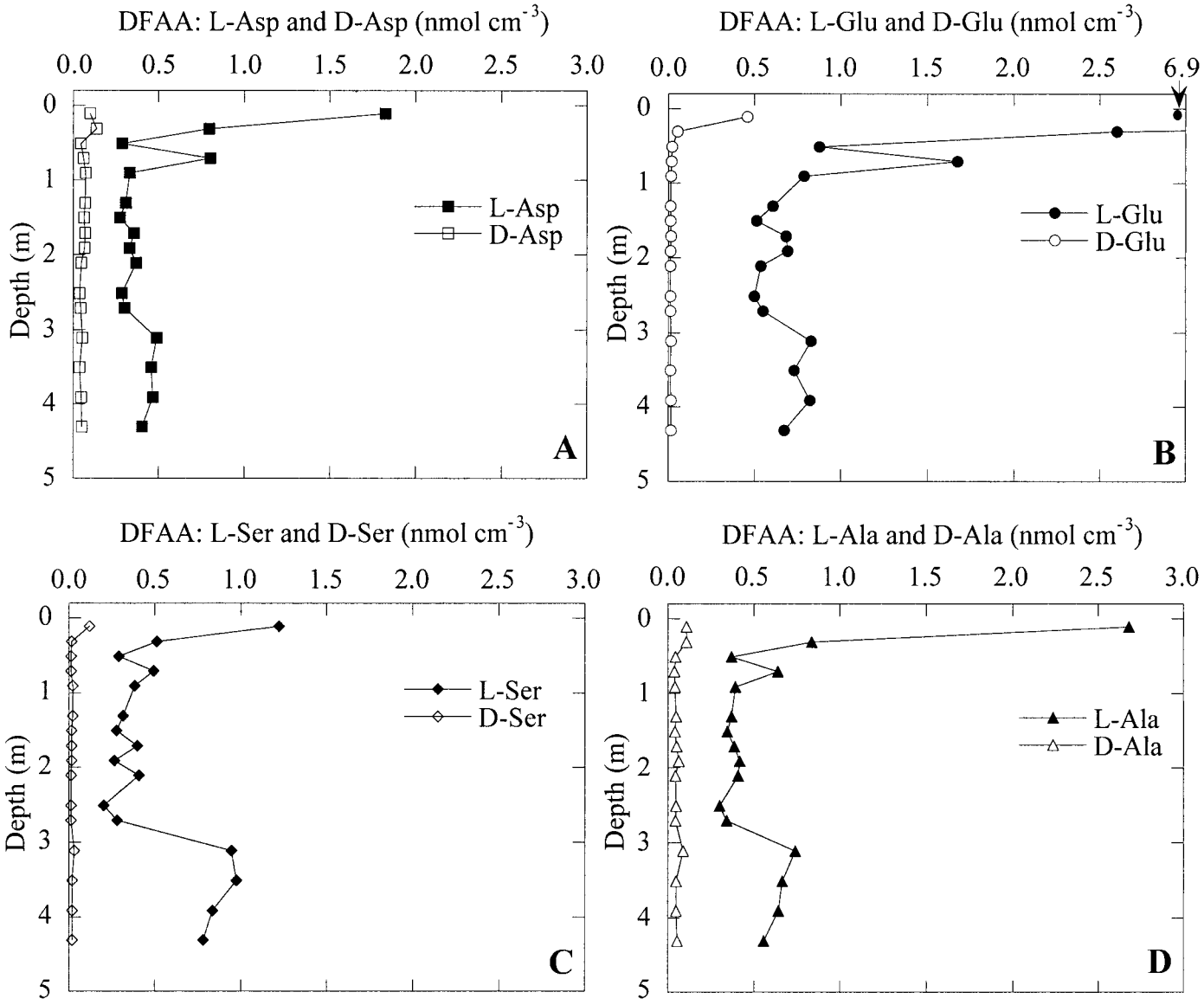


Fig. 3. Depth profiles of D- and L-isomers of four DFAA: (A) Asp, (B) Glu, (C) Ser, and (D) Ala. Single concentration measurements were performed. The analytical uncertainty is <5%.

found to vary between 21 and 76 μmol g dw (upper 66 cm in Buzzards Bay, Massachusetts, USA, sediment; Henrichs and Farrington 1987), and 22–37 μmol cm^{-3} (surface sediment from Knebel Vig, Denmark; Lomstein et al. 1998). These concentration ranges agree with the present pools of 8–59 μm cm^{-3}. The THAA peak at 11 cm depth (58 μmol cm^{-3}) is in accordance with a previous analysis of THAA in the upper 6 cm of the sediment layer from the same location (63 μmol cm^{-3}; Pedersen et al. 1999). The declining concentration of THAA-N with depth indicated a degradation of THAA to a depth of at least 4.3 m; hence, a continuous bacterial activity may have occurred for more than 2,000 yr at this depth.

Composition of THAA, DCAA, and DFAA—Glu, Ala, Asp, Ser, and Gly dominated the molar composition of DFAA, DCAA, and THAA. The mole percentage of Gly increased through the pools of DFAA, DCAA, and THAA. A similar trend has previously been observed in the surface sediment of Knebel Vig, Denmark (Lomstein et al. 1998). Furthermore, Cowie and Hedges (1992) measured an order of dominating THAA (Gly, Asp, Ala, Glu, and Ser) almost similar to Aarhus Bay in the sediment of Dabob Bay, Washington, USA. The tendency of a decreased mole percentage of Glu and Asp and an increased mole percentage of Gly with increased depth is commonly observed in marine sediments (e.g., Brown et al. 1972; Cowie et al. 1992).

The increase in mole percentage of the nonprotein THAA, α-ABA, γ-ABA, and Orn, with sediment depth probably was related to microbial activity. α-ABA is produced from dehydration of threonine (Thr), γ-ABA is formed from decarboxylation of Glu (Lee and Cronin 1982), and Orn is a fermentation product of arginine (Arg). The increase of these amino compounds with sediment depth, combined with the

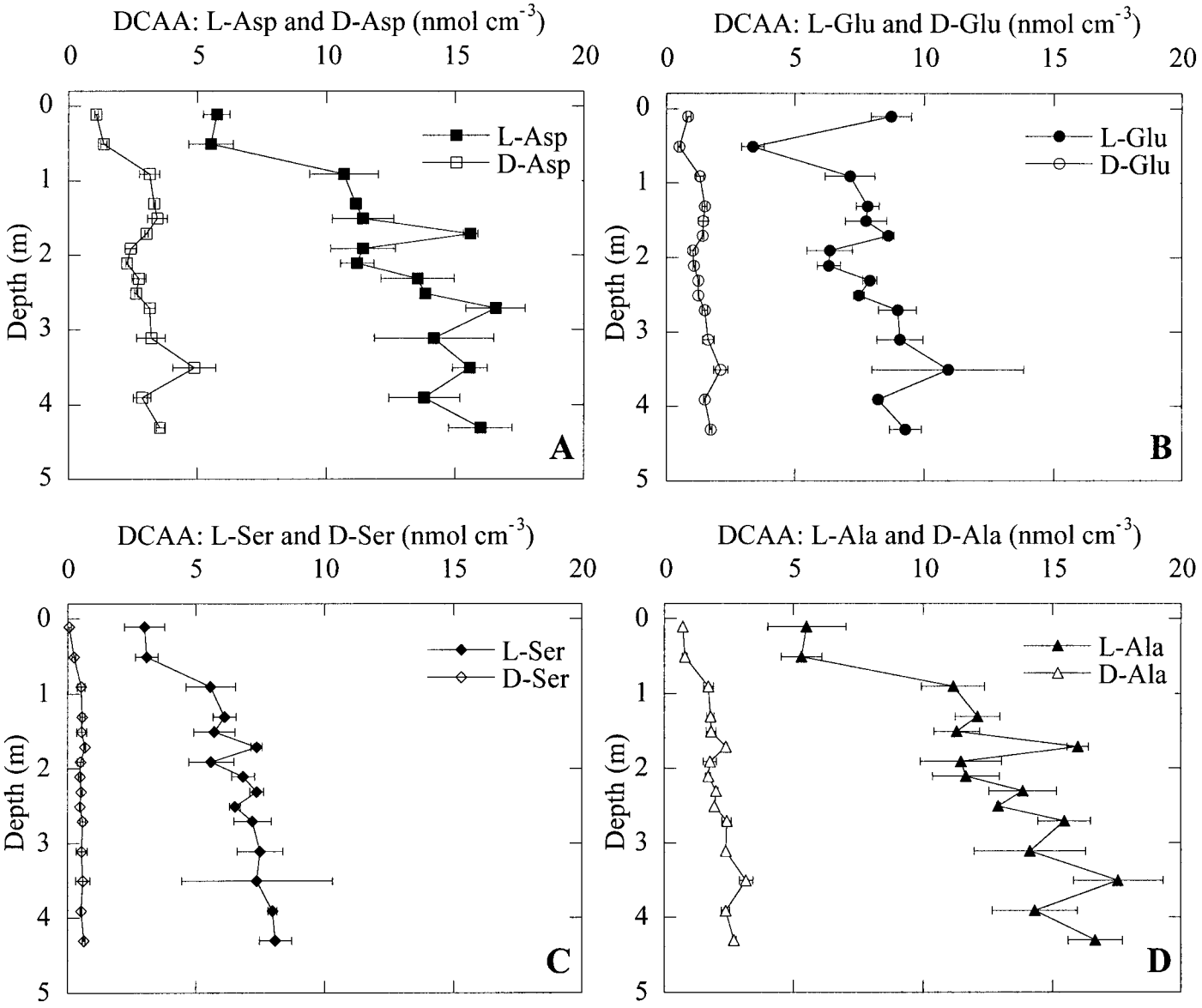


Fig. 4. Depth profiles of D- and L-isomers of four DCAA: (A) Asp, (B) Glu, (C) Ser, and (D) Ala. Mean values ± 1 SD (n = 3) shown.

inverse down-core behavior of their parent amino acids, suggests that the changes in composition were related to microbial metabolism. Cowie et al. (1992) found similar down-core compositional changes in the mole fraction of Glu and γ-ABA in the sediment of Saanich Inlet. In addition, Dauwe and Middelburg (1998) observed an increase in the mole percentage of γ-ABA in relatively labile (Broad Fourteens and Brouwerhavenschegat [B], <0.22 mole-%) and more refractory (Skagarrak, 1.01 mole-%) North Sea sediment.

Indications on bacterially derived D-*amino acids*—D-amino acids of the THAA pool peaked at a sediment depth of 11 cm, with D-Asp being most important, followed by D-Ala, D-Glu, and D-Ser. The maximum abundance of D-amino acids in the upper sediment most likely reflects a high bacterial density and, with this, a high content of peptidoglycan. Supporting this, Jørgensen et al. (1990) found a maximum bacterial density of 4.5×10^8 cells cm^{-3} within the upper 10 cm of sediment at the same location. Bacteria have previously been suggested to be the source for D-Asp, D-Ala, and probably also D-Glu in marine surface sediment (Pollock and Kvenvolden 1978), as well as in the water column (Lee and Bada 1977; McCarthy et al. 1998).

Do the measured concentrations of D-amino acids in the sediment reflect intact bacterial cells, empty cell sacs (in sensu Moriarty and Hayward 1982), or bacteria-free peptidoglycan? To answer this question, the bacterial density in the sediment was estimated from the D-Ala content of the THAA pool and the D-Ala content of the cultured G^+ and G^- bacteria (Table 2). The resultant ''cell numbers'' at sediment depths of 11 cm were 1.7×10^{11} cells cm^{-3} (based on the G^- AT strain) and 9.6×10^{10} cells cm^{-3} (based on

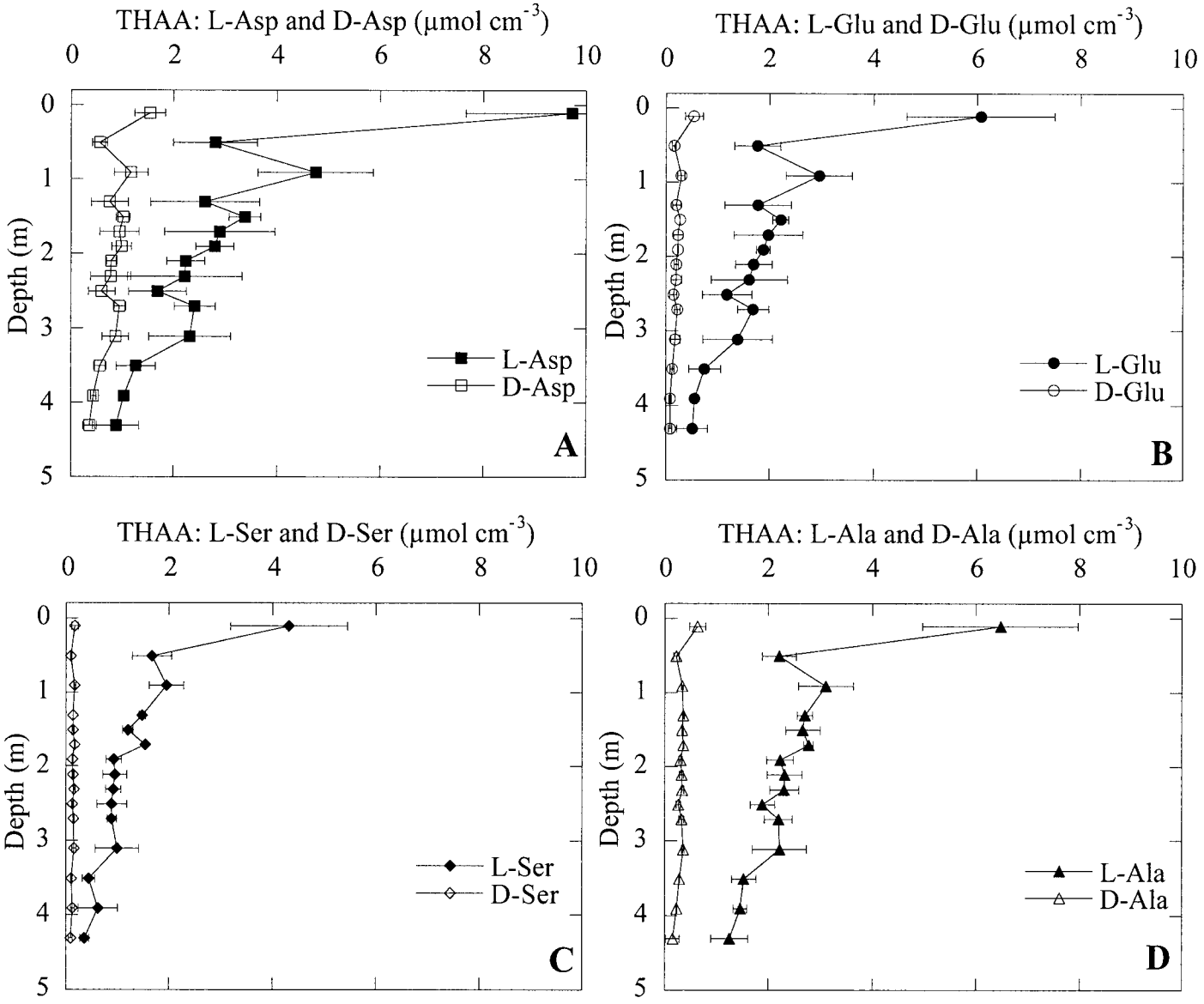
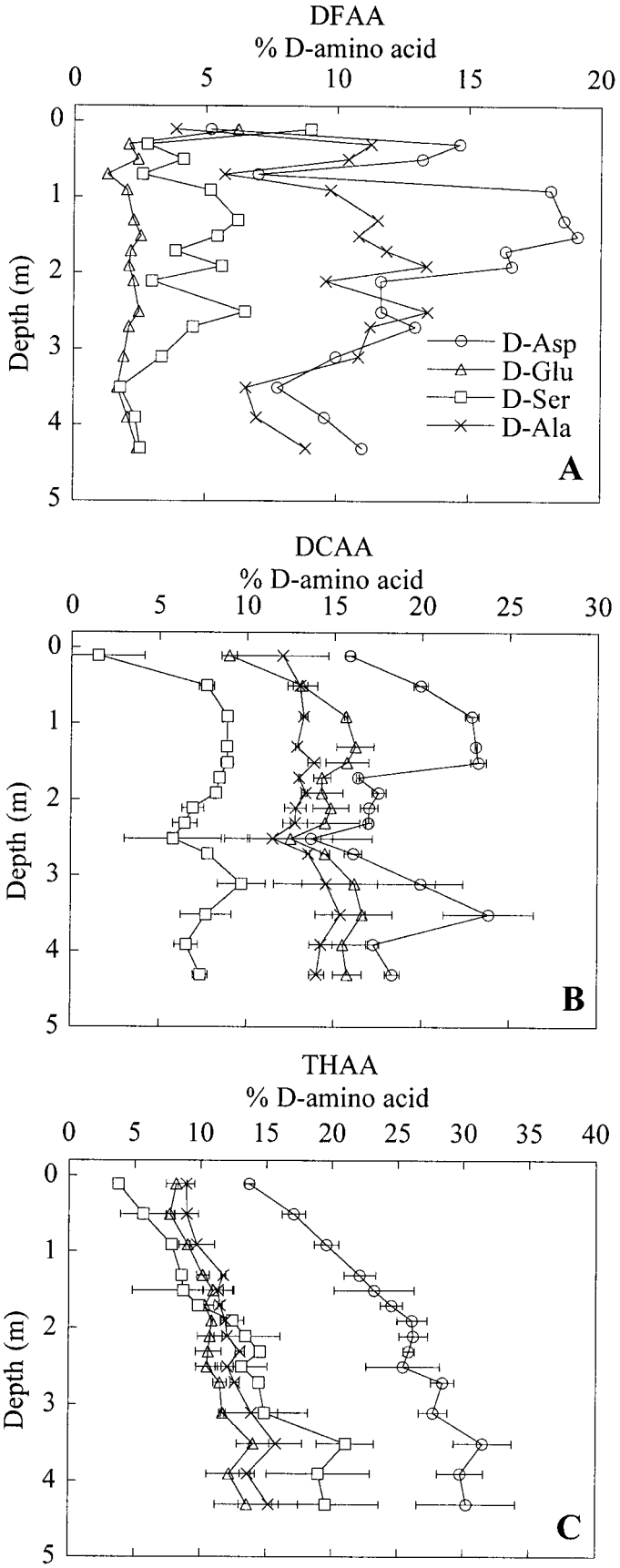


Fig. 5. Depth profiles of D- and L-isomers of four THAA: (A) Asp, (B) Glu, (C) Ser, and (D) Ala. Mean values ± 1 SD (n = 3) shown.

the G^+ BJ strain). At sediment depths of >11 cm, the respective "cell numbers" were 3.9–9.6 × 10^{10} cells cm^{-3} and 2.2–5.5 × 10^{10} cells cm^{-3}. The number of G^- cells probably was higher than the number of G^+ cells in the sediment, because Moriarty and Hayward (1982) found that anoxic sediment contained 70% G^- and 30% G^+ cells. The estimated bacterial "cell numbers" were ~1,000 times higher than bacterial numbers obtained by epifluorescence microscopy in the upper 2 m of this sediment (Jørgensen et al. 1990). Although the estimated bacterial densities probably are biased by a higher cell biomass of the laboratory strains than the sediment bacteria and by expected differences between aerobic estuarine bacteria (the AT and BJ strains) and anaerobic sediment bacteria, this 1,000-fold difference most likely indicates that most D-Ala of the THAA fraction consisted of empty cell sacs and cell wall fragments, including peptidoglycan, which persisted in the sediment for a long time after cell death.

Because D-Ala and D-Glu are reported to be the dominant D-amino acids in peptidoglycan (Schleifer and Kandler 1972; Prescott et al. 1996), we were surprised to find that D-Asp was the most important D-amino acid of the THAA, DCAA, and DFAA pools at most sediment depths and that the three analyzed bacterial strains contained more D-Asp and D-Glu than D-Ala (Table 2). However, a high content of D-Asp has been found in some bacterial strains, including *Streptococcus* sp., *Lactobacillus* sp., and *Synechococcus bacillaris* (Stalton 1964; Brückner et al. 1994; McCarthy et al. 1998). Variability in peptidoglycan structures and in the species composition among marine bacterial communities clearly is poorly defined, as stressed by McCarthy et al. (1998).



Racemization—The increasing proportion of D-isomers of THAA with depth might, in addition to a bacterial origin, be the result of chemical racemization (Bada 1982). Racemization of sediment amino acids was studied in Saanich Inlet by Kvenvolden and Peterson (1970), who found that the dominant peptidoglycan amino acids Ala and Glu appeared to racemize at a higher rate than other amino acids. In order to examine if racemization could have influenced the presently measured D-amino acids, the age of the sediment was estimated. In the calculation of racemization rates, the equation and racemization constants of Ala and Asp by Bada (1982) was applied to THAA from a depth of 3.5 m, assuming a sediment temperature <10°C. The calculation estimated the sediment age to be 15,000 or 28,000 yr, depending on whether the percentage of D-Ala or D-Asp, respectively, was applied. The age of the sediment at 3.5 m was estimated at 1,750 yr based on extrapolation of a Pb-210 sediment dating by Pejrup et al. (1996). This suggests that racemization was not a major process in the formation of D-amino acids in the Aarhus Bay sediment. Similarly, Lee and Bada (1977) concluded that racemization during typical oceanic mixing periods (570 and 1,700 yr in the North Atlantic and South Pacific, respectively) could not account for the D:L ratios of amino acids found in oceanic waters. Furthermore, the conclusions by Kvenvolden and Peterson (1970) on racemization of sediments in Saanich Inlet was invalidated by Pollock and Kvenvolden (1978), who found a high content of D-amino acids in THAA from surface sediment of Saanich Inlet, indicating a bacterial D-amino acid production rather than a racemization.

Preservation of D-*amino acids in the sediment*—The amino acid analysis of Aarhus Bay sediment suggests that there was a selective degradation of specific amino acids: (1) the higher proportion of D-enantiomers in the THAA pool with increasing sediment depth suggest that D-amino acids, to a larger extent than L-amino acids, resisted degradation; (2) the increasing proportion of D-amino acids in the THAA pool relative to the dissolved pools (DCAA and DFAA) suggest that dissolved amino acids were degraded at a higher rate than particulate amino acids. Whether L- and D-forms of combined and free amino acids of the dissolved pool are used at similar rates by sediment bacteria is unknown. Bacteria in soils apparently do not have a preference for D- or L-forms of free amino acids (O'Dowd and Hopkins 1998) and this may also be true for marine sediments.

The apparent selective removal of L- to D-amino acids in Aarhus Bay sediment probably reflects that D-isomers of the THAA pool occur in a form that is less accessible to biological degradation than L-amino acids. We hypothesize that THAA are composed of at least two pools with different degradability: (1) structural biopolymers including D- and L-amino acids of bacterial cell walls and peptidoglycan and (2) ''conventional'' proteinaceous material that is largely

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Fig. 6. Depth profiles of the percent D-amino acids of four amino acids in the (A) DFAA pool, (B) DCAA pool, and (C) THAA pool. Mean values ± 1 SD ($n = 3$) shown.

composed of L-amino acids. With time, the conventional proteinaceous material is degraded (hydrolyzed) by microorganisms to a larger degree than peptidoglycan, leading to an enrichment of THAA with D-amino acids. This selective degradation of THAA might explain the observed D-amino acid enrichment over time and depth in the sediment.

Contribution of peptidoglycan to THAA and PON—The contribution of peptidoglycan-N to the pool of THAA-N in oceanic waters has been estimated as 45–80% (McCarthy et al. 1998). These authors also suggested that peptidoglycan remnants were a major source of DON in the ocean. In an attempt to determine the relative contribution of peptidoglycan ($THAA_{pep}$) to the total THAA pool in the present sediment, we applied two different assumptions: (1) the percentages of D- and L-isomers of Asp, Glu, Ala, and Ser in sediment peptidoglycan ($THAA_{pep}$) were assumed to be identical to the respective percentages in peptidoglycan of the G^+ *S. aureus* (Table 1) and (2) the percentage of the respective D-amino acids in sediment peptidoglycan was 50% as suggested by McCarthy et al. (1998). The total sediment peptidoglycan pool ($THAA_{pep}$) was calculated as D-THAA (measured in the sediment) + L-$THAA_{pep}$ (measured as D-THAA multiplied by the peptidoglycan L : D ratios from the respective assumptions). The calculated proportion of $THAA_{pep}$ = ($THAA_{pep} \times 100$)/THAA pool is shown for each assumption in Fig. 7A.

According to assumption 1 (based on D- and L-isomer composition of peptidoglycan in *S. aureus*), the percent $THAA_{pep}$ of the total THAA increased with depth from 39% at 0.1 m to 90% at 3.5 m (Fig. 7A). However, because bacterial populations in anoxic sediments are dominated by G^- bacteria (Moriarty and Hayward 1982), the content of $THAA_{pep}$ based on the calculation with the G^+ bacterium *S. aureus* probably is biased.

Assumption 2 (D-amino acids made up 50% of peptidoglycan) estimates a lower content of peptidoglycan in the THAA pool, ranging from 9% at 0.3 m to 18% at 3.5 m (Fig. 7A). This contribution of peptidoglycan amino acids to the THAA pool is significantly lower than the values obtained from the D- and L-isomer composition of *S. aureus*. Assumption 1 is supported by McCarthy et al. (1998), who found that $THAA_{pep}$ was a major source of THAA in the ocean. However, to decide whether assumption 1 or 2 most likely mirrors the actual input of bacterial cell wall material in the sediment, more studies on composition of bacterial communities and their peptidoglycan content in sediments are required.

The relative contribution of $THAA_{pep}$ to the PON pool was estimated from the same assumptions as above. The percent $THAA_{pep}$-N of PON increased steeply from 0.1 to 1.0 m (Fig. 7B). Below 1.0 m deep, the proportion of $THAA_{pep}$-N decreased to 6% (assumption 1) and 1% (assumption 2) at 4.3 m deep. Hence, peptidoglycan of bacterial cell walls appeared to be a minor source of PON in this marine sediment. The declining proportion of peptidoglycan THAA below 1.0 m deep suggests that organic nitrogen compounds other than peptidoglycan were more resistant to biological degradation.

The different estimates of the contribution of peptidoglycan to the THAA and PON pools from assumption 1 and 2

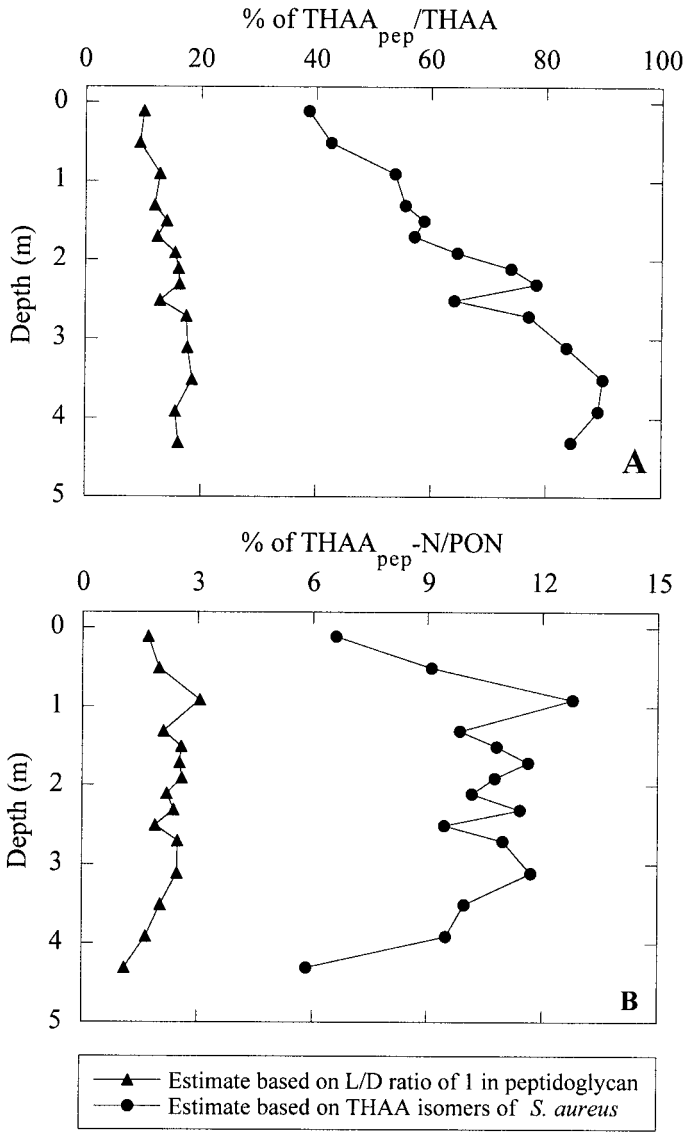

Fig. 7. (A) Peptidoglycan amino acids ($THAA_{pep}$) as percentage of THAA and (B) peptidoglycan amino acid N content ($THAA_{pep}$-N) as percentage of PON in the sediment. The calculations were based on an assumed L : D ratio of 1 in peptidoglycan and proportions of D- and L-amino acids in peptidoglycan of *S. aureus*. See text for further information.

clearly demonstrate that the present knowledge of the role of bacterial cell walls in biogeochemical processes in marine sediments is limited. In order to test the validity of the two assumptions, the occurrence of G^+ and G^- bacteria in marine sediments and their potential for degradation must be examined. Despite these reservations, our study suggests that analysis of peptidoglycan amino acids in marine sediments may provide new information on the fate of prokaryotes in anoxic marine environments.

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