Universitat de Barcelona

Facultat de Biologia

Departament de Microbiologia

ECOPHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF ESTUARINE MICROBIAL MATS

Caracterización Ecofisiológica y Molecular de Tapetes Microbianos de Estuario

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Dr. Ricardo Guerrero Moreno, Professor of the Department of Microbiology (Faculty of Biology) of the Universitat de Barcelona, certifies that the research work "**Ecophysiological and Molecular Characterization of Estuarine Microbial Mats**" presented as PhD Thesis by Laura Villanueva Álvarez has been performed under his direction in the mentioned department, and that satisfies the necessary requirements to apply for the PhD degree of the Universitat de Barcelona. And for the record, he signs this certificate dated October 10th 2005.

El Dr. Ricardo Guerrero Moreno, catedrático del Departamento de Microbiología de la Facultad de Biología de la Universidad de Barcelona, certifica que el trabajo de investigación "**Ecophysiological and Molecular Characterization of Estuarine Microbial Mats**" presentado como Tesis Doctoral por Laura Villanueva Álvarez realizado bajo su dirección en dicho departamento, y que reúne los requisitos necesarios para optar al grado de Doctor por la Universidad de Barcelona. Y para que así conste, firma la presente certificación con fecha 10 de octubre de 2005.

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"If I could do it all over again, and relive my vision in the twenty-first century, I would be a **microbial ecologist**. Ten billion bacteria live in a gram of ordinary soil, a mere pinch held between thumb and forefinger. They represent thousands of species, almost none of which are known to science. Into that world I would go with the aid of modern microscopy and molecular analysis. I would cut my way through clonal forests sprawled across grains of sand, travel in an imagined submarine through drops of water proportionately the size of likes, and track predators and prey in order to discover new life ways and alien food webs. All this, and I need venture no farther than ten paces outside my laboratory building. The jaguars, ants, and orchids would still occupy distant forests in all their splendor, but now they would be joined by an even stranger and vastly more complex living world virtually without end. For one more turn around I would keep alive the little boy of Paradise Beach who found wonder in a scyphozoan jellyfish and barely glimpsed monster of the deep"

Edward O. Wilson

"Verba volant, scripta manet"
("Words fly away, the written remains";
"Las palabras vuelan, lo escrito permanece")

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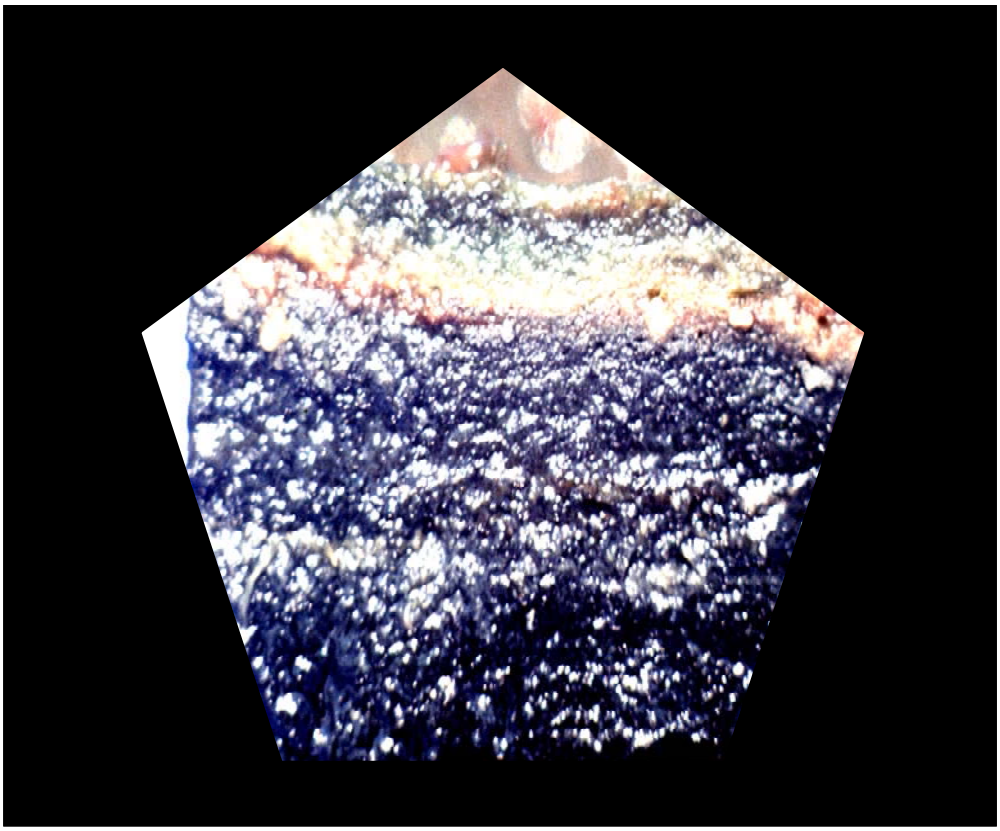

Figure C. Vertical section of an Ebro delta microbial mat and the microbial populations in horizontal layers.

ABBREVIATIONS

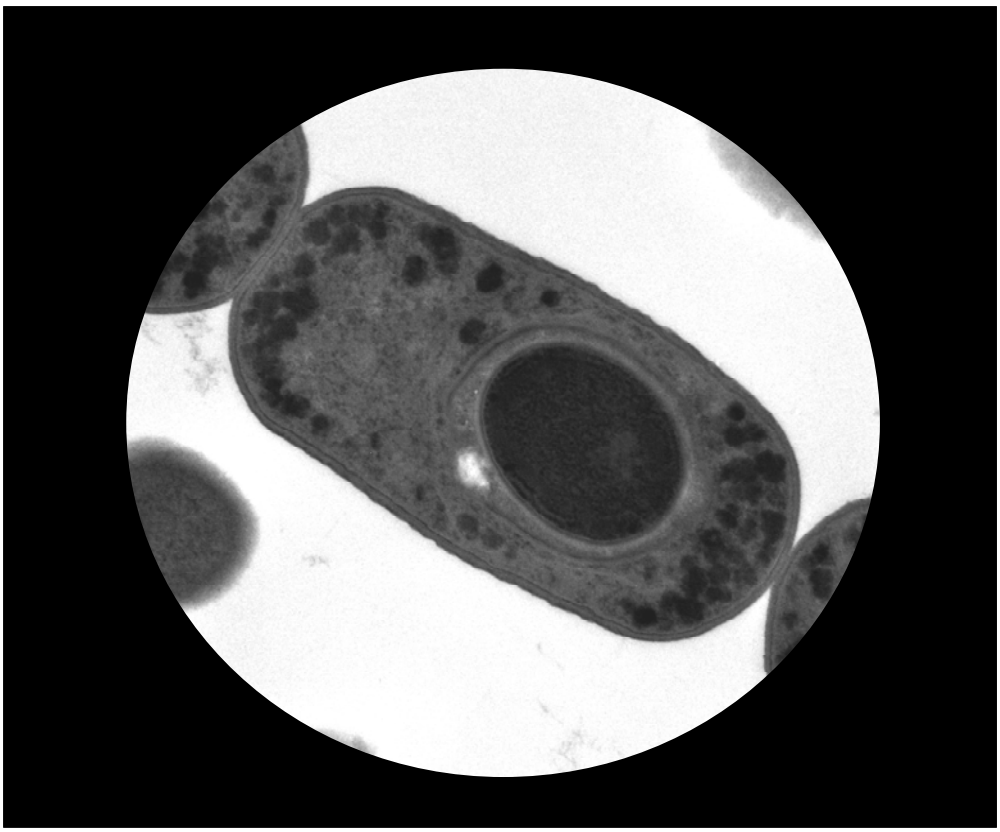

Figure A. Transmission electron micrograph of *Clostridium* sp. EBD (micrograph by the author and published on the cover of *Int. Microbiol*. Vol. 8, year 2005)

A

amu: atomic mass unit

AODC: Acridine orange direct counts

APCI-MS MS: Atmospheric pressure chemical ionization tandem MS

API: Atmospheric pressure ionization

APS: Ammonium persulfate

ArPG: Archaeol phosphatidylglycerol

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

B- C

BD: Bioenergetic index

BPE: Bovine phosphatidyl ethanolamine

BSTFA: *N,O*-bis(trimethylsylil)trifluoroacetamide

BW: Backward

ca: *circa* (about)

CDGE: Constant denaturing gradient electrophoresis

CECT: Spanish Type Culture Collection

CFB: *Cytophaga-Flavobacterium-Bacteroides* phylum

CMC: Carboxymethyl cellulose

CO: Carbon monoxide

CoA: Coenzyme A

Cps: counts per second

CRH: Corticotrophin releasing hormone

D

D: Divergence index

DAPI: 4',6'-diamino-2-phenylindole dihydrochloride

DCMU: 3-(3',4'-dichlorphenyl)-1,1-dimethylurea

DG: Diglycerides

DGFA: Diglyceride fatty acid(s)

DGGE: Denaturing gradient gel electrophoresis

DIC: Dissolved inorganic carbon

DMA(s): Dimethylacetal(s)

DMK(s): Demethylmenaquinone(s)

DMS: Dimethylsulfide

DMSO: Dimethylsulfoxide

DMSP: Dimethylsulfoniopropionate

DNA: Deoxyribonucleic acid

dNTPs: Deoxynucleotide triphosphates

DO: Dissolved oxygen

DO: Optical density

DOM: Dissolved organic matter

DP: Declustering potential

DPDS: *N,N*-dimethyl-*p*-phenylenediamine

DSMZ: Deutch Sammlung von mikroorganismen und zellkulturen (German collection of microorganisms and cell cultures)

E-F

EDTA: Ethylenediamine-N,N,N',N'-tetraacetic acid

E.g.: *Exempli gratia* (as example)

EPS: Exopolysaccharide

ESI: Electron spray ionization

ES-MS²MS: Electrospray tandem mass spectrometry

FA(s): Fatty acid(s)

FAME(s): Fatty acid methyl ester(s)

FIA: Flow-injection analysis

FISH: Fluorescence *in situ* hybridization

FITC: Fluoresceine-isothiocyanate

FW: Forward

G-H-I

GC: Gas chromatography

GC–FID: Gas chromatography with a flame ionization detector

GC–MS: Gas chromatography tandem mass spectrometry

Gly: Glycolipids

GMT: Greenwich mean time

GNSB: Green non-sulfur bacteria

GSB: Green sulfur bacteria

H': Shannon-Weaver index of diversity

HA(s): Hydroxyalkanoic acid(s)

HP: Hewlett-Packard

HPLC: High Pressure Liquid Chromatography

i.e.: *id est* (that is)

IPL: Intact polar lipid

IPTG: Isopropyl-β-D-thiogalactopyranoside

IS: Ion transfer voltage

J-K-L

KDO: Ketodeoxyoctanate

LB: Luria-Bertani broth

LCB: Long chain bases

LC-MS²MS: Liquid chromatography tandem mass spectrometry

LIT: Linear ion trap

LPS: Lipopolysaccharide

LPS-OH FA(s): Hydroxy fatty acid(s) of the lipopolysaccharide

M-N-O

MBrFA: Medium-branched fatty acids

MBSTFA: *N*-tert-butyl-dimethylsilyl-*N*-methyltrifluoroacetamide

MCL: Medium chain length

MD: Microbial divergence index

MN: Mineral medium for cyanobacteria

MK(s): Menaquinone(s)

MS²MS: Mass spectrometry tandem mass spectrometry

MT: Methanetiol

MW: Molecular weight

m/z: Mass-to-charge ratio

NCBI: National center for biotechnology information

Neu: Neutral lipid fraction

NOM: Nitrogenated organic matter

ODA: Oil displacement activity

O/N: Overnight

P

PBS: Phosphate Buffered Saline

PC: Phosphatidylcholine

PCR: Polymerase chain reaction

PG: Phosphatidylglycerol

PHA(s): Polyhydroxyalkanoate(s)

phaC: Coding gene for the PHA-synthase

PHB: Polyhydroxybutyrate

Ph-B: Photosynthetic biomass

P3HB: Poly-3-hydroxybutyrate

PHV: Polyhydroxyvalerate

PI: Phosphatidylinositol

PLFA(s): Phospholipid fatty acid(s)

PPG(s): Polypropyleneglycols

PQ: Plastoquinone

PS: Phosphatidylserine

PSB: Purple sulfur bacteria

PUFA(s): polyunsaturated fatty acid(s)

Q-R

Q: Quinone

RCM: Reinforced *Clostridium* medium

rDNA: Ribosomal deoxyribonucleic acid

RNA: Ribonucleic acid

RQ: Rhodoquinone

RT: Room temperature

S-T

SAC: Sicilic acid chromatography

SCL: Short chain lenght

SEM: Scanning electron microscopy

SET: Serial endosymbiosis theory

SDS: Sodium dodecyl sulphate

SLB(s): Signature lipid biomarker(s)

sn: Stereospecific number

SQD(s): Sulfoquinovosyldiacylglycerol(s)

SRB: Sulfate-reducing bacteria

SWYP: Sea water yeast peptona

Tº: Temperature

TBT: Tributyrin

TEM: Transmission electron microscopy

TEMED: *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine

TGGE: Temperature gradient gel electrophoresis

Tm^1: Theoretical melting temperature (at 50 mM Na^+) of each primer

Tm^2: Melting temperature at which the amplification reaction was performed

TMS: Trimetylsylil

T-RFLP: Terminal restriction length polymorphism

Tris: Tris(hydroxymethyl)amino methane

TSB: Tryptic soy broth

U-V-W-X-Y-Z

UIPAC: International union of pure and applied chemistry

UQ: Ubiquinone

UPGMA: Unweighted pair-group method with arithmetic mean

UV: Ultraviolet

VOSC: Volatile organosulfur compounds

X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABSTRACT

Microbial mats are prokaryotic communities that are thought to represent the present-day analogues of the first ecosystems on Earth. Their study reveals microbial strategies for survival under a broad range of environments. Here, we report the combination of different methods such as lipid analysis, nucleic-acid based techniques, and the isolation and characterization of microbial members to determine changes in the physiological status, viable biomass and community composition in microbial mats. The combination of lipid analysis and DNA based methods has provided information about the temporal dynamics of populations and has revealed the importance of heterotrophic bacteria, green non-sulfur bacteria, as well as fermentative bacteria. The application of quinone profiling method has been useful for taxonomic purposes, biomass estimation and microbial redox state. We have observed important differences in the community structure and redox status in microbial mats from different locations that were apparently very similar. In addition, we have performed a preliminarily study about the detection of intact polar lipids and *Archaeal* members in mat samples. The mentioned approaches were also applied to microbial mat samples along a circadian cycle and a daily pattern of physicochemical responses was observed.

Moreover, the importance the heterotrophic bacteria in the regulation of metabolic processes in the photic zone was investigated and two strains were isolated. One of them, *Pseudoalteromonas* sp. EBD, revealed important metabolic capacities and cooperative interactions with cyanobacteria. On the other hand, a member of the *Sphingomonas* genus has been also characterized and its importance in the nutrient cycling and in the polyhydroxyalkanoate dynamics will be investigated. Finally, the morphological succession of microbial populations in the transition zones oxygen–sulfide has been investigated. Molecular screenings have provided information about the microbial composition and have permitted the design of probes for the detection of the observed microorganisms in mats.

I. INTRODUCTION

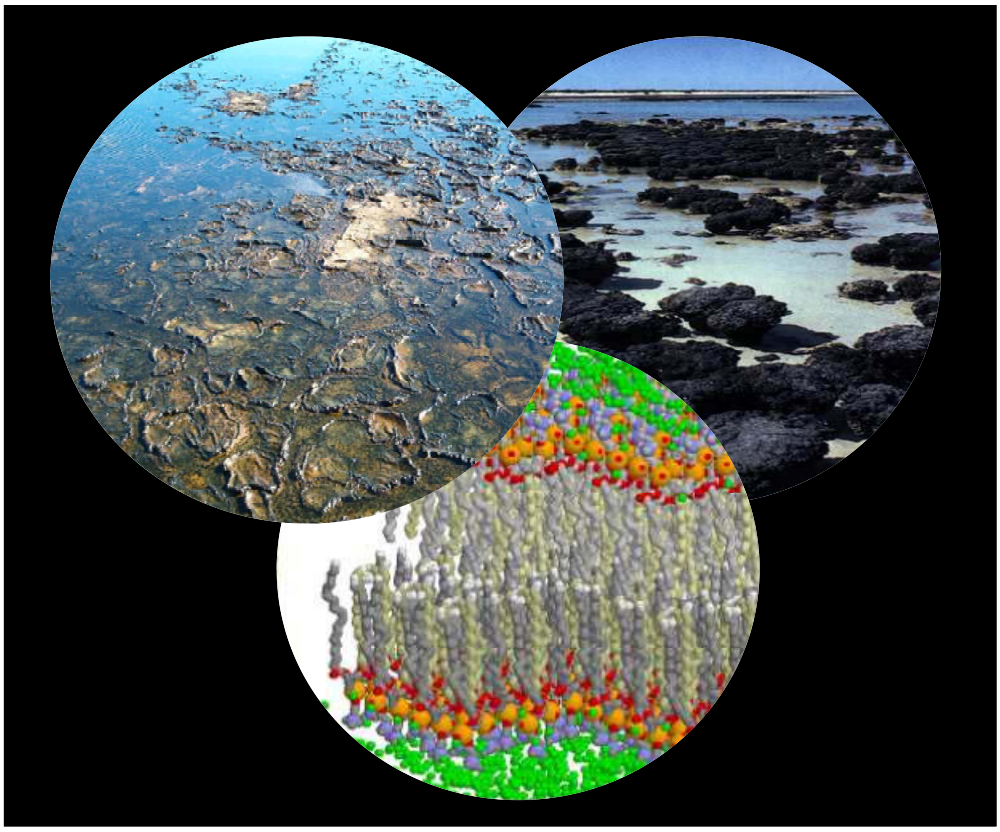

Figure I. “Give a place to stand, and I will move the Earth” Archimedes (ca. 235 BC).

Top left: Ebro delta microbial mats (Spain) / Top right: Shark Bay stromatolites (Australia) / Center: Phosphatidyl choline lipid bilayer.

I. INTRODUCTION

1. Microbial mats: the dense living carpet of Gaia

- The earliest ecosystems on Earth
- Structure and location

  Location and types of microbial mats
  Sediment stabilization and lithification

- Biogeochemistry, microenvironment and nutrient cycling

  Microenvironmental conditions
  Carbon and oxygen cycling in microbial mats
  Sulfur cycle in microbial mats
  Iron cycling
  Nitrogen cycling
  Gas production

- Major mat-building microorganisms and minority populations
- Future perspectives

1. Microbial Mats: the dense, living carpet of Gaia

- The earliest ecosystems on Earth

Microbial mats are stratified microbial communities that develop in the environmental microgradients established at the interfaces of water and solid substrates. They form a laminated multilayered biofilm (Davey and O'Toole, 2000) and largely alter the environmental microgradients in the interface as a result of their metabolism. The develop of these microbial communities causes steep gradients and the establishment of a well-defined diffusion boundary layer immediately proximal to the multilayered biofilm. Microbial mats are probably the oldest biota on Earth, as witnessed by the oldest known microfossils being found in lithified microbial mats: Stromatolites, which have been dated to over 3.5 billion years old (Tyler and Barghoorn, 1954; Awramik, 1984). These microfossils found at Gunflint and Warrawoona (Lowe, 1980), are sedimentary structures made mostly of calcium carbonate or flint, and are formed by communities of bacteria (especially photosynthetic bacteria).

Stromatolites were the most dominant sedimentary structures in rocks of the Precambrian era, together with the vast deposits of the Banded Iron Formations (Walter *et al*., 1976). In the Archaean and Proterozoic eras microbial mats were very abundant and may well have been responsible for the primordial oxygen build-up in the atmosphere enabling the later evolution of higher forms of life. Diversification of new life forms, and the establishment of trophic chains for the recycling of nutrients (ecopoiesis or the origin of ecosystems; Guerrero, 1998), made it possible the persistence of life and the evolution to eukaryotic cells (eukaryopoiesis) (Fig. I.1.1).

The persistence and abundance of stromatolites throughout most of geological time demonstrate the evolutionary success of the microbial mat ecosystem. Much of the understanding and interpretation of ancient stromatolites has been derived from investigations of these structures within their geological context and by applying information obtained from the study of modern microbial mats. In fact, biological studies on microbial mats include: identification and isolation of individual members;

studies on the physiological requirements and ecological ranges of microorganisms; distribution of physicochemical parameters, gradients and microorganisms; microbial associations and communities; ecology of the mat; and the overall understanding of microbial mats as miniature ecosystems. Microbial mats are an extremely dynamic and complex ecosystem, and highly conserved over the last 2 billion years, for this reason they provide a unique opportunity to study the evolution of a microbial community.

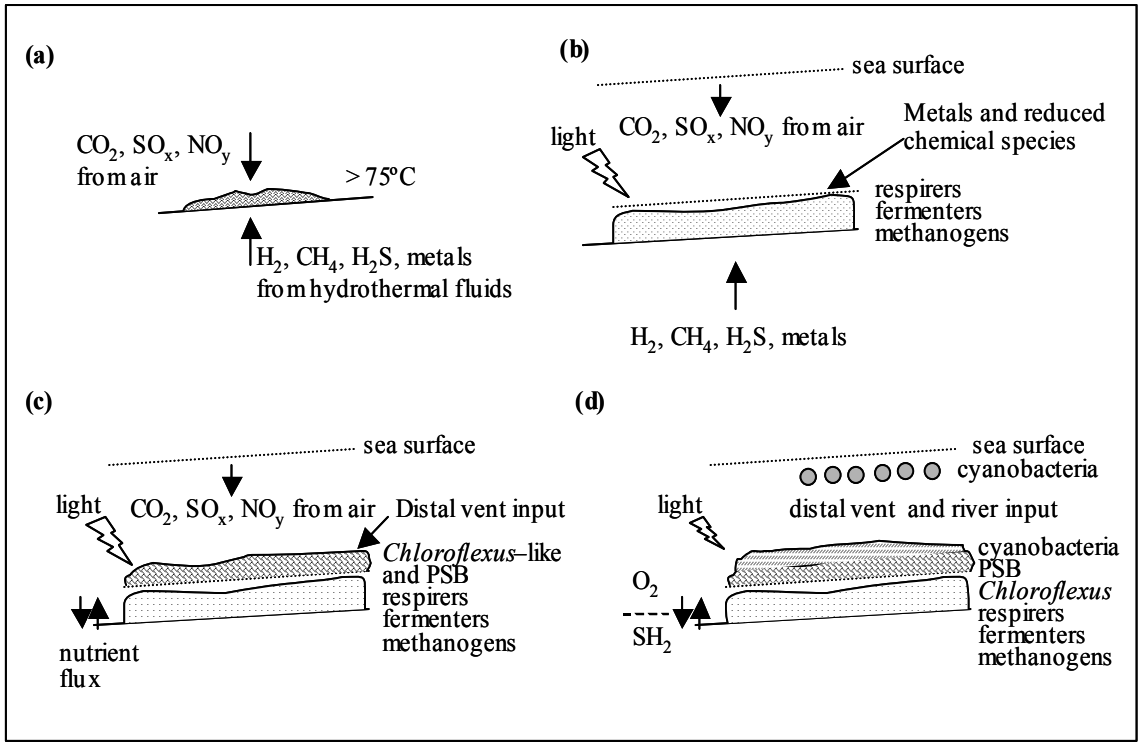

Figure I.1.1. Possible archaean metabolic evolution of microbial mats.

(a) Earliest Archaean (ca. 4 Gyr ago?): hyperthermophilic biofilms near hydrothermal vents. (b) Early Archaean (prior to 3.8 Gyr ago?): first photosynthesis in organisms close to vents. The primitive pigments, formely used for thermotaxis, may have adapted and played supplementary photosynthetic function using bacteriochlorophyll. This would allow colonization of mesothermophilic habitats in the photic zone. Associated with these early photosynthetizers would have been other microorganisms exploiting organic matter by fermentation and respiration. (c) Early Archaean: anaerobic and microaerobic photosynthesis further form vents and development of green and purple sulfur bacteria (PSB). (d) Mid-late Archaean (ca. 3.5–3.6 Gyr ago?): cyanobacterial mats and plankton (Nisbet and Fowler, 1999).

- Structure and location

Location and types of microbial mats

Microbial mats develop in a wide variety of environments such as hot springs, hypersaline ponds, dry and hot deserts, alkaline lakes and coastal intertidal sediments (Cohen *et al*., 1984; Cohen and Rosenberg, 1989; Stal, 1994) (Table I.1.1). Particularly, multicellular organisms are excluded from such environments and it has been conceived that the absence or limited activity of grazing organisms is an important requisite for the development of microbial mats (Farmer, 1992). Interestingly, other non-typical mats have been discovered in which primary production is entirely or significantly due to anoxygenic photoautotrophic bacteria (Ward *et al*., 1992; Castenholz *et al*., 1992), and in some cases due to extensive mats of non-photosynthetic, sulfide-oxidizing autotrophs such as *Thermothrix* or *Beggiatoa* (Nelson *et al*., 1989). Grazing invertebrates are absent or rare in these habitats, but in most of these cases even cyanobacteria are absent, usually due to intolerance of high sulfide levels at higher temperatures or to low pH or darkness.

Other interesting discoveries have been the revelation of cyanobacterial mats dominate the benthic communities of ponds and small lakes in south Antarctica (Vincent *et al*., 1993), the presence of microbial mats in tropical scleractinian corals (Rützler and Santavy, 1983), or in ultraoligotrophic lakes (Castenholz, 1994). However, mats persistent enough to develop 'laminae' sometimes develop in marine intertidal habitats with normal salinity and regular wettings, but often these are habitats where sulfide concentrations reach high enough levels to discourage most invertebrate grazers. Such mat communities are known as 'sulfureta' e.g. mats in the Orkney Islands (van Germerden *et al*., 1989). Also, extensive mats dominated by typical cyanobacteria (e.g. *Microcoleus*) have developed in the Persian Gulf in association with widespread intertidal crude oil where grazers have been exterminated by the pollutants (Sorkhoh and Al-Hasan, 1992; Al-Hasan *et al*., 1998; Cohen, 2002).

Table I.1.1. Types and location of microbial mats.

Classification	Types	Example
COASTAL	**Sandy beaches**	Orkney Islands, U□ (van Germerden *et al.*, 1989)
Supralittoral: Regularly exposed, often by daily tidal fluctuations	**Estuarine or delta**	Ebro delta, Spain (Mir *et al*., 1991)
	Marshes	Sippewissett, Cape Cod, USA (Gilson *et al*., 1984)
	Hypersaline ponds	Salins-de-Giraud, France (Caumette *et al*., 1994)
	Atolls	French Polynesian '□opara' (Defarge *et al*., 1994)
	Dunes	Meijendel dune, The Hague (Jelgersma *et al*., 1970)
	Mangrove swamp	Bido Salterns, Cuba (Margulis *et al*., 1986)
MARINE	**Marine mats**	Friesian Islands, Holland (Patterson *et al*., 1994)
Submerged mats: Exposed only seasonally		Mellum Islands, Germany (Stal *et al.*, 1985)
		Laguna Figueroa, Baja CA (Horodyski *et al*., 1977)
		Guerrero negro, Baja CA (Javor and Castenholz, 1984)
		Shark Bay and Spencer Gulf, Australia (Bauld, 1984)
		Gulf of Aqda, Egypt (□rumbein and Cohen, 1974)
		Solar Lake, Egypt (□rumbein *et al.*, 1977)
	Hyperscums	Fishponds, Israel (van Rijn and Shilo, 1985)
	Hydrothermal vents	Guaymas Basin, Pacific (Belkin and Jannasch, 1989)
INLAND	**Alkaline lakes**	Big Soda Lake (Oremland and Des Marais, 1983)
	Neutral lakes	Great Salt Lake, Utah (Rushforth and Felix, 1982)
	Hot springs	Yellowstone, Wyoming (Castenholz, 1984)
	Antarctic ponds	McMurdo ice shelf, Antarctica (Vincent *et al*., 1993)
	Deserts	Desert crusts, Utah (García-Pichel *et al*., 2001)
	Active volcanoes	Loihi Seamount (Moyer *et al*., 1994)
	Hypersaline lagoons	Chiprana lagoon, Spain (Montes, 1990)
	Sulfur springs	Hamei Mazor, Israel (Oren, 1989)

Sediment stabilization and lithification

Microbial mats develop as a result of microbial growth and activity, sediment trapping and binding in the organic matrix, and sedimentation. Important environmental parameters for the development of these kinds of ecosystems are grain size of the substratum, capillary attraction of water, penetration of light, sedimentation, erosion rates, and grazing pressure. As a result of these processes, the annual elevation of the mat surface may range from 1–2 mm (van Germerden, 1993).

Microbial mat organisms release high molecular-weight mucous secretions called exopolymers (EPS). The exopolymer matrix can: (i) slow the diffusion of ions, (ii) bind and store nutrients, (iii) reduce desiccation, (iv) protect cells against toxic compounds, (v) enhance the cohesiveness and macro-physical stability of the mat and maintain the microspatial organization of non-motile microbial cells, and (vi) maintain cyanobacteria hydrophobicity (Decho, 1990). Mats may be viewed as systems embedded in a semi-solid organic matrix resulting from the excretion of EPS by resilient microorganisms. EPS act as a laminar diffusional barrier, and aided by porewater allow that oxygen respiration rates can exceed oxygen diffusion. This fact is essential for the establishment of localizated anoxic microzones in the upper aerobic zone and a 'sulfuretum' in the lower layers, harbouring microaerophilic or anaerobic taxa (Paerl *et al*., 2000).

Cyanobacterial cells with their EPS have a very important role as the site of precipitation of carbonates (Defarge *et al*., 1996), acting as a reactive interfaces or templates for heterogeneous nucleation. Moreover, the presence of calcium carbonate closely associated with cyanobacterial cells indicate that they may participate in the formation of this biomineral; indeed calcification is a common phenomenon in microbial mats and seems to be influenced and controlled by their microbial members. It has been suggested that lithification in laminated mats does not occur at the surface rather at the bottom after the cyanobacteria have died. Mineralization (e.g. calcification) of dead cyanobacterial material is probably due o carbonate precipitation by heterotrophic bacteria living on and from the organic material of the sheaths (Merz-Preiβ, 2000).

- Biochemistry, microenvironment and nutrient cycling

Microenvironmental conditions

To understand the function of a microbial mat community, the physical and chemical microenvironment in which the microorganisms live must be known well and in detail. The community just below the mat surface experiences steep vertical gradients of light intensity and redox conditions that change markedly during the diel cycle. Indeed, motile photosynthetic organisms optimize their position with respect to the resultant light gradient.

Oxygenic photosynthesis ceases at night, the upper layers of the mat become highly reduced and sulfidic (Jørgensen, 1994). Counteracting gradients of oxygen and sulfide shape the environment and provide daily-contrasting microenvironments that are separated in a scale of a few millimeters (Fig. I.1.2; Revsbech *et al*., 1983). Radiation hazards (UV, etc.), as well as oxygen and sulfide toxicity, elicit motility and other physical responses. The combination of benefits and hazards of light, oxygen, and sulfide promotes the allocation of the various essential mat processes to the periods of light and dark (Bebout *et al.*, 1994) and to various depths of the mat.

- Light microenvironment

The light flux penetrating the mat can be measured both as downward irradiance (the total down-welling light that passes through a horizontal plane) and as scalar irradiance (the sum of all light that converges upon a given point within the mat) (Des Marais, 2003). Due to the high density of photosynthetic organisms, bacterial mucilage, and mineral particles in microbial mats, light absorption is dominated by the light-harvesting pigments of the phototrophic bacteria, and light is strongly scattered. Because absorption and scattering of light are quite substantial within the mat, scalar irradiance can differ substantially from downward irradiance (Jørgensen and Des Marais, 1988).

Previous studies (Jørgensen *et al*., 1987) have observed how the mat matrix affects the penetration of light and the physiology of the living community. For example, cyanobacteria that uses light that has been filtered by overlying diatoms exhibit greatest photosynthetic activity at wavelengths between 550 and 650 nm (Jørgensen *et al.*, 1987), a region that lies between the maximum absorption for the chlorophyll *a*.

The shortest wavelengths of the solar spectrum (UV, 280–400 nm) represent a small percentage of the total incident irradiance; however they may produce important biological effects. The solar UV has been recognized as an important environmental stress factor that cause inhibition of both primary productivity and induce changes in species composition. The impact of UV radiation on microbial mats vary in space and time, and peaking during noon and in the summer season, but clearly is an important factor at least for the top phototrophic layers. Some factors tend to increase the effectiveness of UV action in microbial mats, for example the density of the microbial assemblages. The compact photosynthetic layers have steep gradients of oxygen with a maximum close to the surface and the effects of the UV irradiation are caused indirectly through the excitation of reactive oxygen species. UV also seems to play a role in determining cyanobacterial composition, and have additional effects on biochemical processes such as photosynthesis, respiration and dinitrogen fixation (García-Pichel and Castenholz, 1994).

- Chemical gradients

The high rates of oxygenic photosynthesis that occur in the narrow photic zone of the mat create steep and variable gradients (Revsbech *et al.*, 1983) in pH and in concentrations of dissolved inorganic carbon (DIC) and O_2 (DO). The oxic zone reflects a dynamic balance between photosynthetic O_2 production and O_2 consumption by a host of sulfide-oxidizing and heterotrophic bacteria. Extremely high rates of oxygenic photosynthesis create DO levels that are nearly five times the value of air-saturated brine. Oxygen production can become negligible at a depth of 0.5 mm, due to light limitation. However, O_2 diffuses farther down to a point at which it overlaps with sulfide diffusing up from below. This interval is typically inhabited by abundant green

non sulfur phototrophic bacteria (e.g. *Chloroflexus*) and by *Beggiatoa*. As sunset approaches, the oxic zone collapses quickly, and the oxic-anoxic boundary approaches the mat surface (Canfield and Des Marais, 1993).

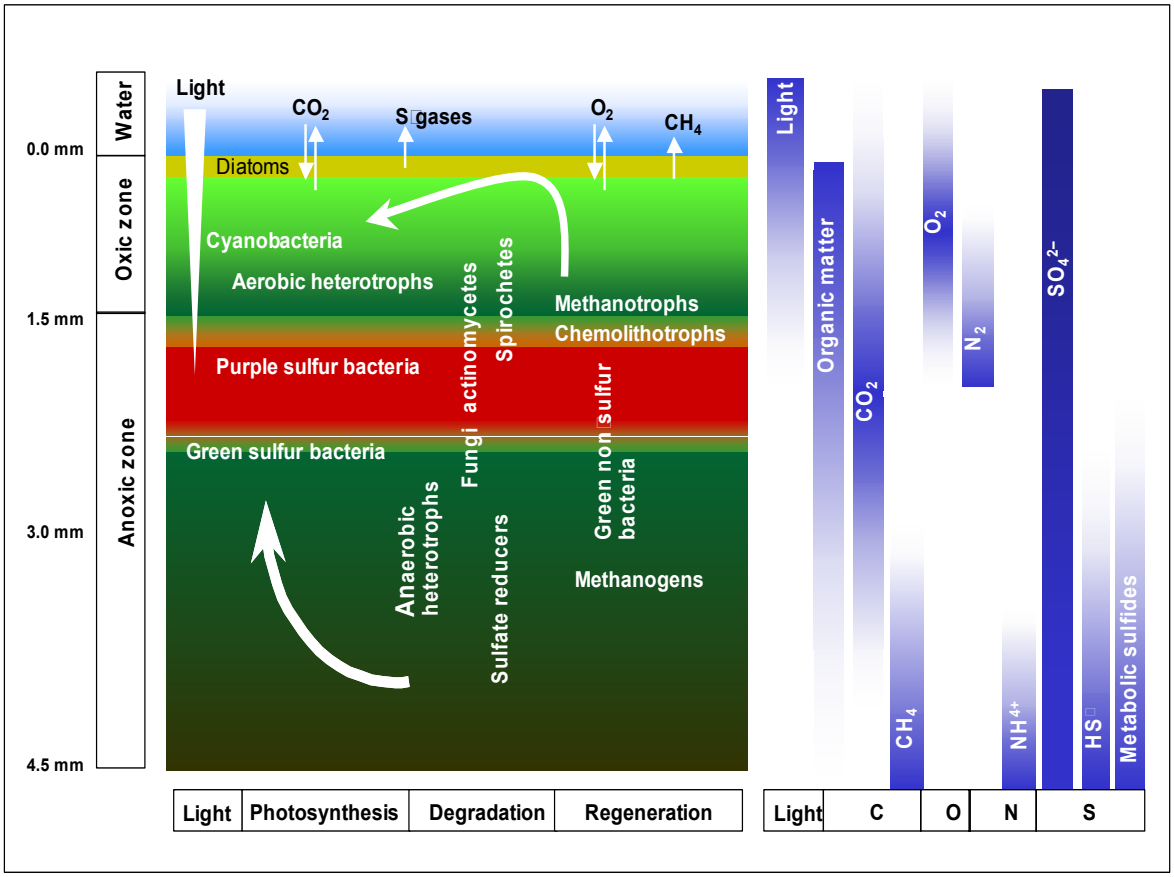

Figure I.1.2. Schematic of a cyanobacterial microbial mat with associated depth-related light and chemical gradients (Navarrete, 1999).

Carbon and oxygen cycling in microbial mats

During the day, light strikes the mat surface, fueling primary carbon fixation by both oxygenic and anoxygenic phototrophs. Some secondary carbon fixation will occur with the growth of non-photosynthetic autotrophic and heterotrophic bacteria. The sources of inorganic carbon (DIC) for primary production include diffusion from the overlying water, from both O_2 and anaerobic respiration in the photic zone (Canfield and Des Marais, 1993), and from heterotrophic activity deeper in the mat. Some dissolved organic carbon (DOC) obtained from the water column or within the mat, may also be incorporated into growing biomass, or may be lost from the mat by diffusion (Fig. I.1.3).

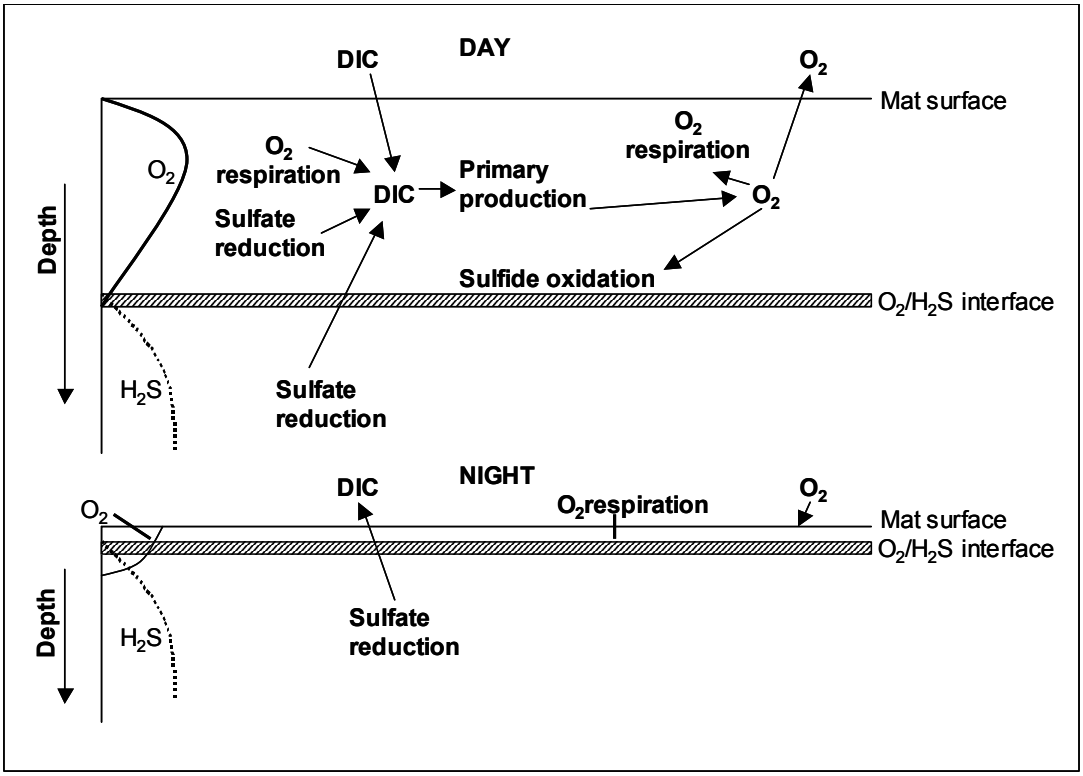

Figure I.1.3. Carbon and oxygen cycling in cyanobacterial mats.

Day: O_2 production from oxygenic photosynthesis may diffuse from the mat, diffuse into the O_2/H_2S interface to oxidize sulfide, or to be used in the aerobic zone for O_2 respiration, or to oxidize any sulfide produced by sulfate reduction in this zone. DIC is used by both oxygenic and anoxygenic phototrophs in primary production. The sources of DIC are diffusion from the overlying brine, diffusion from below the interface, and liberated in the oxic zone by O_2 respiration and sulfate reduction. Night: O_2 diffuses into the mat and is used to oxidize organic carbon (O_2 respiration) and sulfide produced by sulfate reduction

During the day, the only source of O_2 in the mat is primary production by oxygenic photosynthesis. Sinks for O_2 include both diffusion out of the mat and deeper into the mat to oxidize reduced chemical species such as sulfide and ammonia. Some O_2 will also used to oxidize both organic matter in the aerobic zone (O_2 respiration), as well as any sulfide produced in the aerobic zone by sulfate reduction (Fründ and Cohen, 1992). At night, the oxidation of organic matter produces DIC that diffuses out of the mat into the overlying water. Carbon oxidation can occur both by aerobic (O_2 respiration) and anaerobic pathways (sulfate reduction as the most important). As during the day, some DIC will also be used in the growth of non-photosynthetic bacteria, and DOC may be cycled within the mat and/or exchanged cross the mat-brine interface.

- Irradiance and temperature regulation of oxygen production rate

Previous studies have shown a clear correlation between O_2 production rate and light intensity (Canfield and Des Marais, 1993; Wieland and □ühl, 2000). Increasing surface irradiance increases light penetration and activates photosynthesis in deeper, light-limited parts of the mat, resulting in a deepening of the photic zone and in a linear increase of O_2 production. The rate of increase in net oxygen metabolism (photosynthesis minus oxygen consumption) is depth dependent, and is determined by the change in oxygen consumption activity due to the increasing supply of photosynthate from the photic zone and the decreasing supply of reduced compounds (from below) due to the oxygenation of deeper layers (Epping *et al.*, 1999).

Rates of O_2 production respond strongly to changes in temperature (Canfield and Des Marais, 1993; Epping and □ühl, 2000). One factor contributing to the temperature response of O_2 production is the fixation of CO_2 by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Photorespiration due to the oxygenase activity of Rubisco is believed to increase with temperature because the affinity constant of Rubisco for O_2 increases more slowly with temperature than for CO_2 (Berry and Raison, 1981). Moreover, according to initial studies about the regulation of Rubisco, a decrease of the photosynthetic activity would be expected with increasing oxygen concentration. However, recent studies have shown that the increased photosynthetic rates at high

oxygen concentration are probably caused by enhanced oxidation of organic matter and concomitant CO_2 production, and this fact can be explained by the turnover of the excreted photosynthate (Grötzschel and de Beer, 2002), by photorespiration or by the Mehler reaction (Wieland and □ühl, 2000a) (Fig. I.1.4).

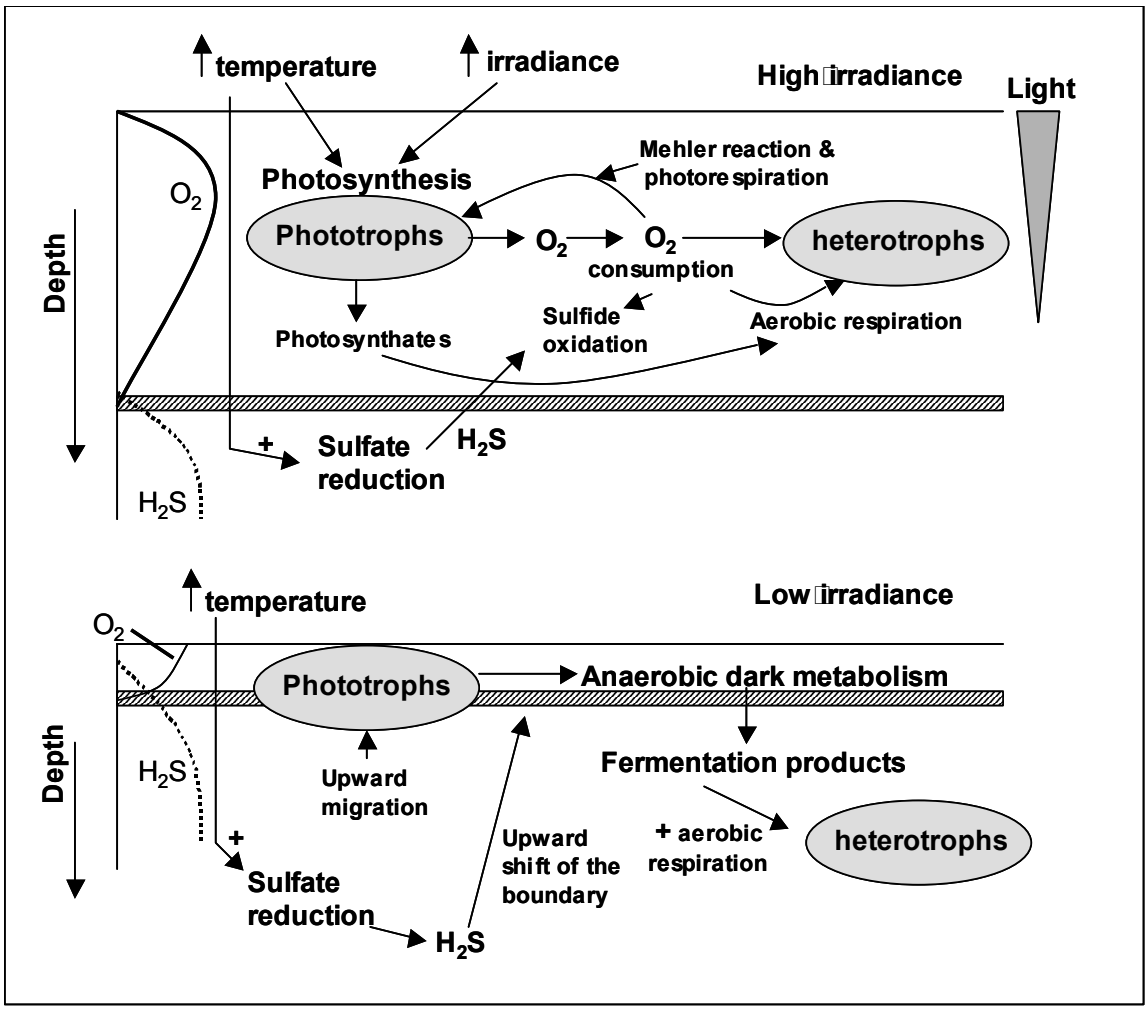

Figure I.1.4. Effects of irradiance and temperature on photosynthesis and oxygen consumption.

Increasing surface irradiance increase light penetration and activates photosynthesis, resulting in a deepening of the photic zone. In the light, O_2 consumption occurs in the photic zone by phototrophs and heterotrophs and in the aphotic zone by aerobic respiration and sulfide oxidation. In low-irradiance consitions, cyanobacteria switch to an anaerobic dark metabolism, and the fermentation products stimulate aerobic respiration when the light turned on and photosynthesis lead to a sufficient O_2 supply. The increase of Tº results in increasing rates of sulfate reduction which raise H_2S. High H_2S enhance diffusive transport towards the mat-water interface resulting in an upward shift of the O_2/H_2S boundary as indicated by the upward migration of *Beggiatoa* sp. (Epping and □ühl, 2000; Wieland and □ühl, 2000a, b).

Sulfur cycle in microbial mats

Microbial mats can be regarded as ideal model systems to study sulfur cycling. Cycling between elemental sulfur and sulfide is referred to as the 'small sulfur cycle' to differentiate this process from the 'large sulfur cycle' in which sulfur is cycled between sulfide and sulfate (Trüper, 1984) (Fig. I.1.5). Sulfate reduction is the key process in generating reduced sulfur compounds that are used by chemolithotrophic bacteria, anoxygenic phototrophic bacteria and sulfate-reducing bacteria (SRB). Chemolithotrophic bacteria obtain energy by oxidizing reduced sulfur compounds and anoxygenic phototrophic bacteria use reduced sulfur compounds as electron donors to fix CO_2 in the light. Sulfide oxidation and sulfide precipitation proceed efficiently at rates high enough to allow development of diatoms, which are very sensitive to sulfide toxicity, on the surface of the mat. Iron-bound sulfides or hydrogen sulfide can be reoxidized biologically or chemically to form thiosulfate (Detmers *et al*., 2001). Moreover, SRB may play an important role in the regulation of the electron flow in the sulfur cycle of microbial mats due to their metabolic versatility in reducing sulfate and thiosulfate and to disproportionate sulfur compounds (Visscher *et al.*, 1992).

Sulfide (H_2S, HS^-, S^{2-}) in marine sediments is generated mainly as a result of dissimilatory sulfate reduction, although sulfur reduction may contribute. Without the participation of O_2, purple sulfur bacteria (PSB) oxidize sulfide to zero-valent sulfur ('elemental sulfur', S^0), stored intracellularly (which is oxidized to sulfate without detectable intermediates). Some cyanobacteria (e.g. *Oscillatoria limnetica*) oxidize sulfide to S^0 (Cohen *et al*., 1975), others (e.g. *Microcoleus chthonoplastes*) to thiosulfate (De Witt and Van Germerden, 1988). Thiosulfate can serve as an electron donor for most chemotrophic and phototrophic sulfur bacteria. Oxidation of thiosulfate by anoxygenic phototrophic bacteria may result in the formation of tetrationate or in the formation of S^0 and SO_4^{2-}. Thiosulfate is also used by some SRB in a energy-conserving disproportionation reaction yielding sulfate and sulfide. In the presence of oxygen, sulfide can be oxidized to sulfate by colorless sulfur bacteria, thiobacilli form zero-valent sulfur as intermediate which is deposited outside the cells, whereas *Beggiatoa* and other large thiobacteria form intracellular sulfur. Unless oxygen is limiting, the end product of sulfide oxidation by colorless sulfur bacteria is sulfate.

Phototrophic bacteria with the ability of chemotrophic oxidize sulfide to S^0, which is stored intracellularly with sulfate as end product (Van Germerden, 1993).

Oxygen-tolerant sulfate reduction has been demonstrated in microbial mats (Minz *et al*., 1999). Indeed, the sulfate reduction rates measured under oxic conditions during daytime often exceed those observed at night under anoxia (Visscher *et al*., 1992). This phenomenon is explained in part by elevated temperatures during the day since sulfate reduction rates generally show a temperature-dependence (Jørgensen, 1994). Ultimately, the high sulfate reduction rates in hypersaline mats are driven by cyanobacterial production *in situ* (Fründ and Cohen, 1992).

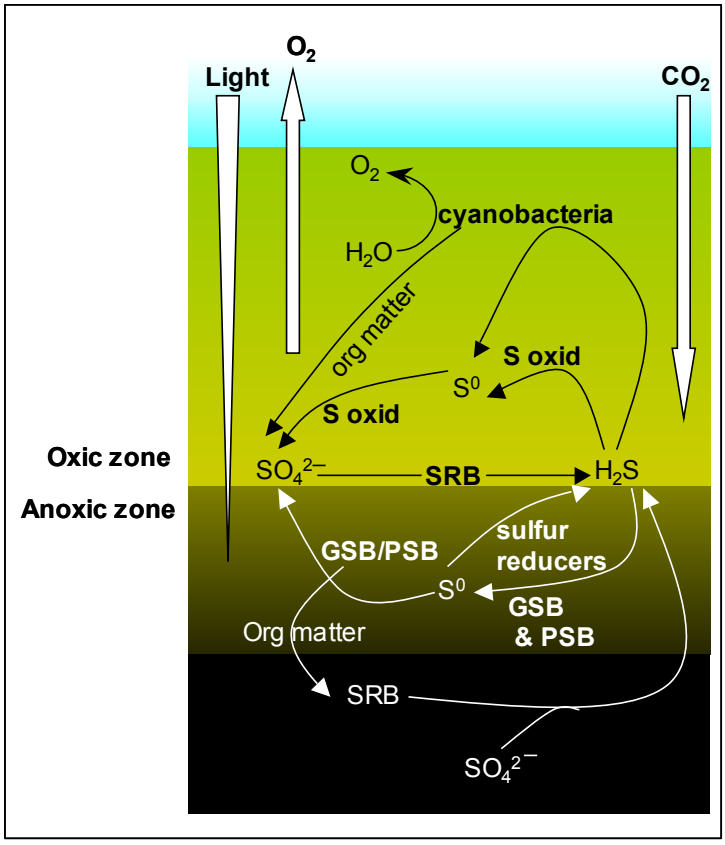

Figure I.1.5. Biochemical sulfur cycle in a sedimentary ecosystem with oxic/anoxic zones (modified from Guerrero *et al*., 2002).

During light conditions, the oxygenic phototrophs (cyanobacteria) perform an active photosynthesis and generate organic matter. The oxygen-tolerant sulfate reducers use this organic matter and activate a daytime sulfate reduction. Sulfide (H_2S) is generated mainly by dissimilatory sulfate reduction by sulfate-reducing bacteria (SRB), although sulfur reducers may contribute. Purple and green sulfur bacteria (PSB/GSB) oxide sulfide to 'elemental sulfur' (S^0), which is eventually stored, and then it is oxidized to sulfate. Some cyanobacteria can also oxidize sulfide to S^0.

- Volatile organic sulfur compounds

When there is a conversion from inorganic to organic carbon by autotrophs, organic biomarkers can accumulate in the lithosphere and reduced C-containing gases (e.g. methane, low-molecular weight fatty acids, and volatile organosulfur compounds, VOSC, as dimethyl sulfide and methanetiol, MT) can enter the atmosphere. Dimethyl sulfide (DMS) is the single most important biogenic contributor of S to the atmosphere (Visscher, 1996), its oxidation leads to the formation of cloud condensation nuclei and is linked to planetary albedo (Charlson *et al*., 1987). Considering the extent of microbial mat predominance on early Earth, even a small contribution could have had a significant impact on the chemistry of the atmosphere (Des Marais and Walter, 1999).

In most marine and hypersaline environments studied to date, the osmolyte dimethylsulfoniopropionate (DMSP) has been the major precursor of DMS production (Jonkers *et al.*, 1998). However, recent studies (Visscher *et al*., 2003) have shown that DMS and MT can be produced biogenically as a result of community metabolism, and not to be formed from the breakdown of DMSP. Alternative sources for DMS include microbial reduction of dimethylsulfoxide (DMSO) and methylation of methanethiol (□iene and Capone, 1988) (Fig. I.1.6).

Iron cycling

Several authors have discussed the possibility of a naturally occurred oxidation of ferrous ion [Fe(II) or Fe^{+2}] without free oxygen and the implications of this process would have for the understanding of precambrian banded iron formations. On the other hand, there have been speculations about a biological oxidation of ferrous ion by photosynthetic microorganisms without free oxygen (Cohen, 1983). Cohen (1983) could measure a Fe^{2+}-dependent photoassimilation of CO_2 by cyanobacteria from mats. Other studies (Widdel *et al*., 1993) have described purple bacteria which grow by using ferrous ion as electron donor for anoxygenic photosynthesis. A prerequisite for iron cycling would be the production of Fe^{+3} by Fe^{+2}-dependent anoxygenic photosynthesis between the oxic/sulfidic layer at suitable light conditions (Fig. I.1.7). This Fe^{+3} would react with the produced sulfide in this layer of high sulfate reduction activity. Sulfate

reduction and FeS precipitation at greater depth underneath the oxic zone of anoxygenic photosynthesis lead to burial and therefore building up of a pronounced FeS and reduced sulfur pool with depth (Wieland *et al*., 2005). Increasing irradiance and therefore increasing oxygenation of the mat during the day led to accumulation of Fe^{+3}. Reduction of Fe^{+3} occur at the same time in the oxic layer via the H_2S produced by sulfate reduction, but Fe(III) predominate due to rapid re-oxidation of the formed Fe(II). During sunset at decreasing O_2 penetration and concentration but at a constant depth of the upper sulfide boundary, the accumulated Fe^{+3} pools reacted with the produced sulfide. After exhaustion of the Fe^{+3} deposits towards the end of the night, FeS and free sulfide accumulated in this layer, which is again re-oxidized during sunrise.

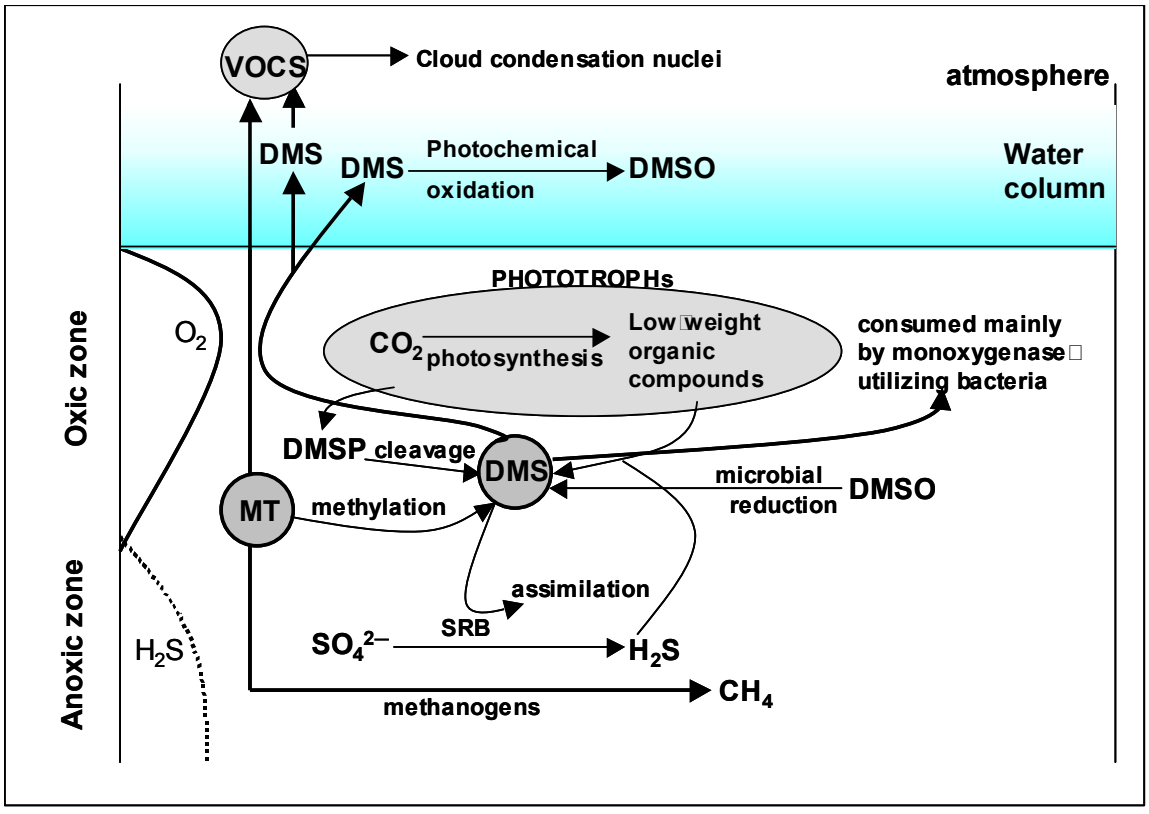

Figure I.1.6. Volatile organic sulfur compounds (VOCS) cycling in microbial mats.

DMS and MT are probably formed by the reaction of photosynthetically produced organic compounds and biogenic H_2S produced by sulfate reduction. DMS can also be formed by microbial reduction of DMSO or by cleavage of DMSP or S-containing amino acids. The major DMS consumers with oxygen are monoxygenase-utilizing bacteria, and under anoxia DMS is consumed by SRB and methanogenic bacteria.

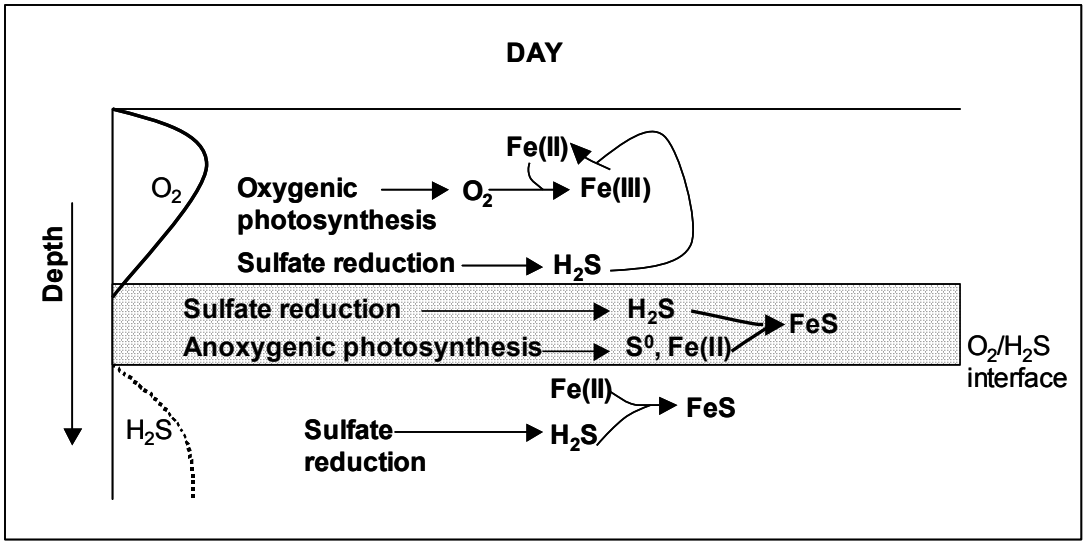

Figure I.1.7. Relationship between iron and sulfur cycle in a microbial mat during daylight conditions (Wieland *et al*., 2005).

Nitrogen cycling

The mat building photosynthetic microorganisms need nitrogen to produce biomass. The availability of combined nitrogen depends on the balance between sources and sinks in the mat (Nielsen and Sloth, 1994). Sources are dissolved nitrogen in the overlying water, nitrogen from mineralization processes, and nitrogen fixation; and sinks are burial of nitrogen, efflux of dissolved nitrogen, and denitrification. The general nitrogen cycle in a microbial mat is outlined in Fig. I.1.8.

Very little or no nitrogen leaves these communities, once it has been brought in via either the process of nitrogen-fixation (N-fixation) or by uptake from the water column. Microbial mats may not be sources of nitrogen to the overlying water column, but rather sinks. It seems unlikely that the N-fixation which occurs in mats directly supports productivity in the overlying water column due to the small fluxes of nitrogen out of these systems (Bebout *et al*., 1994). The remarkable success of mats in these nitrogen-limited environments has been attributed to the ability of specific groups of microorganisms (anoxygenic and oxygenic phototrophs, chemolithotrophic and heterotrophic bacteria; for details see Paerl *et al*., 1994) to 'fix' (reduce) atmospheric nitrogen (N_2), thereby providing nitrogen biologically available. Nitrogen fixation is a

prokaryotic process, confined to specific eubacterial and cyanobacterial genera (Paerl, 1990). The enzyme complex, nitrogenase, responsible for the conversion of N_2 in NH_4^+, only function under anoxic conditions, forcing confinement of N_2 fixation to obligate anaerobes, microaerophiles, and among oxygenic cyanobacteria in O_2-devoid cells (heterocysts) or intracellular regions supporting localized O_2 depletion. Some environmental factors control the N_2 fixation in mats, e.g. irradiance, temperature, nutrient limitation, end product suppression by ammonium, and oxygen inhibition (Paerl *et al*., 1994).

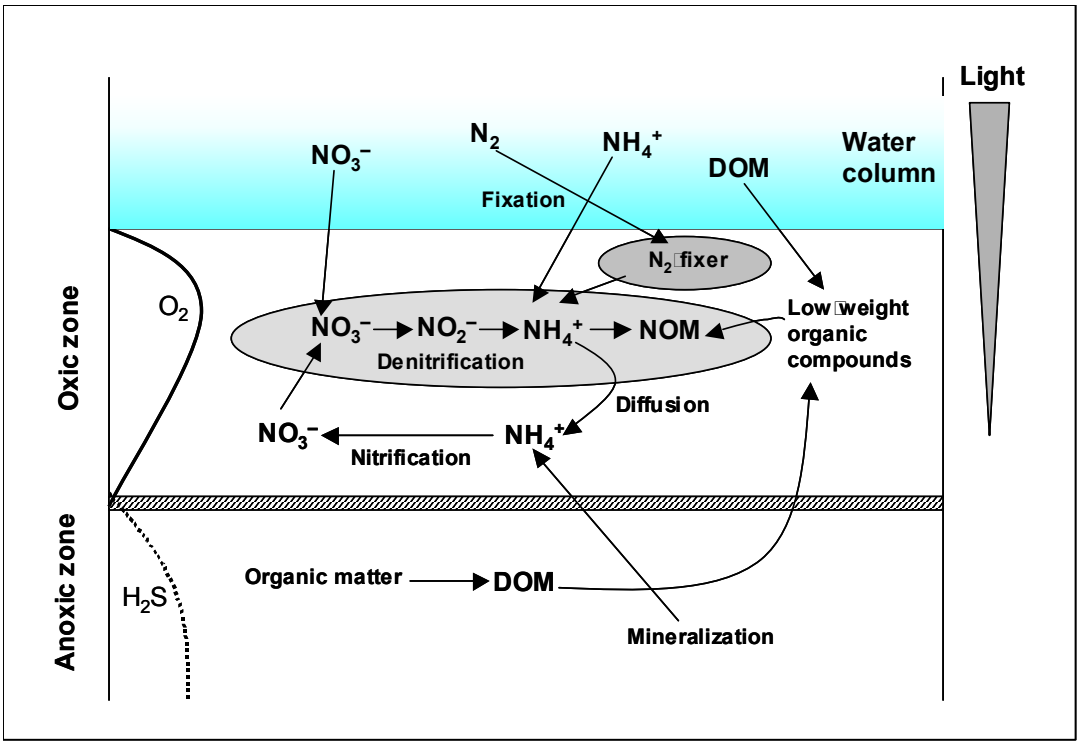

Figure I.1.8. Nitrogen cycling in microbial mats.

Photosynthesis is restricted to the upper photic zone where O_2 producing phototrophs assimilate combined nitrogen from the sediments below or from the overlying water. Ammonia is directly incorporated into organic compounds while NO_3^- and NO_2^- have to be reduced first to ammonia by assimilatory processes. The oxic zone where the nitrifying bacteria are active extends below the photic zone. The nitrification process is fueled by ammonia from mineralization processes in the oxic and anoxic zones or diffusing from the overlaying water. Denitrification activity is restricted to the anoxic zone and must depend on the diffusion of NO_3^- or NO_2^- from the overlying water column or from the nitrification zone (Nielsen and Sloth, 1994). NOM: nitrogenated-organic matter; DOM: dissolved organic matter.

Gas production

Hoehler *et al*. (2001) observed that subtidal mats generated carbon monoxide (CO), methane (CH_4), and significant quantities of hydrogen (H_2). Rates of emission of CO correlated with rates of photosynthesis, implicating cyanobacteria, diatoms, or both sources. Emission rates of H_2 were greatest at night, consistent with fermentation under anoxic conditions. These fluxes of reduced gases are significant for at least three reasons. First, microorganisms that inhabit cyanobacterial mat benefit from abundant products of photosynthesis. Therefore, the advent of oxygenic photosynthesis billion years ago perhaps triggered an evolutionary transformation and diversification of the anaerobical microbial world. Second, the proximity of mats to the atmosphere allows a substantial fraction of reduced gases to escape biological recycling and to enter and alter atmospheric composition (Hoehler *et al*., 2001). Third, if analogous microbial ecosystems exist on habitable planets, they should influence the composition of their atmospheres (Des Marais, 2003).

These studies (Hoehler *et al*., 2001) have been observed that mat communities exhibit a CO production tied to cyanobacterial photosynthesis. On the other hand, mats also generate large quantities of H_2 during dark and anoxic conditions in a process closely dependent on oxygenic photosynthesis. Cyanobacterial photosynthesis can be indirectly responsible for this activity, by fueling night-time production of H_2 by the O_2-sensitive processes of nitrogen fixation and fermentation. Moreover, CH_4 production in microbial mats is stimulated by increasing H_2 concentrations, because of the fact that sulfate-reducing bacteria efficiently out-compete CH_4-producing methanogens (*Archaea*) for the common substrate H_2. As a result, the very large flux of H_2 in microbial mats is interesting in a geochemical context, as a potential mechanism for oxidation of the Earth's surface. Net oxidation of the planet via oxygenic photosynthesis is only possible when the reducing power generated is effectively removed from the system, and this removal could be partially due to the H_2-flux into the atmosphere and subsequent oxidations of large reservoirs of reduced iron and sulfur, which must have precede actual atmospheric oxygenation.

In modern marine microbial mats, primary production is remineralized mainly by aerobic respiration and by sulfate reducing activity (Canfield and Des Marais, 1993); methanogenesis is quantitatively unimportant (Oremland and □ing, 1989). This fact is due to the limited scale to which light penetrates the densely packed assemblages of photosynthetic organisms resulting in the limitation of the photosynthetic activity within millimeters. Almost all the organic carbon becomes available and is remineralized in this narrow region (Canfield and Des Marais, 1993) with two important consequences for methane production. First, methanogens as strict anaerobes are excluded from the oxic zone and therefore from the zone of active carbon cycling. Additionally, at the photosynthetic zone, diffusion is highly efficient and this means that sulfate can be rapidly re-supplied to the zone of carbon cycling even when the sulfate concentration in the overlying water is low (Bebout *et al*., 2004).

- Mat-building microorganisms and minority populations

The major groups that are distributed in different depth layers of the mat have been widely studied. The driving force of most microbial mats is photosynthesis by cyanobacteria. Subsequently, dissimilatory sulfate-reducing bacteria (SRB), using excretion-, lysis-, and decomposition products of cyanobacteria, produce sulfide. The sulfide can be reoxidized to sulfate by colorless sulfur bacteria and purple sulfur bacteria (PSB). Aerobic heterotrophic bacteria are functionally important as their activity leads to oxygen depletion, and fermentative microorganisms provide growth substrates for SRB. In microbial mats, these metabolically different groups of microorganisms live together in a layer of 5–10 mm thickness. Those species making up the phylogenetic groups (Table I.1.2) perform specific interrelated metabolic functions in the community but little is know about microorganisms that are not distributed in layers and that represent only a small fraction of the community. An extremely dynamic community sustains a functionally stable ecosystem, and a large number and diversity of minority populations likely contribute significantly to these dynamics (Fernández *et al*., 1999).

Table I.1.2. Examples of major groups inhabiting microbial mats.

Group	Examples of genera	References
DIATOMS	*Navicula* sp., *Nitzschia* sp.	Mir *et al*., 1991
CYANOBACTERIA **Unicellular**	*Aphanothece* sp., *Microcystis* sp., *Gloeocapsa* sp., *Synechocystis* sp., *Chroococcus* sp., *Pleurocapsa* sp.	García-Pichel *et al.*, 1998; Wieland *et al*., 2003; Fourçans *et al.*, 2004
Filamentous	*Phormidium* sp., *Microcoleus* sp., *Lyngbya* sp., *Spirulina* sp., *Oscillatoria* sp., *Pseudoanabaena* sp.,	Urmeneta *et al.*, 2003; Vincent *et al.,* 2004; Solé *et al*., 1998
PURPLE SULFUR BACTERIA	*Chromatium* sp., *Thiocapsa* sp., *Thioflavicoccus* sp., *Thiorhodococcus* sp., *Halorhodospira* sp., *Rhodospirillum* sp., *Ectothiorhodospira* sp., *Thiocystis* sp., *Allochromatium* sp.	Zaar *et al*., 2003; Imhoff and Pfenning 2001; Pfenning *et al.*, 1997; Caumette *et al.*, 2004; Hirschler-Rea *et al.,* 2003
PURPLE NON SULFUR BACTERIA	*Rhodobacter* sp., *Rhodoferax* sp., *Roseospira* sp., *Roseospirillum* sp., *Rhodomicrobium* sp.	Heising *et al.*, 1996; Jung *et al*., 2004; Guyoneaud *et al.*, 2002; Glaeser and Overmann 1999.
GSB (Green sulfur)	*Chlorobium* sp.	Caumette, 1989
GREEN NON SULFUR BACTERIA	*Chloroflexus* sp., *Oscillochloris* sp.	□lappenbach and Pierson 2004; Nübel *et al.*, 2001
SULFUR-OXIDIZING BACTERIA	*Beggiatoa* sp., *Thiomicrospira* sp., *Thiobacillus* sp., *Thiovulum* sp.	Mills *et al.,* 2004; Brinkhoff and Muyzer 1997; Thar and □ühl 2002;
HETEROTROPHIC BACTERIA **Aerobic and anaerobic organoheterotrophs**	*Marinobacter* sp., *Halomonas* sp., *Roseobacter* sp., *Psychroflexus* sp., *Pseudoalteromonas* sp., *Spirochaeta* sp., *Titanospirillum velox, Mobilifilum chasei, Aeromonas* sp., *Pseudomonas* sp., *Vibrio* sp., *Bacillus* sp., *Clostridium* sp., *Halanerobium* sp.	Jonkers and Abed, 2003; Teske *et al.,* 2000; Margulis *et al.,* 1993; Guerrero *et al.,* 1999; Margulis *et al*., 1990; Donachie *et al*., 2004a,b; Spring *et al.*, 2003; Ollivier *et al.*, 1994
SULFATE-REDUCING BACTERIA	*Desulfovibrio* sp., *Desulfobacter* sp., *Desulfococcus* sp., *Desulfosarcina* sp., *Desulfonema* sp.	Teske *et al*., 1998; Muβmann *et al.*, 2005
ARCHAEA	*Methanobacterium* sp., *Methanococcus* sp., members of the *Halobacteriales* order etc.	Cytryn *et al.*, 2000; Elshahed *et al.*, 2004; Ochsenreiter *et al*., 2002

- Future perspectives

Study of microbial communities has raised questions about the composition, structure and stability of these communities and about the activity and function of the individual inhabitants. Traditional microbiological techniques and conventional microscopy are insufficient means to answer these questions (Muyzer and de Waal, 1994). Most of the bacteria in natural samples cannot be detected by conventional microscopy, because they adhere to sediment particles. Activity measurements of bacteria in sediments have been performed, but they lack specificity to discriminate between the actions of different species. Physiological experiments have also been used to characterize isolated species. However, it is now widely recognized that less than 20% of the naturally occurring bacteria have been isolated and characterized.

The application of molecular biological techniques offers new opportunities for the analysis of structure and species composition of microbial communities. Since these techniques are not dependent upon the enrichment or pure culture isolation, they promise a complete accounting of the community structure and direct access to the study of microorganisms at the levels of population and single cells (Stahl and Capman, 1994). Thus, the integration of molecular techniques (DNA, lipid analysis etc.) with more standard or classical approaches (e.g. microscopy, microelectrodes, stable isotopes, radiotracers, analytical chemistry) should provide better overview of the dynamic and composition of microbial mats and other microbial communities.

The study of microbial mats, including their community composition, metabolic relationships and physiological status, can expand our knowledge of these first microbial ecosystems to have evolved on Earth. Likewise, their ecological success and their broad array of metabolic activities suggest that microbial mat ecosystems will have useful applications in the bioremediation of polluted sites as well as in the biogeneration of useful products (Bender and Phillips, 2004). Photosynthetic microbial mats contain ecophysiological strategies to support life under a broad range of environmental conditions (Paerl *et al*., 2000) and can be used to characterize the requirements for microbial life on Earth and, potentially, on other planets.

I. INTRODUCTION

2. The Signature Lipid Biomarker (SLB) approach

- Classification of lipids

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1. 3. Aldehydes and vinyl-ether lipids
1. 4. Amino compound-containing lipids
1. 5. Amino alcohols and ceramides
1. 6. Terpenoid lipids

2. Complex lipids

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2. 3. Lipoamino acids

- Lipid Biomarkers and the SLB approach

1. The polar lipid fraction

1.1. Viable biomass
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1.3. Community composition

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3. Objectives and structure of this work

2. The Signature Lipid Biomarker (SLB) approach

- Classification of lipids

General text books usually describe lipids as a group of naturally occurring compounds, which have in common a ready solubility in such organic solvents as hydrocarbons, chloroform, benzene, ethers and alcohols. They include a diverse range of compounds, like fatty acids and their derivatives, carotenoids, terpenes, steroids and bile acids. Lipids may be relatively simple molecules, as for example the fatty acids themselves, or more complex and contain phospho- or sulpho- groups, amino acids, peptides, sugar or even oligosaccharides.

The diversity of lipids signifies a variety in function. Lipids can act as storage material in animal, plant and microbial cells, where the lipids typically occur in the form of triacylglycerols in eukaryotic cells (also found in the bacteria *Corynebacterium* and *Mycobacterium*, Daniel *et al*., 2004; Wältermann *et al*., 2005), and as poly-β-hydroxyalkanoates in certain prokaryotes, and they are also responsible for the structure of cell membranes. Besides these well-known roles, lipids carry out many other functions. They are associated with the photosynthetic processes in plants and microorganisms, providing not only chlorophyll itself but many of the quinones and pigments associated with the process of converting light energy into chemical energy. Lipids, besides their universal role in the structure of membranes, also participate in the organization of bacterial cell envelopes, as components both the lipoteichoic acids associated with the inner cytoplasmatic membrane of Gram-positive bacteria and of the lipopolysaccharides and lipoproteins of the outer membrane of Gram-negative bacteria.

Various systems of classifying lipids have been published. A common and practically useful system is the division into ‘neutral’ or ‘apolar’ lipids, and ‘polar’ or ‘amphiphilic’ lipids. To simplify the study of lipid classes, we propose a classification based on the classification proposed by the Cyberlipid center (see ‘Useful websites’ section) and Ratledge and Wilkinson (1988). In the following pages, this lipid classification will be explained giving more emphasis to those kinds of microbial lipids that can be analyzed in the Signature lipid biomarker (SLB) approach or that can be

important as biomarkers of biological activity, for that reason some lipid categories have been avoided in this division. For more information, see the references mentioned above.

In the following scheme, we propose a classification based on two major classes,

1. Simple lipids
    - 1.1. Fatty acids
    - 1.2. Simple fatty esters
        - *Acylglycerols*
        - *Wax esters and fatty alcohols*
        - *Polyhydroxyalkanoates*
    - 1.3. Aldehydes and vinyl ether lipids
    - 1.4. Amino compound-containing lipids
    - 1.5. Aminoalcohols and ceramides
    - 1.6. Terpenoid lipids
        - *Steroids and related lipids*
        - *Carotenoids*
        - *Polyprenoids*
        - *Chlorophylls*
        - *Isoprenoid quinones*
        - *Isopranyl ethers and ether lipids*
2. Complex lipids
    - 2.1. Phospholipids
        - *Glycerophospholipids*
        - *Sphingophospholipids*
    - 2.2. Glycolipids
        - *Lipopolysaccharides*
    - 2.3. Lipoaminoacids

1. Simple Lipids

1. 1. Fatty acids

Fatty acids are of the widest distribution in all living cells. They are rarely found in their free form, but are linked to a variety of molecules of which glycerol is the most common. To describe precisely the structure of a fatty acid molecule, one must give the length of the carbon chain (number of carbon), the number of double bonds and also the exact position of these double bonds; this will define the biological reactivity of the fatty acid molecule and even of the lipid containing the fatty acids studied.

Saturated fatty acids

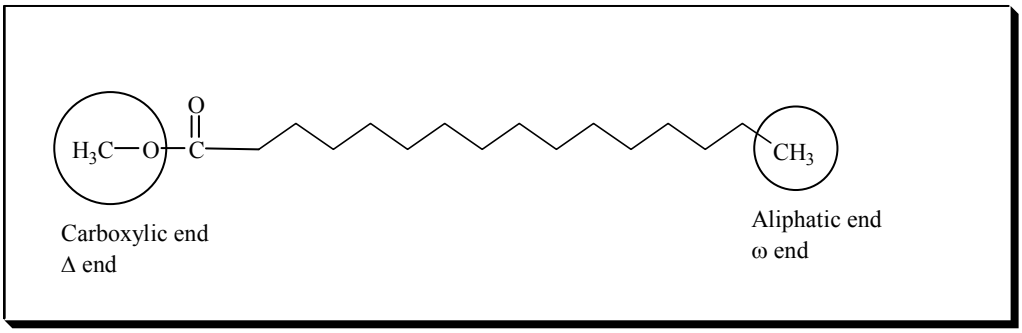


Figure I.2.1. Formula of a saturated, straight-chain fatty acid (16:0).

Table I.2.1 list most of the commonly saturated, straight-chain fatty acids which have the general formula shown in Fig. I.2.1.

Branched saturated fatty acids

Branching of the fatty acids is usually confined to the appearance of one or two methyl groups on the alkyl chain. The nomenclature for these acids follows the following guidelines: the number before the colon indicates the total number of carbon atom, and to denote branching, the prefix ‘br’ is used. When the methyl group is at the penultimate (sometimes referred to as ω-1) carbon atom (distal from the carboxyl group), the fatty acids are termed *iso* fatty acids; when the methyl group is on the third

(*ω*-2) carbon from the end, the fatty acids are referred to as *anteiso* fatty acids (Fig. I.2.2). Some of the more common, branched chain fatty acids are listed in Table I.2.2

Table I.2.1. Some saturated, straight-chain fatty acids.

Systematic name	Trivial name	Shorthand designation
Decanoic acid	Capric acid[1]	10:0
Undecanoic acid		11:0
Dodecanoic acid	Lauric acid	12:0
Tridecanoic acid		13:0
Tetradecanoic acid	Myristic acid	14:0
Pentadecanoic acid		15:0
Hexadecanoic acid	Palmitic acid	16:0
Heptadecanoic acid	Margaric acid	17:0
Octadecanoic acid	Stearic acid	18:0
Nonadecanoic acid		19:0
Icosanoic acid[2]	Arachidic acid	20:0
Docosanoic acid	Benhenic acid	22:0
Tetracosanoic acid	Lignoceric acid	24:0
Hexacosanoic acid	Cerotic acid	26:0
Octacosanoic acid	Montanic acid	28:0
Triacontanoic acid	Melissic acid	30:0
Dotriacontanoic acid	Lacceroic acid	32:0

[1]No longer used in view of posible confusion with caproic (6:0) or caprylic (8:0) acids.
[2]The previous spelling of the name (eicosanoic acid) is still widely used.

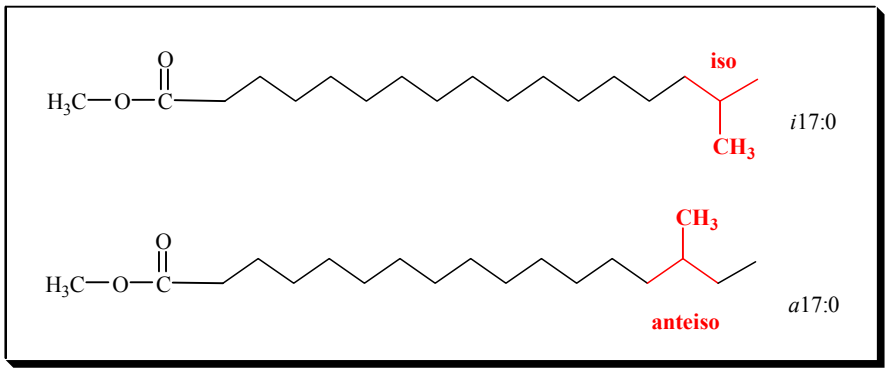


Figure I.2.2. Examples of *iso* and *anteiso* branching in a saturated fatty acid (17:0).

Besides simple branching, a fatty acid may possess a cyclopropane ring or, more exceptionally, in microorganisms a cyclopropene ring. In order to denote a branching by a cyclopropane ring, the prefix '*cy*' is used. Other alicyclic fatty acids are the *ω*-cyclohexyl and *ω*-cycloheptyl compounds which occur mainly in thermoacidophylic

bacteria (Hippchen *et al*., 1981) but also in some mesophiles (Kawaguchi *et al*., 1986). An example is given in Figure I.2.3.

Table I.2.2. Some saturated, branched-chain fatty acids.

Carbon atoms	Systematic name	Trivial name	Shorthand designation		
			Non-specific	*Iso*/ *anteiso*	Specific
15	13-Methyltetradecanoic acid	Isopentadecanoic acid	br15:0	*i*15:0	13Me14:0
16	14-Methylpentadecanoic acid	Isopalmitic acid	br16:0	*i*16:0	14Me15:0
16	13-Methylpentadecanoic acid	Anteisopalmitic acid	br16:0	*a*16:0	13Me15:0
18	16-Methylheptadecanoic acid	Isostearic acid	br18:0	*i*18:0	16Me17:0
19	10-Methyloctadecanoic acid	Tuberculostearic acid	br19:0	-	10Me18:0

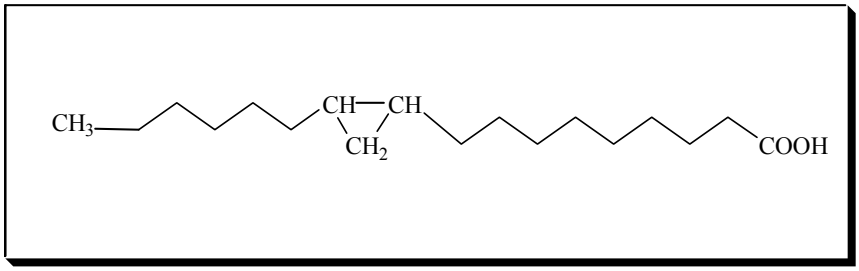


Figure I.2.3. An example of an alicyclic fatty acid (*cy*19:0).

Unsaturated fatty acids

Straight chain fatty acids with one (monoenoic) or more (polyenoic) double bonds have been isolated from most microorganisms. The double bond usually has the *cis* configuration. Two alternative systems for designating the type of unsaturation are used, though in both systems the position(s) of the double bond(s) is indicated immediately after the numeral indicating the number of double bonds. For example, a fatty acid such as linoleic acid, *cis*, *cis*-octadeca-9,12-dienoic acid, can be represented as *cis*, *cis*-18:2(9,12) or 18:2(9*c*, 12*c*).

Occasionally, one system for locating the double bond by counting from the ω or methyl end of the chain is used. The system employing an (ω-x) prefix is comparable with the system described above, in that the carbon atom specified thereby as the first in

the double bond is that nearest the carboxyl group. In the second system, which employs an (ω *n*) suffix or sometimes even an (*n* x) suffix, the double-bonded carbon atom specified is that nearest the methyl end. An example is detailed in the following figure.

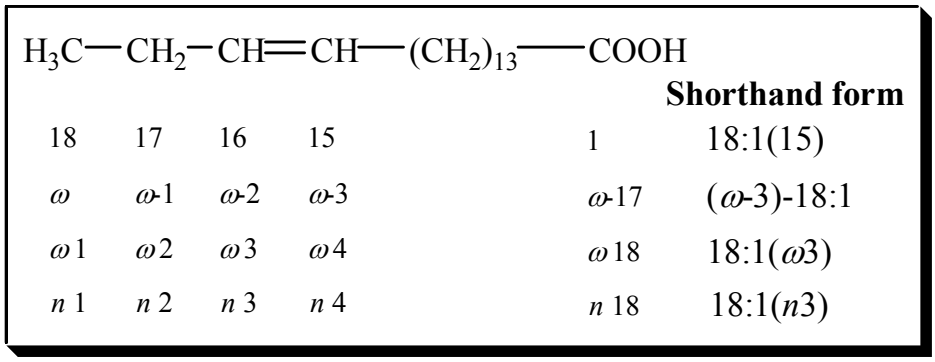


Figure I.2.4. Systems for designating unsaturated fatty acids.

A further disadvantage of these systems is that in polyunsaturated fatty acids only the position of the 'first' double bond is specified, it then being assumed that additional double bonds are methylene-interrupted (see Fig. I.2.6). Thus, the isomeric octadecatrienoic acid 18:3(6,9,12) (Table I.2.3) is often referred to as the ω-6 (or *n*6). Table I.2.3 list some of the most common mono-, di-, and poly-enoic fatty acids, together with alternative schemes of designation.

Table I.2.3. Some unsaturated fatty acids.

Systematic name	Trivial name	Shorthand designations
Monoenoic fatty acids		
cis-Hexadec-9-enoic acid	Palmitoleic acid	*cis*-16:1(9), (ω-7)-16:1, 16:1ω7
cis-Octadec-9-enoic acid	Oleic acid	*cis*-18:1(9), (ω-9)-18:1, 18:1ω9
Dienoic fatty acids		
cis, *cis*-Octadeca-9,12-dienoic acid	Linoleic acid	*cis*,*cis*-18:2(9,12), (ω-6)-18:2, 18:2ω6
trans, *trans*-Octadeca-9,12-dienoic acid	Linelaidic acid	*trans, trans*-18:2(9,12), (ω-6)-18:2, 18:2ω6
Polyenoic fatty acids		
cis, cis, *cis*-Octadeca-9,12,15-trienoic acid	α- linolenic acid	*cis,cis*,*cis*-18:3(9,12,15), (ω-3)-18:3, 18:3ω3
cis, cis, *cis*-Octadeca-6,9,12-trienoic	γ- linolenic acid	*cis,cis*,*cis*-18:3(6, 9,12), (ω-6)-18:3, 18:3ω6

Monoenic fatty acids

Mono-unsaturated normal fatty acids are widespread in the living world where they occur mostly as the *cis*-isomer. The monoenoic fatty acids have the following general structure:

$$CH_3(CH_2)_xCH=CH(CH_2)_yCOOH$$

As a general rule, they tend to have an even number of carbon atoms and the unique double bond may be in a number of different positions. The double bond can exist in two stereoisomeric forms: the *cis* (or *Z* configuration) and the *trans* (or *E*) configuration (Fig. I.2.5).

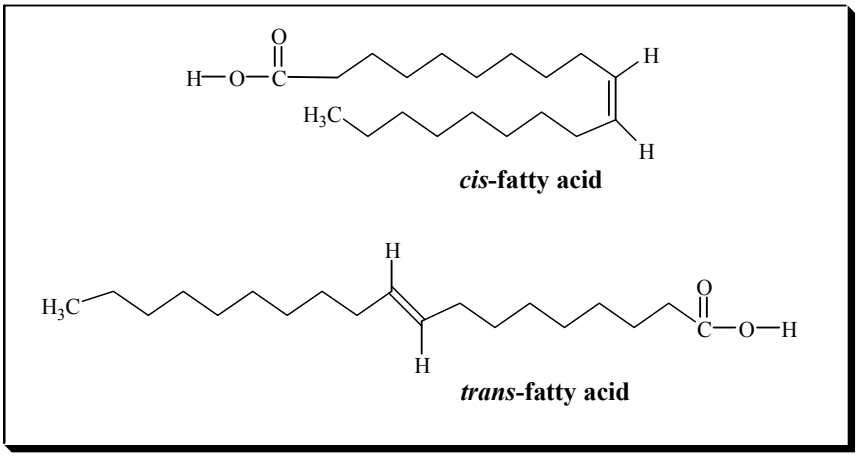


Figure I.2.5. Steroisomeric form of the double bond in a saturated fatty acid.

Polyenoic fatty acids

These fatty acids (also called polyunsaturated fatty acids, PUFA) have 2 or more *cis* double bonds which are frequently separated from each other by a single methylene group (methylene-interrupted polyenes). Some rare polyenoic fatty acids may have also a *trans* double bond. Some other polyunsaturated fatty acids undergo a migration of one of their double bonds which are not again methylene-interrupted and are known as conjugated fatty acids (Fig. I.2.6).

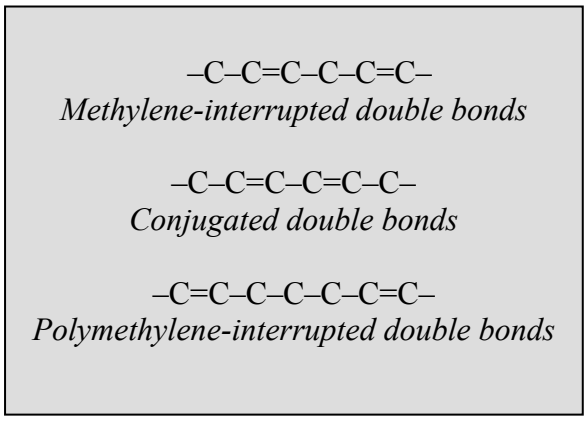


Figure I.2.6. Polyenoic fatty acids.

This classification was established according to the position of double bonds.

Oxyfunctionalized fatty acids

A wide group of fatty acids with a second oxygen-cointaining functional group occur in microorganisms. The hydroxyl group may occur at various positions in the carbon chain which can be saturated or monoenoic, and may contain more than one additional function. Some examples are listed in Table I.2.4.

Table I.2.4. Some oxyfunctional fatty acids.

Systematic name	Trivial name	Shorthand designations
Hydroxy fatty acids		
2-Hydroxyhexadecanoic acid	2-hydroxypalmitic acid	2-OH-16:0
3-Hydroxyoctadecanoic acid	3-hydroxystearic acid	3-OH-18:0
Oxo fatty acids		
8,9-Dihydroxy-13-oxodocosanoic acid		8,9-di-OH-13-oxo-22:0
Epoxy fatty acids		
cis-12,13-Epoxy-*cis*-octadec-9-enoic acid	Vernolic acid	12,13-epoxy-*cis*-18:1(9)

In some bacteria, complex hydroxy, branched-chain fatty acids, named mycolic acids, are described. Mycolic acids are the major component of the cell wall of Actinomycetes forming a distinct suprageneric taxon that encompasses the genera *Mycobacterium*, *Gordonia*, *Nocardia* and *Rhodococcus* (Fig. I.2.7).

Moreover, a large variety of bacteria are able to synthesize polyesters (polyhydroxyalkanoates) forming linear chains of esterified 3-hydroxy acids (Lee,

1996). The structure, function and ecophysiological role the polyhydroxyalkanoates is detailed in the 'Simple fatty esters' section.

Apart from that, hydroxy fatty acids typically are constituents of lipopolysaccharides (LPS), which are endotoxins located in the outer membrane of gram-negative bacteria (for more details see section 2.2 Glycolipids).

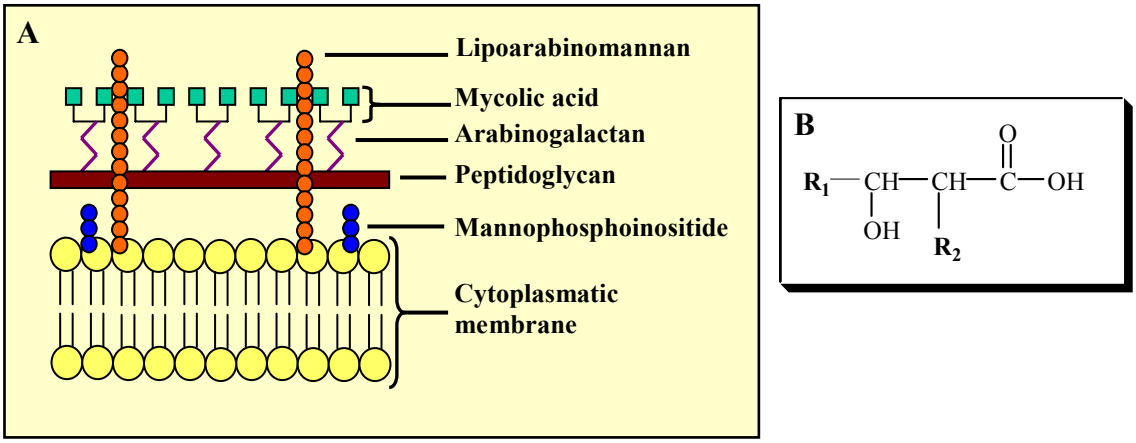


Figure I.2.7. Mycobacterial cell wall.

(A) Mycolic acid position in a mycobacterial cell wall.
(B) Mycolic acid structure: High molecular weight fatty acids with a hydroxyl group at position 3, a long carbon chain at position 2 (R_2), and other groups on R_1 (keto-, methoxy-, carboxy-). Total carbon atoms: aprox. 60–80.

1. 2. Simple fatty esters

Acylglycerols

Free fatty acids do not usually accumulate intracellularly because of their toxic effects (binding to and inactivating many enzymes and other proteins), but occur linked to various alcohols and amines. The structures of the simple acylglycerols are given in Fig. I.2.8.

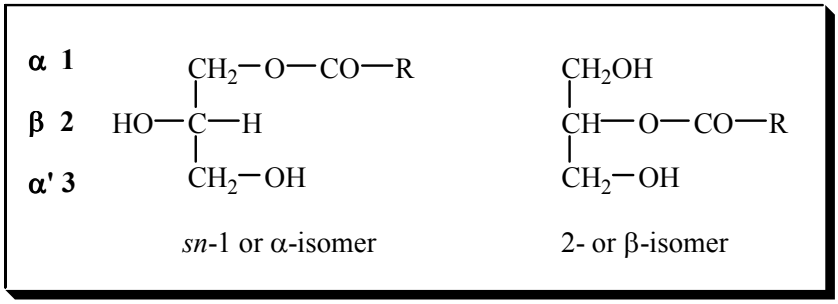


Figure I.2.8. Acylglycerol structure.

Isomers of a simple acylglycerol. The nomenclature of 'glycerides' with terms as mono-, di-, and triglyceride is ambiguous because all of these compounds involve a single glycerol moiety.

Wax esters and fatty alcohols

Fatty alcohols are aliphatic alcohols that occur naturally in free form (component of the cuticular lipids) but more usually in esterified (wax esters) or etherified form (glyceryl ethers). Long-chain alcohols are known as major surface lipid components (waxes) with chains from C_{20} up to C_{34} carbon atoms. Multibranched alcohols have been found mainly in geological sediments under saturated forms. Examples are:

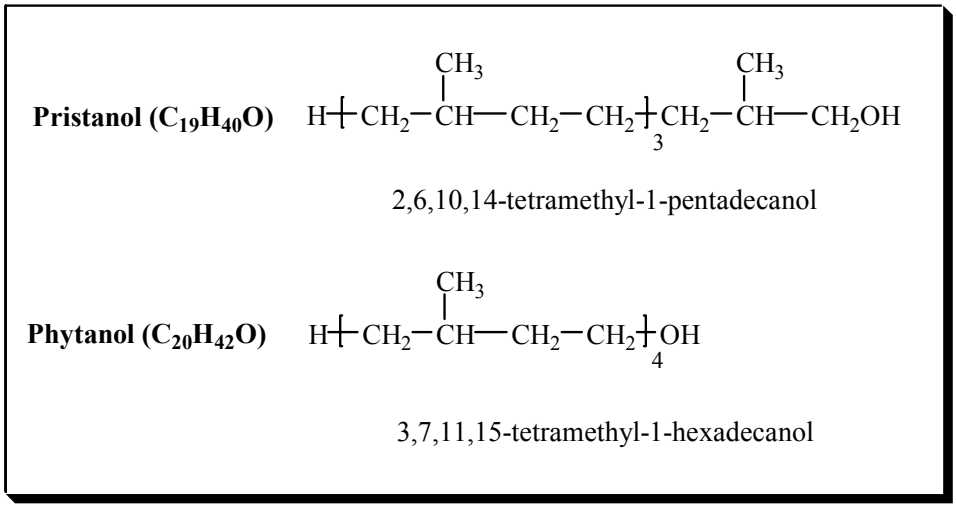


Figure I.2.9. Examples of long-chain alcohols: pristanol and phytanol.

Waxes are water-resistant materials made up of various substances including hydrocarbons, ketones, diketones, primary and secondary alcohols, aldehydes, sterol esters, alkanoic acids, terpenes (squalene) and monoesters (wax esters), all with long or very long carbon chains. Wax ester formation may also occur with dicarboxylic acids, forming diesters or polyesters. Examples of various mono-, di- and polyesters are given in Fig. I.2.10.

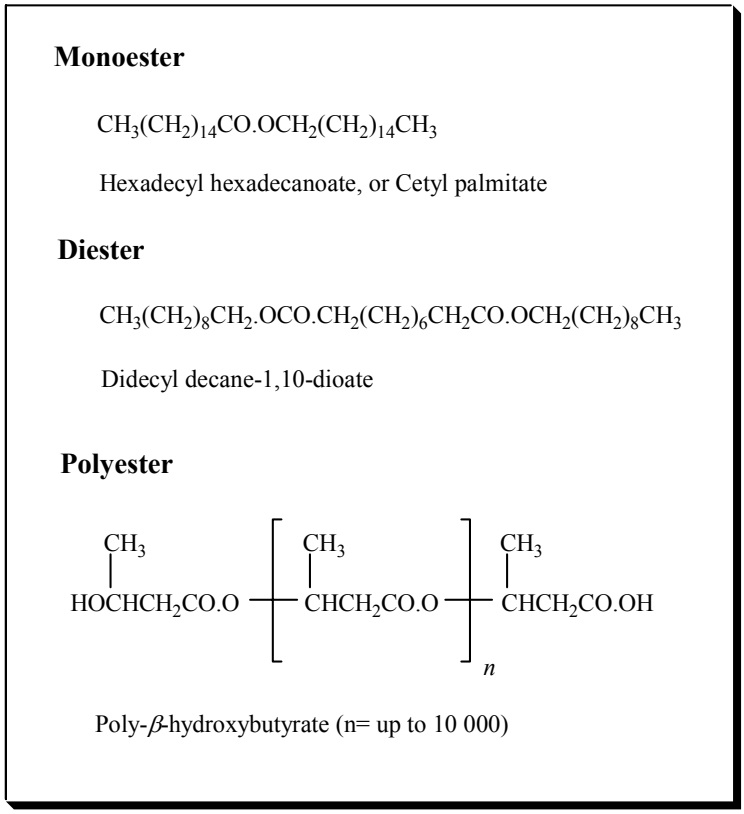


Figure I.2.10. Examples of wax esters and polyesters.

Polyhydroxyalkanoates

Polyhydroxyalkanoates are sometimes (but erroneously) considered to be a carbohydrate, but its solubility characteristics are those of a lipid. Polyhydroxyalkanoates (PHAs), for a long time thought to be exclusively represented by poly-β-hydroxybutyric acid [P(3HB)], the polyester first observed and characterized by Lemoigne at the Institute Pasteur (Lemoigne, 1923), now constitute a family of natural water-insoluble stereospecific polyesters of a wide range of different D(–)-

hydroxyalkanoic acids (HAs), being characterized by the general chemical structure reported in Fig. I.2.11.

Polymers of HAs have been found in a wide variety of prokaryotes and also in many eukaryotic plants and animal cells, but only prokaryotes are able to accumulate high molecular weight PHAs in the form of cytoplasmatic amorphous granules with almost no osmotic activity. The increasing interest in the development of biodegradable plastic able to compete, at least to some extent, with the non-biodegradable, highly polluting, petrochemical polymers has given in recent years a tremendous impetus to researches on the high molecular weight PHAs of bacterial origin (Lee 1996; Steinbüchel and Valentin, 1995).

PHAs are thermoplastic polymers with material properties ranging from brittle to flexible to rubbery, according to the presence of different kinds of hydroxyalkanoic acids. From a general point of view, the most attractive characteristics of these polymers are their material properties which are similar to those of conventional synthetic plastics, their biodegradability, their hydrophobicity and the possibility to use renewable resources for their production.

According to the terminology proposed by Anderson and Dawes (1990) and by Steinbüchel *et al*. (1992), PHAs can be divided into three classes, depending on the number of atom carbons in the monomer units; short chain length (SCL), medium chain length (MCL), and long chain length polyhydroxyalkanoates (LCL-PHAs), composed by hydroxyacids with 3–5, 6–14 or more than 14 carbons, respectively. At the present time, up to 91 different monomer units have been reported as constituents of PHAs, even if most of them are present only in a very limited number of cases and/or at vey low concentrations (Steinbüchel and Valentin, 1995). Among SCL-PHAs, P(3HB) is the most commonly found in bacteria. Some possible applications of PHAs as plastic goods, related to their more significant properties, and their actual commercial uses are summarized in Table I.2.5. A detailed explanation about the microbial occurrence of these polymers in microorganisms, their biosynthesis and degradation and their ecological significance, are detailed in the ‘Glycolipid fraction’ section of the ‘Lipid biomarkers and the SLB approach’.

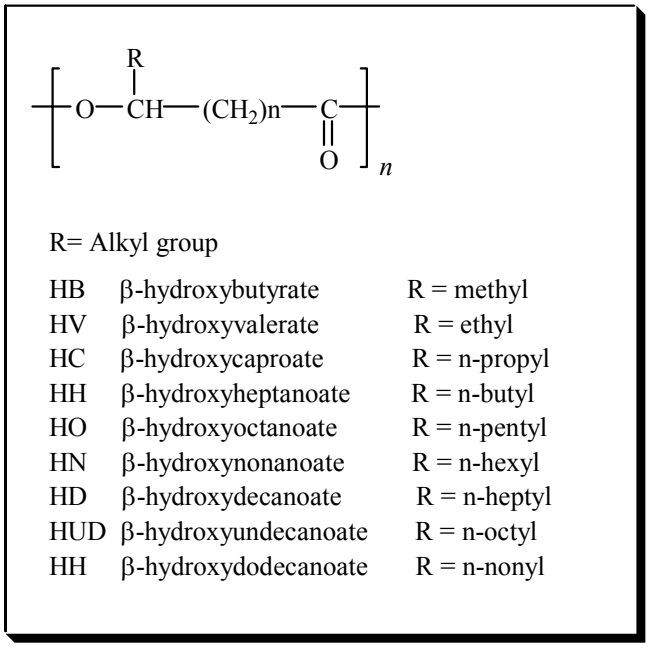


Figure I.2.11. General structure of polyhydroxyalkanoates.

The polymerization number *n* can reach values up to 30 000. The value "n" is generally 1 (in poly-3-hydroxyalkanoates), but in a few cases it has also assumed the value of 2 (in poly-4-hydroxyalkanoates) and 3 (in poly-5-hydroxyalkanoates) (Steinbüchel and Valentin, 1995; Lee, 1996). The R-pendant group includes the H-atom and a large variety of C-atom chains (Steinbüchel and Valentin, 1995).

Table I.2.5. Some actual and potential industrial applications of PHAs.

Application field	Uses	Properties useful for specific uses
Agriculture	Controlled release of pesticides, plant growth regulators, herbicides, fertilizers	Biodegradability, retarding properties
	Covering foils	Biodegradability
	Seed encapsulation	Biodegradability
Disposables	Razors, trays for food, utensils, etc.	Biodegradability, good mechanical properties
Hygiene products	Diapers, feminine hygiene products	Moisture resistance, biodegradability
Medical	Absorbable sutures, surgical pins, staples, bone plates, films around bone fractures	Biocompatibility, biodegradability
Packaging	Bottles, films for food packaging, paper coating	Biodegradability, good liquid barrier, low O_2 permeability

1. 3. Aldehydes and vinyl-ether lipids

Short-chain aldehydes are produced from fatty hydroperoxides but are also found in vegetals. Long-chain aldehydes occur in free form but are frequently included in complex lipids in the form of vinyl ether (known as alk-1-enyl ether or as plasmalogen analogs of glycerides or phospholipids), for example, the alk-1-enyl-acyl derivative (1-alk-1'-enyl-, 2-acyl-*sn*-glycero-3-phosphorylcholine) also named choline plasmalogen. As the first carbon of glycerol is linked to the carbon chain through a [–C–O–C=C–] vinyl ether bond, these lipids are known as ether-linked lipids or ether lipids.

The alk-1'-enyl, acyl derivative (ethanolamine plasmalogen) occurs widely in nature. It is practically absent in plants and bacteria.

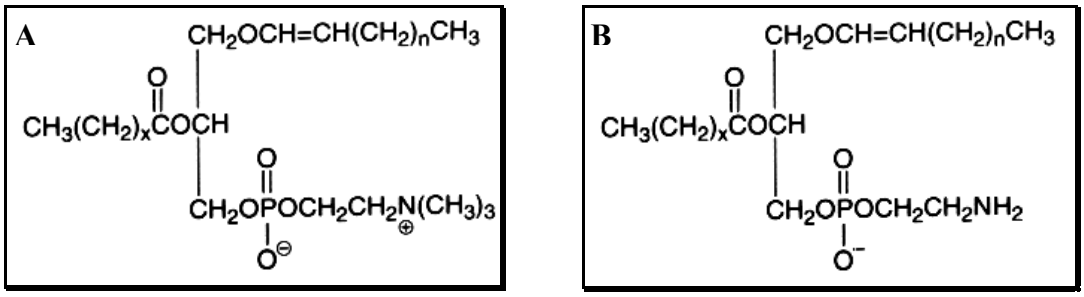


Figure I.2.12. Plasmalogens.

(A) Alk-1´-enyl acyl derivative of 2-acyl-*sn*-glycero-3-phosphorylcholine or Choline plasmalogen. (B) Derivative of 1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine, Ethanolamine plasmalogen.

The first carbon chain is of the vinyl ether group, *n* is usually equal to 13–15. The second carbon chain is an esterified fatty acid with × being equal to 14–16 and one or two double bonds. The vinyl ether bond is very sensitive to acid treatment which generates a free long-chain aldehyde. When the acid treatment is made in the presence of methanol, it generates dimethylacetals. These derivatives are stable and they can be analyzed by gas liquid chromatography (see 'General Material and Methods' chapter).

1. 4. Amino compound-containing lipids

Amino acid-containing lipids *(simple Lipoamino acids and lipopeptides)*

Some bacteria species are known to contain in their inner and outer membranes amphipatic lipids based on one amino acid linked to a fatty acid through an amide bond and sometimes another through an ester bond.

- Lipids containing serine

The best known is serratamic acid or hydroxydecanoyl serine (Fig. I.2.13). This compound was detected in *Serratia* species of bacteria (Cartwright, 1957). It was suggested that this compound may contribute to the virulence of the bacteria (inhibition of phagocytosis, hemolytic activity). Another form was described in an opportunistic pathogen *Flavobacterium* (Kawai *et al*., 1988). In this compound (named 'flavolipin' and with a high hemaglutinating activity), serine is amide-linked to 3-hydroxyisoheptadecanoic acid which is esterified to isopentadecanoic acid.

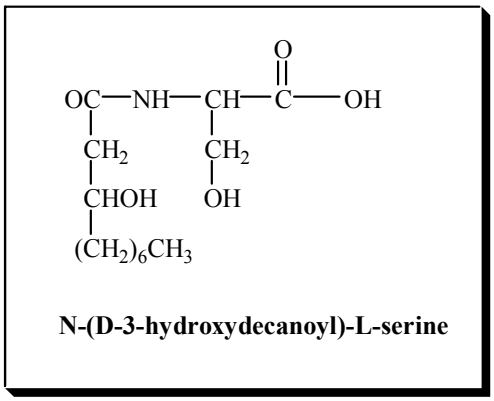


Figure I.2.13. Structure of *N*-(D-3-hydroxydecanoyl)-L-serine or Serratamic acid.

- Lipids containing ornithine

They are found among others in several species of *Bordetella*, *Pseudomonas, Flavobacterium* and *Achromobacter*.

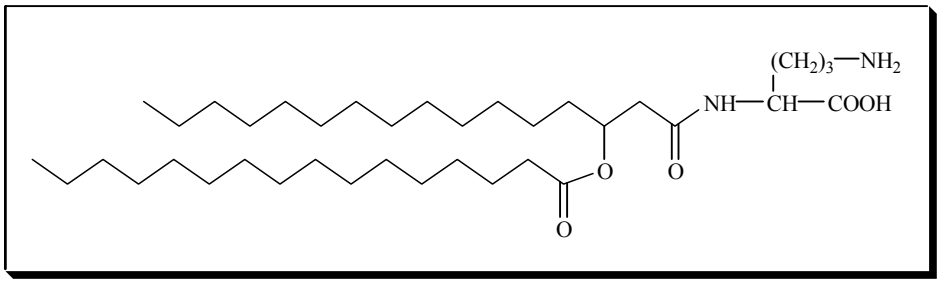


Figure I.2.14. Structure of *N*-[3-hexadecanoyloxy)hexadecanoyl]-ornithine.

This ornithine-containing lipid (Fig. I.2.14) synthesized by several bacteria species (*Bordetella, Pseudomonas, Achromobacter*), was recently found to be a strong stimulant for macrophages (Kawai *et al*., 1999). Other forms were found in various *Flavobacterium*, opportunistic pathogens (Kawai *et al*., 1988). In these compounds, ornithine is amide-linked to a hydroxylated fatty acid (3-hydroxyisoheptadecanoic acid) which is itself esterified to isopentadecanoic acid or 2-hydroxyisopentadecanoic acid. They were shown to exhibit high hemagglutinating activity.

- Lipids containing glycine

A glycine-containing lipoamino acid (Fig. I.2.15) was described for the first time in a gliding bacterium, *Cytophaga johnsonae*.

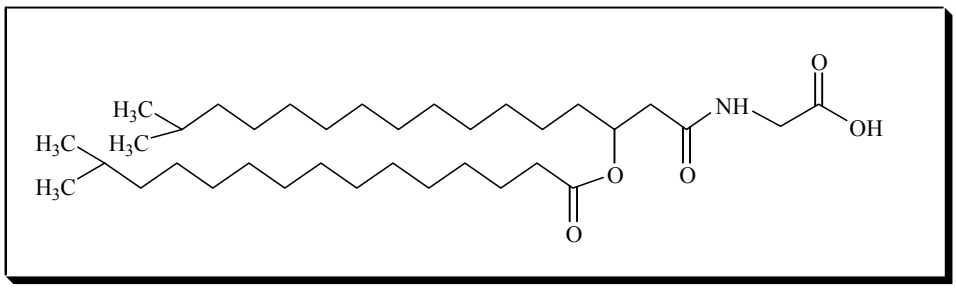


Figure I.2.15. Structure of a glycine-containing lipoamino acid.

Its structure showed an *iso*-3-hydroxy heptadecanoic acid, amide linked to glycine and esterified to isopentadecanoic acid; it formed about 6% of the total bacteria lipids (Kawazoe *et al*., 1991).

- Lipids containing leucine

Lipstatin, a new and very potent inhibitor of pancreatic lipase was isolated from *Streptomyces toxytricini* (Weibel *et al*., 1987). Lipstatin contains a beta-lactone ring, which carries two aliphatic residues with chain lengths of 6 and 13 carbon atoms. One of the side chains contains two isolated double bonds and a hydroxy group esterified to *N*-formyl-leucine.

- Lipopeptides

These lipids occur in *Mycobacteria* and *Nocardia* species of bacteria and have the basic structure of *N*-acyl olipeptides. This class of lipids often occurs in the form of glycosides derivatives, as for example the glyco-peptidolipid in *Mycobacteria* (Laneelle and Asselineau, 1968).

1. 5. Amino alcohols and ceramides

Amino alcohols

These amino alcohols occur largely in complex form (amides of fatty acids) as sphingolipids (ceramides, sphingomyelin, cerebrosides and complex glycolipids). More than 60 long-chain bases (or sphingoid bases) were described in bacteria, plants and animals with 12 to 20 carbon atoms , 2- to 3- hydroxy groups and zero to 2 double bonds, some may be phosphorylated or sulfated (Fig. I.2.16, and Table I.2.6). Ceramides are amides of fatty acids with long-chain di- or trihydroxy bases.

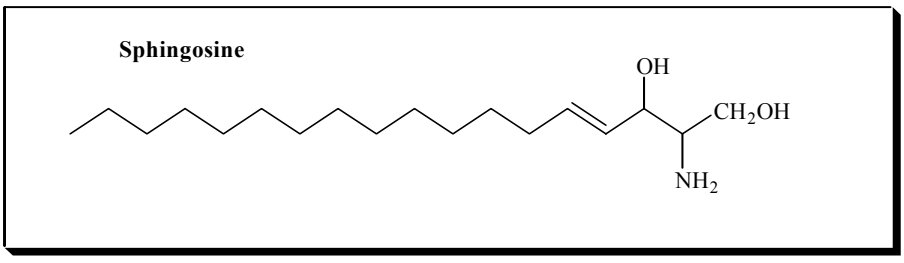


Figure I.2.16. Example of a ceramide: sphingosine structure.

Table I.2.6. Amino alcohols: Long-chain sphingoid bases.

Name	Alternative names	Formula
Sphingosine *d*18:1	(4*E*)-Sphingenine, 4-*trans*-sphingenine, 2*D*-amino-*trans*-octadec-4-ene-1,3*D*-diol, D-*erythro*-2-amino-*trans*-octadec-4-ene-1,3-diol, (2*S*,3*R*,4*E*)-2-amino-octadec-4-ene-1,3-diol	$C_{18}H_{37}NO_2$
Dihydrosphingosine *d*18:0	Sphinganine, 2*D*-amino-octadec-1,3*D*-diol, D-*erythro*-2-amino-octadec-1,3-diol, (2*S*,3*R*)-2-amino-octadec-1,3-diol	$C_{18}H_{39}NO_2$
C_{20}-Dihydrosphingosine *d*20:0	Eicosasphinganine, 2*D*-aminoeicosane-1,3-diol	$C_{20}H_{43}NO_2$
Phytosphingosine *t*18:0	4*D*-hydroxysphinganine, 2*D*-amino-octadecane-1,3*D*,4*D*-triol, (2*S*,3*R*,4*R*)-2-amino-octadecane-1,3,4-triol	$C_{18}H_{39}NO_3$
C_{20}-Phytosphingosine *t*20:0	4-hydroxyeicosasphinganine, 2*D*-aminoeicosane-1,3,4-triol	$C_{20}H_{43}NO_3$
Dehydrophytosphingosine *t*18:1	4*D*-hydroxy-8-trans-sphingenine, 2*D*-amino-trans-octadec-8-ene-1,3,4-triol, D-*ribo*-2-amino-octadec-8-ene-1,3,4-triol, (2*S*,3*R*,4*E, 8E*)-2-amino-octadec-8-ene-1,3,4-triol	$C_{18}H_{37}NO_3$
Sphingadienine *d*18:2	4,8-sphingadienine, 2*D*-aminooctadeca-4,8-diene-1,3-diol	$C_{18}H_{35}NO_2$

These amino alcohols are frequently represented by a simplified nomenclature similar to that used for fatty acids but with additional *d* or *t* to designate di- and trihydroxy bases, respectively.

1. 6. Terpenoid lipids

Terpenes

Terpenes are a wide group of natural hydrocarbons whose structure is based on various but definite numbers of isoprene units (Fig. I.2.17; Table I.2.7). Many of them are also oxygen-containing compounds (terpenoids). They are constituents of essential oils, resins, waxes, rubber, and several bioactive molecules such as alkaloids, quinones, vitamins, carotenoids and phenols belong to that chemical group.

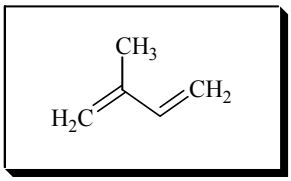


Figure I.2.17. Isoprene units, base structure of terpenes.

Table I.2.7. Classification of terpenoid lipids[1].

Class	Carbon number	Isoprene units	Examples
Hemiterpenoids	5	1	Isoprene
Monoterpenoids	10	2	Citronellol, geraniol
Sesquiterpenoids	15	3	Trichodermin, farnesol
Diterpenoids	20	4	Isopranyl ethers, phytol, geranylgeraniol, gibberellic acid
Sesterterpenoids	25	5	Ophiobolin A
Triterpenoids	30	6	Squalene, steroids, hopanoids
Tetraterpenoids	40	8	Carotenoids, some isoprenoid quinones
Polyterpenoids	> 40	> 8	Isoprenoid quinones, polyprenols, some carotenoids

[1]A detailed explanation about the structure and functions of the examples mentioned in this table can be found in Ratledge and Wilkinson, 1988.

Steroids and related lipids

- Free steroids

Steroids are modified triterpenes derived from the basic hydrocarbon squalene. Examples are cholest-5-en-3β-ol (cholesterol), (22*E*)-ergosta-5,7,22-trien-3β-ol (ergosterol), (22*E*)-stigmasta-5,22-dien-3β-ol (stigmaesterol), cholesta-8(9),24-dien-3β-ol (zymosterol), and ergosta-7,24(28)-dien-3β-ol (episterol).

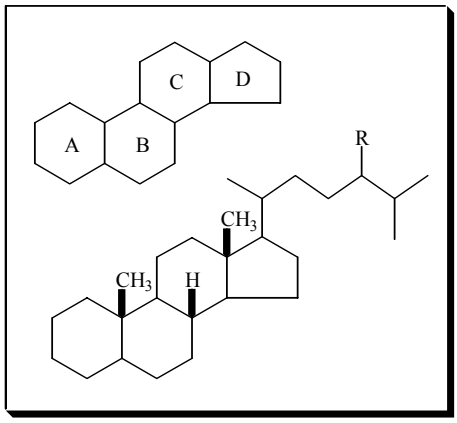


Figure I.2.18. Nuclear framework of all steroids: 1,2-cyclopentanoperhydrophenanthrene.
R= H (cholestane); R= CH_3 (ergostane); R= CH_3CH_2 (stigmastane).

- Sterol esters and glycosides

As well as occurring free, sterols are also found as fatty acids and as glycosides. Among the prokaryotes, sterols are always found in cyanobacteria and *Prochloron* species, but rarely in other bacteria and not at all in anoxyphotobacteria. Some bacteria, such as halobacteria, that do not synthesize sterols, are capable of producing the universal sterol precursor squalene; other bacteria which are considered more evolved, can cyclize squalene to lanosterol (*Methylococcus*, *Methylobacterium*). The production of sterols is considered a trademark of the eukaryotes; but with better analytical methods very small amounts of sterols have been found in some bacteria, especially in those with a high G+C type DNA, e.g. *Escherichia coli*, species of *Azotobacter*, *Streptomyces*, *Micromonospora* and *Methylococcus* (Taylor, 1984; Nes and McKean, 1977).

Carotenoids

Carotenoids are probably the most widespread of natural pigments. They are involved in the photosynthetic responses of algae and photosynthetic bacteria as well as plants, in the photo-protection of these and non-photosynthetic microorganisms, and in the synthesis of vitamin A in animals. Carotenoid hydrocarbons are termed *carotenes*, and their oxygenated derivatives *xanthophylls*.

Polyprenoids

The multiprenyl (polyprenyl) chains incorporated into the isoprenoid quinones are related to a group of polyisoprenoid alcohols (polyprenols) (Table I.2.8).

Table I.2.8. Classification of saturated polyisoprenoids (isopranols).

n	Number of isoprene units	Number of carbons	Name
0	2	10	Geraniol
1	3	15	Farnesol
2	4	20	Geranylgeraniol
3	5	25	Geranylfarnesol
7	9	45	Solanesol
8–11	10–13	50–65	Castaprenols-Ficaprenols

The unsaturated isoprenoid alcohols (prenols or polyprenols) are also known as terpenols. A partially saturated terpenol which is bound via ester bonds to the tetrapyrrol macrocyclic system of chlorophyll is phytol (Fig. I.2.19). This covalent bond is resistant to hydrolysis but is susceptible to photoinduced processes (Rontani *et al*., 1999). The ketone 6,10,14-trimethylpentadecane-2-one was described to originate from the abiotic oxidation of free phytol; formation resulting from aerobic bacterial activity is also possible (Rontani *et al*., 1998). Because the formation of this isoprenoid ketone involves oxidative pathways, it might be regarded as an indicator of oxidative processes in aquatic systems unless bacterial activities resulting in rapid biodegradation prevail.

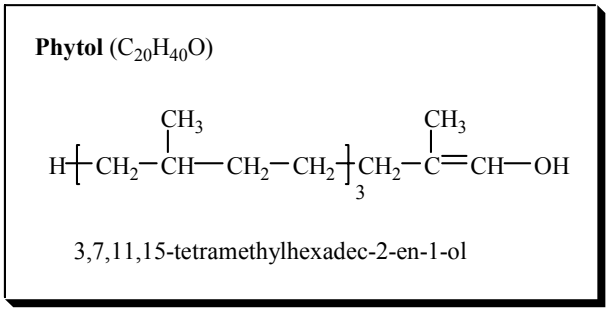


Figure I.2.19. Phytol: 3,7,11-tetramethylhexadec-2-en-1-ol.

Chlorophylls

Chlorophylls, the light-gathering photosynthetic pigments found in both eukaryotes and prokaryotes, also incorporate isoprenoid residues (C_{10} to C_{20}). The chlorophyll of cyanobacteria is chlorophyll *a* (Chl*a*), and both Chl*a* and Chl*b* are found in prochlorphyta, but in other prokaryotic phototrophs, the related bacteriochlorphylls (Bchl) occur. Whereas the isoprenoid residue in eukaryotic chlorophylls is the monoenoic C_{20} phytyl group (derived from (7*R*,11*R*)-phytol), the corresponding residue in bacteriochlorophylls may be phytyl, granyl, farnesyl, or other groups.

Isoprenoid quinones

The recently increased interest in the isoprenoid quinones has greatly expanded our knowledge of these compounds in bacteria (Collins and Jones, 1981) and has lead to the discovery of many novel quinones. The isoprenoid quinones are involved with electron transport and the process of oxidative phosphorylation, and are thus associated with the plasma membranes of bacteria, the mitocondrial membranes of eukaryotic organisms, and the thylacoid (photosynthetic) membranes of phototrophic organisms.

The two main types of isoprenoid quinones are the ubiquinones and the menaquinones, which are respectively, examples of benzoquinones and naphtoquinones. Reduction of the oxo groups (total or partial) produces hydroquinones (quinols) and semiquinones, respectively. Quinones incorporate multiprenyl (polyprenyl) side-chains, which may vary from 1 to 16 isoprenoid units in length. Apart from characteristic variations in the extent of polymerization, isoprenoid quinones differ in their degree of saturation of the side-chain, in nuclear substitution, and in other structural features

illustrated below. Both the quinone type and the structural details of the side-chain appear to be a significant taxonomic criterium, an indicator of the biomass and of the redox state (the significance of quinones as biomarkers of microbial communities in terms of quantity, quality and activity are widely explained in the 'Neutral lipid fraction' section of 'Lipid biomarkers and the SLB approach').

- Naphtoquinones

Naphthoquinones can be divided further into two main types on the basis of structural considerations; these are the phylloquinones and the menaquinones (Fig. I.2.20). Phylloquinone, or vitamin K_1, was first isolated in 1939 from alfalfa and was shown by MacCorquodale *et al*. (1939) in degradation and synthetic studies to be 2-methyl-3-phytyl-1,4-naphthoquinone. The first representative of the menaquinone family (formerly designated vitamin K_2) was isolated by McKee *et al*. (1939). Today, naturally occurring menaquinones form a rather large class of molecules, and the length of the C-3 isoprenyl side-chains in these molecules varies from 1 to 14 isoprene units (Collins and Jones, 1981).

As an example of the various menaquinone forms, MK-7 has 6+1=7 isoprenoid units or 35 carbons in the side chain and can be called vitamin K_2 or menaquinone-7. This could be called also 2-methyl-3-all-trans-farnesyl digeranyl-1,4-naphthoquinone. Farnesol and geraniol are the alcohols with 3 (15 carbons) and 2 (10 carbons) isoprenoid units, respectively.

Demethylmenaquinones (Fig. I.2.20), which lack the ring methyl substituent (C-2), have also been isolated from bacteria (Lester *et al*., 1964). To date, demethylmenaquinones with polyprenyl side-chains varying in length from one to nine isoprene units have been described (Hammand and White, 1969).

- Benzoquinones

The second major class of bacterial isoprenoid quinones is the benzoquinones, of which there are two main types, the ubiquinones and the plastoquinones (Fig. I.2.21).

Ubiquinones are also known as coenzyme Q, mitoquinones or 2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinones, and are the most common benzoquinones.

Ubiquinones are present in all aerobic organisms, bacteria, plants and animals. Ubiquinones contain a 2,3-dimethoxy-5-methyl-1,4-benzoquinone nucleus with a polyprenyl side-chain in position 6.

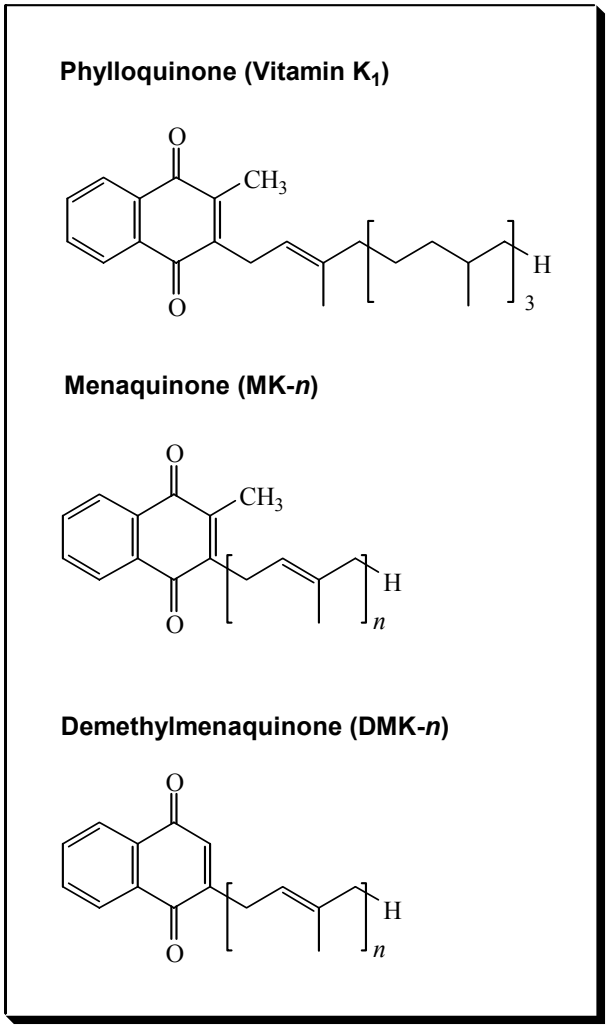
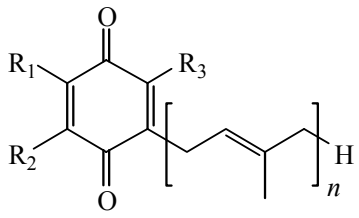


Figure I.2.20. Naphtoquinone structures.

In the recommended abbreviation MK-*n*, *n* is the number of prenyl units in the side-chain attached at position 3 of the naphtoquinone nucleus. The corresponding compounds lacking the 2-methyl substituents are referred as demethylmenaquinones, DMK-*n*. partial saturation of the polyprenyl side-chain is indicated by the suffix (H_x), and the site of saturation is indicated by Roman numbering of the isoprene units, starting next to the quinone nucleus. Thus phylloquinone (Vitamin K_1, 2-methyl-3-phytyl-1,4-naphtoquinone), could be abbreviated as MK-4(II,II,IV-H_6).

Benzoquinones



Ubiquinones (Q-*n*) $R_1 = R_2 = CH_3O$; $R_3 = CH_3$
Plastoquinones (PQ-*n*) $R_1 = R_2 = CH_3$; $R_3 = H$
Rhodoquinones (RQ-*n*) $R_1 = CH_3O$; $R_2 = NH_2$; $R_3 = CH_3$

Figure I.2.21. Benzoquinone structures.

There are several plastoquinones with side chains of different length in position 5. They are designated as plastoquinone-*n* where *n* is the number of carbon atoms in the side chain or better as PQ-*n* where *n* indicates the number of isoprenoid units, *n* varies from 6 to 9. Plastoquinone is found not only in the photosynthetic tissues of higher plants but also in red, brown, and green algae and in cyanobacteria (Threlfall and Whistance, 1971), while the purple rhodoquinones (RQ-*n*; n= 8–10, Fig. I.2.21) are produced by various phototrophs from the family Rhodospirillaceae (Imhoff, 1984). Unusual benzoquinones include the epoxyubiquinone from *Rhodospirilum rubrum*, and a series of methylene-ubiquinones, from *Methylomonas rubra*, present in some methanotrophs (Collins and Green, 1985).

Apart from plastoquinone A (PQ-A, PQ-9), whose role in photosynthesis is well characterized (Więckowski and Bojko, 1997), other plastoquinones like plastoquinone B (PQ-B) and C (PQ-C) were isolated in the 60's by column and thin-layer chromatography, mainly by Barr and collaborators (Barr *et al*., 1967) (Fig. I.2.22). Recent studies, have demonstrated that PQ-C is a natural component of photosynthetic membranes (Kruk *et al*., 1998).

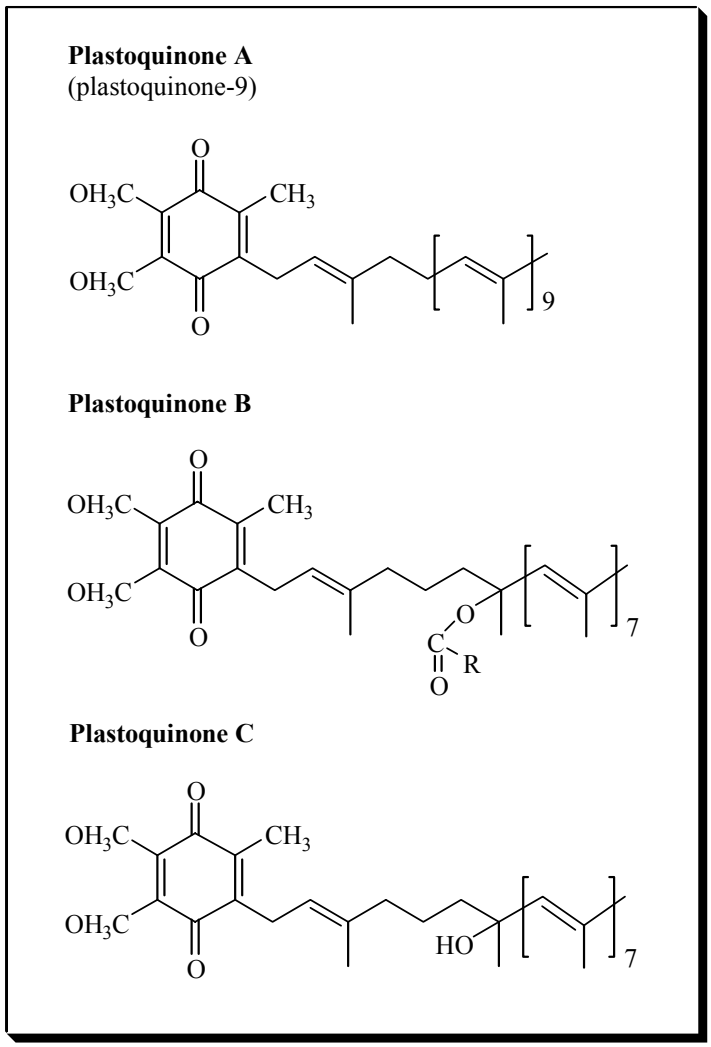


Figure I.2.22. Plastoquinones.

Chemical structure of PQ-A, PQ-B, and PQ-C. The exact association of the OH group with a specific isoprene unit (shown here with the second), has not been established for the individual components of the PQ-B and PQ-C series.

Isopranyl ethers

Prominent amongst the features which distinguish the archaebacteria from eubacteria and eukaryotes is the structure of their membrane phospho- and glycolipids. One of the most distinctive facts about archaeal lipids is the occurrence of compounds based on isopranyl glycerol ethers in place of acylglycerols. Moreover, the configuration of the glycerol residue, as in 2,3-di-*O*-phytanyl-*sn*-glycerol (‘archaeol’) is the opposite of that found in the conventional acyl counterparts. (Fig. I.2.23).

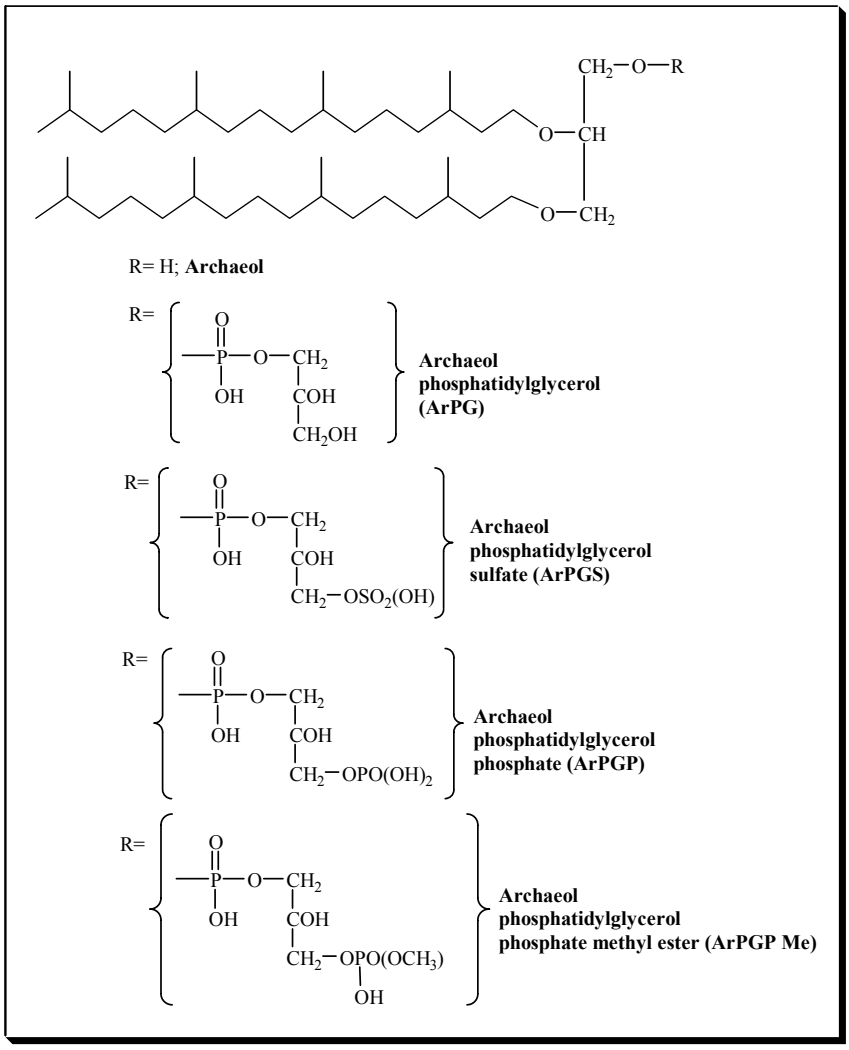


Figure I.2.23. Archaeol ether lipid.

Core ether lipid, diether of archaeol (with the phospho-or glyco- head groups removed).

Diether lipids were first discovered by Kates and collaborators (reviewed by Kates, 1978) to comprise the major portion of the total lipids of the extreme halophile *Halobacterium cutirubrum*. Following the discovery of a tetraether structure in *Thermoplasma acidophilum* (Langworthy, 1977), a predominance of ether lipids has proven to be a characteristic of archaeobacteria (Makula and Singer, 1978; Tornabene and Langworthy, 1979), distinguish this grouping of organisms from the eubacteria and eukaryotes. The ether core structure of *H□cutirubrum* was fully elucidated as 2,3-diphytanyl-*sn*-glycerol, of opposite sterochemistry to the glycerolipids of non-

archaeobacteria (Kates, 1988). There are exceptions also in archaebacterial lipid chemistry, for example rarely eubacterial obligate anaerobes contain plasmalogen glycerophospholipids (see 'Complex lipids' section) and glycerol diethers. Even the archaeobacterial *R* configuration is found in a minor monoether geranylgeranyl glycerol from the brown algae *Diplophus fasciola* (Amico *et al*., 1977).

The archaeal membrane phospholipids and glycolipids are derived entirely from the saturated, C_{20}-C_{20}-isopranyl glycerol diether, *sn*-2,3-diphytanylglycerol ('archaeol', Nishihara *et al*., 1987) (Fig. I.2.24) and/or its dimmer, dibiphytanyldiglycerol tetraether ('caldarchaeol', Nishihara *et al*., 1987) (De Rosa *et al*., 1991; Langworthy, 1985) (Fig. I.2.25). In extreme halophiles, the major phospholipid is the archaeol analogue of phosphatidylglycerol phosphate methyl ester (PGP-Me); the glycolipids are sulfated and/or unsulfated glycosyl archaeols with diverse carbohydrate structure characteristic of taxons on the generic level. In methanogens, polar lipids are derived both from 'archaeol' and 'caldarchaeol', and thermoacidophiles contain essentially only 'caldarchaeol'-derived polar lipids (with one or two cyclopentane rings or without cyclization). Characteristic for the archaeal kingdom *Crenarchaeota*, however, is the occurrence of 'caldarchaeols' with three to eight cyclopentane rings.

Specifically, tetraether lipids predominantly consist of glycerol dialkylglycerol tetraethers (GDGTs), which contain two glycerol head groups linked by two biphytanyl moieties with 0 to 4 cyclopentane rings. Although theoretically many combinations of biphytanyl moieties are possible, so far only 9 GDGTs have been identified in the membrane lipids of *Archaea* (Hopmans *et al*., 2000).

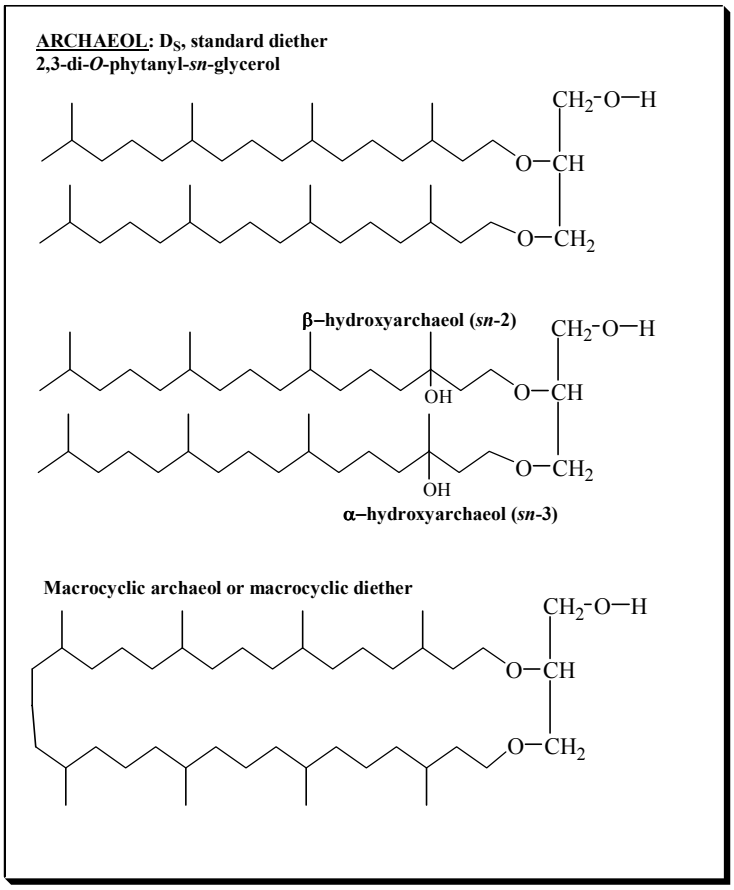


Figure I.2.24. Archaeol ether lipids.

The nomenclature of ether used here is the following: D_S, standard diether, 'archaeol' or 2,3-di-*O*-phytanyl-*sn*-glycerol; T_S, standard tetraether, 'caldarchaeol' or 2,2′,3,3′-tetra-*O*-dibiphytanyl-*sn*-diglycerol; D_{OH}, 3- or 3′-hydroxydiether or hydroxyarchaeol; D_M, macrocyclic diether, macrocyclic 'archaeol', or 2,3-di-*O*-cyclic-biphytanyl-*sn*-glycerol.

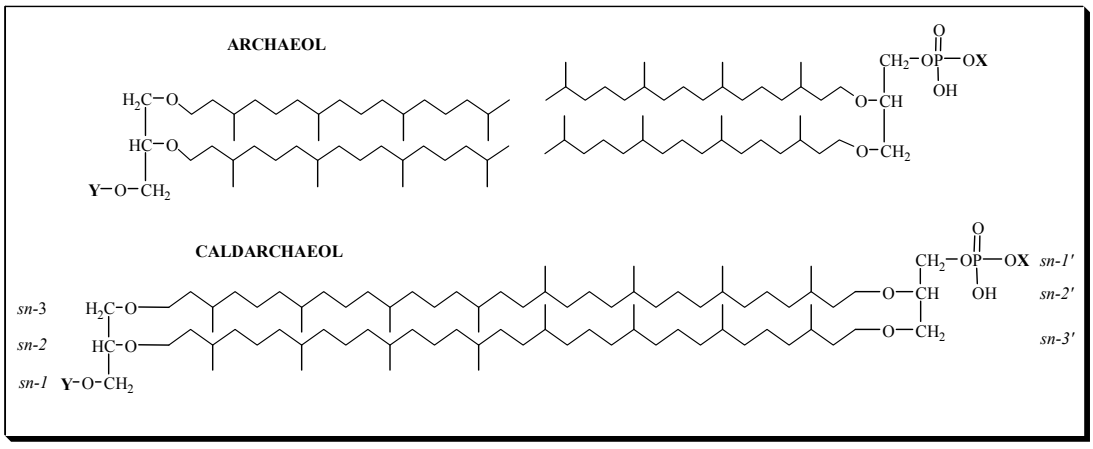


Figure I.2.25. Archaeol and caldarchaeol ether lipids.

X: Phospholipid polar head group; Y: Glycolipid sugars

2. Complex lipids

They contain frequently three or more chemical identities (i.e. glycerol, fatty acids and sugar, one long chain base, one fatty acid and one phosphate group, etc.) and have polar properties. These important lipids are widely distributed in plants, bacteria and animals. They are the major constituents of cell membranes but are found also in circulating fluids. They can be classified into three main groups:

2. 1. Phospholipids

They are defined as lipids with a phosphate residue, one glycerol, or an aminoalcohol or a fatty alcohol, without or with one or two fatty chains. Most classifications contain a category for the glycerol-containing phospholipids (Glycerophosphatides) and one for the sphingolipids (Sphingosyl phosphatides).

Glycerophospholipids

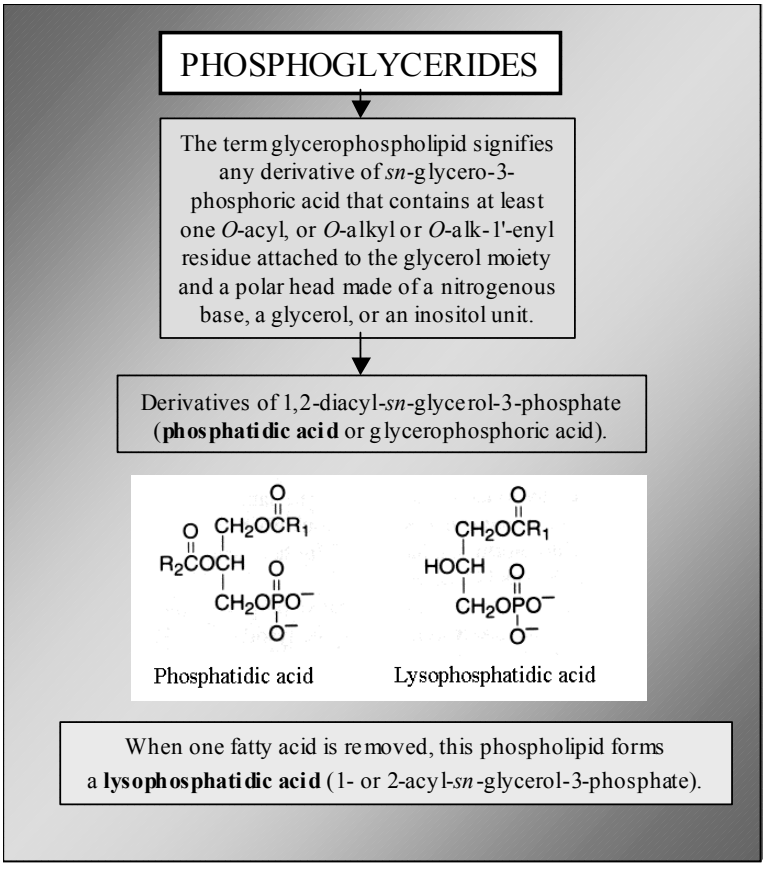


Figure I.2.26. Definition and base structure of phosphoglycerides.

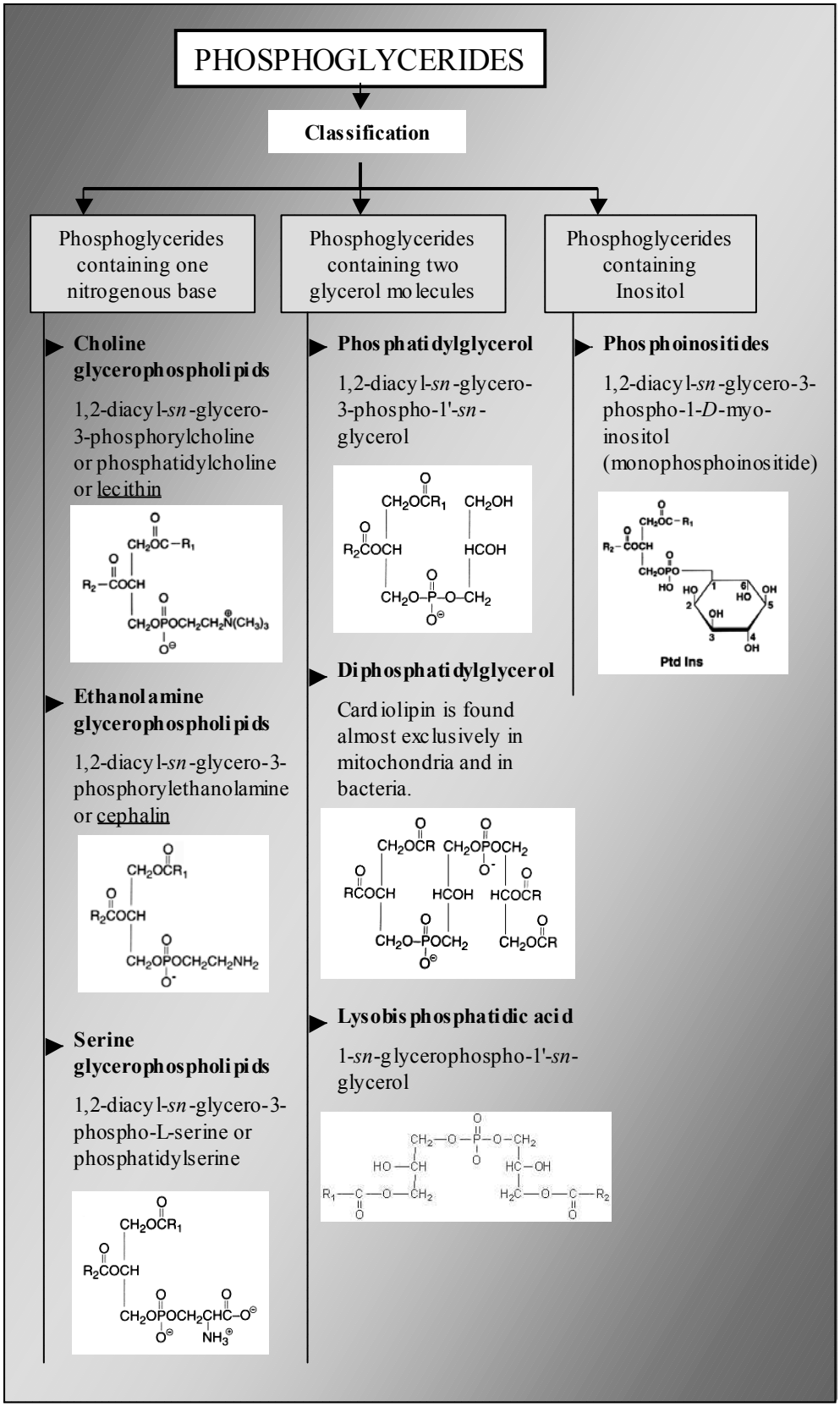


Figure I.2.27. Classification of phosphoglycerides.

Sphingophospholipids

The term sphingolipid (sphingosyl phosphatide) refers to lipids containing phosphorus and a long-chain base. This group consists of phosphorus-containing sphingolipids (mainly sphingomyelin) but containing a ceramide (see section '1.5. Amino alcohols and ceramides') linked to a phosphate group, itself esterified to a small polar head group (choline, ethanolamine, glycerol). The ceramide part is formed by a long-chain fatty acid linked to the amino group (i.e. *N*-acyl or amide) of a long-chain base (Table I.2.6). In sphingomyelin the long-chain base is sphingosine, dihydrosphingosine (in animals, and bacteria), and phytosphingosine (in plants).

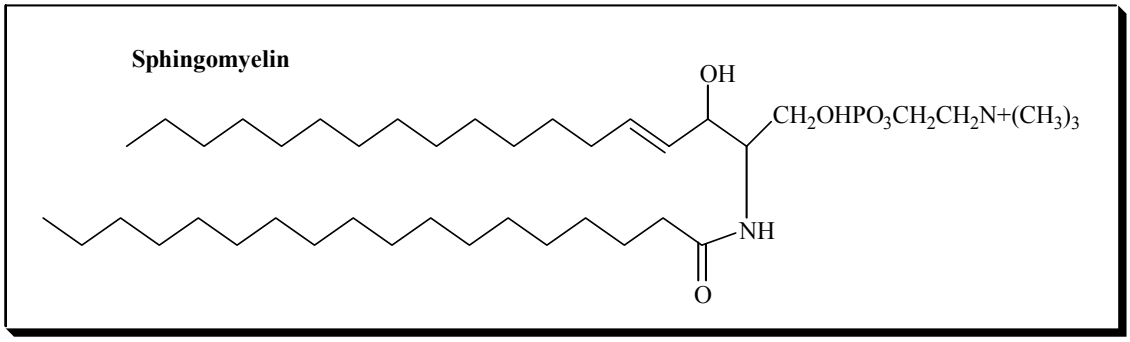


Figure I.2.28. Example of a sphingolipid (sphingosyl phosphatides): sphingomyelin.

A sphingolipid analogue of phosphatidyl ethanolamine, has been reported for the first time in the bacteria *Bacteroides ruminicola* (Kunsman *et al*., 1973). Shortly later, its correct composition was described and evidence was reported that this sphingolipid represented a significant proportion of the lipids (about half of phospholipids) in another anaerobe, *Bacteroides melanogenicus* (now *Prevotella melaninogenica*), in which a ceramide phosphorylglycerol has been also isolated (LaBach and White, 1969). The presence of this rare lipid, similar to phosphatidylglycerol found extensively in bacteria, has been confirmed later in other *Bacteroides* species (Kato *et al*., 1995). The presence of sphingolipids has also been extensively studied in lipid extracts from several species of *Sphingobacterium* (Naka *et al*., 2003) and *Sphingomonas* (Kawasaki *et al*., 1994).

2. 2. Glycolipids

This class of lipids is heterogeneous and includes various types of long chain derivatives of sugars which may contain most frequently in bacteria, plants and animals either a glycerol (a diacylglycerol), a ceramide backbone, a sterol or a phosphorylated polysaccharide-lipid complex. Some of these glycolipids may be composed of a carbohydrate moiety linked to a single fatty acid or fatty alcohol. A great variety of glycolipids is also found in bacteria where specific lipopolysaccharides are also present.

Lipopolysaccharides

The lipopolysaccharide consists of two portions, the core polysaccharide and the *O*-polysaccharide. In *Salmonella*, where it has been best studied, the core polysaccharide consists of ketodeoxyoctonate (KDO), seven-carbon sugars (heptoses), glucose, galactose, and *N*-acetylglucosamine. Connected to the core is the *O*-polysaccharide, and the lipid portion of the lipopolysaccharide, referred to as lipid A, consists of fatty acids connected by ester linkage to a disaccharide composed of *N*-acetylglucosamine phosphate. Fatty acids commonly found in lipid A include caproic, lauric, myristic, plamitic, and stearic acids.

2. 3. Lipoamino acids

Several classes of complex lipids devoid of phosphorus have one amino acid linked to both a long-chain alcohol and a fatty acid or to a glycerolipid, they are sometimes named lipoamino acids. Simple forms of these lipoaminoacids containing only amino acid and fatty acid(s) are described in the section '1. 4. Amino compound-containing lipids'. Two groups of complex lipoamino acids are known, lipids having an amino acid with *N*-acyl and/or ester linkages, and lipids having a glycerol and an amino acid with ether linkage. In this section, only the *N*-acyl and ester derivatives of amino acids are explained and classified according to their amino acid moiety, as follows:

Lysine-containing lipids

Some of them are known as Siolipin A. In these compounds lysine is *N*-linked to a fatty acid (normal or hydroxylated, R_1) and to a fatty alcohol (R_2). They are found in *Streptomyces* species of bacteria. Later, this compound was described in polar lipids of group B *Streptococci* (Fischer, 1977), in *Caulobacter crescentus* (Jones *et al*., 1979), in *Bacillus subtilis* (Deutsch *et al*., 1980), and was shown to be a major component in *Staphylococcus aureus* and *S. intermedius* (Nahaie *et al*., 1984). It was also detected in *Vagococcus fluvialis* (Fisher *et al*., 1998) and in several species of *Listeria* (Fisher *et al*., 1999). It has been suggested that lysylphosphatidylglycerol may selectively protect bacteria against antimicrobial polypeptides (Ganz, 2001).

Lysylcardiolipin, a cardiolipin species substituted on the hydroxyl group of the middle glycerol moiety with a lysyl residue has been described first in several species of *Listeria* (gram-positive bacteria; Peter-Katalinic *et al*., 1998) which could be valuable as chemotaxonomic marker.

Ornithine-containing lipids

In these lipids ornithine is linked to a fatty acid by an amide link and to a long-chain fatty alcohol by an ester link. They occur in photosynthetic purple bacteria (Benning *et al*., 1995; Linscheid *et al*., 1997), *Pseudomonas* sp. (Kawai *et al*., 1988); *Thiobacillus* sp. (Hilker *et al*., 1978), *Paracoccus denitrificans* (Thiele *et al*., 1980), *Mycobacterium tuberculosis* (Lanéelle *et al*., 1990), *Desulfovibrio gigas* (Makula and Finnerty, 1975), *Flavobacterium* (Kawai *et al*., 1988), etc.

Alanyl-containing lipids

An alanylphosphatidylglycerol has been first discovered in *Clostridium welchii* (Macfarlane, 1962) and later in many gram-positive bacteria (O'Leary *et al*., 1988). In *Vagococcus fluviatilis,* alanylcardiolipin and alanyl bis(acylglycerol)phosphate were isolated and characterized (Fisher *et al*., 1998).

Proline-containing lipids

In search of surfactants, it was shown that among several lipoaminoacids synthesized by coupling stearic acid with the α-amino group of an amino acid, *N*-stearoyl proline had the most efficient surface-active properties. Thus, it has a potential utility as biostatic additive in commercial products (Sivasamy *et al*, 2001).

- Lipid Biomarkers and the SLB approach

Biological marker compounds or 'biomarkers'– at least in the organic geochemical literature – are commonly considered to be small to medium molecular weight compounds (Peters and Moldowan, 1993). Most work has been done with the lipid fractions, extracted from organisms (biolipids) and sediments (geolipids). Such lipid biomarkers can be expected to reflect both the source and the biochemical process involved.

Microbial lipid analysis is a relatively sensitive, quantitative method to detect most of the microorganisms present in a particular environment. The method is based on the liquid extraction and separation of microbial lipids from environmental samples, followed by quantitative analysis using gas chromatography/mass spectrometry (GC/MS). Several unique classes of lipids, including sterols, diglycerides (DG), respiratory quinones (Q), poly-β-hydroxyalkanoates (PHA), phospholipid fatty acids (PLFA), lipo–amino acids, plasmalogens, acyl ethers, sphingolipids and lipopolysaccharide hydroxy fatty acids (LPS–OHFA), can be used as signature lipid biomarkers (SLB) in order to characterize microbial populations (White *et al*, 1998). Using this methodology, microbial population changes due to physical or chemical environmental perturbations can be followed over time. A scheme proposed for SLB/environmental nucleic-acid probe analysis is diagrammed in Fig. I.2.29.

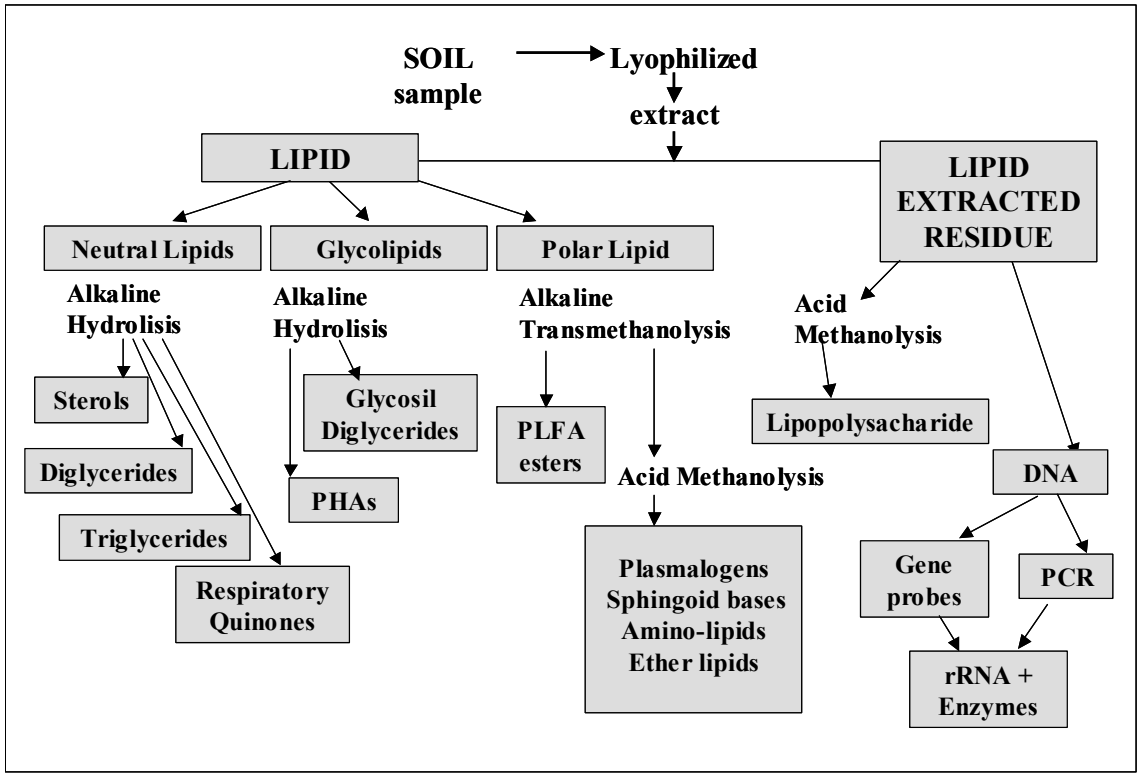


Figure I.2.29. The Signature Lipid Biomarker (SLB) approach.

Signature lipid biomarker/ environmental nucleic-acid probe analysis showing each of the lipid fractions that can be identified and quantified. PHA (polyhydroxyalkanoates); PLFA (phospholipid fatty acid); DNA (desoxy-ribonucleic acid); PCR (polymerase chain reaction); rRNA (ribosomal ribonucleic acid).

The SLB approach explanation will be divided in three parts: Polar lipid, glycolipid and neutral lipid fraction, which are the phases obtained after the separation of the total lipid extract by silicic acid chromatography. These categories include the lipid compounds that can be quantified in each fraction and their ecological significance for microbial ecology studies.

1. The polar lipid fraction

Most lipids have some polar character in addition to being largely nonpolar. Generally, the bulk of their structure is nonpolar or hydrophobic, meaning that does not interact well with polar solvents. Another part of their structure is polar or hydrophilic and will tend to associate with polar solvents. This makes them amphiphilic molecules

(having both hydrophobic and hydrophilic portions). The polar lipid fraction of the SLB approach contains the phospholipid fatty acids (PLFA), lipo-amino acids, plasmalogens, acyl esthers, and sphingolipids.

All living cells are surrounded by a membrane containing polar lipids, and the lysis of the cell membrane results in cell death. Since the major polar lipids in sediments are phospholipids, the fatty acids of the phospholipids are one of the most important SLB classes. The identification and quantification of total phospholipid as ester-linked fatty acids (PLFA, phospholipid fatty acids) is particularly useful to measure: (i) Viable microbial biomass, (ii) community physiological status, and (iii) microbial community composition.

1.1. Viable biomass

Microbial biomass has been traditionally quantified from the number of stained cells in a sample or cells detected by viable count with subsequent conversion of cell number to carbon content. However, an effective and quantitative way to measure microbial biomass *in situ* is to measure cellular components of the microorganisms. In order to be a good marker of biomass, a cellular component has to be universally distributed, to present a short residence time after death-induced release, and to be expressed at a relatively constant level among the microbial community and throughout the growth cycle. For example, phospholipids fit these requirements.

The determination of the total phospholipid ester–linked fatty acids (PLFA) provides a quantitative measure of the viable or potentially viable biomass. The viable microorganisms have an intact membrane, which contains PLFAs. Cellular phospholipases hydrolyze and release the phosphate group within minutes to hours following cell death (White *et al*., 1979). The resulting diglyceride contains the same signature fatty acids as the original phospholipid, at least for days to years in the subsurface sediments (Fig. I.2.30). Consequently, a comparison of the ratio of phospholipid fatty acid to diglyceride fatty acid (DG) profiles provides a measure of the viable to non-viable microbial abundance.

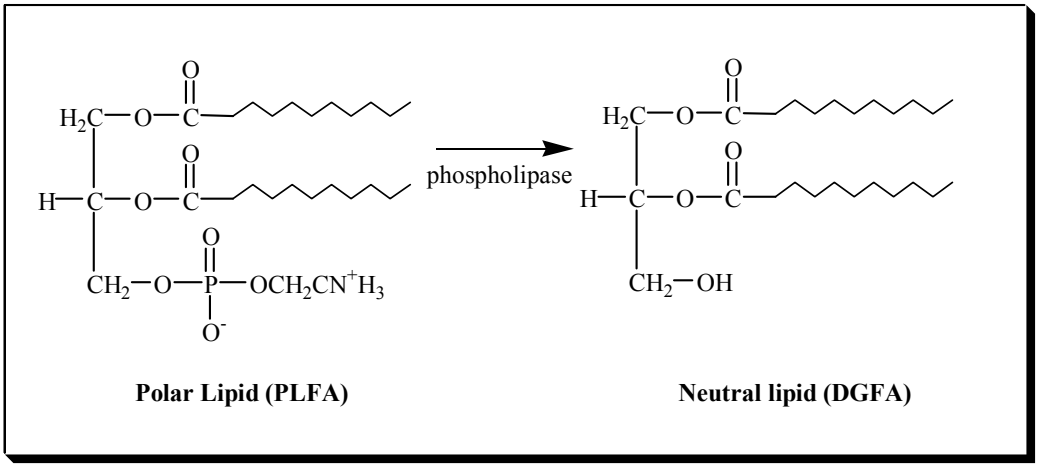


Figure I.2.30. Conversion of phospholipid fatty acid (PLFA) to diglyceride fatty acid after cell death.

PLFA (phospholipid fatty acid); DGFA (diglyceride fatty acid).

A study of subsurface sediment showed that viable biomass as determined by PLFA was equivalent (but with a much smaller standard deviation) to that estimated by intracellular ATP, cell wall muramic acid, and very careful conducted acridine orange direct counts (AODC) (Balkwill *et al*., 1988). One of the problems with biochemical biomass measures is that the results are determined as the quantity of component (e.g. pmol PLFA) per gram of soil or sediments. Problems of converting viable biomass in chemical terms to numbers of microbes has been discussed (White *et al*., 1995) because there is no universally applicable conversion factor for estimating the PLFA per bacterial cell or the number of cell per gram of dry weight of bacteria. This problem results from observations that most environments harbor microorganisms of widely differing volumes and shapes. Bacterial biovolumes can vary over 3 orders of magnitude (Guckert *et al*., 1985), and the volume of a viable cell can vary with nutritional status. Conversion factors for eukaryotic PLFA to biomass or biomass to cell number are even more problematic. Quantifying fungal biomass based on PLFA and sterol content present a major problem since mycelia often exist as large multinucleated cells with a huge biomass, much of which is not active. In essence, converting lipid phosphate or PLFA values to cell-based carbon content or cell numbers is problematic, and results should be interpreted cautiously (White *et al*., 1995).

1.2. Community physiological status

Microbial adaptation is the process whereby microorganisms respond to changes in the environment, and thus maintain homeostasis. The primary point of contact between microbes and chemicals is the cell envelope, especially the outer and cytoplasmatic membranes. It is assumed that maintenance of a certain fluidity of either membrane is a prerequisite for active life, meeting changes in environmental factors like changes in temperature or the presence of toxic compounds at potentially damaging concentrations. Cells frequently respond to such influences by quantitatively, and sometimes qualitatively, changing their membrane composition. The technical term for this phenomenon is homeoviscous adaptation (Cossins *et al*., 1986). Changes in the membrane lipids, especially in the fatty acid composition of the lipid bilayer, are generally believed to play a major role in this process (Marr *et al*., 1962; Ingram, 1976; Okuyama *et al*., 1991; Suutari and Laakso, 1993; Dubois-Brissonet, 2000).

Without these adaptive adjustments, key physical properties (e.g. membrane fluidity) of the environment of enzymes localized in the lipid phases would be changed. This could have severely negative effects on their activity, together with loss of cytoplasmatic membrane integrity and inhibition of the membrane protein and barrier functions, followed by collapse of the proton motive force and ATP synthesis (Sikkema *et al*., 1994 and 1995).

Many subsets of the microbial community respond to specific conditions in their microenvironment by changing their lipid composition. The proportion of poly-β-hydroxyalkanoates (PHA) in bacteria (Findlay and White, 1987; Nickels *et al*., 1979) or triglyceride (in microeukaryotes) (Gehron *et al*., 1982) relative to PLFA provides a measure of the nutritional/physiological status. Also, an increase in the ratio of *trans*/*cis* monoenoic PLFAs indicates a toxic/sublethal stress on bacterial communities (Guckert *et al*., 1986; Heipieper *et al*., 1992).

Changes in membranes in response to starvation, thermal variation, and physicochemical stress

Insights into microbial community physiological status can be obtained by analyzing lipid biomarkers. Changes that are typically found in PLFA profiles when gram-negative bacteria are starved include an increase in the ratio of saturated to unsaturated fatty acids (Guckert *et al*., 1986; Kieft *et al*., 1994), and increase in the ratio of the *trans*- to *cis*-monoenoic unsaturated fatty acids, and increase in the moles percent of cyclopropyl fatty acids (Guckert *et al*., 1986). Cyclopropyl fatty acids, which are mainly found in gram-negative microorganisms (Ratledge and Wilkinson, 1988), are formed by modifications of existing membrane lipids, often as the microorganisms enter the stationary phase. MacGarrity and Armstrong (1975) found that cyclopropane fatty acids in *E coli* increased during the transition period from the late exponential to the stationary phase. Diefenbach *et al*. (1992) also noted that this transition period was characterized by an increase in the degree of membrane fluidity. A less fluid membrane, by limiting transport and respiration, facilitated conservation of energy (MacGarrity and Armstrong, 1975).

Factors such as oxygen depletion, decreasing pH, a high concentration of Mg^{2+}, high temperature, and poor nutrient quality have been shown to stimulate the production of cyclopropyl fatty acids in pure culture studies and environmental communities (Guckert *et al*., 1986; Petersen and Klug, 1994). For example, anaerobic incubation of a prokaryotic estuarine sediment community has been shown to increase the proportion of cyclopropyl fatty acids to aerobically incubated sediments (Guckert *et al*., 1985).

The aspect of bacterial lipids most extensively studied in relation to thermal adaptation is fatty acid composition (Suutari and Laakso, 1994; Taylor *et al*., 1998). The expected response with an increase in the temperature would be a decrease in the concentration of *cis*-unsaturated and branched-fatty acids and a corresponding increase in the concentration of straight-chain saturated fatty acids, which have higher phase transition temperature. This, however, is complicated by the fact that very few organisms are capable of removing a double bond; therefore the conversion of unsaturated to saturated fatty acids can take place only in connection with membrane

lipid turnover or growth. In the presence of higher, i.e. toxic, concentrations the cells cannot react and are thus notable to adapt to such conditions or they even die (Kabelitz *et al*., 2003).

Bacteria adapt to an increase in their membrane fluidity by increasing the degree of saturation of their phospholipid fatty acids and in some cases, changing from *cis* to *trans* the configuration of their unsaturated fatty acids (Killian et al., 1992; Loffhagen *et al*., 2004). Though the change from the *cis* to the *trans* unsaturated double bond does not have the same decreasing effect on membrane fluidity as a conversion to saturated fatty acids it still causes a substantial effect on the rigidity of the membrane.

Heipieper *et al*. (1992), who studied the adaptation of a *Pseudomonas* strain to phenol, observed conversion of *cis* fatty acids to their *trans* form. The authors suggest that the *cis*-to-*trans* conversion increases membrane ordering and consequently decreases the membrane fluidity, which is in accordance with physicochemical studies on the behaviour of *trans* fatty acid (Okuyama *et al*., 1991). Thus, a decrease in the ordering of the phospholipid molecules caused by phenol is balanced by changing the configuration of the fatty acids from *cis* to *trans*.

1.3. Community composition

The presence of certain groups of microorganisms can be inferred by the detection of unique lipids that originate from specific biosynthetic pathways (Edlung *et al*., 1985; Dowling *et al*., 1986; Hedrick *et al*., 1991 etc.). Consequently, the analysis of SLB classes provides a quantitative definition of a microbial community.

In addition to biomass measurements and physiological status, the quantification of PLFA obtained from lipid analysis provides insight into microbial community composition. Because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, the PLFA are effective taxonomic markers. However, despite its versatility PLFA analysis has limitations for the analysis of Gram-negative bacteria community structure.

Terminally-branched saturated PLFA are common to Gram-positive bacteria, but also are found in Gram-negative bacteria, such as the sulfate-reducing bacteria, and some Gram-negative facultative anaerobes (Kaneda, 1991). Monoenoic PLFA are found in most all Gram-negative microorganisms. Branched monoenoic unsaturated PLFAs and mid-chain branched saturated PLFAs are usually found in anaerobic bacteria. Specific groups of bacteria form monoenoic PLFA with the unsaturation in an atypical position, such as 18:1ω8*c* in the type II methane-oxidizing bacteria (Nichols *et al*., 1985). Polyenoic PLFAs usually indicate the presence of microeukaryotes, but also are common in cyanobacteria (Potts *et al*., 1987; Ratledge and Wilkinson, 1988).

Branched-chain monoenoic PLFA are common in the anaerobic *Desulfovibrio*-type sulfate-reducing bacteria, both in culture and in manipulated sediments (Edlund *et al*., 1985). They are also found in a large proportion of the actinomycetes, which contain mid-chain branched saturated PLFA, in particular 10Me18:0. Environments with a higher quantity of 10Me16:0 in comparison with 10Me18:0 is often a feature of the anaerobic Gram-negative *Desulfobacter*-type sulfate-reducing bacteria (Dowling *et al*., 1986). Although normal (straight-chain) saturated PLFA are found in both prokaryotes and eukaryotes, proportionally, bacteria generally contain more of the 16 carbon moiety (16:0), whereas the microeukaryotes contain more of the 18 carbon moiety (18:0), and this kind of PLFA are considered as ubiquitous (Table I.2.9).

The analysis of other lipids such as the sterols (for the microeukaryotes, nematodes, algae, protozoa) (White *et al*., 1980), glycolipids (phototrophs, gram-positive bacteria), or the hydroxy fatty acids from the lipid A component of the lipopolysaccharide of gram-negative bacteria (Bhat and Carlson, 1992), sphinganines from sphingolipids (Fredickson *et al*., 1995), plasmalogen-derived dimethylacetals (Tunlid and White, 1991), and alkyl ether polar lipids derived from *Archaea* (Hedrick *et al*., 1991) can provide a more detailed community composition analysis.

Table I.2.9. Examples of signature lipids biomarkers[1].

Domain	Group	Markers[2]
BACTERIA	**Common markers**	15:0, *i*15:0, *a*15:0, *i*16:0, 16:1ω9, 16:1ω5, *i*17:0, *a*17:0, 18:1ω7t, 18:1ω5, *i*19:0, *a*19:0
	Aerobic bacteria	16:1ω7c, 18:1ω7, 16:1ω7*t,* 18:2ω6
	Facultative bacteria	17:1ω6
	Anaerobic bacteria	17:0, *cy*17:0, *cy*19:0
	Mycobacteria	Micocerosic acids, hydroxy alcohols
	Sulfate-reducing bacteria	10Me16:0, *i*17:1ω7, *i*15:0, *a*15:0
	Cyanobacteria	18:2ω6
	Actinomycetes	10Me18:0, 11Me16:0, 12Me18:0, MBr FA
	Psychrophilic bacteria	20:5, 22:6
	Anaerobic phototrophs	16:0, 16:1, 18:1, 14:0 (Chlorobiaceae)
	Sulfur-oxidizers	*i*15:0, *a*15:0
ARCHAEA	**Common markers**	Archaeol, and Caldarchaeol
	Methanotrophs	Type I: 16:1ω5*c,* 16:1ω8*c* Type II: 18:1ω8*c,* 18:1ω8*t*
EUKARYA	**Fungi**	18:2ω6, 18:3ω3, 18:3ω6, sterols, 16:1
	Diatoms	16:1ω3*t,* 16:2ω4, 16:3ω4, 20:5ω3
	Green algae	16:1ω13*t,* 18:1ω9, 17:0, 17:1
	Plants	18:1ω11, 26:0, 18:3ω3, 20:5ω3
	Protozoa	20:2ω6, 20:4ω6, 20:3ω6, plasmalogens

[1]References: White *et al*., 1995; Russel and Nichols, 1999. More examples of signature lipid biomarkers can be found in Ratledge and Wilkinson, 1988. [2]Abbreviations: MBr FA, Mid-chain branched fatty acids.

2. The glycolipid fraction

In the SLB approach, the glycolipid fraction is obtained after the elution of the total lipid extract with acetone. As it shows in Figure I.2.29, not only glycolipidic components are recovered in this fraction, but also polyhydroxyalkanoates that can be quantified. As it has been previously explained in section '1.2. Simple fatty esters', polyhydroxyalkanoates are polyesters of hydroxy fatty esters synthesized by numerous bacteria as intracellular carbon and energy compounds and accumulated as granules in the cytoplasm of cells, and that are good candidates for biodegradable plastics because of their mechanical properties and bacterial production.

A wide variety of microorganisms can accumulate polyhydroxyalkanoates (PHAs) such as *Cupriavidus necator*, some *Bacillus* and *Pseudomonas* species, etc. (Rehm, 2003). Some cyanobacteria accumulate substantial amounts of PHA as *Spirulina platensis* and *Spirulina maxima* (Vincenzini *et al*., 1990). Nearly all microorganisms involved in the sulfur cycle accumulate high quantities of PHA, for example the purple sulfur bacteria *Chromatium* (Esteve *et al*., 1990), the sulfur-oxidizing bacteria *Beggiatoa* (Güde *et al*., 1981; Strohl *et al*., 1981) or the sulfate-reducing bacteria *Desulfovibrio saporovorans* (Nanninga and Gottschal, 1987).

The bacteria accumulate PHA compounds if the carbon and/or energy source exceeds the cell metabolic capacity because the mineral nutrient or oxygen supply is limiting (Dawes *et al*., 1973). Some bacteria undergo unbalanced growth and cannot divide when exposed to adequate carbon and terminal electron acceptors because of other limitations such as a lack of essential nutrients (e.g. phosphate, nitrate, trace elements). These bacteria form PHA, as a carbon storage compound and when the essential component becomes available, they catabolize the PHA and form PLFA as they grow and divide. PHA/PLFA ratios can range from 0 (dividing cells) to over 40 (carbon storage). Ratios greater than 0.2 usually indicate the beginning of unbalanced growth in at least part of the microbial community (White *et al*., 1995).

Hence, the ability to accumulate and degrade intracellular storage PHA helps prokaryotes to survive and compete in natural microbial communities (Beccari *et al*.,

1998; López *et al*., 1995). The level of PHA in a community can change rapidly due to variations in nutritional status (Elhottová *et al*., 1997), and the ratio of PHA concentration to the total concentration of microbial biomass can serve as an important marker of the growth and nutritional status of microbial communities (Tunlid and White, 1992).

2.1. Microbial synthesis and degradation of polyhydroxyalkanoates

Polyester synthases are the key enzymes of polyester biosynthesis and catalyse the conversion of (*R*)-hydroxyacyl-CoA thioesters to polyesters with the concomitant release of CoA (coenzyme A) (Fig. I.2.31). These polyester synthases have only recently been biochemically characterized. At present, 59 polyester synthase structural genes from 45 different bacteria have been cloned and the nucleotide sequences have been obtained (Rehm, 2003). Polyester synthases can be assigned to four classes based on their substrate specificity and subunit composition.

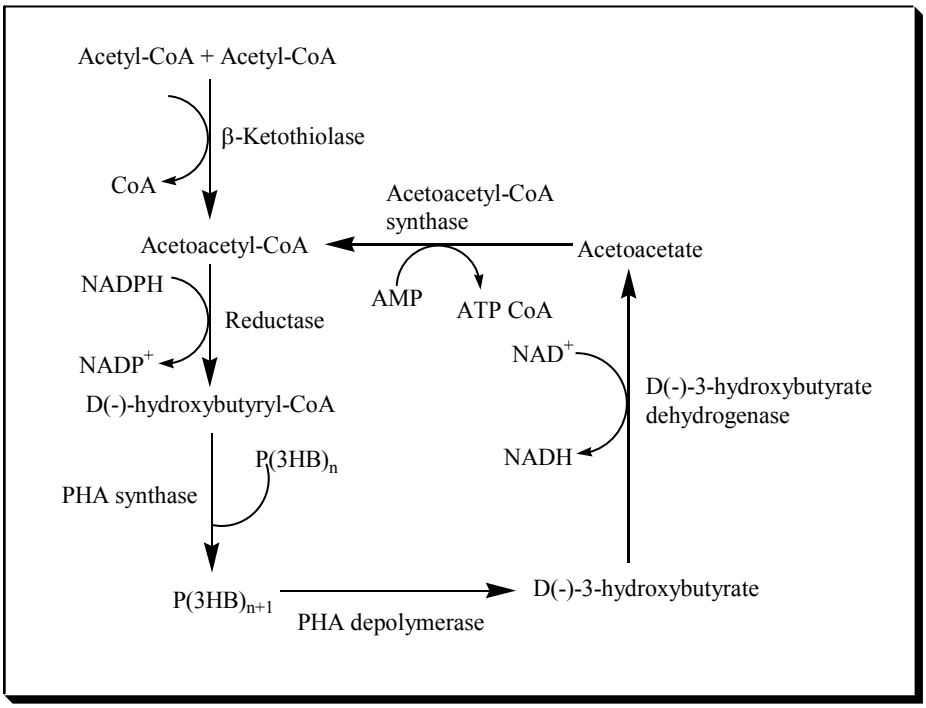


Figure I.2.31. Metabolic pathway involved in the synthesis and degradation of PHB in *Cupriavidus necator* (Vandame and Coenye, 2004; formerly *Alcaligenes eutrophus, Ralstonia eutropha* and *Wautersia eutropha*) (Steinbüchel, 1991).

Class I and class II PHA synthases comprise enzymes consisting of only one type of subunit (*PhaC*) with molecular (Qi and Rehm, 2001). According to their in vivo and in vitro substrate specificity, class I PHA synthases (e.g. in *Wautersia eutropha*) preferentially utilize CoA thioesters of various (*R*)-3-hydroxy fatty acids comprising 3 to 5 carbon atoms, whereas class II PHA synthases (e.g. in *Pseudomonas aeruginosa*) preferentially utilize CoA thioester of various (*R*)-3-hydroxy fatty acids comprising 6 to 14 carbon atoms (Amara and Rehm, 2003). Class III PHA synthases (e.g. in *Allochromatium vinosum*) comprise enzymes consisting of two different types of subunits: (i) the *PhaC* subunit and (ii) the *PhaE* subunit. These PHA synthases prefer CoA thioesters of (*R*)-3-hydroxy fatty acids comprising 3 to 5 carbon atoms (Yuan *et al*., 2001). Class IV PHA synthases (e.g. in *Bacillus megaterium*) resemble the class III PHA synthases, but *PhaE* is replaced by *PhaR* (McCool and Cannon, 2001). Exceptions to this classification are the synthases from *Thiocapsa pfennigii* (two different subunits with strong similarity to the *PhaC* subunit), from *Aeromonas punctata* and from *Pseudomonas* sp. 61-3 (*PhaC1* and *PhaC2*) (Fukui and Doi, 1997; Matsusaki *et al*., 1998; Liebergesell *et al*., 2000) (Fig. I.2.32).

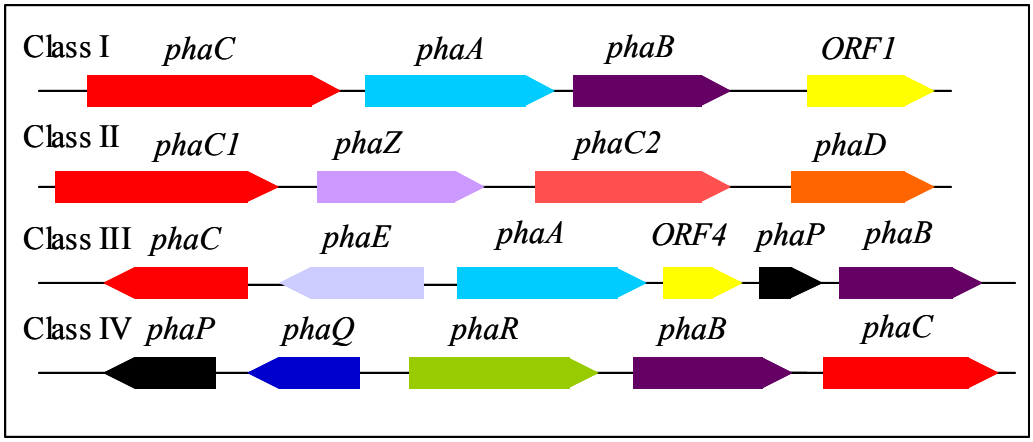


Figure I.2.32. Genetic organization of representative PHA synthase genes.

PhaC/C1/C2, gene encoding PHA synthase; *phaE*, gene encoding subunit of PHA synthase; *phaA*, gene encoding β-ketothiolase; *phaB*, gene encoding acetoacetyl-CoA reductase; *phaR*, gene encoding regulator protein; ORF, open reading frame with unknown function; *phaZ*, gene encoding PHA depolymerase; *phaD*, open reading frames with unknown function.

3. The neutral lipid fraction

In the SLB approach, the neutral lipid fraction is obtained after the elution of the total lipid extract with the less polar solvent of the analysis (chloroform). Sterols, diglycerides, triglycerides and respiratory quinones can be identified and quantified in this fraction (Figure I.2.29). Sterols (see section '1.6. Terpenoid lipids'), are found primarily in eukaryotic microorganisms and many studies have demonstrated their usefulness as indicators of lipid contributions from different eukaryotic organisms in an environmental sample (Boon *et al*., 1979; Lee *et al*., 1980). Their apparently greater resistance to degradation of sterols compared with fatty acids (Johns *et al*., 1978) further enhances their value as biological markers (Volkman *et al*., 1981).

Diglycerides are the breakdown products of phospholipids. Upon cell death, the phosphate group of the phospholipids is degraded by phospholipases leaving the diglyceride with intact fatty acids (see the 'Polar Lipid fraction' section, Viable microbial biomass). The resulting diglycerides contain the same signature lipid fatty acids as the original phospholipid, at least for days to years in the subsurface sediments. Consequently, a comparison of the ratio of phospholipid fatty acid profiles to diglyceride fatty acid profiles provides a measure of the viable to non-viable microbial abundance and composition. Triglycerides are fat storage compounds found in inclusion bodies in eukaryotes and in some bacteria (*Mycobacterium* and *Corynebacterium*, Daniel *et al*., 2004), and indicate an excess of carbon source in the environment.

Isoprenoid or terpenoid quinones are lipid-soluble substances found in almost all species of organisms (see section '1.6. Terpenoid lipids', Isoprenoid quinones). The most important biological aspects of quinones are their functions as electron carriers in respiratory chains and photosynthetic electron transport systems coupled with proton translocation. In addition to their biological importance, quinones have attracted attention in connection with their significance in microbial systematics, because of their inherent structural variations that have a chemotaxonomic significance (Crane, 1965). In general, most Gram-positive bacteria and anaerobic Gram-negative bacteria contain only menaquinones (MKs), whereas the majority of strictly aerobic Gram-negative bacteria contain exclusively ubiquinones (UQs). Both types of isoprenoid quinones

occur only among some facultative anaerobic Gram-negative bacteria (Søballe and Poole, 1999). It is also known that the α-, β- and γ-Proteobacteria have UQs, whereas the Gram-positive bacteria and most δ- and ε-Proteobacteria contain MKs (Iwasaki and Hiraishi, 1998). A detailed list of the distribution of quinones in different groups of microorganisms is shown in Table I.2.10.

In recent years, the quinone profiling method (Hiraishi, 1999) has been successfully applied to the determination of microbial community structures in various environments such as wasterwater environments (Hiraishi *et al*., 1998), natural aquatic systems (Urukawa *et al*., 2000), hot springs (Hiraishi *et al*., 1999), soil (Katayama *et al*., 1998), compost (Tang *et al*., 2004), etc. Moreover, recent studies have shown that quinone content correlates to the microbial biomass and total bacterial counts (Saitou *et al*., 1999; Hiraishi *et al*., 2003).

Quinones for evaluating the redox state of microbial communities

In 1986, Hedrick and White demonstrate the value of quinone analysis in ecological studies. They proposed bacterial respiratory quinones as sensitive indicators of the aerobic versus anaerobic metabolisms in microbial populations. Indeed, there are three reasons to believe that the quinone content of a bacterial community would shift with changes in the availability of oxygen is: (i) because of the fact that some energy-yielding reactions are more or less available depending on the redox carrier, bacteria with the appropriate quinone may have an energetic advantage and overgrow their competitors (Holländer, 1976) (ii) because aerobic bacteria tend to have UQs and anaerobic bacteria tend to have MKs, and some facultative gram-negative bacteria have DMKs (demethylmenaquinones) as well as UQs and/or MKs (Collins and Jones, 1981) (iii) that to maintain a respiratory chain for synthesis of ATP requires certain quantity of respiratory quinones per gram of bacterial quinones, whereas substrate level phosphorylation does not (Höllander *et al*., 1977). Recent studies (Peacock *et al*., 2003), have applied the ratio of ubiquinones to menaquinones and quinones to total PLFA as a measure of the respiratory status of a microbial community in the environment.

Table I.2.10. Distribution of quinone structural types in different phylogenetic groups[1].

Domain	Group	Main Quinone type[2]	
ARCHAEA	**Common quinones**	MK-8 and MK-8(H_2)	
EUBACTERIA	**Cyanobacteria**	Phylloquinone (K_1) and PQ-9	
	Green non-sulfur bacteria	*Chloroflexus*-like, MK-10 and MK-8	
	***Thermus-Deinococcus* group**	MK-*n* (n≤8)	
	Cytophaga-Flavobacterium-Sphingobacterium	MK-6, MK-7	
	Bacteroides	MK-9, MK-10, MK-11, 12,13	
	Green sulfur bacteria	MK-7	
	Plactomycetales	MK-6	
	Gram positive bacteria (low G+C)		
	Bacillales	MK-7	
	Lactobacillales	MK-7, MK-8, MK-9, MK-10	
	Gram positive bacteria (high G+C)	Actinobacteria MK≥9, MK-*n*(Hx)	
	α-Proteobacteria Q-10, and some contain also MK-9 or MK-10	*Rhodospirillum*	Q-9, Q-10, MK-9 MK-10, RQ-*n*
		Acetobacter	Q-8, Q-9
		Rhodomicrobium	Q-9, Q-10, RQ-*n*
		Rhodopseudomonas	Q-10, MK-9, MK-10
	β-Proteobacteria Q-8 and some MK-8	*Cupriavidus*	Q-8
	γ-Proteobacteria Q-8, Q-9 or Q-10 to Q-14	*Chromatium*	Q-8, Q-7, MK-8
		Beggiatoa	Q-8
		Thiomicrospira	Q-8
		Escherichia	Q-8, DMK-8
		Pseudomonas	Q-9, Q-10
	δ- and ε-Proteobacteria MK-6, MK-7 or saturated MK	*Desulfovibrio*	MK-6
		Helicobacter	MK-6

[1]References: Collins and Jones, 1981; Hiraishi, 1999; Ratledge and Wilkinson, 1988. Q-*n* and MK-*n*, Ubiquinone and menaquinone with *n* isoprenoid units. [2]Abbreviations: MK-*n*(Hx), menaquinone with the side chain saturated with x hydrogen atoms. PQ, plastoquinone. RQ, rhodoquinone.

4. The expanded SLB analysis

Figure I.2.29 represent the diagram of the possibilities for lipid analysis and how can it allows insight not only in the community composition and viable biomass but also into the physiological status of the microbial community. One of the most important points of the expanded SLB analysis is that can be expanded to include analysis of DNA. The DNA probe analysis offers powerful insights because of the specificity in the detection of genes coding for enzymes and/or for 16S rDNA for organisms identification. The DNA recovered from the lipid extraction is of high quality and suitable for enzymatic amplification (Macnaughton and Stefen 2001). As a result, new techniques based on combining lipid analysis and PCR of rDNA, with subsequent separation of the amplicons by DGGE (denaturing gradient gel electrophoresis), can be applied in order to obtain a complete picture of the activities, dynamics and diversity of a microbial community (Stefen *et al*., 1999). Moreover, the future application of other techniques, e.g. Real-time PCR, analysis of the expression of certain genes, and microarrays, would provide an even better understanding of complex ecosystems such as those of microbial mats.

Further insight can be provided by the carbon isotopic compositions ($\delta^{13}C$ values) of lipids as determined by compound-specific isotope analyses (Pancost and Sinninghe Damsté, 2003). The controls on the carbon isotopic compositions of individual prokaryotic lipids are diverse and include the source of substrate carbon, the biological mechanism of carbon assimilation (van der Meer *et al*., 2001), and pathways of lipid biosynthesis (Teece *et al*., 1999). Many studies have utilized the unique structures and $\delta^{13}C$ values of prokaryotic lipids to elucidate ancient sedimentary processes or to study modern ecosystems (Koopmans *et al*., 1996; van der Meer *et al*., 2001; Zhang *et al*., 2005).

3. Objectives and structure of this work

This PhD thesis is integrated in the general objectives of the Microbial Ecogenetics group of the Department of Microbiology of the University of Barcelona. Our research group is focused on the study of microbial mats as a complex microbial community in which ecophysiological relationships are established between their members. As it has been explained before (Introduction, section '1. Microbial Mats: the dense, living carpet of Gaia'), microbial mats are a model of study of biochemical cycles, microbial interactions, survival strategies under extreme conditions and evolution of microbial communities. In this sense, previous studies in our research group have been focused on the ecology of anoxygenic phototropic bacteria in microbial mats and lakes (Mas-Castellà *et al*., 1996) and dynamics of bacteria involved in the sulfur-cycle, distribution and geological characteristics of estuarine microbial mats (Guerrero *et al*., 1993a; Rampone *et al*., 1993), identification of new bacterial species in microbial mats (Guerrero *et al*., 1993b; Guerrero *et al*., 1999), study of the photosynthesis and respiratory activity in microbial mats (Urmeneta *et al*., 1998), isolation and characterization of cyanobacteria (Urmeneta *et al*., 2003), evaluation of ecophysiological changes in microbial mats by signature lipid biomarkers (Navarrete, 1999; Navarrete *et al*., 2000; Navarrete *et al*., 2004), and the study of the dynamic and microbial members implicated in the synthesis and degradation of polyhydroxyalkanoates as reserve compounds (Urmeneta, 1995; Urmeneta *et al*., 1995; Rothermich *et al*., 2000).

Since 1999, the Microbial Ecogenetic group is interested in the ecophysiological characterization of microbial communities by molecular techniques based on DNA and signature lipid biomarkers, as combined methods that provide a better knowledge of this kind of microbial communities. This interest is the result of collaboration with Prof. D. C. White from the Center of Biomarker Analysis (Knoxville, Tennessee, USA), and more recently with Dr. Roland Geyer from the UFZ Center for Environmental Research (Leipzig-Halle, Germany). Moreover, this thesis is applied for being an European PhD accomplishing the normative of the European universities that demands a minimum stay of three months in another european country. In this case, the stay was performed in the 'Consiglio Nazionale delle Ricerche. Istituto di ricerca sulle acque' in Rome (Italy)

under the direction of Dr. Valter Tandoi and in the 'UFZ Center for Environmental Research' (Leipzig-Halle, Germany) under the direction of Dr. Roland Geyer.

Therefore, the general objective of this thesis was the ecophysiological and molecular characterization of estuarine microbial mats from different locations, and the isolation and characterization of new microbial species involved in the physiological relationships in this microbial ecosystem as a model. The detailed objectives and the distribution in chapters are summarized as follows:

CHAPTER III

- Validation of the signature lipid biomarker approach in microbial mat samples.

CHAPTER IV

- Vertical characterization of microbial mats by signature lipid biomarkers (SLB), and microbial composition by PCR-DGGE (Denaturing Gradient Gel Electrophoresis).

CHAPTER V

- Evaluation of the redox state and community composition of microbial mats.

CHAPTER VI

- Ecophysiological changes and composition of mats in a circadian cycle by SLB.

CHAPTER VII

- Characterization of heterotrophic bacteria isolated from the photic zone.

CHAPTER VIII

- Bacterial succession in microbial mat sulfur blooms, and characterization and relationships between microbial members.

CHAPTER IX

- General conclusions.