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ORIGINAL PAPER

Occurrence and bacterial cycling of D amino acid isomers in an estuarine environment

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Abstract Abundance of D isomers of amino acids has been used in studies of organic matter diagenesis to determine the contribution of bacterial biomass to the organic matter, especially in marine sediments. However, fluxes of D amino acids in pelagic waters are poorly known. Here we present seasonal changes (March–September) in concentrations of dominant D amino acids in the pool of dissolved free and combined (hydrolysable) amino acids (DFAA and DCAA) in the shallow Roskilde Fjord, Denmark. The amino acid dynamics are related to pelagic bacterial density and activity and abundance of viruses. D isomers made up 3.6 and 7.9% of the DFAA and DCAA (average values), respectively, and had similar seasonal variations in concentrations. In batch cultures (0.7- and 0.2-m filtered water in a 1:9 mixture) microbial activity reduced L+D DCAA concentrations in seven of ten sampling dates, while DCAA were released at the remaining three sampling times. NH_4^+ balance (uptake or release) in the cultures correlated significantly with variations in concentrations of D-DCAA, but not with the total DCAA pools. Abundance of viruses did not correlate with density or production of bacteria in the fjord, but covaried with mineralization of total C, DCAA and PO_4^{3-} in the batch cultures. The content of D amino acids in bacterial biomass in the cultures varied from 6.7 to 12.5% and correlated with the D isomer concentration in the fjord, except for D-Ala. In an additional six-day batch culture study, DCAA and D-DCAA were assimilated by the bacteria during the initial 36 h, but were released between 36 and 42 h simultaneous with a decline in the bacterial density. Our results demonstrate that peptidoglycan components contribute to natural amino acid pools and are assimilated by bacterial assemblages. This cell wall "cannibalism" ensures an efficient recycling of nutrients within the microbial community. Significant positive correlations between viral abundance and bacterial mineralization of organic matter in the fjord indicated that viral lysis contributed to this nutrient recycling.

Keywords DFAA · DCAA · D isomers · Bacteria · Virus · N cycling

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Introduction

In biogeochemical studies, the dominant function of bacteria is assumed to be transformation of

organic and inorganic compounds, e.g., Azam et al. (1983), but also bacteria themselves contribute to the organic matter pool. The significance of bacterial cells in organic matter has been determined from the abundance of D amino acid (D-AA) isomers, originating from cell wall peptidoglycan (Pollack and Kvenvolden 1978). In marine sediments, the proportion of D-AA in the pool of dissolved combined (hydrolysable) amino acids (DCAA) has been shown to increase with the sediment age (Grutters et al. 2002; Pedersen et al. 2001). The higher proportion of D-AA in deeper strata has been assumed to reflect a low biodegradability of bacterial peptidoglycan, leading to an accumulation of intact peptidoglycan or peptidoglycan components during organic matter diagenesis. A spontaneous racemization of L to D isomers cannot explain the increased abundance of D amino acid isomers in deep sediments, at least not in sediments being only a few thousand years old, as the time required for a complete racemization has been estimated to $>10^4$ years (for Ala and Asp (Bada 1982)).

A slow degradation of bacterial cell wall material has been supported by the finding of a persistent porin-like protein (membrane channel protein in Gram-negative bacteria) in oceanic water (Tanoue et al. 1995). Using radiolabeled peptidoglycan from a Gram-negative bacterium, Nagata et al. (2003) also observed that mineralization of peptidoglycan by marine bacteria was 2–21 times lower than that of bacterial proteins, and that polysaccharides in peptidoglycan were more recalcitrant than its peptide component. However, individual peptidoglycan D amino acid isomers constitute a potential nutrient source to bacteria if other nutrients are low. This was shown by Perez et al. (2003) who observed an increased uptake of D-Asp with water depth in the oligotrophic North Atlantic at depths up to 1,000 m.

D amino acid isomers have been found to constitute 5 and 15% of peptidoglycan amino acids in a Gram-negative and a Gram-positive bacterium, respectively (Jørgensen et al. 2003). The difference in peptidoglycan structure between Gram-positive and Gram-negative bacteria appears to influence the degradability. Jørgensen et al. (2003) observed that peptidoglycan of Gram-positive bacteria (thick, uniform peptidoglycan layer) was more degradable than that of Gram-negative bacteria (thin peptidoglycan structure in a complex, multilayered structure). D isomers of amino acids have also been identified in eukaryotic organisms such as algae and fungi (Brückner et al. 1994; Lee and Bada 1977) and thus, it cannot be excluded that some algae seasonally and locally contribute D-AA to the ambient water. Potentially, a substantial input of D-AA to the dissolved amino pool may originate from viral lysis of bacteria. Viral lysis constitutes a significant loss factor for aquatic prokaryotes and between 10 and 40% of pelagic bacterial production is lost as lysates due to viral activity, e.g., Weinbauer (2004). Previous studies have demonstrated that bacterial cells disintegrate following viral lysis and that most of the material is transformed into dissolved organic matter (<0.2 μm size fraction), which is again available for bacterial uptake (Riemann and Middelboe 2002), emphasizing the potential influence of viruses for the dynamics of D-AA in aquatic environments. The virus particles as such appear not to be significant to ambient pools of dissolved amino acids at typical concentrations in seawater (Kuznetsova et al. 2005). In addition to viral attack, disintegration of bacterial cell walls may also results from protist grazing on bacteria. Nagata and Kirchman (2001) observed production of liposome-like particles containing bacterial membrane fragments after flagellate gazing on bacteria, but the significance of this process in natural environments remains to be studied.

In general, amino acids constitute an important portion of dissolved organic nitrogen (DON) in natural waters, typically making up 4–9% of all DON and 12–29% of the high-molecular-weight (>1,000 D) DON pool (Benner 2002). The dissolved amino acid pool is dominated by DCAA and only a minor portion are truly dissolved free amino acids (DFAA) (Bronk 2002). L amino acids are the most abundant isomers in DON and appears to reflect the composition in living cells (Lee and Bada 1977; McCarthy et al. 1998).

Reports on concentrations and composition of D amino acid isomers in aquatic environments typically have presented scattered and episodic observations, or have been used to demonstrate

new analytical approaches for detection of isomers. In an attempt to provide a deeper understanding on dynamics of D-AA in a natural, pelagic environment, seasonal changes in D amino acid isomers were measured in a shallow fjord and related to the total DFAA and DCAA pools, the abundance and activity of bacteria and viral density. In addition, uptake and production of D-DCAA by natural bacterial populations from the fjord were followed in batch cultures. The results demonstrate that natural pools of D-AA are dynamic and that bacteria are major contributors as well as consumers of these amino acids.

Material and methods

Sampling

Water was collected at Station 60 at 1-m depth (water depth was 6 m) in the shallow and eutrophic estuary Roskilde Fjord in eastern Denmark. Station 60 is located in the sheltered, western part of the fjord. Residence of the water at this station is unknown, but has been estimated to vary from 4 weeks to >1 year according to the local county authorities. In 1997, Roskilde Fjord water was used for a batch culture (microcosm) study of bacterial N cycling (Experimental set-up 1). In 1999, fjord water was sampled at 1–2 week intervals (a total of 20 samplings) from March to September to characterize seasonal changes in microbial carbon (C) and nitrogen (N) dynamics (Experimental set-up 2).

Experimental set-up 1: bacterial microcosms

In order to enhance the nutrient-to-bacteria ratio in the batch cultures, 0.7-μm pore size GF/F filtered water (Whatman International, Maidstone, UK) and sterile filtered water (0.2-μm pore size; Whatman Polycap filter capsules (including a glass fibre prefilter)) was mixed in a 1:9 ratio. Volumes of 2.5-l of the mixed water were transferred to each of nine 5-l glass bottles. In order to examine the impact of N enrichment on the bacterial N cycling, three of the bottles were enriched with NH_4^+ to a final concentration of 40 μM, while other three bottles received free amino acids to a final concentration of 10 μM (AA-S-18 Amino Acid standard solution; http://www.sigma-aldrich.com). The last three bottles remained unamended. The nine bottles were incubated on rotary shakers in the dark at 20°C. One hour after the beginning of the incubations and at various time points during the 140-h incubation period, subsamples were taken for measurement of chemical and microbiological parameters (see below). Samples for analysis of amino acids and inorganic nutrients were filtered through 0.2-μm Minisart membrane filters (http://www.sartorius.com) and frozen immediately for later analysis.

Experimental set-up 2: seasonal field and laboratory studies

Water samples from Station 60 were transported in acid-washed 5-l polyethylene bottles in a cooler to the laboratory and processed within 1 h. Samples for analysis of amino acids and inorganic nutrients were passed through 0.2-μm membrane filters and frozen. Mineralization of C, N and phosphorus (P) were studied in a 1:9 mixture of 0.7- and 0.2-μm filtered water as above. The mixed water was transferred to six 100-ml BOD bottles (filled completely and capped tightly) and to three 500-ml glass bottles each receiving 350 ml water samples and were left lightly capped on a shaking table at room temperature (20–23°C).

Chemical analysis

Dissolved nitrogen was determined as total dissolved nitrogen (TDN), including both inorganic N (DIN) and organic N (DON) on a Dohrman DN 1900 analyser (Teledyne Technologies, Los Angeles, USA). PO_4^{3-}, NH_4^+, NO_3^- and urea were measured on an AlpKem FlowSolution IV autoanalyser (OI Analytical, College Station, USA) using standard methods provided by the manufacturer, except for urea which was measured with the monoxime method according to Price and Harrison (1987) and using fast cooling as suggested by Mulvaney and Bremner (1979). Chlorophyll *a* was measured by spectrometry of acetone extracts of particulate matter in 200-ml water and collected on GF/C filters, according to Jespersen and Christoffersen (1987).

DFAA and DCAA were quantified by high performance liquid chromatography (HPLC) and fluorescence detection using two methods. Total amounts of DCAA (after hydrolysis, see below) and DFAA were detected as fluorescent primary amines after derivatization with *o*-phthaldialdehyde (OPA) according to Lindroth and Mopper (1979) and Jørgensen et al. (1993). In addition to protein amino acids (except for Pro and Cys that were not quantified), the detection included ornithine (Orn), α-aminobutyric acid (α-ABA), γ-aminobutyric acid (GABA), and *m* and LL-isomers of diaminopimelic acid (DAPA; amino acid in peptidoglycan interbridges in Gram-negative bacteria). All DFAA concentrations are presented as single concentrations as the variation between replicate analyses (injections) was within the method (machine) variability of maximum <5%.

For the analysis of DCAA, triplicate water samples were freeze-dried and subsequently hydrolyzed by a microwave technique (Jørgensen and Jensen 1997). The hydrolyzed samples were re-dissolved in 1.25-M borate buffer adjusted to pH 9.5, filtered through 13 mm diameter 0.2-μm pore size filters and analyzed by HPLC. Concentration of DFAA were subtracted from the DCAA concentrations.

D and L isomers of Asp, Glu, Ser and Ala were measured by the method of Mopper and Furton (1999), with the exception that *N*-isobutyryl-L-cysteine (IBC) was used as a chiral agent (Brückner et al. 1994).

The HPLC columns used were a 3.9 × 150 mm Nova-Pak C18 steel column (Waters Associates, USA) for the OPA method, and a 4.6 × 250 mm Discovery C18 steel column (Supelco, USA) for the IBC method. The detection of amino acid isomers focused on Asp, Glu, Ser and Ala because these amino acids have been found to be dominant isomers in natural and biological material, including bacterial peptidoglycan (Brückner et al. 1994; Jørgensen et al. 2003; Pedersen et al. 2001). Further, the HPLC separation of these isomers could be optimized to produce well-separated peaks in a single chromatographic run.

Concentrations of inorganic and organic N species, PO_4^{3-}, chlorophyll *a*, temperature and salinity in Roskilde Fjord water during the studied periods are summarized in Table 1.

Microbiological analyses

Bacteria were counted in water samples preserved with formaldehyde (2% final conc.) by epifluorescence microscopy after staining with acridine orange according to Hobbie et al. (1977), and bacterial densities were calculated from the mean number of cells at 10 independent locations on each filter. A total of at least 200 cells were counted. Bacterial production was measured by incorporation of 10 nM ^{3}H-thymidine and 100 nM ^{3}H-leucine in each of triplicate 5 ml water samples and a killed control (formaldehyde at 2% final conc.) according to Fuhrman and Azam (1980) (thymidine), and Kirchman et al. (1985) and Jørgensen (1992) (leucine). After incubation times of 45–60 min at in situ temperature, the bacteria were filtered onto 0.2 μm membrane filters and radioassayed by liquid scintillation counting. Viruses were quantified according to Noble and Fuhrman (1998). Subsamples of 0.5–2 ml were filtered onto 0.02 μm Anodisc filters (Whatman), placed on a drop of

Table 1 Background information on Roskilde Fjord during the sampling times in 1997 and 1999

	June 1997	March–September 1999
Chlorophyll *a*	2.5 μg l^{-1}	3–6 μg l^{-1} (April: 4–12 μg l^{-1})
NH_4^+	13 μM	<2 μM
NO_3^-	1.8 μM	<3 μM (March–April: >12 μM)
PO_4^{3-}	3.0 μM	From <1 μM in spring to 11 μM in August
Urea	0.5 μM	<0.5 μM (2 μM peaks in June and July)
DON	40 μM-N	35–90 μM-N
DFAA	260 nM	110–640 nM
DCAA	3,400 nM	2,800–8,700 nM (see Fig. 1c)
Temperature	18°C	3°C (March)–22°C (August)
Salinity	10 ppt	10–12 ppt

0.2% SYBR-Green I (Molecular Probes) for 15 min and mounted on a glass slide. Three hundred to six hundred viruses were counted on each slide by epifluorescence microscopy.

Mineralization of dissolved organic matter was determined by standard BOD technique. The bottles were incubated in the dark for 7 days after which the oxygen consumption was determined as the decrease in O_2 concentration relative to an initial sample. Net mineralization of N and P was determined from differences in concentrations of NH_4^+ and PO_4^{3-} during a 7-day incubation of fjord water in the 1 (0.7 μm):9 (0.2 μm) batch cultures. In addition, samples for analysis of changes in DCAA concentrations in the batch cultures during the incubations were collected in spring 1999 and were assumed to represent DCAA degradation (or production) in the fjord water.

Composition of amino acid isomers in bacteria growing in the batch cultures was measured by filtration of 200-ml water through 0.2-μm pore-size polycarbonate filters (http://www.gewater.com). The bacteria were harvested after 48-h incubation to minimize the risk of flagellate growth and its possible bias on determination of the bacterial amino acid content. Bacteria on the filters were hydrolyzed by the microwave method after which the amino acids were dissolved in borate buffer and treated as above.

Statistical analyses

Statistical analyses were performed with SigmaStat 3.1 (http://www.systat.com). Correlations in seasonal changes, e.g., simultaneous increases or decreases in concentrations or rates during the studied period, were tested using Pearson product moment correlation coefficients. Since these correlations indicate similarities in changes but do not show actual relations between two concentrations or rates, e.g., whether concentrations of D-DCAA made up a constant proportion of DCAA on the different sampling dates from March to September, correlations between actual concentrations or rates were tested using linear regressions. Variables including percentage values were subjected to arcsin square root transformation before the analyses.

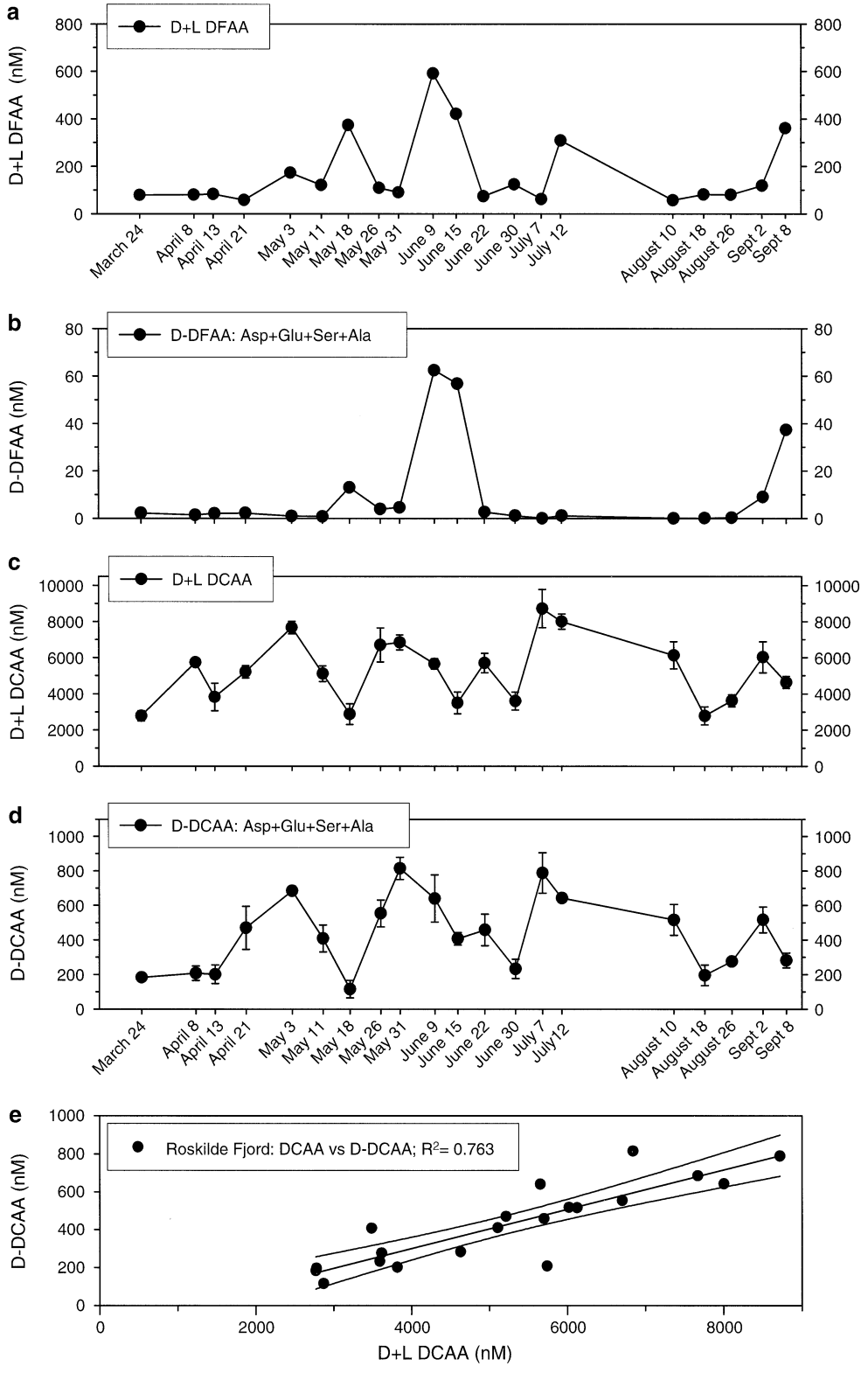
Results

Seasonal changes in Roskilde Fjord

Concentrations of DFAA (including both D and L isomers) fluctuated between 57 and 591 nM, while D-DFAA (Glu, Ser and Ala; D-Asp was excluded due to absence in most samples) ranged from 0.3 to 62 nM (Fig. 1a, b). D-DFAA made up 3.6% ± 3.9 (SD) of the DFAA. The specific D isomers ranged from 0.8% (Glu) to 7.3% (Ala) of the total (D+L) amounts of the three amino acid isomers (Table 2). Dominant individual DFAA were Ser, Asp, Gly, Glu and Ala, making up an average of 21% (Ser) to 8% (Ala) of the DFAA (Table 2). Trace amounts (<1.5 nM) of GABA and LL-DAPA were occasionally observed. Concentrations of all D-DFAA and all DFAA showed similar seasonal changes ($P < 0.01$; Table 3) and were significantly correlated ($R^2 = 0.776$). In contrast, concentrations of the individual D amino acids did not correlate with the total pools of D-DFAA or DFAA ($R^2 < 0.134$) or have similar seasonal variations ($P > 0.1$; not shown).

Concentrations of all (D+L)-DCAA varied from 2,800 to 8,700 nM, while the four D-DCAA (Asp, Glu, Ser and Ala) ranged from 115 to 814 nM (Fig. 1c, d). D-DCAA made up 7.9% ± 2.3 (±1 SD) of the DCAA pool. Concentrations of all D-DCAA and DCAA had similar variations with respect to seasonal changes ($P < 0.02$, Table 3) and concentrations ($R^2 = 0.763$; Fig. 1e), but the single D isomer concentrations did not correlate with the total DCAA pool or sum of the four D-DCAA ($R^2 < 0.213$). The D isomers made up from 8.5% (Ser) to 26% (Asp) of the total (D+L) amounts of these amino acid (Table 2). The most abundant amino acids in the DCAA pool (as average proportion of all DCAA) were Gly, Asp, Ala, Glu and Ser, making up an average of 27% (Gly) to 9.4% (Ser) (Table 2). Dominant non-protein amino acids among the DCAA were LL-DAPA, GABA, *m*-DAPA and α-ABA, making up 3.1, 1.4, 1.1 and 0.1%, respectively, of all DCAA.

Bacterial density in the fjord ranged from 2.7 to 11.1×10^9 l^{-1} from March to September, but on most of the sampling days the number varied



◀**Fig. 1** Seasonal changes in concentrations of all D+L-DFAA (**a**) and four D-DFAA (**b**), and all D+L dissolved combined amino acids (DCAA) (**c**) and four D-DCAA (**d**) in Roskilde Fjord in 1999. Single concentrations (DFAA) or mean concentrations of triplicate analyses (±1 SD) (DCAA) are shown. Correlation (linear regression) between concentrations of all D+L DCAA and D-DCAA, including 95% confidence interval, is shown in panel **e**

between 6 and 9×10^9 l^{-1} (Fig. 2a). A larger variation was measured in the incorporation of ^{3}H-thymidine and ^{3}H-leucine (Fig. 2b). During the five month period, the incorporation varied up to 23-fold, with rates of 1.3–30 pmol l^{-1} h^{-1} (thymidine) and 7–160 pmol l^{-1} h^{-1} (leucine). No seasonal trends were obvious, but the seasonal changes in incorporation of thymidine and leucine were similar ($P< 0.05$) (Table 3). The bacterial density only correlated with thymidine incorporation ($P< 0.05$) (Table 3).

Virus abundance showed a general increase over the investigation period from a minimum of 2.9×10^9 viruses l^{-1} in March to a maximum density of 65×10^9 viruses l^{-1} in mid-August, and with a distinct peak of 45×10^9 viruses l^{-1} in May (Fig. 2c). By late August, viral abundance again decreased to $20–30\times 10^9$ viruses l^{-1}. No correlations between densities of virus and bacterial abundance and production were found, but the number of viruses correlated with several bacterially mediated degradation processes as discussed later.

Batch culture studies of microbial cycling of DCAA relative to N and P mineralization

Microbial utilization or production of DCAA was studied from March to early June in batch cultures. On seven of ten sampling days, DCAA were degraded by the bacteria, reducing the ambient DCAA pools by 3–80% (Fig. 3a). On the remaining 3 sampling days (May 3, 26 and 31), a production of DCAA occurred in the batch cultures, corresponding to 2–30% of the ambient DCAA pools. Seasonal changes in the DCAA mineralization correlated with variations in bacterial number and production, and with variations in the DCAA and D-DCAA pools ($P< 0.05$; Table 3). Further, the actual DCAA flux (nmol DCAA consumed or produced during 7 days) correlated with the DCAA concentrations ($R^2 = 0.546$; Fig. 4a)

Analysis of amino acid composition of bacteria in the batch cultures showed that D isomers made up from 6.7 to 12.5% (mean was 8.8%) of bacterial amino acids (Fig. 3b). D-Asp, D-Glu, D-Ser and D-Ala on average made up 30, 27, 3 and 40% of the D isomers in the bacteria, respectively (data not shown). The proportion of specific D isomers in the bacteria correlated with the percentage of D-Asp in the DCAA pool, but not with the other D amino acids ($P< 0.02$; Table 3).

Net release or uptake of N by bacteria in the batch cultures was estimated from the NH_4^+ balance in the cultures. Typically there was an uptake of N in March, April and May and a release of N from June to September (Fig. 3c). Seasonal changes in the N mineralization correlated with seasonal variation in concentrations of D-Asp, D-Glu and D-Ser ($P< 0.02$) and with the D isomer content of the bacteria ($P< 0.02$; Table 3). The N mineralization rate further correlated with concentrations of the D isomers, although the correlation coefficient for D-Ala was low (Fig. 4b). In contrast to that, there were no correlations between concentrations of the total D+L DCAA pools and the mineralization of N (Table 3; Fig. 4c).

Cycling of phosphorus in the batch cultures (PO_4^{3-} uptake or release; data not shown) dem-

Table 2 Proportion of (a) dominant DFAA and DCAA in Roskilde Fjord (the shown percentages include both L and D isomers), and (b) proportion of D isomers among the four analyzed amino acid isomers in the DFAA and DCAA pools

	(a) Dominant DFAA and DCAA (% of total pools)					(b) D isomers (%D of L+D isomers)			
	Asp	Glu	Ser	Gly	Ala	D-Asp	D-Glu	D-Ser	D-Ala
DFAA	19 ± 5.1	9.3 ± 3.6	21 ± 3.5	16 ± 2.9	8.1 ± 2.2	nd	0.8 ± 0.2	3.9 ± 0.7	7.3 ± 1.1
DCAA	13 ± 4.3	11 ± 3.1	9.4 ± 3.0	27 ± 4.5	11 ± 2.8	26 ± 2.9	12 ± 2.3	8.5 ± 1.9	12 ± 2.8

Mean values for the studied period (±1SD) are shown. nd = no data

Table 3 Pearson product moment correlation coefficients of seasonal changes in field observations and in parallel batch culture studies of Roskilde Fjord from March to September 1999

	Virus density	Leucine incorp.	TdR incorp.	DFAA (nM)	D-DFAA (nM)	DCAA conc.	D-DCAA conc.	%D Asp[a]	%D Glu[a]	%D Ser[a]	%D Ala[a]	%D in bacteria	DCAA mineral.	N (NH_4^+) mineral.	P (PO_4^{3-}) mineral.	C (BOD) mineral.
Bacteria density	0.106	0.397	**0.430***	0.041	0.073	0.038	0.266	0.170	–0.040	–0.264	–0.222	0.274	**0.612***	–0.005	–0.143	0.403
Virus density		–0.255	0.351	–0.137	–0.073	0.120	0.230	**0.522****	0.339	0.247	0.386	0.086	**0.598***	0.162	**0.421***	**0.521***
Leucine incorp.			**0.463***	–0.205	–0.261	0.277	0.238	–0.270	–0.289	–0.458	–0.338	0.522	**0.685***	–0.337	–0.238	0.267
TdR incorp.				–0.202	–0.388	**0.469***	**0.433***	0.083	0.018	–0.165	0.013	–0.036	**0.721****	–0.203	–0.095	**0.480***
DFAA (nM)					**0.874*****	–0.096	0.047	0.049	**–0.486****	–0.438	–0.370	–0.153	–0.213	–0.160	0.005	0.011
D-DFAA (nM)						–0.171	0.056	–0.395	–0.032	0.039	0.042	–0.302	–0.216	–0.089	0.024	0.028
DCAA conc.							**0.883*****	0.181	0.066	0.017	0.076	–0.241	**0.624***	–0.032	**–0.511***	0.269
D-DCAA conc.								0.365	0.155	0.051	0.141	–0.258	**0.733****	0.063	**–0.466***	0.425
%D Asp[a]									**0.626*****	**0.627*****	**0.713*****	**0.575****	0.421	**0.536****	0.107	0.232
%D Glu[a]										**0.860*****	**0.851*****	0.221	0.404	**0.726*****	0.103	0.103
%D Ser[a]											**0.906*****	0.217	0.391	**0.599*****	0.124	–0.167
%D Ala[a]												0.233	0.508	0.376	0.156	–0.040
%D in bacteria													0.189[b]	**0.511****	0.302	0.132
DCAA mineral.														0.054	0.309	0.426
N (NH_4^+) mineral.															0.087	–0.197
P (PO_4^{3-}) mineral.																0.052

*$P < 0.05$; **$P < 0.02$; ***$P < 0.01$

[a]D isomers in the DCAA pool

[b]Few simultaneous observations

Statistically significant correlations are shown in bold

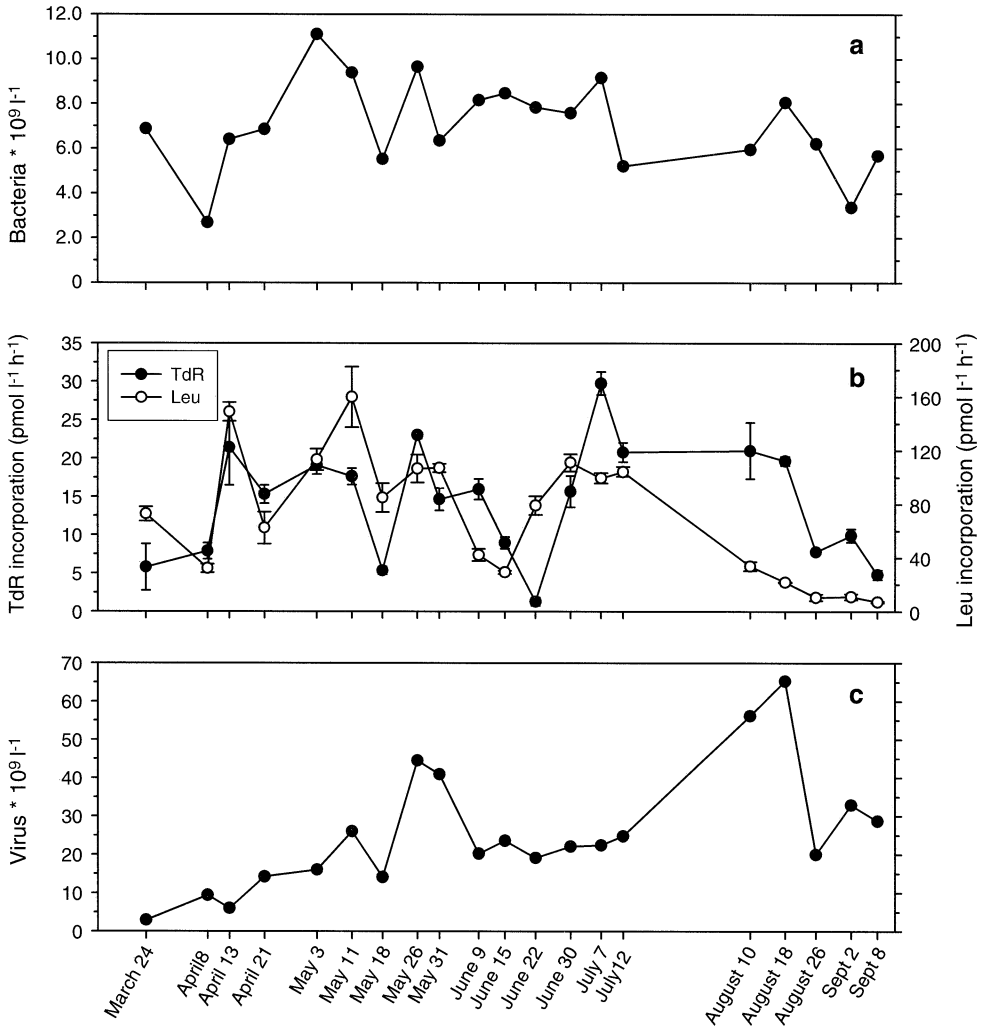


Fig. 2 Densities of bacteria (**a**), bacterial production (incorporation of thymidine (TdR) and leucine (Leu)) (**b**), and virus abundance (**c**) in Roskilde Fjord 1999. Mean numbers (bacterial and viral density) or means ± 1 SD shown (n = 3) (bacterial production)

onstrated a positive seasonal correlation between release of PO_4^{3-} and viral abundance, but the release of PO_4^{3-} correlated negatively with seasonal concentration changes of both DCAA and D-DCAA ($P < 0.05$; Table 3). The viral abundance further correlated with seasonal changes in the mineralization of DCAA and C (BOD) ($P < 0.05$; Table 3). Finally, there was a negative correlation between P mineralization and concentrations of DCAA and D-DCAA ($P < 0.05$) and a positive correlation between BOD and thymidine incorporation ($P < 0.05$) (Table 3).

Bacterial growth and cycling of DCAA and D-DCAA in microcosms

Bacterial density in the microcosms increased from initially 0.8×10^9 cells l^{-1} to maxima of 6×10^9 l^{-1} (control and $+NH_4^+$ microcosms at 30 h) and 9×10^9 l^{-1} (+DFAA microcosms at 42 h) (Fig. 5a). A temporary reduction in bacterial numbers occurred in all the microcosms from 30 to 36 h. At 140 h, the densities were reduced to 2.4×10^9 cells l^{-1} ($+NH_4^+$ and +DFAA microcosms) and 2.4×10^9 cells l^{-1} (control).

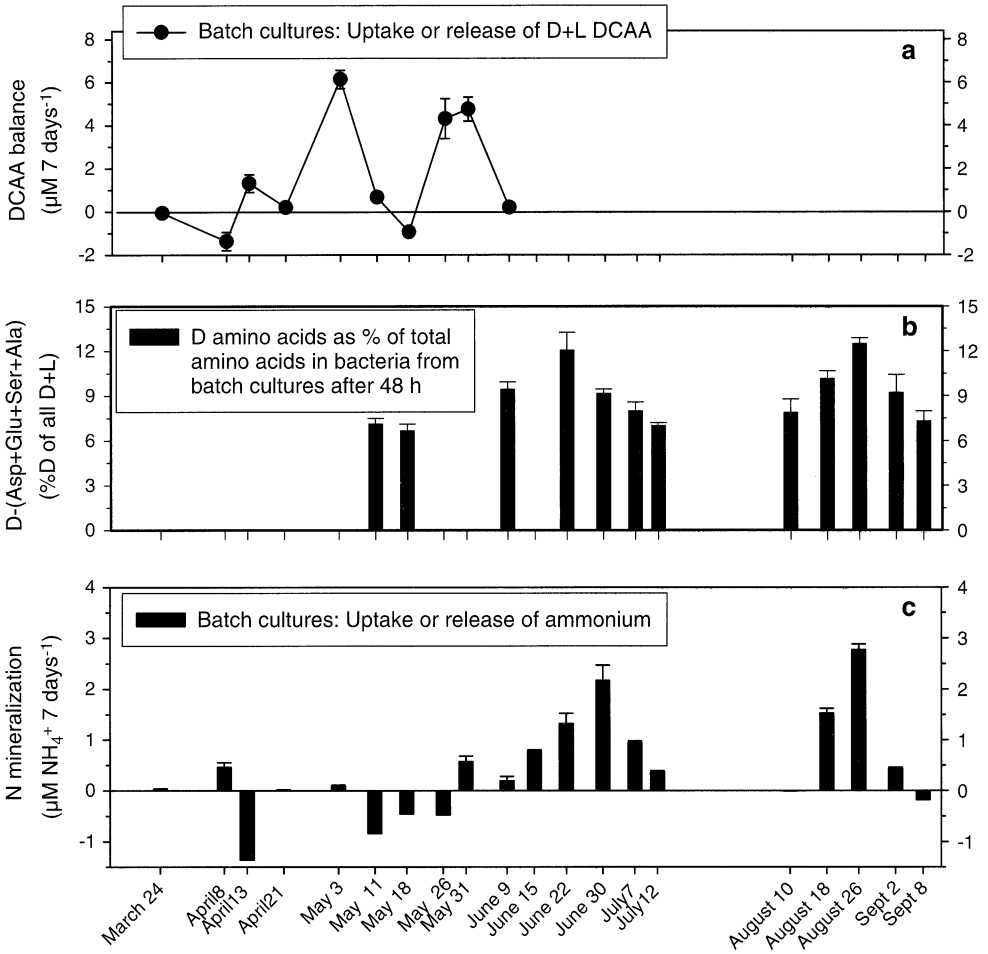


Fig. 3 Roskilde Fjord batch cultures. Seasonal changes in utilization of DCAA (uptake or production) (**a**), in content of D amino acids in bacteria (**b**), and in mineralization of N (production or release of NH_4^+) (**c**). Negative values for the DCAA utilization and N mineralization indicate an uptake. Means ±1 SD shown (n = 3)

The concentration of NH_4^+ in the microcosms mirrored the bacterial densities. During the increase in cell numbers, NH_4^+ was reduced by 6–10 μM (22 μM in the +NH_4^+ microcosms), but during the decline in bacterial numbers from 30 to 36 h, a release of NH_4^+ of 7–12 μM occurred (Fig. 5b).

Bacterial uptake reduced the total DCAA concentrations by 27, 44 and 52% in the control, NH_4^+- and DFAA-enriched microcosms, respectively, during the initial 36 h (Fig. 5c, d, and e). D-DCAA was more efficiently utilized than the total DCAA and were reduced by 51, 73 and 78% in the respective cultures. Thus, addition of NH_4^+ and DFAA stimulated the uptake of both L and D amino acids. The bacterial uptake of DCAA between 0 and 36 h was followed by a net release of DCAA in the DFAA- and NH_4^+-enriched cultures of 1,150–1,150 nM, but no changes were found in the control cultures. D isomers among the released DCAA made up 50–120 nM. From 42 to 140 h, the only change in amino acid concentrations was a minor increase in DCAA in the controls. Although the bacterial density increased after 36 h, DCAA appeared not to be a major nutrient source to the bacteria during that period. In the microcosms, the four D amino acids typically made up about 40% (D-Asp), 22% (D-Glu), 16% (D-Ser) and 22% (D-Ala) of the total D isomer concentrations and only minor changes occurred during the incubation (data not shown).

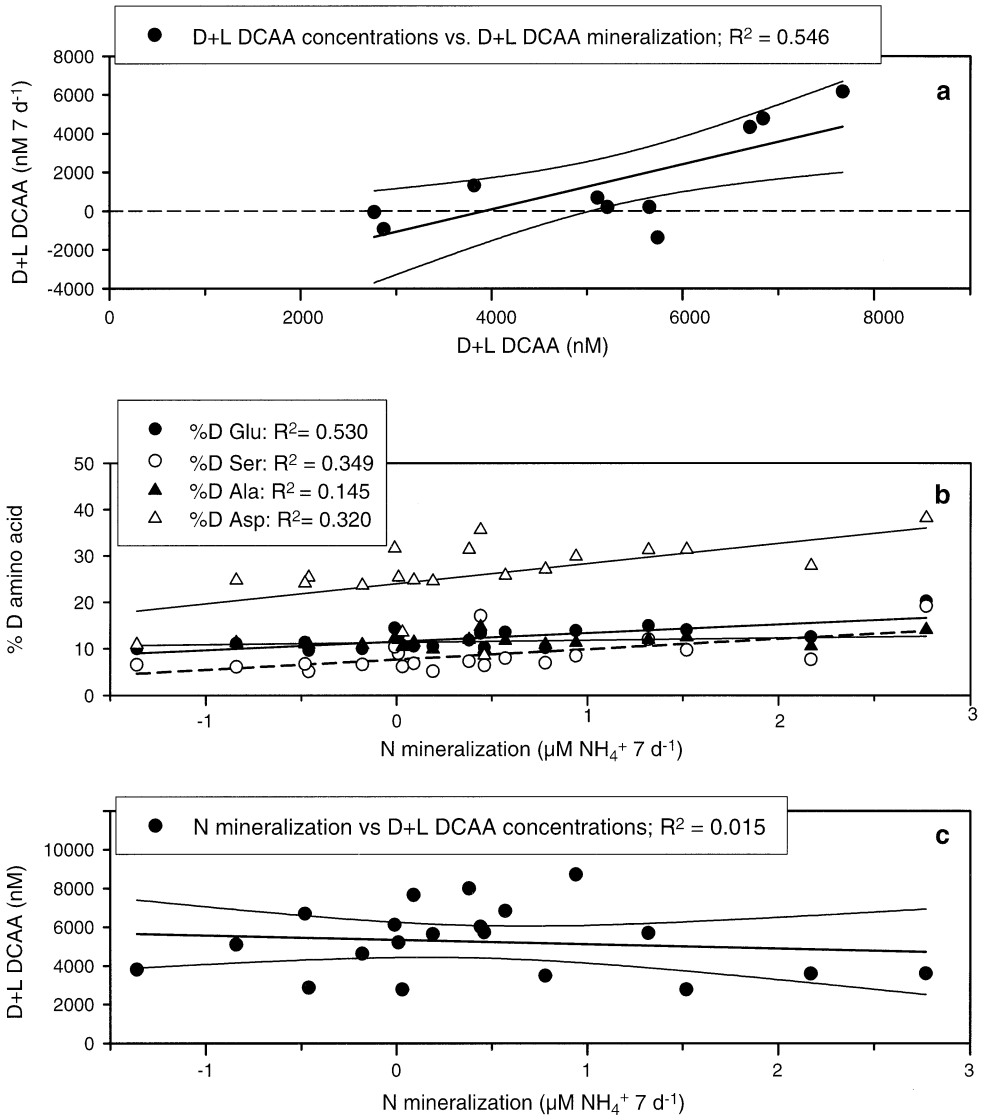


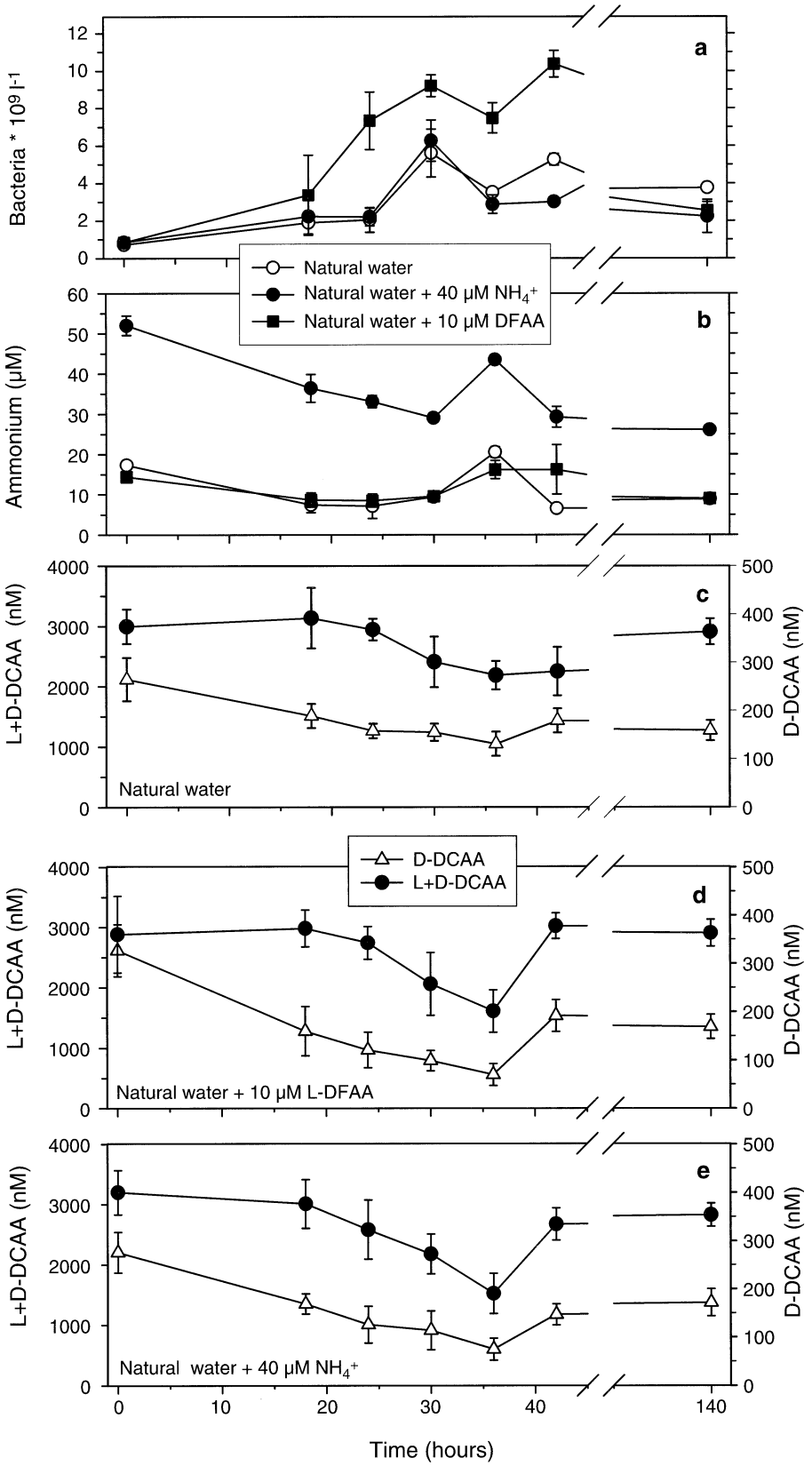
Fig. 4 Batch culture studies: correlations (linear regressions) between ambient concentrations and mineralization rates (uptake or release) of all D+L dissolved combined amino acids (DCAA) (**a**), between N mineralization (production or release of NH_4^+) and proportions of the individual D amino acids (**b**) and all D+L DCAA (**c**). Confidence intervals (95%) of the regressions are shown in panels (**a**) and (**c**)

Discussion

The seasonal abundance of D amino acid isomers in Roskilde Fjord water demonstrates that D isomers were inherent components of the dissolved organic matter pool in the water and that bacteria, in addition to degrading organic matter, also contribute organic substances to the ambient nutrient pool. The data further suggest that viral-mediated disintegration of peptidoglycan can be a supplementary source of D isomers as well as an important mediator in P cycling.

Occurrence of D-DFAA and D-DCAA

The four D isomers made up a smaller portion of DFAA (average of 3.6%) than of DCAA (average of 7.9%) but there were large variations between the individual amino acids. Thus, D-Asp was not detectable as a DFAA, but it made up



◀**Fig. 5** Observations from Roskilde Fjord microcosms, showing (1) bacterial density (**a**) and concentrations of NH_4^+ (**b**), (2) concentrations of all L+D-DCAA and four D-DCAA (Asp, Glu, Ser and Ala) in batch cultures with untreated fjord water (**c**), in fjord water enriched with L isomers of 18 DFAA (**d**) and in fjord water enriched with NH_4^+ (e). Means ± 1 SD shown (one sample from each of three batch cultures)

26% of the total amount of DCAA-Asp. At the other end of the range, however, D-Ala made up a relatively comparable fraction within the two pools (7.3% of DFAA-Ala and 12% of DCAA-Ala).

Concentrations of D isomers among DFAA have not previously been published, but have been estimated to make up <5 nM in Atlantic waters (Perez et al. 2003). For DCAA, the proportion of D isomers has been reported to range from about 5% (D-Glu) to 35% (D-Ala) (Jørgensen et al. 1999b; Lee and Bada 1977; Perez et al. 2003; Stepanaukas et al. 2000). D-Ser appears to be among the least abundant DCAA, but a low D isomer proportion has also been measured for the amino acids Val and Leu in sea water (Lee and Bada 1977). Analysis of high-polymer organic matter (1–100 nm size) in oceanic waters supports that D-Ala is an abundant D isomer among amino acids in dissolved marine organic matter (McCarthy et al. 1998).

A direct comparison of amino acid isomers in different studies, however, may be problematic when considering the spectrum of chromatographic and derivatization techniques applied as well as the variable procedures used for filtration and possible concentration of the water samples. Further, selected amino acid isomers have been measured in most studies rather than, e.g., isomers of all protein amino acids. In the present study, D isomers of Asp, Glu, Ser and Ala were selected since these amino acids were all found in purified peptidoglycan of a Gram-positive and a Gram-negative bacterium, where they occurred at a ratio of 1.00/1.58/6.35/5.24 for Ser/Asp/Glu/Ala (Jørgensen et al. 2003). This amino acid composition contradicts the general idea that D amino acid isomers in peptidoglycan typically consist of D-Glu and D-Ala (Madigan and Martinko 2006), but it most likely reflects that peptidoglycan has only been characterized in relatively few bacteria. It should be mentioned that some thermophilic *Archaea* contains D amino acid isomers, but their function is unknown (Matsumoto et al. 1999).

Total concentrations of both DFAA and DCAA in Roskilde Fjord agreed with those previously measured in other estuaries, e.g. in Delaware estuary (Keil and Kirchman 1991; Middelboe et al. 1995), except that DCAA concentrations in the fjord periodically exceeded maximum concentrations obtained in Delaware estuary. The occurrence of the two DAPA isomers (LL- and *m*-DAPA), together making up 4.2% of the DCAA and detected at trace amounts among DFAA, supports the presence of peptidoglycan components in Roskilde Fjord water. DAPA occurs in peptide interbridges of Gram-negative bacteria and have been found in amounts close to D-Ser in peptidoglycan (Jørgensen et al. 2003).

The concentration of D isomers in the DFAA and DCAA pools covaried with the total amounts of these amino acids in Roskilde Fjord ($R^2 > 0.76$). This suggests that the nature of D isomers of DFAA and DCAA isomers was comparable to other amino acids, that is, they were produced and degraded at rates comparable to amino acids originating from general organic matter cycling. A similar observation was made in an analysis of the same four specific D isomers in 50 rivers entering the Baltic Sea (Jørgensen et al. 2003). In this study D isomers in the rivers covaried with the total DON pool and made up about 0.5% of the DON. Despite this covariation between DON and D isomers, some experiments suggest that a differential microbial degradation of D isomers may occur in natural waters as discussed below. Further, it should be emphasized that although pools of dissolved L and D amino acids covary in natural waters, this does not necessarily indicate a similar degradation rate of peptidoglycan and proteins. The occurrence of almost intact bacterial "cell sacs" in marine sediments (Moriarty and Hayward 1982), the observation of slow degradation of peptidoglycan relative to bacterial proteins (Nagata et al. 2003) and the preservation of cell walls of a Gram-negative bacterium for >1 month in sea water with actively growing bacteria (Jørgensen et al.

2003), together indicate that peptidoglycan typically is more resistant to bacterial degradation than proteins.

Degradation of DCAA and D-DCAA

The positive correlations between seasonal changes in DCAA mineralization and (1) concentrations of DCAA and D-DCAA, (2) bacterial production (leucine and thymidine incorporation), and (3) viral densities (see further comments on impact of viruses below) indicate that DCAA and D-DCAA were essential components in the bacterial degradation of organic matter in the fjord. Also, the decline in L- and D-DCAA concentrations in the microcosms during intense bacterial production supports that DCAA were a significant substrate source for the bacterioplankton. Another interesting observation was the high degree of correlation (correlation coefficients of 0.54–0.73) between N mineralization (production of NH_4^+) and concentrations of three D-DCAA (D-Asp, D-Glu, D-Ser), but lack of correlations between N mineralization and the total DCAA pool. This suggests that peptidoglycan amino acids may have had an important role in the pelagic N mineralization in the fjord water.

Supporting that D-DCAA were indeed utilized by bacteria, the measured BOD values (presumed to represent an estimate of the labile organic matter pool) was positively correlated to the D-DCAA concentration, and even showed a stronger correlation to D-DCAA (0.43) than to all DCAA (0.27). A likely explanation to this relationship might be that peptidoglycan amino acids, despite being reported as relatively recalcitrant, were in fact more labile than amino acids in the DCAA pool (DCAA lability is discussed later). In contrast to this positive relation between D-DCAA and organic matter degradation in the eutrophic Roskilde Fjord, a reverse relation between concentrations of D-DCAA and bacterial biomass production was found along an estuary-to-open sea gradient in the mesotrophic Gulf of Riga (Jørgensen et al. 1999b). Apparently the relation between organic matter lability and D amino acid pools can be variable and may depend on site-specific conditions such as the general composition of the organic matter.

The close correlations between bacterial production (thymidine incorporation) and (1) D-DCAA and DCAA concentrations and (2) BOD support the suggestion above that the bacterial production in the fjord had a strong influence on occurrence and cycling of DCAA. However, when relating the DCAA uptake to the BOD values in the batch culture experiments, it can be estimated that DCAA-C on average only met about 7% of the biological oxygen consumption (assuming a respiratory quotient of 1, bacterial growth efficiency of 25%, and that that each DCAA contained three C atoms). Despite the fact that organisms other than bacteria may have consumed O_2 in the BOD bottles, e.g. protists grazing on bacteria, this estimate suggests that DCAA probably were not major sources of C to the bacteria. Speculatively, DCAA-N rather than DCAA-C may have been regulating the bacterial production. In batch culture studies of estuarine bacteria, a reverse relation between bacterial utilization of DCAA and general availability of N, including DFAA and NH_4^+, has previously been found (Jørgensen et al. 1993; 1999a; Middelboe et al. 1995). In Roskilde Fjord, however, it is unclear whether DCAA dynamics were coupled to the microbial N status or seasonal changes in the relative composition of various N sources. In the present microcosms, the changes in DCAA indicated a stimulated DCAA utilization with increasing amounts of N, with DCAA uptake increasing from 30 to 50% in microcosms enriched with NH_4^+, relative to control cultures. Possibly, in these microcosms DCAA served as a C source to the bacteria, as has been found in other microcosm studies in which DCAA uptake was stimulated during carbon limitation (Middelboe et al. 1995).

Observations by Keil and Kirchman (1993) indicate that DCAA in marine environments may consist of different pools with a variable lability to bacteria. In our study, up to half of the DCAA were taken up by bacteria in the microcosms. It might be speculated that the portion of DCAA not being degraded consisted of peptidoglycan components, as peptidoglycan amino acids have been reported relatively recalcitrant to marine bacteria (McCarthy et al. 1998). On the other hand, Nagata et al. (2003) found that peptide

interbridges in peptidoglycan were degraded significantly faster than the glycan backbone (*N*-acetyl-glucosamine and *N*-acetyl-muramic acids). This is supported by Jørgensen et al. (2003) who found that about half of all D amino acids in added peptidoglycan were utilized within 8 days in bacterial microcosms, while glucosamine remained unchanged. A partial degradation of peptidoglycan D amino acids may also have occurred in our microcosm study, where naturally occurring D isomers were reduced by 50–80% within the initial 36 h, but increased again after the transient reduction in bacterial density and remained at a higher level.

It is not obvious why the released D amino acids in the microcosm study were not degraded. Speculatively, the released peptidoglycan (only fragments <0.2 μm were included in the analysis) had a structure that was resistant to enzymatic attack. Enzymatic processes involved in uptake of extracellular peptidoglycan are still unidentified. Hydrolases that are known to break covalent bonds in peptidoglycan are typically located in the cell wall (Cibik et al. 2001; Smith et al. 2000). If cell-bound enzyme systems are responsible for degradation of extracellular peptidoglycan, a physical contact between the bacterial cells and peptidoglycan is necessary, but the mechanisms remain to be identified. An alternate pathway for degradation of peptidoglycan may involve a suite of extracellular enzymes such as peptidases (for removal of the peptide interbridges) and lysozyme-like enzymes (for breakage of the $\beta(1–4)$ bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid). Both peptidases and $\beta(1–4)$-cleaving enzymes are common exoenzymes in natural waters (Karner et al. 1992; Zubkov and Sleigh 1998).

Sources of D-DCAA

The production of D amino acids in the bacteria-only microcosms shows that peptidoglycan components were released by the bacterial community. D-amino acids detected in situ in Roskilde Fjord water most likely also originated from bacterial cell walls, but the specific source of the D-DCAA was not determined. Several processes may have led to production of dissolved peptidoglycan fragments in the microcosms as well as in the fjord water, including: (1) loss of wall fragments during cell division (Giesbrecht et al. 1997); (2) damage on cell walls of living bacteria due to peptidoglycan-degrading enzymes produced by other bacteria (Mercier et al. 2000); (3) peptidoglycan-hydrolyzing enzymes produced by bacteriophages (Moak and Molineux 2004; Rydman and Bamford 2002; Ugorakova and Bukovska 2003) and leading to colloidal-sized cell wall debris (Riemann and Middelboe 2002); (4) activity of peptidoglycan-degrading hydrolases (autolysins) causing cell mortality (Lewis 2000); and (5) protist grazing on bacteria (Nagata and Kirchman 2001).

Most of the above-mentioned processes have so far only been examined in laboratory studies and it is uncertain to which extent they actually contribute quantitatively to the pool of D-DCAA in natural waters. Also, it is not known if the measured D-DCAA, e.g., in Roskilde Fjord, were located in intact peptidoglycan fragments (smaller than 0.2 μm) or, e.g., in free peptide interbridges released after activity of amidase and/or endopeptidase (Giesbrecht et al. 1998). The abundance of D amino acids in high molecular weight dissolved organic matter as observed by McCarthy et al. (1998) may indicate that most peptidoglycan amino acids occur in larger fragments rather than in shorter peptides. Further studies are required to substantiate this notion.

The present study did not indicate a strong relation between viral abundance and occurrence of D amino acids in the water (correlation coefficients of 0.25–0.39) and a statistically significant correlation was only found for D-Asp among DCAA ($P< 0.02$). Possibly, effects of virus infection and turnover time of D-DCAA may occur at different time scales or staggered in time, causing a lack of coincidence. Supporting an expected relation between viral abundance and D amino acid isomers, recent laboratory studies with a marine bacterium–virus model system demonstrated the release of both DFAA and DCAA (including D isomers) following viral lysis of the host bacteria Middelboe and Jørgensen (2006). In a natural aquatic environment, release of amino acids following viral lysis may be less distinct and obscured by microbial uptake.

D amino acids in bacteria, DCAA and DFAA

The proportion of D amino acids in bacteria from the seasonal batch cultures (6.7–12.5% of all amino acids were D isomers) agrees well with the content of D isomers in purified peptidoglycan of a Gram-negative bacterium (*Pseudomonas* sp.) and a Gram-positive bacterium (*Bacillus* sp.) of 4.9 and 15%, respectively (Jørgensen et al. 2003). Considering that bacteria also contain amino acids in proteins, the present proportion of D amino acids is relatively high, but may reflect prevalence of Gram-positive bacteria in the cultures. Unfortunately, no molecular studies were performed to confirm this, but recent studies indicate that Gram-positive bacteria such as *Actinobacteria* actually can be abundant in estuarine environments (Kirchman et al. 2005).

Peptidoglycan in bacteria from the seasonal batch cultures had a higher content of D amino acids than the DCAA and DFAA pools. D-Asp, D-Glu, D-Ser and D-Ala made up the following mean percentages of each amino acid in (1) bacteria from the batch cultures, (2) DCAA and (3) DFAA: D-Asp, 30–26–0 (i.e., bacteria–DCAA–DFAA); D-Glu, 27-12-1; D-Ser, 3-9-4; D-Ala, 22–12–7. Thus, except for Ser, there was a clear reduction in D to L isomers from bacteria to DCAA and on to DFAA. This differentiation undoubtedly reflects a "D isomer dilution effect" due to production of L-only isomers from bacterial and non-bacterial sources, but it also reflects a selective degradation of D isomers as was observed for DCAA within the initial 36 h in the microcosms (Table 3). The significant reduction in D isomers from DCAA to DFAA may indicate a significant production of L-DFAA from biological processes, but it may also indicate a fast uptake of free D isomers as observed for D-Asp in the Atlantic (Perez et al. 2003). It should be mentioned that composition of D amino acids in bacteria from the batch cultures may not necessarily be identical to the composition in bacteria in the fjord and may have biased the comparison.

Viral infection and nutrient cycling

Viruses are known to influence bacterial cycling of carbon and mineral nutrients in pelagic systems. During viral-induced cell lysis particulate organic matter is transformed to labile dissolved material that stimulates the bacterial mineralization (Gobler et al. 1997; Middelboe 2006). The observed positive correlations between (1) virus abundance and (2) rates of bacterial mineralization of DCAA, total DOC (BOD) and P ($P < 0.05$) during the seasonal study support that production of viruses stimulated the bacterial turnover of carbon and nutrients. This is the first indication of an in situ coupling between virus abundance and specific rates of microbial mineralization based on a number of independent measurements, and it emphasizes the potential role of viruses in pelagic nutrient cycling. However, such correlations do not provide evidence for a direct coupling between these parameters and should be viewed with care due to the complex interactions that control bacterial activity and viral densities. The viral abundance in Roskilde Fjord did not correlate with total bacterial abundance or overall activity of the bacteria, suggesting that the observed correlations for specific bacterial mineralization rates were not simply the result of a general increase in viral numbers with increasing bacterial abundance and activity. A complex relationship between viral abundance and nutrient cycling was further indicated by lack of general correlations between virus abundance and concentrations of D-DFAA (except for D-Asp) and D-DCAA. This suggests that viral lysis of bacterial cells was not a primary contributor to the bulk D isomers.

Conclusions

The production and uptake of D isomers of amino acids in batch cultures with Roskilde Fjord bacteria and the occurrence of D amino acids in the fjord water together confirm that bacteria are essential elements in shaping both size and composition of organic matter pools in natural waters. The absence of the accumulation of D isomers in intact fjord water supports the hypothesis that dissolved peptidoglycan amino acids were recycled by bacteria, but it cannot be excluded that particulate peptidoglycan

components were more resistant to microbial attack and accumulated in the water. The existence of a peptidoglycan salvage pathway within microbial populations ensures that important nutrients are recycled and remain in the microbial nutrient pool. The presence of D amino acid isomers in dissolved and particulate detrital matter might be considered used as markers of bacterial processes and bacterial activity in future studies.

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