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ORIGINAL ARTICLE

Degradation of cyanobacterial biomass in anoxic tidal-flat sediments: a microcosm study of metabolic processes and community changes

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To follow the anaerobic degradation of organic matter in tidal-flat sediments, a stimulation experiment with ^{13}C-labeled *Spirulina* biomass (130 mg per 21 g sediment slurry) was conducted over a period of 24 days. A combination of microcalorimetry to record process kinetics, chemical analyses of fermentation products and RNA-based stable-isotope probing (SIP) to follow community changes was applied. Different degradation phases could be identified by microcalorimetry: Within 2 days, heat output reached its maximum (55 μW), while primary fermentation products were formed (in μmol) as follows: acetate 440, ethanol 195, butyrate 128, propionate 112, H_2 127 and smaller amounts of valerate, propanol and butanol. Sulfate was depleted within 7 days. Thereafter, methanogenesis was observed and secondary fermentation proceeded. H_2 and alcohols disappeared completely, whereas fatty acids decreased in concentration. Three main degraders were identified by RNA-based SIP and denaturant gradient gel electrophoresis. After 12 h, two phylotypes clearly enriched in ^{13}C: (i) *Psychrilyobacter atlanticus*, a fermenter known to produce hydrogen and acetate and (ii) bacteria distantly related to *Propionigenium*. A *Cytophaga*-related bacterium was highly abundant after day 3. Sulfate reduction appeared to be performed by incompletely oxidizing species, as only sulfate-reducing bacteria related to *Desulfovibrio* were labeled as long as sulfate was available.
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Introduction

Coastal marine environments such as tidal-flat sediments have a high nutrient input from the land and the open sea. They are characterized by high primary production rates and an intense heterotrophic activity (Wilms *et al.*, 2006a). The high microbial activity leads to oxygen depletion within the first few millimeters of the sediment, below anoxic conditions are prevailing. Anaerobic organic matter degradation comprises different phases. After hydrolysis of polymers by exoenzymes, the monomers are fermented to short-chain fatty acids, alcohols, H_2 and CO_2 (Schmitz *et al.*, 2006). As long as sulfate is available, the oxidation of fermentation products is mainly coupled to sulfate reduction and not to methanogenesis (Jørgensen, 1982). The terminal processes sulfate reduction and methanogenesis are well studied (for example, Llobet-Brossa *et al.*, 2002; Beck *et al.*, 2011), whereas the initial fermentation processes and the involved organism are poorly understood. In most studies, molecular tools were used to investigate the terminal oxidizers. As sulfate reducers and methanogens can be easily detected by specific important genes, which either encode for the dissimilatory sulfate reductase or the methyl-coenzyme M reductase, the recent studies focused on these groups (Wilms *et al.*, 2007). It is well known that the terminal oxidizers make only a minor part of the community, whereas the fermentative microorganisms are more abundant (Schink, 2002; Köpke *et al.*, 2005; Wilms *et al.*, 2006b). Thus, owing to a lack of a fermentation important gene, the largest part of the community remains unexplored. Here we used an approach, combining microcalorimetry and RNA-based stable-isotope probing (SIP), to investigate the metabolic activity of fermenting organisms in a stimulation experiment as follows: sediment samples were enriched with ^{13}C-labeled, autoclaved *Spirulina* cell material as a substrate simulating a burial of organic matter. High substrate concentrations in intertidal sediments are not uncommon. High loads of organic material (for

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example, algal mats or clams) are dislodged and buried by tidal flow or storm events (Neira and Rackemann, 1996). As metabolic activity is always coupled to the production of heat, the degradation process can be monitored by microcalorimetry. This technique allows real-time detection of heat flows in the range of micro-watts (Larsson *et al.*, 1991; Teeling and Cypionka, 1997). In a microcalorimeter, it is possible to detect successive degradation phases and therefore to determine the optimal sampling time points.

Instead of DNA, we used RNA-based SIP to identify active community members. RNA is an excellent proxy for SIP studies (Manefield *et al.*, 2002; Whiteley *et al.*, 2007), as it is turned over independently from cell division and the RNA content of cells reflects their activity. Thus, the incorporation of ^{13}C into RNA is much faster than into DNA.

The combination of microcalorimetry, fermentation product analysis and rRNA-SIP enabled us to answer the questions: Which phases of anaerobic degradation can be distinguished? What are the main fermentation processes and who are the important players involved?

Materials and methods

Sample collection

Sediment samples were collected from the back barrier tidal-flat area of Spiekeroog island, Germany (53°44.178′N and 07°41.974′E), in July 2010. Anoxic sediment from 1 to 5 cm depth was transferred into a sterile 500 ml glass bottle. Before the experiment, the sediment was incubated at 20 °C for 2 weeks in the presence of sulfate to decrease the organic carbon content of the sediment. The sulfate concentration decreased twice below 15 mM and was refilled to 30 mM. During this incubation, the sediment was stored in gas-tight bottles, under nitrogen atmosphere in the dark. Every second day, the atmosphere was flushed with N_2 to remove gaseous sulfide to keep the sulfide concentration low.

Cultivation and microcalorimetry

Slurries were prepared from 18 g of sediment and 3 ml of artificial seawater (containing per liter of distilled water, 20 g NaCl, 3 g $MgCl_2 \times 6H_2O$, 0.15 g $CaCl_2 \times 2H_2O$, 0.3 g KCl, 0.25 g NH_4Cl, 0.2 g KH_2PO_4). The sulfate concentration was adjusted (from approximately 2 mM before the experiment) to 30 mM. This material was transferred to 23 ml glass vials and mixed with 130 mg wet autoclaved *Spirulina*-^{13}C (Isotec, Berlin, Germany). Approximately 10% of the *Spirulina* dry weight consists of saturated hydrocarbons ranging from C16 to C24 (Munifah *et al.*, 2009). Carbohydrates account to 15–25% cellular dry weight and consist mainly of a branched polysaccharide, composed of only glucose and structurally similar to glycogen (Ciferri, 1983). The prokaryotic cells were not disrupted after autoclaving. Under the microscope, large cylindrical cells of the *Spirulina* filaments were visible. Probably, a part of the cell constituents was lysed and increased the accessibility of *Spirulina* carbon. Autoclaving was necessary to exclude a contamination with living *Spirulina* cells and potential *Spirulina* degraders, which might be contaminants of the cell material.

The vials were sealed with rubber stoppers and flushed with N_2. The pH was 7.3 at the beginning of the experiment. *Spirulina*-free slurries served as controls. Four cultures (three *Spirulina*-amended samples and one *Spirulina*-free control) were incubated in a heat-conduction microcalorimeter (2277 Thermal Activity Monitor; ThermoMetric, Järvalla, Sweden) at 20 °C for 24 days. The heat production was recorded at intervals of 300 s using the program DIGITAM 3.0 (Sci Tech Software; ThermoMetric). The cultures were sampled at specific points when heat was produced, resulting from the degradation of *Spirulina* cells. Triplicates of ^{13}C-*Spirulina*-amended samples were analyzed. To demonstrate that ^{13}C labeling of the substrate had no influence on the experiment, a control experiment with ^{12}C-*Spirulina* (GSE-Vertrieb GmbH, Saarbrücken, Germany) was performed under the same conditions with single measurements. No significant difference for all measured metabolites and total cell counts could be detected between ^{13}C-*Spirulina* and ^{12}C-*Spirulina* degradation (Supplementary Figures S3 and S4).

Chemical analyses

Concentrations of fermentation products in the pore water were analyzed by high-performance liquid chromatography (Sykam, Fürstenfeldbruck, Germany) using an Aminex HPX-87H column (Biorad, München, Germany) at 60 °C. The eluent was 5 mM H_2SO_4 at 0.5 ml min^{-1}. Organic acids were detected by an UV–VIS detector (UVIS 204; Linear Instruments, Reno, NV, USA) at 210 nm. Alcohols were detected by a refractive-index detector (Smartline 2300; Knauer, Berlin, Germany). Before injection, the pore water was filtered through a 2 μm filter.

Sulfate concentrations were measured by an ion chromatograph (Sykam) with an anion separation column (LCA A24; Sykam) at 60 °C, followed by conductivity detection (S3115; Sykam). The eluent consisted of 0.64 g sodium carbonate, 0.2 g sodium hydroxide, 150 ml ethanol and 2 ml modifier (0.1 g 4-hydroxybenzonitrile/10 ml methanol) filled up to 1 l with distilled water. The flow rate was set to 1.2 ml min^{-1}. Before analysis, the samples were diluted 1–100 in the eluent without the modifier.

The concentrations of gaseous compounds were determined by an 8610C gas chromatograph (Schambeck SFD GmbH, Honnef, Germany). Analysis was carried out with argon (1 ml min^{-1}) as carrier

gas and at a column oven temperature of 40 °C. For the analysis of molecular hydrogen and methane, a molecular sieve 13 × packed column was used, whereas carbon dioxide was separated by a HayeSep D packed column. A thermal conductivity detector (256 °C) and a flame ionization detector (380 °C) were connected in a series for the detection of the gases. Sulfide concentrations were determined photometrically as described by Cord-Ruwisch (1985).

Determination of total cell numbers

Total cell counts were obtained by SybrGreen I staining (Lunau *et al.*, 2005), modified as follows. For sample fixation, 0.5 cm^3 of sediment was transferred to 4.5 ml of fixing solution (63 ml distilled water, 30 ml methanol, 2 ml of 25% glutaraldehyde solution, 5 ml Tween-80) and incubated at room temperature overnight. For detaching cells from particles, the sediment slurries were incubated for 15 min at 35 °C in an ultrasonic bath (Bandelin, Sonorex RK 103 H, Berlin, Germany, 35 kHz, 2 × 320 W per period). Homogenized aliquots of 20 µl were equally dispensed on a clean microscope slide in a square of 20 × 20 mm^2. The slide was dried on a heating plate at 40 °C. A drop of 12 µl staining solution (190 µl Mowiol, 5 µl SybrGreen I, 5 µl 1 M ascorbic acid in Tris-acetate-EDTA buffer) was placed in the center of a 20 × 20 mm^2 coverslip, which was then placed on the sediment sample. After 10 min of incubation, 20 randomly selected fields or at least 400 cells were counted for each sediment sample by epifluorescence microscopy.

RNA extraction and quantification

Total RNA was extracted from 1 g sediment by using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cell disruption, 1 g sediment and 1 ml RLT buffer were added to 1 g glass beads (0.18 mm diameter; Sartorius, Göttingen, Germany). Samples were homogenized for 90 s using a Mini Beadbeater (Biospec Products, Bartlesville, OK, USA).

For quantification, 100 µl of RiboGreen solution (diluted 1:200 in TE buffer; pH 7.5) was added to 100 µl of RNA extract (each sample diluted 1:100 in TE buffer; pH 7.5) and subjected to a microtiter plate. Serial dilutions (200:1 ng μl^{-1}) of *Escherichia coli* 16S and 23S rRNA (Roche, Grenzach-Wyhlen, Germany) were treated as described above and served as a calibration standard in each quantification assay. Fluorescence was measured at an excitation of 485 nm and an emission of 520 nm.

Isopycnic centrifugation and gradient fractionation

Density gradient centrifugation was performed with cesium tri-fluoroacetate as described by Lueders *et al.* (2004). Centrifugation conditions were 20 °C > 60 h at 125 000 g_{av}. Centrifuged gradients were fractionated from top to bottom into 14 equal fractions (~500 µl). The density of a small aliquot (35 µl) of each collected fraction was determined at 20 °C using a digital refractometer (DRBO-45ND, Müller Optronic, Erfurt, Germany). From cesium trifluoroacetate gradient fractions, nucleic acids were precipitated with 500 µl of isopropanol. Precipitates from gradient fractions were washed once with 150 µl of 70% ethanol and were re-eluted in 25 µl elution buffer for subsequent determination of RNA concentration.

Quantitative reverse transcription-polymerase chain reaction

Bacterial rRNA from gradient fractions was quantified by real-time polymerase chain reaction (PCR) (Rotor-Gene 3000 Cycler, Corbett Research, Sydney, Australia) using the primers Ba519f/Ba907r (Stubner, 2002) in combination with the OneStep RT-PCR Kit (Qiagen). Each 25 µl PCR reaction contained 15.9 µl nuclease-free water, 5 × RT-PCR buffer (Qiagen), 0.4 mM dNTP mix (Qiagen), 0.2 µM of each primer, 0.1 µl of a 1–500 diluted SybrGreen I solution (Molecular Probes, Eugene, OR, USA), 1 µl OneStep Enzyme Mix (Qiagen) and 1 µl standard (8.2×10^9–8.2×10^2 gene copies per µl) or environmental target RNA. Thermal cycling comprised a reverse transcription step for 30 min at 50 °C, followed by an initial denaturation step for 15 min at 95 °C, 40 cycles of amplification (10 s at 94 °C, 20 s at 54 °C, 30 s at 72 °C and 20 s at 82 °C) and a terminal step (2 min at 50 °C). After each run, a melting curve was recorded between 50 °C and 99 °C to ensure that only specific amplification had occurred. 16S and 23S rRNA of *E. coli* (Roche Diagnostics GmbH, Risch, Switzerland) served as standard for bacterial gene targets.

Denaturing gradient gel electrophoresis analysis

Partial 16S rRNA genes were amplified using the OneStep RT-PCR Kit (Qiagen) with bacterial primers GC-357f and Ba907r as given in Muyzer *et al.* (1995). Thermal cycling included a reverse transcription step for 30 min at 50 °C, followed by an initial denaturation step for 15 min at 95 °C, 30 cycles of RNA amplification (30 s at 94 °C, 45 s at 57 °C, 60 s at 72 °C) and a terminal elongation step (10 min at 72 °C).

The PCR amplicons and loading buffer (40% (wt vol^{-1}) glycerol, 60% (wt vol^{-1}) 1 × Tris-acetate-EDTA and bromophenol blue) were mixed in a ratio of 1:2. Denaturing gradient gel electrophoresis (DGGE) was carried out as described by Süß *et al.* (2004) using an INGENYphorU-2 system (Ingeny, Leiden, The Netherlands) and a 6% (wt vol^{-1}) polyacrylamide gel containing denaturant gradients of 50–70% for separation of PCR products. The gels were stained for 2 h with 1 × SybrGold (Molecular Probes, Leiden, The Netherlands) in 1 × Tris-acetate-EDTA buffer and washed for 20 min in distilled

water before UV transillumination. A control DGGE was conducted as described above with fractions of ^{12}C-amended samples in comparison to ^{13}C-amended samples to show that the community structure of ^{12}C- and ^{13}C-amended samples were developing equally (Supplementary Figure S5).

Sequence analysis

DGGE bands were excised for sequencing and treated as described previously (Del Panno *et al.*, 2005) without the second denaturing gel for purification. For reamplification of the bands, the same primer pairs as described above were used without the GC clamp. The bacterial reamplification comprised 26 PCR cycles (annealing temperature 55 °C), whereas the archaeal reamplification comprised 32 PCR cycles (annealing temperature 48 °C). For subsequent sequence analysis, PCR products of DGGE bands were purified using the QIAquick PCR purification kit (Qiagen) and were commercially sequenced by GATC Biotech (Konstanz, Germany). The partial 16S rRNA sequences of the DGGE bands were compared to those in GenBank using the BLAST function (Altschul *et al.*, 1998). All partial 16S rRNA gene sequences obtained in this study have been deposited in the EMBL database under accession numbers HE578749–HE578778.

Results

Phases of activity

The addition of ^{13}C-labeled *Spirulina* biomass (130 mg per 21 g sediment slurry) rapidly induced microbial activity (Figure 1). The maximum heat output (55 μW) measured by microcalorimetry was reached already after 1 day and even after 22 days a heat output of 6 μW was detectable. At least three different phases could be discriminated: two major peaks in the beginning (from days 0 to 5 and from days 5 to 10) and the phase of decreasing heat production from day 10 to the end of the experiment. The two main peaks showed several activity shoulders, indicating that biomass degradation involved a series of different microbial processes or/and organisms.

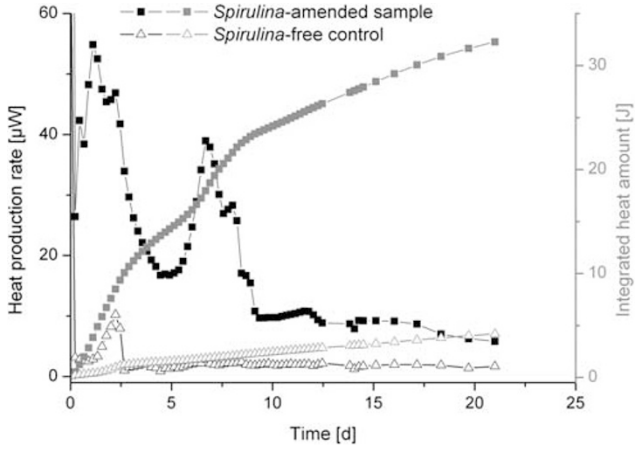

Figure 1 Heat production resulting from ^{13}C-*Spirulina* biomass degradation (black symbols, one representative sample out of three replicates; all showed a similar curve progression); filled symbols: *Spirulina*-amended sample; open symbols: substrate-free control; and gray symbols: integrated heat production.

The integrated heat amount (Figure 1) did not reach a plateau, which indicated that the biomass was not completely degraded and that the community was still active at the end of the experiment. Most of the heat production was due to the *Spirulina* degradation. The integrated heat mounted to 33 J, whereas the *Spirulina*-free control produced only 4 J within 22 days. The initial activity in the *Spirulina*-free control might be induced by mixing of the sediment during transfer to the microcalorimeter vials.

Fermentation products

The degradation resulted in a release of typical fermentation products like acetate, propionate, butyrate, ethanol, valerate, CO_2 and H_2 (Figures 2a–d). The main products, acetate and CO_2, increased rapidly within the first 5 days. Thereafter, this accumulation decelerated and the concentrations of ethanol and H_2 even decreased. At the end of the experiment, the alcohols were completely consumed, whereas the fatty acids were still present. Between days 16 and 24, the concentrations of all fermentation products (except CO_2 and valerate) decreased, indicating the depletion of electron donors derived from the added biomass. The decreasing concentrations (especially of alcohols) and the production of methane showed that secondary fermentations coupled to methanogenesis had started. According to the production of fatty acids, the pH decreased from 7.3 to 6.3 (Supplementary Table S1).

Sulfate was almost completely consumed within the first 7 days (Figure 2a). Sulfate was converted to sulfide in almost equimolar amounts (Supplementary Table S1). As only dissolved sulfide was measured, the missing part of the sulfide is probably present as H_2S in the head space and a small part might also have precipitated, and thus evade detection. Methane production started as soon as sulfate was depleted. This observation is in accordance with the well-known facts about substrate affinity and competition between sulfate reducers and methanogens (Oremland and Taylor, 1977; Cord-Ruwisch *et al.*, 1988).

In the *Spirulina*-free control, sulfate consumption was similar to that of *Spirulina*-amended samples (Figure 2a), indicating that the sediment still contained degradable organic matter. Small amounts of acetate and ethanol (about 100 μmol, each) were also produced in these samples (data not shown). Most of the HPLC peaks could be identified. An exception was one peak, which occurred from days 3 to 22. Comparing the very large signal of the UV

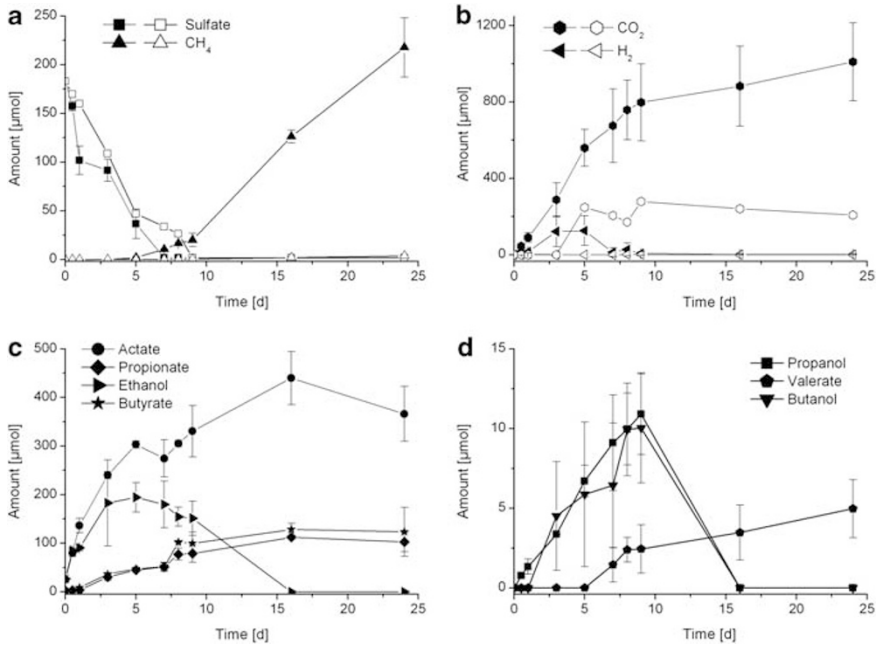

Figure 2 Chemical analyses of ^{13}C-*Spirulina*-amended samples (filled symbols, mean values of triplicates) and *Spirulina*-free controls (open symbols, single measurements). (**a**) CH_4 production and sulfate consumption, (**b**) CO_2 and H_2, (**c**) major fermentation products, and (**d**) minor fermentation products. Error bars are given for the *Spirulina*-amended samples.

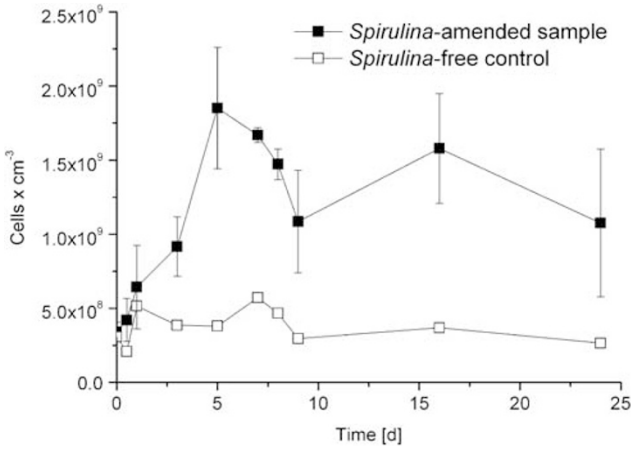

Figure 3 Total cell counts of ^{13}C-*Spirulina*-amended samples (mean values of triplicates) and of *Spirulina*-free controls (single measurements). Error bars are given for the *Spirulina*-amended samples.

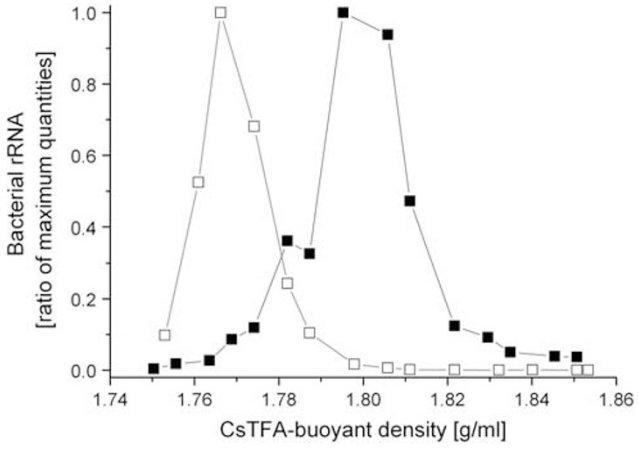

Figure 4 Quantitative distribution of bacterial rRNA in SIP centrifugation gradients after 12 h (one representative sample out of three replicates). Template distribution within gradient fractions was quantified by RT-qPCR and normalized for each gradient (Lueders *et al.*, 2004). Each square represents one gradient fraction. Open squares: fractions of the ^{12}C-*Spirulina*-amended samples; filled squares: fractions of the ^{13}C-*Spirulina*-amended samples; CsTFA: cesium trifluoroacetate.

detector to the signal of the RI detector, it seems probable that the unknown substance was an aromatic compound.

Total cell counts and community analysis

Although the stimulation induced significant heat output, the total cell numbers increased only slightly from approximately 5×10^8 to 2×10^9 cells per cm^3 (Figure 3). After 5 days, the total cell numbers began to decrease. Without the addition of *Spirulina* biomass, the cell numbers remained relatively constant at about 10^8 cells per cm^3.

Separation of ^{13}C-labeled and -unlabeled rRNA by density gradient centrifugation showed surprisingly large amounts of highly ^{13}C-labeled rRNA already after 12 h, which indicates a fast turnover of the added biomass (Figure 4). This efficient separation allowed an analysis of the labeled and unlabeled fractions, with only minor disturbances due to overlaying signals.

The most prominent ^{13}C-labeled DGGE bands (Figure 5) were affiliated to *Psychrilyobacter*

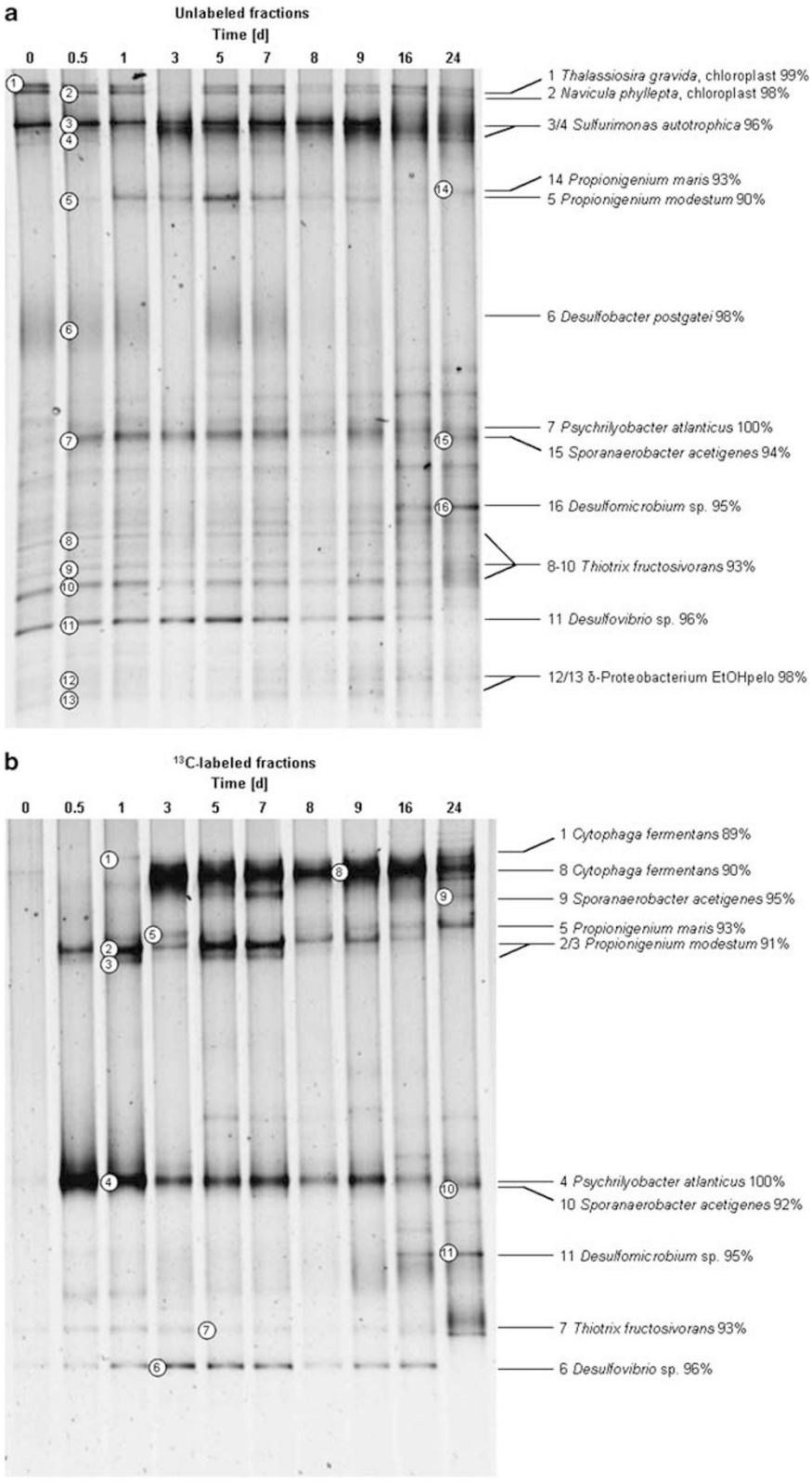

Figure 5 DGGE-community profiles of ^{13}C-labeled *Spirulina*-amended samples (one representative sample out of three replicates, respectively). The lanes in (**a**) show the profiles of the non-labeled community (light fractions). (**b**) The profiles of the ^{13}C-labeled community (heavy fractions). Fraction 4 with a buoyant density of approximately 1.765 g ml^{-1} served as unlabeled fraction for DGGE analysis and lane 10 with a buoyant density of approximately 1.805 g ml^{-1} as ^{13}C-labeled fraction. For representative bands, the closest cultivated relatives are given.

atlanticus (similarity 100%), to the genus *Propionigenium* (similarity to *P. modestum* 91%) and to *Cytophaga* (similarity to C. *fermentans* 89%). *P. atlanticus* and C. *fermentans* are typical primary fermenting bacteria (Bachmann, 1955; Zhao *et al.*, 2009), whereas *P. modestum* is known to be a secondary fermenting organism (Schink, 2006). Relatives of *P. atlanticus* and *Propionigenium* were already ^{13}C labeled after 12 h. The *Cytophaga*-related bacterium showed enhanced ^{13}C labeling after 3 days.

Sulfate reducers related to *Desulfovibrio* showed the strongest ^{13}C-labeling between days 3 and 7, when the sulfate concentration was decreasing rapidly and primary fermentation products were available as electron donors. The ^{13}C-labeled community pattern reflected the multi-phase biomass degradation, which could already be monitored by microcalorimetry. A successive appearance and disappearance of bands could be observed. As expected, only faint bands of the ^{13}C-labeled fractions were visible at the very beginning of the experiment, showing that there was little background label.

The light ^{12}C fractions derived from the ^{13}C-*Spirulina*-amended samples were analyzed for the community composition as well (Figure 5). They showed a significant higher diversity throughout the whole experiment than the community of the corresponding heavy fractions. Most of the ^{13}C-labeled organisms were already visible in the light RNA fractions before stimulation. This means that the *Spirulina*-carbon-assimilating bacteria were already abundant in the natural sediment. All of the sequenced organisms were fermenting or sulfate-reducing bacteria, most of them typically found in marine environments. Interestingly, the rRNA of diatoms was found throughout the whole experiment. Their presence was already observed in a previous study with tidal-flat sediments conducted by Logemann *et al.* (2011). However, the diatoms did not incorporate ^{13}C atoms.

Discussion

In this study, the determination of metabolic activity and chemical quantification combined with phylogenetic analyses were used to investigate the anaerobic degradation of complex organic matter. Different phases of primary and secondary fermentation as well as sulfate reduction followed by methanogenesis were detected. Important players involved in the initial steps were identified.

Experimental design and comparison to other studies

Most of the microbial activity was caused by the added biomass. High substrate concentrations are not unusual for tidal-flat sediments. They are caused, for example, by burial of algal blooms during storm events. Total organic matter concentrations reach values up to 2% dry weight in intertidal sand flats of the German Wadden Sea (Neira and Rackemann, 1996). This is about twice as high as the *Spirulina* amount added in our study. In comparable studies (Hull, 1987; Neira and Rackemann, 1996), even higher substrate concentrations (up to 67% dry weight) were applied to simulate the formation of black spots. A large part of cyanobacterial mats in tidal-flats consists of *Spirulina* spp. (Gerdes *et al.*, 1993); thus, *Spirulina* was an appropriate organism for our study. The experimental conditions led to about equimolar productions of sulfide and methane. Methane ebullition is not untypical for Wadden Sea sediments, provided that high organic loads cause sulfate depletion (Giani and Ahrensfeld, 2002; Røy *et al.*, 2008). Probably, the community did not shift far away from natural conditions owing to sulfide accumulation. In natural sediments of our sampling site, up to more than 15 mM were measured (Al-Raei *et al.*, 2009). In the control experiment without *Spirulina* addition, the same amount of sulfide was built as in the stimulated experiment. Although the sulfate-reducing organisms and the archaeal community were already investigated in previous studies (Wilms *et al.*, 2006b, 2007), our study focused on the initial steps of degradation.

Most previous studies of fermentation processes in sediments were carried out with defined monomers instead of complex organic matter. King and Klug (1982) determined glucose uptake rates in sediments of a eutrophic lake and analyzed the fermentation products resulting from glucose degradation. They found acetate, propionate and lactate as major fermentation products and only minor amounts of butyrate or valerate. Sansone and Martens (1982) studied the turnover of volatile fatty acids in marine, sulfate-reducing surface sediments, but did not analyze the communities involved. Webster *et al.* (2006) used ^{13}C-labeled acetate, glucose and pyruvate to identify the main consumers of these defined compounds. The identity of the major glucose utilizers remained unclear, whereas in ^{13}C-acetate and ^{13}C-pyruvate slurries, the *Desulfococcus* or *Desulfosarcina* were detected. However, the focus of this study was to compare SIP of DNA and phospholipid fatty acids.

Another interesting study using ^{13}C-labeled *Spirulina* was conducted by Gihring *et al.* (2009) by performing a comparable experiment under essentially different conditions. They used oxic coastal surface sediment and observed oxygen consumption and denitrification, whereas fermentation, sulfate reduction and methanogenesis only played a minor role. Correspondingly, they found completely different active communities than we did in our study.

Overall reaction equation

Based on the end products and the sulfate consumed (Figure 2 and Supplementary Figure S2), the

following equation was established:

$$2530.5\langle CH_2O\rangle + 183SO_4^{2-} \rightarrow 366\,\text{acetate} + 102\,\text{propionate} + 123\,\text{butyrate} + 5\,\text{valerate} + 183HS^- + 214CH_4 + 761.5CO_2 + 547.5H_2O + 413H^+$$

As the products contain only carbon, oxygen and hydrogen, a simple formula $\langle CH_2O \rangle$ was used for *Spirulina* biomass. CO_2, protons and water were used to balance the overall equation. Under standard conditions, the equation has a $\Delta G'$ of approximately 104 J. The measured heat amount (33 J) was roughly in the same order of magnitude. Possible explanations for the discrepancy may be that the entropy was not regarded and that a significant acidification occurred during the experiment that might have dissolved carbonates. Furthermore, a few unidentified metabolites were produced, which were not included in our equation.

Process assessment

The finding that the total cell counts began to drop after 5 days might be explained by cell lysis by phages, as the viral counts and the activity in aquatic ecosystems (Weinbauer and Höfle, 1998) and sediments (Danovaro *et al.*, 2008; Engelhardt *et al.*, 2011) are high. Owing to the anoxic conditions, we did not expect eukaryotic grazers and they were not detected by microscopy.

More than half of the carbon derived from the added *Spirulina* (130 mg biomass assumed to consist of 4333 μmol $\langle CH_2O \rangle$) was detected in the products (Supplementary Figure S1). Most of the carbon was present in reduced form as fermentation products, indicating that the degradation was incomplete. The finding that the amount of fatty acids was only slowly decreasing after day 8 shows that a steady-state situation, which keeps the concentration of fatty acids low, was not yet achieved. After sulfate was depleted, the methanogens were consuming the accumulated acetate. As acetoclastic methanogenic archaea are slowly developing (Beckmann *et al.*, 2011a, b), it is likely that the acetate concentration would decrease if the experiment is conducted for a longer time.

As initial steps, primary and secondary fermentation could be distinguished. During primary fermentations, H_2 and several acids and alcohols were produced. Later on, H_2 and the alcohols were consumed rapidly, whereas the amount of fatty acids was decreasing more slowly.

Two of the important players, probably involved in primary fermentation, were *P. atlanticus* and a relative of *C. fermentans*. Both have previously been found in marine sediments (Bachmann, 1955; Zhao *et al.*, 2009). *P. atlanticus* is a primary fermenting bacterium that produces acetate and hydrogen as main fermentation products (Zhao *et al.*, 2009). It showed an increase in ^{13}C incorporation at the beginning of the experiment. This organism was probably responsible for acetate and hydrogen production. *C. fermentans* generally ferments sugars to acetate, propionate and succinate (Bachmann, 1955). However, in our case, the physiological classification is uncertain, as the detected bacterium is only distantly related to *C. fermentans*.

Although a large part of the electrons (20%) was transferred to sulfide, only two active sulfate-reducing bacteria (as indicated by ^{13}C labeling in combination with sulfate consumption) were identified, which were not highly abundant. This is in accordance to the study of Beck *et al.* (2011), who found that sulfate-reducing bacteria only count up to approximately 5% of the bacterial community in tidal-flat sediments. During the phase of rapid sulfate depletion, *Desulfovibrio*-related bacteria were detected. That *Desulfovibrio*-related bacteria were outcompeting other SRB is not surprising as they have higher growth rates than other sulfate reducers (Widdel, 1986). The *Desulfovibrio*-related bacteria probably used hydrogen as electron donor, as it showed its highest activity in the phase of hydrogen excess, and *Desulfovibrio* is known to utilize hydrogen and to produce acetate from organic substrates (Voordouw, 1995). Sulfate-reducing bacteria known to oxidize their substrates completely to CO_2 did not emerge. They commonly grow more slowly than 'incomplete oxidizers' and had no chance to develop before sulfate depletion. Sulfate was also depleted after 9 days in the substrate-free control, indicating that the added biomass was not causing the absence of 'complete oxidizers'. Surprisingly, a *Desulfomicrobium*-related bacterium became labeled at the end of the experiment when sulfate was already depleted.

In the absence of sulfate, the degradation of organic matter becomes a three-step process—including secondary fermentation and methanogenesis. Accordingly, in our experiment, the produced fatty acids and especially alcohols were degraded. This indicates that secondary fermentation played an important role. The metabolism of syntrophic bacteria gains only little energy (Schink, 1997), which leads to low cell numbers. As DGGE analysis only detects the most abundant organisms (Muyzer and Smalla, 1998), it is not surprising that only two potentially secondary fermenters were identified, which were distantly related to *Propionigenium*. Both, *Propionigenium modestum* and *Propionigenium maris* produce propionate and CO_2 from succinate (Schink and Pfennig, 1982; Janssen and Liesack, 1995). Succinate might be supplied by the *C. fermentans*-related bacterium.

Concluding remarks

Our study provides a comprehensive analysis of organic matter degradation in a tidal-flat model system. Kinetics as well as the overall chemical reactions and the populations catalyzing them were unraveled. Although so far mainly populations involved in the terminal steps had been analyzed,

our approach enabled us to detect the important players involved in primary and secondary fermentations. Studies with radioactively labeled substrates would be suited to determine the metabolic processes at lower tracer concentrations. However, to detect the active members of the bacterial community (for example, by microautoradiography-fluorescence *in situ* hybridization), one would need to know their important genes in advance (Okabe *et al.*, 2004). Our study displayed the growing populations independent of this precondition.

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