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Utilization of marine sedimentary dissolved organic nitrogen by native anaerobic bacteria

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Abstract

Bioavailability and chemical composition of dissolved organic nitrogen (DON) in marine pore water were followed and related to bacterial activity in a 14-d anoxic decomposition experiment. For the experiment 0.2-μm filtered pore water from a coastal marine sediment was inoculated with native sediment bacteria. The initial composition of DON was characterized by a high contribution of dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA), and urea, which together accounted for 82% of the DON pool. During the experiment, 54% of the DON was used by bacteria, indicating that DON was readily available to bacterial degradation. The consumption of DFAA and DCAA accounted for more than half of the DON consumed. The unidentified DON pool accounted for 33% of the net DON consumption, and the unidentified DON pool was fully depleted by the end of the experiment. Systematic changes in the amino acid composition occurred with time of incubation, as demonstrated by use of a principal component analysis based on the mole percent contribution of amino acids. The results indicated that amino acids of both the DFAA and the DCAA pools were useful indicators of the diagenetic state of DON. The present study presents the first direct evidence of short-term (within 14 d) changes in DFAA and DCAA of the DON pool. These changes were similar to decompositional changes in the molecular composition of amino acids observed in traditional studies of bulk sediment diagenesis.

At present, the majority of marine dissolved organic nitrogen (DON) has not been characterized at a molecular level. However, the chemical identity of DON may be a key in understanding the mechanisms by which DON is formed and used. Several different strategies have been used to gain insight into these topics. Amon and Benner (1996) proposed a size-reactivity continuum model, which links the physical size of organic matter to its diagenetic state. The model suggests a decrease in size with increasing diagenesis and chemical alteration, but it does not explain a mechanism for the observed production of biorefractory low-molecular-weight (LMW) dissolved organic matter (DOM) or the observed bacterial production of recalcitrant DOM (Ogawa et al. 2001).

^{15}N nuclear magnetic resonance analysis of oceanic DON by McCarthy et al. (1997) indicated that the enormous reservoir of fixed nitrogen that is not readily identified by conventional biochemical techniques exists in amide form. McCarthy et al. (1997) argued that these amides were derived directly from degradation-resistant biomolecules. Furthermore, McCarthy et al. (1998) suggested that the enrichment of oceanic DON with D-isomers of amino acids indicated that peptidoglycan remnants derived from bacterial cell walls constitute a major source of DON throughout the sea. It is generally accepted that LMW compounds such as amino acids, amines, and urea account for only a minor fraction of total oceanic DON.

The limited number of studies on the chemical composition of sedimentary DON indicate that dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA), and urea may account for a larger fraction (39%–58%) of the DON pool in sediments than in the water column (Lomstein et al. 1998, unpubl. data).

The availability of DOM to bacterial degradation has been

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investigated in various environments such as DON in a spring flood (Stepanauskas et al. 2000), rainwater (Seitzinger and Sanders 1999), the Gulf of Riga (Jørgensen et al. 1999), and DOM from sea ice (Amon et al. 2001). However, studies of the microbial availability of sedimentary DON are scarce, and they have typically focused on the availability and turnover of a few known compounds as for instance DFAA or urea (e.g., Christensen and Blackburn 1980; Therkildsen and Lomstein 1994; Lomstein et al. 1998). It may be worth noting that processes that control the reactivity and composition in the water column and sediment may be different. Thus, care should be taken in extrapolating information on factors controlling water-column DON to sedimentary DON.

The aim of the present experiment was to study the availability of DON in coastal marine sediments for anaerobic sediment bacteria. Microorganisms and chemical pore-water constituents were monitored over time. In particular, both DFAA and DCAA were measured at the molecular level, and these results were framed within the context of the overall DON and unidentified DON components. In addition, the Dauwe degradation indices (Dauwe et al. 1999) were, for the first time, applied in a study of the progressive anaerobic utilization over short periods of time within the dissolved phase. Pore-water DON was isolated from the sediment, to avoid a continuous production of DON from hydrolysis of particulate organic nitrogen, which would have obscured the consumption of DON. Pore-water DON was isolated from the sediment by centrifugation, and the DON pool was obtained by 0.2-μm filtration. Filtered anoxic pore water was inoculated with an assemblage of natural anaerobic sediment bacteria. Net production of bacterial cells and virus-like particles (VLP) was followed through the course of the 14-d incubation in addition to carbon oxidation, net changes in DON, DCAA, DFAA, urea, and NH_4^+, and the proportion of D-amino acids isomers to the respective D + L amino acids within the DCAA and DFAA pools.

Materials and methods

Sampling of sediment—The sediment was collected in Norsminde Fjord (56°01′04″N, 10°15′02″E), Denmark, in January 1999. Water temperature at the sampling site was 4°C, the water depth was ~30 cm, and the salinity of the pore water was 13 psu. The sediment was sampled by hand in Plexiglas tubes (6 cm inner diameter [i.d.]). Water overlying the sediment and the top 1 cm of the sediment were removed after sampling. Sediment from the upper 1–6-cm depth was sieved (1.0-mm mesh size) in the laboratory, to remove macrofauna and larger particles. No precautions were taken to avoid the introduction of oxygen during sieving, given that Blaabjerg and Finster (1998) have shown that the activity of sulfate reducing bacteria is not affected by exposure to 20% oxygen for up to 8 h. Sieving of the sediment took <2 h. Anoxic conditions in the sediment were reestablished during storage of the sediment. The sediment was stored in the dark at 4°C for up to 3 d.

Preparation of incubation bag—An incubation bag (15 × 18 cm) was made of gas-tight laminated plastic film NEN/PE 80/100. The bag was produced from a single sheet of plastic film by use of an impulse heat-sealer (Elwis-Pack; Andertech International) and mounted with a pipe stub and a rubber stopper according to the method of Hansen et al. (2000). The plastic film had a double-layered structure with an outer 80-μm nylon layer and an inner 100-μm membrane of gas-tight polyethylene. The bag was sterilized by washing with 70% (v : v) ethanol followed by ultraviolet irradiation for 12 h. The bag was then pasteurized three times at 70°C for 30 min on days 0, 1, and 4 before it was filled with pore water.

DON isolation and incubation—Pore water was extracted by centrifugation of the sediment at 1,000 × *g* for 5 min under a N_2 atmosphere. The pore-water supernatant was centrifuged again at 4,000 × *g* for 5 min under a N_2 atmosphere. Further handling of the pore water took place in a glove bag filled with N_2 to avoid oxidation. The pore water was filtered successively through GF/C and GF/F filters and finally through a sterile 0.2-μm pore size Minisart filter. In between handling, the pore water was stored in an ice bath under a N_2 atmosphere. The pore water was frozen at −20°C immediately after the final 0.2-μm filtration. Pore-water extraction and filtration lasted ~2 h. Thawed 0.2-μm filtered pore water was transferred to the incubation bag by aseptic techniques. Gas bubbles were carefully removed with a syringe through the rubber stopper, and the pH was adjusted to pH 7.9 with 0.2-μm filtered 0.2 M NaOH, to keep CO_2 in solution during incubation. The incubation bag was preincubated for 2 d prior to inoculation. Throughout the preincubation period, ΣCO_2 and pH remained constant, which indicated that there was no mineralization before native bacteria were inoculated. The bag was kept in the dark at 4°C from the time it was filled with pore water and during the experiment.

The bacterial inoculum was prepared by mixing 10 ml sediment with 40 ml of 0.2-μm filtered anoxic pore water in a 50-ml centrifuge tube. The headspace was flushed with a mixture of $N_2 : CO_2$ (90% : 10% v : v). The sediment and pore water mixture was vortexed and subsequently sonicated (2 × 30 s) to detach bacteria from sediment particles. The sediment particles were allowed to settle for ~4 min. The supernatant (32 ml) was transferred to the incubation bag, and the resultant final volume was 401 ml. During incubation, 700 μl 0.2 M NaOH was added to the pore water to avoid production of CO_2 (g).

Sampling procedures—The total incubation time after inoculation was 14 d. On days 0, 1, 6, and 14, samples were taken for the determination of urea, total dissolved nitrogen (TDN), DFAA, and total hydrolyzable amino acids (THAA). Samples for urea, TDN, and DFAA were passed through sterile 0.2-μm Minisart filters and stored at −20°C for later analysis. The concentration of THAA was determined in samples of unfiltered pore water ($THAA_{total}$) and in pore-water samples filtered through a sterile 0.2-μm Minisart filter ($THAA_{<0.2\ \mu m}$). Samples (1 ml) for THAA analysis were mixed with 12 M HCl in a 1 : 1 v : v ratio and stored at room temperature for later analysis. All pore water–HCl samples for THAA determination were stored in preweighted glass vials closed with aluminium ColorSeal mounted with a bu-

tyl/PTFE septum. Samples for bacterial enumeration were taken on days 0, 1, 6, 10, and 14, preserved in formalin (2% v : v final concentration), and stored at −20°C for later analysis. Samples for ΣCO_2 were taken one to five times per day, and samples for pH and NH_4^+ were taken one to two times per day. Samples for ΣCO_2, pH, and NH_4^+ were filtered through 0.2-μm Minisart filters before analysis. Samples for ΣCO_2 and pH analysis were analyzed within 30 min, and NH_4^+ samples were stored frozen at −20° C for later analysis.

Urea and DON analysis—The concentration of urea-N was determined in triplicates by the manual diacetyl monoxime method (Mulvenna and Savidge 1992). $OD_{515\ nm}$ was measured on a Bio-Tek Microplate Autoreader model EL 309. Milli-Q water was used as blank. Concentrations of TDN were determined in 50-μl samples ($n = 5$) as NO on a modified Antek 7000 system as described in Lomstein et al. (1998). Calibration standards were made from Tris buffer and the areas of TDN samples were always >100 times higher than the area of blanks (Milli-Q water). Concentrations of DON were calculated as the difference between the concentration of TDN and NH_4^+. The net decrease in the unidentified DON pool was calculated as the difference between the change in DON and the summed changes in urea and $THAA_{<0.2\ \mu m}$.

Amino acid analysis—The concentration of DFAA was measured by high-performance liquid chromatography (HPLC, Waters Chromatographic Systems) of fluorescent *o*-phthaldialdehyde (OPA)–derived products ($n = 3$) according to the method of Lindroth and Mopper (1979). The concentrations of identified amino acids were calculated from individual five-point calibration curves made from a mixture of the amino acids standard solution AA-S-18 (Sigma-Aldrich) to which was added β-alanine (β-Ala), taurine, and ornithine (Orn). β-glutaric acid served as an internal standard. The concentrations of δ-amino valeric acid (δ-Ava) and unidentified amino acids were calculated by use of the average calibration curve of the standards. Milli-Q water was used as blanks.

Hydrolysis of the sample-HCl mixture was carried out at 105°C for 24 h. After hydrolysis, the samples were vortexed, and 100 μl of the hydrolysate ($n = 2$) from each sample was transferred to new glass vials. The samples were neutralized by repeated drying under vacuum at 50°C and redissolution in Milli-Q water. After the last drying, samples were dissolved in Milli-Q water. The concentration of THAA in the dissolved hydrolysate was analyzed as DFAA. Blanks were prepared as samples, with the exception that pore water was omitted. To identify additional peaks in the amino acid chromatograms, single standards of δ-Ava, citrulline, glucosamine, and muramic acid were included in addition to the AA-S-18 standard solution. $THAA_{>0.2\ \mu m}$ was determined as the difference between $THAA_{total}$ and $THAA_{<0.2\ \mu m}$. DCAA was determined as the difference between $THAA_{<0.2\ \mu m}$ and DFAA.

D and L isomers ($n = 1$) of aspartate (Asp), glutamate (Glu), serine (Ser), and alanine (Ala) were measured in the DFAA and DCAA pool according to the method of Mopper and Furton (1991), with the following modifications: (1) N-isobutyryl-L-cysteine (IBLC) was used as an additional chiral agent (Brückner et al. 1994); (2) 100 μl OPA/IBLC reagent was mixed with a 400-μl sample or standard solution by the WISP autosampler (autotransfer function with two mixing cycles, a delay time of 1 min, and an injection volume of 145 μl); (3) in eluent A, the concentrations of sodium acetate trihydrate and sodium dihydrogen potassium monohydrate was 90 and 11.5 mM, respectively; (4) the pH of eluent A was adjusted to 6.45; (5) β-aminoglutaric acid was used as an internal standard; (6) D and L enantiomers were separated on a 3.5-μm 4.6 × 150 mm $XTerraRP_{18}$ (Waters) HPLC column thermostated at 25°C; (7) the exitation wavelength was 230 nm; and (8) the gradient cycle was 60 min in total. The amino acid derivates were separated by a linear gradient from 100% to 84% A within 1 min, followed by an isocratic hold for 10 min. Between 10 and 27 min, eluent B was increased by a linear gradient to 50% B, followed by a linear gradient to 10% A within 4 min. The gradient was terminated by a linear gradient to 100% A in 4 min, followed by an isocratic hold for 14 min at 100% A. The flow rate was 0.65 ml min^{-1}. Care was taken to ensure that the pressure was <2,000 psi before each cycle was initiated.

Bacteria counts—Bacteria were counted by epifluorescence microscopy on black polycarbonate filters after staining with acridine orange. Prior to filtration, the formalin-preserved samples (50 μl) were mixed with 1 ml 1.0 M KCl to detach aggregated bacteria cells. After 10 min, the sample mixture was passed through a black 0.2-μm pore size polycarbonate filter (25 mm i.d., Osmonics), which was stained with 0.06% w : v acridine orange for 5 min (Finster et al. 1998). The cells were counted in a Zeiss Axioplan epifluorescence microscope at 100× magnification. One polycarbonate filter was prepared from each sample, and 31,250 μm^2 were counted on each filter (>6,700 cells mm^{-2}). The number of bacteria on blank filters (<300 cells mm^{-2}) was subtracted from the number of bacteria counted in samples. Blank filters were prepared with Milli-Q water instead of pore water.

Counts of VLP—The abundance of VLP in the pore-water samples was measured by epifluorescence microscopy after SYBR-Green staining, according to the method of Noble and Furhman (1998). From duplicate samples, 100-μl subsamples were added to 2 ml 0.02-μm filtered seawater to increase the filtration volume and collected onto 25 mm 0.02-μm filters (Anodisc; Whatman). The filters were stained for 15 min on a drop of 0.25% SYBR-Green I (Molecular Probes), rinsed with 0.02-μm filtered distilled water, and mounted with an antifade solution (Noble and Fuhrman 1998). The concentration of VLP-N was estimated under the assumption of a mean VLP diameter of 60 nm, a VLP-specific density of 1.4 g cm^{-3} (Laskin and Lechevalier 1973), an assumed carbon content of 50% (Bratbak et al. 1992), and a molar C : N ratio in VLP of 5 (Middelboe unpubl. data).

ΣCO_2, pH, and NH_4^+ analysis—ΣCO_2 was analyzed on a small-volume flow injection system (Hall and Aller 1992). Sulphide was removed before ΣCO_2 analysis by reaction

with 0.5 M Na_2MO_4. The sample : Na_2MO_4 ratio was 20 : 1 v : v, in accordance with the methods of Lustwerk and Burdige (1995). The pore water–$Na_2MoO_4 \cdot 2H_2O$ mixtures were vortexed in clean 1.5-ml glass tubes sealed with a Teflon screw-cap and allowed to react for 15 min. Samples (50 μl) were injected ($n = 2$ or 3) into the ΣCO_2 flow-injection system (Hall and Aller 1992). pH was measured in small vials with a glass Hamilton Minitrode. NH_4^+ ($n = 3$) was analyzed spectrophotometrically by the salicylate-hypochlorite method (Bower and Holm-Hansen 1980). Milli-Q water was used as blanks.

Adsorption experiment—At the end of the incubation, the amount of THAA adsorbed to the inner wall of the incubation bag ($THAA_{wall}$) was determined by washing the incubation bag with 10 ml 12 N HCl after it was drained. The washing solution was collected, and the amount of THAA was determined as described above.

The adsorption of organic matter to the walls of the plastic bag and possible biofilm formation were investigated in detail in an additional experiment. Four bags (8 × 8 cm) were prepared according to the procedure described above. The first bag was washed with 2.5 ml of 12 M HCl immediately after pasteurization to determine the preexperimental content of $THAA_{wall}$. The remaining three bags were filled with 0.2-μm filtered pore water and inoculated with sediment bacteria. Incubation conditions and bag surface area in relation to pore-water volume in these bags were similar to the original incubation bag. Samples for pH and bacterial counts were taken as described above. One of the bags was used for analysis of bacterial biofilm formation. This bag was emptied after 14 d of incubation and cut into fragments of ~1 × 0.5 cm. The wall fragments were stained with 0.06% w : v acridine orange for 5 min, rinsed twice with Milli-Q water, and mounted on an object slide, followed by epifluorescence microscopy as described above. The remaining two bags were treated as follows: samples were taken from both bags to determine $THAA_{total}$ and $THAA_{<0.2\,\mu m}$ on days 1 and 14 (bag 3) and day 28 (bag 4). Bag 3 was emptied on day 14 and washed with 2.5 ml 12 M HCl to determine the content of $THAA_{wall}$, as described above. Bag 4 was emptied on day 28 and divided into two parts by welding with the impulse heat-sealer. One half (50 cm^2) was washed with 1 ml 12 M HCl, to measure the content of $THAA_{wall}$ as described above. The other half was cut into fragments (~0.8 × 0.8 cm) that were either stained with acridine orange as described above or used for scanning electron microscopy (SEM). Samples for SEM were prepared as follows: pieces of the bag material were mounted on aluminum stubs by use of double-sided carbon tabs, dried in a desiccator, and coated for 5 min in an Edwards Sputter Coater S150B. Specimens were stored in a desiccator prior to viewing in a CamScan scanning electron microscope at an accelerating voltage of 20 kV.

Statistical methods—Bacterial counts of 10 subfields on the polycarbonate filters were grouped by date. The counts were assumed to have a normal distribution around the means. The error of variance among groups was tested by Levene's test. The change in bacteria density with time of incubation was tested by a one-way model 1 analysis of variance (ANOVA; Sokal and Rohlf 1995). The distributions of amino acids in $THAA_{wall}$ samples from the incubation bag and from the small bags were analyzed by a two-way model 1 ANOVA (Sokal and Rohlf 1995).

Principal component analysis (PCA) was performed by use of the mole percentage composition of individual amino acids in the DCAA and the DFAA pools on day 0, 1, 6, and 14 as the original data matrix. The statistical package SPSS 9.0 was applied for the analysis. PCA was used for the detection of structure in the relationships between variables, as suggested by Dauwe et al. (1999). Only the first derived principal component was taken into account, because it contained the maximum variance. Using this approach, we estimated a degradation index for DCAA and DFAA at the different time points in our experiment. The Dauwe degradation index was estimated as the component score by the Dauwe et al. (1999) equation:

$$DI = \sum_i \left[\frac{\mathrm{var}_i - \mathrm{AVGvar}_i}{\mathrm{STDvar}_i}\right] \times \mathrm{fac{\cdot}coef}_i \quad (1)$$

where var_i was the non-standardized mole percentage of amino acid *i*, AVGvar_i and STDvar_i were the average and standard deviation of the nonstandardized mole percentage of all amino acids, respectively, and $\mathrm{fac{\cdot}coef}_i$ was the factor coefficient for amino acid *i*. The factor coefficients range from +1.0 to −1.0. Amino acids with the highest contribution to the principal component have factor coefficients close to +1.0 or −1.0. More negative degradation indices represent a higher degree of decomposition.

Results and discussion

Composition of DON—The initial concentration of DON in the pore water was 664 μM-N, which is an intermediary concentration relative to DON pools measured by others in shallow marine sediments (15–2,792 μM-N; Table 1, Fig. 1). In the present study, DFAA-N + DCAA-N + urea-N comprised a major fraction of the total DON pool (82%), compared with <59% in similar marine sediments (Table 1). The contribution of nitrogen from VLP in the pore water to the DON pool was not quantitatively important, because VLP-N was estimated to contribute <0.1% of DON (data not shown). This proportion did not change during the incubation.

The observed large contribution of amino acid nitrogen to total DON may be indicative of relatively fresh DON. A likely source of fresh DON could be the mixing of recently produced microalgal material into the sediment by macrofauna. Sediments in temperate shallow areas (water depth <1 m) often sustain microphytobenthos during the winter season. Andersen (2001) found chlorophyll *a* contents of 30–40 $\mu g\ g^{-1}$ dry weight in January in two temperate microtidal mudflats. Furthermore, Therkildsen and Lomstein (1993) found Chl *a* contents of >40 μg Chl *a* cm^{-3} fresh sediment in December 1990 at the same sampling site as described herein. In addition, the sediment at the sampling site has been found by others to be highly bioturbated by polychaetes and crustacea (Therkildsen and Lomstein 1993), which ensures the downward mixing of fresh microphyto-

Table 1. Concentrations and composition of DON, including DFAA, DCAA, urea, and unidentified DON components in sediments at contrasting sampling sites. ND, not determined.

Sampling site	DON (μM-N)	DFAA-N % of DON	DCAA-N % of DON	Urea-N % of DON	Unidentified-N % of DON	Reference
Norsminde Fjord, Denmark	664	25	50	7	18	This study
Knebel Vig, Denmark	113–335	10	27	2	61	Lomstein et al. (1998)
Chesapeake Bay	15 to ~160	ND	ND	ND	ND	Burdige and Zheng (1998)
Løgstør Broad, April, Denmark	435–2,810	31	26	2	42	Lomstein unpubl. data
Løgstør Broad, August, Denmark	468–2,944	14	36	3	47	Lomstein unpubl. data

benthic material into the sediment. Similarly, Amon et al. (2001) suggested that a large contribution of amino acids (and neutral sugars) to the pool of DOM by microalgae was indicative of fresh DOM in arctic sea ice. Similar to marine pore water, sea ice is a high-DON environment. The unidentified DON may have been composed of amides, which have been found to be dominant in high-molecular-weight DON in the ocean (McCarthy et al. 1997). However, RNA may also have been an important component of the unidentified DON. Lomstein et al. (1998) estimated that an input of fresh microalgae material to the sediment could potentially supply the sediment with a significant amount of RNA-N compared with the unidentified DON pool.

Bacterial growth and overall decomposition of DON—There was significant bacterial growth, increase in VLP, and DON decomposition after inoculation. This was indicated by increasing bacterial cell numbers, numbers of VLP, ΣCO_2 production plus a net reduction in DON, and net production of NH_4^+ during incubation (Figs. 1, 2A–2C). Bacterial cell numbers increased by $2.5 \times 10^7 \pm 1.1 \times 10^7$ cells ml^{-1} during the first 10 d of incubation, after which time bacterial density stabilized. This may have been due to the depletion of a compound limiting bacterial growth.

In parallel to the increase in bacterial cell numbers, the number of VLP increased from 1.4×10^8 to 2.7 10^8 VLP ml^{-1} during the first 10 d of incubation, with a final increase to 2.8×10^8 VLP ml^{-1} on day 14 (Fig. 2A). The observed numbers of VLP and bacteria are in accordance with densities found in sediments along a eutrophication gradient in two subtropical estuaries (10^7–10^9 VLP cm^{-3} and 10^7–10^8 bacteria cm^{-3} of sediment, respectively; Hewson et al. 2001). However, until now, there have only been a limited number

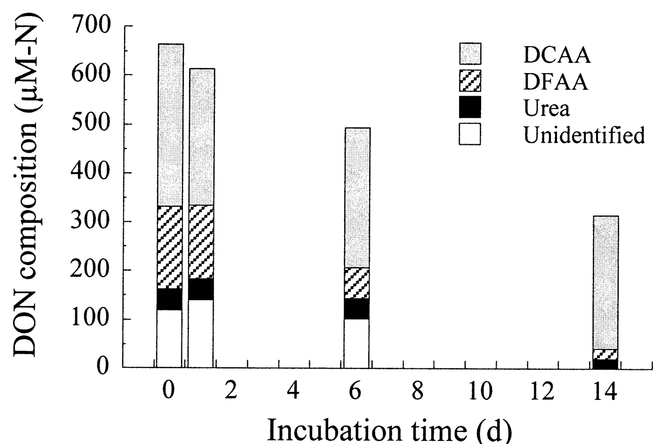

Fig. 1. Composition of DON during the time course of incubation. The concentration of unidentified DON was estimated as the difference between total DON and (DCAA-N + DFAA-N + urea-N).

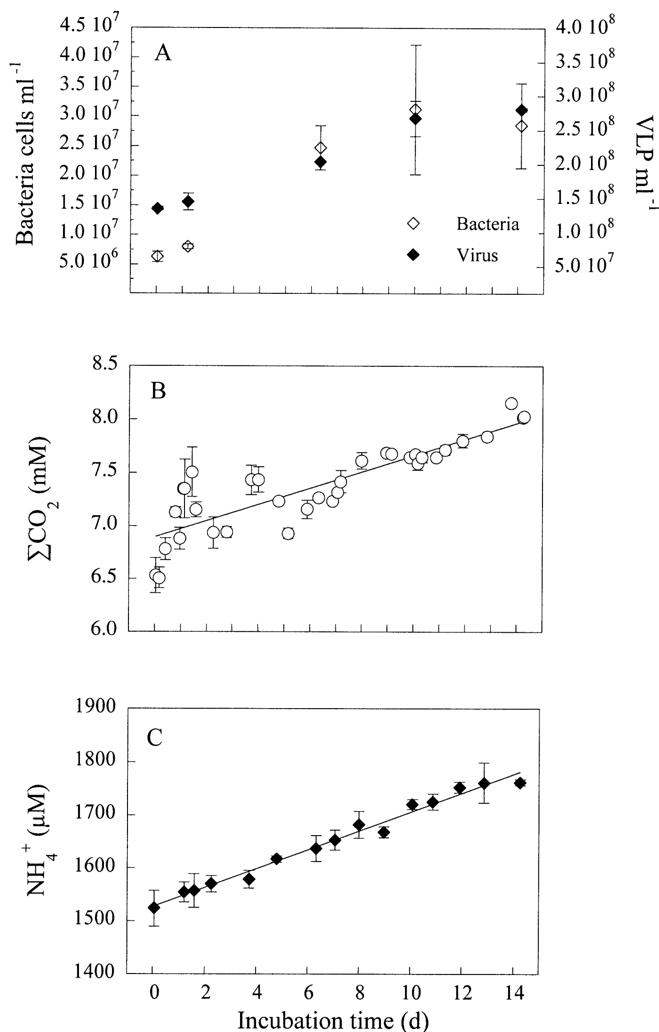

Fig. 2. (A) Abundance of bacteria and VLP, (B) concentration of ΣCO_2, and (C) concentration of NH_4^+ during degradation of sedimentary DON. Error bars represent the standard deviation (n = 2 or 3 for bacterial counts and n = 3 for NH_4^+).

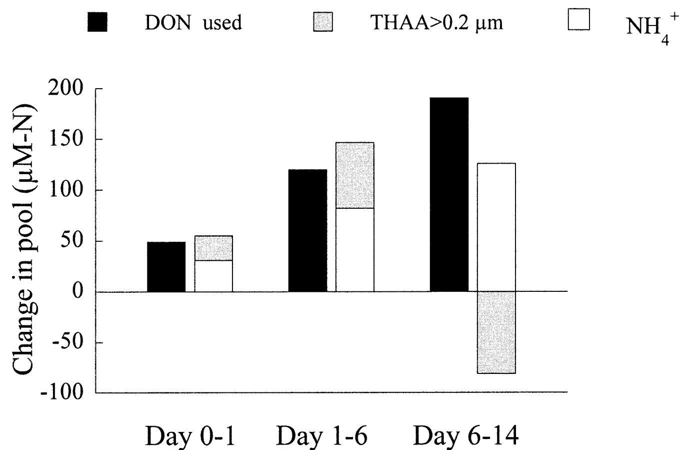

Fig. 3. Molar-N changes in DON, $THAA_{>0.2\ \mu m}$, and NH_4^+ at different time intervals during sedimentary DON degradation.

of studies on the abundance and ecological impact of viruses in marine sediment pore waters (e.g., Drake et al. 1998; Hewson et al. 2001).

The observed carbon oxidation rate of 76 μM-C d^{-1} was ~15% of the average carbon oxidation rate obtained by Therkildsen and Lomstein (1993) at the same location and time of the season. For comparison, it was assumed that the carbon oxidation rate given by Therkildsen and Lomstein (1993) was evenly distributed within the upper 12 cm of the sediment. However, these two carbon oxidation rates are not directly comparable, because the purpose of the present experiment was to examine the bacterial availability of sedimentary DON isolated from the particulate organic nitrogen pool rather than to simulate in situ bacterial respiration due to DON degradation. The ΣCO_2 concentrations during the first 6 d of the experiment were variable compared with the remaining part of incubation (Fig. 2B). At present, we do not have any explanation for this variability.

During the 14-d incubation, bacteria degraded 361 $\pm$ 49 μM-N (54%) of the initial DON pool. This fraction of DON available to bacteria is in accordance with observations of DON availability from different environments such as a spring flood in Sweden (45%–55% degradation in 14 d; Stepanauskas et al. 2000) and rainwater DON (46% degradation in 3 d; Seitzinger and Sanders 1999) but is higher than the degradation of DON in May and July in the brackish Gulf of Riga (4%–29% degradation in 8 d; Jørgensen et al. 1999). The degraded DON was recovered as net NH_4^+ production and $THAA_{>0.2\ \mu m}$ (assumed to represent bacterial amino acids) during the first 6 d of incubation (Fig. 3). During the remaining 8 d of incubation, 66% of the degraded DON (191 $\pm$ 36 μM-N) was recovered as NH_4^+ (126 $\pm$ 25 μM). However, during the same period, there was an unexplained net decrease in $THAA_{>0.2\ \mu m}$ of 87 $\pm$ 47 μM-N. Thus, it was only 39 $\pm$ 53 μM-N of the DON degraded that could be recovered as NH_4^+ + $THAA_{>0.2\ \mu m}$. The adsorption experiment did not reveal any THAA, bacteria, or NH_4^+ on the walls of the incubation bags (data not shown). In addition, it was not likely that denitrification could have been of any importance in the removal of nitrogen, because the bags and welded seams did not allow oxygen penetration (Hansen et al. 2000) and thus nitrification. However, NH_4^+ and DON concentrations on day 14 followed the changes in the respective concentrations in the preceding period, whereas that was not the case for $THAA_{>0.2\ \mu m}$. Although it was not possible to find any mistakes in the analysis of $THAA_{>0.2\ \mu m}$ on day 14, it may be that $THAA_{>0.2\ \mu m}$ was underestimated.

Utilization of DON components—Changes in DON composition also demonstrated that DFAA and the unidentified DON were readily available for bacterial degradation. All of the unidentified DON was decomposed during incubation (119 μM-N). DFAA decreased by 88% (from 169 to 21 μM-N), and urea-N decreased by 52% (from 43 to 21 μM-N). In contrast, the DCAA-N pool was only reduced by 17% (from 333 to 275 μM-N, Fig. 1). In arctic sea ice, Amon et al. (2001) showed that THAA (DFAA + DCAA) decreased by 68% during a 220-h incubation, which is higher than the 39% molar decrease in DFAA + DCAA in the present study. The higher availability of DFAA + DCAA in sea ice than in pore water may be due to direct input of photosynthesis-based DON of high quality compared with marine sediments with more diverse routes of organic nitrogen input.

Changes in the molar composition of amino acids during bacterial degradation—Most information on the degradative changes in the molecular composition of amino acids is derived from geochemical studies of particulate organic matter diagenesis (e.g., Cowie and Hedges 1994; Dauwe and Middelburg 1998; Dauwe et al. 1999) rather than from analysis of changes in DOM composition in water and sediment. However, in the recent study by Amon et al. (2001), the dynamics of amino acid composition in sea ice were determined during decomposition.

In the present experiment, the DCAA pool was initially dominated by Glu, Asp, and glycine (each >26 μM; Fig. 4A). The utilization of individual DCAA amino acids varied, and there was a net production of δ-Ava, Ala, and some unidentified amino acids during incubation (Fig. 4B). As examples, >40% of the initial Glu, tyrosine (Tyr), and Orn were utilized, whereas there were 318%, 217%, and 107% increases in the concentrations of δ-Ava, Ala, and unidentified amino acids, respectively. Because of the production of specific amino acids, the relatively low net consumption of DCAA (10%) during the course of incubation masked a major redistribution of individual amino acids within the DCAA pool. If only the utilized amino acids are considered, the decrease in DCAA amino acids was 36%. The selective utilization of Glu and Tyr is in accordance with the findings in biogeochemical studies of diagenetic changes in bulk sediment THAA, where Glu and Tyr (and phenyl alanine) show strong depletion with increasing state of decomposition (Dauwe and Middelburg 1998). Dauwe and Middelburg (1998) argued that these amino acids are concentrated in diatom cells and can thus be considered as readily available for bacterial decomposition. The accumulation of the nonprotein amino acid, δ-Ava, and unidentified amino acids agrees with the general expectation that nonprotein amino acids are much less reactive than protein amino acids or are intermediates produced in the course of protein degradation (e.g., Cowie and Hedges 1992; Dauwe and Middelburg

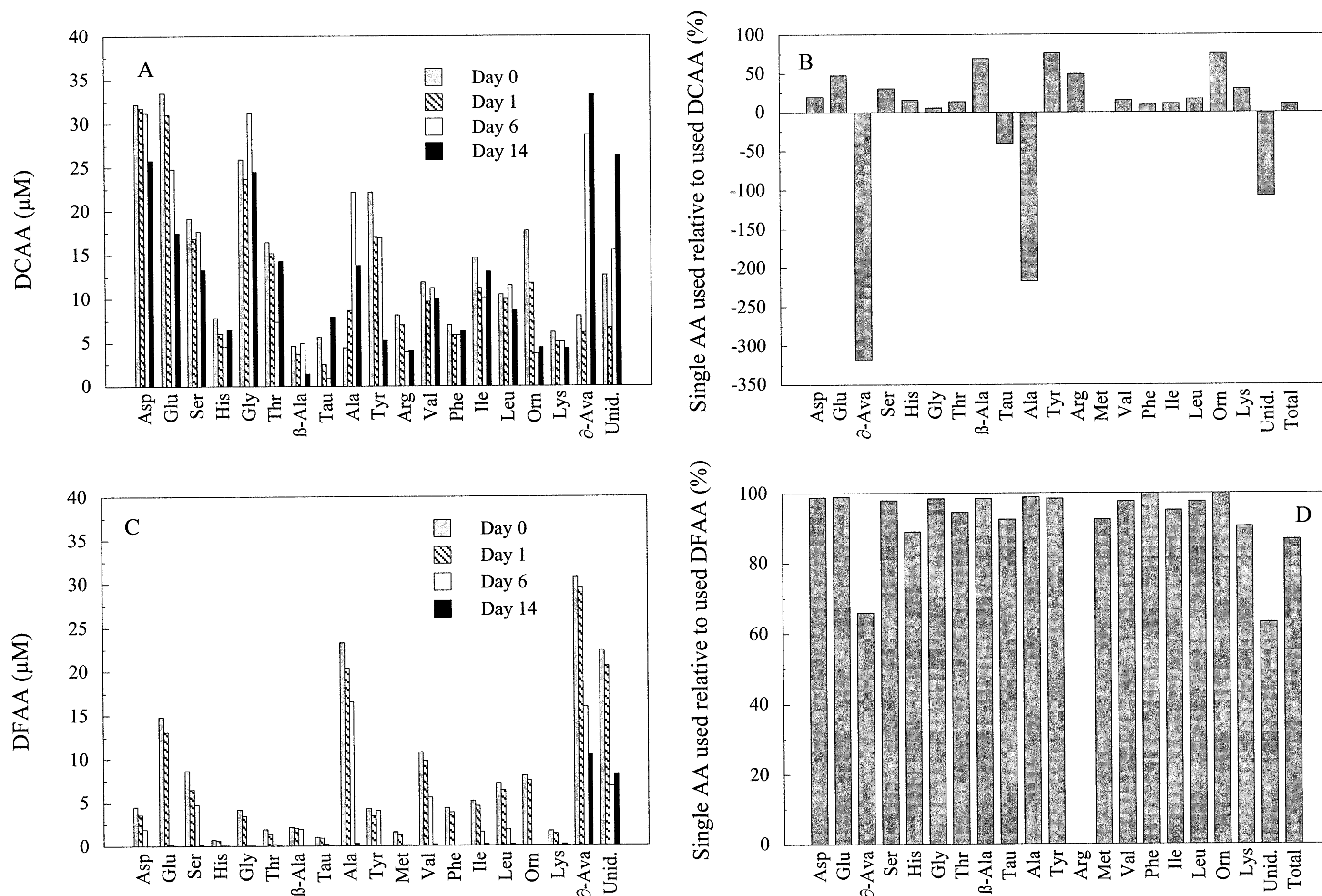

Fig. 4. (A) The concentration of individual DCAA on days 0, 1, 6, and 14 of sedimentary DON degradation. The concentration of Met was below the detection limit. (B) Use of individual amino acids (AA) relative to the used DCAA. Negative values represent net production of the AA in question. (C) The concentration of individual DFAA on days 0, 1, 6, and 14 of sedimentary DON degradation. The concentration of Arg was below the detection limit. (D) Use of individual AA relative to the used DFAA.

1998). Because many nonprotein amino acids (e.g., β-Ala and γ-amino butyric acid [γ-Aba]) are formed during the breakdown of protein counterparts (e.g., Asp and Glu), the former will be produced in the course of overall degradation and thus exhibit reduced net reactivity. This does not mean, however, that, once formed, nonprotein amino acid intermediates are necessarily less reactive than their protein counterparts. The accumulation of Ala was somewhat unexpected. Again, apparent low net reactivity could result from simultaneous production.

Initially, the DFAA pool was dominated by Glu, Ala, valine, δ-Ava, and unidentified amino acids (each >10 μM; Fig. 4C). Most individual amino acids were reduced by $>90\%$ during the 14-d incubation, except for δ-Ava, histidine (His), and the unidentified amino acids, which were reduced by 66%, 89%, and 63%, respectively (Fig. 4D). Obviously, most of the individual protein and nonprotein DFAA were readily available for decomposition by anaerobic bacteria.

The amino acid composition of the bioavailable DON (individual utilized DFAA and DCAA in relation to total used DON) was dominated by Orn, Glu, Ala, Tyr, and δ-Ava (each contributed $>5\%$, Fig. 5). Because most amino acids decreased during incubation, the relative contribution of each individual amino acid was low. In total, DFAA and DCAA accounted for 71% of the bioavailable DON. In contrast, Amon et al. (2001) found that amino acids only accounted for $\sim$15% of the bioavailable DOM pool (amino acids yield) in sea ice.

D-amino acids—Production of D-amino acids is primarily restricted to bacterial synthesis of peptidoglycan and racemization during ageing of dead organic matter. However, because the formation of a racemic mixture of L- and D-isomers from L-isomers requires a period of 10^4–10^6 yr, D-amino acids in marine sediments have been suggested to originate from bacteria rather than racemization (Pollock and Kvenvolden 1978). Recent studies of amino acid enantiomers in seawater and marine sediments support that most D-isomers of amino acids actually are derived from bacteria (McCarthy et al. 1998; Amon et al. 2001; Pedersen et al. 2001).

In the present study, the initial concentration of total dissolved free D-amino acids (Glu, Ser, and Ala) was 9 μM

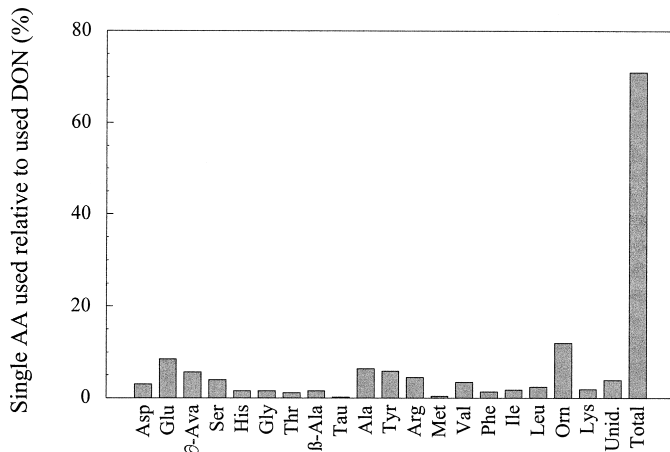

Fig. 5. Relative contribution of individual AA of the DCAA + DFAA pool to the used DON pool during the 14-d of sedimentary DON degradation.

and did not change during incubation. The percentages of the D-forms of these three amino acids were 23%, 10%, and 33%, respectively, where %D is the molar percentage of the D-amino acid in relation to the sum of the D- and the corresponding L-amino acid. The %D of total Glu and Ala in the present study were thus higher than has been found elsewhere in pore water of an Aarhus Bay sediment, where %D-Glu, %D-Ser, and %D-Ala were 6%, 9%, and 4%, respectively, at 10 cm depth (Pedersen et al. 2001).

In the present study, the proportion of D-Glu and D-Ala in the DFAA pool increased from 23%D and 33%D, respectively, on day 0 and 36%D and 51%D, respectively, after 6 d. The proportion of D-Glu and D-Ala remained at this level to the end of incubation (Fig. 6A). %D-Ser was <10% and remained rather constant, except for a low abundance on day 1. The D-amino acids D-Glu and D-Ala were utilized at a lower net rate than their L-amino acid counterparts. This was shown by a constant concentration of these D-DFAA with time of incubation in parallel to an increase in the relative proportion of D-Glu and D-Ala to L-Glu and L-Ala, respectively. The reduced net uptake of DFAA D-isomers was unexpected, given that O'Dowd and Hopkins (1998) found that free D-amino acids in soils were utilized at rates similar to those of L-amino acids. Thus, there is a need for future and more comprehensive studies on free D-amino acids to judge whether chiral properties per se affect their availability.

Individual concentrations of D-amino acids in the DCAA pool were low and variable (from undetectable to 3 μM) during incubation compared with the respective L-amino acid forms (8–31 μM). Hence, the %D-Ala and %D-Glu in the DCAA pool varied within the range of 2.4%–12.3% during incubation (D-Asp and D-Ser were not detected, Fig. 6B). Apparently, combined D-amino acids, e.g., in intact or partially degraded peptidoglycan from bacterial cell walls, did not account for a quantitatively important fraction of the DON pool in the present study.

Diagenetic alterations of DFAA and DCAA—Recent and comprehensive studies by Dauwe and Middelburg (1998)

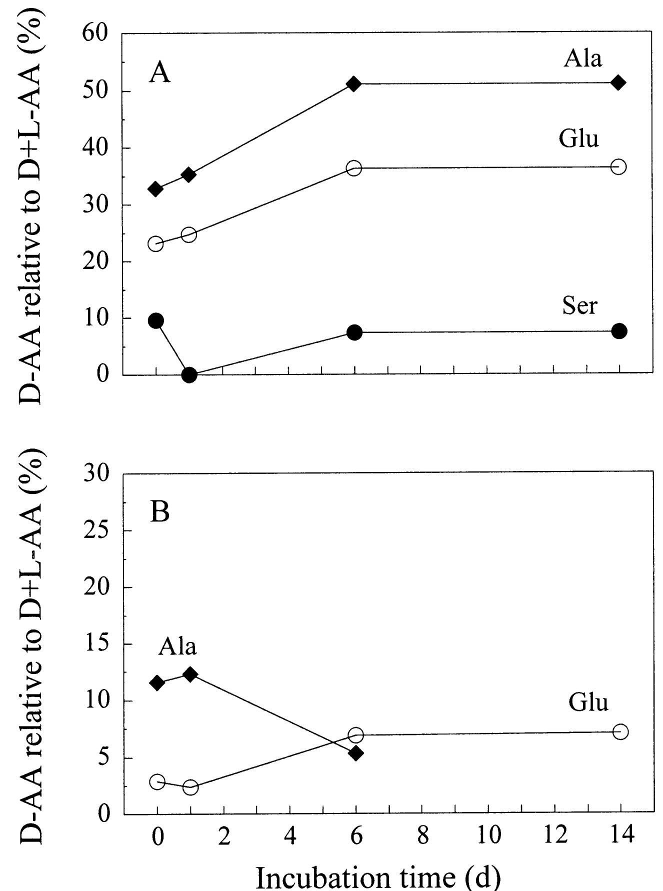

Fig. 6. The relative contribution of D-AA to the total D + L-AA content during sedimentary DON degradation in the (A) DFAA pool and (B) DCAA pool. The %D-Ala of the DCAA pool on day 14 is missing. The concentration of D-Ser in DCAA and the concentration of D-Asp in DFAA and DCAA were below the detection limit. All data points represent $n = 1$.

and Dauwe et al. (1999) have linked amino acid composition to organic matter degradation by use of the Dauwe degradation index (DI). Dauwe et al. (1999) found a progressively decreasing DI from labile particulate source material (DI, 1.0–1.5) to particulate refractory material (DI down to −2.2) and concluded that more negative DI is a quantitative reflection of progressive diagenesis. In agreement with this observation, we found a systematic decrease in DI during early diagenesis of the organic matter (Fig. 7). In the present study, DI decreased linearly with time of incubation from 0.8 to −1.3 and −1.2 in the DFAA and DCAA pools, respectively, by the end of incubation. Similarly, Amon et al. (2001) observed a decrease in DI (based on the changes in amino acid composition of DOM-THAA) from 0.5 to −1.1 during the 220-h decomposition of sea ice DOM. The similarity in DI values of amino acid degradation in different environments stresses that, although amino acid composition and bacterial communities may vary, fundamental similarities exist in decomposition of amino acids in organic matter.

In both the DFAA and DCAA pool, δ-Ava and the unidentified amino acids were identified as degradation marker molecules, because they had the lowest and most negative

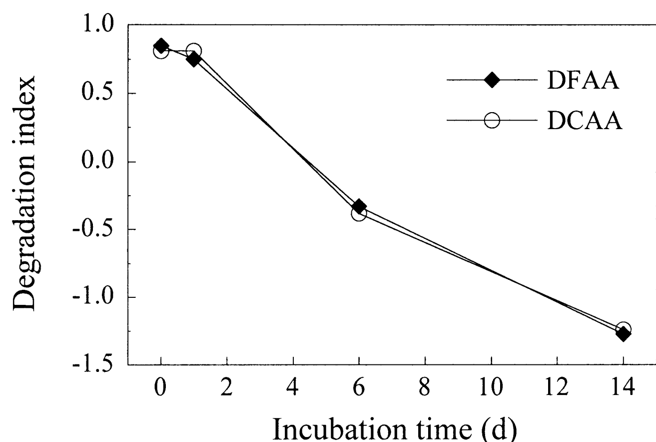

Fig. 7. Degradation index of the DCAA and the DFAA pool as a function of time of sedimentary DON degradation. The degradation index was estimated as the first principal component score, and it was based on the change in mole percentage of individual amino acids.

factor coefficients (Table 2). This was caused by their relative increase in mole percentage with time of incubation. Nonprotein amino acids such as γ-Aba and β-Ala of the THAA pool had the second and the third most negative factor coefficients in North Sea sediments with increasing degradation status of THAA (Dauwe and Middelburg 1998).

For DFAA and DCAA, 57% and 52%, respectively, of the variability was explained by the first principal component axis, which is interpreted to represent organic matter degradation (Dauwe and Middelburg 1998). In accordance with the present study, Amon et al. (2001) found that 65% of the variability in DOM-THAA amino acids was explained by the first principal component axis. The results of the present study indicate that changes in the amino acid composition of both the DFAA and the DCAA pools are useful indicators of the diagenetic state of DON. Furthermore, the present study demonstrated that the nature of DON changed from labile (DI close to +1, sensu Dauwe et al. 1999) to more degraded (DI = -0.3) states within ~1 week. However, the continuous linear production of NH_4^+ combined with the linear decrease in DI with time of incubation indicates that refractory pore-water DON may score a lower DI than what is found in bulk sediment organic matter. Nevertheless, through the coupling of the DI and DON degradation, there is an association of amino acid compositional changes with degradation time. The presently successful application of the DI may be due to the high contribution of amino acids to the DON pool. However, it should be noted that not all circumstances of sedimentary DON degradation were reproduced in the present experiment. Most important, mineral grains were not included so that we could avoid a continuous production of DON from particulate organic nitrogen. The latter would have obscured the measured consumption of DON. Mineral grains possibly affect the reactivity of amino acids (e.g., Aufdenkampe et al. 2001). They found that the basic amino acids His, arginine (Arg), and lysine (Lys) and the hydrophobic amino acids Tyr and methionine (Met) preferentially sorbed to suspended fine particulate organic matter of the river waters in the Amazon Basin. Furthermore, nonprotein amino acids preferentially remained dissolved. If sorption in the sediment originally containing the pore water

Table 2. Parameters of the principal component analysis as based on the relative concentrations (mole percentage) of individual amino acids in the DFAA and DCAA pools during degradation. The average (Avg) and standard deviation (SD) of individual amino acids were estimated from the original matrix. The factor coefficients (FC) are eigenvector scores of the first principal component, and the DI is the component score of each sample. The first principal component axis explained 57% and 52% of the variance in the DFAA and DCAA pools, respectively. The first principal component axis was interpreted to present DON degradation.

	DFAA			DCAA			DI		
Amino acid	FC	Avg	SD	FC	Avg	SD	Time (d)	DFAA	DCAA
Asp	0.071	2.18	1.31	0.081	12.20	1.30	0	0.85	0.81
Glu	0.085	4.92	5.09	0.100	10.76	2.86	1	0.75	0.81
δ-Ava	−0.086	29.22	14.54	−0.100	7.66	5.70	6	−0.33	−0.38
Ser	0.049	4.66	2.87	0.095	6.75	0.83	14	−1.27	−1.24
His	0.040	0.38	0.12	0.030	2.50	0.52			
Gly	0.084	1.41	1.36	−0.031	10.59	1.06			
Thr	0.074	0.75	0.42	0.038	5.40	1.70			
β-Ala	0.026	1.58	1.27	0.071	1.47	0.59			
Tau	0.089	0.50	0.16	−0.042	1.70	1.27			
Ala	0.039	14.27	10.33	−0.068	4.95	2.97			
Tyr	0.019	3.05	2.63	0.094	6.14	2.72			
Arg	—	—	—	0.091	2.32	0.83			
Val	0.061	5.99	3.33	0.065	4.30	0.12			
Phe	0.086	1.38	1.60	0.015	2.53	0.15			
Ile	0.091	2.60	0.99	0.009	4.95	0.73			
Leu	0.092	3.21	1.77	0.045	4.10	0.40			
Orn	0.085	2.64	3.05	0.090	3.76	2.55			
Lys	0.044	0.71	0.49	0.101	2.09	0.24			
Met	0.063	0.6	0.37	—	—	—			
Unidentified	−0.072	19.88	13.34	−0.095	6.15	3.42			

used in the present experiment was comparable to sorption in the Aufdenkampe et al. (2001) study, it is not likely that the mineral grains would have affected the reactivity of amino acids to any significant degree. This is because His, Arg, Lys, Tyr, and Met contributed relatively little to the DCAA and DFAA pools, respectively. In total, His, Arg, Lys, Tyr, and Met accounted for 8.4–16.5 and 2.1–7.0 mole percentage of the DCAA and the DFAA pools, respectively, during the course of incubation. In addition, the sediment at the study site was medium sand, which is known to sorb relatively little organic carbon compared with silty sediments as those used in the Aufdenkampe et al. (2001) study. In future studies, it would be interesting to study whether the same trends in DI and DON degradation would be observed under oxic conditions or in the presence of mineral grains and to identify the DI threshold value for refractory DON below which no further NH_4^+ production occurs.

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