Bioreactor Janssand
Fermentation processes in tidal-flat sediments of the German North Sea

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Zusammenfassung

Der anaerobe Abbau organischen Materials in Wattsedimenten umfasst mehrere Schritte. Die terminalen Abbauschritte, Sulfatreduktion und Methanogenese, sind relativ gut aufgeklärt, während wenig über die einleitenden Fermentationsprozesse bekannt ist. Der Fokus dieser Studie liegt auf diesen ersten Schritten und soll folgende Fragen beantworten: Welche Phasen des anaeroben Abbaus können identifiziert werden? Welches sind die vorherrschenden Fermentationsprozesse und welche Organismen sind im Wesentlichen daran beteiligt?

Im Rahmen dieser Arbeit wurde ein neuer experimenteller Ansatz entwickelt, bei dem Mikrokalorimetrie zur Ermittlung verschiedener Abbauschritte, chemische Analysen zur Aufklärung der Fermentationsprozesse und „stable-isotope probing“ (SIP) zur Identifizierung der aktiv substratassimilierenden Organismen miteinander kombiniert wurden. Es wurden Abbauversuche mit drei verschiedenen Modellsubstraten durchgeführt.

Im ersten Teil dieser Studie wurde unmarkiertes Zellmaterial von *Haloferax volcanii* und *Saccharomyces cerevisiae* verwendet, um die Abbauraten von archaeellen intakten polaren Lipiden (IPLs) mit Etherbindung mit denen von bakterienähnlichen IPLs mit Esterbindung zu vergleichen. Im Laborexperiment wurden die IPLs mit Esterbindung schneller abgebaut als die IPLs mit Etherbindung. Neben den IPLs wurde auch organisches Material verwertet, das aus dem Sediment stammte. Die Analyse der Bakteriengemeinschaft mittels RT-PCR (Reverse Transkriptase PCR) basierter DGGE (Denaturierende Gradienten-Gelelektrophorese) zeigte, dass sulfatreduzierende und fermentierende Bakterien abundant waren.

Im zweiten und dritten Teil dieser Studie wurden ^{13}C-markierte Substrate verwendet, um die aktiv fermentierenden Bakterien mittels SIP zu identifizieren. Im zweiten Teil wurde Glucose, im dritten Teil Biomasse von *Spirulina* als Substrat verwendet. Die SIP Experimente wurden in einem abgeschlossenen System mit ausgehungertem Sediment durchgeführt. Daraufhin wurden Reaktionsgleichungen erstellt, die die Prozesse am Ende des Experiments beschreiben:

$$58.75\ \text{Glucose} + 89\ SO_4^{2-} + 17\ H^+ \rightarrow 63\ \text{Acetat} + 7\ \text{Propionat} + 2\ \text{Butyrat} + 89\ HS^- + 7\ CH_4 + 180\ CO_2 + 183.5\ H_2O$$

Eine vereinfachte Biomasseformel wurde für die Biomasse von *Spirulina* verwendet:

$$2530.5 <CH_2O> + 183\ SO_4^{2-} \rightarrow 366\ \text{Acetat} + 102\ \text{Propionat} + 123\ \text{Butyrat} + 5\ \text{Valerat} + 183\ HS^- + 214\ CH_4 + 761.5\ CO_2 + 547.5\ H_2O + 413\ H^+$$

Der Hauptverwerter der Glucose war eng mit *Psychromonas macrocephali* verwandt. Ein naher Verwandter dieses Bakteriums, *Psychromonas antarcticus*, fermentiert Glucose zu Acetat, Formiat, Ethanol, Lactat, CO_2 und Butyrat. Drei Hauptverwerter der *Spirulina* Biomasse wurden identifiziert: (i) *Psychrilyobacter atlanticus*, ein fermentierendes Bakterium, das H_2 und Acetat produziert, (ii) Bakterien, die weitläufig mit sekundären Gärern der Gattung *Propionigenium* verwandt sind und (iii) ein entfernter Verwandter von *Cytophaga*.

Summary

The anaerobic organic matter degradation in tidal-flat sediments is a multi-phase process. The terminal degradation steps, methanogenesis and sulfate reduction, are well studied. The initial fermentation processes, however, are poorly understood. The present study focused on these first steps and aimed at answering the following questions: Which phases of anaerobic degradation can be distinguished? What are the predominant fermentation processes? Which are the involved key players?

A new experimental approach was developed which combines microcalorimetry to detect different degradation phases, chemical analyses to identify the fermentation processes and stable-isotope probing (SIP) to determine the actively substrate assimilating organisms. Degradation experiments with three different model substrates were conducted.

In the first part of this study, unlabeled cell material of *Haloferax volcanii* and *Saccaromyces cerevisiae* was used to compare the degradation rates of ether-bound archaeal and ester-bound bacteria-like intact polar lipids (IPLs). IPLs are commonly used as biomarkers for viable cells. In the laboratory experiment ester-bound IPLs were degraded faster than ether-bound IPLs. The degradation of the IPLs was accompanied by the degradation of organic matter originating from the sediment. The community analysis via RT-PCR (Reverse Transcriptase-PCR) based DGGE (Denaturing Gradient Gel Electrophoresis) showed a high abundance of sulfate-reducing and fermenting bacteria.

For the second and third part of this study, ^{13}C-labeled substrates were used to identify the actively fermenting bacteria via SIP. The substrate used for the second part was glucose, whereas *Spirulina*-biomass was used for the third part. The SIP experiments were conducted in closed systems with starved sediment. Reaction equations were established to summarize the processes at the end of the experiments:

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

A simplified biomass formula was used for *Spirulina*-biomass:

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

The main glucose assimilating organism was closely related to *Psychromonas macrocephali*. Another close relative, *Psychromonas antarcticus* ferments glucose to acetate, formate, ethanol, lactate, CO_2 and butyrate. Three main degraders of *Spirulina*-biomass were identified as: (i) *Psychrilyobacter atlanticus*, a fermenter known to produce H_2 and acetate, (ii) bacteria distantly related to secondary fermenting bacteria of the genus *Propionigenium* and (iii) a remote *Cytophaga*-related bacterium.

List of publications

The results published in this dissertation have been submitted to international journals:

1. J. Logemann, **J. Graue**, J. Köster, B. Engelen, J. Rullkötter, and H. Cypionka, *A laboratory experiment of intact polar lipid degradation in sandy sediments* (2011), submitted to Biogeosciences Discuss., 8, 3289-3321

   Concept: J. L., J. G., J. K., B. E., J. K., H. C. ; IPL and fatty acid analyses: J. L.; Molecular biological analyses: J. G.; Fermentation products, methane and sulfate analyses: J. G.; First draft of manuscript: J. L., J. G.; Revision: H. C., B. E., J. K. J. R.

2. **J. Graue**, Sara Kleindienst, Tillmann Lueders, Heribert Cypionka, Bert Engelen, *Identifying fermenting microorganisms in anoxic tidal-flat sediments by a combination of microcalorimetry and ribosome-based stable-isotope probing* (2011), submitted to Applied and Environmental Microbiology

   Concept: B. E., S. K., J. G., T. L., H. C.; Electron micrographs: S. K.; Comparison of nucleic acid extractions: S. K.; Molecular biological analyses: J. G.; Chemical analyses: J. G; First draft of manuscript: S. K; J. G., B. E., Revision: B. E., H. C., T. L.

3. **J. Graue**, B. Engelen and H. Cypionka, *Degradation of cyanobacterial biomass in tidal-flat sediments: A combined study of metabolic processes and community changes* (2011), submitted to ISME Journal

   Concept: J. G., H. C.; Molecular biological analyses: J. G.; Chemical analyses: J. G.; First draft of manuscript: J. G.; Revision: H. C., B. E.;

Further publications

M. Beck, T. Riedel, **J. Graue**, J. Köster, N. Kowalski, C. S. Wu, G. Wegener, Y. Lipsewers, H. Freund, M. E. Böttcher, H.-J. Brumsack, H. Cypionka, J. Rullkötter, and B. Engelen (2011) *Imprint of past and present environmental conditions on microbiology and biogeochemistry of coastal Quaternary sediments*. Biogeosciences, 8, 55-68

M. Seidel, **J. Graue**, B. Engelen , J. Köster, H. Sass, and J. Rullkötter, *Intact polar membrane lipids in tidal flat sediments indicate active microbial communities impacted by advective pore water transport*, submitted to Geochimica et Cosmochimica Acta

Abbreviations

BES	2-Bromoethanesulfonic acid
BLAST	Basic Logical Alignment Search Tool
BSA	Bovine Serum Albumin
bp	Base pair
C	Carbon
°C	Degree Celsius
d	Day
Δ	Delta
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside Triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Resource Center for Biological Material)
dsr	Dissimilatory sulfite reductase
EDTA	Ethylene Diamine Tetraacetic Acid
e. g.	Exempli gratia (for example)
EMBL	European Molecular Biology Laboratory
et al.	*Et alii* (and others)
f	forward
Fig.	Figure
FISH	Fluorescence *In Situ* Hybridization
g	Gram
g	Gravitational acceleration
G	Gibbs free energy
G_0	Gibbs free energy under standard conditions
G_0`	Gibbs free energy under standard conditions at pH 7
GC	Gas Chromatograph
h	Hour
HPLC	High Performance Liquid Chromatography
IPL	Intact Polar Lipid
J	Joule

k	Kilo-
l	Liter
µ	Micro-
m	Milli-
M	Molar
mcr	Methylcoenzyme M reductase
min	Minute
n	Nano-
NCBI	National Center for Biotechnology Information
NVT	Near Vertical Tube
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
qPCR	Quantitative Polymerase Chain Reaction
r	Reverse
RNA	Ribonucleic Acid
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
RT	Reverse Transcriptase
s	Seconds
S	Svetberg
SDS	Sodium Dodecyl Sulfate
SIP	Stable-Isotope Probing
sp.	Species (singular)
spp.	Species (plural)
Tab.	Table
TAE	Tris-EDTA
Tris	Trishydroxymethylaminoethane
UV	Ultraviolet
UV-VIS	Ultraviolet-Visible
wt/vol	Weight per volume
W	Watt

1 Introduction

1 Introduction

1.1 High microbial activity in tidal-flat sediments

Tidal-flats are characterized by a high nutrient input from the adjacent mainland through tidal outlets and rivers. The resulting high primary production in the water column induces a high microbial activity and a fast turnover of organic matter (Poremba *et al*., 1999; van Beusekom and de Jonge, 2002). However, up to 80 % of the organic matter is deposited in intertidal sediments (Freese *et al*., 2008). This material is an important substrate source for subsurface microorganisms (McInerney and Bryant, 1981; Stal, 2003). The rest of the organic matter has previously been mineralized in the water column.

1.2 Bioreactor Janssand

A representative sampling site for Wadden Sea sediments is site Janssand, a typical sandplate located in the backbarrier area of Spiekeroog Island (Fig. 1). This area was intensively studied within the frame of the research group "BioGeoChemistry of tidal flats".

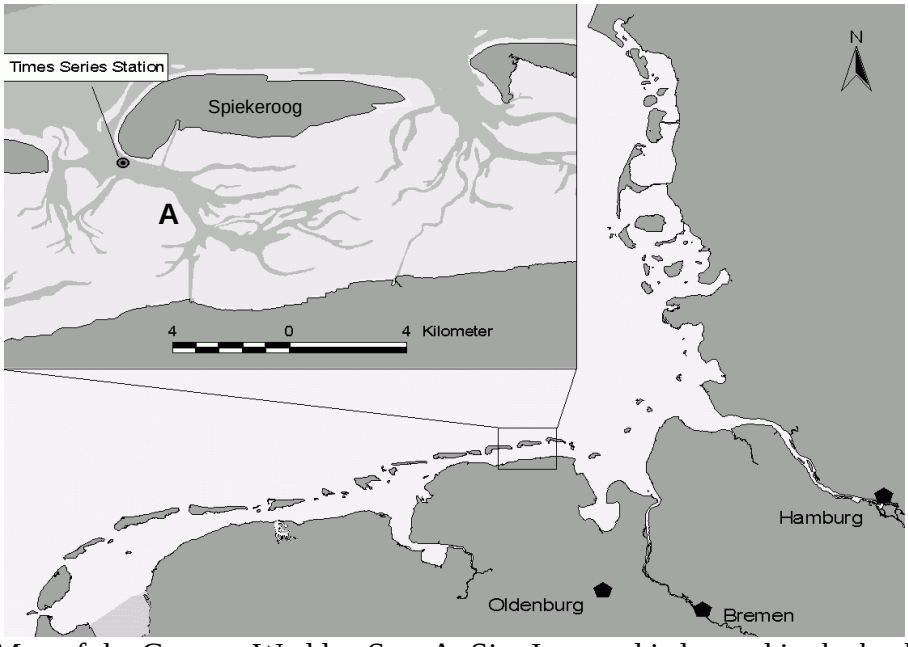

Figure 1: Map of the German Wadden Sea; A: Site Janssand is located in the backbarrier area of Spiekeroog Island.

Sandy sediments often have low organic carbon contents. Therefore, it was assumed that the microbial activity in these sediments is low. However, recent studies showed that sandy intertidal sediments are characterized by an intense advective pore water flow, which supplies the microbial community of these sediments with nutrients from the water column (Huettel and Webster, 2001; Billerbeck *et al*., 2006; Beck *et al*., 2008). Correspondingly, the microbial activity in sandy sediments can be as high as in organic-rich mud-flats (Huettel and Rusch, 2000; Ishii *et al*., 2004; Musat *et al*., 2006). Due to the high rates of organic matter remineralization, site Janssand is often considered as an open-system bioreactor.

1.3 Successive utilization of organic matter

The anaerobic degradation of organic matter in sediments is a multi-phase process (Fig.2). The first step is the hydrolysis of polymers to monomers by exo- or ectoenzymes. The monomers are the substrates for primary fermenting microorganisms, which produce short chain fatty acids, alcohols, H_2 and CO_2. The following steps depend on the availability of sulfate: In the presence of sulfate, the fermentation products are terminally oxidized by sulfate reducers. When sulfate is depleted the primary fermentation products are degraded to acetate, CO_2 and H_2 during secondary fermentation. Under standard conditions, this is an endergonic process, which only provides energy if H_2 is diminished. The secondary fermentation products are utilized by hydrogenotrophic and acetoclastic methanogens.

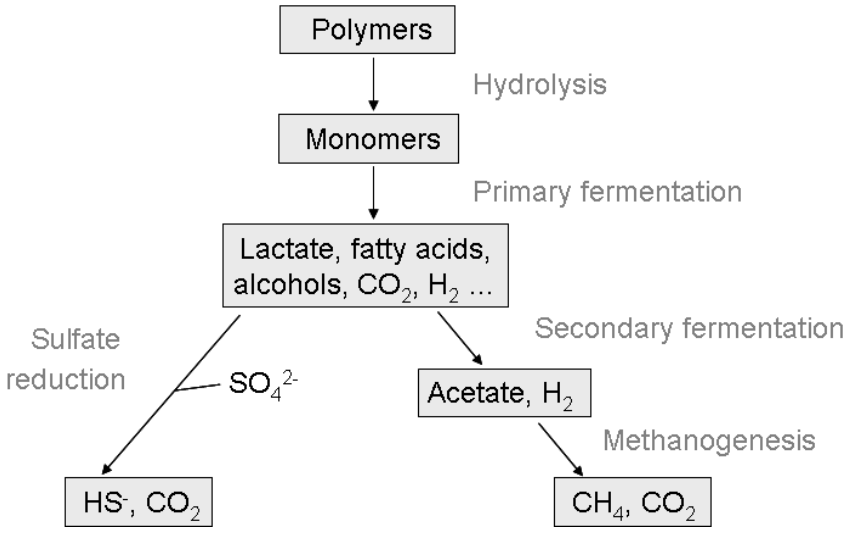

Figure 2: Anaerobic degradation of organic matter in presence and absence of sulfate

1.4 Vertical distribution of electron acceptors

The substrate degradation is coupled to the reduction of electron acceptors, which are successively consumed according to their decreasing redox potential and the corresponding energy yield. The highest energy amount is gained by oxygen reduction. Accordingly, oxygen is depleted within the first few millimeters of tidal-flat sediments (Böttcher *et al*., 2000) and below anoxic conditions are prevailing. Iron and nitrate are reduced in the first centimeters. Below, sulfate becomes the predominant electron acceptor. Sulfate diffuses from the sea water (sulfate concentration ~28 mM) to sediment layers in which other electron acceptors are already depleted (Jørgensen, 1977). Generally, methanogenesis becomes the most important terminal oxidation process when sulfate is depleted. Methanogens commonly use electron donors also consumed by sulfate reducing bacteria. Since the latter have a higher substrate affinity and sulfate reduction is energetically favored, methanogenesis is suppressed in the presence of sulfate (Oremland and Taylor, 1977; Cord-Ruwisch *et al*., 1988).

1.5 Experimental approach

The microbial activity and community structure of tidal-flat sediments in the German North Sea have been intensively studied over the last 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). Recent studies focused on sulfate-reducing bacteria and methanogens (Llobet-Brossa *et al*., 2002; Wilms *et al*., 2007) as these organisms can be easily detected by key genes for dissimilatory sulfate reduction (*dsr*) and methanogenesis (*mcr*). However, sulfate-reducing bacteria and methanogenic archaea only represent a small part of the subsurface community (Beck *et al*., 2011). The most abundant microorganisms in anoxic sediments are fermentative microorganisms (Köpke *et al*., 2005; Wilms *et al*., 2006). Due to the high diversity of fermenters and respective metabolic pathways, no specific key genes for fermentation are available. Thus, a different strategy is required to analyze fermentation processes and the key players involved.

In this study a new approach was developed which combines microcalorimetry to determine different phases of heat production, chemical analyses of fermentation products

and stable-isotope probing to identify the substrate assimilating bacteria. This combination of different methods offers the opportunity to answer the questions: Which phases of anaerobic degradation can be distinguished? What are the predominant fermentation processes? Which are the involved key players?

1.5.1 Balancing and quantification of fermentation processes by microcalorimetry combined with chemical analyses

The successive anaerobic degradation of organic matter is often carried out by microorganisms which are consecutively active. The metabolic activity is always accompanied by heat production, which can be monitored by microcalorimetry. This technique allows a non-invasive detection of heat flows in the range of micro-watts (Larsson *et al.*, 1991; Teeling and Cypionka, 1997) and makes it possible to follow temporal dynamics of fermentation processes. The conduction of degradation experiments in a microcalorimeter allows the identification of different degradation phases and therefore the determination of optimal time points for sampling of successive activities. Furthermore, processes can be quantified via the integration of heat production.

Chemical analyses of fermentation and terminal oxidizing processes at specific time points make it possible to identify different degradation processes and to quantify the fermentation products. As a result, the overall processes can be balanced in a reaction equation.

1.5.2 Identification of actively fermenting organisms via RNA-based stable-isotope probing

Stable-isotope probing (SIP) is a culture-independent approach which links function to phylogeny. In SIP experiments ^{13}C-labeled substrates are assimilated by microorganisms, which results in incorporation of heavy carbon atoms in bacterial biomass - including biomarkers such as phospholipid fatty acids (PLFA), DNA and RNA. These labeled biomarkers are extracted and subsequently separated according to their mass or density (Radajewski *et al.*, 2000).

RNA is an optimal proxy for SIP experiments: The RNA content of cells reflects the cell activity and RNA is synthesized independently from cell replication. Additionally, RNA is information-rich and large databases of 16S rRNA sequences are available for comparison. RNA-based SIP has already been applied to identify dechlorinating microbes in tidal-flat communities from the North Sea (Kittelmann and Friedrich, 2008).

1.6 Intact polar lipids (IPLs) as biomarker for living cells

Intact polar lipids were used as model substrates to analyze fermentation processes and to determine their relative degradation rates. IPLs build the major part of cytoplasmic membranes (Pitcher *et al*., 2009) and have been commonly used as biomarker for living organisms (Zink *et al*., 2003; Ertefai *et al*., 2008; Rossel *et al*., 2008).

In general, bacterial and eukaryotic cytoplasmic membranes mainly contain ester-bound IPLs whereas the membranes of *Archaea* consist of ether-bound IPLs. This difference between bacterial and archaeal IPLs is used to quantify *Bacteria* and *Archaea* in sediments and water samples (Biddle *et al*., 2006; Rossel *et al*., 2008).

1.7 Thesis outline

The aim of this study was to analyze fermentation processes and to identify the actively fermenting bacteria in tidal-flat sediments with the help of three model substrates. This thesis comprises three successive experiments which are based on each other: The first experiment was conducted with unlabeled cell material. As the RNA-based community analysis also displayed a part of the inactive community, the second experiment was done with ^{13}C-labeled monomers. For the third experiment, complex ^{13}C-labeled organic matter was used to simulate the degradation of a natural substrate.

For the first part of this study, unlabeled complex cell material of *Haloferax volcanii* and *Saccharomyces cerevisiae* was used as substrate for a degradation experiment, which was conducted over a period of 100 days. The relative degradation rates of the corresponding archaeal ether-bound and bacteria-like ester-bound intact polar lipids were compared. During the course of the experiment fermentation processes, methanogenesis

and sulfate reduction were analyzed. The community changes were followed by RT-PCR based DGGE. This experimental approach did not only detect the actively degrading bacteria but also a part of the inactive background community. Therefore, ^{13}C-labeled glucose was used as substrate for the second part of this study. Exclusively, the actively glucose assimilating bacteria were detected by RNA-based SIP. Like in the first part, fermentation and terminal oxidizing processes were analyzed. Additionally, microcalorimetry was used to detect different degradation phases. The conduction of the degradation experiment in sealed, gas tight vials with a defined substrate allowed the balancing of the processes in an overall reaction equation.

Natural substrates in marine sediments are usually more complex. Thus, ^{13}C-labeled *Spirulina* cell material was used as substrate for the third publication to simulate natural conditions. With a simplified biomass formula a reaction equation was established for the degradation of complex organic matter.

2 Publications

2.1 Publication 1

A laboratory experiment of intact polar lipid degradation in sandy sediments

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Biogeosciences Discussions

A laboratory experiment of intact polar lipid degradation in sandy sediments

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Abstract

Intact polar lipids (IPLs) are considered biomarkers for living biomass. Their degradation in marine sediments, however, is poorly understood and complicates interpretation of their occurrence in geological samples. To investigate the turnover of IPLs, a degradation experiment with anoxic sandy sediments from the North Sea was conducted. Intact cells of two organisms that do not naturally occur in North Sea sediments were chosen as IPL sources: (i) *Saccharomyces cerevisiae*, representative for ester-bound acyl lipids that also occur in *Bacteria*, and (ii) the archaeon *Haloferax volcanii*, representative for ether-bound isoprenoid lipids. Surprisingly, IPLs with phosphoester-bound head groups showed approximately the same degradation rate as IPLs with glycosidic head groups. Furthermore, the results indicate a relatively fast degradation of *S. cerevisiae* IPLs with ester-bound moieties (analogs of bacterial membrane lipids) and no significant degradation of archaeal IPLs with ether-bound moieties. Pore water and 16S rRNA-based DGGE analysis showed only a minor influence of the IPL source on microbial metabolism and community profiles. Due to our results, the IPL-based quantification of *Archaea* and *Bacteria* should be interpreted with caution.

Introduction

Intact polar lipids (IPLs) have widely been used as biomarkers for living organisms in sediments and water columns for several years (e.g. Zink *et al*., 2003; Sturt *et al*., 2004; Biddle *et al*., 2006; Ertefai *et al*., 2008; Rossel *et al*., 2008). Additionally, IPLs are applied as chemotaxonomic markers as some of these molecules are representative for specific microbial clades. The chemotaxonomic information of IPLs is based on the combination of various head groups with different side chains that are attached to a glycerol backbone by two different bonding types. In general, bacterial and eukaryal cytoplasma membranes contain intact polar lipids with unbranched ester-bound fatty acid moieties. In contrast, archaeal IPLs contain ether-bound isoprenoid moieties. This difference between bacterial and archaeal IPLs is used to quantify *Bacteria* and *Archaea* in sediments and water samples (e.g. Rossel *et al*., 2008). Thus, IPL analysis is valuable as an alternative or complementary technique to standard microbiological methods. On a higher

chemotaxonomic level ammonia-oxidizing bacteria can be identified by the presence of ladderane lipids (Kuypers *et al*., 2003; Sinninghe Damsté *et al*., 2005). Intact polar lipids with mixed ether/ester-bound moieties attached to the glycerol backbone were found in some strains of sulfate-reducing bacteria (Rütters *et al*., 2001). Separate $\delta^{13}C$ analysis of polar head groups or unpolar core lipids of IPLs can also be used to gain information on the metabolism of their producers (Lin *et al*., 2010; Takano *et al*., 2010).

However, one fundamental assumption underlies most of these applications: Intact polar lipids are considered to degrade rapidly after cell death (White *et al*., 1979; Harvey *et al*., 1986). Harvey *et al*. (1986) examined the degradation of both, a glycosidic ether lipid and ester-bound phospholipids. Based on their findings the authors assumed that intact polar lipids with glycosidic head groups show a higher stability against degradation than intact polar lipids with phosphoester head groups. Based on the results of Harvey *et al*. (1986) modeling was used by Lipp *et al*. (2009) and Schouten *et al*. (2010) to assess the potential preservation of fossilized IPLs of planktonic origin during sediment burial.

The quantification of *Bacteria* and *Archaea* in the deep marine biosphere by IPL analysis and fluorescence *in situ* hybridization (FISH) in comparison to catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) and quantitative polymerase chain reaction (q-PCR) analysis is controversially discussed in several studies (e.g. Biddle *et al*., 2006; Lipp *et al*., 2008). A reason for the contradictory abundances of *Archaea* and *Bacteria* in these studies may be that ether-bound archaeal IPLs are more stable than their ester-bound bacterial counterparts, which in turn may lead to an overestimation of archaeal cell numbers. However, the lack of a broad study on the degradation of intact polar lipids, which includes lipids with phosphoester and glycosidic bound head groups as well as ester- and ether-bound moieties, that occur in *Bacteria* and *Archaea* respectively, makes it necessary to revisit the degradation of IPLs to ensure the robustness of this proxy.

We designed a degradation experiment to answer three general questions: What are the degradation rates of IPLs? Are there differences between ester- and ether-bound intact polar lipids and what is the influence of the bonding type of the head group upon lipid degradation? How does the microbial community change over time and what are the main degradation products? The main degradation experiment was accompanied by two controls: The first control was intended to assess any processes that are not mediated by microorganisms and lead to the degradation of the added IPLs. This control is subsequently named “abiotic control”. The second control was used to investigate the influence of the addition of inactive biomass on the microbial community and to monitor the development

of the microbial community without any further substrate addition under laboratory conditions. This control is subsequently named “untreated control”.

Material and Methods

Experimental setup

The incubation vessels for the degradation experiment and the untreated control had a total volume of 2.5 liters each and were filled with 3 kg wet sediment (water content 20 % wt). The sediment used in this experiment had been freshly collected in November 2009 on the Janssand, a tidal flat located approximately 3 km south of Spiekeroog island, North Sea, Germany (53°44.178’N and 07°41.974’E). For sampling, the top cm of the oxic surface sediment was removed until only black anoxic sediment was visible. The underlying sediment was transferred into plastic containers that were sealed by a lid, transported to the laboratory and stored at 4 °C for one week prior to further use. The total organic carbon (TOC) content was 0.23 %. It was calculated as the difference between total carbon (Vario EL Cube, Elementar Analysensysteme GmbH, Germany) and inorganic carbon (analyzed in a UIC CO_2 coulometer). A total of 2.5 g *Saccharomyces cerevisiae* biomass (elemental composition: 45.1 % C, 7.9 % H, 7.9 % N and 0.4 % S; % of dry mass) as source for ester-bound IPLs and 1.25 g of *Haloferax volcanii* (18.9 % C, 3.6 % H, 4.7 % N and 0.7 % S; % of dry mass) as source for ether-bound IPLs were added to the sediment in the incubation vessels used to study IPL degradation. The experimental parameters for the untreated control were the same as for the degradation experiment but no inactive cell material was added. To prevent contamination with microorganisms due to frequent sampling in the abiotic control, 100 ml bottles instead of one 2.5 l incubation vessel were used. The bottles contained 50 g of sediment, 50 mg, dry mass inactive biomass of *S. cerevisiae* and 25 mg, dry mass inactive *H. volcanii* biomass and were closed with rubber stoppers. The incubation vessels of the abiotic control were autoclaved after addition of the intact polar lipid containing cell material.

Source material for intact polar lipids

As source for intact polar lipids two different organisms were used which do not occur in the North Sea sediment. As source for ether-derived IPLs a pure culture of *Haloferax volcanii* (DSM No. 16227) was grown at 37 °C in "*Haloferax sulfurifontis* medium" (DSMZ No. 1018). This archaeon provided two kinds of structurally different molecules – first, two IPLs with ether-bound isoprenoid moieties (PGP-Me, PG; Fig. 1) and a phosphoester head group and second, one IPL with ether-bound isoprenoid moieties and a glycosidic head group (S-GL-1; Fig. 1). The cells were harvested at the end of the exponential growth phase. None of the *H. volcanii* IPLs occur in the natural sediment and thus all of them could be used as tracers to monitor the degradation of ether-bound IPLs. Commercially available *Saccharomyces cerevisiae* (baker´s yeast; Fala GmbH, Germany) was used as source for IPLs with ester-bound acylic moieties and phosphoester head groups. *S. cerevisiae* cells harbour a broad variety of IPLs which were partly used as tracers because these IPLs were not synthetized by the natural sediment microbial community. The *S. cerevisiae* and the harvested archaeal cells were freeze dried and stored at -20 °C. Before use in the experiment, *H. volcanii* and *S. cerevisiae* cells were pasteurized at 100°C for 30 min. Thereafter, part of the cells was transferred to fresh medium ("*Haloferax sulfurifontis* medium" and yeast extract medium, respectively) and analyzed for growth to exclude that the cells are still alive and able to grow.

Identification of *S. cerevisiae* lipids was achieved by HPLC-MS/MS experiments and by comparison to commercially available standards. The lipids of *H. volcanii* were identified by MS/MS experiments and comparison with published results of lipid structures (Sprott *et al.*, 2003). In this experiment we monitored the concentrations of 16 intact polar lipids - 13 ester-bound and 3 ether-bound ones.

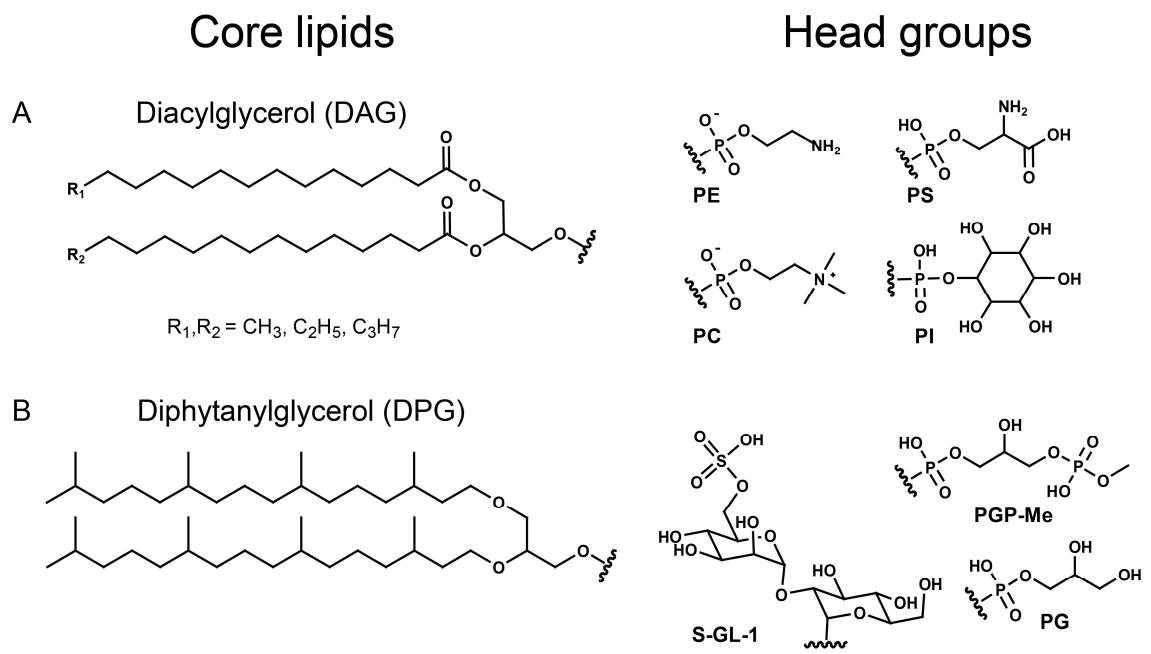

Figure 1: Chemical structures of IPLs characteristic for the two organisms used in this study: A: diacylglycerol ester-bound core lipids of *Saccharomyces cerevisiae* bound to: phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatydylserine (PS); B: isoprenoid ether-bound core lipids of *Haloferax volcanii* bound to: archaetidylglycerol (PG-Ar), archaetidylglycerol methylphosphate (PGP-Me) and sulfono diglyco diacylglycerol S-GL-1 (nomenclature according to Sprott *et al*., 2003).

Incubation parameters and sampling

After starting the experiment, the incubation vessels were stored at room temperature in the dark. Before sampling, the sediment was homogenized for a minimum of 20 min on a mixing device. Head-space gas samples were taken with a syringe directly before sampling the sediment. For sediment and pore water sampling, the incubation vessels were transferred into a glove box with an oxygen-free nitrogen (99 %) and hydrogen (1 %) atmosphere. Samples were taken as triplicates, aliquots of 8-12 g sediment for IPL analysis and 3-5 g sediment for RNA extraction. Pore water (1-2 ml) was extracted with rhizones (Rhizon CSS 5 cm, Rhizosphere Research Products, Wageningen, the Netherlands) from the samples that were collected for IPL analysis. Previous investigations had ensured the absence of IPLs in the pore water samples. Until further processing, all samples were stored at - 20 °C. The samples for RNA extraction were kept at – 80 °C. After sampling, the head space of the incubation vessels was flushed with nitrogen to remove traces of hydrogen that was used in the glove box to sustain the oxygen-free atmosphere.

Lipid extraction

The wet sediments were extracted using a modified Bligh & Dyer method according to Sturt *et al*. (2004). In the first three extraction steps, a single-phase mixture of methanol, dichloromethane and phosphate buffer (2:1:0.8, v/v/v) was used. In the following three extraction steps, trichloroacetic acid was used instead of phosphate buffer. The combined extracts were collected in a separatory funnel. Phase separation was achieved by addition of dichloromethane and water to a final mixture of 1:1:0.9 (v/v/v, methanol, dichloromethane, aqueous phase). The organic phase containing the IPLs was removed and the aqueous phase was washed three times with dichloromethane. The dry extracts were stored in a freezer at - 20 °C until further use.

HPLC-MS

Intact polar lipids were analyzed by HPLC (2695 separation module, Waters, Milfort, USA) coupled to a time-of-flight mass spectrometer equipped with an electrospray source (Micromass, Q-TOF micro, Waters, Milfort, USA). HPLC separation was achieved on a diol phase (Lichrospher100 Diol 5 µ, CS - Chromatographie Service, Langerwehe, Germany) using a 2×125 mm column. A flow rate of 0.35 ml·min^{-1} was employed with the following solvent gradient: 1 min 100 % A, increasing over 20 min to 35 % A, 65 % B using a concave curvature, followed by 40 min of reconditioning (Rütters *et al*., 2001). Eluent A was a mixture of *n*-hexane, *i*-propanol, formic acid and a solution of 25 % ammonia in water (79:20:1.2:0.04 by volume), eluent B was *i*-propanol, water, formic acid and a solution of 25 % ammonia in water (88:10:1.2:0.04 by volume). After addition of an injection standard (O-PE, phosphatidyl ethanolamine diether with two C_{16} acyl moieties), the extracts were dissolved in the starting eluent and directly analyzed. In this study, we exclusively report the change of those compounds that were added with the inactive cell material and did not occur in the natural sediment. Due to the lack of analytical standards for the archaeal glycolipids used, it was not possible to determine the absolute concentrations of these compounds. Instead, ratios of peak areas of the monitored compounds to the peak area of the injection standard for each sample were calculated. Subsequently, the calculated values are named relative concentrations. Since all samples had the same matrix background, this procedure should give reasonable results without any

influence of changing ionization. The analytical error varied between 0.5 and 7 % depending on the investigated IPL and was determined by repeated analysis of the same samples taken at three different times.

The limit of detection in general depends on the ionization efficiency for every analyzed compound and typically lies between 2 - 10 ng per injection and IPL for the used mass spectrometer (Micromass Q-TOF micro, Waters, Milfort, USA).

Chemical analyses of microbial metabolites

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. Organic acids were detected by an UV-VIS detector (UVIS 204; Linear Instruments, Reno, USA) at 210 nm. Alcohols were detected by a refractive-index detector (Knauer, Berlin, Germany). Prior to injection, the pore water was filtered through a 2 µm filter.

Sulfate concentrations were measured by an ion chromatograph (Sykam, Fürstenfeldbruck, Germany) with an anion separation column (LCA A24; Sykam, Fürstenfeldbruck, Germany) at 60 °C followed by conductivity detection. 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. Prior to analysis the samples were 1:100 diluted in eluent without 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) as carrier gas and at a column oven temperature of 40 °C. For analysis of molecular hydrogen and methane a molecular sieve 13X 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 *et al*. (1985). The pH was adjusted at each sampling point to values between 7 and 7.5 by

addition of hydrochloric acid. Hydrogen sulfide was expelled by flushing the headspace with CO_2. After each CO_2 flushing the headspace was replaced with N_2.

Determination of total cell numbers

Total cell counts were obtained after SybrGreen I staining according to a protocol of Lunau *et al*. (2005), which was adapted to sediment samples. 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 % aqueous glutardialdehyde 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 (35 kHz, 2x320 W per period; Sonorex RK 103 H, Bandelin, Mörfelden-Walldorf, Germany). Homogenized aliquots of 20 µl were equally dispensed on a clean microscope slide in a square of 20×20 mm. The slide was dried on a heating plate at 40°C. A drop of 12 µl staining solution (190 µl Moviol, 5 µl SybrGreen I, 5 µl 1 M ascorbic acid in TAE buffer) was placed in the center of a 20×20 mm 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 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 Puffer 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, USA).

For quantification, 100 µl of RiboGreen (Invitrogen, Eugene, USA) solution (diluted 1:200 in TE buffer; pH 7.5) were added to 100 µl of RNA extract (each sample diluted 1:100 in TE buffer; pH 7.5) and transferred to a microtiter plate. Serial dilutions (200 ng μl^{-1} to 1 ng μl^{-1}) of *E .coli* 16S and 23S ribosomal-RNA (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.

Quantitative reverse transcription PCR (qRT-PCR)

Bacterial and archaeal 16S rRNA gene copy numbers were determined by quantitative reverse-transcription PCR using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany). The primer pairs 519f/907r and s-D-Arch-0025-a-S-17/s-D-Arch-0344-a-S-20 were used to quantify bacterial and archaeal RNA, respectively. Primer sequences for these two domains are given in Wilms *et al*. (2007). Each 25 µl PCR reaction contained 15.9 µl nuclease-free water, 5×RT-PCR Puffer (Qiagen, Hilden, Germany), 0.4 mM dNTP Mix (Qiagen, Hilden, Germany), 0.2 µM of each primer, 0.1 µl of a 1 to 500 diluted SybrGreen I solution (Molecular Probes, Eugene, OR, USA), 1 µl One Step Enzyme Mix (Qiagen, Hilden, Germany) and 1 µl standard (10^9 to 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 for bacterial RNA quantification or 48 °C for archaeal RNA quantification, 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 ribosomal RNA of *E. coli* (Roche Diagnostics GmbH, Grenzach-Wyhlen, Germany) served as standard for bacterial gene targets. A PCR product was used as standard for quantification of *Archaea*. Archaeal primer sequences and PCR conditions are given in Wilms *et al*. (2007). For each amplification, the OneStep RT-PCR Kit was used according to the manufacturer's instructions. All PCRs contained a reverse transcription step (30 min, 50 °C) prior to amplification.

Denaturing gradient gel electrophoresis (DGGE) analysis

Partial 16S rRNA were amplified using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany) with bacterial primers GC-357f and 907r as given in Muyer *et al*. (1995) and archaeal primers S-D-Arch-GC-0344-a-S-20 und 907r as described previously (Wilms

et al., 2006). Each forward primer contained a GC-clamp (Muyzer *et al*., 1993). 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 for bacterial RNA amplification or 48 °C for archaeal RNA amplification, 60 s at 72 °C), and a terminal step (10 min at 72 °C). The PCR amplicons and loading buffer (40 % glycerol, 60 % [wt/vol] 1x 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, Netherlands) and a 6 % (wt/vol) polyacrylamide gel containing denaturant gradients of 50 to 70 % for separation of bacterial PCR products. For separation of archaeal PCR products a denaturant gradient of 30 to 80 % was prepared. The gels were stained for 2 h with 1×SybrGold (Molecular Probes, Leiden, Netherlands) in 1×tris-acetate-EDTA buffer and washed for 20 min in distilled water prior to UV transillumination.

Sequence analysis

DGGE bands were excised for sequencing and treated as described by Del Panno *et al*. (2005) without the second denaturing gel for purification. For reamplification of the bands the same primers 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 cycles (annealing temperature 48 °C). For subsequent sequence analysis, PCR products of DGGE bands were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and 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*., 1997).

Results

The monitored microbial processes demonstrated similar trends in the degradation experiment and the untreated control. In contrast, the abiotic control showed no sign of sulfate reduction, methanogenesis, IPL degradation, fermentation or any other microbial activity as demonstrated by the stability of all measured parameters (data not shown). This indicates that the degradation of IPLs was exclusively mediated by microorganisms.

Sulfate and methane data

Sulfate and methane are part of the terminal mineralization processes and their concentrations were monitored to assess the current status of the experiment.

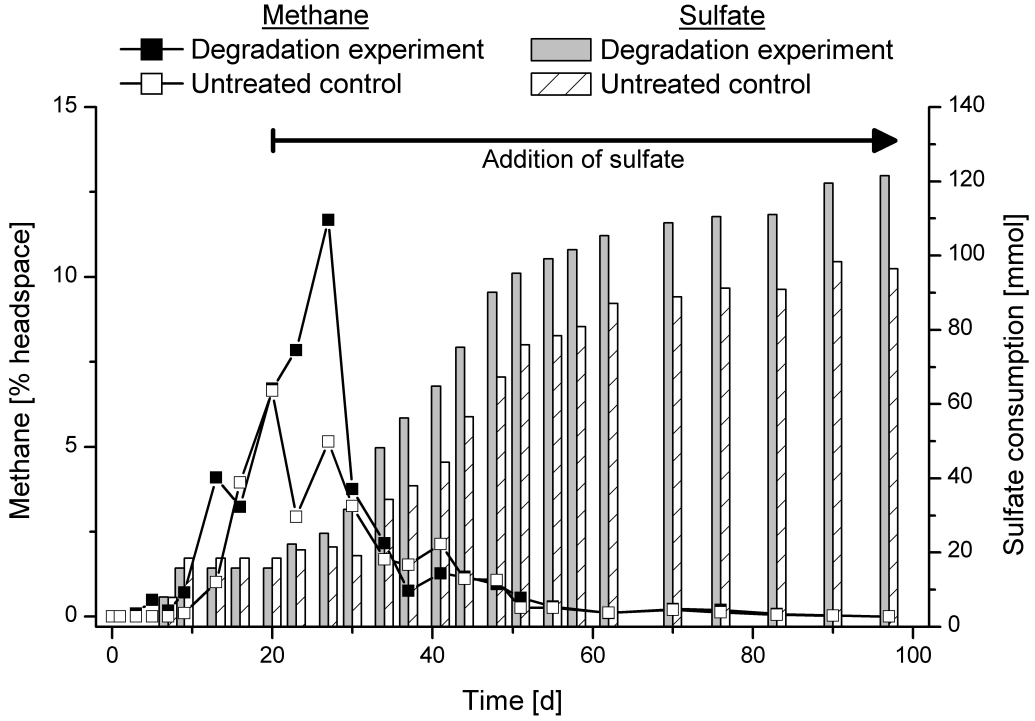

Figure 2: Sulfate consumption and methane production in the degradation experiment and the untreated control. Sulfate consumption for each time point was calculated by addition of measured day-to-day losses. Methane concentrations are given in percentage of incubation vessel headspace. After day 20 sulfate was refilled when the concentration decreased below 10 mM as indicated by the arrow.

Sulfate was completely consumed within the first 9 days (Fig. 2). Between day 9 and day 20, the sulfate concentration remained below the detection limit, until sulfate was refilled. Sulfate was replenished to mimic the natural environment. At the end of the experiment,

sulfate was consumed more slowly than at the beginning, indicating the depletion of electron donors. The sulfate concentration decreased to 9 mM at day 97. Large amounts of methane were only detected in the absence of sulfate. The reason for this is, that methanogens commonly use the electron donors also used by sulfate-reducing bacteria. Since sulfate reduction is energetically favored methanogenesis is suppressed in the presence of sulfate (Oremland and Taylor, 1977). Gaseous sulfide (produced by sulfate reduction) was removed from the headspace during every sampling by purging with nitrogen. The concentration of dissolved sulfide in the pore water remained relatively low until day 27 (6 mM). The maximum concentration was reached at day 76 (38 mM). Since this is a toxic concentration for most microorganisms, the headspace was purged with CO_2 to release the hydrogen sulfide. After flushing, the CO_2 in the head space was replaced with N_2. The pH increased constantly in the degradation experiment and the untreated control and was adjusted to values between pH 7 and 8 by addition of hydrogen chloride. Oxygen was never detected in any incubation vessel.

Degradation of intact polar lipids

The relative concentrations of ester-bound IPLs decreased in the course of the experiment whereas the relative concentrations of ether-bound IPLs remained stable. The head groups had no significant influence on the observed degradation pattern. The relative concentrations of ester-bound IPLs with PC and PI head groups showed no significant change in the first days of the degradation experiment. Beginning at day 5, they decreased rapidly over 5 days followed by a phase of moderate loss until day 90. In case of PE and PS (Fig. 3 C and D) higher relative concentrations were found at day 5. In the following period the signal decreased over the rest of the experiment. In contrast to this, the relative concentrations of all ether-bound IPLs (Fig. 4) scattered but did not decrease significantly until the end of the degradation experiment.

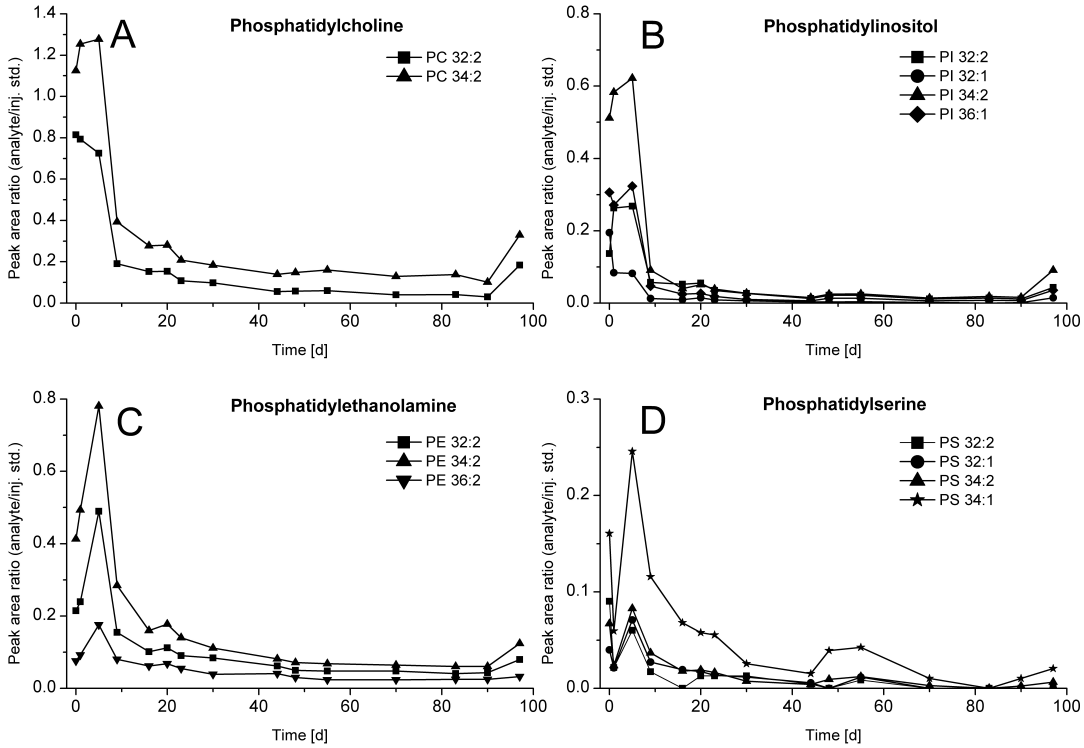

Figure 3: Relative concentrations of ester-bound IPLs with different head groups in the degradation experiment vs. time in days. Core lipid structures are given as sum of fatty acids (e.g. 32:2) where 32 represents the number of carbon atoms and 2 represents the number of double bonds in the core lipid structure.

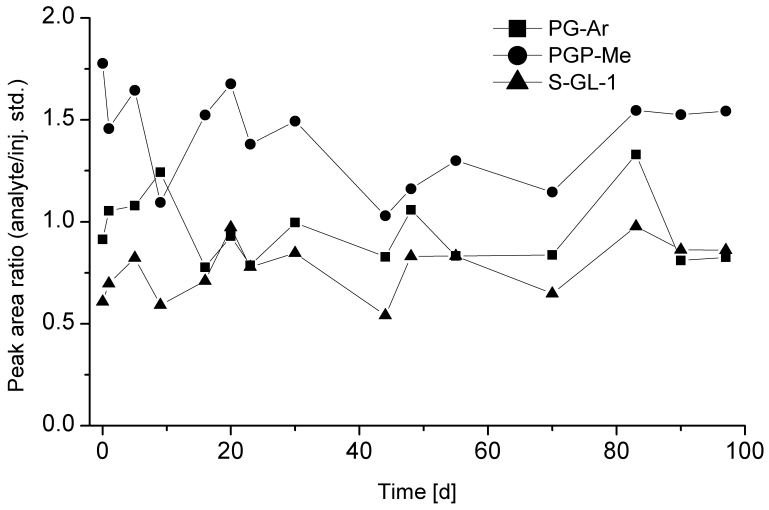

Figure 4: Relative concentrations of ether-bound IPLs with different head groups in the degradation experiment vs. time in days. Abbreviations: archaetidylglycerol (PG-Ar), archaetidylglycerol methylphosphate (PGP-Me), and sulfono diglyco diacylglycerol S-GL-1 (according to Sprott *et al*., 2003).

The relative concentrations of ester- and ether-bound IPLs in the abiotic control showed a slight decrease relative to day 0 in the course of the experiment (Fig 5). The small decrease in the abiotic control at day 1 was probably caused by inhomogeneity since the drop occurred in both ester- and ether-bound IPL quantifications.

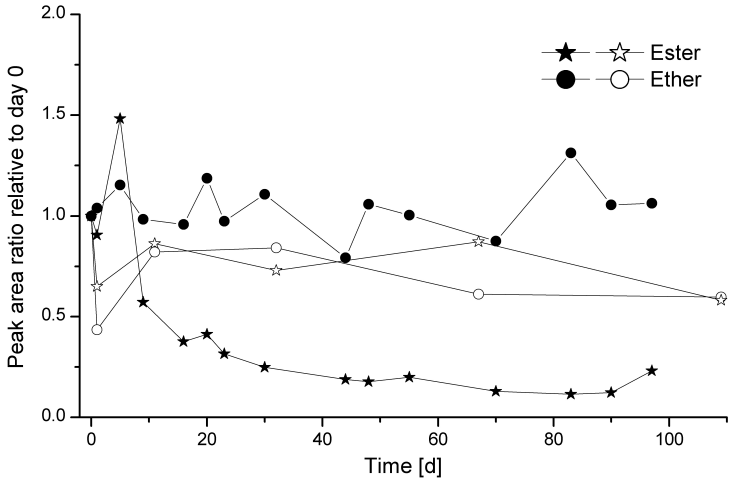

Figure 5: Sum of ester- and ether-bound IPLs in the degradation experiment and the abiotic control given as relative concentrations relative to day 0. Closed symbols: degradation experiment; open symbols: untreated control.

Intact polar lipids in the untreated control

Samples from the untreated control were taken in the same sampling intervals as in the degradation experiment. Most of the IPLs added to monitor the degradation were not detected in the untreated control. Exceptions were the IPLs PE 34:2 and PE 36:2, but compared to the corresponding PEs in the added biomass their total amount was low (less than 3 %). However, other IPLs such as SQDG (sulfoquinovosyl diacylglycerol) and ester-bound PG were identified in the untreated control. Additionally, ester-bound PEs with side chains different from those in the degradation experiment were found (31:0, 31:1, 33:1, 33:2, 35:2). SQDG was the most prominent IPL in the untreated control. It showed increasing abundances from day 0 to day 23 and then a decrease to the starting value at a moderate rate after a major drop between days 23 and 27. PE and PG showed an increase between day 0 and day 5 and returned to the starting values in the course of the experiment.

Microbial fermentation

Fermentation products were analyzed to monitor the utilization of organic matter.

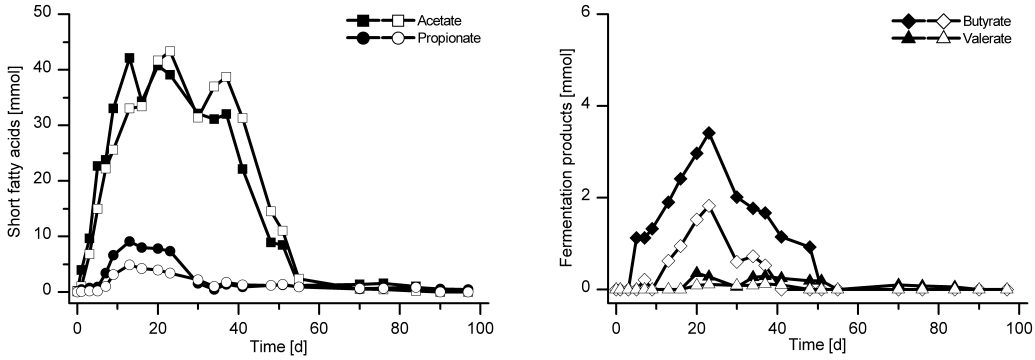

Figure 6: Pore water concentrations of acetate, propionate, butyrate and valerate in mmol vs. time in days. Closed symbols: degradation experiment; open symbols: untreated control.

The data show the net balance of fermentation products since they are produced and consumed simultaneously (Fig. 6). In the beginning of the experiment, the short-chain fatty acids were produced faster than consumed as indicated by their increasing concentrations. The main fermentation product was acetate followed by propionate, butyrate and valerate. During the final phase of the experiment, the fermentation products were utilized at the same rate as they were produced or the consumption was faster than the formation. Ethanol was also produced but could not be quantified due to signal overlapping with those of other unknown organic compounds from the sediment matrix. The graphs for short-chain fatty acids in the degradation experiment and the untreated control shared the same characteristics. The accumulation of short-chain fatty acids tended to be slightly faster in the degradation experiment but overall the added cell material had only a minor influence on the shape of the production curves of short-chain fatty acids. This indicates that the experiment was not disturbed by the addition of biomass as external substrate.

Succession in microbial abundance, diversity and activity

The development of the microbial communities was monitored to obtain background information for the degradation of IPLs.

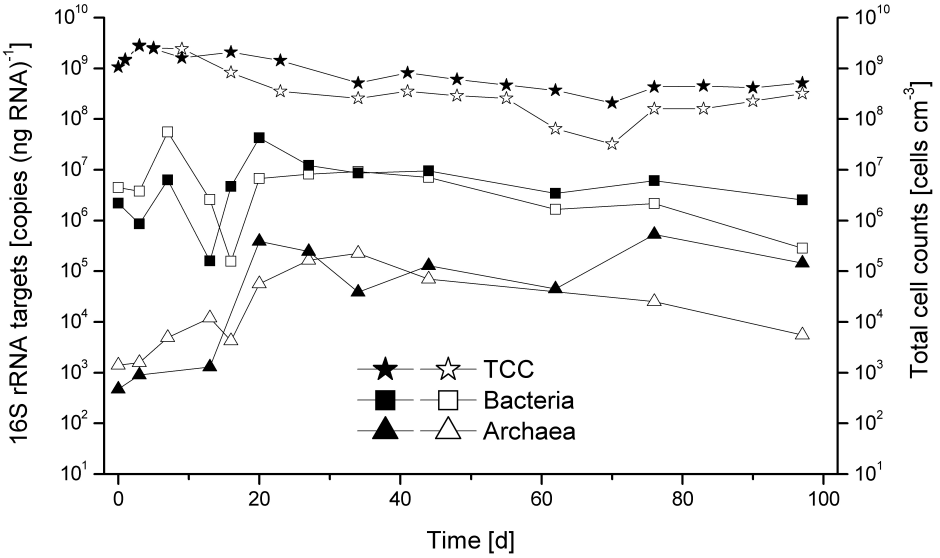

Figure 7: Bacterial and archaeal 16S rRNA copies and total cell counts (TCC) in the course of the experiment. The number of bacterial and archaeal 16S rRNA targets are given in copies per ng of extracted RNA, whereas the total cell counts are given in cells per cm³ sediment. Closed symbols: degradation experiment; open symbols: untreated control.

The total cell counts of the degradation experiment and the untreated sediment showed the same trend and decreased only slightly during the experiment (Fig. 7). The total cell numbers of the degradation experiment were marginally higher than those of the untreated control. The numbers of bacterial 16S rRNA targets were one to two orders of magnitude higher than those of *Archaea*. Between day 7 and day 16, the bacterial 16S rRNA copy numbers dropped significantly and returned to their previous values. The number of archaeal 16S rRNA copies showed a generally increasing trend. After day 20, both the number of bacterial and archaeal 16S rRNA targets remained relatively constant. The RNA content of the sediment ranged from 80 to 4800 ng cm^{-3}. Ravenschlag *et al*. (2000) determined a rRNA-content of 0.9 to 1.4 fg rRNA per cell for two sulfate-reducing bacteria from surface sediments. Using these values to assess the total cell numbers for our study, the calculated values range between 8.9×10^7 to 3.4×10^9 cells cm^{-3}, which fits nicely to our total cell counts.

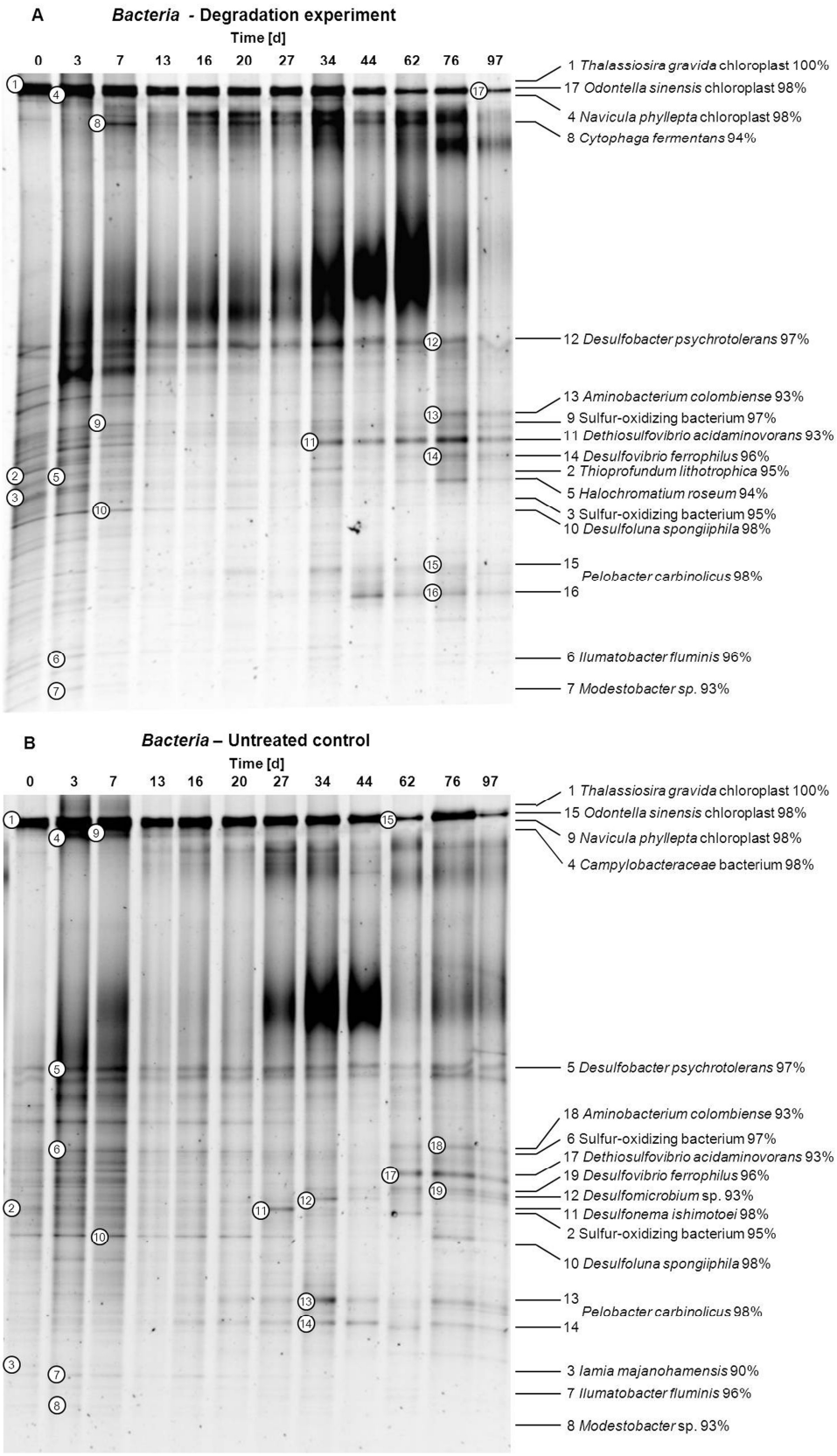

Figure 8: DGGE community profiles of *Bacteria* and closest cultivated relatives of the sequenced DGGE bands with similarity in % compared to data from the EMBL database. A: degradation experiment B: untreated control.

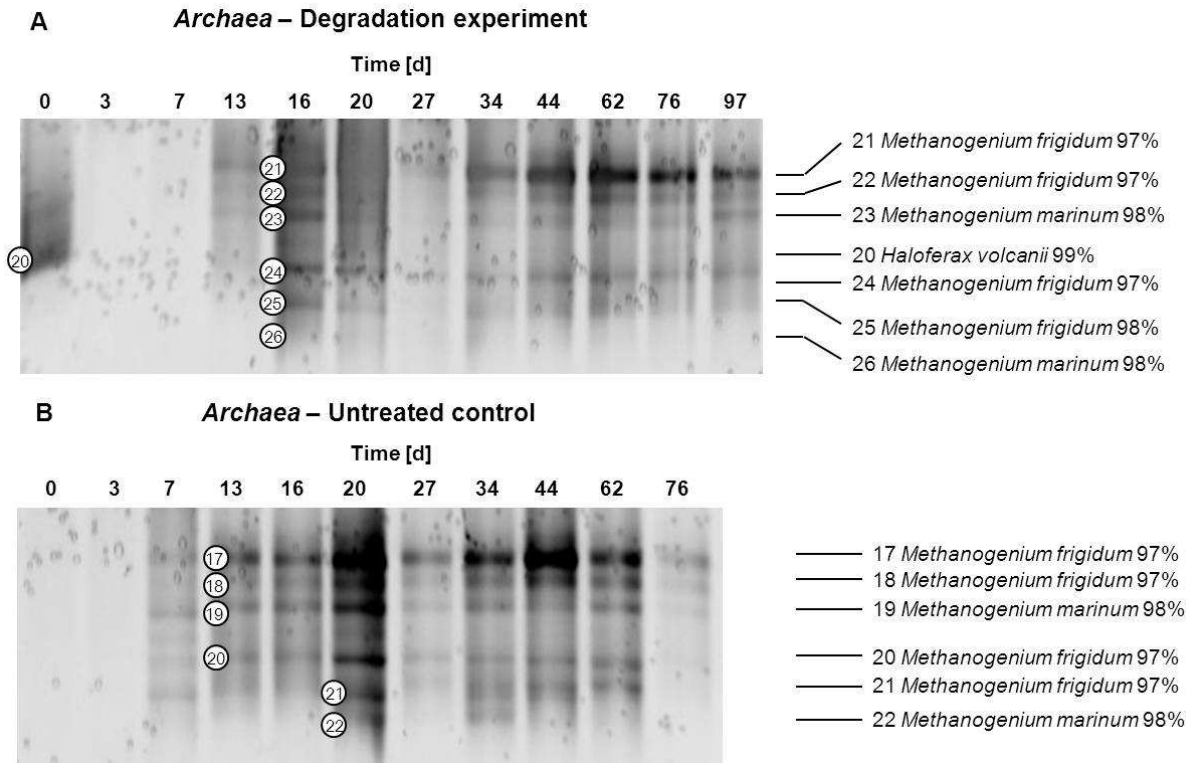

Figure 9: DGGE community profiles of *Archaea* and closest cultivated relatives of the sequenced DGGE bands with similarity in % compared to data from the EMBL database. A: degradation experiment B: untreated control.

The community profiles of the degradation experiment and the untreated sediment looked similar to each other. Initially, the community structure was highly diverse. This diversity decreased in the course of the experiment probably due to a diminishing substrate spectrum as indicated in Fig. 6. Sequencing of representative bands indicated the presence of different fermenting and sulfate-reducing bacteria. The most conspicuous organisms were relatives of *Pelobacter* sp. and *Dethiosulfovibrio acidaminovorans*. These bacteria were highly abundant during the end phase of the experiment, when acetate and propionate were absent, indicating an enhanced activity in this period. A sequence of a *Cytophaga fermentans*-related organism was detected after one week, also hinting toward an increased activity of that organism from that time on. Additionally, this bacterium showed a higher abundance in samples from the degradation experiment. The abundance of other organisms, most of them being sulfate-reducing bacteria like a *Desulfobacter psychrotolerans*-related organism, appeared to be relatively constant over time, since sulfate was available almost during the entire experiment. Sulfur-oxidizing bacteria were only detected at the beginning of the experiment. Sequences affiliated to diatom chloroplasts were found during the complete course of the experiment.

The archaeal community pattern showed minor differences between the degradation experiment and the untreated control (Fig. 9). *H. volcanii* could only be detected at the very first sampling point in the degradation experiment. This indicated that the *H. volcanii* cells were successfully disintegrated during the experiment.

For both, the degradation experiment and the untreated control six bands were detected throughout the whole experiment. All sequences were closely related to two different organisms, either *Methanogenium frigidum* or *Methanogenium marinum*. The community patterns reflect the quantification of *Archaea* by qPCR: In the first week when the number of archaeal 16s rRNA gene targets was lowest, only very faint bands were detected in the DGGE profile. In the beginning of the experiment, when sulfate was still present, no methanogenic archaea could be detected (Fig. 9). Only after depletion of sulfate, the rRNA of methanogens was found. The presence and activity of these organisms was supported by the methane production that was observed in the absence of sulfate. In all samples, the content of eukaryotic RNA was too low to obtain sufficient PCR-products to prepare a DGGE with eukaryotic primers.

Total organic carbon

The carbon content was analyzed at five time points of the degradation experiment and the untreated control. The difference in organic matter between the degradation experiment and the untreated control reflected the amount of organic carbon that was added to the degradation experiment with the inactive cell material of *S. cerevisiae* and *H. volcanii.* Although we added 1.36 g of cell-derived organic carbon which increased the TOC content of the natural sediment (0.23 % C_{org}) by roughly 25 %, no pronounced difference was visible between the degradation experiment and the untreated control for all other parameters.

Effects of sediment-derived organic carbon on microbial processes

The increasing concentrations of fermentation products and total cell counts within the first week demonstrate a stimulation of microbial activity. This was likely caused by a temperature increase from 4 °C (storage temperature) to 20 °C in the laboratory. The

addition of biomass caused a faster increase of methanogenesis and fermentation. The added biomass also resulted in slightly increased values of sulfate consumption, methane production and fermentation (Figs. 2 and 6). The absolute amount of IPLs that were added with the biomass of *H. volcanii* and *S. cerevisiae* was low compared to other organic compounds present in the sediment itself. Therefore, the fermentation products do not only reflect the products of IPL degradation but mainly the degradation products of the organic matter which originated from the natural sediment.

Discussion

In this experiment ester-bound bacteria-like IPLs were faster degraded than ether-bound archaeal IPLs. The bonding type of the head group had no influence on the degradation rate.

Assessment of experimental conditions

On the first view the scattering for ether-bound IPLs appears to be much higher than for ester-bound IPLs. This different behavior is an artifact of the data processing applied. To monitor the degradation of IPLs the relative concentration for each monitored IPL was calculated. With this method, a scattering of IPL signals with values near the detection limit becomes much less visible than the scattering of IPL signals with values in the range of the injection standard. This explains the smooth degradation curve for ester-bound IPLs in Figure 3 after the first 10 days. Nevertheless, these analytical limitations do not have any impact on the general results of this study.

Haloferax volcanii and *Saccharomyces cerevisiae* do not naturally occur in tidal flat sediments. Thus, it could be assumed that the observed degradation rates of IPLs could be different from those originating from the natural community. However, the source of the ether-bound and ester-bound IPL should have no influence on the degradation rate since the chemical structure and the bonding types of the added material can also be found in IPLs that naturally occur in Wadden Sea sediments (Rütters *et al*., 2001).

One could argue that the different degradation pattern observed for ether- and ester-bound IPLs are caused by a selective protection of ether-bound IPLs in intact *H. volcanii*

cell material and on the other hand disrupted cells of *S. cerevisiae*. This is unlikely because the rRNA of *H. volcanii* related archaea was only found at the very beginning of the experiment. Additionally, cultivation attempts with pasteurized cells showed no growth which indicates that the *H. volcanii* cells were killed during the experiment.

As displayed in Fig. 4, the results of ether-bound IPL analysis showed a non-systematic scattering. Adsorption/desorption processes of IPLs to/from the sediment matrix may be an issue. Sediment inhomogeneity is also a possible explanation. We tried to minimize this effect by the design of the incubation vessel and intense mixing on a mixing device prior to every sampling. In addition to this, directly before opening the incubation vessels in the anaerobic chamber, the sediment was resuspended by shaking. Other reasons for scattering IPL values may be varying extraction efficiencies or changing matrix effects during ESI-ionization which may add some scattering in addition to inhomogeneity.

Influence of bonding types and moieties on IPL degradation

The degradation pattern and hence the stability of all investigated ester-bound bacterial-like IPLs was approximately the same (Fig. 3 A-D). It can be assumed that the degradation of ester-bound intact polar lipids was independent from the type of head group, since the core structures of these IPLs are generally the same. A similar behavior was observed for the ether-bound archaeal IPLs. But in contrast to the ester-bound bacterial IPLs, the investigated archaeal IPLs had two structurally different types of head groups – one glycosidic (S-GL-1) and two phosphoester (PG-Ar and PGP-Me; Fig. 1) head groups. Based on the study of Harvey *et al*. (1986) glycosidic ether lipids are commonly assumed to be more stable than phospholipids with ester-bound moieties. However, Harvey and coauthors investigated the degradation of a glycosidic ether lipid and a phosphoester lipid. They found a higher stability of the glycosidic ether lipid. In general, compared to glycosidic bonds phosphoester bonds are chemically more labile. In combination with the findings of Harvey and coworkers this could lead to the assumption that the bonding type of the head group has a major influence on the IPL degradation rate. But in fact, the study of Harvey *et al*. is not suitable to answer the question which part of an IPL-molecule (head group or moiety bonding type) is responsible for the observed differential stability of bacterial and archaeal IPLs. According to our results, we assume that the differences in chemical stability of IPLs play only a minor role during the degradation of IPLs, at least in

the investigated system. Since the head groups had no influence on the IPL degradation rates the structurally different core lipids are assumed to cause the observed degradation rates.

In general complex molecules like lipids and proteins are hydrolyzed by exo- or ectoenzymes which are released by prokaryotic cells. Therefore, we conclude that microbiological enzymatic processes are the driving force in IPL degradation rather than expected chemical stabilities alone.

Microbial activity

Originally, the experiment was planned as an enrichment culture for lipid-degrading bacteria. It turned out, however, that the added cell material had only little influence on the community structure and the metabolic activity. This had a positive side-effect, since the processes which originally occur in tidal flat sediments were not disturbed and superposed. As a consequence the selected set-up is suitable to reflect the natural IPL degradation in such sediments.

Most of the *Archaea* in sediments are known to be methanogens (Wilms *et al*., 2007) or nitrifying archaea (Wuchter *et al*., 2007). They have a narrow substrate spectrum limited to simple molecules and are not known to degrade lipids. Probably, the main lipid degraders are *Bacteria* which are more abundant than *Archaea* in surface sediments (e.g. Beck *et al.*, 2011).

Chloroplast RNA of diatoms was found throughout the whole course of the experiment. Since no oxygen and no light for algal growth were available in the experiment, the diatoms might be inactive in a resting state. The RNA of chloroplasts is exceptionally stable, because the RNA in this cell organelle is surrounded by four membranes (Kroth and Strotmann, 1999). Another reason for the stability of chloroplast RNA may be that the diatoms were in fact active and survived by a heterotrophic kind of metabolism. Facultative heterotrophy is known for many diatoms, including several *Navicula* species (Lewin, 1953; Sherr and Sherr, 2007). Heterotrophically growing diatoms continue to synthesize their photosynthetic pigments in the dark and return immediately to photosynthesis under light exposure (Lewin and Hellebus, 1970). This indicates that chloroplast RNA is present even if no light is available.

The question if ester-bound lipids are recycled and used for membrane synthesis by *Bacteria* cannot be answered by our experimental-setup. However, enzymes are described for *Eukarya* and *Prokarya* that facilitate bidirectional ATP-independent flipping of polar lipids across cytoplasmic membranes (Sanyal and Menon, 2009). The incorporation of extrinsic cell building blocks is energetically useful for microorganisms because it is more efficient to recycle existing molecules than breaking them down to smaller molecules to synthesize them "*de-novo*". Takano and coworkers (2010) added ^{13}C-labeled glucose to marine sediment and found that the ^{13}C was incorporated into the glycerol backbone of archaeal membrane lipids whereas the isoprenoid core lipids remained unlabeled. This indicates a recycling of comparable large membrane building blocks. To answer the question if IPLs can be recycled as intact molecules, stable-isotope probing (SIP) or ^{14}C-labeling would be suitable tools to follow the degradation and incorporation of IPLs and their resulting products.

Conclusions

The quantification of *Bacteria* and *Archaea* in the deep marine biosphere by IPL and FISH analysis led to the assumption that this habitat is dominated by *Archaea* (Biddle *et al*., 2006; Lipp *et al*., 2008). In contrast, catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) and quantitative polymerase chain reaction (q-PCR) suggest a predominance of *Bacteria* (Schippers *et al*., 2005; Inagaki *et al*., 2006). Our experiment shows that these interpretations have to be considered with caution, since ether-bound archaeal IPLs were clearly more stable than ester-bound bacteria-like IPLs over a period of 100 days. In nature, ether-bound IPLs may even be preserved for longer time periods since the enhanced temperature in the laboratory accelerated the degradation processes. Therefore IPL based quantification may lead to an overestimation of archaeal cell numbers.

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2.2 Publication 2

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

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Abstract

A novel approach was developed to follow the successive utilization of organic carbon in anaerobic food chains by microcalorimetry, chemical analyses of fermentation products and stable-isotope probing (SIP). The fermentation of ^{13}C-labeled glucose was monitored over 27 days by microcalorimetry in a stimulation experiment with tidal-flat sediments (59 µmol glucose per 21 g sediment slurry). 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 pre-isolation of ribosomes resulted in rRNA with an excellent quality. Already after one day, heat production reached its maximum (225 µW) followed by a second peak (up to 46 µW) between day one and three. Glucose was completely consumed within two days and was mainly fermented to acetate (up to 90 µmol). Ethanol (max. 46 µmol), formate (max. 23 µmol) and hydrogen (max. 2.5 µmol) were detected intermittently. The amount of propionate (7 µmol) which was built within the first three 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 three 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 (39). 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 (6, 20, 25) and molecular methods (5, 30). Studies on the anoxic subsurface revealed that

bacterial communities in the sediment column are dominated by fermentative bacteria (17, 41), as typically found also in other anoxic sediments (32). 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 (18). 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 (40). However, due to 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.

One approach to study metabolic activities is the stimulation of microbial communities in enrichment cultures by adding different substrates and to follow organic matter degradation (36). 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 (19) 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 non-invasive determination of optimal time points for sampling of successive activities in carbon degradation. Furthermore, processes can be quantified via the integration of heat production.

While microcalorimetry helps to distinguish the different degradation processes, the active degraders can be identified 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 (29). In pioneering SIP-experiments, ^{13}C-labeled phospholipid fatty acids (PLFA) were analyzed (4), while later SIP has also been applied to labeled DNA (29), RNA (21, 22) and proteins (15). Gihring *et al*. (13) used DNA-based SIP to identify phytodetritus degrading and denitrifying bacteria in oxic coastal surface sediment. Alos, rRNA-based SIP has already been applied to identify dechlorinating microbes in tidal-flat communities from the North Sea (16).

In our study, we introduce a novel combination of microcalorimetry in tandem with stable-isotope probing 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*. (10) to investigate active members of

microbial communities (9, 11, 12). Separating ribosomes from other cell structures results in a concentration and purification of ribosomal RNA. Furthermore, due to the intact structure of the ribosomes, highly labile RNA is protected against degradation by nucleases.

Material 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. Sediment from 1 cm - 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, the sediment was incubated at 20°C for two weeks in the presence of sulfate to decrease the organic carbon content of the sediment. When sulfate was depleted, it was refilled to 30 mM.

For evaluating the ribosome-extraction method, pure cultures of *Phaeobacter gallaeciensis* (DSMZ 17395) and *Micrococcus luteus* (DSMZ 20030) were incubated at 20 °C in HEPES-buffered peptone glucose media and complex lactate media, respectively. Cultures of *Methanosarcina barkeri* (DSMZ 800) were grown at 38 °C in mineral media (38). 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 rRNA amount (9).

Cultivation and microcalorimetry

A slurry was prepared from 18 g of surface sediment (0-5 cm) 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 to 30 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. The vials were sealed with rubber stoppers and flushed with N_2. Glucose-free slurries served as controls. Four cultures were incubated (three glucose-amended, one glucose-free) in a

heat-conduction microcalorimeter (2277 Thermal Activity Monitor, ThermoMetric, Järvalla, Sweden) at 20°C for 27 days. Additional samples were incubated at 20°C outside the microcalorimeter. The heat production was recorded at intervals of 300 seconds using the program DIGITAM 3.0 (Sci Tech Software, ThermoMetric, Järvalla, Sweden). The cultures were sacrificed for sampling at specific points when characteristic heat production resulting from the degradation of glucose was observed. 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.

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. Organic acids were detected by an UV-VIS detector (UVIS 204; Linear Instruments, Reno, USA) 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, Fürstenfeldbruck, Germany) with an anion separation column (LCA A24; Sykam, Fürstenfeldbruck, Germany) at 60 °C followed by conductivity detection (S3115, Sykam, Fürstenfeldbruck, Germany). 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. 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) as carrier gas and at a column oven temperature of 40 °C. For analysis of molecular hydrogen and methane, a molecular sieve 13X 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 (7).

Extraction of nucleic acids

Ribosomes were extracted according to Felske *et al*. (10). Briefly, the cells were mechanically disrupted, followed by three steps of differential centrifugation: The first two steps serve the purpose to remove the cell debris and the third step with a higher acceleration to pelletize the ribosomes. Thereafter the RNA of the resulting ribosome pellet was extracted with the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions. RNA extracts were controlled for DNA contaminations using the universal 16S rRNA primers 357f/907r (27) 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 manufacturers protocols (Molecular Probes, Eugene, USA). In contrast to the original DNA quantification instructions, only the tenth part of each volume and 1 µl of the extracted DNA and Lambda-DNA in different concentrations from 100 $ng \cdot \mu l^{-1}$ to 1 $ng \cdot \mu l^{-1}$ were used.

Isopycnic centrifugation and gradient fractionation

Density gradient centrifugation was performed with cesium tri-fluoroacetate (CsTFA) as described by Lueders *et al*. (21). 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).

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 (34). 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 (21).

Denaturing gradient gel electrophoresis (DGGE) analysis

Partial 16S rRNA genes were amplified using the OneStep RT-PCR Kit (Qiagen) with bacterial primers GC-357f and 907r as described by Muyzer *et al*. (27). 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 step (10 min at 72 °C).

The PCR amplicons and loading buffer (40 % [wt/vol] glycerol, 60 % [wt/vol] 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*. (35) using an INGENYphorU-2 system (Ingeny, Leiden, The Netherlands) and a 6 % (wt/vol) polyacrylamide gel containing denaturant gradients of 50 to 70 % for separation of bacterial 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 prior to UV transillumination.

Sequence analysis

DGGE bands were excised for sequencing and treated as described by Del Panno *et al*. (8) except the second denaturing gel for purification. For reamplification of the bands, the same primers pairs as described above were used 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, Hilden, Germany) and were commercially sequenced by GATC Biotech. The partial 16S rRNA sequences of the DGGE bands were compared to those in GenBank using the BLAST function (1).

Results

Pre-experiments to asses 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).

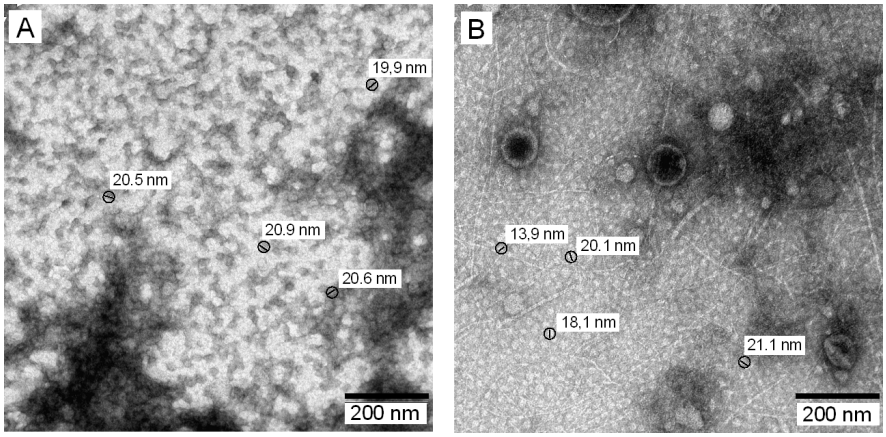

Fig. 1. Electron micrograph of ribosome preparations. A: pure culture of *Phaeobacter gallaeciensis*, B: surface sediment of a German tidal-flat. Diameters of ribosome-like particles are indicated.

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 *Phaeobacter 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) were roughly in the same order of magnitude: *Phaeobacter gallaeciensis* (Gram-negative): 533; *Micrococcus luteus* (Gram-positive): 1214 and *Methanosarcina 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 one day, followed by a broader peak (day one to day three) and a phase of decreasing heat production (day three to the end of the experiment). The integrated heat output was 22 Joule 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.

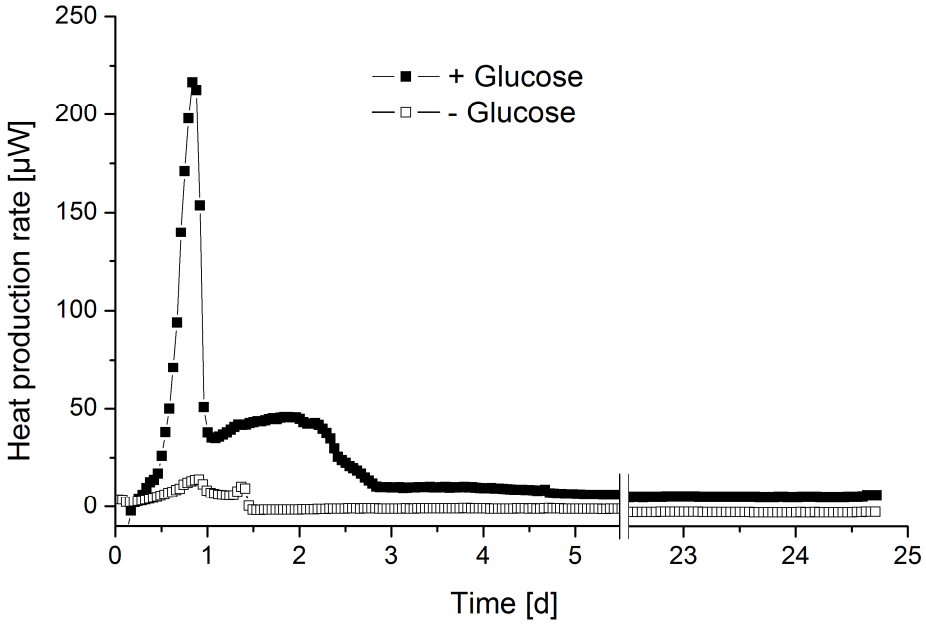

Fig. 2. Heat production measured by microcalorimetry during the stimulation experiment. Heat production values remained constant between day 5 and 23 in the glucose-amended sediment (closed symbol) and the glucose-free control (open symbol).

Glucose was completely consumed within the first two days in the amended samples (Fig. 3 A). The substrate degradation resulted in the production of typical fermentation products like acetate, ethanol, formate, propionate, H_2 and CO_2 (Fig. 3 A-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. 3 B and C). Propionate was produced more slowly and reached a plateau after three days. Sulfate was rapidly depleted 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 pre-incubated in the presence of sulfate for two weeks at 20 °C. 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 substrates by mixing during the preparation of sediment slurries. Methanogenesis started as soon as sulfate was depleted (Fig. 3 D). 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:

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

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.

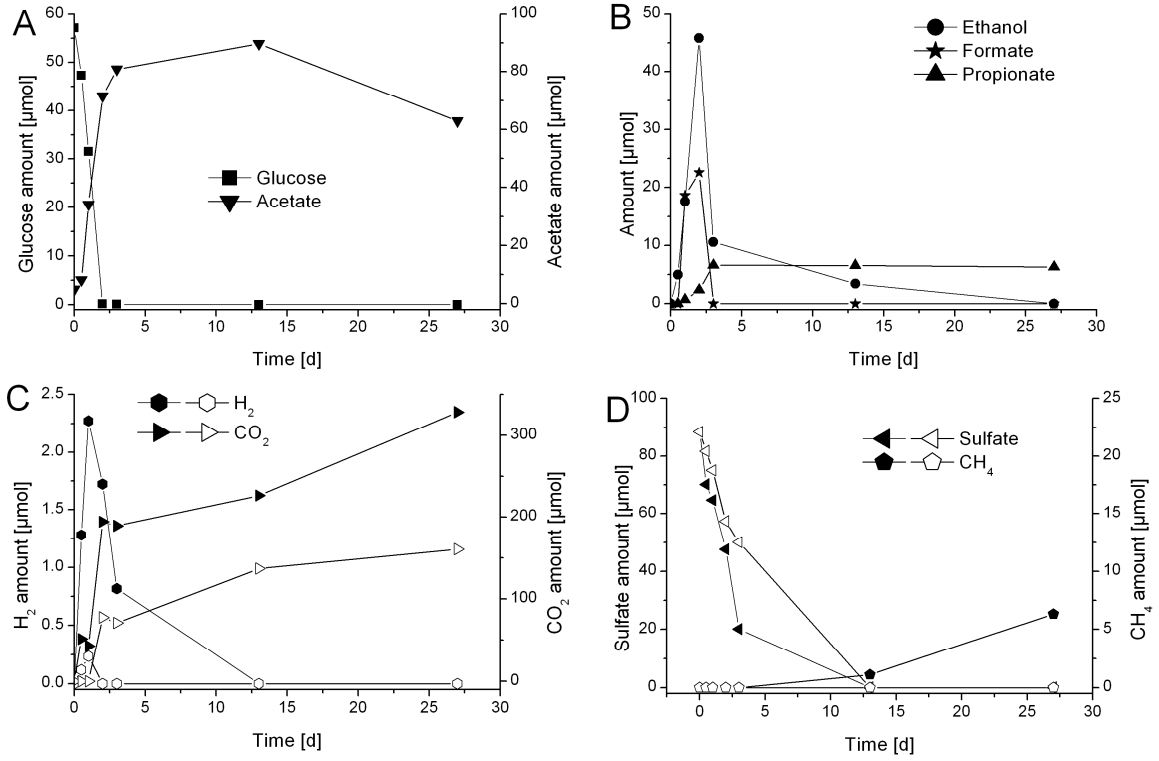

Fig. 3. Chemical analyses over the course of the experiment (referring to 21 g sediment slurry). (A) Glucose consumption and acetate production. (B) Distribution of dissolved fermentation products: ethanol, formate and propionate. (C) Distribution of gaseous fermentation products: H_2 and CO_2. (D) CH_4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

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 one day (Fig. 4), which indicates fast substrate assimilation. This efficient separation allowed an analysis of the labeled and unlabeled fractions with only minor disturbances due 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.

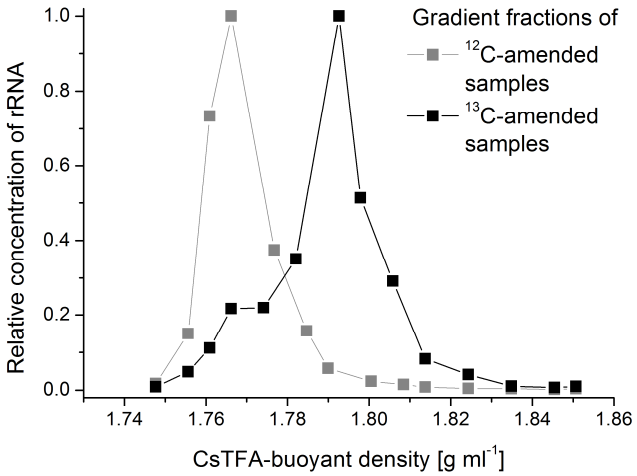

Fig. 4. Quantitative distribution of bacterial rRNA in SIP centrifugation gradients after one day of glucose fermentation. Domain-specific template distribution within gradient fractions was quantified by RT-qPCR and normalized for each gradient (21). CsTFA: Caesiumtrifluoracetate

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 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.* Exemplarily, the communities of the ^{13}C-glucose degradation experiment and the ^{12}C-glucose control at day three were compared. No significant differences were observed (data not shown).

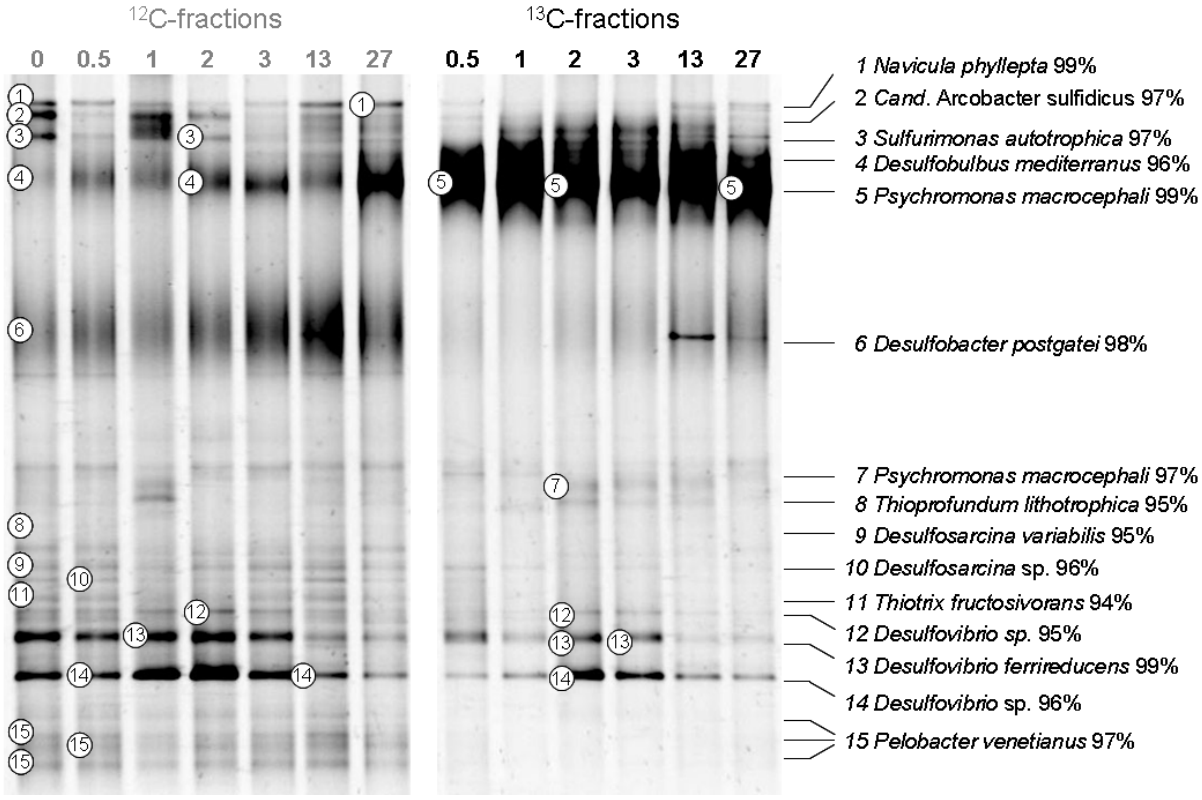

Fig. 5. DGGE-community profiles of unlabeled (grey) and ^{13}C-labeled bacteria (black) over the time course of the experiment. The "light" and the "heavy" RNA fractions originate from the same RNA extract for each time point. The time is given in days (numbers above lanes). Closest cultivated relatives and sequence similarities (%) are given.

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 *Psychromonas macrocephali* was identified by DGGE analysis of density-resolved rRNA and subsequent sequencing of DGGE bands.

The course of fermentation

The key player involved in primary fermentation was closely related to *Psychromonas macrocephali*. The type strain was originally isolated from an organic-rich marine sediments (23). In the study of Miyazaki *et al*. (23), *P. macrocephali* was found to be facultatively anaerobic and capable to form acids from glucose. 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 (24). The same products (except lactate) were identified in our study.

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 (28), 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 to the study of Beck *et al*. (3), 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, since the sulfate consumption is correlated to the phase of hydrogen excess. *Desulfovibrio* is capable of utilizing hydrogen for incomplete oxidation of organic substrates to acetate (37). 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 (31) and therefore low cell numbers are sustained. DGGE analyses, however, only detect the most abundant bacteria within communities (26). 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 µmol 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. *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 towards 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 (14). The indigenous microbial communities were probably adapted to a high load of organic matter as 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 (2). Therefore, the relatively high concentration of 9 mM glucose used in our study may not have caused a substrate shock (33). 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 free 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.

Especially when successive processes and trophic interactions in anoxic microbial food webs are analyzed, microorganisms do not necessarily react with cell division but with boosting their cell machinery which results in a higher ribosome content. This is reflected e.g. 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, while 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.

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2.3 Publication 3

Degradation of cyanobacterial biomass in tidal-flat sediments: A combined study of metabolic processes and community changes

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Abstract

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 two days, heat output reached its maximum (55 µW), while primary fermentation products were formed (in µmol): 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, while fatty acids decreased in concentration. Three main degraders were identified by RNA-based SIP and denaturant gradient gel electrophoresis (DGGE). After 12 h two phylotypes were massively ^{13}C-labeled (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 three. 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.

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 exo- or ectoenzymes, the monomers are fermented to short chain fatty acids, alcohols, H_2 and CO_2. Thereafter the oxidation of fermentation products is mainly coupled to sulfate reduction and less to methanogenesis. The terminal processes sulfate reduction and methanogenesis are well studied (e. g. Jørgensen, 1982; Llobet-Brossa *et al*., 2002), 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 key genes which either encode for the dissimilatory sulfate reductase (dsr) or the methyl-coenzyme M reductase (mcr) 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, while the fermentative microorganisms are more abundant (Schink, 2002; Köpke *et al*., 2005; Wilms *et al*., 2006b). Thus, due to a lack of a fermentation key gene, the largest part of the community remains unexplored. Here we used a new approach, combining microcalorimetry and RNA-based stable-isotope probing (SIP) to investigate the metabolic activity of fermenting organisms: Sediment samples were stimulated with ^{13}C-labeled, autoclaved *Spirulina* cell material as substrate. Thus, we simulated the burial of algal material which is quite common during storm events.

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 stable-isotope probing (SIP) to identify active community members. RNA is an excellent proxy for stable-isotope probing 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 key players involved?

Material 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 cm - 5 cm depth was transferred into a sterile 500 ml glass bottle. Prior to the

experiment, the sediment was incubated at 20°C for two weeks in the presence of sulfate to decrease the organic carbon content of the sediment. When sulfate was depleted it, was refilled to 30 mM.

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 × $6H_2O$, 0.15 g CaCl×$2H_2O$, 0.3 g KCl, 0.25 g NH_4Cl, 0.2 g KH_2PO_4). The sulfate concentration was adjusted to 30 mM. This material was transferred to 23 ml glass vials and mixed with 130 mg wet autoclaved *Spirulina*-^{13}C (Isotec, Germany). The vials were sealed with rubber stoppers and flushed with N_2. *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 seconds using the program DIGITAM 3.0 (Sci Tech Software, ThermoMetric, Järvalla, Sweden). The cultures were sampled at specific points when heat was produced resulting from the degradation of *Spirulina* cells. To demonstrate that ^{13}C-labeling of the substrate had no influence on the experiment, a control experiment with ^{12}C-*Spirulina* was performed under the same conditions. No significant difference for all measured metabolites could be detected between ^{13}C-*Spirulina* and ^{12}C-*Spirulina* degradation.

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. Organic acids were detected by an UV-VIS detector (UVIS 204; Linear Instruments, Reno, USA) 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 2 µm filter.

Sulfate concentrations were measured by an ion chromatograph (Sykam, Fürstenfeldbruck, Germany) with an anion separation column (LCA A24; Sykam, Fürstenfeldbruck, Germany) at 60 °C followed by conductivity detection (S3115, Sykam, Fürstenfeldbruck, Germany). 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. Prior to analysis the samples were 1 to 100 diluted in eluent without 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) as carrier gas and at a column oven temperature of 40 °C. For analysis of molecular hydrogen and methane a molecular sieve 13X 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 (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% Glutardialdehyde solution, 5 ml Tween 80) and incubated at room temperature over night. 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, 35 kHz, 2x320 W per period). Homogenized aliquots of 20 µl were equally dispensed on a clean microscope slide in a square of 20×20 mm. The slide was dried on a heating plate at 40°C. A drop of 12 µl staining solution (190 µl Moviol, 5 µl SybrGreen I, 5 µl 1 M ascorbic acid in TAE buffer) was placed in the center of a 20×20 mm 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) according to the manufacturer's instructions. For cell disruption, 1 g sediment and 1 ml RLT Puffer 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, 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 ng µl−1 to 1 ng µl−1) of *E.coli* 16S and 23S ribosomal-RNA (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 (CsTFA) 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). 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).

Quantitative Reverse Transcription-PCR (qRT-PCR)

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) in combination with the OneStep RT-PCR Kit (Qiagen). Each 25 µl PCR reaction contained 15.9 µl nuclease-free water, 5×RT-PCR Puffer (Qiagen), 0.4 mM dNTP Mix(Qiagen), 0.2 µM of each primer, 0.1 µl of a 1 to 500 diluted SybrGreen I solution (Molecular Probes, Eugene, OR, USA), 1 µl One Step Enzyme Mix (Qiagen) and 1 µl standard (8.2×10^9 to 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 ribosomal RNA of *E.coli* (Roche Diagnostics GmbH) served as standard for bacterial gene targets.

Denaturing gradient gel electrophoresis (DGGE) analysis

Partial 16S rRNA genes were amplified using the OneStep RT-PCR Kit (Qiagen) with bacterial primers GC-357f and 907r 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] glycerol, 60% [wt/vol] 1x 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 % (wt/vol) polyacrylamide gel containing denaturant gradients of 50 to 70% for separation of PCR products. The gel was 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

DGGE bands were excised for sequencing and treated as described by Del Panno *et al*. (2005) without the second denaturing gel for purification. For reamplification of the bands, the same primers pairs as described above were used without 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, Hilden, Germany) 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).

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 one 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 day 0 to 5 and from day 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 and organisms.

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 Joule, while the *Spirulina*-free control produced only 4 Joule 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.

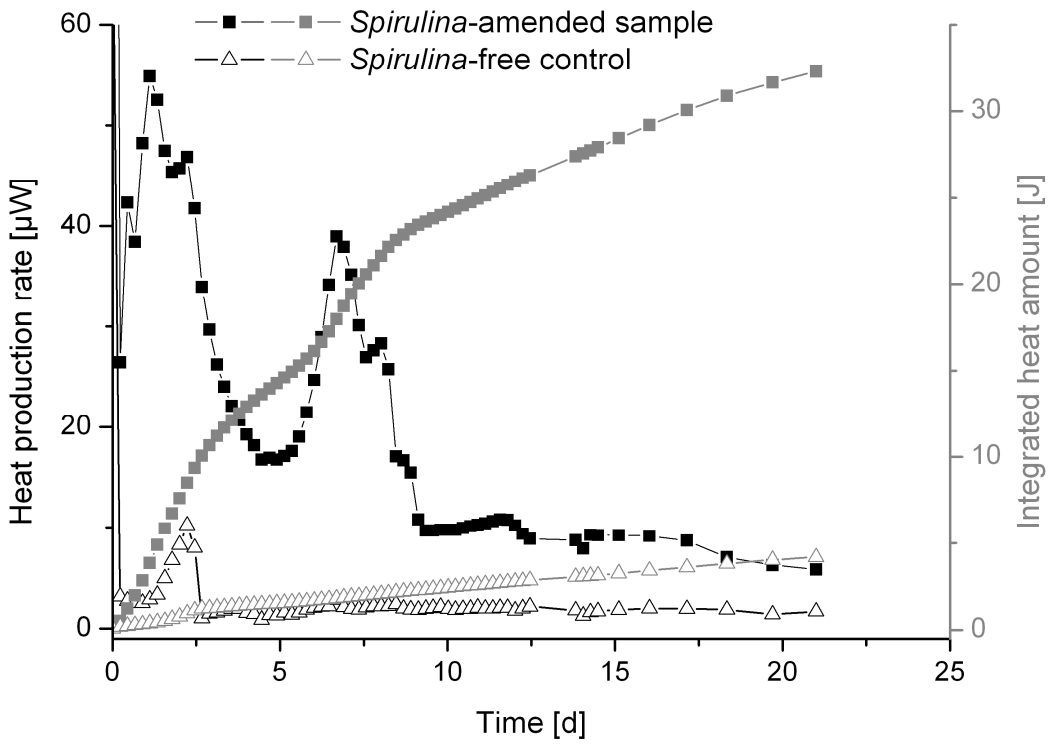

Figure 1 Heat production resulting from *Spirulina*-biomass degradation (black symbols), filled symbols: *Spirulina*-amended sample, open symbols: substrate free control, grey symbols: integrated heat production

Fermentation products

The degradation resulted in a release of typical fermentation products like acetate, propionate, butyrate, ethanol, valerate, CO_2 and H_2 (Figure 2 A - D). The main products, acetate and CO_2 increased rapidly within the first five 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 day 16 and day 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 showed that secondary fermentations coupled to methanogenesis had started.

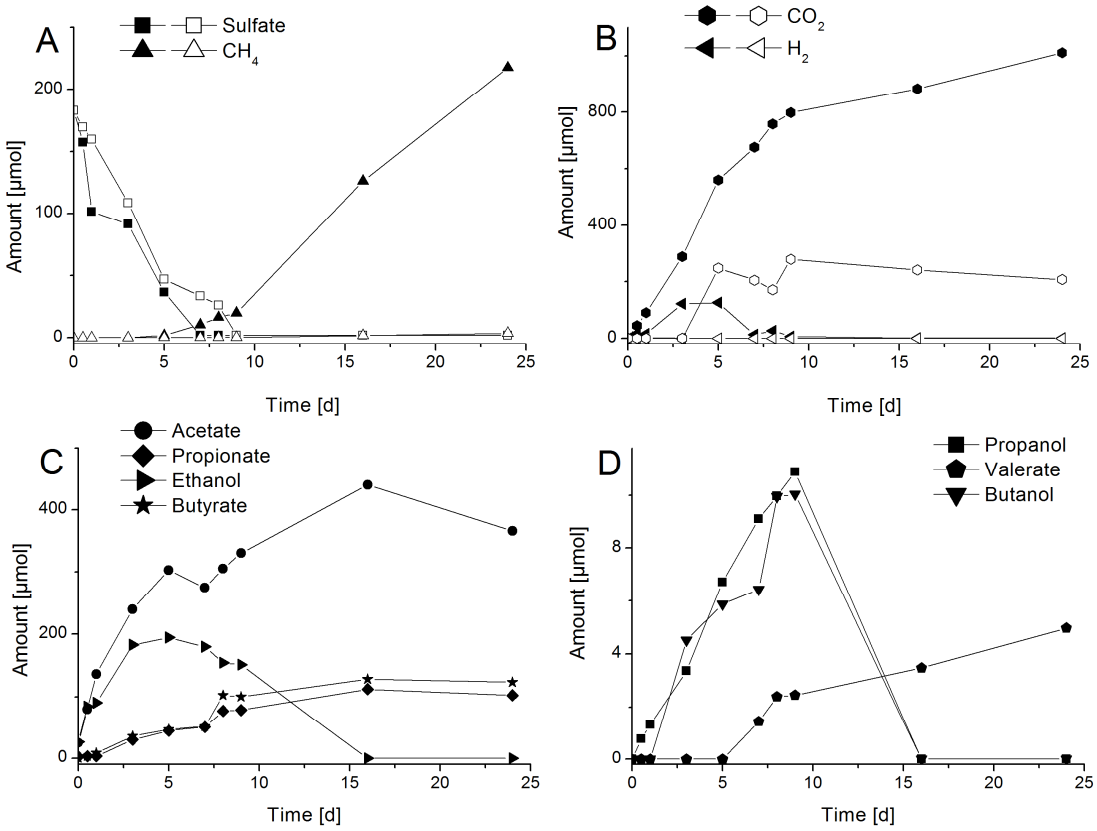

Figure 2 A: CH_4 production and sulfate consumption, B: CO_2 and H_2, C: Major fermentation products, D: minor fermentation products, filled symbols: *Spirulina*-amended samples, open symbols *Spirulina*-free control

Sulfate was almost completely consumed within the first seven days (Figure 2 A). Sulfate was converted to sulfide in almost equimolar amounts (data no shown). 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 2 A) 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 day 3 to day 22. Comparing the very large signal of the UV 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

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

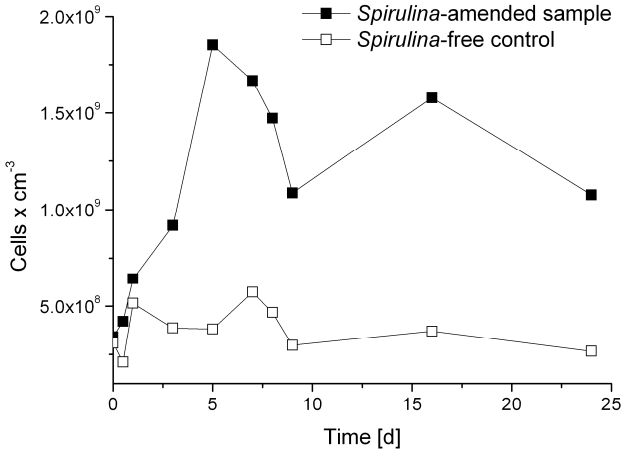

Figure 3 Total cell counts of *Spirulina*-amended samples and of *Spirulina*-free controls

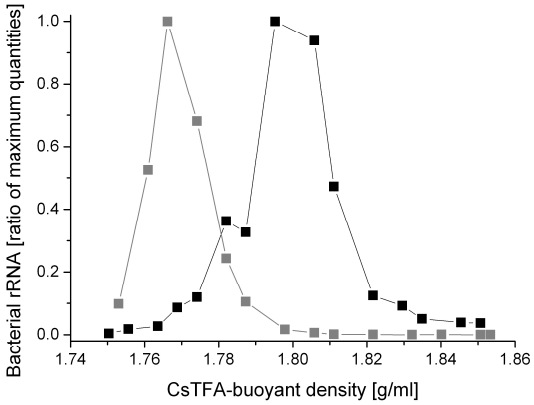

Figure 4 Quantitative distribution of bacterial rRNA in SIP centrifugation gradients after 12 hours. Template distribution within gradient fractions was quantified by RT-qPCR and normalized for each gradient (Lueders *et al*., 2004). Each square represents one gradient fraction. Grey: fractions of the ^{12}C *Spirulina*-amended samples, black: fractions of the ^{13}C *Spirulina*-amended samples, CsTFA: Caesiumtrifluoracetate

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 hours, which indicates a fast turn-over of the added biomass (Fig 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 bands (Figure 5) were affiliated to *Psychrilyobacter 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). The thickest bands, affiliated to *Psychrilyobacter atlanticus* and *Propionigenium*, were already ^{13}C-labeled after 12 hours. The *Cytophaga*-related bacterium showed an enhanced activity after three days.

Sulfate reducers related to *Desulfovibrio* showed the highest activity between day 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 been 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 ^{13}C-labeled community. Most of the active organisms, detected in the ^{13}C-labeled fractions were already visible in the light RNA fractions prior to stimulation. This means, that the stimulated 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.

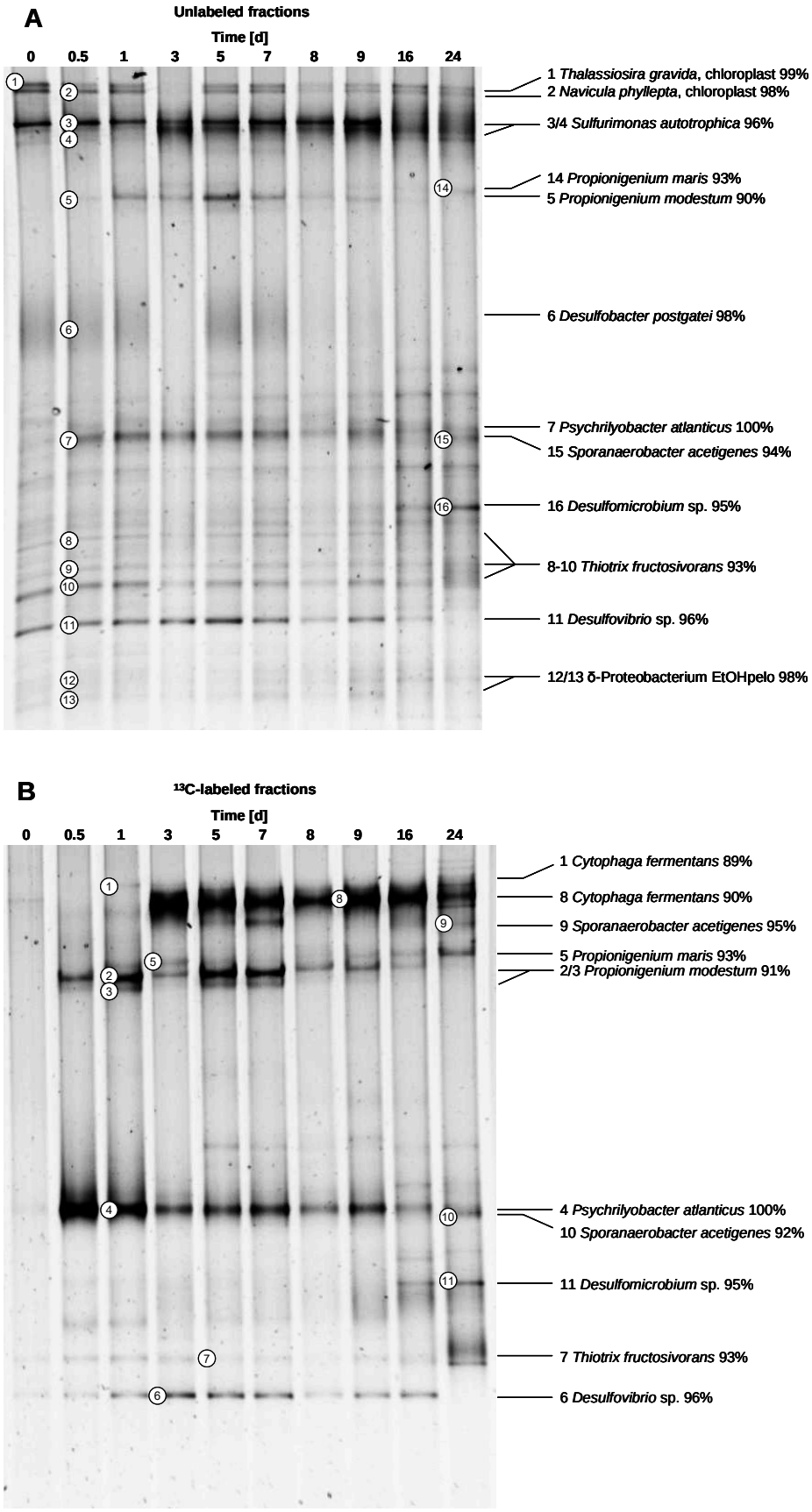

Figure 5 DGGE-community profiles of ^{13}C-labeled *Spirulina*-amended samples. The lanes in Figure A show the profiles of the non-labeled community Figure B show the profiles of the ^{13}C-labeled community. Fraction 4 with a buoyant density of approximately 1.765 [g ml^{-1}] served as unlabeled fraction 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

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. Key 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 by burial of algal blooms during storm events. In comparable studies even higher substrate concentrations were applied (Hull, 1987; Neira and Rackemann, 1996). 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). While the sulfate-reducing organisms and the archaeal community were already investigated in previous studies (Wilms *et al*., 2006b; Wilms *et al*., 2007), our study focused on the initial steps of degradation.

Most previous studies of fermentation processes in sediments were done 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 analyse 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 focus of this study was to compare stable-isotope probing of DNA and phospholipid fatty acids (PLFA).

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 S2) the following equation was established:

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

As the products contain only carbon, oxygen and hydrogen, a simple formula $<CH_2O>$ was used for *Spirulina*-biomass. CO_2, protons and water were used to balance the overall equation. Under standard conditions, the equation has a $\Delta G_0´$ 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 five days might be explained by cell lysis by phages, since 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. Due 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 $<CH_2O>$) was detected in the products (Figure S1). While CO_2 was only a smaller part of these products, most of the carbon was present in

reduced form as fermentation products. Thus, the degradation was incomplete, confirmed by the heat formation measurements.

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 key players, probably involved in primary fermentation, were *Psychrilyobacter atlanticus* and a relative of *Cytophaga fermentans*. Both have previously been found in marine sediments (Bachmann, 1955; Zhao *et al*., 2009). *Psychrilyobacter atlanticus* is a primary fermenting bacterium which produces acetate and hydrogen as main fermentation products (Zhao *et al*., 2009). It showed enhanced activity at the beginning of the experiment. This organism was probably responsible for acetate and hydrogen production. *Cytophaga fermentans* generally ferments sugars to acetate, propionate and succinate (Bachmann, 1955). However, in our case the physiological classification is uncertain, since the detected bacterium is only distantly related to *Cytophaga fermentans*.

Although a large part of the electrons (20 %) was transferred to sulfide, only two active sulfate-reducing bacteria 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 a *Desulfovibrio*-related bacterium was detected. This bacterium probably used hydrogen as electron donor, since 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 prior to sulfate depletion. 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. Since 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 *Cytophaga fermentans*-related bacterium.

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Supplementary Information

Figure S1

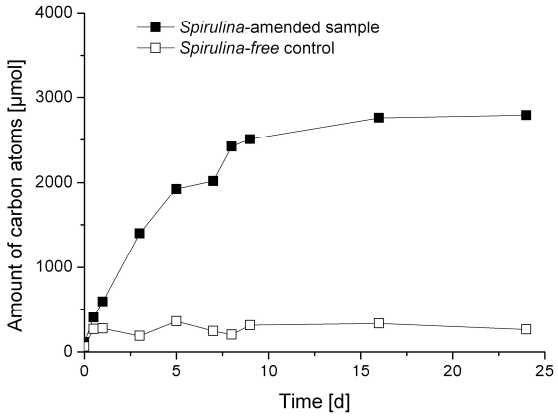

Figure S1 Accumulated amount of carbon atoms in the products formed.

Figure S2

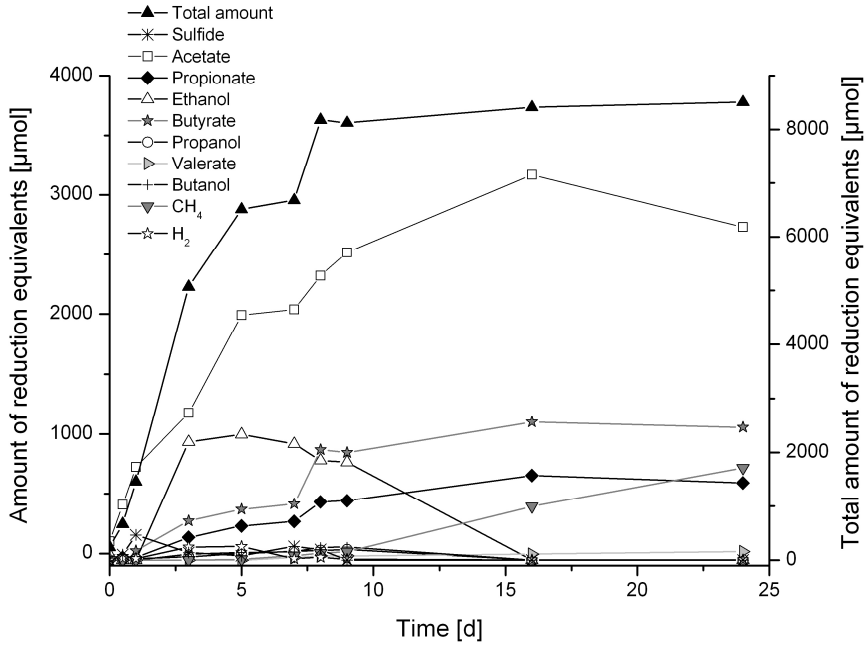

Figure S2 Amount of reduction equivalents derived from each quantified metabolite (left y-axis) and the sum of these reduction equivalents over time (right y-axis). The reduction equivalents derived from the *Spirulina*-free control were subtracted, respectively.

3 Discussion

3 Discussion

In this study, chemical quantifications combined with phylogenetic analyses were used to investigate the anaerobic degradation of complex organic matter. In starved marine sediments different phases of degradation were detected. Via chemical analyses, these phases were identified as primary and secondary fermentation as well as sulfate reduction followed by methanogenesis. The respective key players involved in the initial steps of ^{13}C-labeled glucose and ^{13}C-labeled *Spirulina* degradation were identified by RNA-based stable-isotope probing.

3.1 Method development

The aim of the IPL degradation experiment was to compare the degradation rates of different IPLs and to determine the subsequent fermentation processes and the involved key players. The experiment was conducted in large vials, which were opened at each sampling time point. This prevents an accurate quantification of methane and gaseous fermentation products. Accordingly, no reaction equation could be established for IPL degradation. Thus, the following glucose and *Spirulina*-biomass degradation experiments were conducted in small gastight vials, which were sacrificed after each sampling.

3.1.1 Fresh sediment versus starved sediment

Originally, the IPL degradation experiment was planned as an enrichment culture for lipid-degrading bacteria. It turned out, however, that the degradation of the added IPLs was superposed by the degradation of organic matter which was present in the sediment prior to substrate addition. Thus, the added cell material had only little influence on the community structure and the metabolic activity. This had a positive side-effect, since the processes which originally occur in tidal flat sediments were not disturbed. Therefore, the experimental set-up was suitable to reflect the natural IPL degradation.

Prior to the glucose and the *Spirulina*-biomass degradation experiment, the sediment was incubated in the presence of sulfate for approximately two weeks to diminish the

organic matter originating from the sediment. Accordingly, the active bacteria assimilated mainly ^{13}C-labeled carbon which resulted in a very efficient ^{13}C-labeling of the bacterial RNA.

3.1.2 Detection of active microorganisms by RT-PCR based DGGE and RNA-based SIP

The microbial community was analyzed by RT-PCR based DGGE during the IPL degradation experiment. RNA based community analyses are often used to determine the active part of a community, since the presence of DNA proves neither the activity nor even the presence of the corresponding cells (Lorenz and Wackernagel, 1987; Josephson *et al.*, 1993). On the contrary, RNA is labile and the RNA content of cells can be correlated with cellular activity (Lee and Kemp, 1994; Wagner, 1994).

In contrast to expectations, the RNA of diatom chloroplasts was detected throughout the whole IPL degradation experiment. Since no oxygen and no light for algal metabolism were available in this experiment, the diatoms might be inactive in a resting state. The RNA of chloroplasts is surrounded by four membranes (Kroth and Strotmann, 1999) and therefore probably exceptionally stable. Another reason for the detection of chloroplast RNA may be that the diatoms were in fact active and survived heterotrophically. Facultative heterotrophy is known for many diatoms, including several *Navicula* species (Lewin, 1953; Sherr and Sherr, 2007) which were also detected in our study. Heterotrophically growing diatoms continue to synthesize their photosynthetic pigments in the dark and return immediately to photosynthesis under light exposure (Lewin and Hellebus, 1970). This indicates that chloroplast RNA is present even if no light is available.

For the second and third part of this study, the experiments were conducted with ^{13}C-labeled substrates for stable-isotope probing. This method allows detecting only the active community members. Again, chloroplast RNA was found throughout the whole course of the experiments. However, the RNA was not ^{13}C-labeled, which indicates that the diatoms were inactive. Therefore, it is unlikely that the diatoms were active in the previous IPL degradation experiment under anaerobic conditions over a period of 100 days. Additionally, the RNA of a relative of the sulfur-oxidizing bacterium *Sulfurimonas autotrophica* was detected for 24 days although no oxygen for sulfur oxidation was

available. The absence of “heavy” RNA indicated that this bacterium was inactive. These findings suggest that RNA is more stable than commonly assumed. Thus, RNA-based studies do not necessarily display the active part of a community and should be combined with stable-isotope probing to ensure that exclusively active microorganisms are detected.

3.2 Different degradation rates of ester-bound and ether-bound IPLs

The quantification of *Bacteria* and *Archaea* in the deep marine biosphere by IPL and fluorescence *in situ* hybridization (FISH) analysis indicated that this habitat is dominated by *Archaea* (Biddle *et al*., 2006; Lipp *et al*., 2008). However, ester-bound bacteria-like IPLs were degraded faster than ether-bound archaeal IPLs in the laboratory experiment. Therefore, IPL-based quantification may lead to an overestimation of archaeal cell numbers and should be interpreted with caution. The IPL degradation experiment was conducted with anoxic, surface sediment which is dominated by bacteria affiliated to *Proteobacteria*, *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, *Spirochaetes* and *Chloroflexi*, and archaea related to *Methanosarcinales*, *Thermococcales*, *Methanomicrobiales* and *Methanobacteriales* (Wilms *et al*., 2006). The community of the deep biosphere, however, consists mainly of bacteria affiliated to *Chloroflexi*, *Gammaproteobacteria* and JS1 candidate group and of archaea related to the Miscellaneous Crenarchaeotic Group (MCG), Marine Benthic Group (MBG) and South African Goldmine Euryarchaeotal Group (SAGMEG) as described by Inagaki *et al*. (2006), Webster et al. (2006), Teske and Sørensen (2008). As our results strongly suggest a microbial degradation of IPLs by enzymes, the community structure probably has a major influence on the degradation pattern. Thus, the observed degradation rates might be different if the experiment is repeated with sediment of the deep biosphere.

The experiment was based on the addition of killed cells. Their status of disintegration was probably influencing the degradation rates, as membrane lipids are protected by their surrounding cell walls. The surface of *Haloferax volcanii* consists of hexagonally packed surface (S) layer glycoprotein (Sumper *et al*., 1990), whereas the cell wall of *Saccharomyces cerevisiae* consists mainly of glucan polymers, chitin and glycoproteins (Levin, 2005). As *H. volcanii* lives in an extreme habitat, one could assume that the cell wall is exceptionally stable and that the cells were still intact during the experiment. This

would lead to a protection against degradation and might explain the enhanced stability of ether-bound IPLs. However, the cell walls of halophilic archaea of the order *Halobacteriales* are only stable at a high NaCl concentration (Mohr and Larsen, 1963; Kushner, 1964). *H. volcanii* additionally needs a high Mg^{2+} concentration to maintain a stable cell wall (Cohen *et al*., 1983). As the *H. volcanii* cells were pasteurized in distilled water, it is highly unlikely that the cells remained intact. This is supported by several other aspects: Approximately, 10^9 *H. volcanii* cells ml^{-1} were added to the sediment. No difference in total cell numbers between the degradation experiment and the untreated control was observed at the beginning of the experiment. 2. The amount of archaeal 16S rRNA of the untreated control was even lower compared to the degradation experiment at the beginning of the experiment. 3. The RNA of *H. volcanii* was only detected at the very first sampling point of the experiment. The rapid degradation of the RNA indicated a disintegration of the *H. volcanii* cells because this study indicated that RNA might be more stable than commonly assumed.

3.3 Degradation of monomers and polymers: a comparison

A few major differences between *Spirulina* degradation and glucose degradation were observed: As expected, the degradation of a complex substrate leads to a broader variety of intermediates. Accordingly, butanol and propanol were produced during *Spirulina* consumption and not upon glucose consumption. Only one main glucose assimilating bacterium was identified, whereas three bacteria were stimulated in the *Spirulina* degradation experiment. Glucose was completely consumed within three days. The Spirulina biomass, however, was not completely degraded during the whole course of the experiment as indicated by the integrated heat production and the overall reaction equation. These findings are not surprising, as the degradation of complex organic matter additionally requires hydrolysis of polymers to monomers. The monomers of the *Spirulina* biomass comprise not only different sugars, but also fatty acids, amino acids and derivates of these compounds.

However, the degradation of the two model substrates revealed some important similarities: Although sulfate reduction played an important role, only few sulfate-reducing bacteria were identified. This is in accordance to the study of Beck *et al*. (2011) who found

that sulfate-reducers account only to ~5% of the bacterial community in tidal-flat sediments. The most abundant sulfate-reducers were in both cases related to the genus *Desulfovibrio* and showed their highest activity in the phase of hydrogen access and rapid sulfate depletion. Thus, these organisms probably used hydrogen as electron donor, as *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 prior to sulfate depletion.

In the absence of sulfate, the degradation of organic matter becomes a three-step process – including secondary fermentation and methanogenesis. Accordingly, during *Spirulina* and glucose degradation, the amount of primary fermentation products (especially alcohols) was decreasing even when sulfate was depleted. This indicates that secondary fermentation played an important role. The metabolism of syntrophic bacteria gains only little energy (Schink, 1997) and thus low cell numbers. Since DGGE analysis only detects the most abundant organisms (Muyzer and Smalla, 1998), it is not surprising that during *Spirulina* degradation only two and during glucose degradation no potentially secondary fermenters were identified.

3.4 Outlook

The IPL degradation experiment was originally planned as an enrichment culture for lipid degrading microorganisms. However, the IPL degradation was superposed by the degradation of organic matter originating from the sediment. Thus, it would be reasonable to repeat the experiment with starved sediment and to combine it with stable-isotope probing as this study indicated that RNA-based DGGE also displays a part of the inactive background community. Additionally, the experiment should be conducted with cell material derived from organisms which naturally occur in tidal-flat sediment. This approach would be most suitable to reflect the natural degradation rates of ether-bound and ester-bound IPLs. A methanogenic archaeon is an appropriate source for ether-bound IPLs as the community profile showed that *Methanogenium frigidum* was the most abundant archaeon. Pure cultures of members of the genus *Methanogenium* are commercially available and can be grown in the presence of ^{13}C-labled CO_2 and acetate to gain

^{13}C-labled IPLs. As representative source for ester-bound IPLs, sulfate-reducing bacteria or *Pelobacter* ssp. can be used. The crucial step for this experimental approach is to get a sufficient amount of ^{13}C-labeled archaeal biomass.

Stable-isotope experiments in combination with microcalorimetry and chemical analyses can be conducted in the presence of different inhibitors. If the terminal oxidizing processes are suppressed, intermediates will probably accumulate which otherwise are immediately consumed and therefore evade analyzing. Molybdate, an inhibitor of sulfate-reduction or/and 2-Bromoethanesulfonic acid (BES), an inhibitor of methanogenesis can be added either when the experiment begins or when the respective processes have reached their highest rates. Instead of inhibiting sulfate-reduction, the experiments can also be conducted with sediment samples originating from sulfate-free layers to stimulate secondary fermenting organisms.

4 References

4 References

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Zusätzlich erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat.

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