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Identification of phytodetritus-degrading microbial communities in sublittoral Gulf of Mexico sands

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Abstract

We identified microbial taxa that catalyze phytodetritus degradation and denitrification in permeable coastal sediments of the northeast Gulf of Mexico. Stable isotope probing experiments were used to track the assimilation of isotopically labeled substrate into bacterial deoxyribonucleic acid (DNA) and directly link the taxonomic identification of benthic microorganisms with particulate organic matter degradation and denitrification activity. Phytodetritus deposition events were simulated in the laboratory by the addition of ^{13}C-enriched, heat-killed *Spirulina* cells to intact sediment core incubations. Immediate increases in O_2 consumption (3-fold), N_2 efflux (16-fold), and dissolved inorganic nitrogen efflux were observed after phytodetritus addition relative to unamended treatments, suggesting that the benthic microbial community was poised to immediately begin oxidizing deposited organic matter. Analyses of 16S ribosomal ribonucleic acid gene sequences amplified from ^{13}C-enriched DNA fractions demonstrated that members of the *Gammaproteobacteria* (*Vibrionales* and *Alteromonadales*), *Deltaproteobacteria*, *Actinobacteria*, *Verrucomicrobia*, and *Planctomycetes* metabolized the phytodetritus amendment. Terminal restriction length polymorphism analyses showed increases in the relative abundance of *Gammaproteobacteria*, *Planctomycetes*, and *Bacteroidetes* with phytodetritus addition. *Alphaproteobacteria* were identified as metabolically active denitrifiers by phylogenetic analysis of nitrous oxide reductase gene sequences from ^{13}C-enriched DNA fractions. This study provides the first identification of microorganisms responsible for organic matter degradation in marine sediments by DNA sequence analysis. Microbial assemblages recognized for high-molecular-weight organic matter oxidation in the marine water column were important in catalyzing these processes in permeable sediments.

High rates of marine primary production in continental margins are fueled largely by nutrients regenerated during mineralization of organic matter in sediments (Wollast 1991). The majority of continental shelf surficial deposits are sandy sediments (Hall 2002) that are low in organic carbon content because of relatively frequent sediment resuspension, highly active microbial communities, and rapid rates of organic matter mineralization (Huettel and Rusch 2000). In the shallow shelf, highly degradable, fresh phytodetritus can settle to the sea floor and is the predominant form of particulate organic matter mineralized in the sandy sediments (Wollast 1991; Jørgensen 1996). Microorganisms act as the primary agents of benthic organic matter decomposition through the production of extracellular hydrolytic enzymes, fermentation, and terminal carbon mineralization coupled with respiratory processes (Jørgensen 2000).

The use of isotopically enriched substrates (e.g., diatoms and glucose) combined with isotopic analysis of biomarkers or tissues of individual organisms has been essential in elucidating the fate of recently deposited organic matter in a variety of coastal and deep-sea environments (Aberle and Witte 2003; Witte et al. 2003). Accumulation of ^{13}C from stable-isotope-labeled phytodetritus into bacteria-specific phospholipid fatty acids (PLFA) showed rapid conversion of deposited organic matter into bacterial biomass (Middleburg et al. 2000; Moodley et al. 2002; Bühring et al. 2006). The specificity of PLFA biomarkers across most groups of microorganisms is low, however, and the ability to differentiate between major groups is limited (Boschker and Middleburg 2002). As a result, direct evidence linking the identity and ecology of benthic microorganisms to phytodetritus degradation is scarce.

Nitrification and denitrification are critical microbially mediated processes in the nitrogen cycle that, when coupled, link the mineralization of nitrogenous compounds to the removal of nitrogen from coastal marine sediments (Hulth et al. 2005). The surface layers of permeable beds typically are well flushed and therefore oxygen penetration can be deep (Reimers et al. 2004; De Beer et al. 2005), suggesting that denitrification activity, which is restricted to anoxic environments, may be low in these layers. On the other hand, flume and in situ studies have shown that in permeable sands zones of pore water up- and downwelling associated with sediment ripples generate alternating oxic and anoxic conditions as these zones move horizontally through the surface layer of the bed (Huettel et al. 1998). This scenario may promote both nitrification and denitrification, though few studies have examined coupled nitrification–denitrification in permeable sediments. Recent studies suggest substantial rates of this process in shelf deposits including sands (Laursen and Seitzinger 2002; Rao et al. 2008). The community composition of denitrifying microorganisms in marine sands has begun to be elucidated in studies targeted to the *nosZ* (nitrous oxide reductase) gene, which encodes for proteins involved in the final step of denitrification, thus directly relating denitrifier ecology to the loss of biologically available nitrogen from the seafloor (Scala and Kerkhof 1999; Hunter et al. 2006).

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However, few studies have progressed beyond a description of community composition to directly link the metabolic activity of denitrifiers to their phylogenetic identity.

The combination of ^{13}C labeling with deoxyribonucleic acid (DNA) extraction and analysis (commonly called "DNA-stable isotope probing," or DNA-SIP) is a powerful technique for identifying active microorganisms involved in specific biogeochemical processes (Boschker and Middleburg 2002; Friedrich 2006). However, DNA-SIP has been rarely applied to studies of benthic marine food webs. As organic matter degradation in margin sediments is a central process in the coastal cycles of matter, determination of the identity and ecology of benthic microorganisms catalyzing decomposition processes in permeable shelf sands is essential for a thorough understanding of the function of these sediments in the coastal ecosystem.

The objectives of this study were to (1) demonstrate the rapid and direct transfer of carbon from deposited phytodetritus into the biomass of microbes colonizing the permeable sands; (2) identify the microbial taxa that catalyze phytodetritus degradation in permeable coastal sediments of the northeast Gulf of Mexico; and (3) demonstrate denitrification activity in the permeable sands and identify within the metabolically active community the taxa responsible for denitrification.

We used SIP experiments to trace the uptake of ^{13}C, added through phytodetritus, into microbial DNA, thereby directly linking the degradation process to specific microbial taxa colonizing the permeable sediment. Phylogenetic analysis of nitrous oxide reductase (*nosZ*) gene sequences from the ^{13}C-enriched DNA fractions revealed the denitrifiers within the active taxa. Our working hypotheses were that (1) phytodetrital ^{13}C are incorporated within days into microbial biomass, reflecting the high degradation rates reported from permeable sediments; (2) the microbial taxa mainly responsible for organic matter degradation in the water column are the dominant taxa in the sedimentary degradation process; and (3) active denitrifiers are present in the sands despite high sediment flushing rates, producing substantial denitrification.

Cyanobacterial *Spirulina* biomass enriched in ^{13}C simulated phytodetritus deposition onto intact sediment cores retrieved from the northeastern Gulf of Mexico, and ^{13}C incorporation into bacterial DNA was assessed using ^{12}C- and ^{13}C-labeled nucleic acid fractionation followed by clonal analysis of and fingerprinting (terminal restriction fragment length polymorphism [TRFLP]) of 16S ribosomal ribonucleic acid (rRNA) and *nosZ* genes. The responses of benthic O_2, N_2, and dissolved inorganic nitrogen (DIN) fluxes to phytodetritus amendment were used to estimate benthic respiration and denitrification rates.

Methods

Site and sampling—Sediment cores were collected from a nearshore environment in the northeastern Gulf of Mexico at St. George Island, Florida (29°44′88.1″N, 084°42′58.6″W) in May 2006. This area is adjacent to an uninhabited region of the island and is considered relatively unaffected by anthropogenic effects. Sediments at the site consist of well-sorted quartz sand (grain median 209 to 220 μm) with a low organic matter content (<0.1%), a permeability of 1.6 to 2.9 $\times$ 10^{-11} m^2, and a porosity of 31% to 37% (Mills et al. 2008). Sediment cores of 10-cm depth were collected by hand at 1.5–2.0-m water depth using 9-cm-diameter, 20-cm-long acrylic core tubes. The sediment temperature was 27°C and the water column salinity was 36 at the time of sampling. Unfiltered seawater (80 liters) was collected from the same location. Cores and water were transported immediately (<2 h) at near in situ temperatures to the Florida State University Coastal and Marine Laboratory where they were placed in a temperature-controlled room (27°C).

Whole-core incubations and chemical analyses—Cores were preincubated by submerging them in a tank filled with seawater from the field site. The water overlying each core was equilibrated with air and circulated continuously overnight using air-lift pumps (Cornwell et al. 1999). A container of site seawater for sample water replacement was equilibrated overnight by bubbling with air. After preincubation and equilibration, three cores were amended with ^{13}C-enriched *Spirulina* biomass (>99% ^{13}C; Spectra Stable Isotopes) at 0.8 mg dry wt cm^{-2}. The *Spirulina* amendments were prepared as thin (~2-mm thickness) disks of slurried biomass and wet sand, treated by brief heating (~80°C for 5–10 s), and frozen on dry ice. Immediately before incubation, one *Spirulina* disk was placed in the seawater overlying each core and allowed to sink to the sediment surface. Four cores were left unamended and one core tube contained only seawater as a blank control. All cores were sealed with gastight caps fitted with sealable sampling ports, and were stirred at 70 revolutions per minute using laboratory stir bars driven by an external magnet. Overlying water samples for DIN, O_2, and N_2 : Ar measurements were withdrawn and replaced with air-equilibrated seawater at 0, 2.5, 8, 14.5, 18, and 22 h.

Samples for N_2 : Ar measurements were preserved with 10 μL of half-saturated $HgCl_2$ in 7-mL vials with ground-glass stoppers. Samples for dissolved oxygen measurements were collected in 7-mL vials with ground-glass stoppers and mixed immediately with Winkler reagents for later titrations. Samples for dissolved ammonium, nitrate, and nitrite were filtered immediately (0.45 μm) and frozen at −20°C until analysis. Sets of one unamended and one ^{13}C-amended core were sacrificed at 14.5, 18, and 22 h. These cores were uncapped, the overlying water was removed, and sediment was collected from the central part of the core by subcoring using four 30-mL syringes with the ends cut off. Sediment subcores were frozen immediately on dry ice and stored at −80°C.

Dissolved N_2 was measured using a membrane inlet mass spectrometer according to Kana et al. (1994). Oxygen was removed from the inflow gas upstream of the mass spectrometer using an in-line quartz column packed with Cu turnings heated to 600°C (Eyre et al. 2002); N_2 concentrations were calculated from measured N_2 : Ar ratios assuming constant Ar concentrations (Kana et al. 1994). Samples for dissolved O_2 were analyzed immediately after each sampling time point using Winkler titrations

(Grasshoff et al. 1983). Dissolved ammonium (NH_4^+) and nitrite (NO_2^-) were determined by colorimetry (Strickland and Parsons 1972; Bower and Holm-Hansen 1980), and NO_x^- ($NO_3^- + NO_2^-$) was determined by chemiluminescence after reduction to NO (Braman and Hendrix 1989).

DNA extractions—Frozen (−80°C) sediment subcores were sectioned into 12 depth intervals: 0–2, 2–4, 4–6, 6–8, 8–10, 10–12.5, 12.5–15, 15–20, 20–25, 25–30, 30–40, and 40–50 mm. Three replicate subcores were sectioned for each time point and treatment; sediment from replicate intervals was combined and homogenized. For TRFLP analyses of DNA-SIP separations, frozen sections from ^{13}C-*Spirulina*-amended cores sampled at 14.5 h were combined and homogenized after slight thawing to make sediment pools corresponding to 0–0.5, 1–2, 2–3, and 3–5 cm. DNA was extracted and purified using a modified method of Scala and Kerkhof (2000) and was resuspended in 100 μL of Tris–ethylenediaminetetraacetic acid (TE) pH 8.0 buffer.

DNA-SIP separations—Cleaned DNA (2.0 μg) was loaded into a 4.9-mL Optimax ultracentrifuge tube (Beckman Coulter) with 400 μL of 20 mg mL^{-1} ethidium bromide and 1.55 g mL^{-1} CsCl in deionized H_2O. Samples were centrifuged at 225,000 × *g* for 48 to 60 h in a NVT-100 rotor (Beckman Coulter) at 20°C. DNA bands were recovered after centrifugation by slicing the tube top and removing the fractions with a 200-μL pipette tip. Exposure to ultraviolet light for visualization was minimized (<3 s) as to limit DNA degradation. Gradient fractions were washed with 1.0 mL of TE-saturated MBG-butanol (Sigma-Aldrich), centrifuged at 5000 × *g* for 3 min, and the organic phase was decanted. This procedure was repeated until no visible signs of ethidium bromide remained. The aqueous phase was augmented with 10 μg of bovine serum albumin (New England Biolabs) and drop dialyzed on 25-mm nitrocellulose filters (Millipore) against 10 mmol L^{-1} Tris-HCl pH 8.0 for 15 min. Samples were recovered, precipitated with ethanol, and resuspended in 20 μL of 10 mmol L^{-1} Tris-HCl.

TRFLP analyses—Polymerase chain reaction (PCR) amplification of 16S rRNA gene sequences was conducted with universal bacterial primers 27f (5′-AGRGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991) at 500 nmol L^{-1}. Primer 27f was labeled with 6-carboxyfluorescein on the 5′ end. PCR cycling conditions were: 2-min initial denaturation at 95°C followed by 27 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 60 s. Amplification of *nosZ* gene sequences was performed as in Mills et al. (2008). Each PCR reaction was verified by agarose gel electrophoresis. Three duplicate PCRs were combined and cleaned using standard spin column cleanup kits (Qiagen) and quantified (NanoDrop Spectrophotometer; Thermo Fisher Scientific). The PCR product (200 ng) was digested with *Mnl*I restriction endonucleases (New England Biolabs) for 3 h according to manufacturer's standard protocol. After digestion, 30 ng of *Mnl*I-digested template were ethanol precipitated and resuspended in 19.7 μL of deionized formamide and 0.3 μL of Generuler ROX 0–500-base pair standard marker (Applied Biosystems). Fragment analysis was conducted at the Florida State University DNA Sequencing Core Facility. Subsequent TRFLP analysis was completed using GeneMapper 4.0 software (Applied Biosystems) using amplified fragment length polymorphism analysis settings. Peaks of less than 50 nucleotide length or representing less than 1% of total peak area were discarded. Further TRFLP peak analysis was conducted as described in Mills et al. (2008).

Cloning and sequencing—16S rRNA and *nosZ* gene sequences were PCR amplified from each SIP gradient fraction using the aforementioned PCR primers and reaction conditions with the exception of the 16S rRNA, which was amplified with unlabeled 27f primer. PCR amplicons were purified with PCR-clean spin columns (Qiagen) and resuspended in 30 μL of H_2O. Products from PCR reactions of all individual intervals were combined and the pooled product was cloned into pCR2.0 vector (Invitrogen) using 3.0 μL of template and chemically transformed into *Escherichia coli* DH10b cells. Putative transformants were verified by blue-white screening and PCR reamplification with M13F/M13R primers according to manufacturer's specifications. Validated amplicons were purified on PCR-clean spin columns before DNA sequencing at the Florida State University DNA Sequencing Core Facility. Sequencing reactions were performed using Applied Biosystems Big Dye 4.0 terminators on an ABI 3730 instrument (Applied Biosystems).

Phylogenetic and statistical analysis—All clone sequences were trimmed of vector sequence contamination using 4Peaks software (Griekspoor and Groothuis, mekentosj.com.) and assembled manually in CAP3 (Huang and Madan 1999). Sequences of highest similarity to those of the present study were retrieved by comparison to the Greengenes 16S rRNA database (DeSantis et al. 2006). Reference *nosZ* sequences were obtained by BLAST analysis (Altschul et al. 1990). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Kimura two-parameter method (16S rRNA) (Kimura 1980) or the Poisson correction method (*nosZ*) (Zuckerkandl and Pauling 1965) and are given in units of the number of respective substitutions per site. All positions containing gaps and missing data were eliminated from the data set. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Sequences for *nosZ* and 16S rRNA genes were submitted to the National Center for Biotechnology Information GenBank database and have accession numbers FJ626871 to FJ626910.

Results

Geochemistry—Dissolved N_2 concentrations (Fig. 1a) showed a linear net flux from the sediment without *Spirulina* amendment (0.30 mmol N m^{-2} d^{-1}); addition of *Spirulina* biomass resulted in efflux (4.77 mmol N m^{-2} d^{-1}) accelerated by a factor of 15.9 relative to unamended cores. Measurements of dissolved oxygen

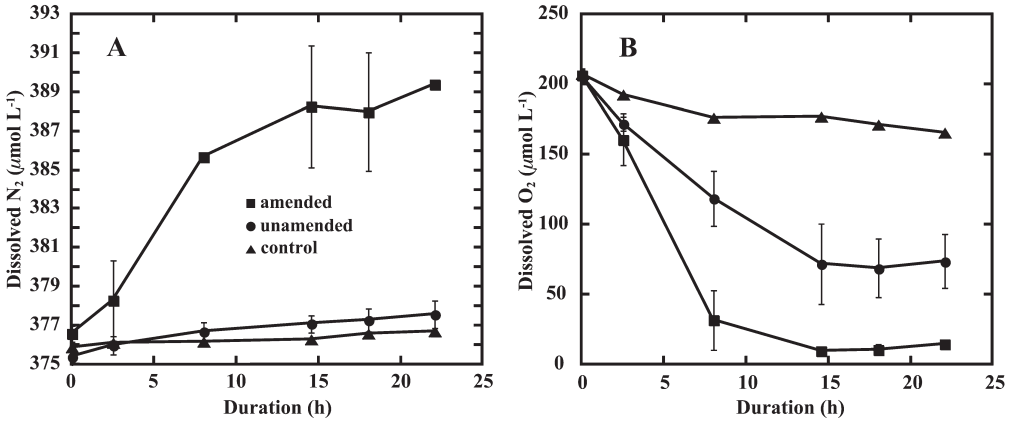

Fig. 1. Average concentrations of (A) dissolved dinitrogen and (B) oxygen in the overlying water of *Spirulina*-amended cores, unamended cores, and control (water-only) experiments. Error bars indicate the standard deviation of replicate cores.

within the core overlying water indicated net O_2 consumption for all cores during the first 14 h of incubation (Fig. 1b); oxygen consumption was accelerated by factor 2.6 with *Spirulina* biomass addition (mean = −48.63 mmol O_2 m^{-2} d^{-1}; $n = 2$) relative to unamended cores (mean = −18.41 mmol O_2 m^{-2} d^{-1}; $n = 4$). Changes of DIN ($[NH_4^+] + [NO_2^-] + [NO_3^-]$) within the overlying water indicated uptake without amendment (−0.073 μmol N m^{-2} d^{-1}) and strong efflux with *Spirulina* addition (2240 μmol N m^{-2} d^{-1}; data not shown).

Oxygen consumption rate measurements were considered as a proxy for carbon mineralization rates with the ratio of oxygen consumption to N_2 production indicative of the percentage of organic carbon used during denitrification (Canfield et al. 1993). These calculations indicated that complete denitrification shifted from 2% of total carbon oxidation without amendment to 10% with *Spirulina* addition.

TRFLP-based assesment of microbial community structures and relative abundances—TRFLP analysis is a method for assessing microbial community structures and dynamics on the basis of detecting variations in 16S rRNA genes. PCR using fluorescently labeled primers is followed by digestion and analysis of terminal restriction fragment (TRF) sizes and intensities using a DNA sequencer. Fluorescently labeled terminal fragments of different size are detected as discrete peaks, each corresponding to a genetic variant (one or more taxa) in the sample. The fluorescence intensity (area) of each peak is proportional to the amount of genomic DNA of each variant in the sample and thus corresponds to relative abundance in the community (Liu et al. 1997; Dunbar et al. 2001). TRFLP peak areas from amended and unamended core profiles were compared for the semiquantitative assessment of changes in the relative abundance of bacterial groups due to phytodetritus addition. By comparison of TRF sizes from the sediment slices with predicted TRF sizes from 16S rRNA gene clone libraries, TRFLP peaks were identified for 51% of TRFs from unamended core profiles and 69% from amended core profiles, on average, at the phylum level. Peaks matching cloned 16S rRNA gene sequences were grouped into 11 phyla and classes. The percentage of peak area belonging to these 11 groups, relative to total peak area for each TRFLP profile, was averaged for six depth intervals and are shown in Table 1. Whereas the majority of phyla showed little relative change (<10%) with *Spirulina* amendment, three groups showed substantial increases: the *Bacteroidetes* (formerly the Cytophaga–Flexibacter–Bacteroides group) increased by 28% at the surface (0 to 0.4 cm); *Gammaproteobacteria* increased up to 54% in relative abundance between 0.4 and 2.0 cm; *Planctomycetes* also showed an increase in relative abundance at the lower depths (2.0 to 5.0 cm).

Clustering of 16S rRNA gene TRFLP fingerprints from ^{12}C and ^{13}C DNA fractions of the ^{13}C-*Spirulina*-amended cores yielded grouping into three clades (Fig. 2). For samples at the top of the cores (0–3-cm depth), community fingerprints of ^{13}C DNA (0–3-cm depth) clustered together, whereas ^{12}C DNA samples (0–2-cm depth) clustered separately. Both ^{12}C and ^{13}C DNA TRFLP fingerprints from deeper in the core (2–5-cm and 3–5-cm depth, respectively) clustered together in a clade separate from the shallower depths.

Phylogeny of Spirulina*-degrading microbial populations*—Cloned sequences of 16S rRNA genes from ^{13}C-enriched DNA fractions extracted from the 14.5-h *Spirulina*-amended cores were analyzed for their phylogenetic affiliation (Fig. 3). A total of 27 distinct phylotypes were identified, 14 of which were most closely related to *Gammaproteobacteria*. Of these *Gammaproteobacteria* taxa, nine were affiliated with the *Vibrionales* order and *Pseudoalteromonadaceae* family. Six *Actinobacteria* phylotypes were detected, in addition to members of the *Deltaproteobacteria*, *Verrucomicrobia*, and *Planctomycetes*. Rarefaction analyses indicated that the screened clones adequately sampled the 16S rRNA gene sequence diversity (Fig. 4).

Identification and quantification of nosZ *genes*—Gene sequences encoding for *nosZ* were analyzed from the *Spirulina*-amended core to identify those organisms most likely to be active in producing N_2 and to assess the link between *Spirulina* degradation and denitrification. The

Table 1. Relative abundances of microbial groups as determined by terminal restriction fragment length polymorphism (TRFLP) for *Spirulina*-amended and unamended cores (parentheses) sampled at 14.5 h. Percentages are the sum of peak areas from each group relative to total TRFLP peak areas. Taxonomic groups and depth intervals having greater than 10% difference between amended and unamended relative peak areas are bolded.

	Percentage TRFLP peak area					
Class or Phylum	0–0.4 cm	0.4–0.8 cm	1.0–1.25 cm	1.25–2.0 cm	2.0–3.0 cm	3.0–5.0 cm
Acidobacteria	0 (6)	1 (4)	1 (5)	1 (2)	1 (6)	1 (4)
Actinobacteria	3 (6)	4 (4)	12 (15)	1 (4)	14 (12)	1 (1)
Alphaproteobacteria	3 (9)	10 (9)	8 (8)	4 (5)	4 (10)	**4 (15)**
Bacteroidetes	**34 (6)**	1 (7)	4 (7)	1 (5)	2 (9)	7 (4)
Chloroflexi	0 (4)	1 (6)	1 (0)	1 (4)	0 (1)	0 (3)
Cyanobacteria	5 (3)	6 (2)	5 (2)	3 (2)	2 (5)	5 (2)
Deltaproteobacteria	0 (0)	2 (0)	1 (1)	1 (1)	0 (2)	1 (1)
Firmicutes	**12 (2)**	3 (10)	**14 (4)**	1 (0)	6 (0)	0 (0)
Gammaproteobacteria	**14 (24)**	**46 (11)**	**25 (12)**	**61 (7)**	7 (12)	**16 (5)**
Gemmatimonadetes	0 (0)	0 (1)	1 (0)	0 (0)	9 (1)	0 (1)
Planctomycetes	0 (2)	1 (2)	2 (2)	1 (3)	**25 (4)**	**16 (1)**

sequences of *nosZ* genes amplified from ^{13}C DNA fractions indicated seven distinct phylotypes. All phylotypes were affiliated with the *Alphaproteobacteria* and were not closely related to sequences from cultivated strains (Fig. 5). Quantitative PCR of *nosZ* gene sequences revealed copy numbers within the range of 4×10^6 and 38×10^6 copies per gram of sediment (Table 2). Little variation in *nosZ* copy number was observed between treatments and across depth profiles. Total copy numbers were higher in *Spirulina*-amended cores; however, this difference was relatively small and not considered significant here.

Discussion

Coastal benthic environments typically receive intermittent pulses of organic matter after phytoplankton bloom events (Gooday 2002), and permeable sediments have been demonstrated to rapidly degrade this material (Huettel and Rusch 2000). Although the role of bacteria in the decomposition of organic matter in marine sediments has long been recognized (Zobell 1941; Jørgensen 2000), the mechanisms regulating organic matter decomposition and relationships between the phylogeny of benthic microorganisms and changes in biogeochemical and ecological function are underexplored. By simulating phytodetritus deposition events using ^{13}C-enriched *Spirulina* biomass amendment in sediment core incubations, tracking ^{13}C assimilation into bacterial DNA, and quantifying changes in water column chemistry, we were able to demonstrate that (1) benthic microbial populations were poised to respond immediately to phytodetritus additions; (2) phytodetrital ^{13}C was assimilated rapidly into microbial DNA; (3) the dominant microbial taxa responsible for phytodetritus degradation in sediments resemble those known for dissolved and particulate organic matter degradation in the water column; and (4) denitrifiers were present and produced substantial levels of N_2 after phytodetritus addition.

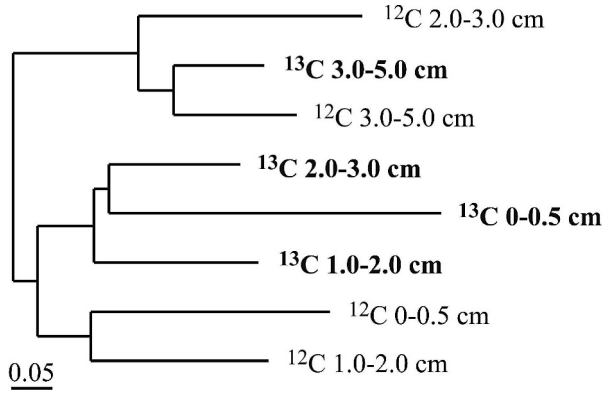

Fig. 2. Clustering dendrogram on the basis of pairwise comparisons of small subunit rRNA gene terminal restriction fragment length polymorphism profiles amplified from ^{12}C and ^{13}C DNA fractions of ^{13}C-*Spirulina*-amended cores at 14.5 h. Samples from ^{13}C DNA fractions are in bold.

Response of the benthic bacterial populations to fresh detritus—This study of shallow, permeable sediments demonstrates an immediate response of increased benthic respiration upon phytodetritus deposition. These results are consistent with oxidation of highly labile carbon immediately after the phytodetritus amendment, followed by slower utilization of less-labile material. Short-term denitrification rates, during the initial phase of labile organic matter utilization, approached some of the highest fluxes reported for marine sediments (Laursen and Seitzinger 2002). The response of benthic microbial processes was also evident in the reversal of the DIN flux, with phytodetritus-amended cores having net DIN release, whereas unamended cores (representing prebloom detritus conditions) had net DIN consumption. Respiration pathways also shifted toward a greater percentage of denitrification with phytodetritus addition. Although the initiation and duration of a phytodetritus deposition event may occur over a longer timescale (from days to weeks), an immediate response is clear from these experiments. The observed short-term upsurge of denitrification in response to a simulated particulate organic matter sedimentation event

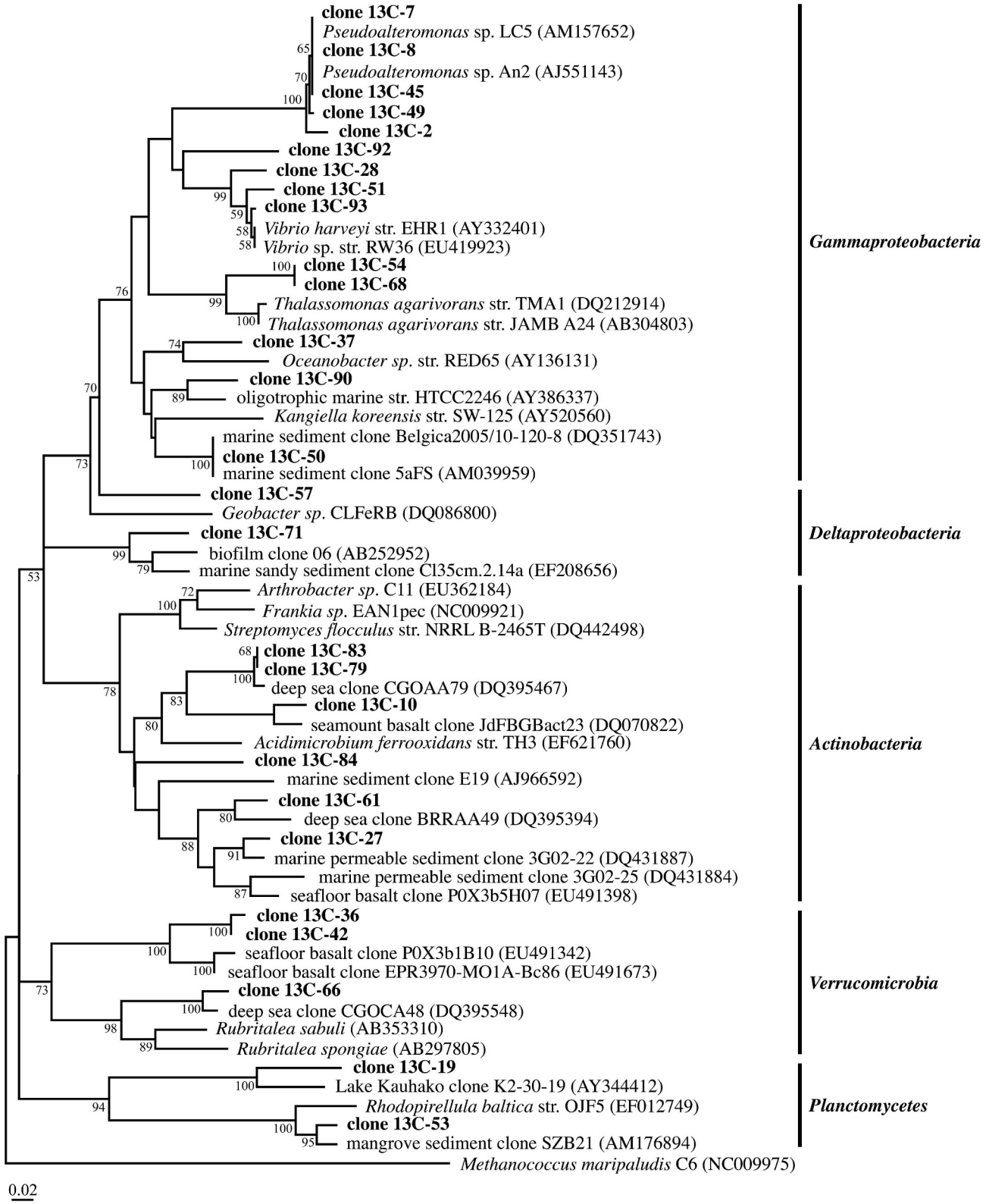

Fig. 3. Neighbor-joining phylogenetic tree of small-subunit rRNA gene sequences. GenBank accession numbers are in parentheses. Bootstrap percentages greater than 50% are shown adjacent to internal nodes. Scale bar indicates the number of base substitutions per site. Phylotypes in bold were derived from the ^{13}C DNA-based clone library of ^{13}C-*Spirulina*-amended cores sampled at 14.5 h.

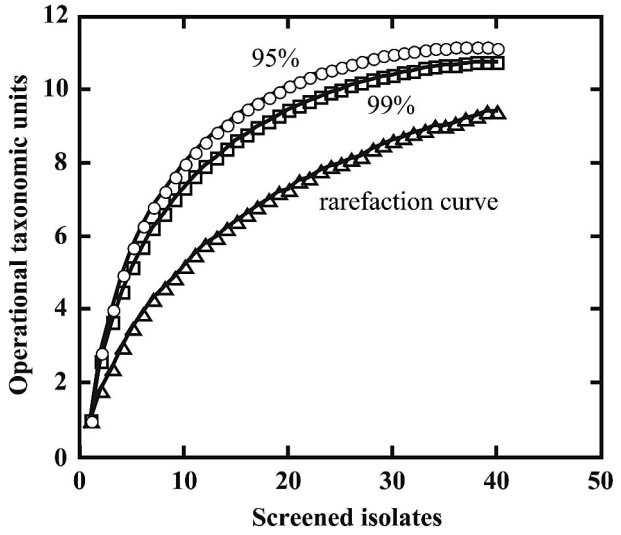

Fig. 4. Rarefaction curve, upper 99% confidence limit, and upper 95% confidence limit calculated for the ^{13}C DNA 16S rRNA gene clone library. Rarefaction was determined at 97% sequence identity threshold using FastGroupII software (http://biome.sdsu.edu/fastgroup/fg_tools.htm).

may point to a larger role of denitrification in permeable sediments over broader scales than was perceived previously.

These rapid biogeochemical responses to detritus deposition suggest that members of the benthic microbial community were poised to begin acting upon and degrading organic matter immediately as it was delivered to the sediment. Rapid degradation of detritus following benthic deposition has been observed in shallow sediments where both labile and less-reactive carbon organic matter reach the sediment (Azam and Long 2001). The immediate onset of respiratory processes (O_2 consumption and denitrification) observed here suggests that populations maintained a state of metabolic readiness and appeared to include those members of the community that perform initial degradation (e.g., hydrolysis reactions, fermentation) and aerobic respiration but also the anaerobic, terminal respiratory populations such as the denitrifiers.

In contrast with fine-grained sediments, coarse-grained sands typically have low organic matter content (<0.05%) because of high rates of organic matter turnover and frequent resuspension and winnowing of the surface layers. Therefore, within coastal marine permeable sediments, organic matter is rapidly oxidized when it becomes available, and the system returns to scarce organic conditions, a likely ecological strategy that is one of "feast or famine." The concept of feast or famine as an adaptive strategy within marine bacterioplankton communities has been put forward by Eilers et al. (2000), who showed that members of the *Vibrio*, *Alteromonas*, and *Colwellia* genera in seawater were found to maintain a high potential for growth during periods of starvation, i.e., these frequently cultivated bacterioplankton adapted to quickly respond to

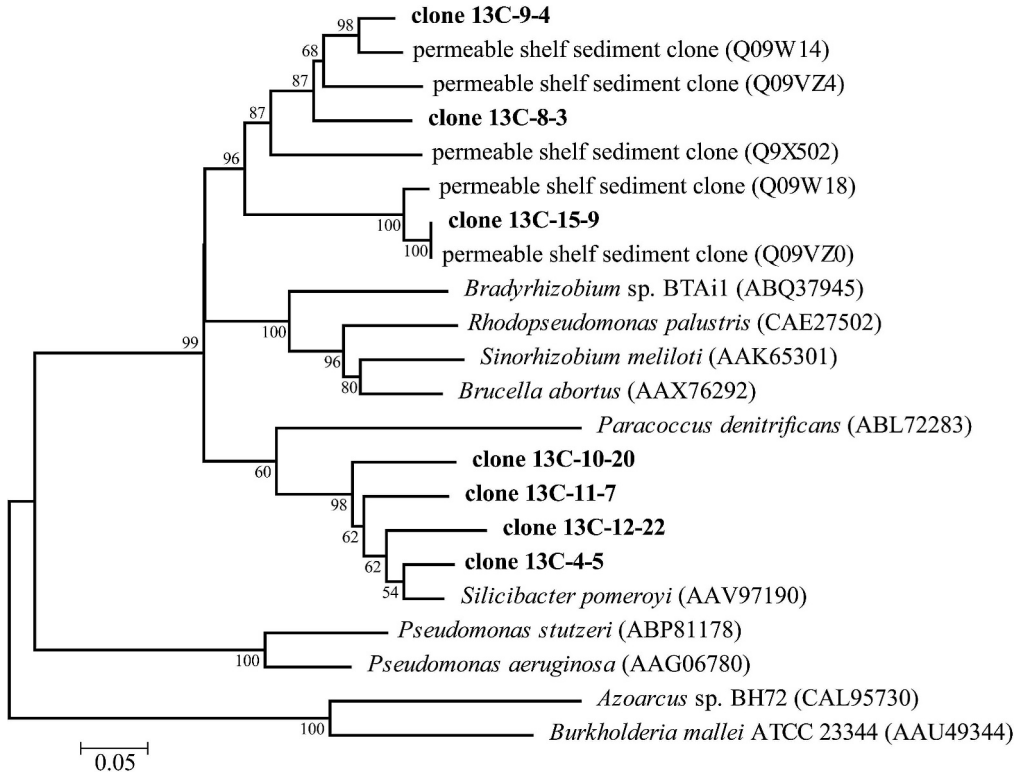

Fig. 5. Neighbor-joining phylogenetic tree on the basis of amino acid sequences inferred from *nosZ* gene sequences. GenBank accession numbers are in parentheses. Bootstrap percentages greater than 50% are shown adjacent to internal nodes. Scale bar indicates the number of amino acid substitutions per site. Phylotypes in bold were derived from the ^{13}C DNA-based clone library of ^{13}C-*Spirulina*-amended cores sampled at 14.5 h.

Table 2. Quantitative PCR results using primers specific for nitrous oxide reductase (*nosZ*) gene sequences.

	nosZ gene copies $\times 10^6$ g^{-1} sediment	
Depth interval (cm)	Unamended cores	Amended cores
0.5–1.0	38	13
1.0–2.0	6	20
2.0–3.0	4	20
3.0–5.0	11	26
Sum	59	79

changes in environmental conditions. Other studies, including Bühring et al. (2006), Moodley et al. (2002), and Witte et al. (2003), also observed rapid increases in benthic respiration after detritus deposition initiated by ^{13}C-enriched substrate amendments. In these studies,^{13}C was incorporated into bacterial PLFAs, revealing that the benthic microbial communities were poised to respond to influxes of organic matter with immediate biomass production.

Identification of Spirulina*-degrading microbial populations*—DNA-SIP and community fingerprinting identified microbial groups mediating phytodetritus degradation in shallow marine sediments. Whereas traditional 16S rRNA gene cloning and sequencing provides an assessment of overall microbial community composition, ^{13}C-DNA-SIP allowed for the identification of the major taxa active specifically in the early stages of phytodetritus degradation. Cluster analysis of TRFLP fingerprints from ^{12}C and ^{13}C DNA fractions (Fig. 2) demonstrated that the microbial populations that assimilated ^{13}C-labeled *Spirulina* in the surface layers were distinctly different from the remainder of the community. Phylogenetic analyses of 16S rRNA gene sequences in ^{13}C-enriched DNA fractions (Fig. 3) identified multiple phylotypes of the *Alteromonadales* and *Vibrionales* (*Gammaproteobacteria*) in the clone libraries, indicating that these taxa utilized *Spirulina*-derived carbon. *Actinobacteria*, *Deltaproteobacteria*, *Planctomycetes*, and *Verrucomicrobia* members were detected in the ^{13}C DNA fractions, thereby directly linking these groups to phytodetritus utilization. Semiquantitative comparisons of TRFLP peak areas (Table 1) provide further, independent evidence that the *Gammaproteobacteria*, *Planctomycetes*, and *Bacteroidetes* increased in relative abundance with the *Spirulina* amendment and are important in phytodetritus degradation.

The identification of *Gammaproteobacteria*, *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia*, and *Bacteroidetes* as the microbial assemblages breaking down and assimilating fresh detritus is consistent with previous evidence that many of these taxa are capable of degrading high-molecular-weight organic compounds. *Gammaproteobacteria* detected in ^{13}C DNA fractions were predominantly of the *Alteromonadales* and *Vibrionales* orders, which, in addition to their ability to maintain a state of metabolic readiness during periods of starvation (Eilers et al. 2000), also have high activity of hydrolases and carry out mixed acid fermentation (Mikhailov et al. 2006). The *Planctomycetes* are a widespread group comprised of many aerobes capable of degrading polymers but also include autotrophs that oxidize ammonium in anoxic environments. Assemblages of *Bacteroidetes*, *Gammaproteobacteria*, and *Planctomycetes* dominate microbial communities associated with, and likely decomposing, marine snow (Delong et al. 1993). *Gammaproteobacteria*, *Planctomycetes*, and *Bacteroidetes* were abundant in shallow marine permeable sediments that experience strong, seasonal phytodetritus deposition and have high rates of benthic organic matter mineralization (intertidal sand flat of the southeastern North Sea; Musat et al. 2006). Fluorescence in situ hybridization (FISH) showed a predominance of *Planctomycetes* and *Bacteroidetes* in sediments expressing carbohydrate and protein hydrolysis (Rusch et al. 2003), providing further evidence of the importance of these groups in the initial degradation of recently deposited complex organic matter.

Results of TRFLP peak area comparisons (Table 1) indicated a relative increase in *Bacteroidetes* at the sediment surface in response to *Spirulina* amendment, suggesting a role in phytodetritus degradation. *Bacteroidetes* were not detected in the ^{13}C 16S rRNA gene clone library, possibly as a result of sediment sample pooling (multiple samples spanning 0 to 5 cm) before ^{13}C DNA separations and dilution of *Bacteroidetes* sequences (abundant only at 0 to 0.4 cm) to below the level detectable here by cloning. However, an independent study of the overall microbial communities at this same site detected multiple taxa of *Bacteroidetes* in both genomic DNA and rRNA-derived clone libraries, demonstrating that these organisms were present and active in the upper layers of sediment (Mills et al. 2008). The *Bacteroidetes* are a broad group of chemoorganoheterotrophs specializing in polymer degradation, and the role of these bacterial groups in catalyzing high-molecular-weight organic matter degradation in the marine water column is recognized (Kirchman 2002). FISH in combination with 16S rRNA gene cloning demonstrated that *Bacteroidetes* can be among the most prominent microbial taxa in shallow, permeable sediments that receive fresh detritus (Llobet-Brossa et al. 1998; Musat et al. 2006). *Bacteroidetes* cell numbers also increased in marine sediments after the amendment of cyanobacterial biomass to simulate recent phytodetritus deposition (Rosselló-Mora et al. 1999). The evidence here showing a response of *Bacteroidetes* to phytodetritus input provides corroborating evidence that this group is important in benthic organic matter degradation.

SIP results shown here provide clear evidence that *Actinobacteria* and *Verrucomicrobia* participate in phytodetritus degradation in marine sediments. Although the importance of *Actinobacteria* in polymer (e.g., cellulose and chitin) degradation in soils is well established, the ecological function of marine *Actinobacteria* is debated (Bull et al. 2005). Marine *Actinobacteria* are present mainly in sediments, most notably near deep-sea gas hydrates (Lanoil et al. 2001; Reed et al. 2002) and in shallow sandy sediments (Hunter et al. 2006; Mills et al. 2008). Multiple strains of *Actinobacteria* were isolated from coastal, suspended aggregates, demonstrating a probable role in

particulate organic matter degradation (Grossart et al. 2004). The function of *Actinobacteria* populations in the environment has not been directly assessed in previous work, however. *Verrucomicrobia* are widely distributed in the oceans (Wagner and Horn 2006) but their ecological function is largely unknown. Only three representatives have been isolated, and all described *Verrucomicrobia* strains are aerobic chemoorganotrophs (Scheuermayer et al. 2006).

Pelagic microbial communities degrade phytodetritus in permeable sediments—Many of the taxa identified here, most notably the *Bacteroidetes*, *Gammaproteobacteria*, *Planctomycetes*, and *Actinobacteria*, are important for using particulate and dissolved organic matter in the marine water column (Kirchman 2002; Grossart et al. 2004). As noted by Rusch et al. (2003), sandy sediments likely enlist microbial taxa known for catalyzing organic matter degradation in the water column because of their permeability and the exchange of pore water with the overlying water. Studies utilizing cloning, FISH, and DNA-SIP have now independently confirmed that bacteria previously studied in the water column are important in benthic detritus degradation in the surface layers of permeable sediments. In contrast, experiments involving ^{13}C substrate uptake with PLFA analysis suggest that anaerobic bacteria are more important for organic matter decomposition in fine-grained, or muddy, coastal and deep-sea benthos (Bühring et al. 2006). In the deeper layers of permeable sediment, not affected by the advective pore-water exchange caused by boundary flow–ripple interaction, the microbial community and its activities are expected to be similar to those of the fine-grained, cohesive sediments.

Identification of potential denitrifying bacteria—Cloning and sequencing of *nosZ* genes from ^{13}C DNA fractions determined those microorganisms that actively processed phytodetritus and have denitrification potential. Such bacteria were all identified as *Alphaproteobacteria*, including taxa closely related to *P. denitrificans* and other microbes previously detected in permeable marine sediments (Fig. 5). Our quantitative PCR results further show that denitrifiers were abundant in the permeable Gulf of Mexico sediments over the depth intervals examined. Although is it possible that facultative denitrifiers could have assimilated ^{13}C during aerobic utilization of phytodetritus, these data nonetheless link the phylogenetic identity to the metabolic activity of these marine denitrifying bacteria. The diversity of denitrifying bacteria was shown previously to be high in permeable sediments, consistent with the high permeability and associated rapid oxygen fluctuations, high rates of organic matter turnover, and enhanced remineralization of nitrogen observed in these environments (Scala and Kerkhof 2000; Mills et al. 2008). Although the utility of functional gene databases for identifying marine denitrifiers beyond high-level classification is limited by a lack of cultivated representatives (Zehr and Ward 2002), members of the *Alphaproteobacteria* have been implicated as important denitrifiers in permeable sediments of the South Atlantic Bight (Hunter et al. 2006) and the northeast Gulf of Mexico (Mills et al. 2008).

This present study for the first time uses nucleic acid-based SIP to determine the taxonomy of microorganisms catalyzing phytodetritus degradation in marine sediment. By linking ^{13}C uptake with 16S rRNA gene phylogeny, the ecological function of *Gammaproteobacteria*, *Planctomycetes*, *Actinobacteria*, *Verrucomicrobia*, and *Bacteroidetes* as initial degraders of organic matter in shallow, permeable sands could be demonstrated. Simulated bloom deposition experiments using intact sediment cores revealed a pulse of increased denitrification coinciding with accelerated oxygen consumption, underlining the role of denitrifiers in the initial degradation process. SIP combined with cloning and sequencing of *nosZ* genes identified potential denitrifying microorganisms responsible for this response. This study focused on the surface layer of permeable sand deposits and the initial, rapid response of the sedimentary microbial community to a labile organic matter pulse. How important is this pronounced initial response relative to the ensuing decomposition of the less labile material, and which microbial groups dominate the postinitial degradation in sand sediments? How important are sediment pore-water flows in controlling the microbial metabolic activites, e.g., coupled nitrification–denitrification? Only a thorough understanding of the microbial processes will permit a reliable assessment of the role of permeable shelf sediments in the coastal cycles of matter.

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