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The structure of marine benthic food webs

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Chapter 6

Fate of ^{13}C labeled bacterial proteins and peptidoglycan in an intertidal sediment

Bart Veuger, Dick van Oevelen, Jack J. Middelburg and Henricus T. S. Boschker. *In revision for Limnology and Oceanography*

6.1 Introduction

Bacteria constitute a major pool of biomass in marine sediments and play a central role in ecological and biogeochemical processes. Although rates of bacterial secondary production and respiration in sediments vary over orders of magnitude, bacterial abundance is relatively constant at $\sim 10^9$ cells ml^{-1} (Schmidt et al., 1998). This suggests that bacterial production is balanced by various loss processes. Such losses include bacterial grazing by benthic fauna (Kemp, 1990), mortality due to viral lysis (Fischer et al., 2003; Hewson and Fuhrman, 2003), programmed cell death (Yarmolinsky, 1995) and other unidentified causes. Generally, < 20 % of bacterial production is removed by grazing (Epstein and Shiaris, 1992; Hondeveld et al., 1995; Sundback et al., 1996). Consequently, after cell death most bacterial C stays in the sediment organic carbon (OC) pool as bacterial cell remnants, where it can be subject to degradation and recycling. Bacterial cells contain a variety of components, some of which will be readily degraded, while others are more resistant to degradation ('refractory'). The refractory nature of peptidoglycan makes it a potentially important contributor to total sediment OM just like 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). Increasing D/L-AA ratios with sediment depth reflect the selective preservation of peptidoglycan relative to protein at longer time scales (10-10.000 years), but do not provide direct information on the degradability of peptidoglycan or its significance as a potential long term sink for bacterial C and N.

An alternative approach that does provide a direct indication of the degradability of peptidoglycan is to study degradation of peptidoglycan in bacterial cultures (Jorgensen et al., 2003) or marine waters (Nagata et al., 2003). This approach showed that peptidoglycan is degraded slower than proteins and can be characterized as 'semi-labile'. However, these studies used peptidoglycan and proteins extracted from cultures and were

performed in vitro. This does not provide a direct indication of the degradability of peptidoglycan and proteins from sediment bacteria in situ, since this is also dependent on their presence in macromolecules and by interactions with particles (Borch and Kirchman, 1999; Arnarson and Keil, 2005).

In this study, we combine the two approaches mentioned above by ^{13}C labeling bacterial biomass in a tidal flat sediment and subsequent analysis of the in situ fate of the ^{13}C in three different bacterial cell components: 1) D-Ala, an amino acid unique to peptidoglycan that has only recently been used as a bacterial biomarker in combination with stable isotope labeling (Veuger et al., 2005). In this study, ^{13}C-D-Ala is used as an indicator for ^{13}C-peptidoglycan. 2) Total hydrolyzable amino acids (THAAs) comprises a pool of 14 HAAs (including D-Ala). Except for D-Ala, these HAAs are common protein amino acids that have very limited biomarker potential, as they are present in all organisms (Cowie and Hedges, 1992). However, as the ^{13}C-glucose labeling in this study specifically tagged bacteria, ^{13}C-THAAs could be used as an indicator for bacterial ^{13}C-proteins. 3) Bacteria-specific phospholipid-derived fatty acids (PLFAs) are a more established group of biomarkers that have been used in various ^{13}C-labeling studies to estimate total bacterial ^{13}C incorporation (Boschker et al., 1998; Middelburg et al., 2000; Pelz et al., 2001). Since PLFAs are rapidly degraded after cell death (Parkes, 1987; Moodley et al., 2000), they are restricted to living organisms, which makes them true 'ecological biomarkers' as opposed to D-Ala, which is more of a 'biogeochemical biomarker' as it is also present in bacterial cell remnants (Boschker and Middelburg, 2002). In this study, bacteria-specific PLFAs are used as an indicator for living bacteria and thereby serve as a reference to determine the fate of bacterial cell remnants (proteins and peptidoglycan).

The unique comparison of ^{13}C in the different bacterial components allowed us to compare the in situ fate of bacterial proteins (THAA) and peptidoglycan (D-Ala) derived from sediment bacteria and to link this with the accumulation of D-Ala in sediments. In addition to analysis of their long term fate, this study also allows direct comparison of ^{13}C incorporation into the different bacterial components. Comparison of estimates of total bacterial ^{13}C incorporation from bacteria-specific PLFAs and D-Ala provides a validation of the use of these bacterial biomarkers and their accompanying conversion factors in stable isotope labeling studies.

6.2 Materials and methods

6.2.1 Experimental setup

The material presented in this publication is part of a larger ^{13}C labeling study (see also chapter 4 and 5). 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 and the upper 10 cm was injected with ^{13}C glucose (1 injection per 6.25 cm^2, 0.4 ml of 24 mmol l^{-1} ^{13}C 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 days), plots were sampled regularly by taking sediment cores (Ø 50 mm). Before removing sample cores from the plots, larger cores (Ø 90 mm) were placed around the sample cores to prevent disturbance of surrounding sediment. Sample cores were transported to the laboratory and sliced into three layers (0-2 cm, 2-5 cm and 5-10 cm) that were analyzed for total C and ^{13}C in POC (by EA-IRMS after removal

of carbonates by acidification), pore water DIC and PLFAs as well as ^{13}C in meio- and macrobennthos. Further details on the experimental setup and analytical procedures can be found in chapter 4. Samples from the 2-5 cm layer from one plot were also analyzed for ^{13}C in HAAs, including D-Ala. Since this paper focuses on comparison of ^{13}C in bacteria-specific PLFAs, D-Ala and THAAs, it only deals with results for the 2-5 cm layer.

6.2.2 PLFA analysis

Lipids were extracted from 3 g of dry sediment in chloroform-methanol-water using a modified Bligh and Dyer method and fractionated on silicic acid into different polarity classes. 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). Further details on PLFA extractions and analysis can be found in Boschker (2004). Data were processed as in Middelburg et al. (2000). Although analysis included a wide range of PLFAs, we only present results for bacteria-specific PLFAs i14:0, i15:0, a15:0, i16:0 and 18:1ω7c.

6.2.3 Amino acid analysis

Samples for GC-c-IRMS analysis of ^{13}C in HAAs (including D-Ala) were processed following 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, 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 further purified by solvent extraction. Amino acid D- and L-enantiomers were separated by gas chromatography using a Chirasil-L-Val column. Flame ionization detection (GC-FID) was used for concentration measurements, while 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 δ^{13}C (‰): $\delta^{13}C = \left(\frac{R_{sample}}{R_{VPDB}} - 1\right) \cdot 1000$, where $R = \frac{^{13}C}{^{12}C}$ and $R_{VPDB} = 0.0112372$.

During derivatization, extra (unlabeled) C atoms are added to the original amino acids, which changes their δ^{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) using the following mass-balance equation: $\delta^{13}C_{AA} = \delta^{13}C_{DAA} \cdot (C_{AA} + C_{IP} + C_{PFA}) - \frac{\delta^{13}C_{IP+PFA} \cdot (C_{IP} + C_{PFA})}{C_{AA}}$, where C_{AA} = number of C atoms in the original amino acid, D_{AA} = derivatized amino acid, C_{IP} = number of C atoms added by esterification with IP and C_{PFA} = number of C atoms added by acylation with PFA. However, the change in δ^{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 amino acid). As this effect is not included in the mass balance equation, it requires empirical correction. This was done by measuring the δ^{13}C of three amino acid standards (D-Ala, D-Glu and D-Ser) before and after derivatization by EA-IRMS, which allowed us to determine empirical $\delta^{13}C_{CIP+PFA}$ values for these three amino acids. These values showed a strong linear correlation with the %C from PFA, which is similar to results by Silfer et al. (1991). Subsequently, this relation was used to determine empirical $\delta^{13}C_{CIP+PFA}$ values for the other amino acids which were then

used to calculate their original δ^{13}C values using the mass balance equation. Our empirical $\delta^{13}C_{CIP+PFA}$ values ranged between -52 and -45 ‰, which is lighter than those in Silfer et al. (1991), because we used PFA for acylation, which contains more C than the TFA used by Silfer et al. (1991) and because the used PFA was very depleted in ^{13}C (δ^{13}C: -55 ‰, measured by EA-IRMS).

After correction, δ^{13}C values were used to calculate $\Delta\delta^{13}$C: $\Delta\delta^{13}C_{sample}$ (‰) = $\delta^{13}C_{sample}$ - $\delta^{13}C_{unlabeled\ sample}$ and the atom percentage of ^{13}C: at%^{13}C = [100 $\cdot$ $R_{standard}\cdot$ (($\delta^{13}C_{sample}$ / 1000) + 1)] / [1 + $R_{standard}\cdot$ (($\delta^{13}C_{sample}$ / 1000) +1)]. Subsequently, at%^{13}C was used to calculate the absolute amount of incorporated excess ^{13}C : excess ^{13}C = (at%$^{13}C_{sample}$ - at%$^{13}C_{control}$)$\cdot$ AA-C concentration$_{sample}$. Amino acid concentrations and excess ^{13}C are expressed in nmol carbon per gram dry sediment (nmol C gdw^{-1}).

6.3 Results and discussion

The aim of the whole ^{13}C labeling experiment was to investigate production and fate of bacterial C in the sediment of the Molenplaat tidal flat. While two companion papers discuss and model the overall turnover and fate of total bacterial ^{13}C (Chapter 4) and bacterial grazing by meio- and macrobenthos (Chapter 5), this paper focuses on the fate of ^{13}C-labeled bacterial proteins and peptidoglycan during the 4.5 months after labeling, using bacteria-specific PLFAs as an indicator for living bacteria.

Below, we first discuss ^{13}C incorporation into the different bacterial components and validate their use to estimate total excess ^{13}C incorporated by bacteria, including their respective conversion factors. Thereafter, we discuss the fate of bacterial proteins (^{13}C-THAAs) versus peptidoglycan (^{13}C-D-Ala) to clarify the role of peptidoglycan as a potential long term sink for bacterial C and its contribution to total sediment OC.

6.3.1 ^{13}C incorporation into PLFAs, D-Ala and THAAs

Injection of ^{13}C glucose into the sediment resulted in rapid and steady ^{13}C enrichment of bacteria-specific PLFAs, D-Ala and THAAs (represented by L-Ala) with the same general trends for these different bacterial components (Fig. 6.1, 6.2 and 6.3). Enrichment was well above natural abundance for D-Ala (δ^{13}C -8 ‰), other amino acids (Leu, Ile, Tyr and Thr+Val: -35 to -30 ‰, L-Ala, Lys, Glu+Phe and Pro: -25 to -20 ‰, Asp, Ser and Gly: $\sim$ -10 ‰), bacteria-specific PLFAs (-19.6 $\pm$ 1.3 ‰) and bulk OC (-24 ‰). The measured range of natural abundance δ^{13}C values for the different HAAs is comparable with that reported by Keil and Fogel (2001).

Trends for ^{13}C incorporation into the five bacteria-specific PLFAs were the same and absolute differences in excess ^{13}C in these PLFAs were proportional to their concentrations (Fig. 6.3). Therefore, 18:1ω7c was used as a representative for the five bacteria-specific PLFAs for comparison with D-Ala and L-Ala/THAAs (Fig. 6.1). Although 18:1ω7c can also be present in certain algae (Moodley et al., 2000), this did not affect results in this study since ^{13}C uptake by algae was negligible (see below).

The strong increase in $\Delta\delta^{13}$C during the labeling period (day 0 to 4) shows that ^{13}C from ^{13}C glucose was readily incorporated into bacterial biomass (Fig. 6.1) and that ^{13}C incorporation rates were the same for cell membranes (PLFAs), proteins (THAAs) and cell walls (D-Ala). This indicates that the experimental time scale (days) exceeded the time required for bacteria to produce complete cells, meaning that results were not biased by differences in synthesis rates for different cellular components.

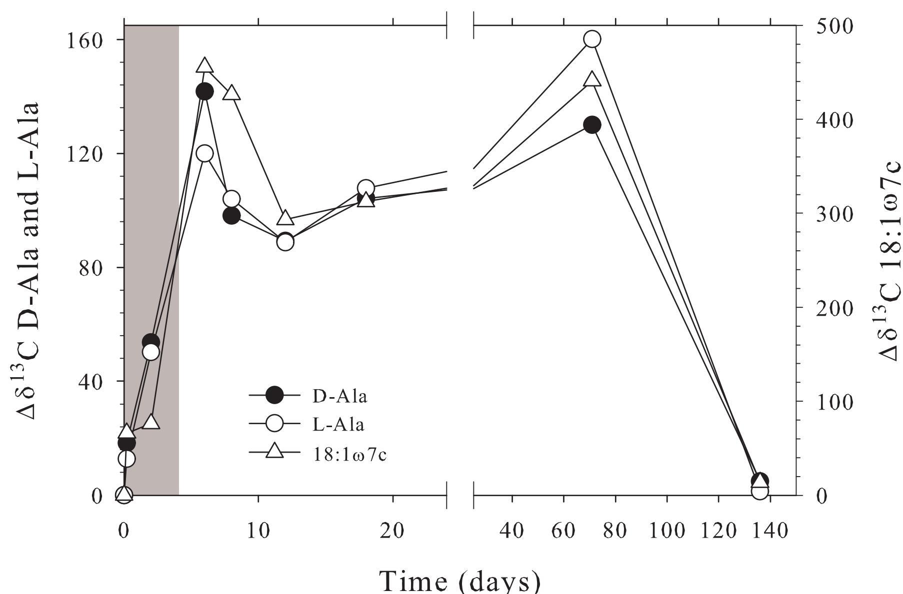

Figure 6.1: $\Delta\delta^{13}$C values for D-Ala, L-Ala and bacteria-specific PLFA 18:1ω7c. Grey area indicates labeling period.

Trends for $\Delta\delta^{13}$C values in D-Ala, L-Ala and bacteria-specific PLFAs were very similar (Fig. 6.1). The close correlation between PLFAs and D-Ala indicates that these two different bacterial biomarkers represented the same active bacterial community. Furthermore, these results confirm that uptake of ^{13}C glucose was dominated by bacteria (i.e. additional uptake by other organisms was negligible), because: 1) Trends for $\Delta\delta^{13}$C values for D-Ala and PLFAs (bacterial biomarkers) were closely correlated with L-Ala (Fig. 6.1). Since L-Ala is a common amino acid that is present in bacteria as well as other organisms and given that bacteria normally show highest turnover rates, additional ^{13}C incorporation by non-bacteria likely would have resulted in a different trend in $\Delta\delta^{13}$C for L-Ala than for the bacterial biomarkers. 2) Excess ^{13}C in D- versus L-Ala (Fig. 6.2B) showed a D/L-Ala ratio of $\sim$ 5 % (until day 71), which is a typical D/L-Ala ratio for a mixed marine bacterial community (Veuger et al., 2005). Substantial ^{13}C incorporation by other organisms would have resulted in a higher excess ^{13}C in L-Ala and thus in a lower excess ^{13}C D/L-Ala ratio. 3) $\Delta\delta^{13}$C values for biomarker PLFAs from other groups of organisms, mainly microphytobenthos (20:5ω3) and benthic fauna (20:4ω6), were an order of magnitude lower than those for the bacteria-specific PLFAs, which confirms that ^{13}C incorporation by organisms other than bacteria was negligible (data not shown).

6.3.2 Estimates of total bacterial ^{13}C uptake

The excess ^{13}C incorporated into bacteria-specific PLFAs, D-Ala and THAAs can be used to estimate total bacterial ^{13}C incorporation, which requires conversion from these specific components to total bacterial biomass. This study provides the opportunity to compare estimates derived from the three different bacterial components and thereby serve as a validation of the use of these components and their respective conversion factors (Fig. 6.4).

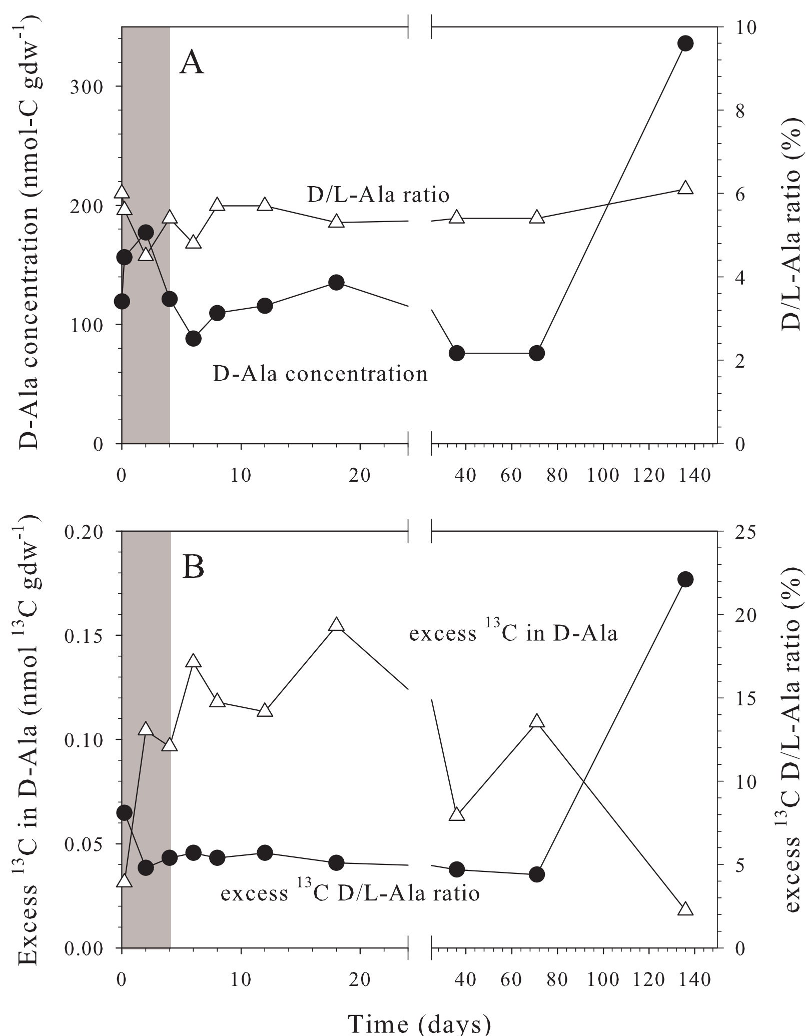

Figure 6.2: A) D-Ala concentrations and ratio between concentrations of D-Ala and L-Ala. B) excess ^{13}C in D-Ala and ratio between excess ^{13}C in D-Ala and L-Ala. Grey area indicates labeling period.

For this comparison, average values for day 6-18 were used (i.e. freshly labeled bacteria) to avoid bias from potential selective removal and/or degradation. Below, we discuss the conversion factors used in Fig. 6.4.

Conversion from excess ^{13}C in bacteria-specific PLFAs to total bacterial excess ^{13}C is the product of two separate conversion steps. The first step is conversion from bacteria-specific PLFA-C to total bacterial PLFA-C. The used value of $\times$3.6 (28 %) is relatively robust (E Boschker, unpub data), has been used in various studies (e.g. Middelburg et al., 2000; Moodley et al., 2000) and was confirmed by the contribution of excess ^{13}C in bacteria-specific PLFAs to excess ^{13}C in total PLFAs in this study (31 $\pm$ 2 %). The second step is conversion from total bacterial PLFA-C to total bacterial-C. Assuming that bacteria in the 2-5 cm layer were predominantly anaerobic, a conversion

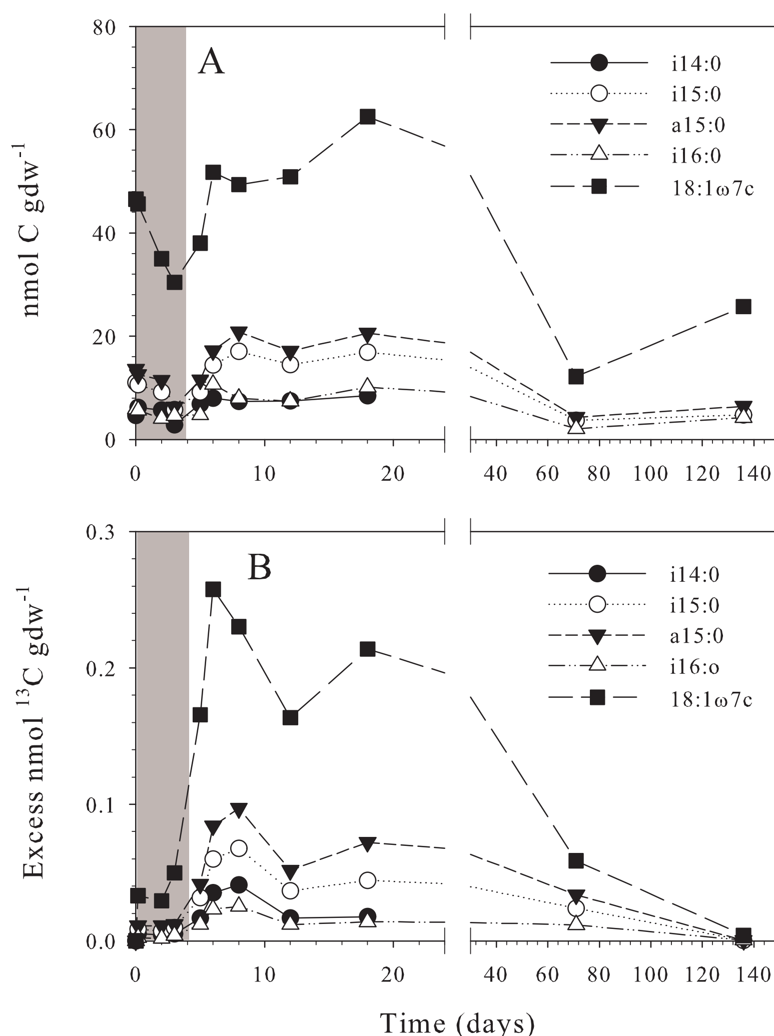

Figure 6.3: A) Concentrations and B) excess ^{13}C for bacteria-specific PLFAs. Grey area indicates labeling period.

of ×26 (3.8 %) was used, which is based on the PLFA content of a mixed bacterial culture grown under anaerobic conditions (Brinch-Iversen and King, 1990). Similar values from Brinch-Iversen and King (1990) have successfully been used in various other studies (e.g. Middelburg et al., 2000; Van den Meersche et al., 2004). For this study, the first and second step together yield a total conversion of ×94 (Fig. 6.4).

In contrast to estimates from the bacterial biomarkers, estimates of total bacterial excess ^{13}C from ^{13}C-THAAs require only a small conversion since THAAs are ∼ 50 % of total bacterial-C (Cowie and Hedges, 1992; Madigan et al., 2000), resulting in a conversion of ×2. Conversion from D-Ala to total bacterial-C can be derived from measured D-Ala contents of bacterial cultures as presented in Veuger et al. (2005). However, these cultures showed rather variable D-Ala contents, while conversion based on the average D-Ala content for these cultures (1.9 mg D-Ala gdw^{-1}) yielded an estimate of total bacterial

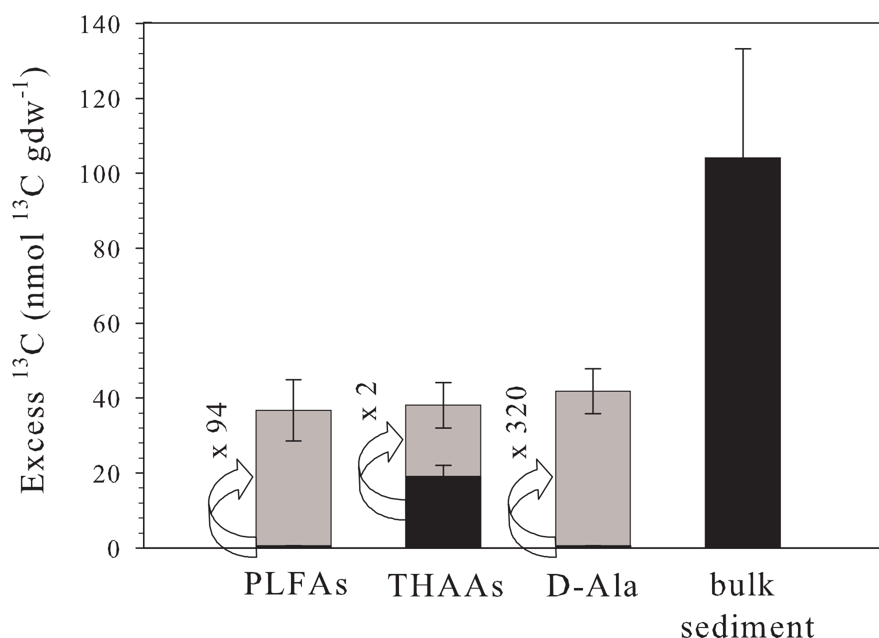

Figure 6.4: Excess ^{13}C in bacteria-specific PLFAs, THAAs, D-Ala and bulk sediment (black bars) and resulting estimates of total bacterial excess ^{13}C (grey bars) for day 6-18 (average $\pm$ stdev). Numbers are used conversion factors (see text).

excess ^{13}C of $\sim$ 86 nmol ^{13}C gdw^{-1}, which is considerably higher than estimates from bacteria-specific PLFAs and THAAs (Fig. 6.4). Therefore, a new conversion factor for D-Ala was derived from three separate steps that involve relatively consistent values. The first step is from D-Ala to L-Ala. Since bacteria appear to have a relatively stable D/L-Ala ratio of $\sim$ 5 % (Fig. 6.2B and Veuger et al. (2005) + references therein), we used a conversion of $\times$20. The second step is from L-Ala to THAA. Cowie and Hedges (1992) found Ala to be 9-15 mole % of the THAA pool of three bacterial cultures (5-9 % C-based). We used a somewhat higher value of 12.6 % (C-based), which was the average contribution of ^{13}C-L-Ala to the ^{13}C-THAA pool for day 6-18 in this study (Fig. 6.5A). Resulting conversion for step 2 is $\times$8. The third step is from THAA to total bacterial-C, which requires a conversion of $\times$2, as already discussed above. The three steps together result in a total conversion of $\times$320, yielding an estimate of total bacterial excess ^{13}C very similar to those from bacteria-specific PLFAs and THAAs (Fig. 6.4). This internal consistency between the different estimates is excellent given the underlying assumptions (as discussed above) and the general uncertainties typically associated with this kind of conversions (e.g. dependence on bacterial cell size, community composition and environmental conditions).

Comparison of estimates of total bacterial excess ^{13}C from excess ^{13}C in bacteria-specific PLFAs, THAAs and D-Ala (Fig. 6.4) served as a useful validation of the use of bacteria-specific PLFAs and D-Ala as bacterial biomarkers and their accompanying conversion factors in ^{13}C labeling studies. Furthermore, it also supports the feasibility of using D-Ala as a bacterial biomarker in ^{15}N labeling studies, although results indicate that the N-based conversion factor of $\times$400 presented in Veuger et al. (2005) may be somewhat too large.

The average estimate of total excess ^{13}C incorporated by bacteria (39 nmol ^{13}C

gdw^{-1}) was considerably lower than the measured excess ^{13}C for the whole sediment (110 nmol ^{13}C gdw^{-1}), indicating that a considerable pool of ^{13}C was present in the sediment next to the ^{13}C incorporated in bacterial biomass. Since the presence of a substantial pool of ^{13}C labeled cell remnants is not likely for days 6-18 (as discussed in following paragraphs) this pool probably comprised ^{13}C-glucose adsorbed to the sediment, which is supported by modeling results (Chapter 4). In addition, ^{13}C labeled compounds excreted by bacteria may also have contributed to this extracellular ^{13}C pool.

6.3.3 Fate of bacterial ^{13}C: General trends

After the positive validation of using excess ^{13}C incorporated in the different bacterial components to estimate total bacterial excess ^{13}C, we now focus on the fate of the bacterial ^{13}C, especially ^{13}C-labeled proteins (^{13}C-THAAs) and peptidoglycan (^{13}C-D-Ala), in the 4.5 months after labeling.

In general, excess ^{13}C in the bacterial components remained high until day 18. After day 18, excess ^{13}C in PLFAs decreased gradually while excess ^{13}C in D-Ala (Fig. 6.2B) and $\Delta\delta^{13}$C values (Fig. 6.1) showed a less rapid and less consistent decrease. For all components, a considerable fraction of the initial excess ^{13}C remained present until day 71. Excess ^{13}C at day 136 was very low for all bacterial components (Fig. 6.1, 6.2 and 6.3), indicating that the bulk of the ^{13}C labeled bacterial biomass had disappeared after 136 days. Loss processes for bacterial ^{13}C in this experiment included cell death, grazing and resuspension. Modeling results for the whole 0-10 cm layer showed that resuspension and grazing only contributed 10 % and 30 % to total loss respectively (Chapter 4). Although the 2-5 cm layer was not in direct contact with the overlying water, resuspension may still have contributed to the removal of bacterial biomass from this layer since the sediment was thoroughly mixed by bioturbating animals (Chapter 5). As these loss processes contributed only $\sim$ 40 % to total loss, this implies that most bacterial ^{13}C was not removed as whole cells but instead remained in the sediment after cell death. These dead bacteria likely provided a pool of fresh organic matter readily available for degradation. In the following paragraphs, we further discuss the fate of the different ^{13}C-labeled bacterial components, especially after 136 days, in more detail.

6.3.4 Fate of bacterial ^{13}C: ^{13}C-THAAs

A first indication as to whether the ^{13}C labeled bacterial biomass had been subject to degradation is provided by the composition of the ^{13}C-THAA pool (Fig. 6.5). Various studies (e.g. Cowie and Hedges, 1992; Keil et al., 2000; Pantoja and Lee, 2003) found that the THAA pool composition of organic matter shows consistent changes during degradation. Therefore, the composition of the THAA pool can be used as an indicator for the extent of organic matter degradation ('diagenetic state') (Dauwe et al., 1999).

The composition of the total sediment THAA pool (i.e. concentrations) remained stable throughout the entire sampling period (data not shown), indicating that the diagenetic state of total sediment organic matter did not change during the experiment. This is consistent with observations that changes in composition of the total sediment THAA pool typically occur at longer time scales since this pool is a mixture of different pools of fresh and degraded organic matter (e.g. Dauwe and Middelburg, 1998). However, the ^{13}C-glucose labeling in this study only tagged a specific pool (bacteria active at the time of labeling) within the total sediment organic matter pool (i.e. no bias from other pools). Therefore, changes in composition of the ^{13}C-THAA pool appeared at a much shorter time

scale (months) than for the total sediment THAA pool: The relative composition of the ^{13}C-THAA pool remained stable until day 71 (except for some variation during the first few days), indicating that no substantial degradation occurred before day 71 (Fig. 6.5). Conversely, a clear change did occur between days 71 and 136 (Fig. 6.5): the relative abundance of Glu+Phe, D-Ala, Ser, Pro and Gly increased while L-Ala, Asp, Leu and Ile decreased in abundance and Tyr and Thr+Val disappeared completely. This decrease for amino acids like Leu and Ile and the increased relative abundance of Gly and Ser are typical for organic matter degradation (Dauwe and Middelburg, 1998; Dauwe et al., 1999; Keil et al., 2000). Therefore, these changes show that the ^{13}C-THAA pool (i.e. bacterial proteins) had been subject to substantial degradation between days 71 and 136. The relatively sudden shift from predominantly living bacteria at day 71 to clearly degraded bacterial remnants at day 136 is striking. However, available data do not allow further clarification.

When considering the differences in relative abundance at day 136 versus day 6-18 (Fig. 6.5B) in more detail, Gly, and to a lesser extent Ser and Pro, actually showed a considerably stronger increase than D-Ala. The refractory behavior of Gly is generally attributed to its presence in diatom cell walls and/or bacterial peptidoglycan (Dauwe and Middelburg, 1998; Keil et al., 2000). The first can be excluded in this study as we specifically labeled bacterial biomass. If Gly would have accumulated only because of its presence in (refractory) peptidoglycan, one would expect it to show a similar refractory behavior as D-Ala since the latter is unique to peptidoglycan. The fact that the increase for Gly (as well as Ser and Pro) was considerably stronger than for D-Ala (Fig. 6.5B) indicates that another mechanism was involved in the preservation of Gly. Potential mechanisms include the presence of Gly in another bacterial component (more refractory than peptidoglycan) and/or accumulation of Gly as a degradation product (Dauwe and Middelburg, 1998). The latter would involve a similar principle as the one underlying the production of non-protein amino acids (γ-ABA en β-Ala) as degradation products (Lee and Cronin, 1982; Cowie and Hedges, 1994; Keil et al., 2000). This is supported by results of Keil and Fogel (2001) who compared natural abundance δ^{13}C values for various HAAs in different pools of marine organic matter and found that δ^{13}C values for Gly showed a different behavior than other HAAs and bulk δ^{13}C. This different behavior was attributed to relatively intensive microbial reworking of Gly. Results from Ziegler and Fogel (2003) suggest that similar mechanisms may apply for Ser, which is consistent with the refractory behavior of Ser in this study (Fig. 6.5B) .

In addition, Pro showed a refractory behavior similar to Gly and Ser. Although Pro is usually not included in other studies (since it cannot be measured with typically used HPLC methods), results from a previous (^{15}N labeling) experiment also indicated a refractory behavior of Pro (Veuger et al., 2005). While Gly and Ser are relatively simple amino acids (2 and 3 C atoms, respectively), Pro is a larger (5 C atoms), more complex, secondary amino acid that therefore seems less likely to accumulate as a degradation product like Gly and Ser. This is supported by results from Keil and Fogel (2001) where $\Delta\delta^{13}$C values for Pro did not indicate relatively intensive microbial reworking, while values for Gly did. Therefore, it seems most likely that Pro accumulated because it was relatively resistant to degradation (rather than due to accumulation as a degradation product), which might be related to the molecular structure (including a cyclic side group) of this secondary amino acid.

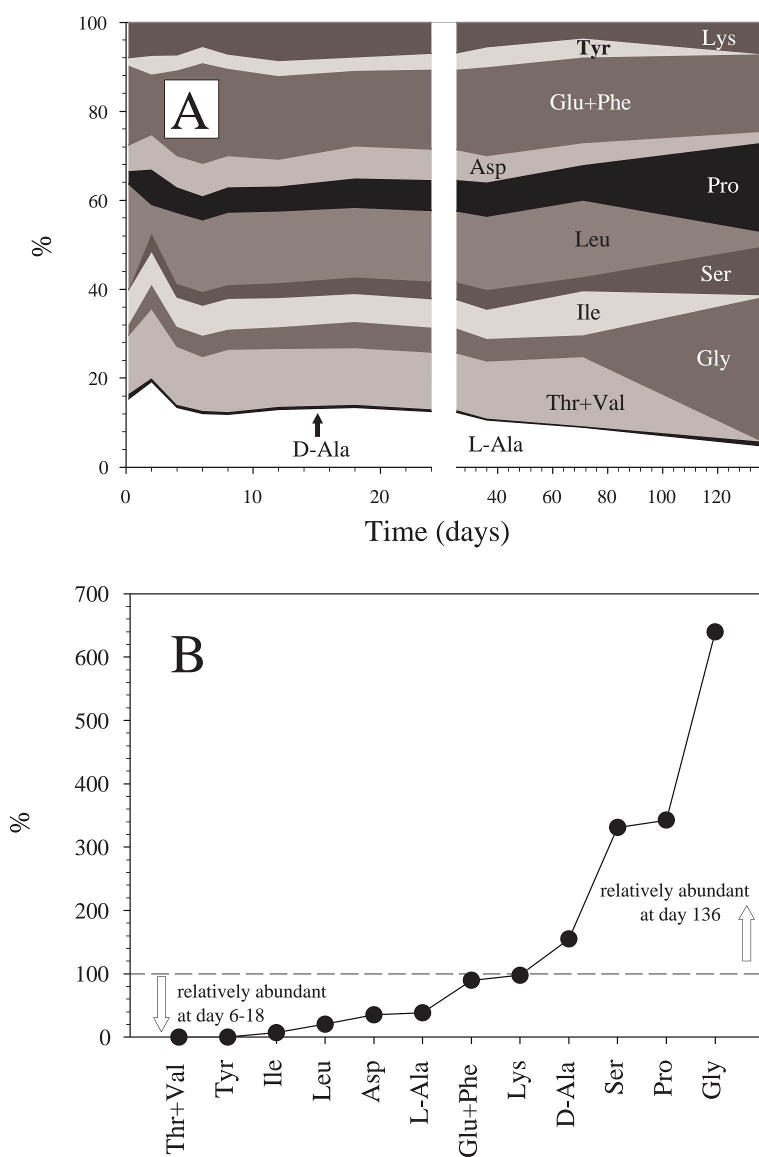

Figure 6.5: A) Relative composition of ^{13}C-THAA pool. B: Comparison of relative abundance of individual ^{13}C-HAAs in ^{13}C-THAA pool for days 6-18 versus day 136. Dashed line at 100 % indicates same abundance at days 6-18 and 136. All amino acids are L-enantiomers, except D-Ala. Glu+Phe and Thr+Val are combined since their GC-c-IRMS peaks overlapped.

6.3.5 Degradation of bacterial proteins versus peptidoglycan

After finding that bacterial ^{13}C-proteins had been subject to substantial degradation, we now discuss how degradation affected ^{13}C-peptidoglycan. A direct comparison of degradation of ^{13}C-labeled proteins versus peptidoglycan is provided by the ^{13}C D/L-Ala

ratio (Fig. 6.2B) where D- and L-Ala represent peptidoglycan and proteins respectively: Until day 71, the excess ^{13}C D/L-Ala ratio remained stable at $\sim$ 5 %, which is typical for living marine bacteria (Veuger et al., 2005). This indicates that ^{13}C-D-Ala and ^{13}C-L-Ala (i.e. proteins and peptidoglycan) were mainly present in living (active and dormant) bacteria or intact dead bacteria until day 71. Between day 71 and 136, the excess ^{13}C D/L-Ala ratio increased to 22 %, which means that ^{13}C-L-Ala disappeared faster than ^{13}C-D-Ala. This relative accumulation of D-Ala is consistent with results from various other studies that found increasing D/L-Ala ratios with increasing degradation (McCarthy et al., 1998; Amon et al., 2001; Dittmar et al., 2001) and sediment depth (Pedersen et al., 2001; Grutters et al., 2002). Although preferential degradation of free L-AAs relative to D-AAs has been shown in soils (O'Dowd et al., 1999), our results concern hydrolyzable (i.e. bound) amino acids, meaning that results reflect differences in degradability of the components containing these HAAs. Therefore, the increased ^{13}C D/L-Ala ratio shows that bacterial proteins (L-Ala) were degraded more rapidly than peptidoglycan (D-Ala). Precise quantification of the difference in degradability of peptidoglycan versus total protein is difficult, since the individual protein HAAs showed a range in degradability (Fig. 6.5B). However, the use of L-Ala as a representative for the whole THAA seems justified by its 'average' behavior during degradation (Fig. 6.5). Given the range from Fig. 6.5B, results indicate that the degradability of peptidoglycan was 1.6 to 23 times lower than that of the bacterial proteins (based on Lys and Ile, respectively). This range is consistent with results by Nagata et al. (2003), who found degradation of peptidoglycan in marine waters to be 2 to 21 times lower than that of proteins. Our results confirm the semi-labile character of peptidoglycan (Jorgensen et al., 2003; Nagata et al., 2003), which is thought to be due to the strong bonds in the polysaccharide matrix and the presence of D-Ala and other D-AAs in the peptide cross links, since peptides containing D-AAs cannot be cut with 'common' enzymes that are used to cut 'common' peptides and proteins (Koch, 1990; McCarthy et al., 1998; Nagata et al., 2003).

6.3.6 Importance of peptidoglycan as a long term sink for bacterial ^{13}C

In order to investigate whether the relatively refractory peptidoglycan served as an important long term sink for the bacterial ^{13}C, we compared excess ^{13}C in different bacterial components for freshly labeled bacterial biomass (average for day 6-18) versus the leftovers at day 136 (Fig. 6.6). Total ^{13}C incorporation in living bacteria for day 6-18 is the average estimate from bacteria-specific PLFAs, D-Ala and THAA (see Fig. 6.4). For day 136, this value was estimated from excess ^{13}C in bacteria-specific PLFAs (being an indicator for living bacteria). Subsequently, fractions of ^{13}C-THAA and ^{13}C-D-Ala present in living bacteria were estimated from excess ^{13}C in living bacteria, using the reversed conversion factors from Fig. 6.4. Excess ^{13}C in peptidoglycan was estimated from excess ^{13}C in D-Ala using a conversion factor of $\times$12 (i.e. C in D-Ala = 8 % of total peptidoglycan-C), which was based on the typical composition of peptidoglycan (containing equal amounts of D-Ala, L-Ala, D-Glu, DAP and the two sugar derivatives NAG and NAM, De Leeuw and Largeau (1993) and Madigan et al. (2000)).

Comparison of excess ^{13}C remaining in the different bacterial components at day 136 with the original excess ^{13}C in freshly labeled bacteria (Fig. 6.6) shows that, at day 136, only $\sim$ 1 % (0.44/39) of the originally labeled bacterial biomass was still present as living bacteria while most of the ^{13}C-THAA and ^{13}C-D-Ala were present in bacterial remnants (87 % and 92 %, respectively). Furthermore, excess ^{13}C in peptidoglycan at day 136

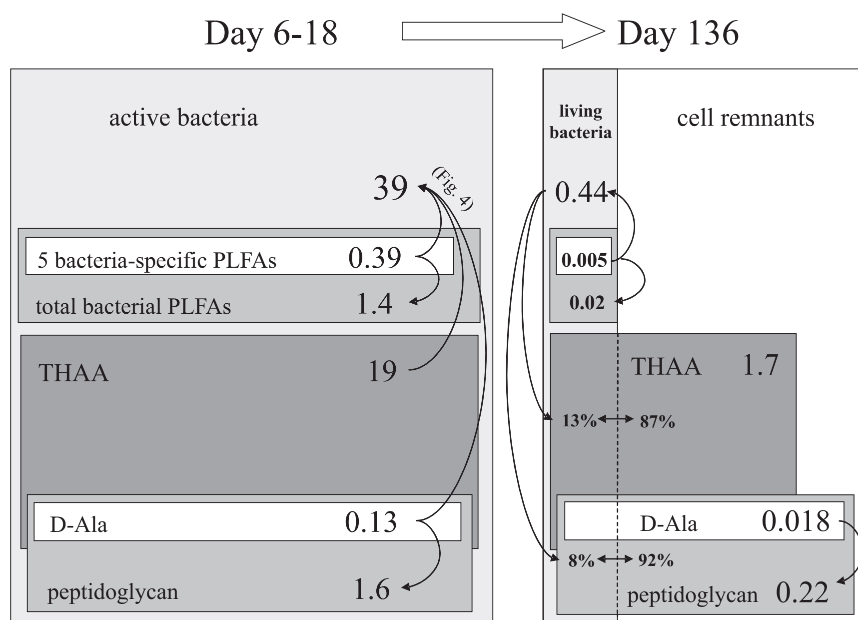

Figure 6.6: Overview of excess ^{13}C (nmol ^{13}C gdw^{-1}) in different bacterial components at day 6-18 (freshly labeled bacteria) versus day 136 (after degradation). Excess ^{13}C in D-Ala, THAAs and bacteria-specific PLFAs were measured. Excess ^{13}C in active/living bacteria, total bacterial PLFAs and peptidoglycan were estimated using conversion factors from Fig. 6.4.

was only 0.6 % (0.22/39) of the original total bacterial ^{13}C at day 6-18. Even if these estimates are corrected for a 3 times lower degradability of the polysaccharide component of peptidoglycan relative to the peptide component (Nagata et al., 2003), excess ^{13}C in peptidoglycan at day 136 still represented only a very small (< 2 %) fraction of the original bacterial ^{13}C.

6.3.7 Substantial contribution of peptidoglycan to total sediment OC?

Although peptidoglycan did not serve as an important long-term sink for bacterial ^{13}C, it might still have been a relatively important contributor to the total sediment OC pool, since this pool consists of a mixture of fresh organic matter (living bacteria and other organisms) and accumulated leftovers that escaped degradation (enriched in D-Ala/peptidoglycan like the ^{13}C pool at day 136). In addition, further recycling of sediment organic matter by bacteria may further increase the contribution of peptidoglycan to the total sediment OC pool. To test this, we compared different (bacterial) C pools in the sediment using measured concentrations of D-Ala, THAA, bacteria-specific PLFAs and total OC (Fig. 6.7). Carbon in living bacteria and peptidoglycan was estimated from bacteria-specific PLFAs and D-Ala respectively using the same conversion factors as in Fig. 6.4. Estimates were tested for potential bias from 18:1ω7c in algae (as discussed before), which was found to be negligible. Unlike for the ^{13}C-THAA pool in Fig. 6.6, the total sediment THAA pool can be derived from various organisms and was therefore not treated as a bacterial-C pool in Fig. 6.7.

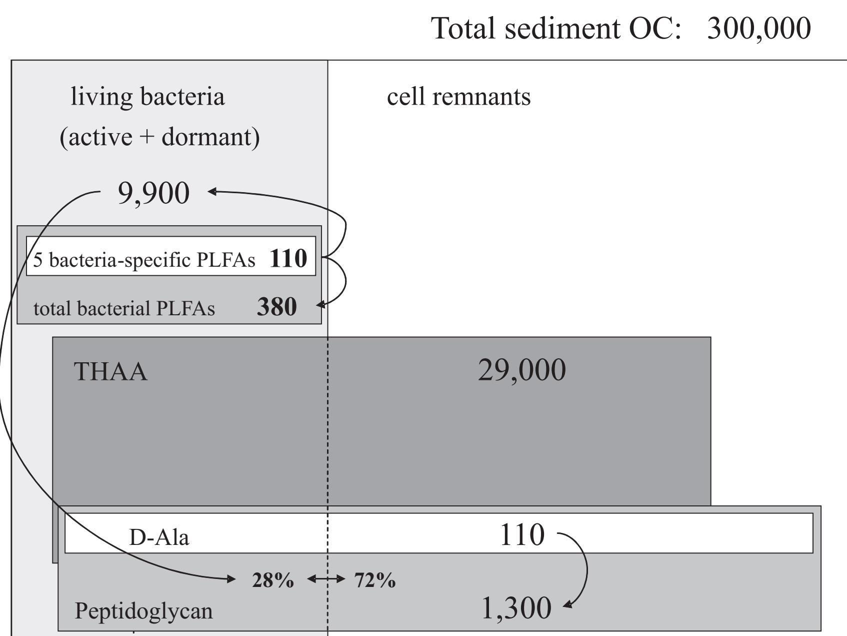

Figure 6.7: Overview of (bacterial) C pools in the whole sediment (averages for day 6-18 in μmol C gdw^{-1}). Concentrations of D-Ala, THAAs, bacteria-specific PLFAs and total OC were measured. Total C in living bacteria, total bacterial PLFAs and peptidoglycan as well as the fraction of D-Ala (and peptidoglycan) in living bacteria were estimated using conversion factors from Fig. 6.4.

The overview in Fig. 6.7 shows that living bacteria were only 3.3 % (9,900/300,000) of the total OC in the sediment and that most D-Ala (72 %) was present in cell remnants. The latter is confirmed by the difference between $\Delta\delta^{13}$C values for the bacteria-specific PLFAs versus D-Ala (Fig. 6.1), where the 3.5$\times$ lower values for D-Ala were the result of dilution of ^{13}C-D-Ala in living bacteria by an (unlabeled) background pool of D-Ala from cell remnants (while no such background pool exists for PLFAs given their rapid turnover). The 3.5$\times$ difference indicates that the background D-Ala pool was 2.5$\times$ larger than the D-Ala pool in living bacteria. This means that D-Ala in living bacteria was 29 % of total D-Ala, which is very similar to the calculated 28 % in Fig. 6.7. This fraction of D-Ala in living bacteria is larger than the fraction ^{13}C-D-Ala in living bacteria at day 136 (8 %, Fig. 6.6), which is consistent with the total sediment D-Ala pool being a mixture of D-Ala in living bacteria (like ^{13}C-D-Ala at days 6-18) and bacterial remnants (like ^{13}C-D-Ala at day 136).

Figure 6.7 also shows that D-Ala was only $\sim$ 0.4 % (110/29,000) of the THAA pool while THAAs were only $\sim$ 10 % (29,000/300,000) of the total sediment OC pool. The latter is consistent with values for shallow marine sediments reported by Dauwe and Middelburg (1998) and Keil et al. (2000). Together, this means that D-Ala was only $\sim$ 0.04 % of the total sediment OC pool, suggesting that the contribution of peptidoglycan was only $\sim$ 0.5 % (0.04 $\times$ 12). Although these values might be underestimates given the higher degradability for the peptide fraction of peptidoglycan compared to the polysaccharide fraction (Nagata et al., 2003), these results clearly show that peptidoglycan-C was only a minor fraction ($<$ 2 %) of the total sediment OC pool. 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 peptidoglycan to be only a minor fraction of total sediment organic matter. Moreover, a similar conclusion was drawn by Sinninghe Damsté and Schouten (1997) who used a number of alternative approaches to show that there is no evidence for a substantial contribution of bacterial biomass to sediment OC.

6.4 In summary

1. ^{13}C from ^{13}C-glucose was readily incorporated into the different cellular components of the sediment bacteria, while uptake by other organisms was negligible.
2. Estimates of total bacterial ^{13}C incorporation from excess ^{13}C in bacteria-specific PLFAs, D-Ala and THAAs yielded very similar results, which served as a positive validation of the use of bacteria-specific PLFAs and D-Ala as bacterial biomarkers, and their accompanying conversion factors, to estimate total bacterial incorporation of ^{13}C- and ^{15}N-labeled substrates.
3. ^{13}C-labeled bacterial biomass could be traced up to 136 days and changes in composition of the ^{13}C-THAA pool showed that ^{13}C-bacterial biomass was degraded substantially.
4. Degradation resulted in an increased relative abundance of ^{13}C-D-Ala compared to ^{13}C-L-Ala and ^{13}C-THAAs, indicating that ^{13}C-peptidoglycan was more resistant to degradation than ^{13}C-proteins. However, in spite of the relatively low degradability of peptidoglycan, it did neither serve as an important long term sink for bacterial ^{13}C nor as an important contributor to total sediment OC.