Tracing viable bacteria in Wadden Sea sediments using phospholipid analysis

Nachweis lebensfähiger Bakterien in Wattsedimenten mittels Phospholipidanalytik

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.... Surprisingly, the benthic (bottom) organisms that reside within marine sediments are extremely diverse.... There are some 10^5 species described from marine sediments and perhaps 10^8 that remain undescribed. But is consideration of and inventorying biodiversity little better than stamp collecting as was once suggested by the physicist Ernest Rutherford ? Among the many reasons for considering living organisms differently from stamps are their roles in maintaining the Earth's life support system....

(Snelgrove et al., 1997)

Parts of this thesis were presented at (international) meetings and conferences by Heike Rütters:

"Intertidal Mudflats", Plymouth, U.K. April 1999	Poster: Analysis of phospholipids as a tool to study microbial communities in Wadden Sea sediments
"Jahrestagung der Deutschen Gesellschaft für Massenspektrometrie (DGMS) 1999", Oldenburg, May 1999	Poster: Chemotaxonomische Klassifizierung von Sulfatreduzierern anhand ihrer Phospholipidmuster
"19th International Meeting on Organic Geochemistry", Istanbul, Turkey September 1999	Oral and poster: Studying microbial communities in Wadden Sea sediments by phospholipid analysis
"Microbiology 2000", Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), München, March 2000	Poster: Tracing sulfate-reducing bacteria in Wadden Sea sediments by phospholipid analysis
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Chapter 2.1

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Chapter 2.2

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Chapter 3.1

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Chapter 3.2

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Chapter 3.3

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Chapter 4

Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2001. Microbial communities in Wadden Sea sediments - clues from the analysis of intact phospholipids. Organic Geochemistry, submitted.

ABSTRACT

This thesis describes a protocol that was developed to analyse different types of lipids from bacteria and sediments. Special emphasis was placed on the qualitative and quantitative analysis of intact phospholipids by HPLC-ESI-MS and -MS/MS, because phospholipids appear to be suitable biomarkers to trace viable bacteria. The specificity of phospholipids as biomarkers was tested by investigating different pure cultures of bacteria. As an example, the lipid composition of 30 different strains of sulfate-reducing bacteria was investigated and compared to the phylogenetic relatedness of these organisms. These investigations revealed that most of the sulfate reducers contain three different phospholipid classes (PE, PG, DPG) substituted with different, characteristic fatty acids. Additionally, phospholipids substituted with straight-chain alkylethers were detected in two sulfate-reducing bacteria, *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*. Membrane lipids with non-isoprenoidal ether-linked side-chains had before only been known from thermophilic bacteria. On the other hand, corresponding hydrolysis products, the so-called monoalkylglycerol ethers, had been found at different locations, e.g. in temperate sediments overlying methane hydrates.

A sediment core with a length of 55 cm, studied in this thesis work, was taken from an intertidal flat close to Neuharlingersiel (NW Germany). Results from geochemical investigations were compared to data obtained by microbiological techniques. Despite the highly dynamic nature of the system, steep gradients were observed in biomass contents. Highest amounts of phospholipids as well as of other types of glycerides were measured at the surface and decreased rapidly within the upper few centimetres. Nevertheless, intact phospholipids could still be detected at a sediment depth of 50 cm indicating the presence of intact cells. In the depth interval between 10 and 20 cm, elevated amounts of phospholipids were detected which probably reflect the presence of high amounts of bacterial biomass at that depth. Intact phospholipids showed a strong shift in microbial communities with depth. At the surface phospholipids dominated which according to their headgroups and fatty acid side-chains were presumably of algal origin. This signal decreased rapidly with depth, and bacterial phospholipids became relatively more abundant. Compounds with isoprenoid side-chains typical for archaea were not detected.

KURZFASSUNG

In der vorliegenden Arbeit wurde eine Methode entwickelt, um verschiedene Lipidtypen aus Bakterien und Sedimenten zu analysieren. Der Schwerpunkt lag hierbei auf der qualitativen und quantitativen Analyse intakter Phospholipide mittels HPLC-ESI-MS und –MS/MS, da Phospholipide potentielle Biomarker für „lebende“ Bakterien darstellen. Die Spezifität der Phospholipide als Biomarker wurde exemplarisch durch die Untersuchung verschiedener Reinkulturen getestet. Dafür wurden die Lipidzusammensetzungen von 30 verschiedenen Sulfatreduzierern untersucht und ihren phylogenetischen Verwandtschaftsbeziehungen gegenübergestellt. Aus diesen Untersuchungen ergab sich, dass die meisten Sulfatreduzierer drei verschiedene Phospholipidklassen (PE, PG, DPG) enthalten, die mit einer Reihe unterschiedlicher, charakteristischer Fettsäuren substituiert sind. Zusätzlich wurden in zwei Sulfatreduzierern Phospholipide mit geradkettigen Alkylether-Seitenketten nachgewiesen. Solche Membranlipide waren bisher nur aus thermophilen Bakterien bekannt. Andererseits waren entsprechende Hydrolyseprodukte, sog. Monoalkylglycerylether, in verschiedenen Sedimenten gefunden worden, so z.B. auch oberhalb von Methanhydraten.

Des weiteren wurde in der vorliegenden Arbeit ein Sedimentkern von 55 cm Länge untersucht, der im zeitweise trockenfallenden Mischwatt vor Neuharlingersiel (Ostfriesische Nordseeküste) genommen wurde. Die mittels geochemischer Verfahren erhaltenen Ergebnisse wurden mit Daten aus mikrobiologischen Untersuchungen verglichen. Dabei zeigte sich, dass das untersuchte Sediment ein stark durchmischtes System ist, in dem sich trotzdem steile Gradienten an Biomasse ausbilden. So wurden die höchsten Gehalte an Phospholipiden und anderen Glyceriden an der Oberfläche gemessen. Schon innerhalb der ersten Zentimeter fielen diese Werte stark ab. Trotzdem wurden selbst in 50 cm Tiefe noch intakte Phospholipide gefunden, d.h. es gab dort noch „lebende“ Zellen. Zwischen 10 und 20 cm Sedimenttiefe zeigten sich deutlich höhere Phospholipidgehalte, die auf eine erhöhte bakterielle Biomasse in diesem Bereich hindeuten. Aus der Untersuchung der intakten Phospholipide konnte eine starke Veränderung der Zusammensetzung der mikrobiellen Gemeinschaft mit der Tiefe festgestellt werden. So wurden in den oberen Sedimentschichten viele Phospholipide gefunden, die vom Typ und den Fettsäureseitenketten her auf einen großen Anteil an eukaryotischen Algen schließen lassen. Mit der Tiefe nahm dieses Signal schnell ab, und es überwogen Phospholipide bakteriellen Ursprungs. Verbindungen mit isoprenoiden Etherseitenketten, wie sie für Archaea typisch sind, wurden nicht nachgewiesen.

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FIGURES

(captions abbreviated)

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(captions abbreviated)

ABBREVIATIONS

bp	base pairs
BP	base peak
DAPI	diaminophenyl indol
DGGE	denaturing gradient gel electrophoresis
DMDS	dimethyl disulfide
DNA	desoxyribonucleic acid
DPG	diphosphatidyl glycerol
ELSD	evaporative light scattering detector
ESI	electrospray ionisation
FAB	fast atom bombardment
FAME	fatty acid methyl ester
FID	flame ionisation detection
GC	gas chromatography
G+C	(content of) guanidine and cytosine (in DNA)
HPLC	high performance liquid chromatography
MALDI	matrix assisted laser desorption ionisation
MPN	most probable number
MS	mass spectrometry
MS/MS	collision-induced fragmentation of quasi-molecular ions
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
n.d.	not detectable
n.q.	not quantifiable
PA	phosphatidic acid
PC	phosphatidyl choline
PCR	polymerase chain reaction
PDME	phosphatidyl dimethylethanolamine
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PI	phosphatidyl inositol
PLFA	polar lipid fatty acids
PMME	phosphatidyl monomethylethanolamine
PXM	phospholipid of type X with mixed, i.e. alkyl and acyl side chains
rDNA	ribosomal desoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	retention time
sn	strereospecific numbering
SRB	sulfate-reducing bacteria
TDS	total dissolved sulfur
TGFA	triglyceride fatty acids
T_{inc}	incubation temperature
TMS	trimethylsilyl

1 INTRODUCTION

1.1 MICROBIAL COMMUNITIES IN INTERTIDAL SEDIMENTS

In open oceans, only a minor fraction of the organic material originally produced in the photic zone is ultimately buried in the sediments (Jannasch and Taylor, 1984; Hedges and Keil, 1995). Most of the organic matter produced is already recycled or degraded in the oxic water column. The fraction reaching the sediment depends, among other things, on the residence time of the organic material in the water column. The residence time itself is controlled by the settling velocity of the organic matter and the water depth (Suess, 1980). Remaining organic material accumulates at the sediment-water interface where it is further degraded by benthic organisms (e.g. Volkman et al., 1980). Microbial activities at the sediment surface are stimulated by the continuous supply of organic material. If oxygen consumption exceeds (diffusive) oxygen supply into sediments, other electron acceptors have to be used. Usually, oxygen is depleted within the upper few millimetres of the sediment, so that sediments are anoxic from a few millimetres or centimetres downwards (Revsbech et al., 1980; Reimers and Smith, 1986; Sørensen and Jørgensen, 1987). In general, electron acceptors for terminal oxidation are used in the order of decreasing redox potential, i.e. oxygen is preferred to nitrate > Fe(III) ≥ Mn(IV) > sulfate > carbon dioxide (Froelich et al., 1979; Sørensen et al., 1979; Nealson 1997). Whereas in open oceans most of the organic matter is degraded under oxic conditions, anoxic processes in sediments become more prominent in coastal areas (Edenborn et al., 1987; Henrichs and Reeburgh, 1987; Canfield and Thamdrup, 1996). In coastal marine systems, sulfate is the most important electron acceptor accounting for over 50% of organic matter mineralisation (Jørgensen, 1982; Skyring, 1987).

Intertidal sediments are highly dynamic systems in which settling particles are resuspended many times before ultimate burial in the sediments (e.g. Reineck, 1980; Paterson and Black, 1999). Additionally, intertidal sediments exhibit a large spatial heterogeneity both vertically and horizontally ("patchiness") (Jickells and Rae, 1997). Depending on the permeability of the sediments, porewater concentrations are not only controlled by diffusion, but also by advection due to the changing water coverage and wave action (Huettel et al., 1996; Malcolm and Sivyer, 1997; Asmus et al., 1998). Furthermore, the sediments often bear high abundances of burrowing macro- and

meiofauna which enhance sediment heterogeneity and mixing by their activities (bioturbation); the fauna also contributes significantly to the porewater exchange (bioirrigation) (Aller, 1982; Fry, 1982; Riisgård and Banta, 1998; Kristensen, 2000).

On sheltered intertidal sandflats, photosynthetic laminated microbial mats of 2 to 20 mm thickness often thrive, e.g., in the so-called "Farbstreifen-Sandwatt" close to the island of Mellum (Hoffmann, 1942; Gerdes et al., 1987; van Gemerden et al., 1989). These microbial mats are characterised by steep redox gradients due to the complex interplay of oxygenic cyanobacteria with anoxygenic phototrophs and sulfate-reducing bacteria.

Under anoxic conditions organic matter is degraded stepwise by a complex microbial community due to constraints by the energy regime (Pfennig and Widdel, 1982; Schink, 1989; Nealson, 1997). Sulfate-reducing bacteria are involved in the terminal step(s) of this degradation sequence. Sulfate reducers are a physiologically and phylogenetically heterogeneous group, but are unified by their ability to use sulfate as the external electron acceptor for respiration (Devereux and Stahl, 1993; Hansen, 1994). Typical electron donors for sulfate-reducing bacteria are fermentation products such as volatile fatty acids, pyruvate and lactate, but also short-chain alcohols and hydrogen (Widdel and Hansen, 1992). Despite of being obligate anaerobes, sulfate-reducing bacteria are not only found in truly anoxic environments, but are also detected in oxic marine surface sediments (e.g. Jørgensen and Bak, 1991; Sahm et al., 1999; Wieringa et al., 2000) and microbial mats (Visscher et al., 1992; Risatti et al., 1994; Minz et al., 1999).

Microbial communities in surface layers of intertidal sediments have been intensely studied (e.g. Federle et al., 1983; Epstein, 1997; Hamels et al., 1998; Rooney-Varga et al., 1998; Böttcher et al., 2000), whereas little is known about processes that occur in the deeper parts (>20 cm sediment depth) and about the microorganisms involved. The importance of microorganisms inhabiting deeper sediment layers has not been recognised for a long time. Bacteria living in subsurface environments face the problem of substrate limitation, because organic matter that is easy to degrade, is usually already consumed at the surface (van Es, 1984; Henrichs and Reeburgh, 1987; Deming and Baross, 1993). Due to the substrate limitation, subsurface bacterial cells are often small, metabolic rates are low, and the bacteria have developed means to survive long starvation periods (Bachofen et al., 1998; White et al., 1998). Nevertheless, subsurface bacteria represent an enormous pool of biomass (>90% of all bacteria live in

the subsurface) with major implications for the global carbon cycle (Parkes et al., 1994; Whitman et al., 1998; Pedersen, 2000).

In general, studying microbial communities involves two questions: "Who is there ?" and "What are they doing ?". Unfortunately, microorganisms cannot be detected and classified just by visual inspection, because they are too small in most cases (usually a few micrometres) and they display only little differences in their morphologies (Krieg and Holt, 1984; Dworkin, 1992). To estimate the size of the total bacterial community in a given sample, microorganisms can be stained using fluorescent dyes, e.g. acridine orange and DAPI, which bind to certain cell components. Subsequently, fluorescing cells are counted using an epifluorescence microscope (Hobbie et al., 1977; Costerton and Geesey 1979; Porter and Feig, 1980; Kepner and Pratt, 1994). Other techniques to estimate total microbial biomass include measurement of total amount of DNA (e.g. Dell'Anno et al., 1998) or RNA (Sahm and Berninger, 1998) and determination of phospholipid phosphorus contents (White et al., 1979; Gillan and Sandstrom, 1985; see 1.2). The former method encounters the problem of a necessary differentiation between DNA present in viable bacteria and "fossil" DNA. "Fossil" DNA can contribute quite significantly to the total amount in some locations (Lorenz and Wackernagel, 1987; Coolen and Overman, 1998; Dell'Anno et al., 1998).

To assess the diversity of organisms present in a given sample, either cultivation-based or cultivation-independent approaches are used. Cultivation-based techniques, such as so-called "most probable number" experiments* (e.g. Cochran, 1950; de Man, 1977; Seyfried and Owen, 1978) with subsequent isolation, allow investigation of physiological capabilities of bacteria from the sample. The great disadvantage of these techniques is that the media and growth conditions are selective and artificial. Additionally, often only a subtle fraction of the bacteria can be cultivated at all (Vester and Ingvorsen, 1998; McDougald et al., 1998; Barer and Harwood, 1999). The reason for this discrepancy is still a matter of research (e.g. Jaspers, 2000 and references therein).

* In "most probable number" experiments the number of bacteria that can grow under the experimental conditions is estimated from parallel serial dilutions of the original sample, subsequent incubation and statistical analysis of the results.

To overcome the problem of low cultivation efficiency, "cultivation-independent" techniques can be employed such as biomarker analysis (see 1.2) or molecular techniques (e.g. Kozdroj and van Elsas, 2001). The latter involve, for example, determination of the variety of the 16S rRNA or DNA sequences in the sample of interest. This variety can be investigated either *in situ* using fluorescently-labelled oligonucleotide probes (Amann et al., 1995) or after extraction of nucleic acids by "dot blot" (Devereux et al. 1996; Sahm and Berninger, 1998). Another possibility is the extraction of DNA, its amplification and sequencing after separation by cloning (Giovannoni et al., 1990) or separation according to sequence differences by denaturing gradient gel electrophoresis (DGGE) (Muyzer, 1998, 1999). The amplification procedure, i.e. the polymerase chain reaction (PCR), may be selective and prone to artefact formation (von Wintzingerode et al., 1997; Head et al., 1998). As phylogeny is not necessarily closely coupled to physiology - e.g. dissimilatory sulfate reduction is carried out by organisms belonging to phylogenetically different groups such as δ–Proteobacteria, low G+C Gram-positive bacteria, some thermophilic bacteria and archaea (Castro et al., 2000) - conclusions about the physiology of an organism drawn from 16S rDNA sequences may be speculative. Often also a high physiological diversity is not adequately reflected in the variety of 16S rDNA sequences (e.g. Sass et al., 1998; Sass, 2001). One possibility to couple physiological and phylogenetic information is to target physiologically important gene sequences such as that of the dissimilatory sulfite reductase instead of 16S rDNA (Minz et al., 1999; Tunlid, 1999).

To investigate metabolic activities of microbial communities, consumption/ metabolism of likely substrates is measured. To facilitate the detection of substrates and their metabolites, labelled compounds are often used (Sorokin, 1962; Wright and Hobbie, 1966). For example, if radiolabelled sulfate ($^{35}SO_4^{2-}$) is applied to sediments, sulfate reduction rates can be estimated from the labelled sulfide generated (Jørgensen, 1978; Fossing and Jørgensen, 1989). Potential activities of different extracellular enzymes, that are necessary for hydrolytic cleavage of polymeric substrates, can be measured using fluorescently-labelled polysaccharides (Arnosti, 1995) or monomeric substrate analogues (Hoppe, 1983). Following the uptake of isotopically labelled (^{13}C or ^{14}C) organic compounds or carbonate allows determination of major "consumers" and their position in the food web (Abraham et al., 1998; Boschker et al., 1998; Roslev et al., 1998; Middelburg et al., 2000).

1.2 PHOSPHOLIPIDS AS BIOMARKERS

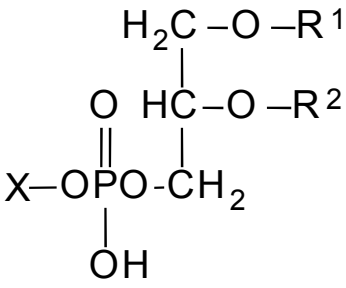
To study microbial community composition, biomarker analysis is often applied. Biomarkers – in this context - are compounds which are known to derive from specific organisms only, so that the presence of that particular organism can be inferred from the detection of this biomarker in a given sample. Different cellular components of microorganisms have been used as biomarkers, such as quinones (Hiraishi et al., 1998; Hiraishi, 1999), hopanoids (Buchholz et al., 1993), β-hydroxy fatty acids (Parker et al., 1982) and total fatty acids (Schutter and Dick, 2000). The usefulness of these compound classes is diminished as their occurrence is not wide-spread or not systematic and they posses only low resolving potential (e.g. Collins and Jones, 1981; Goossens et al., 1986; Kannenberg and Poralla, 1999; Oka et al., 2000). For the investigation of microbial communities, it is essential that the biomarkers allow a differentiation between viable cells and allothigenic or authigenic detritus. Phospholipids appear to be ideal candidates of biomarkers for viable cells, because they

- are major membrane lipids (ca. 5% of cell dry weight) of nearly all organisms (Madigan et al., 1999) allowing already for detection of a few cells – prokaryotic and eukaryotic;
- are known to be only stable in intact cells and are hydrolysed within weeks after cell death (White et al., 1979; Harvey et al., 1986) thereby indicating only "viable" biomass.

Therefore, phospholipids – measured as lipid phosphate or "phospholipid" fatty acids – have often been applied to determine total biomass and community composition in environmental samples such as marine sediments (e.g. Rajendran et al., 1992; Parkes et al., 1993; Guezennec and Fiala-Medioni, 1996), terrestrial soils (e.g. Bååth et al., 1992; Zelles et al., 1997; Frostegård et al., 1997) or biofilms (e.g. Tunlid et al., 1989). Usually, conversion of lipid phosphate or "phospholipid" fatty acids to cell numbers or biomass is done using an average conversion factor which may lead to over- or underestimation of biomass due to large variations in cellular phospholipid contents (e.g. Brinch-Iversen and King, 1990; Petersen et al., 1991; Onstott et al., 1998).

"Phospholipid" fatty acids are not only used as a tool to measure total biomass, but also for community level profiles (e.g. Findlay and Dobbs, 1993; Hedrick et al., 2000). Direct linkages between variations in fatty acids observed, e.g. by altering

environmental conditions, and abundances of certain types of microorganisms are often contradictory, because fatty acids unique to a distinct group of organisms are rare and there is usually a great overlap in fatty acid patterns (Haack et al., 1994; White et al., 1996; Zelles, 1999). On the other hand, fatty acid patterns (after whole-cell hydrolysis) are important in the chemotaxonomic classification of isolated microorganisms (Asselineau and Asselineau, 1990; Hutchinson et al., 2000; MIDI Inc., http://www.midi-inc.com). Additionally, polar lipid patterns can be used in bacterial taxonomy (Krieg and Holt, 1984). In most bacteria, phospholipids are the major compound class of the polar lipids (e.g. Brinch-Iversen and King, 1990; White et al., 1997). The chemotaxonomic information is contained in the so-called headgroup and in the side-chains (Fig. 1).



X = headgroup,
e.g. glycerol, ethanolamine, choline, serine, inositol

R^1, R^2 = side-chains,

e.g. R^1, R^2 = acyl (usually C_{14}-C_{19})	- diacyl phospholipid
R^1 = alkyl, R^2 = acyl	- O-alkyl acyl phospholipid (alkyl ether phospholipid)
R^1 = 1'-alkenyl, R^2 = acyl	- alkenyl acyl phospholipid (vinyl ether phospholipid, plasmalogen)

Fig. 1 Molecular structures of phospholipids

Headgroups often encountered in bacteria are short-chain alcohols, e.g. glycerol, ethanolamine, choline, as well as amino acids, e.g. serine, and sugars or cyclic polyols such as galactose, inositol (Thiele, 1979; Lechevalier and Lechevalier, 1988). Typically, phospholipids are substituted with two different fatty acids with chain lengths of 14 to 19 carbon atoms. Often not only saturated straight-chain fatty acids are found, but also monounsaturated, branched or fatty acids with a cyclopropyl ring (Lechevalier and Lechevalier, 1988). These fatty acids are preferentially linked to the central carbon atom of the glycerol backbone (e.g. Suutari and Laakso, 1994). Besides ester-linked fatty

acids, some bacteria also contain fatty aldehydes as side-chains in their phospholipids, which are then called plasmalogens (Thiele, 1979). Ether-bound alkyl substituents are commonly only found in archaea and in some thermophilic bacteria (Kates, 1997; Langworthy et al., 1983). Ether side-chains of archaeal phospho- and glycolipids are usually built-up from isoprenoid units, whereas thermophilic bacteria display straight-chain or singly branched substituents. Recently, ether diglycerides have been detected in temperate sediments combining "typical" archaeal and bacterial features (Schouten et al., 2000).

In the literature, data on phospholipid types and whole-cell fatty acid composition of many organisms are available (see Ratledge and Wilkinson, 1988, and references therein), whereas information on phospholipid molecular structures are rare because of the time-consuming procedures that used to be involved in structure determination of phospholipids (i.e. preparative thin layer chromatography and subsequent hydrolysis) (Olsson and Salem, 1997). Nowadays, structures of intact phospholipids can be directly determined using mass spectrometry (MS) with a soft ionisation technique, such as fast atom bombardment (FAB), matrix assisted laser desorption ionisation (MALDI) or electrospray ionisation (ESI), and analysis of intact phospholipids were used to classify bacterial isolates (Fenselau, 1994; Cole and Enke, 1994). Additionally, Fang et al. (2000) showed that intact phospholipids have a higher discriminating potential for different *Pseudomonas* sp. than PLFA patterns alone. The higher resolution can be attributed to a more detailed information obtained by analysis of intact phospholipid species, because, in contrast to a set of 20 to 50 different fatty acids (Zelles, 1999), organisms contain a much higher diversity of different side-chain combinations at the various phospholipid types present. Additionally, all kinds of side-chains can be analysed by ESI-MS, i.e. ester and ether bonds as well as vinyl ether bonds of plasmalogens (Han and Gross, 1995) thereby enlarging the diversity of phospholipid species that can be used for classification.

In this thesis, the following expressions are used to distinguish the various levels of information:

(Total) fatty acid pattern: Distribution of fatty acids measured after whole-cell hydrolysis, i.e. it includes fatty acids derived from all cellular components (membrane lipids, lipopolysaccharides, storage compounds etc.).

Polar lipid fatty acid (PLFA) pattern: Distribution of fatty acids obtained after hydrolysis of the polar lipid fraction, i.e. a fraction defined by polarity which may contain not only phospholipids, but, depending on the sample, may also comprise other compounds of similar polarity.

Phospholipid (class or type) pattern: Distribution of different types/classes of phospholipids. These types/classes are defined according to the headgroups present irrespective of side-chains. Examples: phosphatidyl glycerol (PG), phosphatidyl ethanolamine (PE) etc.

Phospholipid (molecular) species pattern: Distribution of individual phospholipids taking into account differences in headgroups and side-chains. Examples: PE with two *n*-$C_{16:0}$ fatty acids, PE with an *n*-$C_{17:0}$ and an *i*-$C_{15:0}$ fatty acid etc.

1.3 OUTLINE OF THIS PROJECT

This thesis is part of the interdisciplinary research project "Bacterial biomarkers in Wadden Sea sediments" which involved a cooperation of the Organic Geochemistry Group and the Paleomicrobiology Group of the Institute of Chemistry and Biology of the Marine Environment (ICBM). The aim of this project was to study microbial communities in Wadden Sea sediments combining microbiological and chemical analytical techniques. As the main sampling location, a nearshore intertidal flat in the backbarrier area of Spiekeroog island close to the town of Neuharlingersiel was chosen (Fig. 2).

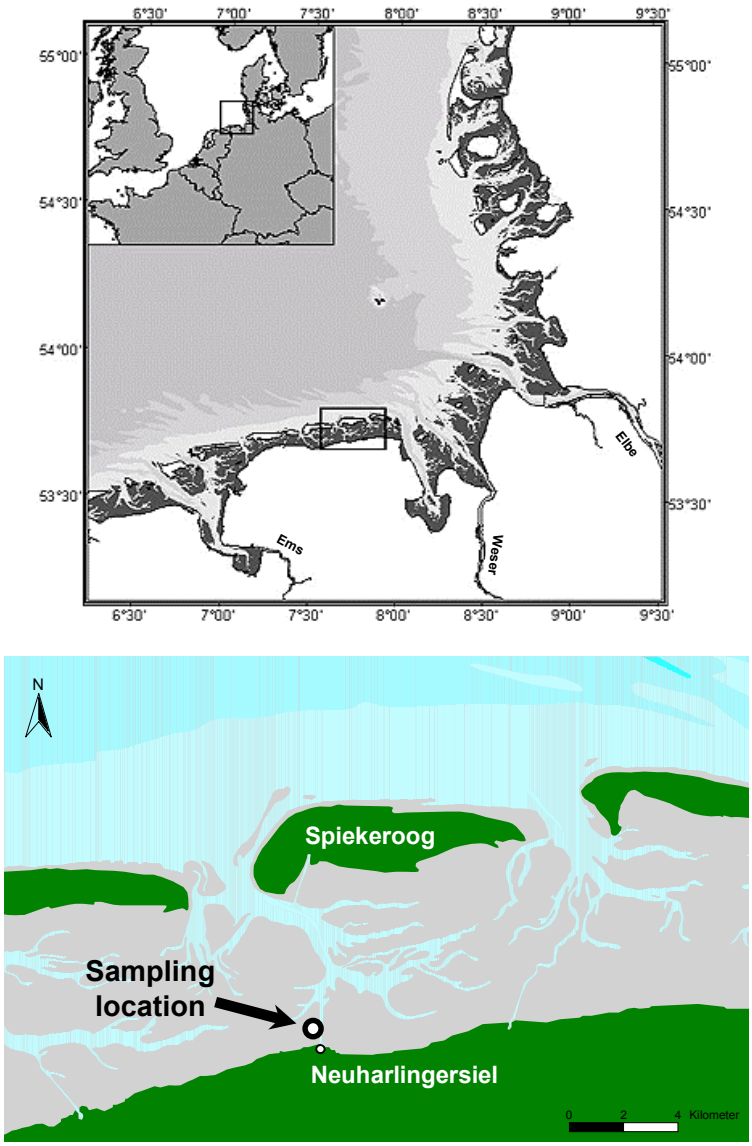


Fig. 2 Maps of German Bight (top) and sampling location in the backbarrier area of Spiekeroog island (bottom).

Special emphasis in the investigations was to be placed on the processes in the deeper parts of the sediment and the microbes involved, because most investigators had so far only investigated the uppermost sediment layers (<20 cm) (see 1.1). Therefore, the major question in this project was: "What's up down there ?" To answer this question, different microbiological techniques for community analysis had to be applied comprising activity measurements, cell counts and cultivation-based approaches as well as molecular techniques. These microbiological data were determined by Henrik Sass and Stefan Dröge.

To complement information about microbial communities and processes in the sediment obtained by microbiological methods, sediments needed to be characterised by

molecular and bulk geochemical parameters. This work was done by the author of this thesis, Heike Rütters. The main focus in this part of the project was to establish and apply phospholipids as biomarkers for viable microbial cells. So far, "phospholipid" fatty acid (PLFA) patterns had been used to study microbial community composition which usually involved extraction of the sample using a modified Bligh-Dyer procedure (Bligh and Dyer, 1959; Vancanneyt et al., 1996), separation of the sample extract in three fractions of different polarity (i.e. neutral lipids, glycolipids and polar lipids) and analyses of fatty acids liberated from each fraction after mild alkaline methanolysis (White et al., 1979; Guckert et al., 1985). By analysing solely fatty acids of the "phospholipid" fraction after methanolysis, only part of the information contained in the original phospholipids is obtained. Furthermore, "phospholipid" fractions from complex environments such as sediments may contain other fatty acid-substituted compounds of similar polarity, which may show a different fatty acid pattern, thereby altering the targeted phospholipid fatty acid pattern (Nielsen and Petersen, 2000; Aries et al., 2001). To circumvent this problem and to obtain maximum possible information about the source organisms, analysis of intact phospholipids from sediment samples appeared desirable.

Intact phospholipids can be analysed by ESI-MS. With the development of the ESI technique, coupling between liquid chromatography (LC) and mass spectrometry became feasible allowing for the analysis of more complex mixtures of compounds (Niessen, 1999). As LC-ESI-MS is a relatively new technique, no protocol existed to analyse intact phospholipids from complex environmental samples such as sediments. Phospholipids from pure cultures of bacteria can be analysed directly from the crude extracts, because phospholipids are the major compounds of such a lipid extract (Tornabene, 1985). Therefore, a protocol had to be developed in this thesis to analyse intact phospholipids from complex environmental samples using LC-MS.

Separate analysis of different glyceride classes (e.g. phospho- and glycolipids, tri-, di- and monoglycerides) and their corresponding fatty acid side-chains allows to gain information about the sources and the quality of the sedimentary organic matter, the microorganisms inhabiting the sediment and about degradation processes occurring (Parrish, 1988). Therefore, the "clean-up" step should not only include a purification of the phospholipids, but should also involve a separation of the sediment extract into various fatty acid-containing compound classes such as phospho- and glycolipids, triglycerides and their degradation products (di-, monoglycerides and free fatty acids).

For the molecular analysis of intact phospholipids, an HPLC separation had to be set up. Owing to the great diversity of different phospholipid molecular species that may be present in complex microbial communities, a column/eluent system was adopted from the literature (Karlsson et al., 1996) and optimised. In this system, phospholipid species are separated according to their headgroups irrespective of their side-chains, i.e. individual phospholipid species are separated in different phospholipid classes. Optimisation of the HPLC separation, firstly, involved adaptation of the literature method to a narrow-bore column to increase detection limits. Secondly, parameters for electrospray ionisation and for MS/MS experiments had to be optimised. The developed "clean-up" procedure and the HPLC-MS method are described in Chapter 2.1. In this paper, the applicability of the developed procedure to the analysis of phospholipids from bacteria and sediment extracts is shown. As an example, the top 11 cm of a sediment core taken from a sandy flat near the island of Mellum are investigated. For comparison, three strains of sulfate-reducing bacteria were analysed for their lipid composition. The author of this thesis, Heike Rütters, developed the clean-up procedure, optimised HPLC-MS and –MS/MS conditions and applied this method to bacteria and sediments. The sulfate-reducing bacteria were cultured by Henrik Sass.

Later on, the eluent system had to be changed due to problems caused by the ion-pairing agent triethylamine (see Chapter 2.2). Localisation and removal of the triethylamine contamination from the mass spectrometer and the HPLC system occupied Thomas Möhring, Jens Griep-Raming and Heike Rütters. The manuscript about the triethylamine contamination was written by H.R. As examples, a TIC chromatogram of an HPLC-MS analysis of a phospholipid standard mixture (analysed by H.R.) and a mass spectrum from the work of Jens Griep-Raming – including the triethylamine signal – are shown. For further analysis of intact phospholipids, triethylamine was replaced by ammonia (aqueous solution) and the HPLC gradient had to be adjusted by H.R. Additionally, for better quantitation, an evaporative light scattering detector was used parallel to the mass spectrometer. The "new" HPLC method is described in Chapter 4.

If phospholipids are not only to be used as a measure for the total viable biomass, but also to get qualitative information about the community composition, the specificity of phospholipid patterns for groups, genera or species of microorganisms has to be tested. To evaluate the specificity of phospholipid patterns, pure cultures of bacteria

were analysed for their phospholipid composition. Special emphasis was placed on the investigation of sulfate-reducing bacteria due to their important role in marine sediments (see 1.1). In Chapter 3.2, the phospholipid composition of 30 different strains of sulfate-reducing bacteria is compared to fatty acid patterns after whole-cell hydrolysis and the phylogenetic relatedness of the organisms. Sulfate-reducing bacteria were grown in cooperation with Henrik Sass, lipid analyses were performed by Heike Rütters. To evaluate the discriminatory potential of fatty acid and phospholipid patterns, lipid data were examined by cluster analysis (performed by H.R.). An example for the usefulness of fatty acid patterns for chemotaxonomic classification of new bacterial isolates can be found in Chapter 3.1. Here, isolation and characterisation of the new sulfate-reducing bacterium was done by Andrea Sass and coworkers, whereas fatty acid patterns were determined and discussed by Heike Rütters.

Analysis of intact phospholipids offers an additional advantage compared to the analysis of (polar lipid) fatty acids, because it allows also the detection of phospholipids with ether-linked side-chains. If only hydrolysis products of phospholipids are analysed, compound classes detected depend on the hydrolysis procedure employed. Usually hydrolysis procedures applied to polar lipids are optimised for cleavage of ester-linked fatty acids, but they may fail to liberate ether-linked side-chains. Until this thesis ether-linked lipids were only known from archaea (Kates, 1997) and thermophilic bacteria (e.g. Langworthy et al., 1983), although monoalkylether lipids had been detected in sediments from different temperate environments. In Chapter 3.3, the presence of ether phospholipids in two mesophilic sulfate reducers was shown by HPLC-MS analysis and confirmed by chemical degradation experiments (ether cleavage). This work was done by Heike Rütters, bacterial biomass was grown and prepared in cooperation with Henrik Sass.

Finally, a sediment core (0-55 cm) from an intertidal flat near Neuharlingersiel (Fig. 2) was investigated in more detail (Chapter 4). Here, various bulk geochemical parameters (TOC, TIC, TS, $\delta^{13}C$, $^{210}Pb_{xs}$) and pore water compositions had to be determined to characterise the "habitat". Microbial communities and the fate of their lipids were to be investigated by analysis of intact phospholipids and other types of glycerides. This work was done by Heike Rütters. Microbiological techniques were applied by Stefan Dröge and Henrik Sass. Some results of the diploma thesis of Stefan Dröge are compared to geochemical data in Chapter 5.

2 DEVELOPMENT OF A METHOD FOR THE ANALYSIS OF INTACT PHOSPHOLIPIDS FROM BACTERIA AND COMPLEX ENVIRONMENTAL MATRICES

2.1 PHOSPHOLIPID ANALYSIS AS A TOOL TO STUDY MICROBIAL COMMUNITIES*

Heike Rütters, Henrik Sass, Heribert Cypionka, Jürgen Rullkötter

2.1.1 ABSTRACT

To complement information on microbial communities in marine sediments that can be obtained using microbiological methods, we developed an analytical procedure to trace microbial lipids in environmental samples. We focused on analysing intact phospholipids as these membrane constituents are known to be biomarkers for viable cells. Analysis of intact phospholipids from a fractionated and preconcentrated sediment extract was achieved using liquid chromatography-electrospray ionisation/mass spectrometry (HPLC-ESI-MS). The combined analysis of phospholipid types and their fatty acid substituents allowed a differentiation between various groups of microorganisms living in the sediment. For comparison three strains of marine sulfate-reducing bacteria were analysed for their lipid content.

* Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2001. Phospholipid analysis as a tool to study microbial communities. Journal of Microbiological Methods, in press.

2.1.2 INTRODUCTION

Investigation of microbial community structures is one of the main issues currently under research by microbial ecologists (Atlas, 1984; Muyzer, 1998). For this aim different strategies are applied, such as isolation, cultivation and molecular techniques based on 16S rRNA sequences. All these methods yield valuable information, but none is free of biases or shortcomings (Bartscht et al., 1999, von Wintzingerode et al., 1997).

Another strategy to describe microbial communities is the analysis of polar lipid fatty acid (PLFA) patterns (Guezennec and Fiala-Medioni, 1996). Fatty acid patterns, determined after alkaline hydrolysis of whole cells, are also commonly used for chemotaxonomical classification of bacterial isolates (e.g. Asselineau and Asselineau, 1990). The fatty acids are mainly derived from membrane (phospho-)lipids and from lipopolysaccharides. Intact phospholipids are known to be hydrolysed within weeks after cell death (White et al., 1979; Harvey et al., 1986). Therefore, their presence in sediments is a good indicator for the existence of viable biomass. As the phospholipid content of bacterial cells is relatively constant over a wide range of growth conditions, quantitative analysis of phospholipids allows to estimate total viable biomass (White and Findlay, 1988). The amount of phospholipids is often assessed by the analysis of polar lipid fatty acids (PLFA) (e.g. Haack et al., 1994) or by measuring the "phospholipid phosphate" content (e.g. White et al., 1979).

The interpretation of variations in PLFA patterns, taken to indicate shifts in the relative proportions of different groups of bacteria, by different authors is often contradictory (e.g. Harvey and Macko, 1997). One reason for this may be the choice of single fatty acids as specific "biomarkers" for certain groups of bacteria. On the other hand, complete PLFA patterns were successfully used for bacterial classification because the result of PLFA cluster analysis closely matched that of 16S rRNA sequence similarities (Kohring et al., 1994).

A main problem for the application of PLFA patterns to sediment samples is the presence of many different fatty acid-bearing biomolecules, such as different types of glycerides of eukaryotic as well as prokaryotic origin and wax esters from higher land plant material (Eglinton and Hamilton, 1967; Volkman and Johns, 1977). Commonly, total sediment extracts are separated by column chromatography into fractions of different polarity. The fraction containing the most polar substances - including phospholipids - is transesterified, and the resulting fatty acid methyl esters are analysed

by gas chromatography (e.g. White et al., 1979). By analysing PLFA patterns, only part of the information contained in the phospholipids is used. Information on phospholipid types, i.e. their different headgroups, is lost.

Direct analysis of intact phospholipids is now possible due to the recent development of soft ionisation techniques and interfaces for coupling high performance liquid chromatography (HPLC) to mass spectrometry (MS) and has recently been applied to extracts of pure cultures of bacteria (Fang and Barcelona, 1998; Black et al., 1997).

In this paper we report a fractionation procedure for different types of glycerides to analyse complex mixtures of intact phospholipids by HPLC-ESI-MS in an extract of a surface sediment from a sandy tidal flat. For comparison, we analysed three different strains of sulfate-reducing bacteria (SRB) for their lipid content.

2.1.3 MATERIALS AND METHODS

Reagents and chemicals

Triolein and α,β-distearyl diglyceride were obtained from Fluka, Germany. All other standard substances (purity ≥98%) were purchased from Sigma-Aldrich, Germany. All solvents used for sample preparation were "residue analysis" grade (Scharlau, Spain). For HPLC analyses the following solvents were used: *i*-propanol (LiChrosolv, Merck, Germany), *n*-hexane (HPLC grade, Fluka, Germany); ultrapure water.

Sulfate-reducing bacteria

Pure cultures of marine strains of sulfate-reducing bacteria were obtained from our own culture collection: *Desulfococcus multivorans*^{T} (DSMZ 2059), *Desulfomicrobium* str. SAL (Sass and Cypionka, unpublished), *Desulfobacter postgatei* 2ac9^T (DSMZ 2034). Pure cultures were grown at 28°C using a mineral medium (Sass et al., 1992) with lactate (*Desulfococcus mulitvorans*, *Desulfomicrobium* str. SAL) or acetate (*Desulfobacter postgatei*) as carbon source. Cells were harvested at the end of their exponential growth phase by centrifugation, washed with saline phosphate buffer, stored at -20°C, and freeze-dried.

Sediment samples

A bulk sediment sample (top 0-11 cm) comprising both the thin oxic surface layer and part of the underlying anoxic section was taken in November 1997 on a tidal flat near the island of Mellum, NW Germany. These sandy tidal flats are covered with laminated microbial mats, known as "Farbstreifen-Sandwatt" (Hoffmann, 1942). The sediment was deep-frozen within 6 h after sampling and stored at -20°C. Afterwards, the sample was freeze-dried, sieved (<2 mm), and ground before extraction. The organic carbon content was determined as the difference between total carbon (determined by combustion in a Leco CS-444 instrument) and inorganic carbon (analysed in a UIC CO_2-coulometer) to be 0.21% of dry weight.

Extraction

The freeze-dried sediment (20 g) and the cell material (30-80 mg) were ultrasonically extracted using a modified Bligh-Dyer procedure (Vancanneyt et al., 1996) with a solvent mixture of methanol/dichloromethane/phosphate buffer (pH 7.4), 2:1:0.8 (v/v), for 10 min in centrifuge tubes, allowing for reextraction of residue after centrifugation and removal of supernatant. The supernatants of the first extraction and the following nine reextractions were collected in a separation funnel. Dichloromethane and phosphate buffer were added to achieve a ratio of methanol/dichloromethane/phosphate buffer of 1:1:0.9 (v/v). After phase separation, the organic phase was removed. The aqueous phase was reextracted five times with dichloromethane. The combined extracts were dried over anhydrous sodium sulfate, evaporated to dryness and stored at -20°C.

Column-chromatographic separation

To separate major compound classes in which fatty acids may occur in the extracts, chromatographic separation on a silica gel column (2 g silica 60, 63-200 µm, Merck, Germany, dried at 110°C for 16 h) was carried out combining the procedures described by Thiele (1979). Eight fractions were obtained by elution with the following solvents: 1) 10 ml *n*-hexane; 2) 25 ml *n*-hexane/diethylether 98:2 v/v; 3) 30 ml *n*-hexane/ diethylether 8:2 v/v; 4) 20 ml diethylether; 5) 10 ml 0.5 vol% acetic acid in diethylether; 6) 20 ml acetone/dichloromethane 1:1 v/v and 10 ml acetone/ dichloromethane 8:2 v/v;

7) 10 ml 5 vol% methanol in dichloromethane and 25 ml acetone; 8) 30 ml dichloromethane/methanol/water 5:4:0.4 v/v. All fractions were evaporated to dryness and stored at -20 °C.

Transesterification, gas chromatography (GC-FID), and gas chromatography-mass spectrometry (GC-MS) analysis

Aliquots of all fractions, except for fractions 1 and 5, were transesterified by mild alkaline hydrolysis as described by White et al. (1979). The methyl esters obtained were analysed by GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph, Hewlett Packard, Waldbronn, Germany) on a DB-5 column (30 m x 0.25 mm, 0.25 µm film thickness, J&W, Folsom, CA, U.S.A.) and by GC-MS using a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA, U.S.A.). Free fatty acids in fraction 5 were transformed into their trimethylsilyl (TMS) ester derivatives using N-Methyl-N-trimethylsilyltrifluoroacetamide (CS Chromatographie Service, Langerwehe, Germany). The TMS esters were analysed by GC-FID and GC-MS as described above. Double bond positions were tentatively assigned by comparison of retention times with those of a standard mixture (Bacterial Acid Methyl Esters CP Mix, Supelco, Bellefonte, PA, U.S.A.) and according to the relative elution order given by Nichols et al. (1989).

HPLC-MS and -MS/MS

Phospholipids were analysed on an HPLC instrument (Thermo Separation Products, San Jose, CA, USA) coupled to an ion trap mass spectrometer equipped with an electrospray source (Finnigan LCQ, Thermoquest-Finnigan, San Jose, CA, USA). HPLC separation was achieved on a diol phase (Lichrospher 100 Diol 5µ, Merck, Germany) using a 2x125 mm column. A flow rate of 0.30 ml/min was employed with the following solvent gradient: 1 min 100 % A, increasing over 20 min to 45 % A, 55 % B using a convex curvature, followed by 30 min of reconditioning; eluent A was a mixture of *n*-hexane/*i*-propanol/formic acid/triethylamine 79:20:0.6:0.08 v/v, B was *i*-propanol/ water/formic acid/triethylamine 88:10:0.6:0.08 v/v (Karlsson et al., 1996; Rütters et al., 2000; Chapter 2.2). The mass spectrometer was set to the negative ion mode with a spray voltage of -4.5 kV and a capillary temperature of 200 °C. MS/MS experiments

were done in the so-called dependent scan mode, i.e. the most intense quasi-molecular species of each full scan was automatically isolated and fragmented. For PC and PI, an octapole voltage of 25 and 30 V had to be applied to prevent adduct formation with eluent components. In MS/MS experiments, helium was used as a collision gas (relative collision energy: 50%). Conditions for MS/MS experiments were optimised to get maximum intensities of so-called lyso-fragments which result from the loss of one acyl group in the ion trap (see Table 3).

2.1.4 RESULTS AND DISCUSSION

Column chromatographic separation of lipid extracts

Since free and bound fatty acids in sediments comprise a very complex mixture of compounds of a wide structural diversity and, accordingly, of different polarities, a liquid chromatographic separation scheme on silica gel was developed which yields eight different fractions by elution with solvents or solvent mixtures of increasing polarity (Table 1).

Table 1 Examples of compound classes separated by column chromatography. Fatty acid-bearing standards used to define the fractionation scheme are in bold font.

Fraction	Solvent mixture	Compound classes
1	10 ml *n*-hexane	aliphatic hydrocarbons
2	25 ml *n*-hexane/diethylether 98:2 v/v	**wax esters (20:0)**, ketones, aldehydes, fatty acid methyl esters, aromatic hydrocarbons
3	30 ml *n*-hexane/diethylether 8:2 v/v	**triglycerides (18:1)**, triterpenoidal ketones, long-chain alcohols
4	20 ml diethylether	**diglycerides (18:0)**, triterpenoidal alcohols, *n*-alcohols
5	10 ml 0.5 vol% acetic acid in diethylether	**free fatty acids (22:1)**
6	20 ml acetone/dichloromethane 1:1 v/v + 10 ml acetone/ dichloromethane 8:2 v/v	**monoglycerides (14:0)**
7	10 ml 5 vol% methanol in dichloromethane + 25 ml acetone	**glycolipids (mainly 18:2)**
8	30 ml dichloromethane/methanol/water 5:4:0.4 v/v.	**phospholipids (mainly 16:0)**

Standard substances with fatty acids of different chain lengths were used to define the cuts between the fractions. Transesterification of the conjugated fatty acids was applied to analyse the fatty acids in each fraction as methyl esters by GC-MS. Particular emphasis was placed on the more polar fractions 3 to 8, i.e. the wax esters were not investigated in this study.

Lipids of sulfate-reducing bacteria

Extracts of sulfate-reducing bacteria (SRB) were dominated by phospholipids; 83-85% of the extractable lipids were recovered in the most polar fraction 8, the so-called "phospholipid fraction", which may, however, comprise other highly polar lipids as well. In all other glyceride fractions, only trace amounts of fatty acids were detected. Fatty acid distributions determined after transesterification of the phospholipid fractions, i.e. PLFA patterns, are displayed in Fig. 3. These PLFA patterns closely resembled fatty acid distributions which were obtained after whole-cell hydrolysis (data not shown).

Polar lipid fatty acid patterns of the three SRB strains investigated, each representing another genus, were significantly different from each other. *Desulfobacter postgatei* mainly contained saturated and unsaturated C_{16} fatty acids. Additionally, a *cyc*-17:0 fatty acid was present in high amounts. The so-called "*Desulfobacter* fatty acid", 10-Me-16:0, accounted for 15.4% of the total "phospholipid" fatty acids in this strain. In *Desulfomicrobium* str. SAL, a variety of saturated and unsaturated C_{16} and C_{18} compounds dominated the fatty acid pattern, whereas in *Desulfococcus mulitvorans*, branched C_{15} and C_{17} fatty acids (the latter including saturated and unsaturated acids) were the most abundant compounds, with the *anteiso*-form being always more abundant than the *iso*-isomer.

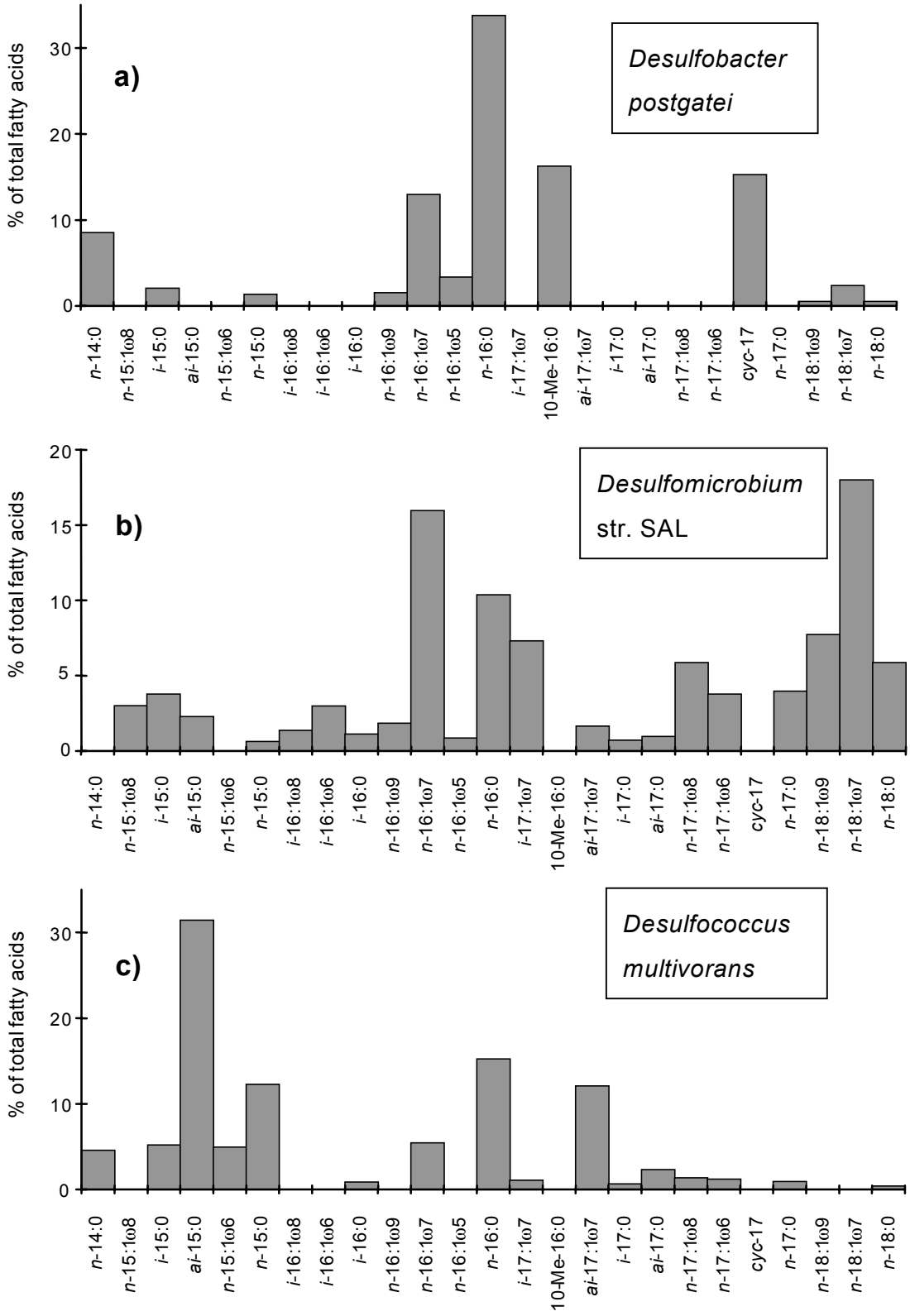


Fig. 3 Relative abundances of fatty acids in the phospholipid fractions (PLFA) of a) *Desulfobacter postgatei*, b) *Desulfomicrobium* str. SAL, c) *Desulfococcus multivorans.* (Fatty acids are denoted as x:y, with x indicating number of carbon atoms and y giving the number of double bonds; structural isomers are denoted by prefixes: *i* = *iso*, *ai* = anteiso, *cyc* = *cyclopropyl*; additional methyl groups are noted with their position.)

Lipids in tidal flat sediments

The distribution of major fatty acids in individual fractions of the tidal flat sediment extract, representing different types of glycerides, was considerably more complex than those of pure cultures of organisms (Table 2). Fraction 3 contained triglycerides and compounds of similar polarity. In marine microorganisms, triglycerides often form oily droplets used as storage compound or sometimes for buoyancy adjustment (Thiele, 1979; Wood, 1988). Some microalgae growing under nitrogen-deficient conditions are especially rich in triglycerides (Volkman et al., 1998). Fatty acids in the triglyceride fraction were abundant components of the investigated surface sediment, indicating a high contribution of eukaryotes and/or cyanobacteria to sediment biomass. The fatty acid pattern was dominated by $C_{16:0}$, $C_{16:1}$, $C_{18:2}$, $C_{18:1}$, $C_{18:0}$ acids, resembling the distribution often found in *Oscillatoria*-type cyanobacteria (Kenyon et al., 1972). Highly polyunsaturated fatty acids typical of eukaryotic algae, e.g. 20:5, 22:6, 18:3, 18:4 (Volkman et al., 1998), and cyanobacteria, such as 18:3, 18:4 (Kenyon et al., 1972), could not be detected. The lack of "algal fatty acids" is in agreement with the fact that the upper layers of the laminated microbial mats that cover sandy tidal flats around the island of Mellum are mainly formed by cyanobacteria, such as *Microcoleus* sp., *Oscillatoria* sp. and *Calothrix* sp., whereas algae are mostly absent (Stal, 1987). The absence of fatty acids typical for eukaryotes, e.g. polyunsaturated C_{16}, C_{18} and C_{20} acids (Erwin, 1973; Wood, 1988), indicated a low biomass of macro- and microfauna.

The "phospholipid fraction" (fraction 8), in addition to the fatty acids found in the triglyceride fraction, contained C_{17} branched fatty acids. Branched fatty acids are commonly considered to be of bacterial origin, e.g. from sulfate-reducing or sulfur-reducing bacteria (Kaneda, 1991). In contrast, purple sulfur bacteria (*Chromatiaceae*) only biosynthesise straight-chain even-carbon-numbered fatty acids such as 16:1, 16:0, 18:1 (18:0) (Imhoff and Bias-Imhoff, 1995). In the cyanobacterium *Anabaena variabilis* only C_{16} and C_{18} phospholipid fatty acids were found (Sato and Murata, 1982). Therefore, it can be concluded from the PLFA pattern in Table 2 that the sediment extract comprised phospholipids from different groups of viable microorganisms. However, it can not be ruled out from the analysis of PLFA, that other fatty acid-containing polar compounds contribute to the fatty acid pattern of this most polar fraction.

Table 2 Major fatty acids of different glycerides in sediment extract (most abundant fatty acids printed in bold).

Compound class	Fatty acids	Relative proportion	Possible origin
Triglycerides	*i*-14:0, *n*-14:0, *i-/ai*-15:0, *n*-15:1ω8, *n*-15:1ω6, *n*-15:0, *i*-16:0, ***n*-16:1ω7**, ***n*-16:0**, *n*-17:1ω8, *n*-17:0, 18:2, *n*-18:1ω9, *n*-18:1ω7, *n*-18:0, *n*-20:0	intermediate	storage compound of eukaryotes, e.g. algae, seeds; also in cyanobacteria, mycobacteria
Diglycerides	like triglycerides; no *n*-20:0	low	degradation of glycerides, indicate cell lysis
Free fatty acids	as above, but no 15:1, no 18:2; additionally three branched 17:0*, *i-/ai*-17:0, *n*-19:0 to *n*-28:0, most abundant: ***n*-16:0**	high	hydrolysis products from various sources
Monoglycerides	-	absent	degradation of glycerides
Glycolipids	*n*-14:0, *i-/ai*-15:0, *n*-15:0, *n*-16:1ω9, ***n*-16:1ω7**; ***n*-16:0**, *n*-18:1ω9, *n*-18:0	low	photosynthetic bacteria, algae**
Phospholipids	like triglycerides, additionally *i-/ai*-17:0, branched-17:1ω7; most abundant: ***n*-16:1ω7**, ***n*-16:0**, ***n*-18:1ω9**	intermediate	membranes of all (micro-)organisms

* Branched-17:0 isomers elute close to retention time of 10-Me-16:0.

** Glycolipids which can be found in archaea are often ether bound and can therefore not be detected by the analytical method applied.

The "glycolipid fraction" (fraction 7) of the sediment extract contained only small amounts of fatty acids. Glycolipids are found in all photosynthetic organisms known (Thiele, 1979). In some anoxygenic photothrophs such as *Chromatiaceae*, glycolipids are only a minor component of the cell's polar lipids (Imhoff and Bias-Imhoff, 1995), but the opposite applies to cyanobacteria (Smith, 1988). Thus, the low abundance of "glycolipid fatty acids" may indicate that photosynthetic organisms represent only a minor fraction of the overall biomass in this sediment sample.

Lysis products of different glycerides, such as mono- and diglycerides (Thiele, 1979), were only trace constituents of the extract. The fatty acid composition of the diglyceride fraction (fraction 4) resembles that of the triglyceride fraction. Diglycerides may be low in abundance because they either were not formed at all, or they were consumed too rapidly to accumulate in significant amounts. The same appears to apply to monoglycerides which could not be detected in fraction 6.

In fraction 5, high amounts of free fatty acids were recovered. They are considered hydrolysis products of various biomolecules. Their high abundance points toward intense breakdown of organic matter in this surface sediment. The free fatty acids include "typical bacterial" fatty acids as discussed above, e.g. branched short-chain (C_{15} and C_{17}) fatty acids, as well as long-chain (C_{20} – C_{30}) *n*-fatty acids with a strong even-over-odd carbon number predominance. Long-chain *n*-fatty acids in the sediment are probably derived either directly from higher land plant material - such as cuticular waxes - or from eroded peats (Lehtonen and Ketola, 1993), although even-numbered long-chain fatty acids have also been discovered in some soil bacteria (Režanka et al., 1991) and in *Desulfotomaculum* sp. (Režanka and Sokolov, 1990). The significant contribution of eroded peats to the sedimentary organic matter in the NW German tidal flats was shown by Volkman et al. (2000) using biomarker and stable carbon isotope analysis as well as radiocarbon dating of extractable lipid fractions.

Phospholipid analysis by HPLC-ESI-MS

High-performance liquid chromatographic conditions were optimised to separate most phospholipid types known to commonly occur in prokaryotes, such as phosphatidyl phosphatidic acid (PA), phosphatidyl glycerol (PG), diphosphatidyl glycerol (DPG), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl serine (PS), and phosphatidyl inositol (PI). When complex microbial communities are to be investigated, a good HPLC separation is necessary to distinguish between the various phospholipid types within a single run. Furthermore, in case of sediment extracts, HPLC helps to separate phospholipids from interfering compounds of similar polarity also present in fraction 8. Under the electrospray ionisation (ESI) conditions chosen, negatively charged quasi-molecular ions $[M-H]^-$ of the various phospholipids were obtained from the ionic species preformed in solution. Only in the case of PC, intensity of ionic species $[M+45]^-$ (adduct with formiate) or $[M-15]^-$ (resulting from the loss of a methyl group) was dependent on the octapole voltage. Structural information of phospholipids could be obtained from MS/MS experiments by collisionally induced dissociation of quasi-molecular ions generated in the electrospray process. The resulting cleavage products revealed the identity of phospholipid type and fatty acid substituents. Fragmentation patterns of standard substances are listed in Table 3.

Table 3 Typical mass spectral fragments of phospholipid standards formed in MS/MS experiments. In the first column, fatty acid combinations of standard substances are specified in parentheses; in the third column relative abundances of fragments normalised to the most intense ion (base peak) are given in parentheses.

Phospholipid	$[M-H]^-$	Fragments [m/z]		Possible fragment composition
Phosphatidic acid (16:0/16:0)	647	409	(13%)	lyso-PA (- ”16:0”)
		391	(100%)	PA - 16:0
		255	(1.2%)	16:0
Phosphatidyl glycerol (16:0/16:0)	721	483	(40%)	lyso-PG (- "16:0")
		465	(28%)	PG - 16:0
		391	(100%)	(PG - 16:0) - glycerol
		255	(11%)	16:0
Diphosphatidylglycerol (18:2/18:2)	1448	831	(15%)	DPG - "diglyceride"
		695	(100%)	DPG - "PG"
		415	(12%)	(DPG - "PG") - 18:2
Phosphatidyl ethanolamine (16:0/16:0)	690	452	(100%)	lyso-PE (- ”16:0”)
		255	(11%)	16:0
Phosphatidyl choline (16:0/16:0)	718 $[M-15]^-$	480	(100%)	lyso-"PC" (- "16:0")
		462	(10%)	"PC" - 16:0
		255	(1.2%)	16:0
Phosphatidyl serine (16:0/16:0)	734	647	(100%)	PS - serine
		391	(29%)	(PS - serine) - 16:0
		255	(0.6%)	16:0
Phosphatidyl inositol (16:0/18:2)	833	577	(14%)	PI - 16:0
		553	(86%)	PI - 18:2
		415	(8.3%)	(PI -16:0) - inositol
		391	(100%)	(PI -18:2) - inositol

Note: - 16:0 indicates loss of fatty acid side-chain with carboxyl group,
- "16:0" represents loss of acyl part of fatty acid only, i.e. "cleavage of ester bond".

Phospholipids in bacterial extracts

By HPLC-ESI-MS analysis of the bacterial extracts, similar phospholipid compositions were found for the three strains investigated. PE was the most abundant phospholipid type in each case. PG and DPG were present in smaller amounts, PG being more abundant than DPG in all strains. As an example, the total ion current (TIC) chromatogram of the phospholipid fraction of *Desulfobacter postgatei* is shown in Fig. 4 together with averaged mass spectra of its phosphatidyl ethanolamine components (Fig. 4b). Each quasi-molecular ion represents a PE species with a distinct fatty acid combination. PE was found to be also the major phospholipid type in two lacustrine sulfate reducers, *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris*, as well as in other Gram-negative bacteria (Wilkinson, 1988). Gram-positive bacteria, in

contrast, contain more PG and DPG and only minor amounts of PE, PI, PA (O'Leary and Wilkinson, 1988).

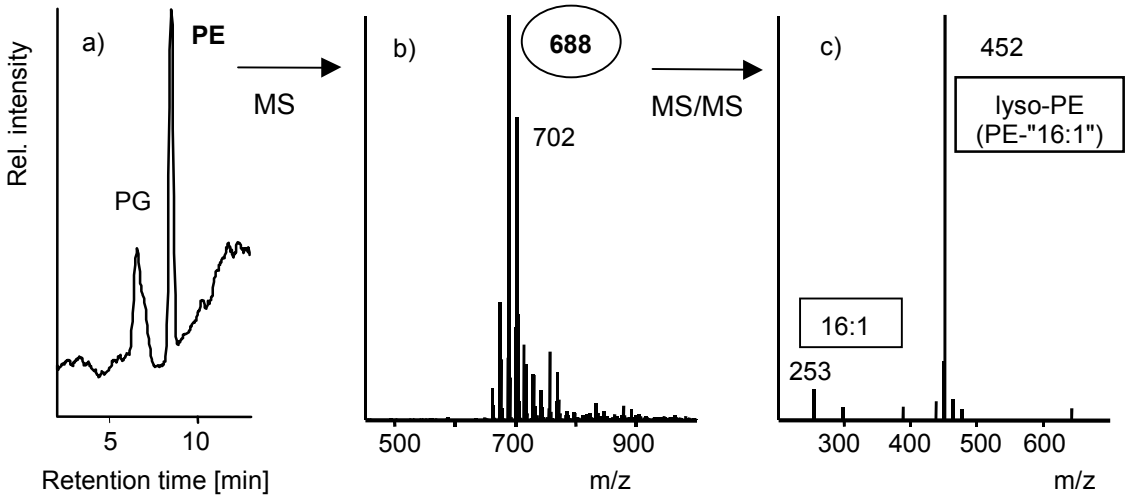


Fig. 4 ESI-HPLC-MS analysis of phospholipid fraction of *Desulfobacter postgatei*. a) Total ion current (TIC) chromatogram; b) averaged mass spectrum of phosphatidylethanolamine (PE) peak, c) collision-induced fragmentation (MS/MS experiment) of PE quasi-molecular ion m/z 688 indicating presence of 16:0 and 16:1 fatty acid substituents.

MS/MS experiments on the most intense quasi-molecular ions were performed to confirm the fatty acid combinations present. As an example, the corresponding fragmentation of PE in such an experiment is shown in Fig. 4c. For all investigated bacterial strains, fatty acid combinations of PE and PG are listed in Table 4. As DPG were not present in all strains, they were not included in this comparison. Fatty acids were tentatively identified by comparison with PLFA patterns after hydrolysis or, in case of the most intense peaks, assigned after MS/MS experiments. Often, only one of the fatty acid substituents could be seen in MS/MS data. Most likely, this signal resulted from cleavage of the fatty acid linked to the *sn*-2 position, because this acid is known to be lost preferentially (Lehmann, 1996).

Table 4 Comparison of fatty acid substituents in phosphatidyl glycerol (PG) and phosphatidyl ethanolamine (PE) of *Desulfobacter postgatei*, *Desulfomicrobium* str. SAL and *Desulfococcus multivorans* (most intense peaks [>80%] are underlined; fatty acids confirmed by MS/MS experiments are printed in bold letters).

Strain	PG	PE
Desulfobacter postgatei	16:1/16:0; 16:1/10-Me-16:0; 16:0/**10-Me-16:0**; *cyc*-17/10-Me-16:0; cyc-17/18:0	14:0/16:1; 15:0/16:1; **16:1**/16:0; 16:1/*cyc*-17; **16:0**/cyc-17; *cyc*-17/*cyc*-17; *cyc*-17/18:1
Desulfomicrobium str. SAL	15:0/16:0; 16:0/16:1; **17:1**/16:0; 16:1/18:1; 16:0/18:1; 18:0/17:1; 18:0/17:0	15:0/16:0; 16:0/16:1; 16:1/17:1; 16:1/17:0; **16:1**/18:1; 17:1/18:1
Desulfococcus multivorans	15:0/15:0; 15:0/16:0; 16:0/16:1; 17:1/16:0	14:0/15:0; 14:0/16:1; 15:0/15:0; 15:0/16:1; 15:0/16:0; 16:0/16:1; 16:0/17:1; 17:1/17:1

A comparison of the PE and PG fatty acid patterns of the three bacterial strains investigated, rendered the following main results:

- Both phospholipid types are mostly substituted with two different fatty acids, one of which is often unsaturated or branched.
- The most abundant fatty acid combinations comprise mainly those fatty acids which also dominate the "PLFA patterns" after hydrolysis.
- Substitution patterns of PG and PE are different, probably reflecting different functions of the two phospholipid types in the cell membrane. There is no clear trend of the variation between PG and PE substitution patterns among the different strains.

Intact phospholipids in sediment extracts

The TIC chromatogram of the surface sediment sample investigated (Fig. 5) showed two major broad peaks besides several smaller ones. From retention times and m/z values of quasi-molecular ions, it was concluded that mainly four different types of phospholipids were present, namely PG and DPG in the first chromatographic peak and PE and PC in the second one, all with a range of different fatty acid substituents. The great diversity of fatty acid chain lengths caused broadening of chromatographic peaks. For PE and PC, coeluting unknown substances further complicated structure

assignment. Mass chromatograms of representative quasi-molecular ions of the four identified phospholipid types and of the unknown compounds are also shown in Fig. 5.

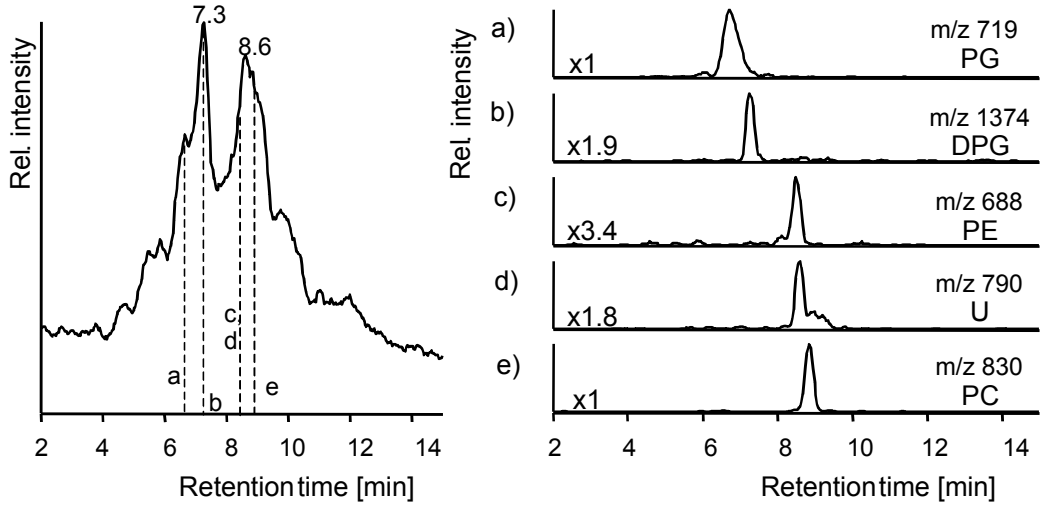


Fig. 5 HPLC-ESI-MS analysis of tidal flat sediment (0-11 cm) extract: Total ion current (TIC) chromatogram (left) and normalised mass chromatograms (right) representing a) PG with a fatty acid combination of 16:0/16:1 (m/z 719), b) DPG with 2x16:0/16:1/18:1 (m/z 1373), c) PE with 16:0/16:1 (m/z 688), d) Unknowns (U) (m/z 790), and e) PC with 18:1/18:1 (m/z 830). Relative abundances can be deduced from enhancement factors.

Mass spectra of the major phospholipid types (Fig. 6) illustrated the dominance of fatty acid substituents with a chain length between C_{15} and C_{18}. PC in addition contained fatty acids with chain lengths $>C_{18}$. In contrast to PG and DPG, averaged mass spectra of PE and PC showed no obvious series of homologues, but several "subsets" representing PE, PC, and coeluting unknown compounds.

From the low relative abundance of PE, which was found to be the dominant phospholipid type in the investigated strains of SRB and also in many other Gram-negative bacteria (Wilkinson, 1988), we conclude that sulfate-reducing bacteria contribute only little to the microbial biomass in the top 11 cm of the tidal flat sediment studied. PG and DPG could derive from a variety of microorganisms. Gram-positive bacteria have PG and DPG as their major phospholipid types (O'Leary and Wilkinson, 1988). PG is the only phospholipid type found in cyanobacteria (Wood, 1988) whereas in most algal families DPG and PG as well as PC, PE, PI are present (Lechevalier and Lechevalier, 1988). PC and PI are often abundant in eukaryotic microorganisms such as ciliates. In *Tetrahymena pyriformis*, besides PC and PE a phosphonolipid analogue of PE was discovered (Wood, 1988). A eukaryotic origin of PC is further supported by

long-chain fatty acid substituents with multiple unsaturations among the PC components of the sediment extract.

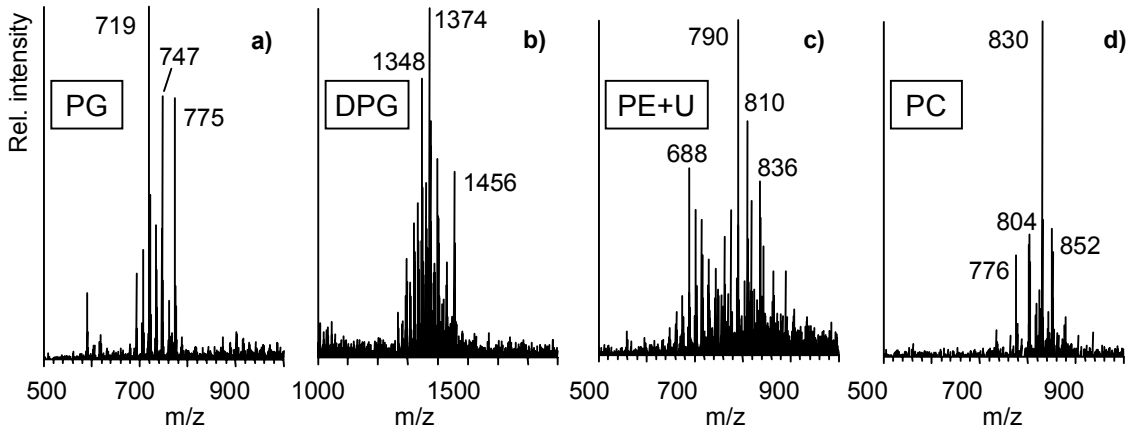


Fig. 6 Averaged mass spectra of four major HPLC-TIC peaks of different phospholipid types in tidal flat sediment extract: a) PG (retention time (RT): 6.2-6.9 min), b) DPG (RT: 7.1-7.4 min), c) PE and unknowns (RT: 8.4-8.7 min), d) PC (RT: 8.8-9.2 min).

2.1.5 CONCLUSIONS

The analytical procedure developed provides a powerful tool to study intact phospholipids in sediment samples as well as fatty acid distributions in other lipid classes. Combining the findings from lipid fatty acid patterns and phospholipid analysis we conclude that different groups of microorganisms contributed to the microbial community in the tidal flat sediment. By applying microbiological techniques together with phospholipid analysis, we hope to improve the correlation between phospholipids in sediments and their source organisms. This will allow a better qualitative and quantitative investigation of sediment microbial communities.

2.1.6 ACKNOWLEDGEMENTS

This project was funded by Deutsche Forschungsgemeinschaft (DFG), grant no. Ru 458/21. H.R. gratefully acknowledges a scholarship by Oldenburgische Landesbank.

2.2 A PERSISTENT MEMORY EFFECT OF TRIETHYLAMINE IN THE ANALYSIS OF PHOSPHOLIPIDS*

Heike Rütters, Thomas Möhring, Jürgen Rullkötter,
Jens Griep-Raming, Jürgen O. Metzger

To the Editor-in-Chief

Sir,

Triethylamine (TEA) as an eluent modifier for high performance liquid chromatography (HPLC) applications is chosen by chromatographers as an ion pair reagent or as a buffer component of intermediate polarity (Meyer, 1992). There are examples in the literature that prove its capability to support ionisation of various compound classes in electrospray ionisation mass spectrometry (ESI-MS) (DeJohn, 1997).

Following Karlsson et al. (1996), we developed an HPLC/ESI-MS and -MS/MS method for the separation and identification of phospholipids from purified bacterial and sediment extracts. Chromatographic separation was carried out on a Thermo Separation Products (TSP, San Jose, CA, USA) HPLC instrument coupled to a Finnigan LCQ ion trap mass spectrometer (Thermoquest-Finnigan, San Jose, CA, USA). Phospholipids were separated according to their headgroups on a LiChrospher 100 Diol 5µ HPLC column (125x2mm; Merck, Darmstadt, Germany) using a convex gradient. Eluents contained a formic acid/TEA buffer (0.6/0.08% v/v, respectively). Measurements in the negative ion mode (spray voltage: -4.5 kV, capillary temperature: 200°C) exclusively yielded $[M-H]^-$ ions. Details of the procedure will be described elsewhere. Figure 7 shows an example of the effective separation and the mass spectra resulting from a single run.

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* Rütters, H., Möhring, T., Rullkötter, J., Griep-Raming, J., Metzger, J.O., 2000. The persistent memory effect of triethylamine in the analysis of phospholipids. Rapid Communications in Mass Spectrometry, 14, 122-123.

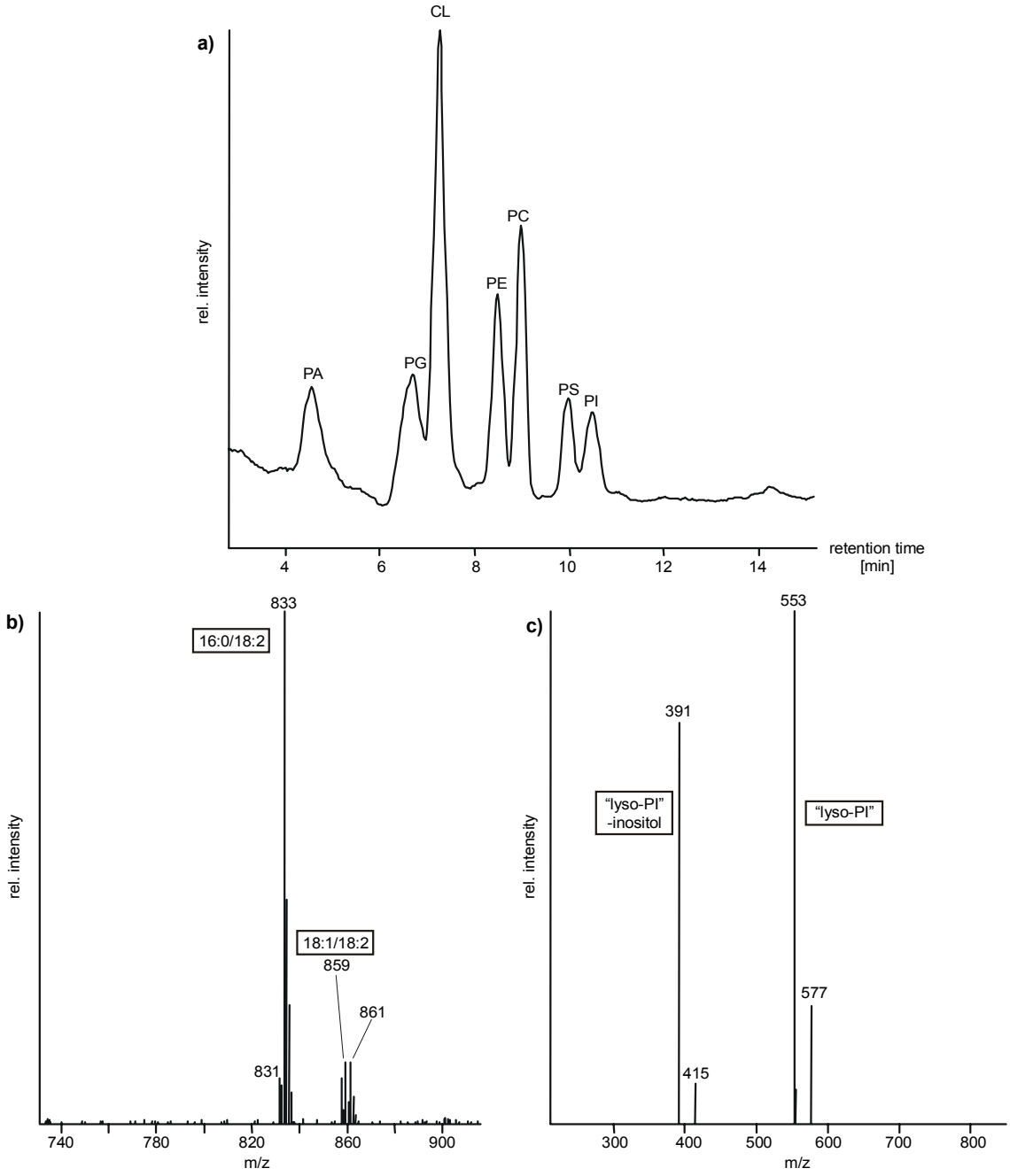

Fig. 7 a) TIC chromatogram of PL standards: phosphatidic acid (PA), phosphatidyl glycerol (PG), cardiolipin (CL), phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl inositol (PI), all Sigma-Aldrich, Germany ; b) quasi-molecular ions of PI (from soybean) showing different fatty acid combinations (fatty acids are denoted as x:y, with x giving the number of carbon atoms and y representing the number of double bonds, c) MS/MS of m/z 833.

Problems arose when the same instrument was used for measurements in the positive ion mode, especially in the low molecular weight range (m/z 50-650). Such analyses were virtually made impossible by a very intense signal at m/z 102, $[M+H]^+$ of triethylamine. Direct infusion of pure solvents by the syringe pump resulted in a

strongly dominant signal of TEA with intensities ranging from 10^6 to 10^7 counts during 10 milliseconds of ion collection time, i.e. close to the maximum ion intensity during normal operation of the instrument. The intensity was independent of the kind of solvent supplied. The problems did not only concern suppression of ionisation of other analytes but also severely increased detection limits, because an ion trap collects only a limited number of ions and cannot exclude a single m/z species and so it was nearly entirely filled with only triethylammonium ions. Therefore, detection of other compounds from diluted solutions, i.e. in the normal ESI concentration range of approx. 1 µg/ml, was strongly hindered. Only at concentrations as high as 100 µg/ml certain compounds could still be detected. Figure 8, as an example, shows the ESI mass spectrum of 2-(9-decenyl)-5-methyl-1,3,4-oxadiazol (positive ion mode, spray voltage: +4.5 kV, solvent supply: 10 µL/min, concentration: ca. 100 µg/ml). Even under these conditions the TEA peak is still significant. However, for many compound classes, measurements at these concentration ranges are not feasible because of preferential formation of adducts and "oligomers", or due to limited sample material.

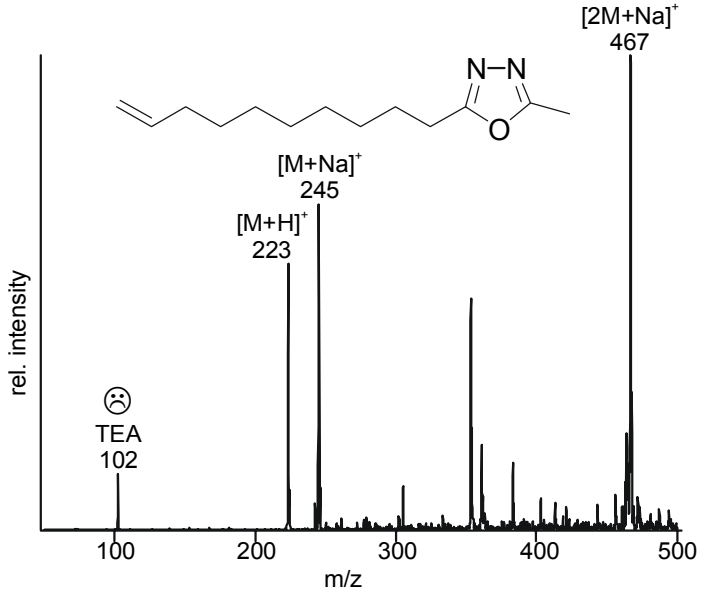

Fig. 8 ESI mass spectrum of 2-(9-decenyl)-5-methyl-1,3,4-oxadiazol (ca. 100 µg/ml in methanol).

As the vacuum unit of the LCQ instrument is not designed for baking, other procedures had to be employed to remove the contaminant (TEA). Even several cleaning cycles of ESI source, heated capillary, API stack and the ion optics using various solvents and replacement of a number of mass spectrometer parts, tentatively identified as the most contaminated ones, did not significantly reduce the intensity of the TEA signal.

Cleaning procedures recommended by the instrument manufacturer (Thermoquest, personal communication) or other researchers working with TEA (DeJohn, personal communication), i.e. sonication with formic acid/acetonitrile 50/50 v/v, did not improve the instrument performance. Finally, we disassembled the whole vacuum unit and all attached parts of the instrument, had all the metal pieces (ion optics, trap, vacuum manifold etc.) cleaned by an MS manufacturer (MassTech, Bremen, Germany) and replaced all teflon, PEEK and other plastic components (including analyser mount, tube lens and skimmer mount, all sealings, tubings etc.). This ultimately removed the TEA contamination to a level below the detection limit.

From this experience we conclude that TEA was strongly adsorbed on the surfaces of the vacuum manifold and parts therein. Ionisation may have occurred by proton transfer from solvent ions generated by the ESI process to desorbed TEA molecules.

We strongly recommend refraining from the use of triethylamine in ion-trap LC-MS analysis, and considering alternative eluent modifiers, if the instrument is ever to be used for the analysis of low-molecular weight substances in the positive ion mode.

3 CHEMOTAXONOMIC CHARACTERISTICS OF BACTERIA

3.1 A SULFATE-REDUCING BACTERIUM FROM A DEEP-SEA SEDIMENT GROWING ON MONO- AND DISACCHARIDES, *DESULFOBULBUS MEDITERRANEUS* SP. NOV.*

Andrea Sass, Heike Rütters, Heribert Cypionka, Henrik Sass

3.1.1 ABSTRACT

A sulfate-reducing bacterium, strain 86FS1, was isolated from a dilution culture inoculated with deep-sea sediment from the continental slope in the western Mediterranean Sea off the coast of Spain. Cells were ovoid, Gram negative and motile. Strain 86FS1 exhibited b- and c-type cytochromes. The organism was able to utilise propionate, pyruvate, lactate, succinate, fumarate, malate, alanine and straight chain alcohols (C_2-C_5) as electron donors for the reduction of sulfate, sulfite or thiosulfate. Major product of carbon metabolism was acetate, with exception that alcohols were oxidised only to the corresponding acid. Fermentative growth on mono- and disaccharides (glucose, fructose, sucrose, cellobiose), glutamate, lactate or pyruvate could also be observed. With lactate and sulfate as growth substrate a growth yield of 7.9 g dry mass/mol lactate was achieved. Growth was observed from 10 °C to 30 °C and NaCl concentrations from 10 to 70 g/l. Strain 86FS1 could respire with oxygen but was highly sensitive to oxygen during growth. After four to twenty hours of aeration with 60 µmol/l O_2 no viable cells could be detected. Sulfide concentrations above 4 mmol/l inhibited growth. The fatty acid pattern of strain 86FS1 resembled those of *Desulfobulbus propionicus* with *n*-14:0, *n*-16:1ω7, *n*-16:1ω5, *n*-17:1ω6 and *n*-18:1ω7 as dominant fatty acids. Due to its phylogenetic position and its phenotypic properties strain 86FS1 could be affiliated to the genus *Desulfobulbus* and is described as a new species, *Desulfobulbus mediterraneus* sp. nov.

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* Sass, A., Rütters, H., Cypionka, H., Sass, H., 2001. A sulfate-reducing bacterium from a deep-sea sediment growing on mono- and disaccharides, *Desulfobulbus mediterraneus* sp. nov. Archives of Microbiology, submitted.

3.1.2 INTRODUCTION

Hitherto most cultures of sulfate-reducing bacteria have been isolated from freshwater or coastal marine habitats, exceptions are those from deep subsurface habitats (Barnes et al., 1998; Motamedi and Pedersen, 1998). However, by molecular techniques several sequences probably derived from sulfate-reducing bacteria were detected in deep-sea sediments (Li et al., 1999; Bidle et al., 1999; Boetius et al., 2000). These sequences could be assigned to members of the families *Desulfobacteriaceae* and *Desulfobulbusaceae*. Many organisms of these two phylogenetic groups have a wide substrate spectrum, including fatty acids or aromatic compounds (Widdel, 1988). However, none of them has been proven to utilise carbohydrates. Among sulfate-reducing bacteria only some *Desulfovibrio* sp. and *Desulfotomaculum* sp. (e.g. Akagi and Jackson, 1967; Ollivier et al., 1988; Zellner et al., 1989; Trinkerl et al., 1990) have been found to grow on monosaccharides.

In the present study we describe a new carbohydrate-utilising sulfate-reducing bacterium which was isolated from sediments from the continental slope off the coast of Spain. This organism grew on mono- and disaccharides. Due to its 16S rDNA sequence and phenotypic traits it could be assigned to the genus *Desulfobulbus*, and is described as *Desulfobulbus mediterraneus* sp. nov.

3.1.3 MATERIALS AND METHODS

Source of organisms

Sediment was sampled from the continental slope off the coast of NW Spain at 41°12.36'N 2°50.02'E during cruise M40/4 of the R/V Meteor to the Mediterranean Sea in February 1998. Undisturbed sediment cores were taken using a multicorer from a water depth of 1268 m. Temperature of the bottom water at this site was about 15°C. The pH of the sediment ranged from 8.2 at the sediment surface to 7.6 in 2 cm depth. Free sulfide could not be detected by needle electrodes along the upper 5 cm. Oxygen penetrated up to 30 mm into the sediment with half air saturation at 7 mm depth.

Desulfobulbus propionicus (DSM 2032) was taken from our own culture collection, while *Desulforhopalus vacuolatus* (DSM 9700) was purchased from the

Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

Enrichment, isolation and cultivation

The upper 2 cm of a sediment core were removed with a sterile spatula and sediment from 2 cm to 4 cm depth was taken by means of sterile syringes which the tips were cut off. The sediment was diluted in the artificial seawater medium described below under anoxic conditions. Growth in the enrichment cultures was followed by microscopy. Strain 86FS1 was isolated from the highest positive dilution step (10^{-3}).

The medium used contained (g/l): NaCl, 24.32; $MgCl_2 \cdot 6H_2O$, 10.0; $CaCl_2 \cdot 2H_2O$, 1.5; KCl, 0.66; Na_2SO_4, 4.0; HEPES, 2.38. The following constituents were added from stock solutions (final concentrations in mol/l): KBr, 0.84; H_3BO_3, 0.4; $SrCl_2$, 0.15; NH_4Cl, 0.4; KH_2PO_4, 0.04; NaF, 0.07. Per litre of medium 1 ml trace element solution SL10 and 0.2 ml of a selenite-tungstate solution (Widdel and Bak, 1992) were added. The pH was set to 7.0 to 7.2 by addition of NaOH and autoclaved. In a separate screw cap bottle 0.2 g $NaHCO_3$ were added to 10 ml of distilled water. The bottle was tightly closed and autoclaved. After autoclaving the medium was cooled under an atmosphere of N_2 (50 hPa), the bicarbonate solution and 10 ml/l of a vitamin solution (Balch et al. 1979) were added. Finally the medium was reduced by the addition of a sterile Na_2S solution to a final concentration of 0.5 mmol/l and the pH adjusted to 7.0 to 7.2 if necessary.

For enrichments 5 mmol/l sodium lactate was added to the medium as electron and carbon source. The enrichment cultures were incubated for 5 weeks at 20°C. For obtaining pure cultures the deep agar dilution method of Widdel and Pfennig (1984).

Desulfobulbus propionicus and *Desulforhopalus vacuolatus* were grown with lactate as electron donor in a freshwater medium and a brackish water medium, respectively (Widdel and Bak, 1992). *Db. propionicus* and *Drh. vacuolatus* were incubated at 25°C and 10°C, respectively.

Morphology and pigments

For phase contrast micrographs the agar slide technique of Pfennig and Wagener (1986) was used. Cell dimensions were determined using a Leitz DMRB microscope (Wetzlar, Germany) equipped with a digital image analysis system (H & K, Berlin, Germany). Flagellae were stained according to Heimbrook et al. (1989). Gram staining was performed after a standard procedure (Gerhardt et al., 1994). Cytochromes were analysed on a spectrophotometer (Lambda 2S spectrophotometer, Perkin Elmer, Überlingen, Germany) in spectra of cell free extracts obtained by use of a French press and subsequent centrifugation. The G + C content of the DNA was determined by HPLC analysis (Mesbah et al., 1989) at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Physiological tests

Substrate utilisation was studied in completely filled screw cap bottles. Growth tests with hydrogen as electron donor were carried out in 100 ml serum bottles filled with 20 ml of the same medium and a gas phase consisting of H_2/CO_2 (80/20, v/v). In positive disproportionation assays the formation of sulfate and sulfide was verified as described by Tabatabai (1974) and Cline (1969). Growth rates were determined in batch cultures by monitoring the increase of turbidity using a turbidimeter (Hach, Loveland, Co).

Determination of growth yields

Cultures were incubated in 1 l screw cap flasks. Due to the high sulfate concentration in the artificial sea water medium growth was limited by the electron donor in all experiments. Cells were harvested at the end of the exponential phase, washed twice with carbonate-free PBS buffer (130 mmol/l NaCl, 10 mmol/l sodium phosphate buffer pH 7.2) and freeze-dried (Sigma 2-4, Christ, Osterode, Germany). The total carbon contents of freeze-dried samples were determined by combustion using an elemental analyser (EA 1108, Carlo Erba, Rodano, Italy). The amount of electron donor utilised was calculated from the sulfide concentration formed during growth. The bacterial dry

mass was calculated from carbon contents assuming an overall composition of cellular biomass of $C_4H_7O_3$ (Widdel and Pfennig, 1981).

Aerobic respiration and oxygen sensitivity

Capacity for aerobic respiration was investigated with washed cells in a multielectrode chamber (Cypionka 1994). Survival of exposure to oxic conditions was tested in 500 ml screw cap bottles were filled with 100 ml medium and sealed with a gas tight rubber septum. The assays were inoculated with freshly grown culture to a density of about 10^6 cells/ml. The headspace of the flasks was purged with a mixture of air, nitrogen and carbon dioxide (25/55/20%), resulting in an oxygen concentration of 5% in the headspace (approximately 60 µmol/l O_2 in the medium). The flasks were shaken during the whole experiment. Four parallels were analysed, two with (1% w/v) and two without sediment particles, respectively. Samples were taken by means of sterile syringes through the rubber septa. Viable counts were determined by the MPN method in microtiter plates, which were inoculated and incubated in an anaerobic chamber.

Analyses of fatty acids

For alkaline hydrolysis, freeze-dried cells (ca. 20 mg) were refluxed over night with 5 ml KOH solution (5 % KOH·$H_2$0 grade "Suprapur", Merck, Darmstadt, Germany, in methanol/water 4:1 v/v) under a nitrogen atmosphere. Afterwards the alkaline solution was transferred to a separation funnel and acidified using hydrochloric acid (2 mol/l). Non-saponifiable remnants were extracted three times with 10 ml dichloromethane each. Dichloromethane extracts were added to the methanol/water-phase in the separation funnel. After phase separation the organic phase was removed and the aqueous phase was reextracted three times with 10 ml dichloromethane each. Combined organic phases were dried over sodium sulfate. After removal of the solvent, aliquots of samples were transformed into their trimethylsilyl (TMS) ester derivatives using N-Methyl-N-trimethylsilyl-trifluoroacetamide (CS Chromatographie Service, Langer-wehe, Germany). The TMS derivatives of fatty acids were analysed by GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph, Hewlett Packard, Waldbronn, Germany) on a DB-5HT column (30 m x 0.25 mm, 0.1 µm film thickness, J&W,

Folsom, CA, U.S.A.) and by GC-MS using a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA, U.S.A.). Double bond positions of monounsaturated fatty acids were confirmed by preparation of dimethyldisulfide adducts (Dunkelblum et al., 1985) and GC-MS analysis using the conditions described above.

Isolation of nucleic acids, PCR and sequencing

Extraction of nucleic acids and amplification of the bacterial 16S rDNA was performed as described by Overmann and Tuschak (1997), using the Bacteria-specific primers 8f and 1492r. The QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) was used to purify the amplification products from nucleotides, primers and Taq polymerase. Sequencing was performed as described previously (Overmann and Tuschak, 1997) using the sequiTherm Long-Read Kit (Epicentre, Madison, Wis., USA) and an automated infrared laser fluorescence sequencer (Li-Cor Model 4000 DNA sequencer).

Phylogenetic analysis

Highest similarities of the novel 16S rDNA sequence of strain 86FS1 were searched using the SIMILARITY RANK tool of the Ribosomal Database Project (Maidak et al., 2000) and the BLAST tool of GenBank (Altschul et al., 1997). For phylogenetic analyses all available total 16S rRNA sequences belonging to the *Desulfobulbusaceae* were used as well as some representative sequences belonging to the *Desulfobacteraceae* and the *Desulfovibrionaceae*. The dataset contained the 16S rRNA sequences (GenBank accession numbers in brackets) of strain 86FS1, *Desulfobulbus* strain BG25 (U85473), "*Desulfobulbus marinus*" 3pr10 (M4411), *Desulfobulbus elongatus* DSM2908^T (X95180), *Desulfobulbus* strain R-PropA1 (AJ012591), *Desulfobulbus propionicus* DSM2032^T (M34410), *Desulfobulbus rhabdoformis* M16^T (U12253), *Desulfotalea arctica* LSv54^T (AF099061), *Desulfotalea psychrophila* LSv54^T (AF099062), *Desulfocapsa sulfoexigens* SB164P1^T (Y13672), *Desulfocapsa thiozymogenes* DSM7269^T (X95181), *Desulfofustis glycolicus* PerGlys^T (X99707), *Desulforhopalus vacuolatus* ltk10^T (L42613), *Desulforhopalus singaporensis* T1^T (AF118453), *Desulfococcus multivorans* DSM2059^T (M34405), *Desulfobotulus*

sapovorans DSM 2055^T (M34402), *Desulfobacter postgatei* DSM684^T (M26633), *Desulfovibrio gigas* ATCC19364^T (M34400), *Desulfovibrio desulfuricans* CSN (AF354664), *Escherichia coli* ATCC11775^T (X80725), delta proteobacterium LSv53 (AJ241014), and the uncultured proteobacteria BD1-2 (AB015515), JTB20 (AB015241), Sva0631 (AJ241011), Sva1036 (AJ140990), Sva1041 (AJ240984) and Sva0103 (AJ240978).

The sequences were aligned with the ClustalW program (Thompson et al., 1994). The phylogenetic tree was based on DNA fragments of 1397 Bp which could unambiguously aligned. For phylogenetic calculation the maximum likelihood program (DNAml) of the PHYLIP 3.57c package (Felsenstein, 1993) was used. Nucleotide positions that differed in more than 50% of all sequences were excluded from the analysis.

The 16S rDNA sequence of strain 86FS1 is available under the accession number AF354663 at GenBank.

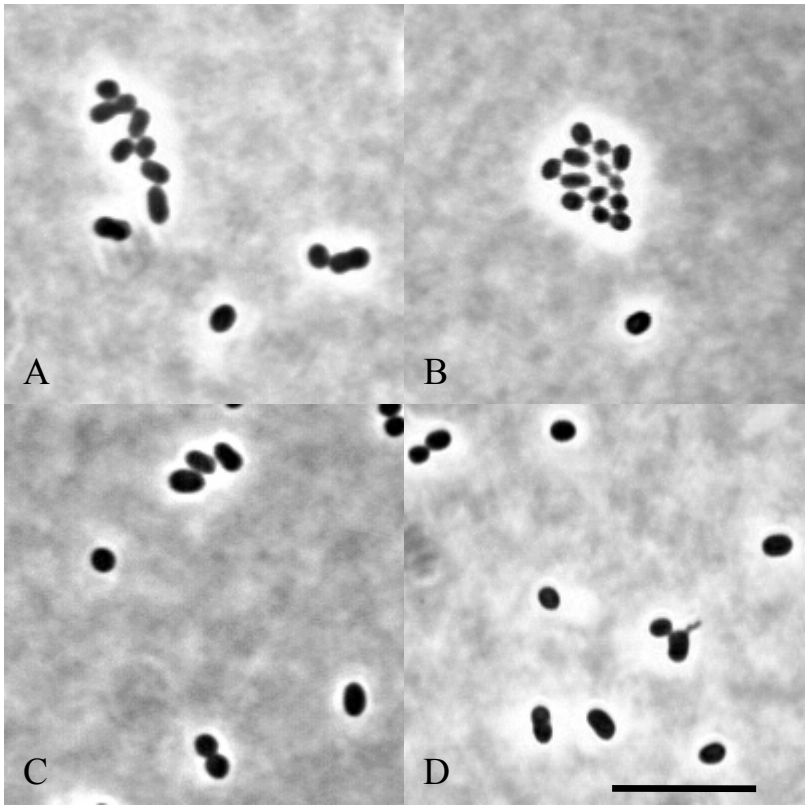

Fig. 9 Phase contrast micrographs of strain 86FS1, bar = 10 µm.

3.1.4 RESULTS

Morphology and physiology

Cells of strain 86FS1 were oval-shaped with rounded ends (Fig. 9) measuring 1.2-1.7 x 1.4-3.2 µm (width x length). Cells of strain 86FS1 stained Gram-negative and were motile by a single or a tuft of up to three flagella inserted in terminal to subterminal position. In cultures at the end of the exponential growth phase dark inclusions, probably storage compounds, could frequently be observed. The sulfite reductase desulfoviridin was not found. In cell-free extracts b- and c-type cytochromes could be detected. The G+C content of the DNA of strain 86FS1 was 58.6 mol%.

The isolate utilised pyruvate, lactate, malate, fumarate, succinate, alanine, glutamate, straight chain alcohols (C_2-C_5). From the carbohydrates tested glucose, fructose, sucrose and cellobiose supported growth (Table 5). Hydrogen was not used as electron donor. Incomplete oxidation of electron donors was deduced from the amount of sulfide formed during growth on lactate. From the amount of sulfide formed (and the typical smell) it was also concluded that *n*-propanol, *n*-butanol and *n*-pentanol were oxidised only to propionate, butyrate and valerate, respectively. Growth with carbohydrates and glutamate was mainly fermentative, only up to 0.5 mmol/l sulfide were formed. Lactate and pyruvate, but not malate or fumarate, were also fermented in the absence of sulfate. Sulfate, sulfite, and thiosulfate were used as electron acceptors. Elemental sulfur and nitrate were not utilised. Autotrophic growth was not observed.

The growth yield obtained with strain 86FS1 (Table 6) with sulfate and lactate or propionate was 7.5 g dry mass/mol electron donor if grown at 20°C. These values are in the range as observed for *Desulforhopalus vacuolatus* (7.5 g dry mass/mol lactate; Isaksen and Teske 1996), but slightly higher than for *Desulfobulbus rhabdoformis* (4.6 g dry mass/mol lactate; Lien et al., 1998) or *Desulfobulbus propionicus* (5.5 g dry mass/mol propionate; Widdel and Bak, 1992).

Strain 86FS1 grew in the range from 15°C to 35°C. Fastest growth was observed at 25°C ($\mu = 0.77\ d^{-1}$) while the growth yield was more or less constant between 10°C and 30°C. The salinity range for growth was found to be 10 to 70 g/l NaCl, with optimal growth at 20 g/l NaCl. Strain 86FS1 did not grow at pH values below 6.3 and above 8.0.

Table 5 Physiological properties of strain 86FS1 and other members of the genus *Desulfobulbus*.

	Strain 86FS1	*Desulfobulbus propionicus*[1)]	*Desulfobulbus elongatus*[2)]	*Desulfobulbus rhabdoformis*[3)]
Motility	+	-	+	-
Temperature range	10-30°C	10-43°C	20-40°C	10-40°C
pH range	6.3-8.0	6.0-8.6	6.0-7.8	n.a.[4)]
mol % G+C	58.6	59.9	59.0	50.6
Electron donors				
H_2	-	+	+	+
Formate	-	-	-	-
Acetate	-	-	-	-
Propionate	+	+	+	+
Pyruvate	+	+	+	+
Lactate	+	+	+	+
Succinate	+	-	-	
Fumarate	+	-	-	+
Malate	+	-	-	+
Ethanol	+	+	+	+
n-Propanol	+	+	+	+
n-Butanol	+	n.a.	-	n.a.
n-Pentanol	(+)	n.a.	n.a.	n.a.
Glucose	+	-	-	n.a.
Fructose	(+)	-	-	n.a.
Cellobiose	+	-	n.a.	n.a.
Sucrose	(+)	n.a.	n.a.	n.a.
Alanine	+	-	n.a.	n.a.
Glutamate	(+)	n.a.	n.a.	n.a.
Electron acceptors				
Sulfate	+	+	+	+
Thiosulfate	+	+	+	+
Sulfite	+	+	+	+
Nitrate	-	+	-	-

[1)] data from Widdel and Pfennig (1982); [2)] data from Samain et al. (1984); [3)] data from Lien et al. (1998); [4)] data not available.

The following substrates were tested but did not support growth of strain 86FS1:

H_2, formate, acetate, fatty acids (C_4-C_9), crotonate, isobutyrate, 2-methyl butyrate, 3-methyl butyrate, glycolate, citrate, tartrate, malonate, 2-ketoglutarate, methanol,

2-propanol, ethylene glycol, 1,2-propanediol, 1,2-butanediol, 2,3-butandiol, glycerol, tween80, acetone, arabinose, xylose, mannose, rhamnose, trehalose, maltose, glucosamine, gluconate, cellulose, asparagine, cyteine, glutamine, glycine, isoleucine, phenylalanine, proline, serine, tryptophane, benzoate, salicylate, nicotinate, choline, betaine, cholate.

Table 6 Growth yield of strain 86FS1 during growth on different substrates. Values are given as g dry mass/mol substrate oxidised.

Substrate	Lactate	Propionate	Ethanol	Propanol	Succinate	Glucose
Growth yield	7.5	7.5	8.1	6.0	11.8	8.3

Maintenance

Strain 86FS1 was transferred at least every four weeks. Longer intervals lead to loss of the culture. Fresh inoculated cultures could sometimes show a lag phase of up to three to four weeks. Long-term preservation by deep-freezing did not work. Strain was sensitive to sulfide and dithionite. At sulfide concentrations above 4 mmol/l growth ceased. On the other hand, strain 86FS1 was sensitive to oxidised conditions and was not able to reestablish reducing conditions.

Aerobic respiration and oxygen tolerance

Strain 86FS1 was able to reduce oxygen with lactate and sulfide. In spite of this the strain showed a high sensitivity towards oxygen. In oxygen exposure experiments without particles no viable cells could be detected already after four hours. Cells survived longer if sediment particles were present. In these assays no decrease of viable counts was detected after four hours, while after 18 h no viable cell were found.

Fatty acid patterns

In strain 86FS1 the following fatty acids were found to dominate: *n*-14:0, *n*-16:1ω7, *n*-16:1ω5, *n*-17:1ω6 and *n*-18:1ω7 (Table 7). *Desulfobulbus propionicus* (this study) and *Desulfobulbus rhabdoformis* (Lien et al., 1998) showed a similar fatty acid pattern

after whole-cell hydrolysis. *Desulforhopalus vacuolatus*, as a close relative to the genus *Desulfobulbus*, contained similar fatty acids, but was clearly distinct in having *n*-16:1ω5 as its most abundant fatty acid with smaller contributions of *n*-16:1ω7 and *n*-17:1ω6 with traces of *n*-14:0 and *n*-18:1ω7. The fatty acid patterns of the *Desulfobulbus* sp. determined in this study are in good agreement with previous work (Parkes and Calder, 1985, Kohring et al., 1994).

Table 7 Relative abundances of fatty acids in strain 86FS1 and related species of sulfate-reducing bacteria (given as % of total fatty acids).

	Strain 86FS1	*Desulfobulbus propionicus*	*Desulfobulbus rhabdoformis*[1)]	*Desulforhopalus vacuolatus*
n-14:1ω5	0.5			
n-14:0	11.4	10.2	9.4	0.7
n-15:1ω8	0.9			
n-15:1ω6	2.4	2.3		2.2
n-15:1ω6t	0.5			
n-15:0	1.9	7.7	13.9	1.6
i-16:1ω6				0.2
i-16:0		0.8		
n-16:1ω7	27.1	10.0	7.9	17.1
n-16:1ω7t	3.5			
n-16:1ω5	9.9	24.4	4.4	51.6
n-16:0	3.0	4.9		10.6
n-17:1ω8	1.1			
n-17:1ω6	11.1	23.7	24.1	11.6
n-18:1ω9		0.6		
n-18:1ω7	26.6	14.5	24.5	0.6
n-18:1ω5				3.2

[1)] data from Lien et al. (1998)

Fatty acids are denoted as x:y, with x indicating the total number of carbon atoms and y giving the number of double bonds. Position of double bond is defined by the number of carbon atoms from the alkyl end of the fatty acid and denoted as e.g. ω7. Geometry of double bonds is usually cis unless indicated otherwise (t for trans). Chain branching is indicated by the prefixes *i*- and *ai*- for branching at the ω-1 and ω-2 position, respectively.

Phylogenetic position

Sequence analysis of the 16S rDNA clearly confirmed the assignment of strain 86FS1 to the genus *Desulfobulbus*. The closest relatives were *Desulfobulbus* strain BG25 (97.0 % similarity), "*Desulfobulbus marinus*" 3pr10 (96.0 % similarity) and *Desulfobulbus rhabdoformis* (95.9 % similarity). A maximum likelihood tree is shown in Fig. 10.

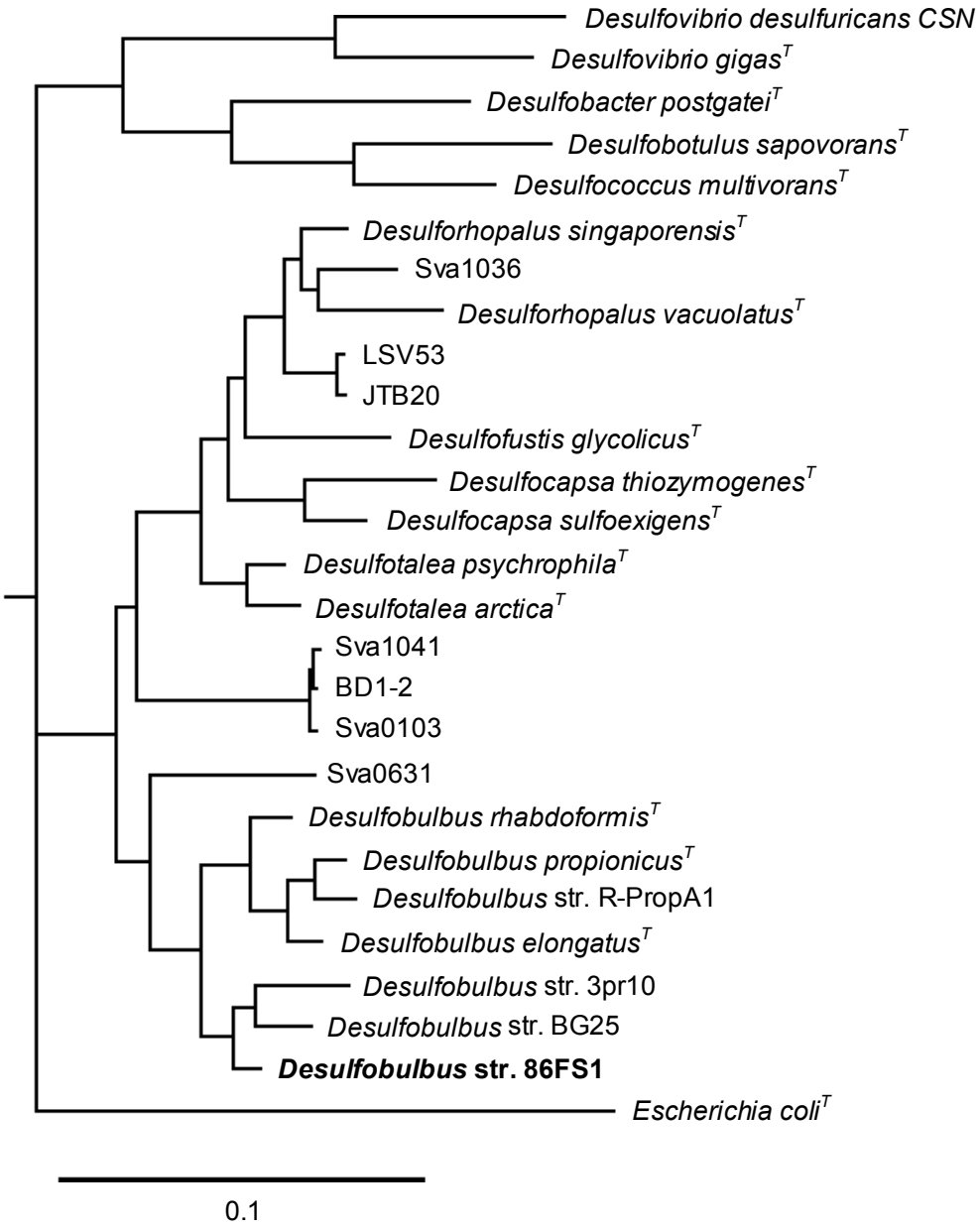

Fig. 10 Phylogenetic position of strain 86FS1 within the δ-Proteobacteria. Maximum likelihood tree based on 1397 alignment positions. The bar represents 0.1 changes per nucleotide position. *Escherichia coli* was used as outgroup.

3.1.5 DISCUSSION

Sulfate-reducing bacteria are ubiquitous in marine sediments (Widdel, 1988). In the present work we describe the physiology and phylogeny of a new sulfate-reducing bacterium isolated from a deep-sea sediment in the western Mediterranean Sea.

A striking feature of strain 86FS1 is its ability of carbohydrate utilisation. Hitherto only a few *Desulfovibrio* species (Ollivier et al., 1988; Trinkerl et al., 1990; Zellner et al., 1989; Reichenbecher and Schink, 1997; Nielsen et al., 1999) as well as two *Desulfotomaculum* species (Akagi and Jackson, 1967; Daumas et al., 1988) are known to grow on sugars. Strain 86FS1 is the first organisms from the *Desulfobulbus* clade growing on carbohydrates and the first sulfate reducer shown to grow on disaccharides. However, strain 86FS1 differs from the other SRB growing on glucose in that it is only fermenting glucose and that only traces of sulfide were formed. Nevertheless, it is obvious that the ability to degrade glucose is probably widespread among sulfate reducers. Polyglucose was detected as a storage polymer in *Desulfovibrio* sp. and *Desulfobulbus propionicus* (Stams et al., 1983). However, the latter does not grow with glucose (Widdel and Pfennig, 1982). One possible reason might be the lack of an uptake system for glucose in these strains.

In deep-sea sediments the material left over by aerobes and reaching the deeper sediment layers is generally more refractory than in coastal sediments. For anaerobic bacteria there are two possibilities to cope with this unfavourable conditions. Moving upwards in the sediments could give access to more easily degradable substrates, but at the risk of oxygen contact. The other opportunity would mean staying down in the anoxic layers, and being adapted to low nutrient conditions. However, there are indications that the active microbial populations are not restricted to the oxic layers at all. Tholosan and Bianchi (1998) investigated a number of surface sediments, some of them at water depths comparable to that strain 86FS1 was isolated from. For total cell counts, potential glutamate mineralisation rates, potential proteolytic activities, they found only minor differences along the uppermost 7 cm. Since our strain was oxygen sensitive, it appears to be more adapted to the conditions at or below the chemocline. Another aspect which points to life in deep-sea sediments where no free sulfide is detected, is the high sensitivity towards sulfide. Sulfide formation here is low and sulfide formed can be scavenged by iron. In coastal marine sediments or microbial mats sulfide concentrations above 1 mmol/l can be exceeded. Since strain 86FS1 was

completely inhibited by sulfide concentrations of 4 mmol/l it is likely that also lower values can decrease viability. Other sulfate-reducing bacteria are inhibited by higher sulfide concentrations. As an example Desulfobulbus propionicus has a 50 % reduced growth rate (IC 50) at neutral pH and a total sulfide concentrations of 7 mmol/l (O'Flaherty et al., 1998) and *Desulfovibrio inopinatus* even survives values above 20 mmol/l (Reichenbecher and Schink, 1997).

Not much information about the types of sulfate-reducing bacteria inhabiting deep-sea sediments are available. Culture-based approaches yielded mainly *Desulfovibrio* sp. (Elsgaard et al., 1995, Barnes et al., 1998) while molecular techniques found mainly sequences affiliating to the families *Desulfobacteraceae* and *Desulfobulbusaceae* (Li et al., 1999; Bidle et al., 1999; Boetius et al., 2000). The molecular techniques detected a variety of sequences phylogenetically distinct from each other with no counterparts in culture collections. From this data it is obvious that there is a large diversity of SRB in deep-sea sediments still to be discovered.

Strain 86FS1 could be affiliated to the genus *Desulfobulbus* by several phenotypical traits (Table 5, Table 7) as well as by its 16S rDNA sequence. Its phylogentic distances to other described species of this genus are in the range of 5 % to 6 % sequence similarity. However, species are defined on the basis of DNA relatedness (Wayne et al., 1987). Stackebrandt and Goebel (1994) adapted DNA-DNA homology values to 16S rDNA similarity values and found a threshold value of about 97 % sequence similarity for the species level. Together with phenotypical features like growth on sugars or dicarboxylic acids, which are missing in other members of the genus, the proposal of strain 86FS1 as a new species, *Desulfobulbus mediterraneus* is justified.

Description of *Desulfobulbus mediterraneus* sp. nov.

Desulfobulbus mediterraneus (me.di.terra'ne.us. M.L. adj. *mediterraneus* from the Mediterranean Sea, pertaining to the habitat the organism was isolated from). Ovoid cells, 1.2-1.7 µm wide and 1.4-3.2 µm long. Gram-negative. Motile. One to three flagella are terminal to subterminal inserted. The G + C content of the DNA is 58.6 mol%. Temperature range from 10°C to 35°C with optimum temperature at 25°C. Growth was observed between pH 6.3 to 8.0 and from 10 to 70 g/l NaCl. Substrates

used for anaerobic respiration are pyruvate, lactate, propionate, malate, succinate, fumarate, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, alanine. Hydrogen does not support growth. Pyruvate, lactate, glucose, fructose, cellobiose, sucrose and glutamate can be fermented. Sulfate, sulfite, and thiosulfate serve as electron acceptors. Oxygen can be used as electron acceptor. Does not grow in presence of oxygen. Catalase activity is present. Vitamins are not required, but promote growth.

Strain 86FS1 is designated as type strain of the species. Strain 86FS1 is deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) under the number DSM 13871.

3.1.6 ACKNOWLEDGEMENTS

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3.2 BIOMARKERS OF SULFATE-REDUCING BACTERIA - COMPARISON BETWEEN FATTY ACID PATTERNS, INTACT PHOSPHOLIPID PROFILES AND 16S rDNA SEQUENCE SIMILARITIES

Heike Rütters, Jürgen Rullkötter, Heribert Cypionka, Henrik Sass [*]

3.2.1 ABSTRACT

In this study, 30 strains of mesophilic sulfate-reducing bacteria were analysed for their fatty acid and phospholipid composition. Strains were selected to represent major phylogenetic branches of δ-proteobacteria, two spore-forming sulfate reducers were included for comparison. The discriminating power of fatty acid patterns was compared to that of phospholipid types and phospholipid molecular species. Most non-sporulating sulfate reducers contained very similar classes of phospholipids, i.e. phosphatidyl ethanolamine, phosphatidyl glycerol and diphosphatidyl glycerol. In *Desulfosarcina variabilis*, *Desulfonema limicola* and *Desulforhabdus amnigenus* additionally phosphatidyl cholines were present. Phospholipid patterns of the two spore-forming bacteria were very distinct. Cluster analysis revealed that phospholipid molecular species followed the phylogenetic relatedness of the sulfate reducers. Therefore, intact phospholipids may be useful biomarkers to trace sulfate-reducing bacteria in complex microbial communities from environmental samples.

[*] Rütters, H., Rullkötter, J., Cypionka, H., Sass, H., 2001. Biomarkers of sulfate-reducing bacteria - comparison between fatty acid patterns, intact phospholipid profiles and 16S rDNA sequence similarities. Microbiology U.K., submitted.

3.2.2 INTRODUCTION

Sulfate-reducing bacteria catalyse the last step in the oxidation of organic matter in natural habitats like sediments or soils (Jørgensen, 1982). They also have a strong economic impact, causing souring of oil reservoirs and biocorrosion (Aeckersberg et al., 1998; Hamilton, 1998). Furthermore, they play a key role in the mobilisation and methylation of heavy metals (Pak and Bartha, 1998), and they also occur in the intestines of various organisms (Gibson et al., 1993; Dzierzewicz et al., 1996). Different approaches have been used to estimate the diversity and relative contribution of sulfate-reducing bacteria to microbial communities. These approaches include cultivation-based methods (Laanbroek and Pfennig, 1981; Sass et al., 1997; Wieringa et al., 2000), molecular techniques (Amann et al., 1992; Minz et al., 1999; Sahm et al., 1999) or biomarker analysis (Dowling et al., 1988; Parkes et al., 1993). Each of these approaches has its advantages but also its limitations (Haack et al., 1994; Amann et al., 1995; von Wintzingerode et al., 1997).

Whole-cell fatty acid or polar lipid fatty acid patterns have been applied for chemotaxonomic classification of clinical bacterial isolates at species or even at subspecies level (Thompson et al., 1993). However, for the use of fatty acids as biomarkers to trace distinct groups of bacteria in mixed communities and complex environments, the specificity of these biomarkers has to be tested carefully. Validation of fatty acid biomarkers usually involves two steps: Firstly, bacteria of interest have to be isolated and analysed for their lipid composition to search for biomarker candidates; secondly, the abundance of biomarker candidates in the original sample and after stimulation/repression of the target organisms is determined (Dowling et al., 1988; Ringelberg et al., 1989). Unfortunately, fatty acids unique to certain phylogenetic or metabolic groups of bacteria are rare, and there is often a great overlap in the (polar lipid) fatty acid composition amongst different species. Therefore, in many cases, fatty acid patterns of environmental samples can only be used as total community profiles, i.e. to distinguish between different microbial assemblages or to follow variations in community structure with changing environmental conditions (Haack et al., 1994; White et al., 1996; Zelles, 1999; Hedrick et al., 2000). However, correlation between substrate utilisation and fatty acid patterns of the organisms involved can be greatly improved by use of ^{13}C- or ^{14}C-labelled compounds (Boschker et al., 1998; Roslev et al., 1998).

The information on microbial community composition can be greatly enhanced when polar lipid fatty acid (PLFA) instead of total fatty acid patterns are analysed, because PLFA derive only from intact cells, thereby allowing to differentiate viable and dead/refractory biomass (White et al., 1979; White et al., 1997). As sedimentary organic matter is a very complex mixture of material from various sources, these "polar lipids" may comprise "phospholipid-related" membrane compounds such as sphingolipids or lipo-amino acids (White et al., 1997), but also other substances of similar polarity. Recently, Aries et al. (2001) showed that the polar lipid fraction of a sediment sample comprised phosphate-free compounds which exhibited a fatty acid composition different from the phospholipids.

One possibility to exclude the interference of non-membrane polar compounds with the PLFA pattern is the direct analysis of intact phospholipids using a combination of high-pressure liquid chromatography and mass spectrometry (Lytle et al., 2000; Rütters et al., 2001a, Chapter 2.1). This approach is advantageous to PLFA analysis as not only the information about the fatty acids present is obtained, but also about combinations of fatty acids in the different types of phospholipids. Fang et al. (2000a) showed that the analysis of intact phospholipids had a higher discriminating potential for different *Pseudomonas* spp. than PLFA patterns alone.

In the present work we investigated the discriminating power of intact phospholipids for 30 strains of sulfate-reducing bacteria, representing major phylogenetic branches with different metabolic capacities, and compared these results to fatty acid patterns and the phylogenetic relatedness of the sulfate reducers.

3.2.3 MATERIALS AND METHODS

Source and Cultivation of Organisms

Pure cultures of known sulfate-reducing bacteria were obtained from our own culture collection or purchased from the DSMZ (Table 8). Two new isolates, strain D1 and strain SFA4, were obtained from the highest positive dilutions of most probable number series inoculated with sediment of intertidal mudflats near Dangast and Neuharlingersiel (Eastfrisian Wadden Sea, Northwest Germany), respectively. Detailed descriptions of these strains will be published elsewhere.

Strains were grown as described in the references or as recommended by the DSMZ (Table 8). Strains D1 and SFA4 were grown in the medium described by Sass et al. (2001; Chapter 3.1). Growth temperatures, electron and carbon sources are given in Table 8. For all strains sulfate was used as the electron acceptor except for *Desulfovibrio desulfuricans* CSN which was grown with nitrate. Cells were harvested at the end of their exponential growth phase by centrifugation, washed with phosphate buffer (pH 7) of appropriate salinity, freeze-dried and stored at -20°C.

Whole-cell hydrolysis, gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS)

For determination of whole-cell fatty acid pattern cells were saponified as described by Sass et al. (2001, Chapter 3.1). In brief, freeze-dried cells (ca. 20 mg) were refluxed over night with methanolic KOH solution. Following acidification, fatty acids were extracted with dichloromethane. After removal of the solvent, aliquots of extracted fatty acids were transformed into their trimethylsilyl (TMS) ester derivatives using N-methyl-N-trimethylsilyl-trifluoroacetamide (CS Chromatographie Service, Langerwehe, Germany). The TMS derivatives of fatty acids were analysed by GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph, Hewlett Packard, Waldbronn, Germany) on a DB-5HT column (30 m x 0.25 mm, 0.1 μm film thickness, J&W, Folsom, CA, U.S.A.) and by GC-MS using a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA, U.S.A.). Positions of double bonds were confirmed using dimethyldisulfide addition as described by Dunkelblum et al. (1985).

Extraction and clean-up, transesterification

Freeze-dried samples were extracted by a modified Bligh-Dyer method using a solvent mixture of methanol/dichloromethane/phosphate buffer (pH 7.4), 2:1:0.8 (v/v). Different lipid classes were separated by column chromatography on silica gel (details given by Rütters et al., 2001a, Chapter 2.1). Phospholipids eluted in the most polar fraction. Aliquots of polar lipid fractions were transesterified by mild alkaline hydrolysis as described by White et al. (1979). The methyl esters obtained were analysed by GC-FID and GC-MS as described above.

Table 8 Strains of sulfate-reducing bacteria used in this study. T_{inc}: Incubation temperature. .

Strain	Reference	Origin	$T_{inc.}$ [°C]	Electron donor
Desulfobacter postgatei^T	DSM 2034	Brackish water	28	Acetate
Desulfobacter hydrogenophilus^T	DSM 3380	Marine mud	28	Acetate
Desulfobacterium autotrophicum^T	DSM 3382	Marine mud	28	Lactate
Desulfosarcina variabilis^T	DSM 2060	Marine mud	28	Benzoate
Desulfococcus multivorans^T	DSM 2059	Sewage treatment plant	28	Lactate
Desulfonema limicola^T	DSM 2076	Estuarine mud flat	28	Succinate, acetate
"Desulfobotulus sapovorans"	DSM 2055	Freshwater mud	28	Butyrate
Desulfobulbus propionicus^T	DSM 2032	Freshwater ditch	28	Lactate
Desulfobulbus strain 86FS1	Sass et al., 2001	Deep-sea sediment	20	Lactate
Desulforhopalus vacuolatus^T	DSM 9700	Brackish sediment	10	Lactate
Desulfotalea strain SFA4	Dröge and Sass, unpublished	Intertidal sediment	20	Propionate, butyrate
"*Desulfoarculus baarsii*"	DSM 2075	Freshwater mud	28	Butyrate
Desulforhabdus amnigenus^T	DSM 10338	Granular sludge	37	Propionate
SRB strain STP23	Sass et al., 1997	Freshwater sediment	28	Lactate
Desulfomicrobium baculatum	DSM 1742	Freshwater pond	28	Lactate
Desulfomicrobium strain STL8	Sass et al., 1997	Freshwater sediment	28	Lactate
Desulfomicrobium apsheronum Ac1.2	Wieringa et al., 2000	Intertidal sandflat	30	Lactate
Desulfomicrobium apsheronum Ac3.2	Wieringa et al., 2000	Intertidal sandflat	30	Lactate
Desulfomicrobium apsheronum Be2.2	Wieringa et al., 2000	Intertidal sandflat	30	Lactate

Table 8 continued.

Strain	Reference	Origin	$T_{inc.}$ [°C]	Electron donor
Desulfomicrobium apsheronum Bu4.1	Wieringa et al., 2000	Intertidal sandflat	30	Lactate
Desulfovibrio oxyclinae^{T}	DSM 11498	Hypersaline mat	28	Lactate
Desulfovibrio desulfuricans CSN	DSM 9104	Sewage treatment plant	28	Lactate
Desulfovibrio vulgaris^{T}	DSM 644	Soil	28	Lactate
Desulfovibrio acrylicus^{T}	DSM 10141	Intertidal sediment	28	Lactate
Desulfovibrio strain D1	Sass and Sass, unpublished	Intertidal sediment	10	Lactate
Desulfovibrio strain Pr1.2	Wieringa et al., 2000	Intertidal sandflat	30	Lactate
Desulfovibrio strain Ac5.2	Wieringa et al., 2000	Intertidal sandflat	30	Lactate
SRB strain La4.1	Wieringa et al., 2000	Intertidal sandflat	30	Lactate
Desulfosporosinus strain STP12	Sass et al., 1997	Freshwater sediment	28	Lactate
Spore-forming SRB strain STP3	Sass et al., 1997	Freshwater sediment	28	Lactate

HPLC-MS and -MS/MS

Phospholipids were analysed on an HPLC instrument (Thermo Separation Products, San Jose, CA, USA) coupled to an ion trap mass spectrometer equipped with an electrospray source (Finnigan LCQ, Thermoquest-Finnigan, San Jose, CA, USA) and to an evaporative light scattering detector (ELSD 500, Alltech, Deerfield, IL, USA) using a flow-splitter (split ratio 1:0.88, with the larger flow feeding the ELSD). HPLC separation was achieved on a diol phase (Lichrospher 100 Diol 5 µ, Merck, Germany) using a 2 x 125 mm column. A flow rate of 0.35 ml/min 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; eluent A was a mixture of *n*-hexane/*i*-propanol/formic acid/ammonia (25% solution in water) 79:20:1.2:0.04 v/v, eluent B was *i*-propanol/water/formic acid/ammonia (25% solution in water) 88:10:1.2:0.04 v/v . The mass spectrometer was set to the negative ion mode with a spray voltage of -4.5 kV and a capillary temperature of 200°C. For MS/MS experiments, an octapole voltage of 45 V was applied to prevent adduct formation with eluent components. For these experiments, helium was used as a collision gas (relative collision energy: 35%). The light scattering detector was operated at a drift-tube temperature of 75°C using a nebulizer gas flow rate of 2.25 l N_2/min. MS data were used for compound identification, quantitation was performed with the ELSD trace after external calibration.

16S rDNA sequencing and phylogenetic analysis

Partial 16S rDNA sequences of the new strains D1 and SFA4 were determined as described by Overmann and Tuschak (1997). Sequencing primers used were 338f and 907r (Lane, 1991). Total or partial 16S rRNA gene sequence data of known strains of sulfate-reducing bacteria were retrieved from Genbank (http://www.ncbi.nlm.nih.gov). The sequences were aligned with the ClustalW program (Thompson et al., 1994). The phylogenetic tree was based on DNA fragments of 440 bp which could unambiguously aligned. For phylogenetic calculation the maximum likelihood program (DNAml) of the PHYLIP 3.57c package (Felsenstein, 1993) was used. Nucleotide positions that differed in more than 50% of all sequences were excluded from the analysis. The dataset

contained the 16S rRNA sequences of all organisms used in this study (GenBank accession numbers in parentheses): *Desulfobacter postgatei*^{T} (M26633), *Desulfobacter hydrogenophilus*^{T} (M34412), *Desulfobacterium autotrophicum*^{T} (M34409), *Desulfosarcina variabilis*^{T} (M34409), *Desulfococcus multivorans*^{T} (M34405), *Desulfonema limicola*^{T} (U45590), *"Desulfobotulus sapovorans"* (M34402), *Desulfobulbus propionicus*^{T} (M34410), *Desulfobulbus* strain 86FS1 (AF354663), *Desulforhopalus vacuolatus*^{T} (AF118453), "*Desulfoarculus baarsii*" (34403), *Desulforhabdus amnigenus*^{T} (X83274), SRB strain STP23 (X83274), *Desulfomicrobium baculatum* (M37311), *Desulfomicrobium* strain STL8 (X99505), *Desulfomicrobium apsheronum* Ac1.2 (AF228120), *Desulfomicrobium apsheronum* Ac3.2 (AF228124), *Desulfomicrobium apsheronum* Be2.2 (AF228136), *Desulfomicrobium apsheronum* Bu4.1(AF228133), *Desulfovibrio oxyclinae*^{T} (U33316), *Desulfovibrio desulfuricans* CSN (AF354664), *Desulfovibrio vulgaris*^{T} (M34399), *Desulfovibrio acrylicus*^{T} (U32578), *Desulfovibrio* sp. Pr1.2 (AF228128), *Desulfovibrio* sp. Ac5.2 (AF228127), SRB strain La4.1 (AF228119), *Desulfosporosinus* strain STP12 (AJ006607), spore-forming SRB strain STP3 (AJ006605). The partial 16S rDNA sequences of strain D1 and strain SFA4 are available at Genbank under the accession numbers AJ318380 and AJ318381, respectively.

Statistical analysis

For comparison of fatty acid and phospholipid patterns with the phylogenetic data, hierarchic agglomerative cluster analyses were performed (NTSYSpc 2.02j software packet; Applied Biostatistics). For fatty acid patterns after whole-cell hydrolysis, relative abundances of major fatty acids were used, i.e. compounds representing <1% of total fatty acids were excluded. To refine the pattern obtained from phospholipid types, a cluster analysis was performed for all strains containing only PE, PG and DPG using presence/absence data of phospholipid molecular species. For quantitative data, the dissimilarity coefficient after Bray and Curtis (1957) was calculated, whereas the Dice similarity coefficient (Dice, 1945) was used for binary data. For clustering, the flexible cluster algorithm (Lance and Williams, 1966, 1967) was chosen with β values adjusted to preserve original distances.

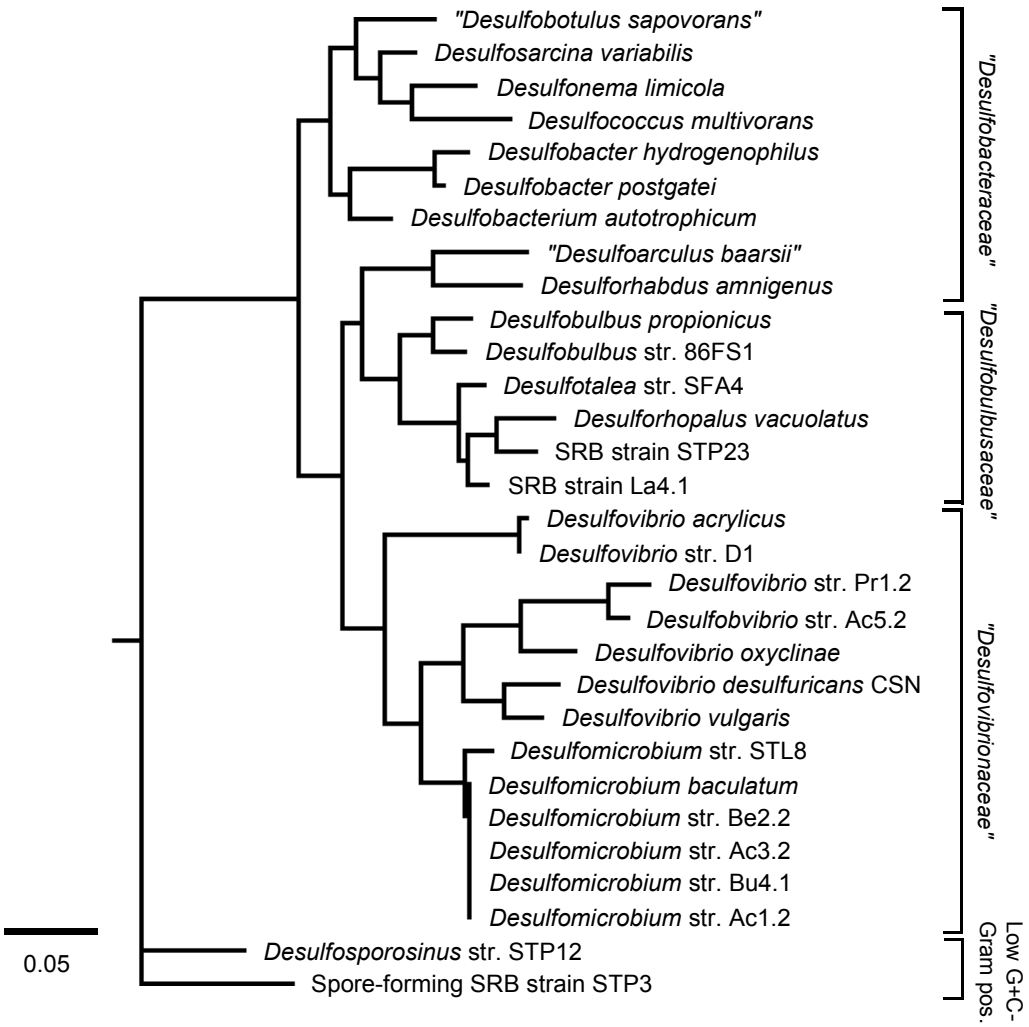

Fig. 11 Maximum likelihood tree of sulfate-reducing bacteria investigated based on 440 alignment positions. The bar represents 0.05 substitutions per nucleotide position.

3.2.4 RESULTS AND DISCUSSION

Phylogeny and taxonomy

Of the pure cultures investigated in this study, two strains belonged to the low-G+C Gram-positives. Strain STP3 was most closely related to the genus *Sporomusa* (95% sequence similarity to *Sporomusa termitida* in 530 bp) while strain STP12 could be grouped with *Desulfosporosinus orientis* (97% sequence similarity in 560 bp) (Sass et al., 1998). The other isolates were affiliated with the δ-*Proteobacteria*: 13 strains

grouped to the *"Desulfovibrionaceae"*, six to the *"Desulfobulbusaceae"* and nine to the *"Desulfobacteraceae"* (Fig. 11).

Sequence analysis of the partial 16S rRNA genes of the new strains D1 and SFA4 clearly confirmed the assignment to the δ-*Proteobacteria*. Strain D1 was closely related to *Desulfovibrio acrylicus* (99.5% sequence similarity in 440 bp). Strain SFA4 could be affiliated to the genus *Desulfotalea* with sequence similarities of 95.4% and 94.5% (440 bp) to *Desulfotalea psychrophila* and *D. arctica*, respectively.

Fatty acid patterns

For comparison of fatty acid patterns, a hierarchic agglomerative cluster analysis was performed with the fatty acid data obtained after whole-cell hydrolysis (Table 9, Fig. 12). These fatty acid patterns were similar to those of the polar lipid fraction (data not shown), the only difference being that hydroxy fatty acids, dimethylacetals or monoglycerol ethers could not be detected after mild methanolysis of the polar lipid fraction. Therefore, these compound classes were not included in the statistical analysis. Monoalkyl glycerol ethers were detected after whole-cell hydrolysis of *Desulfosarcina variabilis* and *Desulforhabdus amnigenus* indicating the existence of alkyl acyl phospholipids. Dimethylacetals, the hydrolysis products of vinyl ether lipids (plasmalogens), were only found in *Desulfovibrio desulfuricans* CSN. Whereas the finding of plasmalogens in *Desulfovibrio* sp. was reported by Makula and Finnerty (1974) and Kamio et al. (1969), alkyl acyl phospholipids have so far only been known from deeply branching thermophilic bacteria (e.g. Langworthy et al., 1983).

In nearly all sulfate-reducing bacteria investigated, β-hydroxy fatty acids with chain lengths between C_{14} and C_{18} were present in minor amounts. Only in strain STP3, β-hydroxy fatty acids with chain lengths of 12 and 13 carbon atoms accounted for 17 and 5.8%, respectively, of total fatty acids after whole-cell hydrolysis, but they were absent in the PLFA.

In general, fatty acid data of known strains of sulfate-reducing bacteria agreed well with literature data (Ueki and Suto, 1979; Dowling et al., 1986; Vainshtein et al., 1992; Kohring et al., 1994; Tourova et al., 1998) taking into account the variability between different batch cultures (±17% according to Vainshtein et al., 1992). For *Desulfobacter*, *Desulfobulbus* and *Desulfovibrio* species certain fatty acids have been

used as biomarkers to trace these genera in the environment. The 10-Me-$C_{16:0}$ fatty acid, claimed to be indicative for *Desulfobacter* spp. (Dowling et al., 1986, 1988), was very

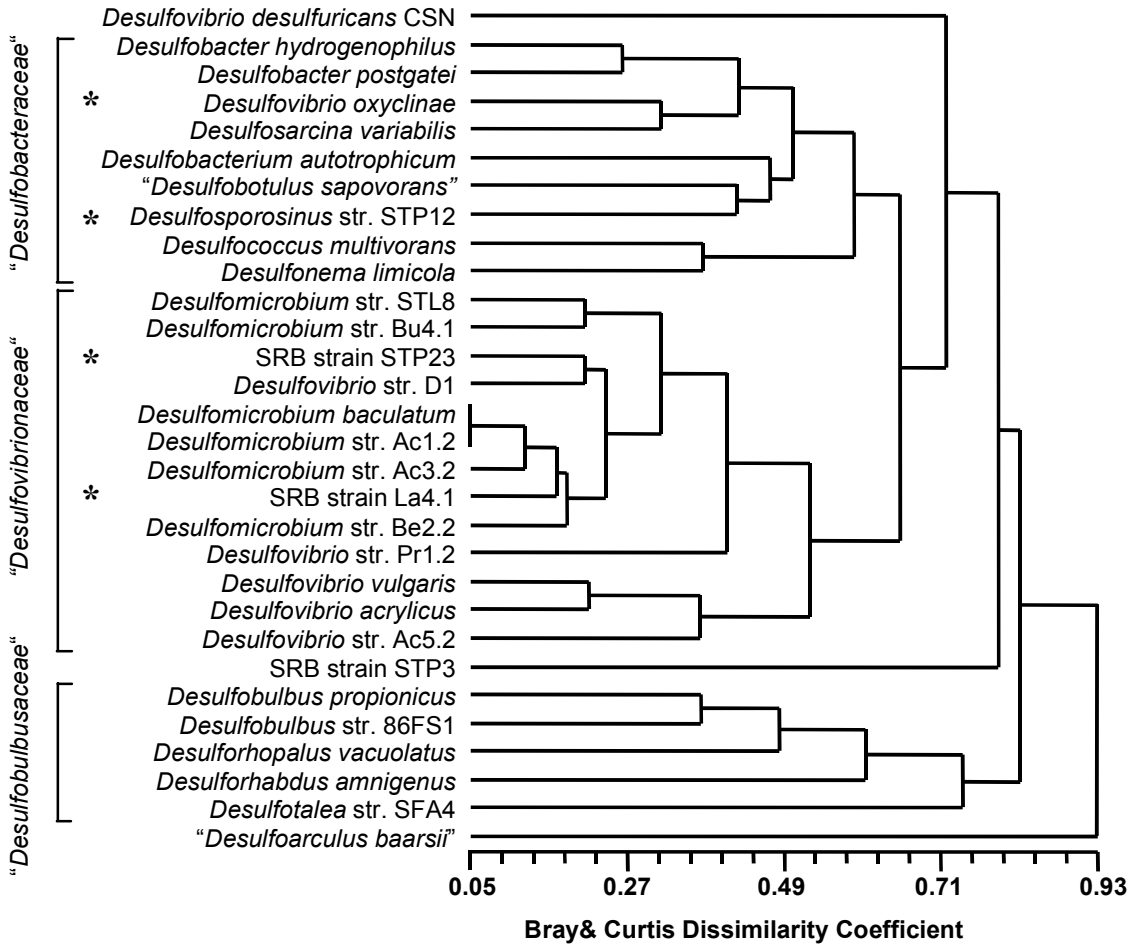

Fig. 12 Dendrogram constructed from dissimilarities of fatty acid patterns, i.e. a dissimilarity coefficient of 0 indicates smallest dissimilarity (or greatest similarity) whereas values approaching unity reflect increasing dissimilarity; strains whose grouping does not match phylogenetic relatedness are marked with an asterisk.

abundant in *Desulfobacter postgatei*, but also accounted for 17% of total fatty acids in the closely related *Desulfobacterium autotrophicum*. This proportion is much higher than that reported by Vainshtein et al. (1992). According to these authors, other *Desulfobacterium* spp. do not contain this "marker" fatty acid. Often, also a cyclo-C_{17} fatty acid has been employed as an additional biomarker for *Desulfobacter* spp. (Dowling et al., 1988). In our study, this fatty acid was abundant in the two *Desulfobacte*r spp., but also in *Desulforhabdus amnigenus* and *Desulfovibrio oxyclinae*, whereas it was absent from *Desulfobacterium hydrogenophilus*. The two *Desulfobulbus* strains investigated showed similar fatty acid patterns "typical" for this genus (cf. Parkes and Calder, 1985). The marker fatty acid *n*-$C_{17:1\omega6}$ (Parkes and Calder, 1985)

accounted for 24 and 11% of all quantified fatty acids, but also many other sulfate reducers contained this fatty acid (Table 9). Therefore, the *n*-$C_{17:1\omega6}$ fatty acid appears not to be exclusively characteristic for *Desulfobulbus* sp.

Fatty acid patterns of *Desulfococcus multivorans* and *Desulfosarcina variabilis* agreed with those reported by Kohring et al. (1994). Major fatty acids of the related *Desulfonema limicola* were *i*-, *ai*- and *n*-$C_{15:0}$ and *n*-$C_{16:0}$ fatty acids, and this resembled the major fatty acid distribution of *D. multivorans*. In contrast to *D. multivorans* and *D. variabilis*, however, *D. limicola* did not contain the *ai*-$C_{17:1\omega7}$ fatty acid, which accounted for 6.5 and 11% in *D. variabilis* and *D. multivorans*, respectively.

"*Desulfobotulus sapovorans*" and "*Desulfoarculus baarsii*" were the only two sulfate reducers investigated which contained significant amounts of fatty acids with an even number of carbon atoms ≥20. The fatty acid pattern of "*Desulfoarculus baarsii*" was unique in that it was dominated by the *i*-$C_{14:0}$ fatty acids; other fatty acids were mainly saturated compounds with an even number of carbon atoms. In contrast to this finding, there are literature reports of high amounts of *n*-$C_{14:0}$ or *i*- and *n*-$C_{14:0}$ fatty acids in this organism (Vainshtein et al., 1992; Kohring et al., 1994). In our investigation, "*Desulfobotulus sapovorans*" contained only 3.2% of the *n*-$C_{18:1\omega7}$ fatty acid which is one of the major fatty acids in the literature data (*ibid.*).

For *Desulforhopalus vacuolatus* our data did not agree with those reported by Knoblauch et al. (1999). Unfortunately, up to now no further literature data are available for comparison. The fatty acid pattern of *Desulfotalea* str. SFA4 did not resemble those reported by Knoblauch et al. (1999) for *Desulfotalea arctica* and *D. psychrophilica*. Fatty acid patterns of STP23 and La4.1, although related to the genera *Desulforhopalus* and *Desulfotalea*, matched those of *Desulfomicrobium/ Desulfovibrio* sp. (see below) more closely. In particular, both isolates exhibit high amounts of *i*-$C_{17:1\omega7}$ fatty acid (12 and 16%, respectively), a feature that is considered characteristic for *Desulfovibrio* spp. (e.g. Boon et al., 1977; Edlund et al., 1985; Vainshtein et al., 1992).

Table 9 Major fatty acids after whole-cell hydrolysis [% of total fatty acids]; dominant fatty acids (≥10%) are printed in bold letters.

	i-14:0	*n*-14:1ω5	*n*-14:0	*i*-15:1ω7	*i*-15:0	*ai*-15:0	*n*-15:1ω8	*n*-15:1ω6	*n*-15:0	*i*-16:1 ω8	*i*-16:1-ω6	*i*-16:0	*n*-16:1ω9	*n*-16:1ω7*	*n*-16:1ω5	*n*-16:0	*i*-17:1ω7	10-Me-16:0	*ai*-17:1ω7	*i*-17:0	*ai*-17:0	*n*-17:1ω8	*n*-17:1ω6	*cyc*-17	*n*-17:0	*n*-18:1 ω9	*n*-18:1ω7	*n*-18:1ω5	*n*-18:0	Σ*n*-20-26:0
Dbc pos	-	0.4	9.8	-	2.8	0.3	-	-	1.2	-	-	0.9	1.8	**13**	3.9	**31**	-	**13**	-	0.3	n.q.	-	-	**13**	0.3	-	2.7	-	1.0	-
Dbc hyd	-	-	**10**	-	6.6	0.6	-	-	0.5	-	-	0.5	-	**30**	3.2	**30**	1.2	3.9	-	1.0	-	-	-	6.5	-	-	3.9	-	1.9	-
Dbm aut	-	-	4.4	-	-	-	-	4.3	6.5	-	-	0.3	-	**22**	-	**20**	-	**17**	-	-	-	2.2	9.5	-	4.5	1.6	4.3	-	4.0	-
Dsc var	0.6	-	2.8	-	2.0	**23**	-	-	0.9	-	2.5	1.2	n.q.	**16**	2.0	**32**	3.1	-	6.5	0.2	1.6	-	n.q.	-	0.7	1.0	2.1	-	2.8	-
Dcc mul	-	-	5.6	-	5.4	**30**	-	4.7	**12**	-	-	0.7	-	4.9	-	**16**	0.9	-	**11**	0.7	2.2	0.9	1.1	-	0.9	-	-	-	2.3	-
Dnm lim	0.3	-	8.7	-	**13**	**15**	-	-	**15**	-	-	1.5	-	1.6	-	**37**	-	-	-	1.7	1.6	-	-	-	1.7	1.2	-	-	2.0	-
Dbt sap	-	1.8	1.7	-	-	-	-	-	0.8	-	-	-	4.3	**23**	-	**31**	-	-	-	-	-	2.6	0.6	-	1.3	**19**	3.2	-	3.2	7.9
Dbb prp	-	-	**10**	-	n.q.	-	n.q.	2.3	7.7	-	-	0.8	n.q.	**10**	**24**	4.9	-	-	-	-	-	n.q.	**24**	-	-	0.6	**15**	-	0.9	-
Dbb med	-	0.5	**11**	-	-	-	0.9	2.9	1.9	-	n.q.	-	n.q.	**31**	9.9	3.0	-	-	-	-	-	1.1	**11**	-	-	-	**27**	-	0.3	-
Drp vac	-	n.q.	0.7	-	-	-	-	2.2	1.6	-	0.2	-	n.q.	**17**	**52**	**11**	-	-	-	-	-	-	**12**	-	n.q.	-	0.6	3.2	0.6	-
SFA4	-	-	0.3	-	-	-	0.1	0.4	1.2	-	-	-	-	5.7	4.8	5.5	-	-	-	-	-	-	4.3	-	1.0	0.8	**74**	0.4	0.8	-
Drc bar	**55**	-	6.8	-	1.2	5.8	-	-	-	-	-	6.7	-	-	-	3.2	-	-	-	-	-	-	-	-		-	-	-	4.9	**16**
Drb amn	-	-	1.7	-	3.3	0.1	n.q.	1.6	**18**	-	-	-	n.q.	0.9	**10**	7.2	n.q.	-	-	0.3	-	1.5	**24**	4.7	**11**	0.8	1.1	-	1.6	-
STP23	0.1	n.q.	0.3	2.5	2.3	4.3	n.q.	-	0.1	3.1	**10**	1.4	1.6	**13**	1.4	5.9	**12**	-	9.2	0.5	2.2	1.1	0.8	-	0.4	4.8	**19**	0.3	1.9	-
La4.1	-	-	0.5	3.9	3.4	3.0	-	-	0.3	1.0	3.2	0.6	2.0	**12**	1.3	5.3	**16**	-	5.2	0.9	1.7	2.3	1.7	-	0.7	8.0	**22**	0.3	3.3	-
Dmb bac	-	-	0.4	1.7	2.5	3.3	-	-	0.3	1.6	4.0	1.3	1.7	**14**	1.6	8.1	6.4	-	3.8	0.7	1.6	4.0	2.4	-	2.5	8.5	**21**	-	8.2	-
STL8	-	-	0.4	1.3	1.3	1.5	-	-	0.3	1.3	4.1	-	2.0	**22**	3.6	5.3	6.4	-	3.9	0.2	0.6	3.1	3.0	-	0.7	8.5	**27**	-	3.6	n.q.
Ac1.2	0.2	-	0.4	2.5	2.5	2.8	-	0.2	0.3	1.4	3.3	1.2	2.2	**14**	1.9	7.9	4.6	-	3.9	0.6	1.5	3.5	2.7	-	2.6	9.1	**21**	0.3	8.3	-

Table 9 continued.

	i-14:0	*n*-14:1ω5	*n*-14:0	*i*-15:1ω7	*i*-15:0	*ai*-15:0	*n*-15:1ω8	*n*-15:1ω6	*n*-15:0	*i*-16:1 ω8	*i*-16:1-ω6	*i*-16:0	*n*-16:1ω9	*n*-16:1ω7	*n*-16:1ω5	*n*-16:0	*i*-17:1ω7	10-Me-16:0	*ai*-17:1ω7	*i*-17:0	*ai*-17:0	*n*-17:1ω8	*n*-17:1ω6	*cyc*-17	*n*-17:0	*n*-18:1 ω9	*n*-18:1ω7	*n*-18:1ω5	*n*-18:0	Σ *n*-20-26:0
Ac3.2	-	-	0.8	2.0	3.4	4.6	-	-	0.5	1.5	3.2	1.4	1.7	9.5	1.1	8.9	7.8	-	4.8	1.3	3.7	2.0	1.4	-	1.6	7.8	**21**	-	9.3	-
Be2.2	-	-	0.9	2.7	2.1	2.6	-	n.q.	1.0	-	1.3	0.2	2.0	**12**	1.2	7.5	9.2	-	4.3	0.7	1.6	9.9	6.0	-	3.1	8.1	**19**	-	5.1	-
Bu4.1	-	-	0.7	1.2	1.0	0.5	-	0.5	0.7	-	-	-	3.1	**28**	2.8	**10**	2.3	-	0.9	0.2	0.3	4.7	3.8	-	2.0	**12**	**21**	-	5.0	-
Dv oyx	2.0	-	4.7	-	3.6	6.4	-	-	1.0	-	3.4	3.8	-	9.0	n.q.	**34**	3.7	-	2.4	0.4	0.6	-	-	2.7	2.9	-	8.7	-	**11**	-
Dv des	-	-	0.4	-	**31**	0.8	-	-	n.q.	-	-	1.5	-	5.0	-	8.9	**35**	-	0.3	**14**	0.2	-	-	-	0.3	-	0.9	-	1.8	-
Dv vul	0.5	-	0.4	1.1	6.2	6.4	-	-	0.3	-	8.6	8.0	-	2.7	-	7.2	**22**	-	6.5	5.1	7.0	0.7	-	-	1.8	-	7.8	-	5.6	-
Dv acr	-	-	0.3	1.0	**11**	3.2	-	-	0.1	-	1.5	3.0	-	4.0	-	9.0	**22**	-	7.8	**13**	6.5	-	-	-	1.1	-	5.8	-	**10**	-
D1	n.q.	0.7	0.6	2.2	2.4	2.0	-	-	0.4	0.7	4.8	2.3	1.1	**14**	0.8	8.1	**15**	-	2.8	1.8	2.4	2.5	0.5	-	0.4	2.1	**23**		4.8	-
Pr1.2	0.4	-	1.5	-	1.5	6.0	-		0.4	-	2.8	0.8	0.9	**22**	-	9.4	2.6	-	1.9	0.7	0.5		-	-	0.6	1.7	**15**	0.4	**28**	1.8
Ac5.2	1.5	-	0.5	-	3.0	**16**	-	-	0.4	-	**10**	4.8	-	8.7	-	**12**	6.6	-	6.7	2.0	3.7	0.8	1.6	-	2.4	0.5	8.7	0.2	4.5	-
STP12	-	1.3	6.0	-	-	-	-	-	0.6	-	-	-	9.8	**42**	2.4	**13**	-	-	-	-	-	-	0.6	-	-	8.5	**12**	1.7	2.1	
STP3	-	-	2.2	-	-	-	5.9	-	-	-	-	-	**55**	**16**	0.6	8.4	1.1	-	-	-	-	3.2	0.4	-	-	3.4	1.7	-	2.2	-

Σ *n*-20-26:0 includes $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$ fatty acids;
n.q.: not quantifiable; *: trans isomer included if present.

All investigated *Desulfomicrobium* strains formed one coherent group in our cluster analysis reflecting the close relatedness within this phylogenetically homogeneous group (Figs. 11 and 12). Relative to this, *Desulfovibrio* sp. displayed rather heterogeneous types of fatty acid patterns. In our cluster analysis, *D. acrylicus* and *D. vulgaris* were grouped together with *Desulfovibrio* strain Ac5.2, whereas *Desulfovibrio* strain D1 clustered with SRB strain STP23 owing to its higher abundance of fatty acids with an even number of carbon atoms. *Desulfovibrio desulfuricans* CSN had a fatty acid pattern similar to *D. acrylicus*, but with a much higher proportion of the *i*-$C_{15:0}$ fatty acid. Therefore, it did not fall within the same group of the other *Desulfovibrio* species in the cluster analysis. Most *Desulfovibrio* sp. investigated had in common that they contained >10% of the "marker" fatty acid *i*-$C_{17:1\omega7}$. In contrast, *Desulfovibrio oxyclinae* and strains Pr1.2 and Ac5.2 possessed much lower contents of this fatty acid. Similarly, Edlund et al. (1985) did not detect the *i*-$C_{17:1\omega7}$ fatty acid in *Desulfovibrio gigas*. Relatively high proportions (up to 10%) of this fatty acid were also detected in all *Desulfomicrobium* spp. investigated. Therefore, the *i*-$C_{17:1\omega7}$ fatty acid may only be used as a biomarker for "*Desulfovibrionaceae*" as suggested by Tourova et al. (1998). With respect to the high abundances of *i*-$C_{17:1\omega7}$ in strains STP23 and La4.1, which phylogenetically do not belong to the "*Desulfovibrionaceae*", even this suggestion appears debatable.

The fatty acid pattern of *Desulfosporosinus* str. STP12 was similar to that reported by Ueki and Suto (1979) for *Desulfosporosinus orientis* (formerly, *Desulfotomaculum orientis*) with high amounts of monounsaturated C_{16} fatty acids. In the cluster analysis it grouped with *Desulfobotulus sapovorans*. The second spore-forming sulfate reducer investigated, strain STP3, contained a very distinct fatty acid distribution with very high abundances of *n*-$C_{16:1\omega9}$ (55%) and *n*-$C_{16:1\omega7}$ (16%). Phylogenetically, this sulfate-reducing strain is related to the *Sporomusa/Selenomonas* cluster, but its fatty acid pattern did not match that of other *Sporomusa* sp. which have $C_{15:1}$, $C_{15:0}$ and $C_{17:1}$ as predominant fatty acids (Strömpl et al., 1999). Although *Sporomusa* and closely related species are affiliated with the low G+C Gram-positive bacteria according to their 16S rRNA gene sequence, they possess a cell wall typical for Gram-negative bacteria (Stackebrandt et al., 1985). Therefore, the β-hydroxy fatty acids, which were found in high abundance after whole-cell hydrolysis, but not among the PLFA in strain STP3, may be derived from the cell-wall lipopolysaccharides. Similarly, Strömpl et al. (1999)

reported high amounts of hydroxy fatty acids (C_{11} to C_{13}) in the *Sporomusa*-affiliated *Anaerosinus glycerini*.

Unsaturated fatty acids with *trans* configuration of the double bond were detected in some sulfate reducers investigated, but usually the ratio of individual *trans/cis* fatty acids was <0.05 indicating healthy/non-stressed bacteria (White et al., 1996). Only in *Desulfomicrobium* strain Bu4.1, *trans/cis* ratios of ≈0.4 were observed, which are still much lower than those reported for starving or stressed bacteria (Guckert et al., 1986; Heipieper et al., 1992). High amounts of *trans* monounsaturated fatty acids (6% of total fatty acids) were reported for a bacterial isolate from mangrove sediments (Gillan et al., 1981). Therefore, we consider the observed levels of *trans* fatty acids in strain Bu4.1 as "normal" values, i.e. not indicating starvation or stress.

Phospholipid patterns

The HPLC gradient was optimised to separate different classes of phospholipids, i.e. to distinguish between different head groups nearly irrespective of the side-chains. The relative retention times of major phospholipid types in our system are: PA<PG<DPG<PDME<PMME<PE<PC<PS<PI. Further elucidation of the molecular structure of each phospholipid species was achieved by MS/MS experiments in the negative ion mode. In these experiments, the preferred fragmentation is the loss of an acyl group (usually the one at the central carbon atom of the glycerol backbone is lost preferentially). Phospholipid molecular species of the sulfate-reducing bacteria were assigned according to their retention times, quasi-molecular ions and fragmentation behaviour in MS/MS experiments.

Table 10 Major phospholipids of sulfate-reducing bacteria [% of total phospholipids]..

	PG (unknown)	PG (unknown2)	PG (alkyl/acyl)	PG	CL	Unknown 1	PE (unknown)	PE (alkenyl/acyl)	PE (alkyl/acyl)	PE	PC (unknown)	PC (alkyl/acyl)	PC	Unknown 2	PS	PI
Dbc pos	-	-	-	45	-	-	-	-	-	55	-	-	-	-	-	-
Dbc hyd	-	-	-	50	-	-	-	-	-	50	-	-	-	-	-	-
Dbm aut	-	-	-	54	9	-	-	-	-	37	-	-	-	-	-	-
Dsc var	-	-	16	24	n.q.	-	-	-	18	29	-	-	13	-	-	-
Dcc mul	-	-	-	45	n.q.	-	-	-	-	55	-	-	-	-	-	-
Dnm lim	-	-	-	39	-	-	-	-	-	33	28		-	-	-	-
Dbt sap	-	-	-	37	-	-	-	-	-	63	-	-	-	-	-	-
Dbb prp	-	-	-	18	n.q.	-	-	-	-	82	-	-	-	-	-	-
Dbb med	-	-	-	38	24	-	-	-	-	38	-	-	-	-	-	-
Drp vac	-	-	-	46	n.q.	-	-	-	-	54	-	-	-	-	-	-
SFA4	-	-	-	50	8	-	-	-	-	42	-	-	-	-	-	-
Drc bar	-	-	-	-	8	-	-	-	-	92	-	-	-	-	-	-
Drb amn	-	-	7	7	n.q.	-	-	-	34	31	-	6	5	-	-	9
STP23	-	-	-	15	13	-	-	-	-	72	-	-	-	-	-	-
La4.1	-	-	-	36	7	-	-	-	-	57	-	-	-	-	-	-
Dmb bac	-	-	-	16	16	-	-	-	-	68	-	-	-	-	-	-
STL8	-	-	-	31	7	-	-	-	-	62	-	-	-	-	-	-
Ac1.2	-	-	-	41	n.q.	-	-	-	-	59	-	-	-	-	-	-
Ac3.2	-	-	-	21	11	-	-	-	-	68	-	-	-	-	-	-

Table 10 continued.

	PG (unknown)	PG (unknown2)	PG (alkyl/acyl)	PG	CL	Unknown 1	PE (unknown)	PE (alkenyl/acyl)	PE (alkyl/acyl)	PE	PC (unknown)	PC (alkyl/acyl)	PC	Unknown 2	PS	PI
Be2.2	-	-	-	24	13	-	-	-	-	62	-	-	-	-	-	-
Bu4.1	-	-	-	26	20	-	-	-	-	54	-	-	-	-	-	-
Dvb oyx	-	-	-	15	10	-	-	-	-	75	-	-	-	-	-	-
Dvb des	-	-	-	12	n.q.	-	-	38	-	50	-	-	-	-	-	-
Dvb vul	-	-	-	21	21	-	-	-	-	58	-	-	-	-	-	-
Dvb acr	39	-	-	19	6	-	-	-	-	36	-	-	-	-	-	-
D1	8	-	-	35	7	-	-	-	-	50	-	-	-	-	-	-
Pr1.2	-	-	-	22	30	-	-	-	-	48	-	-	-	-	-	-
Ac5.2	-	-	-	32	15	-	-	-	-	53	-	-	-	-	-	-
STP12	-	20	-	25	-	-	21	-	-	24	-	-	-	10	-	-
STP3	-	-	-	-	-	20	-	-	-	68	-	-	-	-	11	-

n.q.: not quantifiable.

a) Phospholipid classes

The major phospholipid type of the non-sporulating, Gram-negative sulfate-reducing bacteria investigated was phosphatidyl ethanolamine (PE), with variable amounts of phosphatidyl glycerol (PG) and diphosphatidyl glycerol (DPG) (Table 10). In addition, in *Desulfonema limicola*, *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, minor amounts of phosphatidyl cholines (PC) were detected. The latter strain also contained phosphatidyl inositols. High amounts of PE with smaller contributions of PG and DPG are typical membrane lipids of many Gram-negative bacteria (Wilkinson, 1988) and were found in other sulfate reducers such as *Desulfomicrobium norvegicum*, *Desulfovibrio vulgaris* (Makula and Finnerty, 1974) and *Desulfotalea* sp. (Knoblauch et al., 1999). Makula and Finnerty (1974) also reported the presence of (lyso) phosphatidyl serine in *Desulfovibrio* and *Desulfomicrobium* spp.; this phospholipid type was not observed in our strains.

Besides diacyl phospholipids, we detected compounds with ether-linked alkyl and alkenyl side-chains in *Desulforhabdus amnigenus*, *Desulfosarcina variabilis* and *Desulfovibrio desulfuricans* CSN, respectively. The presence of these side-chains was confirmed by the detection of monoalkyl ethers and dimethyl acetals after whole-cell hydrolysis (see above). In case of *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, ether cleavage was performed with the polar lipid fraction for identification of ether side-chains (Rütters et al., 2001b; Chapter 3.3).

Desulfovibrio acrylicus and *Desulfovibrio sp.* D1 contained PG species with unknown side-chains (m/z 651, 637, 623). Since loss of either a $C_{15:0}$ or a $C_{17:0}$ fatty acid was observed in MS/MS experiments, the second substituent should be a C_9-C_{11} acyl or a C_{10}-C_{12} alkyl moiety, if the structures are not completely different. No corresponding hydrolysis products, i.e. short-chain fatty acids or monoalkyl glycerol ethers, were detected after whole-cell hydrolysis.

The two spore-forming sulfate reducers investigated, *Desulfosporosinus* strain STP12 and SRB strain STP3, exhibited completely different phospholipid class patterns. In *Desulfosporosinus* strain STP12, PE and PG with diacyl and with one unknown side-chain each (the other one being a $C_{16:1}$ fatty acid) are the most abundant phospholipids. Furthermore, an unknown phospholipid with a quasi-molecular ion at m/z 1434, eluting shortly after PC, accounted for 10% of total phospholipids. In contrast, typical low G+C Gram-positive bacteria, such as *Clostridium* sp., have phosphatidyl glycerol as their

major phospholipid type (Drucker et al., 1996). In the sulfate-reducing bacterium strain STP3, PE was the most abundant component with phosphatidyl serine and an unknown phospholipid type accounting for 11% and 20% of the total phospholipids, respectively. The unknowns had a retention time similar to PE, but mass-to-charge values of quasi-molecular ions ranged between m/z 1113 and 1155. For comparison, lyso-DPG, being of similar molecular weight, elutes ca. 2 min later. PE and PS were also the major phospholipid types in *Dendrosporobacter quericolus* and *Anaerovibrio* sp. which are phylogenetically closely related to the *Sporomusa/ Selenomonas* group (Strömpl et al., 1999, 2000).*b) Phospholipid molecular species*

Since the phospholipids thought to be typical for non-sporulating SRB are also often encountered in other Gram-negative bacteria, phospholipid classes may not be appropriate biomarkers for the study of complex microbial communities in environmental samples, i.e. without isolation of bacteria prior to lipid analysis. As many researchers have shown that phospholipid species analysis provides a good discriminatory tool for bacterial groups, genera or species (Nunes et al., 1992; Black et al., 1997; Fang et al., 2000a), we analysed phospholipid molecular species composition of our SRB. Different types of phospholipids were always substituted with similar fatty acid combinations (data not shown). These fatty acids resembled the dominant fatty acids obtained after whole-cell hydrolysis. To evaluate the discriminatory power of phospholipid molecular species analysis, presence/absence data of most abundant phospholipid species (>20% in a particular phospholipid class) were used for a cluster analysis (Fig. 13). Phospholipid molecular species were differentiated by their molecular weights only, because exact fatty acid combinations could not be determined for all phospholipid species by MS/MS experiments in the dependent-scan mode. The cluster structure obtained after flexible clustering revealed that the relationships within the phospholipid molecular species closely followed fatty acid patterns as can be seen from comparison of Figs. 12 and 13.

Most *Desulfomicrobium* sp. and most *Desulfovibrio* sp. showed similar phospholipid molecular species patterns and therefore can be found in one subcluster. Also the SRB strains STP23 and La4.1 fell within this group of "*Desulfovibrionaceae*" although these two organisms are affiliated with the *Desulfobulbus*/ *Desulforhopalus*/*Desulfotalea* group. Representatives of these three genera formed a second distinct cluster. Furthermore, the two investigated *Desulfobacter* sp. grouped

closely together due to the lack of DPG in both strains and their similar fatty acid distribution. The last "group" was formed by a rather heterogeneous assemblage of *Desulfovibrio desulfuricans* CSN, *Desulfovibrio oxyclinae*, *Desulfobacterium autotrophicum* and *Desulfococcus multivorans*, although *D. desulfuricans* contained the unusual plasmalogens. The peculiar fatty acid pattern of *Desulfoarculus baarsii* lead to its isolated position in this cluster analysis.

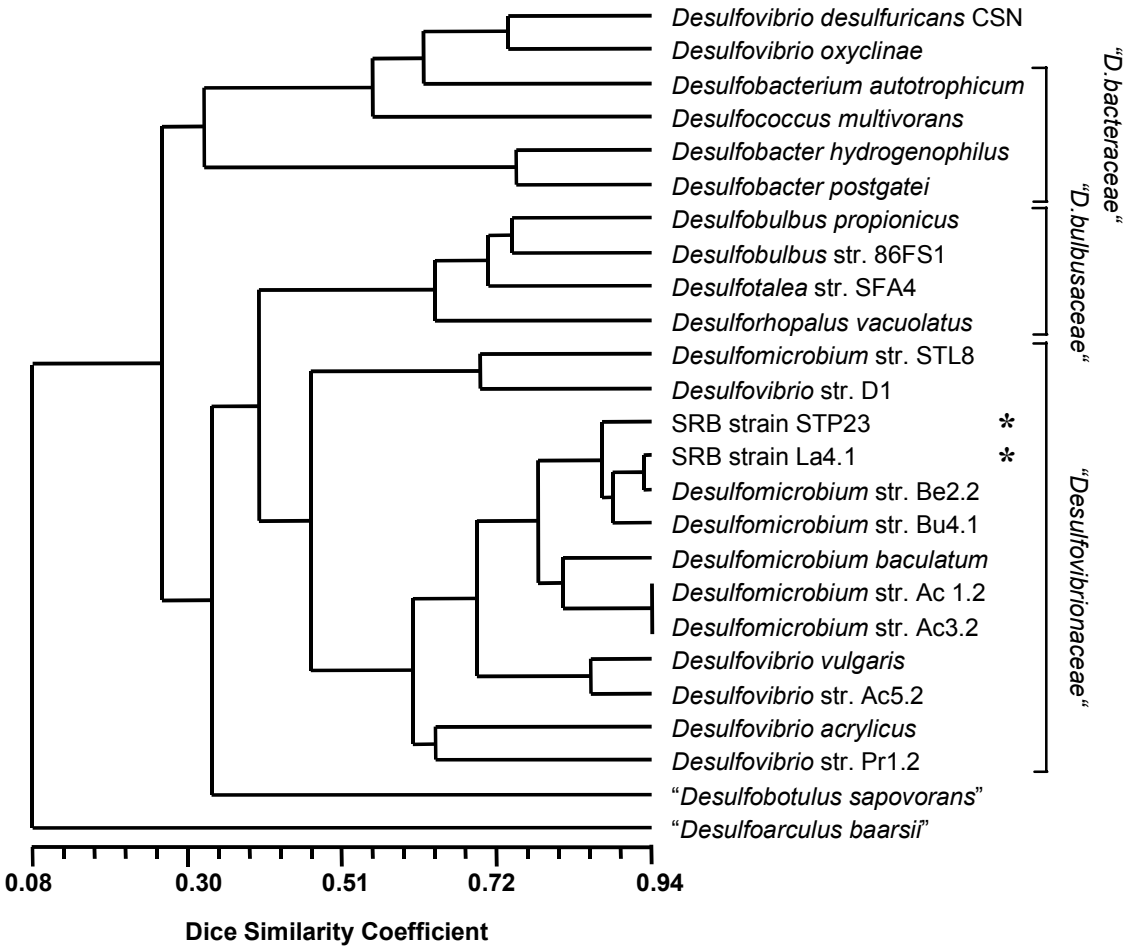

Fig. 13 Dendrogram constructed from similarities of presence/absence data of phospholipid molecular species, i.e. the greater the value of the similarity coefficient the more similar to each other are the patterns compared; strains whose grouping does not match phylogenetic relatedness are marked with an asterisk.

Lipid composition of bacteria as a function of environmental conditions.

Phospholipids are major membrane lipids of bacteria. For optimal membrane functioning the phospholipid bilayer is maintained in the liquid-crystalline state.

Various biochemical mechanisms have evolved for adjustment of membrane fluidity to changing environmental conditions (temperature, pH, salinity, solutes etc.) such as variation of phospholipid composition, (fatty acid) side-chains, cellular lipid content and the presence of other compounds such as steroids/hopanols (Guckert et al., 1986; Hood et al., 1986; Heipieper et al., 1992; Suutari and Laakso, 1994). On the other hand, membranes are involved in various cellular processes, and these processes require the presence of specific phospholipids: anionic phospholipids provide organised sites in the membrane at which multisubunit complexes can be formed involving membrane proteins as well as cytoplasmic enzymes. The zwitterionic PE is important for correct folding and functioning of many membrane proteins, and it is a major non-bilayer-forming phospholipid. Formation of non-lamellar phases is a prerequisite for cell division, transport of macromolecules into the cell. Therefore, organisms usually display a great diversity of phospholipid classes and species (Dowhan, 1997). The phospholipid head group composition is usually less sensitive towards changing growth conditions than the fatty acid composition (Cole and Enke, 1994; Dowhan, 1997; Olsson and Salem, 1997). Examples of minor changes in phospholipid class composition are higher abundances of DPG at the expense of PG when cells enter the stationary phase (Dowhan, 1997) or when salinity decreases (Wilkinson, 1988). Therefore, phospholipid class composition should provide valuable, stable chemotaxonomic information (Cole and Enke, 1994).

In the literature, there is a continuing discussion about the variability of membrane fatty acid composition in response to changing environmental conditions. Systematic investigation of the variability of fatty acid patterns with changing growth conditions revealed that a) different genera of SRB possess different susceptibilities to variations of their carbon source and growth temperature depending - among other things - on their fatty acid synthetases (Taylor and Parkes, 1983; Dowling et al., 1986; Vainshtein et al., 1992; Kohring et al., 1994; Aeckersberg et al., 1998), b) in many cases fatty acid patterns - although variable in the relative proportions - remain distinguishable (Haack et al., 1994; Kohring et al., 1994).

In general, variation of fatty acid side-chains is one mechanism amongst others to adapt membrane properties to environmental conditions. For example, cyclopropyl fatty acids are preferentially biosynthesised in the stationary growth phase and decrease susceptibility of membrane lipids toward oxidative degradation (Silvius and McElhaney, 1979). The isomerisation of *cis* to *trans* monounsaturated fatty acids

represents one mechanism adjusting membrane fluidity when environmental conditions inhibit cell growth and lipid synthesis (Keweloh and Heipieper, 1996). Membranes are usually maintained in the liquid-crystalline state, i.e. between gel-liquid transition temperature and the transition temperature from lamellar to non-lamellar phases, by varying phospholipid fatty acid composition either by biosynthesis or incorporation of exogenic fatty acids (Suutari and Laakso, 1994; Dowhan, 1997).

Large differences in growth temperatures may account for the variation in fatty acid patterns between literature data and our measurements as well as for dislike fatty acid patterns of closely related strains. For example, *Desulfovibrio* strain D1 (grown at 10°C) exhibited high levels of monounsaturated *cis*-C_{16} and -C_{18} fatty acids whereas the fatty acid pattern of its close relative *D. acrylicus* (grown at 28°C) was dominated by large amounts of *iso*-branched C_{15} and C_{17} fatty acids. According to Suutari and Laakso (1994), introduction of a *cis*-monounsaturated fatty acid is the most efficient way to lower the gel-to-liquid-transition temperature. As a second example, *Desulforhopalus vacuolatus* was grown on lactate at 10°C in our study, whereas Knoblauch et al. (1999) cultured it at 4°C using the same substrate. The observed difference is mainly seen in a very high abundance of the *n*-$C_{17:1\omega6}$ fatty acid (60% of total fatty acids) in their analysis in contrast to large amounts of *n*-$C_{16:1\omega5}$ fatty acid (52%) observed by us, which does not follow the expected temperature adaptation mechanisms, i.e. variation of branching and unsaturation (Suutari and Laakso, 1994).

If strains are cultured under standardised growth conditions, variability in their fatty acid patterns may reflect genetic differences (Dzierzewicz et al., 1996), as can be seen, e.g., in the coincidence between a relatively large phylogenetic heterogeneity of *Desulfovibrio* spp. and large variations in fatty acid patterns within this genus (Ueki and Suto, 1979). In general, relatedness of SRB as indicated by 16S rDNA similarities matches the "traditional" taxonomic classification according to chemotaxonomy and physiology (Devereux et al., 1989; Widdel and Bak, 1992; Kohring et al., 1994). Also for other bacteria phylogenetic relatedness is often reflected in the similarities of their fatty acid patterns (Thompson et al., 1993; Vancanneyt et al., 1996). Among the mesophilic Gram-negative sulfate-reducing bacteria two families have been proposed, namely the "*Desulfovibrionaceae*" and the "*Desulfobacteraceae*" (Devereux et al., 1990). As *Desulfobulbus*, *D.rhopalus, D.fustis* and *D.capsa* sp. represent a fairly deeply branching group within the "*Desulfobacteraceae*", they have been proposed to form a third "family", the "*Desulfobulbusaceae*" (Rooney-Varga et al., 1998; Castro et al.,

2000). Because fatty acid patterns of their representatives were distinct from those of "*Desulfovibrionaceae*" and other members of the "*Desulfobacteraceae*", our data would support the establishment of a new family in accordance with the major metabolic properties of the organisms (Widdel and Bak, 1992). Fatty acid patterns of strain STP23 and strain La4.1, which are phylogenetically affiliated to the "*Desulfobulbusaceae*", were different from those of the known "*Desulfobulbusaceae*", but resembled patterns of "*Desulfovibrionaceae*" more closely than those of "*Desulfobacteraceae*" and thus still favour a separation of "*Desulfobulbusaceae*" and "*Desulfobacteraceae*".

Combining the information of phospholipid types and fatty acid side-chains means integrating over two variables which are influenced by different factors. Often, the phospholipid fatty acid composition is altered more rapidly than phospholipid class composition owing to specific involvement of certain phospholipid classes in essential cellular processes (see above). Therefore, it is not surprising that the topology of the dendrogram (Fig. 13) only partially reflects the phylogenetic relatedness inferred from the 16S rDNA sequence similarities of the SRB investigated. To separate data of taxonomic value, i.e. properties unique to an organism, from data that correlate particular qualities of microorganisms, i.e. represent a common adaptive strategy to environmental parameters, Cole and Enke (1994) suggested to analyse molecular species of each phospholipid class separately. For our data, statistical analysis of PE molecular species lead to a cluster (data not shown) that did not reflect the phylogenetic relatedness more closely than the one combining all phospholipid classes.

In general, by applying a hierarchic cluster algorithm, a hierarchic structure in the data analysed is implied. Properties such as phospholipid molecular species patterns, which are also influenced by environmental parameters, will only partly follow evolutionary lineages, because similar adaptive strategies for changing environmental conditions may have developed in different groups of microorganisms.

3.2.5 ACKNOWLEDGEMENTS

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3.3 MONOALKYL ETHER LIPIDS IN THE SULFATE-REDUCING BACTERIA *DESULFOSARCINA VARIABILIS* AND *DESULFORHABDUS AMNIGENUS* [*]

Heike Rütters, Henrik Sass, Heribert Cypionka, Jürgen Rullkötter

3.3.1 ABSTRACT

In this study, cellular lipid compositions of two mesophilic sulfate-reducing bacteria were analysed by high performance liquid chromatography-mass spectrometry (HPLC-MS). In *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, alkylether-containing phospholipids were detected which had previously only been found in significant amounts in deeply branching hyperthermophilic bacteria and archaea. Combining information from HPLC-MS analysis and chemical degradation experiments, ether lipids were identified as 1-alkyl-2-acyl-phosphatidyl ethanolamines, glycerols and cholines. In *Desulforhabdus amnigenus*, *n*-penta-, *n*-hexa- and *n*-heptadecyl ethers were present (in order of decreasing abundance), whereas *Desulfosarcina variabilis* solely contained *n*-hexadecyl ether side-chains.

[*] Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2001. Monoalkyl ether lipids in the sulfate-reducing bacteria *Desulfosarcina variabilis* and *Desulforhabdus amnigenus.* Archives of Microbiology, submitted.

3.3.2 INTRODUCTION

For chemotaxonomic classification of microorganisms whole-cell fatty acid patterns after alkaline hydrolysis are widely applied (e.g. Vainshtein et al., 1992; Vancanneyt et al., 1996). Fatty acid patterns of polar cellular lipids are often used to distinguish different kinds of viable microorganisms in the analysis of *in situ* microbial community structures (e.g. Guckert et al., 1985; White et al., 1996). Compounds detected include saturated and unsaturated, branched and straight-chain fatty acid as well as cyclopropyl and hydroxyl-substituted fatty acids. The presence of aldehydes has also been reported (e.g. Knoblauch et al., 1999). All ester-bound compounds are liberated from lipopoly-saccharides and cellular polar lipids by the saponification procedure. In contrast, amide and most ether bonds can only be cleaved under acidic conditions (Goossens et al., 1986; Hanahan, 1972).

In the last few years, direct structural analysis of intact phospholipids has become possible using electrospray ionisation-mass spectrometry, which is now widely applied to characterise bacterial isolates (e.g. Black et al., 1997; Fang and Barcelona, 1998). Using this technique, information about phospholipid types and their corresponding side-chains is obtained and this information allows a better taxonomic differentiation than fatty acid patterns alone (Fang et al., 2000a). Additionally, not only easily-hydrolysable ester-linked fatty acids, but also ether-linked side-chains can be detected by intact phospholipid analysis (e.g. Han and Gross, 1995).

Whereas reports of bacterial phospholipid types and fatty acid patterns as well as reports of ether-linked isoprenoidal side-chains in archaeal lipids are numerous (e.g. Fang et al., 2000b; Kates, 1997; Koga et al., 1998; Ratledge and Wilkinson, 1988), there are only few observations of ether linkages in bacteria: Vinyl ether lipids (plasmalogens) were proposed to be common in strict anaerobes as they were detected, e.g., in *Clostridium* spp. and other rumen bacteria, but also in soil bacteria grown under anoxic conditions and a *Desulfovibrio* sp. (Fischer et al., 1994; Goldfine and Hagen, 1972; Kamio et al., 1969). Alkyl-acyl-phospholipids are said to occur as traces in bacteria that also contain plasmalogens (e.g. Paltauf, 1994; Goldfine and Hagen, 1972). The presence of non-isoprenoidal glycerol ether lipids was noticed in the myxobacterium *Stigmatella aurantiaca* and the halophilic sulfate reducer *Desulfohalobium retbaense* (Asselineau and Asselineau, 1990; Ollivier et al., 1991). In contrast, large proportions (i.e. ≥90% of the core lipids) of saturated, non-isoprenoidal

mono- and dialkyl glycerol ether lipids have so far only been discovered in hyperthermophilic bacteria such as *Aquifex pyrophilus* (Huber et al., 1992) and *Thermodesulfobacterium commune* (Langworthy et al., 1983). Therefore, the presence of substantial amounts of saturated ether lipids seemed to be confined to archaea and deepest phylogenetic branches within the *Bacteria* (Gambacorta et al., 1994).

Evidence for the presence of ether lipids in non-thermophilic bacteria comes from reports of the ubiquitous distribution of tetraether lipids in "cold" environments; these tetraethers combine structures of archaeal lipids (isoprenoid side-chains and cyclopentyl or -hexyl groups within the side-chains) and bacterial lipids (mono- or dimethyl alkyl chains) within a single molecule (Schouten et al., 2000). Furthermore, in "cold" methane seep sediments from the Eel River Basin and the Santa Barbara Basin, the occurrence of $\delta^{13}C$-depleted, non-isoprenoidal monoalkyl and dialkyl ethers has recently been reported (Hinrichs et al., 2000; Orphan et al., 2001). Anaerobic oxidation of methane in seep sediments and in sediment sections overlying methane hydrates is thought to be mainly carried out by syntrophic consortia of methane-oxidising archaea and sulfate-reducing bacteria (Boetius et al., 2000; Hinrichs et al., 1999). The mono- and dialkyl ether lipids found by Orphan et al. (2001) were suggested to originate from yet unknown types of bacteria additionally involved in the anaerobic oxidation of methane.

We investigated the lipid composition of mesophilic sulfate-reducing bacteria using HPLC-ESI-MS and -MS/MS. In two strains, ether-containing phospholipids were detected. In this paper we report detailed evidence for the presence of alkyl-acyl-phospholipids in *Desulforhabdus amnigenus* and *Desulfosarcina variabilis*.

3.3.3 MATERIALS AND METHODS

Organisms

Pure cultures of sulfate-reducing bacteria were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany): *Desulfosarcina variabilis*^{T} (DSMZ 2060) and *Desulforhabdus amnigenus*^{T} (DSMZ 10338). Pure cultures were grown at their optimum growth temperatures of 28 and 37°C, respectively, using growth media recommended by the DSMZ with propionate (*Desulforhabdus amnigenus*) or benzoate (*Desulfosarcina variabilis*) as electron donor

and carbon source. Cells were harvested at the end of their exponential growth phase by centrifugation, washed with phosphate buffer (pH≈7) of appropriate salinity , freeze-dried and stored at -20°C.

Extraction and clean-up

Freeze-dried samples were ultrasonically extracted by a modified Bligh-Dyer method using a solvent mixture of methanol/dichloromethane/phosphate buffer (pH 7.4), 2:1:0.8 (v/v). Different lipid classes were separated by column chromatography on silica gel (2 g silica 60, 63-200 µm, Merck, Germany, dried at 110°C for 16 h). Eight fractions were obtained by elution with the following solvents: 1) 10 ml *n*-hexane; 2) 25 ml *n*-hexane/diethylether 98:2 v/v; 3) 30 ml *n*-hexane/diethylether 8:2 v/v; 4) 20 ml diethylether; 5) 10 ml 0.5 vol% acetic acid in diethylether; 6) 20 ml acetone/ dichloromethane 1:1 v/v and 10 ml acetone/dichloromethane 8:2 v/v; 7) 10 ml 5 vol% methanol in dichloromethane and 25 ml acetone; 8) 30 ml dichloromethane/methanol/ water 5:4:0.4 v/v. Phospholipids eluted in the most polar fraction. All fractions were evaporated to dryness and stored at -20 °C.

Transesterification, gas chromatographic (GC-FID) and gas chromatographic-mass spectrometric (GC-MS) analysis

Aliquots of polar lipid fraction were transesterified by mild alkaline hydrolysis as described by White et al. (1979). The methyl esters obtained were analysed by GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph, Hewlett Packard, Waldbronn, Germany) on a DB-5HT column (30 m x 0.25 mm, 0.1 µm film thickness, J&W, Folsom, CA, U.S.A.) and by GC-MS using a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA, U.S.A.).

Whole-cell hydrolysis

Freeze-dried cells (ca. 20 mg) were saponified over night with 5 ml KOH solution (5% KOH·H_20 grade "Suprapur", Merck, Darmstadt, Germany, in methanol/water 4:1 v/v) under a nitrogen atmosphere. Afterwards the alkaline solution was transferred to a separation funnel and acidified using hydrochloric acid (2 mol/l). Non-saponifiable

remnants were extracted three times with 10 ml dichloromethane each. Dichloromethane extracts were added to the methanol/water-phase in the separation funnel. After phase separation the organic phase was removed and the aqueous phase was reextracted three times with 10 ml dichloromethane each. Combined organic phases were dried over anhydrous sodium sulfate. After removal of the solvent, aliquots of samples were transformed into their trimethylsilyl (TMS) ester derivatives using N-methyl-N-trimethylsilyl-trifluoroacetamide (CS Chromatographie Service, Langerwehe, Germany). The TMS derivatives of fatty acids were analysed by GC-FID and GC-MS as described above. Positions of double bonds were confirmed using dimethyldisulfide addition as described by Dunkelblum et al. (1985).

HPLC-MS and -MS/MS

Phospholipids were analysed on an HPLC instrument (Thermo Separation Products, San Jose, CA, USA) coupled to an ion trap mass spectrometer equipped with an electrospray source (Finnigan LCQ, Thermoquest-Finnigan, San Jose, CA, USA) and to an evaporative light scattering detector (ELSD 500, Alltech, Deerfield, IL, USA) using a flow-splitter (split ratio 1:0.88, with the larger flow feeding the ELSD). HPLC separation was achieved on a diol phase (Lichrospher 100 Diol 5µ, Merck, Germany). HPLC separation was achieved on a diol phase (Lichrospher 100 Diol 5µ, Merck) using a 2x125 mm column. A flow rate of 0.35 ml/min 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. Eluent A was a mixture of *n*-hexane/*i*-propanol/formic acid/ammonia (25% solution in water) 79:20:1.2:0.04 v/v, eluent B was *i*-propanol/water/formic acid/ammonia (25% solution in water) 88:10:1.2:0.04 v/v . The mass spectrometer was set to the negative ion mode with a spray voltage of -4.5 kV and a capillary temperature of 200 °C. MS/MS experiments were done in the so-called dependent scan mode, i.e. the most intense quasi-molecular species of each full scan was automatically isolated and fragmented. For MS/MS experiments, an octapole voltage of 45 V had to be applied to prevent adduct formation with eluent components; helium was used as collision gas (relative collision energy: 35%). The light scattering detector was operated at a drift-tube temperature of 75°C using a nebulizer gas flow rate of 2.25 l N_2/min.

Ether cleavage

Ether cleavage was performed according to Wenzel (unpublished results, 1998): An aliquot of the polar lipid fraction was refluxed with 3 ml of hydroiodic acid (57%, Aldrich, Taufkirchen, Germany) and 2 ml of acetic acid (99.8%, Riedel de Haen, Seelze, Germany) for 3 h under a nitrogen atmosphere. The resulting solutions were transferred into separation funnels; *n*-hexane and water were added. Water phases were reextracted with 10 ml of *n*-hexane. Combined organic phases were washed with 5% $NaSO_4$ solution and twice with water. The resulting organic phase was dried over anhydrous sodium sulfate and then reduced to dryness. Crude material was separated by column chromatography on silica gel (silica gel 100, 63-200 µm, Merck, Darmstadt, Germany) into an apolar (elution with 10 ml *n*-hexane) and a polar fraction (elution with 15 ml dichloromethane/10% methanol/50 µl acetic acid (99%). Fractions obtained were transformed into their TMS esters as described above. The TMS derivatives were analysed by GC-MS on an HP-1 column (60 m x 0.25 mm, 0.25 µm film thickness, Hewlett Packard, Waldbronn, Germany) using a Finnigan MAT 95Q mass spectrometer (Finnigan-Thermoquest, San Jose, CA, U.S.A.) set to a resolution of about 1000. Blanks were run to check for contamination.

3.3.4 RESULTS

HPLC-MS analysis

The HPLC conditions were optimised to separate different phospholipid types, which means that the headgroup is the main factor controlling retention time. Nevertheless, differences in fatty acid side-chains lead to minor shifts in retention times (<0.1 min/C atom), so that compounds with longer side-chains elute earlier than those with shorter substituents. Major changes in the side-chains - such as introduction of an ether bond - shift retention times significantly (ca. 0.6 min). For phosphatidyl choline (PC) standards with ether and ester-linked side-chains, the following order of retention times was observed: di-O-hexadecyl < O-hexadecyl-palmitoyl < dipalmitoyl. The structurally related compounds, dipalmitoyl phosphatidyl monomethyl ethanolamine and dipalmitoyl dimethyl ethanolamine, elute shortly before dipalmitoyl ethanolamine

(PE) and nearly 1.5 min before the dipalmitoyl choline standard, so that a coelution of O-alkyl-PC or -PE and other (isobaric) compounds can be excluded.

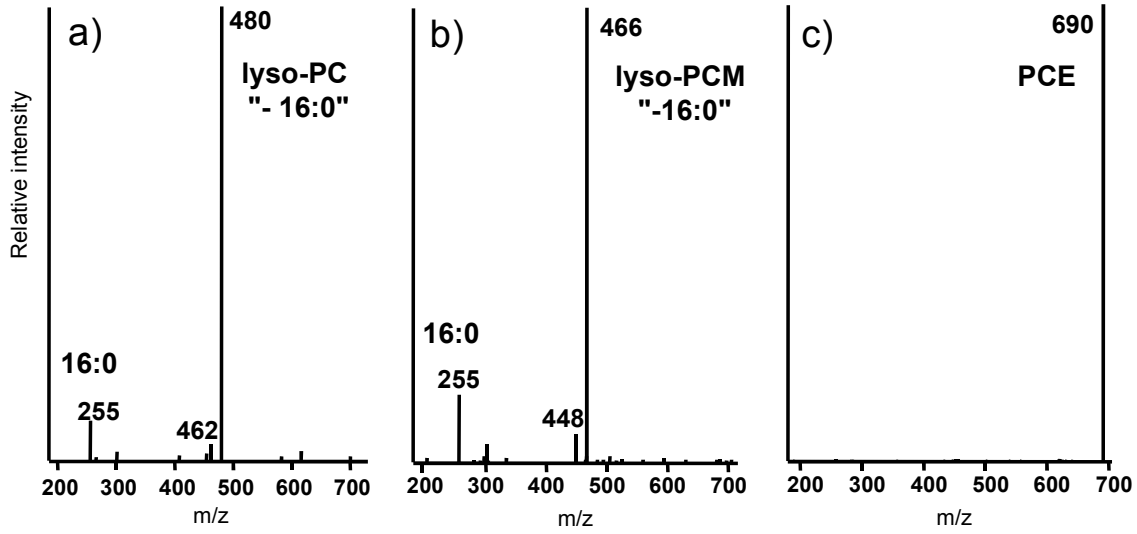

Fig. 14 MS/MS spectra of phosphatidyl choline "molecular species" $[M-15]^-$;
a) dipalmitoyl phosphatidyl choline (m/z 718), b) O-hexadecyl-palmitoyl phosphatidyl choline (m/z 704), c) dihexadecyl phosphatidyl choline (m/z 690).

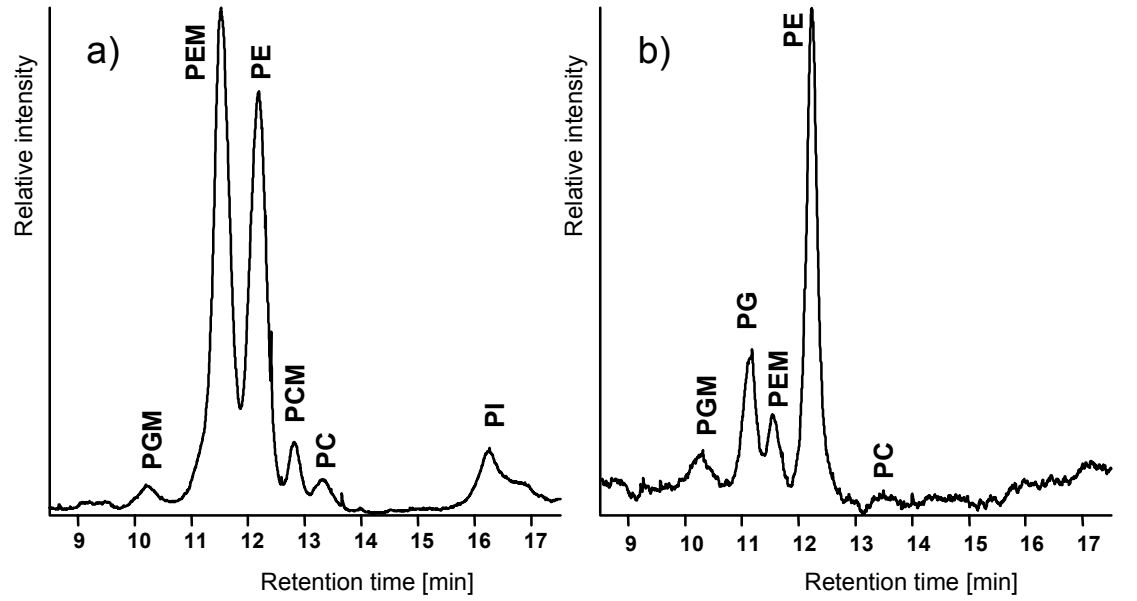

Fig. 15 HPLC-ELSD chromatogram of phospholipid fraction of a) *Desulforhabdus amnigenus* and b) *Desulfosarcina variabilis*.

In MS/MS experiments, diacyl phospholipids exhibit fragments resulting from the loss of the fatty acid from the central glyceryl carbon atom (Fig. 14a), whereas di-O-alkyl phospholipids give no fragments under the MS/MS conditions chosen (Fig. 14c). Mixed 1-O-alkyl-2-acyl compounds show MS/MS-spectra very similar to that obtained from diacyl phospholipids, i.e. loss of the fatty acid from the central carbon atom yields a prominent fragment (Fig. 14b).

Table 11 Major phospholipids of *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*; fatty acids confirmed in MS/MS experiments are printed in bold letters.

		Desulforhabdus amnigenus	*Desulfosarcina variabilis*
	m/z	side-chains	side-chains
PGM	705	**17:1**/O-15:0	-
	693	16:0/O-15:0	**15:0**/O-16:0
	679	15:0/O-15:0	tr
PG	747	**17:1**/17:0	tr
	733	tr	16:1/17:0
	721	tr	**16:0**/16:0
	719	tr	**16:1**/16:0
	707	tr	**15:0**/16:0
PEM	688	**17:1**/O-16:0	tr
	674	**17:1**/O-15:0	16:0/O-16:0
	662	tr	**15:0**/O-16:0
	660	16:1/O-15:0	tr
PE	716	**17:1**/17:0	tr
	714	17:1/17:1	tr
	702	**16:1**/17:0	**17:1/16:0**
	690	17:0/15:0	tr
	688	**17:1/15:0**	**16:1/16:0**
	676	tr	**15:0**/16:0
PCM	776	**17:1**/O-16:0	-
	762	**17:1**/O-15:0	-
PC	804	**17:1**/17:0	-
	802	17:1/17:1	-
	778	tr	16:0/16:0
	776	17:1/15:0	-
PI	821	**17:1**/16:0	-
	807	**17:1**/15:0 + **16:1**/16:0	-
	793	**17:1**/14:0	-

-: not detected; tr: minor component with unknown side-chains

In the two investigated strains of sulfate-reducing bacteria, phosphatidyl ethanolamine is the major phospholipid type (Fig. 15a, b). In *Desulfosarcina variabilis* minor amounts of phosphatidyl glycerols (PG) and traces of phosphatidyl cholines are present (Fig. 15b). In *Desulforhabdus amnigenus*, phosphatidyl inositols (PI) were detected besides smaller amounts of PG and PC (Fig. 15a). Whereas in *Desulfosarcina variabilis* phospholipids with ether-linked alkyl-chains account for 38% of all phospholipids, they dominate the phospholipid pattern of *Desulforhabdus amnigenus*, alkyl-acyl-PE being the most abundant compounds (34% of all phospholipids). Likely combinations of alkyl side-chains and fatty acid substituents of major phospholipids of the two investigated strains of sulfate-reducing bacteria are given in Table 11.

Fatty acid side-chains were determined by MS/MS experiments, numbers of carbon atoms in alkyl side-chains were calculated from "lyso fragments" (see Fig. 14). In *Desulfosarcina variabilis* only hexadecyl substituents were found, whereas HPLC-MS and -MS/MS data indicated the presence of penta- and hexadecyl ethers in *Desulforhabdus amnigenus*. Plasmalogens, i.e. alkenyl ether phospholipids, are not present in either one of these bacteria.

Whole-cell hydrolysis and fatty acid patterns

Polar lipid fatty acid (PLFA) patterns of both investigated strains closely match the fatty acid patterns obtained after whole-cell hydrolysis (Table 12). As hydroxy fatty acids were not detected among the PLFA, they were not included in this comparison. Major fatty acids of *Desulforhabdus amnigenus* are straight-chain saturated and monounsaturated $C_{15,}$ C_{16}, and C_{17} fatty acids. In *Desulfosarcina variabilis* mainly *n*-$C_{16:0}$, *ai*-$C_{15:0}$ and *n*-$C_{16:1\omega7}$ were found.

After whole-cell hydrolysis, glycerol monoethers were detected in both samples in addition to fatty acids liberated by saponification. These glycerol monoethers are hydrolysis products from mixed ether/ester phospholipids. In *Desulfosarcina variabilis* only one monoether, namely 1-O-hexadecyl glycerol, was found (identified by m/z 205 characteristic of 1-O-alkyl glycerols; Grönneberg and Albone, 1976). In *Desulforhabdus amnigenus* additionally 1-O-penta- and heptadecyl glycerols are present, which form a series of homologues indicating analogous branching in all three compounds. 2-O-Alkyl ethers could not be detected. The exclusive presence of 1-O-alkyl ethers is in accordance with the data obtained from HPLC-MS/MS analyses.

Table 12 Relative amounts of fatty acids in *Desulfosarcina variabilis* and *Desulforhabdus amnigenus* - comparison of whole-cell hydrolysis, transesterification of polar lipid fatty acid fraction and ether cleavage**.**

	Desulforhabdus amnigenus			*Desulfosarcina variabilis*		
Fatty acid	Whole-cell hydrolysis	PLFA	polar fraction after HI treatment	Whole-cell hydrolysis	PLFA	polar fraction after HI treatment
i-14:0	n.d.	n.d.	n.d.	0.6	0.4	n.d.
n-14:0	1.7	1.5	+	2.8	2.2	+
i-15:0	3.3	3.1	+	2.0	2.6	+
ai-15:0	0.1	0.4	n.d.	23	20	++
n-15:1ω6	1.6	1.6	n.d.	n.d.	n.d.	n.d.
n-15:1ω4	5.6	5.4	n.d.	n.d.	n.d.	n.d.
n-15:0	18	18	++	0.9	1.0	+
i-16:1ω6	n.d.	n.d.	n.d.	2.5	1.7	n.d.
i-16:0	n.d.	n.d.	n.d.	1.2	1.3	n.d.
n-16:1ω7	0.8	1.2	n.d.	16	17	n.d.
n-16:1ω5	10	10	n.d.	2.0	1.6	n.d.
n-16:0	7.2	7.2	++	32	35	++
i-17:1ω7	n.d.	n.d.	n.d.	3.1	4.1	n.d.
ai-17:1ω7	n.d.	n.d.	n.d.	6.5	5.6	n.d.
i-17:1ω5	5.9	6.2	n.d.	n.d.	n.d.	n.d.
ai-17:1ω5	0.2	0.2	n.d.	n.d.	n.d.	n.d.
i-17:0	0.3	0.4	n.d.	0.2	0.4	n.d.
ai-17:0	n.d.	n.d.	n.d.	1.6	1.8	n.d.
n-17:1ω8	1.5	1.7	n.d.	n.d.	n.d.	n.d.
n-17:1ω6	24	25	n.d.	n.d.	n.d.	n.d.
cyc-17	4.7	4.2	n.d.	n.d.	n.d.	n.d.
n-17:0	11	10	+	0.7	1.3	+
n-18:1ω9	0.8	0.5	n.d.	1.0	0.7	n.d.
n-18:1ω7	1.1	1.3	n.d.	2.1	0.8	n.d.
n-18:0	1.6	1.4	+	2.8	2.8	n.d.

n.d.: not detectable; ++: major compound, +: minor compound.

Ether cleavage

To confirm the presence of ether-bound alkyl side-chains in *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, ether bonds were cleaved using hydroiodic acid (HI). Resulting alkyl iodides were directly analysed by GC-MS without further reduction to

hydrocarbons by lithium aluminium hydride. The procedure was checked using dialkyl- and diacyl-PC standards and by running blanks. The mass spectrum of hexadecyl iodide obtained in the apolar fraction after HI treatment of dihexadecyl-PC can be seen in Fig. 16. In the case of dipalmitoyl-PC, the $C_{16:0}$ fatty acid was recovered in the polar fraction after "acid hydrolysis" by HI treatment.

In *Desulforhabdus amnigenus*, penta-, hexa- and heptadecyl iodides were detected in the apolar fraction after HI treatment, whereas in *Desulfosarcina variabilis* only hexadecyl iodide was present in amounts well above the background level (Fig. 17). These chain-length distributions obtained after ether cleavage are in good agreement with the results of whole-cell hydrolysis and HPLC-MS/MS analyses of intact phospholipids, although in the latter no significant amounts of O-heptadecyl phospholipids were detected in *Desulforhabdus amnigenus* (Table 11). As the hexadecyl iodide from the lipids of our bacteria has the same retention time in the GC-MS analysis as the *n*-hexadecyl iodide generated by HI treatment of the PC standard, it can be concluded that the two investigated strains of sulfate-reducing bacteria contain straight-chain alkyl substituents.

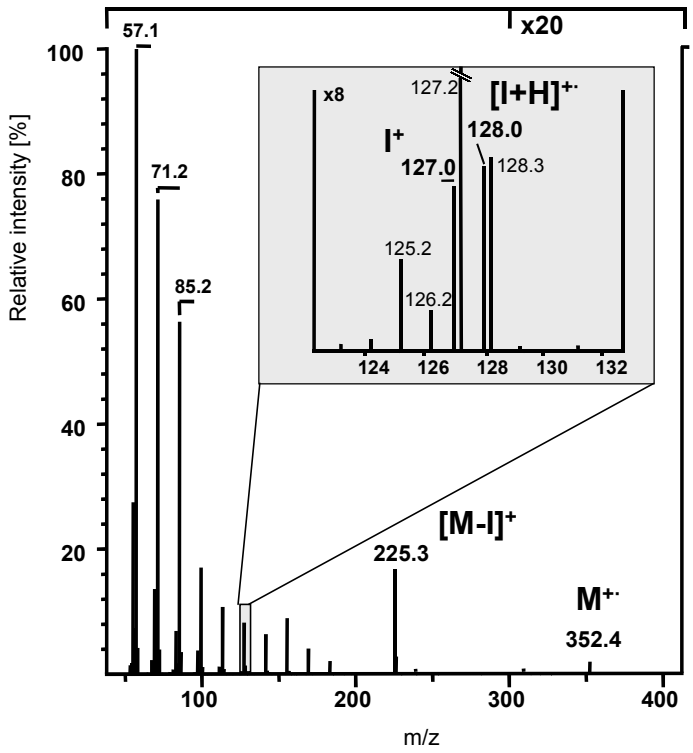

Fig. 16
Mass spectrum of hexadecyl iodide obtained in apolar fraction after ether cleavage of a di-O-hexadecyl-phosphatidyl choline standard; presence of monoisotopic iodide can be deduced from signals of $[I]^+$ with m/z 127.0 and $[I+H]^{+\bullet}$ with m/z 128.0 in addition to "normal" alkyl fragments $[C_9H_{19}]^+$ with m/z 127.2 (see area enlarged).

When distributions of fatty acids recovered in the polar fraction after HI treatment are compared to those after whole-cell hydrolysis (Table 12), the lack of unsaturated and cyclopropyl fatty acids in the former is striking, but can be readily explained by the fact

that double bonds and cyclopropyl rings are labile towards strong acid treatment (e.g. Vollhardt and Schore, 1998). The distribution patterns of all major saturated fatty acids remained unchanged by the ether cleavage procedure.

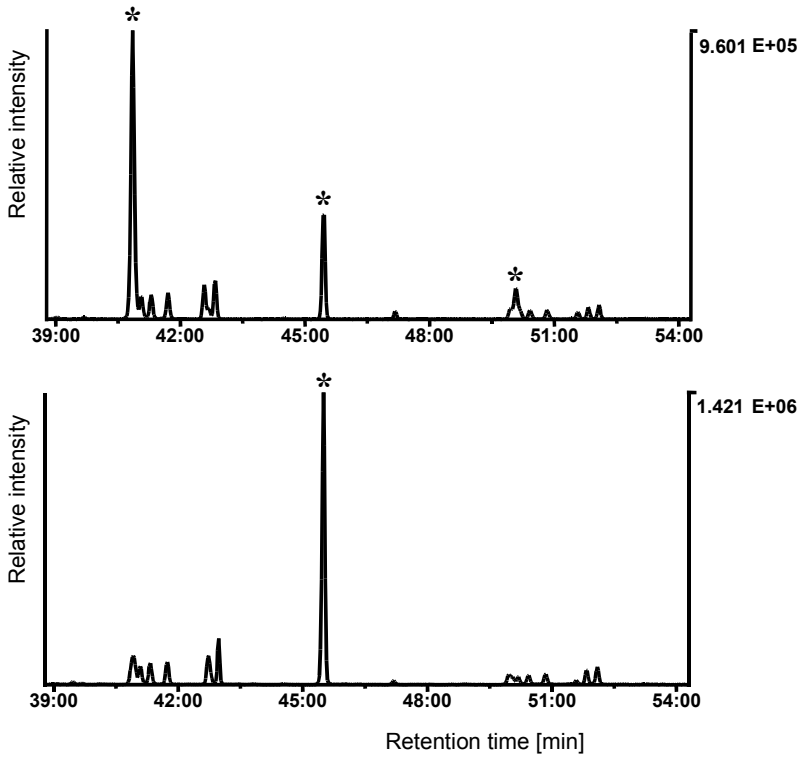

Fig. 17 Mass chromatograms (m/z 57+127) of apolar fractions after HI treatment of *Desulforhabdus amnigenus* (top) and *Desulfosarcina variabilis* (bottom). Peaks of *n*-penta-, *n*-hexa- and *n*-heptadecyl iodides are marked with asterisks.

3.3.5 DISCUSSION

Our fatty acid data for *Desulforhabdus amnigenus* are in good agreement with the PLFA pattern reported by Oude Elferink et al. (1998), although an unknown monounsaturated C_{18} fatty acid accounted for 14.5% of total fatty acids in their analysis, whereas we could only detect 1.3% of *n*-$C_{18:1\omega7}$ among the PLFA. Our polar lipid fatty acid pattern of *Desulfosarcina variabilis* is similar to that described by Kohring et al. (1994) although we found a much higher abundance of the *ai*-$C_{15:0}$ fatty acid. The occurrence of monoethers was not reported by either of these authors. The phospholipid patterns of *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, i.e. PE as most abundant phospholipid type with minor amounts of PG and other phospholipid types, is in agreement with that observed for many sulfate-reducing bacteria (Rütters et al.,

unpublished data) and other Gram-negative bacteria (Wilkinson, 1988). In our two strains of sulfate-reducing bacteria, alkyl-acyl-containing phospholipid types occur together with the corresponding diacyl phospholipids as it has been observed for plasmalogens as well (Goldfine and Langworthy, 1988).

Until present, the biosynthesis of large amounts of alkyl ether lipids seemed to be confined to archaea and the most deeply branching thermophilic bacteria (Gambacorta et al., 1994). The introduction of ether bonds into the membrane lipids may be advantageous for an organism living in extreme environments, because these bonds are chemically more stable than ester bonds, and they cannot be hydrolysed by lipases and phospholipases which are not specialised toward cleavage of ether bonds (Paltauf, 1994). Furthermore, introduction of ether phospholipids alters phase-transition behaviour of the lipid membrane and may therefore be involved in the adjustment of membrane fluidity and permeability to environmental parameters such as temperature changes (Goldfine, 1982).

The presence of significant amounts of mono- and dialkyl ethers in environmental samples (Orphan et al., 2001) and the detection of alkyl ether phospholipids in mesophilic sulfate-reducing bacteria (this study) show that these ether lipids may be more common membrane lipids of bacteria than previously thought. One reason for the few reports of alkyl ether lipids in bacteria may be related to the analytical procedures commonly employed: To analyse ether moieties from polar lipids by gas chromatography, either the ether bond has to be cleaved directly by strong acid treatment giving alkyl iodides or the phosphate/sugar headgroup (and the fatty acid side-chain) has to be removed by acid or strong alkaline hydrolysis yielding mono- or dialkyl glycerol ethers. Therefore, Langworthy et al. (1983) and Huber et al. (1992) detected alkyl ether lipids in hyperthermophilic bacteria after hydrolysis of extracted lipids with methanol/hydrochloric acid, whereas for investigation of PLFA patterns polar lipid fatty acids are usually transesterified under mild alkaline conditions as described by White et al. (1979), which will not remove the phosphate group. As the alkyl-acyl-phospholipids form a significant proportion of the total phospholipids of our investigated sulfate-reducing bacteria (38% and 47% in *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*, respectively) and possibly of other bacteria as well, sole analysis of polar lipid fatty acids in environmental samples may seriously underestimate viable biomass.

The occurrence of monoalkyl ethers with 14 to 18 carbon atoms in anoxic sediments of methane seeps (Orphan et al., 2001) may partly be explained by the degradation of bacterial ether phospholipids, e.g., from sulfate-reducing bacteria involved in the anaerobic oxidation of methane. Using 16S rRNA clone libraries Orphan et al. (2001) found that sequences related to *Desulfosarcina* were the most common phylotypes in their methane seep sediments. As the type strain *Desulfosarcina variabilis* only contains hexadecyl ether lipids, other (sulfate-reducing) bacteria seem to be involved in the methane oxidation process as well. In the closest relatives of *Desulfosarcina variabilis*, *Desulfonema limicola* and *Desulfococcus multivorans*, no phospholipids with ether-linked side-chains were detected (Rütters et al., unpublished results). The second strain of sulfate-reducing bacteria containing ether lipids, the freshwater strain *Desulforhabdus amnigenus*, is not closely related to *Desulfosarcina sp.* If its closest relatives, the *Syntrophobacter* spp., or representatives of other phylogenetic groups are capable of biosynthesising ether lipids, remains the subject of further research.

3.3.6 ACKNOWLEDGEMENTS

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4 MICROBIAL COMMUNITIES IN WADDEN SEA SEDIMENTS - CLUES FROM THE ANALYSIS OF INTACT PHOSPHOLIPIDS*

Heike Rütters, Henrik Sass, Heribert Cypionka, Jürgen Rullkötter

4.1 ABSTRACT

In this study various types of glyceride lipids were investigated in a 50 cm sediment core from an intertidal flat close to Neuharlingersiel, NW Germany. In particular, we focussed on the HPLC-MS(MS) analysis of intact phospholipids as biomarkers for viable microorganisms. Highest abundances of intact microbial lipids were found in the surface layer of the sediment, where phospholipid types and fatty acid patterns indicated a strong contribution of eukaryotic algae. Lipids of microorganisms appeared to be recycled or degraded rapidly within the uppermost 10 cm of the sediment as abundances of intact phospholipids and triglycerides rapidly decreased with depth. Also, the sedimentary contents of their degradation products (e.g. di- and monoglycerides, free fatty acids) showed a strong decline in the top layers. In the depth interval of 10-20 cm, phospholipid contents and species indicated a high abundance of bacterial biomass. In the deepest samples, intact phospholipids still were detected suggesting the presence of viable bacteria at a sediment depth of 50 cm.

* Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2001. Microbial communities in Wadden Sea sediments - clues from the analysis of intact phospholipids. Organic Geochemistry, submitted.

4.2 INTRODUCTION

Tidal flat areas are known to be highly dynamic systems in which settling particles are resuspended several times before ultimate sedimentation (e.g. Reineck, 1980). Mixing also takes place inside the sediments by the activities of burrowing animals (e.g. Aller, 1982; Riisgård and Banta, 1998). Quantity and quality of organic matter in the uppermost sediment layers depend on the supply from different sources, like marine algae or terrestrial plants, in the system studied here also eroded peat (Volkman et al., 2000). Endobenthic organisms like algae, protozoa and bacteria are additional sources of sedimentary organic matter (e.g. Gong and Hollander, 1997; Harvey and Macko, 1997; Postma, 1988).

Most of the early diagenetic transformation and degradation processes in the intertidal sediments are mediated by (micro-)organisms (Jickells and Rae, 1997; Postma, 1988). Many attempts have been made to study microbial communities in tidal flat sediments by cultivation-based approaches or molecular biological methods (Llobet-Brossa et al., 1998; Wieringa et al., 2000). Another common method to study microbial communities is the analysis of polar lipid fatty acid (PLFA) patterns (e.g. Bobbie and White, 1980; Findlay et al., 1989; Ringelberg et al., 1989; White et al., 1997). To determine PLFA patterns, lipid extracts are usually separated by column chromatography into fractions of different polarity. Phospholipids are eluted in the most polar fraction, the so-called polar lipid fraction (often also named phospholipid fraction). After hydrolysis of this fraction, resulting (polar lipid) fatty acids are analysed by gas chromatography (e.g. White et al., 1979; Guckert et al., 1985).

As Aries et al. (2001) could show using preparative thin-layer chromatography with a staining reagent specific for phosphate groups, sedimentary polar lipid fractions consist not only of phospholipids but also of other (non-phosphate-containing) polar lipids. Therefore, PLFA patterns obtained after hydrolysis may be distorted by a contribution of fatty acids from other polar lipids. In our opinion, the direct analysis of intact phospholipids provides a good tool to circumvent this problem. Additionally, analysis of intact phospholipids provides information about both fatty acids and phospholipid types which allows a better differentiation and identification of microorganisms (Fang et al., 2000a). Therefore, we developed an HPLC-MS(MS) method to analyse intact phospholipids from a fractionated sediment extract (Rütters et

al., 2001a, Chapter 2.1). The fractionation scheme also allows us to study fatty acid patterns in other types of glycerides in the sedimentary organic matter, e.g. triglycerides, as well as possible lipid degradation products such as di- and monoglycerides and free fatty acids.

As most of the easily degradable organic matter is generally believed to be recycled in surface sediments (e.g. Deming and Baross, 1993; van Es, 1984), many researchers have investigated microbes in the upper few centimetres of tidal flat sediments in the Wadden Sea area (Böttcher et al., 2000; Hamels et al., 1998). In contrast, very little is known about the communities and processes occurring in the deeper parts of the sediments and the microbes involved. Therefore, we investigated microbial communities and the fate of microbial lipids in a 55 cm sediment core taken from a nearshore tidal flat in the German Wadden Sea analysing different types of glyceride lipids by GC-MS and HPLC-MS/MS.

4.3 METHODS

Sediment samples

A sediment core (55 cm length) was taken in October 1998 from a tidal flat near the harbour of Neuharlingersiel, NW Germany. At the time of sampling, the sediment surface was largely covered by benthic diatoms. The top 3 cm of the sediment core were brownish in colour -representing the oxidised zone (e.g. Kristensen, 2000) - followed by a mixed/patchy brown and black layer. From 7 cm to a sediment depth of 33 cm, sediments were black (caused by presence of iron monosulfides, indicating the top of reduced zone) and showed a greyish colour (caused by presence of pyrite) for the rest of the core. Down to a depth of ca. 20 cm several individuals of different polychaetes were observed.

The top layers (0-1 cm and 1-5 cm) were sampled, and from 7 cm downwards the sediment was cut into 4 cm thick slices. All sections were deep-frozen within 6 h after sampling and stored at -20°C. Afterwards, aliquots of the samples were freeze-dried, sieved (<2 mm), and ground before extraction. Remaining sediment aliquots were used for pore water analysis (see 2.2). The organic carbon content was calculated as the

difference between total carbon (determined by combustion in a Leco CS-444 instrument) and inorganic carbon (analysed in an UIC CO2-coulometer).

The isotopic composition of bulk organic matter ($\delta^{13}C$ value) was analysed on an elemental analyser (Carlo Erba EA 1108) coupled to an isotope-ratio monitoring mass spectrometer (Finnigan MAT 252) via a Conflo II split interface after removal of carbonate by acid treatment. Values are quoted relative to the Vienna-PDB standard.

Grain-size distributions were determined using a laser particle sizer (Fritsch Analysette 22). Note that proportions of different grain size fractions are given as vol%. Median values were calculated from the vol%-based grain size distribution curves.

Activities of radionuclides in sediment samples were measured using a Canberra gamma spectrometer equipped with a germanium well detector. Lead excess (Pb_{xs}) values were calculated as difference between activities of ^{210}Pb and ^{214}Pb to account for supported lead production, i.e. ^{210}Pb originating from the decay of ^{226}Ra (from ^{238}U decay) in the sediment (Appleby et al., 1986; Appleby and Oldfield, 1992).

Pore water sampling

For pore water samples, wet sediments were squeezed over polycarbonate membrane filters (Isopore membrane filters, 0.4 µm HTTP, Millipore) by centrifugation using teflon inserts for the centrifuge tubes similar to the pore-water sampler described by Saager et al. (1990).

Concentrations of major elements in pore water samples were determined by X-ray spectrometry as described by Wehausen et al. (1999). By this method, the sum of dissolved sulfur species is measured and expressed as mg sulfate/l (then converted to mmol dissolved sulfur/l). Salinity values were measured using a conductivity electrode.

Extraction of sediment samples

The freeze-dried sediment samples (40 g) were ultrasonically extracted using a modified Bligh-Dyer procedure (Vancanneyt et al., 1996) with a solvent mixture of methanol/ dichloromethane/phosphate buffer (pH 7.4), 2:1:0.8 (v/v) (for details see Rütters et al., 2001a, Chapter 2.1).

Column-chromatographic separation

To separate major compound classes in which fatty acids may occur in the extracts, liquid chromatography on a silica gel column (2 g silica 60, 63-200 µm, Merck, Germany, dried at 110°C for 16 h) was carried out (Rütters et al., 2001a, Chapter 2.1). Fractions obtained contain the following compound classes: 1) aliphatic hydrocarbons, 2) wax esters, ketones, aldehydes, aromatic hydrocarbons, 3) triglycerides, triterpenoid ketones, mid-chain alcohols, 4) diglycerides, triterpenoid alcohols, *n*-alcohols, 5) free fatty acids, 6) monoglycerides, 7) glycolipids, 8) phospholipids.

Transesterification, gas chromatographic (GC-FID) and gas chromatographic-mass spectrometric (GC-MS) analysis

Aliquots of all fractions, except for fractions 1), 2), 4) and 5), were transesterified by mild alkaline hydrolysis as described by White et al. (1979). The methyl esters obtained were analysed by GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph) on a DB-5HT column (30 m x 0.25 mm, 0.1 µm film thickness, J&W) and by GC-MS using a Finnigan MAT SSQ 710B mass spectrometer. Free fatty acids and diglycerides in fractions 4) and 5) were transformed into their trimethylsilyl (TMS) ester derivatives using N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). The TMS derivatives were analysed by GC-FID and GC-MS as described above. Positions of double bonds were tentatively assigned by comparison with retention times of standards.

HPLC-MS and -MS/MS

Phospholipids were analysed on an Thermo Separation Products HPLC instrument coupled to a Finnigan LCQ ion trap mass spectrometer equipped with an electrospray source and to an evaporative light scattering detector (Alltech ELSD 500) using a flow-splitter (split ratio 0.88:1, with the larger flow feeding the ELSD). HPLC separation was achieved on a diol phase (Lichrospher 100 Diol 5µ, Merck) using a 2x125 mm column. A flow rate of 0.35 ml/min 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. Eluent A was a mixture of *n*-hexane/*i*-propanol/formic acid/ammonia (25% solution in water) 79:20:1.2:0.04 v/v, eluent B was *i*-propanol/

water/formic acid/ammonia (25% solution in water) 88:10:1.2:0.04 v/v . The mass spectrometer was set to the negative ion mode with a spray voltage of -4.5 kV and a capillary temperature of 200 °C. MS/MS experiments were done in the so-called dependent scan mode, i.e. the most intense quasi-molecular species of each full scan was automatically isolated and fragmented. For MS/MS experiments, an octapole voltage of 45 V had to be applied to prevent adduct formation with eluent components; helium was used as collision gas (relative collision energy: 35%). The light scattering detector was operated at a drift-tube temperature of 75°C using a nebulizer gas flow rate of 2.25 l N_2/min.

Mass spectra (full scan and MS/MS) were used for compound identification and determination of fatty acid substituents. Quantitation was done after external calibration of ELSD signals with different phospholipid standards: phosphatidyl glycerol, ethanolamine, choline, serine, inositol and diphosphatidyl glycerol (all purchased from Sigma Aldrich). Quantitation limits for standards ranged between 32 and 50 ng phospholipid (absolute amounts) for diphosphatidyl glycerol and phosphatidyl serine, respectively.

4.4 RESULTS AND DISCUSSION

4.4.1 BULK PARAMETERS

$^{210}Pb_{xs}$ contents and grain-size distributions

In case of undisturbed sediments with a constant rate of sedimentation, an exponential decrease of $^{210}Pb_{xs}$ contents with depth can be observed due to the decay of the radionuclide. Plotting of $^{210}Pb_{xs}$ contents on a logarithmic scale versus depth results in a straight line from which sedimentation rates and sediment ages can be calculated (Appleby and Oldfield, 1992). This technique was successfully applied before, e.g., to a microtidal mudflat in the Danish Wadden Sea area and to selected European estuaries (Andersen et al., 2000). In the depth profile of the Neuharlingersiel sediment core (Fig. 18), Pb_{xs} contents are highly variable, no clear trends evolve and no distinct zonation indicates the mixing depth. Therefore, it seems likely that supply of material to the sediment is neither constant nor continuous and that sediments are intensely reworked by bioturbation leading to the observed variability in Pb_{xs} values.

Grain-size distributions of sediments can also influence their Pb_{xs} contents (Andersen et al., 2000; Appleby and Oldfield, 1992). Therefore, the depth profile of median values of grain-size distributions is included in Fig. 18. Median grain sizes show values between 22 µm and 31 µm, i.e. sediments are dominated by coarse silt. Grain-size distribution curves of our sediments (data not shown) indicate that differences in median values are mainly caused by variation in coarse silt/fine sand rather than clay contents. "Clay" contents (grain size <8 µm when laser particle sizers are used according to Konert and Vandenberghe, 1997) are relatively constant throughout the core with values of 17-20 vol%. From the lack of any correlation of median values with Pb_{xs} contents, we conclude that grain-size distribution is not the main factor controlling Pb_{xs} contents of our sediments.

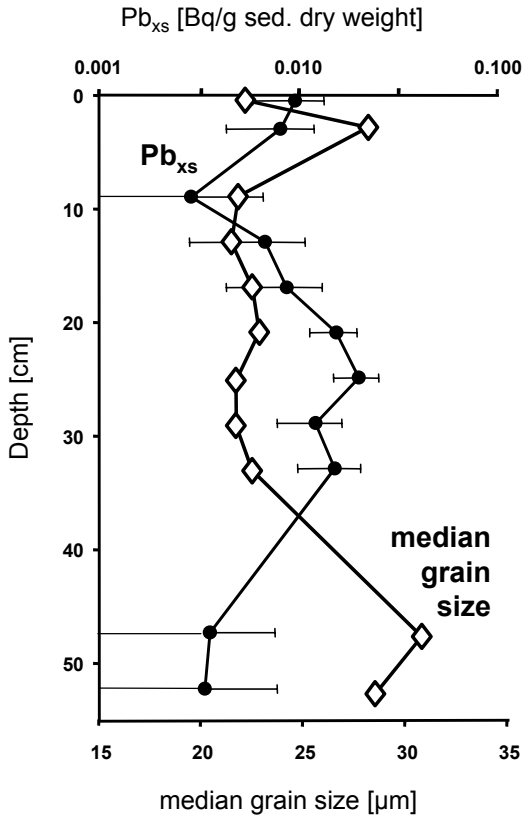

Fig. 18
Depth profiles of sediment Pb_{xs} data (black circles) and median grain size (open diamonds) for a sediment core from the NW German Wadden Sea.

Organic carbon contents and $\delta^{13}C$ values of bulk organic matter

Organic carbon contents of the investigated sediments are variable (Fig. 19), but generally low (<1% of sediment dry weight). In contrast to the sediment core taken nearby from an intertidal sandflat in the backbarrier area of Spiekeroog island, NW Germany, investigated by Volkman et al. (2000), organic carbon contents in our sediment core are only moderately correlated with the proportion of mud, i.e. grain size <63 µm (correlation coefficient r^2 = 0.63). Factors other than sorptive interactions between the sediment mineral matrix and the organic matter components appear to control TOC contents in the Neuharlingersiel sediments.

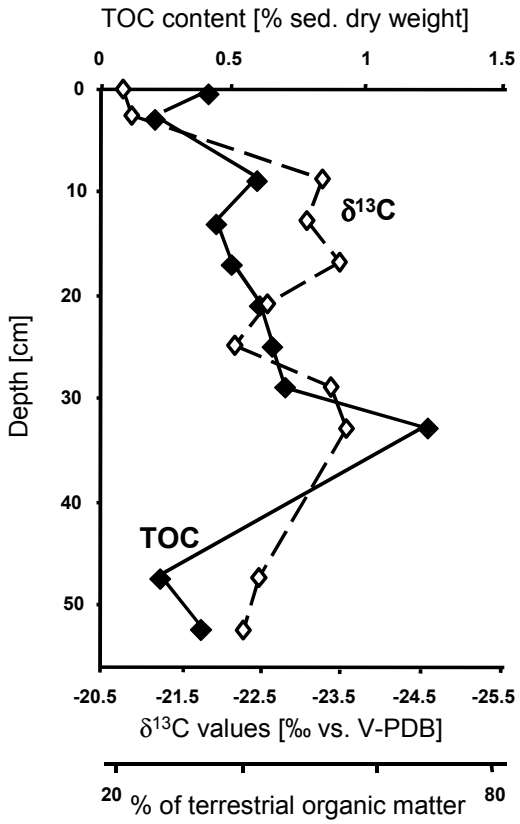

Fig. 19
TOC values (black diamonds) and bulk isotopic composition ($\delta^{13}C$ values) (open diamonds) for a sediment core from the NW German Wadden Sea. Proportions of terrestrial organic matter were estimated using a simple 2-component mixing model with values of -19‰ and -27‰ for the marine and terrestrial end members respectively (Volkman et al., 2000).

Carbon isotopic composition ($\delta^{13}C$ values) of bulk organic matter can also be seen in Fig. 19. The $\delta^{13}C$ values range between -22.1 and -23.5‰, probably representing a mixed signal of marine and terrestrial organic matter (Fry and Sherr, 1984), but a similar value of -23.8‰ was also determined for salt marsh plants of the Brittany coast (Dauby et al., 1998). In the uppermost samples of our Neuharlingersiel sediment core, isotopically heavier values of -20.7 and -20.8‰ were observed and reflect a

predominance of marine organic matter. Dauby et al. (1998) determined values between -15.1 and -22.5‰ for marine animals of an intertidal community with polychaetes/ crustaceans yielding intermediate values of -18‰; algae such as chlorophyceae and phaeophyceae gave values between -16‰ and -18‰. Similar values for chlorophyceae were also measured by Böttcher et al. (1997) on backbarrier tidal flats from Spiekeroog island. In our core, no sections with very light isotopic values (≤ -24‰) were found which would match those reported by Volkman et al. (2000) for a core taken nearby. Very light isotopic values would indicate a strong terrestrial influence on organic matter composition which in this environment is mainly caused by eroded fossil peat (Volkman et al., 2000).

4.4.2 PORE WATER CONCENTRATIONS OF DISSOLVED INORGANIC CONSTITUENTS

Pore water concentrations of total dissolved sulfur species (TDS) were relatively constant in the upper 25 cm of the sediment with a mean value of 25.9 ± 1.9 mmol/l and then started to decrease to 22.4 mmol/l at 36 cm sediment depth. Chloride concentrations as well as salinity values remained nearly constant throughout the core, showing values of 16-18 g chloride/l and salinity values around 29-31. Therefore, processes other than the dilution with ground water are responsible for the decrease of pore water TDS concentrations.

One month later, in November 1998, pore water samples were also obtained from deeper sections of another sediment core at the same location. In that core, TDS concentrations ranged from 30.7 mmol/l at the top and 19.8 mmol/l at 37 cm to 18.5 mmol at 50 cm sediment depth. In the November core, dissolved oxygen could only be detected in the top 3 mm of the sediment, whereas hydrogen sulfide was present from 15 cm downwards with values around 4 mmol sulfide/l at a sediment depth of 35 cm (Sass and Dröge, unpublished data). So "sulfate" concentrations (after correction of TDS values for sulfide concentrations) are still very high at a depth of 50 cm in comparison to the value of 3-4 mmol/l at which sulfate may become limiting for marine sulfate-reducing bacteria (Boudreau and Westrich, 1984).

4.4.3 BIOMARKERS

Free fatty acids

The vertical distribution of contents of free fatty acid groups can be seen in Fig. 20. A largely constant content of long-chain fatty acids with 23-34 carbon atoms and a strong even/odd carbon number predominance was found throughout the sediment core (values mostly vary between 3 and 8 µg/g sediment dry weight). These fatty acids probably stem from decaying cuticular waxes of higher land plant material, i.e. in this region from eroded peats. The presence of ω-hydroxy fatty acids with chain lengths of C_{20} - C_{28} in all samples (data not shown) underlines the strong influence of peat organic matter (Lehtonen and Ketola, 1993).

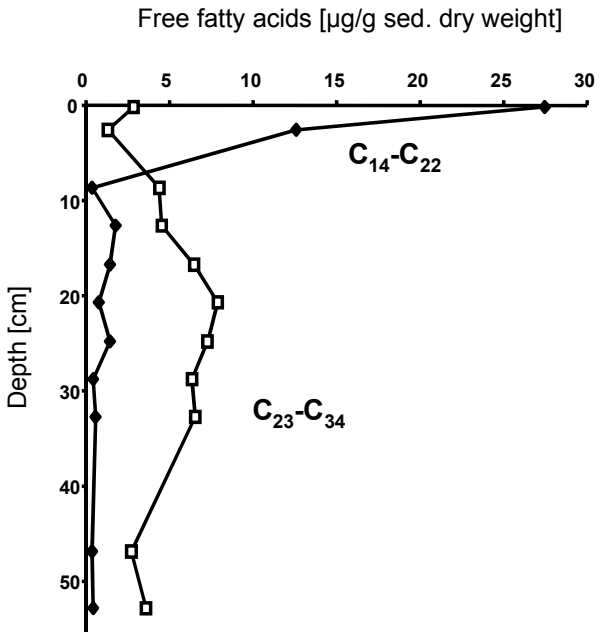

Fig. 20
Contents of different pools of free fatty acids and their variation with depth in a sediment core from the NW German Wadden Sea (C_{14}-C_{22} fatty acids include branched-chain and (poly-) unsaturated fatty acids; C_{23}-C_{34} fatty acids comprise only saturated, straight chain fatty acids).

In the two uppermost samples, high abundances, i.e. 28 and 13 µg/g sediment, of fatty acids with a chain length of 22 carbon atoms or less were observed. (Poly-) unsaturated C_{16}, C_{18}, C_{20} and C_{22} fatty acids are especially abundant, pointing to microorganisms as their source. As intact bacteria rarely contain significant amounts of free fatty acids and there are only a few reports of free fatty acids in algae (e.g. Goutx et al., 1990), we consider these "microbial" fatty acids as hydrolysis products of microbial lipids. From the low abundance of free fatty acids ($\leq C_{22}$) in the deeper sections of the sediment core, we conclude that the "microbial" fatty acids are recycled or rapidly degraded within the

upper few centimetres of the sediment. Free fatty acids with chain lengths between 14 and 22 carbon atoms comprise 49% of all quantified lipids in the Neuharlingersiel surface sediment (note that pigments were not included in our analyses). Such a high proportion of free fatty acids is indicative of *in situ*-degradation of microbial lipids according to Parrish (1988). Similarly, Baldi et al. (1997) found high proportions of free fatty acids during a mucilage phenomenon in the Adriatic Sea in which extensive cell lysis was observed.

Different residence times for the two groups of fatty acids (C_{14-22} and C_{23-34}) may be influenced by molecular properties such as water solubility and degree of unsaturation. In laboratory experiments under oxic and anoxic incubation conditions, Sun et al. (1997) found that ^{14}C-labelled polyunsaturated fatty acids are more rapidly decarboxylated in sediments than monounsaturated and saturated ones. Another factor controlling the fate of fatty acids may be the mode in which they occur in the sediments, i.e. their association with certain (resistant/inaccessible) components of the sediment matrix. The mode of occurrence within sediments is also claimed to be the main factor controlling the degradability of "unbound" fatty acids, i.e. fatty acids which can be liberated by alkaline hydrolysis from solvent-extractable lipids (Sun et al., 2000; Haddad et al., 1992).

Triglyceride fatty acids

High amounts (6.4 µg/g sediment dry weight) of the sum of "triglyceride fatty acids" (TGFA) were detected in the surface sediments (Fig. 21). Major fatty acids of this fraction are *n*-$C_{16:0}$, *n*-$C_{16:1\omega7}$ and $C_{20:5}$, with smaller contributions of *n*-$C_{14:0}$, *n*-$C_{15:0}$, *n*-$C_{18:1\omega9}$ and *n*-$C_{18:1\omega7}$ fatty acids. Polyunsaturated fatty acids with 16, 18 and 22 carbon atoms were also found among "TGFA". In the deeper sediment sections, the saturated *n*-C_{16} and *n*-C_{20} fatty acid dominate, whereas *n*-$C_{14:0}$, *n*-$C_{18:0}$, *n*-$C_{16:1\omega7}$ and *n*-$C_{18:1\omega9}$ fatty acids are present in smaller amounts. Only traces of polyunsaturated "TGFA" were observed in the deeper parts of the sediments.

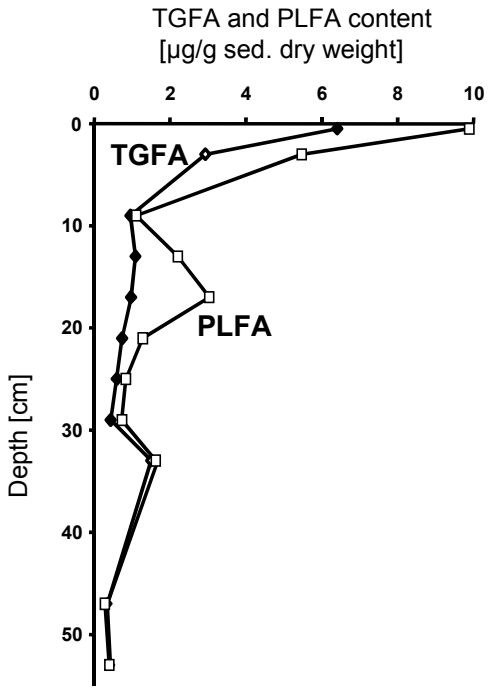

Fig. 21
Total "triglyceride fatty acids" (TGFA) (black diamonds) and "polar lipid fatty acids" (PLFA) (open squares) - variation with depth.

To ensure that fatty acid methyl esters (FAME) in "triglyceride fractions" are derived from triglycerides and not from other (e.g. sterol) esters of similar polarity, non-hydrolysed "triglyceride fractions" of the sediments were directly derivatised with MSTFA and analysed by gas chromatography. The absence of fatty acid methyl esters before hydrolysis and the lack of newly-generated alcohol components with high carbon numbers after hydrolysis, gives strong evidence that virtually all FAME found in the "triglyceride fraction" indeed come from triglycerides.

Triglycerides are major storage lipids of higher eukaryotes and are found in plant seeds or in animal adipose tissues (Thiele, 1979). They are also important constituents of micro-eukaryotes such as microalgae and protozoa like ciliates (Lechevalier and Lechevalier, 1988; Wood, 1988). Highly polyunsaturated C_{20} and C_{22} fatty acids, namely $C_{20:5\omega3}$ and $C_{22:6\omega3}$, are abundant in the total lipids of certain algae such as diatoms, dinoflagellates, eustigmatophytes and prymnesiophytes (Volkman et al., 1998; Mourente et al., 1990), but in algal triglycerides saturated and monounsaturated fatty acids are usually dominant (Sargent et al., 1987). For example, Kayama et al. (1987) could only detect a $C_{20:5}$ fatty acid among the triglyceride fatty acids of diatoms but not in dinoflagellates, although the latter contained this fatty acid in their polar lipids. (Poly-)unsaturated fatty acids with 16 or 18 carbon atoms occur in cyanobacteria, certain algae (Merritt et al., 1991; Volkman et al., 1998) and other eukaryotic organisms (e.g. Thiele, 1979; Erwin, 1973).

In the surface sediments of Neuharlingersiel, the high abundance of a $C_{20:5}$ fatty acid points to a strong contribution of algal triglycerides. The overall TGFA pattern of the surface sediments closely matches the fatty acid pattern described by Kayama et al. (1987) for the triglycerides of a marine diatom species. Diatoms were found to be the main primary producers on nearshore tidal flats close to Neuharlingersiel in 1994 and 1996 (Niesel, 1997), and they were also observed on the surface of our Neuharlingersiel sediments on the day of sampling.

Fatty acids with an odd number of carbon atoms and chain-branching were also detected among the TGFA in the surface sediments. These fatty acids are generally assigned to a bacterial origin (Kaneda, 1991; Boon et al., 1975), but only few bacterial groups, e.g. some marine *Pseudomonas* sp. (Goutx et al., 1990) and *Actinomycetes* (Lechevalier and Lechevalier, 1988), are known to synthesise triglycerides. On the other hand, many eukaryotes often contain trace amounts of branched fatty acids with an odd number of carbon atoms in their storage lipids (Thiele, 1979).

The TGFA pattern of samples from deeper parts of the Neuharlingersiel sediment is dominated by non-specific fatty acids, e.g. saturated and monounsaturated C_{16} and C_{18} acids besides *n*-$C_{20:0}$ (Harvey and Macko, 1997 and references therein; Sargent et al., 1987). Therefore, no direct information about their likely source organisms can be obtained. These TGFA may stem from microeukaryotes such as ciliates or heterotrophic flagellates which could still be detected at a sediment depth of 10 cm in an estuarine tidal flat in the Dutch Wadden Sea (Hamels et al., 1998).

Di- and monoglycerides

Intact 1,2- and 1,3-diglycerides with saturated and monounsaturated C_{16} and C_{18} fatty acids were found in the two uppermost samples (0.53 and 0.25 µg/g sediment dry weight in samples from 0-1 cm and 1-5 cm, respectively). In the sample from 11-15 cm depth, 0.09 µg diglycerides/g sediment were detected. In all these samples, the 1,2-diglyceride with a fatty acid combination of $C_{16:0}/C_{16:1}$ is the major component. Diglycerides are probably degradation intermediates from the hydrolysis of phospho- and glycolipids as well as from triglycerides although some bacteria were reported to contain diglycerides as cell components (Thiele and Kehr, 1969).

Further breakdown of glycerides may lead to the formation of monoglycerides. Significant amounts of fatty acid methyl esters were only detected after hydrolysis of

monoglyceride fractions of the uppermost samples. Their fatty acid patterns resemble those of the triglyceride fractions, with noteworthy higher abundances of a $C_{16:3}$ fatty acid.

Polar lipid fatty acids (PLFA) and glycolipid fatty acids

"Glycolipid fatty acids" accounted for 5% of total quantified lipids in the surface sample of our sediment core. In the deeper core sections, no significant amount of fatty acids could be detected in this fraction. (Diacyl-)glycolipids are abundant in plants, algae and green phototrophic bacteria and are often associated with their chloroplasts/ photosynthetic membranes (Smith, 1988; Wood, 1988). Glycolipids with ether-linked isoprenoidal side-chains are major polar lipids of archaea (Kates, 1997). The fatty acids pattern of our surface sediment "glycolipid" fraction is very similar to that of the triglyceride fraction with a higher abundance of the $C_{16:3}$ fatty acid. High proportions of $C_{16:3}$ were, e.g., found in the glycolipids of a diatom species (Kayama et al., 1987). Therefore, also the "glycolipid" fatty acid pattern indicates a high contribution of algal biomass to the surface sediment at Neuharlingersiel.

The depth profile of total "polar lipid fatty acids" is shown in Fig. 21. Similar to other types of glyceride lipids, highest contents of PLFA were found in the uppermost 5 cm of the sediment column. Between 10 and 20 cm sediment depth, PLFA contents are significantly higher than those of triglyceride fatty acid, which may indicate a high abundance of prokaryotes in that depth interval. Bacteria are known to have phospholipids as their major lipid class (e.g. Thiele, 1979), with minor amounts of triglycerides present in some groups (<10% of total cell lipids; Goutx et al., 1990). In marine phytoplankton relative amounts of triglycerides and phospholipids vary with growth stage and physiological condition of the algae with triglycerides being abundant in stressed or senescent cells (Volkman et al., 1998; Baldi et al., 1997).

In the surface sample, *n*-$C_{16:0}$, *n*-$C_{16:1\omega7}$, *n*-$C_{18:1\omega7}$ and a $C_{20:5}$ fatty acid are the most abundant compounds (Fig. 22). A series of polyunsaturated C_{22} fatty acids is also present in higher proportions than in the other glyceride fractions. Polyunsaturated fatty acids were not found in samples from deeper parts of the sediment (>5 cm), except for the "algal" $C_{20:5}$ fatty acid of which minor quantities were still detectable in sediments down to 20 cm depth. Transport of fresh algal material from the surface into the sediments by the activities of burrowing animals such as polychaetes may account for

the presence of that fatty acid in deeper parts of the sediment column (e.g. Blair et al., 1996). Polyunsaturated C_{22} fatty acids with 5 or 6 double bonds are commonly observed in different algae such as dinoflagellates, cryptomonads and haptophytes (Kayama et al., 1987), but high abundances of various $C_{22:x>2}$ fatty acids were also reported for marine copepods (Volkman et al., 1980).

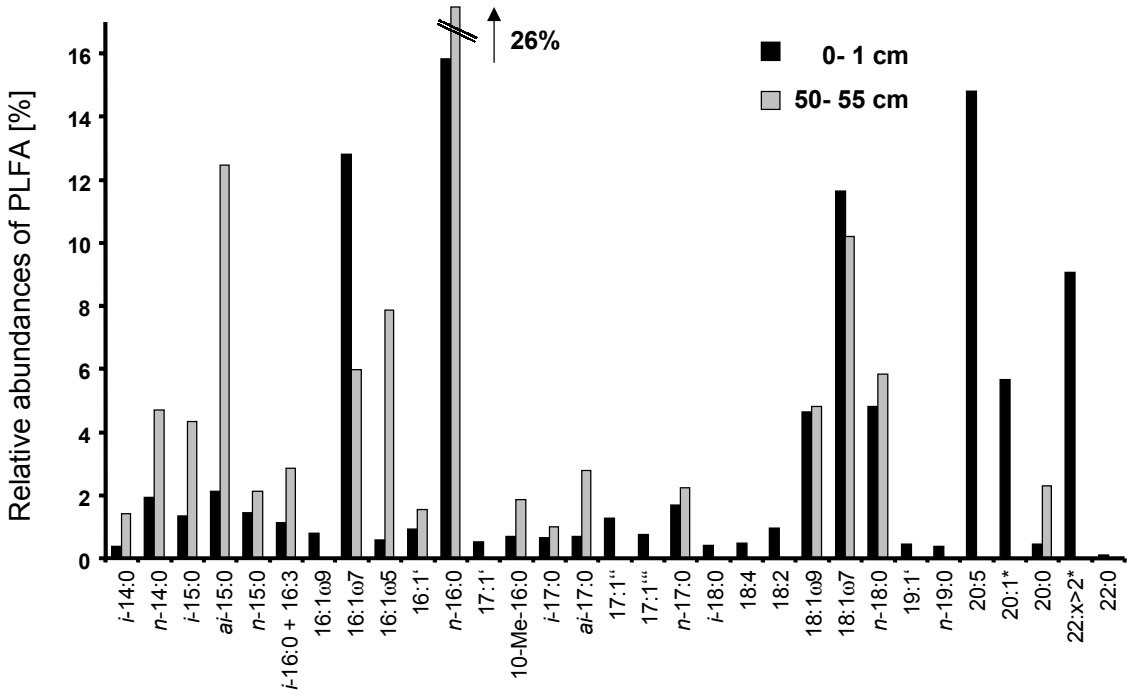

Fig. 22 Relative abundances of polar lipid fatty acids in the surface sample (0-1 cm; black bars) and the deepest sample (50-55 cm; grey bars). Dashes are used to distinguish between different fatty acids with unknown double bond position. Fatty acids marked with an asterisk represent the sum of different isomers.

In the surface sediment sample, traces of 10-methyl-$C_{16:0}$ fatty acid were detected in the PLFA fraction, whereas in the deeper parts of the sediment core this fatty acid accounted for 2-3% of total PLFA. The 10-methyl-$C_{16:0}$ fatty acid is considered to be indicative of *Desulfobacter* spp. (Taylor and Parkes, 1983; Dowling et al., 1986), but it was also found in *Desulfobacterium* spp. (Rütters et al., unpublished data) and in Actinomycetes (Balkwill et al., 1998). In a Danish coastal marine sediment (0-14 cm) investigated with slot-blot hybridisation, *Desulfobacterium* spp. formed the second most abundant group of sulfate-reducing bacteria, whereas signals for *Desulfobacter* spp. were below detection limit (Sahm et al., 1999).

Depth profiles of selected PLFA from our core are presented in Fig. 23. In the samples from the depth interval of 10 to 20 cm, high contents of fatty acids typical for

anaerobic bacteria, e.g. monounsaturated (ω7) C_{16} and C_{18} fatty acid (Gillan and Johns, 1986), were observed. More remarkably, the highest contents of the *ai*-$C_{15:0}$ fatty acid of the entire core occurred in that depth interval. Branched fatty acids are major fatty acids of some bacteria, e.g. of Gram-positive bacteria according to White et al. (1996), but the *ai*-$C_{15:0}$ fatty acid is also abundant in the sulfate-reducing bacteria *Desulfococcus multivorans* and *Desulfosarcina variabilis* (Rütters et al., 2001a, Chapter 2.1, and unpublished data). The presence of all these "bacterial" fatty acids gives strong evidence of bacteria being very abundant in that depth interval.

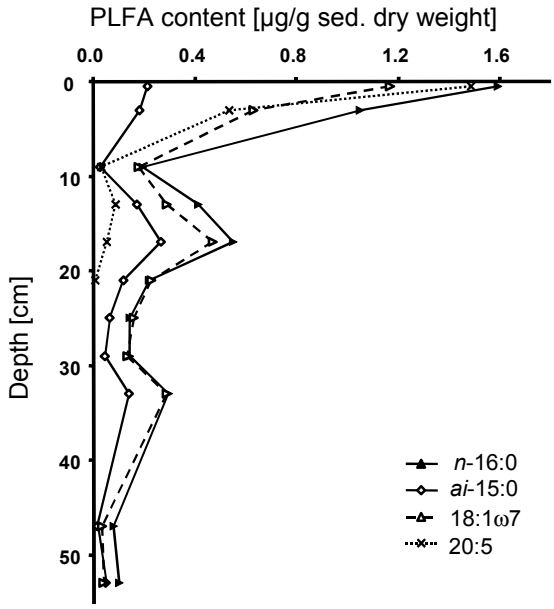

Fig. 23
Depth profiles of contents of selected fatty acids in the polar lipid fraction.

At a sediment depth of 50 cm, the saturated *n*-C_{16} fatty acid is the most abundant PLFA (Fig. 22). Besides monounsaturated fatty acids with 16 and 18 carbon atoms, relatively high amounts of branched C_{15} and C_{17} fatty acids were found with an especially high abundance of *ai*-$C_{15:0}$. Those "bacterial" polar lipid fatty acids point to the presence of viable bacteria at that depth (ca. 50 cm). The $C_{16:1\omega5}$ fatty acid may be indicative of *Cytophaga*-like bacteria (White et al., 1996).

Phospholipid analysis

A comparison between amounts of polar lipid fatty acids and contents of intact phospholipids in the sediments can be seen in Fig. 24. Both depth profiles show high contents at the surface and a sharp decline with depth. This is also the case when PLFA and phospholipid amounts are normalised to sedimentary organic carbon contents. Therefore, viable biomass represents a larger proportion of sedimentary organic matter at the surface (phospholipids: 5.8 mg/g TOC; PLFA: 2.5 mg/g TOC) than in the deeper sediment sections (>23 cm) where more refractory organic matter seems to be present (phospholipids: 0.2 mg/g TOC; PLFA: 0.1 mg/g TOC).

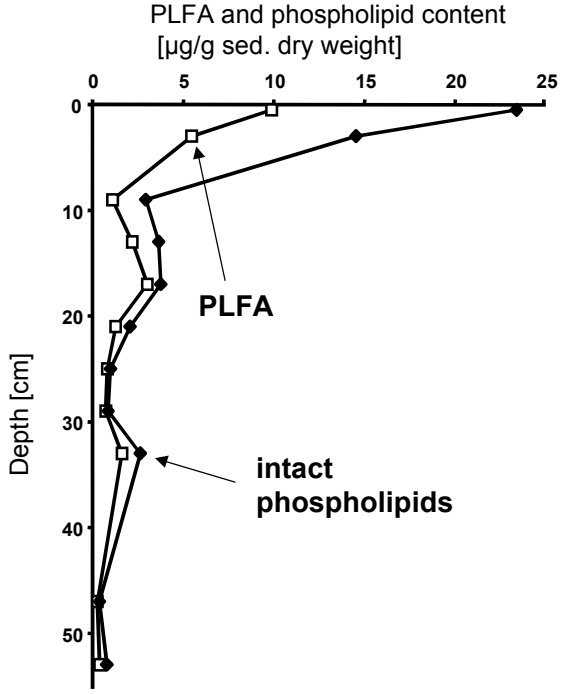

Fig. 24
Comparison of phospholipid contents as determined by PLFA analysis (open squares) and direct analysis of intact phospholipids (black diamonds).

Measured contents of intact phospholipids are always higher than PLFA values (Fig. 24). Assuming a molecular mass of, e.g., 722 g/mol for an "average" phospholipid with two $C_{16:0}$ fatty acid side-chains (molecular mass 256 g/mol each), a conversion factor of 1.3 can be calculated from the weight difference between intact phospholipids and their "PLFA". Especially at the surface, the difference between phospholipid and PLFA content is larger than expected from their weight difference. The presence of ether lipids, such as protozoan or archaeal/bacterial ether lipids (Paltauf, 1994), may in part account for that difference, because phospholipids with ether-bound side-chains

would contribute to the phospholipid pool, but would not yield fatty acids on alkaline hydrolysis. HPLC-MS analysis of intact phospholipids also indicates the presence of ether lipids with non-isoprenoidal side-chains in the surface samples (Fig. 25). In some sections of the deeper parts of the sediment core, PLFA and phospholipid content curves merge which may be caused by a contribution of non-phospholipid fatty acids derived from other polar compounds to the PLFA (Aries et al., 2001).

Table 13 Total cell counts (determined by acridine orange direct counting) and microbial cell numbers (calculated from PLFA and phospholipid contents)

Sediment depth for PL and PLFA analysis	PL [µg/g sed. dry weight]	PLFA [µg/g sed. dry weight]	calculated cell numbers* [cells/g sed. dry weight]	total cell counts [cells/cm^3]	Sediment depth for total cell counts
0-1 cm	23	10	22 x 10^8	35 ± 9 x 10^8	0 - 0.5 cm
1-5 cm	14	5	12 x 10^8	7 ± 4 x 10^8	5 - 5.5 cm
50-55 cm	0.8	0.4	0.9 x 10^8	2 ± 1 x 10^8	50 - 50.5 cm
abundance (0.5 cm)/ abundance (50 cm)	29	26	-	18	

*: calculated after Rajendran et al. (1992) with an average content of 100 µmol PLFA/g bacteria dry weight and a conversion factor of 5.9x10^{12} cells/g bacteria dry weight and assuming an average molecular weight of 270 g/mol for the PLFA.

In Table 13, a comparison is made between total cell counts (cells/cm^3 sediment) determined after staining with acridine orange for sediment depths of 0.5 cm, 5 cm and 50 cm and cell numbers calculated from PLFA and phospholipid contents (cells/g sediment). Applying conversion factors used by Rajendran et al. (1992), counted and calculated cell numbers agree reasonably well, taking into account the different normalisations (g^{-1} and cm^{-3}), the assumptions made in conversion of PLFA contents to cell numbers and the standard deviation of the acridine orange direct counting method. Differences in the decline of biomass - expressed as the ratios of PLFA or phospholipid abundances or of cell counts at depths of 0.5 cm and 50 cm - may be explained by either a high abundance of phototrophic microorganisms at the surface or a significant proportion of spores in the deeper sediment parts. Phototrophs would contribute to the phospholipid pool of the surface sediment, but would not be included in the total cell counts due to their autofluorescence. In contrast, spores can be stained with acridine

orange (data not shown) and therefore would be counted as bacterial cells. The spores may not contribute, however, to the phospholipid pool, because they contain only minor amounts of phospholipids (Madigan et al., 1999), and they may also be resistant to the extraction method employed (Macnaughton et al., 1997). Differences in cell volumes and hence in phospholipid contents between bacteria living at different depth intervals in the sediments may also be important.

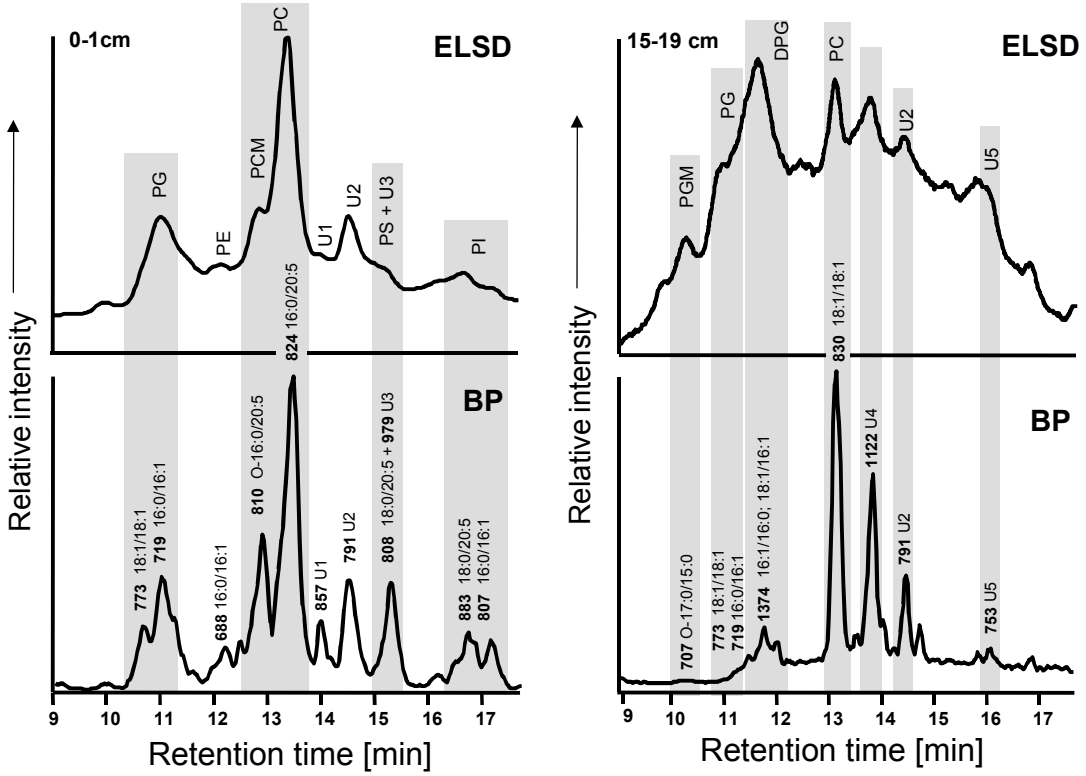

Fig. 25 HPLC-ELSD chromatogram (top) and base peak (BP) plot from HPLC-MS analysis (bottom) of phospholipid fraction of a surface sediment sample (0-1 cm) (left) and a sediment sample from a depth of 15-19 cm (right). Phospholipid types are denoted as follows: PG: phosphatidyl glycerol; DPG: diphosphatidyl glycerol; PE: phosphatidyl ethanolamine; PCM: phosphatidyl choline probably with ether - and ester-linked side-chains (alkyl substituents are marked by "O-" followed by numbers of carbon atoms and double bonds); PC: phosphatidyl choline; PS: phosphatidyl serine; PI: phosphatidyl inositol; U1 to U5: unknown polar lipids. In the base peak plot mass/charge ratios of most abundant quasi-molecular ions and fatty acid side-chains are given. Fatty acid side-chains are assigned according to fragmentation behaviour in MS/MS experiments.

In Fig. 25, HPLC-ELSD chromatograms and intensities of most abundant quasi-molecular ions from HPLC-MS analysis (so-called base peak plot) of phospholipid fractions are displayed for the surface sample (0-1 cm) and for the sediment section

from 15-19 cm. HPLC conditions were optimised to separate phospholipids according to their headgroups, i.e. to distinguish between different phospholipid types. At the surface, contents of phospholipids are generally higher, with phosphatidyl glycerol (PG) and phosphatidyl choline (PC) being the most abundant phospholipid types with minor contributions of diphosphatidyl glycerol (DPG), phosphatidyl ethanolamine (PE), serine (PS) and inositol (PI). Furthermore, a significant amount of possibly mixed phosphatidyl cholines with ester and ether side-chains (marked as PCM) were found. PCM were tentatively identified by comparison of retention times and fragmentation behaviour with those of standard substances, i.e. dipalmitoyl-, di-O-hexadecyl- and O-hexadecyl-palmitoyl-phosphatidyl choline.

PC, PS and PI are often found in eukaryotes (Mangnall and Getz, 1973) although they were also reported to occur in some bacteria (Lechevalier and Lechevalier, 1988). In the base peak plot, mass-to-charge ratios of the most abundant quasi-molecular ions are attributed to their likely fatty acid combination as determined by MS/MS experiments. The most abundant molecular species of PC (and PCM) as well as of PS and PI in the surface sample are substituted with the "algal" 20:5 fatty acid supporting the eukaryotic origin of these phospholipids. Also PG with a fatty acid combination of 16:0/20:5 and 16:1/20:5 were detected in this sample. In the marine diatom *Navicula incerta*, PG, PC and PI are the major phospholipid types with minor amounts of diphosphatidyl glycerols (DPG) and PE being present (Kayama et al., 1987). Therefore, diatoms appear to be major contributors to microbial communities in the Neuharlingersiel surface sediment. The high amount of PG with C_{16} and C_{18} side-chains may indicate a contribution of other phototrophs, because PG is the major phospholipid type of cyanobacteria (Nordbäck et al., 1998) and green algae (Wood, 1988) both of which do not contain C_{20} fatty acids (e.g. Volkman et al., 1989; Merritt et al., 1991). The proportion of prokaryotic biomass (excluding the phototrophs) appears to be small in the surface sample, because main phospholipid types of bacteria like PE and DPG (O'Leary and Wilkinson, 1988; Wilkinson, 1988; Goutx et al., 1990) are only present in minor amounts. As phospholipid contents reflect cell volumes, smaller (bacterial) cells can only make a minor contribution to an overall biomass dominated by larger (algal) cells, even if bacteria and algae were present in equal numbers.

The presence of non-isoprenoidal, ether-linked alkyl substituents provides additional evidence of a eukaryotic contribution to the viable biomass, because they occur mainly in combination with C_{20} or C_{22} polyunsaturated fatty acids. Plasmalogens

with an alkenyl side-chain were not discovered in our surface sediments. In the literature, the presence of plasmalogens is reported for protozoa and some anaerobic bacteria (Snyder, 1972; Mangnall and Getz, 1973; Lechevalier and Lechevalier, 1988; Paltauf, 1994), but little information is given about alkyl-ether lipids in microorganisms. One reason for this could be related to problems in analysing these ether side-chains which are more difficult to hydrolyse than the enol ethers of plasmalogens (e.g. Thiele, 1979). Direct (mass spectrometric) analysis of intact phospholipids could circumvent this problem.

In the deeper parts of the sediment core (>10 cm), the HPLC-ELSD chromatograms reveal a broad hump which can be resolved using the base peak plot (see Fig. 25). In the sample from 15-19 cm sediment depth as well as in the other samples, phosphatidyl cholines, phosphatidyl glycerols and diphosphatidyl glycerols were detected. In addition, a significant number of different unknown compounds (U_X) is present. Phospholipid types and fatty acid substituents indicative of eukaryotes could not be found anymore. In PC, the most abundant combination of fatty acid substituents is 18:1/18:1 which probably reflects a bacterial origin of those PC. Additionally, alkyl-acyl-PG (PGM) were found in these deeper parts of the sediments with O-17:0/15:0 being the most abundant side-chain combination in the depth interval from 15-19 cm. In this depth interval, contents of PGM, PG and DPG were increased relative to the sediment sections above and below. These results support the idea that a high bacterial biomass is present in the sediment section from 10 to 20 cm in which significantly more PLFA than TGFA were found (see 3.3). Surprisingly, only traces of phosphatidyl ethanolamines, which are the major phospholipid type of many sulfate-reducing bacteria (SRB) (Rütters et al., 2001a, Chapter 2.1, and unpublished results), were detected below the surface sediments, although the pore water sulfate profile indicates activity of SRB in the depth interval between 20 and 30 cm. Similarly, Llobet-Brossa et al. (1998) found highest abundances of SRB (6.5% of total cell counts) at a sediment depth of 2 cm, followed by a 3-fold decrease within then next few centimetres in an estuarine mudflat in the Jade Bay, NW Germany.

4.5 SUMMARY AND CONCLUSIONS

Tidal flat sediments in the Wadden Sea area of NW Germany are well mixed systems with a strong contribution of peat material to the sedimentary organic matter. Despite the strong mixing, sharp gradients were observed in the depth profiles of marker molecules for intact microorganisms, such as intact phospholipids and PLFA, and also of total cell counts. This indicates that microbial communities in tidal flat sediments which form these gradients readjust rapidly after perturbation of their sedimentary environment.

The sharp decrease with depth in abundances of intact glyceride lipids and their hydrolysis products points to a rapid recycling or degradation of these microbial lipids within the upper 10 cm of the sediment.

In the depth interval between 10 and 20 cm, higher abundances of phospholipids relative to other types of glycerides were found, which indicate a high bacterial biomass at that depth. The presence of phosphatidyl choline with two $C_{18:1}$ fatty acid substituents as most abundant phospholipid species supported the dominance of bacterial biomass at that depth interval. Therefore, by structural analysis of intact phospholipids more detailed information could be obtained about the microbial communities present at various sediment depths than by PLFA or phospholipid type analyses alone.

At a sediment depth of 50 cm, intact phospholipids were still detectable and indicate the existence of viable microorganisms at greater depth. Because "fresh" and therefore easily metabolisable organic matter appears to be consumed already within the uppermost centimetres of the sediment, it remains unknown what fuels the microbial communities in the deeper parts of the sediments. This will be the subject of further research.

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5 SUMMARY AND CONCLUDING DISCUSSION

In this thesis, the applicability of intact phospholipids as biomarkers to study microbial community composition was evaluated. As a first step, a protocol was developed to analyse intact phospholipids and other types of glycerides from pure cultures of bacteria and complex environmental samples such as sediments. This protocol involved a) extraction of the sample using a modified Bligh-Dyer procedure, b) separation of the extract into eight fractions containing different types of glycerides using column chromatography on silica gel, c) analysis of different fractions directly using HPLC-ESI-MS or, if necessary after transesterification, by GC-FID and GC-MS.

As a validation, 30 strains of sulfate-reducing bacteria were analysed for their lipid contents and their fatty acid compositions. Fatty acid patterns are important for chemotaxonomical classification of new bacterial isolates. Accordingly, it was shown in this thesis, that the fatty acid pattern of a sulfate-reducing bacterium isolated from a deep-sea sediment in the western Mediterranean Sea by A. Sass supported its classification as a new member of the genus *Desulfobulbus*.

All non-sporulating sulfate reducers investigated contained very similar phospholipid types, namely phosphatidyl ethanolamine (PE) as the major compound with smaller contributions of phosphatidyl glycerol (PG) and diphosphatidyl glycerol (DPG); in three strains additionally phosphatidyl choline was present. The two investigated Gram-positive sulfate reducers possessed very distinct phospholipid types. Therefore, high abundances of PE with smaller amounts of PG and DPG appeared to be a "typical" phospholipid pattern for sulfate-reducing bacteria. Unfortunately, these phospholipid types are also encountered in various other Gram-negative bacteria (Wilkinson, 1988). Therefore, not only information about phospholipid types, but also about fatty acid combinations present in the different phospholipid types has to be used as biomarkers to trace sulfate-reducers in environmental samples. The discriminatory power of phospholipid molecular species for chemotaxonomic classification of sulfate-reducing bacteria was compared to that of fatty acid patterns after whole-cell hydrolysis and the phylogenetic relatedness of these organisms. Similarities of both, fatty acid patterns as well as phospholipid molecular species, were investigated by cluster analysis. The clustering obtained by this statistical treatment matched the phylogenetic relatedness of the sulfate reducers investigated. Therefore, analysis of phospholipid

molecular species combines features that most sulfate reducers have in common, i.e. phospholipid types, with a high resolving potential to distinguish between sulfate reducers and other Gram-negative bacteria and to differentiate between groups of sulfate reducers.

Additionally, the analysis of intact phospholipids allows not only the detection of ester-linked fatty acid side-chains, but also of ether-linked fatty alcohols and vinyl ether-linked aldehydes. Thereby, the variety of different phospholipid molecular species that can be used as biomarkers, is enhanced. In two sulfate-reducing bacteria, *Desulfosarcina variabilis* and *Desulforhabdus amnigenus,* ether-bound alkyl side-chains were detected, whereas *Desulfovibrio desulfuricans* CSN contained alkenyl ethers (plasmalogens). The presence of the former was confirmed by chemical degradation with hydroiodic acid and analysis of the resulting alkyl iodides. From the latter, hydrolysis products, i.e. dimethylacetals, were observed after whole-cell hydrolysis. The presence of straight-chain alkyl-ether lipids in mesophilic sulfate-reducing bacteria such as *Desulfosarcina variabilis* is remarkable, because these compounds had so far only been detected in deeply branching hyperthermophilic bacteria. On the other hand, their hydrolysis products, monoalkyl glyceryl ethers, had been reported from various "cold" environments like the sediments overlying methane seeps (Hinrichs et al., 2000, Orphan et al., 2001). From the carbon isotopic composition, these authors concluded that the alkylether glycerides may derive from organisms involved in the anaerobic oxidation of methane. Therefore, the finding of alkylether phospholipids in non-thermophilic sulfate-reducing bacteria may be one additional hint for the involvement of sulfate reducers (or maybe even of *Desulfosarcina* sp. ?) in the anaerobic oxidation of methane. Strong evidence for the involvement of *Desulfosarcina*-related sulfate reducers in the anaerobic methane oxidation has indeed been given by Boetius et al. (2000) using *in situ* hybridisation, but clearly further research is needed to unambiguously assign the source of the monoalkylether lipids present in sediments adjacent to methane seeps.

The usefulness of phospholipids as biomarkers to study microbial communities was demonstrated in this thesis for sediment samples from two different tidal flats. Firstly, a sandy surface sediment from the island of Mellum was studied. Here, phospholipid types and fatty acids present in the different types of glycerides indicated a high contribution of cyanobacteria and eukaryotic algae to the overall biomass. High

contents of free fatty acids pointed to a strong degradation of microbial lipids. Solely among the free fatty acids, compounds with a chain length $>C_{22}$ were detected which presumably derived from decaying matter of higher land plants.

Sediments and their microbial communities were studied in more detail at the Neuharlingersiel site, revealing

- highest abundances of microorganisms in the top layer as indicated by highest contents of phospholipids; here, phospholipids substituted by a highly polyunsaturated fatty acid (i.e. $C_{20:5}$) presumably of algal origin dominated;
- rapid recycling of microbial lipids in the top 10 cm inferred from a rapid decrease with depths of intact lipids (phospholipids, triglycerides) and their degradation products (di- and monoglycerides, free fatty acids);
- high content of prokaryotic biomass at a depth of 10-20 cm as deduced from the much higher abundance of phospholipids relative to triglycerides in that interval; here, phospholipids indicated a predominance of bacterial biomass;
- presence of intact phospholipids also at 50 cm sediment depth, phospholipid content was only ≈1/30 of that in the top layer;
- relatively constant porewater sulfate concentrations in the upper 20 cm and a steady decrease further downcore giving values of ca. 19 mmol/l at 50 cm sediment depth, i.e. no sulfate limitation at that depth.

Stefan Dröge investigated a parallel sediment core from Neuharlingersiel in his diploma thesis (Dröge, 2000) using microbiological techniques. The following results about the microbial communities present at different depths are important for the interpretation of biomarker data:

- Highest total cell numbers were counted at the surface. This number declined rapidly with depth (i.e. decreased from 0.5 to 50 cm by a factor of 18). In October 1998, only three depth intervals were counted, i.e. 0.5, 5 and 50 cm sediment depth.
- Even at a sediment depth of 50 cm, significant potential activities of extracellular enzymes were measured, indicating the presence of an active microbial community.
- At 50 cm depth, MPN experiments after pasteurisation indicated a high relative proportion of spores, accounting for 38% and 22% of bacteria that could be cultured

under oxic and anoxic incubation conditions, respectively, using a "universal" medium.

These results from microbiological investigations and those from biomarker studies point in the same direction, indicating highest microbial abundances at the surface, but also viable, potentially active cells at a sediment depth of 50 cm. Unfortunately, microbiological investigations were only carried out at three depth intervals, so that no further information about the microorganisms that inhabited the depth interval between 10 and 20 cm is available (a depth, at which very high phospholipid contents were observed).

In the deeper parts of the sediment a high proportion of spores was observed. Spores are very durable resting states, which some bacteria can form when environmental conditions become unfavourable for growth. These spores are very resistant to heat and dryness and can persist for thousands of years without loss of viability (Gest and Mandelstam, 1987). Therefore, a high abundance of spores indicates that relatively more inactive bacteria are present at a depth of 50 cm. The detection of spores supports one possible explanation for the discrepancy in the decline of phospholipid contents and total cell counts from the surface to 50 cm. As spores may be stained by acridine orange (Sass and Rütters, unpublished results), they will contribute to the total cell counts of the 50 cm sample. On the other hand, spores have only a small phospholipid content and, additionally, extraction of their phospholipids is inefficient unless more vigorous conditions are chosen such as extraction under elevated temperature and pressure, e.g., in an accelerated solvent extractor (Macnaughton et al., 1997).

Further investigations of the diversity of microbial communities at different depths revealed (Dröge, 2000):

- Molecular techniques indicated a high number of different bacterial populations with comparable abundances throughout the sediment core. "Molecular fingerprints" of samples from 0.5 and 5 cm depth were surprisingly similar.
- MPN experiments revealed a generally higher cultivation efficiency under oxic relative to anoxic conditions, but whereas the cultivation efficiency under oxic conditions decreased with sediment depth, it increased under anoxic incubation.

- A total of 35 strains were isolated from three different depth intervals of the sediment. Most of the bacteria isolated under oxic conditions could also grow anaerobically and vice versa. Among the "true" anaerobes two sulfate reducers were obtained (isolated from 5 cm sediment depth on volatile fatty acids).

The high number of facultative organisms, i.e. bacteria that can grow under oxic as well as under anoxic conditions, probably reflects a great physiological versatility that may be advantageous for organisms to survive in such highly dynamic, strongly mixed sediments with changing water coverage and variable oxygen penetration depth.

6 OUTLOOK

Analysis of intact phospholipids with HPLC-MS appears to be a very promising tool to study microbial communities. Recently, Lytle et al. (2000) established a method for analysis of intact phospholipids in a soil extract and applied it to study transport of bacteria with per-^{13}C labelled phospholipids in subsurface environments (Lytle et al., 2001).

Because HPLC-ESI-MS is a relatively new technique, literature data on phospholipid molecular species of different microorganisms – eukaryotes and prokaryotes – are still scarce. Therefore, to improve the correlation between phospholipids detected in the sediment and the organisms they come from, a larger data set is needed including bacterial isolates from different physiological and phylogenetic groups, but also eukaryotic microorganisms such as benthic algae, ciliates, nematodes and gastropods which inhabit the sediments under investigation. Additionally, examining the depth distributions of different microeukaryotes may help to explain phospholipid and fatty acid depth profiles, e.g. the presence of the "algal" $C_{20:5}$ fatty acid in the polar lipid fraction down to a depth of 20 cm.

To investigate microbial communities in the deeper parts of the sediments (≥50 cm), the analytical procedure has to be further optimised to improve detection limits. The presence of non-phospholipid material in the polar lipid fractions is obvious in the deeper samples, as HPLC chromatograms display a rather broad hump. This non-phospholipid material may be composed of (humic) substances with higher molecular weights, but similar polarity. An indication for the presence of humic-like substances is the intense yellowish/brownish colour of the polar lipid fraction. If these non-phospholipid compounds are of higher molecular weights than phospholipids, then one possible further clean-up step could be a size fractionation, e.g. by size-exclusion chromatography (cf. Tornabene, 1985).

In general, microbiological and geochemical techniques complement each other very nicely. Combining the results of various investigations will help to understand how microbes survive and thrive in such complex and dynamic systems as intertidal sediments.

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8 APPENDIX

8.1 SUPPLEMENTARY DATA

Table A1 Bulk parameters of sediment core from Neuharlingersiel.

Depth [cm]	Water content [%]	Median grain size [µm]	TOC [%]	TIC [%]	TS [%]	$\delta^{13}C$ [‰ vs. PDB]
0-1	28.5	22.3	0.39	0.23	0.09	-20.7
1-5	22.9	28.5	0.20	0.18	0.07	-20.8
7-11	22.8	22.0	0.58	0.49	0.27	-23.2
11-15	21.4	21.6	0.42	0.38	0.22	-23.0
15-19	21.4	22.6	0.48	0.41	0.24	-23.4
19-23	21.9	23.0	0.59	0.51	0.27	-22.5
23-27	22.7	21.8	0.63	0.55	0.37	-22.1
27-31	23.9	21.9	0.68	0.59	0.30	-23.3
31-35	31.8	22.6	1.21	0.97	0.63	-23.5
45-50	19.5	31.3	0.22	0.42	0.20	-22.4
50-55	23.0	28.9	0.37	0.54	0.28	-22.2

Table A2 Total normalised fatty acid contents [µg/g TOC] in different fractions of sediment samples.

Fraction	0-1 cm	1-5 cm	7-11 cm	11-15 cm	15-19 cm	19-23 cm	23-27 cm	27-31 cm	31-35 cm	45-50 cm	50-55 cm
Σ triglyceride fatty acids	1600	1470	165	260	203	125	94	63	125	159	116
Σ free fatty acids	7670	7200	9180	1630	1760	1570	1470	1080	632	1670	1240
Σ short-chain fatty acids (C_{14}-C_{20})	6840	6360	92	435	303	137	197	79	56	252	161
Σ long-chain fatty acids (C_{21}-C_{34})	838	836	826	1190	1460	1430	1280	998	576	1410	1080
Σ phospholipid fatty acids	2510	2740	190	525	632	218	132	107	134	129	107
Σ phospholipids	5850	7270	509	870	787	354	158	129	220	187	218

Table A3 Normalised fatty acid contents [µg/g TOC] of triglyceride fractions of sediment samples.

Fatty acid	0-1 cm	1-5 cm	7-11 cm	11-15 cm	15-19 cm	19-23 cm	23-27 cm	27-31 cm	31-35 cm	45-50 cm	50-55 cm
$C_{14:0}$	75.5	59.8	9.4	8.0	14.6	9.7	6.4	2.4	8.8	3.8	3.5
$C_{15:0}$	84.5	126	7.5	12.5	8.9	4.2	3.1	1.7	2.6	6.3	4.0
$C_{16:0}$	419	385	47.5	68.5	55.0	34.8	23.1	14.5	27.0	45.6	36.2
$C_{17:0}$	23.1	35.9	3.5	5.9	3.6	2.5	1.5	1.0	1.2	3.6	2.0
$C_{18:0}$	37.2	35.4	13.5	16.2	14.5	12.5	8.2	7.4	9.4	10.3	9.4
$C_{20:0}$	17.7	24.5	26.5	35.1	33.5	23.1	21.2	16.9	36.1	28.9	19.2
$C_{22:0}$	5.2	n.d.	7.0	7.6	7.8	5.5	5.1	4.1	7.6	6.4	4.9
i-$C_{15:0}$	7.7	12.1	n.d.	1.8	2.1	1.7	1.1	0.4	0.8	n.d.	1.0
ai-$C_{15:0}$	8.6	13.2	n.d.	2.5	3.2	2.3	1.7	0.9	1.1	n.d.	1.1
i-$C_{16:0}$	n.d.	n.d.	n.d.	3.1	n.d.	n.d.	n.d.	n.d.	4.4	3.1	2.3
$C_{16:3}$	35.3	35.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
i-$C_{17:0}$	7.0	7.9	0.9	1.1	0.7	0.6	0.3	n.d.	0.3	n.d.	0.3
ai-$C_{17:0}$ + $C_{17:1}$	50.2	77.1	4.9	9.3	5.5	2.8	1.6	n.d.	1.4	3.5	1.9
$C_{16:1\omega7}$	485	451	17.8	41.7	25.8	11.3	5.8	3.0	9.6	19.1	11.0
$C_{18:2}$	24.4	15.1	3.8	4.2	3.4	1.7	1.6	1.7	1.6	3.2	2.4
$C_{18:1\omega9}$	58.1	52.2	13.4	13.9	12.7	6.3	6.1	4.4	5.1	11.1	8.4
$C_{18:1\omega7}$	59.9	39.1	5.6	16.2	5.7	3.7	2.0	1.1	2.4	3.7	2.3
$C_{20:1}$	40.4	27.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{20:5}$	147	61.2	3.8	9.4	6.1	2.6	1.4	0.8	2.9	4.7	3.1
$C_{22:X>2}$	27.1	9.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detectable; X : unknown degree of unsaturation;

*: comprises several fatty acids with different degrees of unsaturation.

Table A4 Normalised fatty acid contents [µg/g TOC] of free fatty acid fractions of sediment samples.

Fatty acid	0-1 cm	1-5 cm	7-11 cm	11-15 cm	15-19 cm	19-23 cm	23-27 cm	27-31 cm	31-35 cm	45-50 cm	50-55 cm
$C_{14:0}$	222	144	8.4	19.5	13.8	7.7	17.0	5.9	4.3	19.1	12.3
$C_{15:0}$	118	176	12.0	24.9	23.2	13.4	14.2	10.0	7.6	24.6	20.9
$C_{16:0}$	1070	1070	10.0	105	73.5	38.4	53.5	26.4	17.2	82.2	45.4
$C_{17:0}$	62.6	83.2	2.4	8.2	8.3	3.0	4.0	2.2	1.2	6.4	3.7
$C_{18:0}$	1510	122	18.2	33.9	23.0	18.5	22.0	14.2	8.0	42.9	30.3
$C_{20:0}$	31.2	27.3	7.6	17.8	16.6	13.4	25.7	7.3	4.0	18.6	15.6
$C_{22:0}$	58.9	47.4	25.8	49.4	55.1	48.6	75.0	31.4	16.9	48.6	40.8
$C_{23:0}$	35.7	29.4	18.0	29.3	34.9	30.9	40.2	20.1	12.1	31.7	27.7
$C_{24:0}$	205	189	158	242	291	270	325	181	103	281	230
$C_{25:0}$	45.8	42.6	35.6	50.7	63.8	59.7	61.5	39.0	23.6	69.7	58.2
$C_{26:0}$	217	226	238	333	403	377	363	284	159	407	293
$C_{27:0}$	33.7	33.5	30.8	42.5	54.2	55.6	44.3	34.1	21.9	58.5	47.9
$C_{28:0}$	141	153	177	241	301	307	222	222	127	269	196
$C_{29:0}$	16.9	19.8	18.2	25.8	32.8	36.5	23.7	22.2	14.0	34.8	29.5
$C_{30:0}$	47.2	54.8	66.3	91.8	116	131	80.6	86.2	50.9	112	83.3
$C_{31:0}$	n.d.	n.d.	10.0	14.7	18.2	19.6	40.6	12.1	7.8	18.0	13.7
$C_{32:0}$	21.5	24.8	32.0	46.8	59.5	63.7	n.d.	43.1	25.7	54.6	40.9
$C_{33:0}$	n.d.	n.d.	3.8	5.3	7.2	9.2	n.d.	5.2	3.5	8.3	6.1
$C_{34:0}$	14.5	15.7	12.3	17.7	21.6	23.5	n.d.	17.4	11.0	21.5	15.9
i-$C_{15:0}$	44.1	59.4	1.7	6.4	6.7	2.1	3.8	1.1	0.8	4.6	2.4
ai-$C_{15:0}$	92.3	128	4.2	18.0	18.7	5.0	12.2	2.1	1.9	8.3	7.7
$C_{16:2}$	250	130	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{16:1\omega7}$	1900	1890	6.0	90.1	45.3	7.7	12.6	2.1	3.2	23.9	6.9
$C_{16:1\omega5}$	36.8	37.1	6.3	3.4	n.d.	n.d.	2.5	n.d.	n.d.	n.d.	n.d.
$C_{16:1\omega?}$	71.0	43.7	n.d.	2.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{17:1\omega8}$	140	206	n.d.	14.8	10.7	3.4	3.2	n.d.	n.d.	n.d.	n.d.
$C_{17:1\omega6}$	52.2	79.0	n.d.	4.4	2.8	4.0	4.1	n.d.	n.d.	n.d.	n.d.
$C_{18:X}$	76.9	60.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{18:2}$	107	134	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{18:1\omega9}$	170	180	7.2	19.2	17.0	11.3	12.1	4.4	3.8	16.1	10.0
$C_{18:1\omega7}$	451	361	7.9	27.7	25.0	8.6	10.3	3.8	4.1	5.5	6.1
$C_{20:X>2}$	217	417	n.d.	17.1	7.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{20:5}$	1330	573	n.d.	21.8	11.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{22:X>2}$*	128	440	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detectable; x : unknown degree of unsaturation;

*: comprises several fatty acids with different degrees of unsaturation.

Table A5 Normalised fatty acid contents [µg/g TOC] of polar lipid fractions of sediment samples.

Fatty acid	0-1 cm	1-5 cm	7-11 cm	11-15 cm	15-19 cm	19-23 cm	23-27 cm	27-31 cm	31-35 cm	45-50 cm	50-55 cm
n-$C_{14:0}$	48.2	51.0	3.5	12.6	16.2	5.7	3.1	2.7	3.9	n.d.	5.1
n-$C_{15:0}$	35.8	53.0	2.9	10.0	10.1	3.2	1.8	1.8	2.0	n.d.	2.3
n-$C_{16:0}$	398	525	33.3	98.6	114	37.5	23.1	21.1	24.5	39.1	28.1
n-$C_{17:0}$	42.6	56.3	14.0	12.1	10.9	4.8	3.4	2.9	2.6	4.3	2.4
n-$C_{18:0}$	121	102	9.7	21.4	24.7	9.8	5.8	7.1	5.5	11.0	6.4
n-$C_{19:0}$	9.6	n.d.	2.5	5.2	3.0	1.5	n.d.	n.d.	0.9	n.d.	n.d.
n-$C_{20:0}$	11.6	13.6	5.2	12.2	15.8	5.8	3.9	4.2	3.5	3.6	2.5
n-$C_{22:0}$	3.0	4.2	3.0	6.5	8.2	3.2	2.0	2.2	1.9	n.d.	n.d.
n-$C_{24:0}$	n.d.	n.d.	3.7	8.0	10.9	4.1	2.3	3.1	2.7	n.d.	n.d.
n-$C_{26:0}$	n.d.	n.d.	3.4	7.9	10.7	3.4	2.2	2.9	2.8	n.d.	n.d.
i-$C_{14:0}$	9.6	14.9	0.7	3.9	6.3	2.3	1.1	0.5	n.d.	n.d.	1.6
i-$C_{15:0}$	33.6	56.0	5.8	18.2	23.9	9.2	4.8	2.9	4.5	2.6	4.7
ai-$C_{15:0}$	53.1	90.5	4.4	40.6	55.4	19.9	10.2	6.5	11.8	7.7	13.6
i-$C_{16:0}$+$C_{16:3}$	28.3	38.7	3.4	9.6	12.7	4.3	2.5	1.9	4.2	3.0	3.1
n-$C_{16:1\omega9}$	19.7	25.2	1.7	8.1	7.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n-$C_{16:1\omega7}$	322	423	18.5	58.1	69.1	20.6	13.8	9.8	13.1	10.6	6.5
n-$C_{16:1\omega5}$	15.0	25.4	4.4	8.3	12.8	5.2	3.8	3.2	2.5	7.3	8.6
n-$C_{16:1\omega?}$	23.4	32.8	2.8	8.2	11.4	3.6	2.2	1.5	2.5	n.d.	1.7
$C_{17:1\omega8}$	13.3	19.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10-Me-$C_{16:0}$	17.5	25.5	0.9	14.1	18.3	7.5	4.1	n.d.	4.4	2.6	2.0
i-$C_{17:0}$	16.0	21.3	2.8	4.4	6.4	1.9	1.2	1.0	1.1	1.3	1.1
ai-$C_{17:0}$	17.4	24.0	3.3	8.0	12.8	3.4	1.5	1.4	2.4	3.5	3.1
$C_{17:1\omega8}$	31.9	48.6	2.1	4.5	4.4	1.5	1.4	0.9	1.0	n.d.	n.d.
$C_{17:1\omega6}$	18.6	28.1	2.3	5.6	7.9	2.4	1.6	1.3	1.5	n.d.	n.d.
i-$C_{18:0}$	10.3	n.d.	n.d.	n.d.	n.d.	n.d.	1.2	n.d.	n.d.	n.d.	n.d.
$C_{18:4}$	12.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{18:2}$	23.9	30.3	5.8	12.1	14.4	6.5	3.9	3.7	4.2	n.d.	n.d.
n-$C_{18:1\omega9}$	116	136	8.4	24.0	27.4	9.7	6.3	5.7	6.7	9.3	5.3
n-$C_{18:1\omega7}$	292	314	30.9	68.1	98.0	38.5	25.3	19.5	24.0	16.9	11.1
$C_{19:1}$	11.2	16.4	1.0	2.3	3.9	1.1	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{20:5}$	371	268	5.5	20.9	11.0	1.3	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{20:1}$	142	131	4.5	11.9	3.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{21:X>2}$	13.8	18.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{22:x>2}$*	228	141	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not detectable; x : unknown degree of unsaturation;

*: comprises several fatty acids with different degrees of unsaturation.

Table A6 Normalised contents [µg/g TOC] of polar lipids in polar lipid fractions of sediment samples.

Phospholipid type	0-1 cm	1-5 cm	7-11 cm	11-15 cm	15-19 cm	19-23 cm	23-27 cm	27-31 cm	31-35 cm	45-50 cm	50-55 cm
PG (unknown)	290	398	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown (m/z 808)	n.d.	n.d.	46	46	71	26	26	19	30	n.d.	n.d.
PGM	456	642	76	79	118	27	24	17	29	87	36
PG	1210	1570	100	282	278	77	n.d.	n.d.	37	n.d.	n.d.
PG+DPGM	n.d.	n.d.	78	n.d.	n.d.	n.d.	32	n.d.	n.d.	n.d.	n.d.
PG+DPG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	113	n.d.	160
DPGM	n.d.	n.d.	n.d.	n.d.	n.d.	20	n.d.	17	n.d.	n.d.	n.d.
DPG	601	1090	138	301	286	186	79	74	n.d.	n.d.	n.d.
PE	295	431	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown (m/z 937)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	170	38
PCM	795	651	50	38	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PC	1280	1470	67	170	105	44	23	21	41	100	22
Unknown (m/z 833 +m/z 1122)	n.d.	n.d.	22	56	109	66	40	29	74	270	143
Unknown (m/z 857)	185	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown (m/z 791)	912	1280	18	115	100	61	21	20	54	120	21
Unknown (m/z 1204)	n.d.	n.d.	n.d.	n.d.	n.d.	66	n.d.	n.d.	n.d.	n.d.	n.d.
PS + Unknown (m/z 979)	405	522	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PI	921	1020	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown (m/z 753)	n.d.	n.d.	64	158	199	n.d.	43	32	43	249	102
Unknown (m/z 1233)	n.d.	n.d.	66	77	75	34	63	20	22	247	101

n.d.: not detectable.

8.2 SELECTED EI MASS SPECTRA

Diglycerides as TMS derivatives

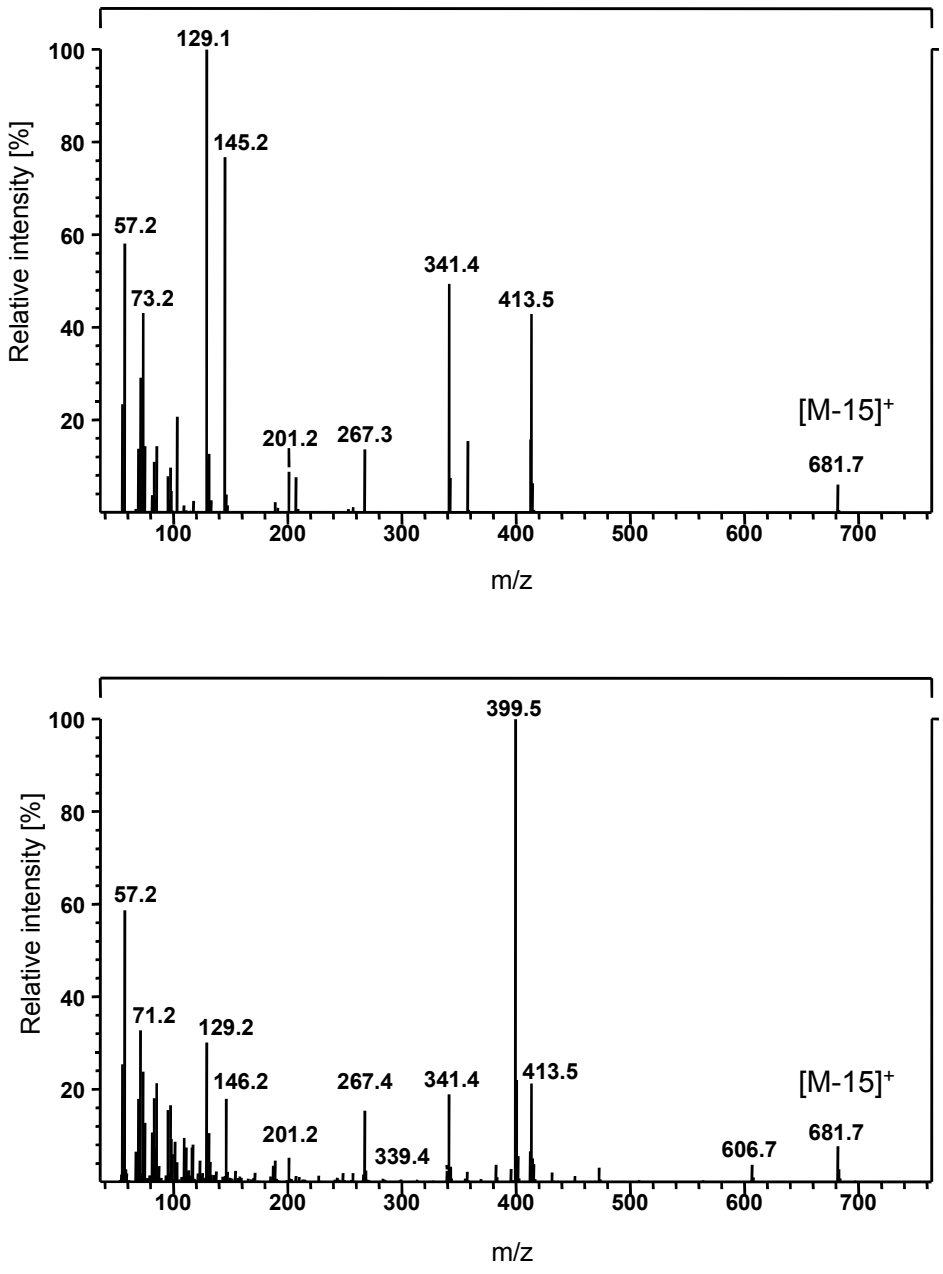

Fig. A1 Mass spectra of 1,2-distearyl diglyceride (top) and 1,3-distearyl diglyceride (bottom).

Monoalkylglycerol ether as TMS derivative

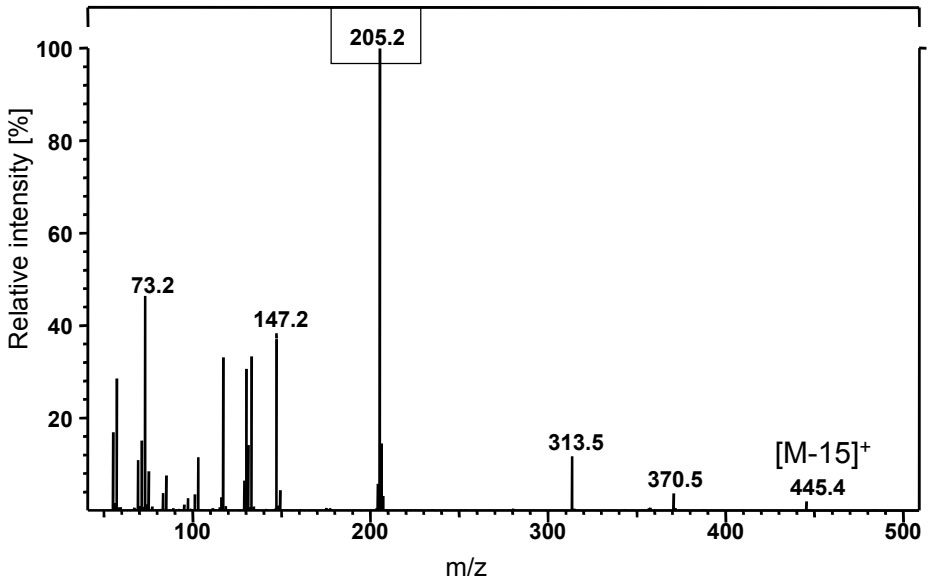

Fig. A2 Mass spectrum of hexadecanylglycerol ether.
Note the characteristic ion at m/z 205 marked with a black frame (Grönneberg and Albone, 1978) !

Polyunsaturated fatty acid methyl ester

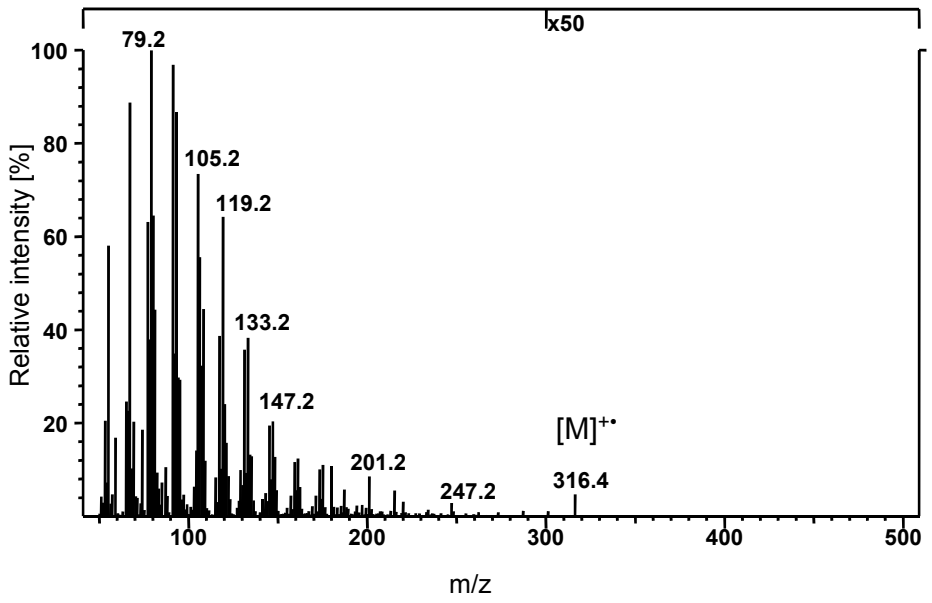

Fig. A3 Mass spectrum of $C_{20:5}$ fatty acid methyl ester.

Double bond determination from DMDS adducts

Diagnostic ions and molecular weights for derivatised fatty acids. i.e. as DMDS adducts (Dunkelblum et al., 1985) and TMS derivatives are summarised in Table A7. Selected mass spectra are shown in Fig. A4.

Fig. A4
Examples of mass spectra of monounsaturated fatty acids after DMDS addition for determination of double bond positions (characteristic ω-fragments are marked with a frame; Nichols et al., 1989).

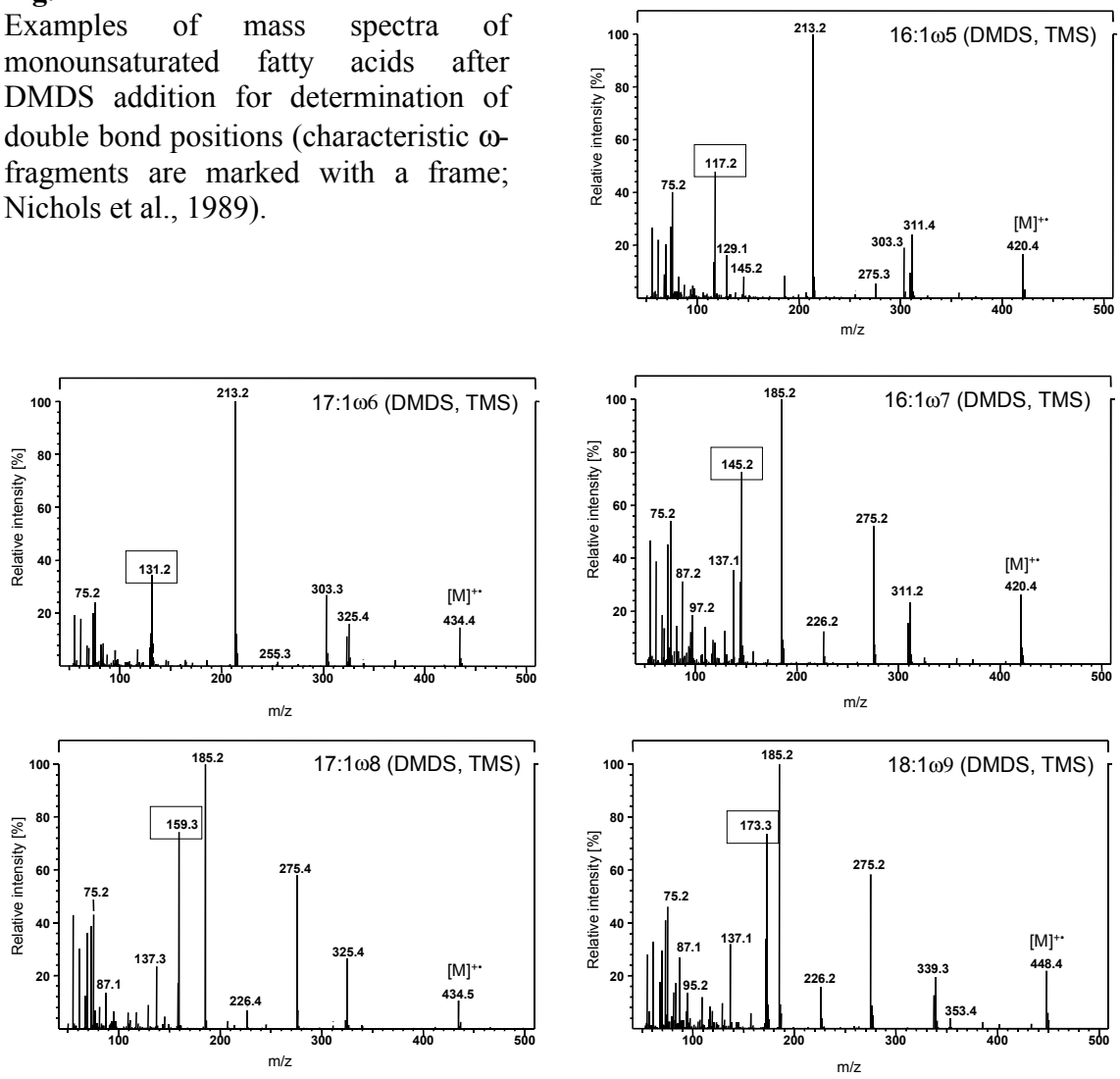

Table A7 Characteristic fragments and molecular weights of fatty acids after DMDS addition and derivatisation with TMS.

Double bond position	ω-fragment [m/z]	Fatty acid	$M^{+\bullet}$ [m/z]
ω4	103	$C_{14:1}$	392
ω5	117	$C_{15:1}$	406
ω6	131	$C_{16:1}$	420
ω7	145	$C_{17:1}$	434
ω8	159	$C_{18:1}$	448
ω9	173	$C_{19:1}$	462
ω10	187	$C_{20:1}$	476

8.3 PHOTOGRAPHS

Fig. A5
Sampling Wadden Sea sediments at Neuharlingersiel (top left and right); cross section of a sediment core taken from a sand flat at the island of Mellum (right).

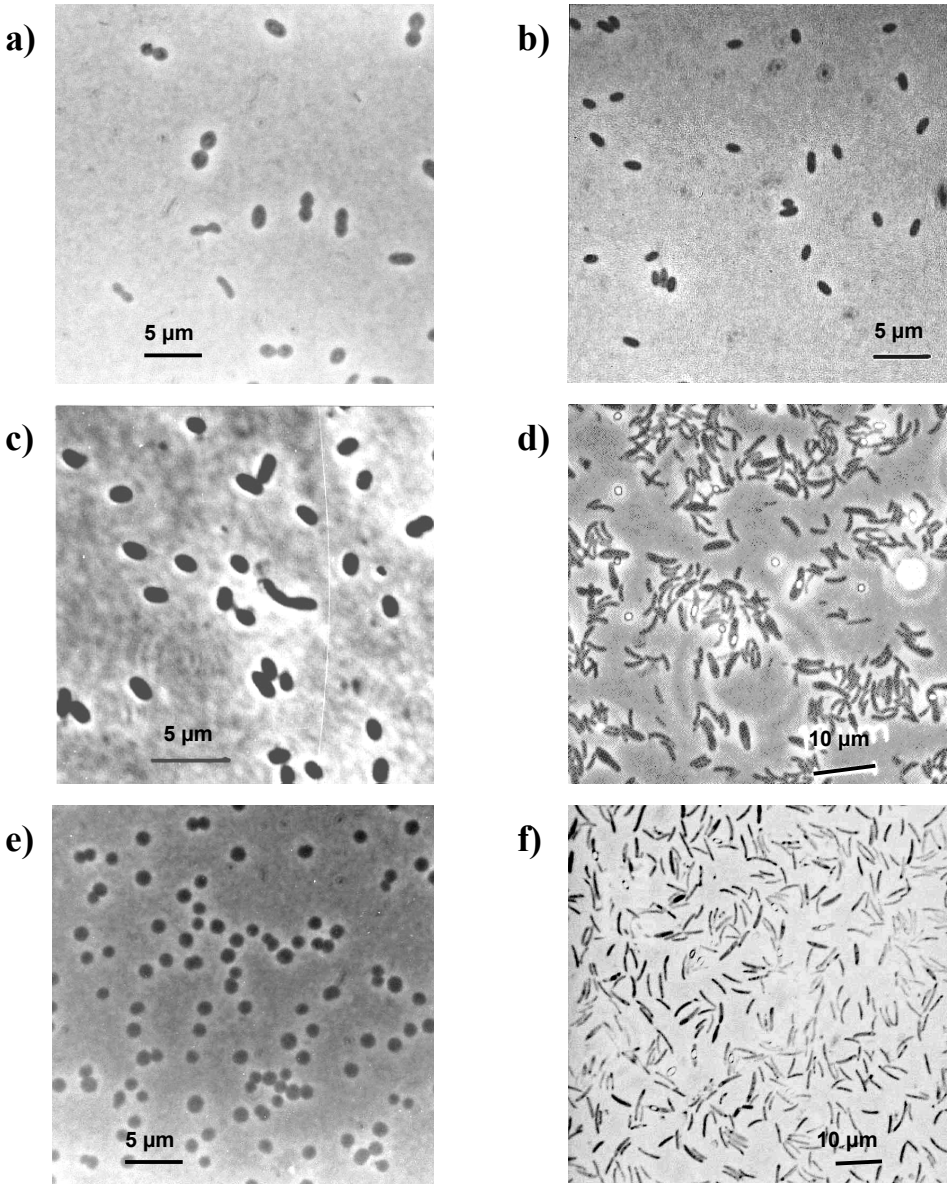

Fig. A6 Morphologies of sulfate-reducing bacteria (Sass, 1997 and unpublished):
a) *Desulfobacter hydrogenophilus*;
b) *Desulfomicrobium* str. STL8;
c) SRB str. STP23;
d) SRB str. STP3;
e) *Desulfococcus multivorans*;
f) *Desulfosporosinus* str. STP12.

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Hiermit versichere ich, dass ich diese Arbeit selbstständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Oldenburg, den 10.7.2001