RESEARCH ARTICLE

Identifying fermenting bacteria in anoxic tidal-flat sediments by a combination of microcalorimetry and ribosome-based stable-isotope probing

Jutta Graue[1], Sara Kleindienst[1], Tillmann Lueders[2], Heribert Cypionka[1] & Bert Engelen[1]

[1]Institut für Chemie und Biologie des Meeres, Carl-von-Ossietzky Universität Oldenburg, Oldenburg, Germany; and [2]Helmholtz Zentrum München, Institut für Grundwasserökologie, Neuherberg, Germany

Correspondence: Bert Engelen, AG Paläomikrobiologie, ICBM – University of Oldenburg, Carl-von-Ossietzky Strasse 9-11, D-26129 Oldenburg, Germany. Tel.: +49 441 798 5376; fax: +49 441 798 3583; e-mail: engelen@icbm.de

Present address: Sara Kleindienst, Max-Planck-Institut für Marine Mikrobiologie, Bremen, Germany.

Received 26 September 2011; revised 13 December 2011; accepted 14 December 2011. Final version published online 9 January 2012.

DOI: 10.1111/j.1574-6941.2011.01282.x

Editor: Max Häggblom

Keywords
microcalorimetry; ribosome-based stable-isotope probing; rRNA; fermentation; glucose; anoxic; tidal-flat sediments.

Abstract

A novel approach was developed to follow the successive utilization of organic carbon under anoxic conditions by microcalorimetry, chemical analyses of fermentation products and stable-isotope probing (SIP). The fermentation of ^{13}C-labeled glucose was monitored over 4 weeks by microcalorimetry in a stimulation experiment with tidal-flat sediments. Based on characteristic heat production phases, time points were selected for quantifying fermentation products and identifying substrate-assimilating bacteria by the isolation of intact ribosomes prior to rRNA-SIP. The preisolation of ribosomes resulted in rRNA with an excellent quality. Glucose was completely consumed within 2 days and was mainly fermented to acetate. Ethanol, formate, and hydrogen were detected intermittently. The amount of propionate that was built within the first 3 days stayed constant. Ribosome-based SIP of fully labeled and unlabeled rRNA was used for fingerprinting the glucose-degrading species and the inactive background community. The most abundant actively degrading bacterium was related to *Psychromonas macrocephali* (similarity 99%) as identified by DGGE and sequencing. The disappearance of *Desulfovibrio*-related bands in labeled rRNA after 3 days indicated that this group was active during the first degradation phase only. In summary, ribosome-based SIP in combination with microcalorimetry allows dissecting distinct phases in substrate turnover in a very sensitive manner.

Introduction

Tidal flats are characterized by nutrient inputs from the open sea and the hinterland resulting in a high microbial turnover and mineralization of organic matter (Wilms *et al.*, 2006a, b). In sediments, oxygen is depleted within the first few millimeters and anoxic conditions are predominant below. The microbial diversity of surface sediment layers has been intensively studied in a German North Sea tidal flat over the last few years by cultivation (Brinkhoff *et al.*, 1998; Llobet-Brossa *et al.*, 1998; Mussmann *et al.*, 2003) and molecular methods (Böttcher *et al.*, 2000; Rütters *et al.*, 2002). Studies on the anoxic sediments revealed that bacterial communities in the sediment column are dominated by fermentative bacteria (Köpke *et al.*, 2005; Wilms *et al.*, 2006a, b), as typically found also in other anoxic sediments (Schink, 2002). The terminal steps of organic matter degradation are performed by sulfate-reducing bacteria and methanogenic archaea, which are dependent on metabolites produced in fermentation processes (Laanbroek *et al.*, 1985). The distribution of sulfate reducers and methanogens was investigated via key genes for dissimilatory sulfate reduction and methanogenesis and reflected the vertical profiles of sulfate and methane (Wilms *et al.*, 2007). However, because of the high diversity of fermenting microorganisms and fermentative pathways, no such key genes are available for this group. Thus, unraveling the ecology of initial carbon degradation and the microorganisms involved requires a different strategy.

FEMS MICROBIOLOGY ECOLOGY

One approach to study metabolic activities is the stimulation of microbial communities in enriched cultures by adding different substrates and to follow organic matter degradation (Teeling & Cypionka, 1997). As metabolic activity is always coupled to the production of heat, substrate utilization can be monitored by microcalorimetry. The technique allows real-time detection of heat flows in the range of micro-watts (Larsson *et al.*, 1991) and is suited to follow the temporal dynamics of fermentation processes, where primary and secondary substrate consumers are often consecutively active. Degradation experiments in a microcalorimeter enable the noninvasive determination of optimal time points for sampling of successive activities in carbon degradation. Furthermore, the heat evolved during the processes can be quantified via the integration of the calorimetric heat production–time curves.

While microcalorimetry helps to distinguish the different degradation processes, the active degraders can be identified by the incorporation of heavy stable isotopes into microbial biomass followed by stable-isotope probing (SIP). In this technique, labeled biomarkers are extracted after incubation under labeled substrate addition and subsequently separated according to their mass or density (Radajewski *et al.*, 2000). In pioneering SIP experiments, ^{13}C-labeled phospholipid fatty acids (PLFA) were analyzed (Boschker *et al.*, 1998), while SIP was later applied to labeled DNA (Radajewski *et al.*, 2000), RNA (Manefield *et al.*, 2002; Lueders *et al.*, 2004), and proteins (Jehmlich *et al.*, 2008). Gihring *et al.* (2009) used DNA-based SIP to identify phytodetritus-degrading and denitrifying bacteria in oxic coastal surface sediment. Also, rRNA-based SIP has already been applied to identify dechlorinating microorganisms in tidal-flat communities from the North Sea (Kittelmann & Friedrich, 2008).

In our study, we introduce a novel combination of microcalorimetry in tandem with SIP of rRNA obtained via a pre-extraction of intact ribosomes from tidal-flat sediments. The extraction of ribosomes from the environment was successfully applied to soil samples before by Felske *et al.* (1996) to investigate active members of microbial communities (Felske *et al.*, 1997, 1998a, b). Separating ribosomes from other cell structures results in a concentration and purification of ribosomal RNA. Furthermore, because of the intact structure of the ribosomes, highly labile RNA is protected against degradation by nucleases.

Materials and methods

Sample collection

Sediment samples were collected during low tide from the back barrier tidal-flat area of Spiekeroog Island, Germany (53°44.178′N and 07°41.974′E), in June 2010. At the time of sampling, the air temperature was 19 °C and the water temperature 17.5 °C. Sediment from 1- to 5-cm depth was transferred into a sterile 500-mL glass bottle. Subsamples for ribosome isolation were stored at −80 °C until processing. Prior to the stimulation experiment, sulfate was added to the sediment and further incubated at 20 °C to decrease the content of degradable organic carbon within the sediment. The sulfate concentration decreased twice below 5 mM and was replenished to 20 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.

For evaluating the ribosome extraction method, pure cultures of microorganism with different cell wall constructions were prepared: *Phaeobacter gallaeciensis* (DSMZ 17395) as representative for Gram-negative bacteria and *Micrococcus luteus* (DSMZ 20030) as representative for Gram-positive bacteria were incubated at 20 °C in HEPES-buffered peptone glucose media and complex lactate media, respectively. Cultures of the archaeon *Methanosarcina barkeri* (DSMZ 800) were grown at 38 °C in mineral media (Widdel & Bak, 1992). Cells of the pure cultures were harvested during the exponential growth phase and counted in a *Thoma* chamber to calculate total cell numbers. Yields of ribosomes per cell were estimated by the measured rRNA amount assuming an average RNA content of 2.5×10^{-12} μg rRNA per ribosome (Felske *et al.*, 1998a).

Cultivation and microcalorimetry

All sample handling prior to stimulation experiment was performed within an anaerobic chamber containing an nitrogen atmosphere with traces of hydrogen (< 0.1%). To eliminate spatial heterogeneity, surface sediment (1–5 cm) was mixed thoroughly. Sulfate concentrations were adjusted to 28 mM. Individual subsamples were prepared as slurries 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 resulting sulfate concentration was 14.3 mM. This material was transferred to 23-mL glass vials and mixed with 57 μmol D-Glucose-$^{13}C_6$ (Isotec, Germany) resulting in a final concentration of 9 mM (6.3 mL pore water volume). The vials were sealed with rubber stoppers and flushed with N_2 to remove residues of H_2. The pH was 7.3 at the beginning of the experiment. Glucose-free slurries served as controls. Four cultures were incubated (three glucose-amended and one glucose-free) in a heat-conduction microcalorimeter

(2277 Thermal Activity Monitor; ThermoMetric, Järvalla, Sweden) at 20 °C for 27 days. In previous experiments, this temperature was determined in MPN enrichments from tidal-flat sediments as the optimum growth temperature (Sass *et al.*, 2003). Additional samples were incubated at 20 °C outside the microcalorimeter. The heat production was recorded at intervals of 300 s using the program DIGITAM 3.0 (SciTech Software; ThermoMetric). The cultures were sacrificed for sampling at specific points when characteristic heat production resulting from the degradation of glucose was observed. Triplicates of ^{13}C-labeled glucose-amended samples were analyzed. To demonstrate that ^{13}C-labeling of the substrate had no influence on the experiment, a control experiment with unlabeled *glucose* was performed under the same conditions with single measurements. No significant difference for all measured metabolites could be detected between ^{13}C-*glucose* and ^{12}C-*glucose* degradation (data not shown).

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) at 210 nm. Alcohols were detected by a refractive index detector (Smartline 2300; Knauer, Berlin, Germany). Prior to injection, the pore water was filtered through a 0.2-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 per 10 mL methanol) filled up to 1 L with distilled water. The flow rate was set to 1.2 mL min^{-1}. Prior to analysis, the samples were 1 to 100 diluted in eluent without modifier.

The concentrations of gaseous compounds were determined by a gas chromatograph (8610C; 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 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 series for detection of the gases. Sulfide concentrations were determined photometrically as described by Cord-Ruwisch (Cord-Ruwisch, 1985).

Extraction of nucleic acids

Ribosomes were extracted according to Felske *et al.* (1996). Briefly, the cells were mechanically disrupted, followed by three steps of differential centrifugation: The first two steps (15 000 ***g*** for 15 min and 30 000 ***g*** for 30 min) serve the purpose to remove the cell debris and the third step with a higher acceleration (100 000 ***g*** for 2 h) to pelletize the ribosomes. As a final step, the ribosome pellet was dissolved in 295 μL TN150-buffer (10 mM Tris–HCl, 150 mM NaCl, pH8) and 5 μL VRC solution (vanadyl ribonucleoside complexes, 200 mM; Fluka, Munich, Germany). Thereafter, the RNA of the resulting ribosome solution was extracted with the All-Prep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA extracts were controlled for DNA contaminations using the universal 16S rRNA gene primers 357f/907r (Muyzer *et al.*, 1995) for PCR. Nucleic acid concentrations were quantified fluorometrically in a microtiter plate reader (FLUOstar Optima; BMG Labtechnologies, Offenburg, Germany) using a 1 : 200 diluted PicoGreen reagent for DNA quantification and a 1 : 200 diluted RiboGreen reagent for RNA quantification according to modified manufacturer's protocols (Molecular Probes, Eugene). One-tenth of each volume and 1 μL of the extracted DNA and Lambda-DNA in different concentrations from 100 to 1 ng μL^{-1} were used.

Transmission electron microscopy (TEM)

A 400-mesh Formvar copper grid (Plano) was carefully placed on a 50-μL drop of the ribosome extract. The ribosomes that were adsorbed on the grid were stained with 50 μL of aqueous uranyl acetate (2%) for 2 min and examined with an EM 902A Transmission electron microscope (Zeiss, Jena, Germany). A high-speed SSCCD camera system (Proscan elektronische Systeme GmbH, Lagerlechfeld, Germany) with ITEMFIVE software was used for images acquisition.

Isopycnic centrifugation and gradient fractionation

Density gradient centrifugation was performed with cesium tri-fluoroacetate (CsTFA) as described by Lueders *et al.* (2004). Centrifugation conditions were 20 °C > 60 h at 125 000 ***g***. 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).

From CsTFA 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 using the RiboGreen assay. Bacterial rRNA from gradient fractions was quantified by real-time PCR (iCycler iQ real-time PCR system; Bio-Rad) using the primers Ba519f/Ba907r (Stubner, 2002). The one-step real-time reverse transcription (RT)-PCR system was based on the 'Access one-step RT-PCR system' (Promega, Germany), as described earlier (Lueders *et al.*, 2004).

Denaturing gradient gel electrophoresis (DGGE) analysis

Partial 16S rRNA gene transcripts were reverse transcribed, and the resulting cDNA was amplified using the OneStep RT-PCR Kit (Qiagen) with bacterial primers GC-357f and 907r as described by 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 bacterial RNA amplification and 35 cycles of archaeal RNA amplification (30 s at 94 °C, 45 s at 57 °C, and 60 s at 72 °C), and a terminal step (10 min at 72 °C).

The PCR amplicons and loading buffer [40% (w/v) glycerol, 60% (w/v) 1× Tris-acetate-EDTA (TAE), and bromphenol blue] were mixed in a ratio of 1 : 2. DGGE was carried out as described by Süß *et al.* (2004) using an INGENYphorU-2 system (Ingeny, Leiden, The Netherlands) and a 6% (w/v) polyacrylamide gel containing denaturant gradients of 50–70% for separation of bacterial PCR products. DGGE gels were run at 60 °C for 20 h. After electrophoresis, 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 prior to UV transillumination.

Sequence analysis of DGGE bands

DGGE bands were excised, subjected to 50 μL of PCR-water, and incubated overnight at 4 °C to elute the DNA. Two microliters of the DNA eluate was taken for reamplification with the same primer pairs as described above without containing the GC-clamp. The reamplification comprised 26 PCR cycles (annealing temperature 55 °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. The partial 16S rRNA gene 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 HE616750–HE616776.

Results

Pre-experiments to assess the quality of ribosomes as source for nucleic acids

Pre-experiments with pure cultures and sediment samples were conducted to evaluate the performance of the rRNA extraction method. Electron micrographs from direct ribosome extracts of pure cultures and tidal-flat sediment samples illustrate the high quality of the ribosome extracts (Fig. 1). A homogeneous pellet of intact ribosome-like particles, with a diameter of app. 20 nm was obtained by the extraction from a pure culture of *P. gallaeciensis* (Fig. 1a). Ribosome-like particles were also present in the pellet extracted from sediments but varied slightly in diameters, as it would be expected for different species. In this environmental sample, some other structures like viruses and vesicles of unknown origin were also visible (Fig. 1b). However, the high amount of ribosomes within both extracts demonstrates the potential of this technique in yielding a high enrichment of phylogenetically relevant rRNAs (up to 30 ng cm^{-3}). To establish the influence of different cell structures on the ribosome extraction efficiency, the ribosome numbers per cell of three pure cultures were determined. The ribosome yield (in ribosomes per cell) was roughly in the same order of magnitude: *P. gallaeciensis* (Gram-negative): 533; *M. luteus* (Gram-positive): 1214; and *M. barkeri* (Archaeon): 467.

Time course of glucose fermentation

The addition of glucose to fresh anoxic surface sediments from tidal flats induced a rapid heat production, which was followed via microcalorimetry (Fig. 2). In the course of the stimulation experiment, at least three different phases were detected: the maximum heat output (225 μW) was already reached after 1 day, followed by a broader peak with its maximum at day two and a phase of decreasing heat production indicating the exhaustion of glucose. The calculated heat output was −22 J in each stimulation experiment. In control experiments without glucose, little heat production was measured, indicating that the observed metabolic activity was caused by the substrate added.

In coincidence with the heat production curves of the calorimetric experiments, glucose was completely consumed within the first 2 days in the amended samples (Fig. 3a). The substrate degradation resulted in the

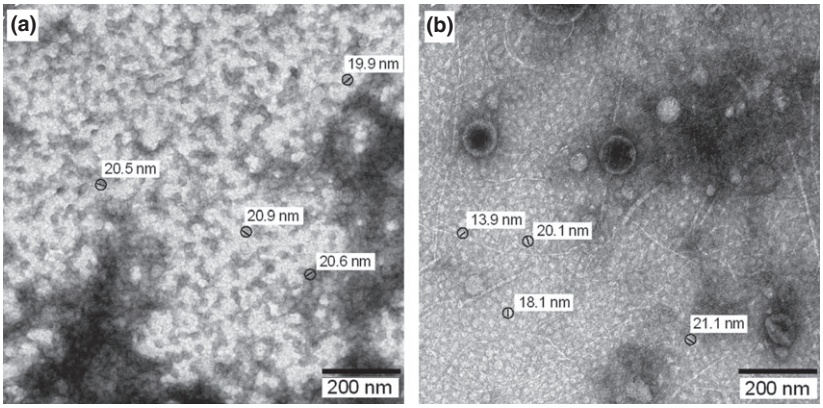

Fig. 1. Transmission electron micrograph of ribosome preparations. (a) Pure culture of *Phaeobacter gallaeciensis*, (b) surface sediment of a German tidal flat. Diameters of ribosome-like particles are indicated.

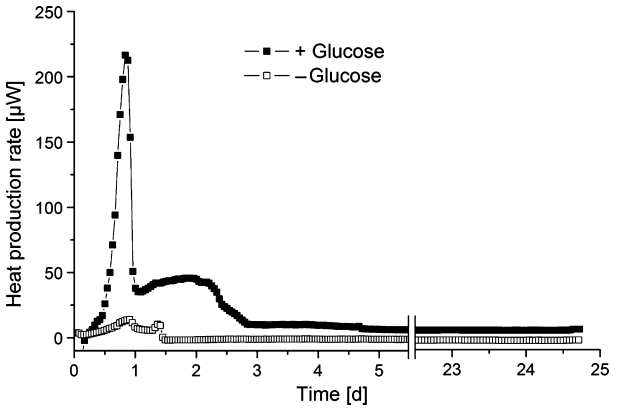

Fig. 2. Heat production measured by microcalorimetry during the stimulation experiment. Heat production values remained constant between day 5 and 23 in the glucose-amended sediment (closed symbol) and the glucose-free control (open symbol).

production of typical fermentation products like acetate, ethanol, formate, propionate, H_2, and CO_2 (Fig. 3a–c). A small amount of butyrate (2 μmol) was only detected at day 27 (data not shown). The most important fermentation product was acetate, which reached values up to 90 μmol. Ethanol, formate, and H_2 accumulated rapidly, but were completely consumed at the end of the experiment (Fig. 3b and c). Propionate was produced more slowly and reached a plateau after 3 days. According to the formation of acidic fermentation products, the pH was decreasing (Table 1). Sulfate was rapidly depleted and converted to sulfide (Table 1) in the glucose-amended samples as well as in the glucose-free control. This indicates that the sediment itself still contained some electron donors prior to glucose addition even though the samples were preincubated for a depletion of indigenous substrates. Additionally, CO_2 was produced in the glucose-free controls, and a transient heat production was observed during the first days. This was probably due to mobilizing indigenous electron donors by mixing during the preparation of sediment slurries. Methanogenesis started as soon as sulfate was depleted (Fig. 3d). The chemical analyses showed no differences between ^{13}C-glucose and ^{12}C-glucose degradation. Based on the end products and the sulfate consumed, the following equation was established for the overall reaction at the end of the experiment:

$$58.75\,\text{glucose} + 89\ SO_4^{2-} + 17\,H^+ \rightarrow 63\,\text{acetate} + 7\,\textit{propionate} + 2\,\textit{butyrate} + 89\,HS^- + 7\,CH_4 + 180\,CO_2 + 183.5\,H_2O$$

CO_2, protons, and water were used to balance the overall equation. Under standard conditions, this equation has a $\Delta G_0'$ of approximately -26 J, which fits nicely to the measured heat amount of -22 J (ΔH).

Identification of active fermenters via SIP

Separation of ^{13}C-labeled and unlabeled rRNA by density gradient centrifugation showed surprisingly large amounts of highly ^{13}C-labeled rRNA already after 1 day (Fig. 4), which indicates fast substrate assimilation. This efficient separation allowed an analysis of the labeled and unlabeled fractions with only minor disturbances owing to overlaying signals.

The most abundant glucose degrader was a relative of *Psychromonas macrocephali* (similarity 99%), which was already highly stimulated after half a day (Fig. 5). The increasing band intensity in the DGGE community pattern indicates an enhanced metabolic activity. Two sulfate-reducing bacteria affiliated to the genus *Desulfovibrio* showed a transiently increased abundance in 'heavy' rRNA at days two and three.

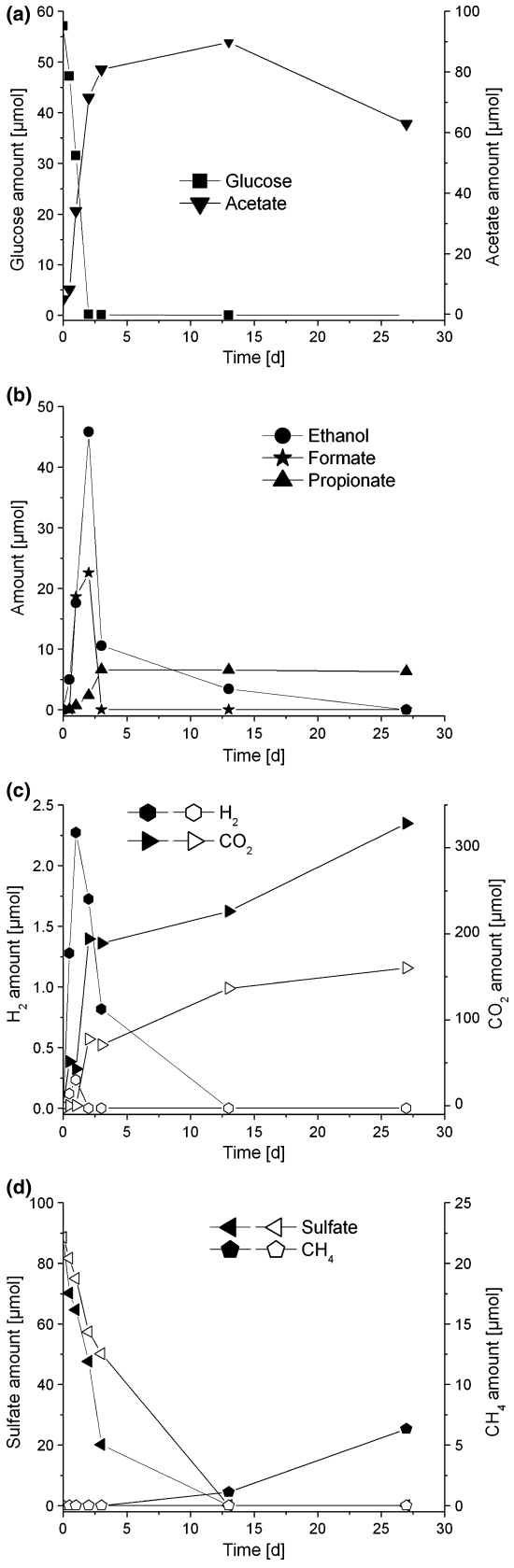

Fig. 3. Chemical analyses over the course of the experiment (referring to 21 g sediment slurry). (a) Glucose consumption and acetate production. (b) Distribution of dissolved fermentation products: ethanol, formate, and propionate. (c) Distribution of gaseous fermentation products: H_2 and CO_2. (d) CH_4 production and sulfate consumption, filled symbols: glucose-amended samples, open symbols glucose-free control.

Table 1. Dissolved sulfide concentrations and pH values of glucose-amended samples and glucose-free controls. Mean values of triplicates are given for the glucose-amended samples; other values are single measurements

	pH		Sulfide (nM)	
Time (d)	+Glucose	−Glucose	+Glucose	−Glucose
0	7.3	7.3	2.1	2.1
0.5	7.7	7.8	3.8	2.3
1	7.2	7.8	5.6	3.3
2	7.0	7.8	6.5	5.5
3	7.1	7.9	9.9	6.1
13	7.1	7.9	12.4	12.2
27	7.1	7.9	12.5	12.4

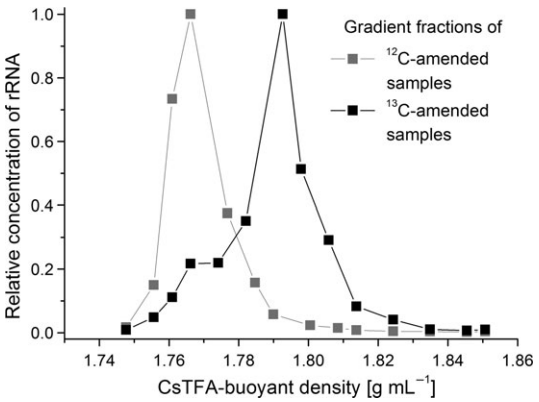

Fig. 4. Quantitative distribution of bacterial rRNA in SIP centrifugation gradients after 1 day of glucose fermentation (one representative sample of three replicates). Domain-specific template distribution within gradient fractions was quantified by RT-qPCR and normalized for each gradient (Lueders *et al.*, 2004). Each square represents one gradient fraction. Gray squares: fractions of the ^{12}C-glucose-amended samples, black squares: fractions of the ^{13}C-glucose-amended samples, CsTFA: Caesiumtrifluoracetate.

The unlabeled fractions represented the background community, which did not incorporate the ^{13}C-labeled carbon. The unlabeled community had a higher diversity than the active community. Most of the unlabeled organisms were affiliated to sulfate-reducing bacteria such as *Desulfovibrio* spp., *Desulfobacter postgatei*, *Desulfosarcina variabilis*, and *Desulfobulbus mediterranus*. As in the ^{13}C-labeled fractions, *Desulfovibrio*-related organisms showed their highest abundance in 'light' rRNA also during the

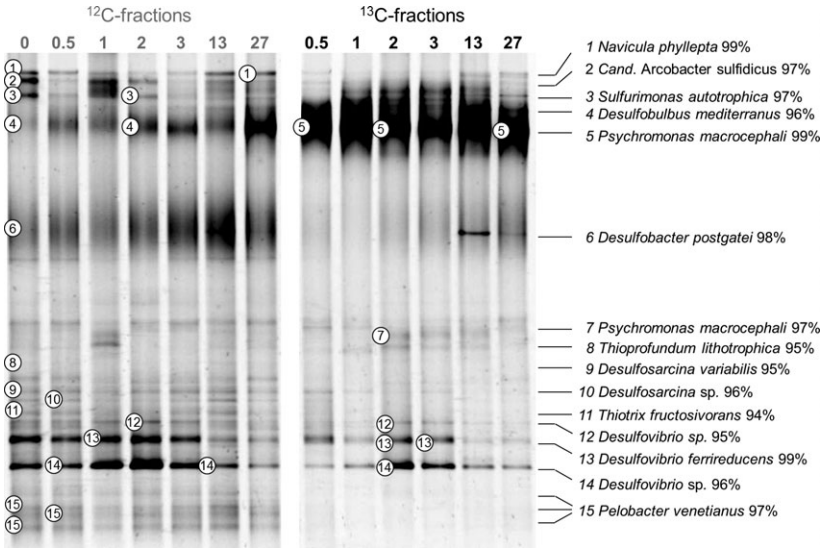

Fig. 5. DGGE community profiles of reversely transcribed partial 16S rRNA gene transcripts. Lanes represent unlabeled (gray) and ^{13}C-labeled bacteria (black) over the time course of the experiment (one representative sample of three replicates, respectively). The 'light' and 'heavy' RNA fractions originate from the same RNA extract for each time point. The time is given in days (numbers above lanes). Closest cultivated relatives and sequence similarities (%) are given.

first days of incubation. This indicates that most of these organisms degraded organic matter, which was already present in the sediment prior to glucose addition. Relatives of organisms that are known to oxidize sulfur compounds were also abundant in the unlabeled fractions. They were affiliated to '*Candidatus* Arcobacter sulfidicus', *Sulfurimonas autotrophica*, *Thioprofundum lithotrophica,* and *Thiotrix fructosivorans.*

A control DGGE was conducted with fractions of ^{12}C-amended samples in comparison with ^{13}C-amended samples to show that the community structure of ^{12}C- and ^{13}C-amended samples were developing equally. Exemplarily, the communities of the ^{13}C-glucose degradation experiment and the ^{12}C-glucose control at day 13 were compared. No significant differences were observed (Fig. 6).

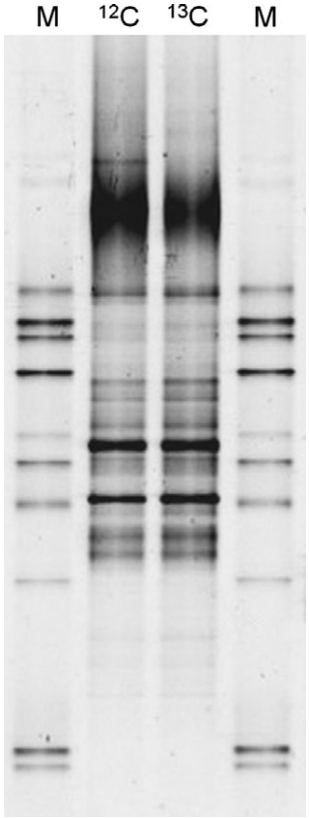

Fig. 6. DGGE community profiles of reversely transcribed partial 16S rRNA gene transcripts. Lanes represent light fractions (fractions 4, respectively) of ^{12}C and ^{13}C glucose-amended samples on day 13, M = marker.

Discussion

In the present study, glucose-degrading bacteria within tidal-flat sediments have been identified by a novel combination of microcalorimetry, chemical analyses of fermentation products and ribosome-based SIP. The ribosome isolation method for sediments turned out to be well suited for SIP. Microbial glucose degradation could be monitored in real time by microcalorimetry to identify sampling points for successive metabolic processes. The most active glucose-degrading bacterium in our experiments, a relative of *P. macrocephali,* was identified by DGGE analysis of density-resolved rRNA and subsequent sequencing of DGGE bands.

The course of fermentation

The key organism involved in primary fermentation was closely related to *P. macrocephali*. The type strain was originally isolated from an organic-rich marine sediments (Miyazaki *et al.*, 2008). In the study of Miyazaki *et al.* (Miyazaki *et al.*, 2008), *P. macrocephali* was found to be facultatively anaerobic and capable to form acids from glucose. Fermentative pathways were not described in detail for the type strain of *P. macrocephali*. A close relative, *Psychromonas antarcticus* (with 98% sequence similarity of the complete 16S rRNA gene), forms the following fermentation products from 100 mol of hexose: acetate (60 mol), formate (130 mol), ethanol (56 mol), lactate (73 mol), CO_2 (15 mol), and 2 mol of butyrate (Mountfort *et al.*, 1998). The same products except lactate were identified in our study (compare to the equation for the overall reaction).

Although half of the electrons derived from the added glucose were transferred to sulfate, only three bands (Fig. 5, band 12–14) of active sulfate-reducing bacteria were identified. These bacteria were all related to the genus *Desulfovibrio*. As *Desulfovibrio* ssp. are known to utilize lactate (Postgate & Campbell, 1966), it is not surprising that lactate was not detected in our experiment. The detection of only three bands of sulfate-reducing bacteria is in accordance with the study of Beck *et al.* (2011), who found that these bacteria only account for a minor proportion of the total bacterial community in tidal-flat sediments (~5%). *Desulfovibrio*-related organisms showed the highest rRNA DGGE band intensity during the phase of rapid sulfate depletion. These bacteria have probably used hydrogen as electron donor, because the sulfate consumption is correlated with the phase of hydrogen excess. *Desulfovibrio* is capable of utilizing hydrogen for incomplete oxidation of organic substrates to acetate (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 prior to sulfate depletion.

Secondary fermenting bacteria were not identified, as secondary fermentation gains only little energy (Schink, 1997), and therefore, low cell numbers are sustained. DGGE analyses, however, only detect the most abundant bacteria within communities (Muyzer & Smalla, 1998). Primary fermentation products (ethanol, formate, and H_2) were mainly consumed in the presence of sulfate, but small amounts of ethanol were also consumed when sulfate was already depleted – indicating the presence of secondary fermenters. Only the acetate values slightly decreased in the absence of sulfate (from 90 to 63 μmol), indicating acetotrophic methanogenesis. Correspondingly, methane was produced (7 μmol). However, only a small amount of the carbon atoms that derived from the acetate were found in methane.

The 'light' ^{12}C-fraction representing the inactive background community showed a higher diversity than the active degraders. For example, chemolithoautotrophic bacteria such as *S. autotrophica* were detected in the unlabeled fractions, indicating that they did not degrade the substrate. In the sediment, these bacteria occur at oxic/anoxic interfaces where reduced sulfur compounds are available as electron donors and oxygen serves at electron acceptor. In our experiment however, these gradients were broken down owing to the homogenization of the material. Thus, the organisms were still present but could not metabolize the substrate as there is no suitable electron acceptor available for them. Even if chemolithoautotrophs were active, they would not be detected by our approach using ^{13}C-labeled organic substrates as they assimilate CO_2 for carbon fixation.

Desulfovibrio-related bacteria seemed to be also stimulated by unlabeled organic matter originating from the sediment, as their bands in the 'light' fractions showed the highest intensity at day two. This is in accordance with the finding that sulfate was consumed and CO_2 was produced in glucose-free controls, which already demonstrated that the sediment contained degradable organic matter prior to glucose addition.

Do stimulation experiments reflect processes within tidal-flat sediments?

At the end of the experiment, most of the rRNA was fully labeled with ^{13}C, which points toward a high microbial activity in the investigated samples already at the onset of the experiment. This is supported by previous investigations that have demonstrated a high microbial turnover in surface sediments of the tidal flat analyzed here (Gittel *et al.*, 2008). The indigenous microbial communities were probably adapted to a high load of organic matter. DOC values at the same study site were determined to range from 0.5 mM at the surface to 3 mM within 1-m depth (Beck *et al.*, 2009). This fresh marine organic material is easily degradable (Freese *et al.*, 2008) and constantly replenished by advective processes (Billerbeck *et al.*, 2006). Therefore, the relatively high concentration of 9 mM glucose used in our study may not have caused a substrate shock (Straskrabova, 1983). The application of similarly high ^{13}C-substrate concentrations might be more problematic in deeper sediment layers with lower microbial activity. However, it should be considered that indigenous microorganisms of tidal flats have to cope with substrates that are harder to degrade than glucose. This affects the complexity of relations between active species as well as the

bioavailability and the energy gained from the substrates utilized. A successive approximation to natural conditions will be achieved when more complex substrates are used for similar SIP experiments.

Advantages of combining microcalorimetry and rRNA-SIP

The main advantage of conducting SIP experiments in a microcalorimeter is the possibility to monitor successive metabolic processes in real time to find optimal time points for sampling. Microcalorimetry and chemical analyses of fermentation and terminal oxidizing processes afford a detailed description of kinetics and processes. The measured $\Delta G'$ and the calculated $\Delta G_0'$ were in the same range. The overall equation nicely fits to the measured amounts of each component. Only the CO_2 in the equation is lower than the measured amount. However, the measured CO_2 might have partly derived from dissolving carbonates. This is supported by the finding that CO_2 was also produced in glucose-free controls.

In general, microorganisms do not necessarily react with cell division after a pulse of substrate but with boosting their cell machinery, which in turn results in a higher ribosome content. This response is specifically targeted when extracting ribosomes prior to RNA-based community analyses. When successive processes and trophic interactions in anoxic microbial food webs are studied, different microorganisms are successively labeled. This is reflected, for example, by the transient appearance of labeled rRNA of *Desulfovibrio*-related bacteria, which were prominent during initial glucose fermentation, but disappeared upon consumption of sulfate. One could assume that after sulfate depletion, a DNA-based SIP approach would still show the presence of respective labeled genomes. Our RNA-based SIP approach with pre-extraction of ribosomes was capable to record decreasing ribosome labeling and thus activity in a very sensitive manner.

Acknowledgements

We thank Sabine Schäfer (HZ, München) for her help in initial SIP experiments, Erhard Rhiel (ICBM, Oldenburg) for the help with the electron micrography and Ramona Appel (MPI, Bremen) for her assistance in analyzing fermentation products. This work was financially supported by Deutsche Forschungsgemeinschaft (DFG) within the Research Group on 'BioGeoChemistry of Tidal Flats' (FOR 432).

References

Altschul S, Madden T, Schaffer A, Zhang JH, Zhang Z, Miller W & Lipman D (1998) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *FASEB J* **12**: 102.

Beck M, Köster J, Engelen B *et al.* (2009) Deep pore water profiles reflect enhanced microbial activity towards tidal flat margins. *Ocean Dyn* **59**: 371–383.

Beck M, Riedel T, Graue J *et al.* (2011) Imprint of past and present environmental conditions on microbiology and biogeochemistry of coastal Quaternary sediments. *Biogeosciences* **8**: 55–68.

Billerbeck M, Werner U, Polerecky L, Walpersdorf E, de Beer D & Huettel M (2006) Surficial and deep pore water circulation governs spatial and temporal scales of nutrient recycling in intertidal sand flat sediment. *Mar Ecol Prog Ser* **326**: 61–76.

Boschker HTS, Nold SC, Wellsbury P *et al.* (1998) Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. *Nature* **392**: 801–805.

Böttcher ME, Hespenheide B, Llobet-Brossa E *et al.* (2000) The biogeochemistry, stable isotope geochemistry, and microbial community structure of a temperate intertidal mudflat: an integrated study. *Cont Shelf Res* **20**: 1749–1769.

Brinkhoff T, Santegoeds CM, Sahm K, Kuever J & Muyzer G (1998) A polyphasic approach to study the diversity and vertical distribution of sulfur-oxidizing *Thiomicrospira* species in coastal sediments of the German Wadden Sea. *Appl Environ Microbiol* **64**: 4650–4657.

Cord-Ruwisch R (1985) A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *J Microbiol Methods* **4**: 33–36.

Felske A, Engelen B, Nubel U & Backhaus H (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. *Appl Environ Microbiol* **62**: 4162–4167.

Felske A, Rheims H, Wolterink A, Stackebrandt E & Akkermans ADL (1997) Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology-UK* **143**: 2983–2989.

Felske A, Akkermans ADL & De Vos WM (1998a) Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription PCR in temperature gradient gel electrophoresis fingerprints. *Appl Environ Microbiol* **64**: 4581–4587.

Felske A, Wolterink A, Van Lis R & Akkermans ADL (1998b) Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). *Appl Environ Microbiol* **64**: 871–879.

Freese E, Köster J & Rullkötter J (2008) Origin and composition of organic matter in tidal flat sediments from the German Wadden Sea. *Org Geochem* **39**: 820–829.

Gihring TM, Humphrys M, Mills HJ, Huettel M & Kostka JE (2009) Identification of phytodetritus-degrading microbial communities in sublittoral Gulf of Mexico sands. *Limnol Oceanogr* **54**: 1073–1083.

Gittel A, Mussmann M, Sass H, Cypionka H & Könneke M (2008) Identity and abundance of active sulfate-reducing

bacteria in deep tidal flat sediments determined by directed cultivation and CARD-FISH analysis. *Environ Microbiol* **10**: 2645–2658.

Jehmlich N, Schmidt F, von Bergen M, Richnow HH & Vogt C (2008) Protein-based stable isotope probing (Protein-SIP) reveals active species within anoxic mixed cultures. *ISME J* **2**: 1122–1133.

Kittelmann S & Friedrich MW (2008) Novel uncultured Chloroflexi dechlorinate perchloroethene to trans-dichloroethene in tidal flat sediments. *Environ Microbiol* **10**: 1557–1570.

Köpke B, Wilms R, Engelen B, Cypionka H & Sass H (2005) Microbial diversity in coastal subsurface sediments: a cultivation approach using various electron acceptors and substrate gradients. *Appl Environ Microbiol* **71**: 7819–7830.

Laanbroek HJ, Blok JP & Steenhuis L (1985) Variability in fermentation patterns of sugar-utilizing bacteria isolated from Anaerobic, intertidal sediments. *Microb Ecol* **11**: 117–125.

Larsson C, Blomberg A & Gustafsson L (1991) Use of microcalorimetric monitoring in establishing continuous energy balances and in continuous determinations of substrate and product concentrations of batch-grown *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **38**: 447–458.

Llobet-Brossa E, Rossello-Mora R & Amann R (1998) Microbial community composition of Wadden Sea sediments as revealed by fluorescence *in situ* hybridization. *Appl Environ Microbiol* **64**: 2691–2696.

Lueders T, Manefield M & Friedrich MW (2004) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6**: 73–78.

Manefield M, Whiteley AS, Griffiths RI & Bailey MJ (2002) RNA stable isotope probing, a novel means of linking microbial community function to Phylogeny. *Appl Environ Microbiol* **68**: 5367–5373.

Miyazaki M, Nogi Y, Fujiwara Y & Horikoshi K (2008) *Psychromonas japonica* sp nov., *Psychromonas aquimarina* sp nov., *Psychromonas macrocephali* sp nov and *Psychromonas ossibalaenae* sp nov., psychrotrophic bacteria isolated from sediment adjacent to sperm whale carcasses off Kagoshima, Japan. *Int J Syst Evol Microbiol* **58**: 1709–1714.

Mountfort DO, Rainey FA, Burghardt J, Kaspar HF & Stackebrandt E (1998) *Psychromonas antarcticus* gen. nov., sp. nov., a new aerotolerant anaerobic, halophilic psychrophile isolated from pond sediment of the McMurdo Ice Shelf, Antarctica. *Arch Microbiol* **169**: 231–238.

Mussmann M, Schulz HN, Strotmann B *et al.* (2003) Phylogeny and distribution of nitrate-storing *Beggiatoa* spp. in coastal marine sediments. *Environ Microbiol* **5**: 523–533.

Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* **73**: 127–141.

Muyzer G, Teske A, Wirsen CO & Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16s rDNA fragments. *Arch Microbiol* **164**: 165–172.

Postgate JR & Campbell LL (1966) Classification of *Desulfovibrio* species, the nonsporulating sulfate-reducing bacteria. *Bacteriol Rev* **30**: 732–737.

Radajewski S, Ineson P, Parekh NR & Murrell JC (2000) Stable-isotope probing as a tool in microbial ecology. *Nature* **403**: 646–649.

Rütters H, Sass H, Cypionka H & Rullkötter J (2002) Phospholipid analysis as a tool to study complex microbial communities in marine sediments. *J Microbiol Methods* **48**: 149–160.

Sass H, Rütter H, Schledjewski R & Freese E (2003) The influence of seasonal changes on microbial communities in the Wadden Sea. *Forschungszentrum Terramare Berichte* **12**: 99–102.

Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* **61**: 262–280.

Schink B (2002) Synergistic interactions in the microbial world. *Antonie Van Leeuwenhoek* **81**: 257–261.

Straskrabova V (1983) The effect of substrate shock on populations of starving aquatic bacteria. *J Appl Bacteriol* **54**: 217–224.

Stubner S (2002) Enumeration of 16S rDNA of Desulfotomaculum lineage 1 in rice field soil by real-time PCR with SybrGreen (TM) detection. *J Microbiol Methods* **50**: 155–164.

Süß J, Engelen B, Cypionka H & Sass H (2004) Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods. *FEMS Microbiol Ecol* **51**: 109–121.

Teeling H & Cypionka H (1997) Microbial degradation of tetraethyl lead in soil monitored by microcalorimetry. *Appl Microbiol Biotechnol* **48**: 275–279.

Voordouw G (1995) The Genus *Desulfovibrio* – the Centennial. *Appl Environ Microbiol* **61**: 2813–2819.

Widdel F & Bak F (1992) Gram-negative mesophilic sulfate-reducing bacteria. *The Prokaryotes*, vol. **IV** (Balows A, Trüper HG, Dworkin M, Harder W & Schleifer K-H, eds), pp. 3352–3378. Springer-Verlag, New York.

Wilms R, Sass H, Köpke B, Köster H, Cypionka H & Engelen B (2006a) Specific bacterial, archaeal, and eukaryotic communities in tidal-flat sediments along a vertical profile of several meters. *Appl Environ Microbiol* **72**: 2756–2764.

Wilms R, Köpke B, Sass H, Chang TS, Cypionka H & Engelen B (2006b) Deep biosphere-related bacteria within the subsurface of tidal flat sediments. *Environ Microbiol* **8**: 709–719.

Wilms R, Sass H, Köpke B, Cypionka H & Engelen B (2007) Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea. *FEMS Microbiol Ecol* **59**: 611–621.