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Fate of peptidoglycan in an intertidal sediment: An in situ ^{13}C-labeling study

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Abstract

We investigated the fate of peptidoglycan, a bacterial cell wall component, in sediment by ^{13}C-labeling the bacterial community of an intertidal mudflat and subsequently tracing the fate of ^{13}C in D-alanine (D-Ala, a bacterial biomarker specific for peptidoglycan), bacteria-specific phospholipid-derived fatty acids (PLFAs, specific for cell membranes of living bacteria), and total hydrolysable amino acids (THAAs, representing bulk proteinaceous material) over a 4.5-month period in situ. Results showed a relatively slow loss of ^{13}C from D-Ala (half lives of 20–67 d) compared with that from bacteria-specific PLFAs (half lives of 13–33 d) during the sampling period and a relatively strong retention of ^{13}C in D-Ala compared with the other compounds after 4.5 months. This provides direct in situ evidence for relative accumulation of peptidoglycan during reworking and degradation of bacterial biomass in sediments. Degradation also resulted in compositional changes of the [^{13}C]THAA pool, including increased relative abundances of glycine, serine, and proline.

Bacteria play a central role in ecological and biogeochemical processes in marine sediments. Not only do bacteria dominate degradation of organic matter, but bacterial biomass itself is also an important active pool of organic carbon (OC) in the sediment. Bacterial numbers in sediments are relatively invariant (Schmidt et al. 1998) because bacterial production is balanced by loss of bacterial biomass by processes such as cell death (e.g., viral lysis) and consumption of bacteria by bacterial grazers. Bacterial cell death produces bacterial remnants consisting of a variety of components that are prone to degradation. Some of these components will be readily degraded, whereas others are more resistant to degradation. One of the more refractory components is peptidoglycan, a unique constituent of bacterial cell walls. Laboratory studies on the degradation of peptidoglycan in bacterial cultures (Jørgensen et al. 2003) and seawater samples (Nagata et al. 2003) showed that peptidoglycan is degraded slower than proteins and can be characterized as semilabile. Peptidoglycan contains D–amino acids (D-AAs), which are specific for peptidoglycan. The abundance of these D-AAs in aquatic systems is usually reported relative to the abundance of their respective L-stereoisomers as D/L-AA ratios, where L-AAs are common protein amino acids occurring in all organisms. Studies on the abundance of D-AAs in seawater dissolved organic matter (DOM) revealed high D/L-AA ratios (McCarthy et al. 1998) and increasing D/L-AA ratios with increasing degradation (Amon et al. 2001; Dittmar et al. 2001), suggesting a relative accumulation of peptidoglycan. Similarly, studies on the abundance of D-AAs in marine sediments showed increasing D/L-AA ratios with increasing depth, which has also been attributed to selective preservation of peptidoglycan during organic matter degradation (Pedersen et al. 2001; Grutters et al. 2002). However, these D/L-AA ratios typically reflect the net result of reworking and degradation of organic matter over long periods of time (10^1–10^4 yr) and do not provide direct information on production and degradability of peptidoglycan. Conversely, laboratory studies of the degradation of peptidoglycan in vitro allow direct assessment of degradation rates, but resulting rates cannot directly be translated to degradation in the field because in situ degradation is dependent on various additional processes such as interactions with other compounds, particles, or both (Borch and Kirchman 1999; Arnarson and Keil 2005). Moreover, retention of bacterial biomass and remnants, including peptidoglycan, in the field is not only determined by degradation but also by other processes such as grazing and resuspension.

This study aims to clarify the fate of bacterial carbon, with particular emphasis on peptidoglycan, in an intertidal sediment. To do this, we linked the two existing lines of research (degradation of peptidoglycan in vitro vs. analysis of D-AA concentrations and D/L-AA ratios in situ) by in situ ^{13}C-labeling of the bacterial community of an intertidal sediment and subsequent analysis of the fate of the ^{13}C-labeled bacterial biomass during a 4.5-month sampling period. Comparison of the fate of ^{13}C in the bacterial biomarker D-alanine (D-Ala, representing peptidoglycan) with that of ^{13}C in bacteria-specific phospholipid-derived fatty acids (PLFAs, representing living bacteria) and ^{13}C in total hydrolysable amino acids (THAAs, representing total proteinaceous material) allowed us to investigate the fate of peptidoglycan compared with other components from ambient bacteria under in situ conditions.

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Materials and methods

Experimental setup—The material presented in this paper is part of a larger ^{13}C-labeling study (Van Oevelen et al. 2006). In May 2003, two 0.25-m^2 sediment plots were selected at a silty part of the Molenplaat intertidal mudflat in the turbid, nutrient-rich, and heterotrophic Scheldt estuary (The Netherlands). Plots were confined by steel frames (8 cm deep), and the upper 10 cm of the sediment was injected with [^{13}C]glucose (one injection per 6.25 cm^2, with ~0.4 mL of a ^{13}C-glucose solution [24 mmol L^{-1}] per injection) at days 0, 2, 3, and 4, resulting in a ^{13}C flux of 15.3 mmol ^{13}C m^{-2} per injection event. During the label addition period and in the days, weeks, and months after label addition (up to 136 d), plots were sampled regularly by taking sediment cores (50 mm inner diameter). Cores were sliced into three layers (0–2 cm, 2–5 cm, and 5–10 cm) that were analyzed for total C and ^{13}C in the bulk sediment, pore-water dissolved inorganic carbon (DIC), PLFAs, and benthic fauna. Further details on the experimental setup and analytical procedures can be found in Van Oevelen et al. (2006). In addition to these analyses, a selection of sediment samples from the 0–2-cm layer of plot 2 and the 2–5-cm layer of plots 1 and 2 were analyzed for ^{13}C in hydrolysable amino acids (HAAs), including bacterial biomarker D-Ala. Because this paper is largely based on the [^{13}C]HAA data, it only deals with the layers that were analyzed for ^{13}C in HAAs.

PLFA analyses—Lipids were extracted from 3 g of dry sediment in chloroform–methanol–water by a modified Bligh and Dyer method and fractionated on silica gel into different classes by polarity. The most polar fraction containing the PLFAs was derivatized by mild methanolysis yielding fatty acid methyl esters (FAMEs) that were analyzed by gas chromatography–combustion–isotope ratio mass spectrometry (GC-c-IRMS; *see* Middelburg et al. [2000] and Boschker [2004] for details). Although PLFA analyses included a wide range of PLFAs, this paper primarily deals with results for bacteria-specific PLFAs i14:0, i15:0, a15:0, i16:0 and 18:1ω7c.

HAA analyses—^{13}C in HAAs, including D-Ala, was analyzed by GC-c-IRMS. Sample preparation and analyses were according to the protocol presented in Veuger et al. (2005). Briefly, samples (1 g) of freeze-dried sediment were washed with HCl (2 mol L^{-1}) and Milli-Q water (removing dissolvable HAAs), followed by hydrolysis in HCl (6 mol L^{-1}) at 110°C for 20 h. After purification by cation exchange chromatography, amino acids were derivatized with isopropanol (IP) and pentafluoropropionic anhydride (PFA), and samples were further purified by solvent extraction. Derivatized D- and L-amino acids were separated by gas chromatography in a Chirasil L-Val column. Concentrations were calculated from separate analyses by GC–flame ionization detection (FID), directly from GC-c-IRMS analyses, or both. A selection of samples was also analyzed with a quadrupole mass spectrometer (GC-MS) to verify peak identity and purity.

^{13}C abundance was measured by GC-c-IRMS and expressed as $\delta^{13}C : \delta^{13}C\ (‰) = [(R_{sample}/R_{VPDB}) - 1] \times 1{,}000$, where $R = {}^{13}C/{}^{12}C$ and $R_{VPDB} = 0.0112372$ (VPDB = Vienna Pee Dee Belemnite). During derivatization, extra (unlabeled) C atoms are added to the original AAs, which change their $\delta^{13}C$ (Silfer et al. 1991; Pelz et al. 1998). It is possible to correct for the effect of the added C (Silfer et al. 1991; Pelz et al. 1998) with the mass balance equation $\delta^{13}C_{AA} = [\delta^{13}C_{DAA} \times (C_{AA} + C_{IP} + C_{PFA})] - [\delta^{13}C_{IP+PFA} \times (C_{IP} + C_{PFA})]/C_{AA}$, where C_{AA} is the number of C atoms in the original amino acid, DAA is the derivatized amino acid, C_{IP} is the number of C atoms added by esterification with IP, and C_{PFA} is the number of C atoms added by acylation with PFA. However, the change in $\delta^{13}C$ during derivatization is also influenced by additional kinetic fractionation during acylation (addition of C from PFA). The effect of this fractionation depends on the %C derived from PFA (i.e., the number of added C atoms from PFA relative to the number of C atoms in the original AA). Because this effect is not included in the mass balance equation, it requires empirical correction. This was done by measuring the $\delta^{13}C$ of three amino acid standards (D-Ala, D-glutamic acid [D-Glu], and D-serine [D-Ser]) before and after derivatization on an elemental analyzer coupled to an IRMS (EA-IRMS), which allowed us to determine empirical $\delta^{13}C_{IP+PFA}$ values for these three AAs. These values showed a strong linear correlation with the percent C from PFA, which is similar to results by Silfer et al. (1991). Subsequently, this correlation was used to determine empirical $\delta^{13}C_{IP+PFA}$ values for the other amino acids, which were then used to calculate their original $\delta^{13}C$ values with the mass balance equation. Empirical $\delta^{13}C_{IP+PFA}$ values ranged between –52‰ and –45‰, which is lighter than those in Silfer et al. (1991), for example, because we used PFA for acylation, which contains more C than the trifluoroacetic anhydride (TFA) used by Silfer et al. (1991) and because the used PFA was very depleted in ^{13}C ($\delta^{13}C$, –55‰, measured by EA-IRMS).

Data treatment—$\delta^{13}C$ values for the different ^{13}C pools were calculated by the formula presented above for the HAAs. Subsequently, $\delta^{13}C$ values were used to calculate $\Delta\delta^{13}C$ as $\Delta\delta^{13}C_{sample}\ (‰) = \delta^{13}C_{sample} - \delta^{13}C_{control\ sample\ (unlabeled)}$ and the atom percentage (at%) of ^{13}C as $at\%{}^{13}C = \{100 \times R_{VPDB} \times [(\delta^{13}C_{sample}/1{,}000) + 1]\}/\{1 + R_{VPDB} \times [(\delta^{13}C_{sample}/1{,}000) + 1]\}$, which was used to calculate excess ^{13}C (absolute amount of incorporated ^{13}C) as excess $^{13}C = [(at\%{}^{13}C_{sample} - at\%{}^{13}C_{control})/100] \times AA - C_{sample}$ concentration. Concentrations of carbon and excess ^{13}C for the different OC pools are all expressed in moles C or ^{13}C per gram dry sediment.

D-Ala data were corrected for the formation of D-Ala from L-Ala by racemization during acid hydrolysis (Kaiser and Benner 2005). Measured concentrations and excess ^{13}C values for D-Ala were corrected by subtracting 1.7% from concentrations and excess ^{13}C for L-Ala, where 1.7% is based on the 1.5–2% range reported for liquid phase hydrolysis of proteins and microbial biomass (Kaiser and Benner 2005).

Abundance of ^{13}C in D-Ala is also presented relative to that of ^{13}C in L-Ala as excess ^{13}C D/L-Ala ratios that were calculated as excess ^{13}C in D-Ala / excess ^{13}C in L-Ala using

the measured excess ^{13}C in D-Ala (i.e., not corrected for hydrolysis induced racemization). Instead, the racemization background (0.015–0.02) is indicated graphically. D/L-Ala ratios for concentrations were calculated the same way (i.e., also not corrected for racemization).

Loss rate constants for loss of ^{13}C from the different compounds after the [^{13}C]glucose injection period were determined by fitting the data with an exponential model by minimizing the sum of squared model data deviations. Standard errors of the model parameters were estimated with the non–linear regression method in the free software package R (The R Foundation for Statistical Computing).

Results

Sediment organic carbon pools—Total OC in the sediment was ~400 ± 30 μmol C g^{-1} for the 0–2-cm layer and ~600 ± 100 μmol C g^{-1} for the 2–5-cm layers. A considerable fraction of total OC consisted of HAA-C with THAA-C concentrations of 94 ± 33 μmol C g^{-1} for the 0–2-cm layer and 54 ± 57 μmol C g^{-1} for the 2–5-cm layers. D-Ala was a stable fraction of the THAA pool with concentrations (corrected for racemization) of 0.35 ± 0.15 μmol C g^{-1} for the 0–2-cm layer and 0.19 ± 0.23 μmol C g^{-1} for the 2–5-cm layers and D/L-Ala ratios of 0.05–0.07 for all samples (not corrected for racemization). Bacterial biomass in the sediment plots was derived from concentrations of bacteria-specific PLFAs i14:0, i15:0, a15:0, i16:0, and 18:1ω7c. Summed concentration of these five bacteria-specific PLFAs were 292 ± 172 nmol C g^{-1} for the 0–2-cm layer and 77 ± 84 nmol C g^{-1} for the 2–5-cm layers. Assuming bacteria-specific PLFAs of ~28% of total bacterial PLFAs and total bacterial PLFA-C of ~6% of total bacterial C (Middelburg et al. 2000) yielded total bacterial C estimates of 27 ± 16 μmol C g^{-1} for the 0–2-cm layer and ~7 ± 7 μmol-C g^{-1} for the 2–5-cm layers. Concentrations of bacteria-specific PLFAs as well as THAAs and D-Ala were rather variable (data not shown), most likely reflecting spatial variability in (microbial) biomass. In addition to bacteria, fauna were an important pool of OC. Benthic macrofaunal biomass (dominated by the bivalve *Macoma balthica* and the polychaetes *Heteromastus filiformis* and *Pygospio elegans*) in the upper 10 cm was 1,624 mmol C m^{-2} (13 μmol C g^{-1} dry sediment), whereas meiofaunal biomass (dominated by nematodes and foraminifera) was smaller at 188 mmol C m^{-2} (1.5 μmol C g^{-1} dry sediment) (*see* Van Oevelen et al. 2006).

^{13}C in bacteria-specific PLFAs—Bacteria-specific PLFAs showed clear ^{13}C enrichment following the injection of [^{13}C]glucose with $\delta^{13}C$ values (up to 700‰) well above natural abundance $\delta^{13}C$ (–21‰ ± 2‰). Trends in excess ^{13}C values for the five individual bacteria-specific PLFAs were very similar, and data are therefore presented as summed excess ^{13}C in the five bacteria-specific PLFAs (Fig. 1). Although 18 : 1ω7c can also be present in certain algae (Moodley et al. 2000), ^{13}C incorporation dynamics for 18 : 1ω7c were very similar to that for the other bacteria-specific PLFAs and different from that for diatom

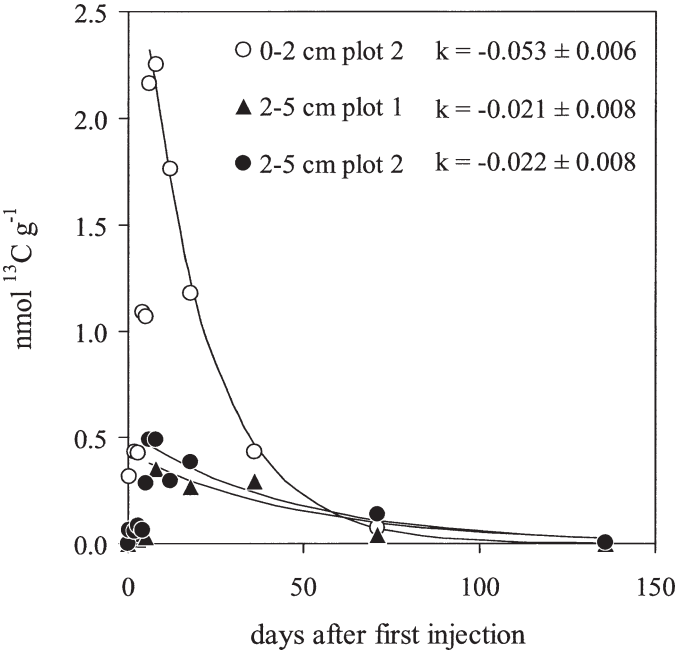

Fig. 1. Excess ^{13}C in bacteria-specific PLFAs (summed excess ^{13}C in i14:0, i15:0, a15:0, i16:0, and 18:1ω7c). Lines are the exponential fits for data between day 6 and day 136 (after label injection period). *k*-Values are loss rate constants (±SE).

PLFA 20 : 5ω3 (data not shown), indicating that ^{13}C-labeled 18 : 1ω7c was bacterial. Excess ^{13}C in bacteria-specific PLFAs was highest on days 6 and 8 (directly after the [^{13}C]glucose injection period), with highest values for the 0–2-cm layer. Average summed excess ^{13}C in the five bacteria-specific PLFAs for the period between days 6 and 18 (representing freshly labeled bacteria) was used to estimate excess ^{13}C incorporated in total bacterial biomass. Using the same conversion factors as for estimates of total bacterial biomass (see previous paragraph) yielded estimates of 107 nmol ^{13}C g^{-1} for the 0–2-cm layer and 18–23 nmol ^{13}C g^{-1} for the 2–5-cm layers.

After the labeling period, excess ^{13}C in the bacteria-specific PLFAs decreased exponentially with a rate constant of 0.053 (5.3% d^{-1}) for the 0–2-cm layer and 0.021–0.022 for the 2–5-cm layers. Corresponding half lives, (ln 2)/*k*, were 13 d and 31–33 d, respectively. At the end of the 136-d sampling period, ^{13}C enrichment of the bacteria-specific PLFAs had decreased to near natural abundance ($\Delta\delta^{13}C < 10‰$). Corresponding excess ^{13}C values were 0.1% of the original excess ^{13}C in bacteria-specific PLFAs in the period directly after [^{13}C]glucose injection (days 6–18) for the 0–2-cm layer and 0.5–2% for the 2–5-cm layers.

In addition to the bacteria-specific PLFAs, ^{13}C enrichment of diatom marker 20 : 5ω3 and fauna marker 20 : 4ω6 were examined to verify that [^{13}C]glucose labeling specifically tagged bacteria. This was confirmed by $\Delta\delta^{13}C$ values for both these markers that were an order magnitude lower than those for the bacteria-specific PLFAs (data not shown).

^{13}C in THAAs—HAAs were enriched in ^{13}C well above natural abundance (corrected natural abundance $\delta^{13}C$

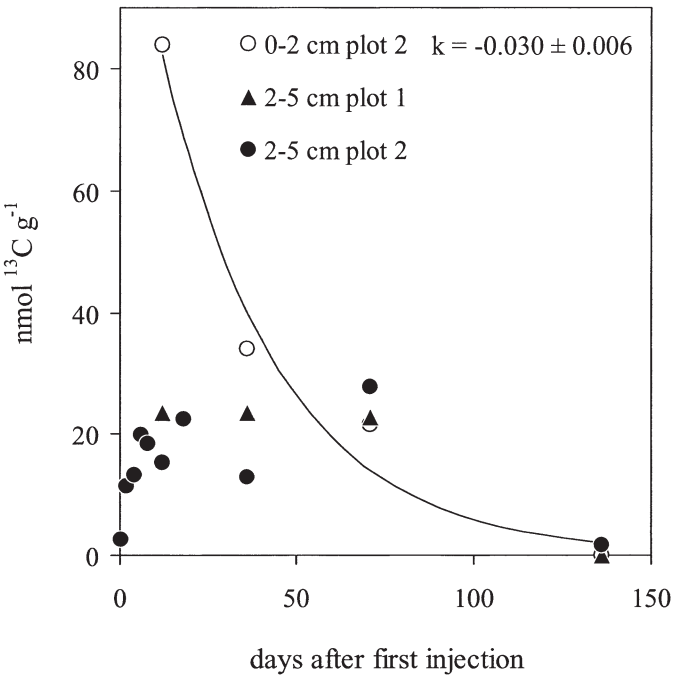

Fig. 2. Excess ^{13}C in THAAs (summed excess ^{13}C in individual HAAs). Line is the exponential fit for data between day 6 and day 136 (after label injection period) for 0–2-cm layer of plot 2. *k*-Value is loss rate constant (±SE).

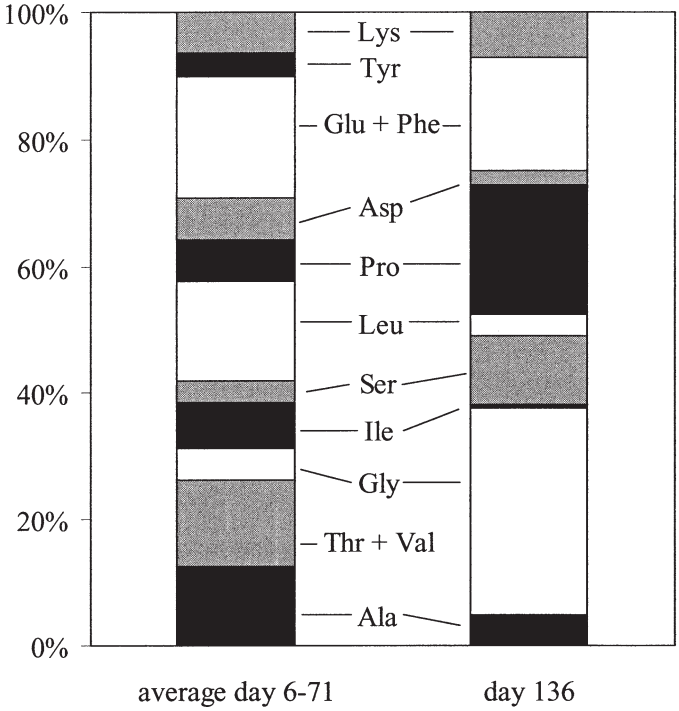

Fig. 3. Composition of [^{13}C]THAA pool (relative contributions of excess ^{13}C in individual HAAs to summed excess ^{13}C in THAAs) for the period between day 6 and day 71 versus day 136. All amino acids are L-AAs. Glu+Phe and Thr+Val are combined because their GC-c-IRMS peaks were not sufficiently separated.

values for individual HAAs ranged from –8‰ to –38‰) with corresponding $\Delta\delta^{13}$C values up to 100–150‰. Trends for excess ^{13}C in the different individual HAAs were similar, except for D-Ala, which is presented separately. Therefore, we only present excess ^{13}C in THAAs (i.e., summed excess ^{13}C in individual HAAs; Fig. 2). Average excess ^{13}C in THAAs in the period between day 6 and day 18 was used to estimate excess ^{13}C incorporated in total bacterial biomass assuming that THAA-C makes up ~50% of total bacterial C (Cowie and Hedges 1992; Madigan et al. 2000). Resulting estimates of ^{13}C incorporation into total bacterial biomass were 168 nmol ^{13}C g^{-1} for the 0–2-cm layer and 38–48 nmol ^{13}C g^{-1} for the 2–5-cm layers.

After the [^{13}C]glucose injection period, excess ^{13}C in THAAs in the 0–2-cm layer decreased exponentially with a rate constant of 0.030 (half-life of 23 d) while excess ^{13}C in THAAs in the 2–5-cm layers remained at a stable level up to day 71 and showed a strong decrease between day 71 and day 136 (Fig. 2). At day 136, excess ^{13}C in THAAs was below the limit of detection for two of the three layers. Only the 2–5-cm layer of plot 2 still contained traces of excess ^{13}C in all HAAs (totaling 1.7 nmol ^{13}C g^{-1}), which corresponded to 9% of the original excess ^{13}C in THAAs in the period directly after [^{13}C]glucose injection (days 6–18). The relative composition of the [^{13}C]THAA pool in this layer after 136 d was clearly different from its composition during the first 71 d of the experiment, with increased relative abundance of glycine (Gly), Ser, and proline (Pro); decreased relative abundance of tyrosine (Tyr), threonine + valine (Thr+Val), leucine (Leu), isoleucine (Ile), L-Ala, and aspartic acid (Asp); and similar abundance of lysine (Lys) and glutamic acid + phenylalanine (Glu+Phe) (Fig. 3).

^{13}C in D-Ala—Timing of ^{13}C incorporation into D-Ala during the label injection period was very similar to that for the other HAAs and the bacteria-specific PLFAs with $\Delta\delta^{13}$C values within the range of values for the other HAAs (up to 140‰ with natural abundance δ^{13}C of –10‰ ± 7‰). Excess ^{13}C D/L-Ala ratios (not corrected for racemization) directly after the [^{13}C]glucose injection period were 0.05–0.07 for the 2–5-cm layers and 0.09 for the 0–2-cm layer (Fig. 4). As for the bacteria-specific PLFAs and THAAs, excess ^{13}C in D-Ala for the period between days 6 and 18 (corrected for racemization) was used to estimate excess ^{13}C incorporated in total bacterial biomass assuming (1) a bacterial D/L-Ala ratio of 0.05 (0.07 minus a racemization background of ~0.02), (2) Ala at ~10% of the THAA pool (Cowie and Hedges 1992; Keil et al. 2000), and (3) THAA-C at ~50% of total bacterial C (*see previous section*). Resulting estimates were 324 nmol ^{13}C g^{-1} for the 0–2-cm layer and 40–80 nmol ^{13}C g^{-1} for the 2–5-cm layers.

During the weeks and months after the [^{13}C]glucose injection period, excess ^{13}C in D-Ala decreased exponentially with a rate constant of 0.035 (half-life of 20 d) for the 0–2-cm layer and 0.010–0.012 (half-life of 58–67 d) for the 2–5-cm layers (Fig. 5). This more rapid decrease of excess ^{13}C in D-Ala relative to that of excess ^{13}C in other HAAs,

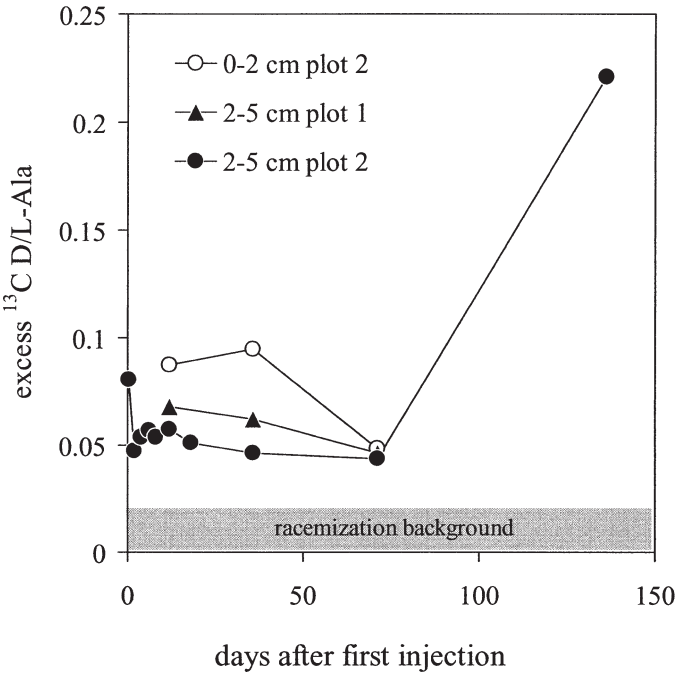

Fig. 4. Excess ^{13}C D/L-Ala ratios (excess ^{13}C in D-Ala/excess ^{13}C in L-Ala) uncorrected for racemization. Racemization background (0.015–0.02) is indicated by the gray box.

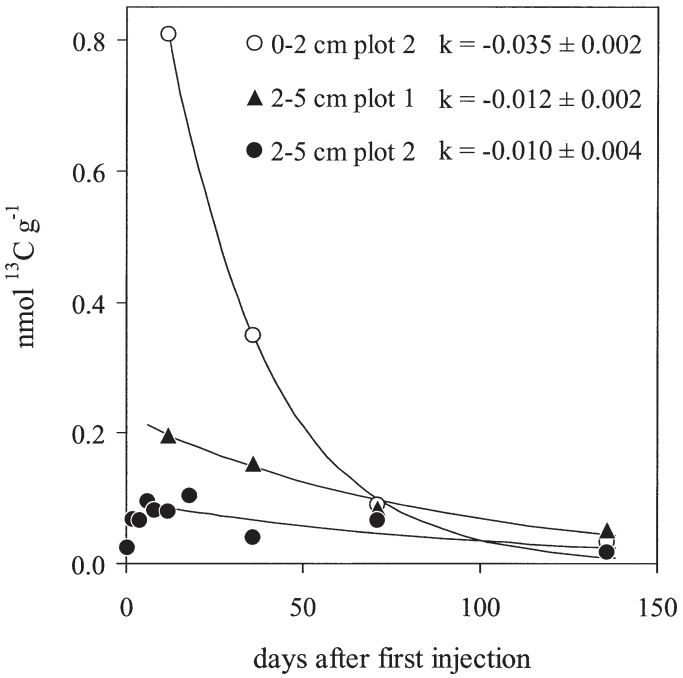

Fig. 5. Excess ^{13}C in D-Ala (corrected for racemization). Lines are the exponential fits for data between day 6 and day 136 (after label injection period). *k*-Values are loss rate constants (±SE).

including L-Ala, resulted in a decrease in excess ^{13}C D/L-Ala ratios between days 12 and 71 (Fig. 4).

After 136 d, excess ^{13}C in D-Ala was 4% of the original excess ^{13}C in D-Ala in the period directly after [^{13}C]glucose injection (days 6–18) for the 0–2-cm layer and 18–27% for the 2–5-cm layers. The excess ^{13}C D/L-Ala ratio for the 2–5-cm layer of plot 2, the only layer for which excess ^{13}C was present in L-Ala, was 0.22 (not corrected for racemization; Fig. 4). Excess ^{13}C in D-Ala relative to that in THAAs (not shown in Fig. 3) increased from 0.5% for the period up to day 71 to 1.0% at day 136. This increase was smaller than the about four times increase in excess ^{13}C D/L-Ala ratio (Fig. 4) because the relative abundance of ^{13}C L-Ala in the [^{13}C]THAA pool actually decreased by about two times between days 71 and 136 (Fig. 3).

Discussion

The data presented in this paper are part of a comprehensive in situ ^{13}C-labeling experiment investigating the fate of bacterial carbon in an intertidal sediment. The main data concerning total ^{13}C and total bacterial ^{13}C for the whole upper 10-cm layer of both sediment plots are presented in a companion paper (Van Oevelen et al. 2006) that focuses at modeling the ^{13}C results to quantify loss processes for ^{13}C from bacterial biomass and the sediment. In addition, a separate paper was dedicated to detailed analysis of the transfer of ^{13}C from bacteria to fauna via grazing (Van Oevelen et al. in press). Van Oevelen et al. (2006) documents strong loss of bacterial ^{13}C from the sediment during the 4.5-month sampling period. Loss processes included grazing (~25% of total loss); exchange processes such as resuspension (~10% of total loss); and cell death by viral lysis, programmed cell death, and other potential causes (~65% of total loss). Cell death resulted in production of bacterial remnants. Degradation of these remnants produced ^{13}C–dissolved organic carbon (DOC), of which part was lost from the sediment (either directly as [^{13}C]DOC or indirectly as [^{13}C]DIC after respiration), whereas another part of the [^{13}C]DOC was recycled back to bacterial biomass (Van Oevelen et al. 2006). As a result of these different loss processes, bacterial ^{13}C had disappeared from the sediment almost completely after 4.5 months.

The material presented here adds to that in the companion papers by focusing on the fate of different ^{13}C-labeled compounds from the ^{13}C-labeled bacterial biomass during the weeks and months after the [^{13}C]glucose injection period. Before discussing the results, it seems useful to clarify some points that should be considered during interpretation of the data. (1) Because $\Delta\delta^{13}$C values represent changes in ratios between ^{13}C and ^{12}C, $\Delta\delta^{13}$C values for the ^{13}C-labeled bacteria might have been "diluted" by production of new, unlabeled bacterial biomass. Therefore, our results are all presented as excess ^{13}C (i.e., absolute amounts of ^{13}C), which is not biased by such dilution and therefore represents the true fate of the ^{13}C-labeled compounds from bacteria that were labeled during the [^{13}C]glucose injection period. (2) PLFAs are turned over within days after cell death (Parkes 1987; Moodley et al. 2000). Therefore, at the experimental timescale of weeks to months, ^{13}C-labeled bacteria-specific PLFAs can be

considered specific for living bacteria. Conversely, HAAs, including D-Ala, can remain present in the sediment as remnants for considerably longer periods of time. Therefore, [^{13}C]THAAs and [^{13}C] D-Ala were not specific for living (bacterial) biomass. (3) HAAs included in this study, except D-Ala, are common protein amino acids that are abundant in proteinaceous biomass of all organisms and are therefore not specific for bacteria. However, during the first 2–3 weeks of the experiment, ^{13}C was predominantly present in bacterial biomass, meaning that during this period, [^{13}C]THAAs represented bacterial proteinaceous biomass. During the following weeks to months, a substantial fraction of ^{13}C was transferred to nonbacterial biomass, meaning that [^{13}C]THAA was no longer specific for bacteria but instead represented total benthic proteinaceous material. (4) Sediment samples for analysis of ^{13}C in HAAs were washed with water and HCl (2 mol L^{-1}) before hydrolysis, which removed dissolved HAAs (Veuger et al. 2005). Therefore, [^{13}C]THAAs and [^{13}C]D-Ala reflect particulate HAAs only (living biomass + particulate remnants).

In the following paragraphs we first discuss incorporation of ^{13}C from [^{13}C]glucose into the different bacterial components and resulting estimates of total bacterial ^{13}C. Subsequently, we focus on the fate of the different ^{13}C-labeled compounds, distinguishing between bacterial ^{13}C (^{13}C in bacteria-specific PLFAs and D-Ala) and ^{13}C in total benthic proteinaceous biomass ([^{13}C]THAAs). The last section deals with retention of [^{13}C] D-Ala in the sediment and its consequences for the accumulation potential of peptidoglycan.

^{13}C incorporation and estimates of total bacterial excess ^{13}C—^{13}C incorporation into the different bacterial cell components confirmed that ^{13}C incorporation from [^{13}C]glucose was initially dominated by bacteria. First, the substantially lower ^{13}C enrichment of biomarker PLFAs for other groups of organisms (algae and fauna) indicated that total ^{13}C incorporation into these groups was substantially lower than that into bacteria. Second, the excess ^{13}C D/L-Ala ratios of 0.05–0.08 during the [^{13}C]glucose injection period (Fig. 4) are within the range of D/L-Ala ratios for bacterial biomass (Veuger et al. 2005 and references therein). In case of substantial ^{13}C incorporation by organisms other than bacteria (algae and/or fauna), excess ^{13}C D/L-Ala ratios would have been lower because ^{13}C incorporation by nonbacterial organisms results in ^{13}C enrichment of L-Ala only.

Although the use of biomarkers in stable isotope labeling studies is primarily a semiquantitative tool, compound-specific results can also be used for estimating label incorporation into total biomass. Excess ^{13}C incorporated into total bacterial biomass was estimated from excess ^{13}C in the two different bacterial compounds (bacteria-specific PLFAs and D-Ala). In addition, a third estimate could be made from excess ^{13}C in THAAs because this pool was also predominantly bacterial during the first 2–3 week of the experiment (as already discussed). This estimate is a useful addition because THAAs represent a much larger fraction of total biomass than the two bacterial biomarkers and hence require only a small conversion step. Estimates of total bacterial excess ^{13}C were in reasonable agreement given the magnitude of the conversion steps from bacteria-specific PLFAs and D-Ala to total bacterial C and the variation and uncertainty inherent in these kinds of conversions. This variation and uncertainty include dependence on bacterial cell size, community composition, and environmental conditions as well as potential heterogeneity in distribution of ^{13}C between different cell components. Moreover, additional bias might have resulted from differences in the turnover of the different ^{13}C-labeled bacterial compounds (as discussed below) during the first 2–3 weeks of the experiment.

Fate of ^{13}C in bacteria-specific PLFAs and D-Ala—Both bacterial biomarkers showed substantial loss of ^{13}C during the months after the [^{13}C]glucose injection period, indicating rapid loss of ^{13}C from bacterial biomass. The substantially shorter half lives for the 0–2-cm layer compared with the 2–5-cm layers (Figs. 1, 5) point to a more rapid turnover of bacterial biomass in the 0–2-cm layer. However, the relatively strong loss of ^{13}C from the 0–2-cm layer probably also included a relatively strong loss by resuspension. Although modeling results showed that resuspension accounted for only ~10% of total loss of bacterial ^{13}C from the whole 0–10-cm layer (Van Oevelen et al. 2006), this fraction might well have been substantially higher for the 0–2-cm layer alone given its direct exposure to the overlying water.

Although trends for excess ^{13}C in the two bacterial biomarkers were roughly similar, the consistent about two times longer half-lives for D-Ala show that ^{13}C D-Ala was retained in the sediment longer than the ^{13}C bacteria-specific PLFAs (Figs. 1, 5). This was also evident from the substantially larger fraction of original excess ^{13}C in D-Ala (days 6–18) still present in D-Ala after 136 days (18–27% for the 2–5-cm layers) compared with the much lower ^{13}C fraction left in bacteria-specific PLFAs (≤2%). The relatively strong retention of ^{13}C in D-Ala could simply have resulted from more rapid degradation of PLFAs (cell membranes) than D-Ala (cell wall peptidoglycan) alone but could also reflect a difference in recycling efficiency for the two compounds. This recycling involves reincorporation of ^{13}C from the [^{13}C]DOC pool resulting from degradation of ^{13}C bacterial remnants into new bacterial biomass, either by direct reincorporation of intact dissolved [^{13}C]PLFAs and [^{13}C]AAs or by new production from other [^{13}C]DOC compounds. Modeling results indicated that about one third of all released [^{13}C]DOC was reincorporated into new bacterial biomass (Van Oevelen et al. 2006). Although recycling could have resulted in reincorporation of ^{13}C in D-Ala (and other HAAs), as well as in bacteria-specific PLFAs, recycling of [^{13}C]AAs might well have been more efficient than recycling of [^{13}C]PLFAs because dissolved amino acids also play an important role as a nitrogen source to the benthic microbial community, including bacteria (Veuger et al. 2005). Because nitrogen is generally a limiting element for microbial production, and given the high microbial affinity for dissolved amino acids as a source of nitrogen (Veuger et al. 2004), recycling of nitrogenous

compounds is likely to be more efficient than for non-nitrogenous compounds such as PLFAs.

Irrespective of the precise mechanisms for retention, our results clearly show a relatively strong retention of ^{13}C in D-Ala and hence confirm the potential for accumulation of peptidoglycan in sediments, as discussed in more detail below.

Fate of ^{13}C in THAAs—The fate of ^{13}C in THAA is discussed separately from the bacterial biomarkers because [^{13}C]THAAs represented ^{13}C in total benthic proteinaceous material, which was different from bacterial biomass and remnants alone. Trends in excess ^{13}C in the THAAs were clearly different for the 0–2-cm versus 2–5-cm layers (Fig. 2). The decrease of ^{13}C in the 0–2-cm layer occurred at a rate similar to that for ^{13}C in D-Ala (half-life of ~20 d), indicating that, for this layer, degradation and subsequent loss of ^{13}C from bulk proteinaceous biomass ([^{13}C]THAAs) was similar to that for peptidoglycan (D-Ala). Conversely, the lack of such a decrease in excess ^{13}C in THAAs for the 2–5-cm layers up to day 71 indicates that there was little to no loss of ^{13}C from total proteinaceous biomass. This stronger retention of ^{13}C in THAAs (including L-Ala) than ^{13}C in the bacterial biomarkers, as reflected in a decrease in excess ^{13}C D/L-Ala ratios (Fig. 5), might imply a lower degradability of the THAAs. However, this seems unlikely because peptidoglycan (D-Ala) has been found to be more refractory than proteins that make up a large fraction of the THAA pool (Jørgensen et al. 2003; Nagata et al. 2003). Moreover, the relative composition of the [^{13}C]THAA pool did not change up to day 71, which strongly suggests that, during this period, the [^{13}C]THAA pool was not subject to substantial degradation (*see discussion below for details*). Therefore, the strong retention of ^{13}C in THAAs while ^{13}C was rapidly lost from bacterial biomass is more likely explained by a substantial transfer of ^{13}C from bacteria to other organisms.

Bacteria-derived ^{13}C could have been incorporated by benthic microalgae (BMA) either via uptake of [^{13}C]DOM resulting from degradation of ^{13}C-labeled bacterial remnants or via uptake of [^{13}C]DIC resulting from respiration. However, substantial transfer of ^{13}C to BMA seems unlikely given that BMA predominate in the photic zone (i.e., the upper few millimeters) of the sediment, which is only a small fraction of the sediment layers analyzed in this study. Moreover, recycling of DOM in the upper millimeters of intertidal sediment is much less efficient than that deeper in the sediment because of diffusional losses of DOM to the overlying water. The negligible role of ^{13}C in BMA (mainly diatoms) in this study was confirmed by the low ^{13}C enrichment for diatom-specific PLFA 20 : 5ω3.

Alternatively, the strong retention of ^{13}C in THAAs and the associated decrease in excess ^{13}C D/L-Ala ratios could have resulted from a ^{13}C transfer from bacteria to fauna via grazing. The clear ^{13}C enrichment of the fauna following ^{13}C labeling of bacterial biomass showed that substantial faunal grazing of ^{13}C-labeled bacteria did occur (Van Oevelen et al. 2006, in press). Because turnover of (macro-) faunal biomass is considerably slower than that of bacterial biomass, transfer of bacterial C to fauna serves as a potential mechanism for retention of bacteria-derived C in the sediment over longer periods of time. However, at day 136, there was no excess ^{13}C in THAAs for two of the three sampled layers, whereas a small fraction (9% of original ^{13}C in THAAs for days 6–18) was left in the 2–5-cm layer of plot 2. These results indicate that, as for bacterial biomass, only a minor fraction of the original ^{13}C was retained in total benthic proteinaceous material over the 4.5-month sampling period.

The relative composition of the [^{13}C]THAA pool for the 2–5-cm layer of plot 2 also showed some interesting changes (Fig. 3). These changes, especially the increased relative abundance of Gly and Ser and decreased relative abundance of Leu and Ile, are consistent with observed changes during degradation of total organic matter in sediments, (e.g., Cowie and Hedges 1992; Dauwe et al. 1999; Keil et al. 2000; Pantoja and Lee 2003). Therefore, results indicate that the remaining ^{13}C-labeled material at day 136 had been subject to substantial degradation, meaning that this material likely consisted of remnants of ^{13}C-labeled bacterial or faunal biomass or both, rather than living biomass. Because our THAA analyses only included particulate HAAs (dissolved material was removed by washing the sediment before hydrolysis), the remnants remaining after 136 d must have consisted of biomass components and/or smaller molecules associated with larger molecules and/or particles by mechanisms similar to those reported by Borch and Kirchman (1999) and Arnarson and Keil (2005). Therefore, the relative accumulation of Gly, Ser, and Pro likely resulted from their presence in larger molecules, particles that were relative resistant to degradation, or both. The relative accumulation of Gly during degradation of total organic matter in sediments is usually attributed to its presence in refractory diatom cell walls or peptidoglycan (Dauwe and Middelburg 1998; Keil et al. 2000), of which the latter might have been relevant for this study. However, the relative accumulation of [^{13}C]Gly was actually about four times stronger than that for ^{13}C D-Ala (specific for peptidoglycan), indicating that the relative accumulation of [^{13}C]Gly could not have resulted from its presence in peptidoglycan alone. Similarly, the relative accumulation of [^{13}C]Ser and [^{13}C]Pro was also stronger (~2×) than for ^{13}C D-Ala. Hence, these results suggest that the relative accumulation of Gly, Ser, and Pro was due to their presence in other compounds or particles that were more refractory than peptidoglycan or both. An alternative explanation for the relative accumulation of Gly and Ser is the formation of degradation products that are relatively rich in "simple" amino acids such as Gly and Ser. This is similar to the production of nonprotein AAs (γ-ABA and β-Ala) as degradation products (Lee and Cronin 1982; Cowie and Hedges 1994; Keil et al. 2000) and is supported by results from Keil and Fogel (2001), who found natural abundance $\delta^{13}C$ values for Gly to be different from those for other HAAs in marine organic matter and attributed this to relatively intensive microbial reworking of Gly. Results from Ziegler and Fogel (2003) suggest that similar mechanisms may apply for Ser. Conversely, Pro is a large, more complex secondary amino acid and therefore less likely to accumulate as degradation product like Gly and Ser. The relatively refractory

character of Pro, which also showed in Veuger et al. (2005), might be related to its molecular structure (including a cyclic side group).

Accumulation potential of peptidoglycan—Although most bacterial ^{13}C was lost during the 4.5 months in situ, results clearly showed an approximately two times slower loss of ^{13}C from D-Ala than from bacteria-specific PLFAs during the sampling period and a relatively high excess ^{13}C in D-Ala compared with that in the bacteria-specific PLFAs and THAAs in the remaining leftovers at the end of the sampling period (day 136). This relatively strong retention of D-Ala (i.e., peptidoglycan) is consistent with the relatively low degradability of peptidoglycan in laboratory experiments (Jørgensen et al. 2003; Nagata et al. 2003). Hence, results show that the potential for accumulation of peptidoglycan is also present during reworking and degradation of bacterial biomass in the highly dynamic upper centimeters of an intertidal sediment. This provides direct evidence that increasing D/L-AA ratios with increasing time or depth as reported for sediment organic matter (OM; Pedersen et al. 2001; Grutters et al. 2002) and seawater DOM (Amon et al. 2001; Dittmar et al. 2001), as well as for OM in soils (Brodowski et al. 2004), can indeed result from the relative accumulation of peptidoglycan (rich in D-AAs) relative to bulk proteinaceous material (L-AAs).

Peptidoglycan is only a small fraction of total bacterial biomass and our ^{13}C results showed that only part of the peptidoglycan from bacterial biomass was retained in the sediment. However the relative accumulation of peptidoglycan over longer periods of time ($\geq$10 yr) combined with potential reworking of sediment OM by bacteria might result in a substantial accumulation of peptidoglycan. This makes peptidoglycan a potentially important contributor to total sediment OM, just as it is thought to be an important contributor to total DOM in seawater (McCarthy et al. 1998; Amon et al. 2001; Dittmar et al. 2001). The contribution of peptidoglycan to total OM in the intertidal sediment in the present study was estimated from measured concentrations of D-Ala and total OC. Peptidoglycan concentrations were estimated from D-Ala concentrations assuming that C in D-Ala makes up ~10% of total peptidoglycan C, which was based on the typical composition of peptidoglycan (equal amounts of D-Ala, L-Ala, D-Glu, diaminopimelic acid, and the two sugar derivatives *N*-acetyl glucosamine and *N*-acetyl muramic acid; Madigan et al. 2000). Resulting estimates showed that peptidoglycan C was only a minor fraction (~1%) of total OC in the sediment. This low contribution is consistent with other HAA-based estimates by Keil et al. (2000), Pedersen et al. (2001), and Grutters et al. (2002), who also found that, despite its relatively refractory character, peptidoglycan was only a minor fraction of total sediment organic matter.

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