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Analysis of ^{15}N incorporation into D-alanine: A new method for tracing nitrogen uptake by bacteria

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Abstract

The quantitative contribution of bacteria to total microbial uptake of nitrogenous substrates is an aspect of the aquatic nitrogen cycle that is still largely unclear, mainly because existing methods are generally inadequate. We investigated the feasibility of measuring ^{15}N incorporation into bacterial D-amino acids by gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) and the potential of this method as a new tool for quantification of ^{15}N uptake by bacteria. The presented method allowed analysis of ^{15}N incorporation into various hydrolysable amino acids (HAAs), including the bacterial biomarker D-alanine (D-Ala), at trace levels. The potential of the method was tested in a ^{15}N labeling experiment in which sediment slurries were incubated with $^{15}NH_4^+$ and a ^{15}N-labeled amino acid mixture. ^{15}N incorporation into D-Ala was used to calculate total bacterial ^{15}N uptake while comparison of ^{15}N incorporation into D-Ala versus L-Ala provided a direct measure for the relative contributions of bacteria versus algae to the total microbial ^{15}N uptake. Subsequently, it was also possible to calculate ^{15}N uptake by algae. Results for the test experiment showed that bacteria accounted for 38% of total $^{15}NH_4^+$ uptake and dominated uptake of the ^{15}N-amino acid mixture (90%). Analysis of ^{15}N incorporation into other (non-biomarker) HAAs yielded useful additional information on the transformation of these HAAs during organic matter degradation. In conclusion, GC-c-IRMS analysis of D-Ala combined with ^{15}N labeling is a unique approach in aquatic sciences that provides a powerful new method for quantification of nitrogen flows through bacteria in natural microbial communities.

The nitrogen cycle plays a central role in aquatic systems, and although it has been studied extensively, various aspects are still poorly understood (Zehr and Ward 2002). One of these aspects is the uptake of nitrogenous substrates by bacteria. Although bacteria were traditionally considered as remineralizers of organic matter, it is now evident that they can also take up various forms of dissolved inorganic and organic N (e.g., Coffin 1989; Kirchman 1994; Kroer et al. 1994). The importance of N uptake by bacteria is especially interesting in comparison with that by algae (the other main group involved in microbial N uptake) as these 2 groups form different parts of the food web. Therefore, the relative contributions of bacteria versus algae can determine the subsequent fate of the N after uptake (Caraco and Cole 2002). However, the quantitative importance of bacterial N uptake in natural microbial communities is still largely unclear due to a lack of adequate methodology. The common technique for measuring microbial N uptake is to measure the uptake of nitrogenous substrates labeled with the stable isotope ^{15}N. Total microbial ^{15}N uptake can generally be measured relatively easy as ^{15}N enrichment of the bulk particulate material. However, specifically measuring ^{15}N uptake by bacteria or algae within the total microbial community is far more difficult. Up to now, two different methods have been used to distinguish N uptake by bacteria and algae. The first method is size fractionation, which involves physical isolation of microbial cells by filtration or flow cytometry with cell sorting (Lipschultz 1995). Although this method can work for oligotrophic waters, its use is limited by an overlap in bacterial and algal cell size range. Furthermore, size fractionation in turbid waters and sediment is prohibited by practical aspects like filter clogging and the close association of bacteria with larger particles. The

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second method involves the use of antibiotics to inhibit bacterial ^{15}N uptake. Although this method can sometimes provide interesting results (e.g., Wheeler and Kirchman 1986; Middelburg and Nieuwenhuize 2000), their interpretation is limited by uncertainties concerning the efficiency and specificity of inhibitors (Oremland and Capone 1988). In response to this lack of a suitable method for measuring ^{15}N uptake by bacteria in natural microbial communities, we developed a new approach that involves the use of biomarkers (compounds unique to a specific group of organisms). In combination with stable isotope labeling, biomarkers provide the possibility to directly link microbial identity (biomarker), biomass (biomarker concentration), and activity (label incorporation into biomarker) (Boschker and Middelburg 2002).

Analysis of isotopic enrichment in biomarkers at trace levels requires compound-specific isotope ratio mass spectrometry (IRMS) (Brenna et al. 1997; Boschker and Middelburg 2002). Compound-specific analysis requires isolation of individual compounds prior to IRMS analysis, which can be done by liquid chromatography (LC) or gas chromatography (GC). Although interfaces for online LC-IRMS analysis are starting to become commercially available, these are, so far, only suitable for ^{13}C analysis. Therefore, the technique for online compound-specific ^{15}N analysis is GC coupled to IRMS via a combustion interface (GC-c-IRMS). The range of compounds suitable for GC-c-IRMS analysis is restricted to smaller molecules that are volatile or can be made volatile by derivatization. A group of biomarkers that meets these requirements are the phospholipid-derived fatty acids. The use of these membrane-associated compounds has yielded interesting results on bacterial and algal uptake of ^{13}C-labeled substrates (Boschker et al. 1998; Middelburg et al. 2000; Moodley et al. 2000). However, phospholipid-derived fatty acids are not suitable for ^{15}N studies as these compounds do not contain N. Therefore, we investigated the potential of D-amino acids (D-AAs) from peptidoglycan as alternative bacterial biomarkers. Peptidoglycan, a cell wall component unique to bacteria, consists of strands sugar derivatives that are cross-linked by peptide bridges consisting of both the D- and L-enantiomers (or "stereoisomers") of alanine (D-Ala and L-Ala), D-glutamic acid (D-Glu), and either diaminopimelic acid (DAP) or L-lysine. In addition to this basic structure, over 100 variations of peptidoglycan have been reported (Madigan et al. 2000), including the presence of other D-AAs. Since bacteria are the only organisms to incorporate D-AAs into their biomass, D-AAs can be used as bacterial biomarkers (e.g., Ueda et al. 1989; Pelz et al. 1998; McCarthy et al. 1998; Grutters et al. 2002). Out of the different D-AAs, D-Ala is the most promising bacterial biomarker for our application since it is the only D-AA that is present in all bacteria (providing a good link with total bacterial biomass). Furthermore, D-Ala has been shown to be most suitable for GC-c-IRMS analysis (Pelz et al. 1998) and to be relative insensitive to racemization (the abiotic formation of D-AAs from their respective L-enantiomers) during acid hydrolysis (Erbe and Brückner 2000). Next to D-Ala, DAP has also been tested and used as a bacterial biomarker amino acid (Pelz et al. 1998; Tobias et al. 2003). Although DAP has the advantage of containing twice as much N as D-Ala, it seems to be less suitable for the current application as DAP is not present in all bacteria (Madigan et al. 2000) and because GC-c-IRMS analysis of DAP in environmental samples proved to be more problematic than D-Ala (Pelz et al. 1998).

^{15}N incorporation into D-Ala was already measured in 1985 by Tunlid et al. in an *Escherichia coli* culture. However, they used gas chromatography-mass spectrometry (GC-MS), which only allows analysis of very high levels of isotope enrichment (typically > 1 atom%). Hence, this technique is not suitable for environmental labeling studies that typically work at trace levels and, therefore, require GC-c-IRMS. Applications of GC-c-IRMS analysis of hydrolysable AAs (HAAs) in aquatic sciences and related fields of research usually involve studies on ^{13}C without chiral resolution (i.e., not distinguishing between D-AAs and L-AAs) (e.g., Uhle et al. 1997; Fantle et al. 1999; Keil and Fogel 2001). Analysis of ^{13}C in D-AAs is restricted to Pelz et al. (1998) and Glaser and Amelung (2002). However, these studies concern the natural abundance of ^{13}C; we are not aware of any relevant studies combining GC-c-IRMS analysis of D-AAs with ^{13}C labeling. Applications of GC-c-IRMS analysis of ^{15}N in HAAs are few: McClelland and Montoya (2002) and McClelland et al. (2003) measured natural abundance of ^{15}N in various HAAs from zooplankton and phytoplankton and Tobias et al. (2003) measured assimilation of ^{15}N from $^{15}NO_3^-$ into DAP. However, these studies used a non-chiral column, which does not allow analysis of D-AAs. Although the analytical aspects of ^{15}N analysis of D-AAs and L-AAs by GC-c-IRMS were already presented by Macko et al. (1997), we are not aware of any applications in aquatic sciences or related fields of research. This seems to be due to the analytical difficulties associated with GC-c-IRMS analysis of ^{15}N compared to ^{13}C resulting from the relatively low N content of amino acids (C/N: 2-9) and the additional reduction step required to convert NO and N_2O to N_2 prior to IRMS analysis (Brenna et al. 1997). To the best of our knowledge, we are the first to combine GC-c-IRMS analysis of D-AAs with ^{15}N labeling and to use this as a method to quantify ^{15}N incorporation by bacteria.

Materials and procedures

The protocol used for extraction of the HAAs from the sediment and subsequent purification of the samples is primarily based on work by Pelz et al. (1998) and Amelung and Zhang (2001) and similar to McClelland and Montoya (2002). All steps were individually validated and fine-tuned to maximize their efficiency and yield. The protocol for sample preparation and analyses are summarized in Fig. 1 and will be described in detail below.

Washing—Samples (1 g) of ground, freeze-dried sediment were suspended in 6 mL 2 M HCl in 10 mL glass test tubes to

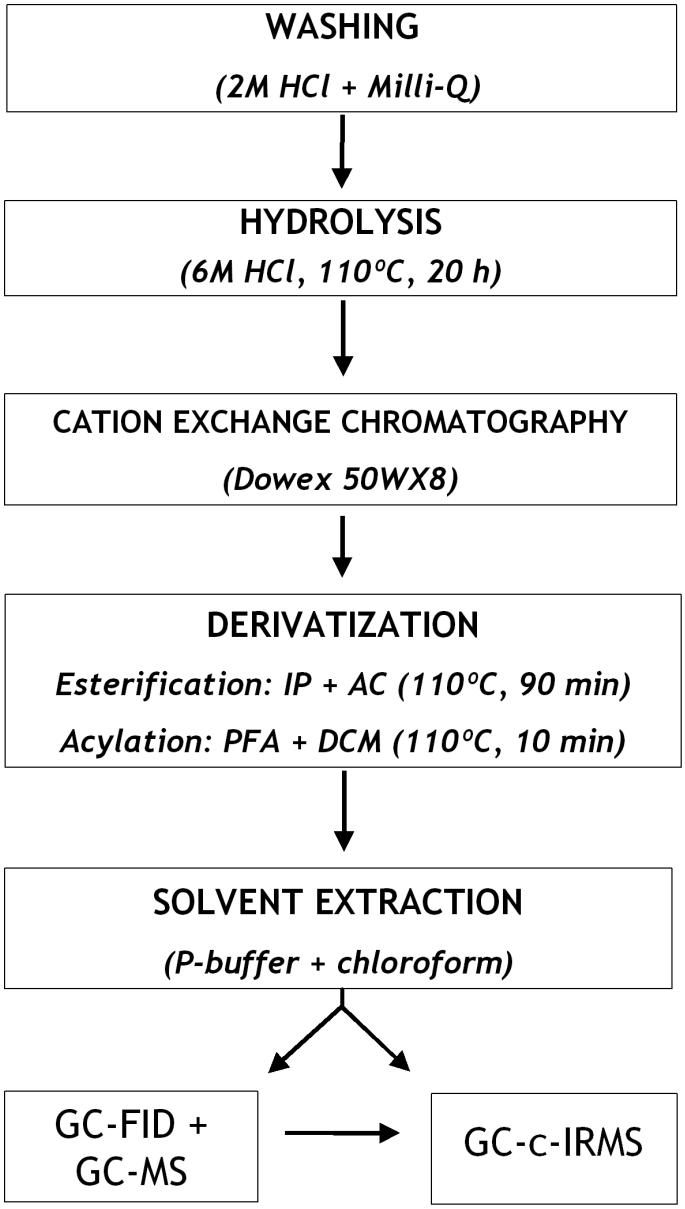

Fig. 1. Schematic overview of the sample preparation protocol.

remove carbonates (Pelz et al. 1998) and to destruct microbial cells. After centrifugation (10 min at 600*g*) pellets were washed with 10 mL Milli-Q water twice to further remove water-soluble compounds, including dissolved and/or adsorbed L-AAs.

Hydrolysis—HAAs were liberated from (microbial) cell walls, proteins, etc. by hydrolyzing the washed pellets in 4 mL 6 M HCl at 110°C for 20 h in test tubes that were sealed tight with Teflon-lined screw caps, after replacing the headspace with N_2. After hydrolysis, samples were cooled to room temperature and spiked with L-Norleucine (Nle), which was used as an internal standard to determine the recovery of the HAAs after further sample processing. Samples were centrifuged (10 min at 600*g*), and supernatants (containing the HAAs) were transferred to glass test tubes (25 mL). Pellets were washed by resuspension in 10 mL Milli-Q water followed by centrifugation (10 min at 600*g*). After repeating the latter step, the resulting Milli-Q supernatants were pooled with the original 6M HCl supernatant, yielding a 24 mL 1 M HCl solution that could directly be purified by cation exchange chromatography.

Cation exchange chromatography—Glass columns were filled with 5 mL of Dowex 50WX8 resin (50-100 mesh) between two pieces of quartz wool. Columns were prerinsed with 50 mL NH_4OH (2 M), 50 mL HCl (2 M), and 50 mL Milli-Q water. After adding the samples (HAAs in 1 M HCl) to the columns, salts and organic contaminations were washed out with 50 mL Milli-Q water. Amino acids were eluted from the column by rinsing with 30 mL 2 M NH_4OH, which was subsequently evaporated to dryness in glass beakers (250 mL) on a heating plate (~ 60°C) under a warm air flow. Dried samples were resuspended in 2 mL acidified Milli-Q water (0.1 M HCl), transferred to glass screw cap vials (12 × 32 mm), and stored at –20°C until derivatization.

Derivatization—The protocol for derivatization (making the amino acids amenable to GC analysis) is primarily based on those of Macko et al. (1997) and Pelz et al. (1998). All steps were tested individually to maximize derivatization efficiency and AA recovery. Samples were evaporated in the 12 × 32 mm vials at 50°C under a gentle flow of N_2. The amino acids were esterified by adding 500 µL isopropanol (IP) that had been freshly acidified with acetylchloride in a 4:1 ratio, and heating for 90 min in a block heater at 110°C. Vials were sealed tight with Teflon-lined screw caps to prevent loss of amino acids during heating. After the esterified samples had been cooled and evaporated at 50°C under a gentle flow of N_2, 200 µL dichloromethane was added to the dried samples and subsequently evaporated to remove residual water and IP (Macko et al. 1997). The AA-IP esters were acylated by adding 150 µL dichloromethane and 50 µL pentafluoropropionic anhydride (PFA) and heating for 10 min in a block heater at 110°C. After acylation, samples were cooled and further purified by solvent extraction.

Solvent extraction—The solvent extraction protocol was based on Ueda et al. (1989): 0.5 mL chloroform and 1 mL P-buffer (KH_2PO_4 + Na_2HPO_4 in Milli-Q water, pH 7) were added to the samples. Firm shaking caused the AA-PFA/IP esters to be transferred to the chloroform fraction while contamination ended up in the P-buffer. Separate testing of this step showed full recovery of the AA-PFA/IP esters in the chloroform fraction. The two solvents were separated by centrifugation (10 min at 600*g*) after which the clear chloroform fraction was transferred to a crimp-cap GC vial, evaporated under a gentle stream of N_2, dissolved in ethyl acetate (25-200 µL) and stored capped at –20°C until analysis.

Concentration measurements—Amino acid concentrations were measured by GC with flame ionization detection (GC-FID) within

24 h after derivatization. A Carlo Erba Mega 2 series GC was equipped with a capillary Chirasil-L-Val column (Alltech, 50m, ID: 0.32 mm, film thickness: 0.2 µm), which is the standard column for separating D- and L-AAs (e.g., Macko et al 1997; Pelz et al. 1998). Helium was used as the carrier gas at a pressure of 150 kPa and samples (0.5-1 µL) were injected in splitless mode (120 s) at 200°C. The oven program started with 10 min at 80°C, then increased with 3°C min^{-1} to 190°C where it was held for 5 min. AA-PFA/IP esters eluting from the column were detected by FID, which yielded peak areas that were tested to be linearly correlated with amino acid concentrations over several orders of magnitude, including relevant concentrations for this study. Peak areas were converted to concentrations using separate conversion factors for all individual amino acids (obtained via analysis of standards) and corrected for losses during sample processing using the recovery of the internal standard.

GC-MS analysis—A selection of samples was measured by GC-MS using the same GC, but coupled to a quadrupole mass spectrometer (Thermo Finnigan Voyager). This technique uses electro ionization to split the compounds eluting from the GC column into characteristic mass fragments. The resulting mass spectra were used for direct identification of the amino acids and to check their peak purity.

GC-c-IRMS analysis—For stable isotope analysis, amino acids were separated on an HP 6890 GC using the same column and settings as for the GC-FID/MS analyses except for the temperature program, which was modified to increase chromatographic performance and decrease analysis time. This modified temperature program started with 2 min at 60°C, followed by a 3°C min^{-1} increase to 120°C, then a 10°C min^{-1} increase to 190°C, and finally 10 min at 190°C. The GC was coupled to a Thermo Delta Plus IRMS via a combustion interface (Thermo type III). Briefly, AA-PFA/IP esters eluting from the GC column were combusted to CO_2, H_2O, N_2, N_2O, and NO in a combustion furnace at 940°C. Subsequently, N_2O and NO were reduced to N_2 in a reduction furnace at 600°C while H_2O and CO_2 were removed. The IRMS detected masses 28 ($^{14}N_2$) and 29 ($^{14}N/^{15}N$) and used the $^{15}N/^{14}N$ ratio (R) for the individual AA-PFA/IP esters to calculate their $\delta^{15}N$ (relative to standard N_2 reference gas pulses that were measured at the start and end of each run): $\delta^{15}N$ (‰) = $[(R_{sample}/R_{standard}) - 1] \times 1000$. Unlike for ^{13}C analysis, the measured $\delta^{15}N$ values are the same as those for the underivatized amino acids since no extra N is added during derivatization and, therefore, do not require correction. ^{15}N enrichment resulting from incorporation of ^{15}N into the amino acids is reported as $\Delta\delta^{15}N$ ($\Delta\delta^{15}N_{sample} = \delta^{15}N_{sample} - \delta^{15}N_{control\ (unlabeled)}$). Furthermore, the atom percentage ^{15}N was calculated, $at\%^{15}N = [100 \times R_{standard} \times (\delta^{15}N_{sample}/1000) + 1]/[1 + R_{standard} \times (\delta^{15}N_{sample}/1000) + 1]$, which was subsequently used to calculate the excess ^{15}N in the amino acids, excess $^{15}N = (at\%^{15}N_{sample} - at\%^{15}N_{control}) \times AA\ concentration_{sample}$.

Assessment

Test experiment—A simple ^{15}N labeling experiment was performed to test and validate the method. Briefly, surface sediment (upper 10 mm) was collected from an intertidal mudflat (Appelzak) in the Scheldt estuary (The Netherlands) in October 2003. In the laboratory, 20 mL samples of homogenized sediment were placed in glass beakers and diluted with 20 mL water from the same location. The resulting slurries were allowed to settle overnight. The next day, slurries were spiked with 0.4 µmol $^{15}NH_4^+$ or 0.4 µmol of a ^{15}N-labeled algal amino acid mixture (Cambridge Isotope Laboratories, NLM-2161). Resulting $^{15}NH_4^+$ and ^{15}N-free AA (FAA) concentrations in the slurries were 1.8× and 6.5× higher than the respective ambient NH_4^+ and DFAA concentrations. Due to this strong increase in total substrate concentrations, the resulting ^{15}N uptake cannot be extrapolated to ambient N uptake. Therefore, measured ^{15}N uptake should be considered as potential uptake for the two substrates. However, these increased concentrations did not affect the main focus of this test experiment, which was to determine the relative contributions of bacteria versus algae to the uptake of ^{15}N labeled substrates by a natural, mixed microbial sediment community. Samples were incubated at ambient temperature and light conditions for 6 h. Incubations were terminated by transferring the slurries to centrifuge tubes for centrifugation. Resulting pellets were frozen (–20°C), freeze-dried, and stored frozen until analysis. In addition to the test experiment, we also analyzed material from various bacterial cultures (pure strains and mixed cultures, *see* Table 2) to determine the D-Ala content and D/L-Ala ratio of bacterial biomass.

Sample preparation—The protocol for washing, hydrolysis, and cation exchange chromatography allowed processing of ~30 samples in 4 work days (not including derivatization and solvent extraction). Results for the sediment samples confirmed the need to wash the sediment with 2 M HCl and Milli-Q water prior to hydrolysis. Washing not only eliminated carbonates that would otherwise interfere with hydrolysis, but also removed contamination (including a compound interfering with GC analysis of D-Ala) and dissolved and/or adsorbed FAAs. This removal of free L-AAs decreased the abundance of the total L-AAs in the samples and, thereby, effectively increased the relative abundance of the D-AAs (that are predominantly present in the HAA fraction and were therefore not removed by washing), which facilitated their analysis. A potential problem related to acid hydrolysis of organic material is racemization (the abiotic formation of D-AAs from their respective L-enantiomers). Although we did not measure racemization during hydrolysis, we are confident that it was negligible because we only detected peptidoglycan-associated D-Ala and D-Glu. In case of significant racemization, other D-AAs also should have been present in detectable amounts, especially D-Asp since Asp is considered to be relatively sensitive to racemization in 6 M HCl (Erbe and Brückner 2000). In addition, similar studies also concluded that the effect of racemization is negligible (Tunlid and Odham 1983; Sonesson et al. 1988; Pelz et al. 1998). Washing the sediment material with Milli-Q water after hydrolysis proved to be an important step that was necessary to recover the significant amount of HAAs

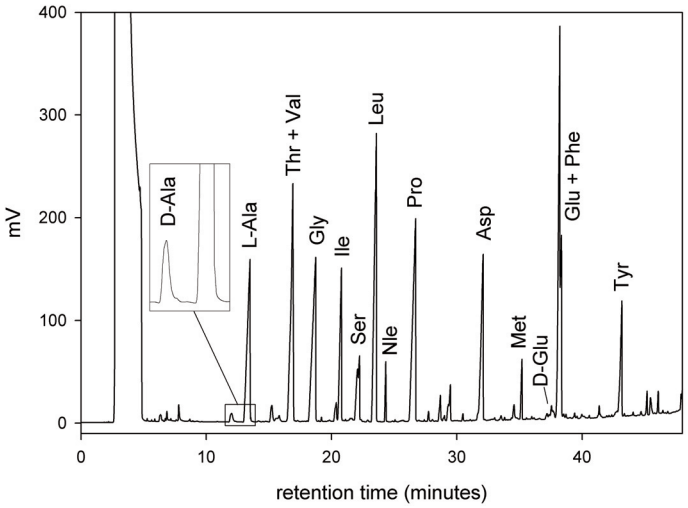

Fig. 2. GC-FID chromatogram for a sediment sample from an intertidal mudflat in the Scheldt estuary. Insert shows magnification of D-Ala peak. Amino acids are all L-enantiomers, unless indicated otherwise. (Nle = internal standard).

still present in the pellet after removal of the 6M HCl. As an additional advantage, dilution of the 6M HCl with the Milli-Q water used for washing resulted in a 1 M HCl solution that could directly be purified by cation exchange chromatography. This eliminated the need for the time-consuming additional step of evaporating the HCl solution and subsequent redilution in Milli-Q water as required for samples in 6 M HCl. Protocols for cation exchange chromatography and derivatization are based on established protocols and will, therefore, not be discussed separately. Additional sample purification by solvent extraction was included because sediment samples still contained considerable amounts of impurities after derivatization. Solvent extraction proved to be simple, fast, and very effective in removing these remaining impurities. We also tested solvent extraction as an alternative for cation exchange chromatography, but found that only the combination of both steps resulted in sufficient purification.

The HAA recovery after purification and derivatization (as determined by the recovery of the internal standard Nle) was 30% to 53% (average 43%). This apparently low recovery was the result of the extensive sample processing, where the overall recovery reflects the sum of smaller losses during the various individual steps. Incomplete recovery introduces the risk of isotopic fractionation (preferential loss of ^{14}N). The latter was tested by comparing the $\delta^{15}N$ of Nle that was derivatized directly (i.e., full recovery) with the $\delta^{15}N$ of Nle that had been added to the sediment samples after hydrolysis (same recovery as for the other amino acids). This yielded $\delta^{15}N$ values of 19.6‰ ± 1.8‰ and 23.1‰ ± 2.9‰, respectively, indicating a fractionation of +3.5‰. Although this fractionation can be an issue for natural abundance studies, it is negligible for ^{15}N labeling studies where labeling clearly exceeded natural abundance levels. Furthermore, this fractionation will not only occur in the labeled samples, but also in the control samples, which means that ^{15}N enrichment (labeled – control) is not influenced by the fractionation. Another potential risk of incomplete recovery is preferential loss of certain amino acids. However, comparison of samples with different recoveries showed no differences in the total HAA (THAA) pool composition (including the D/L-Ala ratio). Therefore, although not completely satisfactory, the overall recovery of 43% is acceptable for labeling studies, such as our test experiment.

GC-FID and GC-MS analyses—GC-FID and GC-MS analyses generally showed good chromatography (Fig. 2) with sharp and well-separated peaks for most amino acids and little contamination, which confirms that sample purification was sufficient. Most of the 20 "common" L-AAs were detected in the sediment samples, except for His, Cys, Trp, and Arg, because these require special derivatization (Erbe 1999). During hydrolysis, Gln and Asn are transformed to Glu and Asp, respectively (Uhle et al. 1997; Erbe 1999). Therefore, peaks for Glu and Asp represent the sum of Glu + Gln and Asp + Asn, respectively. Peaks for Thr and Val as well as those for L-Glu and Phe were not always separated sufficiently and are, therefore, reported as one. The measured THAA concentration (sum of individual amino acids) was 5.6 mg per gram dry sediment, which is similar to values reported for various other sediments (Dauwe and Middelburg 1998; Keil and Fogel 2001; Grutters et al. 2002).

Out of the different D-AAs that could be used as bacterial biomarkers, only D-Ala and D-Glu were detected in the sediment samples (as identified by GC-MS). D-Ala was the first amino acid to elute from the GC column (retention time ~12 min), directly followed by L-Ala (~13 min), which resulted in optimal chromatographic performance (good peak separation and low column bleed). D-Glu eluted later (~37 min), resulting in poorer separation from L-Glu and higher column bleed. In addition, an unidentified compound co-eluted with D-Glu, which prohibited quantification of D-Glu. The average D-Ala concentrations in the sediment samples was 32 μg g^{-1}, which is somewhat higher than the range of 2 to 20 μg g^{-1} reported by Grutters et al. (2002). The D/L-Ala ratio (concentration D-Ala/concentration L-Ala × 100) in the test sediment was 5.2% ± 0.2%, which is similar to values for marine sediment surface layers reported by Pedersen et al. (2001) and Grutters et al. (2002).

GC-c-IRMS analyses—GC-c-IRMS analyses of the amino acids showed lower peak separation than GC-FID/MS analyses due to peak broadening in the combustion interface. During earlier measurements (using the same temperature program as for the GC-FID/MS analyses), separation between D-Ala and L-Ala was critical due to this reduced peak separation. However, our most recent measurements (with the adjusted temperature program and general optimization of GC-c-IRMS performance) showed improved peak separation with a clear and well-separated peak for D-Ala (Fig. 3). Injected amounts of D-Ala were ~1 nmol

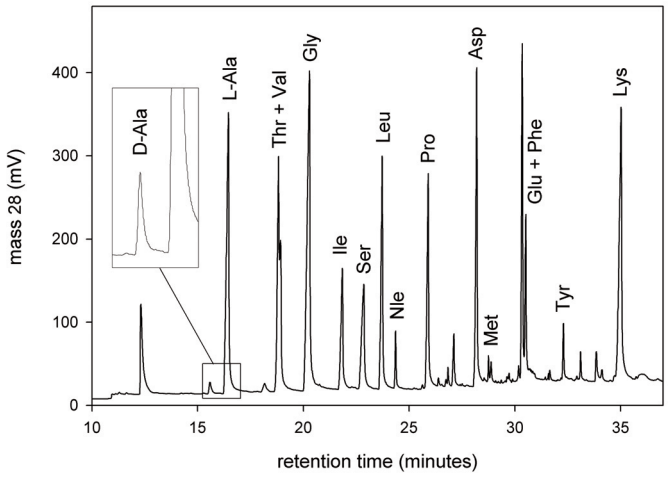

Fig. 3. GC-c-IRMS chromatogram for a sediment sample from an intertidal mudflat in the Scheldt estuary. Insert shows magnification of D-Ala peak. Amino acids are all L-enantiomers, unless indicated otherwise.

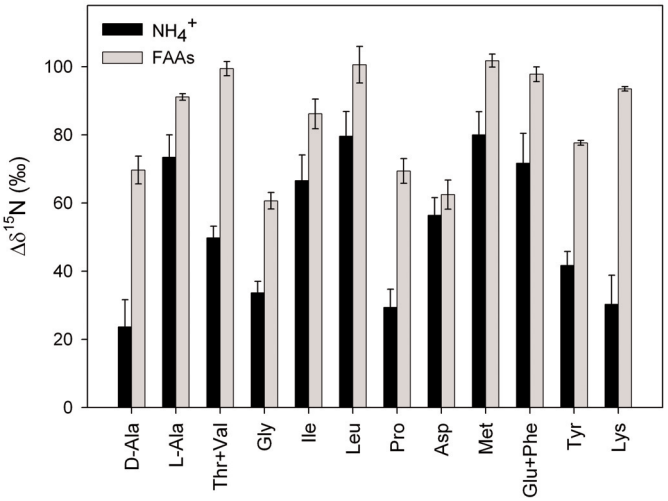

Fig. 4. ^{15}N enrichment ($\Delta\delta^{15}N$) of individual HAAs after incubation with $^{15}NH_4^+$ (black bars) and the ^{15}N-labeled FAA mixture (gray bars). Amino acids are all L-enantiomers, unless indicated otherwise. Error bars indicate experimental variation (standard deviation for replicate incubations).

(~ 0.1 µg), which resulted in co-injection of ~200 nmol (~25 µg) THAAs. The feasibility of GC-c-IRMS analysis of ^{15}N in ~1 nmol D-Ala has already been reported by Macko et al. (1997). The injected amount of sample was less than 1% of the total sample derived from 1 g dry sediment. When assuming an injection volume of 1 µL and a minimum sample volume of 10 µL ethyl acetate, this means that the minimum amount of sample required for analysis is ~100 mg dry sediment.

^{15}N enrichment in D-Ala and the other HAAs—We were able to measure ^{15}N enrichment ($\Delta\delta^{15}N$) for D-Ala and various other HAAs with consistent $\delta^{15}N$ values well above natural abundance (Fig. 4) and little variation (Table 1). Standard deviations for the L-AAs in the unlabeled samples (Table 1) are similar to those reported for natural abundance $\delta^{15}N$ analyses by McClelland and Montoya (2002). The level of variation for D-Ala was somewhat higher (Table 1), but still very acceptable for ^{15}N labeling studies, such as our test experiment.

We are confident that the measured ^{15}N enrichment of the amino acids in the samples incubated with the ^{15}N-FAAs actually represents incorporation of the ^{15}N into the HAAs without bias from the added ^{15}N-FAAs, because (1) washing the sediment with 2 M HCl and Milli-Q water likely removed most of the adsorbed and dissolved amino acids. (2) Our results show clear incorporation of ^{15}N from $^{15}NH_4^+$ (without potential bias from the added ^{15}N-substrate). Since the microbial preference for FAAs is generally considered to be similar to or even higher than for NH_4^+ (Veuger et al. 2004), uptake of the ^{15}N-FAAs likely was similar to or even higher than for $^{15}NH_4^+$.

From ^{15}N in D-Ala to total bacterial ^{15}N uptake—The most straightforward way to interpret the results obtained with the method is to directly use $\Delta\delta^{15}N$ values for D-Ala as a semi-quantitative measure for bacterial ^{15}N incorporation. This can provide interesting results with respect to trends in bacterial uptake of a ^{15}N-labeled substrate over time. However, the potential of the method would be greatly enhanced if ^{15}N incorporation into D-Ala can be used to calculate total bacterial ^{15}N uptake. Calculation of total bacterial ^{15}N uptake requires a conversion factor based on the D-Ala content of bacterial biomass. The latter was measured in 5 of our bacterial cultures (Table 2), which yielded an average value of 1.9 mg D-Ala per gram dry biomass. The measured values are similar to those measured by Sonesson et al. (1988) (Table 2). Assuming that dry bacterial biomass consists of ~12% N (Madigan et al. 2000), this yields a D-Ala content of 0.25% (moles-N in D-Ala/moles-N in total bacterial biomass × 100) and a corresponding conversion factor of 400×. Application of this conversion factor to the measured excess ^{15}N in D-Ala yielded estimates of 12 and 36 nmol ^{15}N g^{-1} for total bacterial uptake of $^{15}NH_4^+$ and the ^{15}N-FAAs, respectively (Table 3).

Bacterial contribution to total microbial ^{15}N uptake—Next to total bacterial ^{15}N uptake (as calculated above), the method can also be used to determine the relative contributions of bacteria versus algae to the total microbial uptake of the $^{15}NH_4^+$ and the ^{15}N-FAAs. One way to do this is by comparing the bacterial uptake with the ^{15}N enrichment of the bulk sediment (measured by EA-IRMS, *see* Table 3) where the latter is considered to represent total microbial ^{15}N uptake. This approach yielded bacterial contributions of 36% and 81% to the total microbial uptake of the $^{15}NH_4^+$ and the ^{15}N-FAAs, respectively. However, these estimates contain some major uncertainties because they are based on (1) a relatively large conversion factor representing the average for the 5 bacterial cultures that showed quite variable D-Ala contents and (2) the assumption that the ^{15}N enrichment of the bulk sediment represents the total microbial ^{15}N uptake. It seems that this was true for our test experiment. However, the ^{15}N enrichment of

Table 1. Analytical variation for GC-c-IRMS analyses

	n	D-Ala, stdev	Other HAAs, stdev
Replicate analysis (same sample measured 2-3 times)			
Natural abundance ($\delta^{15}N$ ~ 15‰)	3	2.5‰	0.2‰-1.5‰ (avs 1.0‰)
Labeled ($\delta^{15}N$ 30‰-150‰)	2	3.2‰	0.1‰-3.8‰ (avs 1.9‰)
Replicate sample preparation + analysis (identical samples processed separately)			
Labeled ($\delta^{15}N$ 30‰-150 ‰)	2	6.3‰	1.3‰-7.5‰ (avs 2.8 ‰)

stdev, standard deviation; avs, average.

the bulk sediment can also include part of the added ^{15}N substrates and/or ^{15}N labeled degradation and excretion products. When the latter occurs, total microbial ^{15}N uptake will be overestimated, resulting in an underestimation of the bacterial contribution.

Fortunately, the method also provides an alternative way to determine the bacterial contribution to total microbial ^{15}N uptake. This approach is based on the relative incorporation of ^{15}N into D-Ala versus L-Ala. Because L-Ala is a common amino acid that is abundant in biomass of all microorganisms (including bacteria) while D-Ala only occurs in bacterial biomass, comparison of ^{15}N incorporation into D-Ala versus L-Ala provides a direct measure for ^{15}N incorporation by bacteria (D-Ala) versus that by the total microbial community (L-Ala). Since comparison of $\Delta\delta^{15}N$ values can be complicated by possible differences in isotope dilution by the (inactive) background pools of D-Ala and L-Ala in the sediment, it is more practical to use excess ^{15}N values (Fig. 5).

In Fig. 5, the measured excess ^{15}N in total L-Ala (representing ^{15}N incorporation by the total microbial community) is compared with the excess ^{15}N in bacterial L-Ala. The latter was calculated from the excess ^{15}N in D-Ala, assuming a bacterial D/L-Ala ration of 5% (excess ^{15}N L-Ala $_{bacteria}$ = excess ^{15}N D-Ala $\times$ 20). Subsequently, excess ^{15}N in total and bacterial L-Ala were used to calculate the bacterial contribution to total microbial ^{15}N uptake (bacterial contribution (%) = [excess ^{15}N L-Ala $_{bacteria}$]/[excess ^{15}N L-Ala $_{total}$] $\times$ 100). This yielded bacterial contributions of 38% and 90% to total microbial uptake of the $^{15}NH_4^+$ and ^{15}N-FAAs, respectively. These estimates are very similar to the estimates derived from the first approach (com-

Table 2. D-Ala contents and D/L-Ala ratios of bacterial cultures

	Gram (G^+/G^-)[a]	D-Ala content (mg g^{-1})	D/L-Ala (%)
Halomonas pacifica (ATCC 27122)	G–	1.3	5.5
Marinobacter hydrocarbonoclasticus (ATCC 27132)	G–	0.9	5.2
Psychrobacter sp. (SW5H)	G– (?)	5.9	18
Pure culture I (isolated from Scheldt water)			5
Pure culture II (isolated from Scheldt water)			4.2
Mixed culture I (inoculated with sediment from Scheldt estuary)		1	4.2
Mixed culture II (inoculated with sediment from Scheldt estuary)		0.3	4
Mixed culture III (inoculated with water from Scheldt estuary)			4
Mixed culture IV (inoculated with water from Scheldt estuary)			5
Mixed culture V (inoculated with water from Scheldt estuary)			5.6
From Sonesson et al. (1988)			
Average for 4 Gram-negative strains (±stdev)	G–	0.7 (±0.1)	5.3 (±1.8)
Average for 4 Gram-positive strains (±stdev)	G+	1.9 (±0.3)	19 (±5)
From Pedersen et al. (2001)			
Bacterium AT	G–		6.9
Bacterium B	G–		5.1
Bacterium BJ	G+		5.9
Peptidoglycan (*S. aureus*)	G+		24.6

[a]Gram-positive, G+; Gram-negative, G–; (?), D/L-Ala indicates G^+ (see text).

Table 3. Overview of excess ^{15}N (nmol $^{15}Ng^{-1}$) in different N pools for the incubations with $^{15}NH_4^+$ and the ^{15}N-FAAs

	$^{15}NH_4^+$	^{15}N-FAAs
D-Ala (measured by GC-c-IRMS)	0.03	0.09
Bacteria	12[a] (38%)[b]	36[a] (90%)[b]
Algae	20[c] (62%)[b]	4[c] (10%)[b]
Total microbial community (bacteria + algae)	32[d]	40[d]
Bulk sediment (measured by EA-IRMS)	34	45

[a]Calculated from excess ^{15}N in D-Ala (× 400).
[b]Relative contributions determined with D-Ala versus L-Ala approach.
[c]Calculated by combining footnotes a and b.
[d]Sum of footnotes a and c.

paring total bacterial ^{15}N uptake with the ^{15}N enrichment of the bulk sediment). However, we prefer the second approach for ^{15}N uptake work in general as it is more direct and based on a relatively small and robust conversion factor (*see* Table 2 and following paragraph).

Bacterial D/L-Ala ratios—The calculation of the relative contribution of bacteria to the total microbial ^{15}N uptake (Fig. 5) relies on the conversion factor of 20×, which was based on an average bacterial D/L-Ala ratio of 5%. This value was derived from our bacterial cultures and additional data from literature (Table 2). All of our mixed cultures and the pure gram negative (G-) strains showed a D/L-Ala ratio of ~5%,

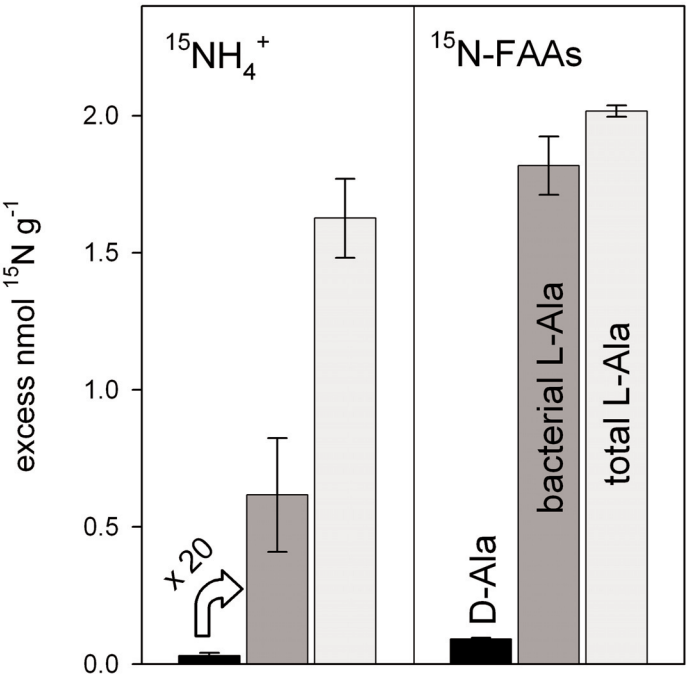

Fig. 5. Measured excess ^{15}N in D-Ala (black bars) and L-Ala (light gray) and calculated values for bacterial L-Ala (dark gray) for incubations with $^{15}NH_4^+$ (left) and ^{15}N-FAAs (right). Values for bacterial L-Ala are calculated from the excess ^{15}N in D-Ala, assuming a bacterial D/L-Ala ratio of 5%. Error bars indicate experimental variation (standard deviation for replicate incubations).

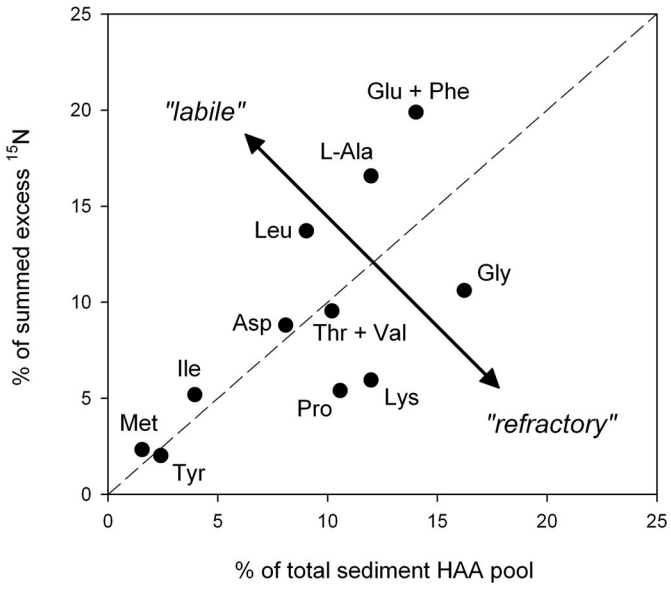

Fig. 6. Relative abundance of individual amino acids in the total sediment HAA pool versus the ^{15}N-labeled HAA pool (excess ^{15}N) after labeling with $^{15}NH_4^+$.

except for *Psychrobacter* sp. However, the fact that the D/L-Ala ratio of *Psychrobacter* sp. is very similar to those reported for 4 Gram-positive (G+) strains by Sonesson et al. (1988) indicates that *Psychrobacter sp*. may have been a G+ bacterium that was identified falsely. The high D/L-Ala ratios for the G+ bacteria from Sonesson et al. (1988) are likely due to the fact that the peptidoglycan layer in G+ bacteria is much thicker than in G- bacteria (Madigan et al. 2000). Although the D/L-Ala ratios for the G+ bacteria are considerably higher than 5%, bacterial communities in aquatic systems are generally thought to be dominated by G– bacteria. Therefore, it seems most likely that the average D/L-Ala ratio of the bacterial community in the sediment was ~5%, which was supported by the measured ratios in our mixed cultures. However, some uncertainty remains as these culture-based values only concern the culturable fraction of the total bacterial community in situ. Because of these potential problems related to the use of culture-based D/L-Ala ratios, we tested an alternative approach that allowed us to directly determine the D/L-Ala ratio of the active bacterial community in the sediment: Since the method presented in this paper also allows analysis of ^{13}C in D- and L-Ala (Pelz et al. 1998), it is possible to incubate the sediment with ^{13}C-glucose and to subsequently measure the incorporation of ^{13}C into D- and L-Ala. Glucose is used as a general C source by bacteria and by bacteria only (i.e., uptake by algae and other organisms is negligible). Therefore, the excess ^{13}C in D-Ala versus L-Ala provides a direct measure for the D/L-Ala ratio of the active (^{13}C-incorporating) bacterial community in the sediment. This approach was applied to sediment from an intertidal mudflat in the Scheldt estuary in the months prior to our

^{15}N labeling experiment and yielded an average D/L-Ala ratio of 5.1% ± 0.5%. These results further support the use of a D/L-Ala ratio of 5%. It seems that the ^{13}C-glucose approach provides a relatively simple and elegant tool to determine the D/L-Ala ratio for the active bacterial community in the same sediment, at the same time and under the same conditions as for the incubations with the ^{15}N labeled substrates.

Bacterial and algal ^{15}N uptake in the test experiment—So far, we calculated the total bacterial ^{15}N uptake (from the excess ^{15}N in D-Ala) and the relative contribution of bacteria to total microbial uptake (comparing the excess ^{15}N in D-Ala versus L-Ala). When these values are known, it is also possible to calculate the absolute ^{15}N uptake by the algae (assuming bacteria and algae were the only 2 groups involved in the uptake of the ^{15}N labeled substrates) (*see* Table 3 for results). Most likely, diatoms were the main contributor to this algal uptake as they were abundant in the sediment and have been shown to be able to take up both NH_4^+ and FAAs (Admiraal et al. 1987; Antia et al. 1991). The sum of the total uptake by the algae and bacteria was only slightly lower than the excess ^{15}N in the bulk sediment. The difference between these two values was most likely due to the presence of ^{15}N labeled substrates adsorbed to the sediment.

Alternative use for non-biomarker amino acids—Although analysis of D-Ala was our primary focus, the method also allowed analysis of ^{15}N incorporation into other (L-) HAAs. Since the HAA pool composition of different groups of organisms is very similar (Cowie and Hedges 1992), these HAAs have limited biomarker potential and, therefore, cannot be used to measure group-specific ^{15}N uptake. However, the excess ^{15}N incorporated into the various HAAs directly reflects their relative contributions to the total HAA pool of the active (^{15}N incorporating) microbial community. This feature of the method can provide useful additional information for studies on organic matter degradation. In these studies, the composition of the HAA pool in the sediment is used as an indicator for the state of organic matter degradation. This is based on the principle that when fresh organic matter is degraded, some HAAs are preferentially degraded ("labile" HAAs) whereas others are less degradable and/or accumulate as degradation products ("refractory" HAAs). As a result, the labile HAAs decrease in relative abundance when degradation progresses while the refractory HAAs become relatively more abundant. In general, the composition of the total HAA pool is measured at different sites, depths, or time points representing different stages of organic matter degradation while cultures of various organisms are used as reference material for fresh organic matter (e.g., Cowie et al. 1992; Dauwe and Middelburg 1998; Pantoja and Lee 2003). The method presented in this paper offers the possibility to compare the composition of the "traditionally" measured total sediment HAA pool (fresh + degraded organic matter) with that of the active microbial community (i.e., fresh organic matter) in a single sediment sample. This is illustrated by the results from our test experiment (Fig. 6) where the relative composition of the total HAA pool in the sediment (calculated from HAA concentrations) is compared with the relative composition of the active (^{15}N incorporating) microbial community (calculated from the excess ^{15}N in the HAAs). When the relative abundance of an amino acid is the same in both pools, it will be on to the dashed 1:1 line in Fig. 6. Gly, Lys, and Pro are clearly below this line (i.e., relatively abundant in the total sediment HAA pool) while Glu + Phe, L-Ala, Leu, and Ile are clearly above this line (i.e., relatively abundant in the microbial biomass = fresh organic matter). This indicates that the HAAs above the line are relatively labile amino acids (easily degraded) while the ones below the line are relatively refractory (accumulating in the total sediment HAA pool). These results are similar to those reported by, for example, Dauwe and Middelburg (1998) and Pantoja and Lee (2003). Although, these results are only a small example, it seems that analysis of stable isotope incorporation into the non-biomarker amino acids may provide a useful new tool for organic matter degradation studies. Applications to further exploit the potential of this tool may include the use of different ^{13}C and /or ^{15}N labeled substrates (labeling different fractions of the total microbial community) and/or tracing changes in the labeled HAA pool composition over time.

Discussion

The presented method proved to be suitable for analysis of ^{15}N incorporation into D-Ala and other HAAs in the sediment. The excess ^{15}N in D-Ala could be used to determine the relative contributions of bacteria versus algae to total microbial ^{15}N uptake as well as to calculate total bacterial ^{15}N uptake (and indirectly algal ^{15}N uptake). Although sediment samples were used for the test experiment, there seem to be no fundamental problems prohibiting the use of the method for analysis of suspended matter in water samples.

These promising first results open the door for various applications in ^{15}N labeling studies where the method can provide a powerful new tool for quantification of bacterial ^{15}N uptake, which will help to clarify the relative importance of bacteria versus algae in the uptake of various nitrogenous substrates. Furthermore, the method can also be used to measure bacterial uptake of ^{13}C labeled substrates. This creates the unique possibility to directly compare bacterial uptake of ^{15}N versus ^{13}C from dual-labeled organic substrates to clarify whether bacteria use these substrates as C and/or N sources. All together, these applications will ultimately help to increase our understanding of the role of bacteria in the cycling of N and C in aquatic food webs.

In addition, the method may provide a useful addition to existing methods for quantification of bacterial production based on incorporation of isotopically labeled amino acids (e.g., Kirchman et al. 1985; Smith and Azam 1992) since it eliminates the potential bias from additional amino acid incorporation by algae, which is inherent to the existing methods.

Finally, the method can also be used as a new tool to study the dynamics of HAAs during organic matter degradation. Not only is it possible to determine the THAA pool composition of fresh organic matter ($^{15}N/^{13}C$ incorporating microbial biomass) produced in situ, but it can also be used to trace the fate of the labeled THAA pool (including D-Ala) over time. The fate of D-Ala during organic matter degradation is especially interesting because it provides an indication for the degradability of peptidoglycan, which is an outstanding topic in aquatic biogeochemistry (e.g., McCarthy et al. 1998; Jørgensen et al. 2003; Nagata et al. 2003).

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