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Optimizing sample pretreatment for compound-specific stable carbon isotopic analysis of amino sugars in marine sediment

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Abstract

Amino sugars are quantitatively significant constituents of soil and marine sediment, but their sources and turnover in environmental samples remain poorly understood. The stable carbon isotopic composition of amino sugars can provide information on the lifestyles of their source organisms and can be monitored during incubations with labeled substrates to estimate the turnover rates of microbial populations. However, until now, such investigation has been carried out only with soil samples, partly because of the much lower abundance of amino sugars in marine environments. We therefore optimized a procedure for compound-specific isotopic analysis of amino sugars in marine sediment employing gas chromatography-isotope ratio mass spectrometry. The whole procedure consisted of hydrolysis, neutralization, enrichment, and derivatization of amino sugars. Except for the derivatization step, the protocol introduced negligible isotopic fractionation, and the minimum requirement of amino sugar for isotopic analysis was 20 ng, i.e. equivalent to ∼ 8 ng of amino sugar carbon. Our results obtained from δ^{13}C analysis of amino sugars in selected marine sediment samples showed that muramic acid had isotopic imprints from indigenous bacterial activities, whereas glucosamine and galactosamine were mainly derived from organic detritus. The analysis of stable carbon isotopic compositions of amino sugars opens a promising window for the investigation of microbial metabolisms in marine sediments and the deep marine biosphere.

1 Introduction

Amino sugars are significant sedimentary components that are mostly derived from microorganisms and invertebrates. For example, the four major amino sugars, glucosamine (GlcN), galactosamine (GalN), mannosamine (ManN) and muramic acid (MurA; Fig. 1), accounted for up to 12 % of total organic carbon (TOC) in grassland soils in North America (Amelung et al., 1999) and ∼ 2 % of TOC in coastal Peruvian surface sediments (Niggemann and Schubert, 2006). As amino sugars are preserved in the form of biopolymers such as peptidoglycan, chitin, and lipopolysaccharides, the amount of amino sugars has been frequently used as a proxy for microbial contributions to soil organic matter, and the ratios between different amino sugars have been employed as indicators of microbial community compositions (e.g. Guggenberger et al., 1999; Zhang et al., 1999; Glaser et al., 2004). In marine environments, amino sugars could be of microbial or animal origin. Prokaryotic biomass is thought to be the major source of GlcN and GalN in marine sediment (e.g. Niggemann and Schubert, 2006; Langerhuus et al., 2012) and seawater (Benner and Kaiser, 2003); however, most amino sugars are not likely associated with peptidoglycan (Aluwihare et al., 2005; Aluwihare and Meador, 2008). Recently, the investigation of amino sugars has been extended to the marine deep biosphere by Lomstein et al. (2012), who used MurA as a tool for indirectly quantifying endospores in subseafloor sediment.

The stable carbon isotopic composition (δ^{13}C) of individual cellular biomarkers can reveal the carbon metabolism and/or lifestyles of the source organisms in the natural environment (e.g. Lin et al., 2010; Schubotz et al., 2011), and incubations with ^{13}C-labeled substrates have been used to probe microbial communities by tracking the production of labeled biomarkers over time (e.g. Veuger et al., 2006; Kellermann et al., 2012; Lin et al., 2012). The latter technique has been widely employed in soil science to investigate the formation and/or turnover kinetics of amino sugars (e.g. Glaser et al., 2005; Decock et al., 2009; Bai et al., 2012). However, to date, the isotopic composition of amino sugars in marine sediment remains poorly explored, partly because of the much lower concentration of amino sugars in marine sediment compared to soils, which results in the need for an efficient pretreatment procedure to enable precise isotopic determination.

Compound-specific δ^{13}C analysis of amino sugars is commonly performed via isotope ratio mass spectrometry (IRMS) after separation of compounds by either gas chromatography (GC; Glaser and Gross, 2005) or liquid chromatography (LC; Bodé et al., 2009). Compared with LC-IRMS, the GC-based method is advantageous as it provides separation of amino sugars in a single measurement and is less sensitive to the sample matrix (McCullagh, 2010; Morrison et al., 2010; Rinne et al., 2012). Substantial effort has been made during the past few decades to optimize the pretreatment procedure for GC-based quantification of amino sugars, which requires three major steps: acid hydrolysis, purification, and derivatization, with hydrolysis being the key step for releasing amino sugars from biopolymers. Besides the most frequently used hydrolysis protocol, namely 6 M hot hydrochloric acid (HCl; Zhang and Amelung, 1996), less destructive procedures involving either hot trifluoroacetic acid (TFA; Neeser and Schweizer, 1984) or sulfuric acid (H_2SO_4; Fox et al., 1983) have been proposed for the simultaneous extraction of neutral and amino sugars. Different purification protocols, such as neutralization with a base solution (Zhang and Amelung, 1996), precipitation of excess acid (Cowie and Hedges, 1984; Neeser and Schweizer, 1984), and deionization of hydrolysate (Cowie and Hedges, 1984), to name a few, have been used to reduce the content of acid and salts that are known to interfere with amino sugar derivatization. Conversion of amino sugars for GC analysis has been achieved via derivatization into alditol acetates (AA; Fox et al., 1983), aldononitrile acetates (ANA; Guerrant and Moss, 1984), or O-methyloxime acetates (Neeser and Schweizer, 1984). However, to perform isotopic analysis of amino sugars at trace levels, a systematic evaluation of these various methods with regard to the product recoveries is necessary.

The goal of this study is to devise a pretreatment protocol for GC-based isotopic analysis of amino sugars in subseafloor sediments. Since deep sediment samples are generally severely limited in size and typically contain substantially lower TOC than the surface sediments from the Peru Margin (Niggemann and Schubert, 2006), which represent the high end-member of TOC content in modern marine sediments, a protocol for sensitive analysis of amino sugars in small sample size is crucial. Hence, we tackled three major analytical tasks. (1) We systematically evaluated existing pretreatment methods for amino sugar analysis to select the method that gave the highest recoveries of products from marine sediments. (2) We introduced the use of a new type of solid-phase extraction (SPE) in the pretreatment protocol and demonstrated enhanced recoveries compared with existing methods. (3) We developed a preparative high performance liquid chromatography (HPLC) method to enrich amino sugars to a desired concentration for proper isotopic assessment. We then applied our protocol to determine the stable carbon isotopic compositions of amino sugars from the selected subseafloor sediment samples.

2 Experimental

2.1 Standards and environmental samples

The amino sugar standards, derivatization reagents, and all other chemicals used in this study were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany) or Merck KGaA (Darmstadt, Germany). The Supelclean™ ENVI-Carb™ Plus SPE cartridges and accessories were obtained from Sigma-Aldrich Chemie GmbH or Carl Roth GmbH (Karlsruhe, Germany).

A batch of surface sediment sample for method optimization was collected from the upper tidal flat of the Wadden Sea near Wremen, Germany, in February 2010 (53°38′0° N, 8°29′30° E). In addition, four marine sediment samples of different types were selected for stable carbon isotopic analysis of individual amino sugars. The seep sample was recovered from the continental margin off Pakistan (Site GeoB 12315-9, 1–2 cm below the seafloor, cmbsf) during the RV *Meteor* cruise M74/3 in November 2007. It was located within the lower part of the oxygen minimum zone and associated with dense microbial mats from sulfide-oxidizing bacteria (Fischer et al., 2012). Two subseafloor samples were retrieved from the Marmara Sea (Site GeoB 15104-2) and the Black Sea (Site GeoB 15105-2) during the RV *Meteor* cruise M84/1 (DARCSEAS) in February 2011 (Zabel et al., 2013). The sample from the Marmara Sea was collected at 2.88 mbsf and was located in a sapropel layer deposited under suboxic bottom-water condition (Çağatay et al., 2000). The Black Sea sample was collected at 1.57 mbsf, where the sediment was highly diluted by terrigenous components and showed a lack of bioturbation. The deepest sediment sample was recovered from the Peru Margin (Ocean Drilling Program Leg 201, 1229A-3H-2, 16.6 mbsf) and had high TOC content (3.8 %).

2.2 Hydrolysis tests

Hydrolysis tests were performed in triplicate in 40 mL glass tubes by adding different acids to ~ 5 g of freeze-dried Wadden Sea sediment. The tubes were filled with nitrogen before being sealed with Teflon-coated screw caps to prevent oxidation of sugars at high temperature during hydrolysis. The evaluated hydrolytic conditions were as follows.

1. The sample was kept at room temperature for 2 h after the addition of 12 M H_2SO_4, followed by dilution to 1.2 M H_2SO_4 and heating at 105 °C for 3 h (modified from Cowie and Hedges, 1984).
2. The sample was hydrolyzed with 4 M TFA at 105 °C for 4 h (Amelung et al., 1996).
3. The sample was treated with 6 M HCl at 105 °C for 8 h (Zhang and Amelung, 1996).

After hydrolysis, the samples were cooled to room temperature, spiked with 100 µg of myo-inositol as an internal standard, and centrifuged at 800 × *g* for 5 min. After being passed through combusted glass microfiber filters (GF/F, 70 mm Φ, Whatman™), the hydrolysates were evaporated to dryness with a rotary evaporator (45 °C, under vacuum) and re-dissolved in 4 mL of MilliQ water.

2.3 Neutralization and desalting

Samples hydrolyzed with H_2SO_4 were neutralized by adding finely ground barium hydroxide ($Ba(OH)_2$; Cowie and Hedges, 1984), whereas TFA-treated samples were freeze-dried overnight to remove the acid. Hydrolysates liberated with HCl were initially subjected to the procedure described by Zhang and Amelung (1996) with slight modification, i.e. the acidic solution was adjusted to pH 6.5–7.0 with 1 M potassium hydroxide (KOH), evaporated to dryness under N_2, re-dissolved in 2 mL methanol (MeOH) and amino sugars were collected in the supernatant after centrifugation. Because treatments with HCl gave the best recoveries (see below), we went on to explore other neutralization and desalting methods that are compatible with HCl. A standard mixture containing 20 µg of each amino sugar was treated with 1 mL of 6 M HCl and the following three methods were performed in triplicate to compare the yields with that of Zhang and Amelung (1996).

1. Silver carbonate (Ag_2CO_3) was added gradually to the mixture to neutralize the HCl. The silver chloride (AgCl) precipitate was removed by centrifugation (cf. Neeser and Schweizer, 1984).

2. After neutralization with 1 M KOH solution, the mixture was desalted by percolating the solution through a glass column filled with 3 g of pre-conditioned Dowex 50WX8 cation exchange resin (100–200 mesh), as described by Amelung et al. (1996). The column was washed with 10 mL of MilliQ water to remove excess salts and amino sugars were subsequently eluted with 10 mL of 2 M ammonium hydroxide (NH_4OH).

3. We evaluated for the first time the applicability of Supelclean™ ENVI-Carb™ Plus SPE cartridges in carbohydrate analysis. ENVI-Carb™ Plus is a microporous amorphous carbon molecular sieve developed for the extraction of highly polar compounds from water. Its predecessor, Supelclean™ ENVI-Carb™, has been used to extract oligosaccharides in aqueous samples (Itoh et al., 2002). Prior to use, the SPE cartridge was pre-conditioned sequentially with 10 mL methylene chloride (DCM), 20 mL MeOH, and 15 mL MilliQ water. The hydrolysate containing amino sugars was neutralized with 1 M KOH and desalted by pulling through the SPE cartridge coupled with a SPE manifold (Carl Roth GmbH, Karlsruhe, Germany) under vacuum. The SPE cartridge was then dried for 10 min and

eluted under vacuum in the reversed direction with 5 mL MeOH followed by 5 mL DCM/MeOH (1 : 1, v : v) to recover the amino sugars.

All the desalted products were evaporated under a stream of N_2, lyophilized overnight, and converted to GC-amenable derivatives for analysis. Quantities and recoveries of individual amino sugars were determined via calibration curves that were generated from pure standards. The Wadden Sea sediment was also used to assess the efficiencies of the neutralization and desalting methods for environmental samples.

2.4 Derivatization and purification

Amino sugar standards were transformed to AA or ANA derivatives in triplicate following the methods of Fox et al. (1983) or Guerrant and Moss (1984), respectively. After being converted to ANA derivatives, amino sugars extracted from the environmental samples were further purified with a self-packed silica gel column (0.5 g; Kieselgel, 0.06–0.2 mm; Carl Roth GmbH, Karlsruhe, Germany; cf. Lin et al., 2010). The best recovery (> 95 % of each amino sugar as ANA derivative) was achieved by elution with 8 mL hexane/ethyl acetate (1 : 4, v : v). The eluent was evaporated to dryness under a N_2 stream and re-dissolved in hexane/ethyl acetate (1 : 1, v : v) prior to analysis.

2.5 Preparation of amino sugar-enriched fractions

A mixture of amino sugar standards and desalted hydrolysate of the Wadden Sea sediment sample were employed for developing separation of amino sugars via preparative HPLC. The sample was re-dissolved in acetonitrile (ACN)/H_2O (7 : 3, v : v) prior to injection. The flow rate was 1 mL min^{-1}, and the eluent gradient ramped steadily from 100 % eluent A to 100 % eluent B over 15 min, then held at 100 % eluent B for 15 min, followed by 10 min re-equilibration with 100 % eluent A. Eluent A was composed of ACN/H_2O/NH_4OH/formic acid (90 : 10 : 0.1 : 0.2, v : v : v : v); eluent B was ACN/H_2O/NH_4OH/formic acid (30 : 70 : 0.1 : 0.2, v : v : v : v). NH_4OH and formic acid were included for online monitoring of the separation of amino sugars by MS and impeding amino sugars partially retained on the stationary phase of the column.

Amino sugars were thereafter separated into two fractions, the first (F1; 5.0–7.6 min) containing the three hexosamines and the second (F2; 7.6–9.6 min) MurA. Fractions collected during preparative HPLC were evaporated to dryness and converted to GC-amenable derivatives to monitor separation efficiency and isotopic fractionation effect.

2.6 Accessible amino sugar concentrations for GC-IRMS analysis

To determine the accessible concentration range of amino sugars for compound-specific δ^{13}C analysis, 10–140 ng of the two minor amino sugars found in natural marine environments (i.e. ManN and MurA) were converted to ANA derivatives and injected as pure standards into the GC-IRMS in triplicate. Additionally, isotopic analysis was validated for realistic conditions with matrix obtained from analysis of marine sediment samples. For this purpose, the δ^{13}C of pure ManN and MurA was measured after addition to the preparative HPLC fractions obtained from separation of sedimentary preparations that were either ManN-free or MurA-free.

2.7 Instrumentation

Separation and online detection of amino sugars were achieved with an Agilent 6130 MSD single quadrupole mass spectrometer coupled to an Agilent 1200 Series HPLC system via a multimode ion source in electrospray ionization mode (Agilent Technologies Deutschland GmbH, Böblingen, Germany) equipped with an Econosphere NH_2 column (250 mm × 4.6 mm, 5 mm particle size; Alltech Associates Inc., Deerfield, IL, USA).

Quantification of amino sugars was accomplished using an Agilent 6890N GC instrument coupled to an Agilent 5973 inert Mass Selective Detector (MSD) with an electron impact (EI) source, whereas compound-specific isotopic analysis of amino sugars was performed with a Trace GC Ultra instrument coupled to a Delta Plus XP isotope ratio mass spectrometer via a Combustion Interface III (Thermo Finnigan MAT GmbH, Bremen, Germany). Two columns with different polarities, an Optima 17MS column (30 m × 0.25 mm, 0.25 µm film thickness; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and an Rxi-5ms column (30 m × 0.25 mm, 0.25 µm film thickness; Restek GmbH, Bad Homburg, Germany), were applied to optimize separation of sugar derivatives.

The ANA method produced the most stable derivatives and satisfying results (see below); the corresponding GC operation conditions were specified here in detail. Separation of ANA derivatives on the Optima 17 MS column was modified from Glaser and Gross (2005). The injector temperature was 250 °C and helium was used as carrier gas at a constant flow rate of 1 $\text{mL}\,\text{min}^{-1}$. The GC temperature program was initiated at 80 °C and held for 4 min, then increased to 250 °C at 30 $^{\circ}\text{C}\,\text{min}^{-1}$ and held for 10 min, and finally raised to 280 °C at 3 $^{\circ}\text{C}\,\text{min}^{-1}$ and held for 5 min. The GC parameters for the Rxi-5ms column were as follows: injector temperature, 250 °C; carrier gas, helium; flow rate, 1.1 $\text{mL}\,\text{min}^{-1}$. The oven temperature was kept at 70 °C for 1 min, ramped to 230 °C at 20 $^{\circ}\text{C}\,\text{min}^{-1}$, held for 20 min, and further increased at 20 $^{\circ}\text{C}\,\text{min}^{-1}$ to 300 °C and held for 5 min. The mass spectrometer was programmed in selective ion monitoring (SIM) mode to target specific mass fragment ions of the derivatives for quantification. The selected ions were m/z 187 and 289 for GlcN, GalN and ManN; m/z 236 and 356 for MurA.

Stable carbon isotopic compositions of the pure amino sugars, internal standards and the acetylation reagent were determined independently by a Flash 2000 Organic Elemental Analyzer coupled to a Delta V Plus isotope ratio mass spectrometer (EA-IRMS) via a Conflo IV interface (Thermo Finnigan MAT GmbH). Quadruplicate measurements by EA-IRMS generated highly accurate δ^{13}C values of each compound.

2.8 Calculations

Isotopic compositions were expressed using the δ notation: $\delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}}1) \times 1000$ ‰, with $R = {}^{13}\text{C}/{}^{12}\text{C}$ and $R_{\text{standard}} = 0.0112372 \pm 2.9 \times 10^{-6}$; the reference standard was Vienna Pee Dee Belemite. We determined the isotopic values only for ANA derivatives of amino sugars, which exhibited the highest yields. The δ^{13}C values and the corresponding standard errors (s_{Total}) of individual amino sugars were calculated following the procedure described by Glaser and Gross (2005), with slight modification. 3-*O*-methyl-D-glucopyranose (3-*O*-Me-Glc) was applied as an internal standard to correct the isotopic fractionation during acetylation of the hydroxyl groups (Lin et al., 2010). The determination of F, a compound-specific correction factor, was based on analysis of ANA derivatives of seven external standard solutions in the concentration range of 1–20 µg per batch for ManN and MurA and 1–60 µg per batch for the other standards.

3 Results and discussion

3.1 Hydrolytic conditions for releasing amino sugar monomers

Table 1 summarizes the results of the hydrolysis tests. Hydrolysis with 6 M HCl provided the highest recoveries of amino sugars from the Wadden Sea surface sediment sample, followed by H_2SO_4 and TFA. GlcN and GalN were the most abundant amino sugars, with concentrations being one order of magnitude higher than those of ManN and MurA. Based on these results, further work aimed to optimize the neutralization and desalting steps that followed the hot HCl treatment. We observed that the HCl method yielded lower recoveries of neutral sugars compared with the other two protocols (data not shown), suggesting that partial destruction of neutral sugars occurred under the harsh hydrolysis condition. The different responses of amino sugars and neutral sugars to strong acids may be attributable to structural differences in the oligo- or polysaccharides as which they are preserved.

3.2 Comparison of neutralization and desalting methods after hydrolysis by HCl

Recoveries of amino sugar standards ranged from 77 % to 98 % using the procedure proposed by Zhang and Amelung (1996), i.e. neutralization with 1 M KOH and desalting with MeOH (Table 2). We did not observe significant differences in amino sugar recoveries using standard compounds after employing either the ENVI-Carb™ Plus SPE cartridge or the method described by Zhang and Amelung (1996). However, for surface sediment samples, recoveries using the former method exceeded those of the latter by a factor of 1.5 to 2 (Table 1), suggesting that the SPE method is less sensitive to the presence of sedimentary matrices. Moreover, we noted that the SPE cartridge allowed for excellent recoveries of neutral hexoses such as glucose, galactose, and mannose (data not shown).

The other two methods tested in this study yielded less satisfying results. Ag_2CO_3 reacts with HCl to form CO_2 and AgCl, which has very low solubility in water and eliminates the need for a desalting step. However, with the exception of ManN, major losses of amino sugars were observed (Table 2), presumably owing to interactions between amino sugars and the AgCl–Ag_2CO_3 mixture. Cation exchange using the Dowex 50WX8 cation exchange resin allows for separation of amino sugars (in the NH_4OH fraction) from neutral sugars (in the H_2O fraction). Recoveries of amino sugars by this method were highly variable (Table 2), partly because of the incomplete evaporation of NH_4OH, which might affect the efficiency of ANA derivatization.

3.3 Derivatization of amino sugars

Although the AA derivatization method has been employed previously to investigate amino sugars (Fox et al., 1995, 1996), it was rather time consuming (~ 1 d; cf. Fox et al., 1983), and the derivative of MurA exhibited poor stability compared with the corresponding hexosamine derivatives (data not shown). The addition of *N*-methylimidazole as a catalyst helped to accelerate the acetylation of hexosamines, but it failed to produce an appreciable signal peak for MurA derivative (Whiton et al., 1985). By contrast, the acetylation time of the ANA derivatization method was reasonably low (20 min), and the derivatives remained stable at −20 °C for up to 1 yr, as confirmed by repeated injections of amino sugar ANA derivatives over the course of 12 months (data not shown). Each amino sugar, including MurA, yielded a single, well-resolved chromatographic peak during GC analysis with either an Rxi-5ms or an Optima 17MS column, when the injected amount was $<$ 120 ng per amino sugar. We therefore concluded that the ANA method was superior to the AA approach for GC-based analysis of amino sugars and subsequently implemented this step in our method optimization. Like Glaser and Gross (2005), we also noted that the elution order of ANA derivatives differed between the two GC columns (Fig. 2a and b). The availability of two alternative separations was advantageous for determining the δ^{13}C values of the minor components, which co-eluted with major peaks when using the Optima 17MS column, but were separable when using the Rxi-5ms column for environmental samples.

3.4 Performance of the preparative HPLC procedure

MurA was the primary target compound when developing the preparative HPLC protocol because it serves as a specific biomarker for bacteria but is typically low in abundance in subseafloor sediments (Fig. 2c). ManN, another minor amino sugar, could not be isolated from GlcN and GalN using this approach. Nevertheless, preparative HPLC was beneficial for isotopic analysis of ManN due to partial removal of the sample matrix, which allowed us to concentrate the sample to a small volume for GC injection. We observed minimal losses of GalN and MurA after preparative HPLC, but recoveries of GlcN and ManN were only 87 % and 71 %, respectively (Table 3). The F2 and the waste fractions contained less than 1 % of the original GlcN and ManN, suggesting that GlcN and ManN were either lost via preferential adsorption on the column or derivatization of these two hexosamines was particularly sensitive to the presence of ammonium formate originating from the HPLC eluents.

3.5 Summary of the protocol and isotope data assessment

Figure 3 summarizes the optimized pretreatment procedure. In brief, amino sugars are released from marine sediments by hot HCl. The hydrolysate then undergoes a series of purification steps, including filtration with combusted glass fiber filters, evaporation under vacuum, neutralization with KOH solution, and desalting with the ENVI-Carb™ Plus SPE cartridge. After freeze-drying overnight, amino sugars in the extract are transformed to ANA derivatives and further purified by a self-packed silica gel column. The recoveries of the whole procedure, estimated from parallel tests ($n = 3$) using a mixture of amino sugar standards, were 111.6 ± 1.9 %, 95.0 ± 9.2 %, 47.0 ± 3.8 % and 102.5 ± 2.6 % for GlcN, GalN, ManN, and MurA, respectively. The lower recovery of ManN may result from successive preferential losses during each step and is probably related to its steric structure, but the underlying mechanism is not yet fully understood.

It is established that derivatization with acetic anhydride to ANA derivatives is associated with an isotope effect (Glaser and Gross, 2005; Decock et al., 2009); this effect can be corrected with the use of a derivatization standard such as 3-*O*-Me-Glc (e.g. Lin et al., 2010). After processing authentic amino sugar standards using our proposed preparation procedure (Fig. 3) and correcting for the isotope effect, the δ^{13}C values measured by GC-IRMS ($\delta^{13}C_{\text{AS, GC}}$) were in good agreement with those obtained by EA-IRMS ($\delta^{13}C_{\text{AS, EA}}$; Table 4), indicating negligible isotopic fractionation during the other sample pretreatment steps. The standard errors of repeated injections for GC-IRMS were less than 1 ‰, but the total errors were up to 1.4 ‰, i.e. about 1 ‰ greater than those reported for the HPLC-based method developed for soils (Bodé et al., 2009). The total errors impose constraints on the isotopic resolving power of our method and should be taken into account during data interpretation.

For samples that exhibited low signals of MurA and/or ManN due to the high background generated by sedimentary matrix or high abundance of the adjacent peak of GalN (i.e. the Peru Margin sample), amino sugar extracts were separated into two fractions (i.e. hexosamine fraction and MurA fraction) via the preparative HPLC procedure described above (Fig. 3), which could be further concentrated for GC-IRMS analysis. Tests using authentic amino sugar standards confirmed that this additional step did not introduce significant isotopic fractionation, as the δ^{13}C values (Table 3) deviated from those obtained by EA-IRMS (Table 4) by less than 0.6 ‰.

The δ^{13}C values of ANA derivatives of ManN and MurA were consistent with the EA-IRMS reference values within the range of 20 to 140 ng of non-derivatized compound, i.e. equivalent to ~ 8–56 ng of amino sugar carbon (Fig. 4), regardless of the presence of sedimentary matrix; we note that these two amino sugars are usually expected to have the lowest concentration in environmental samples (e.g. Guggenberger et al., 1999; Niggemann and Schubert, 2006; Carsten et al., 2012). At quantities below 20 ng, indistinct peaks precluded proper evaluation of the isotopic composition. The minimum threshold of 20 ng of amino sugar on GC-IRMS is one order of magnitude lower than the values reported for HPLC-IRMS (Bodé et al., 2009) and enables the isotopic analysis of amino sugars at trace levels, as required by their low abundances in subseafloor sediment.

3.6 Abundance and isotopic composition of individual amino sugars in marine sediment

Figure 5 summarizes the concentrations and δ^{13}C values of individual amino sugars from four marine sediment samples determined according to the protocol described in Fig. 3. GlcN, accounting for 43.7–63.0 % of the total amino sugars, was the most abundant amino sugar in all investigated samples, followed by GalN (29.0–39.7 %) and ManN (4.4–12.4 %), while MurA was always < 5 %. The δ^{13}C values of GlcN and GalN were similar and fell between $\delta^{13}C_{TOC}$ and $\delta^{13}C_{DIC}$ in sediment collected from the Black Sea, Marmara Sea, and Peru Margin. In the cold seep sample from the Pakistan Margin, GalN was depleted in ^{13}C by 9 ‰ compared to GlcN. This sample was collected from a microbial mat composed of anaerobic methanotrophic archaea (ANME) group 2 and associated bacterial partners (Yoshinaga et al., 2012). These organisms were likely the predominant sources of TOC in the seep sample, which also exhibited the lowest $\delta^{13}C_{TOC}$ (−40.0 ‰). ManN exhibited δ^{13}C values ranging from −31.5 ‰ to −10.9 ‰ and showed no consistent trend relative to $\delta^{13}C_{TOC}$ and $\delta^{13}C_{DIC}$, or to the other amino sugars. MurA spanned the widest range in δ^{13}C (from −46.0 ‰

to −13.6 ‰), and was more depleted than $\delta^{13}C_{TOC}$ in the Pakistan Margin, Black Sea, and Peru Margin samples.

GlcN and GalN are typically the major amino sugars found in sediment and preserved in the form of peptidoglycan, lipopolysaccharides, and pseudopeptidoglycan from prokaryotes (Schleifer and Kandler, 1972; Kandler et al., 1998; Madigan and Martinko, 2005). Moreover, GlcN has also been detected in most fungal cell walls and invertebrate exoskeletons as chitin (Müller et al., 1986; Liang et al., 2007; Davis et al., 2009). It is generally believed that the hexosamines persist after cell death and are stable in soils (Glaser et al., 2004). Assuming a similar behavior in marine sediment, the hexosamines are likely derived from a diverse assemblage of pelagic and sedimentary organisms and have been preserved in the sediment. The lack of a significant discrepancy between the $\delta^{13}C$ values of GlcN and GalN (usually < 3.5 ‰) implies a common, likely allochthonous source in the Black Sea, Marmara Sea, and Peru Margin sediments. The distinct isotopic compositions of GlcN (−28.2 ‰) and GalN (−37.2 ‰) in the Pakistan Margin cold seep sample can be best explained by an increasing fraction of these amino sugars from autochthonous microbes that utilize ^{13}C-depleted carbon for biosynthesis, as known from other biomolecules at similar sites (e.g. Lin et al., 2010; Schubotz et al., 2011). This process, and others, such as organic matter diagenesis or zones of enhanced microbial activity in specific sediment horizons, could be further examined by application of this method to define the heterogeneity of $\delta^{13}C$ signatures of individual hexosamines in the downcore sediment profiles.

In contrast to the hexosamines with their diverse origin, MurA is a diagnostic bacterial biomarker because it is exclusively derived from peptidoglycan in bacterial cell walls (Schleifer and Kandler, 1972) and is presumed to rapidly degrade after cell death (Moriarty, 1977). The relatively high abundance of MurA (4.2 % of total amino sugars) and its strong ^{13}C-depletion ($\delta^{13}C$ of −46.0 ‰) in the Pakistan Margin sample are consistent with our expectations of cold seep sediment that hosts microbial biomass fueled by methane (Hinrichs et al., 1999). Likewise, the ^{13}C-depleted MurA in the Black Sea sample is probably an indigenous signal resulting from bacteria that utilize relatively

^{13}C-depleted carbon at this depth (1.57 mbsf), which is close to the sulfate-methane transition zone, based on this site's methane profile (Zabel et al., 2013). Although compound-specific isotopic analysis of amino sugars is more demanding than that of membrane lipids, these observations of putative indigenous bacterial signals in the form of MurA make this compound a valuable addition to the targets selected for isotopic analysis of microbial biomass in the deep marine biosphere, which so far has been based on lipids and intact cells and biased towards detection of signals of the Archaea (cf. Biddle et al., 2006; Schubotz et al., 2011).

4 Conclusion

Methods for the carbon isotopic analysis of amino sugars have been developed for soils but not for marine sediments, where amino sugar concentrations tend to be lower. We tested various steps in the workflow of amino sugar analysis in order to establish a robust protocol for the stable carbon isotopic analysis of amino sugars in marine sediments. A combination of the most effective steps, including a new SPE protocol for the post-hydrolysis clean-up and a new step for enrichment of amino sugars via preparative HPLC, resulted in a protocol optimized for GC-based isotopic analysis of amino sugars at trace levels (limit of detection = 20 ng; equivalent to ~ 8 ng of amino sugar C). Moreover, use of the protocol did not introduce significant isotopic fractionation during sample preparation, except for the derivatization step, which can be accounted for with a derivatization standard. Applying this protocol, we determined for the first time the carbon isotopic composition of amino sugars in marine sediment samples. The stable carbon isotopic values of hexosamines indicated a major contribution from organic detritus, whereas MurA was more sensitive to an indigenous and active bacterial community. This method thus enables investigation of the stable carbon isotopic compositions of amino sugars to infer microbial metabolism in the deep marine biosphere.

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Table 1. Amino sugars liberated from surface sediment by different hydrolytic procedures. The sample used in this test was collected from the Wadden Sea. All the amino sugars were quantified by GC-MS in SIM mode ($n = 3$); the ions selected for detection were m/z 187 and 289 for GlcN, GalN and ManN; m/z 236 and 356 for MurA.

Hydrolysis and neutralization methods	Reference	GlcN ($\mu g\,g^{-1}$ dw)	GalN ($\mu g\,g^{-1}$ dw)	ManN ($\mu g\,g^{-1}$ dw)	MurA ($\mu g\,g^{-1}$ dw)
12–1.2 M H_2SO_4; $Ba(OH)_2$	Cowie and Hedges (1984)	8.8 ± 1.1	4.7 ± 0.8	0.7 ± 0.1	0.1 ± 0.2
4 M TFA; 105 °C, 4 h; evaporation	Amelung et al. (1996)	1.2 ± 0.1	1.0 ± 0.4	0.3 ± 0.5	0.2 ± 0.1
6 M HCl; 105 °C, 8 h; KOH–MeOH	Zhang et al. (1996)	17.6 ± 0.8	15.7 ± 0.6	1.5 ± 0.2	0.5 ± 0.2
6 M HCl; 105 °C, 8 h; KOH–SPE	This study	34.8 ± 0.3	26.0 ± 0.7	2.2 ± 0.3	0.7 ± 0.02

Abbreviations: GlcN, glucosamine; GalN, galactosamine; ManN, mannosamine; MurA, muramic acid.

Table 2. Recoveries of HCl-treated amino sugar standards after different neutralization and desalting procedures ($n = 3$).

Methods	Reference	GlcN (%)	GalN (%)	ManN (%)	MurA (%)
KOH–MeOH	Zhang et al. (1996)	95.4 ± 4.7	86.5 ± 4.2	77.3 ± 9.6	97.8 ± 10.7
Ag_2CO_3	Neeser and Schweizer (1984)	26.4 ± 6.7	12.0 ± 1.0	82.8 ± 6.4	7.0 ± 0.6
Dowex 50WX8 H_2O	Amelung et al. (1996)	7.0 ± 1.5	8.7 ± 1.9	8.5 ± 1.8	7.1 ± 0.2
Dowex 50WX8 NH_4OH	Amelung et al. (1996)	104.5 ± 29.9	63.7 ± 23.9	71.6 ± 23.7	107.7 ± 28.5
KOH–SPE	Our proposed method	103.6 ± 6.6	92.7 ± 5.5	89.4 ± 4.9	95.0 ± 2.4

Table 3. Recoveries and δ^{13}C values of amino sugar standards after separation by preparative HPLC. Standard errors of GC-IRMS measurements (s_{GC}) are reported for individual amino sugars and the total analytical error of δ^{13}C values (s_{Total}) was calculated according to error propagation ($n = 3$).

Fraction	Amino sugars	Recovery (%)	GC-IRMS		
			$\delta^{13}C_{\text{AS, GC}}$ (‰)	s_{GC} (‰)	s_{Total} (‰)
1	GlcN	86.6 ± 1.5	−20.6	0.26	1.57
	GalN	113.8 ± 3.0	−20.1	0.36	1.73
	ManN	70.8 ± 2.8	−19.8	0.75	1.44
2	MurA	105.8 ± 6.7	−20.9	0.35	1.02

Table 4. δ^{13}C values of amino sugar standards analyzed by EA-IRMS ($\delta^{13}C_{AS,EA}$) or according to pretreatment procedure described in Fig. 3 prior to GC-IRMS analysis ($\delta^{13}C_{AS,GC}$). s_{EA} and s_{GC} stand for standard errors of EA- or GC-IRMS measurements, respectively. s_{Total} is the total analytical error of δ^{13}C values of individual amino sugars calculated according to error propagation ($n = 3$).

Compounds	EA-IRMS Pure standard		GC-IRMS Hydrolyzed standard		
	$\delta^{13}C_{AS,EA}$ (‰)	s_{EA} (‰)	$\delta^{13}C_{AS,GC}$ (‰)	s_{GC} (‰)	s_{Total} (‰)
3-*O*-Me-Glc	−21.62	0.02	−21.6	0.32	0.95
Ino	−30.25	0.02	−29.2	0.65	1.54
GlcN	−20.02	0.03	−19.9	0.41	1.34
GalN	−20.06	0.02	−20.1	0.29	1.36
ManN	−20.32	0.05	−20.1	0.98	1.32
MurA	−20.77	0.02	−20.8	0.30	0.84
Acetic anhydride	−38.55	0.03	–	–	–

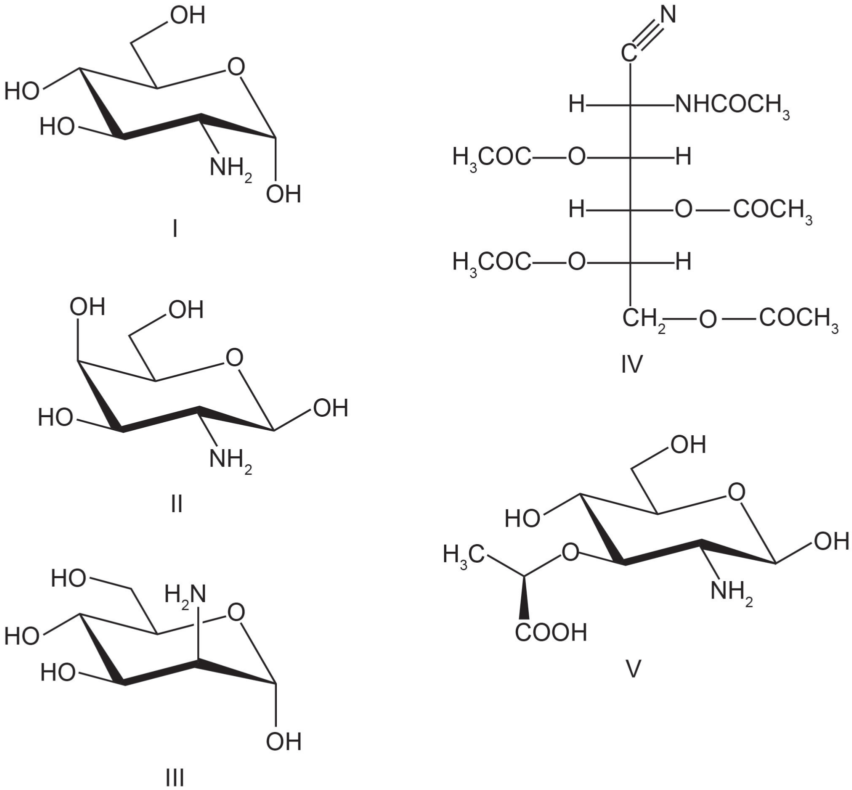

Fig. 1. Structures of amino sugars and aldononitrile acetate (ANA) derivative: glucosamine (I), galactosamine (II), mannosamine (III), ANA derivative of glucosamine (IV) and muramic acid (V).

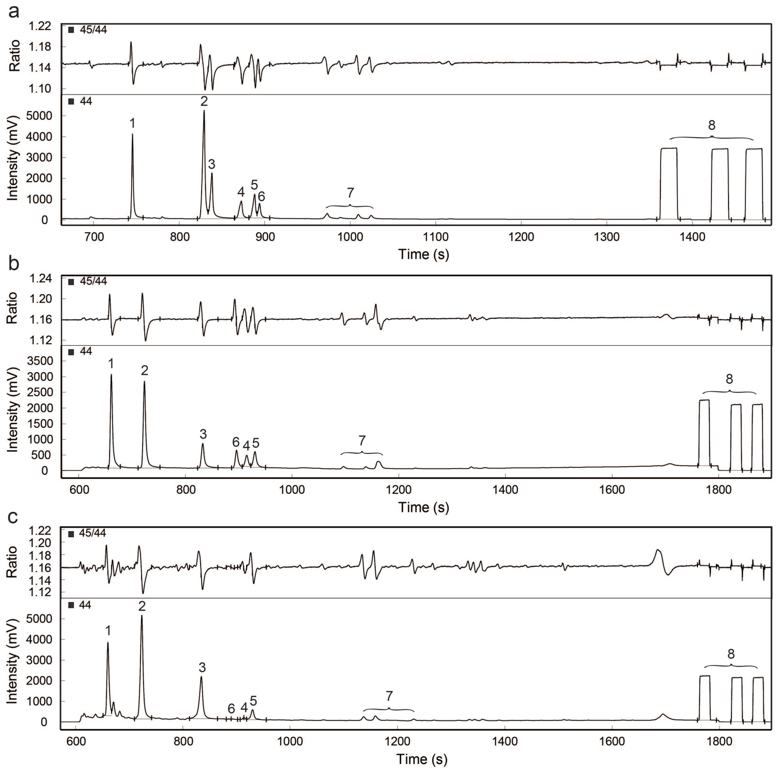

Fig. 2. GC-IRMS chromatograms of a standard mixture containing 100 ng of each amino sugar separated by an Rxi-5ms column **(a)** and an Optima 17MS column **(b)**, respectively, and a marine sediment sample from the Peru Margin separated by an Optima 17MS column **(c)**. Key to peak numbers: aldononitrile derivatives of 3-*O*-methyl-D-glucopyranose (internal standard) (1), myo-inositol (2), glucosamine (3), mannosamine (4), galactosamine (5), muramic acid (6), unknown compounds (7) and reference CO_2 gas (8).

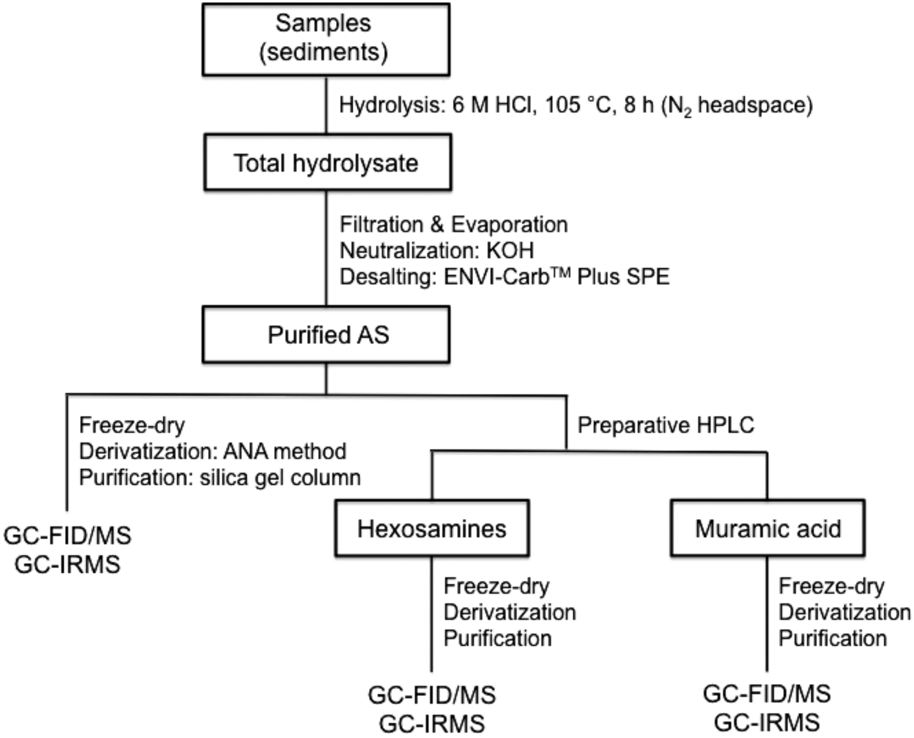

Fig. 3. Schematic of the optimized procedure for isotope analysis of amino sugars. Each step has been validated and/or optimized in this study.

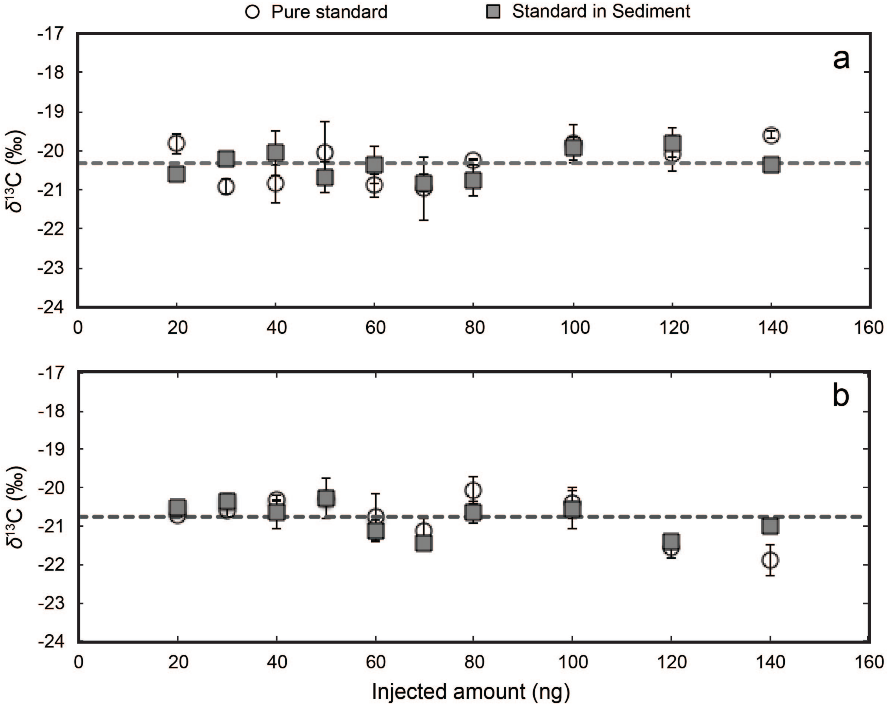

Fig. 4. Stable carbon isotopic analysis of ManN **(a)** and MurA **(b)** in a range of 20 to 140 ng. Open circles represent the $\delta^{13}C_{AS,GC}$ values of pure standards. Solid squares designate the $\delta^{13}C_{AS,GC}$ values of the same standards spiked into sediment extract that did not contain the corresponding amino sugar. The dashed lines are the isotopic values of ManN and MurA determined by EA-IRMS, respectively ($n = 3$). The error bars represent for the total analytical errors.

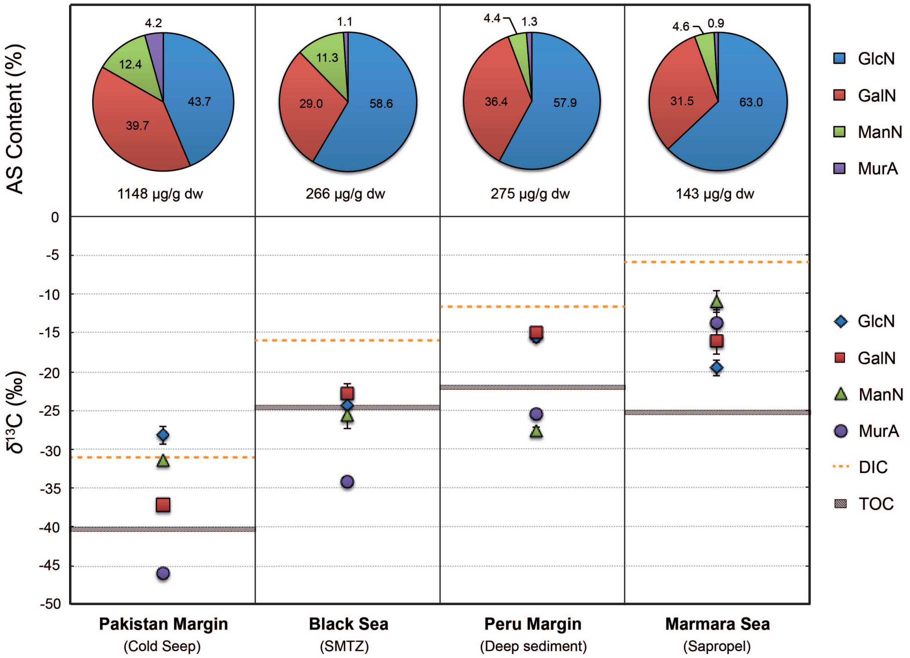

Fig. 5. Concentrations and stable carbon isotopic values of amino sugars released from selected marine sediment samples. The depositional settings of the sediment samples are indicated in parentheses. Error bars indicate the total analytical errors (s_{Total}) of δ^{13}C values of individual amino sugars calculated according to error propagation. The isotopic values of dissolved inorganic carbon (DIC) and total organic carbon (TOC) are also plotted for comparison.

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