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The contribution of macrophyte-derived organic matter to microbial biomass in salt-marsh sediments: Stable carbon isotope analysis of microbial biomarkers

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Abstract

Stable carbon isotope ratios of bacterial biomarkers were determined to infer sources of organic carbon used by bacteria in the sediments of three salt marshes. Biomarkers studied were polar lipid-derived fatty acids (PLFA), mainly bacteria-specific, methyl-branched i15 : 0 and a15 : 0. Experiments showed that isotopic fractionation between substrate and biomarkers was relatively constant (−4 to −6‰, on average) compared to the wide range in $^{13}C/^{12}C$ ratios of carbon sources found in the studied marshes. At the *Spartina* site of the Waarde Marsh (The Netherlands), biomarker $^{13}C/^{12}C$ ratios were depleted by approximately 6‰ more than expected for bacteria growing on *Spartina* litter and were similar to an unvegetated control sediment. This pattern suggested that local macrophyte production was of little importance and that other material (probably of algal origin) was the dominant carbon source for bacterial growth. *Spartina* contributed about half of the carbon in bacterial PLFA at the Kattendijke Marsh (The Netherlands) and dominated at the Great Marshes (U.S.). The variation in bacterial carbon sources in these marshes was probably related to estimated inputs of nonmacrophyte organic matter to the sediment. At the Waarde Marsh, a clear plant species effect was found as coupling between plant and bacteria was more important in *Scirpus maritimus* than in *Spartina anglica*. The contribution of local plant production to bacterial biomass in salt-marsh sediments is highly variable between marshes and depends on the input of nonmacrophyte material by sedimentation in comparison to local plant input, which in turn may differ among plant species.

Tidal salt marshes are highly productive ecosystems whose productivity is dominated by macrophytes. This high productivity supports an active microbial community both on standing dead shoots and in salt-marsh sediments (Teal and Kanwisher 1961; Howarth 1993; Newell 1993). Some of the highest mineralization rates have been detected in salt-marsh sediments, and it is generally believed that a close coupling exists between macrophyte productivity and microbial processes in these ecosystems (Howarth 1993). For instance, Howes et al. (1984) found that root and rhizome productivity was similar to sedimentary carbon mineralization rates in *Spartina* vegetation. Sulfate reduction, the dominant mineralization process in salt-marsh sediment (Howes et al. 1984; Howarth 1993), is influenced by the growth phase of the vegetation (Hines et al. 1989).

Besides macrophyte-derived material, other sources of organic matter may be important in salt-marsh sediments. In many of the European and American marshes that have a mineral sediment, the stable carbon isotope ratio of the sediment organic matter does not reflect the macrophyte material produced in these systems. For example, being a C4 genus, *Spartina* spp. have a relatively heavy carbon isotope signature of around −13‰ ($\delta^{13}C$ notation), whereas the associated sediment organic matter is more depleted in ^{13}C and ranges from −17 to −24‰ in a number of marshes (Haines 1976; Ember et al. 1987; Creach 1995; Middelburg et al. 1997). Although isotope effects during decomposition (Benner et al. 1987; Ember et al. 1987) or production by chemoautotrophic bacteria (Peterson et al. 1980) may explain some of this large difference, the most likely explanation is that a substantial part of the sediment organic matter is derived from a mixture of sources that have a depleted carbon isotope signature, like phytoplankton, benthic algae, or terrestrial material (Middelburg et al. 1997).

Stable isotope ratios have been used extensively to study sources of organic matter used by the meio- and macrofauna in salt-marsh ecosystems (e.g., Haines 1976; Peterson et al. 1985; Currin et al. 1995). These studies suggest that local macrophyte material has an important though variable contribution to the diet of these organisms. Although microorganisms are the main consumers of organic matter in salt marshes, stable isotopic compositions of microorganisms have been used only to a limited extent to directly infer their carbon sources in salt marshes (Creach 1995). This is partially caused by the difficulty of isolating microbial biomass or biomass related compounds from environmental samples. Isotope ratios of bacteria in natural systems have been studied by extracting DNA from estuarine water samples or sediments (Coffin et al. 1990; Creach 1995). Recently, Pelz et al. (1998) showed that carbon isotopic ratios of a unique bacterial amino acid (D-analine) could be determined to study sources of organic matter assimilated by bacteria in sediments and soils.

To infer sources of organic matter used by the indigenous bacteria in salt-marsh sediments, we have studied carbon iso-

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topic ratios of PLFA by gas chromatography–isotope ratio monitoring mass spectrometry (GC-IRMS [Hayes et al. 1990; Boschker et al. 1998]). PLFA have been widely used to study microbial communities in sediments, as they have several advantages over other types of biomarkers. They include several compounds, mainly methyl-branched fatty acids, that are found only in bacteria. Other types occur in all groups of organisms or predominantly in eukaryotes (Ratledge and Wilkinson 1989; Kaneda 1991). PLFA are primarily found in the cellular membrane, a structural component of the microbial cell. PLFA are readily turned over when an organism dies and are therefore associated with recently formed biomass (Parkes 1987; Tunlid and White 1992). Although the biomass of heterotrophic microorganisms has a stable carbon isotope ratio similar to the substrate on which the microbes have grown (Blair et al. 1985; Coffin et al. 1990; Hullar et al. 1996), the PLFA and lipids in general are depleted 3–6‰ with respect to the total biomass and substrate (DeNiro and Epstein 1977; Monson and Hayes 1982; Blair et al. 1985; Hayes 1993). To be able to link stable carbon isotope signatures of PLFA to carbon sources that support microbial growth, this isotopic fractionation between substrate and PLFA must be known and be relatively constant compared to the differences in isotope signatures of the primary producers in salt marshes.

In this study, carbon sources used by bacteria in *Spartina* sediments and the adjacent unvegetated sediments were studied in three different marshes. At one marsh, we also studied *S. maritimus* vegetation as representative of a C3 type of plant species with a clearly different carbon isotopic composition from that of *Spartina.* In addition, experiments were performed to study isotopic fractionation between substrate and microbial PLFA. Our results will be discussed in terms of the importance of macrophyte-derived material in comparison to other sources of organic matter that support bacterial growth in salt-marsh sediments and in terms of the implications for the general functioning of these ecosystems.

Materials and methods

Sites and sampling—From March 1996 to March 1997, samples were taken at the salt marsh near Waarde, Westerschelde Estuary, SW Netherlands. The Westerschelde Estuary is highly polluted and eutrophic (Heip and Herman 1995). Salinity at the sampling site was approximately 20‰. Three sites were sampled at the Waarde Marsh: (1) an *S. anglica*-dominated vegetation that covers most of the lower marsh, (2) patches of *S. maritimus* that are found between the *Spartina* vegetation, and (3) the unvegetated mudflat just outside the marsh. Sampling sites were within a 50-m distance of each other. The vegetated sites were at approximately the same tidal height, whereas the mudflat was situated 50 cm lower. Sediment cores were taken using a 7-cm-diameter stainless steel tube, and three depth intervals were sampled (0–8, 15–20, and 25–30 cm). The 0–8-cm interval contained most of the root biomass in the *Spartina* vegetation, whereas the root distribution in *Scirpus* vegetation was more diffuse. In the field, rhizomes were removed from a homogenized sediment subsample, and approximately 4 g wet sediment was directly added to tubes containing the PLFA extraction mixture (*see below*). The remainder of the subsample was used for carbon content and stable isotope analysis of sediment organic matter. On several occasions, sediment closely associated with roots, hereafter named rhizosphere samples, was analyzed for PLFA. For this, roots with adhering sediment were removed from the sediment with a pair of pincers and were directly added to the PLFA extraction mixture. Samples of above- and belowground plant biomass were also collected and rinsed over a 1-mm sieve to remove adhering sediment. Sediment and plant material was lyophilized and ground for analysis.

In August 1996, samples from an *S. anglica* vegetation and the adjacent mudflat were also taken from the marsh near Kattendijke at the Oosterschelde Estuary, SW Netherlands. The Oosterschelde Estuary receives very little riverine input and is less polluted than the Westerschelde Estuary (Nienhuis and Smaal 1994). Salinity at the sampling site was 31‰. At the Kattendijke Marsh, *Spartina* grows in recently formed patches of 5 to 10 m in diameter. The tidal heights of the two sites were similar. Sampling was as for the Waarde Marsh.

Several sediment samples taken in *Spartina alterniflora* vegetations and creek bottoms at the Great Marshes near Barnstable (Massachusetts, U.S.) were also analyzed for PLFA and sediment characteristics. Samples were taken in the summer of 1992 using a 3.5-cm in internal diameter polyvinyl chloride tube, and the top 10 cm was lyophilized and homogenized. The Great Marshes is an oligotrophic system situated directly on Cap Cod Bay (Redfield 1972).

Fractionation experiments—We performed several experiments to study the magnitude and variability of the isotopic fractionation between the substrate and microbial PLFA.

In a first experiment, organic substrates with a known carbon isotope ratio were incubated with a microbial inoculum under both aerobic and anaerobic, sulfate-reducing conditions. Aboveground *Spartina* litter (0.5 g; $\delta^{13}C = -13.3 \pm 0.3$‰) and cellulose (0.5 g, microcrystalline, Sigma Chemical; $\delta^{13}C = -24.7 \pm 0.3$‰) were incubated in 250 ml of 0.2-μm–filtered seawater supplied with nitrogen (25 mg of NH_4^+-N per liter) and phosphorus (5 mg of PO_4-P per liter) and were inoculated with a small amount of sediment (approximately 1% additional carbon) sampled in the *Spartina* vegetation at Waarde. Standing, aboveground *Spartina* litter was collected at Waarde and was dried and milled to pass a 0.5-mm screen. The material was extracted with hot water and methanol/dichloromethane (2 : 1 [v : v]) to remove easily extractable compounds and lipids before use as a substrate in the incubations. Incubations (18°C) were continuously shaken to ensure homogeneous conditions. Dissolved oxygen (aerobic incubations) and sulfate (anaerobic incubations) were present at the end of the experiment (data not shown). After 7 (cellulose) and 28 d (*Spartina*), particulate material was collected by centrifugation (10,000 rpm, 20 min) and extracted for PLFA as described below. Total PLFA concentrations increased at least 20-fold during the incubation period.

We also determined carbon isotopic ratios of bacterial PLFA in the rhizosphere of *S. anglica* cultures grown on

organic matter-free sediment. Sediment was sampled at an intertidal mudflat of the Oosterschelde Estuary and consisted mainly of fine sand with some clay. Organic matter was removed by combusting the sediment at 450°C for 4 h. Pots (17.5 by 17.5 cm) were filled with the combusted sediment and planted with *Spartina* seedlings that had been collected at the Waarde Marsh. Plants were grown in a climate room (18°C, 80% humidity, 16 : 8 light : dark [LD]) for 6 months before sediment with roots was collected for PLFA analysis. Pots were placed in a half-Hoagland nutrient solution in 0.2-μm filtered seawater diluted with demineralized water to give a salinity as at the Waarde Marsh, which was refreshed every month. The nutrient solution was covered with floating black polyethylene beads to avoid algal growth on the sediment. As a control, pots with no plants were run.

Characterization of macrophyte and sediment organic matter—Carbon and nitrogen contents of macrophytes and sediments were determined using a Carlo-Erba NA 1500 CN analyzer following an in situ HCl acidification procedure (Nieuwenhuize et al. 1994). Carbon isotopes have been determined using a Fisons NA1500 elemental analyzer coupled online (via a continuous flow interface) to a Finnigan Delta S mass spectrometer. All stable carbon isotope data are reported in the δ notation relative to Vienna-Pee-Dee Belemnite.

PLFA extraction and analysis—Lipids were extracted with a modified Bligh and Dyer extraction using 50-ml screw-cap centrifuge tubes (Findlay et al. 1989). The total lipid extract was fractionated on silicic acid (Merck) into different polarity classes by sequential eluting with chloroform, acetone, and methanol. PLFA in the methanol fraction were derivatized using mild alkaline methanolysis to yield fatty acid methyl esters (FAME) (Guckert et al. 1985). Internal FAME standards of both 12 : 0 and 19 : 0 were used. Identification of FAME was based on the comparison of retention time data with known standards. Additional identification was gained by GC-MS on a Hewlett-Packard Mass Selective Detector (HP 5970). PLFA short-hand nomenclature is according to Guckert et al. (1985).

Isotopic composition of individual FAME was determined by using a Varian 3400 gas chromatograph equipped with a Varian septum-equipped programmable injector, which was coupled via a type II combustion interface to a Finnigan Delta S isotope ratio mass spectrometer. An apolar analytical column (Hewlett-Packard Ultra-2, 50 m × 0.32 mm × 0.17 μm) was used with helium as carrier gas. Oven temperature was kept at 80°C for 1 min and then temperature programmed from 80 to 130°C at 40°C min^{-1} and subsequently from 130 to 260°C at 3°C min^{-1}. Several samples that were also analyzed on a polar column (Scientific Glass Engineering BPX-70, 50 m × 0.32 mm × 0.25 μm) yielded similar stable carbon isotope ratios.

To obtain the actual PLFA ratio, carbon isotope ratios of FAME were corrected for the one carbon atom in the methyl group that has been added during derivatization:

$$\delta^{13}C_{PLFA} = ((n + 1)\cdot\delta^{13}C_{FAME} - 1\cdot\delta^{13}C_{methanol})/n \quad (1)$$

where n is the number of carbon atoms in the PLFA. The methanol that was used for derivatization had a δ^{13}C ratio of −45.6 ± 0.9‰ (N = 4) as determined by GC-IRMS. Equation 1 is valid only if no isotopic fractionation occurs during derivatization (Rieley 1994). We therefore compared mild alkaline methanolysis with acidic derivatization using BF_3-methanol (Supelco), a method for which Eq. 1 has been validated (Abrajano et al. 1994; Rieley 1994). Results for both methods were within 1‰ for PLFA in a mixture of polar lipids containing 12 : 0, 14 : 0, and 19 : 0 fatty acids (Sigma) and a sediment sample (average difference ± SD = −0.1 ± 0.4‰, n = 14).

Compound-specific isotopic analysis by GC-IRMS is most reliable for baseline separated compounds (Hayes et al. 1990). The dominant bacterial markers (i15 : 0 and a15 : 0) were not separated under the chromatographic conditions used (about 10% peak overlap). Results of i15 : 0 and a15 : 0 were therefore combined by using an isotope balance:

$$\delta^{13}C_{i+a15:0} = (a_{i15:0}\cdot\delta^{13}C_{i15:0} + a_{a15:0}\cdot\delta^{13}C_{a15:0})/a_{i+a15:0} \quad (2)$$

where a is the peak area of the PLFA as determined by GC-IRMS. Combining the results of these two compounds should not have affected the interpretation of our results, as both are specific bacterial markers and have a similar distribution in bacteria (Ratledge and Wilkinson 1989; Kaneda 1991).

Results

Fractionation experiments—In incubations with *Spartina* litter and cellulose, PLFA showed a considerable variation in isotope fractionation compared to the source material (Fig. 1A). Aerobic incubations constantly gave somewhat more positive isotopic ratios compared to anaerobic incubations. The even-numbered, straight-chain PLFA (14 : 0, 16 : 1, 16 : 0, 18 : 1, and 18 : 0) showed large variations in isotope ratio and were clearly ^{13}C enriched, with the exception of anaerobic *Spartina* incubations, compared to other PLFA. A number of these compounds even showed a positive fractionation. Regardless, in all incubations, the isotopic fractionation between PLFA and carbon source was relatively constant for the bacterial biomarkers i14 : 0 ($\Delta\delta^{13}$C = −5.0 ± 0.8‰) and i + a15 : 0 ($\Delta\delta^{13}$C = −6.1 ± 1.1‰ [AVG ± SD, n = 12]). Uneven-numbered, straight-chain PLFA (15 : 0 and 17 : 0) that are found in substantial amounts in bacteria, but that have a less exclusive bacterial distribution than methyl-branched PLFA, gave similar fractionations (Fig. 1A).

We also cultured *S. anglica* on organic matter-free sediment, where only plant-derived organic matter could have supported bacterial growth. All detected PLFA were isotopically depleted compared to plant roots (Fig. 1B). The bacterial biomarkers, i14 : 0 and i + a15 : 0, showed similar average isotopic fractionation as in the incubation experiments (*see above*) of, respectively, −4.3 and −5.1 ± 1.5‰. No PLFA could be detected in the sediment of control incubations without plants.

In summary, the results of these experiments suggest that the bacterial PLFA, i14 : 0 and i + a15 : 0, were, respectively,

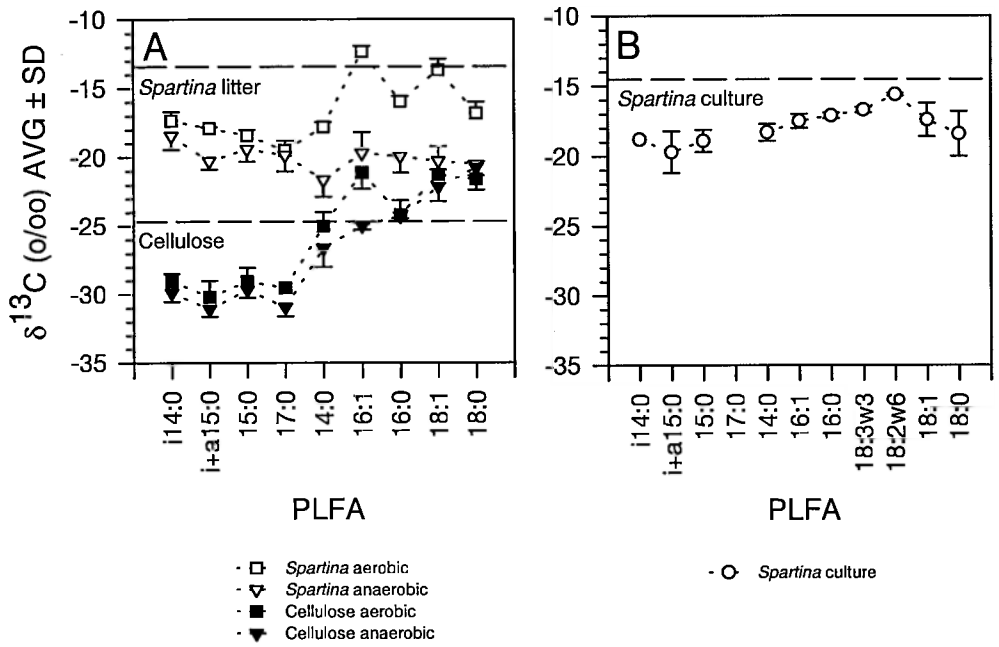

Fig. 1. Results of experiments to determine carbon isotopic fractionation between microbial PLFA and substrates used by the bacteria. Shown are stable carbon isotope ratios of PLFA as detected in incubation experiments with *Spartina* litter and cellulose under both aerobic and anaerobic conditions (A) and as found in sediment samples from a *Spartina* culture grown on organic matter-free sediment (B). The dashed lines give the average stable carbon isotope ratios of the substrates (*Spartina* litter: −13.3 ± 0.3‰; cellulose: −24.7 ± 0.3‰; and *Spartina* culture: −14.5 ± 0.2‰).

Table 1. Organic carbon content (OC), atomic carbon/nitrogen ratios (C/N), and stable carbon isotope ratios ($\delta^{13}C$) of macrophytes and sediments (AVG ± SD).

Site	Macrophyte* $\delta^{13}C$ (‰)	Sediment† % OC	Sediment† C/N	Sediment† $\delta^{13}C$ (‰)
Waarde Marsh				
Spartina anglica		1.83±0.25	15.7±1.8	−22.2±1.1
Scirpus maritimus	−12.7±0.2	1.36±0.45	14.7±1.5	−24.4±0.6
Mudflat	−24.8±0.9	0.67±0.25	13.9±0.5	−25.7±0.8
Kattendijke Marsh				
S. anglica		0.40±0.24	12.6±3.8	−19.8±1.4
Mudflat	−12.8±0.2	0.17±0.04	10.0±2.0	−20.0±0.3
Great Marshes				
Spartina alterniflora		28.1±6.4	26.5±6.7	−14.0±0.5
Creek bottom	−12.5±0.6‡	1.2±0.7	11.3±1.6	−17.8±0.7
Culture				
S. anglica	−14.5±0.2	ND§	ND	ND

* Data for belowground biomass are shown. Aboveground biomass was on average 0.6‰ lighter. Macrophytes contained between 30 and 45% C and had a C/N ratio of 35–50.
† Sediments showed little variation with depth or sampling date: therefore, depth average data are presented.
‡ Rhizome and root data from Fogel et al. (1989) and Benner et al. (1987).
§ ND, not determined.

−4.7 ± 0.8 and −5.6 ± 1.8‰ (AVG ± SD) depleted compared to the substrate used by the bacteria.

Macrophyte and sediment characteristics—Results of organic carbon and total nitrogen content and stable carbon isotope ratios in macrophytes and sediments are given in Table 1. *Spartina* spp. had a typical C4 isotopic signal of around −12.7‰. This was not reflected in the sediment organic matter at *Spartina* sites of Waarde and Kattendijke Marshes, which showed more depleted ratios of −22 and −20‰, respectively.

Waarde Marsh—The PLFA concentration patterns were similar for all marshes, and a typical PLFA chromatogram is shown in Fig. 2. PLFA with even-numbered carbon (14:0, 16:1, 16:0, 18:1, and 18:0) were more depleted than branched and uneven-numbered compounds (i14:0, i + a15:0, 15:0, and 17:0) at the Waarde Marsh (Fig. 2), which was, however, not found for sediments from other sites. This is exactly opposite to the situation found in the fractionation experiments (Fig. 1). All samples contained considerable amounts of PLFA that are only found in bacteria (e.g., i14:0, i15:0, a15:0, i16:0, several PLFA with 17 carbon atoms, and 18:1ω7c). Of these bacterial markers, only i14:0 and i + a15:0 were sufficiently separated from other compounds to allow for an accurate carbon isotopic ratio determination by GC-IRMS.

At the *Spartina* site, bacterial PLFA, i14:0 and i + a15:0 (Fig. 3A,B), showed isotopic ratios between −21 and

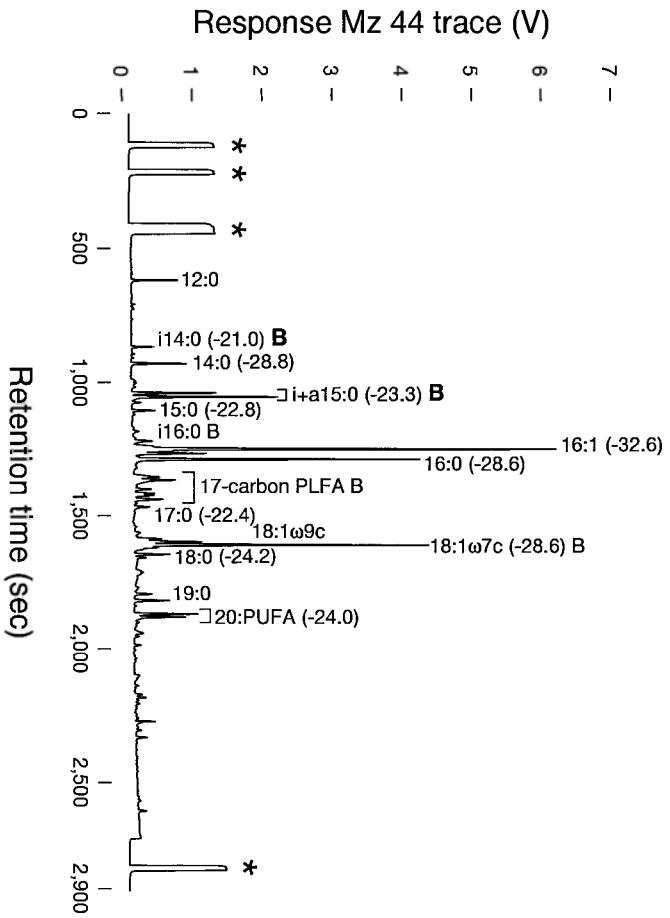

Fig. 2. A typical chromatogram of PLFA in sediment as recorded by GC-IRMS (Waarde Marsh, *Spartina* site, 0–8 cm, July 1996). Peak identity and stable carbon isotope ratios of PLFA are shown. Reference gas pulses are indicated with stars and specific bacterial compounds with "B" (bold **B**s are the main bacterial biomarkers used in this study).

−26‰ in the bulk sediment during the whole study period and were therefore more depleted than expected if bacteria were growing on *Spartina*-derived material. Moreover, ratios were very similar for the *Spartina* and the mudflat site. *Spartina* rhizosphere samples showed the same trend. Only the deeper rhizosphere samples taken in September 1996 may indicate a contribution from *Spartina*-derived material. Isotopic ratios for i14 : 0 were somewhat more variable than for i + a15 : 0, which may be the result of its much lower concentration (Fig. 2).

At the *Scirpus* site, carbon isotope ratios of the bacterial biomarkers were more depleted than at the mudflat sediments on several occasions and therefore shifted in the direction as expected for bacteria growing on *Scirpus*-derived material (Fig. 3A,B). Especially for the deeper samples taken in September 1996, i + a15 : 0 had ratios close to the expected values. Many of the *Scirpus* rhizosphere samples showed a clear macrophyte-influenced signal, which was mostly closer to the expected ratio than bulk sediments from the same sample.

Results of bulk sediment for 15 : 0 showed similar trends as the more specific bacterial markers, although several *Spartina* rhizosphere samples did show a contribution from macrophyte material (Fig. 3C). In Fig. 3D, carbon isotopic data are shown for 16 : 0; a nonspecific PLFA that generally occurs in most bacteria and eukaryotes. For instance, 16 : 0 accounted for about 25% of the total PLFA in *Spartina* roots, which suggests that it is not a good marker to detect transfer from macrophytes to the bacterial community in the sediment since a direct contribution from root PLFA can not be excluded. However, the similarity of carbon isotope ratios for 16 : 0 in bulk sediments from the *Spartina* and mudflat sites excludes both a direct contribution from plant roots and a contribution from microorganisms growing on *Spartina*-derived material, confirming the results from the bacterial PLFA.

In March 1996, washed, sediment-free *S. anglica* roots sampled at Waarde were also analyzed for PLFA. PLFA profiles were dominated by compounds that are generally found in macrophytes (16 : 0, 18 : 1ω9c, 18 : 2, and 18 : 3; Hitchcock and Nichols 1971). Only minor amounts of other PLFA (<1.5% mol mol^{-1}) were detected. Plant PLFA were depleted by about 3.5‰ compared to the total root biomass, and isotope ratios ranged from −15.2 to −17.2‰. Similar ratios were found by Canuel et al. (1997) for *S. alterniflora*.

Comparison between marshes—At the other two marshes, the i + a15 : 0 PLFA in *Spartina* sediments was clearly enriched in ^{13}C content compared to unvegetated sediments. For the Kattendijke Marsh, isotopic ratios of i + a15 : 0 in the marsh sediments were about halfway between the Kattendijke mudflat site and Great Marshes *Spartina* site. At the Great Marshes, i + a15 : 0 was on average 1.5‰ more enriched in ^{13}C than expected when bacteria were growing on *Spartina*-derived material, but the difference was not significant (t-test, $P > 0.05$). Other nonmacrophyte PLFA showed similar trends (data not shown).

Discussion

Isotopic fractionation in PLFA and selection of biomarkers—Using PLFA to infer carbon isotopic composition of the indigenous bacterial biomass could have a potential drawback since lipids such as PLFA tend to be isotopically depleted compared to the total biomass and the substrate used by the bacteria (Hayes 1993). PLFA extracted from sediment usually contain several major compounds that are only produced by bacteria, notably i14 : 0, i + a15 : 0, and 18 : 1ω7c (Fig. 2). We restricted our analysis of specific bacterial biomarkers to the methyl-branched PLFA i14 : 0 and i + a15 : 0, as it was not possible to separate 18 : 1ω7c chromatographically from 18 : 1ω9c, which is a major PLFA in macrophytes (Hitchcock and Nichols 1971). In our fractionation experiments, PLFA i14 : 0 and i + a15 : 0 showed an average offset of between −4 and −6‰ compared to the carbon source, which is in the range of what is found for lipids in bacteria (DeNiro and Epstein 1977; Monson and Hayes 1982; Blair et al. 1985). The variation in this isotopic fractionation was relatively small compared to the range of ^{13}C/^{12}C ratios of carbon sources found in the marshes. Hence, this isotopic fractionation can be used with confidence to

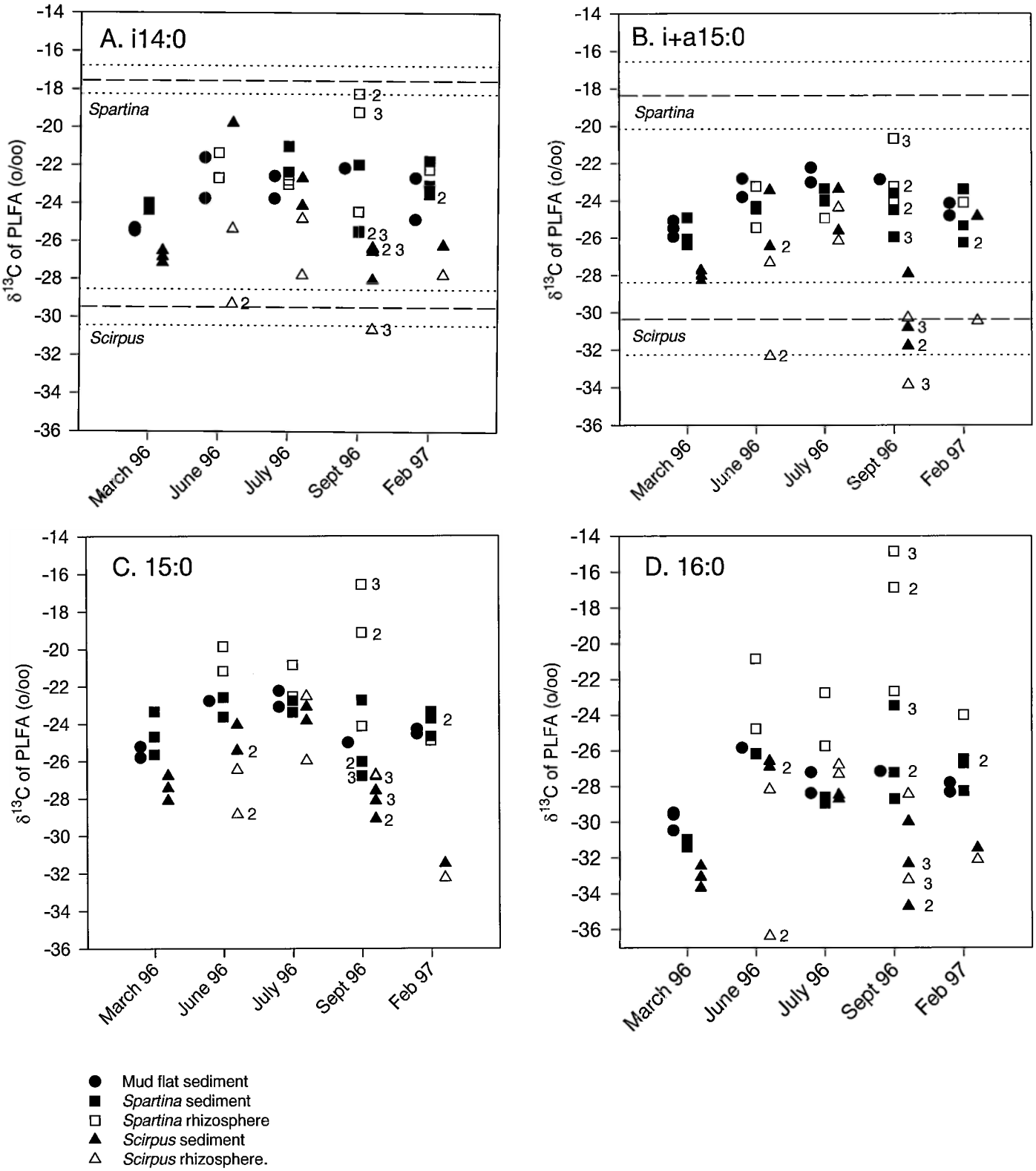

Fig. 3. Carbon isotope ratios of PLFA in the sediments of the Waarde Marsh. Shown are data for the bacterial biomarkers i14:0 (A) and i + a15:0 (B) for the more general compound 15:0 (C) and for the omnipresent 16:0 (D). The dashed lines in Fig. 3A,B show the expected stable carbon isotope ratio when bacteria were growing on *Spartina*- or *Scirpus*-derived material (AVG ± 1 SD are indicated), which are based on the stable isotope ratios in *Spartina* or *Scirpus* roots (Table 1) corrected for isotopic fractionation in bacterial lipid metabolism (Fig. 1). Numbers 2 and 3 next to symbols indicate sediment depth layers of 15–20 and 25–30 cm, respectively; the top layer (0–8 cm) is not marked.

correct measured PLFA isotope ratios to obtain the ratio of the carbon source used by the bacteria in sediments.

In contrast, even-numbered PLFA (14 : 0, 16 : 0, 18 : 0, 16 : 1, and 18 : 1) showed a more variable carbon isotopic fractionation of +4 to −8‰ (Fig. 1). Moreover, this range may even be larger, as these even-numbered PLFA were considerably more depleted than other PLFA in Waarde sediments (Fig. 2). Canuel et al. (1997) also found that even-numbered fatty acids were more depleted than uneven-numbered fatty acids in a coastal sediment. The wide range and sometimes positive fractionation found for these PLFA was rather unexpected, although Pelz et al. (1997) also found a positive offset in some bacterial fatty acids. In the current view, the mechanism of isotopic fractionation in lipid synthesis will always lead to negative shifts (DeNiro and Epstein 1977; Monson and Hayes 1982; Blair et al. 1985; Hayes 1993). However, this mechanism is based on a number of pure culture studies that used a limited number of model organisms, mainly *Escherichia coli*. In sediments, the microbial community will be very diverse, both in species composition and in metabolic capabilities, and it is not clear how this diversity will influence isotopic fractionation in fatty acid metabolism. Isotopic fractionation in microbial metabolism may also be influenced by such factors as temperature and growth rate or stage (Hayes 1993). The large variation in isotopic fractionation found for even-numbered PLFA shows that their isotope ratios should be interpreted with care.

Carbon sources at the Spartina *marsh of Waarde*—Our bacterial biomarker data (Fig. 3A,B) indicate that local macrophyte-derived material was of minor importance for bacterial growth in *Spartina* sediments at the Waarde Marsh during the whole study period. This conclusion is based on two observations. First, isotopic ratios of bacterial PLFA at the *Spartina* site were substantially more depleted than expected for bacteria growing solely on *Spartina*-derived material. Secondly, isotopic ratios were very similar for the *Spartina* and unvegetated sites, which suggests that bacteria in both sediments were using a similar substrate. Moreover, the isotopic ratios of PLFA in rhizosphere samples suggested that even in sediments closely associated with living roots, bacterial populations were mainly growing on organic matter not derived from the root itself. One may argue that only a portion of the bacterial populations is covered when methyl-branched marker PLFA like i14 : 0 and i + a15 : 0 are studied. However, these PLFA occur in a variety of bacterial groups (Kaneda 1991), and isotopic ratios of other generally occurring PLFA like 15 : 0 and 16 : 0 (Fig. 3C,D) also exclude *Spartina* as a major substrate for the bacteria in the sediment of the Waarde Marsh.

The question remains what may have been the dominant carbon source for bacteria in both the *Spartina* and mudflat sediments at the Waarde Marsh. If a fractionation of −5.6‰ for the i + a15 : 0 PLFA is used (average from data in Fig. 1), we obtain an isotope ratio of −19.6 ± 1.2‰ (N = 23; range = −17.6 to −21.8) for the bacterial substrate. This is in the range of the isotope signatures found for suspended organic matter or phytoplankton in the more saline part of the Westerschelde Estuary (−18 to −22‰; Middelburg and Nieuwenhuize 1998) and for the nearby North Sea (Salomons and Mook 1981; Dauby et al. 1992). In addition, production by benthic microalgae is important on the mudflats in the Westerschelde Estuary (Heip and Herman 1995). Benthic algae can show a wide carbon isotope range (−13 to −27‰; Creach 1995; Currin et al. 1995). However, sediments from a nearby mudflat in the Westerschelde that is covered by benthic algae also show a carbon isotope ratio of −22‰ with little temporal variation (Middelburg pers. comm.). Alternatively, it may be argued that bacteria at the *Spartina* site were growing on a mixture of C4 and C3 macrophyte-derived materials. However, this is not likely, as the relatively low C/N ratio of the sediment organic matter points toward a substantial algal component (Table 1; compare Waarde Marsh with the peaty Great Marshes). Furthermore, macrophyte root material enters the sediment at depth, and it seems unlikely that there is extensive mixing of consolidated sediments from different sites of the marsh. Our results therefore suggest that mainly algal-derived material supported bacterial growth at the *Spartina* and mudflat sites of the Waarde Marsh, although small contributions from other sources can not be excluded.

So far, we have used stable carbon isotope ratios of bacterial PLFA to infer relative contributions of *Spartina* material to bacterial growth. With some additional data, however, it is possible to make a preliminary estimate of the absolute amounts of organic carbon transferred from the plants to the sediment in the Waarde Marsh (Table 2). In salt marshes, the aboveground production may not enter the sediment, but it is partially degraded while still standing (New-

Table 2. A carbon budget for the *Spartina* site at the Waarde Marsh, The Netherlands.

	Total rate (g C·m^{-2}·y^{-1})	Contribution from *Spartina*-derived material (%)	Rate based on *Spartina*-derived material (g C·m^{-2}·y^{-1})
Sediment			
Respiration	600*	<10‡	<60
Burial	105†	30–45§	30–50
Total			30–110
Macrophyte			
Belowground production			>600‖

* Based on measured sediment CO_2 fluxes (Middelburg et al. 1997) that most likely include both sediment and root respiration.

† Calculated from the sediment accumulation rate (0.9 cm·y^{-1} [Zwolsman et al. 1993]), the sediment organic carbon content (Table 1), and specific weight (calculated from composition of fresh sediment).

‡ Estimated from the carbon isotopic ratios of bacterial i + al5 : 0 PLFA at the *Spartina* site (0–8 cm horizon: Fig. 3B) using a two-source isotopic mixing model, with the isotopic ratio of the bacterial PLFA at the mudflat site and the expected isotopic ratio for growth on *Spartina* as end members.

§ Calculated from the carbon isotope ratios of the sediment (Table 1) using a two-source mixing model with the *Spartina* roots and the mudflat sediment as end members. Lower value calculated with data on *Spartina* roots in Table 1: higher value calculated by assuming that only lignin was buried (*Spartina* lignin $\delta^{13}C$ = −17.4‰ [Benner et al. 1987]).

‖ Based on aboveground biomass production (Middelburg et al. 1997) and assuming that belowground production is at least similar to aboveground production (Hemminga et al. 1996).

ell et al. 1985) or eroded from the marsh sediment (Dame 1994). However, more than half of the annual production is allocated belowground in the root system of *Spartina* species (Schubauer and Hopkinson 1984; Hemminga et al. 1996), and it seems unlikely that much of this root material is lost by erosion. Root-derived organic matter that actually enters the sediment therefore must be either mineralized by microorganisms or buried as sediment organic matter (Table 2). Although there are uncertainties in the data presented in Table 2, they suggest that transfer of material from the roots to the sediment can only explain between 5 and 20% of the carbon that is allocated in the belowground biomass during a growing season. The most likely explanation is that organic matter allocated to the roots is mainly used again by the plant itself. In perennial grasses such as *Spartina*, a substantial part of the carbon allocated belowground is laid down as storage products like sucrose and starch, which remain mobile and can be used for root respiration or regrowth of shoots in spring (Lytle and Hull 1980). In addition, it can not be excluded that some net growth of roots and rhizomes occurred at the Waarde Marsh during the study period. Our estimates of organic carbon transfer between *Spartina* and sediment (30–110 g C m^{-2} yr^{-1}) are in the same range as found for rhizodeposition studies in terrestrial grasses (Whipps 1990), suggesting that they are not unrealistically low. Thus, in the Waarde Marsh, the low contribution of local *Spartina* to microbial carbon mineralization processes seems to be caused by a combination of two effects: relatively high inputs of nonmacrophyte material and relatively small transfer of organic matter between plant and sediment.

Comparison between Spartina *marshes*—In the two other *Spartina* marshes studied, a clear contribution from the local plant production was detected. The *Spartina* samples from the Great Marshes had isotopic ratios for i + a15:0 that were close to the expected values if bacteria were growing on C4-derived material, whereas at the Kattendijke Marsh, more than half of the carbon in bacteria PLFA came from *Spartina* (Fig. 4). The variation in carbon sources used by the bacteria in these marshes is inversely related to the sedimentation rate of nonmacrophyte organic matter to the sediment, as is reflected in the sediment composition of these marshes (Middelburg et al. 1997). The Waarde Marsh is situated on the highly eutrophic Westerschelde Estuary fed by the polluted River Schelde (Heip and Herman 1995). In addition, salt-marsh accretion rate is high (0.9 cm yr^{-1}; Zwolsman et al. 1993), mainly due to the sedimentation of silts with a relatively high organic matter content (Table 1). The marshes on the Oosterschelde (Kattendijke Marsh) have an accretion rate similar to the Waarde Marsh (Oenema and DeLaune 1988; Callaway et al. 1996), but the Oosterschelde receives little direct riverine input (Nienhuis and Smaal 1994). At the Kattendijke Marsh, this results in a more sandy sediment with a much lower organic matter content than the Waarde Marsh (Table 1). The carbon isotopic composition of the sediment organic matter at both of these marshes suggests that a substantial part is not derived from *Spartina* material (Table 1). The Great Marshes have a much lower sediment accretion rate (0.15–0.2 cm yr^{-1}; de Rijk 1995) than the two European marshes, which is mainly the result

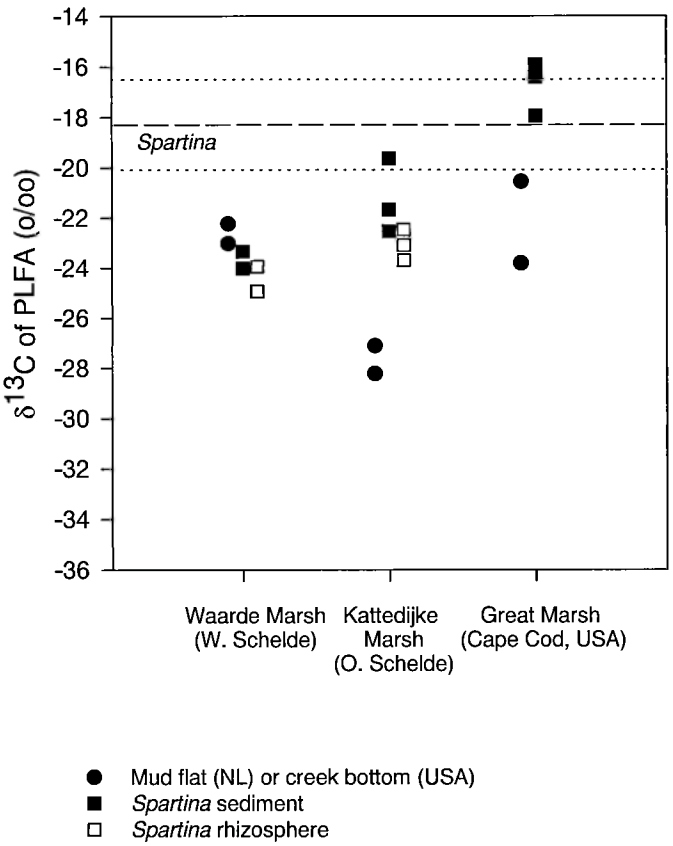

Fig. 4. Stable carbon isotope ratios in the bacterial biomarker PLFA i + a15:0 as determined in the sediment of several *Spartina* salt marshes and the adjacent unvegetated sediments. The dashed lines show the expected stable carbon isotope ratio for bacteria growing on *Spartina*-derived material (AVG ± 1 SD are indicated, *see legend for Fig. 3*). The Waarde Marsh data are from July 1996.

of the *Spartina* peat accumulation (Redfield 1972). It is an even more oligotrophic system that probably receives very little external input, consisting mostly of organic poor silt and sand (Redfield 1972; de Rijk 1995). It therefore seems that the input and availability of nonmacrophyte material to the sediments of salt marshes is an important factor in determining the relative importance of local macrophyte material for bacterial mineralization processes.

Also in absolute terms, the carbon cycle at the Waarde Marsh seems to contrast with data from *Spartina* marshes on the East Coast of the U.S. As discussed above, only a small part of the annual belowground production can be traced in the sediments of the Waarde Marsh. However, our results from the Great Marshes suggest that bacterial growth in this marsh was mainly based on local plant production, and others have shown that sediment carbon mineralization rates are similar to the annual belowground production in East Coast marshes (Howarth and Teal 1979; Howes et al. 1985). This suggests that most of the belowground production in these East Coast marshes eventually enters the sediment, where it is mineralized by microorganisms. We can only speculate on the reasons for this difference in functioning of the *Spartina* vegetation at the Waarde Marsh and the Great Marshes; however, there are several distinct differenc-

es. First, the species studied are different: *S. anglica* at the Waarde Marsh and *S. alterniflora* at the Great Marshes. Although belowground productivity data for *S. anglica* are scarce, a comparison of ratios between below- and aboveground productivity suggests that *S. alterniflora* invests more carbon in its rooting system than *S. anglica* (Hemminga et al. 1996). Secondly, a substantial part of the aboveground biomass may enter the sediment at the Great Marshes, resulting in a coupling between plant and carbon mineralization that is not only linked through the belowground biomass. This, however, seems unlikely since macroremains in the *Spartina* peat at the Great Marshes are mainly derived from root and rhizome material and only to a small extent from shoots and leaves (de Rijk 1995). Thirdly, the age of the marshes may be a factor, as the Great Marshes site is much older (>1,000 yr; Redfield 1972) than the Waarde Marsh (35–40 yr; de Jong pers. comm.), which may have had less time to accumulate large amounts of plant litter or to reach a steady state with respect to its belowground biomass. Finally, the higher input of plant material at the Great Marshes may be related to its oligotrophic character. It is well known that terrestrial plants invest more carbon in their root biomass when they grow on nutrient-poor soils (Brouwer 1962).

Comparison of S. anglica *with other plant species*—At Waarde, *S. maritimus* sediments were also studied because this plant is a C3 type with a more depleted carbon isotopic signal of −25‰. Several of the rhizosphere samples and deeper sediment layers from the *Scirpus* site showed a clear contribution of depleted, C3-derived organic matter in the microbial biomass (Fig. 3). Rhizosphere samples in many cases showed a higher contribution of C3-derived material than the associated bulk sediment, which is in agreement with a local *Scirpus* source. The depth distribution with a higher contribution from plants in deeper sediments also agrees with a local plant source. Algal-derived organic matter that enters the sediment from the top will become progressively older and therefore probably more resistant to bacterial degradation in deeper sediment layers. However, fresh root-derived organic matter enters the sediment at depth and will therefore have a similar degradability in the whole sediment column. It may be argued that the relatively high importance of local *Scirpus* material is the result of a lower input of nonmacrophyte material. The organic carbon content at the *Scirpus* site was indeed lower than at the *Spartina* site (Table 1), but this difference was rather small. Furthermore, the *Scirpus* site was situated between *Spartina* vegetation and at the same tidal height, arguing for a similar sedimentation of suspended material from the estuary. The relatively high contribution from local plant material is therefore most likely due to a higher input from roots in *Scirpus* compared to *Spartina*, suggesting that coupling between plant and sediment is a species-specific process.

A direct link between plant-derived organic matter and microbial processes in sediments has been detected for a limited number of other wetland plant species. Minoda and Kimura (1994) showed that methane produced in the rhizosphere of rice plants is from 15 to 90% derived from recently fixed organic matter, depending on the growth stage of the plants. Several seagrass species also show a tight coupling. About 11% of the carbon fixed by a tropical seagrass vegetation is transferred to the sediment microbial biomass within 6 h (Moriarty et al. 1986). A diel variation in sedimentary bacterial productivity and sulfate reduction rates has been detected in seagrass vegetations, which implies a link between exudation of organic matter by roots and photosynthesis (Moriarty and Pollard 1982; Pollard and Moriarty 1991). Moreover, sulfate reduction rates are much higher in meadows of *Zostera* spp. compared to nearby unvegetated sites (Isaksen and Finster 1996; Holmer and Nielsen 1997). In summary, these data support our finding that there may be species-specific differences in coupling between plant and microbes as found for *S. maritimus* and *S. anglica* at the Waarde Marsh.

Conclusions

We have shown that stable carbon isotope ratios of bacterial PLFA can be used to study sources of organic matter utilized by bacteria in ecosystems. As bacteria or microorganisms in general belong to the main consumers in sediments, this approach can be used to trace carbon flow in estuarine and coastal environments where potentially important sources of organic matter show a large range of carbon isotopic ratios.

We used this approach to study the origins of organic carbon used by bacteria in salt-marsh sediments, concluding that the importance of local macrophyte production is highly variable between marshes and between plant species. In marshes with a relatively high sedimentation rate of nonmacrophyte organic matter, like the Waarde Marsh, algal-derived material is probably the dominant source used by bacteria in the sediments of *Spartina* vegetations, and local plant production is of minor importance. A clear plant species effect was found, as coupling between plant and bacteria was more important in *S. maritimus* than in *S. anglica*.

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