Biomarkers in Organic Geochemistry
On the bacterial contribution to sedimentary organic matter and the formation of pristane in relation to maturity

Hans Goossens

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft
op gezag van de Rector Magnificus, prof. Drs. P. A. Schenck
in het openbaar te verdedigen ten overstaan van
een commissie door het College van Dekanen daartoe aangewezen
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Hans Goossens

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prof. Drs. P.A. Schenck

Toegevoegd promotor Dr. J.W. de Leeuw

voor *Froukje, Maarten en Frank*

En daar dook die fnuikende gedachte weer op dat de zee helemaal geen wetten kende. Maar hij wees dat idee haastig van de hand. Hij wilde begrijpen. Hij moest in het reine komen met de zee om ervan te kunnen houden en om zijn zelfrespect te kunnen bewaren.

Tove Jansson, Papa moem en de mysteriën van de zee
Zwarte Beertjes 1361, p.150

CONTENTS

CHAPTER 1

Introduction

Many investigations in organic geochemistry try to establish the relation of recognizable chemical compounds present in geological samples, the so called chemical fossils, to their precursors. The term chemical fossil is widely used for compounds whose carbon skeletons are structurally related to those of contemporary biosynthesized compounds. The term biomarker is also used to indicate the biological origin of a compound. The concept of chemical fossils goes back to the work of Treibs (Treibs, 1934a,b) who showed the presence of porphyrins in geological samples and related those compounds to the chlorophylls in photosynthetic organisms. Since the gas chromatograph came into use in the late fifties, the field of organic geochemistry has developed rapidly and many biomarkers have been established.

Pristane and phytane

historical background

Despite this progress, there is an ongoing discussion since the early sixties on the origin of the most extensively discussed biomarkers in organic geochemistry: pristane and phytane. The presence of these acyclic C_{19} and C_{20} isoprenoid alkanes (structures I and II) in geological matter was recognized in the early days of gas chromatography from peaks in the gas chromatograms of alkane fractions of oils between the regular patterns of *n*-alkanes. Their structures were assessed in the early sixties (Dean and Whitehead, 1961; Bendoraitis *et al.*, 1962).

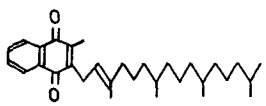

I


II

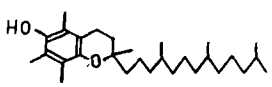
III

Most ancient sediments and crude oils contain relatively major quantities of pristane and phytane. The abundance of these two compounds over the other acyclic isoprenoid hydrocarbons and the structural similarity with the phytyl side chain of the chlorophyll molecule (structure III) were taken as indications that phytol from chlorophyll is the common precursor, mainly because of the abundant presence of chlorophyll in the biosphere. It was shown that the presence of phytadienes and pristane in zooplankton (Blumer *et al.*, 1963; Blumer and Thomas, 1965) and in oils from fishes and whales results from the metabolic conversion of dietary phytol (Avigan and Blumer, 1968).

Other precursors have been proposed as well, however. Vitamins K (naphtoquinone-4; structure IV) and E (tocopherols; structure V) have also been proposed but it was thought that the low proportions of these compounds relative to chlorophyll would preclude a substantial contribution (Oro *et al.*, 1965; Maxwell *et al.*, 1973). As a result of this discussion there was a general agreement that phytol is the common precursor of phytane and pristane.



IV



V

From their discovery in the early sixties until the present time, pristane and phytane have been considered 'biomarkers', initially as indicators of the biogenic origin of ancient organic matter (up to $2.7*10^9$ yr; Oro *et al.*, 1965; Johns *et al.*, 1966) and later as indicators of environmental conditions at times of deposition (see below). The pristane/C_{17} *n*-alkane ratio has been proposed as an indicator of the depositional

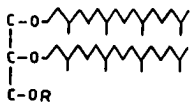
environment (Lijmbach, 1975). Pristane and phytane have also been used to indicate the type of kerogen present in sediments: plots of the ratios pristane / C_{17} *n*-alkane versus phytane / C_{18} *n*-alkane of a suite of samples may reveal the type of kerogen (Connan, 1981).

Many studies have been performed to establish possible diagenetic pathways by which the phytyl side chain is converted into pristane or phytane (Bayliss, 1968; de Leeuw *et al.*, 1974; de Leeuw *et al.*, 1977; Brooks and Maxwell, 1974; Ikan *et al.*, 1975). Phytane may be generated by reduction of phytol whereas the formation of pristane requires oxidation of phytol and subsequent decarboxylation and reduction. The reactions involved have been summarized by Didyk *et al.* (1978). In this view, phytol is the common precursor of pristane and phytane which are generated in relatively oxidative or reductive environments, respectively. Consequently, the pristane / phytane ratio was (and is) used as an indicator of the oxicity of the environment at the time of deposition (Didyk *et al.*, 1978), although recent developments indicate that this interpretation of the pristane / phytane ratio is erroneous (ten Haven *et al.*, 1987; chapters 6, 7 and 8).

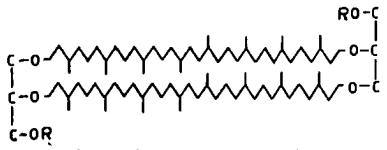
recent developments

During the late seventies important developments occurred.

In 1978 it was found that the membrane lipids of methanogenic bacteria mainly consist of compounds containing acyclic isoprenoid chains (Makula and Singer, 1978; Tornabene and Langworthy, 1979). These compounds contain two phytanyl groups (isoprenoid C_{20}) ether linked to a glycerol molecule (diphytanyl-ether; DPE (Structure VI)). Also a tetraether analogue exists in which two DPE molecules are linked together via the phytanyl chains thus forming a cyclic structure (dibiphytanyl-tetraether; compound VII). The C_{40} chains between the two glycerol moieties consist of two phytanyl chains which are linked head to head. These compounds had been identified in membrane lipids of extremely halophilic bacteria (Kates *et al.*, 1965), present in hypersaline environments, and in thermoacidophilic bacteria (Langworthy *et al.*, 1972, 1974; De Rosa *et al.*, 1974, 1977). The latter species live under the extreme conditions in thermal springs where the temperature is very high (80-90 °C) and the pH is very low (pH = 1-2) (Langworthy, 1982). The discovery of these ether-lipids in methanogenic bacteria made clear that these compounds are omnipresent.



VI



VII

In addition to the etherlipids, acyclic isoprenoid hydrocarbons with varying chain lengths were observed in methanogenic and extremely halophilic bacteria, though in low abundance (Kramer *et al.*, 1972; Holzer *et al.*, 1979).

In the same period the taxonomy of the prokaryotes was revised on basis of 16S rRNA compositions and a new bacterial kingdom was distinguished, the Archaebacteria (Woese *et al.*, 1978; Fox *et al.*, 1980), which comprises methanogenic, extremely halophilic and extremely thermoacidophilic bacteria. Thus, all archaebacterial species share a common feature: they contain the di- and/or tetra-etherlipids described. These etherlipids do not occur outside this kingdom.

When etherlipids were identified in sediments and oils of varying age and maturity (Chappe *et al.*, 1979, 1982), it became clear that bacteria might also contribute significantly to the quantities of the acyclic isoprenoids present in sedimentary materials. The possible link between archaebacterial etherlipids from extremely halophiles and phytanyl skeletons in sediments had already been noticed in a study of Dead Sea sediments (Anderson *et al.*, 1977).

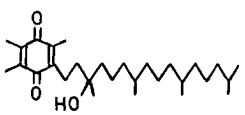
Simultaneously, other specific bacterial markers, the extended hopanoids, were found both in bacteria and sediments (Ourisson *et al.*, 1979; 1984, for a review).

Thus, it became clear that the bacterial contributions to the organic matter in sediments probably had been underestimated. A special study into the contribution from bacteria to sedimentary organic matter was therefore appropriate.

The scope of the study

Initially, this study was intended to reveal the possible archaebacterial contribution to the acyclic isoprenoids pristane and phytane in sediments and oils. The available literature on the lipid composition of archaebacteria showed, *grosso modo*, a uniform lipid composition in this group of bacteria. The lipids mainly consist of di-etherlipids or tetra-etherlipids or mixtures of both. Fatty acids are absent or only present in very low amounts. Some species contain additional amounts of acyclic isoprenoid hydrocarbons with varying chain lengths (Holzer *et al.*, 1979). The major hydrocarbons have chain lengths of C_{25} or C_{30} and only minor amounts of shorter hydrocarbons ($\leq C_{20}$) have been found. The di- and tetra-etherlipids occur only in archaebacteria, but the presence of isoprenoid hydrocarbons is not restricted to the archaebacterial kingdom. These compounds have been reported for a variety of species (Suzue *et al.*, 1968; Taylor and Davies, 1973; Amdur *et al.*, 1978; Taylor, 1984).

A more general inventory of bacterial lipids possibly contributing to the amounts of pristane and/or phytane in sediments shows that isoprenoid chains occur in various compounds in bacteria. In addition to the etherlipids and the hydrocarbons mentioned above isoprenoid chains are present in pigments like different bacteriochlorophylls and carotenoids, and in quinone-like structures.
The chlorophylls in the vast majority of photosynthetic prokaryotes contain phytol as the esterified alcohol but some variation occurs among the photosynthetic-S-bacteria (Gloe *et al.*, 1975; Caple 1978). The chain length of the carotenoids is at least C_{30} and these chains are highly unsaturated.
The quinone-like structures have side chains consisting of a varying number of isoprene units which usually contain several double bonds (Collins and Jones, 1981). In some species quinones occur with a C_{20} chain: vitamin k_1 (phylloquinone) containing 4 isoprene units, one with a double bond, has been reported to occur in some cyanobacteria. Menaquinone and ubiquinone may also occur with C_{20} side chains, but these chains contain 4 double bonds (Collins and Jones, 1981). Many bacteria have been reported to contain tocopherylquinone, a compound which contains a saturated isoprenoid C_{20} chain (structure VIII; Hughes and Tove, 1982), but the amounts are low.



VIII

The distributions of acyclic isoprenoid hydrocarbons normally met in geological materials do not reflect the variations of chain lengths seen in organisms. Usually, pristane and phytane are the most abundant ones in geological materials but acyclic isoprenoid hydrocarbons occur within a range which may extend from C_{14} to C_{40}. In recent sediments the variation is greater and pristenes, phytenes, phytadienes, squalenes and other (un-) saturated isoprenoid hydrocarbons may be included. If bacterial carotenoids or quinones would contribute substantially to the acyclic isoprenoid hydrocarbons in sediments different chain length distributions would be expected.
As far as the literature on prokaryotic lipids suggested at the start of the project, only DPE could be a source of pristane and phytane in addition to phytol, apart from, possibly, other unknown contributing precursors.

There were good reasons to broaden the scope of the study intended beyond the archaebacterial contribution to acyclic isoprenoids.

A. The methods used to screen the archaebacteria for acyclic isoprenoids would also yield information about the other lipid components present.
B. Compounds containing an isoprenoid chain occur also in non-archaebacterial species.
C. The bacterial contribution to the organic matter in sediments is an underdeveloped part of organic geochemistry. There were strong indications that these contributions were underestimated.
D. A better recognition of bacterial organic matter could be highly valuable for organic geochemistry since the composition of bacterial organic matter is very narrowly related to environmental conditions. A more specific recognition of prokaryotic organic matter could give clues to the characterization of the depositional environment and to the conditions during diagenesis.

Therefore, it was decided to direct the study towards the recognition of bacterial organic matter in sediments emphasizing the contribution of acyclic isoprenoid hydrocarbons. As a consequence, species from other bacterial kingdoms were included in the study. Besides non-bacterial precursors of acyclic isoprenoids were also considered (see hereafter).

The bacterial contribution to sedimentary organic matter

This study intended a survey of lipids in bacteria and in bacteria-rich sediments from an organic geochemical point of view. Generally, organic geochemistry reveals the information hidden in geological materials in terms of structures of individual compounds. Many compounds in bacteria and sediments consist of several components linked together by bonds of varying nature. The nature of these bondings may provide important additional information.

analyses of sediments
Sediment analyses have been performed in various ways. In most cases a thorough extraction with organic solvent(s) is performed after which the compound classes are separated using thin layer or column chromatography. Hereafter separation of the individual components is achieved by gas chromatography. The identification of the compounds is based on mass spectrometric data.
Several variations are possible. One of the variations is that the sample receives some treatment before or during extraction. Such treatments augment the amount of lipid extracted by the liberation of "bound" lipids. Saponification of the sample in alkaline solutions of varying strength is classic (Bligh and Dyer, 1959), but also other means have been used like acid treatment (Cranwell, 1978; Parker *et al.*, 1982; Klok *et al.*, 1984) or heat treatment (Kawamura and Ishiwatari, 1981, 1982). These studies indicated that many compounds occur in different modes like "free", "bound", or "tightly bound" and "base extractable" or "acid-extractable". The "bound" mode of occurrence seems to enhance the preservation of compounds compared to the "free" mode. For β-OH-fatty acids, the different modes of occurrence helped distinguish between different sources of these compounds (Cranwell, 1981). The distinction of different modes of occurrence could thus be helpful in the recognition of source organisms.

analyses of bacteria
The lipids of bacteria have been widely investigated. Many studies have been performed, mainly from a taxonomic point of view. The methods used are similar to those used to investigate sediments, *i.e.* extraction, compound class separation and gas chromatography or gas chromatography-mass spectrometry. General reviews on bacterial lipids were published by Lechevalier (1977) and Goldfine (1982). Numerous reports have been published on the lipids of specific groups of bacteria (*e.g.* Kaneda, 1977; Batrakov and Bergelson, 1978; Kenyon, 1978; Langworthy, 1982), on specific parts of bacterial cells like lipopolysaccharides (LPS) (Lüderitz *et al.*, 1982; Galanos *et al.*, 1977) or on specific compounds like triterpenoids (Taylor, 1984), quinones (Collins and Jones 1981; Hiraishi *et*

al., 1984) or hopanoid triterpenes (Rohmer *et al.*, 1984). Studies aiming at "total lipid profiles" are scarce, however.

The lipids of eubacterial species are mainly diglycerides with varying polar groups at the C_3 position of the glycerol molecule. The usefulness of lipids in bacterial taxonomy is frequently based upon the structure of the polar groups of the diglycerides. Due to the many functionalities in these groups, however, there is little hope that they remain recognizable in sediments during and after early diagenesis. They may be reactive in the mixed organic matter. Moreover, the components of the polar groups (phosphate, sulphate and saccharides) occur also in other compounds in the cell whose compositions are highly variable and determined by short term changes in the environment. The distributions of the intact lipids in a sediment would be very informative but, in general, loss of the polar group is the first step in degradation. Thus, for the purpose of recognition of prokaryotic organic matter in sediments the lipid part is more appropriate since this part has a higher potential of survival. Unfortunately, structural differences are usually smaller in the lipid part. As a result, specification of the prokaryotic origin of sedimentary compounds to the (groups of) bacterial species present has seldom been achieved (Ward *et al.*, 1985).

Only few bacterial markers are known and most are not specific for distinct groups or species. In addition to the well established bacterial markers like di- and tetra-ethers and extended hopanoids, branched chain compounds and β-OH-fatty acids are considered to have a bacterial origin despite the fact that these latter two compound types may be synthesized by eukaryotes.

methods used

In this study bacterial and sedimentary organic matter have been analyzed by identical methods, to avoid problems when comparing the results of analyses of bacteria and sediments. The methods were developed to give maximum yield of the extractions in order to approximate as much as possible "total lipid profiles" of bacteria and sediments. In addition, the method distinguishes between different modes of occurrence.

In this way the maximum of information was obtained for the comparison of bacterial lipids with sedimentary lipids and for the recognition of bacterial lipids in sediment samples.

We have not tried to assess absolute amounts of the components in the extracts. Absolute quantitation was omitted since the results, although analytically precise, may be biased by the (unknown) heterogeneity in the sampled sediments. In our opinion, absolute quantitation would not add valuable information at the present level of recognizing prokaryotic organic matter in sediments. The relative amounts of the compounds in different extracts of one sample can be compared, however.

Another important point for the interpretation of the results is the difference between genotypic and phaenotypic variations. Differences of the chemical compositions of bacterial species may be attributed to genetic differences (genotypic variation) between the species but the chemical composition of a single species is not constant under different growth conditions (phaenotypic variation). The influence of growth circumstances on the lipid composition of prokaryotes has been investigated only fragmentary. Although some relationships have been assessed like the influence of growth temperature on the level of unsaturation of fatty acid moieties in glycerides (see Lechevalier, 1977), much is unknown, for example, of the effects of starvation on the lipid composition of prokaryotic cells or communities. We payed no attention to this point since it would have been beyond the scope of this study. Moreover, the growth circumstances of bacteria in sediments are hardly known. Since we investigated bacterial species which were selected to represent very distinct bacterial groups according to 16S rRNA systematics (Fox *et al.*, 1980; Kandler, 1981) the lipid compositions found presumably represent genotypic rather than phaenotypic variations.

description of chapters 2 to 5

The analytical scheme is outlined in chapter 2. To liberate bound lipids the method applies a sequence of treatments and extractions after extraction of the free occurring

apolar compounds. The sequential set-up yields extracts containing compounds with defined modes of occurrence in the original material. The extracts are named after the method used, *i.e.* Ether-extract, Ether/OH^--extract, OH^--extract and H^+-extract. The sample is saponified prior to the acid treatment. Thus, the compounds receiving the acid treatment contain only alkali-resistant bonds. This reduces the number of possible precursors of the H^+-labile compounds. In this way information is obtained about the probable bonding type of the lipid moieties identified in the extracts: The OH^--labile compounds are considered mainly ester-bound and the H^+-labile compounds mainly amide-bound or linked via a glycosidic bond. Information on the mode of occurrence, therefore, enhances the specificity of a compound identified.

Chapters 3 and 4 show the results of this approach when applied to different sediments which were rich in prokaryotes.
Chapter 3 shows the results for the sediment of a eutrophic, stratified lake in the Netherlands: Lake Vechten. The sediment layer investigated is rich in methanogenic bacteria.
The samples described in chapter 4 have been taken in a strictly prokaryotic community in a gypsum precipitation basin in a salt work area in southern Spain. In this hypersaline environment only salt resistant bacteria are present, among those extremely halophilic archaebacteria.
In chapter 5 the compositions of the H^+-extracts of bacteria and sediments from different environments are compared.

concluding remarks
The sequential approach appeared very useful, as is apparent from the distributions of the H^+-labile β-OH-fatty acids. Both OH^--labile and H^+-labile β-OH-fatty acids may occur in sediments but the H^+-labile β-OH-fatty acids can be ascribed specifically to prokaryotes since they have been found only in prokaryotic cell types in contrast to OH^--labile β-OH-fatty acids which may occur also in eukaryotes.
In general, the H^+-labile lipid moieties present in the sediments investigated bear mainly a prokaryotic character. Although the recognition of individual species or groups of species proved impossible, there is considerable variation in the distributions of the H^+-labile β-OH-fatty acids. These distributions provide detailed information and hold therefore promises as fingerprints of prokaryotic communities.

Acyclic isoprenoid hydrocarbons

The results of the screening of the bacteria, partly described in chapters 2 and 5, indicated that the acyclic isoprenoid hydrocarbons pristane and phytane are not present as such in the species investigated. Some potential precursors of acyclic isoprenoids were found. The archaebacteria contained diphytanylether and/or dibiphytanyltetraether. In some of the bacteria (*Methanobacterium thermoautotrophicum*, *Desulfovibrio gigas* and *Halobacterium halobium*) we found squalenes (C_{30} isoprenoid hydrocarbons) with different levels of unsaturation. The phototrophic species showed the presence of phytol or in some cases farnesol (tri-unsaturated C_{15} isoprenoid alcohol). However, no previously unknown potential precursors of isoprenoids in sediments were found.

description of chapters 6 to 8

tocopherol, a precursor of pristane
Van Graas *et al.*, (1981) suggested that natural pristane is derived from the same precursor which generates prist-1-ene upon analytical pyrolysis since the amounts of the pyrolysis product prist-1-ene decreased and those of pristane (present as such) increased in a set of samples with increasing maturity from the Paris Basin. Prist-1-ene is a major pyrolysis product of most immature kerogens (Larter *et al.*, 1979; Van de Meent *et al.*,

1980a). The only compound known at that time to generate prist-1-ene upon pyrolysis is esterified phytanic acid (Van de Meent, 1980b), but phytanic acid is only present in trace amounts (if at all) in sediments beyond the early stages of diagenesis.
The presence of free tocopherols in ancient sediments (Brassell *et al.*, 1983) made clear that the common view on the precursor (pristane and phytane are both derived from the same precursor phytol) might be (in part) erroneous. The mass spectrum of α-tocopherol shows very clearly the loss of a fragment with a mass identical to pristene. Tocopherols are also known as vitamins E and have been studied thoroughly in the past. Repeating an experiment already done in 1937 by Fernholz (Fernholz, 1937), showed that α-tocopherol generates prist-1-ene upon thermal treatment and flash pyrolysis showed that the molecule falls apart in two compounds, prist-1-ene and a tetramethyl-quinone. This work, which is further described in chapter 6, shows that tocopherols are likely precursors of pristane in geological organic matter. The literature indicates that tocopherols occur in photosynthetic tissues and are thus almost as widespread as chlorophylls although their amounts are much smaller in living organisms. In view of their structures, however, the survival potential of tocopherols is expected to be much higher than that of phytol. The high amounts of pristane in mature oils and sediments probably illustrate that rates of biodegradation may strongly influence the composition of ancient materials.

the pristane formation reaction
In chapters 7 and 8 the logical consequence of the suggestion that the pyrolysis product prist-1-ene and pristane represent respectively the precursor and the product of a reaction occurring during diagenesis and catagenesis is elaborated. The relationship appeared to fit an Arrhenius relation describing the effects of time and temperature on the rate of pristane formation. This approach was successful in a series of samples from the Mahakam Delta, and the values of the pseudo-kinetic parameters of the Arrhenius relation could be assessed (Chapter 7). It appeared possible to use these values without modification in a reconstruction of the thermal history of the Paris Basin. Thus, their values were confirmed (Chapter 8).

concluding remarks
Notwithstanding the practical value of this independent molecular maturity parameter for the assessment of maturity and for the reconstruction of thermal histories of basins, some important questions remain. The extremely low values of the pseudo-kinetic parameters of the pristane formation reaction are not understood. Detailed investigations are required into the chemical and physical processes possibly involved, to answer these questions. A better understanding of the kinetics of the pristane formation reaction would not only yield information about the important conditions for maturation, but could also reveal whether pristane is derived from a single (type of) precursor. If the assumption that there is only one (type of) precursor of pristane, which is underlying the Pristane Formation Index, proves correct, the final proof that pristane and phytane are derived from different precursors has been given.

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CHAPTER 2

Lipids and their mode of occurrence in bacteria and sediments I: A methodological study of the lipid composition of Acinetobacter calcoaceticus LMD 79-41

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Abstract

A general analytical procedure distinguishing different modes of occurrence of lipids is described. This procedure was developed using the gram-negative eubacterium *Acinetobacter calcoaceticus* LMD 79-41 as a test substance. Hydrolysis under alkaline and acidic conditions revealed that under alkaline conditions not all lipid moieties were released whereas acid hydrolysis generated artificially formed esters. Artefact formation was minimized by saponification after the acid treatment. Maximal information is obtained by a sequence of extractions and hydrolyses, thus distinguishing free extractable, OH^--labile and H^+-labile lipid moieties. The extract obtained after the acid treatment contained mainly β-hydroxy fatty acids which were shown to be derived from lipopolysaccharide (LPS) and therefore were linked via amide bonds. Whereas esterified β-hydroxy fatty acids are present in prokaryotes and eukaryotes, amide-linked β-hydroxy fatty acids have been found only in prokaryotes and therefore have a potential value as bacterial markers.

Introduction

In organic geochemical studies of recent sediments, distribution patterns of lipids and structures of lipid components are widely used as markers for the environment of sedimentation and for the stage of early diagenesis. During the past few years there has been a growing recognition of the important contribution to the organic matter in sediments by prokaryotic organisms. Several classes of compounds have been ascribed (at least in part) to prokaryotic sources, e.g. ether-bound isoprenoids to Archaebacteria (Chappe *et al.*, 1982), extended hopanoids to various eubacterial species (Ourisson *et al.*, 1984; Rohmer *et al.*, 1984) and several types of hydroxy fatty acids to bacterial cell walls (Boon *et al.*, 1977; Cranwell, 1981a; Kawamura and Ishiwatari, 1982; Parker *et al.*, 1982; Cardoso and Eglinton, 1983; Matsumoto and Nagashima, 1984; Klok *et al.*, 1984). For a more profound evaluation of the significance of bacterial organic matter in sediments and for a further differentiation between groups of bacteria an extensive search for specific bacterial lipids as potential biomarkers is desired.

The chemical composition of bacteria has been widely investigated and there is an extensive literature dealing with their lipids (for reviews see: Lechevalier, 1977; Goldfine, 1982; Langworthy, 1982; Taylor, 1984). Most studies, however, deal with either taxonomic problems or the chemical composition of specific parts of the cell and report on only a part of the lipids present. Relatively few attempts have been made to perform a complete analysis of the lipid composition of bacterial species, taking into account the mode of occurrence of the lipids.

In general, the lipid analysis of bacteria and sediments involves an extraction with organic solvents, often after saponification, to obtain both free and esterified lipids. After extraction, the different compound classes are separated by thin layer- or column-chromatography and subsequently analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

It is known, however, that many bacteria contain lipid moieties which are liberated by acid only, such as the amide bound fatty acids and hydroxy fatty acids present in the lipopolysaccharide (LPS), in the so called ornithine lipids (OL) and in bacterial sphingolipids. In general, however, such an acid treatment is only applied when specific parts of the bacterial cell known to contain amide bound lipids are investigated (Wilkinson, 1977; Galanos *et al.*, 1977; Lüderitz *et al.*, 1982).

Only in a few cases acid hydrolysis has been applied in the analysis of organic compounds present in sediments (Cranwell, 1978, 1981a,b; Parker *et al.*, 1982; Klok *et al.*, 1984; Cardoso *et al.*, 1983). In those cases where β-hydroxy fatty acids were analyzed, increased amounts were observed. These have been ascribed to LPS present in the sediment (Parker *et al.*, 1982; Klok *et al.*, 1984), demonstrating a prokaryotic source.

It is clear from the above that a full lipid analysis of bacteria or prokaryotic organic matter in sediments should include an acid treatment. Therefore, it seemed worthwhile to investigate the effects of hydrolysis under different conditions on prokaryotic organic matter and to develop a procedure for the complete analysis of lipid compositions of bacteria which is also applicable to the analysis of sedimentary organic matter.

To benefit optimally from the potential information, we developed a general analytical procedure to discriminate lipids which are extractable as such, lipids which are released upon subsequent base treatment and those which are liberated by subsequent acid treatment.

The behaviour of amide-linked lipids was investigated in more detail by applying this sequential procedure to isolated LPS.

In this paper we report on the results obtained for *Acinetobacter calcoaceticus* LMD 79-41, since this species contains different classes of compounds such as wax esters, fatty acids and hydroxy fatty acids in different modes of occurrence. *Acinetobacter* is an ubiquitous species in waters, soils and sediments and is known for its ability to degrade long chain hydrocarbons (Makula *et al.*, 1975; Brade and Galanos, 1982).

In the next paper (Goossens *et al.*, 1988) we report on the results obtained for sedimentary organic matter after application of the sequential analytical procedure described here, to recognize the contribution of bacterial organic matter to sediments.

Experimental

Organisms

Acinetobacter calcoaceticus LMD 79-41 cells were obtained from the Laboratory of Microbiology of the Delft University of Technology. They were grown in chemostat at a dilution rate of 0.1 (hr^{-1}) on a mineral medium containing only spore elements and 30 mM acetate as a carbon source. The material was harvested by collecting the overflow of the culture vessel for one week. After centrifugation the cells were stored at -20 °C. The material was washed three times with double distilled water and lyophilized before analysis.

LPS-isolation

LPS was isolated from *Acinetobacter calcoaceticus* LMD 79-41 by a modified phenol-water extraction as described by Brade and Galanos (1982). We purified the extracted LPS only to the "crude LPS" stage.

Analysis

A general outline of the procedures applied is given in Fig.1. Details of the procedures are described below. Procedures A, B, C and D all started with lyophilized material.

Ether extraction
100-200 mg of lyophilized cells were extracted five times by suspending the material in 4 ml of diethyl ether and treating it ultrasonically for 1 minute (Branson B12 Sonifier, microtip). The supernatants obtained by centrifugation (4000 g, 2 min.) were combined and derivatized.

Saponification
Dry material was saponified by refluxing for 1 hour in 4 ml of 1N KOH in CH_3OH (96%). After cooling and centrifugation (4000 g, 2 min.), the supernatant was transferred to a separatory funnel. The residue was washed with 2 ml 2N HCl/methanol (1:1), 2 ml H_2O/methanol (1:1) and 2 ml of methanol, respectively. All washings were added to the contents of the separatory funnel where the pH was adjusted to 3, if necessary. The residue was washed with 4 ml of CH_2Cl_2. This washing was added to the others and a phase separation was accomplished by adding 6 ml H_2O. The CH_2Cl_2 layer was removed and the washing with CH_2Cl_2 and phase separation was repeated two times. The combined CH_2Cl_2 layers were dried on anhydrous Na_2SO_4 and derivatized. The water layer and the residue were combined and lyophilized.

Acid treatment
Freeze dried material was sealed in a glass ampoule with 4 ml 4N HCl and heated for 6 hours at 100 °C. After cooling and opening of the ampoule the pH was adjusted to 3 with 15 M KOH. The solution was extracted (1 min., ultrasonically) five times with 4 ml of diethyl ether. The extracts were combined, dried on anhydrous Na_2SO_4 and derivatized. If the acid treatment was to be followed by saponification, the contents of the ampoule were neutralized (pH = 6-7), freeze dried and saponified as described above.

Derivatization
Before derivatization, the extracts were reduced to a small volume by evaporation under a stream of N_2. Carboxyl groups were esterified with diazomethane in diethyl ether. After methylation, the solvent was evaporated under nitrogen and free hydroxyl groups were converted into trimethylsilyl ethers with Trisil-Z (Pierce). The derivatized sample was diluted with a small volume of cyclohexane/pyridine/HMDS (98/1/1), (HMDS = 1,1,1,3,3,3-hexamethyldisilazan). Very polar compounds were removed from the derivatized sample by chromatography over Lipidex-5000 (Packard) with cyclohexane/pyridine/HMDS (98/1/1) as the eluent. The eluted sample was evaporated to dryness and dissolved in a known amount of ethyl acetate.

Gas chromatography and gas chromatography-mass spectrometry
Gas chromatography was performed on a Carlo-Erba 4160 instrument with flame ionization detection and on column injection using a fused silica CPSil 5 capillary column (Chrompack) with a length of 26 m (inner diameter 0.32 mm, film thickness 0.13 μm). Helium was used as a carrier gas. Samples were injected at 100 °C. After injection the temperature was quickly raised to 130 °C and from there programmed at a rate of 4 °C/min to 340 °C. Gas chromatography-mass spectrometry was performed on a Varian 3700 gas chromatograph connected to a Varian Mat 44 quadrupole mass spectrometer operated at 80 eV with a cycle time of 1.5 seconds.

Results

The lipid composition of *Acinetobacter calcoaceticus* LMD 79-41 was analyzed by four parallel procedures (Fig.1.):
A. Extraction after saponification of the starting material
B. Extraction after acid treatment of the starting material
C. Extraction after acid treatment and subsequent saponification
D. A sequence of extractions and hydrolyses

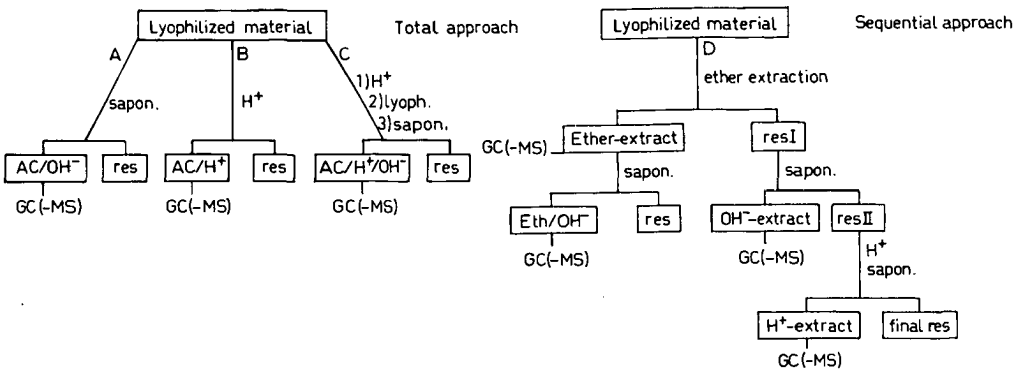

Fig.1. Scheme of analysis; for details: see text.

Extracts were derivatized and injected directly into the GC system without any preseparation. No attempts have been made to establish the absolute amounts of compounds, *i.e.* no internal standards were added to the extracts. Nevertheless, the amounts of a compound in different extracts can be made directly comparable by multiplying the peak intensities of that particular compound in the different gaschromatograms with scaling factors which compensate for differences in extract volumes, injection volumes and attenuations. These factors are 1, 9 and 1.5 for the Ether-extract, the OH^--extract and the H^+-extract, respectively.
Peak numbers in the gas chromatograms correspond to the numbers in Table 1. Identifications are based upon comparison of relative GC retention times and mass spectral data with those of standards.

Table 1. Identifications of the peaks in the gas chromatograms. Abbreviations: FAME = fatty acid methyl ester; TMS = trimethylsilyl; *n* = normal. For structures see Fig.4.

1 *n*-$C_{12:0}$FAME	20 *n*-10-OTMS-$C_{16:0}$FAME	39 $C_{30:0}$ waxester
2 *n*-β-OTMS-$C_{10:0}$FAME	21 *n*-$C_{18:1}$-OTMS	40 β14-β14
3 Δ^2-$C_{12:1}$FAME	22 *n*-$C_{18:0}$-OTMS	41 β12-18:1
4 β-OCH_3-$C_{12:0}$FAME	23 *n*-10-oxo-$C_{18:0}$FAME	42 β12-18:0
5 *n*-$C_{14:0}$FAME	24 *n*-9-OTMS-$C_{18:1}$FAME	43 α-OTMS-$C_{30:0}$ waxester
6 α- and β-OTMS-$C_{12:0}$FAME	25 *n*-9-OTMS-$C_{18:0}$FAME	44 β-OTMS-$C_{30:0}$ waxester
7 Δ^2-$C_{14:1}$FAME	26 *n*-10-OTMS-$C_{18:0}$FAME	45 $C_{32:2}$ waxester
8 *n*-$C_{15:0}$FAME	27 β12-12	46 $C_{32:1}$ waxester
9 β-OCH_3-$C_{14:0}$FAME	28 β12-α12	47 $C_{32:0}$ waxester
10 *n*-$C_{16:1}$FAME	29 α12-β12	48 C_{33}waxester
11 *n*-$C_{16:1}$FAME	30 β14-12	49 $C_{34:2}$ waxester
12 *n*-$C_{16:0}$FAME	31 $C_{28:0}$ waxester	50 $C_{34:1}$ waxester
13 *n*-β-OTMS-$C_{14:0}$FAME	32 β14-α12	51 $C_{34:0}$ waxester
14 *n*-$C_{16:1}$-OTMS	33 α12-β14	52 unknown
15 *n*-$C_{16:0}$-OTMS	34 β12-16:1	53 unknown
16 $C_{17:1}$FAME	35 β12-16:0	54 $C_{36:2}$ waxester
17 *n*-$C_{18:1}$FAME	36 α-OTMS-C_{28} waxester	55 $C_{36:1}$ waxester
18 *n*-$C_{18:1}$FAME	37 β-OTMS-C_{28} waxester	56 $C_{36:0}$ waxester
19 *n*-$C_{18:0}$FAME	38 $C_{30:1}$ waxester	57 unknown

Procedures A, B and C
Figs. 2 and 3 show the lipid compounds of whole cells of *Acinetobacter calcoaceticus* (AC), extractable after base-treatment (AC/OH^-) and acid-treatment (AC/H^+), respectively. Different classes of compounds were observed: straight chain fatty acids ($C_{12:0}$, $C_{16:1}$, $C_{16:0}$, $C_{18:1}$, $C_{18:0}$), α-, β- and mid-chain hydroxy fatty acids (C_{12}, C_{14}, C_{16}, C_{18}), straight chain alcohols ($C_{16:1}$, $C_{16:0}$, $C_{18:1}$, $C_{18:0}$) and wax esters in the C_{32} to C_{36} range.

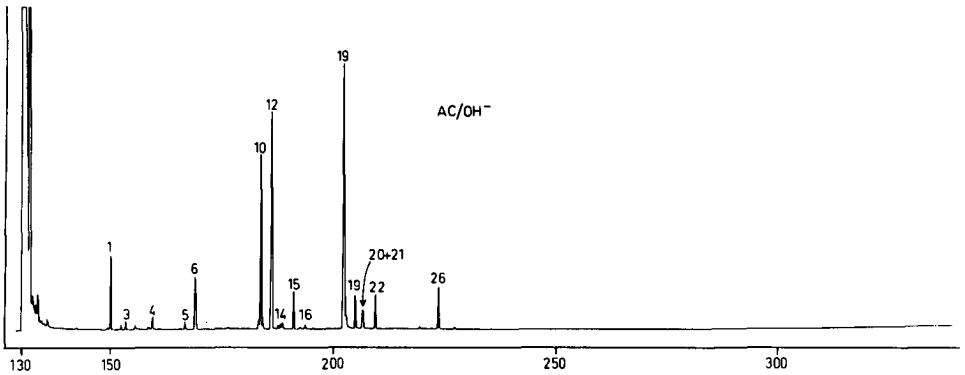

Fig.2. Gas chromatogram of extractable compounds after saponification of whole cells.

Fig. 3 shows a number of compounds (marked *) which are only observed after acid treatment. Based upon their mass spectra they were tentatively identified as esters composed of different combinations of fatty acids, hydroxy fatty acids and alcohols, and they were shown to be formed, at least in part, arteficially.

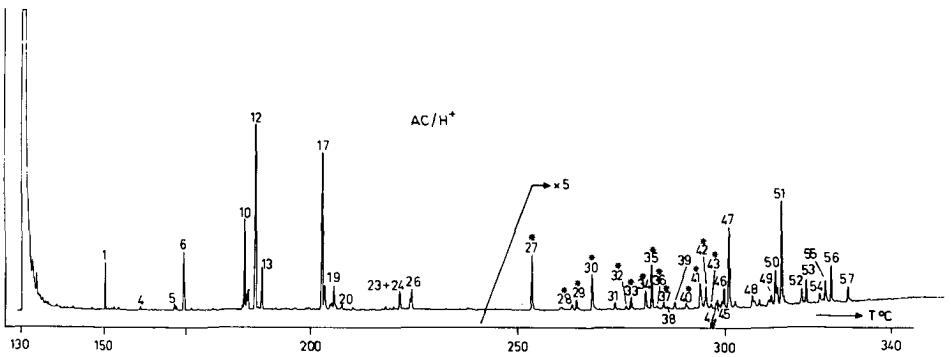

Fig.3. Gas chromatogram of extractable compounds after H^+-treatment of whole cells (* = artefacts, see text).

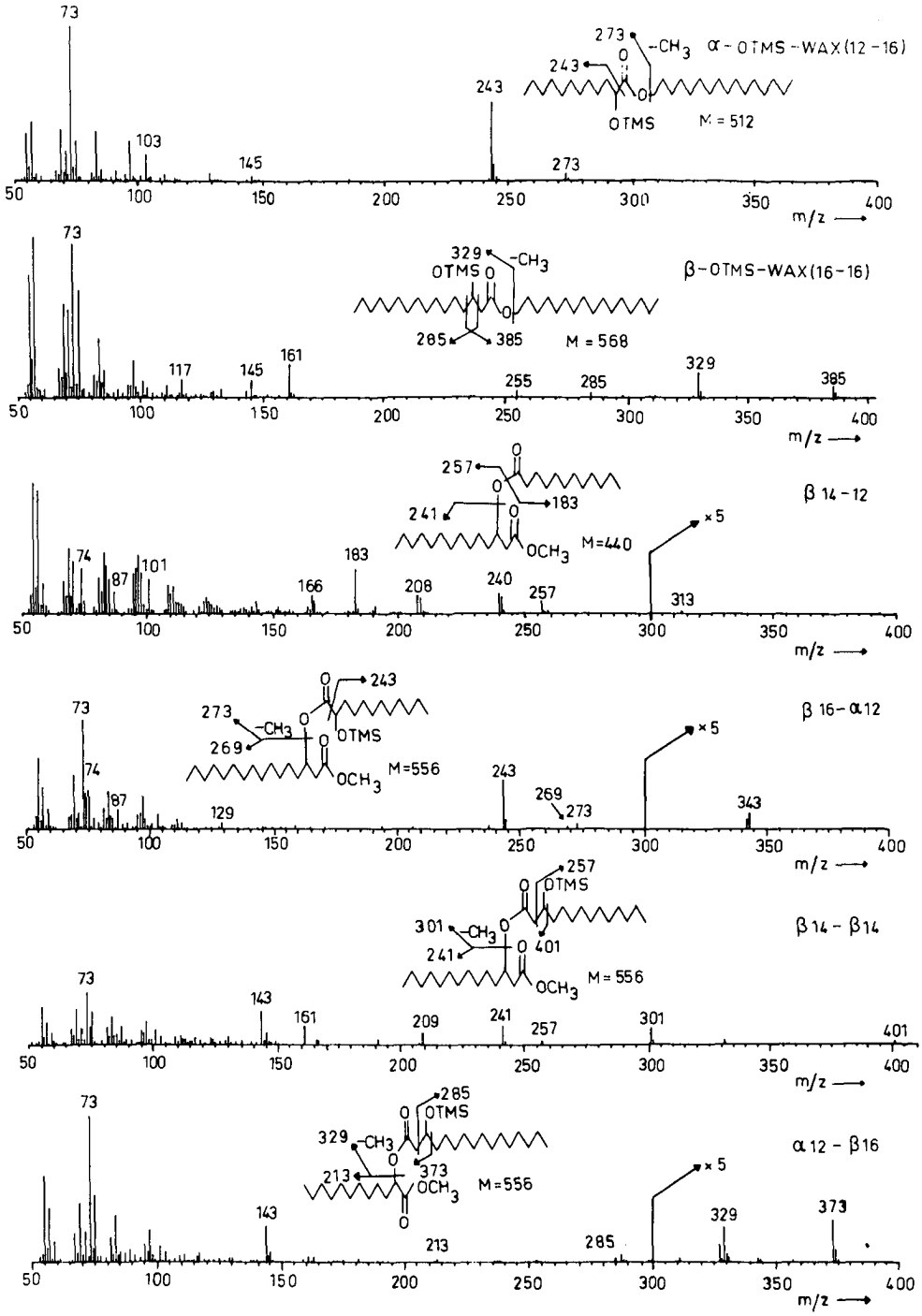

Fig.4. Mass spectra of arteficial esters formed during H^+-treatment.

The structures and the possible artificial formation of these compounds was proved by three separate experiments:

1) Application of the acid treatment to a standard mixture of *n*-β-OH-$C_{16:0}$FA and *n*-α-OH-$C_{12:0}$FA.
2) Application of the acid treatment to a standard mixture of *n*-α-OH-$C_{12:0}$FA and *n*-$C_{16:0}$ alcohol.
3) Application of the acid treatment to a standard mixture of *n*-β-OH-$C_{16:0}$FA and *n*-$C_{16:0}$ alcohol.

The products generated in these experiments showed mass spectra identical to those of compounds observed in the extract obtained by procedure B. Typical examples of structures and mass spectra are reproduced in Fig.4.

To obtain a correct profile of the lipids released upon acid treatment the combined extract and residue after acid treatment were saponified ($AC/H^+/OH^-$, Fig.5). Differences between the lipids released after acid- and base-treatment are clear from comparison of Fig.2 with Fig.5 Hydroxy fatty acids are present in higher quantities in the $AC/H^+/OH^-$-extract than in the AC/OH^--extract.

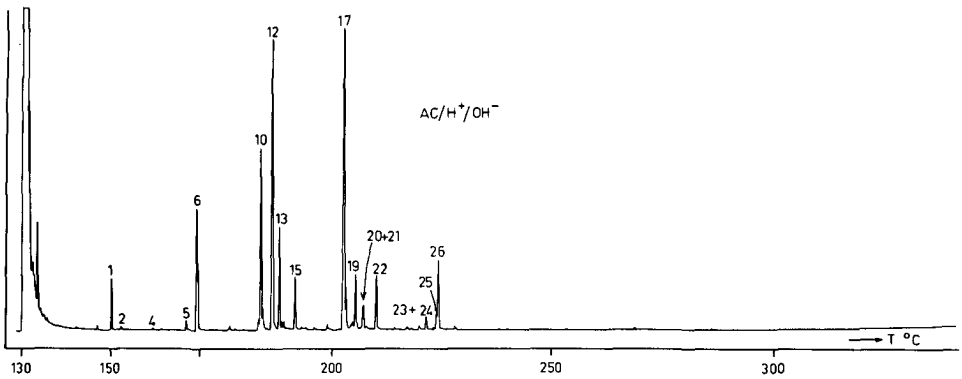

Fig.5. Gas chromatogram of extractable compounds after H^+-treatment and subsequent saponification of whole cells.

Procedure D

Application of the sequential procedure yielded three fractions (Fig.6):

1) apolar lipids which are extractable as such with ether (Fig.6a)
2) lipids which are released from the residue of the first extraction by saponification (Fig.6b)
3) lipids which are released from the residual matter of the second extraction by acid treatment followed by saponification (Fig.6c)

To be sure that all residual material was used in the next step of the sequential procedure, the water/methanol layer and the extracted residue were combined and lyophilized before the subsequent hydrolysis was carried out.

The possible occurrence of complex lipids not amenable to GC analysis in the first extract was investigated by saponification (results not shown). The appearence of alcohols and an increased amount of some fatty acids was observed and readily explained as the result of hydrolysis of the wax esters in the direct ether extract.

The bar-graph in Fig.7 shows the relative amounts of the major components in *Acinetobacter* in their different modes of occurrence.

Fig.8 shows the results of the sequential procedure applied to lipopolysaccharide (LPS). As a part of the isolation procedure of LPS free lipids were removed, leaving only two fractions to be obtained by sequential analysis (LPS-OH^- and LPS-H^+).

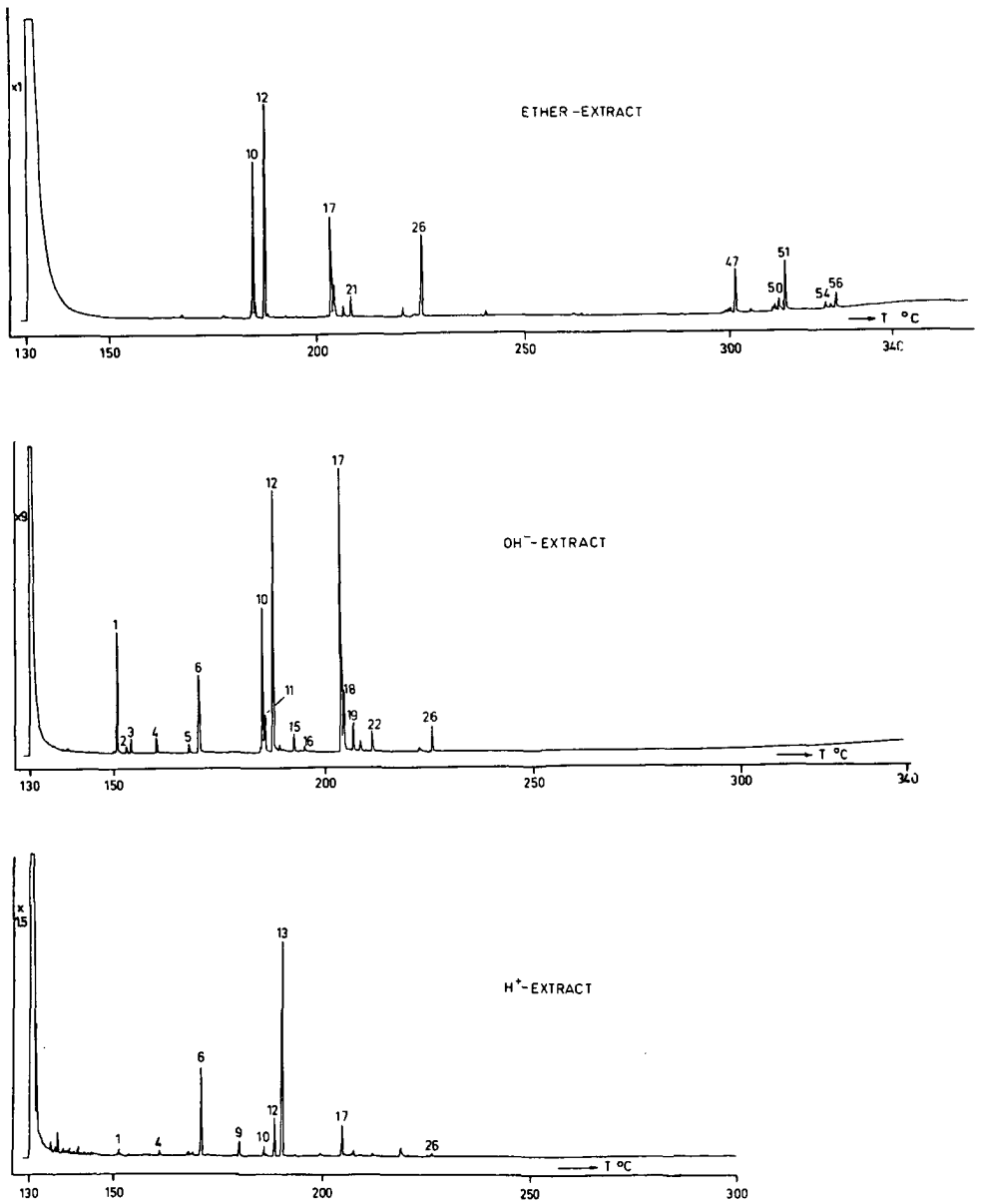

Fig.6. Gas chromatograms of the extracts obtained by sequential analysis.

Discussion

Procedures

Total lipid approach

The "total lipid profile" of *Acinetobacter calcoaceticus* LMD 79-41 (Fig.5) represents all lipids, present as such or as building blocks of *Acinetobacter* organic matter, that are amenable to gas chromatographic analysis (*i.e.* chain length less than about 50 C atoms). To obtain this "total lipid profile", base and acid labile bonds have been hydrolyzed prior to extraction. Application of the ether bond cleaving reagent BCl_3 (results not shown) did not release new compounds, thus, components escaping our analysis are considered non hydrolyzable high molecular weight lipids or lipid containing substances. On the other hand, lipid moieties more volatile than the C_{14} fatty acid methyl ester (FAME) are not quantitatively recovered from the hydrolysates due to partial evaporation during sample work-up.

Comparison of Figs. 2 and 5 clearly demonstrates that by application of an acid hydrolysis more lipid moieties are released from the organic matter: the amount of n-β-OH-C_{12}FA (compound 6) would have been underestimated when the acid treatment was omitted while *n*-β-OH-C_{14}FA (compound 13) would have been missed completely. Acid hydrolysis of lipid containing organic matter is commonly done in investigations dealing with the chemical composition of lipopolysaccharides (LPS) of bacteria (Wilkinson, 1977; Galanos *et al.*, 1977; Lüderitz *et al.*, 1982). It is used to hydrolyse the amide bond by which hydroxy fatty acids are bound to the polymer. These amide bonds are not hydrolyzed by alkali (Mayberry, 1980). The conditions of the acid hydrolysis are necessarily rather drastic, however, and a careful examination of the effects on the lipid moieties is required. Artefact formation is known to occur under these conditions (Wilkinson, 1974; Drewry *et al.*, 1973). The high temperature part of the gaschromatogram of the AC/H^+-extract (Fig.3) shows a series of peaks (marked by an asterisk) representing esters. These esters were synthesized separately by applying the acid hydrolysis procedure to mixtures of standard compounds containing hydroxyl- and carboxylgroups. The structures assigned to the peaks in Fig.3 were verified by these syntheses (Fig.4). The arteficial formation of these esters is also clear from the fact that the distribution pattern of the free fatty acids is reflected in the relative amounts of these esters.

Yet we cannot firmly conclude that all of these asterisk marked esters are artefactually formed since 3-O-acyl-fatty acids are reported to occur in the LPS of gram-negative bacteria (Galanos *et al.*, 1977; Wilkinson, 1977; Brade and Galanos, 1982; Wollenweber *et al.*, 1984) and probably were present in our material also (see below).

The wax ester pattern in Fig.3 does not reflect the free fatty acid pattern, indicating that wax esters at least partly survive the acid treatment. The acid treatment obviously does not lead to complete hydrolysis.

The problem of artefact formation can be solved to a great extent by "re-hydrolysis" under alkaline conditions (Fig.5). Such a "re-hydrolysis" has to be applied to the combined extract and residual matter to prevent a substantial loss of lipid moieties due to formation of high molecular weight esters not amenable to GC-analysis and esterification of lipid moieties to the residue. However, information about the presence and structures of hydrolyzable complex lipids like, in this case, the wax esters is lost by this second alkaline hydrolysis.

Sequential approach

Via these total lipid approaches information is obtained about the structures of the lipid moieties present and their distribution pattern in the sample. However, the presence of *n*-β-OH-C_{14}FA after acid hydrolysis only, indicates that the mode of occurrence (*i.e.* the type of bonding) of this compound is different from that of the other components. Therefore, the sequential procedure shown in Fig.1 was applied to differentiate the lipid moieties present in the starting material according to their mode of occurrence. The mode

of occurrence is a third aspect of information obtainable by lipid analysis in addition to the structures of the components and their distribution pattern in the sample.

The sequential approach has some additional advantages: 1) the extracts become less complex, reducing the chance of coelution upon GC-analysis; 2) information about complex neutral lipids such as wax esters is retained; 3) artefact formation is reduced considerably since the acid treatment is applied after removal of most of the hydroxyl group bearing compounds by saponification and extraction in the second step of the procedure.

In each step of the procedure a separation is induced between material which is soluble and insoluble in a relatively apolar solvent (diethyl ether or dichloromethane). All material which is not in the organic extract (*i.e.* water layers and residues) is carefully combined and lyophilized before analysis in the next step to prevent losses.

In the first step free, relatively apolar lipids are extracted (Ether-extract). These may include complex lipids, like wax esters provided they have a relatively apolar nature. We chose to use diethyl ether as the solvent, in stead of some mixture containing methanol, to exclude the diglycerides, which are the most abundant lipids in bacteria, from the first extract. In this way, as many of the esterified moieties as possible remained in the residual matter to be extracted after saponification.

In the second step the residual material is saponified. During this treatment OH^--labile bonds are cleaved, releasing lipids from insoluble matter or from free occurring compounds with polar groups, which made them inextractable in the previous step. After extraction of the lipid moieties released (OH^--extract) the acid treatment is applied to the combined, lyophilized residual matter. Again lipid moieties are released, now by cleavage of H^+-labile bonds. Subsequently a saponification is done for reasons discussed above. Extraction yields the third extract (H^+-extract).

In bacteria the OH^--labile bonds are mainly ester bonds so that the second extract contains mainly lipid moieties which occurred esterified in the original organic matter.

Two bonding-types occurring in bacteria need acid to be broken: amide bonds and glycosidic ether bonds. Amide bonds are known to occur in LPS, in ornithine lipids (OL) and in bacterial sphingolipids. Glycosidic ether bonds occur in carotenoids (Taylor, 1984), in alkyl resorcinols, which are present in the cyst membrane of *Azotobacter vinelandii*, (Rensch and Sadoff, 1983), and in other glycolipids (Langworthy *et al.*, 1976; Langworthy, 1982).

Comparison of results

The relative amounts of the lipid moieties present in the extracts obtained by the total approaches A and B are shown in Figs. 2 and 5. The main part of the lipids consists of even carbon numbered straight chain fatty acids ($C_{12:0}$, $C_{16:1}$, $C_{16:0}$, $C_{18:1}$, $C_{18:0}$) and hydroxy fatty acids (*n*-α-OH-C_{12}FA, *n*-β-OH-C_{12}FA, *n*-β-C_{14}FA, 10-OH-C_{16}FA, 10-OH-C_{18}FA). The differences between the two approaches (increased amounts of *n*-α-OH-C_{12}FA and *n*-β-OH-C_{12}FA and *n*-$C_{16:0}$FA and the very presence of *n*-β-OH-C_{14}FA upon acid treatment) are easily explained by the results obtained with the sequential approach (Figs. 6 and 7). The increased intensities of some compounds after acid treatment are due to cleavage of H^+-labile bonds.

The first extract obtained by the sequential approach (Fig.6a) shows the presence of the wax ester series, indicating that information about hydrolyzable complex neutral lipids is retained. This extract might contain also hydrolyzable complex lipids not amenable to gas chromatographic analysis such as triglycerides, but saponification of the first extract gave no indications for their presence. However, we checked the absence of such compounds only by saponification of the extract whereas, theoretically, also an acid treatment should be done. We deliberately omitted such an additional treatment since H^+-labile bound lipids are only known to occur in substances not directly extractable by ether because of their relatively polar nature (LPS, OL, carotenoids with glycosidic ether bonds, etc.).

The OH^--extract (Fig.6b) showed a composition somewhat similar to the Ether-extract. Several compounds were not present in the first extract *i.e.* *n*-C_{12}FA, *n*-α-OH-C_{12}FA

and n-β-OH-C_{12}FA, whereas the relative amount of n-$C_{18:1}$FA was much higher. This fraction also contained some alcohols originating from other sources than the wax esters. The presence of Δ^2-$C_{12:1}$FA and β-OCH_3-C_{12}FA is discussed below. The lipid composition of the H^+-extract (Fig.6c) is clearly different. This extract is characterized by the dominance of the β-hydroxy fatty acids (C_{12} and C_{14}) and the virtual absence of the otherwise dominant fatty acids.

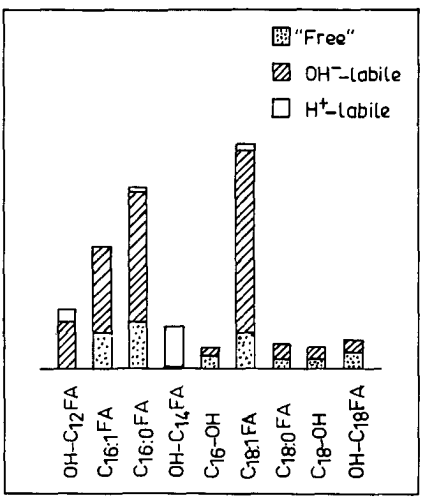

Fig.7. Relative amounts of major compounds in their different modes of occurrence.

Figs. 6 and 7 demonstrate that, whereas some compounds occur only in one mode (n-C_{12}FA, n-α-OH-C_{12}FA, n-β-OH-C_{14}FA), others occur in several modes in different relative amounts. For example, n-β-OH-C_{14}FA occurs almost exclusively linked via an H^+-labile bond, whereas the presence of n-β-OH-C_{12}FA in the OH^-- and H^+-extract indicates at least two bonding types for this component. Both figures (Figs. 6 and 7) clearly demonstrate the specificity of the H^+-extract despite its relatively low lipid content. The source of these β-hydroxy fatty acids, which are only released by acid treatment, is the LPS polymer. This is clear from Fig. 8, which shows the gas chromatograms obtained by application of the sequential procedure to the LPS fraction isolated from the same organism. Again the specificity of the H^+-labile lipids is clearly demonstrated. LPS is known to contain amide-bound β-hydroxy fatty acids, whereas in ester bonding fatty acids, α- and β-hydroxy fatty acids may occur (Galanos *et al.*, 1977). Our results (n-$C_{12:0}$FA, n-α-OH-$C_{12:0}$FA and n-β-OH-C_{12}FA esterified and n-β-OH-C_{12}FA and n-β-OH-C_{14}FA amide bound) are in good agreement with these data.

The OH^--labile and H^+-labile lipid fractions (Figs. 6b,c and Figs. 8a,b) contain different amounts of β-OCH_3-fatty acids and Δ^2 mono-unsaturated fatty acids. When the standard compounds n-α-OH-C_{12}FA and n-β-OH-C_{16}FA and a mixture of both were subjected to saponification conditions these compounds were not formed. However, the LPS polymer may contain hydroxy fatty acids in which the hydroxyl group is esterified to a fatty acid moiety (Drewry, 1973; Wilkinson, 1977; Galanos *et al.*, 1977; Wollenweber *et al.*, 1984). Alkaline conditions can cause β elimination of the substituting O-acylgroup yielding Δ^2-unsaturated fatty acids (Galanos *et al.*, 1977). The presence of Δ^2-$C_{12:1}$FA in the OH^--labile lipid fraction of whole cells and LPS (Figs. 6b and 8a, resp.) strongly suggests the original presence of these O-acyl substituted C_{12} fatty acid residues ester linked in the LPS of *Acinetobacter calcoaceticus* LMD 79-41. In the

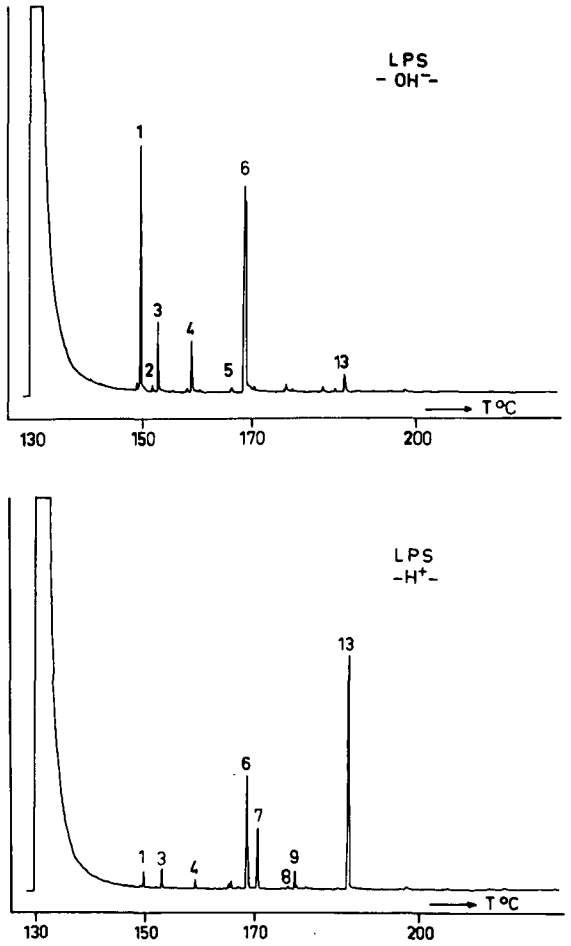

Fig.8. Gas chromatograms of the extracts obtained by sequential analysis of LPS of *Acinetobacter calcoaceticus* LMD 79-41.

H^+-labile lipid fraction (Fig. 8b) the Δ^2-unsaturated fatty acids and the methoxy fatty acids are present in proportion to the corresponding hydroxy fatty acids, indicating a possible arteficial formation, the former probably via β elimination during saponification of O-acyl substituted fatty acids formed during the acid treatment. Methoxy fatty acids were formed only in very low amounts when the conditions of acid or base treatment were applied to the mixture of standard compounds mentioned above.

Based upon these observations with the standards we cannot rule out completely the possibility that methoxy- and Δ^2 unsaturated fatty acid moieties are present as such in this bacterial species.

Another point of uncertainty exists about the mid chain functionalities encountered in several C_{18} fatty acids. Apart from 10-OH-C_{18}FA we identified 9,10-di-OH-C_{18}FA, 9,10-epoxy-C_{18}FA, 10-oxo-C_{18}FA and a mono-unsaturated OH-C_{18}FA, all in relatively

low amounts. Whether 9,10-di-OH-C_{18}FA, 10-oxo-C_{18}FA and the OH-$C_{18:1}$FA are generated from probably present 9,10-epoxy-C_{18}FA and 10-OH-C_{18}FA during acid treatment or whether they are real constituents of this organism remains unclear, at this stage.

Our results are in agreement with previous investigations into the lipid composition of *Acinetobacter calcoaceticus* (Adams *et al.*, 1970; Gallagher, 1971; Thorne *et al.*, 1973; Fixter and Fewson, 1974; Jantzen *et al.*, 1975; Makula *et al.*, 1975; Bryn *et al.*, 1977; Brade and Galanos, 1982)) except that *n*-$C_{18:2}$ FA and cyclopropyl fatty acids were not encountered in this study.

We are not aware of any complete analysis of the lipid composition of *Acinetobacter*. Either specific compound classes are investigated or specific parts of the cell like LPS or the cell wall.

Some specific observations should be noted like the presence of the mid chain hydroxy fatty acids and the predominance of saturated wax esters. The presence of mid chain hydroxy fatty acids (9- and 10-OH-$C_{16:0}$FA and -$C_{18:0}$FA) has not been reported before in *Acinetobacter*. Mid chain hydroxy fatty acids are uncommon constituents of bacteria although the enzyme system for their synthesis has been demonstrated in many genera (Fulco, 1983). The site and function in the cell is unknown.

The occurrence of wax esters in the genus *Acinetobacter* is well known (Gallagher, 1971; Fixter and Fewson, 1974; Bryn *et al.*, 1977). However, the predominance of the saturated wax esters is remarkable, since in most studies mainly unsaturated ones have been found. Saturated wax esters may be related to adverse growth conditions (Fixter and Fewson, 1974). In our case the cells were collected in the overflow vessel of a continuous culture system for about one week so the cells were probably under conditions of starvation. In sediments, wax esters are commonly found (Boon and de Leeuw, 1979; Volkman *et al.*, 1981; Cranwell, 1983) and the saturated ones predominate. It would be interesting to investigate the possibility that saturation of wax esters under conditions of starvation is an alternative explanation for the general predominance of saturated wax esters in sediments.

Geochemical implications

The lipid analysis of prokaryotic organic matter, whether in sediments or as such, should include an acid treatment to liberate lipid moieties withstanding saponification. Problems of artefact formation can be minimized by subsequent saponification of formed esters. It is advantageous to perform the extractions and hydrolyses sequentially. In this way the mode of occurrence (*i.e.* the bonding type) of the lipid moieties is revealed, extending the information content of lipid analyses with a third feature besides the structures of the components present and their distribution patterns in the sample. Some components occur in different modes while others show only one type of linkage. The H^+-labile lipid fraction contains almost exclusively β-hydroxy fatty acids which were shown to be derived from LPS and thus were amide bound. Amide-bound hydroxy fatty acids are known to occur only in LPS, ornithine-lipids (OL) and sphingolipids (SL). The former two are exclusively present in prokaryotes, whereas the latter is rare in bacteria but widespread among fungi, higher plants and animals. The sphingolipids of eukaryotes, however, contain only fatty acids or α-hydroxy fatty acids in amide linkage (Weete, 1974; Gurr and James, 1980). Therefore, amide bound β-hydroxy fatty acids are specific markers of prokaryotic organic matter.

Hydroxy fatty acids have been found in recent sediments (Boon *et al.*, 1977; Volkman *et al.*, 1980; Cranwell, 1981a; Parker *et al.*, 1982; Kawamura and Ishiwatari, 1982; Cardoso and Eglinton, 1983; Klok *et al.*, 1984). The β-hydroxy fatty acids are generally ascribed to bacterial sources. However, in sediment analyses often only saponification is performed to release bound lipids. In those cases the exclusively acid labile lipids are simply overlooked, and the β-hydroxy fatty acids are, as a consequence, highly underestimated.

In the next article of this issue we report on the analyses of a lacustrine sediment (Goossens *et al.*, 1988). The results show that the sequential procedure provides valuable information on sedimentary lipids, indeed.

Acknowledgements

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CHAPTER 3

Lipids and their mode of occurrence in bacteria and sediments II: Lipids in the sediment of a stratified, freshwater lake.

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Abstract

In the course of a systematic study into the recognition of prokaryotic organic matter in sediments, a sample from a very recent, lacustrine sediment (Lake Vechten, the Netherlands) was analysed by a method applying sequential extractions and hydrolyses. Three different modes of occurrence of lipids (free, OH^--labile and H^+-labile) are distinguished. Most lipid moieties occur in different modes. The free compounds are primarily even, long chain fatty acids and alcohols (> C_{20}) and sterols, the latter showing low values of the Δ^5-stenol/5α(H)-stanol ratio. The OH^--labile components are mainly derived from the plant macromolecules cutin and suberin. The extract containing exclusively H^+-labile lipids shows abundant β-hydroxy fatty acids which occurred probably amide-bound in the original organic matter. These H^+-labile β-hydroxy fatty acids are specific for bacteria and their distribution is considered a fingerprint of the prokaryotic community.
The lipids in the sediment are dominated by markers of higher plant material. Since the input of higher plant material is calculated to be of minor importance, the organic geochemical record does not reflect the relative contributions to the carbon budget of the lake. This indicates that the biodegradability of incoming organic matter and the mode of occurrence of the lipids in it are major factors determining the composition of lipids in sediments.

Introduction

A profound investigation into the chemical composition of prokaryotic organic matter within the scope of organic geochemistry is required not only because there is a substantial bacterial contribution to the organic matter in sediments but also because the chemical composition of bacterial organic matter reflects the environmental conditions to a large extent. Therefore, detailed information about the chemical composition of bacterial organic matter might give clues to the environment of deposition.

Some suites of compounds present in the geosphere can be used as bacterial markers since their prokaryotic source has been firmly established (ether-linked isoprenoid alkyl chains, Chappe *et al.*, 1982; extended hopanoids, Ourisson *et al.*, 1984; Rohmer *et al.*, 1984). For some other compounds a prokaryotic origin has been assumed (iso- and anteiso-branched compounds, β-hydroxy fatty acids; Eglinton *et al.*, 1968; Boon *et al.*, 1977a,b; Cranwell, 1981a,1982; Kawamura and Ishiwatari, 1982; Cardoso and Eglinton, 1983). In all these cases the bacterial origin is inferred from the mere molecular structure. However, due to the uniformity of biosynthetic routes in nature, many compounds, including branched compounds and β-hydroxy fatty acids, occur in phylogenetically very different organisms (Downing, 1961,1976; Weete, 1974,1976; Perry *et al.*, 1979; Matsumoto and Nagashima, 1984). Consequently, these compounds are less specific and their structure as such is not informative enough to establish the origin beyond doubt.

During the past few years it has become clear that the information present in the lipid content of sediments is not only expressed in the very structure of the components or in their distribution patterns but also in their modes of occurrence. By discriminating different modes of occurrence additional information has been obtained, which helped establish the origin of compounds (Cranwell, 1978,1981a,b; Chappe *et al.*, 1982). Moreover, the mode of occurrence may be a factor which determines the biodegradability and, consequently, if and how lipid moieties survive the early stages of diagenesis. Eyssen *et al.* (1973), for example, showed that Δ^5 unsaturated sterols are only hydrogenated by bacteria when they occur as free substrates, whereas the presence of ether-linked isoprenoid alkyl chains in Messel shale ($60 * 10^6$ yr.) clearly demonstrates that the ether linkage, present in the archaebacterial ether-lipids, is resistant to biodegradation (Chappe *et al.*, 1982). In general, "bound" lipids seem to be better preserved than "free occurring" compounds (Nishimura and Koyama, 1977; Cranwell, 1978,1981a,b; Kawamura and Ishiwatari, 1984, Albaigès *et al.*, 1984).

In most organic geochemical studies, "bound" lipids are liberated by saponification of the residue which remains after extraction with a mixture of organic solvents. By this approach mainly esterified lipid moieties are released. In the few studies where acid was applied to release the "bound" lipids (Cranwell, 1978,1981a,b; Parker *et al.*, 1982; Klok *et al.*, 1984; Cardoso *et al.*, 1983) relatively high amounts of β-hydroxy fatty acids were observed (Cranwell, 1981a; Parker *et al.*,1982; Klok *et al.*,1984).

Different modes of occurrence for "bound" lipids have also been observed by Kawamura and Ishiwatari (1982,1984). They subjected the residue which remained after extraction of "free" and "bound" lipids (released by saponification) to a heat treatment, which resulted in the release of "tightly bound" lipid moieties.

This indicates that "bound" lipids occur in different modes which can be distinguished by relatively simple means, such as hydrolysis under alkaline or acid conditions. As a consequence, a further differentiation of "bound" lipids is possible.

The extensive literature on bacterial lipids (for reviews see: Lechevalier, 1977; Goldfine, 1982; Langworthy, 1982; Taylor, 1984) makes clear that many complex lipid structures occur in bacteria, which are mainly di-acyl-glycerides (in archaebacteria di-alkyl-glycerides) with a wide range of polar groups linked to the glycerol moiety at position 3. In addition, many other compounds are present which consist of varying lipid moieties (fatty acids, hydroxy fatty acids, alcohols) linked to one another or to polar groups. The linkages present in these substances are ester-bonds, amide-bonds, glycosidic-ether-bonds and ether-bonds. The latter occur primarily in the ether-lipids of archaebacteria.

Since amide- and glycosidic-ether-bonds are alkali-resistant but are cleaved under acid conditions (Mayberry, 1980), lipids linked via these two bonding types can be distinguished from those which are ester-linked. When saponification and acid hydrolysis are performed sequentially, extracts are obtained which contain exclusively OH^--labile lipid-moieties and H^+-labile lipid moieties, respectively.

Such a sequential approach, distinguishing free, OH^--labile- and H^+-labile lipid moieties, was applied to the gram-negative bacterium *Acinetobacter calcoaceticus* as a test substance. The results of this analysis are presented in the preceding paper (Goossens *et al.*, 1988).

In the present paper we report on the results obtained by applying the same analytical procedure to a sediment sample, taken from Lake Vechten, the Netherlands. Lake Vechten is a very small (4.7 ha), slightly eutrophic lake which is stratified from June to November. In the anoxic hypolimnion an active phototrophic bacterial community is present near to the chemocline. The lake was formed in 1941 by excavation of sand for the construction of a nearby highway. It is not directly connected to other waterbodies and receives water only from rain and horizontal ground water flows (Steenbergen and Verdouw, 1982). During 40 years of existence about 25 cm of sediment has formed which is rich in organic carbon (10-20% org C on a dry weight basis in the deeper layers) (Cappenberg and Verdouw, 1982). We selected this lake because it is isolated from other waterbodies and because its limnological parameters have been followed for many years so that a lot of background

information is available (Gulati and Parma, 1982). In addition, methanogenesis has been demonstrated in the sediment and the sample studied here was taken at the depth of maximal methane production (Cappenberg and Verdouw, 1982).

A comparison of the H^+-labile lipid moieties of thirteen selected bacterial species and three sediment samples from different environments is reported elsewhere (Goossens *et al.*, 1986).

Experimental

Sampling site

The sediment sample was taken in Lake Vechten, the Netherlands, at maximum depth (10 m) in October 1984 before the autumn turnover. The core (6 cm diameter) was sectioned immediately after sampling. The samples were stored at - 20 °C and lyophilized before analysis. In this study the 4 - 6 cm depth section was analyzed. The results of the extensive research carried out in this lake have been recently summarized (Gulati and Parma, 1982).

Isolation/extraction

5.0 grams of the lyophilized sample were subjected to exactly the same procedure as described by Goossens *et al.* (1988), with adjusted volumes of solvents. Here, only a short description is given.

The sample is first extracted with diethyl ether, which yields the Ether-extract. The residue of this extraction is saponified (1N KOH/methanol (96%), 1 hr., reflux) and extracted by a modified Bligh and Dyer (1959) method, yielding the OH^--extract. The residue remaining after this extraction and the H_2O/methanol fraction are combined, lyophilized and treated with 4 N HCl (6 hrs., 100 °C). Hereafter, the acid treated matter is saponified again and extracted identically to the second step of the procedure, yielding the H^+-extract. Half of the Ether-extract is also saponified, which yields the Ether/OH^--extract.

The compounds in the extracts were derivatized with diazomethane to convert carboxyl groups into methylesters (FAME) and with BSTFA (Merck) (1 hr., 40 °C) to convert hydroxyl groups into trimethylsilyl ethers. Between methylation and silylation the extract was chromatographed on a little column, filled with about 3 cm of silicagel 60 (Merck, 70 - 230 mesh) with 4 ml of hexane/ethylacetate (3:1, v/v) as the eluent.

The β-hydroxy fatty acids in the H^+-extract were isolated by thin layer chromatography on Kieselgel 60 (Merck) according to Skipski *et al.* (1965).

Gas chromatography and gas chromatography-mass spectrometry

Gas chromatography was performed on a Carlo Erba 4160 instrument with on column injection and flame ionisation detection (FID). A fused silica CPSil 5 capillary column (l=26 m, i.d.=0.32 mm, film thickness 0.13 μm)(Chrompack) was used with He as the carrier gas. After injection of the sample (in ethyl acetate) at 100 °C, the temperature was rapidly raised to 130 °C and then programmed at a rate of 4 °C/min to 330 °C.

Gas chromatography-mass spectrometry was performed on a Varian 3700 gas chromatograph connected to a Varian Mat 44 quadrupole mass spectrometer with EI ionisation at 80 eV. Mass spectra were recorded from m/z 50 to 500 with a cycle time of 1.5 s.

Results

The Lake Vechten sediment was analysed by the sequential method, as described for *Acinetobacter calcoaceticus* (Goossens *et al.*, 1988). Four extracts were obtained: The Ether-extract, containing the ether-extractable lipids; the saponified Ether-extract (Ether/OH^-); the OH^--extract, containing the lipid moieties which are released by saponification of the residue remaining after the ether-extraction; and the H^+-extract

(obtained by acid treatment and subsequent saponification of the second residue), in which the H^+-labile lipid-moieties are present.
The extracts were derivatized and analyzed by GC-FID and GC-MS without any preseparation of compound classes. Only very polar compounds were removed during the derivatization procedure

The amounts of a compound in different extracts can be made directly comparable by multiplying the peak intensities of that particular compound in the different gaschromatograms with scaling factors which compensate for differences in extract volume, injection volume and attenuation. These factors are 1, 1, 11 and 1.5 for the Ether-extract, the Ether/OH^--extract, the OH^--extract and the H^+-extract, respectively. Identifications are based upon comparison of mass spectral data and relative retention times with those of standards and with literature data. β-Hydroxy fatty acids were identified using the similarity of the elution patterns of fatty acids and β-hydroxy

Table 1. Identifications of the peaks in the gas chromatograms. Abbreviations: FAME = fatty acid methyl ester; TMS = trimethylsilyl; *n* = straight chain; *i* = iso branched; *ai* = anteiso branched. Explanation of -"71" and -"355" in the text.

1 *n*-$C_{12:0}$FAME
2 unknown
3 β-OTMS-$C_{10:0}$FAME
4 Δ^2-$C_{12:1}$FAME
5 *i*-$C_{13:0}$FAME
6 *ai*-$C_{13:0}$FAME
7 *i*-$C_{14:0}$FAME
8 *n*-$C_{14:0}$FAME
9 *n*-β-OTMS-$C_{12:0}$FAME
10 *n*-Δ^2-$C_{14:1}$FAME
11 *i*-$C_{15:0}$FAME
12 *ai*-$C_{15:0}$FAME
13 *i*-β-OTMS-$C_{13:0}$FAME
14 *ai*-β-OTMS-$C_{13:0}$FAME
15 *n*-$C_{15:0}$FAME
16 *i*-Δ^2-$C_{15:1}$FAME
17 *ai*-Δ^2-$C_{15:1}$FAME
18 *i*-$C_{16:0}$FAME
19 *n*-$C_{16:1}$FAME
20 *n*-$C_{16:1}$FAME
21 *i*-β-OTMS-$C_{14:0}$FAME
22 *n*-$C_{16:0}$FAME
23 *n*-β-OTMS-$C_{14:0}$FAME
24 *i*-$C_{17:1}$FAME
25 10-methyl-$C_{16:0}$FAME
26 Δ^2-$C_{16:1}$FAME
27 *n*-$C_{16:0}$-OTMS
28 *i*-$C_{17:0}$FAME
29 *ai*-$C_{17:0}$FAME
30 *n*-$C_{17:1}$FAME
31 *i*-β-OTMS-$C_{15:0}$FAME
32 *ai*-β-OTMS-$C_{15:0}$FAME
33 *n*-$C_{17:0}$FAME
34 *n*-β-OTMS-$C_{15:0}$FAME
35 β-OTMS-$C_{16:0}$FAME-"71"
36 *n*-$C_{18:2}$FAME
37 *n*-$C_{18:1}$FAME
38 *n*-$C_{18:1}$FAME
39 *i*-β-OTMS-$C_{16:1}$FAME
40 *n*-$C_{18:0}$FAME
41 *n*-β-OTMS-$C_{16:0}$FAME
42 *n*-$C_{18:0}$-OTMS
43 phytol
44 *i*-β-OTMS-$C_{17:0}$FAME
45 *ai*-β-OTMS-$C_{17:0}$FAME
46 *n*-β-OTMS-$C_{17:0}$FAME
47 *n*-$C_{16:0}$di-FAME
48 ω-OTMS-$C_{16:0}$FAME
49 *i*-β-OTMS-$C_{18:0}$FAME
50 β-OTMS-$C_{18:1}$FAME-"355"
51 *n*-C_{23}alkane
52 *n*-$C_{20:0}$FAME
53 *n*-β-OTMS-$C_{18:0}$FAME
54 6-OTMS-$C_{15:0}$di-FAME
55 7-OTMS-$C_{15:0}$di-FAME
56 *n*-$C_{20:0}$-OTMS
57 *n*-$C_{21:0}$FAME
58 *n*-$C_{22:0}$aldehyde
59 7- and 8-OTMS-$C_{16:0}$di-FAME
60 unknown
61 ω-OTMS-$C_{18:1}$FAME
62 *n*-$C_{21:0}$-OTMS
63 9,16- and 10,16-di-OTMS-$C_{16:0}$FAME
64 *n*-C_{25}alkane
65 *n*-$C_{22:0}$FAME
66 *n*-β-OTMS-$C_{20:0}$FAME
67 *n*-$C_{22:0}$-OTMS
68 unknown
69 *n*-$C_{23:0}$FAME
70 *n*-$C_{24:0}$aldehyde
71 *n*-$C_{20:0}$di-FAME
72 ω-OTMS-$C_{20:0}$FAME
73 *n*-C_{27}alkane
74 *n*-$C_{24:0}$FAME
75 *n*-b-OTMS-$C_{22:0}$FAME
76 *n*-$C_{24:0}$-OTMS
77 9,10,18-tri-OTMS-$C_{18:0}$FAME
78 *n*-$C_{25:0}$FAME
79 *n*-$C_{26:0}$aldehyde
80 *n*-$C_{22:0}$di-FAME
81 ω-OTMS-$C_{22:0}$FAME
82 *n*-C_{29}alkane
83 *n*-$C_{26:0}$FAME
84 *n*-β-OTMS-$C_{24:0}$FAME
85 *n*-$C_{26:0}$-OTMS
86 *n*-$C_{27:0}$FAME
87 5β(H)-cholestan-3β-ol
88 *n*-$C_{24:0}$di-FAME
89 5α(H)-cholestan-3α-ol
90 ω-OTMS-$C_{24:0}$FAME
91 5α(H)-cholest-22-en-3β-ol
92 cholesta-5,22-dien-3β-ol
93 5α(H)-cholest-22-en-3β-ol
94 *n*-$C_{28:0}$FAME
95 cholest-5-en-3β-ol
96 5α(H)-cholestan-3β-ol
97 *n*-$C_{28:0}$-OTMS
98 24-methyl-cholesta-5,22-dien-3β-ol
99 24-methyl-5α(H)-cholest-22-en-3β-ol
100 (ω-1)-OTMS-$C_{25:0}$FAME
101 *n*-$C_{29:0}$FAME
102 24-methyl-cholest-5-en-3β-ol
103 24-methyl-5α(H)-cholestan-3β-ol
104 *n*-$C_{26:0}$di-FAME
105 24-ethyl-cholesta-5,22-dien-3β-ol
106 24-ethyl-5α(H)-cholest-22-en-3β-ol
107 ω-OTMS-$C_{26:0}$FAME
108 *n*-$C_{30:0}$FAME
109 24-ethyl-cholest-5-en-3β-ol
110 24-ethyl-5α(H)-cholestan-3β-ol
111 *n*-$C_{30:0}$-OTMS
112 (ω-1)-OTMS-$C_{27:0}$FAME
113 di-O-phytanyl-glycerol (DPE) (TMS-derivative)

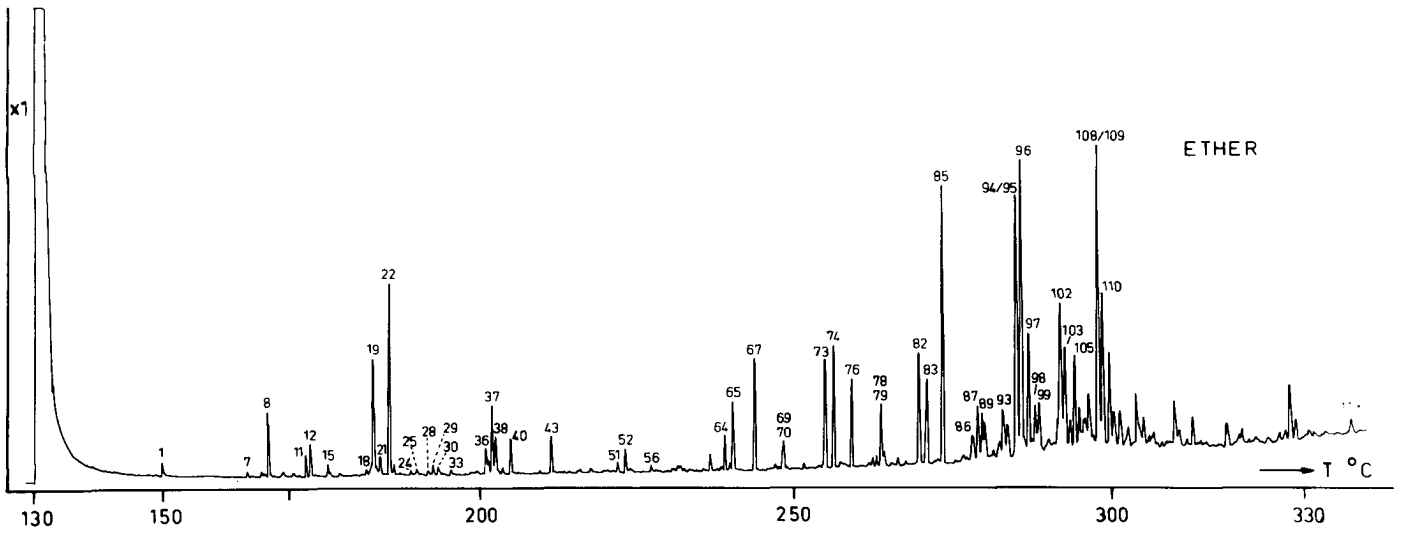

Fig.1 Gas chromatogram of the Ether-extract.

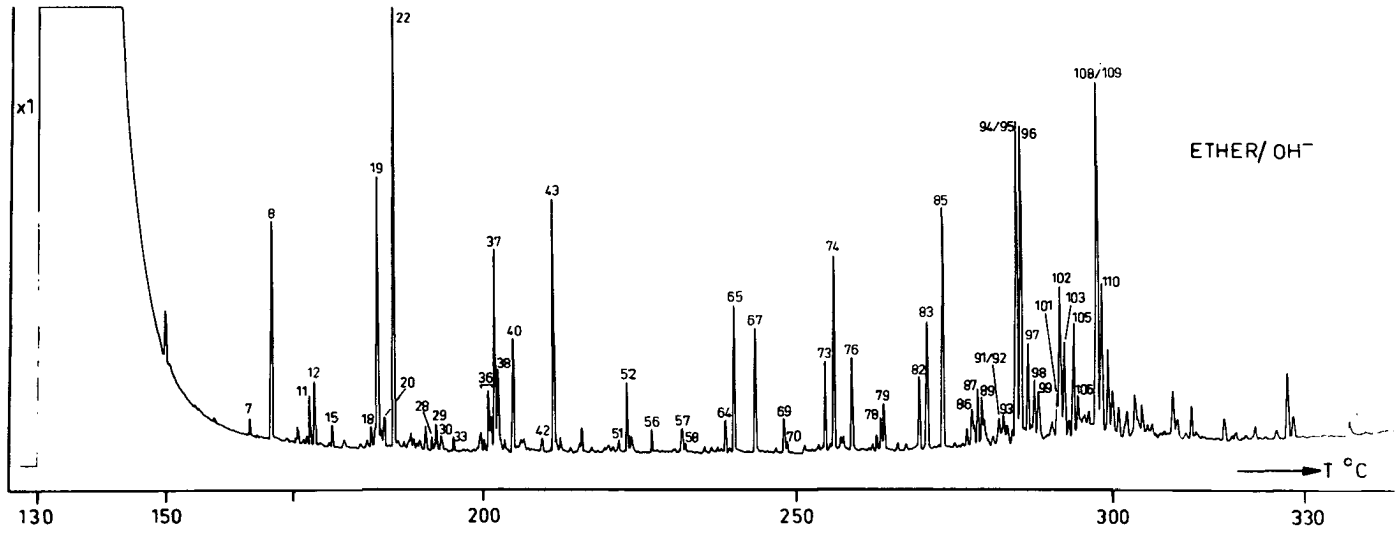

Fig.2 Gas chromatogram of the saponified Ether-extract.

fatty acids (Boon *et al.*, 1977b), since the mass spectra of iso(*i*)-, anteiso(*ai*)- and normal(*n*)-β-hydroxy fatty acids show no characteristic differences.
The gas chromatograms of the four extracts are shown in Figs.1 to 4.
Numbers in the gas chromatograms correspond to numbers in Table 1., which lists the compounds identified.
Distribution patterns of *n*-alcohols, fatty acids and β-hydroxy fatty acids in the different extracts are presented in Fig.5.

Ether-extract
The Ether-extract (Fig.1) contains primarily long-chain compounds (> C_{20}) and *n*-$C_{16:0}$FA. Homologous series of *n*-fatty acids, *n*-alcohols and *n*-alkanes and sterols are the major compounds. A series of sterols (C_{27} to C_{29}) is present showing relatively high amounts of the 5α(H)-compounds. Due to coelution of *n*-C_{28}FA and *n*-C_{30}FA with cholest-5-en-3β-ol (cholesterol) and 24-ethyl-cholest-5-en-3β-ol (peaks 94/95 and 108/109), respectively, the relative amounts of these compounds cannot be read directly from Fig.1. GC-analysis of the same sample using a slightly different temperature program (130 °C to 315 °C at 4 °C/min., data not shown) resulted in separation of these sterols from the fatty acids. The ratio cholest-5-en-3β-ol/5α(H)-cholestan-3β-ol is about 1, whereas the ratio 24-ethyl-cholest-5-en-3β-ol/24-ethyl-5α(H)-cholestan-3β-ol is about 1.7 in this extract. Mass chromatograms showing the distribution patterns of Δ^5 stenols (*m/z* 129), stanones (*m/z* 231) and 5α(H)-stanols (*m/z* 215), respectively, are given in Fig.7.
Peak 89 (Fig.1.) indicates the presence of 5α(H)-cholestan-3α-ol (Mermoud *et al.*, 1985).
Several triterpenoid compounds eluted with the sterols and also at higher temperatures. We observed diplopterol, bishomohopanoic acid and some triterpenoid alcohols. Due to the complexity of the extract identification of other compounds was not possible.
The fatty acid distribution is bimodal maximizing at *n*-$C_{16:0}$FA and *n*-$C_{24:0}$FA, mainly consisting of long chain fatty acids with even carbon number (see also Fig.5). Relatively large amounts of *n*-$C_{16:1}$FA and *n*-$C_{18:1}$FA are present. Polyunsaturated fatty acids were not detected, except several *n*-$C_{18:2}$FA isomers. Methyl-branched fatty acids were only found in low amounts; *i*- and *ai*-$C_{15:0}$FA were most abundant. A series of C_{17} fatty acids, consisting of *i*-$C_{17:1}$FA, 10-Me-$C_{16:0}$FA, *i*- and *ai*-$C_{17:0}$FA and *n*-$C_{17:1}$FA was identified, but only trace amounts are present.
The alcohols present are primarily straight-chain compounds (C_{18} to C_{28}) with a strong even over odd predominance and C_{26} as major constituent (see also Fig.5). Moreover phytol is present in moderate amounts.
Moderate amounts of *n*-alkanes are present, all with long chains, *n*-C_{27} and *n*-C_{29} alkanes being most abundant.
A series of long chain, even-carbon-numbered aldehydes (*n*-C_{22} to *n*-C_{28}) eluted just after the *n*-fatty acid methyl esters with odd carbon number. *n*-C_{26} aldehyde is most abundant.
Minute amounts of some β-hydroxy fatty acids are present.

Ether/OH$^-$-extract
Saponification of the Ether-extract yielded increased amounts of the fatty acids, mainly those with short chains (< C_{20}), and of phytol (Fig.2). No increases were observed in the amounts of the sterols, the *n*-alcohols or the other compounds.

OH$^-$-extract
The major compound classes present in the OH$^-$-extract (Fig.3) are fatty acids and hydroxy fatty acids.
The fatty acids show a bimodal distribution, the shorter fatty acids being the more abundant compounds (Fig.5). A series of dicarboxylic acids (C_{16} to C_{26}) with even carbon number was identified.

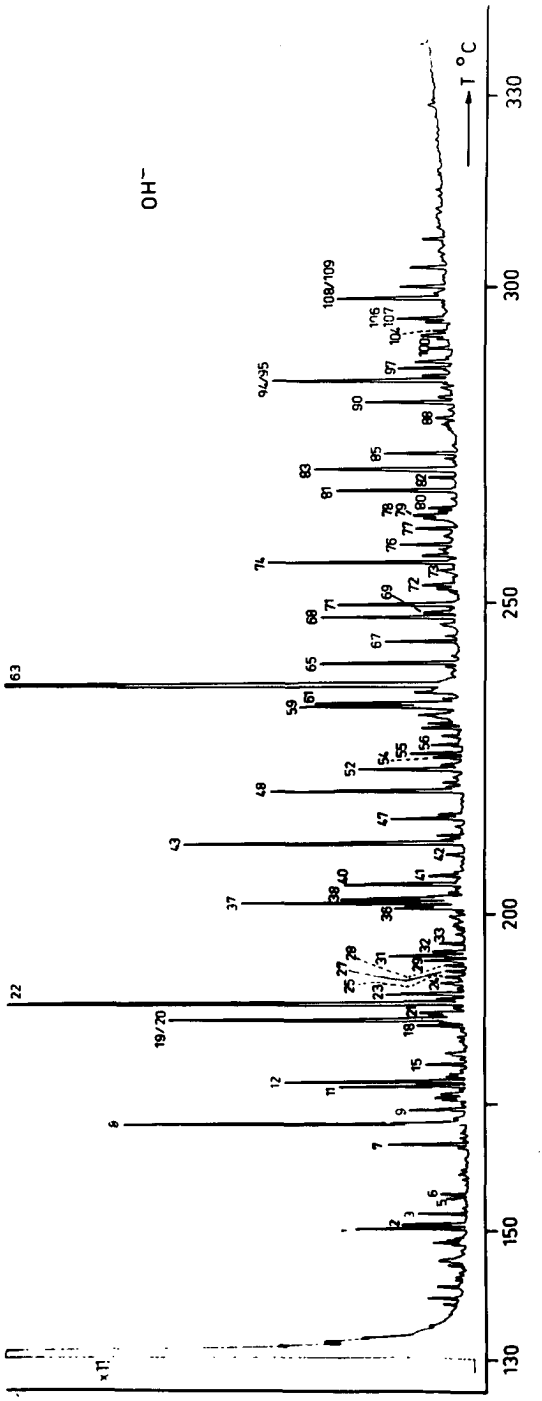

Fig.3 Gas chromatogram of the OH^--extract.

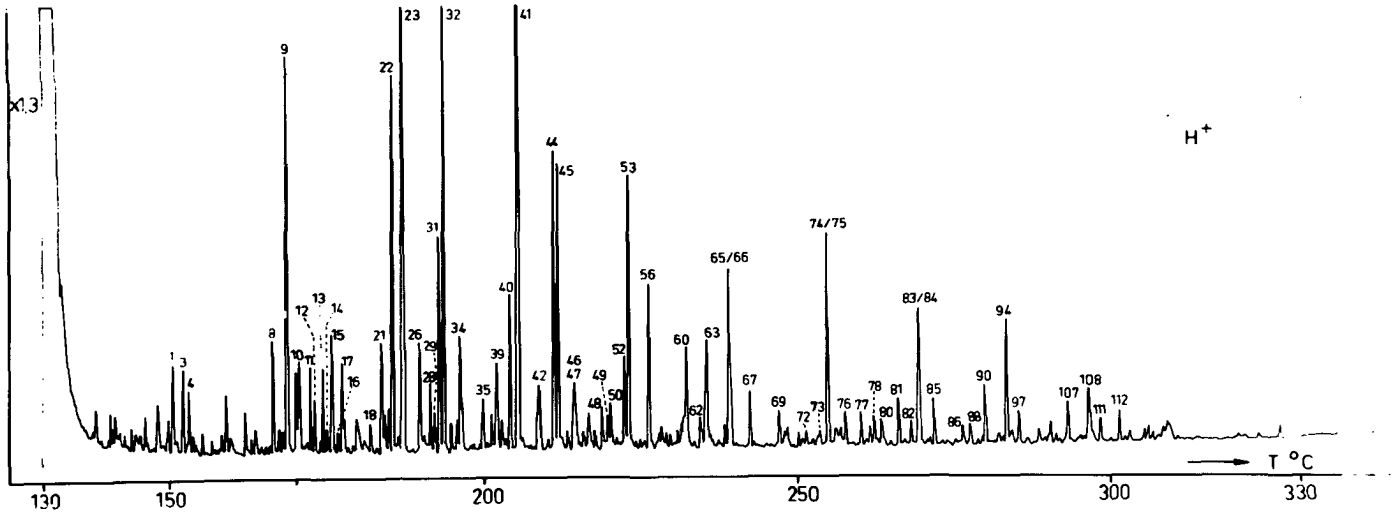

Fig.4 Gas chromatogram of the H^+-extract.

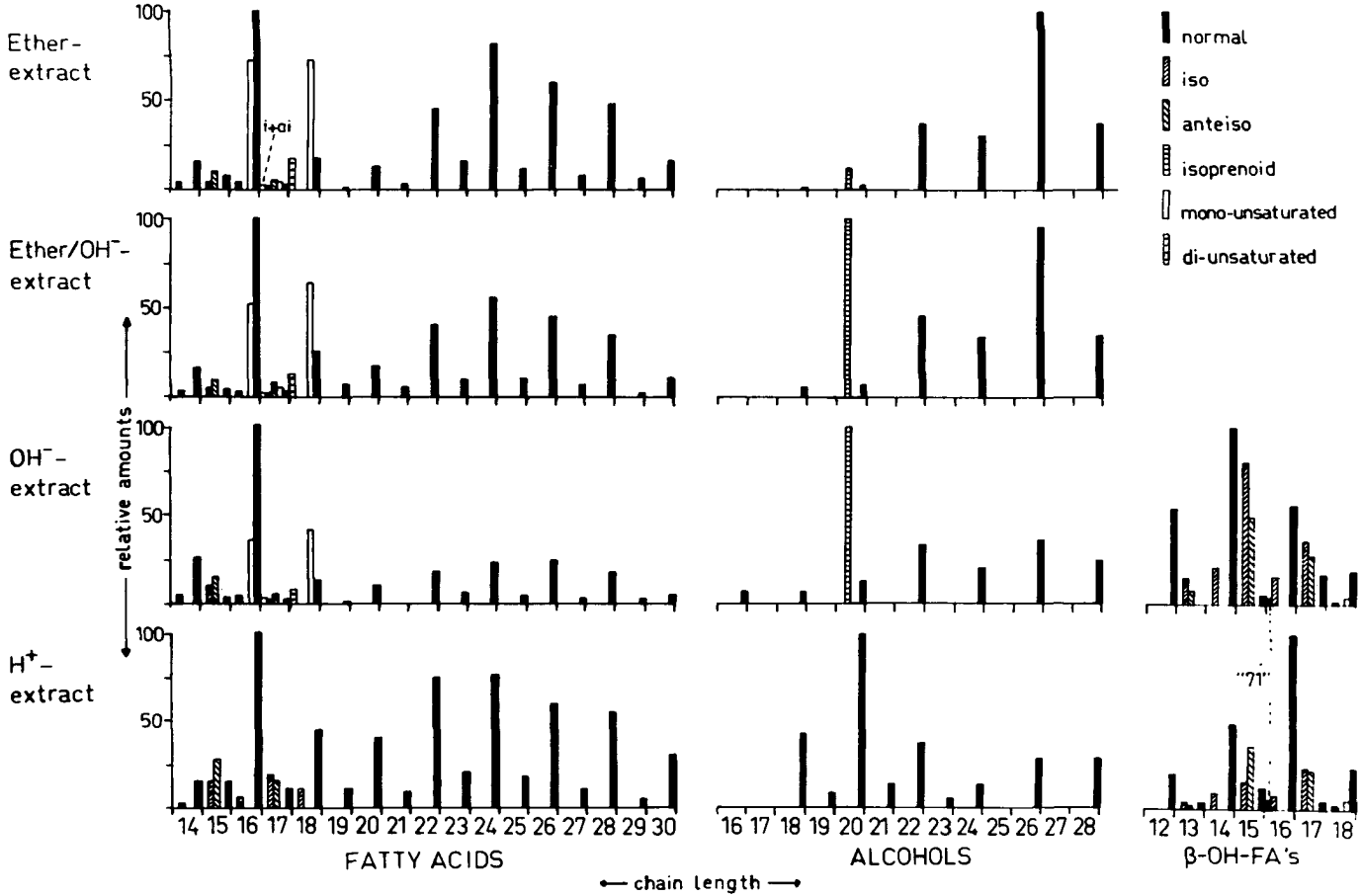

Fig.5 Distributions of fatty acids, alcohols and β-hydroxy fatty acids in the different extracts (relative amounts per extract).

The major peak in the gas chromatogram of this extract (peak 63, Fig.3) corresponds to di-OH-$C_{16:0}$FA, present as a mixture of 9,16-di-OH-$C_{16:0}$FA and 10,16-di-OH-$C_{16:0}$FA; mixtures of hydroxy dicarboxylic fatty acids (peaks 54, 55 and 59, Fig.3) and 9,10,18-tri-OH-$C_{18:0}$FA (peak 77) were also detected.
Considerable amounts of even, straight-chain ω-hydroxy fatty acids were found. The series ranges from $C_{16:0}$ to C_{26} but the amount of ω-OH-C_{18}FA is uncertain due to coelution with the abundant di-OH-C_{16} fatty acids. We also identified ω-OH-$C_{18:1}$FA in this extract. ω-OH-C_{16}FA and ω-OH-C_{22}FA are most abundant.
β-Hydroxy fatty acids with chain lengths between C_{10} and C_{18} are also present in the OH^--extract. Their distribution pattern is given in Fig.5.
Phytol is abundant, whereas only low amounts of *n*-alcohols occur. The saturated, even *n*-alcohols between C_{16} and C_{28} are all present, but unsaturated alcohols were not observed. *n*-Alkanes (*n*-C_{27} and *n*-C_{29}) and sterols are present in relatively low amounts.

H^+-extract
The H^+-extract (Fig.4) contains primarily β-hydroxy fatty acids, which are much more abundant than the fatty acids. Together, these two compound classes account for almost all the GC-amenable components in this extract. Their distribution patterns are shown in Fig.5. The β-hydroxy fatty acids range from C_{10} to C_{24} and are present in high amounts. Two components deserve special attention (peaks 35 and 50, Fig.4): β-OH-$C_{16:0}$FA (peak 35) elutes before *i*-β-OH-C_{16}FA which indicates uncommon branching in the former compound. Its mass spectrum is identical to those of *n*- or *i*-β-OH-C_{16}FA, except for an enhanced intensity of a fragment ion with *m/z* 71. The other compound (peak 50) elutes at the retention time of *n*-β-OH-$C_{18:1}$FA, but in its mass spectrum a fragment ion with *m/z* 355 (M^+-29) is strongly present, whereas M^+-15 (*m/z* 369), normally strongly present in the mass spectra of silylated β-hydroxy fatty acids, only shows a very weak intensity. To verify the mass spectra of these compounds and to check the possible presence of other uncommonly branched β-hydroxy fatty acids in the H^+-extract, a second aliquot of the sample was subjected to TLC separation. The thus purified H^+-labile β-hydroxy fatty acid fraction provided mass spectra without possible contributions by coeluting compounds from other compound classes. The enhanced intensity of a fragment ion with *m/z* 71 and the presence of a fragment ion with *m/z* 355 in the mass spectra of the two compounds were verified. At a very low level, traces of other uncommonly branched and monounsaturated β-hydroxy fatty acids were also observed.
The ω-hydroxy fatty acids in this extract show a similar chain length distribution to those in the OH^--extract, although the relative amounts are slightly different.
The fatty acid distribution in the H^+-extract (Fig.5) shows the presence of the even, long chain fatty acids and of branched and *n*-fatty acids between C_{12} and C_{18}. The amount of *n*-C_{15}FA is relatively high. Unsaturated fatty acids are present, all with the double bond at the 2 position. Their distribution reflects that of the β-hydroxy fatty acids, which indicates that these acids are artificially formed from β-hydroxy fatty acids during the acid treatment and subsequent saponification (Goossens *et al.*, 1988).
In the H^+-extract a suite of *n*-alcohols was also identified (C_{17} to C_{30}), among which *n*-C_{20} is dominant (Fig.5).
Series of dicarboxylic acids (C_{16}, C_{20}, C_{22}, C_{24} and C_{26}) and of *n*-alkanes (C_{27}, C_{29}, C_{30}, C_{31} and C_{33}), present in trace amounts, were also identified.

Discussion

In this study, the extractable organic matter of a sample from the Lake Vechten sediment is characterized by the lipid compositions of four extracts. The sequential analysis applied differentiates the lipid moieties according to their mode of occurrence. In organic geochemical studies the labels "free" and "bound" are often used when different modes of occurrence are distinguished. However, by application of this sequential method,

we aim to differentiate "bound" modes of occurrence. In addition, the question arises whether "free" or "bound" should be used to name, for example, a wax ester which occurs in a free mode as an intact compound while, simultaneously, it contains a "bound" fatty acid and alcohol moiety. To avoid obscurities, the names of the extracts (Ether-extract, Ether/OH^--extract, OH^--extract, H^+-extract) refer to the chemistry of the analytical procedure applied, since the bonding type of the constituents in the sediment is a matter of interpretation.

The sequential analysis distinguishes modes of occurrence on the basis of the nature of the bonds by which the lipid moieties are linked within the "mother"-compounds. Therefore, diethyl ether is used instead of a mixture containing methanol to extract free lipids, because in this way diglycerides, containing two esterified fatty acid moieties, remain in the residue to be saponified. Mayberry (1980) separated ester- and amide-linked lipid moieties quantitatively applying a sequential analysis to *Bacteroides*. The analysis of *Acinetobacter* whole cells and LPS (Goossens *et al.*, 1988) also demonstrates a good separation of ester- and amide-linked lipid moieties.

The results of this sequential method, as applied to the Lake Vechten sediment sample, show that this method yields good results for sedimentary organic matter, too.

Although no independent evidence is presented for the type of linkage of the lipid moieties in the different extracts, it is reasonable to assume that, analogous to the analyses of bacteria, the OH^--extract contains primarily lipid moieties which were esterified in the starting material and that the H^+-extract contains mainly lipid moieties which were amide-bound.

Figs.1 to 4 show that most compound classes are represented in more than one extract, demonstrating that the same compound can occur in different modes.

Hydrocarbons

Acyclic hydrocarbons occur in all extracts, primarily in the Ether-extract. The *n*-C_{27} and *n*-C_{29} alkanes are much more abundant than the shorter homologues.

The abundance of long chain *n*-alkanes (> C_{20}) with odd carbon numbers is indicative for the contribution of higher plant material to the sediment (Kolattukudy, 1980). The presence of hydrocarbons in the OH^-- and H^+-extracts, albeit in low amounts, points either to incomplete extractions or to inclusion of the components in (in)organic matter. The latter possibility seems more probable in view of the apolar nature of the compounds and the exhaustive extraction procedure.

Acyclic isoprenoid hydrocarbons were not detected, although these compounds were expected in this methanogenic sediment, since isoprenoid C_{25} hydrocarbons and isoprenoid C_{30} hydrocarbons have been found in pure cultures of methanogenic bacteria (Holzer *et al.*, 1979; Risatti *et al.*, 1984; Goossens, unpublished results).

Alcohols

All extracts contain a series of acyclic alcohols with different distributions (Fig.5). The alcohols, except phytol, are even, straight chain compounds. We did not observe unsaturated alcohols.

In the Ether-extract almost no C_{16} and C_{18} alcohols and only a small amount of phytol occur in a free mode. The strong dominance of long chain alcohols indicates a high contribution of higher plant material (Kolattukudy, 1980). The relative amount of *n*-C_{26} alcohol is remarkably high.

Saponification of the Ether-extract changed the relative amounts only slightly, in favour of the short-chain compounds. The phytol concentration, however, increased substantially, probably as a result of saponification of intact chlorophyll or phaeophytin, although it is also possible that phytol occurs esterified to other relatively apolar moieties as is the case in phytylesters (Gellerman *et al.*, 1975).

Saponification of the residue yielded a different distribution pattern: phytol is by far the most abundant alcohol in the OH^--extract and the amount of *n*-C_{26} alcohol is much reduced. The long chain alcohols are prominent but the dominance over the short-chain alcohols is less pronounced than in the Ether-extract.

In the H^+-extract the short-chain alcohols are present in about the same abundance as the long-chain alcohols, whereas the n-C_{20} alcohol dominates. Incomplete extraction of alcohols in the previous step of the procedure seems improbable since we found only small residual amounts of the major compounds present in the OH^--extract. In addition, the C_{20} alcohol is much more abundant in the H^+-extract than it is in the OH^--extract. Moreover, phytol or its degradation products (phytadienes), are absent in the H^+-extract, although phytol is the major alcohol in the OH^--extract. The origin of these H^+-labile alcohols is unknown. The only linkage imaginable is the glycosidic-ether-bond. However, we are not aware of compounds occurring in organisms, which show alkyl chains linked to the oxygen atom at position 1 of a saccharide moiety.

The presence of phytol in three different extracts, the major part being in the OH^--extract, suggests that in this sediment a lot of intact or only partly degraded chlorophyll is present, in which the phytyl side chain is still attached.

Sterols

Sterols were present in both the Ether-extract and in the OH^--extract. We consider the ether-extractable compounds to occur in a free state. High amounts of cholest-5-en-3β-ol and 24-ethyl-cholest-5-en-3β-ol and the corresponding 5α(H)-stanols were observed in the Ether-extract. The amounts of 24-methyl-cholest-5-en-3β-ol and 24-methyl-5α(H)-cholestan-3β-ol were much lower. We also identified 5β(H)-cholestan-3β-ol, 24-ethyl-5β(H)-cholestan-3b-ol, the di-unsaturated sterols cholesta-5,22-dien-3β-ol, 24-methyl-cholesta-5,22-dien-3β-ol and 24-ethyl-cholesta-5,22-dien-3β-ol and their 5α(H) counterparts. Apart from these regular series of common sterols also 5α(H)-cholestan-3α-ol and 24-ethyl-5α(H)-cholestan-3α-ol were identified as well as traces of 24-methylene-5α(H)-cholestan-3β-ol and 4α,23,24-trimethyl-5α(H)-cholest-22-en-3β-ol (dinosterol).

Saponification of the Ether-extract did neither change the sterol distribution pattern or increase the amounts, indicating that no significant amounts of steryl esters were present.

The sterols in the OH^--extract (Fig.3) show a similar distribution pattern, although the amounts are much lower than in the Ether-extract. The lower intensities of the 5α(H)-stanols should be noted. The OH^--labile sterols occurred esterified either to a polar compound or to a non extractable macromolecule.

The relative amounts of the major compounds (cholest-5-en-3β-ol and 24-ethyl-cholest-5-en-3β-ol and the corresponding 5α(H)-stanols) in the extracts are shown in Fig.6. The ratios cholest-5-en-3β-ol/5α(H)-cholestan-3β-ol and 24-ethyl-cholest-5-en-3β-ol/24-ethyl-5α(H)-cholestan-3β-ol are about 1.1 and 2.0, respectively, in the Ether-extract and 4.8 and 6.1 in the OH^--extract.

Steroid compounds are very often used in organic geochemical studies as indicators of input sources and of conditions during diagenesis (Huang and Meinschein, 1976; Mackenzie *et al.*, 1982; Robinson *et al.*, 1984a,b; Boon *et al.*, 1979; Volkman, 1986). Although the value of 4-desmethylsterols as indicators of input sources appears to be limited (Volkman, 1986), the specific alterations of the structures of nuclei and side chains in early diagenesis reflect diagenetic conditions. A general observation is the co-occurrence of Δ^5-unsaturated sterols, 5α(H)-stanols and 5β(H)-stanols in recent sediments. This occurrence of saturated and unsaturated species of the same molecule can be ascribed to different processes.

Gaskell and Eglinton (1975) showed that in sediments hydrogenation of unsaturated sterols occurred, possibly as a result of bacterial transformation of the free occurring sterols (Eyssen *et al.*, 1973). The hydrogenation was shown by tracer experiments to proceed via the steroid ketone as an intermediate (Gaskell and Eglinton, 1975; Mermoud *et al.*, 1984). Recently, also a direct hydrogenation pathway has been proposed (Mermoud *et al.*, 1984).

Sterols may also be reduced in the watercolumn during passage through the gut of higher organisms or in a layer of photosynthetic bacteria, especially *Chlorobium* (Robinson *et al.*, 1984a)(see below).

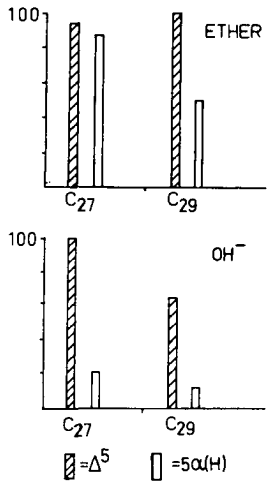

Fig.6. Relative amounts (%) of major Δ^5-unsaturated and α(H)-saturated sterols in Ether-extract and OH^--extract.

Also, a direct input of saturated sterols can be considered. In plankton the 5α(H)-stanols amount to, in general, 5-20 % of the total sterol fraction (Gagosian *et al.*, 1980) but growth conditions may change this percentage drastically (Ballantine *et al.*, 1979). A natural population of the dinoflagellate *Peridinium lomnickii* has been shown to contain high amounts of 5α(H)-cholestan-3β-ol (Robinson *et al.*, 1984b). A direct input of saturated sterols from algae and other organisms (Nishimura and Koyama, 1977) is therefore an alternative explanation for the relatively abundant presence of saturated sterols in sediments.

In studies which distinguish different modes of occurrence generally different distributions have been observed for "free" and "bound" sterols (Cranwell, 1981b; de Leeuw *et al.*, 1983; Albaigès *et al.*, 1984;). These different compositions are understandable as such, assuming that only free substrates are biohydrogenated, as was observed by Eyssen *et al.* (1973). However, the wide range of 5α(H)-stanol/Δ^5-sterol ratios for pairs of corresponding free 5α(H)-stanols and Δ^5-sterols in the same sediment (de Leeuw *et al.*, 1983), strongly suggests that a direct input of 5α(H)-stanols can be important.

In this study we observed sterols as free occurring compounds (Ether-extract) and esterified to polar moieties or macromolecules.

The OH^--labile sterols show ratios of Δ^5-sterols/5α(H)-stanols similar to those of algal material, which supports the view that esterified sterols resist hydrogenation by bacterial activity (Eyssen *et al.*, 1973). Probably, the OH^--labile sterols reflect the composition of the input material much better than the sterols which are directly extractable.

Fig.7. shows the distributions of freely occurring Δ^5-unsaturated sterols, 5α(H)-stanols, 5β(H)-stanols and the steroid ketones. The similar distributions of Δ^5-sterols and stanones and the relatively high amounts of 5α(H)-stanols (Fig.6) suggest that extensive reduction of the free Δ^5-sterols takes place. The distribution of 5α(H)-stanols is somewhat different and may indicate either different rates of reduction of C_{27} and C_{29} sterols or an additional direct input of 5α(H)-cholestan-3β-ol. The latter explanation is more probable in view of the fact that high amounts of 5α(H)-cholestan-3β-ol may occur in a natural algal population (Robinson *et al.*, 1984b). A direct input of 5α(H)-stanones is rather unlikely since the distribution patterns of 5α(H)-stanones and 5β(H)-stanones are similar and 5β(H)-stanones are thought to be formed only by bacterial action on Δ^5-unsaturated sterols.

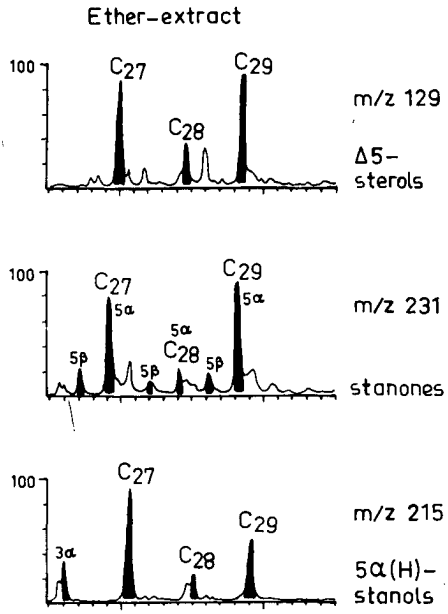

Fig.7. Mass chromatograms of fragment ions with m/z 129, 231, and 215 as indicators of Δ^5-stenols, steroid ketones and 5α(H)/5β(H)-stanols in the Ether-extract and in the OH^--extract.

Low Δ^5-sterol/5α(H)-stanol ratios for freely occurring steroids and the presence of 5α(H)-cholestan-3α-ol and 24-ethyl-5α(H)-cholestan-3α-ol have been observed also in Priest Pot (Robinson *et al.*, 1984a). Lake Vechten is comparable to this lake in that it also contains an anoxic hypolimnion with dense populations of photosynthetic bacteria (Steenbergen, 1982). In Priest Pot the reduction of Δ^5 sterols begins in the anoxic hypolimnion, probably by the activity of the photosynthetic bacterium *Chlorobium*, although it cannot be precluded that *Chlorobium* acts only as an indicator of the chemocline environment and that the reduction is accomplished by other bacteria. Possibly, the occurrence of 3α(OH)-stanols is related to the reduction in such an environment since these sterols have only been found, as yet, in environments harbouring photosynthetic bacteria (Robinson *et al.*, 1984a; Mermoud *et al.*, 1985).
In view of the dominance of the dinoflagellate *Ceratium hirundinella* during the stratification period the amounts of 4-methylsteroids are surprisingly low (see below).

Fatty acids
Fatty acids occur in all extracts (Figs. 1 to 5). The distributions are bimodal. The long chain fatty acids (> C_{20}) show a strong even/odd predominance and represent a contribution by higher plants (Kolattukudy, 1980; Harwood, 1980).
The short-chain fatty acids (< C_{20}) are primarily straight chain components with even carbon number. Iso- and anteiso-branched fatty acids are present but their amounts are comparatively low. *n*-$C_{16:0}$FA is the major fatty acid in all extracts. Also high amounts of mono-unsaturated C_{16} and C_{18} fatty acids are present in the Ether- and the OH^--extract. At least two $C_{18:1}$ fatty acids are present which were almost separated under the GC conditions used. These are probably isomers with different double bond positions which we did not further identify. A small amount of phytanic acid eluted together with the two $C_{18:1}$ isomers. Unsaturated fatty acids did not occur in the H^+-labile mode except the artificially formed Δ^2-fatty acids.

The relative amounts of the fatty acids in the Ether-extract increased upon saponification. Both long- and short-chain fatty acids became more abundant (Fig.2). This increase is probably due to hydrolysis of triglycerides and/or other relatively apolar compounds.
In the Ether-extract the long-chain fatty acids dominate whereas the OH^--labile fatty acids show higher amounts of the short-chain ones. The abundance of the short-chain fatty acids results from the hydrolysis of diacyl-glycerides which are the most abundant membrane lipids in most organisms. *n*-$C_{16:0}$FA was over three times more abundant than any other fatty acid in this extract. The branched C_{15} fatty acids are present in considerable amounts in this OH^--extract.
Among the fatty acids which are present in low amounts some have special characteristics: We could identify *i*-, and *ai*-$C_{17:1}$FA, 10-Me-$C_{16:0}$FA and *n*-$C_{17:1}$FA (peak 30) in the Ether- and OH^--extract but not in the H^+-extract. Branched and straight chain $C_{17:1}$ fatty acids have been found in sulphate reducing bacteria (Boon *et al.*, 1977a; Taylor and Parkes, 1983; Edlund *et al.*, 1985; Goossens, unpublished results) and have been proposed as markers for sulphate reducing bacteria. However, branched $C_{17:1}$ fatty acids have been identified in an aerobic heterotrophic bacterium isolated from a marine surface sediment (Perry *et al.*, 1979).
In the H^+-extract the amount of *n*-$C_{18:0}$FA is relatively high. The proportion of branched fatty acids is higher than in the other extracts. The amounts of branched C_{15} and C_{17} fatty acids are about equal, whereas in the other extracts the C_{15} fatty acids are more important.
The fatty acids extracted after acid treatment probably occurred amide-bound in the starting material (Goossens *et al.*, 1988). Amide linkages only occur in a limited number of compounds: lipopolysaccharides, ornithine lipids and sphingolipids (see also Goossens *et al.*, 1986). The short-chain fatty acids may have been derived from any of these structures, although the relatively high proportion of branched fatty acids suggests that their origin is mainly bacterial. We expect the longer-chain fatty acids to be derived from higher plant material in view of the similar distribution pattern of these fatty acids in the different extracts. Therefore, the presence of these long chain fatty acids may point to inclusion phenomena or to incomplete saponification of plant macromolecules.
Polyunsaturated fatty acids were only detected in very low amounts in the OH^--extract. Analyses of the fatty acids present in intact polar lipids in samples taken at different depths in the water column indicate that these fatty acids are rapidly metabolized in the epilimnion of this lake (Fredrickson *et al.*, 1986).

Hydroxy fatty acids

ω-Hydroxy fatty acids
Different distributions of even ω-hydroxy fatty acids were found in a homologous series (C_{20} to C_{26}) in the OH^--extract and in the H^+extract. In the OH^--extract *n*-ω-OH-C_{16}FA was most abundant, followed by *n*-ω-OH-C_{22}A, whereas *n*-ω-OH-C_{24}FA was the most abundant H^+-labile ω-hydroxy fatty acid. These different relative amounts make incomplete extraction a less probable explanation for the presence of ω-hydroxy fatty acids in the H^+-extract. Esterified ω-hydroxy fatty acids are known constituents of the plant polymer suberin (Kolattukudy, 1980; Harwood, 1980) and the origin of the ω-hydroxy fatty acids in the OH^--extract is therefore rather clear. It might be speculated that the H^+-labile ω-hydroxy fatty acids are also derived from plant material, but we are not aware of compounds occurring in higher plants which release ω-hydroxy fatty acids on acid treatment only. One possibility might be that these compounds are part of glycolipids in which the ω-hydroxy fatty acids are glycosidically linked via their hydroxy group to the oxygen atom at the C_1 position of a saccharide moiety.

(ω-1)-, di- and tri-hydroxy fatty acids
Some (ω-1)-hydroxy fatty acids were identified in the OH^--extract but the amounts are low.

A mixture of 9,16- and 10,16-di-OH-C_{16}FA (peak 63) is present in the OH^--extract in very high amounts. These compounds are major constituents of the cutin polymer (Kolattukudy, 1980; Harwood, 1980) and thus indicative for the input of plant material into this sediment. The H^+-extract also contains these components probably due to non complete extraction. We also identified 9,10,18-tri-OH-C_{18}FA (peak 77) in both extracts. This component is also a cutin constituent (Eglinton and Hunneman, 1968; Harwood, 1980; Kolattukudy, 1980). However, the clearly different relative amounts of di-OH-C_{16}FA and tri-OH-C_{18}FA in the OH^--extract and in the H^+-extract suggests that there is a real source of H^+-labile tri-OH-C_{18}FA.

β-Hydroxy fatty acids

The β-hydroxy fatty acids present in Lake Vechten sediment occur primarily in the H^+-extract (Figs. 4 and 5). In the Ether-extract only minute amounts of *n*-β-OH-C_{14}FA, *i*- and *ai*-β-OH-C_{15}FA, *i*- and *ai*-β-OH-C_{17}FA and *n*-β-OH-C_{18}FA were found. Consequently, most of the β-hydroxy fatty acids are not occurring as free compounds. The distributions are dominated by components with less than 20 carbon atoms, although β-hydroxy fatty acids up to C_{24} are present in the H^+-extract. The uncommon β-OH-C_{16}FA "71" (peak 35, Fig.4) has not yet been found in bacteria, but other, H^+-labile β-hydroxy fatty acids with similar characteristics (elution before the iso-branched β-hydroxy fatty acid of equal carbon number and enhanced intensity of fragment-ion with *m/z* 57, 71 or 85 in the mass spectrum) have been observed in *Chlorobium vibrioforme* and *Desulfovibrio gigas* (Goossens *et al.*, 1986).

The distributions in the OH^--extract and in the H^+-extract are different. In both extracts the even compounds predominate, but in the OH^--extract *n*-β-OH-C_{14}FA is most abundant whereas in the H^+-extract *n*-β-OH-C_{16}FA is the major β-hydroxy fatty acid. *i*- and *ai*-C_{15} and *i*- and *ai*-β-OH-C_{17}FA are also present abundantly, but their relative amounts differ in the two extracts.

In organic geochemical studies the presence of β-hydroxy fatty acids is generally interpreted as a contribution from bacteria. Although this is probably justified for the greater part of the β-hydroxy fatty acids present, other, eukaryotic sources cannot be precluded since β-hydroxy fatty acids have been found in fungi (Pohl and Wagner, 1972; Tulloch and Spencer, 1964; Weete, 1974,1976) and algae (Matsumoto and Nagashima, 1984). However, this variety of possible sources holds only for esterified β-hydroxy fatty acids. Amide-bound β-hydroxy fatty acids have only been found in bacteria, *i.e.* in LPS (Galanos *et al.*, 1977; Wilkinson *et al.*, 1977; Lüderitz *et al.*, 1982; Wollenweber *et al.*, 1984), in ornithine-lipids (OL) (Lechevalier, 1977; Batrakov and Bergelson, 1978) and in bacterial sphingolipids (Mayberry *et al.*, 1973; Goldfine, 1982) (see also Goossens *et al.*, 1986).

β-Hydroxy fatty acids which are present in the H^+-extract were probably amide-bound in the original material, since the other possible bond (a glycosidic ether bond in which the hydroxy group occurs ether-linked to the oxygen atom at the C_1 position of a saccharide moiety) is uncommon and has been found only in one bacterium (Lechevalier, 1977).

All gram-negative eubacteria contain LPS whereas OL is present in a variety of gram-negative and gram-positive eubacteria. Therefore, the H^+-labile β-hydroxy fatty acid distribution can be considered the "fingerprint" of (part of) the bacterial population. Since the chemical composition of individual bacteria and the species composition of bacterial communities are highly dependent on the environment, these "fingerprints" can be used as indicators of environmental conditions (Goossens *et al.*, 1986). Very little is known about the diagenetic fate of amide-bound β-hydroxy fatty acids. Saddler and Wardlaw (1980) found a high degradation rate of LPS under both aerobic and anaerobic incubation conditions. On the other hand, H^+-labile β-hydroxy fatty acids were present abundantly in a 200,000 year old mediterranean sapropel (ten Haven *et al.*, 1986) and it can be speculated that the "tightly bound" β-hydroxy fatty acids observed in a 200 m core of Lake Biwa in Japan (Kawamura and Ishiwatari, 1984) were amide bound. This suggests that H^+-labile β-hydroxy fatty acids can survive the earliest stages of diagenesis.

Scope and limitations of the sequential approach

The main compounds in the different extracts occur in modes consistent with the structures from which they are thought to originate. Only with respect to minor compounds (especially hydrocarbons and alcohols) uncertainties exist whether the different modes of occurrence result from different bonding types or from other phenomena like inclusion or incomplete extraction. The presence of so called "bound hydrocarbons" is a general phenomenon in studies dealing with different modes of occurrence (Cranwell, 1978,1981b; Albaigès *et al.*, 1984; Leenheer *et al.*, 1984).

The H^+-labile mode of occurrence has not been studied previously in sedimentary organic matter. Although in some studies acid was applied to liberate "bound" lipids (Cranwell, 1978,1981a,b,1982; Klok *et al.*, 1984; Cardoso *et al.*, 1983), OH^--labile lipids and H^+-labile lipids were not distinguished. Due to lack of comparable analyses, we cannot conclude whether the presence of alcohols, ω-hydroxy fatty acids and tri-OH-C_{18}FA in the H^+-extract results from incomplete extraction or inclusion or points to, as yet, unknown compounds in organisms.

The organic part of the sediment of Lake Vechten seems to be dominated by higher plant material. This is concluded from the dominant presence of long chain fatty acids with even predominance, *n*-alkanes with strong odd predominance, *n*-alcohols and aldehydes, all well-known plant wax constituents, and the presence of ω-hydroxy fatty acids, diacids, di- and tri-hydroxy fatty acids and hydroxy diacids, all known as cutin and suberin building blocks.

Specific markers of algae were not detected. These organisms may contribute to several classes of compounds such as sterols, fatty acids, alcohols, alkanes and possibly ester-linked β-hydroxy fatty acids but no conclusion can be reached about their contribution. Possibly, identification of the double bond positions in the unsaturated fatty acids could be helpful to solve this problem. However, the distributions of the main components in the extracts suggest that the contribution of algal material is relatively low.

Although methanogenesis has been demonstrated at this depth in the sediment (Cappenberg and Verdouw, 1982), which was one of the reasons to select this sediment sample, the presence of methanogenic bacteria could hardly be recognized: Acyclic isoprenoid hydrocarbons, which occur in methanogenic bacteria (Holzer *et al.*, 1979; Langworthy, 1982; Risatti *et al.*, 1984; Goossens, unpublished results) were not detected. Due to the absence of halophilic archaebacteria in this environment, diphytanyl diether lipid could be used as a marker of methanogenic bacteria. Only low amounts were present. Since we did not cleave ether-linkages in this study, the other marker of methanogenic bacteria, dibiphytanyl tetraether lipid (Mancuso *et al.*, 1985), could not be detected for experimental reasons. The presence of only low amounts of the markers of methanogenic bacteria points to the predominant presence of other (plant) material in this sediment.

The dominant amount of higher plant markers in the sediment seems to be in conflict with the preliminary carbon budget of the lake (Gons, 1981). The input into the carbon cycle by plants was estimated to be of minor importance: leaf fall from surrounding trees was calculated to be maximally 7% of the input by phytoplankton primary production, whereas the contribution by waterplants amounted to only 9% of the carbon input. Phytoplankton primary production is the main source of carbon in the system (54% of annual input). During the stratification period the phytoplankton is dominated by the dinoflagellate *Ceratium hirundinella* which can account, at times, for 95% of the total phytoplankton biovolume (Blaauboer, 1982). A natural, almost pure population of this species was studied by Cranwell (1976). 4-Methyl steroids and 4,4'-dimethyl steroids only accounted for less than 10% of the steroid fraction, indicating that not every dinoflagellate bloom can be recognized from its 4-methyl steroid contribution to the sediment. The main sterol (85% of the desmethyl-steroid fraction) was cholesterol. Also 24-methylene-cholestanol was present in the fresh algal material.

The sediment of Lake Vechten does not contain these specific sterol markers in considerable amounts, cholesterol excepted, although this was expected if phytoplankton were the main source of carbon in the lake. Cholesterol and 5α(H)-cholestan-3β-ol were present in high amounts, but these compounds are very common in nature and may also come

from other sources. A very low amount of 24-methylene-cholestanol was recognized, indeed, but this compound is not very specific. This indicates that the contribution by *Ceratium* is obscured almost completely by other contributions with a higher plant character.
A possible explanation is an enhanced preservation of plant lipids. The morphometry of the lake and the fact that the lake is surrounded by trees supports this explanation: Leaf fall from the surrounding trees provides a direct input of plant material which is transported downwards along the sloping bottom of the lake. Plant leaves are, compared to algae, relatively inaccessible to decomposing microorganisms and are also less readily resuspended into the oxic lake water after turnover of the lake in autumn. As a consequence, the sediment may become selectively enriched with plant material. However, the very high sedimentation rate (25 cm in 40 years) and the high organic carbon content of the sediment (10-20% Dry Weight in the deeper layers) suggest an additional, very important source of plant derived organic matter. Whether this material enters the lake sediment via the sloping bottom (for instance soil from the surrounding pastures) or via the extensive ground water flows (the renewal time for the lake water is 2 years (Steenbergen and Verdouw, 1982)) can, at the moment, only be a matter of speculation.

Concluding remarks
The results of this study indicate that a further differentiation of the "bound" mode of occurrence is possible by relatively simple means.
Discriminating different modes of occurrence indicates that most compounds occur in several modes. The four extracts obtained by this sequential analysis provide a general view on the more important contributions to the sedimentary organic matter. More specific information could be obtained by isolation of classes of compounds which are present in low amounts.
The H^+-treatment liberates primarily β-hydroxy fatty acids from prokaryotic compounds. These H^+-labile β-hydroxy fatty acids have been generally overlooked in organic geochemical studies. However, their distributions can be used as fingerprints of bacterial populations and hence as indicators of environmental conditions. The presence of H^+-labile β-hydroxy fatty acids in a 200,000 years old sapropel (Ten Haven *et al.*, 1986) suggests that they can survive the earliest stages of diagenesis.
The predominance of plant material in the sediment of this lake suggests that relative amounts of markers of different source organisms do not reflect actual inputs and that specific degradation rates may play a major role in determining the lipid composition of sediments.

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CHAPTER 4

Lipids and their mode of occurrence in bacteria and sediments III: The lipid composition of a strictly prokaryotic community in a natural, hypersaline environment.

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Abstract

An attempt to extend the possibilities of recognizing specific groups of prokaryotes via chemical markers was made by investigating two samples from a hypersaline environment. A sequential analytical procedure was used which distinguishes different modes of occurrence of the lipid moieties. Two samples were taken in a gypsum precipitation basin in a salt work area, one representing the gypsum crust containing cyanobacteria, phototrophic sulphur bacteria and accompanying bacteria and another sample representing the underlying mud which was rich in sulphate reducing bacteria. The communities were strictly prokaryotic as was apparent from the absence of sterols from the samples. The lipid composition was relatively simple showing high amounts of fatty acids and of phytol. Large differences between the lipid compositions of crust and mud indicate a rapid turnover of free occurring and esterified fatty acids. The contributions from extreme halophilic archaebacteria, cyanobacteria, phototrophic sulphur bacteria and sulphate reducers are discussed. Distributions of α- and β-hydroxy fatty acids which are released exclusively by an acid treatment, are used as fingerprints of the prokaryotic communities.

Introduction

The chemical recognition of prokaryotic organic matter in sediments is, as yet, only possible in a very general way. In most studies of recent sediments bacterial contributions are noticed without further specification. Only a few molecular markers are known to be exclusive for bacteria , *i.e.* the extended hopanoids (Rohmer *et al.*, 1984; Ourisson *et al.*, 1984) and the isopranyl ether lipids (Langworthy, 1982).
The extended hopanoids occur in a variety of species and are thus not indicative for specific groups of microorganisms. The isopranyl ether lipids, however, occur only in archaebacteria. Since the three main groups of archaebacteria, *i.e.* methanogens, extreme halophiles and thermoacidophiles, inhabit very different environments, some additional information about the environment of deposition is sufficient to establish the source organisms of sedimentary isopranyl ether lipids.
Yet, a better recognition of specific groups of bacteria is desirable since the composition of bacterial organic matter is strongly influenced by environmental conditions and could therefore reveal details of the environment of deposition and of the conditions during diagenesis.
To achieve some differentiation in the recognition of prokaryotic organic matter, information is required about the chemical composition of bacteria as such, preferably sampled from natural habitats, and of sediments which are rich in prokaryotic organic matter. Sediments enriched in prokaryotes are found where extreme conditions with respect to temperature (*e.g.* hot springs), salinity (hypersaline environments such as sabkha's) or anoxicity (methanogenic sediments) prevail. The systems at the extremes of temperature and salinity are less complex than those at more temperate conditions (Ward *et al.*, 1985). The

reduced number of species provides a better chance of recognizing contributions from specific bacterial groups, as is evident from the study of hot spring communities (Ward *et al.*, 1985).

The hypersaline environment is particularly interesting because both primary production and decomposition are mainly accounted for by prokaryotic organisms. In addition, there is a growing interest in the organic geochemical characterization of oils and sediments from hypersaline environments and characteristic compounds of unknown origin have been identified (Damsté *et al.*, 1986; Ten Haven *et al.*, 1985). Therefore, a detailed study of the various communities occurring in present hypersaline environments may yield valuable information.

The various stages of hypersalinity and their concomitant communities occur separately in salt work areas where sea salt is produced by stepwise evaporation of sea water. These areas provide the opportunity to sample sediments containing specific communities, which have developed under rather constant and well defined, natural conditions.

In this paper we report on the lipid composition of sediments, developed at very high salinity (320 g/l), comprising an exclusively prokaryotic community. Two samples have been analyzed, one representing the phototrophic community living in a gypsum crust and another taken from the mud underneath this crust, representing the crust material during the first stages of diagenesis. A sequential analytical procedure has been applied to distinguish different modes of occurrence of lipids (Goossens *et al.*, 1988a,b).

Experimental

Samples

The samples were taken from a gypsum precipitation basin in the salt work area of Roquetas de Mar (Spain). The salinity of the water in this basin (about 30 cm deep) was 320 g/l (Dronkert, 1986). The strong pink colour of the water indicated the abundant presence of extreme halophilic archaebacteria. A gypsum crust of about 3 cm thick had developed. Under this crust a black mud with gypsum crystals, which gave off a strong smell of H_2S during sampling, was present.

Two samples were taken: A piece of the gypsum crust was cut out from under water with a spade and packed in aluminium foil. The underlying mud was sampled and stored in a precleaned polyethylene bottle. Both samples were kept frozen (-20 °C) until lyophilization. Prior to analysis the dry samples were ground in a ball mill.

Lipid analysis

The samples were analyzed applying a sequence of extractions and hydrolyses as described before (Goossens *et al.*, 1988a,b). In short, a sample is extracted with diethyl ether (Ether-extract). The residue of this extraction is saponified (1N KOH/methanol (96%), 1 hr., reflux) and extracted by a modified Bligh and Dyer (1959) method, yielding the OH^--extract. The residue and the H_2O/methanol fraction remaining after this second extraction are combined, lyophilized, and treated with 4N HCl (6 hrs., 100 °C). Hereafter, the acid treated matter is saponified again and extracted in the same way as in the second step of the procedure, yielding the H^+-extract. Half of the Ether-extract is also saponified, which yields the Ether/OH^--extract.

Extracts were analyzed by gas chromatography and gas chromatography-mass spectrometry after derivatization with CH_2N_2 to convert carboxyl groups into fatty acid methylesters (FAME) and with BSTFA (Merck) to silylate free hydroxyl groups.

Gas chromatographic analyses were performed on a Carlo Erba 4160 instrument equipped with an on column injector and a flame ionization detector (FID). Separation was achieved on a Chrompack CPSil5 fused silica capillary column (25m, i.d. 0.32 mm, film thickness 0.12 mm) with He as a carrier gas. After injection of the sample (in ethylacetate) at 100 °C, the temperature was rapidly raised to 130 °C and from there programmed at a rate of 4 °C/min to 325 °C and kept at the final temperature for 5 minutes.

GC-MS was performed on a Varian 3700 gas chromatograph connected to a Varian Mat 44 quadrupole mass spectrometer operated in the electron impact mode with an ionization energy of 80 eV. Mass spectra were recorded from *m/z* 50 to *m/z* 500 with a cycle time of 1.5 sec.

Identifications are based upon comparison of mass spectra and relative retention times with those of standards and with literature data. The identity of cyclopropyl-C_{17}FA (*cy*-C_{17}FA) and *cy*-C_{19}FA was verified by hydrogenation with H_2 using platinum as a catalyst.

Results

The different stages of hypersalinity with their characteristic communities can be recognized in man made salterns where sea salt is produced by evaporation of seawater. The physical separation of the different stages of evaporation and the relatively constant salinities in the basins provide the opportunity to sample communities developed under well defined conditions.

The hypersaline environment is very restrictive to life's processes and only adapted organisms are able to cope with its extreme conditions. Nevertheless, within these salty environments different communities exist in which the proportion of eukaryotic organisms decreases with increasing salinity. At very high salt concentrations (>240 g/l) usually only prokaryotes are found (Cornee, 1983; Holtkamp, 1985), although the eukaryotic algae *Dunaliella viridis* and *Dunaliella salina* may be present abundantly at concentrations up to >340 g/l (Larsen, 1980; Imhoff *et al.*, 1979). In some cases the brine shrimp *Artemia salina* and the brine fly *Ephydra* are observed (Larsen, 1980).

In this paper we report on samples taken at very high salinity (320 g/l, Dronkert, 1986) from a large basin with a gypsum crust on the bottom, formed by precipitation from the turbid water.

The organisms living in this environment colour the water pink and create very bright coloured bands between the gypsum crystals.

Upon cross-sectioning of the gypsum crust a very slimy orange-brown layer, a green band and a strongly purple-red coloured band were observed from top to bottom, the latter covering the underside of the crust.

The leathery consistency which is so typical for the cyanobacterial mats occurring at lower salinities, was missing but the orange-brown layer was very slimy. This slime formation is probably caused by the high salt concentration. Some cyanobacteria increase their slime production as an adaptation to increased salinity (Gerdes *et al.*, 1985).

The colours in the gypsum crust are associated with cyanobacteria and purple sulphur bacteria (Cornee, 1983; Holtkamp, 1985; Gerdes *et al.*, 1985; Krumbein, pers. comm.).

Microscopy revealed that the orange-brown layer was formed by the cyanobacteria *Synechococcus* and *Gloeothece*; *Dunaliella* (an eukaryotic alga) was not observed. The green band was dominated by ensheathed filaments, probably *Microcoleus*. Also some *Spirillum* like species were observed. This green cyanobacterial layer was not coherent and showed no mat structure. The red colour was associated with little coccoid cells, but these photosynthetic purple bacteria could not be identified further by microscopy.

The pink colour in the water results from the abundant presence of the extreme halophilic archaebacteria *Halobacterium* and *Halococcus*, although *Dunaliella* may contribute. This latter species, however, was not observed in the brine where we took the samples.

Since *Dunaliella* and diatoms were not observed, the samples represent a strictly prokaryotic system which was kept under more or less constant conditions for an extended period of time (about 40 years).

The mud under this crust was dark black and contained a lot of gypsum crystals. It smelled heavily with H_2S during sampling.

Two samples have been analyzed : a piece of the integral gypsum crust which contained all the coloured bands, representing the phototrophic organisms, and a sample from the anaerobic mud.

Table 1. Identifications of the peaks in the gas chromatograms.
FAME: fatty acid methyl ester; TMS: trimethylsilyl; *n*: straight chain; *i*: iso-branched; *ai*: anteiso-branched; *cy*: cyclopropyl; me: methyl; *ip*: isoprenoid. * HHFA = hexahydrofarnesylacetone; ** DPE = diphytanylether (see text)

1 *i*-$C_{14:0}$FAME	32 *i*-Δ^2-$C_{17:1}$FAME	63 *n*-$C_{20:0}$di-FAME
2 *n*-$C_{17:1}$alkene	33 α-OCH_3-$C_{16:0}$FAME	64 ω-OTMS-$C_{20:0}$FAME
3 *n*-C_{17}alkane	34 β-OTMS-$C_{16:0}$FAME-71	65 *n*-C_{27}alkane
4 *n*-$C_{14:0}$FAME	35 *n*-$C_{18:2}$FAME	66 *n*-$C_{24:0}$FAME
5 *n*-α+β-OTMS-$C_{12:0}$FAME	36 *n*-$C_{18:1}$FAME	67 *n*-β-OTMS-$C_{22:0}$FAME
6 Δ^2-$C_{14:1}$FAME	37 *n*-$C_{18:1}$FAME	68 *n*-$C_{24:0}$-OTMS
7 *n*-$C_{14:0}$-OTMS	38 *i*-α+β-OTMS-$C_{16:0}$FAME	69 *ip*-$C_{30:6}$alkene
8 *n*-$C_{18:1}$alkene	39 *n*-$C_{18:0}$FAME	70 *n*-$C_{25:0}$FAME
9 *i*-$C_{15:0}$FAME	40 *n*-α+β-OTMS-$C_{16:0}$FAME	71 *n*-$C_{22:0}$di-FAME
10 *ai*-$C_{15:0}$FAME	41 *ip*-$C_{20:0}$FAME-OTMS	72 ω-OTMS-$C_{22:0}$FAME
11 *n*-$C_{15:0}$FAME	42 Δ^2-$C_{18:1}$FAME	73 *n*-C_{29}alkane
12 *n*-α+β-OTMS-$C_{13:0}$FAME	43 *n*-$C_{18:0}$-OTMS	74 *n*-$C_{26:0}$FAME
13 HHFA *	44 *ip*-$C_{20:1}$-OTMS	75 *n*-b-OTMS-$C_{24:0}$FAME
14 *n*-$C_{16:2}$FAME	45 *i*-α+β-OTMS-$C_{17:0}$FAME	76 *n*-$C_{26:0}$-OTMS
15 *i*-$C_{16:0}$FAME	46 *cy*-$C_{19:0}$FAME	77 *n*-C_{30}alkane
16 *n*-$C_{16:1}$FAME	47 *ai*-α+β-OTMS-$C_{17:0}$FAME	78 *n*-$C_{27:0}$FAME
17 *n*-$C_{16:1}$FAME	48 *n*-$C_{19:0}$FAME	79 *n*-$C_{24:0}$di-FAME
18 *i*-β-OTMS-$C_{14:0}$FAME	49 *n*-$C_{16:0}$di-FAME	80 ω-OTMS-$C_{24:0}$FAME
19 *n*-$C_{16:0}$FAME	50 *n*-α+β-OTMS-$C_{17:0}$FAME	81 *n*-C_{31}alkane
20 phtalate	51 ω-OTMS-$C_{16:0}$FAME	82 *n*-β-OTMS-$C_{24:0}$FAME
21 *n*-α+β-OTMS-$C_{14:0}$FAME	52 *n*-$C_{20:0}$FAME	83 *n*-$C_{28:0}$FAME
22 br-$C_{17:1}$FAME	53 *n*-α+β-OTMS-$C_{18:0}$FAME	84 *n*-$C_{28:0}$-OTMS
23 Δ^2-$C_{16:1}$FAME	54 *n*-$C_{20:0}$-OTMS	85 *n*-$C_{29:0}$FAME
24 10-me-$C_{16:0}$FAME	55 *cy*-α-OTMS-$C_{19:0}$FAME	86 ω-OTMS-$C_{26:0}$FAME
25 *n*-$C_{16:0}$-OTMS	56 *n*-$C_{21:0}$FAME	87 *n*-C_{33}alkane
26 *i*-$C_{17:0}$FAME	57 *n*-$C_{18:0}$di-FAME	88 *n*-$C_{30:0}$FAME
27 *ai*-$C_{17:0}$FAME	58 *n*-$C_{25:0}$alkane	89 *n*-$C_{30:0}$-OTMS
28 *cy*-$C_{17:0}$FAME	59 *n*-$C_{22:0}$FAME	90 *n*-$C_{32:0}$FAME
29 *i*-α+β-OTMS-$C_{15:0}$FAME	60 *n*-α+β-OTMS-$C_{20:0}$FAME	91 DPE **
30 *ai*-α+β-OTMS-$C_{15:0}$FAME	61 *n*-$C_{22:0}$-OTMS	92 DPE (desilylated)
31 *n*-$C_{17:0}$FAME	62 *n*-$C_{23:0}$FAME	

Lipids were extracted by a sequential analytical procedure yielding four extracts per sample : the Ether-extract; the Ether/OH^--extract; the OH^--extract and the H^+-extract (Goossens *et al.*, 1988a,b). The extracts were derivatized and analyzed by GC-FID and GC-MS without separation of compound classes.
This sequential procedure provides a general description of the lipid moieties and their modes of occurrence in the samples (Goossens *et al.*, 1988a,b).

Figs. 1 to 3 show the gas chromatograms of three fractions. Numbers in the gas chromatograms refer to Table 1, in which the structural identifications are summarized. Since the saponified Ether-extracts (Ether/OH^--extracts) did not contain other compounds than the Ether-extracts, only the gas chromatograms of the Ether/OH^--extracts are shown (Fig.1). The main difference between these two extracts is that the amounts of fatty acids and alcohols increased upon saponification, especially the amount of phytol, which is virtually absent from the Ether-extracts.
Distributions of the most abundant compound classes (*i.e.* fatty acids and hydroxy fatty acids) in the different extracts are represented in Figs. 4 and 5. The distributions of the hydroxy fatty acids in Fig. 5 are inferred from the distributions of the diagnostic

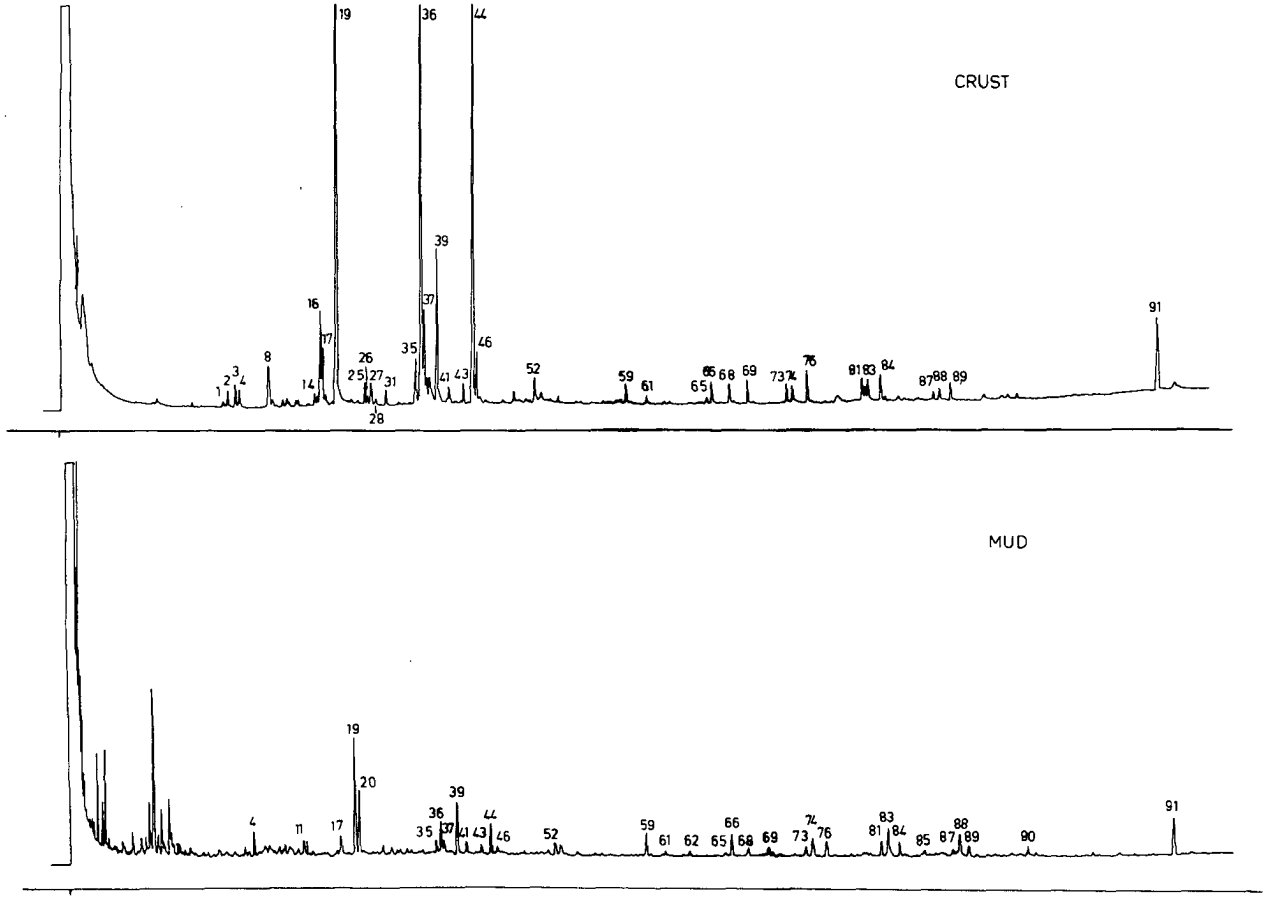

Fig.1 Gas chromatograms of the ether/OH^--extracts of crust and mud sample.

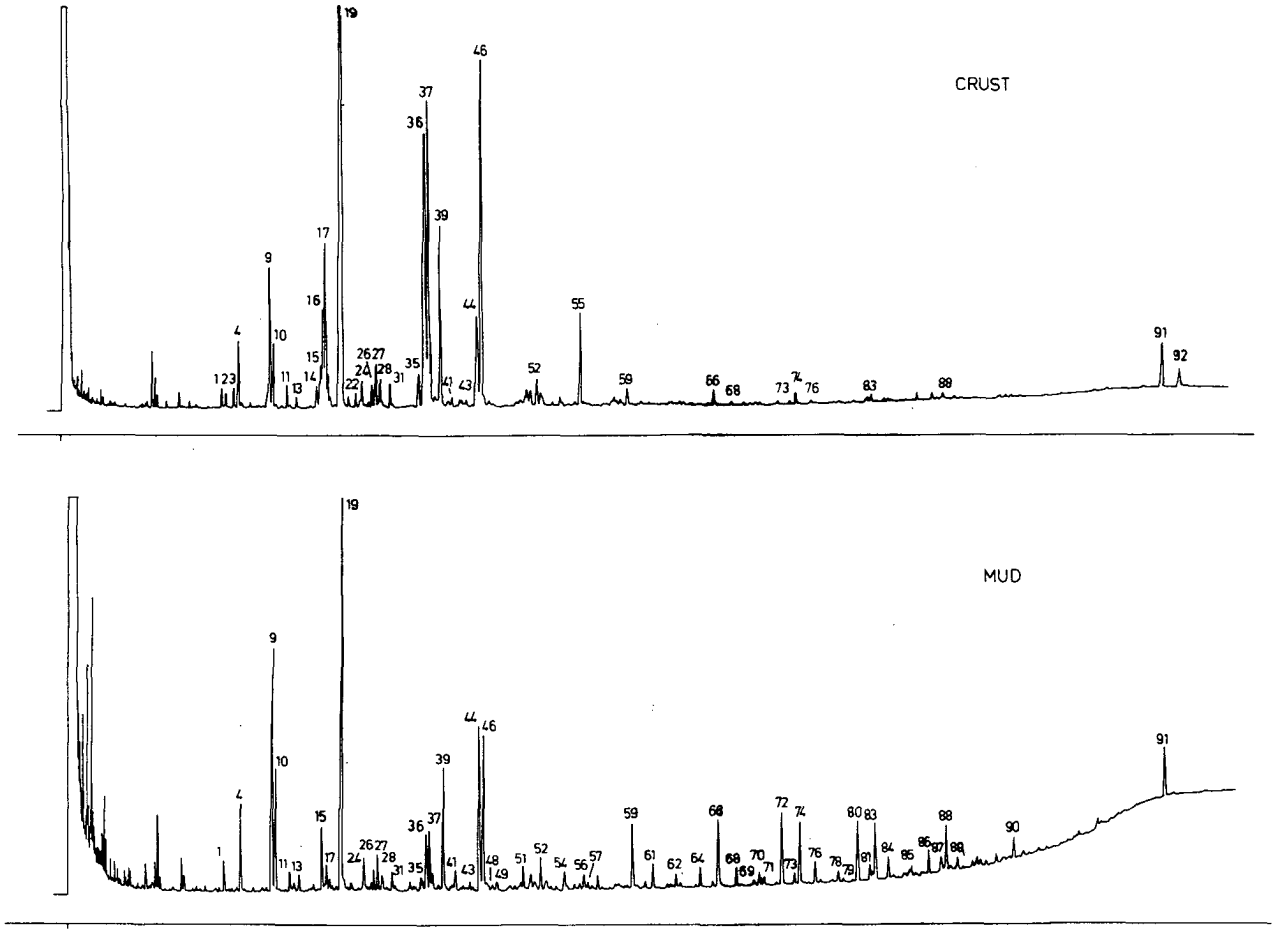

Fig.2 Gas chromatograms of the OH^--extracts of crust and mud sample.

ions *m/z* 175 and M^+-59 for β- and α-hydroxy fatty acids, respectively. Fig. 5 shows the distributions of the intensities of these ions expressed as percentages of the highest intensities in the respective mass chromatograms. The ratios of the maximal intensities (I-max) of the diagnostic ions (set at 100% for each frame in Fig. 5) of β- and α-hydroxy fatty acids (I-max-β/I-max-α) are 3.2 and 4.4 for the crust and the mud extract, respectively.
When a compound occurs in more than one extract of a sample the relative amounts present in the different extracts can be inferred from the gas chromatograms by multiplying the peak intensities of that particular compound with the appropiate scaling factors: 1.5, 4, and 1 for the Ether/OH^--extract, the OH^--extract and the H^+-extract of the crust-sample and 2, 3.3, and 1 for the similar extracts of the mud sample, respectively.

The presence of a cyclopropylgroup was verified by hydrogenation of the extract. The relative retention times of the cyclopropyl fatty acids remained unchanged in contrast to those of the unsaturated fatty acids. These unsaturated compounds disappeared from the extract while the amounts of the corresponding saturated fatty acids increased proportionally.

Gypsum crust

Fatty acids
The main compounds in the extracts of the gypsum crust are fatty acids. The distributions in the three extracts (Fig.4) show that most fatty acids occur in different modes. The composition of the extracts is rather different. The major components are saturated and mono-unsaturated C_{16} and C_{18} fatty acids which are present mainly in an OH^--labile mode (Fig.4). This means that they occur ester-bound in the original sample. The distributions of the $C_{18:1}$FA isomers (compounds 36 and 37) show large differences in the different extracts. The OH^--extract contains also a high amount of *cy*-C_{19}FA and considerable amounts of *i*- and *ai*- branched C_{15}fatty acids. The H^+-labile fatty acids are minor but the presence of unsaturated fatty acids in the H^+-labile mode is remarkable since in most analyses of bacteria only saturated fatty acids were found in the H^+-extract (Goossens *et al.*, 1986). Apart from the branched C_{17} fatty acids low amounts of some specific compounds were observed in the OH^--extract, such as *i*-$C_{17:1}$FA, 10-Me-$C_{16:0}$FA, *n*-$C_{17:1}$FA and *cy*-C_{17}FA. These compounds are very minor ones in the Ether/OH^--extract and are virtually absent in the H^+-labile lipid fraction. A specific increase of 10-Me-$C_{16:0}$FA in the OH^--extract compared to the other extracts was noticed. In all extracts only very low amounts of long chain fatty acids ($>C_{20}$) were present.

Alcohols
Phytol (compound 44) is the most abundant alcohol in the crust sample. The other alcohols (*n*-C_{14} to *n*-C_{30}) and dihydrophytol are only present in low concentrations. The alcohols occur primarily in the Ether/OH^--extract in which the even, *n*-alkanols (C_{16}-C_{30}) are all present in about equal amounts. These straight chain alcohols are virtually absent from the Ether-extract (results not shown). They occur therefore esterified within rather apolar compounds. The concomitant increase of fatty acids and alcohols upon saponification of the Ether-extract points to waxesters as likely precursors.
Phytol was absent from the Ether-extract and from the H^+-extract. The presence of a high amount in the Ether/OH^--extract suggests that intact chlorophyll is extracted. The presence of dihydrophytol in all extracts probably indicates that there is a direct source of dihydrophytol in addition to a possible diagenetic transformation of free phytol. The alcohols observed in the H^+-extract are saturated, even straight chain components (C_{14}-C_{20}) and dihydrophytol. In the H^+-extract the concentration of the shorter alcohols was somewhat higher than in the other extracts.

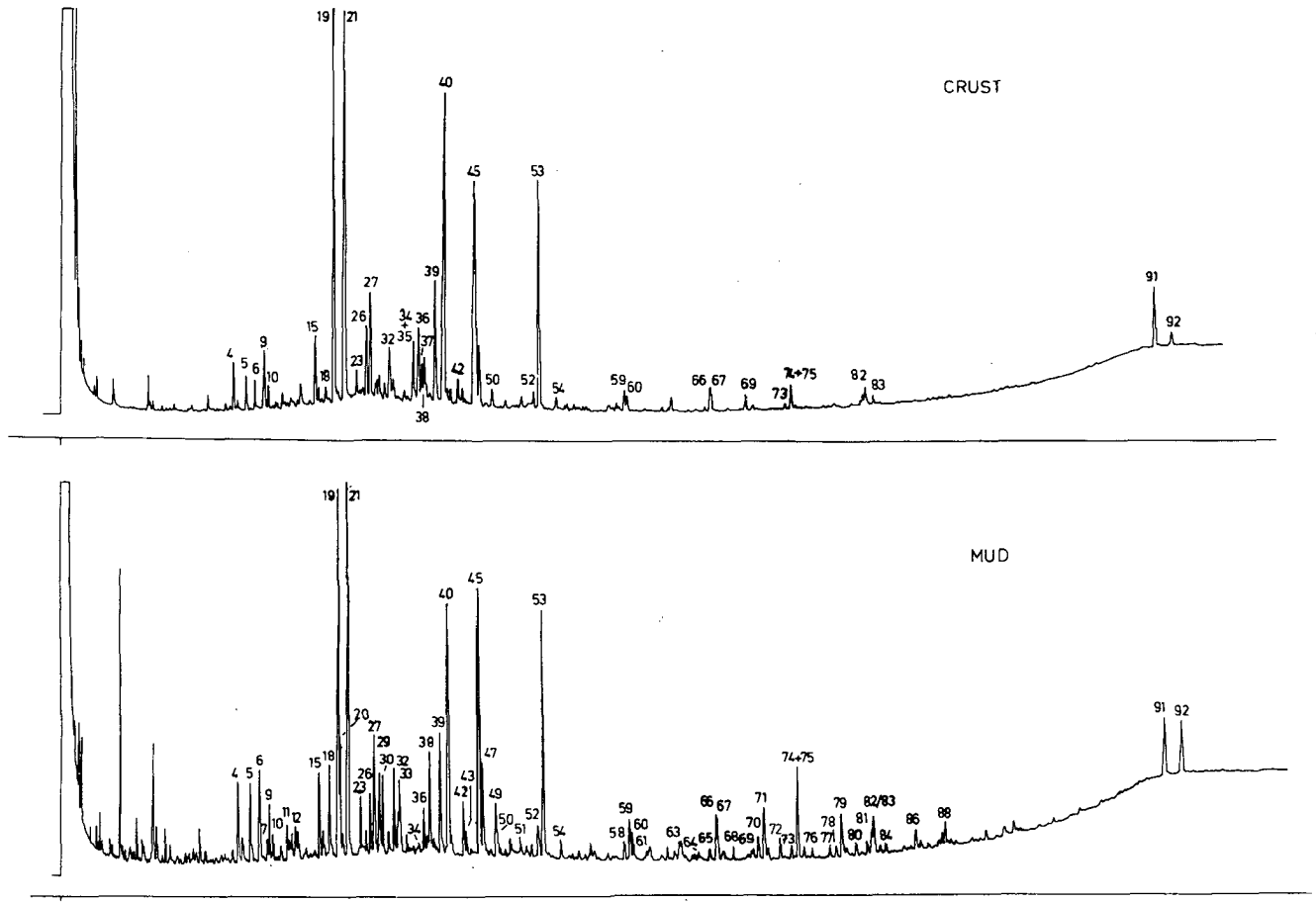

Fig.3 Gas chromatograms of the H^+-extracts of crust and mud sample.

Hydroxy fatty acids
Hydroxy fatty acids occurred exclusively in the H^+-extract, with one exception. A relatively high amount of OH^--labile *cy*-α-OH-C_{19}FA (compound 55, Fig.2) was found, which was not observed in any other extract. We observed this compound also in the OH^--extract of the orange-brown layer which was sampled separately as well (unpublished results).
Most hydroxy fatty acid peaks in the gas chromatograms represent mixtures of α- and β-hydroxy fatty acids. The β-hydroxy fatty acids are more abundant than the α-hydroxy fatty acids. The even, straight-chain compounds and *i*-β-OH-$C_{17:0}$FA were most abundant (Fig.5). In the H^+-extract an uncommonly branched β-hydroxy fatty acid occurs (compound 34), which is characterized by an enhanced intensity at *m/z* 71 in its mass spectrum (Goossens *et al.*, 1986).

Other compounds
Other compounds to be mentioned are diphytanylether (DPE) and some hydrocarbons. The membrane lipids of extreme halophilic archaebacteria consist of DPE, in which two phytanyl chains occur ether-linked to a glycerol moiety.
The Ether-extract contained relatively high amounts of *n*-C_{17} and *n*-C_{18} alkenes, of *n*-C_{17} alkane and also of squalene. The C_{17} hydrocarbons were also observed in the OH^--extract but the C_{18} alkene was not present. Squalene occurred also in the H^+-extract and is probably derived from the halobacteria, which synthesize a suite of hydrogenated squalenes (Langworthy, 1982; Goossens *et al.*, unpublished results). We identified only squalene in the extracts; the more reduced C_{30} isoprenoid alkanes were not observed.
At higher temperatures, a series of triplets of odd *n*-alkanes, even *n*-fatty acids and *n*-alkanols eluted which are ascribed to higher plant material (see below). These triplets are apparent in the Ether/OH^--extract. In the other extracts their concentrations are very low.
In the OH^--extract a low amount of hexahydrofarnesylacetone (HHFA) was found.

Mud

Fatty acids
In contrast to the crust, the mud contains primarily saturated fatty acids with relatively high amounts of *i*- and *ai*-C_{15} fatty acids, *cy*-C_{19}FA and *i*-C_{16}FA in addition to *n*-C_{16}FA and *n*-C_{18}FA. The fatty acids are mainly esterified. In the Ether-extract only low amounts were observed although the intensities of fatty acids and alcohols increased somewhat upon saponification. In the H^+-extract fatty acids are minor components (except *n*-C_{16}FA) and their distribution shows a relatively high proportion of *i*- and *ai*-C_{17} fatty acids in contrast to those of the Ether/OH^-- and OH^--extract. Branched $C_{17:1}$ fatty acids are virtually absent whereas the amount of esterified 10-Me-C_{16}FA is somewhat higher than in the crust.

Alcohols
Phytol is relatively abundant in the Ether/OH^-- and OH^--extract and not present in an H^+-labile mode whereas the shorter alcohols (even *n*-C_{14}-*n*-C_{20} and dihydrophytol) are present in all extracts in very low amounts.
The longer even, *n*-alkanols (C_{20} - C_{30}) are all present in about equal amounts.

Hydroxy fatty acids
α- and β-hydroxy fatty acids are only present in the H^+-extract where they are major compounds. Although the highest amounts were detected in the range C_{12} to C_{18}, substantial amounts of compounds with longer chains (C_{20}-C_{24}) are present. The uncommonly branched β-OH-C_{16}FA "71" is only present in trace amounts in the mud.
The distribution of β-hydroxy fatty acids is different from that of the crust: The contribution of branched species is higher in the mud, whereas the relative amount of *n*-

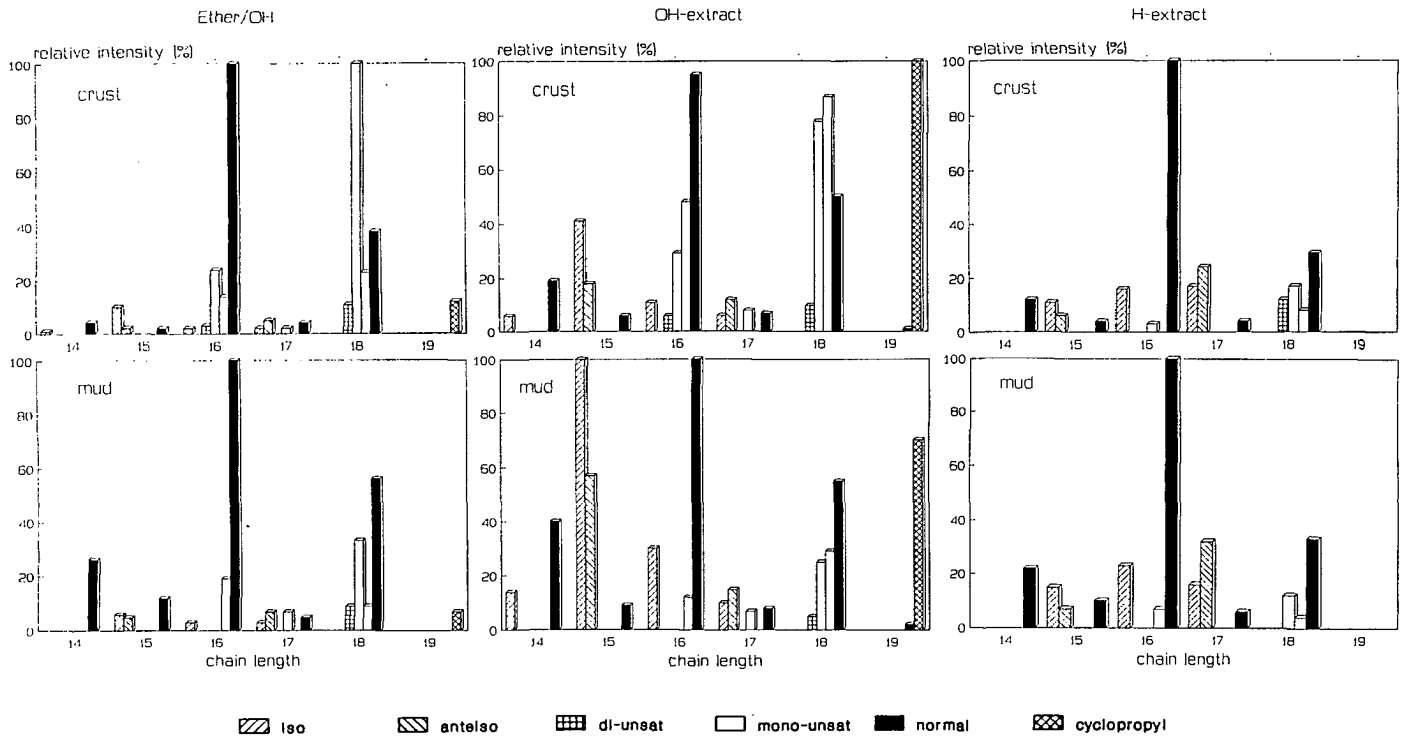

Fig.4. Distributions of fatty acids in the different extracts of crust and mud (relative intensities per extract). The intensities of n-$C_{16:0}$FA in the Ether/OH^--extract of the crust and in both OH^--extracts exceed 100 %.

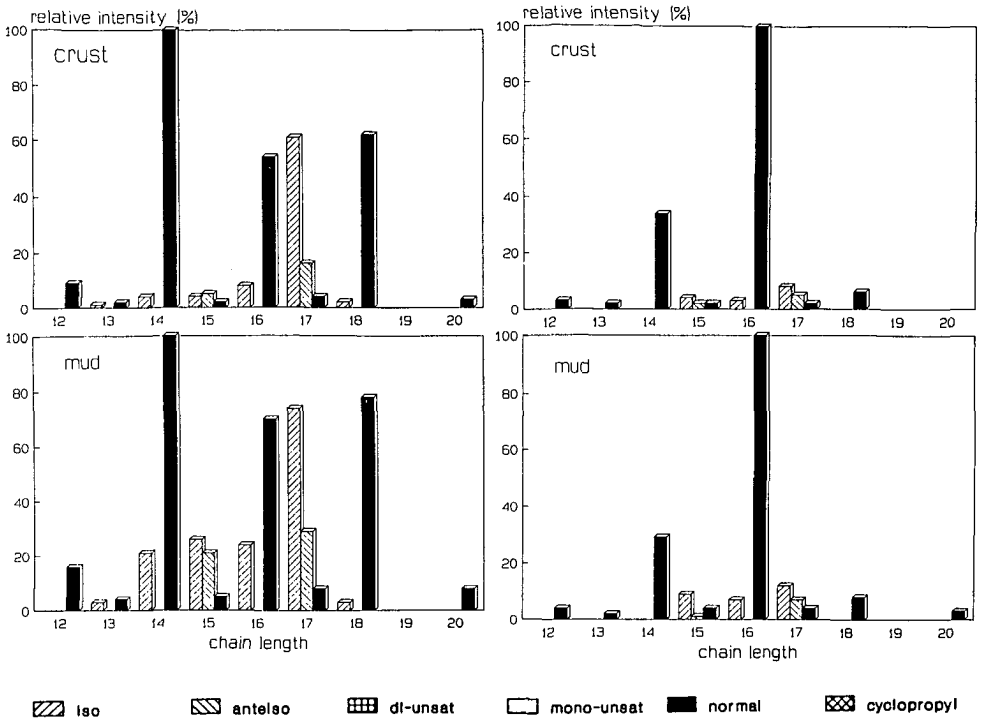

Fig.5 Distributions of α- and β-OH-fatty acids in the H^+-extracts of crust and mud; left: β-OH-fatty acids; right α-OH-fatty acids. (relative intensities per extract of the diagnostic ions M^+-59 for α- and m/z 175 for β-OH fatty acids).

β-OH-C_{14}FA is somewhat lower in the mud.
The α-hydroxy fatty acid pattern also shows a small increase of branched species.
The H^+-extract also contains the uncommonly branched β-hydroxy fatty acid (compound 34).
The α- and β-hydroxy fatty acids with chains ($>C_{20}$) occur only in low amounts.

Higher plant lipids
In all extracts triplets of odd *n*-alkanes (C_{25}-C_{33}), even fatty acids (*n*-C_{22}-C_{32}) and alcohols (*n*-C_{22}-C_{32}) were observed. In addition, a series of diacids (C_{16}-C_{24}) and ω-hydroxy fatty acids (C_{16}, C_{20}-C_{26}) are present. The suite of ω-hydroxy fatty acids present in the mud is absent from the crust.
All these compounds indicate the presence of higher plant material in the mud (Kolattukudy, 1980; Harwood, 1980).

Other compounds
DPE occurs in all extracts in considerable amounts.
The C_{17} hydrocarbons (compounds 2 and 3), present in the crust-extract, could not be detected in the mud. In the mud-extract an increased amount of *n*-C_{25} alkane was observed.
In both extracts low amounts of hexahydrofarnesylacetone (HHFA) were found. Noteworthy is the high amount of α-OCH_3-C_{16}FA (compound 33) which was not observed in the other extracts. Since this compound is virtually absent from the H^+-extract of the crust, artificial formation from α-OH-C_{16}FA is unlikely. Potential mother molecules are not known.

Discussion

The extreme conditions of the environment investigated, can be inferred from the geochemical record primarily from the absence of a set of biomarkers. The virtual absence of steroid compounds is a clear indication of the prokaryotic nature of the organic matter in these samples, since almost all eukaryotes contain sterols (Nes and McKean, 1977). Although some cyanobacterial species have been reported to synthesize sterols (Reitz and Hamilton, 1968; Volkman, 1986), the species present in this environment apparently are not able to or the extreme conditions inhibit the production of detectable amounts.

All extracts contain substantial amounts of DPE which occurs, therefore, in three different modes, *i.e.* as free compound, esterified to a polar group and glycosidically linked to a saccharide moiety. This is in agreement with the literature on the lipid compositions of extreme halophilic archaebacteria (Kates *et al.*, 1965; Kates, 1973; Langworthy, 1982). In this environment DPE originates probably from extreme halophiles. The presence of methanogens, which also may contain DPE (Makula and Singer, 1978; Mancus *et al.*, 1985), cannot be precluded, however, since these may occur also in hypersaline environments (Giani *et al.*, 1984).

The distributions of the fatty acids in the samples show marked differences (Fig.4). Both free and esterified unsaturated fatty acids are less intense in the mud than in the crust.

The substantial differences in the distributions of esterified fatty acids in crust and mud indicate that different prokaryotic communities are sampled and that organic matter from the crust is degraded rapidly in the underlying mud. This rapid degradation is also illustrated by the absence of n-C_{17} and n-C_{18} alkenes and cy-α-OH-C_{19}FA from the mud sample. The low amount of ether-extractable lipids in the mud also points to a rapid turnover of free compounds.

The distributions of the H^+-labile fatty acids show much smaller differences. This might mean that these (primarily amide-bound) fatty acids are less easily degraded, either because they are part of a more resistant macromolecule or because the amide linkage resists biodegradation.

The distributions of H^+-labile α- and β-hydroxy fatty acids are also less different than the esterified fatty acid distributions, but specific increases of some branched β-hydroxy fatty acids (i-β-OH-C_{14}FA, i- and ai-β-OH-C_{15}FA and i-β-OH-C_{16}FA) in the mud were observed. A higher proportion of α-hydroxy fatty acids was present in the crust, although in both samples β-hydroxy fatty acids are dominant.

In all extracts long chain alkanes, fatty acids and alcohols are present showing the typical distributions of higher plant material (Kolattukudy, 1980). In the crust-extracts the amounts are very low except in the Ether/OH^--extract. The parallel increases of the amounts of fatty acids and alcohols upon saponification of the Ether-extract suggests the presence of waxesters in the original material, since no compounds with dual functionalities which could have functioned as cross linkers in a macromolecule, were observed.

In the OH^--extract of the mud ω-hydroxy fatty acids and dicarboxylic acids are present in substantial amounts. These compounds are characteristic for the plant macromolecule suberin (Kolattukudy, 1980).

There is no direct input of higher plant material into the basins in this part of the salt works, which can also be concluded from the absence of sterols. The ground between the basins was covered with gypsum crystals. Plant growth occurs only in and around basins at much lower salinities which are far away. We must assume either an eolian input or transport through many basins by the water, probably via the surface-microlayer. This illustrates the high resistance to biodegradation of the material, which also explains the higher concentration of it in the mud. The presence of substantial amounts of this plant material in this prokaryotic environment stresses the importance of rates of biodegradation for the interpretation of lipid compositions of sediments.

The presence of alkanes and suberin components in the H^+-extracts may be explained in different ways:

A) Poor extraction in the previous step of the sequential procedure.
This seems improbable since the distributions of compounds are different in the OH^--extracts; phytol, for example, is virtually absent in the H^+-extracts whereas it is a major compound in the OH^--extracts and the ratio of the amounts of dicarboxylic acids and ω-hydroxy fatty acids in the mud extracts is reversed.
B) Incomplete saponification of higher plant material.
C) Inclusion of higher plant material in (in)organic matter.
Both B) and C) are possible explanations. The saponification of higher plant macromolecules is known to be a slow process. Inclusion of organic matter during the formation of the gypsum crystals may occur. The volume of water, which is necessary to open the gypsum crystals, is kept low to avoid high volumes of organic solvents. Transportation of some organic matter to the fraction receiving the H^+-treatment is thus possible but if that were the case, we would expect similar distributions of these compounds in the OH^--extract and the H^+-extract.
Inclusion in organic matter is another possibility, since we observed similar phenomena (H^+-labile alkanes, for example) during the analysis of a lacustrine sediment (Goossens *et al.*, 1988b). Moreover, the observation of "bound alkanes" in studies distinguishing different modes of occurrence is very common (Cranwell, 1978, 1981b; Albaigès *et al.*, 1984; Leenheer *et al.*, 1984; Shaw and Johns, 1985).
At present, we cannot conclude which explanation is most likely. The different distributions of the major compounds in the extracts (*e.g.* phytol and the fatty acids $<C_{20}$), however, suggest that inclusion does not seriously affect the interpretation of the modes of occurrence of the major compounds.

Recognition of source organisms

Halophilic archaebacteria
The abundant presence of extreme halophilic archaebacteria can be recognized from the presence of DPE, which occurs in different modes. These organisms are known to synthesize also high amounts of isoprenoid C_{30} alkanes at varying levels of unsaturation (Langworthy, 1982; Taylor, 1984; Goossens *et al.*, unpublished results). Surprisingly, squalene is the only C_{30} isoprenoid hydrocarbon present. This was observed in a sample of the hypersaline Gavish Sabkha as well, in which partially reduced squalenes were only present in trace amounts (De Leeuw *et al.*, 1985). Either squalene is contributed by other eubacterial species (Taylor, 1984) or the archaebacteria present in this environment produce primarily squalene. The latter possibility could result from the presence of different, as yet unknown, archaebacterial species or from physiological responses to the environment.

S-cycle bacteria
Judging from the abundant presence of purple-S-bacteria and the strong smell of H_2S from the mud, the sulphur cycle is an important process in this environment. Two groups of bacteria are discussed which mutually provide the indispensable compounds: the H_2S producing organisms (sulphate and sulphur reducers) and the sulphide oxidizing organisms.

Sulphate reducers
Many sulphate and sulphur reducing species have been investigated (Makula and Finnerty, 1974, 1975; Boon *et al.*, 1977; Ueki and Suto, 1979; Taylor and Parkes, 1983; Edlund *et al.*, 1985; Dowling *et al.*, 1986). The results seem to support the view that branched $C_{17:1}$ fatty acids are specific for *Desulfovibrio* species (except for *Desulfovibrio gigas*; Boon *et al.*, 1977), that 10-Me-C_{16}FA is specific for *Desulfobacter* species (Dowling *et al.*, 1986) and that *n*-$C_{17:1}$FA is specific for *Desulfobulbus* (Taylor and Parkes, 1983). However, the former two compounds have been observed also in other bacterial species although not in major amounts (Perry *et al.*, 1979; Goossens *et al.*, unpublished results). Moreover, a number of sulphate reducers do not contain specific compounds and it has also been shown that major shifts in lipid composition can result

from changes in the availability of compounds which are not metabolized but act as chain initiators in lipid synthesis (Dowling *et al.*, 1986). This may explain why the intensities of $C_{17:1}$fatty acids are low in this gypsum environment and why there is not a major difference in concentration of these "markers" between crust and mud. Other possible explanations are that as yet unspecified species keep the S-cycle going or that the specific fatty acids are not synthesized under the prevailing conditions.

Sulphide-oxidizers
The bright purple band at the underside of the crust illustrates the abundance of purple-S-bacteria. These phototrophic bacteria use H_2S or other reduced sulphur compounds as electron donors in the photosynthetic process, by which reduced sulphur compounds are oxidized to sulphate (Trüper, 1978).
The lipids of many phototrophic purple-S-bacteria have been investigated (reviewed by Kenyon, 1978). The fatty acids of phototrophic bacteria are mainly even, straight chain compounds. As a general rule, *n*-$C_{18:1}$FA is most abundant whereas the amount of *n*-$C_{18:0}$FA is low and *n*-$C_{16:1}$FA and *n*-$C_{16:0}$FA are present in moderate to high amounts. The fatty acid composition of the crust is in agreement with these general characteristics. The high amount of *cy*-C_{19}FA may be related to the presence of *Ectothiorhodospira halophila*. This compound is rare in phototrophic bacteria but *E. halophila* has been reported to contain substantial amounts (Kenyon, 1978). This species may occur in hypersaline environments (Larsen, 1980).
The *cy*-α-OH-C_{19}FA present in the OH^--extract of the crust originates from the orange brown layer in which the species *Synechococcus* and *Gloeothece* were recognized. The relatively high amount suggests that this compound originates from an abundantly present species and therefore one (or both) of the cyanobacterial species are likely source organisms. However, *cy*-α-OH-C_{19}FA occurs esterified to an ornithine lipid in *Thiobacillus thiooxidans* (Knoche and Shively, 1969). This organism derives its energy from the oxidation of reduced sulphur compounds and might therefore occur in this environment, although it is questionable whether it is able to cope with high salt concentrations.

Phototrophic bacteria
Further recognition of the phototrophic species in the samples is not possible due to lack of specificity of the even, straight chain fatty acids which dominate the lipids of the phototrophic bacteria. Some species contain uncommon alcohols substituting phytol in the bacteriochlorophylls (Steiner *et al.*, 1981; Gloe *et al.*, 1975; Caple *et al.*, 1978). In our samples only straight chain alcohols, phytol and dihydrophytol were observed, in contrast to the sample from the Gavish Sabkha which contained considerable amounts of farnesol, phytadienol and geranylgeraniol in addition to phytol (De Leeuw *et al.*, 1985).
Cyanobacteria often synthesize C_{17} and C_{18} hydrocarbons in significant quantities (Han *et al.*, 1968). Probably, the C_{17} and C_{18} hydrocarbons present in the crust extracts originate from the cyanobacteria.

Heterotrophic bacteria
The ability to degrade organic matter under aerobic or anaerobic conditions is present in many genera and therefore recognition of specific species is not to be expected. Nevertheless, the increase in concentration of *i*- and *ai*-C_{15} fatty acids in the OH^--extract of the mud compared to the crust seems to point to decomposing bacteria. These fatty acids have been found in relatively high concentrations in gram positive bacteria and also in anaerobic heterotrophic bacteria cultured from a marine sediment (Perry *et al.*, 1979). In addition, a major increase in the concentrations of these fatty acids was observed in the top layer of a cyanobacterial mat in Solar Lake comparing the very top layer (0.0-0.5 mm !) with the layer directly underneath (0.5-3.0 mm) (Boon and De Leeuw, 1986). Although the common nature of these fatty acids preclude a positive assignment, it seems that the relatively high concentrations of these fatty acids observed in the mud are related to the first stages of decomposition by heterotrophic bacteria.

Prokaryotic community

The H^+-extracts of both samples contain high amounts of α- and β-hydroxy fatty acids. H^+-labile β-hydroxy fatty acids occur in the Lipopolysaccharides (LPS) of bacteria and their distributions are fingerprints of the prokaryotic community (Goossens *et al.*, 1986). Although the occurrence of H^+-labile α-hydroxy fatty acids is not restricted to bacteria since they may be present in sphingolipids from eukaryotic organisms, such a source can be precluded in this strictly prokaryotic environment. Due to lack of comparable data we cannot assign the H^+-labile α-hydroxy fatty acids to specific organisms. It is likely, however, that H^+-labile α-hydroxy fatty acids are very specific since they are present in only a few bacterial species (Lechevalier, 1977; Goldfine, 1982). In addition, we found those in only one out of thirteen bacterial species, *i.e.* in the sulphate reducer *Desulfovibrio gigas* (Goossens *et al.*, 1986), but the presence of substantial amounts in the crust sample suggests that also other species contain these compounds in this mode of occurrence. The number of different α-hydroxy fatty acids present also points to various sources. H^+-labile α-hydroxy fatty acids were not observed in a freshwater sediment (Goossens *et al.*, 1988b).

Differences between the hydroxy fatty acid distributions in crust and mud are mainly a decreased proportion of α-hydroxy fatty acids and an increased proportion of branched α- and β-hydroxy fatty acids in the mud.

Since acid has been used only occasionally to release "bound" lipids (Cranwell, 1978, 1981a,b; Parker *et al.*, 1982; Cardoso *et al.*, 1983; Klok *et al.*, 1984; Goossens *et al.*, 1986, 1988b) we can compare our results with few other investigations. Only in the studies by Goossens *et al.* (1986, 1988a,b) esterified and amide-linked hydroxy fatty acids were distinguished. H^+-labile α-hydroxy fatty acids occurred also in a cyanobacterial mat sample taken in the same salt work area (Goossens *et al.*, 1986). Both the gypsum sample and the cyanobacterial mat sample contain high amounts of branched OH-C_{17} fatty acids.

Conclusions

The lipid composition of the sediment in this extreme, hypersaline environment is relatively simple and differs from the compositions of cyanobacterial mats (Philp *et al.*, 1978; Boon *et al.*, 1983; De Leeuw *et al.*, 1985). No sterols or other polycyclic structures were observed and no other isoprenoid hydrocarbons or alcohols than the common compounds squalene, phytol and dihydrophytol were found. Consequently, biological markers for hypersaline environments having cyclic structures probably relate to conditions of deposition at lower salinities than those of gypsum precipitation. In addition, the absence of isoprenoid hydrocarbons from our samples suggests that the isoprenoid hydrocarbons present in cyanobacterial mats (Philp *et al.*, 1978; Boon *et al.*, 1983; De Leeuw *et al.*, 1985) are not likely to originate from extreme halophilic archaebacteria.

The contribution from extreme halophilic archaebacteria is indicated by the presence of DPE in the extracts and probably also by the presence of enhanced amounts of squalene. However, the absence of hydrogenated squalenes in the extracts shows that the data obtained from laboratory cultures cannot be used in a straightforward manner to interprete the results of sediment analyses.

The presence of higher plant material in substantial amounts in the mud stresses that selective preservation is an important factor determining the lipid compositions of sediments.

The low amounts of the compounds which have been proposed as markers for sulphate reducers indicate that substantial sulphate reduction in a sediment is not necessarily reflected in the presence of these marker molecules.

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CHAPTER 5

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Bacterial contribution to sedimentary organic matter; a comparative study of lipid moieties in bacteria and Recent sediments

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Abstract—A systematic study of the lipid composition of thirteen bacterial species and three Recent sediments (methanogenic sediment, cyanobacterial mat and evaporative gypsum crust) was undertaken in an attempt to recognize bacterial organic matter in sediments. A sequential method, which distinguishes between three different modes of occurrence of lipid moieties (free, OH^-- and H^+-labile), was applied. The acid-labile fractions are discussed.

The three main groups of bacteria, archaebacteria, gram-positive eubacteria and gram-negative eubacteria, are easily distinguished. Methanogenic and extremely halophilic archaebacteria are characterized by the presence of diphytanyl glyceryl ether and the absence of fatty acids. The gram-positive eubacteria contain primarily iso- and anteiso-branched fatty acids whereas the gram-negative bacteria and sediments are dominated by β- and α-hydroxy fatty acids. A wide variety of H^+-labile hydroxy fatty acids was observed which included several, as yet unknown, structures.

β-Hydroxy fatty acids in this H^+-labile mode of occurrence are exclusively present in bacteria. Their distribution patterns in sediments are considered "fingerprints" of past and present bacterial populations. The specific differences in β-hydroxy fatty acid compositions observed in the different bacteria and the three sediments investigated, suggest that amide-linked β-hydroxy fatty acid patterns are useful as markers of bacterial populations and therefore of environmental conditions.

Key words: Bacteria, Recent sediments, analysis of lipids, mode of occurrence of lipids, hydroxy fatty acids, markers for environmental conditions; bacterial markers, methanogenic sediment, cyanobacterial mat, gypsum crust

INTRODUCTION

Many of the compounds present in Recent and ancient sediments and crude oils are thought to originate from prokaroytic organisms. However, a bacterial source has been firmly established only for a limited number of compounds. For instance, ether-linked isoprenoid alkyl moieties occur in various sediments and crude oils (Chappe *et al.*, 1979, 1982; Michaelis and Albrecht, 1979). These structures obviously originate from the isoprenoid ether lipids which occur exclusively in cell walls of archaebacteria (Langworthy, 1982, for a review). A second example is the group of the "extended hopanoids" occurring widely in the geosphere. These compounds are certainly derived from bacterio-hopanetetrol and other extended hopanoids which are present in various bacterial species (Förster *et al.*, 1973; Ourisson *et al.*, 1979; Rohmer *et al.*, 1984; Taylor, 1984). However, numerous other compounds encountered cannot be ascribed to a bacterial origin with certainty, because these compounds or their precursors are observed in both prokaroytes and eukaryotes. The presence of iso- and anteiso-branched compounds, for instance, is generally interpreted as a contribution from bacteria, although they have also been observed in fungi (Weete, 1980), plants (Kolattukudy, 1980) and mammals (Downing, 1961, 1976).

At present, bacteria are considered to be important contributors to sedimentary organic matter. Since environmental conditions are expressed to a large extent in the species composition of bacterial populations and in the chemical composition of the individual cells, a better insight into the composition of prokaryotic organic matter might yield information on the environment of deposition and on conditions during diagenesis.

Therefore, a systematic study of organic matter present in bacteria is required for a more detailed interpretation of the results of analyses of sedimentary lipids.

Bacterial lipids have been investigated widely and intensely (see for reviews; Lechevalier, 1977; Goldfine, 1982; Langworthy, 1982; Taylor, 1984). The major part of eubacterial lipids consists of diacylglycerides in which fatty acids occur esterified to a glycerolmolecule with a polar group at position three. In other substances, such as lipopolysaccharide (LPS) and ornithine lipid, ester- and amide-linked lipid moieties occur. In some cases, glycosidically bound lipid moieties have been observed. Obviously, lipid moieties are present in different modes. Unfortunately, in most studies dealing with total lipid compositions of bacteria, the different modes of occurrence have not been distinguished, whereas in those studies which differentiate between these

modes, only isolated fractions of the cell were investigated.

The mode of occurrence of lipid moieties provides valuable information in organic geochemical investigations. In the first place, this information enhances the specificity of observed structures and can be helpful to establish the source. Cranwell (1981) showed, by differences in stereochemistry, that free β-hydroxy fatty acids were mainly generated by biodegradation of fatty acids, whereas bound ones were derived from microorganisms. In the second place, the mode of occurrence is an important factor determining the rate of biodegradation of lipids. For example, Eyssen *et al.* (1973) showed that sterols are only microbially transformed when they occur in a free state. An enhanced preservation of "bound" compounds compared to "free" ones has been noticed (Cranwell, 1981; Kawamura and Ishiwatari, 1984).

In recent investigations of sedimentary lipids "free" and "bound" lipids are sometimes distinguished (Cranwell, 1978; de Leeuw *et al.*, 1983; Albaigés *et al.*, 1984; Kawamura and Ishiwatari, 1984; Leenheer *et al.*, 1984). Since in most studies the bound lipids are liberated by saponification, it can be assumed that mainly ester linkages are cleaved. Although the major part of bacterial lipid moieties is released by saponification indeed, a substantial proportion of the lipid moieties remains bound. The amide- and glycosidic bonds which occur in LPS, ornithin lipids and other amino- and glycolipids of prokaryotes and in sphingolipids of pro- and eukaryotes (Weete, 1974; Lechevalier, 1977; Gurr and James, 1980; Goldfine, 1982) are only hydrolyzed under acidic conditions (Mayberry, 1980).

In organic geochemical studies, H^+-labile lipid moieties are overlooked with a few exceptions where an acid treatment was applied (Cranwell, 1978, 1981; Parker *et al.*, 1982; Klok *et al.*, 1984). Obviously, a full analysis of prokaryotic organic matter, whether in sediments or as such, should include an acid treatment.

To obtain as much information as possible from lipid analyses of bacteria and sediments, we tried not only to identify the structures of the lipid moieties present, but also their modes of occurrence. By a procedure of sequential extractions and hydrolyses we distinguished between ether soluble, OH^-- and H^+-labile lipid moieties (Goossens *et al.*, 1986a).

For this study, the sequential analysis was performed on a number of bacteria and sediments. The bacteria were selected to represent distinct bacterial groups according to 16*S* rRNA systematics (Fox *et al.*, 1980; Kandler, 1981), taking into account their presence in specific environments. The sediment samples investigated were analysed by exactly the same procedure. They were taken in environments where prokaryotes are abundant: a methanogenic freshwater sediment (Lake Vechten, The Netherlands), a cyanobacterial mat and a hypersaline evaporation system (both from the salt works at Roquetas de Mar, Spain).

In this paper we compare the compositions of extracts containing exclusively H^+-labile lipid moieties of bacteria and sediments. The results of the complete analyses will be published elsewhere.

EXPERIMENTAL

Samples

Prokaryotes were obtained from different sources: material of *Halobacterium halobium*, *Desulfovibrio gigas* DSM 496, *Chromatium vinosum D*, *Thiocystis violacea* strain 2311 DSM 208, *Ectothiorhodospira halochloris*, *Chlorobium vibrioforme* (forma thiosulphatophilum strain 8327, DSM 263) and *Rhodobacter sulfidophilus* W4 were supplied by Prof. H. G. Trüper (University of Bonn, Germany) and *Methanobacterium thermoautotrophicum* ΔH by Prof. G. D. Vogels (University of Nijmegen, The Netherlands); *Anacystis nidulans* and *Bacillus subtilis* LMD 70–64 were obtained from the collection of the Laboratory of Microbiology at the Delft University of Technology, The Netherlands; *Acinetobacter calcoaceticus* LMD 79–41 was a gift by B. J. van Schie (Delft University of Technology, The Netherlands); *Streptomyces sp.* was isolated from a piece of decaying Messel shale; *Micrococcus lysodeikticus* was obtained commercially from Sigma (St. Louis, U.S.A.). The Lake Vechten sediment sample was taken in Lake Vechten, Bunnik, The Netherlands (see Gulati and Parma, 1982). The sample investigated was the 4–6 cm section of a 6 cm diameter core taken in October 1984. The core was sectioned immediately after sampling and the sections were frozen and stored at −20°C until lyophilization prior to analysis of the sample. Two samples were taken in the salt works at Roquetas de Mar (southern Spain) in November 1984. One sample was taken in a basin where cyanobacterial mats covered the bottom. The sample was cut out with a knife, packed in aluminium foil and kept frozen until lyophilization prior to analysis. The other sample was taken in a basin with a higher salinity where gypsum precipitated from the water.

A piece of the gypsum crust was sampled from underwater with a spade, packed in aluminium foil and kept frozen until lyophilization. Prior to analysis the lyophilized sample was ground in a ball mill.

Lipid analysis

Details of the analytical procedure employed are published elsewhere (Goossens *et al.*, 1986a). In short, a sequential procedure of extractions and hydrolyses was applied, which distinguishes between free, OH^-- and H^+-labile lipid moieties. First, free, relatively apolar compounds were extracted with ether; OH^--labile lipid moieties were liberated by saponification of the residue (1M KOH in methanol, 96%; reflux, 1 hr) and extracted with CH_2Cl_2 by a modified Bligh and Dyer (1959) method. The residue remaining after saponification and extraction and the water/methanol layer were combined, lyophilized and treated with acid (4N HCl, 100°C, 6 hr). The acid-treated matter, including the residue, was saponified prior to extraction to prevent losses by the acid-catalyzed formation of high-molecular-weight esters or by esterification of lipid moieties to the residual matter. The H^+-labile lipid moieties released were then extracted by the modified Bligh and Dyer method. This analytical scheme minimizes the formation of artifacts, although the formation of Δ^2 unsaturated fatty acids could not be avoided completely. These compounds are generated by base-catalyzed elimination of the fatty acid moiety from 3-acyl fatty acids, which are formed under acid conditions from β-hydroxy fatty acids and fatty acids (Goossens *et al.*, 1986a).

Carboxyl groups were methylated with diazomethane in diethylether and hydroxyl groups were silylated with Trisil-Z (Pierce) or BSTFA (Merck).

Gas chromatographic analyses were performed on a Carlo Erba 4160 instrument with on-column injection and flame ionization detection. Separation was achieved on a Chrompack CPSil 5 fused silica capillary column (25 m, i.d. 0.32 mm, film thickness 0.12 μm) with He as carrier gas. After injection of the sample (in ethyl acetate) at 100°C the temperature was rapidly raised to 130°C and then programmed at a rate of 4°C/min to 300 or 330°C.

Gas chromatography–mass spectrometry was performed on a Varian 3700 gas chromatograph connected to a Varian Mat 44 quadrupole mass spectrometer with electron impact ionisation at 80 eV. Mass spectra were obtained from m/z 50 to 500 with a cycle time of 1.5 sec.

RESULTS AND DISCUSSION

In this paper we discuss the lipid moieties of bacteria and sediments which are exclusively linked by an H^+-labile bond. The lipid moieties present in the extracts were investigated by GC and GC–MS. Components were identified by comparing GC retention times and mass spectral data with those of standard compounds and with literature data. In some cases tentative structures are given based upon the mass spectrometric fragmentation pattern. The compounds identified are summarized in Table 1. The numbers correspond to those indicated in Figs 1–8. Since the mass spectra of α- and β-OTMS-methyl esters do not contain sufficient information to distinguish iso (*i*)- or anteiso (*ai*)-branched or normal (*n*)-hydroxy fatty acids, these assignments are based upon relative retention times analogous to the procedure followed for the identification of hydroxy fatty acids by Boon *et al.* (1977).

In the sequential analytical procedure we used ether to extract the "free", relatively apolar lipids and CH_2Cl_2 for the extraction of OH^--labile lipid moieties. Therefore, H^+-extracts may not only contain lipid moieties which were bound to insoluble macromolecules but also those which were linked to a relatively polar group via an H^+-labile bond.

It has been shown by IR measurements that ester- and amide-linked lipid moieties are released separately by a sequential based and acid treatment (Mayberry, 1980). Considering the types of bonding which occur in bacteria it can be concluded that the lipid moieties released upon acid treatment in the sequential analysis should have been linked via an amide- or a glycosidic bond. Therefore, fatty acids in the H^+-extract occurred exclusively as amide linked lipid moieties in the original material. Hydroxy fatty acids could have been amide-linked or bound via the hydroxyl group to C-1 of a sugar moiety in a glycosidic bond. However, such a glycosidic bond is not common in bacteria (Lechevalier, 1977).

The amide-linked lipid moieties present in prokaryotes and eukaryotes are fatty acids and hydroxy fatty acids. They occur in spingolipids (Weete, 1974; Gurr and James, 1980), lipopolysaccharides (Galanos *et al.*, 1977; Wilkinson *et al.*, 1977; Lüderitz *et al.*, 1982; Wollenweber *et al.*, 1984), ornithine lipids (Lechevalier, 1977; Batrakov and Bergelson, 1978)

Table 1. Identifications of the peaks indicated in Figs 1–8

	1	n-$C_{12:0}$mE	t	34	i-Δ^2-$C_{17:1}$ mE
	2	β-OTMS-$C_{10:0}$ mE		35	n-β-OTMS-$C_{15:0}$ mE
	3	n-Δ^2-$C_{12:1}$ mE	t	36	ai-Δ^2-$C_{17:1}$ mE
	4	β-OCH_3-$C_{12:0}$ mE		37	β-OTMS-$C_{16:0}$ mE"71"
	5	i-$C_{14:0}$ mE		38	n-$C_{18:2}$ mE
	6	n-$C_{14:0}$ mE		39	i-$C_{18:0}$ mE
	7	β-OTMS-$C_{12:0}$ mE		40	n-$C_{18:1}$ mE
	8	α-OTMS-$C_{12:0}$ mE		41	i-β-OTMS-$C_{16:0}$ mE
a	10	n-Δ^2-$C_{14:1}$ mE		42	i-α-OTMS-$C_{16:0}$ mE (Fig. 8. n.i.)
a	12	i-$C_{15:0}$ mE		43	β-OTMS-$C_{16:1}$ mE
	13	ai-$C_{15:0}$ mE		44	n-$C_{18:0}$ mE
	14	i-β-OTMS-$C_{13:0}$ mE		45	β-OTMS-$C_{16:0}$ mE
	15	ai-β-OTMS-$C_{13:0}$ mE (Fig. 6. n.i.)		46	α-OTMS-$C_{16:0}$ mE
	16	n-$C_{15:0}$ mE		47	n-Δ^2-$C_{18:1}$ mE
	17	β-OCH_3-$C_{14:0}$ mE		48	n-$C_{18:0}$-OTMS
t	18	i-Δ^2-$C_{15:1}$ mE (Fig. 6. n.i.)		49	i-β-OTMS-$C_{17:0}$ mE
t	19	ai-Δ^2-$C_{15:1}$ mE		50	i-α-OTMS-$C_{17:0}$ mE
	20	β-OTMS-$C_{13:0}$mE		51	ai-β-OTMS-$C_{17:0}$ mE
	21	i-$C_{16:0}$ mE		52	ai-α-OTMS-$C_{17:0}$ mE
	22	n-$C_{16:1}$ mE		53	n-$C_{19:0}$ mE (Fig. 7. n.i.)
	23	i-β-OTMS-$C_{14:0}$ mE		54	n-β-OTMS-$C_{17:0}$ mE
	24	n-$C_{16:0}$ mE		55	n-α-OTMS-$C_{17:0}$ mE
	25	β-OTMS-$C_{14:0}$ mE		56	β-OTMS-$C_{18:0}$ mE"57"
	26	ai-$C_{17:1}$ mE		57	α-OTMS-$C_{18:0}$ mE"57"
	27	n-Δ^2-$C_{16:1}$ mE	a	61	i-β-OTMS-$C_{18:0}$ mE
	28	i-$C_{17:0}$ mE		62	i-α-OTMS-$C_{18:0}$ mE
	29	ai-$C_{17:0}$ mE		63	β-OTMS-$C_{18:1}$ mE (Fig. 6. n.i.)
	30	i-β-OTMS-$C_{15:0}$ mE		64	n-$C_{20:0}$ mE
	31	i-α-OTMS-$C_{15:0}$ mE		65	β-OTMS-$C_{18:0}$ mE
	32	ai-β-OTMS-$C_{15:0}$ mE		66	α-OTMS-$C_{18:0}$ mE
	33	n-$C_{17:0}$ mE	t	67	$\Delta^{2,X}$-$C_{20:2}$ mE
				68	n-$C_{30:0}$-OTMS
				69	n-Δ^2-$C_{20:1}$ mE
				70	β-OTMS-$C_{19:0}$ mE"71"
				71	unknown
				72	n-$C_{21:0}$-OTMS (Fig. 6. n.i.)
				73	9,16-di-OTMS-$C_{16:0}$ mE
				74	β-OTMS-$C_{20:0}$ mE"85"
				75	β-OTMS-$C_{20:1}$ mE
				76	n-$C_{22:0}$ mE
				77	β-OTMS-$C_{20:0}$ mE
				78	n-$C_{22:0}$-OTMS
			t	79	β,X-di-OTMS-$C_{20:0}$ mE
				80	unknown
				81	n-$C_{24:0}$ mE
				82	β-OTMS-$C_{22:0}$ mE
				83	α-OTMS-$C_{22:0}$ mE
				84	n-$C_{24:0}$-OTMS
				85	n-$C_{25:0}$ mE
				86	$C_{22:0}$ di-ME
				87	ω-OTMS-$C_{22:0}$ mE
				88	n-$C_{26:0}$ mE
				89	β-OTMS-$C_{24:0}$ mE
				90	α-OTMS-$C_{24:0}$ mE
				91	n-$C_{26:0}$-OTMS
				92	ω-OTMS-$C_{24:0}$ mE
				93	n-$C_{28:0}$ mE
				94	unknown
				95	n-$C_{28:0}$-OTMS
				96	ω-OTMS-$C_{26:0}$ mE
				97	n-$C_{30:0}$ mE
				98	n-$C_{30:0}$-OTMS
				99	unknown
				100	di-O-phytanyl-glycerol(DPE) (TMS-derivative)

a: numbers 9, 11, 58, 59, 60 are not used

Abbreviations: *mE = methyl ester; n = straight chain; i = iso-branched; ai = anteiso-branched; t = tentative identification;* TMS = trimethylsilyl, n.i. = not indicated in the figures.

and in other aminolipids (Braun, 1975). Amide-linked hydroxy fatty acids are encountered exclusively in sphingolipids, lipopolysaccharides and ornithine lipids. In sphingolipids of eukaryotic organisms (Gurr and James, 1980), only α-hydroxy fatty acids have been encountered (Downing, 1961; Gurr and James, 1980). In prokaryotes sphingolipids have only been reported in the genus *Bacteroides* (Goldfine, 1982) and in *Acholeplasma axanthum* (Mayberry *et al.*, 1973).

LPS and ornithine lipids occur exclusively in prokaryotes: LPS in the outer layer of the gram-negative cell wall and ornithine lipids in the cell membrane of some gram-negative and gram-positive species. In these substances β- and occasionally α-hydroxy fatty acids occur in an amide linkage.

β-Hydroxy fatty acids occur in bacteria and sediments also in an OH^--labile mode, indicating an ester bond. Their distribution pattern is, in that case, often different from that of the amide-linked one (Goossens *et al.*, 1986a,b). Since ester-linked β-hydroxy fatty acids also occur in eukaryotes (Tulloch and Spencer, 1964; Matsumoto and Nagashima, 1984) their presence in sediments cannot be ascribed exclusively to prokaryotic sources.

From the above it is clear that amide-linked β-hydroxy fatty acids have been encountered only in bacteria and therefore can be considered very specific markers for prokaryotes. This means that differences in distribution patterns of amide-linked β-hydroxy fatty acids in sediments offer possibilities for recognizing differences in populations of bacteria and hence differences in environments of deposition or in conditions during diagenesis.

Bacterial analyses

According to the phylogeny based upon 16*S* rRNA systematics (Fox *et al.*, 1980; Kandler, 1981), two prokaryotic kingdoms can be distinguished: the archaebacteria and the eubacteria. The latter kingdom can be subdivided further into gram-negative and gram-positive eubacteria. The results of the bacterial analyses are therefore discussed in three different sections.

Archaebacteria. The archaebacteria differ very much from the eubacteria in many respects (Fox *et al.*, 1980). Major characteristics concerning the lipid composition are the virtual absence of fatty acids and the abundance of alkylglycerides containing ether-linked isoprenoid alkyl moieties (see Langworthy, 1982, for a review). Figure 1 shows the gas chromatogram of the H^+-extract of *Halobacterium*. This trace shows only one compound eluting at relatively high temperature. It was tentatively identified as diphytanyl glycerol ether (DPE). This compound is the major lipid in *Halobacterium* and other extreme halophilic archaebacteria (Langworthy, 1982). Since it occurs in the H^+-extract, the polar group attached to the molecule before the acid treatment was probably a saccharide moiety linked via a glycosidic bond. A similar gas chromatogram was obtained for the H^+-extract of *Methanobacterium* (results not shown). Here, too, DPE was the major compound indicating that this compound is not specific for extreme halophilic archaebacteria. DPE has been observed in other methanogenic archaebacteria as well (Langworthy, 1982; Mancuso *et al.*, 1985).

Gram-positive eubacteria. With the exception of the genus *Streptomyces*, where several ornithine lipids are present (Batrakov and Bergelson, 1978), substances containing H^+-labile lipid moieties have not been reported in gram-positive bacteria. Figure 2 shows the gas chromatogram of the H^+-extract of *Streptomyces*. Only *n*-, *i*- and *ai*-fatty acids are present. These fatty acids are probably derived from an ornithine lipid. No hydroxy fatty acids were observed. A similar distribution pattern of fatty acids was obtained for the H^+-extracts of *Micrococcus lysodeikticus* and *Bacillus subtilis* (results not shown), although the amounts present were very low. This indicates that in gram-positive bacteria compounds containing amide-linked fatty acids may be present.

Gram-negative eubacteria. The gas chromatograms of the H^+-extracts of the non-phototrophic organisms *Acinetobacter calcoaceticus* and *Desulfovibrio gigas* (Fig. 3), the purple phototrophs *Chromatium vinosum*, *Ectothiorhodospira halochloris*, *Thiocystis violacea* and *Rhodobacter sulfidophilus* (Fig. 4) and the green phototrophs *Chlorobium vibrioforme* and *Anacystis nidulans* (Fig. 5) show that within the group of gram-negative eubacteria a wide variety of H^+-labile lipid moieties is present.

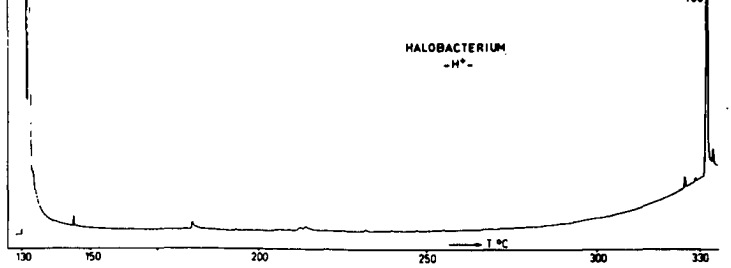


Fig. 1. Gas chromatogram of the H^+-extract of the archaebacterium *Halobacterium halobium*.

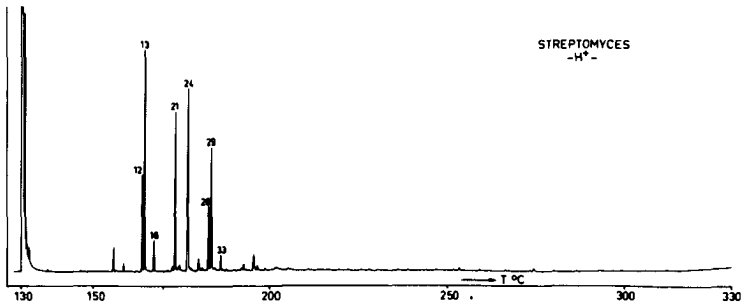


Fig. 2. Gas chromatogram of the H^+-extract of the gram-positive eubacterium *Streptomyces* sp.

The lipid compositions of the non-phototrophs *Acinetobacter calcoaceticus* and *Desulfovibrio gigas* have little in common (Fig. 3): *Acinetobacter* shows a simple lipid pattern with only n-C_{12} β-OH-FA and n-C_{14} β-OH-FA, whereas in *Desulfovibrio* branched and straight-chain α- and β-hydroxy fatty acids and also fatty acids occur. The relative amounts of α- and β-hydroxy fatty acids are almost equal for all hydroxy fatty acids with two exceptions: the greater part of i-C_{16}OH-FA carries the hydroxy group at the β position whereas in the branched C_{18}-OH-FA (peak 57) the hydroxy group is at the α position. In the mass spectrum of the latter compound an enhanced intensity at m/z 57 was observed (see below).

That the phototrophic species have a physiological property in common is not apparent from their H^+-labile lipid patterns (Figs 4 and 5). Only the *Chromatium* and *Ectothiorhodospira* traces are similar, in that they contain both mainly n-C_{14} β-OH-FA. In the cyanobacterium *Anacystis* (renamed *Synechococcus* sp.) n-C_{16} β-OH-FA is the only compound present. Unfortunately, n-C_{14} β-OH-FA and

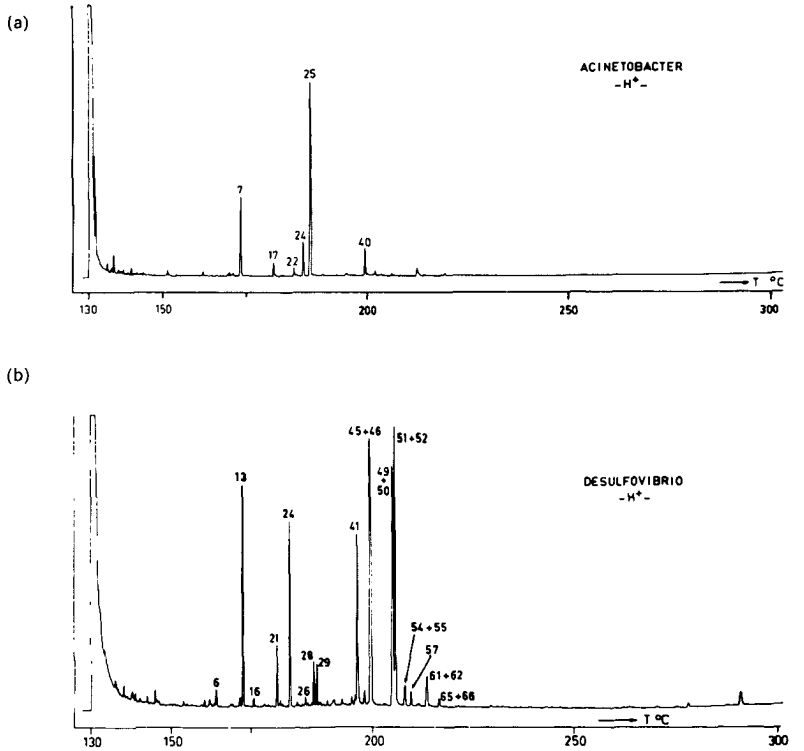
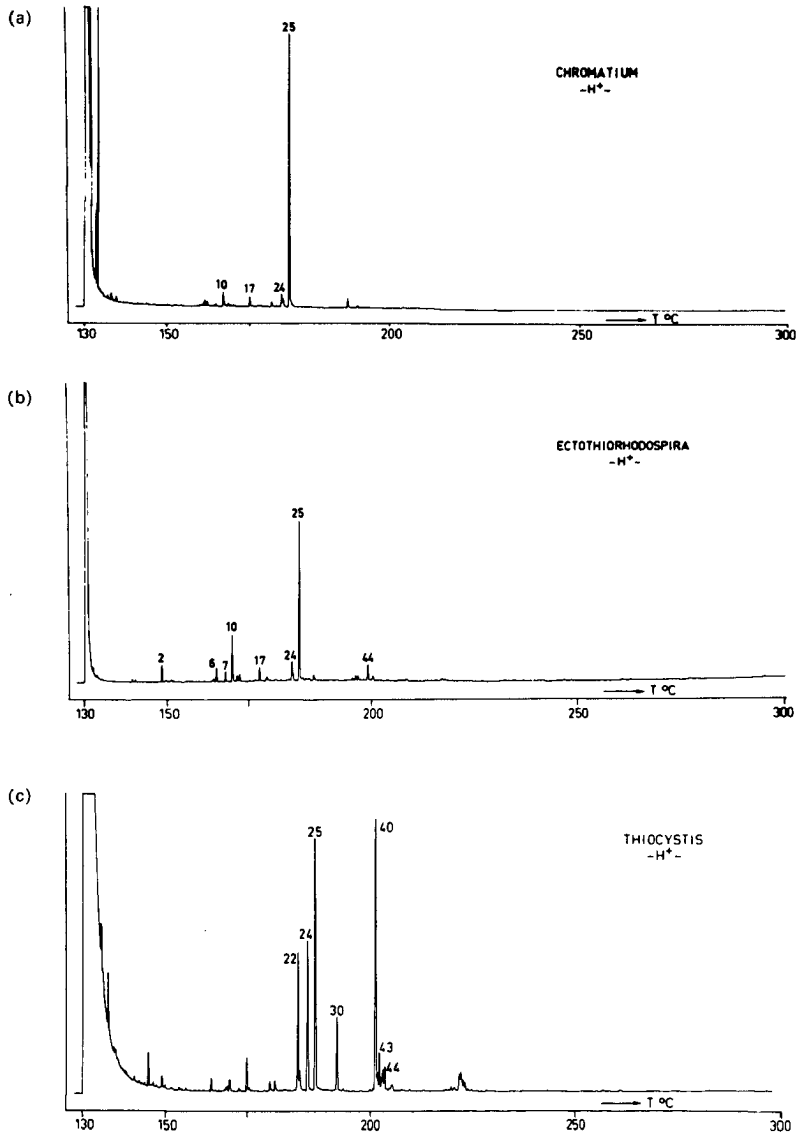


Fig. 3. Gas chromatograms of the H^+-extracts of *Acinetobacter calcoaceticus* and *Desulfovibrio gigas*.

n-C_{16} β-OH-FA are the most commonly occurring β-hydroxy fatty acids in gram-negative bacteria and therefore, these compounds are not useful as markers of specific bacteria.

The other organisms have more complex lipid patterns which are very different from one another. In the *Thiocystis* extract, $C_{18:1}$ FA is the most abundant compound and a considerable amount of $C_{16:1}$ FA is also present. This is remarkable since in all other H^+-extracts of gram-negative bacteria fatty acids are only present in rather low amounts, if at all, and are mainly saturated. n-C_{14} β-OH-FA, i-C_{15} β-OH-FA and n-$C_{16:1}$ β-OH-FA are also present.

The *Rhodobacter* extract has an unusual composition. The major component is n-C_{14} β-OH-FA accompanied by a high amount of n-$C_{20:1}$ β-OH-FA and by varying amounts of the saturated n-C_{18}, n-C_{20} and n-C_{22} β-hydroxy fatty acids. The unsaturated n-C_{20} β-OH-FA has been reported to occur in the sphingolipid of *Acholeplasma axanthum* (Mayberry



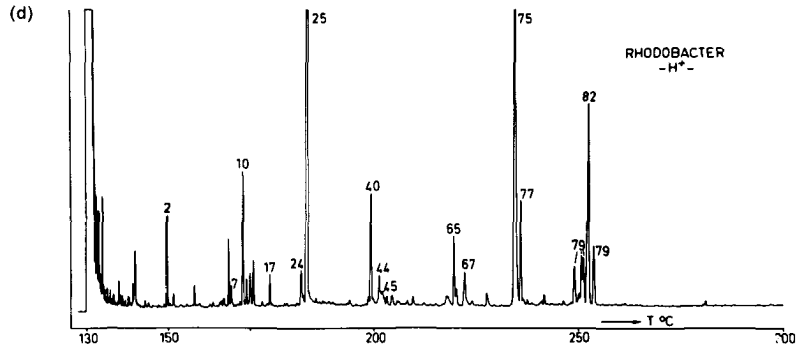


Fig. 4. Gas chromatograms of the H^+-extracts of *Chromatium vinosum*, *Ectothiorhodospira halochloris*, *Thiocystis violacea* and *Rhodobacter sulfidophilus*.

et al., 1973) but such long-chain ($\geq C_{20}$) hydroxy fatty acids are not common in gram-negative bacteria. In addition, four compounds were found which are possibly di-OH-FA's, with one OH-group at the β position and the other at an, as yet, unknown position.

In the H^+-extract of *Chlorobium* (Fig. 5) the straight-chain compounds n-C_{14} β-OH-FA and n-C_{16} β-OH-FA are most abundant. At higher temperatures several β-hydroxy fatty acids eluted which probably contain methyl branches at uncommon positions since their retention times are different from those of *i*-, *ai*- or *n*-hydroxy fatty acids. In their mass spectra enhanced intensities were observed of fragment ions with m/z values 57, 71 and 85 for the chain lengths C_{18}, C_{19} and C_{20}, respectively. This suggests

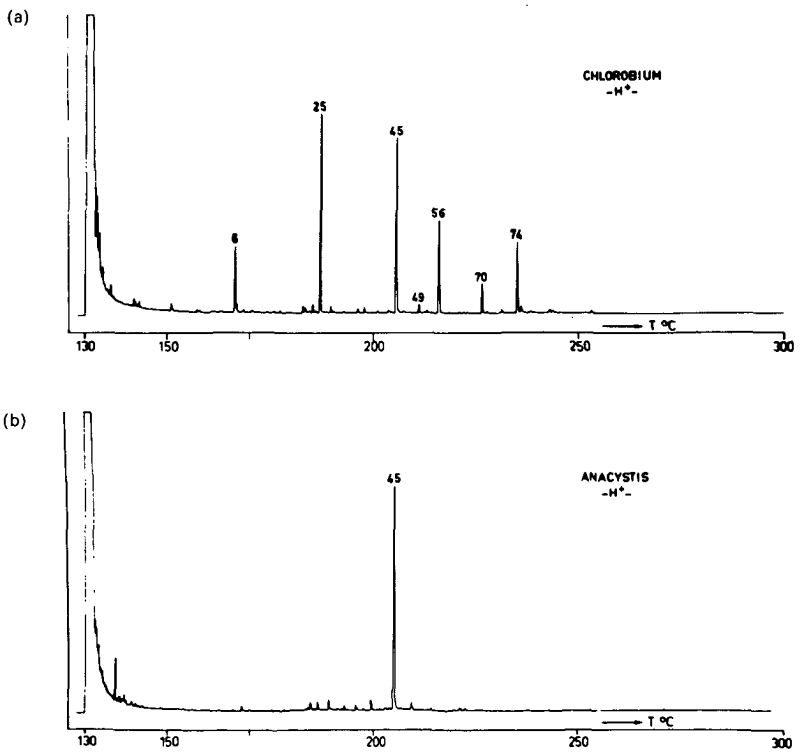


Fig. 5. Gas chromatograms of the H^+-extracts of *Anacystis nidulans* (*Synechococcus* sp.) and *Chlorobium vibrioforme*.

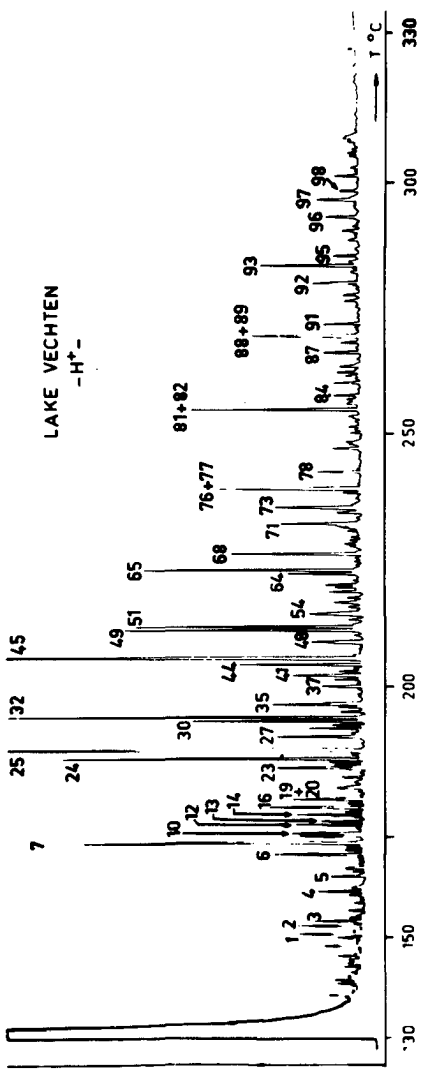


Fig. 6. Gas chromatogram of the H^+-extract of Lake Vechten sediment.

a methyl group at positions ω-4, ω-5 and ω-6, respectively. The enhanced intensity of m/z 57, as observed here in C_{18} β-OH-FA, was also observed in the branched C_{18} α-OH-FA of *Desulfovibrio*, suggesting similar alkyl chains in these two compounds, since their relative retention times are also identical.

Figures 3, 4 and 5, summarized in Table 2, demonstrate that the H^+-labile lipid patterns in the group of gram-negative eubacteria range from very simple in *Chromatium*, *Anacystis* and *Acinetobacter* to rather complex in *Rhodobacter* and *Desulfovibrio*. Some of the H^+-extracts are very characteristic by the presence of special structures like the n-$C_{20:1}$ β-OH-FA in *Rhodobacter* and the branched-chain hydroxy fatty acids in *Chlorobium* and *Desulfovibrio* or by the combination of compounds like α- and β-hydroxy fatty acids in *Desulfovibrio* and of β-hydroxy fatty acids and unsaturated fatty acids in *Thiocystis*. However, in all H^+-extracts, β-hydroxy fatty acids are the dominant compounds and either n-C_{14} β-OH-FA or n-C_{16} β-OH-FA is a major component.

An important source of the H^+-labile lipid moieties is the lipopolysaccharide (LPS) molecule. This complex compound has been investigated in detail in a variety of gram-negative species (Wilkinson, 1977; Galanos *et al.*, 1977; Lüderitz *et al.*, 1982; Wollenweber *et al.*, 1984). In the lipid A part of the LPS molecule, saturated β-hydroxy fatty acids occur in amide linkage to a disaccharide. These β-hydroxy fatty acids mainly have straight chains with 10, 12, 14, 16, 17 or 18C atoms or are iso-branched c_{13} or C_{17} (Lüderitz *et al.*, 1982). The C_{15} chain has only been observed in *Veillonella* sp. and *Bacteroides fragilis* (Galanos *et al.*, 1977; Wollenweber *et al.*, 1984). In most cases, only one β-hydroxy fatty acid is present but in some rare cases mixtures of up to four occur (Lüderitz *et al.*, 1982). The simple patterns observed in some of our bacterial analyses resemble the amide-linked β-hydroxy fatty acid composition of LPS described in the literature. In the case of *Acinetobacter*, the H^+-labile lipid moieties were shown to be derived from LPS (Goossens *et al.*, 1986a), which indicates an amide linkage. The complexity of the extracts of *Rhodobacter*, *Chlorobium* and *Desulfovibrio*, however, suggests that molecules other than LPS may be present which contain H^+-labile lipid moieties, e.g. ornithine lipids or other glyco- or aminolipids. In *D. gigas*, the presence of an ornithine lipid has been demonstrated (Makula and Finnerty, 1975). The structure of a lipoprotein present in *Escherichia coli* has been investigated in detail (Braun, 1975). It contains one amide-linked fatty acid per molecule. In the so-called rhamnolipid of *Pseudomonas* a glycosidically linked β-hydroxy fatty acid has been found (Lechevalier, 1977). The presence of other amphiphilic molecules at the cell surfaces of bacteria has been shown as well (Wicken and Knox, 1980). However, these complex compounds are not well characterized, in most cases, with respect to the type of linkage of the lipid moieties. Unfortunately,

very little is known about the influence of growth conditions on the lipid composition of LPS and other amino- and glycolipids. Under normal physiological conditions at least the lipid A composition of LPS seems to be rather stable (Lüderitz *et al.*, 1982). In addition, the limited number of species investigated with respect to H^+-labile lipids prevents general conclusions. Therefore, the specificity of H^+-labile compounds and their distribution patterns remains to be established. However, the diversity of structures and distribution patterns observed in our analyses of selected bacteria is promising.

Sediment analyses

Three different sedimentary environments with a high contribution of prokaryotic organic matter were selected to compare their lipid contents.

Lake Vechten is a small, slightly eutrophic, freshwater lake, stratified in summer with an anoxic hypolimnion. At the chemocline an abundant population of phototrophic bacteria is present. Methanogenesis has been demonstrated in the sediment. An overview of the extensive research carried out on this lake has been published recently (Gulati and Parma, 1982). The sediment sample was taken at the end of the season (October 1984) just before the autumn turnover. The other two sediment samples were taken in November 1984 in the salt works area at Roquetas de Mar (Spain), where sea salt is produced by evaporation of sea water. Here, the different stages of a sabkha-like evaporation system are created next to each other. A sedimentological description of this area is given by Dronkert (1977). One sample was taken in a basin where cyanobacterial mats covered the bottom. A piece of this very consistent, laminated sediment (about 6 cm thick) was cut out as representative of a cyanobacterial mat. The other sample was taken at the salinity level where gypsum precipitated from the water. In the gypsum crust, taken from under water and about 3 cm thick, strongly coloured bands of orange brown slimy organisms, green cyanobacteria and purple phototrophic bacteria were visible from top to bottom. The sample consisted of the whole gypsum crust including the coloured organisms. The overlying water in this basin was coloured deep pink due to abundant extreme-halophilic archaebacteria.

Lake Vechten. The H^+-extract of Lake Vechten sediment (Fig. 7) is characterized by the presence of high amounts of β-hydroxy fatty acids with chain lengths from C_{10} to C_{24}. The highest amounts are found between C_{12} and C_{18}, n-C_{14} β-OH-FA and n-C_{16} β-OH-FA being the major compounds. There is a strong predominance of straight chain components with even carbon-number. Also, many branched β-hydroxy fatty acids are present but only i- and ai-C_{15} β-OH-FA and i- and ai-C_{17} β-OH-FA were found in considerable amounts. The amount of ai-C_{15} β-OH-FA is much greater than that of i-C_{15} β-OH-FA whereas the amounts of i- and ai-C_{17}

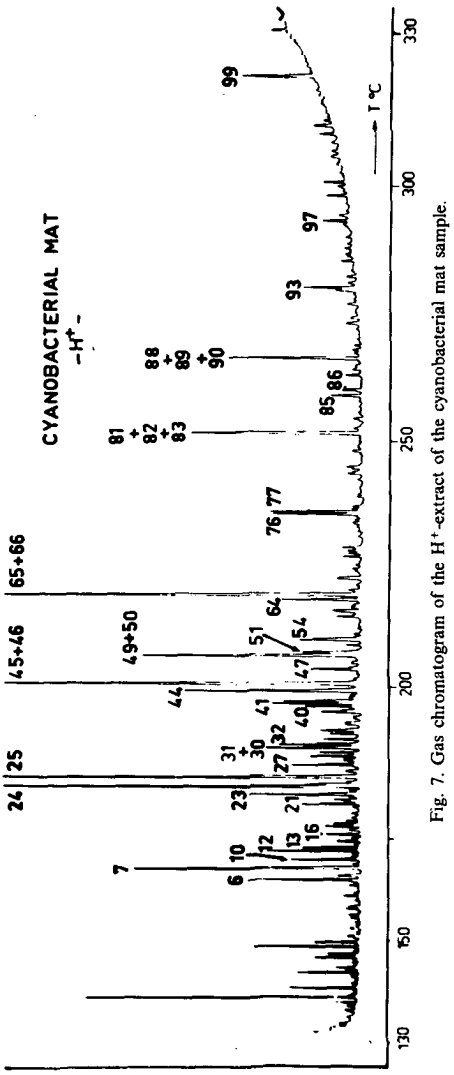


Fig. 7. Gas chromatogram of the H^+-extract of the cyanobacterial mat sample.

β-OH-FA are almost equal. The other minor β-hydroxy fatty acids may contain very specific information in spite of their low amounts, since some of these probably contain methyl branches at uncommon positions judging from their retention times which preclude *i*- or *ai*-branching. The branched C_{16} β-OH-FA (peak 37) showed an enhanced intensity of the fragment ion at m/z 71 in its mass spectrum analogous to the C_{19} β-OH-FA (peak 70) observed in *Chlorobium*. Apart from these branched hydroxy fatty acids also the unsaturated n-$C_{18:1}$ β-OH-FA and n-$C_{20:1}$ β-OH-FA were identified. At higher elution temperatures n-C_{22}, n-C_{24} and n-C_{26} ω-OH-FA were observed (peaks 87, 92 and 96, respectively).

The H^+-labile fatty acids in Lake Vechten show a bimodal distribution pattern with relatively large amounts of $C_{16:0}$ FA and $C_{24:0}$ FA. The even numbered fatty acids predominate. They are all saturated except $C_{18:1}$ FA which occurs in a very small amount.

Straight-chain saturated alcohols are present, the C_{20} compound being the most abundant. Their source is unknown. They may be derived from glycosidically linked alkyl chains.

Cyanobacterial mat. The H^+-labile lipid moieties present in the cyanobacterial mat sample (Fig. 8) are also dominated by hydroxy fatty acids, although $C_{16:0}$ FA is the major compound. Here, both α- and β-hydroxy fatty acids coelute in different proportions at different chain lengths; only in n-C_{14} OH-FA and n-C_{20} OH-FA the hydroxy groups are exclusively at the β-position. In n-C_{16} and n-C_{18} OH-FA hydroxy groups occur mainly at the β-position and in n-C_{22} and n-C_{24} OH-FA mainly at the α-position. The amount of n-C_{24} OH-FA is very low. n-C_{14}, n-C_{16} and n-C_{18} OH-FA account for almost the total amount of hydroxy fatty acids. The branched hydroxy fatty acids are present in relatively low amounts, with i-C_{17} OH-FA being the most abundant one.

The fatty acids in this extract mainly have a straight chain. The amount of $C_{16:0}$ FA is very high. Branched fatty acids are only present in low amounts. All fatty acids are saturated with the exception of two $C_{18:1}$ FA isomers and of $C_{16:1}$ FA, which is present in trace amount. At longer chain lengths only straight-chain fatty acids were observed up to C_{30}.

Alcohols were not detected. The component eluting at high temperature (peak 99) could not be identified.

Gypsum crust. Again, the hydroxy fatty acids are the main components in the H^+-labile extract of this sample (Fig. 8). Both α- and β-hydroxy fatty acids are present. The distribution pattern is dominated by straight-chain hydroxy fatty acids, but a relatively high amount of i-C_{17} OH-FA is also present. The amount of branched C_{17} OH-FA's is much higher than that of the other branched hydroxy fatty acids. c_{13} OH-FA's are almost absent. In the higher region ($>C_{18}$) only a small amount of C_{20} β-OH-FA was detected.

The fatty acid pattern shows a large amount of

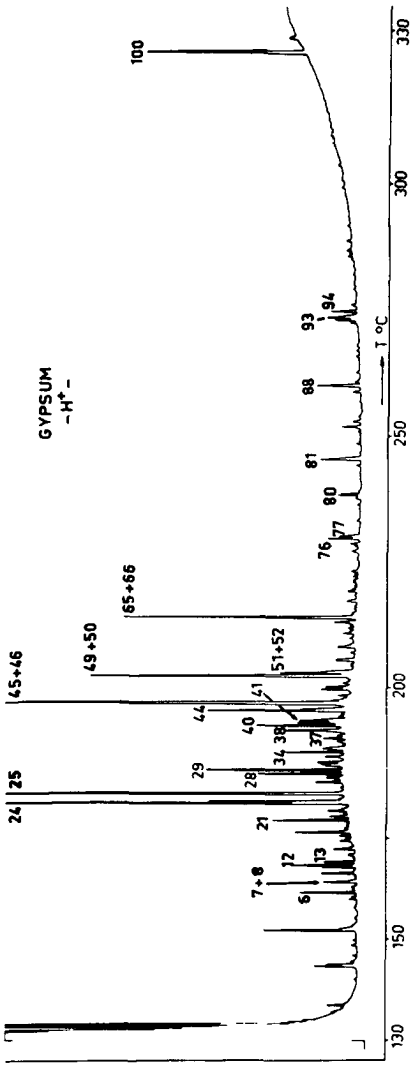
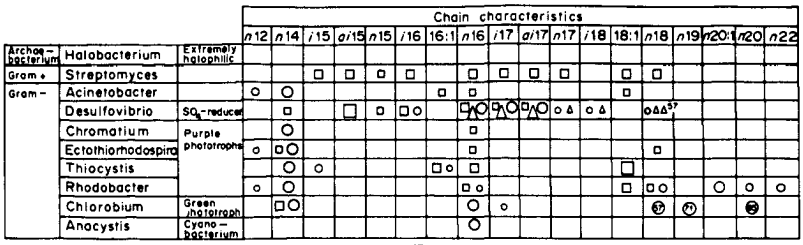


Fig. 8. Gas chromatogram of the H^+-extract of the evaporative gypsum crust.

Table 2. Summary of fatty acids and hydroxy fatty acids present in the bacteria investigated



Δ = α-OH-FA, ○ = β-OH-FA, □ = FA: major (Δ○□), average (Δ○□) and minor (Δ○□) amounts of compounds in the same extract

$C_{16:0}$ FA. The other fatty acids present have a straight chain or are *i*- (C_{15}, C_{16} and C_{17}) or *ai*- (C_{15} and C_{17}) branched. The straight chain fatty acids show a bimodal distribution but the overall concentration of the fatty acids $<C_{20}$ is much higher than that of the longer ones. In this sample unsaturated fatty acids ($C_{16:1}$-, $C_{18:2}$- and $C_{18:1}$ FA) are also present in substantial amounts. No alcohols were detected.

The relatively abundant compound eluting at high temperature was tentatively identified as DPE. The high amount of this component is consistent with the abundance of extreme-halophilic archaebacteria in the overlying water.

Comparison of results. In all three sediments hydroxy fatty acids are the major H^+-labile lipid moieties. As mentioned above, amide-linked β-hydroxy fatty acids only occur in lipids of gram-negative and some gram-positive bacteria. Presumably, a large proportion of the bacteria present in sedimentary environments are gram-negative (Moriarty and Hayward, 1982). Therefore, we may consider the distribution patterns of amide-linked β-hydroxy fatty acids as fingerprints of former and present bacterial populations. Extreme-halophilic and methanogenic archaebacteria are represented by diphytanylether (DPE) in the H^+-extracts.

There are only a few reports in the literature on sediment analyses applying an acid treatment to liberate bound lipid moieties. In those cases where an acid treatment was done (Cranwell, 1981; Klok *et al.*, 1984) an increased amount of β-hydroxy fatty acids was observed. Mainly branched and straight-chain C_{12} to C_{18} hydroxy fatty acids were found. Although the presence of ester-bound hydroxy fatty acids cannot be precluded in these studies, the results do agree well with ours. Another method which liberates high amounts of "tightly bound" hydroxy fatty acids is heat treatment (Kawamura and Ishiwatari, 1981, 1982, 1984). This method was applied to residues of sediments which were extracted after saponification. These residues are thus comparable to our residues II, the starting material for the acid treatment (Goossens *et al.*, 1986a). The abundance of tightly bound β-hydroxy fatty acids (Kawamura and Ishiwatari, 1981, 1982, 1984) suggests that an acid treatment would have yielded similar results and that the "tight bond" is an amide linkage.

The presence of hydroxy fatty acids in sediments seems to be a general phenomenon. Since the major part of these compounds is present in an H^+-labile mode, their amounts are generally underestimated in organic geochemical studies (Cardoso and Eglinton, 1983; Matsumoto and Nagashima, 1984) or their presence is even overlooked.

The three environments investigated represent on the one hand a common freshwater situation where primary production is mainly accounted for by eukaryotic organisms (algae and plants) and on the other two stages of hypersalinity where prokaryotes are dominant. In the cyanobacterial mat, diatoms (eukaryotes) contribute to some extent to the organic matter. This was recognized by microscopic observations of the sample and by the presence of a complex sterol pattern in the ether extract (Goossens *et al.*, unpublished results). In the gypsum precipitation stage of salt works, *Dunaliella* (a green alga) is the only eukaryote which is able to live in the water (Cornée, 1983), but we did not observe this species.

The hydroxy fatty acid patterns are strongly dominated by the even-numbered straight-chain C_{12}-, C_{14}-, C_{16}- and C_{18}-hydroxy fatty acids. The source of the β-hydroxy fatty acids probably is the LPS molecule (Lüderitz *et al.*, 1982). The value of these compounds as markers for specific groups of bacteria is rather low, since they are always present in high amounts in bacteria as well as in sediments. Yet, there are considerable variations in their distribution patterns: the amount of *n*-C_{12} OH-FA is very low in the gypsum, and the cyanobacterial mat contains a high amount of *n*-C_{18} OH-FA.

Significant differences can be observed in the relative amounts of the branched hydroxy fatty acids: whereas *i*- and *ai*-C_{15} OH-FA are very high in the Lake Vechten sediment, they are almost absent in the gypsum. C_{17} OH-FA's are present in considerable amounts in all samples, but the relative amounts of *i*- and *ai*-C_{17} OH-FA differ considerably. This is also the case for *i*- and *ai*-C_{15} OH-FA. Mass fragmentograms, using the diagnostic ions *m/z* 175 and 129 to detect β- and α-hydroxy fatty acids, respectively,

revealed the presence of other acids, not *i*-, *ai*- nor *n*-hydroxy fatty acids, as well as mono-unsaturated β-hydroxy fatty acids in the extracts. However, their amounts are so low that these compounds are not obvious in the gas chromatograms except peak 37 in the Lake Vechten and gypsum analyses. The *i*- and *ai*-branched compounds and especially the uncommonly branched hydroxy fatty acids may be more valuable as markers of distinct bacterial groups than the straight-chain compounds, since our bacterial analyses indicate that they are less common.

α-Hydroxy fatty acids are absent from the Lake Vechten sample but present in high amounts in the salt works samples. *Desulfovibrio gigas* was the only bacterium investigated which contained H^+-labile α-hydroxy fatty acids. However, amide linked α-hydroxy fatty acids also occur in eukaryotic sphingolipids (Downing, 1961; Weete, 1974; Gurr and James, 1980). The contribution of eukaryotes was relatively low in the Salina samples and therefore it is tempting to ascribe the α-hydroxy fatty acids in the hypersaline samples to sulfate reducers since a strong smell of H_2S clearly indicated the presence of an active sulfate reduction process. However, such an assignment can only be tentative and has to be proven by other means.

The specificity of the hydroxy fatty acid patterns has yet to be established, since we do not know the variability of these patterns in samples from similar environments. However, the diversity observed in the analyses of bacteria and the pronounced variations in the sediment distributions hold promises that specific environmental conditions can be recognized from the H^+-labile β-hydroxy fatty acid patterns.

Few systematic studies have been carried out on the diagenetic fate of β-hydroxy fatty acids. Saddler and Wardlaw (1980) found a rapid degradation of LPS in laboratory-incubated estuarine sediments under oxic and anoxic conditions. Amide-linked hydroxy fatty acids disappeared even more rapidly than ester-bound ones. However, the analysis of a 200,000 year old Mediterranean sapropel layer (ten Haven *et al.*, 1986) revealed the abundance of H^+-labile β-hydroxy fatty acids with a distribution pattern very similar to those in the analyses shown above. One possible explanation of this abundance is the presence of living bacteria in this layer, in which case the β-hydroxy fatty acid pattern could be indicative of the diagenetic conditions. It is also possible, however, that, in nature, the biodegradation of substances containing H^+-labile β-hydroxy fatty acids is slow or incomplete. A second indication of resistance against biodegradation is derived from the enhanced preservation of "tightly bound" compared to "bound" and "unbound" β-hydroxy fatty acids as can be concluded from the study of a 200 m core of the sediment of Lake Biwa in Japan by Kawamura and Ishiwatari (1984). β-Hydroxy fatty acids in this amide-linked mode of occurrence seem to be relatively resistant to degradation. Therefore, they are promising as bacterial markers. Analyses of older sediments are under way.

This study indicates that amide bound β-hydroxy fatty acids can serve well as specific indicators for the presence of bacteria in sediments. Since H^+-labile β-hydroxy fatty acids generally occur in a large proportion of the bacteria in sediments, their distribution patterns can be considered fingerprints of past and present prokaryotic populations. Although the specificity of the structures and the lipid compositions in the H^+-extracts of the sequential analyses of bacteria and sediments remains to be established, the diversity observed suggests that a further differentiation of sources of bacterial organic matter will be possible.

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CHAPTER 6

Tocopherols as likely precursors of pristane in ancient sediments and crude oils

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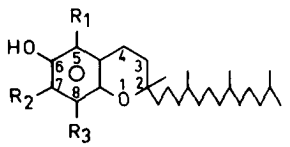
Pristane and phytane, C_{19} and C_{20} isoprenoid alkanes respectively, are major components of many ancient sediments and crude oil. The phytyl side chain of the chlorophyll a molecule was believed to have been the natural source of these hydrocarbons, but the mechanism by which phytane and pristane are generated from the phytyl moiety of chlorophyll, under geological conditions, remained unclear. Here we suggest that tocopherols, also known as E vitamins, are likely sources of pristane found in ancient sediments and crude oils, as both flash pyrolysis and thermal degradation of α-tocopherol yield prist-1-ene as a major product. The organic moieties in the kerogens from which prist-1-ene is generated were previously shown to be similar to those yielding pristane under natural conditions[1]. As a consequence, pristane and phytane might very well originate from different precursors.

Fifty years ago, Alfred Treibs[2] demonstrated the presence of porphyrins in ancient sediments and crude oils. These compounds indicated the biological origin of petroleums: their common tetrapyrrolic skeleton suggested that they were derived from chlorophylls biosynthesized in photosynthetic organisms. Thus, the abundance of phytane and pristane found in extracts of ancient sediments and crude oils in the early days of gas chromatography[3-4], was quite reasonably thought to have come from the major chlorophylls a and b, which have a phytyl side chain with an isoprenoid carbon skeleton similar to that of phytane and pristane.

The abundance of these isoprenoid hydrocarbons in geological materials has stimulated organic geochemists to investigate possible diagenetic pathways by which these compounds can be formed from phytol. Simulation experiments with phytol[5-6], and also *in situ* incubation experiments with ^{14}C-phytol[6-7], have established the conversion of phytol to other functionalised isoprenoids in recent sediments. Unsaturated isoprenoid hydrocarbons such as phytene, phytadienes and phytyl dimers and trimers are main products when phytol is heated to 60 °C under different conditions[6]. In thermodegradation experiments with intact sediments at relatively high temperatures (100 - 300 °C)[8-9], phytane, pristane and other isoprenoids are produced: these are all considered to be degradation products of the phytyl side chain of the chlorophyll molecule. It is questionable, however, whether such thermodegradation studies accurately simulate natural diagenetic processes. During the past twenty years other isoprenoid compounds have been found in organisms and may also be considered as direct or ultimate sources of acyclic isoprenoid hydrocarbons in sediment samples - for example, phytylesters in aquatic mosses[10], pristane and pristenes in zooplankton[11] and diphytanylethers in archaebacteria[12-13].

A detailed study of a suite of Paris Basin samples of increasing maturity showed a good correlation between maturity and the concomitant disappearance of the pyrolysis product prist-1-ene and the generation of pristane[1]. This phenomenon indicates that a common precursor, present in the kerogen, generates pristane during maturation, and prist-1-ene on flash pyrolysis. The ability of several isoprenoids to generate prist-1-ene on flash pyrolysis has been assessed to find possible biological precursors for pristane.

Dihydrophytyl-palmitate[18], chlorophyll a[18], diphytanylethers, phytol and dihydrophytol (J.W. de Leeuw *et al.*, unpublished results) were found to yield mainly phytenes or phytadienes. Prist-1-ene is either not present or is only a trace component in the pyrolysates, except for that from palmitylphytanate[18]. Although esterified phytanic acid is present in very recent sediments[19-20], it is either absent or present in trace amounts in sediments beyond the early stages of diagenesis. Thus, the isoprenoid compounds investigated by flash pyrolysis are not considered to be major sources of pristane in ancient sediments and crude oils. In addition, the type of structure which generates prist-1-ene on flash pyrolysis differs from that which yields straight chain hydrocarbons as these occur as a series of alk-1-enes and alkanes, whereas pristane is absent even when prist-1-ene is the most abundant component in the pyrolysates[14].



	α	γ	δ
R_1	$-CH_3$	$-H$	$-H$
R_2	$-CH_3$	$-CH_3$	$-H$
R_3	$-CH_3$	$-CH_3$	$-CH_3$

Fig.1 Structure of tocopherols

Recently, tocopherols (see Fig. 1) have been found in a wide variety of sediment extracts[21-22]. Their common occurrence in sediments suggests that these compounds - either free or bound - may have an important role in the genesis of isoprenoid hydrocarbons. Therefore, α-tocopherol was pyrolysed under conditions routinely used to investigate kerogens by Curie-point pyrolysis-gas chromatography-mass spectrometry[14]. Two major pyrolysis products were encountered: 2,3,5,6-tetramethyl-2,5-cyclohexadiene-1,4-dione (duroquinone) and prist-1-ene (see Fig. 2). The compounds were identified by comparison of their retention times and mass spectra with those of reference compounds.

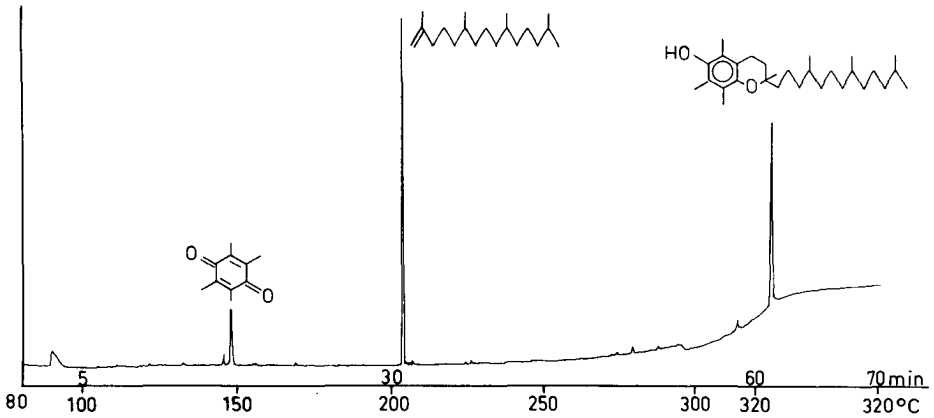


Fig.2 Pyrolysis gas chromatogram of α-tocopherol. Conditions: Curie point pyrolysis at 610 °C; gas chromatographic separation on a fused silica column (length=25 m; ϕi=0.31 mm) coated with CPsil 5 (Chrompack); temperature programme: 80-320 °C at 4 °C min^{-1}, held at 320 °C for 15 min; flame ionization detector (FID) detection).

A thermal degradation experiment with α-tocopherol was also undertaken (N_2; 350 °C; 6 h) and the results were compared with those obtained by Fernholz in 1937[23]. Our results support those of Fernholz to some extent: prist-1-ene and duroquinone were major products here, whereas the other components present such as C_{13}, C_{16} and C_{18}-isoprenoid alkanes, were probably secondary products of prist-1-ene. Figure 3 shows the mechanism proposed for both the flash pyrolysis and thermal degradation. Because tocopherols are highly reactive compounds[24], which can easily react with each other and with other naturally occurring compounds, it is thought that the majority of tocopherols will be incorporated into a complex macromolecular structure, probably via the phenolic hydroxyl group or the 5-methyl group which is very reactive under oxidative conditions. However, their major pyrolysis product should nevertheless be prist-1-ene formed by the intramolecular rearrangement indicated in Fig. 3. The stereochemistry of pristane in immature sediments[25] is also consistent with that of α-tocopherol[26].

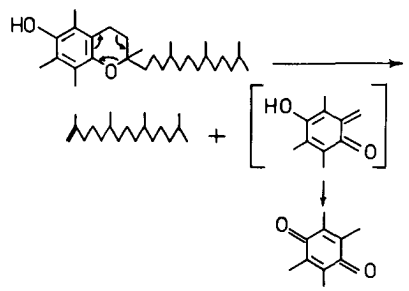


Fig. 3 Proposed degradation mechanism of α-tocopherol.

The flash pyrolysis results obtained for α-tocopherol and the presence of free tocopherols in many sediments[23-24] suggest that tocopheryl moieties can be major sources of pristane in ancient sediments and crude oils. Tocopherols are relatively abundant in most photosynthetic organisms such as higher plants, algae and cyanobacteria[27-31]. Recently, many bacteria have been reported to contain tocopherol derivatives[32]. Amounts of 10 to >1000 mg kg^{-1} dry body weight are not exceptional for the concentration of free tocopherols in organisms. These quantities might be underestimates as almost nothing[29] is known about the occurrence of non-extractable tocopheryl moieties in biological systems.

The relatively great abundance of chlorophylls in photosynthetic organisms (molar ratio tocopherol/chlorophyll = 0.01-0.12[27]) does not imply that other compounds, such as tocopherols, can be ruled out as precursors of pristane in the geosphere: the potential survival of a compound during early diagenesis may be more important. Early in diagenesis, phytol is liberated from the tetrapyrrole macrocycle of chlorophylls and is a labile compound. A very high percentage of the phytol biosynthesized by primary producers is probably metabolized within the food web. In tocopherols, by contrast, the isoprenoid moiety is bonded via a carbon-carbon bond to an aromatic nucleus, favouring survival during early (bio)chemical diagenesis. Moreover, tocopherols may escape biodegradation once incorporated into macromolecular structures.

In conclusion, although tocopherols can now be considered as likely precursors for pristane, in view of their pyrolytic behaviour, their relatively high concentrations in organisms and their expected survival by incorporation into kerogen, the generation of phytane from this source is less likely because α-cleavage next to the aromatic moiety is highly unfavourable. As a consequence, pristane and phytane may very well originate wholly or in part from different precursors.

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CHAPTER 7

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The Pristane Formation Index, a new molecular maturity parameter. A simple method to assess maturity by pyrolysis/evaporation-gas chromatography of unextracted samples

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Abstract—A set of eleven samples from the Handil borehole, Mahakam delta, Indonesia, has been analyzed to test the hypothesis that the Pristane Formation Index (PFI = [pristane]/{[pristane] + [pristenes]}) can be used as a molecular maturation parameter. By pyrolysis/evaporation-gas chromatography (py/ev-GC) of unextracted samples the amounts of the product (pristane) and of the precursor(s) (measured as pristenes) of the pristane formation reaction(s) were measured simultaneously. PFI shows very high correlations with maturity parameters such as vitrinite reflectance, Carbon Preference Index (CPI), *T*-max and with burial depth. Calculation of the pseudo-kinetic parameters of the Arrhenius equation $k = A \exp(-E/RT)$, using published values for effective time of burial (t_{eff}) and temperature gradient, yields values of 59 kJ mol^{-1} and $2.2 * 10^{-7}$ s^{-1} for E and A, respectively. The value of E is not very sensitive to variations in the assumed values of t_{eff} and temperature gradient; the value of A is extremely low in all cases. The values of the pseudo-kinetic parameters are much lower than those of the isomerization and aromatization reactions and thus offer interesting possibilities for the reconstruction of thermal histories of basins.

INTRODUCTION

MANY ORGANIC GEOCHEMICAL studies deal with the assessment of the level of maturity of specific samples. The maturity reflects the stage of transformation of the organic matter as a result of the temperature history and is, therefore, a key parameter in organic geochemistry. Several parameters are used to estimate the maturity. For example, the reflectance of the organic maceral vitrinite is related to temperature history and is widely used as a maturity parameter. However, the accuracy of the measurement is dependent on the amount of vitrinite present and therefore, reliable values are only obtained for kerogens of type III. Moreover, the relationship of reflectance and temperature history is an empirical one and cannot be defined more specifically, since the molecular basis of the changes in reflectance with temperature and time is not known.

Other maturity parameters such as the *T*-max value which is derived from Rock-Eval analysis and the Carbon Preference Index (CPI), for example, show the same lack of specificity (TISSOT and WELTE, 1984).

A much better definition of the relationship between the temperature history of a sample and the value of a maturity parameter is obtained when the parameter reflects the extent to which a specific chemical reaction has proceeded. This approach has been followed by MACKENZIE and MCKENZIE (1983) and MACKENZIE *et al.* (1980, 1981, 1984), who studied the 20R-20S isomerization of steranes, the aromatization of monoaromatic steroid hydrocarbons and the 22R-22S isomerization of 17α(H)-homohopane.

The relationship of temperature (T) and reaction rate (k) for a specific monomolecular chemical reaction following first order kinetics, is given by the Arrhenius equation $k = A \exp(-E/RT)$, where E and A are the activation energy and the frequency factor, respectively, and R is the gas constant. By integration of this rate over time, the amount of product formed within that time span can be calculated. In reverse, if the kinetic parameters E and A of the Arrhenius equation of a specific reaction are known, the reaction extent can be used to reconstruct the temperature history (MACKENZIE and MCKENZIE, 1983; MACKENZIE *et al.*, 1984). The well-defined relationship between chemical reaction extent and temperature and time allows a rather detailed reconstruction of the thermal history of a basin, as has been shown in several studies (MACKENZIE and MCKENZIE, 1983; MACKENZIE *et al.*, 1984; RULLKÖTTER *et al.*, 1986; HONG *et al.*, 1986).

The molecular approach has proved successful and it would be worthwhile to find additional, suitable reactions, preferably with different activation energies and frequency factors. An indication of such a reaction appears from the work by VAN GRAAS *et al.* (1981), who analyzed the identical set of samples from the Paris Basin which has been used for the molecular parameter work by MACKENZIE *et al.* (1980, 1981). VAN GRAAS *et al.* (1981) found that the increase of the amount of pristane with maximal burial depth was accompanied by a decrease of the pyrolysis product prist-1-ene. It was tentatively concluded that under natural conditions pristane is formed from the same precursor(s) which generate prist-1-ene upon analytical pyrolysis. If, indeed, the pyrolysis product prist-1-ene and the diagenetic product pristane reflect the precursor(s) and the product of the pristane formation reaction, then the Pristane Formation Index (PFI = [pristane]/{[pristane] + [pristenes]}) could be used as a new molecular maturity parameter.

To test the hypothesis we analyzed a suite of samples from the Handil borehole in the Mahakam delta, Indonesia. This set of samples was chosen because the temperature history of the basin is relatively simple in comparison with that of the Paris Basin (MACKENZIE and MCKENZIE, 1983) and because the nature of the organic matter is rather uniform (MONTHIOUX *et al.*, 1985; MONTHIOUX, 1986). In addition,

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information about other maturity parameters of these samples was available (MONTHIOUX, 1986; M. MONTHIOUX, unpublished).

In a specific sample the amount of pristane and the amount of the pyrolysis product prist-1-ene (and prist-2-ene, see below) can be measured simultaneously by pyrolysis/evaporation-gas chromatography (py/ev-GC) of the unextracted sample. During the flash heating the pristane present is evaporated and prist-1-ene (and prist-2-ene, see below) is produced by pyrolysis of the precursor(s). The compounds are quantitated during the subsequent gas chromatographic analysis. This simple analytical procedure and the fact that prist-1-ene is a major pyrolysis product of almost all immature sediments (LARTER *et al.*, 1979; VAN DE MEENT *et al.*, 1980) would render PFI a suitable maturity parameter.

EXPERIMENTAL

Pyrolysis/evaporation-gas chromatography (py/ev-GC) was carried out using the equipment described by VAN DE MEENT *et al.* (1980). The samples were pyrolyzed during 10 s using wires with a Curie-temperature of 610°C. Chromatographic conditions were as follows: column: CPSil 5, chemically bonded; temperature program: 0°C (5 min)–300°C (30 min) with a gradient of 3°C/min. The compounds of interest were identified by relative retention time and by py/ev-GC-MS analyses of several selected samples. Py/ev-GC analyses were carried out in duplicate. Peak areas of the selected components were used for quantification using a Maxima datasystem. The average values of the duplicate analyses were used for further calculations. The error in these values was estimated at *ca.* 5%.

SAMPLE DESCRIPTION

The samples investigated are borehole cuttings from the Handil 627 bore hole located in the Mahakam Delta, Indonesia. These samples, covering a depth range from 1300 to 3100 meter, and other samples from this borehole have been investigated by MONTHIOUX (1986). The depths, CPI-values, vitrinite reflectance data and *T*-max values of the eleven samples are given in Table 1.

RESULTS AND DISCUSSION

The samples were analyzed by Curie-point-pyrolysis/evaporation-gas chromatography to measure simultaneously the relative amounts of both the free occurring compound pristane and the exclusive pyrolysis products prist-1-ene and (if present) prist-2-ene (CRISP *et al.*, 1986).

Table 1. Sample description (data from MONTHIOUX, 1986; M. MONTHIOUX, unpublished) and PFI.

Sample	depth (m)	CPI	Vitr.	T-max (°C)	PFI
1	1290	-	0.41	344	0.09
2	1390	2.57	0.38	347	0.13
3	1730	1.94	0.45	359	0.22
4	1920	2.14	0.43	365	0.26
5	2020	1.95	0.47	369	0.36
6	2480	1.68	0.52	384	0.58
7	2600	1.44	0.58	389	0.63
8	2750	1.43	0.63	394	0.75
9	2835	1.38	0.60	396	0.78
10	2950	1.26	0.59	400	0.84
11	3100	1.14	0.65	405	0.95

REGTOP *et al.* (1986) recently showed that prist-2-ene is produced from prist-1-ene under the catalytic influence of clay. Therefore, both compounds are considered pyrolysis products from the same precursor and the sum of prist-1-ene and prist-2-ene was used as a measure of its amount. Other pristene isomers were not present.

Table 1 gives the values of the Pristane Formation Index (PFI), which is the amount of product formed, expressed as fraction of the sum of precursor and product, *i.e.* PFI = [pristane]/{[pristane] + [prist-1-ene] + [prist-2-ene]}.

In Fig. 1 the regression plot of PFI as a function of vitrinite reflectance is shown. PFI and vitrinite reflectance show an excellent correlation. Given the experimental error of both parameters (PFI *ca.* 5%, vitrinite reflectance much lower) the correlation is almost as high as can be obtained. Other maturity parameters are also highly correlated with PFI as is indicated by the high values of the correlation coefficients for linear regression (Table 2). Judging from these correlations PFI seems to be an excellent indicator of maturity.

The values of PFI range from 0.09 to 0.95, which could mean that at least 85% of the amount of pristane present in the deepest sample originated from only one (type of) precursor. Extrapolation of the PFI *vs.* vitrinite reflectance plot (Fig. 1) appears to pass through PFI = 0, implying also that essentially all the pristane is derived from one type precursor, and that there is no free pristane present in the system before maturation starts.

This and the high correlations of PFI with other maturity parameters suggest that pristenes and pristane represent the precursor and the product of one reaction.

Under natural conditions, of course, the pristenes, generated by thermal degradation of the precursor, require some hydrogen donor for the reduction to pristane. However, since there is no accumulation of pristenes in natural samples, the hydrogenation of pristene(s) is a much faster process than the thermal degradation of the precursor. The latter process limits the rate of pristane formation and the reduction of pristenes is therefore not of interest, in our considerations.

If we accept, for the time being, that PFI is a fair estimate of the reaction extent of the pristane formation reaction, some reasonable assumptions are sufficient to calculate the values of the kinetic parameters E and A of the Arrhenius equation $k = A \exp(-E/RT)$.

These assumptions relate to the temperature history of the samples. Analogous to MACKENZIE and MCKENZIE (1983), the temperature history was approximated by an effective time of burial, t_{eff} (defined as the time the sample spent within 15°C from the maximum temperature) and the maximum burial temperature. This maximum temperature can be calculated from the maximum burial depth using estimates of the surface temperature and the temperature gradient in the basin.

As a first approximation, we took the values used by MACKENZIE and MCKENZIE (1983) for the Handil borehole, *i.e.* 16 Ma for t_{eff}, a surface temperature of 27°C and a temperature gradient of 34°C/km to calculate the burial temperatures from the sample depths.

Figure 2 shows the Arrhenius plot of the reaction. Assum-

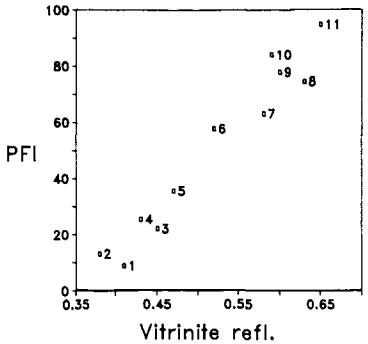

FIG. 1. Regression plot of Pristane Formation Index (PFI) (expressed as %) on % vitrinite reflectance. Average experimental error of PFI is estimated at 5%.

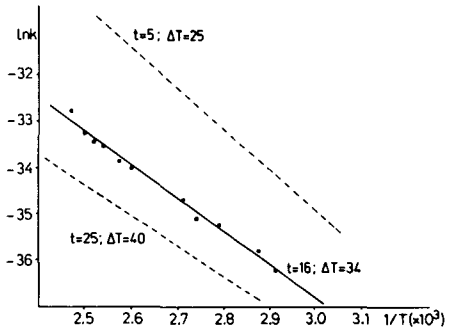

FIG. 2. Arrhenius plot of ln k *versus* T^{-1}; dots indicate values calculated from observed PFI values with the formula $k = -1/t_{eff} \ln (1 - PFI)$; t_{eff} is 16 Ma; the burial temperatures are calculated with a temperature gradient (ΔT) of 34°C km^{-1} and a surface temperature of 27°C. Broken lines show the Arrhenius plots if extreme values of t_{eff} and temperature gradient are assumed (see Table 3).

ing that the reaction was monomolecular and followed first order kinetics the values of k were calculated using the expression $k = -1/t_{eff} \ln (1 - PFI)$, analogous to MACKENZIE and MCKENZIE (1983). From this plot the values of the kinetic parameters of the Arrhenius equation were derived: $E = 59$ kJ mol^{-1} and $A = 2.2 \times 10^{-7}$ s^{-1}.

The correlation coefficient of this linear regression is extremely high: $r = 0.995$, which justifies the original assumption that PFI is a good estimate of the reaction extent of the pristane formation reaction.

To investigate how sensitive these values are to assumptions of t_{eff} and temperature gradient, both the values of burial time and temperature gradient were varied. 5 Ma and 25 Ma were taken as extremes for t_{eff} and 25°C/km and 40°C/km as extreme values for the temperature gradient. Table 3 shows that the values of E are between 74 and 55 kJ · mol^{-1} and thus are not very sensitive to variations in the two variables.

Although frequency factor A changes 4 orders of magnitude (from $2.5*10^{-4}$ to $1.7*10^{-8}$), its value remains extremely low.

The values of the kinetic parameters of the pristane formation reaction are extremely low compared to those of the reactions studied by MACKENZIE and MCKENZIE (1983).

With the activation energy that low, even room temperature would be sufficient to drive the reaction if the frequency factor were not so extremely low.

A low value for the activation energy is in agreement with observations that the pristane formation reaction has relatively low thermal requirements: during laboratory pyrolysis of oil shales prist-1-ene is formed at lower temperatures than most other pyrolysis products (CRISP *et al.*, 1986) and it is also known that under natural conditions pristane is found "early in the oil window" (TISSOT and WELTE, 1984).

The value found for the frequency factor is not easily explained. For a simple bond cleavage reaction the frequency factor is typically in the order of 10^{14} s^{-1}. The observed value of A indicates that a very large decrease in entropy is required to reach the transition state of the thermal degradation reaction. The change of entropy during activation ($\Delta S\ddagger$) can be calculated from A with the formula: $A = (k*T)/h \exp(\Delta S\ddagger/R)$ where k is Boltzmann's, h is Planck's and R is the gas constant. Substitution of $2.2*10^{-7}$ for A yields a value for $\Delta S\ddagger$ of about -370 J mol^{-1} K^{-1}, which is very low and indicates that the transition state is strongly constrained.

An explanation might be in the nature of the reaction. We tried to estimate the order of magnitude of A for a retro-Diels-Alder reaction since we proposed a retro-Diels-Alder mechanism for the formation of pristene from tocopherols (GOOSSENS *et al.*, 1984). In addition, a relatively low value of A is expected, since the reaction mechanism is concerted, requiring the simultaneous cleavage of two bonds.

The entropy of activation for retro-Diels-Alder reactions in the gasphase is between about 30 and -20 J mol^{-1} K^{-1}

Table 2. Correlation coefficients of linear regression of PFI on a number of maturity parameters (values from MONTHIOUX, 1986; M. MONTHIOUX, unpublished).

PFI	x				
CPI	0.9193	x			
T-max	0.9146	0.8694	x		
Depth	0.9183	0.9456	0.8752	x	
Vitr.	0.9470	0.9356	0.8709	0.9281	x
	PFI	CPI	T-max	Depth	Vitr.

Table 3. Values of the kinetic parameters E and A under different assumptions of t_{eff} and temperature gradient. The surface temperature was 27°C in all cases.

Gradient (°C/km)	t_{eff} (Ma) 5	10	16	20	25
25	E=74	74	74	74	74
	2.5×10^{-4}	1.2×10^{-4}	7.5×10^{-5}	6.2×10^{-5}	4.9×10^{-5}
30	65	64	64	64	64
	5.7×10^{-6}	2.7×10^{-6}	1.7×10^{-6}	1.4×10^{-6}	1.1×10^{-6}
34	59	59	59	60	60
	6.9×10^{-7}	3.5×10^{-7}	2.2×10^{-7}	2.0×10^{-7}	1.0×10^{-7}
40	55	55	55	55	55
	8.6×10^{-8}	4.3×10^{-8}	2.7×10^{-8}	2.2×10^{-8}	1.7×10^{-8}

E in kJ mol^{-1}
A in s^{-1}

(SAUER and SUSTMANN, 1980), corresponding to values for A of about $5 * 10^{11}$ to $2 * 10^{14}$, respectively. Since the reaction, in our case, takes place in the solid phase a correction is necessary. The order of magnitude of this correction can be estimated by comparing the values of $\Delta S\ddagger$ of retro-Diels-Alder and Diels-Alder reactions. If we assume that the transition states of both reactions are similar and that the difference between the $\Delta S\ddagger$ values only results from the opposite direction of the two reactions (the retro-Diels-Alder reaction produces two compounds from one and the Diels-Alder-reaction makes one compound from two, by similar reaction mechanisms), then the difference (about 140 J mol^{-1} K^{-1}) can be used to estimate the contribution to the change in entropy by the differences of the rotational and translational degrees of freedom. In the solid phase these differences will be small and the value of $\Delta S\ddagger$ of the retro-Diels-Alder reaction should thus be corrected. Even if the total difference (140 J mol^{-1} K^{-1}) is adopted to correct the entropy of activation, the value of A would drop by only about 7 orders of magnitude. Therefore, it is clear that the observed low value of A cannot be explained by this reaction mechanism.

This calculation also shows that bonding of the precursor into the kerogen, which might restrict its ability to achieve the required transition state orientation for reaction to occur, cannot explain the low value of A.

Strong catalysis could be a possible explanation.

In fact, it is not very clear what is represented by the kinetic parameters calculated. Their values are thought to be determined by the molecular structure of the precursor and the mechanism of the reaction. However, the calculated values may include contributions from other processes. It is not sure, for example, that the assumptions for the calculation (first order kinetics, monomolecularity) are correct. The fact that the Arrhenius plot is linear does not prove that the reaction is monomolecular. If the reaction were bimolecular the Arrhenius plot could also be linear. Other reactions contributing to the measured amounts of pristene and/or pristane, would produce, theoretically, a non-linear Arrhenius plot but the amount of the second precursor or product or the values of the kinetic parameters of the second reaction and the burial time and temperature would determine whether the deviations from linearity would be large enough to be noticed. Moreover, the low values of the kinetic parameters could also result from other processes than chemical reactions. If catalysis is important, diffusion through the solid phase of catalyst and/or precursor to the reaction site might limit the rate of the reaction and thus determine the kinetic parameter values.

Therefore, E and A as calculated in this study are not kinetic parameters in a true chemical sense as calculated from laboratory studies. Consequently, the kinetic parameters should be considered pseudo-kinetic parameters. A better understanding of what is represented by these pseudo-kinetic parameters would help understand the important conditions for maturation of organic matter.

It should be stressed, however, that this uncertainty does not affect the value of PFI as a kinetic maturity parameter. The results presented indicate that the correlations with conventional maturity parameters are very high. In addition, measurements of PFI in samples from the Paris Basin (type II kerogen) yield values of the pseudo-kinetic parameters similar to those obtained in this study (GOOSSENS *et al.*, 1988).

The same holds for the isomerization reactions studied by MACKENZIE *et al.* (1980, 1981) which have frequency factors of 0.006 s^{-1} and 0.016 s^{-1}. These parameters have proved valuable in basins with different types of kerogen, and no influence of sample composition was found (MACKENZIE and MCKENZIE, 1983). Anomalies have been observed, though, in samples from hypersaline environments (TEN HAVEN *et al.*, 1985, 1986a,b).

Laboratory thermal degradation studies (of tocopherols, for example) are needed to find out whether catalysis could explain the observed low values of the frequency factor and the activation energy.

Although the observed low values of the pseudo-kinetic parameters make it impossible to use A and E to unravel the chemical nature of the precursor, strong catalysis of the reaction would not preclude a retro-Diels-Alder mechanism for pristane formation since such a reaction can be strongly catalyzed by a Lewis acid (MAGNUS *et al.*, 1986).

The pseudo-kinetic parameter values obtained are much lower than those for the isomerization of steranes and hopanes and the aromatization of steranes (MACKENZIE and MCKENZIE, 1983). This offers interesting possibilities for the reconstruction of thermal histories. In Fig. 3 the Arrhenius plots of the pristane formation reaction (PF) and of the isomerization (SI for sterane and HI for hopane isomerization) and aromatization (SA) reactions are shown, using the values of MACKENZIE and MCKENZIE (1983) for E and A. The PF line crosses the lines of the other reactions in a temperature range (65–100°C) where the reactions have not gone to completion on a geological time scale. Crossing of the lines in Fig. 3 means that, at that temperature, the rates of the reactions reverse in order. The values of reaction extents measured in the same sample reveal the thermal history because the extents of the reactions are determined by the same thermal history and by the specific values of the pseudo-kinetic parameters. Cross plots of the reaction extents measured in a set of sam-

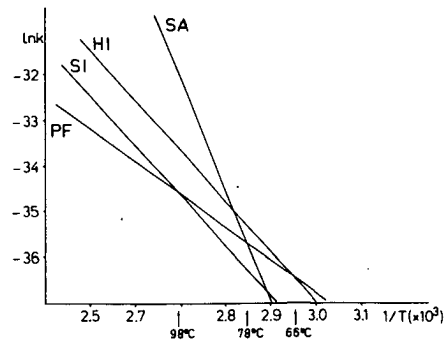

FIG. 3. Arrhenius plots of the molecular maturation reactions. PF = pristane formation (E = 59 kJ mol^{-1}, $A = 2.2 * 10^{-7}$ s^{-1}; SI = sterane isomerization (E = 91 kJ mol^{-1}, A = 0.006 s^{-1}); HI = hopane isomerization (E = 91 kJ mol^{-1}, A = 0.016 s^{-1}); SA = steroid aromatization (E = 200 kJ mol^{-1}, $A = 1.8 * 10^{14}$ s^{-1}); (Values from MACKENZIE and MCKENZIE, 1983).

In the present study we assessed the PFI values for a suite of samples from the Paris Basin for which the values of other molecular maturity parameters have been established by Mackenzie *et al.* (1980, 1981). The identical set of samples has been studied also by Van Graas *et al.* (1981). The samples represent one stratigraphic zone and are of similar age. They comprise the same type of organic matter, but experienced different thermal regimes in the past.

The study was performed for various reasons: First, to compare the pseudo-kinetic parameters determined in the Mahakam Delta with those calculated for a different basin. Since the value of the frequency factor of the pristane formation reaction is extremely low, a strong catalytic influence was suggested (Goossens *et al.*, 1988). Comparison of the values of the pseudo-kinetic parameters obtained for type II kerogen (Paris Basin) and for type III kerogen (Mahakam Delta) should reveal whether there is a strong influence of the sample composition.

Second, the rate of the pristane formation reaction relates to thermal history very differently from the rates of isomerization or aromatization reactions. Due to its low activation energy, the reaction is not frozen at low temperatures like the other reactions. On the other hand, the extremely low value of the frequency factor precludes large changes in reaction rate with temperature. The different values of the kinetic parameters for the pristane formation reaction render cross plots of PFI and other maturity parameters informative for the reconstruction of thermal history.

The thermal history of the Paris Basin is more complex than that of the Mahakam Delta. The eastern part of the basin has been uplifted substantially in the past (Mackenzie and McKenzie, 1983). Estimates of the amount of uplift range from 700 m to 2.6 km (Mackenzie and McKenzie, 1983), the latter being based upon the reaction extents of isomerization and aromatization reactions.

This suite of Paris Basin samples, thus, represents a good set to evaluate the value of PFI as a molecular maturity parameter and to assess the extent of uplift in the eastern part of the Basin.

Experimental

The methods used have been described previously (Goossens *et al.*, 1988). In short, unextracted samples were analyzed by Curie-point pyrolysis/evaporation-gas chromatography. The free occurring compound pristane is evaporated during the flash heating and measured simultaneously with the pyrolysis products prist-1-ene and prist-2-ene. The sum of the quantities of prist-1-ene and prist-2-ene represents the amount of precursor, whereas the amount of product of the reaction is represented by the quantity of pristane. The Pristane Formation Index (PFI) is, by definition, PFI = [pristane] / {[pristane] + [prist-1-ene] +[prist-2-ene]}. The results constitute mean values of duplicate measurements with an average difference of < 5% .

Identification of compounds is based upon relative retention times and py/ev-GC-MS analyses of selected samples.

Results and Discussion

Observed and theoretical values of PFI

The PFI-values obtained for samples from the Paris Basin are presented in Table 1, together with present sample depths and the values of the molecular maturity parameters measured by Mackenzie and McKenzie (1983): 20R - 20S isomerization of steranes (SI), 22R - 22S isomerization of 17α(H)-homohopane (HI) and aromatization of monoaromatic steroid hydrocarbons (SA). If PFI is indeed a reliable estimate of the reaction extent of the pristane formation reaction, then an Arrhenius plot can be constructed by calculation of the reaction rate k from PFI values and burial time, and by estimation of the burial temperatures from the sample depths. For the Mahakam Delta an Arrhenius plot was obtained

ples, like the aromatization-isomerization plots by MACKENZIE and MCKENZIE (1983), can be used to reconstruct the thermal history of a basin.

Cross plots of PFI and other molecular maturity parameters extend the possibilities to reconstruct thermal histories. In the first place, three more plots can be made (PFI *versus* SA, SI and HI), yielding three additional estimates of the temperature history. In the second place, the slopes of the Arrhenius plots of PF and SA are so different that similar reaction rates occur only when temperatures are within a relatively narrow range. Observations plotting around the diagonal of the PFI-SA plot thus provide relatively accurate estimates of t_{eff} and burial temperature. This reasoning, of course, does not hold when uplift or subsidence occurred during the past. Thirdly, the pristane formation reaction is less dependent on temperature than the other reactions (Fig. 3) due to the low value of A. Therefore, measurable values of PFI can be expected over a wider range of burial temperatures if the basin is not too old.

Unfortunately, only few data on other molecular maturity parameters are available for samples from the Handil borehole. However, we have established the PFI values of the samples from the Paris Basin, previously investigated by MACKENZIE *et al.* (1980) and by VAN GRAAS *et al.* (1981), for which the values of the isomerization reactions and the aromatization reaction have been established (GOOSSENS *et al.*, 1988).

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CHAPTER 8

The Pristane Formation Index, a molecular maturity parameter. Confirmation in samples from the Paris Basin

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Summary

The value of the recently proposed molecular maturity parameter PFI (Pristane Formation Index) is evaluated by analysis of a suite of samples from the Paris Basin with known values of the molecular maturity parameters based on the sterane and hopane isomerization (SI and HI) and sterane aromatization (SA) reactions. The values of the pseudo-kinetic parameters E and A of the Arrhenius equation k= A exp (-E/RT), calculated for pristane formation in the Mahakam Delta, were used to compare the observed PFI values with calculated ones. The results indicate that the samples from the eastern part of the Paris Basin, which are at the surface at present, have been at higher temperatures for about 10 - 30 Ma, whereas the deeper samples, located around the center of the Basin, have not undergone considerable temperature changes in the past. These results are in agreement with the conclusions based on SI, SA and HI alone. Thus, the values of the pseudo-kinetic parameters E (59 kJ mol^{-1}) and A (2.2 * 10^{-7} s^{-1}) for the pristane formation reaction can be applied in a different basin with a different type of kerogen. This confirms the value of PFI as a molecular maturity parameter.

Introduction

In many recent studies of immature sediments the level of maturity is measured via the reaction extent of some specific chemical reactions (Mackenzie and McKenzie, 1983; Mackenzie *et al.*, 1984; Rullkötter *et al.*, 1986; Hong *et al.*, 1986). The isomerization of steranes and hopanes and the aromatization of steranes have proved to be useful indicators of the thermal history of some basins, although the kinetics are not fully understood (Moldowan and Fago, 1986; Lewan *et al.*, 1986; Tannembaum *et al.*, 1986) and anomalies have been observed in samples from hypersaline environments (Ten Haven 1986; Ten Haven *et al.*, 1985, 1986). Recently, we proposed the extent of the pristane formation reaction (the Pristane Formation Index, PFI) as an independent maturity parameter (Goossens *et al.*, 1988). The values of PFI in samples from the Mahakam Delta showed an excellent correlation with convential maturity parameters as vitrinite reflectance, T-max and Carbon Preference Index (CPI) and with burial depth. Assuming an effective time of burial and a temperature gradient in the Mahakam Delta, the values of the activation energy E and the frequency factor A of the Arrhenius equation (k=A exp(-E/RT)) for the pristane formation reaction were calculated. They amounted to 59 kJ mol^{-1} and 2.2 * 10^{-7} s^{-1} for E and A, respectively (Goossens *et al.*, 1988). These pseudo-kinetic parameter values are very different from those of the other reactions used to measure maturation and, therefore, crossplots of PFI and other molecular maturity parameters should contain valuable information about the thermal history of basins. For the Mahakam Delta samples, however, little information is available about the values of other molecular maturity parameters (Mackenzie and McKenzie, 1983).

Sample	Sample name	present depth (m)	PFI	SI	HI	SA
1	Semecourt	0-30	0.13	0.04	0.03	0.02
2	Jouy	0	0.11	0.15	0.03	0.00
3	G6-2	9	0.07	0.19	0.28	0.10
4	G6-5	22	0.13	0.17	0.28	0.11
5	Colombotte	0-30	0.26	0.26	0.70	0.30
6	Creveney	0	0.21	0.39	0.70	0.17
7	Der	1165	0.43	0.39	0.78	0.72
8	Cesarville	2020	0.72	0.72	0.92	0.74
9	Coupvray	2040	0.84	0.81	1.00	0.71
10	Grandville	1757	0.85	0.85	1.00	0.86
11	Belou	2142	0.75	0.81	1.00	0.76
12	Montmirail	2388	0.97	0.93	1.00	0.90

Table 1. Sample descriptions and the values of the molecular maturity parameters : pristane formation index (PFI), sterane isomerization (SI), hopane isomerization (HI) and sterane isomerization (SA). All values are expressed as % of the value of completed reaction (1, 0.54, 0.61 and 1 for PFI, SI, HI and SA, resp.) (All data, except PFI, are from Mackenzie and McKenzie, 1983).

with an extremely high correlation coefficient (r=0.995) from which the values for E and A were calculated.

For the Paris Basin, the approximation of the temperature history by an effective time of burial (t-eff= the time spent within 15 °C from the maximum burial temperature) and the maximum burial temperature is complicated by substantial uplift in the eastern part of the basin. Neglecting these complications for the moment, the observed PFI values can be used to locate the samples within an Arrhenius plot and, thus, compare their positions with the theoretical relationship derived from the Mahakam Delta study. To produce this plot we used the age of the Paris Basin (180 Ma; Mackenzie and McKenzie, 1983) for t-eff and calculated k with the formula k = -1/t-eff * ln(1-PFI). We assumed further that the

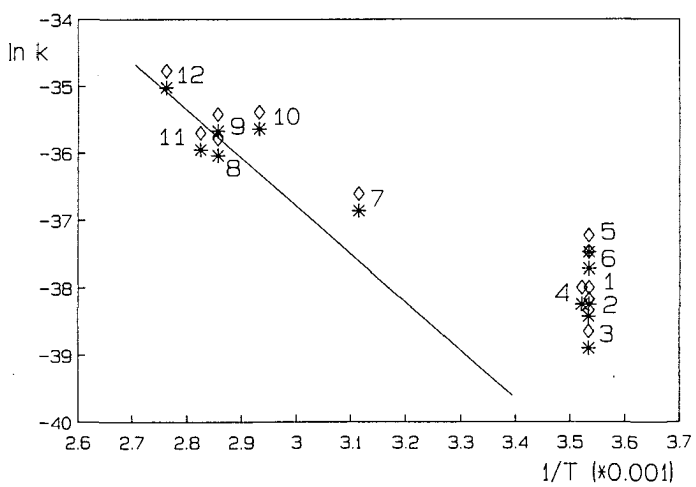

Fig.1. Arrhenius plot of the pristane formation reaction (PF). k = -1/t-eff * ln(1-PFI); t-eff is 180 Ma (*) or 140 Ma (◇); Temperatures are calculated from sample depth (see text). The line is the theoretical relation using values for E and A of 59 kJ mol^{-1} and $2.2*10^{-7}$ s^{-1}, respectively.

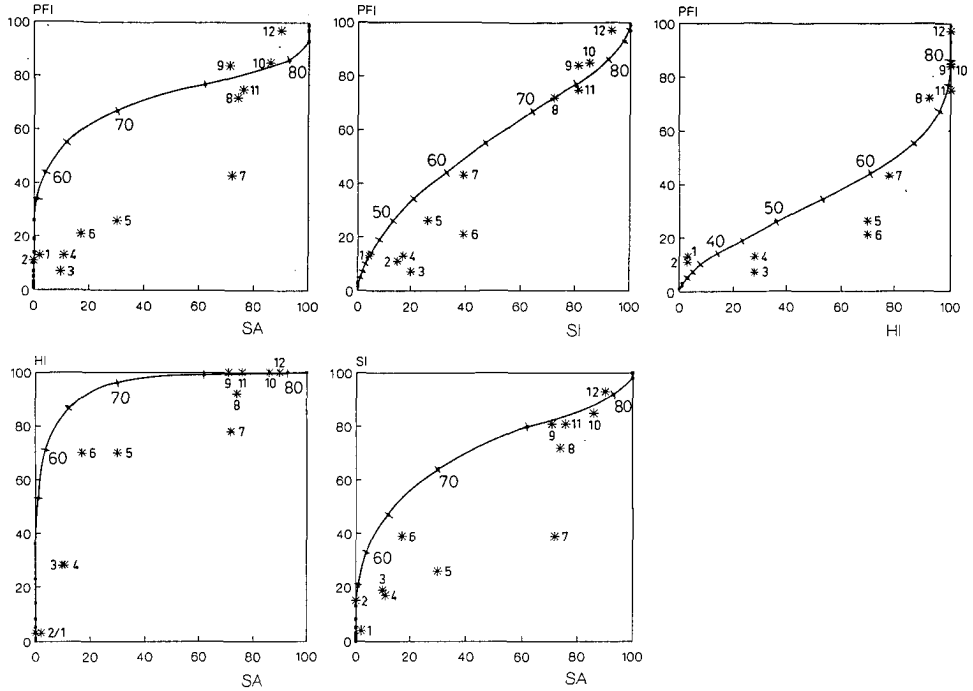

Fig.2. Cross plots of the values of the molecular maturity parameters for the individual samples. The curves show the theoretical positions after 180 Ma at the temperatures indicated (intervals of 5 °C).

samples had been at their present temperature throughout t-eff, which were calculated from the present burial depths, assuming a temperature gradient of 33 °C km^{-1} and a surface temperature of 10 °C (all values derived from Mackenzie and McKenzie, 1983, Table 6).
Fig.1 shows that the values obtained for the deeper samples (8 to 12) from the Paris Basin are in reasonable agreement with the plot of the Arrhenius equation using values for the pseudo-kinetic parameters from the Mahakam Delta study. The values of ln(k) for samples 1 to 7 are all too high, indicating that the pristane formation reaction has proceeded faster than predicted by the assumption of burial for 180 Ma at present temperatures. By taking the age of the basin for t-eff the period of subsidence is neglected. However, the reaction rate is not very sensitive to time due to the low frequency factor. Assuming 140 Ma for t-eff adds only about 0.25 to the ln k values in Fig.1.
As will be shown below, the discrepancies in Fig.1 can be explained in terms of an increased reaction rate during the period these samples were at higher temperatures prior to uplift (Mackenzie and McKenzie, 1983).
The results presented hereafter justify the assumption that PFI is a fair estimate of the extent of the pristane formation reaction and show that the values of the pseudo-kinetic parameters as calculated from the Mahakam Delta observations, are also valid for the Paris Basin. This indicates that the presence of different types of kerogen in the two basins does not affect the rates of the pristane formation reaction.

Cross plots with other molecular maturity parameters
Using the values of the molecular maturity parameters measured by Mackenzie *et al.* (1983), several cross plots can be made. These plots are shown in Fig.2. Since the activation

energies of the sterane and hopane isomerization reactions are identical, the HI-SI cross plot is uninformative and is not shown.
In addition to the positions of the individual samples theoretical curves are given for burial for 180 Ma at the temperatures indicated.
Given the experimental error (estimated at < 5% for PFI; Mackenzie and McKenzia (1983) report maximal experimental errors of 8%, 14% and 15% for SA, HI and SI, respectively), samples 1 and 2, and 8 to 12 plot in good agreement with the theoretical positions. It was observed by Mackenzie and McKenzie (1983) that the sterane aromatization reaction (SA) does not go to 100% completion. As a result, the fit is poorer when values are plotted against SA values. However, in all plots samples 8 to 11 plot in the 70 - 80 °C range and sample 12 plots in the > 80 °C range. Thus, temperature limits read from these plots, are rather narrow.
Sample 3 to 7 do not fit the theoretical curves, indicating that the rate of at least one of the two reactions has been different from that predicted by assumption of constant temperature for 180 Ma.

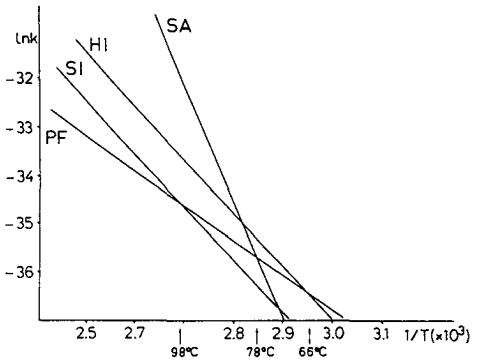

Fig.3. The Arrhenius plots for the different reactions: pristane formation (PF), sterane isomerization (SI), hopane isomerization (HI) and sterane aromatization (SA).

The effect of uplift
In evaluating these cross plots it should be realized that the values of the pseudo-kinetic parameters of the pristane formation reaction ($E=59$ kJ mol^{-1}, $A=2.2*10^{-7}$ s^{-1}) are significantly lower than those of the other reactions (SI: $E=91$ kJ mol^{-1}, $A=0.006$ s^{-1}; HI: $E=91$ kJ mol^{-1}, $A=0.016$ s^{-1}; SA: $E=200$ kJ mol^{-1}, $A=1.8*10^{14}$ s^{-1}). As a consequence of its lower activation energy the pristane formation reaction is not frozen after uplift to shallow depths, in contrast to the other reactions; the temperature needed to cause a 5 % reaction after 180 Ma is 60 °C for SA, 48 °C for SI, 39 °C for HI and only 26 °C for PFI. This trend is apparent from the Arrhenius plots for the different reactions (Fig.3), which show that at relatively low temperatures (< 60 °C), the reaction rate of the pristane formation reaction is considerably higher than that of the other reactions. On the other hand, its low frequency factor causes a much smaller increase of reaction rate with temperature when compared to the other reactions. Therefore, the PFI-value measured in a sample is determined not only by the period the sample was buried, but also by the period after uplift during which the sample was at near surface temperatures. Thus, both periods must be taken into account in the approximation of the thermal history.
The theoretical reaction extents were calculated allowing for changesin temperature. Hence, the thermal history was divided into two periods: one during which the sample was at a relatively high temperature and the second during which it was at surface temperature

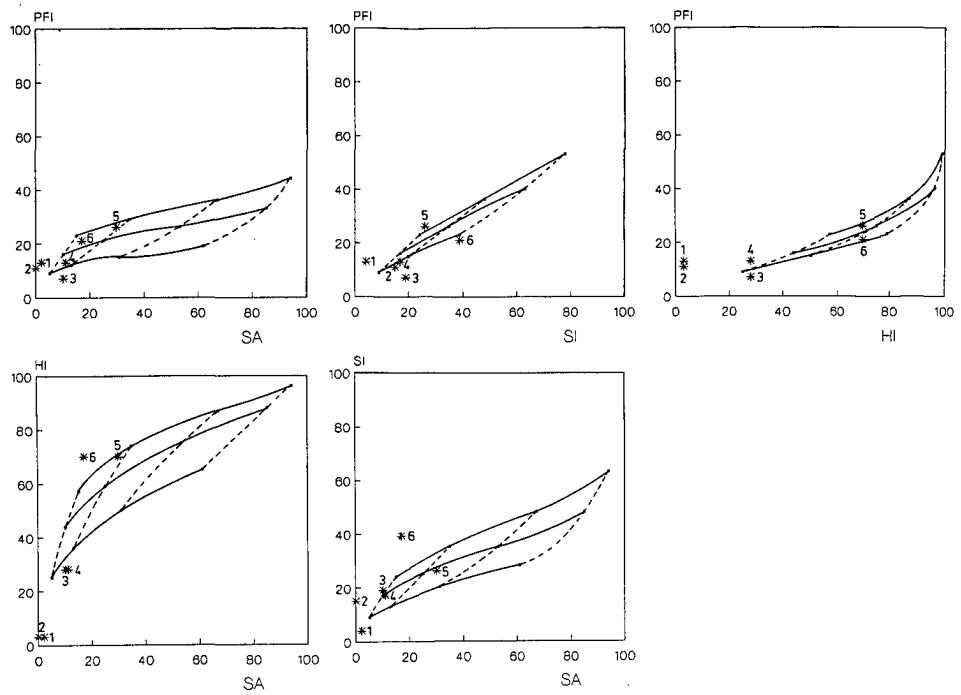

Fig.4. The positions of the samples affected by uplift and theoretical positions when uplift to the surface is taken into account. Drawn curves indicate the length of the subsided period (lower: 10 Ma; middle: 20 Ma; upper: 30 Ma); Broken curves indicate the temperature (from left to right: 75 °C, 80 °C, 85 °C and 90 °C in the plots SA-PFI, SA-SI and SA-HI; and 75 °C, 85 °C and 95 °C in the plots SI-PFI and HI-PFI).

(10 °C). Both the length and temperature of the first period were varied. The second period represented the remainder of the time given the total age of the Basin is 180 Ma. In this way the period of subsidence is approximated by an effective time of subsidence at an effective temperature.

The theoretical positions in the cross plots of a sample which has been uplifted to the surface are shown in Fig.4. The curves indicate the length of the effective period of burial and the prevailing temperature. As is clear from Fig.4, the positions of samples 3 to 6 can be explained, with few exceptions, by asing that the samples were at relatively high temperatures for a short period of time. Sample 7 does not fit into this simulation since its present temperature is 50 °C, whereas that in the simulation had been set at 10 °C after uplift. Taking the present temperature (50 °C) as the temperature after uplift, a period of subsidence of 10-15 Ma at a temperature of about 90 °C was calculated for sample 7.

For samples 8 to 12 only the temperatures were calculated assuming that these samples have not been uplifted.

Table 2 summarizes the best estimates of burial time and temperature for each sample. The estimates were generated by a program which calculated the time and temperature yielding the minimal distance between the theoretical and observed values of the parameters in a specific cross plot for a specific sample. Given the experimental error in the values of the maturity parameters, the agreement of the various estimates is good. For

Sample	PFI-SA	PFI-SI	PFI-HI	SI-SA	HI-SA	Combined
1	65/29	54/49	45/48	74/5	53/8	47/42
2	58/36	86/7	46/37	64/46	47/15	47/52
3	82/5	95/4	87/4	74/25	79/8	80/8
4	77/14	86/8	65/29	75/21	81/7	79/9
5	80/25	74/37	81/25	81/20	80/26	80/25
6	77/24	99/7	89/12	74/49	74/43	75/41
7	89/16	76/39	75/41	92/9	93/8	91/11
8	76	72	71	76	77	76
9	76	76	79	76	76	76
10	79	78	79	78	79	78
11	77	75	74	77	77	77
12	90	86	90	80	79	81

Table 2. Best estimates of temperature and time of the subsided period; Indicated are: temperature (°C)/time (Ma) (for sample 7 the temperature after uplift was 50 °C); for samples 8 to 12 only the temperature is shown, the time was estimated at 180 Ma. Column 1-5: estimates from the positions in the indicated cross plots. The last column (combined) gives the best estimates when all cross plots are taken into account. (see text).

each sample, we also calculated the time and temperature yielding the best fit in all cross plots, by minimizing the sum of the distances of theoretical and observed values for that sample in the various cross plots (Table 2, last column). These times and temperatures were used to calculate the theoretical positions of the samples as shown in Fig.5.

As a conclusion we can state that for all samples, except for 1 and 2, the calculated temperatures are between 70 and 80 °C or slightly higher (samples 7 and 12) for some period of time.

Estimates for samples 1 and 2 are not reliable since only the pristane formation reaction shows any progress, together with the sterane isomerization for sample 2. However, the values of the kinetic parameters for both isomerization reactions require that the hopane isomerization is always faster than the sterane isomerization (see also Fig.3). Since the hopane isomerization shows less progress in samples 1 and 2, the values of SI and/or HI for these samples are questionable.

The PFI values of samples 1 and 2 point to a short period at an elevated temperature of about 50 °C. Although the positions of samples 1 and 2 in the cross plots (Fig.2) suggest a temperature history of 180 Ma at about 40 °C, it is concluded from the PFI-HI plot that the temperature after uplift was, at most, 30 °C. In addition, samples 1 and 2 plot too high in the Arrhenius plot (Fig.1) which indicates that the reaction rate has been higher than predicted by the present temperature. Therefore, the progress in the pristane formation reaction is thought to result from a short period at moderately elevated temperatures.

The differences between samples 3 to 6 seem to relate to the length of the period of burial rather than to temperature. Samples 3 and 4 are closely similar, in agreement with their geographical proximity. Samples 5 and 6 are both from the most eastern margin of the basin. These samples have been at similar temperature for a somewhat longer period. Sample 6 shows a very high value for SI. Sample 7 shows a good agreement of theoretical and observed values and has been at a higher temperature than the other samples, although for only briefly. Samples 8-12 show good agreement between observed and theoretical values, taking into account that the sterane aromatization does not go to completion.

Conclusions

In conclusion, the observed values of the molecular maturity parameters are in agreement with a model of the thermal history of the basin consisting of two phases: one period (less than about 30 Ma) with high temperatures (about 80 °C) and a second period (more than 150 Ma) during which temperatures did not change. Uplift of the eastern part of the

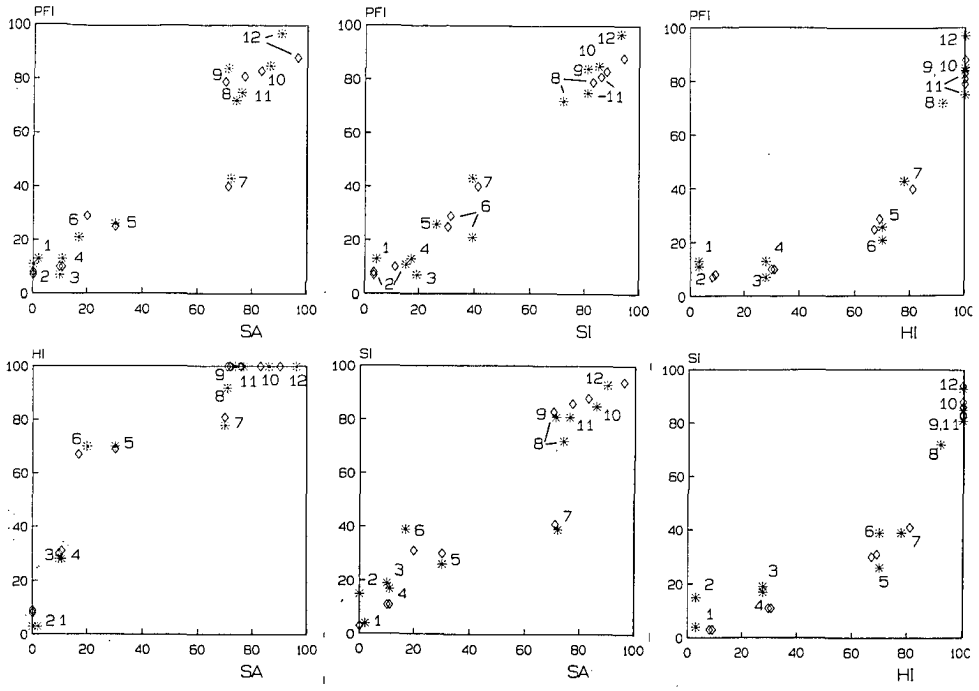

Fig.5. Cross plots showing the positions of observed values (*) and theoretical values (◇) using the best estimates from table 2 for the thermal histories of the samples (The theoretical values for samples 8 and 9 are identical).

basin occurred between these periods. The amount of uplift can be calculated from the burial temperatures derived from the cross plots, but it requires information about the past temperature gradients. However, if we assume a temperature gradient of 33 °C km^{-1} and a surface temperature of 10 °C, the maximum depth for most samples is estimated at between 2000 and 2200 m. Only samples 7 and 12 have probably been at somewhat greater depths (<2400 m). These results are in good agreement with those obtained by Mackenzie and McKenzie (1983), who used their extension model to calculate the thermal history of the Paris Basin and thereby lend support to their view that the uplift of its eastern part was substantially greater than previously assumed.

The estimates derived from the cross plots of PFI versus other parameters are in agreement with those by Mackenzie and McKenzie (1983) derived from the cross plots of the other parameters, only. Thus, the values of PFI observed in the Paris Basin, can be explained using values of the pseudo-kinetic parameters E and A calculated from the Mahakam Delta observations. This approach confirms the values of E and A and indicates that, at least in these two basins, the observations are not affected by the type of kerogen or by other differences in sample composition.

Acknowledgements

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SUMMARY

Twenty five years after the discovery of the isoprenoid C_{19} and C_{20} alkanes pristane and phytane in ancient sediments and oils, their origin has not been established beyond doubt. As a consequence of the general agreement that the phytyl side chain of the chlorophyll molecule is their common precursor, the ratio of the amounts of pristane and phytane has been used as an indicator of the oxicity of the depositional environment.
The discovery of etherlipids in archaebacteria and in oils and ancient sediments in the late seventies suggested a possible contribution by archaebacteria. Moreover, the assessment of the extended hopanoids as bacterial markers in the same time pointed to a serious underestimation of the bacterial contribution to sedimentary organic matter. In addition, recognition of bacterial remains in geological materials is only possible in a very general way.

This study was directed towards the recognition of bacterial organic matter in sediments emphasizing the contribution of acyclic isoprenoid hydrocarbons. Also non-bacterial precursors of acyclic isoprenoids were considered.
A general, systematic survey of lipids in prokaryotes and in bacteria-rich sediments was performed (Chapters 2 to 5). The same method was used for the analyses of bacteria and sediments. Different modes of occurrence of the lipid moieties were distinguished by sequential extractions after base and acid treatment. In this way fractions were obtained containing "free occurring" apolar lipids, OH^--labile lipid moieties, and lipid moieties which could be released exclusively by an acid treatment. The extracts were analyzed by gas chromatography and gas chromatography-mass spectrometry.
The method was developed using bacterial organic matter. The artefact formation accompanying the acid treatment was investigated in detail and a procedure with minimal artefact formation was assessed (chapter 2).
Several prokaryotic species, selected to represent distinct species according to 16 S rRNA systematics, were analyzed.
Sediment samples from the methanogenic zone of a stratified, freshwater lake (Lake Vechten, the Netherlands) and from hypersaline environments in a salt work area (Salinas de Roquetas de Mar, Spain), *i.e.* from a cyanobacterial mat and from a gypsum precipitation basin. The H^+-labile lipid moieties appeared to be the most characteristic. All gram-negative eubacteria, which are generally predominating in sediments, contained H^+-labile β-OH-fatty acids. The fact that these compounds were exclusively released by an acid treatment indicates that they occur bound via an amide-linkage or via a glycosidic bond. These compounds occur in Lipopolysaccharides (LPS), ornithine-lipids (OL) and bacterial sphingolipids. β-OH-fatty acids in this mode of occurrence (H^+-labile) are exclusive for prokaryotes.
The results show that the diversity of the distributions of H^+-labile β-OH-fatty acids is substantial both in bacteria and in sediments which indicates that these distributions may be useful as fingerprints of bacterial populations.

In the extracts of the bacterial species several compounds were identified containing acyclic isoprenoid chains like phytol, isoprenoid $C_{20:2}$ alkenol, farnesol, partly hydrogenated squalenes, di-phytanyl glyceryl etherlipids and di-biphytanyl di-glyceryl tetra-etherlipids. However, no previously unknown potential precursors of isoprenoids in sediments were found.
It was clear from analyses of kerogens that a compound which generates prist-1-ene upon analytical pyrolysis is a quantitatively inmportant precursor of pristane. In chapter 6 the work is described which shows that α-tocopherol generates prist-1-ene upon thermal degradation and that it is likely that tocopherols are precursors of pristane in ancient sediments and oils. The amounts of tocopherols in photosynthetic tissues are low compared to those of chlorophyll but during diagenesis the relative amounts are probably changed in favour of the tocopherols by selective preservation, which is likely in view of the structures of tocopherols and chlorophyll.

The pyrolysis product prist-1-ene and the diagenetically produced pristane could represent the precursor and the product of one reaction. This hypothesis was tested with a series of samples from the Mahakam delta, Indonesia (chapter 7). The dependency of the rate of the pristane formation reaction on temperature could be derived from an Arrhenius plot of the reaction extent, the Pristane Formation Index PFI, versus the effective burial temperature. The kinetic parameters calculated proved extremely low: 59 KJ mol^{-1} for the activation energy and $2.2*10^{-7}$ s^{-1} for the frequency factor. These low values could not be explained by reaction mechanisms and the kinetic parameters are therefore considered pseudo-kinetic parameters. The low values indicate that the molecular maturity parameter approach, although successful, is poorly understood. Nevertheless, the low values were used, without adjustment, to reconstruct the thermal history of the Paris Basin; the results are in agreement with a reconstruction based upon different molecular maturity parameters (chapter 8). Since the Paris Basin is much older than the Mahakam delta and contains a different type of kerogen this agreement is considered a confirmation of the low values of the pseudo-kinetic parameters. It illustrates the practical value of the independent maturity parameter PFI.

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SAMENVATTING

25 jaar na de ontdekking van de isoprenoide C_{19} en C_{20} koolwaterstoffen pristaan en phytaan in oude sedimenten en aardolie is hun herkomst nog niet met zekerheid bekend. De algemeen heersende opvatting dat de phytyl keten van het chlorophyl molecuul de gemeenschappelijke "precursor" is, heeft er toe geleid dat de pristaan/phytaan verhouding wordt gebruikt als een grootheid die een aanwijzing geeft voor de oxiciteit van het afzettingsmilieu.
De ontdekking van etherlipiden in archaebacteriën en in oliën en oude sedimenten, aan het eind van de jaren zeventig, was een aanwijzing dat er mogelijk ook een bijdrage uit archaebacteriën was. Bovendien werd in die tijd duidelijk - door de vaststelling dat de verlengde hopanoiden "markers" zijn voor bacteriën - dat er wel eens sprake kon zijn van een aanzienlijke onderschatting van de bacteriële bijdrage aan het organisch materiaal in sedimenten. Het herkennen van bacterieresten in geologisch materiaal is echter alleen mogelijk in zeer algemene zin.

Het in dit proefschrift beschreven onderzoek was gericht op het herkennen van bacterieel organisch materiaal in sedimenten waarbij de nadruk lag op de bacteriële bijdrage aan de acyclische isoprenoide koolwaterstoffen. Ook niet-bacteriële precursors van acyclische isoprenoiden werden in de studie betrokken.
Een algemeen, systematisch onderzoek werd verricht naar de lipiden in prokaryoten en in bacterie-rijke sedimenten (Hoofdstukken 2 tot en met 5). Voor de analyse van bacteriën en sedimenten werd dezelfde methode gebruikt. Door opeenvolgende extracties voor en na base en zuur behandelingen kon onderscheid gemaakt worden tussen verschillende wijzen van voorkomen van lipiden. Zo werden fracties verkregen met "vrij voorkomende" lipiden en met lipiden die uitsluitend konden worden vrijgemaakt door een base-, respectievelijk zuurbehandeling (OH^-- en H^+-labiele lipiden). De extracten werden geanalyseerd met behulp van gaschromatografie en gaschromatografie-massaspectrometrie. Deze methode werd ontwikkeld aan de hand van analyse van bacterieel organisch materiaal. De artefact vorming die bij de zuur-behandeling kan optreden werd uitgebreid onderzocht waaruit een procedure voortkwam waarbij artefact vorming minimaal is (hoofdstuk 2).
Verscheidene bacteriesoorten werden geanalyseerd, die waren geselecteerd op grond van verschillen in 16S rRNA samenstelling. Sediment monsters werden genomen uit de methanogene zone van een sediment uit een gestratificeerd zoet water meer (Plasje Vechten, Nederland) en in hypersaliene milieus in een zeezoutbedrijf (Salinas de Roquetas de Mar, Spanje), met name uit een cyanobacteriële mat en uit een gips precipitatie bekken. De H^+-labiele lipiden bleken de meest karakteristieke te zijn. Alle gram-negatieve eubacteriën, die meestal het talrijkst zijn in sedimenten, bevatten H^+-labiele β-hydroxy-vetzuren. Uit het feit dat deze alleen door een zuurbehandeling konden worden vrijgemaakt, kan worden afgeleid dat ze in het uitgangsmateriaal gebonden voorkomen via een amidebinding of via een glycosidische binding. β-hydroxy-vetzuren met zo'n bindingstype komen voor in Lipopolysacchariden (LPS), ornithine-lipiden (OL) en in bacteriële sphingolipiden. Zij zijn specifiek voor bacteriën.
De resultaten laten zien dat er grote verschillen zijn in de verdelingen van de H^+-labiele β-hydroxy-vetzuren zowel in bacteriën als in sedimenten. Daarom kunnen zulke verdelingen gebruikt worden als "vingerafdrukken" van bacteriële populaties.

In de extracten van de bacteriën werden verschillende verbindingen aangetoond die een acyclische isoprenoide keten bevatten zoals phytol, phytadienol (isoprenoide $C_{20:2}$ alcohol), farnesol, gedeeltelijk gehydrogeneerde squalenen, diphytanyl-glyceryl-etherlipiden en di-biphytanyl-diglyceryl-tetraetherlipiden. Er werden echter geen nieuwe potentiële precursors van isoprenoiden in sedimenten gevonden.
Uit de analyse van kerogenen was bekend dat een kwantitatief belangrijke "precursor" van pristaan bij analytische pyrolyse pristeen-1 vormt. In hoofdstuk 6 wordt beschreven dat α-tocopherol bij thermische degradatie pristeen-1 vormt en dat tocopherolen waarschijnlijk precursors zijn van pristaan in oude sedimenten en aardolie. De hoeveelheden toco-

pherolen zijn in photosynthetische weefsels weliswaar laag ten opzichte van die van chlorofyl maar gezien de chemische structuren van tocopherol en chlorofyl is het waarschijnlijk dat tocopherol minder snel wordt afgebroken.
Het pyrolyse product pristeen-1 en het door diagenese gevormde pristaan zouden de "precursor" en het product van de pristaan vormingsreactie kunnen vertegenwoordigen. Deze hypothese werd op haar geldigheid getoetst door analyse van een serie monsters uit de Mahakam delta in Indonesïe (Hoofdstuk 7). Het verband van de reactiesnelheid met de temperatuur kon worden afgeleid uit een grafiek voor de Arrhenius vergelijking waarin de voortgang van de reactie (de Pristane Formation Index, PFI) werd uitgezet tegen de effectieve "burial temperature". De berekende kinetische parameters bleken extreem laag te zijn: een activeringsenergie van 59 KJ mol^{-1} en frequentiefactor van 2,2 * 10^{-7} s^{-1}. Deze lage waarden kunnen niet worden verklaard met een bepaald reactiemechanisme en daarom worden de kinetische parameters beschouwd als pseudo-kinetische parameters. De lage waarden laten zien dat de moleculaire parameters voor het bepalen van de rijpheid van sedimenten weliswaar met succes worden toegepast maar niet goed worden begrepen. Desalniettemin kon met de gevonden waarden, zonder enige aanpassing, een reconstructie van de temperatuur historie van het Bekken van Parijs gemaakt worden die in overeenstemming is met een reconstructie gebaseerd op andere rijpingsparameters (hoofdstuk 8). Omdat het Bekken van Parijs veel ouder is dan de Mahakam delta en bovendien een ander type kerogeen bevat, wordt deze overeenstemming gezien als een bevestiging van de lage waarden van de pseudo-kinetische parameters. De praktische waarde van de onafhankelijke rijpingsparameter PFI wordt daarmee onderstreept.

Dankwoord

Op deze plaats past een woord van dank. De voltooiing van dit proefschrift markeert het einde van een periode waarin ik erg veel heb geleerd, en niet alleen van de organische geochemie, waarvan ik niets wist toen ik naar Delft kwam. Deze dankbetuiging betreft daarom niet alleen de werkzaamheden die zoveel mensen hebben verricht voor het hier beschreven onderzoek, maar vooral ook de persoonlijke betrokkenheid. Het was bijzonder prettig en stimulerend te werken in de organische geochemie groep met zijn vaste en wisselende leden. Ik ben heel erg blij de positieve houding van de mensen in deze groep ten opzichte van het werk en ten opzichte van elkaar, persoonlijk te hebben meegemaakt.
Mijn dank gaat uit naar al die mensen die voortdurend bereid bleken hun steentjes bij te dragen. Slechts enkele van de nauwst betrokkenen wil ik hier noemen: Allereerst Irene Rijpstra, die op onvoorstelbaar soepele wijze, in halve dagen, steeds weer nieuwe extracten, gaschromatogrammen en GC-MS runs klaar had. Verder dank ik prof. Schenck voor zijn nauwgezette en positieve commentaar. Zijn rode pennetje heeft mij vele malen het verschil tussen bedoeling en betekenis duidelijk gemaakt. De bijdrage van Jan de Leeuw was groter dan hier vermeld kan worden. Behalve door zijn onuitputtelijke voorraad ideeën en zijn vermogen om in een berg gegevens structuren te herkennen ben ik vooral getroffen door zijn blijvend levendige interesse in andere vakgebieden. Ik denk nog vaak terug aan onze gezamelijke monstertocht in Spanje, waar we in een paar dagen tijd in de aanvankelijk onbekende hypersaliene wereld een duidelijke structuur leerden herkennen.

Tenslotte ben ik blij, zeker nu het boekje klaar is, dat Froukje, Maarten en Frank mij voortdurend vele andere aspecten van het leven hebben laten zien.
Het spijt mij voor de kinderen dat het geen leuk boekje is geworden.

Curriculum vitae

Hans (Johannes Gustaaf Carl Maria) Goossens werd in 1951 in Amsterdam geboren waar hij ook ging studeren na het behalen van het gymnasium-β diploma. Hij studeerde scheikunde (S_2) met als hoofdvak microbiologie en als bijvak limnologie. Na zijn studie deed hij bij het Delta Instituut voor Hydrobiologisch Onderzoek te Yerseke van 1979 tot 1982 onderzoek aan de activiteit van bacteriën in het Grevelingenmeer. Daarna volgde het in dit proefschrift beschreven onderzoek. Sedert 1986 werkt hij bij het Waterloopkundig Laboratorium, sector waterbeheer en milieu, waar hij zich bezig houdt met ecosysteemmodellering en met de modellering van accumulatie en effecten van toxische stoffen.

Hij is (co-)auteur van de volgende artikelen die niet in dit proefschrift zijn opgenomen:

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