

## Bacterial release of dissolved organic matter during cell growth and decline: Molecular origin and composition

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### Abstract

Heterotrophic bacterial growth and the chemical composition of dissolved organic matter (DOM) produced by bacteria from freshwater and marine environments were monitored during experiments with artificial media containing glucose as the sole carbon source. Glucose was quickly consumed, and DOM was released during bacterial growth. Percentages of extracellular release of DOM from bacteria ranged from 14% to 31%, indicating that bacterial production and growth efficiency are underestimated when only cellular carbon is measured. Relatively high concentrations of D-alanine (D-Ala) were observed in DOM released during exponential growth, whereas the concentrations of muramic acid and other D-amino acid components of peptidoglycan were not detected or were in low concentration. The selective release of D-Ala occurred during cell growth and division when peptidoglycan is cleaved and newly synthesized subunits are incorporated into the cell wall via transpeptidation. Most of the D-Ala released during exponential growth was rapidly consumed. Following exponential growth, bacterial abundance decreased due to grazing and possibly viral lysis. The DOM remaining in the incubations after one or more months included a mixture of D-amino acids commonly found in peptidoglycan and the amino sugars glucosamine and galactosamine, which were highly resistant to decomposition. The percentage of D-amino acids was much higher in DOM than in cells due to the preferential release of D-amino acids and decomposition of L-amino acids. The final concentrations of dissolved organic carbon (DOC) ranged from 20 to 30  $\mu\text{mol L}^{-1}$  regardless of the initial concentration of glucose or the source of inoculum. The observed abundances of D-amino acids and amino sugars in DOM from diverse aquatic environments indicate a bacterial source and common decomposition processes.

Heterotrophic bacteria have long been recognized for their critical roles in the transformation and mineralization of organic matter in aquatic and terrestrial environments. It is estimated about half of the photosynthetic production in the ocean is processed by heterotrophic bacteria in the microbial loop (Ducklow 2000). Heterotrophic bacterial production is often 10–20% of primary production in aquatic environments (Cole et al. 1988; Ducklow 2000), but relatively few studies have considered heterotrophic bacteria as an important source of dissolved organic matter (DOM), the most abundant form of organic carbon in most aquatic ecosystems (Hedges 1992; Wetzel 1992). Given the global abundance and production of heterotrophic bacteria (Whitman et al. 1998), it is logical they would be a major source of DOM, but it is difficult to distinguish and quantify the biological origins of DOM in natural environments.

The biochemical composition of bacteria is largely the same as other organisms, but several biomolecules that are unique to bacteria can be used to trace their contributions to DOM. Specific bacterial membrane proteins, such as porin P (Tanoue 1995), and specific lipid components of membrane lipopolysaccharides (Wakeham et al. 2003) are unique to Gram negative bacteria and are widely distributed in the ocean. Gram negative and Gram positive

bacteria synthesize a unique cell wall biopolymer, peptidoglycan, which consists of glycan strands of a repeating disaccharide (*N*-acetyl-glucosamine and *N*-acetyl muramic acid) that are cross-linked by small peptides consisting of both L- and D-enantiomers of specific amino acids (Schleifer and Kandler 1972). Muramic acid (Benner and Kaiser 2003) and the D-enantiomers of amino acids in peptidoglycan (Lee and Bada 1977; McCarthy et al. 1998) have also been identified in DOM. These studies demonstrate the ubiquitous nature of bacterially derived DOM (bacterial DOM) in aquatic environments.

The mechanisms for the release of DOM from bacteria are poorly understood. Experiments by Ogawa et al. (2001) demonstrated that heterotrophic bacteria rapidly consume labile compounds (glucose or glutamate) and release DOM with a complex chemical composition. The rapid (<48 h) release of bacterial DOM in these experiments is consistent with a mechanism linked to cell division during exponential growth. Grazing of bacteria by protozoans (Strom et al. 1997) and viral lysis (Middelboe and Lyck 2002) could release DOM from bacteria, but most grazers were removed from the incubations by filtration, and both grazing activity and viral lysis would be expected to lag behind bacterial growth.

Phylogenetic studies using 16S rRNA (ribosomal ribonucleic acid) show that bacterial community structure varies significantly among aquatic environments (Pernthaler et al. 1998; Crump et al. 1999). There are several studies indicating that biodegradation by heterotrophic bacteria plays an important role in shaping the composition of DOM (Brophy and Carlson 1989; Stoderegger and Herndl 1998), but the role of bacterial community structure in

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shaping the chemical composition of DOM is unknown. In this study, incubation experiments were conducted to investigate the release and chemical composition of DOM in artificial culture media with natural bacterial assemblages collected from three aquatic ecosystems (lake, estuary, and coastal ocean). Glucose was the sole carbon source, and ammonium, nitrate, and phosphate were added as nitrogen and phosphorus sources. The release and removal of bacterial cell wall material were monitored using bulk C and N measurements as well as amino acid and amino sugar measurements. Mechanisms of bacterial DOM release are proposed during and after the exponential growth phase, and differences in the composition of DOM among the experiments and environments suggest that community structure and growth conditions influence DOM chemical composition.

## Materials and methods

*Study area and sample collection*—Water samples were collected from three ecosystems: (1) Lake Murray (LM), a freshwater lake located northwest of Columbia, South Carolina, (2) Winyah Bay (WB), an estuary near Georgetown, South Carolina, and (3) Folly Beach (FB), South Carolina, an ocean beach located southeast of Charleston, South Carolina. Water samples were transported to the laboratory in Columbia immediately following collection in acid-washed 10-liter carboys. They were filtered through a precombusted GF/F filter (at 450°C for 4 h) with low vacuum pressure (<25 kPa) to remove most eukaryotes. Subsamples were collected for enumeration of bacteria and grazers, and measurement of the concentrations of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), ammonium ( $\text{NH}_4^+$ ), nitrate and nitrite (N+N), and enantiomers of total dissolved amino acids (TDAA), and total dissolved amino sugars (TDAS). Paraformaldehyde was added to bacteria and grazer samples at a final concentration of 2%, and the samples were stored at 4°C until counting. The counting was made within 24 h. A total of 100  $\mu\text{L}$  of 1 mol  $\text{L}^{-1}$  HCl was added to each DOC and TDN sample (15 mL), and the sample was stored in the dark at 4°C.  $\text{NH}_4^+$  samples were measured within 24 h of sampling. N+N, TDAA, and TDAS samples were collected in acid-washed 60-mL HDPE bottles (Nalgen) and stored frozen until analysis.

*Incubation experiments*—The artificial media were prepared with water treated in a Milli-Q ultraviolet (UV) plus purification unit. NaCl, KCl,  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , and  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  were added to the media. NaCl,  $\text{Na}_2\text{SO}_4$ , and KCl were combusted (at 450°C for 4 h) to remove organic carbon before use. For LM and WB experiments, glucose (510  $\mu\text{mol C L}^{-1}$ ), ammonium sulfate (102  $\mu\text{mol N L}^{-1}$ ), and dibasic phosphate (10.2  $\mu\text{mol P L}^{-1}$ ) were added as C, N, and P nutrients. For the FB experiment, the medium contained 300  $\mu\text{mol C L}^{-1}$  as glucose, 30  $\mu\text{mol N L}^{-1}$  as sodium nitrate, and 6  $\mu\text{mol P L}^{-1}$  as dibasic phosphate. All media were sterilized using a microwave according to the procedure by Keller et al.

(1988). Once the media were cooled, the pH was adjusted to 8 with either 1 mol  $\text{L}^{-1}$  HCl or NaOH and transferred to acid-washed and combusted (450°C for 4 h) 2-liter glass flasks. Each treatment was run in duplicate. The GF/F filtered waters of LM, WB, and FB were inoculated into the media at a 1 : 50 dilution. Subsamples for TOC, TN, and molecular analyses were collected immediately following inoculation and mixing. After sample collection, the flasks were sealed with precombusted aluminum foil and incubated in the dark on a shaker table (130 revolutions per minute [rpm]) at room temperature (18–23°C).

On days 2, 5, 9, 24, 50, 93, and 154 for LM and WB experiments and days 1, 2, 3, 5, 8, 16, and 38 for FB experiments, subsamples for bacteria and grazers were collected directly from the water. The subsamples for DOC, TDN,  $\text{NH}_4^+$ , N+N, and molecular-level analyses were collected after filtration through a 0.22- $\mu\text{m}$  Durapore membrane filter (Millipore). All samples were stored as described previously. In addition, particulate total amino acids (PTAA) and particulate total amino sugars (PTAS) were collected from the FB incubation using GF/F filters. All filters were frozen until analysis.

*Sample analyses*—Abundances of bacteria and grazers were determined by epifluorescence microscopy using the DAPI (4'-6-diamidino-2-phenylidole) method (Porter and Feig 1980). Subsamples of bacteria were stained with DAPI, then filtered through 0.2- $\mu\text{m}$  black Millipore polycarbonate filters mounted on prewetted 0.45- $\mu\text{m}$  Nuclepore membrafil cellulosic filters. Subsamples for grazers were stained on 0.4- $\mu\text{m}$  black Millipore polycarbonate filters mounted on 0.8- $\mu\text{m}$  Nuclepore membrafil cellulosic filters. At least 300 bacteria per sample were counted, and 40 fields were enumerated for the abundance of grazers.

DOC and TDN samples were measured by high-temperature combustion using a Shimadzu TOC-V analyzer. All values were corrected using Milli-Q UV plus water as a blank (Benner and Strom 1993). Blank C and N values ranged from 3 to 7  $\mu\text{mol L}^{-1}$  and from 0 to 0.8  $\mu\text{mol L}^{-1}$ , respectively. Ammonium was measured following the modified method by Solorzano (1969). Nitrate and nitrite were measured by vanadium (III) reduction with chemiluminescence detection using an Antek 745 analyzer (Cox 1980).

D- and L-enantiomers of amino acids were determined using reverse-phase high-performance liquid chromatography (HPLC) and pre-column derivatization with *o*-phthalaldehyde (OPA) and *N*-isobutryl D- and L-cysteine as described by Kaiser and Benner (2005). Dissolved amino acids were subjected to vapor-phase hydrolysis in a microwave with 6 mol  $\text{L}^{-1}$  HCl at 150°C for 32.5 min. Particulate amino acids were hydrolyzed with 6 mol  $\text{L}^{-1}$  HCl at 110°C for 20 h in sealed ampoules. Ascorbic acid (0.12  $\mu\text{mol L}^{-1}$ ) was added to all samples before hydrolysis. Racemization of enantiomers during hydrolysis was corrected as described by Kaiser and Benner (2005). Free glucose concentrations were determined using a Dionex 500 high-performance anion-exchange chromatography (HPAEC) system with pulsed amperometric de-

tection (PAD) following the procedure described by Skoog and Benner (1999). Amino sugars were analyzed by HPAEC-PAD following the procedures described by Kaiser and Benner (2000). Samples were hydrolyzed with 3 mol L<sup>-1</sup> HCl at 100°C for 5 h. The amino sugar, muramic acid, was analyzed by reverse-phase HPLC and pre-column derivatization with OPA and *N*-isobutryl L-cysteine.

*Statistics and other calculations*—A *t*-test was used to compare two factors, and a one-way ANOVA (analysis of variance) *F*-test was used to compare multiple factors. The percentage of individual D-amino acid, such as Ala, was calculated as

$$\%D-Ala = \frac{[D-Ala]}{[(D-Ala) + (L-Ala)]} \times 100, \quad (1)$$

where [D-Ala] is the concentration of D-Ala, and [D-Ala + L-Ala] is sum of D- and L-Ala concentrations. Exponential decay coefficients (*k*) were calculated as

$$k = - \frac{\ln(A_{initial}/A_{final})}{(t_{initial} - t_{final})}, \quad (2)$$

where *A* is concentration, and *t* is time. The degradation index of organic matter using mol% amino acid composition was calculated by the following formula proposed by Dauwe et al. (1999):

$$DI = \sum_i \left[ \frac{\text{var}_i - \text{AVG var}_i}{\text{STDvar}_i} \right] \cdot \text{fac.coef.}_i, \quad (3)$$

where DI is the degradation index, var<sub>*i*</sub> is mol% of the individual amino acid, AVGvar<sub>*i*</sub> and STDvar<sub>*i*</sub> are the mean mol% and standard deviation of each amino acid, respectively, and fac.coef.<sub>*i*</sub> is the factor coefficient from table 1 in Dauwe et al. (1999).

## Results

*Chemical composition of DOM in Lake Murray (LM), Winyah Bay (WB), and Folly Beach (FB)*—Bulk DOC and TDN concentrations were highest in LM and lowest in FB samples (Table 1). Total dissolved amino acids (TDAA) and amino sugars (TDAS) followed a similar pattern. The concentration of muramic acid (Mur) was below the limit of detection in all systems. Carbon-normalized yields of TDAA and TDAS ranged from 1.3% to 1.8% and 0.4% to 0.7%, respectively. Dauwe degradation indices were low in all systems and ranged from -1.44 to -1.76. The ratio of glucosamine (GlcN) to galactosamine (GalN) was fairly constant among environments (1.6 to 2.0). The percentage of D-amino acids (%D-AA) of Asp, Glu, Ser, and Ala was lowest in LM and highest in FB. Overall, these data indicate many similarities in the composition of DOM from different environments with varying contributions of organic matter from vascular plants, soils, and plankton. The data also indicate that the DOM in all systems was diagenetically altered.

Table 1. Salinity, bacterial abundance, concentrations of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and total dissolved amino acid (TDAA), carbon-normalized yield of TDAA (%DOC), Dauwe degradation index value, percentage of D-amino acids (%D-Asp, %D-Glu, %D-Ser, and D-Ala), total dissolved amino sugar (TDAS) concentration, carbon-normalized yield of TDAS (%DOC), concentrations of galactosamine (GalN), glucosamine (GlcN), and muramic acid (Mur), and the ratio of GlcN to GalN (GlcN : GalN) in Lake Murray (LM), Winyah Bay (WB), and Folly Beach (FB).

	LM	WB	FB
Salinity	0	15	32
Bacteria (×10 <sup>5</sup> mL <sup>-1</sup> )	33.0	8.3	10.8
DOC (μmol L <sup>-1</sup> )	309	257	205
TDN (μmol L <sup>-1</sup> )	22.3	16.0	9.5
TDAA (nmol L <sup>-1</sup> )	1481	901	769
TDAA (%DOC)	1.8	1.3	1.4
Dauwe degradation index	-1.76	-1.44	-1.69
%D-Asp	10	21	46
%D-Glu	9	17	31
%D-Ser	4	11	31
%D-Ala	13	26	30
TDAS (nmol L <sup>-1</sup> )	341	187	150
TDAS (%DOC)	0.7	0.4	0.4
GalN (nmol L <sup>-1</sup> )	125	72	50
GlcN (nmol L <sup>-1</sup> )	216	115	101
Mur (nmol L <sup>-1</sup> )	bd	bd	bd
GlcN : GalN	1.7	1.6	2.0

bd, below detection.

*Bulk C and N concentrations and bacterial abundance in batch culture experiments*—Glucose was consumed within 2 d in WB and FB experiments and within 24 d in the LM experiment (Table 2). Concentrations of DOC produced by bacteria in LM, WB, and FB experiments were 56, 110, and 25 μmol L<sup>-1</sup>, respectively, after glucose was completely consumed (Fig. 1). Based on the initial DOC concentrations in the three ecosystems and the dilution ratio of 1 to 50, about 4 to 6 μmol L<sup>-1</sup> DOC and 0.2 to 0.4 μmol L<sup>-1</sup> TDN were derived from the inocula at the beginning of the experiments. At the end of the experiments, DOC concentrations ranged between 25 and 30 μmol L<sup>-1</sup>. TDN decreased during exponential growth in all experiments due to the decrease in NH<sub>4</sub><sup>+</sup> in the LM and WB experiments and nitrate in the FB experiment (Table 2). N+N concentrations were <1 μmol L<sup>-1</sup> in LM and WB experiments and ~4 μmol L<sup>-1</sup> after day 1 in the FB experiment. NH<sub>4</sub><sup>+</sup> was the dominant inorganic nitrogen species throughout bacterial growth and decline. Bacteria grew rapidly and reached peak abundance within 2 d in WB and FB experiments and within 9 d in the LM experiment (Table 2; Fig. 2). Following exponential growth, bacterial abundance declined in all experiments (Fig. 2). Initial abundance of bacterial grazers was below the detection limit (~500 mL<sup>-1</sup>) in all experiments. Grazer abundance increased to ~1,500 mL<sup>-1</sup> on day 5 in WB and FB experiments, and ~1,000 mL<sup>-1</sup> on day 24 in LM

Table 2. Bacterial abundance and concentrations of DOC, TDN, dissolved organic nitrogen (DON), ammonium ( $\text{NH}_4^+$ ), nitrate and nitrite (N+N), and glucose over the 154-d incubation of Lake Murray (LM) and Winyah Bay (WB) treatments and the 38-d incubation of Folly Beach (FB) treatment. DON was calculated from the subtraction of N+N and  $\text{NH}_4^+$  from TDN. Because day 93 samples for DOC and TDN from LM and WB were contaminated, they were not reported.

	Day							
	0	2	5	9	24	50	93	154
LM								
Bacteria ( $\times 10^6 \text{ mL}^{-1}$ )	0.1	0.2	0.6	7.4	4.8	6.0	5.1	2.9
DOC ( $\mu\text{mol L}^{-1}$ )	529	511	490	157	37	24	–	26
TDN ( $\mu\text{mol L}^{-1}$ )	104.0	103.4	102.6	86.7	81.9	84.5	–	96.9
DON ( $\mu\text{mol L}^{-1}$ )	0.3	1.7	2.3	11.3	6.1	7.8	–	6.1
$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	103.7	101.7	100.3	75.0	75.6	76.6	82.8	90.6
N+N ( $\mu\text{mol L}^{-1}$ )	bd	bd	0.1	0.2	0.1	0.1	0.1	0.3
Glucose ( $\mu\text{mol C L}^{-1}$ )	517	499	480	101	bd	bd	bd	bd
WB								
Bacteria ( $\times 10^6 \text{ mL}^{-1}$ )	0.1	28.2	22.9	5.6	5.7	7.4	6.4	2.1
DOC ( $\mu\text{mol L}^{-1}$ )	513	115	63	79	43	37	–	31
TDN ( $\mu\text{mol L}^{-1}$ )	105.2	39.1	48.7	59.0	68.1	75.2	–	92.3
DON ( $\mu\text{mol L}^{-1}$ )	bd	6.7	10.9	14.0	12.0	12.1	–	3.7
$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	104.6	32.0	37.6	44.5	55.5	62.4	75.8	87.9
N+N ( $\mu\text{mol L}^{-1}$ )	0.5	0.4	0.1	0.5	0.6	0.7	0.6	0.7
Glucose ( $\mu\text{mol C L}^{-1}$ )	514	bd	bd	bd	bd	bd	bd	Bd
FB								
	Day							
	0	1	2	3	5	8	16	38
Bacteria ( $\times 10^6 \text{ mL}^{-1}$ )	0.1	0.5	15.0	11.0	4.1	2.6	2.3	1.2
DOC ( $\mu\text{mol L}^{-1}$ )	310	288	25	22	24	31	28	25
TDN ( $\mu\text{mol L}^{-1}$ )	30.3	28.8	9.2	12.5	18.4	21.5	24.7	25.0
DON ( $\mu\text{mol L}^{-1}$ )	bd	bd	1.3	1.1	1.5	2.1	3.1	2.8
$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	bd	0.4	4.2	7.3	12.9	15.5	17.6	18.1
N+N ( $\mu\text{mol L}^{-1}$ )	30.3	28.4	3.8	4.0	4.0	3.9	4.0	4.1
Glucose ( $\mu\text{mol C L}^{-1}$ )	302	278	bd	bd	bd	bd	bd	bd

bd, below detection.

experiment (data not shown). The abundance of grazers was below detection by the end of the experiments. The abundance of viruses was not measured in these experiments.

*Dissolved components of peptidoglycan*—The concentrations of TDAA increased during exponential growth and decreased or remained constant after bacteria reached stationary phase (Figs. 1, 2). In contrast, the concentrations of TDAS increased throughout the experiments (Fig. 1). Degradation indices from amino acid compositions of DOM were around  $-0.58$ ,  $-0.73$ , and  $-2.78$  during exponential growth of bacteria in WB, FB and LM, respectively. These values changed very little throughout the experiments. In all three experiments, concentrations of D-Ala increased exponentially and mirrored increases in bacterial abundance (Fig. 2). Following exponential growth, D-Ala concentrations decreased sharply. Concentrations of D-glutamic acid (D-Glu) and D-aspartic acid (Asp) were relatively low, and, with the exception of D-Glu in the WB experiment, which followed a different pattern from that of D-Ala (Fig. 2). The %D-Ala was also elevated during cell growth relative to cell decline in all experiments, whereas the %D-Asp did not change between growth phases (Fig. 3). The %D-Glu was elevated during cell

growth in the WB experiment. The selective release of D-Ala during cell growth suggests a specific release mechanism(s).

The dynamics of three components of peptidoglycan, D-Ala, GlcN, and Mur, were surprisingly different in DOM from all experiments (Fig. 4). Mur concentrations were below the limit of detection in all experiments. D-Ala concentrations increased during exponential growth and then declined to fairly low concentrations ( $<10 \text{ nmol L}^{-1}$ ) throughout the experiments. GlcN concentrations increased during the experiments, reaching concentrations of  $55\text{--}180 \text{ nmol L}^{-1}$ . GalN, a common amino sugar in bacteria but not a component of peptidoglycan, increased in concentration during the experiments and followed a pattern similar to that of GlcN. The ratio of GlcN : GalN remained fairly constant (2–4) throughout the experiments. The similar dynamics of GlcN and GalN suggest a common biomolecular origin.

*Comparison of POM and DOM from the FB experiment*—In the FB experiment, amino acid (AA) and amino sugar (AS) concentrations were determined in bacterial cells (i.e., particulate organic matter [POM]) as well as in DOM. The Dauwe degradation indices were 1.23 in POM and  $-0.73$  in DOM, indicating very different compositions of amino

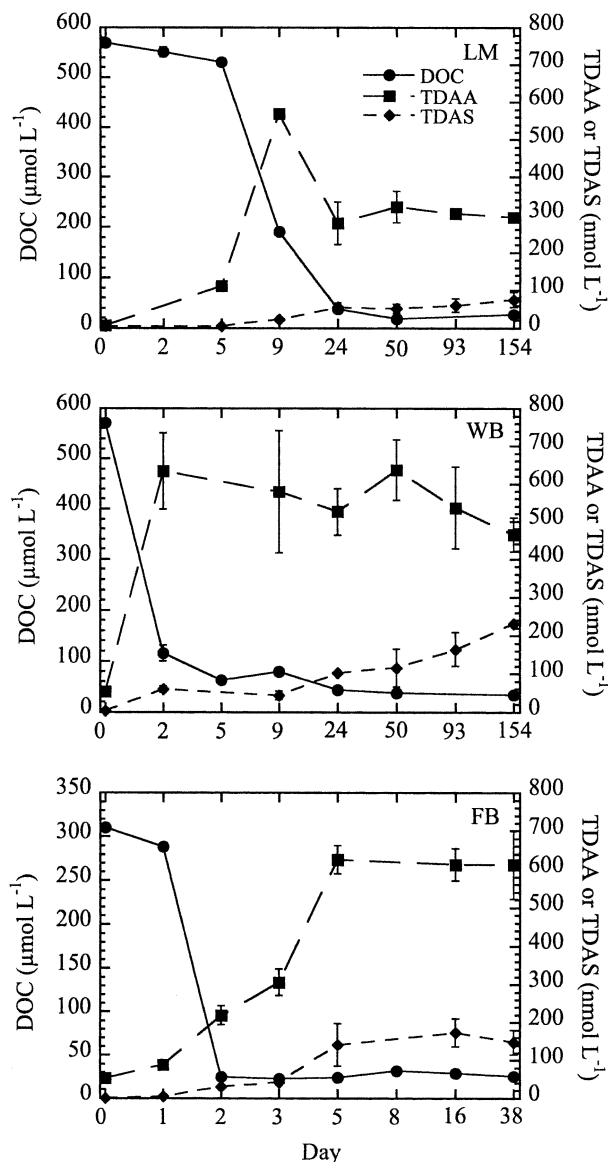


Fig. 1. Concentrations of dissolved organic carbon (DOC), total dissolved amino acids (TDAA), and total dissolved amino sugars (TDAS) ( $\pm$  mean error) during experiments with a bacterial inoculum from Lake Murray (LM), Winyah Bay (WB), and Folly Beach (FB).

acids between bacterial POM and DOM. The %D-AA was compared between POM and DOM during exponential growth (day 2) and during cell decline (days 16 and 38) (Fig. 5). In POM, the %D-AA from all experiments was  $<5\%$  during cell growth and decline, indicating that most bacteria were Gram negative. The %D-Ala and %D-Glu in Gram positive cells is typically  $>20\%$  and  $>15\%$ , respectively. In contrast, the %D-AA in the DOM was much higher and variable between cell growth and decline. During cell growth, the %D-AA in the DOM ranged from 11% to 35%, and during cell decline values ranged from 9% to 12%.

Total amino acids (TAA) and total amino sugars (TAS) showed varying dynamics during the FB experiment

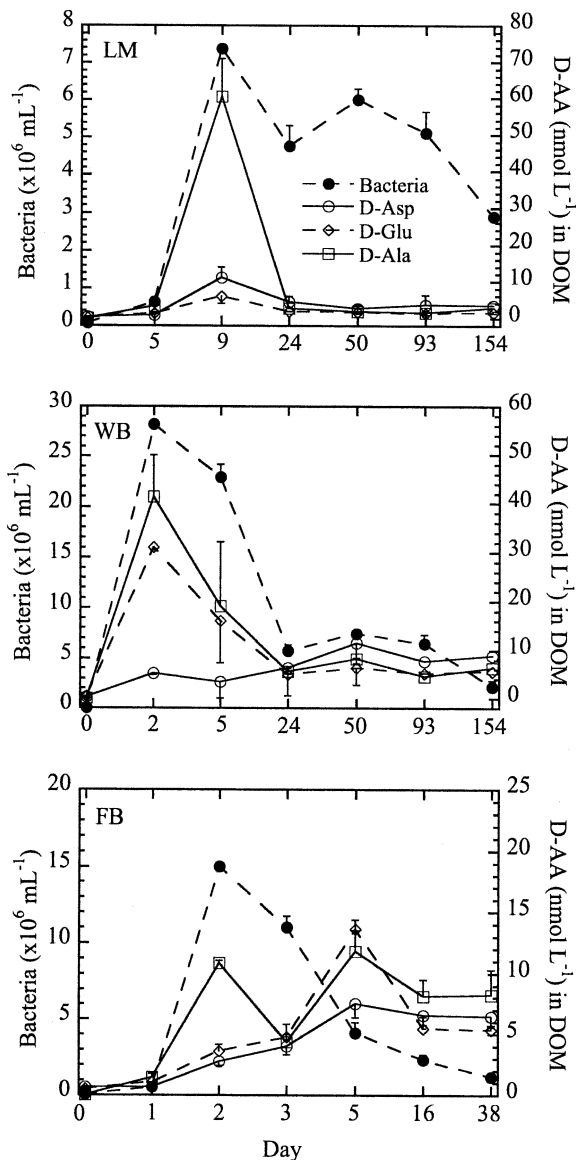


Fig. 2. Bacterial abundance ( $\pm$  mean error) and concentrations of the dissolved D-amino acids (D-AA) D-aspartic acid (D-Asp), D-glutamic acid (D-Glu), and D-alanine (D-Ala) ( $\pm$  mean error) during LM, WB, and FB experiments.

(Fig. 6A). TAA concentrations decreased following exponential growth (day 2), while TAS concentrations remained constant. These results indicate minimal decomposition of TAS during the experiment. Total particulate amino acid (TPAA) and total particulate amino sugar (TPAS) concentrations decreased after exponential growth (Fig. 6B), while TDAA and TDAS increased (Fig. 6C). Increases in TDAA and TDAS concentrations were derived from their particulate counterparts. Concentrations of TDAA were fairly constant after 5 d, indicating a balance between production and removal.

The concentrations of three components of peptidoglycan, D-Ala, GlcN, and Mur, increased in POM during exponential growth and then decreased (Fig. 7A), following a pattern similar to that of bacterial abundance (Fig. 2).

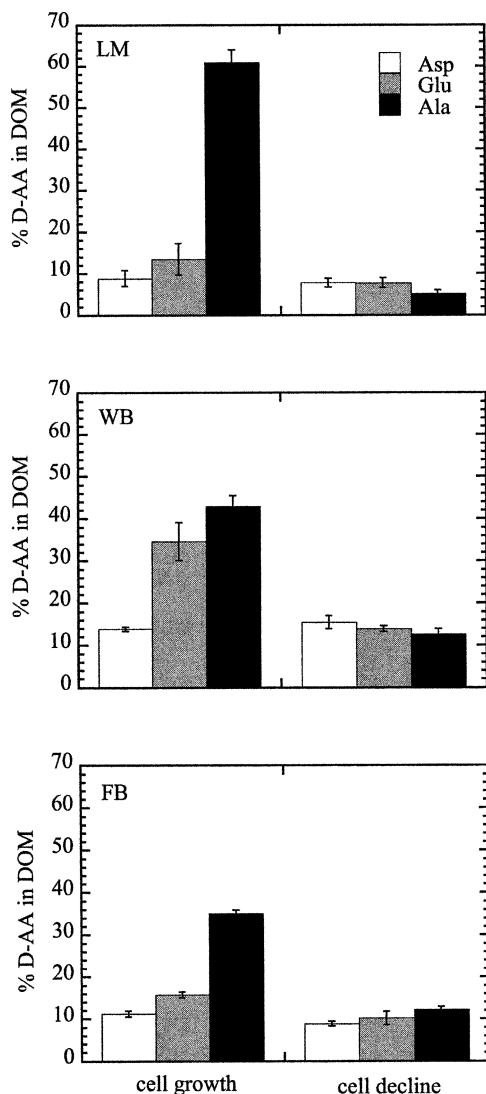


Fig. 3. Percentages of D-amino acids (%D-AA; D-Asp, D-Glu, D-Ala) during exponential growth phase and cell decline ( $\pm$ SE) in LM, WB, and FB experiments.

The ratios of GlcN : Mur as well as GlcN : D-Ala (not shown) increased over time, while the ratio of D-Ala : Mur remained fairly constant (1.2–1.7), indicating the close association between D-Ala and Mur in peptidoglycan. The high GlcN : Mur ratios (6–15) demonstrate that most GlcN was not associated with peptidoglycan. The amounts of Mur and D-Ala per cell were fairly constant, whereas GlcN and GalN increased over time (Fig. 7B).

Exponential decay coefficients ( $k$ ) ( $d^{-1}$ ) for POM (i.e., bacterial cells) and eight biochemical components of POM were calculated for losses between days 2 and 16 (Fig. 7C). Decay coefficients for bacteria, D-Asp, D-Glu, D-Ala, Mur, and TPAA ranged between 0.10 and 0.13  $d^{-1}$  with no statistical differences among them (one-way ANOVA  $F$ -test,  $p$ -value  $> 0.1$ ). Decay coefficients for GlcN, GalN, and TPAS were significantly lower than the six previously mentioned POM components (one-way ANOVA  $F$ -test,  $p$ -value  $< 0.05$ ). The decay coefficient for GalN (0.03  $d^{-1}$ )

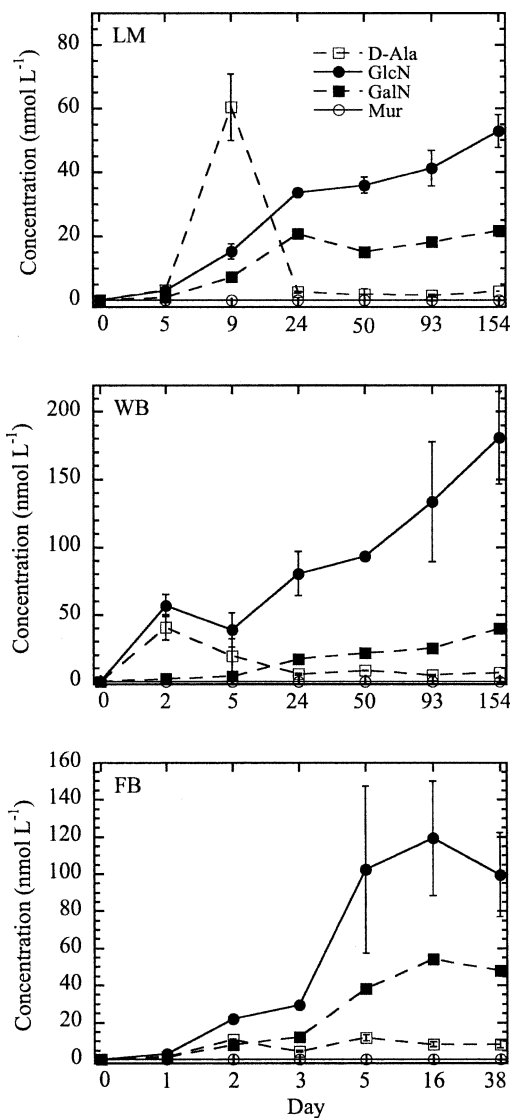


Fig. 4. Concentrations of D-Ala, glucosamine (GlcN), galactosamine (GalN), and muramic acid (Mur) ( $\pm$ mean error) during LM, WB, and FB experiments. Mur concentration was always below the limit of detection.

was also significantly lower than GlcN and TPAS (one-way ANOVA  $F$ -test,  $p$ -value  $< 0.05$ ), indicating its high resistance to degradation.

## Discussion

*Release of DOM during cell growth and division*—Bacteria quickly consumed glucose and directly released DOM during exponential growth in all experiments. The impacts of grazing and viral lysis on DOM release were considered to be minimal during the exponential growth phase of bacteria. The release of DOM during phytoplankton growth has been reported for over three decades (Nagata 2000 and references here in), and the percentage of extracellular release (PER) typically ranges between 2% and 10%. Since the amount of carbon bacteria assimilated was equal to the amount of glucose added to the

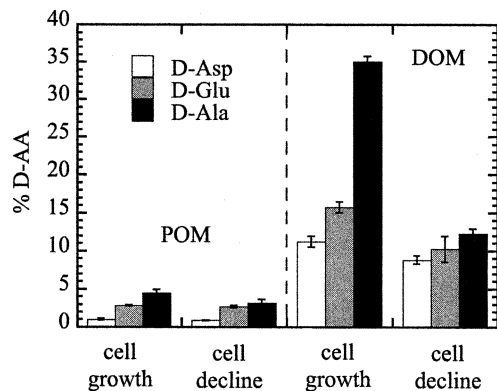


Fig. 5. Comparison of the %D-AA ( $\pm$ mean error) between DOM and POM during cell growth and cell decline in FB experiment.

incubations, we could estimate PER by heterotrophic bacteria during exponential growth if we estimated bacterial production. Bacterial growth efficiency (BGE) on glucose is typically between 0.4 and 0.6 (del Giorgio and Cole 1998), and was estimated to be 0.5 in the present study. Glucose concentrations were  $510 \mu\text{mol C L}^{-1}$  in the LM and WB experiments, and  $300 \mu\text{mol C L}^{-1}$  in the FB experiment, and the estimated bacterial particulate organic carbon (POC) production was  $255 \mu\text{mol C L}^{-1}$  in LM and WB, and  $150 \mu\text{mol C L}^{-1}$  in FB. The PER ( $= \text{DOC}_{\text{produced}} / [\text{DOC}_{\text{produced}} + \text{POC}_{\text{produced}}] \times 100$ ) by heterotrophic bacteria was calculated using the concentrations of DOC released by bacteria during the experiments. The DOC concentrations chosen for these calculations were measured at peak bacterial abundance (day 2 for WB and FB and day 9 for LM), and in the case of the LM experiment the bacterial DOC concentration was corrected for the remaining glucose. The calculated PERs ranged from 14% to 31% among experiments. The PERs for heterotrophic bacteria were somewhat higher than those commonly observed in phytoplankton. Heterotrophic bacteria are smaller than phytoplankton and therefore have higher surface area to volume (S/V) ratios. Their high S/V ratio is an advantage for nutrient uptake, but it could also contribute to higher extracellular release.

The extracellular release of DOM from heterotrophic bacteria affects carbon flow and energetics in aquatic environments. Bacterial production can be significantly underestimated if extracellular release is not included in production, just as phytoplankton production is underestimated when the release of DOM is not measured. None of the methods currently used to determine bacterial production accounts for the release of DOM, thus it appears bacterial production has been continually underestimated in aquatic environments. The release of DOC during growth by heterotrophic bacteria also indicates that bacterial growth efficiency (BGE) has been underestimated because BGE is calculated from bacterial production (BP) and bacterial respiration (BR) using the following formula:  $\text{BGE} = \text{BP} / (\text{BP} + \text{BR})$ .

*Mechanisms of release of D-Ala during exponential growth*—The selective release of D-Ala was observed

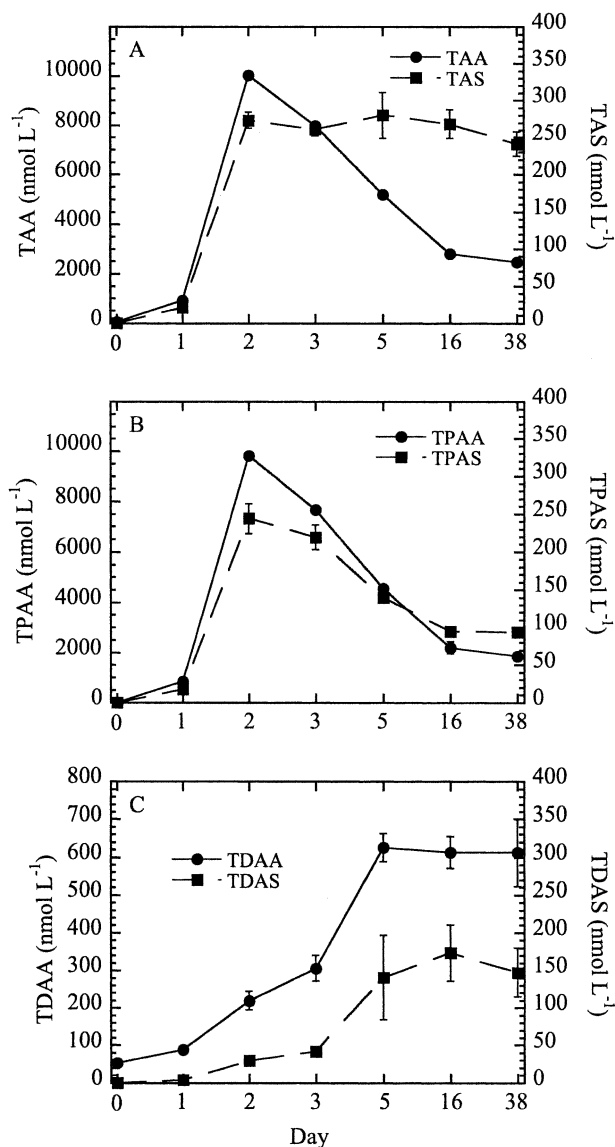


Fig. 6. (A) Concentrations of total amino acids (TAA) and total amino sugars (TAS), (B) total particulate amino acids (TPAA) and total particulate amino sugars (TPAS), and (C) total dissolved amino acids (TDAA) and total dissolved amino sugars (TDAS) in the FB experiment.

during exponential growth in all experiments. When bacteria grow, peptidoglycan bonds must be cleaved for cell division. During this process, cell integrity and viability are maintained, and losses of cellular materials are minimized. Goodell (1985), Goodell and Schwarz (1985), and van Heijenoort (2001) summarized the release mechanisms of D-Ala during cell division in *Escherichia coli*, and the release mechanisms are illustrated in Fig. 8. When a cell divides, various enzymes cleave peptidoglycan structures. Small peptides, especially dipeptides that mainly consist of D-Ala and diaminopimelic acid (DAPA), are released from the cell, while other peptides (tri-, tetra-, and pentapeptides) are recycled or lose D-Ala and are then recycled (Jacobs et al. 1997). Glycan components (Mur and GlcN)

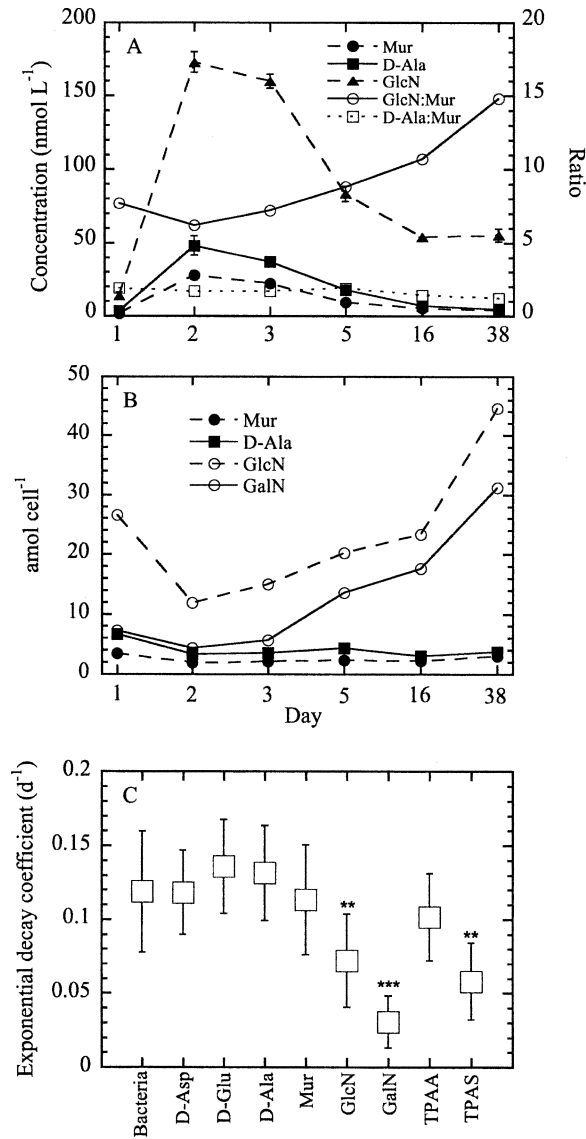


Fig. 7. Cellular compositions and decay coefficients for bacteria in the FB experiment. Concentrations of Mur, D-Ala, GlcN, and the ratios of GlcN : Mur and D-Ala : Mur (A), the amount (amol [10<sup>-18</sup> mol]) of Mur, D-Ala, GlcN, and GalN per cell (B), and exponential decay coefficients (d<sup>-1</sup>) of various components between day 2 and 16 (C). \*\* shows that coefficients are statistically different (ANOVA *F*-test, *p* < 0.05) from coefficients with no asterisk. \*\*\* shows that the coefficient is statistically different (ANOVA *F*-test, *t*-test, *p* < 0.05) from the others.

of peptidoglycan are efficiently recycled during cell division (Fig. 8A).

When cells grow and elongate, newly synthesized peptidoglycan subunits are incorporated into the cell wall via transpeptidation (Fig. 8B). Transpeptidation results in the release of free D-Ala from the peptide side chain (van Heijenoort 2001). These processes, the cleavage and synthesis of peptidoglycan, appear to be responsible for the observed increases in D-Ala concentrations during exponential growth of bacteria. In addition, these mechanisms of D-Ala release are consistent with the observation

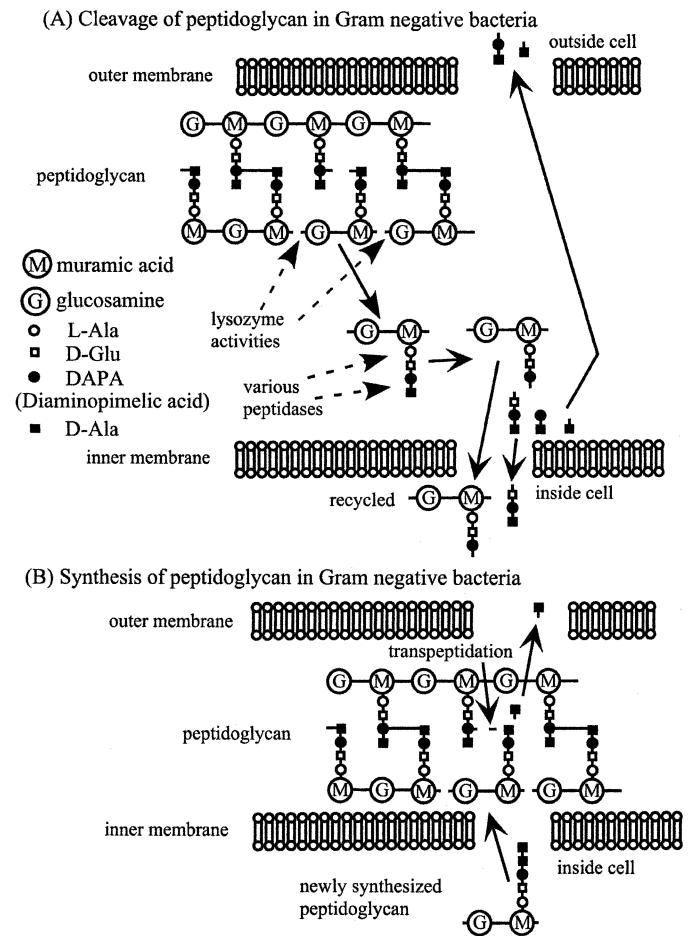


Fig. 8. Mechanisms of D-amino acid release from Gram negative bacteria during cell growth and division. (A) When peptidoglycan elongates, several enzymes, including lysozyme, cleave peptidoglycan. Other enzymes, peptidases, cleave peptide bonds. Small peptides, such as dipeptides and mono-peptides, are released from the cell while tripeptides and glycan components are recycled in the cell. (B) During cell growth and peptidoglycan synthesis, a D-Ala molecule is cleaved from the pentapeptide side chain during transpeptidation and is released from the cell.

that other D-AA and glycan components of peptidoglycan were much less abundant in DOM than D-Ala during exponential growth.

In the FB experiment, ~18% of the total D-Ala produced during exponential growth was released as DOM. During the growth of *E. coli*, 10–50% of D-Ala is released from cells depending on cellular and experimental conditions (Park 1995; Jacobs et al. 1997). Thus, the observed release of D-Ala during exponential growth of a natural assemblage of marine bacteria was well within the range observed in a pure culture of *E. coli*.

The release of D-Ala during bacterial cell division and the turnover rate of D-Ala in coastal seawater (FB) were calculated based on the following assumptions: (1) bacterial death during exponential growth can be ignored, and the number of cell divisions is the same as the increase in bacterial abundance, (2) D-Ala released from cells is not utilized during the period of exponential growth, and (3) all

bacteria in the experiment release the same amount of D-Ala per cell division. In the FB experiment, bacterial abundance on day 2 was  $1.46 \times 10^{10}$  cells  $L^{-1}$ , there was  $3.3 \times 10^{-18}$  mol D-Ala per cell, and  $7.4 \times 10^{-19}$  mol D-Ala was released per cell division. Assuming one bacterial cell division per day in coastal seawater at FB, the amount of D-Ala released during bacterial growth is  $0.8 \text{ nmol } L^{-1} \text{ d}^{-1}$  (D-Ala release per cell division [ $7.4 \times 10^{-19}$  mol D-Ala]  $\times$  bacterial abundance in FB [ $1.08 \times 10^9$  cells  $L^{-1}$ ]). The ambient D-Ala concentration in FB seawater was  $33.4 \text{ nmol } L^{-1}$ , so the estimated turnover rate of D-Ala was  $\sim 0.024 \text{ d}^{-1}$ . Nagata et al. (2003) investigated the degradation of a purified peptidoglycan in seawater experiments and estimated the peptide component was degraded within 10 d ( $\sim 0.10 \text{ d}^{-1}$ ). The lower turnover of peptidoglycan in this study is likely due to its chemical and physical associations with other cellular components.

*Release of DOM during cell decline*—Following exponential growth, bacterial abundance decreased due to grazing and possibly viral lysis. Grazing and viral lysis are likely to result in DOM that has different compositional signatures than DOM released directly during cell growth and division. Protozoan grazers engulf bacteria into food vacuoles and selectively digest and assimilate molecules, releasing unassimilated materials outside the cell. Only a few eukaryotic organisms are known to express enzymes that can hydrolyze D-AA (Asano and Lübbühren 2000), so it seems likely that protozoan grazers selectively assimilate L-AA from bacteria and release DOM that is enriched in D-AA. Thus, grazers could be responsible for increases in concentrations of D-AA in the WB and FB incubations following the decline in bacterial abundance. Perez et al. (2003) reported that the presence of grazers increased the gross release of D-Asp and stimulated the uptake by heterotrophic bacteria. The interaction between bacteria and grazers could be an important factor in controlling the concentrations of D-AA in aquatic ecosystems. In contrast, viral lysis should be nonselective in the release of both L- and D-AA from bacteria because lysis results in cell rupture without utilization of proteins and peptides.

*Decomposition of bacterial POM and DOM*—The concentrations of several components of peptidoglycan were followed in both the DOM and POM fractions. Our results indicate that Mur was below detection in DOM, whereas GlcN accumulated throughout the experiments. Concurrent with the observed increases in GlcN concentrations, we observed increases in concentrations of GalN, which is not a component of peptidoglycan. Most of the GlcN in DOM was resistant to decomposition. D-Ala concentrations increased rapidly in DOM during exponential growth of bacteria, but following exponential growth, D-Ala concentrations decreased to relatively low concentrations. Of the D-AA, only D-Glu in the WB experiment followed a similar pattern as D-Ala. Overall, these results clearly indicate that intact peptidoglycan subunits were not significant components of bacterial DOM in these experiments. Furthermore, analyses of Mur and D-AA in DOM from the lake, estuary,

and coastal ocean waters indicated intact peptidoglycan subunits were minor components of DOM. It is also interesting to note that the relatively high concentrations of GlcN in DOM were not derived from peptidoglycan. The close association of GlcN with GalN in bacterial DOM indicates a different macromolecular origin. The GlcN : GalN ratios observed in bacterial DOM are the same as those observed in natural organic matter from a variety of environments (Amelung 2003; Benner and Kaiser 2003), indicating the common occurrence of these amino sugars in DOM and their potential bacterial origin.

The amounts of Mur and D-Ala per cell (POM) were stable during the experiment, whereas the amounts of GlcN and GalN per cell steadily increased. The GlcN : Mur ratio was  $\sim 6$  in POM during exponential growth, indicating over 80% of the GlcN in bacteria was not associated with peptidoglycan. This observation is consistent with studies of pure cultures of bacteria (Benner and Kaiser 2003). It appears GlcN and GalN accumulated outside the cytoplasm, perhaps in membrane-bound components. They have been found in glycoproteins (Messner 1997), extracellular polymers (Giroldo and Vieira 2002), antibiotics (Kudo et al. 2005), and the O-antigen and lipid A components of lipopolysaccharides (Trefzer et al. 1999). These amino sugars had the lowest decay coefficients measured in POM during our experiments, indicating their resistance to decomposition. The resistance of GlcN to decomposition during incubation experiments was also found by Ogawa et al. (2001) and Jørgensen et al. (2003). The total GlcN concentration in their experiments increased over time. Decreases in the GlcN and GalN concentrations in POM were largely matched by increases in their concentrations in DOM.

*Comparison of the chemical composition of DOM among environments and incubations*—The compositions of DOM in the lake, estuary, and coastal environments displayed both similarities and differences. The C-normalized yields of AA (1.3–1.8%) and AS (0.4–0.7%), the Dauwe degradation indices ( $-1.8$  to  $-1.4$ ), and the ratios of GlcN : GalN (1.6–2.0) were similar among the three environments. For comparison, C-normalized yields of AA and AS ranged from 1% to 3% and 0.4% to 0.6% in the surface ocean, respectively (Benner 2002). The ratios of GlcN : GalN in surface seawater ranged from 1 to 2 (Benner and Kaiser 2003; Davis and Benner 2005). These results indicate that common diagenetic processes play an important role in shaping the chemical composition of DOM in different aquatic environments. There were large differences in the %D-AA among environments. The %D-Asp, %D-Glu, %D-Ser, and %D-Ala were highest ( $>30\%$ ) in the coastal seawater, lowest (4–13%) in the lake, and intermediate (11–26%) in the estuary. Similarly high %D-AA values have been reported in the ocean (Lee and Bada 1977; McCarthy et al. 1998; Dittmar et al. 2001). Previous analyses of %D-AA in river (Stepanuskas et al. 2000) and brackish waters (Jørgensen et al. 1999) are similar to estuarine values presented herein. The %D-AA in DOM could be influenced by microbial community structure as well as rates and mechanisms of D-AA release and

utilization. Additional studies are needed to further explore the observed differences in %D-AA among freshwater and marine environments.

The chemical compositions of DOM in the incubation experiments also showed some similarities with the compositions of DOM in the aquatic environments from which the inocula were obtained. However, the major differences observed in %D-AA among environments were not apparent among incubation experiments. Instead, relatively minor differences in the %D-AA of Asp, Glu, and Ala were observed among the experiments. It is likely that the use of glucose as the sole C source in the experiments resulted in bacterial communities that were different from those in the environments from which they were collected. The similarities in %D-AA in DOM among experiments could simply reflect the role of substrate in shaping bacterial community structure and the composition of DOM in the incubations. Despite these shortcomings, experimental approaches, such as those described herein, have been critical for demonstrating the roles of bacteria in DOM production and utilization. It is clear that heterotrophic bacteria are an important source of DOM in natural aquatic environments. The chemical signature of bacteria is evident in DOM from all environments examined, but there is much yet to understand about factors responsible for shaping the composition of DOM and regulating its bioavailability and reactivity.

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