

LIPIDS IN MARINE ENVIRONMENT

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BY

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CERTIFICATE

This is to certify that the thesis entitled "**Lipids in marine environment**" submitted by **Ms. Ranjita R. Harji** for the award of the degree of Doctor of Philosophy in Microbiology is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any university or institution.



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*A list of abbreviations used included
in the thesis.*

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STATEMENT

As required under the university ordinance 0.19.8 (vi), I state that the present thesis entitled "*Lipids in marine environment*" is my original contribution and the same has not been submitted for any other degree or professional qualification on any previous occasion. To the best of my knowledge the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been provided to the funding agencies and the suggestions, if any, have been duly incorporated.



(Ranjita R. Harji)

**DEDICATED TO MY
PARENTS
&
GUIDE**

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List of Publications

List of abbreviations used

Acridine orange direct counts (AODC)

Anteiso branched PLFAs (Anteiso)

Bacterial acid methyl ester (BAMEs)

Basal salt solution (BSS)

Basal salt solution with glucose yeast extract and peptone (BSS-GYP)

Branched C15 PLFA (Br15)

Branched C17 PLFA (Br17)

Br15 (iC15:0+aC15:0)

Br17 (iC17:0+aC17:0)

Branched chain FAs (Br-FAs)

Carbon isotope composition ($\delta^{13}\text{C}_{\text{oc}}$)

Carbon preference index (CPI)

Chloroform (CHCl_3) Dark incubation 1 (DK1)

Dark incubation 2 (DK2)

Dissolved inorganic carbon (DIC)

Distilled water (D/W)

Dry weight (dw)

Eicosapentaenoic acid (EPA)

Electron ionization (EI)

Equatorial Indian Ocean (EIO)

Fatty acids (FAs)

Fatty acid methyl ester FAME

Gas chromatography (GC)
Gas chromatography isotope ratio mass spectrometry (GC-irms)
Gas chromatography mass spectrometry (GCMS)
Hydrochloric acid (HCl)
Iso branched PLFAs (Iso)
Lipid phosphate (L-PO₄)
Lipopolysaccharide (LPS)
Marine carbon (MC)
Methanol (MeOH)
Monounsaturated fatty acids (MUFAs)
Monounsaturated PLFAs (MUFAs)
Nitrogen (N)
N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)
Organic carbon (OC)
Organic carbon normalized values (OC-normalized)
Organic matter (OM)
Oxygen Minimum Zone (OMZ)
Pee Dee Belemnite (PDB)
Phosphorus (P)
Phosphatidylcholine (PC, GPCho or lecithin)
Phosphatidylethanolamine (PE or GPEtn)
Phosphatidylserine (PS or GPSer).
Polyunsaturated fatty acids (PUFAs)
Polyunsaturated PLFAs (PUFAs)

Saturated fatty acids (Sat-FA)
Saturated PLFAs (Sat)
Sea surface temperatures (SST)
Sodium acetate (NaAc)
Synthetic sea water (SSW)
Terminally branched saturated iso and anteiso fatty acids (Br-FAs)
Terminally branched saturated iso and anteiso PLFAs (Br)
Terrestrial carbon (TC)
Total fatty acids (TFAs)
Total lipid extracts (TLE)
Transition temperature (T_m)
Tributyltin-chloride (TBT)
Unsaturation index of C37 alkenones (U_{37}^K)
Unsaturated fatty acids (UFAs)
Unsaturated PLFAs (Unsat)
Zobell marine agar (ZMA)

General Introduction

1. Introduction

1.1 Organic matter (OM) in marine environment

Marine ecosystems cover approximately 71% of the Earth's surface and contain approximately 97% of the planet's water. Marine environments generate 32% of the world's net primary productivity (Alexander and David, 1999). Microscopic plants called phytoplankton are the primary sources of OM in the ocean ("primary production"). OM produced in surface ocean waters provides the fuel for the food chain (zooplankton, fishes and mammals including humans). Organisms in the deep sea are almost entirely dependent upon sinking living and dead OM which sinks at approximately 100 m per day. The vast majority of OM (~99%) is eaten and respired somewhere within the water column (Zang et al., 2000; Hernes et al., 2001). Only a small fraction (<1%) of OM produced photosynthetically in the ocean by microscopic plants (phytoplankton) passes to an average ocean depth of 4000 m (Suess, 1980; Martin et al., 1987). This small fraction settles to the seafloor largely packaged within biological aggregates (fecal pellets, marine snow) where it accumulates as a sediment deposit. Continuation of this particle rain over time yields a sediment record. OM in marine environment exists as dissolved organic matter, suspended particulate organic matter, sinking organic matter, or as sedimentary organic matter.

Amino acids, carbohydrates and lipids are the major components of the OM of all organisms. Of these, lipids constitute the smallest component of OM of the organisms. Lipids are broadly defined as hydrophobic or amphiphilic molecules. They are water-insoluble organic molecules that can be extracted from cells and tissues using organic solvents, e.g., methanol, chloroform, ether, benzene, etc. The amphiphilic nature of some lipids allows them to form structures such as vesicles, liposomes, or membranes in an aqueous environment. Hence lipids can function as structural, storage, and transport

components. These compounds form protective coating on the surface of many organisms and are involved in cell recognition, species specificity, and tissue immunity. Some fat soluble vitamins and hormones have intensive biological activity (Lehninger, 1993). Based on their structure, they are broadly classified as simple and complex lipids. They are a broad group of naturally occurring molecules such as fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E and K), monoglycerides, diglycerides, phospholipids, and others. There are several different families or classes of lipids but all derive their distinctive properties from the hydrocarbon nature of a major portion of their structure. Lipids originate entirely or in part from two distinct types of biochemical subunits or "building blocks": ketoacyl and isoprene groups (Fahy et al., 2005). Based on this they are classified as follows.

1.2 Major groups of lipids

1.2.1 Fatty acyls

Fatty acyls, a generic term for describing fatty acids (FAs), their conjugates and derivatives, are a diverse group of molecules synthesized by chain-elongation of an acetyl-CoA primer with malonyl-CoA or methylmalonyl-CoA groups in a process called fatty acid synthesis. They are made of a hydrocarbon chain that terminates with a carboxyl group; this arrangement confers the molecule with a polar, hydrophilic end, and a nonpolar, hydrophobic end that is insoluble in water (Vance and Vance, 2002; Brown, 2007). Examples are eicosanoids, derived primarily from arachidonic acid and eicosapentaenoic acid, which include prostaglandins, leukotrienes, and thromboxanes. Other major lipid classes in the FA category are the fatty esters and fatty amides. Fatty esters include important biochemical intermediates such as wax esters, FA thioester coenzyme A derivatives, FA thioester ACP derivatives and FA carnitines. The fatty

amides include N-acyl ethanolamines, such as the cannabinoid neurotransmitter anandamide (Fezza et al., 2008).

1.2.2 Glycerolipids

Glycerolipids are composed mainly of mono-, di- and tri-substituted glycerols (Coleman and Lee, 2004), the most well-known being the FA esters of glycerol (triacylglycerols), also known as triglycerides. In these compounds, the three hydroxyl groups of glycerol are each esterified, usually by different FAs.

1.2.3 Phospholipids

Phospholipids are ubiquitous in nature and are key components of the lipid bilayer of cell membranes, as well as being involved in metabolism and cell signaling. Phospholipids may be subdivided into distinct classes, based on the nature of the polar headgroup at the *sn*-3 position of the glycerol backbone in eukaryotes and eubacteria, or the *sn*-1 position in the case of archaeobacteria (Ivanova et al., 2007). Examples of phospholipids are phosphatidylcholine (also known as PC, GPCCho or lecithin), phosphatidylethanolamine (PE or GPEtn) and phosphatidylserine (PS or GPSer).

1.2.4 Sphingolipids

Sphingolipids are a complex family of compounds (Merrill and Sandhoff, 2002) that share a common structural feature, a sphingoid base backbone that is synthesized *de novo* from the amino acid serine and a long-chain fatty acyl CoA, then converted into ceramides, phosphosphingolipids and glycosphingolipids. The glycosphingolipids are a diverse family of molecules composed of one or more sugar residues linked via a glycosidic bond to the sphingoid base. Examples of these are the simple and complex glycosphingolipids such as cerebrosides and gangliosides.

1.2.5 Sterol lipids

Sterol lipids, such as cholesterol and its derivatives, are an important component of membrane lipids (Bach and Wachtel, 2003), along with the phospholipids and sphingomyelins. The steroids, all derived from the same fused four-ring core structure, have different biological roles as hormones and signaling molecules. The secosteroids, comprising various forms of vitamin D, are characterized by cleavage of the B ring of the core structure (Bouillon et al., 2006).

1.2.6 Prenol lipids

Prenol lipids are synthesized from the 5-carbon precursors isopentenyl diphosphate and dimethyl allyl diphosphate that are produced mainly via the mevalonic acid pathway (Kuzuyama and Seto, 2003). The simple isoprenoids (linear alcohols, diphosphates, etc.) are formed by the successive addition of C5 units, and are classified according to number of these terpene units. Carotenoids are important simple isoprenoids that function as antioxidants and as precursors of vitamin A (Rao and Rao, 2007). Vitamin E and vitamin K, as well as the ubiquinones, are examples of this class. Prokaryotes synthesize polyprenols (called bactoprenols) in which the terminal isoprenoid unit attached to oxygen remains unsaturated, whereas in animal polyprenols (dolichols) the terminal isoprenoid is reduced (Swiezewska and Danikiewicz, 2005).

1.2.7 Saccharolipids

Saccharolipids describe compounds in which fatty acids are linked directly to a sugar backbone, forming structures that are compatible with membrane bilayers. In the saccharolipids, a monosaccharide substitutes for the glycerol backbone present in glycerolipids and phospholipids. The most familiar saccharolipids are the acylated glucosamine precursors of the Lipid A component of the lipopolysaccharides in Gram-negative bacteria. Typical Lipid A molecules are disaccharides of glucosamine, which are derivatized with as many as seven fatty-acyl chains.

1.2.8 Polyketides

Polyketides are synthesized by polymerization of acetyl and propionyl subunits by classic enzymes as well as iterative and multimodular enzymes that share mechanistic features with the fatty acid synthases. They comprise a large number of secondary metabolites and natural products from animal, plant, bacterial, fungal and marine sources, and have great structural diversity (Walsh, 2004; Caffrey et al., 2008). Many commonly used anti-microbial, anti-parasitic, and anti-cancer agents are polyketides or polyketide derivatives, such as erythromycins, tetracyclines, avermectins, and antitumor epothilones (Minto and Blacklock, 2008).

1.3 Factors affecting production of lipids in marine microorganisms

Production of lipids is influenced qualitatively and quantitatively by several environmental parameters including salinity, oxygen, pH, toxic chemicals and other parameters such as growth phase, temperature, concentration of substrates and nutrients. Microbial stress indicators such as cy17:0/16:1n7c, cy19:0/18:1n7c, and monounsaturated fatty acids/saturated fatty acids (MUFA/Sat-FA) have been extensively studied to monitor changes to microbial cell membrane composition due to variation in temperature (Suutari and Laakso, 1994), substrate availability (Kieft et al., 1994), water limitations (Moore-Kucera and Dick, 2008), and various toxic compounds including organometallic compounds (Heipieper et al., 1992; Heipieper et al., 1994; Weber and de Bont, 1996; White and Tobin, 2004; Bernat and Długoński, 2007; Akerblom et al., 2007). For the algal culture light intensity, photoperiod and the intensity of UVB irradiation (Brown et al., 1996; Tzovenis et al., 1997; Skeratt et al., 1998; Oliveira et al., 1999) and carbon dioxide concentration (Pronina et al., 1998) also influence lipid concentration and composition.

1.3.1 Effect of pH

An increase in unsaturated fatty acids (UFAs) with increasing culture pH has been reported in many fungi. An initial culture pH 6.0-7.6 is found to be optimal for UFAs such as eicopentaenoic acids (EPA) production in fungi and algae (Yongmanitchai and Ward, 1991; Bajpai et al., 1992).

1.3.2 Effect of aeration

Microorganisms require molecular oxygen for the desaturation mechanism in the biosynthetic pathways of polyunsaturated fatty acids (PUFAs), so availability of oxygen generally determines the degree of unsaturation of fatty acids produced (Erwin, 1973). Increased oxygen tension in the medium was found to elevate UFA content in the dinoflagellate *Gyrodinium cohnii* (Harrington & Holz, 1966). Growth of *Euglena gracilis* grown under anaerobic conditions induced formation of saturated fatty acids (Sat-FAs) (Yongmanitchai and Ward, 1989).

1.3.3 Effect of light intensity

The light intensity and photoperiod can affect FAs in a number of microalgae (Brown et al., 1996; Tzovenis et al., 1997). It has been reported that low light intensity enhanced formation and accumulation of PUFAs in many diatoms and euglenids, such as *Cyclotella menaghiniana*, *Nitzschia closterium* and *Euglena gracilis* (Erwin and Bloch, 1963, 1964; Constantopoulos and Bloch, 1967; Sicko-Goad et al., 1988). However, for the green alga, *Chlorella aghiniana*, *Nitzschia closterium* and *Euglena gracilis* (Erwin and Bloch, *minutissima* (Seto et al., 1984) and for the red alga *Porphyridium cruentum* (Renzanka et al., 1987), the effect of light intensity was reversed. In many photosynthetic algae, the absence of light was found to enhance the formation of ω -6 FAs (FA and suppress the formation of ω -3 FAs particularly EPA (Erwin, 1973). Levels of ω 3 FAs increased with increasing light intensity in *Chaetoceros gracilis* Schütt (Mortensen et al., 1988). Quality of light i.e., light spectrum had certain effects on the proportion of

arachidonic acid in some marine algae. White light was suitable for *Enteromorpha intestinalis*, whereas red light was better for *Sargassum salicifolium* (Radwan et al., 1988).

1.3.4 Temperature

Microorganisms inhabit environments with temperature ranging from below 0 °C up to 121 °C (Blöchl et al., 1995, 1997, Rothschild and Mancinelli, 2001; Kashefi and Lovley, 2003, Wagner et al., 2007). Thus, it appears that microorganisms show a high adaptability towards extremely variable ambient temperatures. Microorganisms can change their cell membrane lipid composition in order to maintain the fluidity and functionality of cell membrane in response to ambient temperature changes, the term called as homeoviscous adaptation (Sinensky, 1974). In cold environments microbial cells have to keep their solid–liquid phase transition temperatures below the ambient temperature to maintain the membrane fluidity. Thus, the energy supply by ambient temperature is always high enough to keep the cell membranes in a dynamic fluid stage.

Several mechanisms are known for bacteria and archaea to alter their cell membrane composition to adapt to low-temperature (Russell and Fukunaga, 1990; Suutari and Laakso, 1994). Bacterial cell membranes are largely formed of phospholipid bilayers. Decreasing environmental temperatures can lead to two main mechanisms in the phospholipid bilayers: (1) an increase in the degree of cis-unsaturated phospholipid fatty acids (PLFAs) and (2) a higher proportion of short chain PLFAs (Suutari and Laakso, 1994). The inclusion of cis double bonds into the PLFA side chains causes a decrease in the melting temperature of the corresponding phospholipid (Russell, 1989; Coolbear et al., 1983). This is, because cis-unsaturation inserts a bend in the otherwise relatively straight FA side chains, preventing a higher compaction of the cell membrane lipids caused by decreasing ambient temperatures (Russell, 1989). As melting temperatures of fatty acyl side chains decreases with decreasing chain length, a higher proportion of short chain

PLFAs also lead to a lowering of the cell membrane melting temperature (Suutari and Laakso, 1994). Iso and anteiso branched chain FAs (Br-FAs) and Sat-FAs in Gram positive bacteria also changes with the growth temperature (Kaneda, 1991). At low temperature the relative amounts of anteiso FAs increases while the iso and Sat-FAs are generally seen to decrease. Thus, the chemical structure of the different phospholipid groups effects the solid to liquid phase transition temperature of the cell membrane in response to ambient temperature changes (Russell, 1989; Chintalapati et al., 2004).

Temperature effects on lipid content depend upon temperature optima of microalgae involved (Cobetas and Lechado, 1989). Acclimation to low temperatures enhances acyl-chain desaturation as a means of modifying membrane properties in response to low-temperature stress (Lynch and Thompson, 1984). *Spirulina* sp. shows higher unsaturation at lower temperatures (15 and 20°C) stress (Lynch and Thompson, 1984). The ω 3 UFAs (C18:3, C20:5 and C22:6) seen to increase with decreasing temperatures (Mortensen et al., 1988). FAs with high degree of unsaturation are required at low temperatures to maintain flexibility and permeability of membrane phospholipids (Suutari & Laakso, 1994). Low temperatures have been shown to favour formation of PUFAs in marine microalgae (Ackman et al., 1968; Mortensen et al., 1988; Oliveira et al., 1999). However, the response to growth temperature varies from species to species, with no overall consistent relationship between temperature and FA unsaturation (James et al., 1989; Thompson et al., 1992b; Renaud et al., 1995). High growth temperatures decrease the production of total PUFA (C20:5n3 and C22:6n3) in tropical Australian microalgae such as *Rhodomonas* sp., and prymnesiophyte NT19 (Renaud et al., 2002). *Chaetoceros* sp. grew well at 35°C, maintaining moderate percentages of PUFA (9.6 %) at that temperature. However, low temperature favours the formation of Sat-FAs in many species of marine microalgae (Ackman et al., 1968; Mortensen et al., 1988; Renaud et al., 1995).

This effect is influenced by changes in the fluidity of cell membrane phospholipid layers depending on the degree of FA unsaturation (Harwood, 1988; Sargent et al., 1989). These effects are indicative of interruptions to the biosynthetic processes of chain elongation and desaturation at higher temperatures.

EPA production is found to be more at low growth temperatures (Shimizu et al., 1988a; Yongmanitchai and Ward 1991; Seto et al., 1984; Bajpai et al., 1992). Psychrophilic microorganisms with optimum temperature below 20°C contain more highly UFAs than mesophiles (Erwin, 1973). Likewise, thermophiles contain negligible amounts of PUFAs. True thermophiles are generally prokaryotes, and hence, PUFAs are usually rare in bacteria. Increased synthesis of UFAs at lower temperatures has been observed in certain blue-green algae, bacteria, eukaryotic algae, yeasts and fungi (Yongmanitchai and Ward, 1989). EPA production is found to be more at low growth temperatures (Shimizu et al., 1988a; Yongmanitchai and Ward 1991; Bajpai et al., 1992). Brown and Rose (1969) postulated that because of increased solubility of oxygen at low temperatures, a greater amount of intracellular molecular oxygen was available which would be required by oxygen-dependent enzymes catalysing the desaturation of long chain UFAs. On the other hand, it has been suggested that the temperature associated production of PUFAs in microorganisms is a way of adaptation to the environment (Marr and Ingrahm, 1962). Seto et al. (1984) observed that *Chlorella minutissima* produced large amounts of EPA only when incubated at low temperatures and suggested that the enzyme activities involved in desaturation and chain elongation might be highly thermolabile.

1.3.5 Nutrients

Nitrogen content of the growth medium affects the proportion of saturated to unsaturated FAs in algae, bacteria and fungi (Yongmanitchai and Ward, 1989; Erwin, 1973). Under nitrogen stress, *Botryococcus braunii*, *Dunaliella bardawal* and *D. salina*

produce a higher percentage of EPA (Ben-Amotz et al., 1985). In contrast, the proportion of PUFAs in the fresh water algae *Scenedesmus* and *Chlorella* increased at high nitrogen concentrations (Weete, 1980). Besides the concentration, sources of nitrogen also play an important part in controlling lipid concentration and composition in microalgae. In diatom *Phaedactylum tricornerutum* UTEX 640, the best nitrogen sources for EPA production were nitrate and urea (Yongmanitchai and Ward, 1991).

Phosphate concentration in the medium in the lower range (0.05 to 0.5 g l⁻¹) had little effect on biomass production of *Phaedactylum tricornerutum* UTEX 640, but maximum EPA content was obtained at these phosphate levels (Yongmanitchai and Ward, 1991). Increasing concentrations of K₂HPO₄ (0.01% to 0.07%; w/v), γ -linolenic acid content increased, while oleic acid was maximum at 0.04% and the unsaturation was also highest at this concentration (Mahajan and Kamat, 1995).

Lipid content in marine diatoms increases under silicate limited conditions (Schiffirin and Chisholm, 1981; Enright et al., 1986b; Taguchi et al., 1987). Marine diatoms react faster to limitation of the supply of metasilicate than to reduction of other macronutrients. Degree of silicate limitations influences the levels of Sat-FAs in the diatom cultures (*Chaetoceros gracilis* Schütt) (Mortensen et al., 1988), while reducing the level of 22:6n3. Total ω 3 to ω 6 ratio does not show any clear silicate-dependent variation (Mortensen et al., 1988). In *Chlorella minutissima*, the supplementation of medium with sodium chloride increased the proportion of EPA (Seto et al., 1984), however in a marine diatom *Navicula* sp. had no effect on EPA content over a wide range of salinity. The FA γ -linolenic acid level increased with increase in NaCl concentration (Mahajan and Kamat, 1995).

1.3.6 Effect of incubation time

Lipid production by a given species depends to a great extent on the developmental stage/growth of that species (Wassef, 1977; Dunstan et al., 1993; Brown et al., 1996; Zhu et al., 1997). Lipid content, total FAs (TFAs) and phospholipids increased from logarithmic to linear growth phase and declined at stationary growth phase in dinoflagellate *Gymnodinium* sp. (Mansour et al., 2003). High proportions of phospholipids during logarithmic phase required to maintain the integrity of membranes. Some stationary phase cultures are reported to contain lower degree of unsaturation (Dunstan et al., 1993; Brown et al., 1996). However, despite the relative changes in the main FAs, the FA composition remained typical of dinoflagellates at all stages of growth, and can still be used as a distinguishing chemotaxonomic characteristics of this alga (Mansour et al., 2003). In *Aspergillus nidulans*, lipid formation was found to accelerate at the later stages of growth. However, decreased content of lipids in longer periods of growth was observed in *Aspergillus nidulans* and *M. elongata* NRRL 5513 (Singh and Walker, 1956; Bajpai et al., 1992). As microbial biomass ages, many microorganisms tend to store their energy source in the form of lipid, and this lipid is usually rich in Sat-FAs and UFAs. With many microorganisms, a general decrease of UFAs as a function of time has been noted (Erwin, 1973). However, in the case of a photosynthetic protist *Ochromonas danica* (Gellerman and Schlenk, 1979), a marine diatom *Phaedactylum tricornutum* (Yongmanitchai and Ward, 1989) and some *Motierella* species (Shimizu et al., 1989a), the concentrations of PUFAs increased significantly as the culture aged. Shimizu et al. (1989a) reported that EPA content in the harvested mycelia of *M. alpina* can be specifically enriched when the mycelia were allowed to stand for further few days. A similar increase was also observed in arachidonic acid content (Shimizu et al., 1989a).

1.3.7 Environmental contaminants

Most environmental pollutants are membrane active molecules. Growing cells of *Pseudomonas putida* are seen to react to the presence of sublethal concentration of phenol and organic solvents such as toluene by increasing the degree of saturation (Heipieper et al., 1992; Heipieper et al., 1994), while ethanol led to decrease in the saturation in *P. putida* (Heipieper et al., 1994). Sat-FAs also increased in *Oceanomonas baumannii* in the presence of phenol (Brown and Cummings, 2001). *P. putida* showed increasing concentrations of trans UFAs associated with decreasing concentrations of cis UFAs when concentrations of phenol and organic solvents increased in the growth medium (Heipieper et al., 1992; Heipieper et al., 1994). A change in the FA composition leading to an increase in the degree of saturation of the membrane lipids is a well known response of bacteria to membrane active substances, such as organic solvents (Weber and de Bont, 1996). Similarly, the enhanced toxicity of organotin compounds is closely related to their interactions with biological membranes (White and Tobin, 2004). Organotin compounds are widely distributed toxicants. These compounds are membrane active molecules, and their mechanism of action appears to be strongly dependent on organotin lipophilicity (Cima et al., 1996; Kleszczyńska et al., 1997). Several studies have shown the toxicity of tributyltins (TBT) on lipids and FAs of microorganisms (Brown and Cummings, 2001; Viegas et al., 2005; Bernat and Długoński, 2007). In filamentous fungus *Cunninghamella elegans* ratio of Sat-FAs to UFAs augmented (Bernat and Długoński, 2007). This was also observed in *Saccharomyces cerevisiae* in the presence of 2,4-dichlorophenoxyacetic acid (Viegas et al., 2005). Increased amount of C16:0 in response to membrane active substances had been reported in *Saccharomyces cerevisiae* and *Cunninghamella elegans* (Viegas et al., 2005; Bernat and Długoński, 2007)

1.4 Indicators of sources of OM in marine environment

Due to the complex nature of OM in natural environmental samples such as water, suspended particulate matter and sediments, a variety of approaches including bulk elemental carbon analysis, carbon isotope composition ($\delta^{13}\text{C}_{\text{oc}}$), and lipid biomarkers (Zimmerman and Canuel 2001; Bouillon et al., 2008) have been used to determine their sources. Stable carbon isotope ($\delta^{13}\text{C}_{\text{oc}}$) signatures of the various carbon sources are often different, and despite some overlap between different sources, they are powerful tracers of carbon inputs in various environments (Fry and Sherr, 1984; Prahl et al., 1994; Meyers, 1994; Schelske and Hodell, 1995). Geochemical lipid biomarkers such as sterols, alcohols, *n*-alkanes, FAs, PLFAs, and alkenones also are often used to identify the carbon inputs from various organisms in marine environments (Volkman et al., 1992a; Tolosa et al., 2003; Wu et al., 2004). For example, long-chain *n*-alkanes and *n*-alkanols are important compounds of higher plant leaf waxes (Eglinton and Hamilton, 1967). Long-chain alkenones and longchain alkyl diols are associated with marine microalgae-haptophytes (including coccolithophorids) and eustigmatophytes (but in the latter case there may be other sources), respectively (Volkman et al., 1980a, b; 1992). The concentrations of these compounds can be used as a proxy for marine productivity. Diatoms are the most important phytoplankton species in spring blooms and upwelling events, if silicate is delivered to the photic zone (Nybakken, 1993; Abrantes and Moita, 1999) and thus indicate nutrient- rich conditions. Lower nutrient conditions favour smaller photosynthetic organisms, like haptophytes and very small flagellates that have a proportionately greater surface area to adsorb the nutrients, but a lower relative need (Nybakken, 1993).

There exists a considerable difference between the concentration and character of biologically synthesized organic matter that is present in geological settings. Many processes act on the organic matter in the relatively short span of time during its synthesis and its permanent burial in sediments. An important component of past oceanographic

(paleoceanographic) investigations is to identify the sources of organic matter present in sediments deposited at different times in the past. A critical question in view of the known decreases that occur to the quantity of sedimenting organic matter is "how accurately does the type of organic matter in sediments reflect the original sources?" Despite the extensive early diagenetic losses of organic matter in general and of some of its important biomarker compounds in particular, one of the bulk identifiers of organic matter sources - the $^{13}\text{C}/^{12}\text{C}$ ratio appear to undergo little change.

1.4.1 Bulk isotopic carbon

Carbon isotopic ratios are useful to distinguish sources of sedimentary OM between marine and continental plant, and to identify OM from different types of land plants. Most photosynthetic plants incorporate carbon into OM using the C_3 Calvin pathway which biochemically discriminates against ^{13}C to produce a $\delta^{13}\text{C}$ shift of $\sim -20\text{‰}$ from the isotope ratio of the inorganic carbon source. Some plants use the C_4 Hatch- Slack pathway, which creates a diffusional isotope shift of $\sim -7\text{‰}$. OM produced from atmospheric CO_2 ($\delta^{13}\text{C} \approx -7\text{‰}$) by land plants using the C_3 pathway consequently has an average $\delta^{13}\text{C}$ value of $\sim -27\text{‰}$ (PDB) and by those using the C_4 pathway $\sim -14\text{‰}$ (O'Leary, 1988). Freshwater algae utilize dissolved CO_2 , which is usually in isotopic equilibrium with atmospheric CO_2 . As a consequence, the carbon isotopic composition of lake-derived OM is typically indistinguishable from that of OM from the surrounding watershed (e.g., Nakai, 1972; Benson et al., 1991). The source of inorganic carbon for marine algae is dissolved bicarbonate, which has a $\delta^{13}\text{C}$ value of $\sim 0\text{‰}$. Marine OM consequently typically has $\delta^{13}\text{C}$ values between -22 and -20‰ . The $\sim 7\text{‰}$ difference between OM produced by C_3 land plants and marine algae has successfully been used to trace the sources and distribution of OM in particulate OM, coastal ocean sediments and deep sea sediments (e.g., Hunt, 1970; Gearing et al., 1977; Harji et al., 2008). However,

the isotopic source signal can become complicated in coastal areas which receive contributions of OM from algae and both C₃ and C₄ vascular plants (e.g., Fry et al., 1977).

Examples of $\delta^{13}\text{C}$ values from different sources are given in Table 1.1.

Table 1.1. Compilation of representative $\delta^{13}\text{C}$ values of different types of primary organic matter sources to sediments of lakes and oceans (reproduced from Meyers, 1994).

Organic matter source	Location	$\delta^{13}\text{C}$ (‰ vs. PDB)	Reference ^a
<i>C₃ land plants:</i>			
Willow leaves	Walker Lake, Nevada, U.S.A.	-26.7	[1]
Poplar leaves	Walker Lake, Nevada, U.S.A.	-27.9	[1]
Pinyon pine needles	Walker Lake, Nevada, U.S.A.	-24.8	[1]
White spruce needles	Michigan	-25.1	[2]
Mangrove leaves	Penang, Malaysia	-27.1	[3]
Palm fronds	Lake Bosumtwi, Ghana	-25.5	[4]
<i>C₄ land plants:</i>			
Salt grass	Walker Lake, Nevada, U.S.A.	-14.1	[1]
Tumbleweed	Walker Lake, Nevada, U.S.A.	-12.5	[1]
Blood grass	Lake Bosumtwi, Ghana	-11.1	[4]
Wild millet	Lake Bosumtwi, Ghana	-10.8	[4]
<i>Lake algae:</i>			
Mixed plankton	Walker Lake, Nevada, U.S.A.	-28.8	[1]
Mixed plankton	Pyramid Lake, Nevada, U.S.A.	-28.3	[2]
Mixed plankton	Lake Michigan, North America	-26.8	[2]
<i>Marine algae:</i>			
Mixed diatoms	Narragansett Bay, Rhode Island, U.S.A.	-20.3	[5]
Mixed nannoplankton	Narragansett Bay, Rhode Island, U.S.A.	-22.2	[5]
Mixed plankton	Penang, Malaysia	-21.0	[3]
Mixed plankton	Dabob Bay, Washington, U.S.A.	-22.4	[6]

^aReference: [1] = Meyers (1990); [2] = P.A. Meyers (unpublished data, 1992); [3] = Rodelli et al. (1984); [4] = Talbot and Johanessen (1992); [5] = Gearing et al. (1984); [6] = Prahel et al. (1980).

The isotopic composition of organic compounds has been used to speculate on sources of sedimentary OM, and on a wide range of paleo-environmental conditions, including temperature, water column stratification, and dissolved inorganic carbon concentrations (e.g. Hayes et al., 1989; Kohnen et al., 1992; Schoell et al., 1992). The OM that is eventually stored in sediments, however, was originally synthesized by various

organisms at different trophic levels using a number of distinct biochemical pathways. These differences, as well as factors such as temperature, growth rate, and DIC concentrations contribute to the considerable variation in isotopic composition observed in OM. Limited understanding of these processes, as well as of the distribution, cycling and degradation of organic compounds, means that studies of the isotopic composition of modern environments is important for correct interpretation of fossil isotopic signatures.

The key step in the isotopic segregation of carbon is at the point of fixation into plants. For both terrestrial and aquatic plants, photosynthetic fractionation of carbon isotopes arises from differential diffusion of isotopic species of CO_2 , H_2CO_3 or HCO_3^- , and from catalytic reactions that vary depending on the fixation pathway. Marine phytoplankton utilize the C_3 fixation pathway (Calvin-Benson cycle), resulting in bulk $\delta^{13}\text{C}$ values in the range -22 to -20‰. The distribution of $\delta^{13}\text{C}$ values using various pathways in organisms and in different species of carbon is shown in Fig. 1.1.

Stable carbon isotope ratios of bulk OM have been long used to evaluate sources of organic matter in sediments and carbon flow pathways in food webs. Interpretation of bulk isotope ratios are complicated by the fact that bulk material represents mixtures of carbon from several sources, and thus isotope ratios are weighted averages. The recent advent of compound specific gas chromatography isotope ratio mass spectrometry (GC-irms) has brought together the source-specificity of biomarkers with the information in isotope ratios. The advantage of this powerful new tool is to help characterize the sources and cycling of lipid biomarkers in several marine environments. Carbon isotope signatures have helped identify the sources such as bacterial, algal, or terrigenous higher plant, sources in particulate matter and coastal and deep-sea sediments. Among paleoceanographers, there is a great interest in using isotopic data for sedimentary biomarkers to evaluate past environmental conditions.

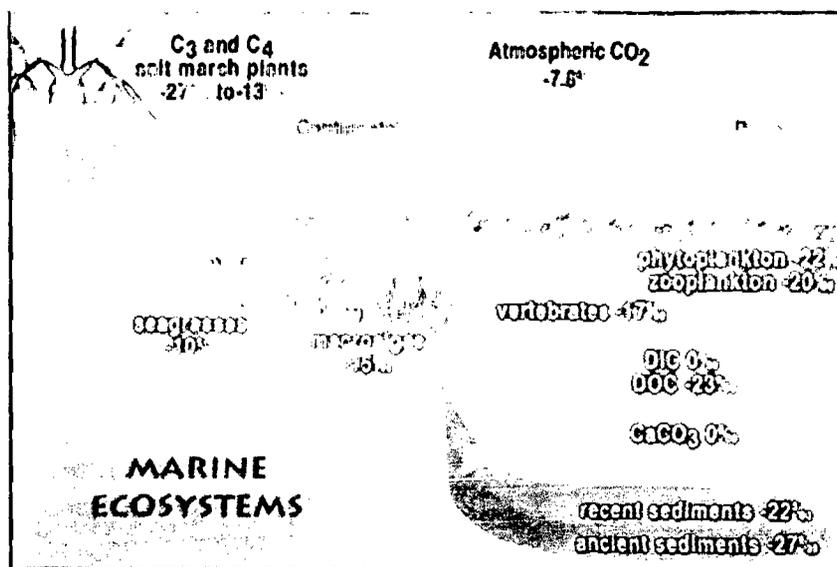


Fig. 1.1 Distribution of $\delta^{13}\text{C}$ values in organisms and in different species of carbon (www.skiio.peachnet.edu/research/biogeochemlab/oso.php).

Although bulk $\delta^{13}\text{C}$ of samples can differentiate between the major terrestrial and algal sources in the marine environment, it is unable to characterize the various functional groups of organisms. Lipid biomarkers on the contrary, are useful compounds in source apportionment. Lipid biomarkers are now widely used to trace OM in the marine environment. Detritus from photoautotrophic biota, components of vascular plants and anthropogenic inputs can be determined using lipid biomarkers.

1.4.2 Lipid biomarkers

Lipids are carbon-rich compounds with a very high energy value, making them important fuels in marine ecosystems. Some molecules have unique structures that are restricted biologically, and represent biomarkers for specific types of organisms. By virtue of their species specificity and diversity make them useful compounds to study sources of OM (e.g. Volkman et al., 1998). The total lipid content in most species of bacteria ranges between 1 and 10% of the dry cell weight (Porter, 1946; Knaysi, 1951). Lipids are comparatively resistant to degradation processes and hence are better preserved in the sediments.

The OM preserved in ocean sediments is comprised of many organic molecules some of which are uniquely traceable to the specific organisms that produced them. Such organic molecules are known as biomarkers. They are complex molecules derived from formerly living organisms (Wang et al., 2006). These compounds are also sometimes called as molecular fossils. When preserved within marine sediment cores encode information about past (paleo) biological, chemical and physical oceanographic conditions. Lipids are receiving increasing amounts of attention in ecological (Sargent et al., 1987) and biogeochemical studies (Saliot et al., 1991; Conte et al., 1995; Villanueva et al., 1997; Guzman-Vega and Mello, 1999). Some of these lipid biomarkers are described below.

1.4.2a Sterols

Sterols are potentially excellent biomarker compounds due to their stability and the diversity of their structures. Their comparative resistance to degradation makes them valuable long-term biomarkers. They are present in all eukaryotes and comparatively rare in prokaryotes, and can be used for OM source identification, chemotaxonomy and for tracing food web (Idler and Wiseman, 1971; Patterson, 1991; Teshima, 1991; Barrett et al., 1995). Sterols are less ambiguous markers of terrestrial plants, phytoplankton, macroalgae, and of human sewage (e.g. Laureillard and Saliot, 1993). For example, fresh domestic wastewater can be identified by high coprostanol/cholesterol and 24-ethylcoprostanol/ β -sitosterol ratios (Quemeneur and Marty, 1992). The C27 sterols (desmosterol, cholesterol) and C28 sterols (24-methylenecholesterol, 24-methylcholesta-5,22-dienol) are mainly of marine origin (Volkman, 1986). The diatom contain sterols such as 24-methylcholesta-5,22- dien-3 β -ol, 24-methylenecholesterol and desmosterol (Volkman et a., 1998). C29 sterols (e.g. 24-ethylcholesterol, ethylcholest-5,22-dienol and 24-methylcholesterol) are associated with terrestrial higher plants (Yunker et al., 1995).

The dinoflagellate contributes C30 sterols. The relative amounts of C27 and C29 sterols can help to identify the contributions of algal and land-plant derived OM in marine sediments (Huang and Meinschein, 1976, 1979), high ratios of C29/C27 sterols in the sediments suggests terrigenous OM. Some examples of sterols are shown in Fig. 1.2. Brassicasterol in sediments is derived mostly from diatoms (Volkman et al., 1998). Changes in the brassicasterol concentration in marine sediments are related to variations in primary productivity, as was demonstrated for Indian Ocean and South Atlantic sediments (Schubert et al., 1998; Hinrichs et al., 1999). Dinosterol (4 β ,23,24-trimethyl-5 β -cholest-22E-en-3 β -ol) is typically found in dinoflagellates where it is generally accepted as a reliable biomarker (Boon et al., 1979; Volkman, 1986; Volkman et al., 1998). Desmosterol and 22-dehydrocholesterol are present in high concentrations in red algae. In higher plants, the dominating sterol is stigmasterol. Other plant sterols include campesterol and β -sitosterol. An important sterol of fungi in general is the C28 compound ergosterol. Ergosterol is a cell membrane component of fungi and its concentration in environmental samples is used as an index to assess fungal biomass (Charcosset et al., 2001; Barajas, 2002).

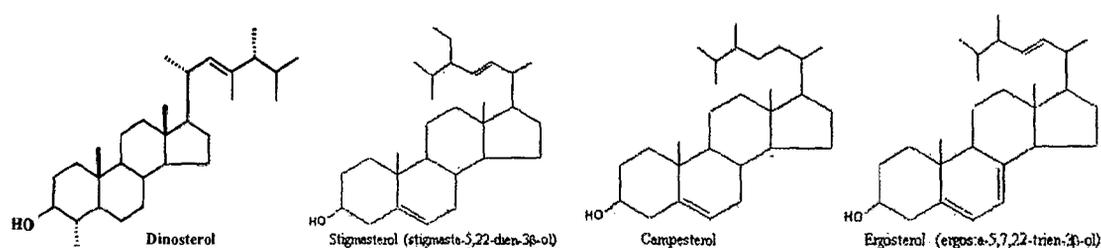


Fig. 1.2. Examples of sterols (reproduced from www.cyberlipid.org/sterols/ster0003.htm)

1.4.2b Alcohols

Long chain (C20 to C30) saturated alcohols are indicators of terrestrial higher plants. Alcohols distribution pattern with the even/odd carbon preference is typical of

epicuticular waxes of higher plants (Eglinton and Hamilton, 1967; Logan et al., 1995; Huang et al., 1995). The alkanediols are synthesized by freshwater and marine eustigmatophyte microalgae (Volkman et al., 1992b, 1999; Gelin et al., 1999), of which one marine genus (*Nannochloropsis gaditana*) also produces keto-ols (Méganelle et al., 2003). Recently, long-chain alkanediols are also detected in rhizosolenoid diatoms belonging to the genus *Proboscia* (Sinninghe Damsté et al., 2003). These compounds are highly abundant in sediments underlying high-productivity upwelling zones such as off Peru (e.g., McCaffrey et al., 1991), and Oman (Ten Haven and Rullkötter, 1991), as well as in the southeast Atlantic (Hinrichs et al., 1999, Versteegh et al., 2000; Sinninghe Damsté et al., 2003), the Arabian Sea (Schulte, 1997; Sinninghe Damsté et al., 2003), the California continental margin (Mangelsdorf, 2000), and also in sapropels of the Mediterranean Sea (Rullkötter et al., 1998; Rinna et al., 2002). In southeast Atlantic sediments, the abundances of C28-C32 alkanediols and C30 keto-ol (n-triacontane-1ol-15-one) are significantly correlated with estimates of paleoproductivity and alkenone concentration (Hinrichs et al., 1999).

1.4.2c n-Alkanes

Biogenic hydrocarbons are typically characterized by homologous series of short-chain *n*-alkanes (C15 to C22) if derived from algae and bacteria. For example, bacteria normally contain *n*-alkanes with an even carbon number predominance and show a strong maxima at one or two carbon numbers in the C14 – C30 range (Han & Calvin, 1969; Nishimura & Baker, 1986). Even-carbon *n*-alkanes in the C14 – C22 range are also known to originate from diatoms (Elias et al, 2000). Blue-green, green, red and brown algae show odd carbon-number predominance in shorter carbon chain length from C14 – C22 and generally produce a simple mixture of hydrocarbons dominated by C15, C17 and C19 *n*-alkanes (Clark & Blumer, 1967). Terrestrial higher plants contain longer chain *n*-alkanes

(C25-C35) and show a strong predominance of odd carbon, especially C25, C27, C28, C29, C31 and C33 (Brassell et al, 1978; Farrington & Tripp, 1977; Wakeham & Farrington, 1980; Doskey & Talbot, 2000; Zhao et al, 2003). The even over odd carbon number preference is better explained with the help of carbon preference index (CPI), which has been used to define the sources of *n*-alkanes (Bray & Evans, 1961; Cooper & Bray, 1963; Kennicutt & Jeffrey, 1981; Doskey & Talbot, 2000; Zhao et al, 2003; Harji et al., 2008). Carbon preference index (CPI) is expressed as a summation of odd carbon number homologues over a range divided by the summation of even carbon number homologues over the same range. For example, petroleum products produces a CPI of ~ 1, organisms such as bacteria and diatoms produces a CPI < 1 (Grimalt & Albaiges, 1987) and higher terrestrial plants show a CPI of 4 to 10. The *n*-alkane composition has been used to assess the sources of organic matter in water, suspended and sedimenting particles (Blumer 1970; Goutx & Saliot 1980; Fernandes & Sicre, 2000) and sediments (Fernandes & Sicre, 2000; Wilson et al, 2001; Harji et al, 2008).

1.4.2d Fatty acids (FAs)

FAs are chemical components that are specific for many organisms. FAs are more sensitive to degradation and modification than most types of lipid biomarkers. Remineralization rate of total fatty acids (TFAs) is four times that of bulk organic matter, and 10 times that of *n*-alkanes of the sinking particles (Meyers and Eadie, 1993). The diversity in the structures of FAs has been used to establish sources of organic material or indicator of specific organisms (Sargent et al, 1987; Budge & Parrish, 1998; Wilson et al, 2001; Najdek et al, 2002; Xie et al 2003). The long-chain FAs (C20 to C30) can be derived from terrigenous higher plants (Eglinton and Hamilton, 1967; Gagosian and Peltzer, 1986; Carrie et al., 1998), whereas FAs in the lower carbon number range (C14–C22) are derived mainly from algae and bacteria (Cranwell, 1974; Claustre et al., 1989;

Table 1.2. Fatty acid distribution in various organisms

Fatty acid	Marker
Normal saturated	Most organisms
Mid-chain branched saturated	Actinomycetes ^a
10me16:0	Gram(+) ^{b,c} ; actinomycetes ^a
10me18:0	Actinomycetes ^{a,d}
Terminally branched saturated: i14:0, i15:0, a15:0, i16:0, i17:0, a17:0	Gram (+) ^{b, c, d, e, f, g}
Branched monounsaturated: i17:1w7	Desulfovibrio ^{a,d,h}
Monounsaturated: 16:1w9c ^{b,g} , 16:1w7c ^{b,d} , 16:1w7t ^{b,g} , 16:1w5c ^b , 18:1w7c ^{b,c,d,f,g} , 18:1w7t ^c , 18:1w5c ^b , cy17:0 ^{b,c,d,f,g} , cy19:0 ^{b,c,d,f,g}	Gram (-)
15:0, 17:0 and branched FAs	Aerobic and anaerobic bacteria
16:1w5c ⁱ , 18:1w9c ^{b,c,e,i} , 20:1w9c ⁱ , 18:2w6 ^{b,c,d,h,j} , 18:3w3 ^h , 20:5w3 ⁱ	Fungi
18:1w9c ^{d,e} , 18:2w6 ^{b,e} , 20:5w3 ^d	Plants
polyunsaturated PLFAs	eukaryotes
16:4w1 ^{j,k} , 20:5w3 ^l	Diatoms
22:6w3 ^l	Dinoflagellates ^{m,n,o}
20:1, 22:1	Zooplankton ^{st^u}
>24:0 ^{p,q,r}	Terrestrial plants

^a White et al. (1997) ; ^b Zak et al. (1996) ; ^c Zogg et al. (1997) ; ^d Ringelberg et al. (1997); ^e Zelles (1997); ^f Bardgett et al. (1996); ^g Frostegård and Bååth (1996); ^h Pinkart et al. (2002); ⁱ Madan et al. (2002); ^j Nichols et al., 1986; ^k Viso & Marty, 1993; ^l Parrish et al., 2000; ^m Parkes & Taylor, 1983; ⁿ Caudales & Wells, 1991; ^o Harvey & Macko, 1997; ^p Harvey, 1994; ^q Santos et al., 1994; ^r Colombo et al., 1997; ^s Ratnayake & Ackman, 1979; ^t Graeve et al., 1994; ^u Albers et al., 1996

Carrie et al., 1998) with the exception of n-C16 and n-C18 acids, both of which are common both in microorganisms and in higher plants. Certain specific fatty acid markers of diatoms (Clauster et al, 1989) and bacteria (Volkman et al, 1980c; Wakeman and Beier, 1991; Haddad et al, 1992; Harvey, 1994) are well documented. Diatoms contains high percentages of PUFAs such as EPA (C20:5n3) (Renaud et al., 1994). Zooplankton feeding predominantly on phytoplankton contains elevated amounts of long-chain MUFAs (20:1 and 22:1) (Ratnayake and Ackman, 1979; Graeve et al., 1994; Albers et al., 1996). Bacteria are the major sources of C15:0 and C17:0, iso-, anteiso- and mid-chain branched FAs, but they can also be a significant source of palmitoleic (16:1n-7) and cis-vaccenic acids (18:1n-7) (Volkman et al., 1980c; Fulco, 1983; Wakeham and Beier, 1991; Haddad et al, 1992; Harvey, 1994). Under favourable environmental conditions, bacteria yield higher proportions of branched-chain over straight-chain 15:0 fatty acid (White et al,

1980). A list of certain FAs is given in Table 1.2. High 16:1/16:0 and $\Sigma 16/\Sigma 18$ ratios indicate relatively higher contribution of diatom to organic matter (Claustre et al, 1989, Mayzaud et al, 1989; Najdek, 1996; Budge & Parrish, 1998). Moreover, the 16:0/24:0 ratio has been used as an indicator of terrestrial input (Gearing & Pocklington, 1990). These well established FA biomarkers are used for identifying sources of OM and assess the fate of OM in water, suspended and settling particles, and sediments (Colombo et al, 1996; Budge & Parrish, 1998; Wilson et al, 2001; Ternois et al, 2001; Xie et al, 2003).

1.4.2e Phospholipid fatty acids (PLFAs)

Total FAs are not useful to assess living microorganisms. This discrepancy can be removed by analyzing PLFAs. In the nature, more than 90% of indigenous microorganisms escape cultivation using the traditional culture-dependent techniques (DeLong and Pace, 2001). Hence the traditional techniques of isolation and culturing have not been adequate for characterization of microorganisms in environmental samples, especially in evaluating the natural microbial diversity (Fang et al., 2000; Delong and Pace, 2001). Thus to detect changes in bacterial biomass and natural microbial communities, culture-independent techniques such as PLFAs, lipid phosphate analysis (White et al., 1979; Pennanen et al., 1996; Pinkart et al., 2002; Petsch et al., 2003), and genetic fingerprinting (Polymenakou et al., 2005), are routinely used. Total PLFAs and lipid phosphate can be used to assess freshly biosynthesized material and individual PLFAs are used to distinguish inputs from bacteria, phytoplankton, zooplankton and other eukaryotes (White et al., 1979; Volkman et al., 1998). Although more specific techniques such as 16sRNA are also used, their use is limited due to high cost of the analysis.

Phospholipids are the main components of cell membranes that perform several important biological functions. Phospholipids are found in the membranes of all cells. Under the conditions expected in natural communities, the bacteria contain a relatively

constant proportion of their biomass as phospholipids (White et al., 1979a, b & c). Phospholipids are not found as storage lipids and have a relatively rapid turnover in some sediment so the assay of these lipids gives a measure of the "viable" cellular biomass (White et al., 1979a; Russell and Nichols, 1999; Fang et al., 2000; Boschker and Middelburg, 2002). The total PLFA, the phosphate of the phospholipids, the glycerol-phosphate and acid-labile glycerol from phosphatidyl glycerol-like lipids that are indicators of bacterial lipids can be assayed to increase the specificity and sensitivity of the phospholipid assay (Gehron and White, 1983; White et al., 1979a, b & c). Estimates of microbial biomass based on polar lipids can be measured from PLFAs and lipid phosphate. Estimates of cell numbers by direct counts and analysis of cell membrane, LPS-OHFAME, muramic acid in the cell wall, and extractible ATP give essentially equivalent numbers for both cell numbers and dry weight biomass (Balkwill et al., 1988) (Table 1.3).

Table 1.3. Equivalence of biomass determinations by membrane lipid and cell wall components, and ATP to direct counts in subsurface sediments (reproduced from Balkwill et al., 1988).

Component	Concentration nmol/g dry wt sediment	Conversion factor $\mu\text{mol/g}$ dry wt cells	Direct count equivalence ^a 10^6 cell/g dry wt sediment	Dry weight cells μg cells/g dry wt sediment
Direct count	-	-	7.4 ± 3.5	1.3 ± 1.1 ^b
<u>Membrane</u>				
PLFAs	0.35 ± 0.08	100	7.0 ± 0.2	3.5 ± 0.1
Lipid phosphate	0.11 ± 0.03	50	4.4 ± 0.1	2.2 ± 0.05
Glycerol phosphate	0.22 ± 0.09	50	8.8 ± 0.3	4.4 ± 0.15
LPS-OHFAME	0.09 ± 0.04	15	12.0 ± 3.0	6.0 ± 1.5
<u>Cell Wall</u>				
Muramic acid	0.22 ± 0.1	58.5	7.5 ± 5.0	3.7 ± 2.5
ATP	$1.39^c \pm 0.42$	$1.7^d/10^7$	8.2 ± 1.1	1.4 ± 0.8 ^e

^a Calculated with 2×10^{13} cells/g dry wt

^b Calculated with a cell volume of $1.07 \mu\text{m}^3$ and 1.72×10^{-13} g/cell

^c ATP given in ng/g dry wt

^d ATP given in ng/ 10^7 cells

^e Calculated assuming 10^7 cells weigh $1.72 \mu\text{g}$

Major taxonomic groups, such as eukaryotes vs. bacteria, can be differentiated using the individual PLFAs. However, focusing on the specific PLFAs certain functional

groups or species of microorganisms can be elucidated (Vestal and White, 1989). Furthermore, these compounds can be extracted from sediments easily and quantitatively (White et al., 1979a; Balkwill et al., 1998). Hence, cellular membrane PLFAs represent a quantitative and sensitive tool to determine the living microbial biomass (White et al., 1979a; Ringelberg et al., 1997; Fang et al., 2000), changes in microbial community composition (Volkman et al., 1980c; Pinkart et al. 2002; Widenfalk et al., 2008), nutritional status of microbial community (Guckert et al., 1986; Kieft et al., 1994), general distribution of microorganisms with different metabolic activities (Kaneda, 1991), heavy metal-contaminated soils (Bååth et al., 1995; Pennanen, 2001), and environmental stress (Fang et al., 2004).

Sterols, alcohols, -alkanes, FAs and PLFAs are useful to assess sources of OM, community structures of organisms and biomass of living organisms. However, these biomarkers are less useful to evaluate past environmental conditions. These palaeoceanographic environmental parameters are well addressed using lipid biomarkers such as alkenones. They are comparatively well preserved in sedimentary record and provide information on various paleoceanographic changes.

1.4.2f Alkenones

The alkenones are a class of unusual, C₃₅-C₄₀ mono-, di-, tri- and tetraunsaturated methyl and ethyl ketones that are synthesized by a limited number of haptophyte (prymnesiophyte) microalgae (Volkman et al., 1980b; 1995; Marlowe et al. 1984; Brassell et al., 1986; Conte et al. 1994; Rontani et al., 2001; 2004; Prah et al., 2006). Marlowe et al. (1990) identified the genus coccolithophorid of class prymnesiophyceae as the exclusive photosynthetic algae that biosynthesize alkenones. *Emiliana huxleyi* which belong to coccolithophorids (Okada and Honjo, 1973) are the recognized biological source of a series of these long-chain unsaturated methyl and ethyl ketones (Marlowe et al.,

1984a) which are widely observed in marine sediments (Brassell et al., 1986). *Emiliana huxleyi* and *Gephyrocapsa oceanica* appear to be the dominant sources of alkenones in today's open oceans and contemporary sediments (Conte et al., 1994; 1998; Volkman et al., 1980b; 1995; Volkman, 2000). In coastal environments, alkenones are biosynthesized by other members of the Haptophyceae such as *Isochrysis galbana* and *Chrysotila lamellose* (Marlowe et al., 1984; Patterson et al., 1994; Rontani et al., 2004).

Generally, *E. huxleyi* is the most dominating microalgae in the waters of the open ocean. As a result, numerous studies have been carried out on lipids of this microalga. Alkenones comprise 5-10% of total cellular carbon of *E. huxleyi* (Prahl, et al., 1988) and have been ascribed to membrane architecture. Recently it has been reported that alkenones are metabolic storage lipids and are localized in cytoplasmic vesicles or in lipid bodies (Eltgroth et al., 2005). These findings indicate that the alkenones are involved with cellular energy storage (Eltgroth et al., 2005).

The alkenones deposited in the marine environment are not completely destroyed in the sedimentary process. Alkenones are valued most because they are useful proxies to assess past oceanographic (paleoceanographic) environment. For example, used as proxies for reconstruction of sea surface temperatures (e.g. Brassell et al., 1986, Prahl and Wakeham 1987, Eglinton et al., 1992) and to measure partial pressures of CO₂ (Jasper and Hayes 1990, Jasper et al., 1994). Due to these environmental applications, alkenones are the most extensively studied lipid class of compounds in marine sedimentary environments.

1.5 Application of lipid biomarkers in oceanographic processes

The application of lipid biomarker in identification of sources and past oceanographic conditions are stated below. Emphasis is given on PLFAs, fatty acids and alkenones. Specifically, these lipid biomarker provides a quantitative means to measure: 1)

viable microbial biomass, 2) microbial community composition, 3) community nutritional status, 4) fate of OM, 5) structure of trophic systems/food webs, 6) sea surface temperature (SST), 7) paleoproductivity, and 8) partial pressure of CO₂.

1.5.1 Viable Biomass

The determination of the total phospholipid ester-linked fatty acids (PLFA) provides a quantitative measure of the viable or potentially viable biomass. The viable microbes have an intact membrane which contains phospholipids (PLFAs). Cellular enzymes hydrolyze and release the phosphate group within minutes to hours following cell death (White et al., 1979a). The lipid remaining is diglyceride (*DG*). The resulting diglyceride contains the same signature fatty acids as the original phospholipid. Consequently, a comparison of the ratio of PLFA profiles to diglyceride fatty acid profiles provides a measure of the viable to non-viable microbial abundance and composition.

1.5.2 Community Composition

The presence of certain groups of microorganisms can be inferred by the detection of unique lipids that originate from specific biosynthetic pathways (White, 1983; Colwell et al., 1985; White, 1986; White, 1988; Tunlid and White, 1991). Identification of microorganisms by PLFA can give valuable insight into microbial community structure. Community-level PLFA profiles have been found to be useful in detecting the responses of soil microbial communities to a variety of land uses or disturbances in other ecosystems (Hedrick et al., 2000; Yao et al., 2000; Fang et al., 2001; Harris, 2003), and certain marker PLFAs can indicate relative amounts of certain functional groups of organisms in soils (Ringelberg et al., 1997; Zelles, 1997). The analysis of other lipids such as the sterols (microeukaryotes-nematodes, algae, protozoa) (White et al., 1980), glycolipids (phototrophs, gram-positive bacteria), or the hydroxy fatty acids from the lipid A component of lipopolysaccharide of gram-negative bacteria (Parker et al., 1982; Bhat and

Carlson, 1992), and alkyl ether polar lipids derived from the *Archae* (Hedrick et al., 1991) can provide a more detailed community composition analysis.

1.5.3 Nutritional Status

Bacterial poly β -hydroxyalkanoic acid (PHA) (Nickels et al., 1979; Doi, 1990) and microeucaryotic triglyceride (Gehron and White, 1982) are endogenous storage lipids. The relative amount of these compounds, as compared to the PLFA, provides a measure of the nutritional status. Many bacteria produce PHA under conditions of unbalanced growth when a carbon source and terminal electron acceptor(s) is present but cell division is limited by the lack of some essential nutrient (Nickels et al., 1979; Doi, 1990). Specific patterns of PLFA can indicate physiological stress (Guckert et al., 1986). Exposure to toxic environments can lead to minicell formation and a relative increase in specific PLFA. Increased conversion from *cis* to trans PLFA occurs in *Pseudomonas* species with exposure to higher concentrations of phenol in the absence of bacterial growth (Heipieper et al., 1992). Prolonged exposure to conditions inducing stationary growth phase induce the formation of cyclopropane PLFA (Tunlid and White, 1991; Guckert et al., 1986; Lennarz, 1970). Respiratory quinone composition can be utilized to indicate the degree of microbial aerobic activity (Hedrick and White, 1986). Environments with high potential terminal electron acceptors (oxygen, nitrate) induce formation of benzoquinones in bacteria in contrast to microbes respiring on organic substrates which form naphthoquinones. Some specific but useful insights come from analysis of organisms like the *Pseudomonas* species which form acyl-ornithine lipids when growing with limited bioavailable phosphate (Minnikin and Abdolrahimzadeh, 1974) while some gram-positive bacteria form increased levels of acylamino acid phosphatidylglycerols when grown at sub-optimal acid pH levels (Lennarz, 1970).

1.5.4 Fate of OM

The ocean margin, including estuarine, coastal and shelf environments, is the primary interface where most OM enters the marine environment, and it is a region where rapid and major transformations of OM can occur. Biodegradation has long been recognized as an important process in the cycling of OM, and there is growing evidence that microbial remineralization of OM could be enhanced in the ocean margin (Cauwet, 2002). The FAs although account for a small fraction, they are important component of the OM. They are degraded in the sediments and thus provide a measure to assess degradation or fate of OM derived from various sources in the marine environment. A variety of factors could be important in promoting microbial remineralization of FAs in these systems, including changes in ionic strength, pH and exposure to solar radiation, as well as physical reworking of sedimentary deposits by benthic organisms, which renews the supply of oxidants and removes inhibitory metabolites (Aller, 1998; Sun et al., 1997). Low levels of PUFAs found in the surficial sediments indicates that most of these labile FAs are effectively recycled during whole settling and depositing processes (Hu et al., 2006). This shows that in estuarine biogeochemical processes, OM derived from phytoplankton is subject to modification by bacterial degradation and/or zooplankton grazing. Most of this type of OM degrades, and only a minor part would ultimately be preserved in sediments. In the marine environment, the OM synthesized in the surficial photic zone is utilized by the various trophic levels and the remainder settles at the sea bottom. It is important to understand the type of OM reaching the sea bottom as a number of deep sea organisms depends on this OM for food. To assess the source of OM and degradative state of the FAs, the proportions of Sat-FAs, MUFAs and PUFAs are useful (Hamm and Rousseau, 2003). Degradation causes a relative increase in the Sat-FAs and decrease of UFAs (De Baar et al., 1983).

paleoproductivity in different oceanic settings (e.g. Brassell et al., 1986; Rostek et al., 1994, 1997; Schubert et al., 1998; Villanueva et al., 1998; Schulte et al., 1999; Schulte and Müller, 2001).

Primary productivity patterns are not uniform in the ocean in either space or time. For example, values in upwelling regions are much higher than those in the central ocean gyres. The total alkenone concentration measured in a given sedimentary sample is related to *E. huxleyi* productivity in overlying surface waters at some point in the past. Temporal variations in the alkenone flux measured in sediment trap time series provide an index for seasonal changes in organic carbon contribution from *E. huxleyi* blooms to the ocean floor. The stratigraphic record for alkenone concentration in dated sediment cores potentially provides information about annual, decadal and longer timeframe changes in *E. huxleyi* productivity.

1.5.7 Estimation of Surface Water Carbon Dioxide Concentrations

In the alkenones carbon atoms exist in two different isotopic forms - ^{12}C and ^{13}C . During photosynthesis, *E. huxleyi* preferentially use carbon dioxide containing ^{12}C as compared to carbon dioxide molecules containing isotopically heavier ^{13}C . This selective photosynthetic uptake of the 'lighter' carbon dioxide molecules is called isotopic fractionation. The magnitude of isotopic fractionation (i.e., the difference between the $^{13}\text{C}/^{12}\text{C}$ composition of the alkenones and dissolved carbon dioxide) is not constant but varies depending upon the dissolved carbon dioxide concentration of surface waters in which *E. huxleyi* grows. Variations in dissolved carbon dioxide concentration of surface waters can result from changes either in the partial pressure of carbon dioxide in the atmosphere or in upwelling of deep ocean waters enriched in carbon dioxide derived from remineralization of settling organic matter.

The quantitative relationship between the availability of dissolved carbon dioxide and isotopic fractionation in *E. huxleyi* has now been calibrated experimentally (Bidigare et al., 1997). Estimates of isotopic fractionation in the past can also be made from combined measurement of $^{13}\text{C}/^{12}\text{C}$ in alkenones and in the calcareous shells of planktonic foraminifera preserved in dated sediment cores. Given such estimates, the dissolved carbon dioxide concentration of surface waters in which *E. huxleyi* grew can be calculated using the calibration relationship. Assuming these calculations are correct, the challenge remains to determine unequivocally whether dissolved carbon dioxide concentrations assessed for a given time at a specific location represent equilibrium values with the atmosphere or supersaturation values due to upwelling. Because this type of information is key to understanding what controls global climate change, this aspect of alkenone biomarker research is currently receiving considerable attention from the paleoceanographic community (see Laws et al., 2002; Benthien et al., 2005; Benthien et al., 2007; Henderiks and Mark Pagani, 2008).

1.5.8 Estimation of Sea-Surface Temperatures (SST)

Unsaturation patterns within the alkenone series change regularly with growth temperature (Brassell et al., 1986; Prahl and Wakeham, 1987; Prahl et al., 1988). The relative concentration of compounds with two and three double bonds biosynthesized by the plant varies in direct response to growth temperature. That is, when growth temperatures get warmer, *E. huxleyi* biosynthesizes alkenones with fewer double bonds (e.g. C37:2) and vice versa. This type of biochemical response to growth temperature is well-known for fatty acids in phospholipids, the classical building blocks of cell membranes in bacteria, plants and animals. The proportion of the triunsaturated C37 alkenone has been shown to increase with decreasing temperature of the water in which the microalgae grow (Marlowe et al., 1984a; Brassell et al., 1986; Prahl and Wakeham,

1987). The observation of such a process in this microalga was utilized to estimate the temperature of water at which this microalgae grew. An unsaturation index of C37 alkenones termed as the “ U_{37}^K ” and defined as the $U_{37}^K = ([C37:2]-[C37:4])/([C37:4]+[C37:3]+[C37:2])$ was initially proposed for paleotemperature estimations (Brassell et al., 1986) and later simplified to $U_{37}^{K'} = [C37 :2]/[C37 :2]+[C37 :3]$ (Prahl and Wakeham, 1987; Prahl et al., 1988; Müller et al., 1998), since the C37:4 alkenone is relatively rare in sediments from tropical to subtropical environments (Prahl and Wakeham, 1987).

Experiments with laboratory cultures of *E. huxleyi* have shown the relationship between $U_{37}^{K'}$ and growth temperature is remarkably linear (Fig. 1.3) (Prahl and Wakeham, 1987). This discovery led to the widespread application of $U_{37}^{K'}$ in the reconstruction of paleo SST (sea surface temperatures) and its variations through time. Field experiments have also shown this relationship applies widely in the modern ocean (Harvey, 2000; Grimalt et al., 2000). Moreover, alkenones are relatively well-preserved in sediments; hence profiles of alkenone unsaturation patterns with depth in dated deep-sea cores provide a temporal record of sea surface temperatures (Farrington et al., 1988; Prahl et al., 1989). Consequently, paleoceanographers now routinely measure $U_{37}^{K'}$ stratigraphically in dated sediment cores to assess climatically driven changes in water temperature at the sea-surface for different parts of the world ocean. This application is performed on timescales ranging from interannual (El Nino) to millennial (Glacial/Interglacial). The $U_{37}^{K'}$ is now widely used to assess paleoenvironment of polar waters (Sikes and Volkman, 1993), tropical Indian Ocean (Rostek, et al., 1997), upwelling regions (Poynter et al., 1989), records of El Nino events (McCaffrey et al., 1990) and also to define the occurrence of glacial terminations (Eglinton et al., 1992).

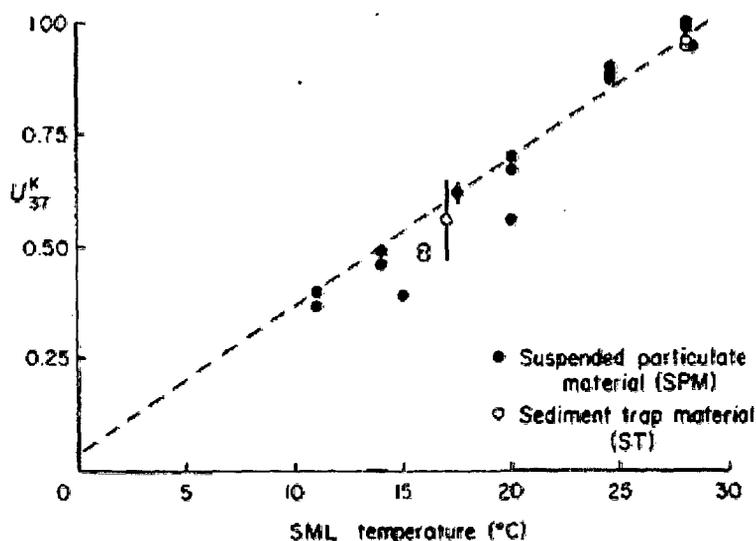


Fig. 1.3 Plot of the unsaturation index, $U_{37}^{K'} = [C37:2]/[C37:2]+[C37:3]$, against surface mixed layer (SML) temperature for natural particulate samples collected at various geographic locations throughout the world's oceans. Solid circles represent data for suspended particulate material (SPM) collected using water bottles or insitu pumps; open circles represent data for vertically transported particulate material (ST) collected in sediment traps. The dashed line represents the temperature calibration curve ($U_{37}^{K'} = 0.033T + 0.043$; $r = 0.997$) determined for cultures of *E. huxleyi* grown under laboratory conditions. Reproduced from Prahl and Wakeham, 1987.

Müller and coworkers (1998) measured alkenone unsaturation index $U_{37}^{K'}$ in modern sediments collected from different parts of the World Ocean and found strong correlation with annual mean sea-surface temperature (SST). Herbert et al. (1998) analyzed alkenones in modern sediments collected along the Chile-Peru margin and the NE Pacific margin and observed strong correlation of unsaturation index and SST. This implies that $U_{37}^{K'}$ not only applies to depositional environments of the open ocean but also applicable to continental boundaries.

For alkenones to be useful as a measure of SST in the geological record, it is essential that any effects of degradation in the water column and in sediments either do not affect the temperature signal established during their initial biosynthesis by the alga (Harvey, 2000; Grimalt et al., 2000), or if there is a change its extent can be reasonably estimated. Alkenone degradation has been observed in the water column and in surface

sediments (Sikes et al., 1991; Freeman and Wakeham, 1992; Conte et al., 1992; Madureira et al., 1995; Hoefs et al., 1998; Harada et al., 2003; Sun et al., 2004; Teece et al., 1998). During degradation unsaturated components could be selectively lost or modified. Such microbially mediated changes will influence the $U_{37}^{K'}$ (Harvey, 2000) and the calculated SST.

Recently, Rontani et al. (2005) isolated bacteria from microbial mats (very rich in alkenones) that were capable of degrading alkenones efficiently under aerobic conditions. They observed a variable selectivity during the microbial attack on C_{37} alkenones resulting in variations of the $U_{37}^{K'}$ index ranging from 0 to + 0.10 that corresponds to an inferred temperature difference of 3°C. This variability could be assigned to the heterogeneity of the inoculum (microbial mats) and to the very large diversity of the aerobic heterotrophic bacteria that can attack the alkenone molecules by various pathways. Thus, it appears extremely audacious and unwise to generalize the results obtained from only one experiment (Teece et al., 1998) to the whole spectrum of aerobic bacterial communities.

1.6 Aim and Scope of the Present Research

OM in aquatic environments is derived from several sources including biogenic and terrestrial. Microorganisms such as microalgae and bacteria are generally the major sources of OM in oceanic environment. Lipids are often employed to assess the sources of OM. Marine waters experience strong seasonal variations in environmental parameters such as temperature, salinity, organic carbon, nutrients, etc. All these parameters are likely to influence concentration and composition of biochemical compounds including lipids. However, effect of these parameters on the lipids concentration and composition in tropical marine bacteria and diatoms are not well addressed, especially those from Indian waters.

In marine environment, living biomass consists of prokaryotes and eukaryotes and their activities strongly influence biogeochemical cycling of OM. However, microbial activities and community structure are known to be influenced by the nature and sources of OM, pollution and nutrient status. Therefore, it is essential to understand the nature and sources of OM and its impact on the abundance and composition of microorganisms. To the best of our knowledge, no such studies are carried out in the Indian waters.

Very little is known about lipids in tropical marine bacteria and microalgae and the distribution, nature and sources of OM in tropical environments such as the Indian Ocean. Therefore, the major aims of the research presented in this thesis were to assess the distribution, nature and sources of OM and to evaluate how alkenone biodegradation can influence the SST. In order to achieve these goals, the research was planned with the following objectives:

- 1) To assess the effect of growth condition on PLFA concentration and composition in some bacteria and diatoms.
2. To identify the sources of OM and microbial community structure in coastal and estuarine sediments.
3. To study the distribution and sources of OM (as FAs) in sediment cores.
4. To assess microbial degradation of alkenones and its implication for paleotemperature reconstruction.

**Effect of growth conditions on the
concentration and composition of
phospholipid fatty acids (PLFA) of some
marine bacteria**

2A.1. Introduction

Microorganisms are widely distributed in various environments due to their ability to adapt to different environments. Such adaptations are carried out by managing the biochemical composition, especially lipid content of their cells (Mateos et al., 1993). Concentration and composition of lipids such as PLFAs of bacterial cell membrane play important role in controlling the impact of changes in chemical and physical properties of the environment (Hazel and Williams, 1990; Šajbidor, 1997). Slight structural modifications to the PLFAs can result in significant changes in the physical properties of the membrane (Cullis et al, 1996). These modifications are important in maintaining both membrane fluidity, integrity and functionality in the face of external perturbations (Russell, 1984).

Bacterial membrane constituents are markedly affected by the growth conditions such as, growth phase, nutrients, growth temperature (McGarrity and Armstrong, 1981; Diefenbach et al., 1992; Annous et al., 1999; Doumenq et al., 1999), carbon source (Doumenq et al., 1999; Tsitko et al., 1999) and salinity (Valderrama et al., 1998). Therefore, PLFA analysis can be considered as a tool to assess the effects of these environmental alterations on bacteria.

Most common modifications in PLFA side chain are changes in the average chain length or degree of unsaturation, with the relative contribution of short chain and unsaturated FAs increasing with decreasing temperature (e.g., Donato et al., 2000; Mannistö and Puhakka, 2001). Similar effects can, however, be obtained by increasing the ratio of anteiso to iso branched FAs (Haque and Russell, 2004; Unell et al., 2007), and increasing the ratio of cis to trans unsaturated FAs (Heipieper et al., 1992; Henderson et al., 1995). Members of the different bacterial phyla modify differently their PLFA patterns in response to growth conditions. A decreasing ratio of MUFA to saturated PLFAs (Sat)

(MUFA/Sat) is typically observed when Gram-negative bacteria are starved (Guckert et al., 1986; Kieft et al., 1994). Moreover, the ratios of MUFA/Sat decline with increasing growth temperatures (Suutari and Laakso, 1994) and change under environmental stresses such as water limitations and metal toxins (Dickens and Anderson, 1999; Li et al., 2007; Moore-Kucera and Dick, 2008). The adaptation in PLFAs prevents the loss of the mechanical and chemical properties of the lipid bilayer.

A comparison of PLFA compositions due to the variations in growth conditions can give clues of the impact of growth conditions on the cells (Joyeux et al., 2004). Hence the relationship between the growth conditions and membrane PLFA composition is very important and interesting. Modifications to the PLFA composition are extensive enough to alter membrane fluidity which affects a number of cellular functions, including carrier-mediated transport, properties of certain membrane-bound enzymes and cell growth. However it is difficult to predict how a given system will respond to a particular type of lipid modification.

In this chapter, effect of changes in the cultivation period, concentration of nitrogen and phosphorus and incubation temperature on PLFA concentration and composition of *Bacillus licheniformis*, *Bacillus subtilis*, and *Aeromonas hydrophila* were studied. Bacteria of the genus *Bacillus* and *Aeromonas* are suitable for such a study because they are ubiquitously distributed in water and sediments. *Bacillus* cultures contain branched (Br) and Sat PLFAs, whereas *Aeromonas* contain monounsaturated (MUFA) and Sat PLFAs. The effects of incubation periods, nutrients and temperature on these bacterial cultures were successfully demonstrated by employing simple indices such as the ratio of Iso/Anteiso, Br15/Br17, Sat/Anteiso, Sat/iso, Sat/Br, for *B. licheniformis* and *B. subtilis* and MUFA/Sat, C16:1/C18:1 for *A. hydrophila*, respectively.

2A.2. Materials and methods

2A.2.1 Bacterial strains and culture medium

The three bacterial cultures, *Bacillus licheniformis*, *Bacillus subtilis* and *Aeromonas hydrophila* were obtained from MCMRD, NIO, Goa laboratory culture collection. After three successive plating on Zobell marine agar (ZMA), each bacterial culture was grown in basal salt solution (BSS) supplemented with 0.1% glucose, 0.05% peptone and 0.01% yeast extract. This medium was designated as BSS-GYP medium. Glucose (10%) was sterilized separately. Suitable aliquot was added to each flask so as to obtain desired concentration (i.e. 0.1%). BSS medium consists of NaCl (25 g), KCl (0.75 g), MgSO₄ (7.0 g), NH₄Cl (1.0 g), K₂HPO₄ (10 %, 7ml), KH₂PO₄ (10%, 3ml) and 1ml trace metal solution (TMS) dissolved in 1000 ml of distilled water (D/W). TMS consists of H₃BO₃ (2850 mg), MnCl₂.4H₂O (1800 mg), FeSO₄.7H₂O (2490 mg), sodium tartarate (1770 mg), CuCl₂ (26.9 mg), ZnCl₂ (20.8 mg), CoCl₂ (40.4 mg), NaMoO₄.2H₂O (25.2 mg) in 1000 ml D/W (D'souza, 2004). The pH was adjusted to 7.5 with 1N NaOH.

2A.2.2 Monitoring growth pattern of the bacterial cultures

B. licheniformis, *B. subtilis* and *A. hydrophila* were grown individually in 100 ml conical flasks containing 20 ml of BSS-GYP medium. Flasks containing the medium were sterilized at 120 °C and 15 lb pressure for 15 min. The cultures were grown at room temperature (28 ± 2 °C) on a rotary shaker (100 rpm) for 24 h. Subsequently, three 1 L flasks containing 200 ml of the BSS-GYP medium was sterilized at 120 °C and 15 lb pressure for 15 min. One flask was inoculated (1%) with *B.licheniformis*, another with *B. subtilis* and third with *A. hydrophila* cultures which were prepared as above. The flasks were maintained at room temperature (28 ± 2 °C) on a rotary shaker (100 rpm) so as to grow the cultures. At specific time intervals 5 ml culture broth was removed, and the optical density of the culture was measured at 540 nm using a Spectrophotometer (UV-1700 Pharma Spec UV-VIS, Shimadzu)

2A.2.3 Effect of incubation period on PLFA concentration and composition of bacteria

Bacterial cultures *B. licheniformis*, *B. subtilis* and *A. hydrophila* were grown in BSS-GYP medium as described above. Each culture was grown in 1L x 3 flasks containing 200 ml of the BSS-GYP medium over a period of 168 h. At specific time intervals culture broth of each culture was sampled. The bacterial cells were harvested by centrifugation for 15 min at 10,000 rpm at 4 °C. Cells were washed with 1.5 % NaCl, lyophilized, homogenized and stored at -20 °C until analysis.

2A.2.4 Effect of nitrogen concentration on PLFA concentration and composition of bacteria

The above bacterial cultures were grown for 24 h in the BSS-GYP medium supplemented with 4.7, 9.3, 14 and 18.7 mM of nitrogen (NH₄-N). The medium was sterilized by autoclaving at 120 °C and 15 lb pressure for 15 min. Flasks were cooled and inoculated with 1 % of *B. licheniformis*, *B. subtilis* and *A. hydrophila* cultures which were pre-grown as described in 2A.2.1. Cultures were grown at room temperature on rotary shaker (100 rpm) for 24 h. Cells of each culture were harvested, washed and lyophilized as above.

2A.2.5. Effect of phosphorus concentrations on PLFA concentration and composition of bacteria

The bacterial cultures were grown as above in the BSS-GYP medium, supplemented with various concentrations of phosphate when other medium components were kept constant. Suitable aliquots of K₂HPO₄ (10% solution) and KH₂PO₄ (10% solution) were added to the BSS medium so as to obtain a total concentration of phosphorus (PO₄-P) of 1.6, 3.1, 4.7 and 6.2 mM-P in the growth medium. The medium was sterilized by autoclaving at 120 °C and 15 lb pressure for 15 min. Flasks were cooled and inoculated (1%) with respective 24 h pre-grown inoculum (1 %). The cultures were

grown for 24 h at room temperature as described above. Cells of each culture were harvested, washed and lyophilized as above.

2A.2.6 Effect of incubation temperatures on PLFA concentration and composition of bacteria

The bacterial cultures were grown for 24 h as mentioned above. For this experiment, the cultures were grown at 27, 30 and 38 °C. After 24 h growth, cells of each culture were harvested, washed and lyophilized as above.

2A.2.7. Isolation and identification of PLFA

2A.2.7.1. Extraction of total lipids

Lipids from the lyophilized bacterial cells were extracted following the modified method of Bligh and Dyer (1959) as described by White et al. (1979). In brief cells were shaken vigorously with chloroform: methanol: PO₄ buffer (1:2:0.8, v/v/v) and kept standing for 24 h. Separation of the organic phase was achieved after obtaining the ratio of chloroform: methanol: water to 1:1:0.9 in a separating funnel. The total lipid extracts (TLE) were pooled, filtered through Whatman No. 1 filter paper, and treated with anhydrous sodium sulphate to remove traces of water. The extract was rotary evaporated at 30 °C under vacuum and made to a known volume (1 ml).

2A.2.7.2. Separation of PLFAs

Organic phase containing the total lipids was reduced to a small volume (1 ml) and transferred onto silicic acid columns (activated at 120 °C for 2 h). The column was eluted first with dichloromethane, followed by acetone and methanol. The methanol fraction (polar fraction) containing the PLFAs were reduced to a small volume using the rotary evaporator. Recovery of the phospholipid standard (phosphatidyl choline) processed following the entire analytical procedure varied from 74 to 89 %. (X= 79.62 %, n=3).

2A.2.7.3 Preparation of Fatty acid methyl esters

Fatty acid methyl esters of the phospholipid fraction were prepared by mild alkaline methanolysis according to White et al (1979). Phospholipid fraction was resuspended in methanol: toluene 1:1 (V/V) to which freshly prepared 0.2 M methanolic KOH was added. The mixture was incubated at 37 °C for 15 min, neutralized to pH 6 with 1 M acetic acid. The PLFAs were recovered using hexane and analyzed using a gas chromatograph (GC) and a gas chromatograph mass spectrometer (GCMS). Solvents were passed through the entire analytical procedure and run on GC and GCMS as blanks to check the probable contamination.

2A.2.7.4. Cleaning of glassware and purification of chemicals

Before use all the glassware used for lipid analysis was washed with tap water, chromic acid, distilled water, ashed at 450 °C and rinsed with methanol and dichloromethane. All the solvents were of analytical grade and were double distilled before use. Sodium sulphate was heated at 450 °C. Silicic acid was washed with methanol, chloroform and dried prior to use.

2A.2.7.5. Capillary GC and GC-MS analysis

GC analyses of the fatty acid methyl esters were done using Agilent GC 6890 equipped with a FID and SP-2560 fused silica capillary column (column length: 100m, film thickness: 0.20 µm, inner diameter: 0.25 mm). The temperature of injector and the detector was maintained at 260 °C. One µl of sample was injected in an on-column injector using nitrogen as a carrier gas at a flow rate of 20 cm sec⁻¹. The column oven temperature was programmed from 140 °C (5 min) to 240 °C (35 min) at a rate of 4 °C min⁻¹.

The quantification of PLFAs was done using the data handling system available on the instrument. The identification of individual PLFA was performed by comparing the retention times of PLFAs in the sample with those of authentic standards (FAME 37 mix,

BAMEs (bacterial acid methyl ester), Supelco, and/or Sigma-Aldrich-Fluka). The peak areas were quantified relative to an internal standard (hexacosanoate methyl ester) added to the sample prior to GC- analysis. Sample values have been corrected for the blank levels.

Peak identification was further verified by analyzing the PLFAs on GC-MS (Shimadzu GC-MS-QP 2010) by comparing the EI spectra of PLFAs of the sample with the spectra of the PLFA of the standards and published data. One μl PLFA sample was injected into a Shimadzu GC-2010 gas chromatograph interfaced with a Shimadzu GC-MS-QP 2010 mass selective detector. The gas chromatograph was equipped with the same 100-m capillary column (SP-2560) as above and the column was programmed as described above for the GC. The injector and source housing temperatures were maintained at 60 °C and 200 °C, respectively. The GC-MS system operated in full scan mode, scanning from m/z 50 to m/z 500. The prefixes “a” and “i” indicate anteiso- and iso-branching in PLFA.

2A.3.0 Results and discussion

2A.3.2. Effect of incubation periods on PLFAs

In *B. licheniformis*, total PLFAs increased from 8157 to 14093 $\mu\text{g/g}$ dw cells over the 72 h of incubation and then decreased (7103 μg^{-1} dw cells) when the incubation period was extended to 168 h (Fig. 2A.1a). In *B. subtilis*, total PLFAs increased from 10605 to 15289 μg^{-1} dw cells over the 168 h of incubation (Fig. 2A.1b). In *Aeromonas hydrophila* the total PLFA concentrations increased from 6470 to 25303 μg^{-1} dw cells over the 168 h of the incubation (Fig. 2A.1c). During the incubation periods, the three different bacteria showed different pattern of total PLFAs. This suggests that growth period can be an important factor in determining total PLFA production in bacteria.

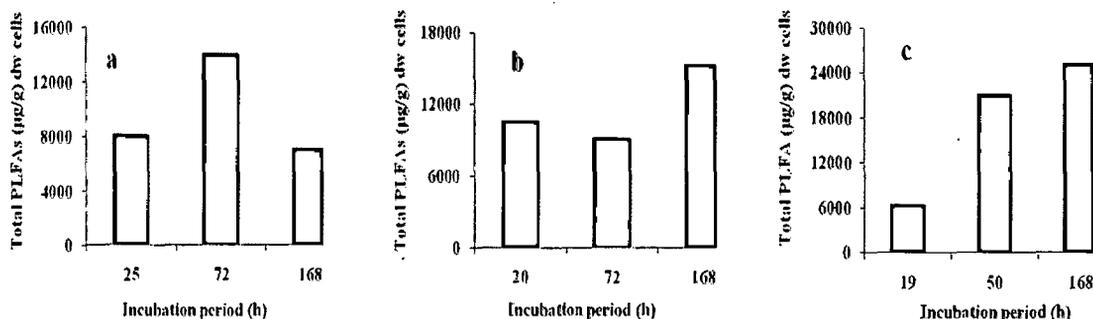


Fig.2A.1 Changes in total PLFA concentrations in *Bacillus licheniformis* (a), *Bacillus subtilis* (b) and *Aeromonas hydrophila* (c) during incubation periods.

A striking feature of *Bacillus licheniformis* and *Bacillus subtilis* was the dominance of terminally branched saturated iso and anteiso PLFAs (Br) with carbon numbers ranging from 14 to 18 (iC14, iC15:0, iC16:0, aC15:0, aC17:0, iC18, aC18:0) (Fig. 2A.2a & 2A.2b). This pattern is typical for *Bacillus* and related genera (Kaneda, 1968; Kaneda, 1991; Haque and Russell, 2004; Lopez et al., 2006; Kim et al., 2007). While in *A. hydrophila* the major PLFAs were MUFAs such as C16:1 and C18:1 and saturated PLFAs (Sat) such as C16:0 (Fig. 2A.2c) (Kämpfer et al., 1994; Huys et al., 1997; Chihib et al., 2005). Br and Unsat PLFAs have special functions, and the amount of these acids must be controlled to meet physiological requirements under given growth conditions (Kaneda, 1977).

In the *B. licheniformis* concentrations of iso and anteiso PLFAs increased over the incubation period of 72 h and then decreased at the 168 h of incubation period. This trend was similar to that observed for the total PLFAs (Fig. 2A.2a). Individual PLFA concentration in the *B. subtilis* also increased over the period of incubation and the variation was almost similar to total PLFAs (Fig. 2A.2b). The major PLFAs (C16:0, C16:1 and C18:1n11) in *A. hydrophila* increased over the period of incubation (Fig. 2A.2c).

In the *B. licheniformis*, the relative contribution of Br PLFAs was more abundant compared to Sat PLFAs over the incubation period (25 h to 168h). The % iso PLFAs were higher than anteiso PLFAs and increased steadily from 37 % to 41% (Table 2A.1), while

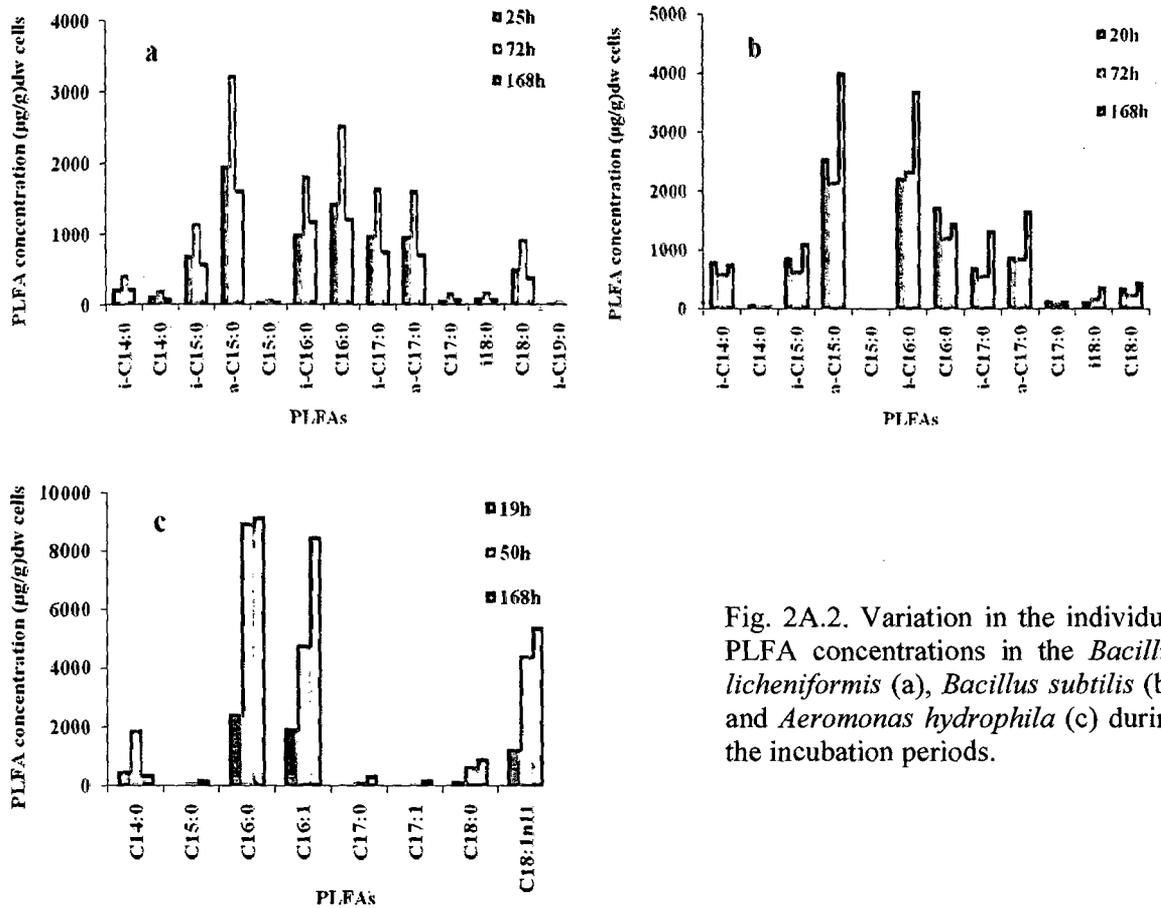


Fig. 2A.2. Variation in the individual PLFA concentrations in the *Bacillus licheniformis* (a), *Bacillus subtilis* (b), and *Aeromonas hydrophila* (c) during the incubation periods.

anteiso decreased over the incubation period. Among the Br PLFAs, Br15 (iC15:0+aC15:0) were generally higher compared to Br17 (iC17:0+aC17:0) over the incubation periods (Table 2A.1). This resulted in higher ratios of iso/anteiso and Br15/Br17. Lower concentration of Sat resulted in lower ratios of Sat/Anteiso, Sat/Iso and Sat/Br in the *B. licheniformis* (Table 2A.1).

In the *B. subtilis*, similarly relative % of iso and Br15 was higher than the anteiso and Br17 PLFAs over the period of incubation (Table 2A.1). Sat were also the minor components, whereas the Br were abundant components during all the incubation periods in *B. subtilis*. Further, Sat decreased while Br increased over the incubation periods. Iso/Anteiso, Br15/Br17, Sat/Anteiso and Sat/Br ratio decreased over the incubation periods (Table 2A.1).

Table 2A.1. Relative % of major PLFA groups: iso, anteiso, BrC15:0 (iso and anteiso C15:0), BrC17:0 (iso and anteiso C17:0), saturated (Sat) and Br (iso and anteiso branched) PLFAs and PLFA ratios in the *Bacillus licheniformis* and *Bacillus subtilis* during the incubation periods.

PLFA groups(%)	Incubation period (h)			Incubation period (h)		
	25	72	168	20	72	168
	<i>Bacillus licheniformis</i>			<i>Bacillus subtilis</i>		
Iso	37	38	41	45	48	48
Anteiso	36	35	33	33	34	38
BrC15:0	33	31	31	32	31	34
BrC17:0	24	23	21	15	16	20
Sat	27	28	26	22	18	14
Br	73	72	74	78	82	86
PLFA ratios						
Iso/Anteiso	1	1.1	1.2	1.4	1.4	1.3
BrC15/BrC17	1.4	1.3	1.5	2.2	1.9	1.7
Sat/Anteiso	0.7	0.8	0.8	0.7	0.5	0.4
Sat/Iso	0.7	0.7	0.6	0.5	0.4	0.3
Sat/Br	0.4	0.4	0.4	0.3	0.2	0.2

Maintenance of proper fluidity of membrane lipids is important for the growth of microorganisms, and this is achieved by varying the fatty acid composition of the membrane lipids (Kaneda, 1977). Iso and anteiso Br PLFAs helps in maintaining cell membrane fluidity. Further, the Br15 are more fluid compared to Br17 at room temperature. This shows that iso and Br15 PLFAs play important role in maintaining the cell membrane fluidity in *B. licheniformis* and *B. subtilis* during various incubation periods. These two *Bacillus* spp requires iso and Br15 PLFAs at higher concentrations than the anteiso and Br17 counterpart in order to maintain fluidity of the membrane and the proper growth of cells. This suggests that both the *Bacillus* cultures perhaps follow the same mechanism to maintain the fluidity of the cell membrane during various stages of growth. Moreover, such regulation could be valuable for cell adaptation to environmental variations (Russell et al., 1995). Iso PLFAs due to their relatively higher boiling points, increase the rigidity of cell membranes, whereas anteiso increases the fluidity in order to maintain the proper fluidity of the cells. It is suggested that increase in certain branched

fatty acids enhances cell membrane rigidity (Syakti et al., 2006). This balance in the fluidity of the membrane is needed for the proper growth of the cells. It is suggested that these changes are a protective mechanism against lipid oxidation due to culture aging (McDaniel et al., 1983).

In *A. hydrophila* Sat PLFAs increased while unsaturated PLFAs (Unsat) decreased over the 50 h incubation period. However, when incubation period further extended to 168 h, Sat PLFAs decreased while Unsat PLFAs increased (Table 2A.2). The ratio of Unsat/Sat and C16:1/C18:1 at the 19 h were high, suggesting Unsat PLFAs are required by the cell during this growth period. At 50 h of the incubation period the decrease in Unsat/Sat was due to relative decrease in C16:1 compared to C16:0 and C18:1 PLFA. This result was in good agreement with previous works (Diefenbach et al., 1992) reporting an increase in Sat FAs when cells entered the stationary growth phase. Increase in C18:1 also shows that this PLFA possibly plays the same role as Sat PLFAs in *A. hydrophila*. A similar result on MUFA decrease was observed for a major fatty acid (16:1), of the psychrophilic *Vibrio* sp., as a response to temperature increase and growth phase (Hamamoto et al., 1994). A recent study on a dinoflagellate (Mansour et al., 2003) reported a similar trend (e.g. a lower abundance of MUFAs in the stationary phase). These results indicated that the growth phase induced significant changes in the PLFA composition possibly to protect cells from aging. The saturated and C18:1 PLFAs probably makes the cells membrane more rigid and thus prevent cell membrane disruption during aging. However, the increase in unsaturation because of C16:1 at 168 h following inoculation was probably the result of new production due to utilization of recycled nutrients by the bacteria. The dynamic states of lipids (the fluidity and the order) are closely related to the functions of biological membranes (Cossins and Sinensky, 1984). In biological systems, Unsat PLFAs may serve to maintain membrane fluidity (Melchior,

1982), influence membrane permeability (Russell, 1988) and supply appropriate quantities of specific phospholipids to optimize enzymatic activities (Farias et al., 1975).

Table 2A.2. Relative percentages of major PLFA groups: saturated (Sat) and unsaturated (Unsat) and PLFA ratios in the *Aeromonas hydrophila* during various incubation periods.

PLFA groups (%)	Incubation period (h)		
	19	50	168
<i>Aeromonas hydrophila</i>			
Sat%	49	56	44
Unsat%	51	44	56
PLFA ratios			
Unsat/Sat	1	0.8	1.3
C16:1/C18:1	1.5	1.1	1.6

The comparison of PLFA profiles in all the three bacteria suggests that iso and Br15 PLFAs were abundant over the incubation period in *Bacillus* spp., while saturated and unsaturated PLFAs were abundant in *A. hydrophila*. These PLFAs are responsible for maintaining the fluidity of the cell membrane.

2A.3.2.3. Effect of nutrients (nitrogen and phosphorus)

In the *B. licheniformis* total PLFAs increased from 4892 (4.7mM-N) to 8469 $\mu\text{g g}^{-1}$ dw cells (18.7mM-N) (Fig. 2A.3a) and 4145 (1.6mM-P) to 8645 $\mu\text{g g}^{-1}$ dw cells (6.2mM-P) (Fig. 2A.3b). The increase in total PLFAs in the *B. licheniformis* was 1.7 fold for nitrogen, whereas, it was 2 fold for phosphorus. In the *Bacillus subtilis*, total PLFAs increased from 2991 (4.7mM-N) to 5539 $\mu\text{g g}^{-1}$ dw (18.7mM-N) (Fig. 2A.4a) and 2326 (1.6mM-P) to 5539 $\mu\text{g g}^{-1}$ dw cells (6.2mM-P) (Fig. 2A.4b). Total PLFA concentration increased from 8200 (4.7mM-N) and 9762 $\mu\text{g g}^{-1}$ dw cells (1.6mM-P) to 16076 $\mu\text{g g}^{-1}$ dw cells in the *Aeromonas hydrophila* (Fig. 2A.5a and b). Increase in nitrogen and phosphorus concentrations in the growth medium of each bacteria showed increased production of total PLFAs. Generally, bacteria, fungi and also saprophagous soil animals are assumed to be controlled mainly by resource availability (Hunt et al. 1987), and

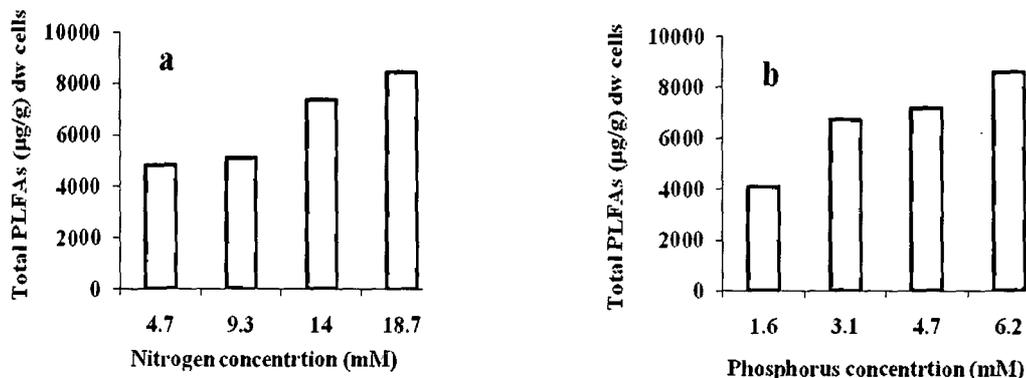


Fig. 2A.3. Changes in total PLFA concentration in the *Bacillus licheniformis* as a function of concentrations of nitrogen (a) and phosphorus (b).

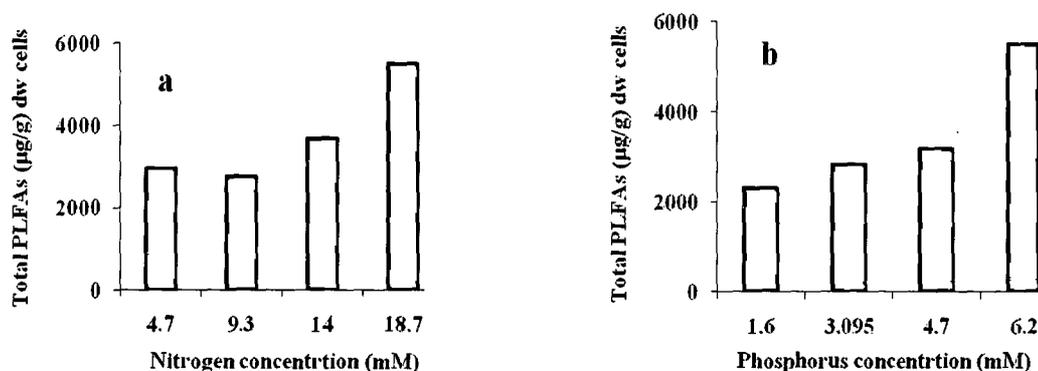


Fig. 2A.4. Changes in total PLFA concentrations in the *Bacillus subtilis* as a function of concentrations of nitrogen (a) and phosphorus (b).

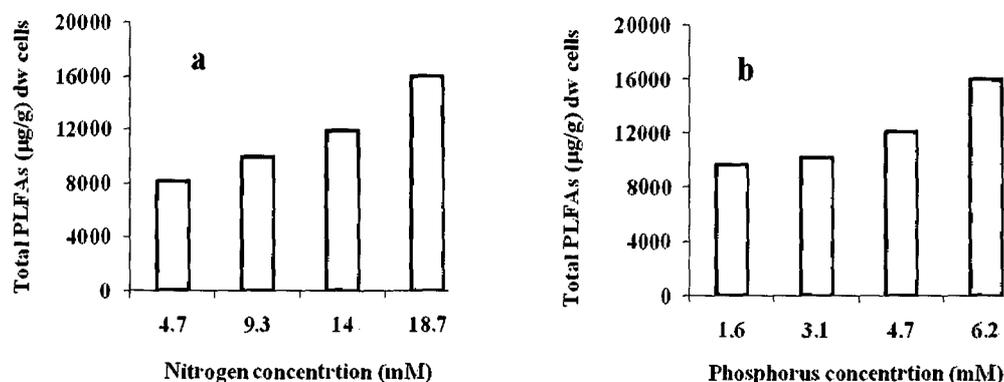


Fig.2A.5. Changes in total PLFA concentrations in the *Aeromonas hydrophila* as a function of concentrations of nitrogen (a) and phosphorus (b).

limited by the availability of carbon and nitrogen (Anderson and Domsch 1978, Gallardo and Schlesinger 1994, Demoling et al. 2007). Increase in microbial biomass after addition of carbon, nitrogen and phosphorous was observed previously (Scheu and Schaefer, 1998; Krashevskaja et al., 2010). It was also suggested that these nutrients in lower concentrations can be a limiting factor for growth (Joergensen and Scheu, 1999; Demoling et al. 2007;

Vance and Chapin, 2001). The increase in total PLFA concentrations with increase in nitrogen and phosphorus suggests the increasing nutrients are required for the proper functioning of the cell membrane and hence proper growth.

Individual PLFAs also increased with the increase in nitrogen and phosphorus concentration in the three cultures. In *B. licheniformis* as the nitrogen concentration increased from 4.7 to 18.7 mM, i-C15:0 and a-C15:0 increased almost 2 folds, while i-C17:0 and a-C17:0 1.9 fold (Fig. 2A.6a). Similar trends in PLFAs were recorded in the *B. subtilis* when grown with different concentrations of nitrogen and phosphorus (Fig. 2A.7a & b). Anteiso C15:0 and iso C16:0 were the most abundant PLFAs in the both the *Bacillus* cultures when grown on different concentrations of nitrogen and phosphorus (Fig. 2A.6 & 2A.7). In *A. hydrophila*, concentrations of the major PLFAs (C16:0, C16:1 and C18:1n11) also increased with the increase in concentration of nitrogen and phosphorus (Fig.2A.8a & 2A.8b).

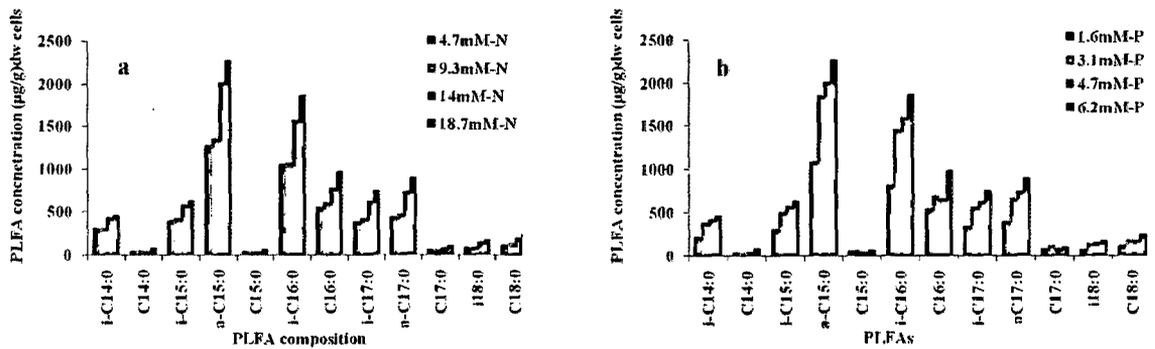


Fig. 2A.6. Effect of nitrogen (a) and phosphorus on the changes in PLFAs in the *Bacillus licheniformis*.

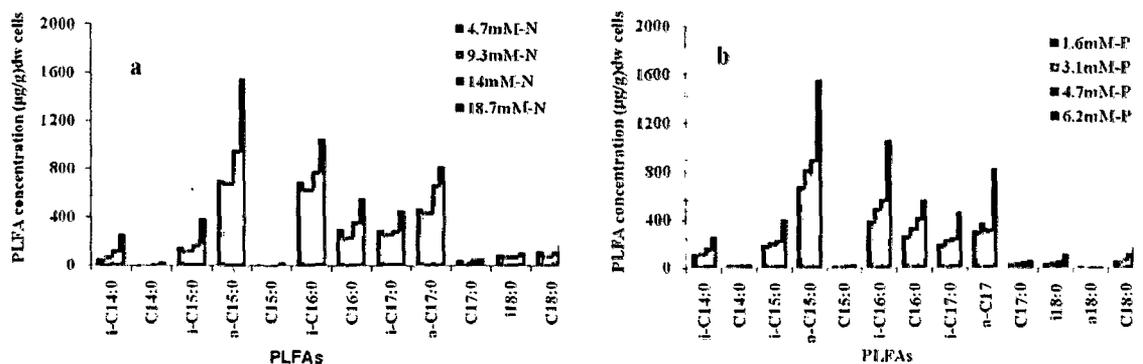


Fig. 2A.7. Effect of nitrogen (a) and phosphorus on the changes in PLFAs in the *Bacillus subtilis*.

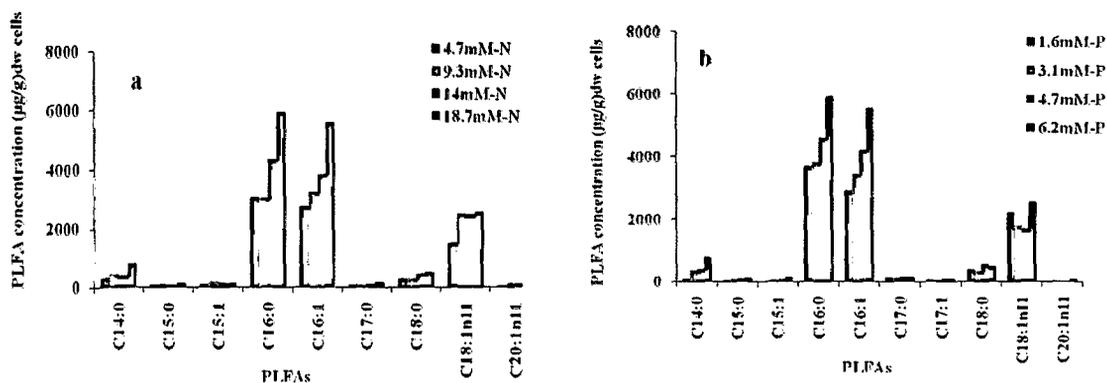


Fig. 2A.8. Effect of nitrogen (a) and phosphorus on the changes in PLFAs in the *Aeromonas hydrophila*.

Irrespective of the nitrogen and phosphorus concentrations used, relative % of iso and Br15 PLFAs were higher than anteiso and Br17 PLFAs in *B. licheniformis* (Table 2A.3). Similarly, the ratios BrC15/BrC17 and iso/anteiso in the *B. licheniformis* did not change much despite changes in concentrations of nitrogen and phosphorus. Saturated PLFAs were less abundant than the branched PLFAs in the *B. licheniformis* (Table 2A.3).

In the *B. subtilis*, relative % of iso PLFAs decreased whereas anteiso PLFAs increased with the increase in nitrogen concentrations resulting in decreased Iso/Anteiso ratio (Table 2A.4). Concentrations of Br15 were higher than Br17 irrespective of the nutrients used. These changes were presumably responsible for the increase in the Br15/Br17 ratio from 1.1 (4.7mM-N) to 1.5 (18.7mM-N) in the *B. subtilis*. Sat PLFAs were also present in minor amounts in the *B. subtilis* grown with different concentrations of nitrogen. With the increase in phosphorus concentration in *B. subtilis* % iso PLFAs increased while anteiso decreased resulting in slight increase of Iso/Anteiso ratio (0.9 to 1) (Table 2A.4). The increase in phosphorus concentration have resulted in slight decrease of Br15 in the *B. subtilis*. The ratio of Br15/Br17 increased initially (1.7 to 1.9) but further decreased (1.5) at higher phosphorus (6.2mM-P) concentration in the *B. subtilis* (Table 2A.4). Similar to *B. licheniformis*, Sat/Anteiso, Sat/Iso and Sat/Br PLFA ratios were very

low during growth with all the nitrogen and phosphorus concentrations used suggesting, saturated PLFAs were present in minor amounts in the *B. subtilis*.

Table 2A.3. Relative % of iso, anteiso, BrC15:0 (iso and anteiso C15:0), BrC17:0 (iso and anteiso C17:0), Br (iso and anteiso branched), saturated and saturated+iso PLFAs and ratios of PLFAs in the *Bacillus licheniformis* during the growth with various nitrogen and phosphorus concentrations.

PLFA groups(%)	Nitrogen concentration				Phosphorus concentration			
	4.7mM-N	9.3mM-N	14mM-N	18.7mM-N	1.6mM-P	3.1mM-P	4.7mM-P	6.2mM-P
Iso	47	46	46	45	43	46	47	45
Anteiso	36	36	38	37	36	37	38	37
Br15	35	35	36	34	34	35	36	34
Br17	18	18	19	19	18	18	19	19
Br	82	82	84	82	79	83	86	82
Sat	18	18	16	18	21	17	14	18
PLFA ratios								
iso/anteiso	1.3	1.3	1.2	1.2	1.2	1.2	1.2	1.2
Br15/Br17	2.0	1.9	1.9	1.8	1.9	1.9	1.9	1.8
Sat/Anteiso	0.5	0.5	0.4	0.5	0.6	0.4	0.4	0.5
Sat/Iso	0.4	0.4	0.4	0.4	0.5	0.4	0.3	0.4
Sat/Br	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2

Table 2A.4. Relative % of iso, anteiso, BrC15:0 (iso and anteiso C15:0), BrC17:0 (iso and anteiso C17:0), Br (iso and anteiso branched), saturated and saturated+iso PLFAs and ratios of PLFAs in the *Bacillus subtilis* during the growth with various nitrogen and phosphorus concentrations.

PLFA groups(%)	Nitrogen concentration				Phosphorus concentration			
	4.7mM-N	9.3mM-N	14mM-N	18.7mM-N	1.6mM-P	3.1mM-P	4.7mM-P	6.2mM-P
Iso	44	44	40	41	40	40	40	41
Anteiso	40	41	44	43	43	42	39	43
Br15	29	30	31	35	37	36	36	35
Br17	26	26	26	23	22	22	18	23
Br	84	86	84	84	83	82	79	84
Sat	16	14	16	16	17	18	21	16
PLFA ratios								
iso/anteiso	1.1	1.1	0.9	1.0	0.9	0.9	1.0	1.0
Br15/Br17	1.1	1.1	1.2	1.5	1.7	1.7	1.9	1.5
Sat/Anteiso	0.4	0.3	0.4	0.4	0.4	0.4	0.5	0.4
Sat/Iso	0.4	0.3	0.4	0.4	0.4	0.5	0.5	0.4
Sat/Br	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2

In the *A. hydrophila*, at the concentration of nitrogen and phosphorus used Unsaturated PLFAs were more abundant and influenced Unsaturated/Saturated and C16:1/C18:1 ratio (Table 2A.5). Small changes in Saturated and Unsaturated PLFAs were recorded with the changes in concentrations of nutrients. This was probably the reason for the decrease in Unsaturated/Saturated ratio and increase in C16:1/C18:1 ratio.

Table 2A.5. Relative % of saturated (Sat) and unsaturated (Unsat) PLFAs and unsaturated/saturated (Unsat/Sat) and C16:1/C18:1 ratios in the *Aeromonas hydrophila* during the growth with various concentrations of nitrogen and phosphorus.

	Nitrogen concentration (mM-N)				Phosphorus concentration (mM-P)			
	4.7	9.3	14	18.7	1.6	3.1	4.7	6.2
PLFA groups(%)								
Sat	46	39	44	47	45	46	48	47
Unsat	54	60	55	53	55	53	51	53
PLFA ratios								
Unsat/Sat	1.2	1.5	1.2	1.1	1.2	1.2	1.1	1.1
C16:1/C18:1	1.8	1.3	1.5	2.1	1.3	1.9	2.4	2.1

The increase in individual PLFA with increase in nitrogen and phosphorus concentration indicates that, the nutrients induce PLFA production. Greater proportion of Unsat PLFAs (16:1n7c and 18:1n7c), was observed previously under the flow of drinking water supplemented with increasing concentrations of phosphorus (0 to 5 $\mu\text{g l}^{-1}$), indicating an increase in gram-negative bacterial biomass (Keinänen et al., 2002). Microbial growth in drinking water was also correlated with the phosphorus present in water (Miettinen et al., 1996; Miettinen et al., 1997; Sathasivan et al., 1997; Sathasivan and Ohgaki, 1999; Charnock and Kjønne. 2000). Addition of inorganic phosphorus often stimulates the growth of bacteria (Vadstein et al. 1988; Vadstein 1998). Unbalanced growth occurs when a suitable carbon source is present, but one or more essential nutrients are absent (Nickels et al., 1979). This suggests that appropriate concentrations of nutrients are needed for the proper functioning of cell membrane and for enhancing bacterial biomass. It is suggested that increased production of PLFAs are required for the proper growth of cells. However, it is observed that the ratios of PLFAs are usually not affected much with the nutrient concentrations used.

2A.3.2.4. Effect of temperatures

During the temperature incubations, total PLFA concentration varied from 545 (27 °C) to 14069 (38 °C) $\mu\text{g g}^{-1}$ dw cells in the *B. licheniformis* (Fig. 2A.9a). At 27 °C the

total PLFA concentration was very low and increased sharply when the incubation temperature was 30°C and 38°C. PLFAs help in proper functioning of the cell growth by permitting the proper fluidity of the cell membrane. It appears that *B. licheniformis* probably grows better at higher temperature (30 °C to 38 °C) as the concentration of total PLFAs increased at these temperatures. In *B. subtilis*, total PLFA concentration varied from 4222 (38 °C) to 5263 (30 °C) $\mu\text{g g}^{-1}$ dw cells (Fig. 2A.9b). In the *B. subtilis*, although the variation in the total PLFAs was not very great, still showed some increase in total PLFAs at 30 °C followed by a decrease at 38 °C. In *Aeromonas hydrophila*, total PLFA concentration varied from 3929 (38 °C) to 7974 (30 °C) $\mu\text{g g}^{-1}$ dw cells (Fig. 2A.9c). *A. hydrophila* showed maximum PLFA concentration at 30 °C and the concentration decreased when temperature increased to 38°C, suggesting at 30 °C optimum production of PLFAs occur.

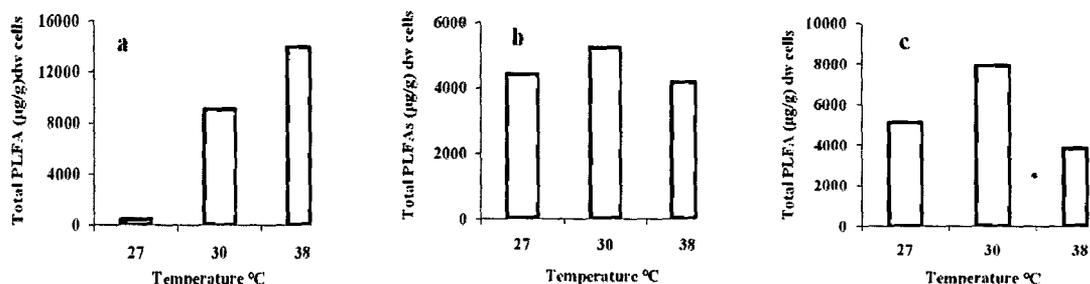


Fig.2A.9: Changes in total PLFA concentrations in the *Bacillus licheniformis* (a), *Bacillus subtilis* (b) and *Aeromonas hydrophila* (c) as a function of temperatures.

Generally bacteria produced low biomass at elevated temperatures (Coultate and Sundaram, 1975; Pirt, 1975). Such responses to temperature may partly explain the lower PLFA production at higher temperatures in *B. subtilis* and *A. hydrophila*. In addition, the selection of different bacterial cultures also have contributed to differences in growth and therefore to higher PLFA at 30°C for *B. subtilis* and *A. hydrophila* and for *B. licheniformis* at 38°C.

The individual PLFA concentrations in the three bacterial cultures varied as the incubation temperature increased (Fig. 2A.10a, b & c). *B. licheniformis* cells grown at 27 °C had less concentration of individual PLFAs as compared to those cells grown at 30 °C and 38 °C and the anteiso PLFA concentration was maximum at 38 °C (Fig. 2A.10a). In the *Bacillus subtilis* anteiso PLFAs (a15:0 and a17:0) were present in higher concentration at 30°C compared to other temperatures, whereas the iso16:0 were higher at 38 °C in the *B. subtilis*. Sat PLFAs in this culture were present in lower concentrations and also decreased at higher temperatures (38 °C).

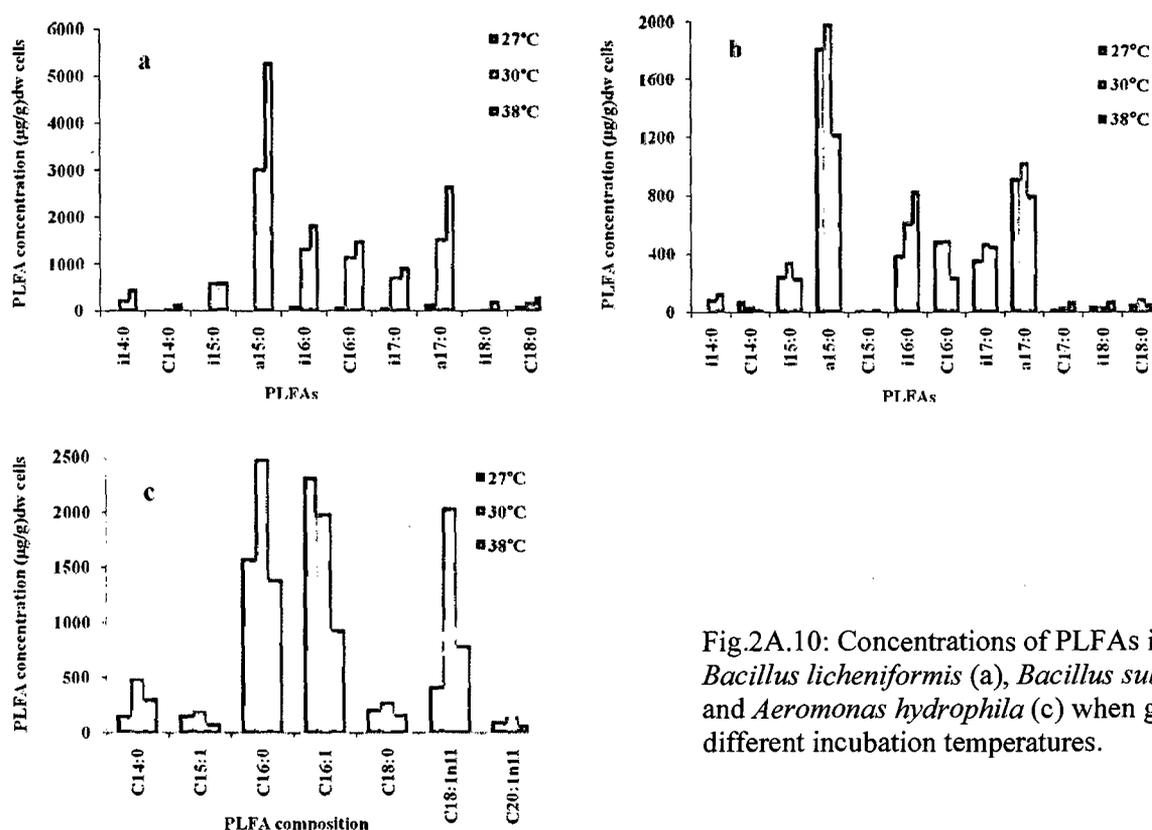


Fig.2A.10: Concentrations of PLFAs in the *Bacillus licheniformis* (a), *Bacillus subtilis* (b) and *Aeromonas hydrophila* (c) when grown at different incubation temperatures.

The melting points of Br PLFAs are generally lower than those of straight-chain PLFAs with the same number of carbons. Particularly, the melting points of anteiso series are 25 to 35 °C lower than those of normal series (Kaneda, 1977). Melting points of the iso series, however, are only slightly lower than those of the normal series. It is suggested that at high temperatures large amounts of the iso PLFAs rather than the Sat PLFAs are

found as lipid constituents (Bauman and Simmonds, 1969). This was also the case in *B. licheniformis*, where the iso increased and Sat PLFAs decreased with increasing temperature. Although Sat and iso-branched PLFAs have similar melting points for the same carbon number, the iso branched PLFAs increase the fluidity of membrane since their phase transition temperatures are lower than their saturated homologous (Kaneda, 1991; Suutari and Laasko, 1994). Indeed, the phase transition temperature (T_m) is more closely related to the membrane fluidity than the average melting point of the membrane PLFAs (Kaneda, 1991). The iso-branched PLFAs play an important role at high temperature growth conditions for many thermophilic Gram-positive bacteria since in their absence fluidity of the membrane will not be maintained and cell will not grow. In addition, it is well established that transport across the membranes of bacteria is dependent on their T_m and on their physical state (Kaneda, 1991; Suutari and Laasko, 1994). This shows that the increase in the iso and anteiso PLFAs in the *B. licheniformis* and iso PLFAs in *B. subtilis* at 38°C was to maintain the proper membrane fluidity in order to maintain growth at higher temperatures.

Individual PLFA concentrations in the *A. hydrophila* showed variable concentrations when grown at the three different temperatures (Fig. 2A.10c). Basically when the incubation temperature was low (27 °C), *A. hydrophila* synthesized PLFA in low amounts but with increase in temperature (30 °C) PLFA concentration also increased. Further increase in temperature (38 °C), PLFA content decreased (Fig. 2A.10c). In *A. hydrophila* at 27 °C higher concentration of C16:1 and lower concentration of C18:1 and C16:0 were observed. It is suggested that PLFA signature depends on the physiological state of the microorganisms (Ourisson et al., 1979), and experimental test variables such as temperature have found to change the PLFA composition (Nichols et al., 1997; White et al., 1997). The decrease in C18:1 at lower temperature is suggested due to partial loss of

the intrinsic β -ketoacyl-ACP-synthase II activity which is involved in C18:1 synthesis as reported for *Escherichia coli* (Garwin et al., 1980) and for *Lactobacillus fermentum* (Suutari and Laasko, 1992). This increase in C16:1 and decrease in C16:0 and C18:1 probably led to a lowering of the gel to liquid-crystalline phase transition temperature, and an increase of the passive permeability of the membrane (Suutari and Laasko, 1994; Russell and Fukunaga, 1990). Fluidity of membrane is strongly influenced by temperature. Bacteria under the changing temperature can undergo possible membrane adaptations by altering the viscosity ('homeoviscous adaptation', Sinensky, 1974).

Br PLFAs are generally involved in maintaining membrane fluidity at lower temperatures (Haque and Russell, 2004). Generally, it is assumed that bacteria use a well defined set of mechanisms to adjust the membrane viscosity to changing temperature such as increase in branched PLFAs in Gram positive bacteria and unsaturated PLFAs in Gram negative bacteria and decrease in Sat at lower temperatures and vice versa at higher temperatures (Hazel and Williams, 1990; Suutari and Laakso, 1994; Denich et al., 2003). In the *Bacillus licheniformis*, decrease in Br PLFA with decreasing temperature and increase with increasing temperature (Fig. 2A.10a) was probably due to a different mechanism the bacteria follows to adapt to the changes in temperature. Freese et al (2008) also observed inconsistent results with *Bacillus* sp. NA402, *Psychrobacter* sp. NC201 and *Shewanella* sp. NA251 with changing temperatures. However, in *B. subtilis* Br PLFAs (especially anteiso) increased at lower temperature (27 °C). Anteiso PLFAs have lower boiling points than saturated and iso PLFAs and hence helps in cell membrane fluidity at low temperatures thus protecting cell membrane against solidifying at lower temperatures. Changes in Br PLFAs, reported to be involved in adaptation to lower temperature, increased with decreasing temperature in *B. subtilis*, whereas in *B. licheniformis* an opposite effect was observed (Freese et al., 2008). However, *B. licheniformis* also showed

lower concentrations of total PLFAs at low temperature than the *B. subtilis*. This shows both the *Bacillus* cultures are adapted differently to temperature changes. Results obtained with *Bacillus* (Haque and Russell, 2004), *Arthrobacter* (White et al., 2000) and *Sphingomonas* spp (Männistö and Puhakka, 2001) also show intrageneric variability in the synthesis of fatty acids in the presence of different growth conditions. In *Bacillus licheniformis* temperature increasing from 30 to 38°C resulted in an increase in iso and anteiso fatty acids. Increase in anteiso and iso PLFAs at 38°C suggests that *B. licheniformis* is in good physiological state and appears to tolerate a higher temperature while doing this. Anteiso acids were more abundant than iso acids in both the *Bacillus* cultures and were similar as in other *Bacillus* spp. (Haque and Russell, 2004).

Increase in relative % of iso and decrease in anteiso PLFAs with increase in temperature from 27 °C to 38 °C resulted in increased ratio of Iso/Anteiso PLFA in *B. subtilis*, (Table 2A.6). Further, Br15/Br17 decreased with the increase in temperature in *B. subtilis* (Table 2A.6). This shows that Br17 chain PLFAs increased over Br15 chain PLFAs at higher temperature. Opposite was observed in *B. licheniformis* where the relative % of iso PLFAs decreased with increase in temperature while anteiso PLFAs increased, resulting in decrease of Iso/Anteiso ratio with increase in temperature (Table 2A.6). The results therefore indicate that there is no unequivocal way as to how bacteria change their membrane PLFA composition in response to changing temperature (Freese et al., 2008). The increase in temperature also laid the Br17 PLFAs to decrease while the Br15 to increase, which in turn increased the Br15/Br17 ratio in *B. licheniformis* (Table 2A.6). Sat/Br PLFA ratio decreased with increase in incubation temperature in both the *Bacillus* cultures. This suggests saturated PLFAs are minor components of *Bacillus* genus, which decrease with increase in incubation temperature.

Table 2A.6. Sum of relative % of iso, anteiso, Br15, Br17, Br, Sat and saturated+iso PLFAs and PLFA ratios iso/anteiso, Br15/Br17, Sat/Anteiso, Sat/Iso and Sat/Br PLFAs during incubation with different temperatures.

Incubation temperature (°C)											
27 30 38				27 30 38				27 30 38			
<i>B. licheniformis</i>				<i>B. subtilis</i>				<i>A. hydrophila</i>			
PLFA groups (%)				PLFA groups (%)				PLFA groups (%)			
iso	34	33	29	iso	24	30	41	Sat	38	43	50
anteiso	30	50	57	anteiso	62	57	48	Unsat	59	56	49
Br15	0	40	42	Br15	47	44	35				
Br17	45	25	26	Br17	29	29	30				
Br	64	83	86	Br	85	87	89				
Sat	36	16	14	Sat	15	13	10				
PLFA ratios				PLFA ratios				PLFA ratios			
Iso/Anteiso	1.1	0.7	0.5	Iso/Anteiso	0.4	0.5	0.9	Unsat/Sat	1.5	1.3	1.0
Br15/Br17	0.0	1.6	1.6	Br15/Br17	1.6	1.5	1.2	C16:1/C18:1	5.3	1.0	1.2
Sat/Anteiso	1.2	0.3	0.2	Sat/Anteiso	0.2	0.2	0.2				
Sat/Iso	1.1	0.5	0.5	Sat/Iso	0.6	0.4	0.2				
Sat/Br	0.6	0.2	0.2	Sat/Br	0.2	0.1	0.1				

C16:1 concentration was maximum at lower temperature (27 °C) which decreased as the temperature increased (30 and 38 °C) in *A. hydrophila* (Fig. 2A.10c). Saturated PLFAs concentrations were lower at 27 °C compared to 30 and 38°C temperature in *A. hydrophila* (Table 2A.6). As the growth temperature increased from 28 °C to 38 °C, the proportion of Unsat PLFAs decreased from 59 % to 49 % because of the marked decrease in C16:1. Increasing growth temperature resulting in a decrease in MUFAs and an increase in Sat FAs was also reported earlier by other workers (Hazel and Williams, 1990; Chihib et al., 2005; Freese et al., 2008). Similar to anteiso PLFAs, MUFAs also have lower boiling points and are liquid at room temperature, while the Sat PLFAs are solid at room temperature. Generally decreasing temperature leads to a reversible change from a 'liquid-crystalline' to a gel-like state, or even to a crystalline array of FAs with restricted macromolecular mobility (Quinn, 1981; de Mendoza and Cronan, 1983). However, elevated temperatures makes FA side chains more disordered and the membrane may change into a non-lamellar state or even melt (Hazel and Williams, 1990). Hence the

increase in Sat PLFAs at higher temperature shows the adaptation of *A. hydrophila* to prevent the cell membrane from melting. In contrast, increase in MUFAs at lower temperature protects the cell membrane from solidifying. Therefore, it can be concluded that C16:1 contributes to the maintenance of membrane fluidity at low temperature (Kamimura et al., 1993) in *A. hydrophila*, whereas branched PLFAs are involved in the fluidity at low temperatures in *B. subtilis*.

**Effects of environmental contaminant,
tributyl tins (TBT) on the PLFA
concentration and composition of some
bacteria**

2B.1. Introduction

Tributyltin-chloride (TBT) is an organotin compound, containing three alkyl groups attached to the tin atom exhibiting biocidal activity against a wide range of organisms. As a result, TBT is used as a biocide in antifouling paints to control/prevent fouling on commercial marine structures, especially boats and ships (White et al., 1999; Hoch, 2001; Rüdél, 2003). From laboratory and environmental monitoring studies, it was realized that TBT is highly toxic to non-target organisms. For example, at a few ng l⁻¹ TBT can cause several harmful effects including high larval mortality, severe malformation of shells and reduced reproduction in oysters (Alzieu, 2000), imposex in dog whelk populations (Evans et al., 2000), growth retardation in mussels (Salazar and Salazar, 1991) and microalgae (Beaumont and Newman, 1986). TBT affects growth, oxygen consumption and membrane organization in bacteria (Martins et al., 2005; Cruz et al., 2007). TBT also affects growth in fungi (Masia et al., 1998). TBT is associated with endocrine disrupting chemicals (EDCs) and can cause developmental aberrations in many organisms (Morcillo and Porte, 2000).

Due to the risk in application of TBT in antifouling coatings, these paints have come under increasing governmental regulation in the United States and many western European countries (Price et al., 1992; Evans et al., 2000). However, some countries continued utilizing this as an antifouling agent (Rüdél et al., 2003). As a consequence, TBT contamination and its impact around the world are still observed at the contaminated sites (Barroso et al., 2000; Bhosle et al., 2004; Bhosle et al., 2006). International Maritime Organisation (IMO) adopted the International Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention). The Treaty was adopted by IMO on October 5, 2001. However, the AFS Convention only came into force on the 17th September 2008 banning globally both the application and presence of TBT-based antifouling paint on ship hulls. Application of TBT-

based paints on ship hulls is responsible for growing pollution of the environments and foods on a worldwide scale (Suzuki et al., 1992).

Effects of TBT are widely studied in various organisms, TBT is toxic to prokaryotic cells (Martins et al., 2005). It has been reported that organotin compounds are toxic to Gram positive and Gram negative bacteria, nevertheless, the former showed increased sensitivity to triorgnotins (Mendo et al., 2003). However, certain Gram positive bacterial strains were shown to be highly resistant to TBT (Cruz et al., 2007). The cytoplasmic membrane and/or intracellular level mechanisms might be involved in the resistance, as suggested by White et al. (1999). TBT being the hydrophobic molecule interacts with the cell membrane leading to its toxicity. Thus membrane adaptation capability determines the survival of the cells (Dercova et al., 2004).

Phospholipid fatty acids (PLFA) are the main components of the cell membrane in microorganisms (White et al., 1979; Russell and Nichols, 1999; Fang et al., 2000). Slight structural modifications to the fatty acids can result in significant modifications to the physical properties of the membrane (Cullis et al., 1996). Fluidity of membrane is strongly influenced by numerous factors including temperature, culture age, pH, and toxic compounds which are most prominent.

Bacillus genus has been previously used as a model organism to assess effect of lipophilic drugs, pollutants and TBT on cell growth, oxygen consumption and lipid composition of cell membrane (Luxo et al., 2000; Monteiro et al., 2003; Martins et al., 2003; Martins et al., 2005). The effect of TBT on the PLFA composition of these bacteria is very important to understand how bacteria respond to this highly toxic chemical. Therefore, the aims of the present study were to evaluate the effect of TBT on growth and PLFA concentration and composition of four marine *Bacillus* cultures.

2B.2. Materials and methods

2B.2.1. Cultures and growth medium

The four bacterial cultures *Bacillus subtilis*, *Bacillus* sp., *Bacillus licheniformis* and *Bacillus pumilus* were obtained from the culture collection of the MCMRD laboratory. The cultures were grown using basal salt solution (BSS) as mentioned in chapter 2A, supplemented with only glucose (0.1 %).

2B.2.2. Preparation of inoculum

Bacterial cultures, *B. subtilis*, *Bacillus* sp., *B. licheniformis* and *B. pumilus* were removed from the slants and were grown individually on ZMA (Zobell marine agar) plates for 24 h. A single colony was picked and transferred on a fresh ZMA plate. For each culture this step was repeated twice. Finally, a single colony of each bacterium was grown individually in the BSS medium supplemented with only 0.1 % glucose. This step was repeated twice before the culture was used as a source of inoculum.

2B.2.3. Effect of TBT on the growth of *Bacillus* cultures

Twelve 5 liter conical flasks, containing 1 liter BSS medium in each were sterilized at 120 °C and 15 lb pressure for 15 min. Flasks were cooled. To each flask glucose was added (sterilized separately) to obtain a final concentration of 0.1 %. Subsequently, to 4 flasks, 1ml methanol was added and these flasks were treated as controls. To the other 4 flasks 500 µl and to the remaining 4 flasks 1000 µl of TBT-Cl (12.1 ng µl⁻¹ in methanol) were added so as to get a final concentration of 6.05 µg l⁻¹ and 12.10 µg l⁻¹ of TBT-Cl, respectively. As required, methanol was added to each flask to adjust its volume to 1ml. One control (without TBT) and 2 TBT flasks (6.05 µg l⁻¹ and 12.10 µg l⁻¹) were inoculated with 1% of either of the 4 *Bacillus* cultures grown as above. All the flasks were incubated in the dark on a rotary shaker (100

rpm) at room temperature (28 ± 2 °C) for 6 days. Growth was monitored by measuring optical density (OD) at 540 nm at every 4 h interval over a period of incubation (Fig. 2B.1). After 6 days, cells of each culture were harvested by centrifuging at 10,000 rpm at 4 °C. The cell pellet was washed twice with 5 mM phosphate saline buffer (PBS) at pH 7.4 to remove unused nutrients. The cells were then lyophilized and kept at -20 °C until used for PLFA analysis.

2B.2.4. Extraction of total lipids, separation of PLFAs, and preparation of fatty acid methyl esters

Extraction of total lipids, separation of PLFAs, and preparation of fatty acid methyl esters were carried out as described in chapter 2A.

2B.2.5. Capillary GC and GC-MS analysis

GC and GC-MS analysis was performed as given in chapter 2A.

2B.3. Results and discussion

2B.3.1. Effect of TBT on the growth of Bacillus cultures

The bacterial cultures showed growth on BSS medium supplemented with various concentrations of TBT. In the presence of TBT, growth phases of all the cultures were influenced. The growth pattern and the cell density of all cultures were also influenced. TBT enhanced lag phase and delayed the development of maximum cell density as compared to control. The lag phase of *B. subtilis*, was extended until 16 h when growth medium was supplemented with $12.1 \mu\text{g l}^{-1}$ of TBT. In *B. subtilis*, the maximum cell density was reduced at $12.1 \mu\text{g l}^{-1}$ of TBT (Fig. 2B.1a). As compared to the control, lag phase of *Bacillus* sp. was delayed by 20 h, and 44 h at 6.05 and $12.1 \mu\text{g l}^{-1}$ of TBT, respectively (Fig. 2B.1b). However, the cell density in *Bacillus* sp. remained mostly unaffected at 6.05 and $12.1 \mu\text{g l}^{-1}$ of TBT

(Fig. 2B.1b). Similarly, in *B. licheniformis* lag phase was highly delayed (36h) at 6.05 and 12.1 $\mu\text{g l}^{-1}$ of TBT, compared to control (Fig. 2B.1c). Decrease in cell density was also observed for *B. licheniformis* when grown at 6.05 and 12.1 $\mu\text{g l}^{-1}$ of TBT (Fig. 2B.1c). *B. pumilus* also showed increase in lag phase (28h) at 6.05 and 12.1 $\mu\text{g l}^{-1}$ of TBT compared to control (Fig.2B.1d).

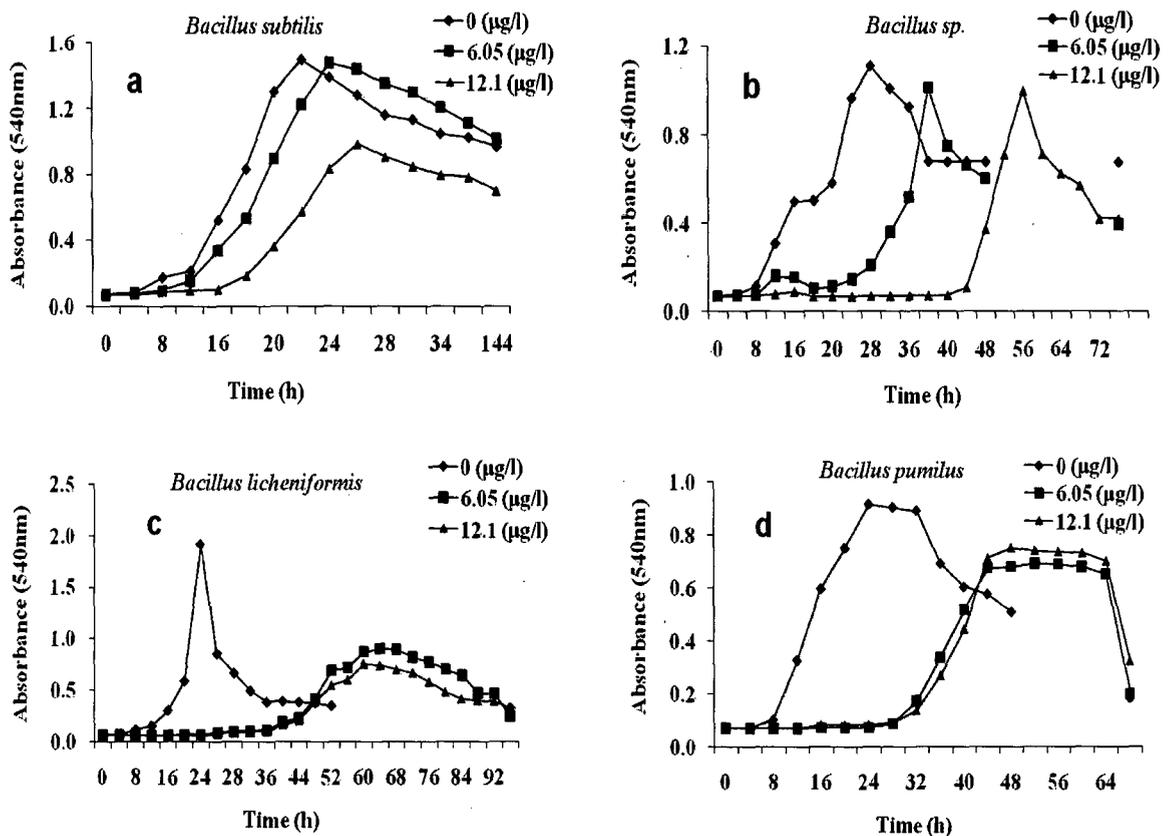


Fig. 2B.1: Growth of the *Bacillus subtilis* (a), *Bacillus sp.* (b), *Bacillus licheniformis* (c) and *Bacillus pumilus* (d) grown in BSS medium supplemented with various concentration of TBT (6.05 and 12.1 $\mu\text{g/l}$).

The increase in lag phase, delay in reaching maximum cell density and overall decrease in the cell density suggests that the TBT has interfered in the metabolism of the carbon (0.1 % glucose). But with the increase in incubation period all the cultures showed growth suggesting bacteria overcame the toxic effect of TBT. Delay in attaining the

maximum growth by these four *Bacillus* cultures suggests that the cultures needed more time to adapt to concentrations of TBT (Blanck and Dahl, 1996). Reduction in growth was observed for *Bacillus stearothermophilus* and *B. subtilis* when grown with greater concentrations (25 nM to 500 nM) of TBT (Martins et al., 2005). Perturbations in the respiratory activity due to TBT may trigger the impairment of bacterial growth (Gadd, 2000; Martins et al., 2005). However, *Bacillus* sp., and *B. pumilus* although showed delayed growth, but later grew better even at higher TBT concentrations ($12.1 \mu\text{g l}^{-1}$), suggesting these two cultures showed higher tolerance towards higher TBT concentrations. *B. subtilis* also showed fairly good growth at most of the TBT concentrations suggesting higher tolerance of this culture towards increasing concentrations of TBT. *B. subtilis* was observed previously to show increasing tolerance towards this xenobiotic (Martins et al., 2005).

2B.3.2. Effect of TBT on the total PLFA concentration

The total PLFA concentrations of the bacterial cells grown without (control) and with TBT is shown in Fig. 2B.2. Generally, PLFA concentrations increased when all the bacterial cultures were grown with $6.05 \mu\text{g l}^{-1}$ of TBT. In *B. subtilis*, concentrations of PLFAs increased with both the concentrations of TBT used in the growth medium. However, for *B. licheniformis*, *B. pumilus* and *Bacillus* sp. PLFA concentrations increased only when $6.05 \mu\text{g l}^{-1}$ of TBT was used in the growth medium. As compared to these cultures, PLFA concentrations were 3 times greater in *Bacillus* sp when it was grown in the presence of $6.05 \mu\text{g l}^{-1}$ of TBT. When cultures were grown at higher TBT concentration ($12.10 \mu\text{g l}^{-1}$), PLFA concentrations decreased in *Bacillus* sp., *B. licheniformis* and *B. pumilus* and the effect was very pronounced in *B. pumilus*.

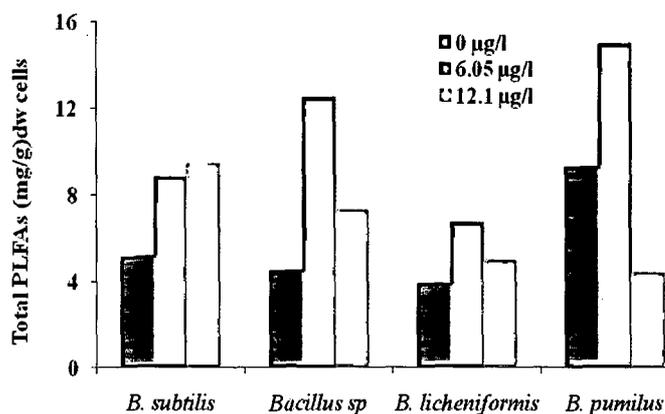


Fig. 2B.2: Effect of TBT on the concentrations of total PLFAs of *Bacillus subtilis*, *Bacillus sp.*, *Bacillus licheniformis* and *Bacillus pumilus*.

Increase in total PLFA concentrations in the presence of $6.05 \mu\text{g l}^{-1}$ of TBT, over the period of cultivation in all the bacterial cultures implies that molecular cell membrane may constitute the main target of this lipophilic xenobiotic (White et al., 1999; White and Tobin, 2004). In order to maintain the proper growth and multiplication of cells, bacterial cultures must have modulated the total PLFA concentrations. White et al (1999) suggested that the cytoplasmic membrane and/or intracellular level mechanisms along with external membrane are involved in the resistance of TBT by the gram positive bacteria (White et al., 1999). Moreover, degradation and/or metabolic utilization as a carbon source (Kawai et al., 1998) and bioaccumulation into the cells without degradation of the compound (Fukagawa et al., 1994; Dykhuizen et al., 1987) are some other factors that may be involved in TBT resistance by bacteria. TBT has been used as a source of carbon by *Pseudomonas sp* and *Aeromonas veronii* (Roy et al., 2004; Cruz et al., 2007). This suggests that the inhibitory effect of toxicants can be overcome by developing adaptive mechanisms in microorganisms. At higher concentration of TBT ($12.1 \mu\text{g l}^{-1}$), however, PLFA concentrations decreased. TBT is the most toxic compound to bacteria. TBT causes leakage of cell constituents and in some cases lysis as observed for *Escherichia coli* and *B. subtilis* (Yamada et al., 1978). Some of these

factors presumably may be associated with the observed decrease in PLFA in these bacterial cultures when grown at higher TBT concentration ($12.1 \mu\text{g l}^{-1}$)

2B.3.3. Effect of TBT on the composition of PLFAs of *Bacillus* cultures

The major PLFAs observed in all the *Bacillus* cultures were i-C14:0, i-C15:0, a-15:0, i-C16:0, C16:0, i-C17:0 and a-C17:0. With increase in TBT concentrations, branched and saturated PLFAs of the cell-membrane of all the *Bacillus* cultures increased. It appears that depending on genetic characteristics, physiological and environmental conditions relative proportions of these PLFAs can vary (Kaneda 1977).

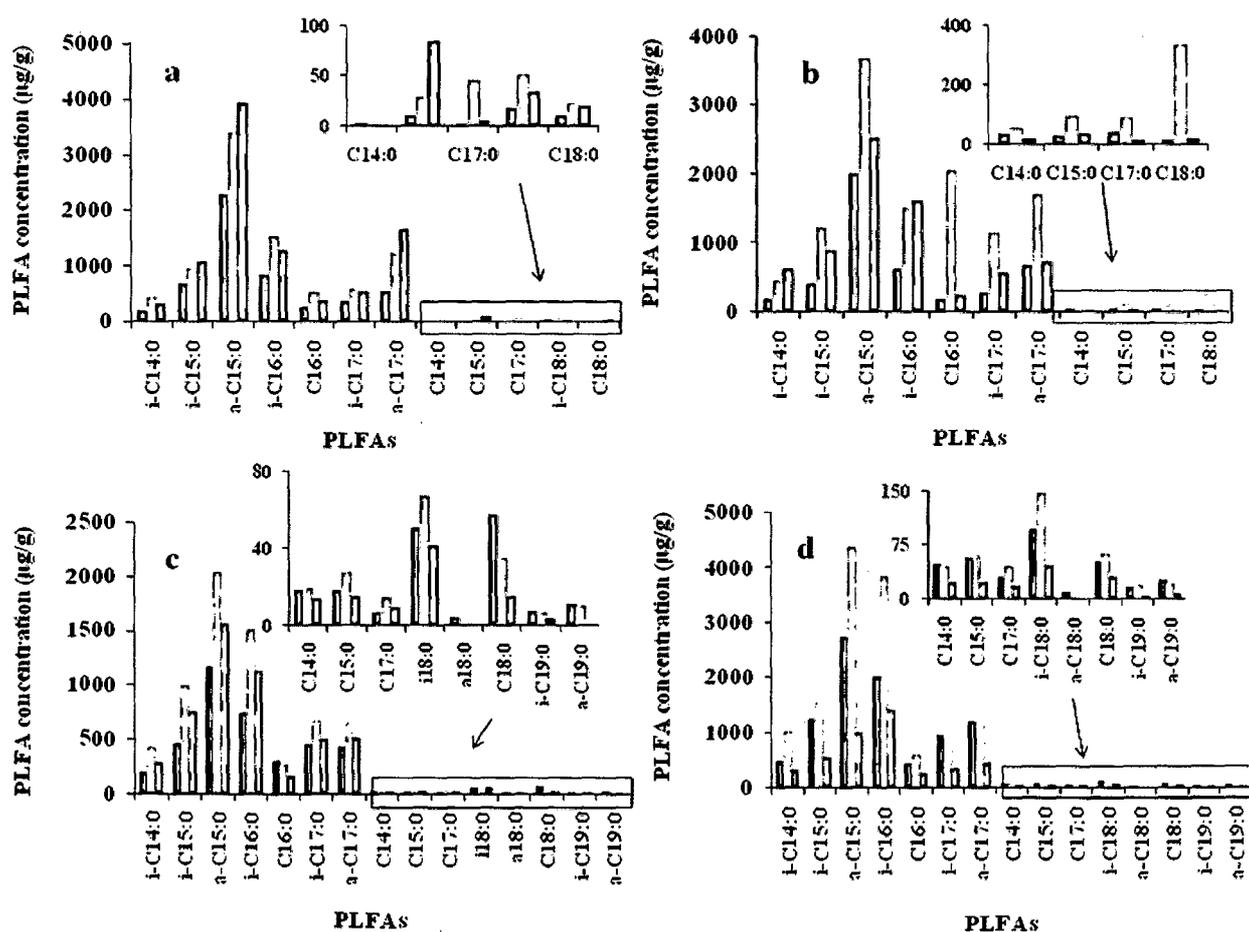


Fig. 2B.3: Changes in the PLFA concentration and composition of the *Bacillus subtilis* (a), *Bacillus* sp. (b), *Bacillus licheniformis* (c) and *Bacillus pumilus* (d) when grown in the BSS medium supplemented with various concentrations of TBT.

The FA profiles of the bacterial cultures *B. subtilis*, *Bacillus* sp., *B. licheniformis* and *B. pumilus* showed high concentrations of branched PLFAs. The presence of these branched PLFA in the *Bacillus* is in agreement with the reports of Kaneda (1968; 1977). Among branched PLFAs, a-C15:0 was the most abundant fatty acid in all the cultures. Major branched PLFAs of the *B. subtilis* such as i-C15:0, a-C15:0 and a-C17:0 increased gradually with increasing TBT concentrations (Fig. 2B.3a). However, i-C14:0 and i-C16:0 decreased when *B. subtilis* was grown with 12.1 $\mu\text{g l}^{-1}$ TBT. The trend for *Bacillus* sp. was similar to that observed for *B. subtilis* when it was grown with 6.05 $\mu\text{g l}^{-1}$ of TBT. However, concentrations of anteiso and iso C15:0 and C17:0 decreased and i-C14:0 and i-C16:0 increased when *Bacillus* sp. was grown at 12.1 $\mu\text{g l}^{-1}$ TBT (Fig. 2B.3b). Branched PLFAs also increased with increase in TBT (from 0 to 6.05 $\mu\text{g l}^{-1}$) in *B. licheniformis* and *B. pumilus* (Fig. 2B.3c and 2B.3d).

Iso and anteiso were the major PLFAs in *Bacillus* genus and a-C15:0 PLFA was the most abundant PLFA in these cultures. As compared to the control, branched PLFA concentrations increased in all the cultures when they were grown in the presence of 6.05 $\mu\text{g l}^{-1}$ of TBT. However, further increase in TBT, concentrations of branched PLFAs decreased. Such increased concentrations of TBT may disrupt the membrane permeability by producing lower concentrations of branched PLFAs, thus becoming toxic to cell. The fluidity of membrane here plays an important role as these branched PLFAs are involved in keeping the membrane fluid (Kaneda, 1977). Branched PLFAs have lower melting points compared to saturated fatty acids and among branched, anteiso have lower melting points than iso (Kaneda, 1977; Kaneda, 1991). Although most useful at lower temperatures, we observed that increasing TBT has perturbed the branched PLFA concentrations. Toxic effects of a variety of pollutants on the cells are suggested to be due to incorporation of the pollutant into the lipid

bilayer affecting its ability by changing the lipid membrane physical properties (Sikkema et al., 1995). Since maintaining the fluidity of the cell membrane is important for the proper functioning of the cell, increase in anteiso and iso PLFAs during the growth of cells with TBT was to keep the cell fluid. Differences in accumulation of the type of PLFAs, suggests that the various *Bacillus* cultures responded differently to the TBT concentrations in the medium and possibly has different mechanisms to adapt to changes in external environments (Bishop et al., 1977; Martins et al., 1990; Klein et al., 1999). Hydrophobicity of TBT suggests that its interaction with membrane lipids play an important role in its toxicity mechanisms,. Therefore membrane adaptation capability could determine the survival of the bacterial cell (Dercova et al., 2004).

In the *B. subtilis*, concentrations of saturated PLFAs particularly C16:0, C18:0, C15:0 and C17:0 increased several folds while C14:0 disappeared when grown in the presence of 6.05 $\mu\text{g l}^{-1}$ and 12.1 $\mu\text{g l}^{-1}$ TBT. (Fig. 2B.3a). In the presence of 6.05 $\mu\text{g l}^{-1}$ TBT, *Bacillus* sp showed several fold increase in C16:0, C18:0, C14:0, C15:0 and C17:0 as compared to the control (Fig 2B.3b). In the presence of TBT, saturated PLFAs accumulated in the *B. subtilis* and *Bacillus* sp. The increase in saturated PLFAs was more obvious in cultures grown with 6.05 $\mu\text{g l}^{-1}$ TBT. The increase in saturated PLFA in *B. subtilis* and *Bacillus* sp. probably lead to the lowering of fluidity and decrease in the passive permeability of the membrane to TBT, since straight chain saturated fatty acids have higher melting points (Russell and Fukunaga, 1990; Kaneda, 1991). Increase in saturated fatty acids and tolerance towards the toxic compounds have been observed previously in *Saccharomyces cerevisiae* (Viegas et al., 2005), *Kocuria varians* (Dercova et al., 2004), and filamentous fungus *Cunninghamella elegans* (Bernat and Dlużoński, 2007). Concentrations of saturated PLFAs were also influenced when *B. licheniformis* was grown with TBT (Fig. 2B.3c). However, 6.05 $\mu\text{g l}^{-1}$ of TBT retarded the

production of saturated PLFAs in *B. licheniformis*. A change in PLFA composition due to the increase in the saturated PLFAs of the membrane lipids is a well known response of bacteria to membrane active substances, such as organic solvents (Weber and de Bont, 1996). Similarly, the xenobiotic used in this study is a membrane active substance, which has altered the saturated PLFA composition of the membrane lipids. In *B. pumilus* slight increase in some of the saturated PLFAs was observed when grown at $6.05 \mu\text{g l}^{-1}$ of TBT. However, when TBT concentration increased to $12.1 \mu\text{g l}^{-1}$, PLFA concentration decreased (Fig. 2A.3d).

The ability of the bacterial cultures to grow with these concentrations of TBT by modulating the PLFAs concentrations suggests the resistance of the bacterial cultures to the increasing concentrations of TBT.

**Effect of cultivation period, nutrients and
incubation temperature on the PLFA
concentration and composition of the marine
microalgae**

2C.1. Introduction

Diatoms are one of the important suppliers of organic matter and energy in the marine ecosystems. They constitute a source of essential FAs and food for marine animals. Variations in the environmental factors can lead to the alteration of FA content and changes in membrane viscosity of algae (Becker, 1994; Šajbidor, 1997). For instance, the microalgal FA composition depends not only on the species and strains used in experiments, but also on factors related to culture conditions including composition of the medium, nutrient supply, pH, etc (Yongmanitchai and Ward, 1991; Fidalgo et al., 1998 ; Lourenco et al., 2002), irradiance and light intensity (Thompson et al., 1990; Brown et al., 1996), salinity (Xu and Beardall, 1997) and temperature (James et al., 1989; Zhu et al., 1997; Oliveira et al., 1999; Li et al., 2003). Several studies have reported that growth phase also has a dramatic effect on the FA compositions of some microalgae (Hallegraeff et al., 1991; Brown et al., 1996; Fidalgo et al., 1998; Liang et al., 2002; Mansour et al., 2003). Change in microalgal nutritional quality may be linked not only to diversity but also to the life history of the algal cells (Thompson et al., 1990).

The PLFA composition of the diatom cell membrane can be used as a characteristic of its growth conditions. Membranes of photosynthetic organisms are known to alter their chemical composition to improve species survival under conditions of low temperature (Wise and Naylor, 1987; Gombos et al., 1992; Wada et al., 1994). An increase in the degree of unsaturation in microbial lipids is thought to be one of the responses of the microorganism to maintain fluidity hence maintaining the function of membranes during acclimatization to low temperatures (Harwood and Jones, 1989). The manipulation of the algal growth environment can alter the growth characteristics and chemical composition of cells cultured under the conditions applied (Brown et al., 1989). Among the different components of the culture

medium, the source and concentration of nitrogen and phosphorus can provoke important changes in the growth and biochemical composition of microalgal species (Droop, 1974; Kaplan et al., 1986; Yongmanitchai and Ward, 1991; Levasseur et al., 1993; Fidalgo et al., 1995). Although, effects of these parameters on total fatty acid composition have been widely reported for marine microalgae (Renaud et al., 2002; Mansour et al., 2003), the potential effects of cultivation period, nitrogen, phosphorus and temperature on microalgal PLFA concentration and composition have barely been investigated. The present study was designed to examine the hypothesis that PLFAs can be also affected with changes in the environmental parameters in the diatoms. In this work, we analyzed the PLFA compositions of two species of marine diatoms, *Skeletonema* sp. and *Amphora* sp. The effect of cultivation period, nutrients nitrogen and phosphorus, and temperature on PLFA concentration and composition was evaluated.

2C.2. Material and methods

2C.2.1. Algal growth medium

The algal growth medium used for the study was f/2 (Guillard and Ryther, 1962). The composition of the f/2 medium includes stock solution-I, which consist of NaNO₃ (7.5g), NaH₂PO₄.H₂O (0.5g), Na₂SiO₃ (1.5-3g) in 100 ml distilled water which makes the stock solution I. Stock solution II consists of trace metals CuSO₄.5H₂O (0.98g), ZnSO₄.7H₂O (2.2g), CoCl₂.5H₂O (1g), MnCl₂.4H₂O (18g) and Na₂MoO₄.2H₂O (0.63g). Each of these trace metals were dissolved in separate 100 ml volumes of distilled water and called as IIa. The FeCl₃.6H₂O (3.15g) and Na₂EDTA (4.36g) was taken up in 950 ml distilled water and called as IIb. To the IIb solution 1 ml each of the IIa was added, and now called as trace metal solution (stock solution-II). Stock solution-III consists of vitamins thiamin-HCl (20mg), biotin (1ml, 10mg biotin + 96ml D/W) and Vitamin B12 (0.1ml, 1,µg VB₁₂ + 1ml D/W) dissolved in 100 ml

distilled water. Stock solution-III should be autoclaved and then stored in a freezer. Finally these stock solutions are mixed as follows;

Stock solution-I	1ml
Stock solution-II	1ml
Stock solution-III	0.5ml
Sea water	997.5ml

The pH of the solution is adjusted to 7.4. The culture media are then autoclaved at 120°C and 15 lb pressure for 15 min.

2C.2.2. Cultures

Microalgal cultures *Skeletonema* sp. and *Amphora* sp. were obtained from the microalgae culture collection of the MCMRD laboratory, National Institute of Oceanography, Goa. Stock cultures of *Skeletonema* sp. and *Amphora* sp. were grown and maintained in 100 ml conical flasks containing 20 ml of f/2 medium. Cultures were grown at room temperature ($\pm 28^{\circ}\text{C}$) under 12 h Light: 12 h dark condition. Illumination was provided with cool white fluorescent lights. Cells were grown under three different experimental conditions representing incubation periods, nutrients nitrates and phosphates and incubation temperatures. Each culture was grown in 1L conical flasks containing 200 ml of the f/2 medium (Guillard and Ryther, 1962).

2C.2.3. Effect of cultivation period on PLFA concentration and composition of diatoms

Diatom cultures *Skeletonema* sp. and *Amphora* sp. were grown in f/2 medium at room temperature under 12 h light : 12 h dark regime for 6, 8, 15, 20, 120 and 240 days. Both algal cultures were pre-grown individually in 20 ml of f/2 medium for 8 d as above. This step was repeated for 2 more times and the algal cells thus grown were used as source of inoculum. Twelve 1 L conical flasks containing f/2 medium were sterilized as above and cooled. Six of

these flasks were inoculated with 1 % inoculum of *Skeletonema* cells grown as above. The remaining six flasks were inoculated with 1 % inoculum of *Amphora* cells grown as above. Both the diatom cultures were grown at room temperature as above over a period of 240 d. Cells were sampled on d 6, 8, 15, 20, 120 and 240. After sampling, the diatom cells were harvested by centrifugation at 10,000 rpm at 4° C for 10 min. Cells were collected and washed twice with 1.5 % Nacl and again centrifuged as above. Cells were collected, lyophilized, homogenized and stored at -20°C until analysis. Lipids from diatoms were extracted, fractionated and analyzed on GC and GC/MS following the methods described in chapter 2A.

2C.2.4 Effect of nitrogen concentrations on the PLFA concentration and composition of diatoms

In order to assess the effect of nitrogen concentrations on the PLFA concentration and composition of microalgae, nitrogen concentration in the f/2 medium was varied from 0.2, 0.4, 0.7 and 0.9 mM by adding suitable quantities of NaNO₃ to f/2 medium. The remaining components of the f/2 medium were kept constant as above. The 8, 1L conical flasks containing 200 ml of the f/2 medium and various concentrations of nitrogen as mentioned above were sterilized by autoclaving at 120 °C and 15 lb pressure for 15 min. The starter culture of *Skeletonema* sp. and *Amphora* sp. were prepared as above. Four flasks were inoculated with 1 % of *Skeletonema* cells and the remaining four flasks were inoculated with 1 % of *Amphora* cells and grown for 10 d at room temperature. The algal cells were collected, lyophilized and stored as above.

2C.2.5 Effect of phosphate concentration on the PLFA concentration and composition of diatoms

Total fatty acid concentration increased with age in *Phaeodactylum tricornutum* and some other microalgae (Taguchi et al., 1987; Siron et al., 1989; Fidalgo et al., 1998).

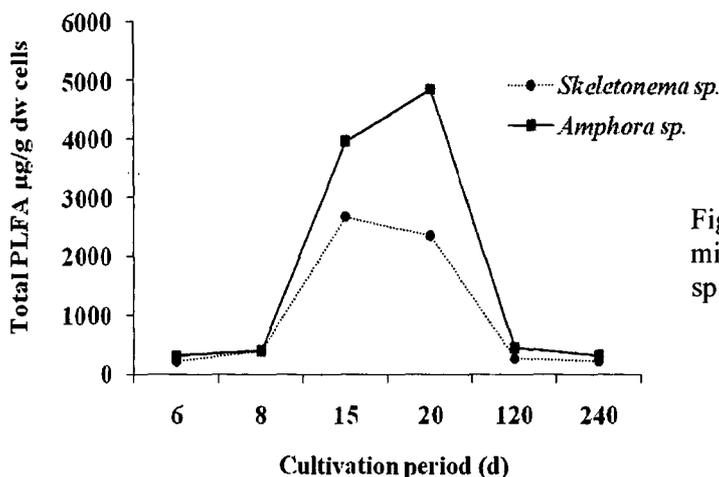


Fig. 2C.1: Total PLFA variation in the microalgae *Skeletonema sp.* and *Amphora sp.* during various periods of incubation.

In the *Skeletonema sp.*, the most abundant PLFAs were C14:0, C16:0, C16:1n7, C18:1n9, C16:2 (9,12-hexadecadienoic acid methyl ester), C18:2n6c, C16:3 (6,9,12-hexadecatrienoic acid methyl ester), C20:4n6 and C20:5n3 (Fig. 2C.2a). PLFAs such as C16 mono- di- and tri-unsaturated were observed in considerable amounts. With the increase in cultivation period the concentrations of these PLFAs increased and reached maximum at d 15 following inoculation. In the diatom *Amphora sp.*, C14:0, C15:0, C16:0, C16:1n7, C18:1n9, C18:2n6c, C20:4n6, C24:0 and C20:5n3 were abundant PLFAs (Fig. 2C.2b). Concentrations of these individual PLFAs also increased with the increase in the cultivation period for the *Amphora sp.* However during 120 d and 240 d the concentration of the individual PLFAs was very low in both the microalgae. Although the concentrations of individual PLFAs changed with the cultivation period, the PLFA composition remained typical of diatoms during all the incubation periods. The major PLFAs present in both the microalgal cultures are in accordance with the major FAs from other marine diatoms (Chriomadha, 1993; Tan and Johns, 1996; Lourenco et al., 2002; Liang, et al., 2002, 2005; Liang and Mai, 2005).

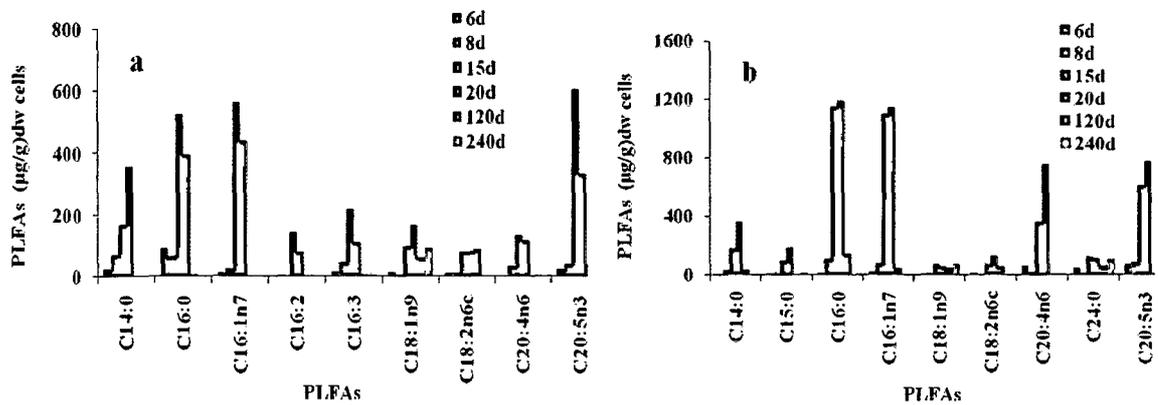


Fig.2C.2. Relative concentrations of PLFA in the microalgae, *Skeletonema* sp. (a) and *Amphora* sp. (b) during various incubation periods.

The biochemical composition of microalgae differs according to the growth phase (Sukenik & Cameli, 1990; Hodgson et al., 1991). Individual PLFAs were grouped based on their degree of saturation, namely, Sat PLFAs, MUFA, PUFA and Unsat PLFAs. The relative percentages (%) of the three classes of PLFAs in the diatoms *Skeletonema* sp. and *Amphora* sp. are shown in Table 2C.1. In the *Skeletonema* sp. highest relative % Sat PLFA (51 %) was observed at d 6 that decreased when incubation period was increased (Table 2C.1). However, with the increase in the cultivation period, % MUFA and % PUFA increased in the *Skeletonema* sp. (Table 2C.1). The ratio of Sat/MUFA and Sat/PUFA decreased from 3.0 (6 d) to 0.05 (120 d), and 2.2 (6 d) to 0.04 (120 d), respectively (Table 2C.1). In general, in the *Skeletonema* sp. with increase in cultivation period, Sat PLFAs decreased while Unsat increased, increasing the Unsat/Sat ratio from 0.8 (6 d) to 43.7 (120 d). Increase in the relative proportions of PUFA was reported previously in dinoflagellate *Gymnodinium catenatum* and other microalgae (Hallegraeff et al., 1991; Shamsudin, 1992). Concentrations of PUFAs in *Ochromonas danica* (Gellerman and Schlenk, 1979), *Phaedactylum tricornerutum* (Yongmanitchai and Ward, 1989) and some *Motierella* species (Shimizu et al., 1989a; Shinmen et al., 1989), increased significantly with the culture age. Incorporation of lower

melting point FAs such as Unsat FAs into cell membrane exerts a fluidizing effect on the membrane (Suutari and Iasko, 1994). Biosynthesis of PUFA takes place mainly during the phase of intense cellular activity and confirms their active part in the photosynthetic processes as was observed in *Phaeodactylum tricoratum* (Siron et al., 1989). The MUFA and PUFA are required in the cell membrane fluidity in order to maintain proper functioning of the cell during active growth of the cells. Hence the increase in the MUFA and PUFA with the increase in cultivation period in *Skeletonema* sp. is understood. Increase in relative % of MUFA was in accordance with previous studies in the other microalgal cultures (Fernandez-Reiriz et al., 1989; Belkoura et al., 2000; Mansour et al., 2003; Liang and Mai, 2005).

In the *Amphora* sp. relative percent of major classes of PLFAs showed interesting trends. In the 6 d old culture of *Amphora*, PUFAs accounted for 56 % while Sat and MUFA contributed 21 % and 7 % of total PLFAs, respectively (Table 2C.1). Similarly, higher concentrations of PUFA were reported in the pre-logarithmic phase culture of *Ellipsoidion* sp. (Xu et al., 2001). Further, Sat and MUFA increased with the increase in the cultivation period in the *Amphora* sp. The increase in Sat resulted in higher ratios of Sat/MUFA and Sat/PUFA (>1) over the period of cultivation except when the cultivation period was extended to 240 d. However, at 240 d of cultivation, MUFA exceeded Sat in the *Amphora* sp. (Table 2C.1). In this culture production of Sat and MUFA increased while that of PUFA reduced over the period of cultivation. Generally Sat FAs increase with the aging of the culture to prevent the cell from dying, while the Unsat FAs are abundant during the initial growth stages (Erwin, 1973). Increase in Sat FAs was associated with reduced PUFA synthesis during aging process (Siron et al., 1989). Further, nutrient deficiency can enhance production of Sat FAs in the aged cultures (Siron et al., 1989). The higher relative % of PUFA during the cultivation period for both the microalgae was also observed (Table 2C.1). PUFAs are likely to be

associated with the photosynthetic membranes of the algae (Sukenik and Carmeli, 1990; Brown et al. 1996; Fidalgo et al., 1998; Zhukova and Aizdaicher, 2001; Tonon et al., 2002). Therefore the presence of PUFA indicates their importance in maintaining the cell membrane function during the ageing process. The differences in the synthesis of relative % of Sat and Unsat PLFAs in both diatom cultures may be ascribed to different microalgal species and their growth conditions (Liang and Mai, 2005).

Table 2C.1: Relative % of saturated (Sat), monounsaturated (MUFA), polyunsaturated (PUFA) and unsaturated (Unsat) PLFAs and Ratios of saturated PLFAs with monounsaturated and polyunsaturated (Sat/MUFA and Sat/PUFA), MUFA/PUFA and unsaturated/saturated (Unsat/Sat) during various periods of cultivation in the *Skeletonema* sp. and *Amphora* sp.

Algae	Incubation period (d)						Algae	Incubation period (d)					
	6	8	15	20	120	240		6	8	15	20	120	240
<i>Skeletonema</i> sp.							<i>Amphora</i> sp.						
PLFAs (%)							PLFAs (%)						
%Sat	51	36	27	34	2	0	%Sat	21	39	39	39	53	35
%MUFA	17	18	26	28	45	63	%MUFA	7	24	31	26	29	36
%PUFA	24	42	48	33	51	27	%PUFA	56	32	29	35	18	25
%Unsat	41	60	74	61	96	90	%Unsat	64	56	60	61	47	61
PLFA ratios							PLFA ratios						
Sat/MUFA	3.0	2.0	1.0	1.2	0.05	0.0	Sat/MUFA	2.8	1.7	1.3	1.5	1.8	1.0
Sat/PUFA	2.2	0.8	0.6	1.0	0.04	0.0	Sat/PUFA	0.4	1.2	1.4	1.1	3.0	1.4
MUFA/PUFA	0.7	0.4	0.5	0.8	0.9	2.4	MUFA/PUFA	0.1	0.7	1.1	0.7	1.6	1.5
Unsat/Sat	0.8	1.7	2.8	1.8	43.7	-	Unsat/Sat	3.1	1.4	1.5	1.6	0.9	1.7

2C.3.2 Effect of nutrients

In both the diatoms, total PLFAs increased with increase in nutrients (Fig. 2C.3). With increase in nitrogen concentrations, total PLFAs also increased and varied from 572 to 1388 $\mu\text{g g}^{-1}$, and 746 to 1638 $\mu\text{g g}^{-1}$ dw in *Skeletonema* sp. and *Amphora* sp., respectively (Fig. 2C.3a). Similarly, with increase in phosphorus concentrations, total PLFAs also increased and varied from 792 to 1388 $\mu\text{g g}^{-1}$, and 599 to 1638 $\mu\text{g g}^{-1}$ dw cells of *Skeletonema* sp. and *Amphora* sp., respectively (Fig. 2C.3b).

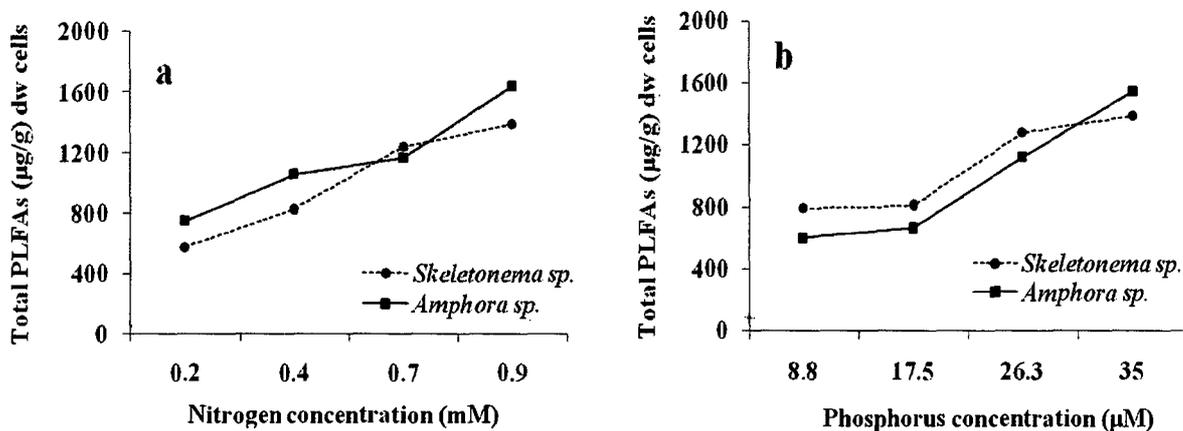


Fig.2C.3: Total PLFA variation in the microalgae *Skeletonema sp.* and *Amphora sp.* during variations in the nitrogen as NO₃-N (a) and phosphorus as PO₄-P (b) concentrations.

Nutrient deficiency can influence the chemical composition of algal cells (Droop, 1974). Both quantity and quality of microalgal lipid fractions appear to vary dramatically with nutrient status (Harrison *et al.*, 1977; Schiffrin & Chishohn, 1981; Enright *et al.*, 1986b). There are also differences among species of the same genera, and such variation in lipid composition may in some cases be of taxonomical interest (Kates & Volcani, 1966; Chuecas & Riley, 1969; Schiffrin & Chisholm, 1981). There are differences in concentrations in both the microalgae studied, however both the cultures showed increase in the production of total PLFAs. In Green algae *Chlorella vulgaris* and *Scenedesmus obliquus* and blue-green algae *Microcystis aeruginos* and *Spirulina platensis* similarly polar lipid fractions increased with increasing nitrogen concentrations (300 to 600 µmol N l⁻¹) (Piorreck *et al.*, 1984). This is because nitrogen as well as phosphorus participates in the elaboration of phospholipids (Tornabene *et al.*, 1983; Piorreck *et al.*, 1984; Suen *et al.*, 1987). In contrast, other studies did not find any appreciable effect on lipid content with respect to nitrogen (Richardson *et al.*, 1969). However, in those studies the nitrogen concentrations used were very high (3000 to 20000 µmol N l⁻¹).

Concentrations of individual PLFAs in *Skeletonema* sp. increased with the increase in nitrogen and phosphorus concentrations. This was particularly evident for the increase in PLFA C14:0, C16:0 and C16:1. However, when nutrient concentrations further increased (0.9mM), concentrations of these PLFAs decreased (Fig. 2C.4a & b). At highest nitrogen concentration (0.9mM) PUFA such as C18:3, C20:4 and C20:5 showed greater concentrations. Phosphorus concentrations had little effect on C14:0 and C16:0 PLFAs. However, C20:5 increased with increasing phosphorus concentration. The abundance of PUFA at various concentrations of nutrients suggest that these fatty acids are required in the cell membrane to provide the cell fluidity.

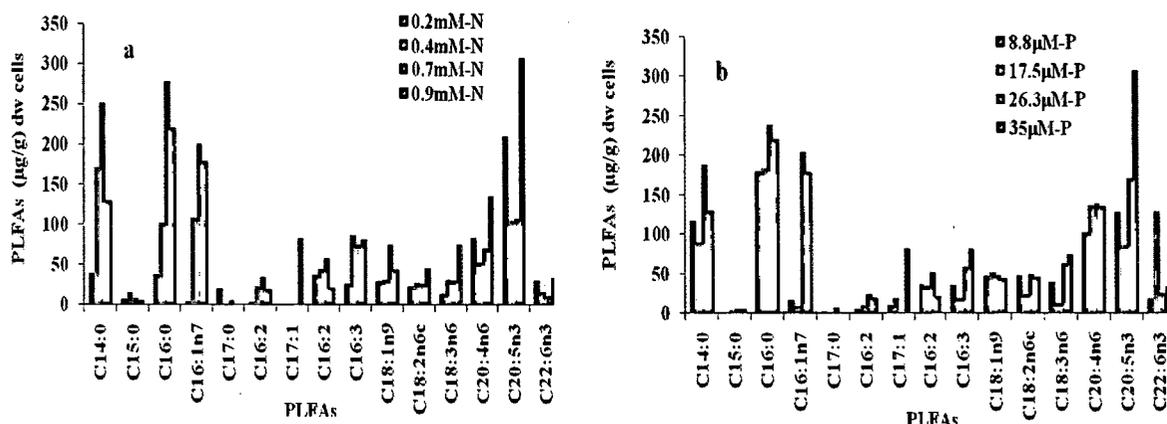


Fig.2C.4. Relative concentrations of PLFA in the microalgae, *Skeletonema* sp. during variation in nitrogen (a) and phosphorus (b) concentrations.

In *Amphora* sp. concentration of C16:1 were low when concentration of nitrogen and phosphorus were low, while C16:0 and C20:5 were comparatively higher (Fig. 2C.5a & b). Basically, at highest concentration of nitrogen and phosphorus individual PLFAs concentration were high in *Amphora* sp. suggesting such high concentrations of nutrients are required for the proper production of these important PLFAs in the diatoms. It is suggested that the source and concentration of nitrogen can in particular cause important changes in the

growth character and biochemical composition of microalgae (Utting, 1985; Fidago et al., 1995).

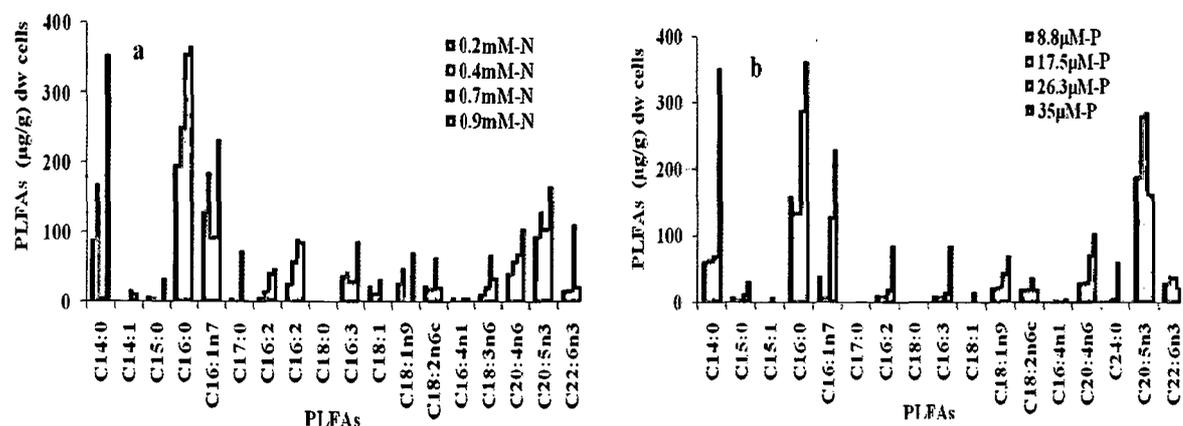


Fig.2C.5. Relative concentrations of PLFA in the microalgae, *Amphora* sp. during variation in nitrogen (a) and phosphorus (b) concentrations.

The relative % of Sat, MUFA and PUFA varied with increasing concentrations of nutrients (Table 2C.2). Relative % of Sat increased (19 to 44 %) with increase in nitrogen concentration (0.2 to 0.7M), in *Skeletonema* sp. In contrast, relative % of saturated PLFAs decreased (38 to 26 %) with increase in phosphorus concentration (8.8 to 35µM) (Table 2C.2). On the other hand, % MUFA increased with increase in nitrogen and phosphorus concentration (Table 2C.2). The concentration of N in the medium has been reported to affect the proportion of Sat and Unsat FAs (Yongmanitchai & Ward, 1991; Molina & Sánchez, 1992; Xu et al. 2001). Proportions of PUFA were relatively higher during various nutrient concentrations in *Skeletonema* sp. The ratio of Sat/MUFA decreased (3.1 to 1.2) due to simultaneous increase in MUFA, while ratio of Sat/PUFA increased (0.2 to 1.3) due to increase in saturated fatty acids. Relative % increase in MUFA while % decrease in PUFA in *Skeletonema* sp. during growth with increasing concentration of nitrogen, resulted in increase in MUFA/PUFA ratio (Table 2C.2). In general, with the increase in the nitrogen concentration in the growth medium Unsat PLFAs decreased while Sat increased, resulting in decreased

Unsat/Sat ratio (4.4 to 1.3) in *Skeletonema* sp. (Table 2C.2). However, the Unsat and specifically the PUFA remained relatively higher to Sat PLFAs at all concentrations of nutrients. Nitrogen and phosphorus are involved in the elaboration of phospholipids. This suggests that the biosynthesis of PUFAs was not disturbed with various concentrations of nutrients. Higher concentration of PUFA was observed at 0.2mM-N for *Seletonema* sp. Similarly, *Dunaliella bardawil* and *D. salina* produced a higher percentage of PUFA under N deficiency (Ben-Amotz et al., 1985).

Relative percent of major classes of PLFAs showed interesting trends in *Amphora* sp. MUFA decreased with increasing concentration of nitrogen (24 to 19 %), while it increased (5 to 19 %) with increasing concentration of phosphorus (17.5 μ M to 35 μ M) in *Amphora* sp. Relative % of Sat PLFAs increased with increasing nitrogen and phosphorus concentrations. The higher concentrations of PLFAs may increase the fluidity of membrane. This may result in increased proportions of Sat PLFAs, which decreases the fluidity of membrane. Generally, Sat/MUFA (1.6 to 3) and Sat/PUFA (1.1 to 1.3) increased while MUFA/PUFA (0.7 to 0.5) and Unsat/Sat (1.5 to 1.2) decreased with increase in nitrogen concentrations (0.2 to 0.7mM) in the growth medium. This shows that higher concentrations of nutrients cause the cell membrane to accumulate more saturated fatty acids and MUFA in the cell membrane.

Under nitrogen stress, *Botryococcus braunii*, *Dunaliella bardawil* and *D. salina* produce a higher percentage of EPA (Ben-Amotz et al., 1985). In *Skeletonema* sp. similarly at lower nitrogen concentration relative % of PUFA were higher. In contrast, the proportion of PUFAs in the fresh water algae *Scenedesmus* and *Chlorella* increased at high nitrogen concentrations (Weete, 1980). However, although there was increase in the PUFA content in *Amphora* sp., the relative % PUFA remained > 35% at all nutrient concentrations. PUFAs were generally higher as compared to MUFA and Sat at most of the nutrient concentration

used. Phosphate concentration in the medium in the range of 0.05 to 0.5 g l⁻¹ ~ had little effect on biomass production of *Phaedactylum tricornutum* UTEX 640, but maximum EPA content was obtained at phosphate levels of 0.1 to 0.5 g l⁻¹ (Yongmanitchai and Ward, 1991). The relatively higher production of PUFA at all the concentrations of nutrients suggest that the algal cultures were in good physiological state and the mechanism of photosynthesis was not limited.

Table 2C.2: Relative % of saturated (Sat), monounsaturated (MUFA) and polyunsaturated PLFAs (PUFA) and ratios of saturated PLFAs with monounsaturated and polyunsaturated (Sat/MUFA and Sat/PUFA), MUFA/PUFA and unsaturated/saturated (Unsat/Sat) during variations of nitrogen and phosphorus concentrations in the *Skeletonema* sp. and *Amphora* sp.

NO3-N (mM)	0.2	0.4	0.7	0.9	PO4-P (µM)	8.8	17.5	26.3	35
<i>Skeletonema</i> sp.					<i>Skeletonema</i> sp.				
PLFAs (%)					PLFAs (%)				
%Sat	19	35	44	26	%Sat	38	34	34	26
%MUFA	6	17	22	22	%MUFA	10	10	20	22
%PUFA	75	48	34	52	%PUFA	53	56	46	52
%Unsat	81	65	56	74	%Unsat	62	66	66	74
PLFA ratio					PLFA ratio				
Sat/MUFA	3.1	2.1	2.0	1.2	Sat/MUFA	4.0	3.4	1.7	1.2
Sat/PUFA	0.2	0.7	1.3	0.5	Sat/PUFA	0.7	0.6	0.8	0.5
MUFA/PUFA	0.1	0.4	0.7	0.4	MUFA/PUFA	0.2	0.2	0.4	0.4
Unsat/Sat	4.4	1.8	1.3	2.9	Unsat/Sat	1.6	1.9	1.9	2.9
<i>Amphora</i> sp.					<i>Amphora</i> sp.				
PLFAs (%)					PLFAs (%)				
%Sat	40	41	37	46	%Sat	39	33	39	49
%MUFA	24	24	12	19	%MUFA	11	5	17	19
%PUFA	36	35	51	35	%PUFA	50	62	43	32
%Unsat	60	59	63	54	%Unsat	61	67	60	51
PLFA ratio					PLFA ratio				
Sat/MUFA	1.6	1.7	3.0	2.4	Sat/MUFA	3.7	6.1	2.3	2.5
Sat/PUFA	1.1	1.2	0.7	1.3	Sat/PUFA	0.8	0.5	0.9	1.5
MUFA/PUFA	0.7	0.7	0.2	0.5	MUFA/PUFA	0.2	0.1	0.4	0.6
Unsat/Sat	1.5	1.4	1.7	1.2	Unsat/Sat	1.5	2.0	1.5	1.0

2C.3.3. Effect of incubation temperature

With increase in incubation temperature total PLFA production decreased gradually in the *Skeletonema* sp., while in *Amphora* sp. it increased till 30°C and then decreased at 38°C (Fig. 2C.6). Microalgal chemical composition is influenced by temperature. For example,

when grown at high temperature lipid content decreased in some species (Thompson et al., 1992a; Renaud et al., 1995). Similarly, in the present study, total PLFA concentrations decreased at higher temperature in *Skeletonema* sp. and *Amphora* sp. Decrease in lipid production at relatively higher growth temperatures was observed in microalgae *Chaetoceros* sp., *Rhodomonas* sp., prymnesiophyte NT19, *Chaetoceros calcitrans*, *C. simplex* and *Nitzschia* spp. (Thompson et al., 1992a; Renaud et al., 1995; Renaud et al., 2002). However, other studies have showed increased microalgal lipid production at high growth temperatures for several microalgal species (Aaronson, 1973; Tomaselli et al., 1988; Thompson et al., 1992a; Oliveira et al., 1999). In contrast, several other microalgae *Rhodomonas* sp. and *Isochrysis* sp. followed non-linear trends, with maximum lipid production at 27 °C (Renaud et al., 2002). Further, reduction in lipid production at extremes of low and high temperature has also been reported previously (Opote, 1974; Thompson et al., 1992a; Renaud et al., 1995). High temperature effect was due to cessation of growth at extreme temperatures by irreversible damage to plant enzymes (Opote, 1974).

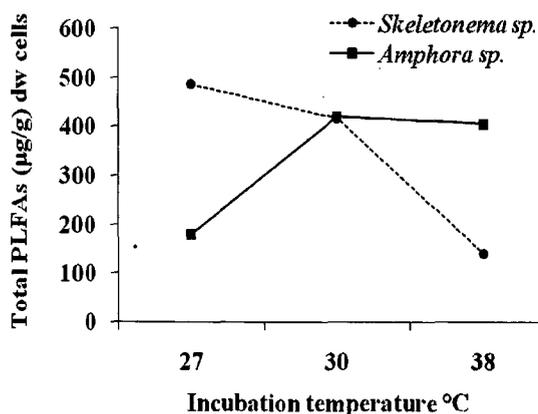


Fig.2C.6: Total PLFA variation in the microalgae *Skeletonema* sp. and *Amphora* sp. during during incubation with different temperatures.

The chemical composition of microalgae can be also influenced by temperature changes (Tomaselli et al., 1988; James et al., 1989; Thompson et al., 1992a, b; Oliveira et al., 1999; Renaud et al., 2002). In the *Skeletonema* sp. the major PLFAs C14:0, C16:0, C16:1,

C18:1 and C20:5 were higher in concentration at lower temperature (27°C) (Fig. 2C.7a). However, at 38°C all the individual PLFAs were low in concentration (Fig. 2C.7a). In *Amphora* sp. reverse was seen with these PLFAs. The concentrations of these PLFAs were higher at 30 and 38°C (Fig. 2C.7b).

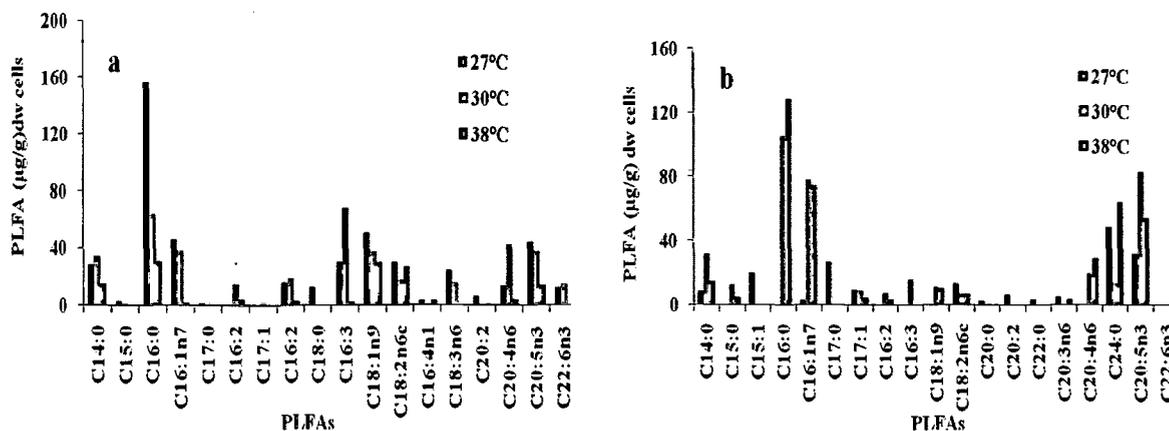


Fig.2C.7. Composition and concentrations of PLFA in the microalgae, *Skeletonema* sp. (a) and *Amphora* sp. (b) during incubation with different temperatures.

The relative percent of the three classes of PLFAs is shown in Table 2C.3. In *Skeletonema* sp. relative % of Sat were highest at 27 °C followed by 38 °C. MUFA were relatively higher at 38 °C, while PUFA and total Unsat were more at 30 °C. The ratio of Sat/MUFA and Sat/PUFA and MUFA/PUFA decreased from 27 °C to 30 °C, however at 38 °C all these ratios showed increase in *Skeletonema* sp. Unsat/Sat ratio was however highest at 30 °C suggesting higher production of unsaturated PLFAs at this temperature (Table 2C.3). The relative percent of the three classes of PLFAs in *Amphora* sp. is shown in Table 2C.3. The trend was similar to *Skeletonema* sp. for relative % of Sat PLFAs, wherein the highest concentration was observed at 27 and 38 °C, respectively. MUFA, PUFA and Unsat PLFAs % increased with increasing incubation temperature but decreased at 38°C in *Amphora* sp. The resulting ratio of Sat/MUFA and Sat/PUFA was higher at 27 and 38 °C, respectively while lower at 30 °C. MUFA/PUFA and Unsat/Sat ratio increased, however Unsat/Sat

decreased at 38 °C in *Amphora* sp., suggesting increase in Sat PLFAs at higher incubation temperature. The decrease in the percentages of the total PUFAs at higher growth temperatures in *Skeletonema* sp. and *Amphora* sp. supports the findings of previous studies (Teshima et al., 1983; Mortensen et al., 1988; Renaud et al., 1995; Renaud et al., 2002). In *Amphora* sp. higher growth temperatures have been shown to favour the formation of saturated fatty acids, which is seen in many species of marine microalgae (Ackman et al., 1968; Materassi et al., 1980; Mortensen et al., 1988; Thompson et al., 1992b; Renaud et al., 1995). This effect is influenced by changes in the fluidity of cell membrane phospholipid layers depending on the degree of fatty acid unsaturation (Harwood, 1988; Sargent et al., 1989), with increased fluidity associated with increase in the number of double bonds. The increase in Sat PLFAs suggests the decrease in the fluidity of cells at higher temperature. These effects are indicative of interruptions to the biosynthetic processes of chain elongation and desaturation at higher temperatures.

Table 2C.3: Relative % of saturated (Sat), monounsaturated (MUFA), polyunsaturated (PUFA) and unsaturated PLFAs (Unsat) and ratios of saturated PLFAs with monounsaturated and polyunsaturated (Sat/MUFA and Sat/PUFA), MUFA/PUFA and unsaturated/saturated (Unsat/Sat) during incubation with different temperatures in the *Skeletonema* sp. and *Amphora* sp.

Algae	Incubation temperature (°C)			Algae	Incubation temperature (°C)		
	27	30	38		27	30	38
<i>Skeletonema sp.</i>				<i>Amphora sp.</i>			
PLFAs (%)				PLFAs (%)			
%Sat	42	24	33	%Sat	50	41	53
%MUFA	20	19	23	%MUFA	18	25	22
%PUFA	38	56	44	%PUFA	32	34	25
%Unsat	58	76	67	%Unsat	50	59	47
PLFA ratio				PLFA ratio			
Sat/MUFA	2.1	1.3	1.4	Sat/MUFA	2.8	1.7	2.4
Sat/PUFA	1.1	0.4	0.8	Sat/PUFA	1.6	1.2	2.1
MUFA/PUFA	0.5	0.3	0.5	MUFA/PUFA	0.6	0.7	0.9
Unsat/Sat	1.4	3.1	2.0	Unsat/Sat	1.0	1.4	0.9

Temperature has a major effect on the types of FAs synthesized in microalgae (Ackman et al., 1968; Satu and Murata, 1980; Thompson et al., 1992b). Microalgal species have a tendency to respond to decreasing growth temperature by increasing the ratio of Unsat to Sat FAs (Ackman et al., 1968; Mortensen et al., 1988; James et al., 1989; Thompson et al., 1992b; Renaud et al., 1995; Oliveira et al., 1999). It was suggested that temperature above 30 °C to 33 °C results in disruption of cell metabolism and death of the cells (Richmond, 1986; Renaud et al., 2002). Ratio of total Unsat/Sat PLFAs in *Skeletonema* sp. was ≥ 1.4 while in *Amphora* sp., this ratio was < 1.4 at the temperature tested for the study. However, response to growth temperature can vary from species to species, with no overall consistent relationship between temperature and FA unsaturation (James et al., 1989; Thompson et al., 1992b; Renaud et al., 1995). This shows that the temperature variation used in the present experiment was unable to modify the ratio drastically in *Skeletonema* sp. (Table 2C.3). Previous studies by Renaud et al. (2002) on the diatom *Chaetoceros* sp. showed no significant difference in the total PUFA over the range of growth temperatures tested (25 to 35 °C). Our results suggest that fatty acids with high degree of unsaturation are required at most of the temperatures studied to maintain the flexibility and permeability of membrane phospholipid layers for proper functioning of the cells in the present cultures.

**Sources of organic matter and microbial
community structure in the sediments of the
Visakhapatnam harbour, east coast of India
and Mandovi-Zuari estuaries, west coast of
India**

3.1. Introduction

Organic matter (OM) in the coastal and estuarine sediments is derived from various sources including phytoplankton, benthic microalgae, terrestrial plants, river runoff, atmosphere, municipal sewage, industrial discharge, and other anthropogenic sources (Hedges and Keil, 1995; Hedges et al., 1997; Wu et al., 2004). Due to the complex nature of OM in such sediments, a variety of approaches including bulk elemental carbon analysis, carbon isotope composition ($\delta^{13}\text{C}_{\text{oc}}$), and lipid biomarkers (Zimmerman and Canuel 2001; Bouillon et al., 2008) have been used to determine their sources. Stable carbon isotope ($\delta^{13}\text{C}_{\text{oc}}$) signatures of the various carbon sources are often different, and despite some overlap between some sources, they are powerful tracers of carbon inputs in various environments (Fry and Sherr, 1984; Prahl et al., 1994; Meyers, 1994; Schelske and Hodell, 1995). Geochemical biomarkers such as *n*-alkanes, alcohols, sterols, alkenones, and fatty acids also are often used to identify the carbon inputs from various organisms in marine environments (Volkman et al., 1992; Tolosa et al., 2003; Wu et al., 2004). However, these geochemical biomarkers and stable carbon isotopes do not differentiate between dead and live organisms.

In marine environment, living biomass consists of a complex mixture of prokaryotes and eukaryotes (Boschker et al., 2001). The microbiota of estuarine and marine sediments comprise of complex communities which perform important functions in aquatic ecosystems including the decomposition of organic matter, recycling of nutrients, and serve as a major food source to benthic organisms (Kemp 1990). Bacteria play a key role in the ecosystem as degraders and scavengers of the different fractions of OM (Boschker et al., 2001). The nature and sources of OM, nutrient status and pollution may influence the microbial community structure (Guckert et al., 1986; Bååth et al., 1995; Pennanen, 2001).

In the nature, more than 90% of indigenous microorganisms escape cultivation using the traditional culture-dependent techniques (DeLong and Pace, 2001). Hence the traditional techniques of isolation and culturing have not been adequate for characterization of microorganisms in environmental samples, especially in evaluating the natural microbial diversity (Fang et al., 2000; Delong and Pace, 2001). Thus to detect changes in bacterial biomass and natural microbial communities, culture-independent techniques such as phospholipid fatty acid (PLFA) analysis (White et al., 1979; Pennanen et al., 1996; Pinkart et al., 2002; Petsch et al., 2003), and genetic fingerprinting (Polymenakou et al., 2005), are routinely used. PLFAs can be used to assess freshly biosynthesized material and individual PLFAs are used to distinguish inputs from bacteria, phytoplankton, zooplankton and other eukaryotes (White et al., 1979; Volkman et al., 1998).

PLFAs are the central component of the microbial cell membranes. They are present in reasonably constant amounts over a wide range of growth conditions and are rapidly hydrolyzed upon cell death (White et al., 1979; Russell and Nichols, 1999; Fang et al., 2000; Boschker and Middelburg, 2002). Furthermore, these compounds can be extracted from sediments easily and quantitatively (White et al., 1979; Balkwill et al., 1998). Hence, cellular membrane PLFAs represent a quantitative and sensitive tool to determine the living microbial biomass (White et al., 1979; Ringelberg et al., 1997; Fang et al., 2000), changes in microbial community composition (Volkman et al., 1980; Pinkart et al 2002; Widenfalk et al., 2008), nutritional status of microbial community (Guckert et al., 1986; Kieft et al., 1994), general distribution of microorganisms with different metabolic activities (Kaneda, 1991), heavy metal-contaminated soils (Bååth et al., 1995; Pennanen, 2001), and environmental stress (Fang et al., 2004).

The Visakhapatnam harbour located along the east coast of India is a natural harbour and is extensively used for various operations. It includes areas differentially impacted by anthropogenic activities (Subba Rao and Venkateswara, 1980; Sarma et al., 1996). It receives large inputs of OM from terrestrial, marine, and anthropogenic sources, and potentially sequesters a significant fraction of OC in its bed (Ganapati and Raman, 1973; Raman, 1995). Several reports are available on hydrography (Varadarajulu and Khadar, 1976) and various aspects of pollution in the harbour (Subba Rao and Venkateswara, 1980; Sarma et al., 1996). Crude oil, iron ore and sulfur dust spilled during loading and unloading operations further influence the physico-chemical conditions of the water in different channels of the harbour. The key factors controlling input, transfer and storage of OM depend on tides, urban and industrial runoff, inadequate flushing and stagnant conditions (Sarma et al., 1982; Kadam and Bhangale, 1993). The effect of pollution stress is greatly seen in the biotic rather than the abiotic components of the harbour.

Mandovi and Zuari estuaries are situated on the west coast of India. These estuaries are well characterized for physical, chemical and biological aspects (Devassy, 1983; Shetye et al., 1995; Alagarsamy, 2006). These estuaries are tropical in nature and experience seasonal changes in climate and physical oceanographic processes. Estuaries and coastal regions receive maximal rainfall and river derived material during the southwest (SW) monsoon (June–September). The river flow during this season is intense and is counteracted by the strong westerly winds, wind-induced waves and currents, and tides. Whereas, during post monsoon (October to May) due to negligible rainfall terrigenous sediment discharge is low, winds are moderate and dominated by the sea breeze cycle and currents are tidally dominated. Saline waters occur several kilometers upstream from the river mouth during the dry season (Shetye et al., 1995). The industrial and mining activities are at a peak during October–May at

several points along the estuaries and discharge nutrients, heavy metals, sewage and other pollutants in the form of organic and inorganic industrial waste into the estuaries (Alagarsamy, 2006; Ramaiah et al., 2007). The human interference, hence can affect the flora and fauna thereby perturbing the health of the estuaries.

The aims of the present study were to 1) investigate the distribution of OM, 2) identify the sources of OM, 3) assess the live microbial biomass, and 4) evaluate the composition of microbial groups in the sediments of the Visakhapatnam harbour, the Mandovi and the Zuari estuaries.

3.2. Materials and methods

3.2.1. Description of the study areas

Visakhapatnam harbour is located at 17°41'825"N to 17°42'313"N and 83°16'881"E to 83°19'793"E, along the east coast of India (Fig. 3.1) at the mouth of river Meghadrigedda. The existence of this natural harbour has transformed the village once called as 'Vizag' into one of the fastest growing industrial cities of the world. It comprises of an inner harbour and outer harbour. There are 24 berths and 4 moorings to accommodate 28 ships at a time. Major industries like Visakhapatnam steel plant, HPCL oil refinery and fertilizer complex are setup near this port. Initially this port was used as a sea outlet for manganese ore. Subsequently, other bulk items such as iron ore, coking coal and oil products were handled by this natural harbour. During the year 2008, the harbour handled ~65 million tons of cargo.

Mandovi estuary is located at 15°35' N and 73°47' E, whereas Zuari estuary is located at 15°25' N and 73°47' E along the west coast of India (Fig. 3.2). Both these estuaries are interconnected by a canal called Cumbarjua canal. These estuaries are the life lines for the coastal population of the state of Goa, and also act as the nursery grounds for the marine fish eggs and larvae to spend time. Both the estuaries are banked by mangrove vegetation. Mandovi and

Zuari estuaries are used extensively for navigation and for the transport of iron and manganese ores.

3.2.2. Cleaning of glassware and purification of solvents

Glassware used for lipid analysis was cleaned as given in Chapter 2A.

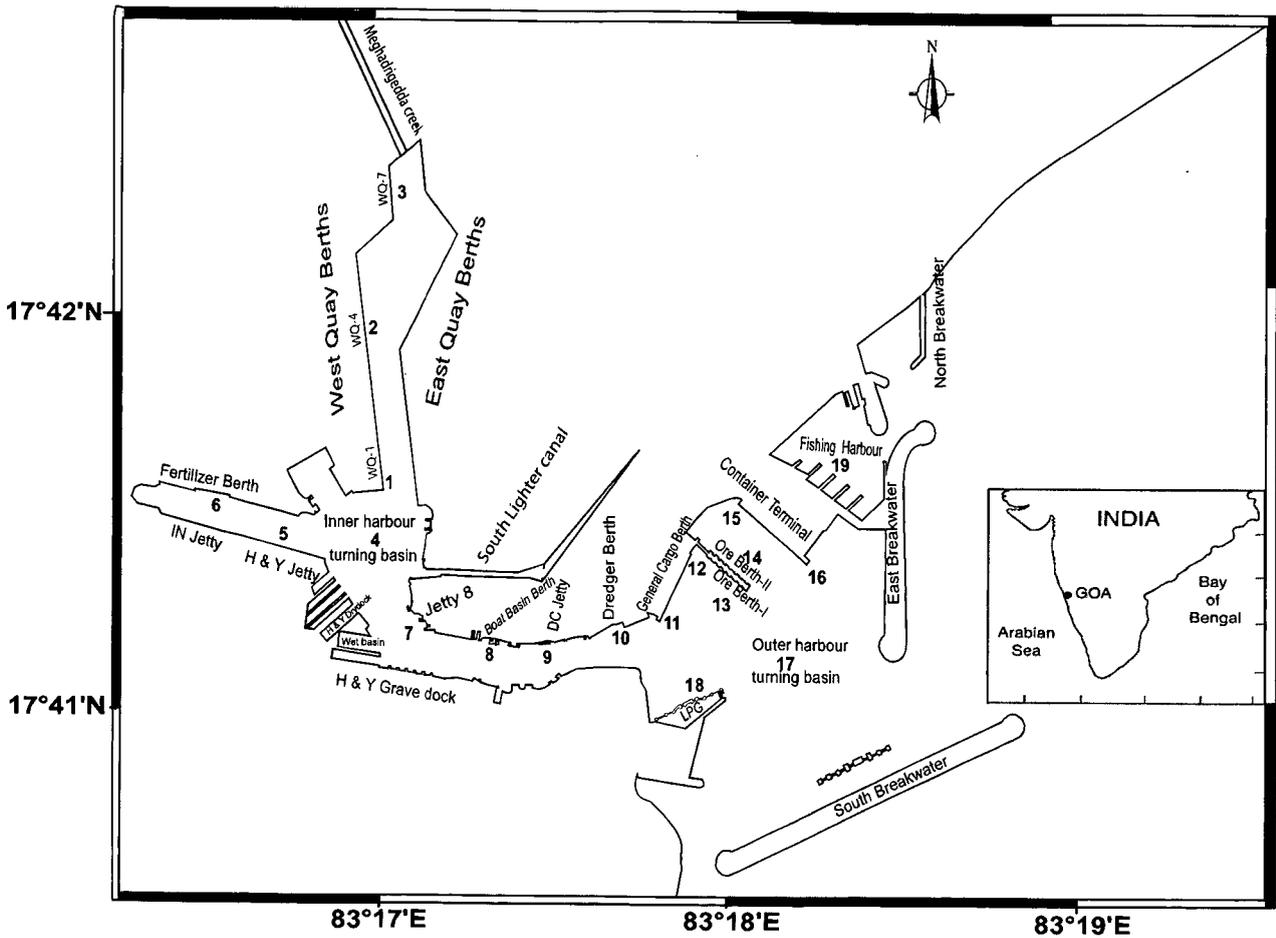


Fig. 3.1. Map of the study area showing the sediment sampling stations in the Visakhapatnam harbour, east coast of India.

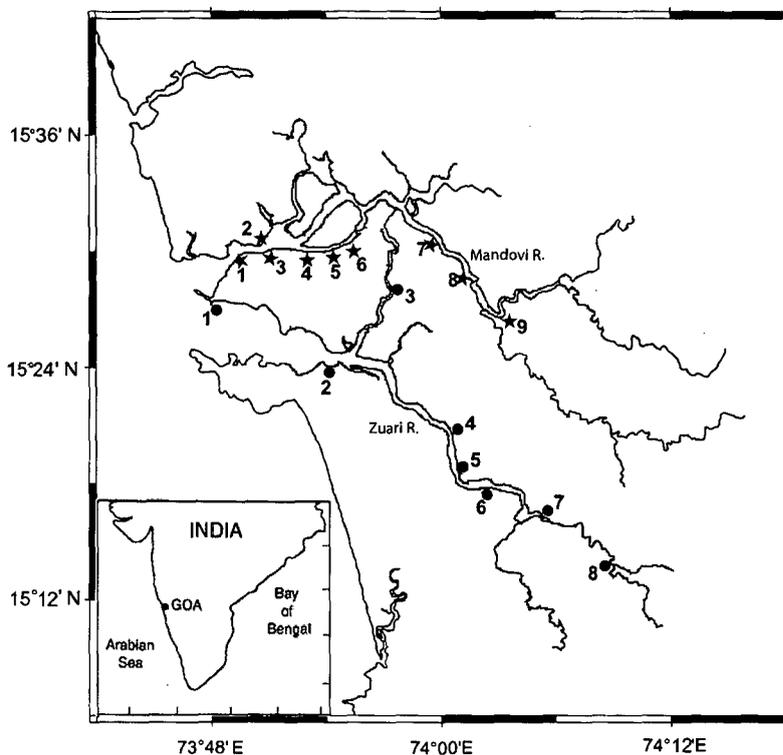


Fig. 3.2. Map showing the sediment sampling stations in the Mandovi and the Zuari estuaries. Solid star: sampling stations in the Mandovi estuary; Solid circle: sampling stations in the Zuari estuary.

3.2.3. Sample collection and analysis

Surficial sediment samples (top 10-15 cm) were collected from 19 stations (#) in the Visakhapatnam harbour (Fig. 3.1 and Table 3.1), 9 stations in the Mandovi estuary and 8 stations in the Zuari estuary (Fig. 3.2 and Table 3.2) using a Van veen grab. The sediment samples were frozen onboard and were transported to the laboratory in an ice box, and stored at $-20\text{ }^{\circ}\text{C}$. The sediments were then lyophilized, homogenized using an agate mortar and pestle to a fine powder and stored in clean vials at $-20\text{ }^{\circ}\text{C}$. The sediments were used for the estimation of OC, $\delta^{13}\text{C}_{\text{oc}}$, total lipids, and PLFAs.

3.2.4. Estimation of bulk geochemical parameters

Subsamples of sediments (100 mg) for OC and $\delta^{13}\text{C}_{\text{oc}}$ content were acidified with HCl (1N) to remove carbonates. Traces of HCl were removed by washing the sediments several

times with distilled water. The sediments were then used for bulk OC and $\delta^{13}\text{C}_{\text{oc}}$ analysis. Concentrations of OC were determined by combusting preweighed samples in a CNS elemental analyzer (NCS 2500, CE instruments). Calibration was carried out using 2, 5-Bis-(5-tetrabutyl-benzoxazol-2-yl) thiopen (BBOT) as a standard. $\delta^{13}\text{C}_{\text{oc}}$ of the sediment sample was performed with the Thermo Finnigan Flash1112 elemental analyzer, linked with a Thermo Finnigan Delta V plus Isotope Ratio MS. The overall analytical precision for replicate samples was within $\pm 1.6\%$ for OC and $\pm 0.2\%$ for $\delta^{13}\text{C}_{\text{oc}}$.

All isotopic compositions are reported as, per-mil (‰) relative to variation (δ) from the PDB standard.

$$\delta^{13}\text{C}_{\text{oc}} = \left\{ \left(\frac{^{13}\text{C}/^{12}\text{C}}{^{13}\text{C}/^{12}\text{C}} \right)_{\text{Sample}} / \left(\frac{^{13}\text{C}/^{12}\text{C}}{^{13}\text{C}/^{12}\text{C}} \right)_{\text{PDB}} - 1 \right\} \times 1000$$

3.2.5. Estimation of terrestrial carbon

To estimate the relative proportions of terrestrial and marine carbon in sediments, a simple two end-member mixing model was employed (Schlunz et al., 1999; Hu et al., 2006).

The following equation was used

$$\text{TC} (\%) = \frac{\delta^{13}\text{C}_{\text{marine}} - \delta^{13}\text{C}_{\text{sample}}}{\delta^{13}\text{C}_{\text{marine}} - \delta^{13}\text{C}_{\text{terrestrial}}} \times 100$$

Where, TC is the terrestrial carbon, $\delta^{13}\text{C}_{\text{sample}}$ is the measured $\delta^{13}\text{C}$ of the sample, $\delta^{13}\text{C}_{\text{marine}}$ is the marine end member (-20.5 ‰, average of $\delta^{13}\text{C}_{\text{oc}}$ -19 to -22 ‰ of marine organic matter, Fry and Sherr, 1984), $\delta^{13}\text{C}_{\text{terrestrial}}$ is the terrestrial end member (most depleted $\delta^{13}\text{C}$ value (-29.32 ‰, from Table 3.1 for the Visakhapatnam harbour and -27.0 ‰ from Table 3.2 for the Mandovi and the Zuari estuaries).

3.2.6. Extraction of lipids

Lipids from the lyophilized sediments were extracted following the modified method of Bligh and Dyer (1959). In brief sediments were shaken vigorously with chloroform: methanol: phosphate buffer (1:2:0.8, V/V/V) and kept standing for 24 h. Separation of the

organic phase was achieved after adjusting the ratio of chloroform: methanol: water to 1:1:0.9 in a separating funnel. The total lipid extracts were pooled, filtered through Whatman No. 1 filter paper, and treated with anhydrous sodium sulphate to remove traces of water. The extract was rotary evaporated at 30 °C under vacuum and made to a known volume (1 ml for Visakhapatnam harbour and 500µl for Mandovi and Zuari estuary). A known aliquot of the extract was used to determine total lipid content (Zaghden et al., 2005; Harji et al., 2008). Lipid extract (20µl) was applied onto the preweighed piece of the Whatman filter paper. The paper was dried in vacuum desiccator and weighed. The weight of the empty Whatman paper was subtracted from the weight of Whatman paper plus solvent extract to get the weight of the total lipids (Harji et al., 2008). The method showed a standard deviation of $\pm 5.3 \%$, based on replicate analyses.

3.2.7. Separation of PLFAs

Separation of PLFAs was done as described in chapter 2A.

3.2.8. Preparation of Fatty acid methyl esters

Fatty acid methyl esters were prepared following the method described in chapter 2A.

3.2.9. Capillary GC and GC-MS analysis

GC and GC-MS analysis was performed as given in chapter 2A.

3.2.10. Statistical analysis

A simple regression analysis was carried out to assess the relationship between different parameters using Microsoft Excel program installed in a personal computer (Sokal and Rohlf, 1981). Stations #2, #10 and #15 of the Visakhapatnam harbour and the station #9 of the Mandovi estuary were excluded from statistical analysis because OC data were not available for these stations. Similarly, the station #8 of the Zuari estuary also was excluded since total lipid data was not available for this station.

Principal component analysis (PCA) based on the distribution of individual PLFAs in the sediments of the Visakhapatnam harbour (Table 3.3), the Mandovi estuary (Table 3.4) and the Zuari estuary (Table 3.5) was carried out to distinguish differences in the PLFA community structure. Absence of PLFA compounds was replaced with zero. The variables were normalized in order to convert the data into a normal distribution with a mean of zero and variance of one, according to the following equation;

$$n_{ik} = (x_{ik} - \bar{x}) / s_i$$

where, x is the concentration of PLFA (i) in sample (k), and s is the standard deviation (Reemtsma and Ittekkot, 1992). PCA was performed using Stat Soft, STATISTICA 6" software package and the principal components were calculated.

3.3. Results and discussion

3.3.1. Bulk parameters

Distribution of OC, $\delta^{13}C_{oc}$, total lipids and total PLFAs in the sediments of the Visakhapatnam harbour, the Mandovi and the Zuari estuaries is shown in Table 3.1 and Table 3.2, respectively. OC varied from 0.6 % (station #4, Turning basin) to 7.6 % (station #5 H&Y Jetty-2/3) in the Visakhapatnam harbour. In the Mandovi estuary, OC varied from 0.7 (station #1) to 4.7 % (station #2) (Table 3.2), whereas in the Zuari estuary, it varied from 0.1 (stations #6 and #8) to 4.8 % (station #4) (Table 3.2), respectively. OC values in these sediments are in good agreement with those reported earlier for other coastal areas of the world (Wang et al., 2006; Harji et al., 2008 and references within).

The sediments displayed a broad range of $\delta^{13}C_{oc}$ values (-29.32 to -23.75 ‰) in the Visakhapatnam harbour (Table 3.1). Highly depleted value of $\delta^{13}C_{oc}$ (-29.32 ‰) was observed at the turning circle (station #17), and the relatively more enriched value (-23.75 ‰) was observed at the Fishery Jetty (station #19). In the Mandovi estuary sediments $\delta^{13}C_{oc}$

values varied from -26.9 ‰ (station #2 Fish jetty) to -22.8 ‰ (station #9 Savoi Verem), while the Zuari estuary sediments displayed values ranging from -27.0 ‰ (station #4 and #5) to -23.1 ‰ (station #8 Sanguem) (Table 3.2). The $\delta^{13}\text{C}_{\text{oc}}$ values varied from -22 to -19 ‰ and -28 to -26 ‰ for marine phytoplankton and terrestrial C3 plants, respectively (Fry and Sherr, 1984; Kendall et al., 2001; Kao et al., 2003). $\delta^{13}\text{C}$ values for C4 plants and soil OM dominated by C4 plants input ranged from -16 to -9 ‰ (Kendall et al 2001). The $\delta^{13}\text{C}_{\text{oc}}$ values recorded for the surface sediments of the Visakhapatnam harbour, Mandovi and Zuari estuaries (Table 3.1 and Table 3.2) were well within the range of values reported for marine (-22 to -19 ‰) and terrestrial C3 plants dominated OM (-28 to -26‰). This implies negligible contribution from C4-based vascular plants.

In order to assess the relative proportions of terrestrial and marine carbon, a two end-member mixing model was used (Schlunz et al., 1999; Hu et al., 2006). Terrestrial carbon accounted for 37 to 100 % of the total OC in sediments of the Visakhapatnam harbour (Table 3.1). Terrestrial carbon was relatively more abundant at most of the stations except at stations #7, #13 and #19. As compared to marine organic matter, terrestrial carbon is relatively more resistant to biodegradation (Meyers, 1994; McCallister et al., 2006). Therefore, preferential removal of marine organic matter may be responsible for the observed abundance of terrestrial organic matter in the sediments of the Visakhapatnam harbor. Terrestrial carbon was also abundant at most of the stations in the Mandovi estuary, while in the Zuari estuary sediments, terrestrial carbon dominance was observed at stations #2, #3, #4, and #5 (Table 3.2). The remaining stations showed mixing of marine and terrestrial C3 plant carbon at various proportions (Table 3.2). The river end stations showed higher amount of phytoplankton derived OC than the sea end stations in both the estuaries probably due to high influx of nutrients which produce the abundance of microalgae at these stations. The river end

stations showed enriched $\delta^{13}\text{C}$ values of marine dominated OM at stations #9 (-22.8) of the Mandovi estuary and at station #8 (-23.074) of the Zuari estuary, respectively (Table 3.2). Both the estuaries experience nearly marine condition during the sampling period (Shetye et al., 1995). Although river run-off is negligible, benthic fluxes of nutrients are higher (Pratihary et al., 2009). This nutrient supply induces growth of phytoplankton, especially diatoms resulting in higher chlorophyll *a*, primary production and bacterial abundance in these estuaries (Matondkar et al., 2007; Pratihary et al., 2009).

Table 3.1: OC, $\delta^{13}\text{C}_{\text{OC}}$, TC, MC, total lipids, total PLFAs and OC normalized total PLFA values in the sediments of the Visakhapatnam harbour, east coast of India.

Station numbers	Sampling site	Site code	OC (%)	$\delta^{13}\text{C}_{\text{OC}}$ (‰)	TC (%)	MC (%)	Total Lipids ($\mu\text{g g}^{-1}$ dw)	Total PLFAs* ($\mu\text{g g}^{-1}$ dw)	Total PLFAs* ($\mu\text{g gOC}^{-1}$)
#1	West Quay Berth-1	VZPWQB-1	1	-25.35	55	45	4200	4.1	409
#2	West Quay Berth-4	VZPWQB-4	-	-	-	-	4050	33.3	-
#3	West Quay Berth-7	VZPSQB-7	6.7	-26.11	64	36	1528	1.9	28
#4	Turning Basin	VZPTB	0.6	-26.05	63	37	1575	0.3	50
#5	H&Y-Jetty 2/3	VZH&Y-2	7.6	-26.27	65	35	14948	7.2	95
#6	Fertilizer Berth	VZPFWRF	7.4	-29.18	98	2	6900	1.1	15
#7	Jetty-8	VZPJT-8	4	-24.6	46	54	4083	15.9	397
#8	Boat Basin Berth-3	VZpBBB-3	1.9	-27.37	78	22	5400	7.1	373
#9	DC Jetty	VZPDCJT	2.4	-28.06	86	14	900	6.5	270
#10	Dredger Berth	VZPDB	-	-	-	-	2724	5.2	-
#11	General Cargo Berth S	VZPGCB-S	2.7	-26.76	71	29	600	1.6	58
#12	General Cargo Berth N	VZPGCB-N	4.4	-27.04	74	26	1575	16.4	372
#13	Ore Berth- 1	VZPORB-1	4.1	-24.35	44	56	2550	12.7	311
#14	Ore Berth-2	VZPORB-2	1.3	-25.06	52	48	300	2.1	161
#15	Container Berth-1	VZPCB-1	-	-26.88	72	28	2600	10.9	-
#16	Container Berth-2	VZPCB-2	3	-25.77	60	40	2250	22.8	761
#17	Turning Circle	VZPTRCL	0.7	-29.32	100	0	300	2.8	399
#18	LPG Berth	VZPLPGB	1.2	-24.88	50	50	1718	0.5	39
#19	Fishery Jetty	VZPFJT	1.3	-23.75	37	63	3825	8.4	643

- = No data; bulk isotopic carbon ($\delta^{13}\text{C}_{\text{OC}}$); TC= terrestrial OC, MC= marine OC; * sum of identified and unidentified PLFAs; dw: dry weight sediment; Total PLFA ($\mu\text{g g}^{-1}$ OC)= OC normalized total PLFAs.

Table 3.2: OC, $\delta^{13}\text{C}_{\text{OC}}$, TC, MC, total lipids, total PLFAs and OC normalized total PLFA values in the sediments of the Mandovi and Zuari estuaries, west coast of India.

Sampling sites	OC	$\delta^{13}\text{C}_{\text{OC}}$	TC	MC	Total lipids	Total PLFAs*	Total PLFAs
	(%)	(‰)	%	%	(mg g^{-1})	($\mu\text{g g}^{-1}$) dw	($\mu\text{g g}^{-1}\text{OC}$)
<u>Mandovi estuary</u>							
#1 Campal creek	0.7	-24.7	65	35	1342	0.5	68
#2 Fish jetty	4.7	-26.9	98	2	4458	10.5	224
#3 Capt. Of ports jetty	2	-25.4	75	25	3292	0.9	45
#4 Ribandar Ferry	2.5	-25.4	75	25	3033	14.9	595
#5 Dempo Shipyard	3.8	-25.8	82	18	942	10.9	287
#6 Old Goa jetty	4.3	-25.4	75	25	1308	12.0	279
#7 Betqui	1.6	-24.4	60	40	508	8.1	504
#8 Bambai	3.3	-24.5	62	38	1133	5.9	178
#9 Savoi Verem	-	-22.8	35	65	2825	4.3	-
<u>Zuari estuary</u>							
#1 Dona Paula	4.6	-23.9	52	48	2992	8.3	180
#2 Cortalim	3.3	-24.8	66	34	2531	26.3	796
#3 Cumbarjua canal	1.1	-26.1	86	14	1742	13.8	1253
#4 Borim	4.8	-27	100	0	2375	43.8	912
#5 Shiroda	2.5	-27	100	0	1275	29.3	1172
#6 Curtorim	0.1	-24	54	46	129	0.6	638
#7 Sanvordem	3.3	-23.6	48	52	1319	48.8	1480
#8 Sanguem	0.1	-23.1	40	60	-	2.1	2059

- = No data; bulk isotopic carbon ($\delta^{13}\text{C}_{\text{OC}}$); TC= terrestrial OC, MC= marine OC; * sum of identified and unidentified PLFAs; dw: dry weight sediment; Total PLFA ($\mu\text{g g}^{-1}\text{OC}$)= OC normalized total PLFAs.

Total lipids varied from $300 \mu\text{g g}^{-1}$ (station #14 and station #17) to $14948 \mu\text{g g}^{-1}$ (station #5) dw sediment in the Visakhapatnam harbour (Table 1). In the Mandovi estuary, total lipids varied from 509 (station #7) to $4458 \mu\text{g g}^{-1}$ dw sediments (station #2) (Table 3.2), while in the Zuari estuary, total lipids varied from 129 (station #6) to 2992mg g^{-1} dw sediments (station #1) (Table 3.2). Total lipid concentrations are in the range of values earlier recorded for the coastal sediments of other areas. For example, for the Marmugoa harbour sediments, the total lipids varied from 233 to $1448 \mu\text{g g}^{-1}$ dw sediments (Harji et al., 2008). Similarly, for the sediments of Sfax coastal zone, Tunisia, the total lipids ranged between

1700 and 9100 $\mu\text{g g}^{-1}$ dw sediment (Zaghden et al., 2005). There was a fair relationship between OC and total lipids in the Visakhapatnam harbour ($r = 0.59$; $n = 16$; $p = 0.01$) and the Zuari estuary ($r = 0.76$; $n = 7$; $p = 0.02$), whereas, a poor relationship ($r = 0.2$; $n = 8$; $p = >0.1$) was recorded between these parameters in the Mandovi estuary.

The concentrations of total PLFA varied between the sampling stations, and values ranged from 0.30 $\mu\text{g g}^{-1}$ (station #4) to 33.3 $\mu\text{g g}^{-1}$ (station #2) dw sediments in the Visakhapatnam harbour (Table 3.1). Concentrations of total PLFAs also ranged from 0.5 to 14.9 $\mu\text{g g}^{-1}$ and 0.6 to 48.8 $\mu\text{g g}^{-1}$ dw sediments, in the Mandovi and Zuari estuary, respectively (Table 3.2). Lower concentrations were recorded at station #1 near the mouth of the Mandovi estuary. Sandy sediments are observed at this station. This indicates that phospholipids are not efficiently preserved in sandy sediments. The observed values are in the range of values reported earlier from different environments. For example, total PLFA concentrations varied from 0.3 to 5.3 $\mu\text{g g}^{-1}$ dw sediments in the Thracian Sea, and from 4.5 to 10.7 $\mu\text{g g}^{-1}$ dw sediments in the Augusta Bay of the Eastern Mediterranean Sea (Polymenakou et al., 2006). Although PLFA concentrations for some of the stations in the Visakhapatnam harbour (Table 3.1) and the Zuari estuary (Table 3.2) were much higher than the above mentioned values, however they were still lower than those reported from eutrophic systems such as the Wadden Sea (36–71 $\mu\text{g g}^{-1}$; Langezaal et al., 2003) and a brackish lagoon in the German Baltic Sea ($>40 \mu\text{g ml}^{-1}$ of sediment; Boschker et al., 2001). Total PLFAs represent the living community of organisms, since they are rapidly degraded following cell death (White et al., 1979; Bouillon et al., 2004; Alfaro et al., 2006; Bouillon and Boschker, 2006). Therefore, the observed variations in PLFAs indicate the spatial differences in living biomass in sediments of the harbour and the estuaries. Higher PLFA concentrations reflect higher viable microbial biomass (Rajendran et al., 1992; Petsch et al., 2003). The abundances of

PLFAs indicate a fairly substantial microbial population at these stations. Higher viable biomass was observed at most of the sediment stations in the Visakhapatnam harbour (Table 3.1), and the Mandovi and the Zuari estuary (Table 3.2).

In order to assess the effect of OC on the PLFAs, concentrations of PLFAs were normalized to OC. OC normalized total PLFAs varied from 15 $\mu\text{g g}^{-1}\text{OC}$ (station #6) to 761 $\mu\text{g g}^{-1}\text{OC}$, (station #16) in the Visakhapatnam harbour sediments (Table 3.1). There was no correspondence between concentrations of total PLFAs and OC in the Visakhapatnam harbour. This was also supported by fairly significant but weak relationship ($r = 0.46$; $n = 16$; $p = 0.02$) between these two parameters. Moreover, irrespective of the carbon sources, the PLFA community was mostly represented by MUFAs, branched PLFAs, and PUFAs (Fig. 3.11).

OC normalized total PLFAs varied from 45 to 595 $\mu\text{g g}^{-1}\text{OC}$ in the Mandovi estuary. In the Zuari estuary, OC normalized total PLFAs varied from 180 to 2059 $\mu\text{g g}^{-1}\text{OC}$ (Table 3.2). There was fairly good relationship between OC and total PLFAs in the Mandovi ($r = 0.63$, $n = 8$, $p = 0.02$) and the Zuari estuary ($r = 0.63$, $n = 8$, $p = 0.05$). This implies that microbial biomass was influenced by OC concentration in both the estuaries. However, irrespective of the carbon source the PLFA community was mostly represented by MUFAs, iso and anteiso PLFAs, methyl branched and cyclopropyl-PLFAs, and PUFAs in both the estuaries (Fig. 3.12 and 3.13).

3.3.2. PLFA composition in the sediments

PLFA analysis is a valuable approach, because it is a biochemical method that provides direct information about the structure of the living microbial community without limitations inherent in the microbial culturing techniques. PLFAs are effective taxonomic markers to characterize the bulk composition of viable microbial communities (White et al.,

1979). However, as there is an overlap in the PLFA composition between many species, it is not possible to assess the presence of individual species. In the sediments of the Visakhapatnam harbour, 38 PLFAs were identified. Concentrations of individual PLFAs varied from 1 ng g⁻¹ (station #18) to 3153 ng g⁻¹ (station #2) dw sediments (Table 3.3). At most of the stations, concentrations of individual PLFA varied, indicating the changes in the composition of PLFA community (Volkman et al., 1980; Elvert et al., 2003; Zhang et al., 2005). Many PLFAs were identified in the Mandovi (37), and Zuari (42) sediments. Concentrations of individual PLFAs varied from 1 ng g⁻¹ (station #1) to 4247 ng g⁻¹ (station #4) in the Mandovi estuary, and from 1 ng g⁻¹ (station #6) and 12784 ng g⁻¹ (station #7) in the Zuari estuary (Table 3.4 and 3.5).

3.3.2. Distribution of PLFAs groups

PLFA groups such as saturated straight chain (Sat-PLFAs), monounsaturated PLFAs (MUFAs), terminally branched saturated and polyunsaturated PLFAs (PUFAs) were present in Visakhapatnam harbour. Saturated PLFAs were generally the dominant PLFAs in most of the sediment samples in the Visakhapatnam harbour (Fig. 3.3). In the present study, the contribution of MUFAs varied from 15.6 to 39.1 % of the total identified PLFAs, and was the second most abundant class of the PLFA community (Fig. 3.3).

In Mandovi and Zuari estuaries PLFAs groups such as Sat-PLFAs, MUFAs, terminally branched saturated, 10-methyl branched and cyclopropyl-PLFAs (Br-PLFAs) and PUFAs were present. Sat-PLFAs were the most abundant and comprised 41 to 58 % and 32 to 48 % of the total identified PLFAs in the Mandovi and Zuari estuary, respectively (Fig. 3.4 and 3.5). Br-PLFAs and MUFAs were the next abundant group of PLFAs in the sediments (Fig. 3.4 and 3.5). The PUFAs were relatively less abundant and accounted for 6 to 18 %, and

Table 3.3: Concentrations of individual PLFAs (ng g⁻¹ dw sediment) in the sediments of the Visakhapatnam harbour, east coast of India.

PLFA (ng g ⁻¹)	Station																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
C12:0	-	74	-	-	-	-	-	-	-	-	-	148	-	-	71	298	-	-	-	
C13:0	-	21	-	-	-	-	11	-	-	-	-	-	44	-	-	-	-	5	-	
C14:0	195	870	136	0	315	74	483	217	205	196	35	626	637	26	360	743	100	0	267	
i-15:0	152	1446	96	24	243	51	747	207	188	191	37	498	554	54	401	739	104	5	375	
a-15:0	140	1060	126	22	259	49	617	155	149	140	37	358	439	46	314	545	73	1	316	
C14:1	-	-	-	-	-	-	14	-	-	-	-	9	-	-	-	-	-	-	-	
C15:0	66	308	37	-	134	27	221	122	100	84	34	216	218	17	117	245	41	-	118	
i-16:0	59	341	39	11	149	28	307	82	63	63	18	186	200	27	152	275	42	-	145	
C15:1	7	36	-	-	76	-	67	93	43	29	-	-	27	-	-	-	-	-	83	
C16:0	593	3153	504	-	1085	185	1958	1045	-	751	204	1709	1613	189	1388	2564	391	6	1068	
i-17:0	76	546	43	11	114	29	354	153	87	106	10	215	218	35	210	370	53	-	161	
C16:1	391	1814	-	37	629	172	905	818	397	616	133	934	811	115	717	1280	292	26	601	
C17:0	58	238	27	5	115	44	150	107	118	85	24	185	162	17	136	316	57	-	117	
C17:1	24	50	-	-	69	-	82	63	-	61	11	115	84	7	62	118	23	60	107	
C18:0	133	448	152	-	283	46	313	214	-	151	15	309	247	55	268	509	72	99	255	
C18:1n8	39	252	-	-	112	-	167	67	29	44	-	152	130	11	118	208	45	-	126	
C18:1n9t	85	632	130	18	163	22	313	191	87	197	57	256	190	78	260	381	86	4	232	
C18:1n9c	125	1126	115	-	268	40	543	368	212	197	48	636	454	96	521	979	124	-	554	
C18:2n6t	-	37	-	-	-	-	26	-	118	6	-	45	-	17	17	46	-	-	-	
Unknown	37	157	32	-	138	14	172	34	162	25	157	48	43	-	67	80	14	-	111	
C18:2n6c	77	10	191	-	287	18	194	108	31	111	3	210	152	27	224	367	53	-	151	
C20:0	17	40	-	-	47	10	43	46	34	20	16	55	41	5	79	77	14	-	40	
C18:3n6	-	-	-	7	-	-	63	-	-	-	-	129	45	-	3	160	301	-	14	39
C20:1	-	19	-	-	-	-	29	-	-	-	-	38	-	6	37	56	-	-	11	
C18:3n3	-	30	-	-	-	-	23	-	-	-	-	141	-	8	65	171	-	-	10	
C21:0	-	-	-	-	-	-	-	-	-	-	-	-	-	7	-	19	-	-	9	
C20:2	-	21	-	11	-	-	23	-	-	-	-	35	-	-	25	46	-	-	17	
C22:0	-	31	-	-	-	-	41	-	-	-	-	31	-	8	29	78	-	-	16	
C20:3n6	-	-	-	-	-	-	-	-	-	-	-	22	-	4	-	23	-	-	-	
C22:1n9	-	-	-	-	-	-	97	-	-	-	-	90	-	-	5	46	-	-	44	
C20:3n3	-	-	-	-	-	-	35	-	-	-	-	-	-	4	-	-	-	-	21	
C20:4n6	-	-	-	-	-	-	-	-	-	-	-	105	-	11	24	57	-	-	-	
C23:0	-	137	-	-	-	-	94	-	-	-	23	-	-	5	-	-	-	-	-	
C22:2	-	-	-	-	-	-	68	-	-	-	-	-	-	12	-	-	-	-	-	
C24:0	-	49	-	-	-	-	49	-	-	-	-	58	-	-	47	76	-	-	17	
C20:5n3	-	-	-	-	-	-	83	-	-	-	-	234	-	6	101	213	-	-	60	
C24:1	-	-	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
C22:6n3	-	39	-	-	-	-	24	-	-	-	34	123	-	16	60	135	-	-	80	

Table 3.4. Concentrations of individual PLFAs (ng g⁻¹ dw sediment) in the sediments of the Mandovi estuary, west coast of India.

PLFA (ng g ⁻¹)	Station								
	1	2	3	4	5	6	7	8	9
C12:0	-	160	3	160	193	119	60	29	341
i-14:0	-	103	8	158	121	122	86	75	27
C14:0	19	523	36	990	821	847	660	272	363
i-15:0	18	590	59	925	345	595	555	459	134
A15:0	17	369	40	278	434	305	241	282	21
C15:0	7	241	13	387	244	328	225	120	105
i-16:0	14	344	39	522	228	276	241	207	55
C15:1	-	37	-	53	-	64	-	-	-
C16:0	95	2745	160	4247	2883	2862	2082	1345	1105
10Me16	-	205	9	152	156	110	151	169	52
i-17:0	-	215	-	335	-	206	185	156	42
C16:1	24	1352	42	1986	1218	1160	1056	688	431
C17:0	13	196	17	281	209	217	195	138	68
i-18:0	10	14	4	27	189	21	18	19	10
Cyclo-9,10- C16	-	119	-	86	101	195	60	58	-
C17:1	-	66	4	112	80	90	65	47	31
C18:0	26	659	53	876	650	556	437	286	167
10Me18	-	186	-	-	181	172	72	66	16
C18:1n9t	-	54	5	104	83	80	60	46	16
C18:1n9c	36	698	12	920	1113	1004	405	274	331
C18:2n6t	-	-	-	-	64	-	-	10	14
Cyclo-9,10- C18	29	499	65	281	409	334	149	98	69
C20:0	4	81	2	129	97	59	80	9	203
C18:3n6	14	120	20	199	112	163	178	78	100
C20:1	-	33	-	57	40	34	35	23	10
C18:3n3	4	91	9	169	79	129	26	21	13
C21:0	16	15	36	-	17	13	11	7	-
C20:2	-	24	6	-	21	43	20	18	15
C22:0	6	60	70	108	36	127	35	39	13
C20:3n6	-	-	-	-	33	-	19	-	3
C22:1n9	-	-	-	-	-	-	6	-	-
C20:3n3	60	324	51	698	270	676	357	211	196
C20:4n6	-	-	9	44	32	31	18	10	15
C23:0	38	53	29	-	-	106	-	52	42
C22:2	6	82	8	-	117	386	61	43	-
C24:0	17	108	83	115	83	144	40	99	86
C24:1	1	47	7	103	60	214	33	31	-

- = not detected

Table 3.5. Concentrations of individual PLFAs (ng g⁻¹ dw sediment) in the sediments of the Zuari estuary, west coast of India.

PLFA (ng g ⁻¹)	Station							
	1	2	3	4	5	6	7	8
C13:0	43	52	24	91	66	-	86	-
i14:0	95	341	116	456	337	4	281	35
C14:0	578	1464	870	2118	1175	35	3132	46
i-15:0	585	2150	766	2514	2069	21	1996	203
a-15:0	469	1228	274	1737	1216	13	1086	108
C15:0	176	498	247	969	791	14	829	78
i-16:0	281	760	250	1008	676	11	883	68
C15:1	-	67	26	250	127	3	-	10
C16:0	1846	6024	4076	10599	6730	198	12784	418
10Me16	434	743	159	1030	732	2	722	69
i-17:0	231	615	205	695	541	12	771	56
C16:1	839	3136	2199	5746	3579	77	6652	190
C17:0	188	418	143	557	466	10	659	51
i18:0	23	52	22	54	47	10	71	19
Cyclo-9,10-C16	-	292	180	471	682	4	382	-
C16:2n6	-	-	111	-	298	-	379	-
C17:1	71	122	71	299	-	2	-	11
C18:0	319	827	421	1744	841	33	1391	45
10Me18	169	-	-	512	281	-	1020	-
C18:1n9t	54	84	55	260	176	1	133	-
C18:1n9c	182	1188	696	1658	1118	43	1736	130
C18:1n11C	587	2093	626	3432	2132	9	5476	185
C18:1	126	114	32	394	117	29	-	-
C18:2n6t	38	120	46	199	139	-	168	-
Cyclo-9,10-C18	250	898	401	2455	1080	16	1763	143
C20:0	32	193	49	191	185	3	175	10
C18:3n6	54	143	24	406	217	9	24	-
C20:1	36	110	56	158	102	2	124	17
C18:3n3	32	446	266	722	425	3	758	-
C21:0	17	20	12	39	18	-	46	-
C20:2	29	122	40	217	84	2	293	-
C22:0	68	153	13	174	182	4	93	11
C20:3n6	32	98	31	96	112	-	92	-
C22:1n9	-	3	16	14	21	4	19	-
C20:3n3	-	80	-	284	163	43	-	-
C20:4n6	147	284	220	362	462	-	867	75
C23:0	81	-	-	-	-	1	-	-
C22:2	11	134	9	166	137	1	81	24
C24:0	27	54	40	189	75	9	212	2
C20:5n3	-	282	616	219	499	-	1474	2
C24:1	-	50	18	81	78	1	-	-
C22:6n3	-	116	174	148	147	0	369	-

- = not detected

4 to 11 % of the total identified PLFAs in the sediments of the Mandovi and Zuari estuary, respectively. Abundance of Sat-PLFAs has been reported from other coastal (estuaries, rivers, bays, etc), and open ocean environments (Polymenakou et al., 2006). Sat-PLFAs are abundant in the cell membranes of most bacteria and eukaryotes, and thus are non-diagnostic (Volkman et al., 1998). In the marine environment, MUFAs, Br-PLFAs mainly of iso and anteiso configurations and PLFAs with cyclopropane rings are mostly produced by bacteria (Wakeham 1995; Rütters et al., 2002). PUFAs are mostly abundant in microalgae (Volkman et al., 1998) and also seen in bacteria (Russell and Nichols 1999; Rütters et al., 2002; Nichols 2003). Abundance of Sat-PLFAs, MUFAs, Br-PLFAs and PUFAs suggests the presence of a diverse type of microbial groups in sediments of these estuaries.

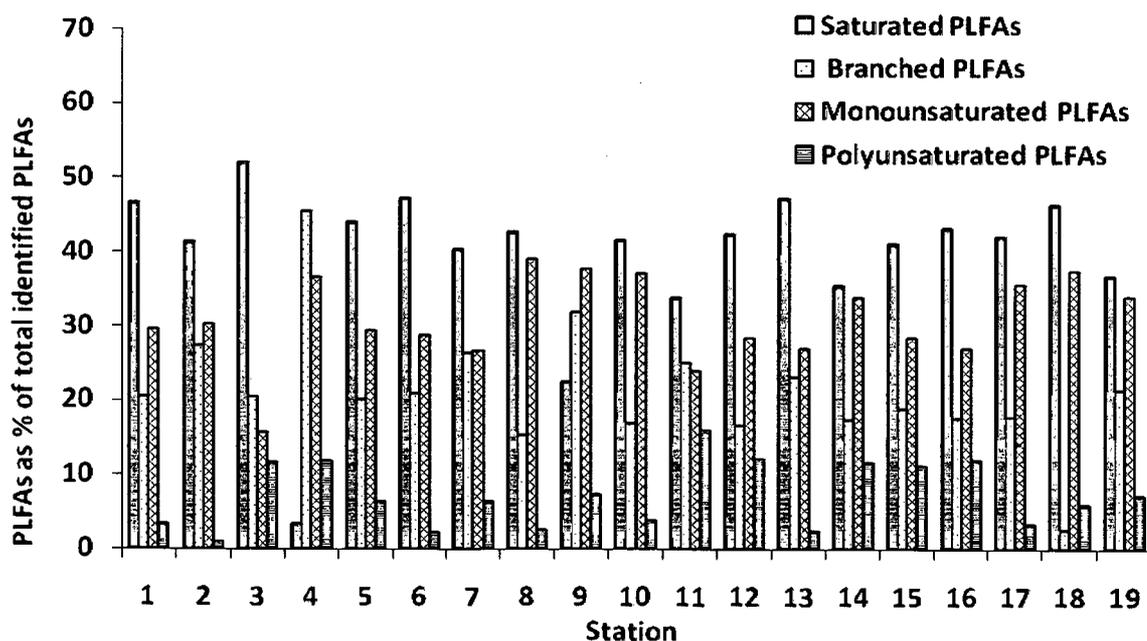


Fig.3.3: Distribution of saturated, branched, monounsaturated and polyunsaturated PLFAs as % of the total identified PLFAs in the sediments of the Visakhapatnam harbour, east coast of India.

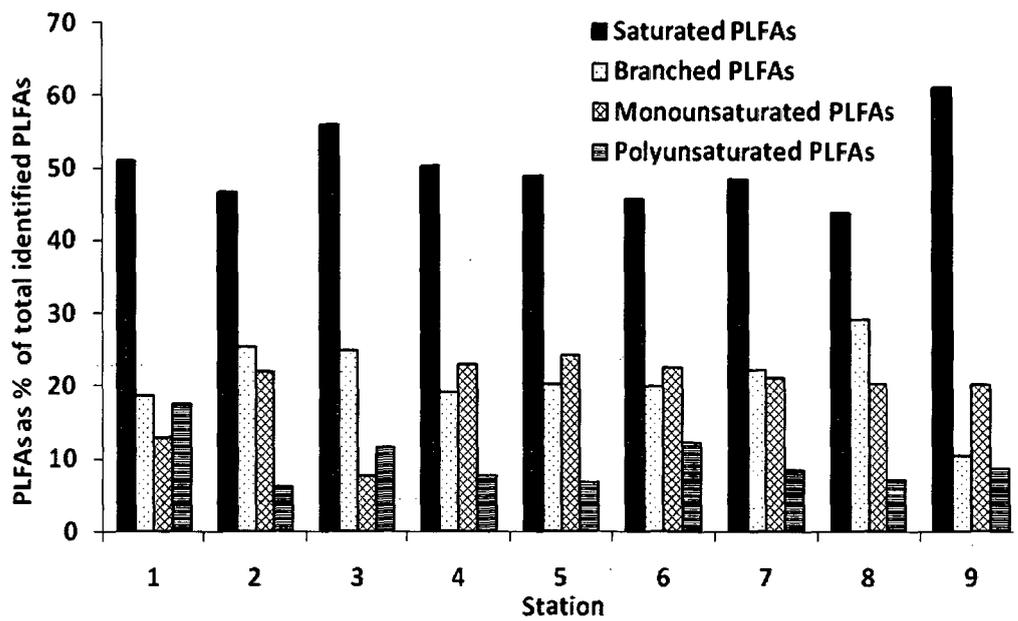


Fig.3.4: Distribution of saturated, branched, monounsaturated and polyunsaturated PLFAs as % of the total identified PLFAs in the sediments of the Mandovi estuary, west coast of India.

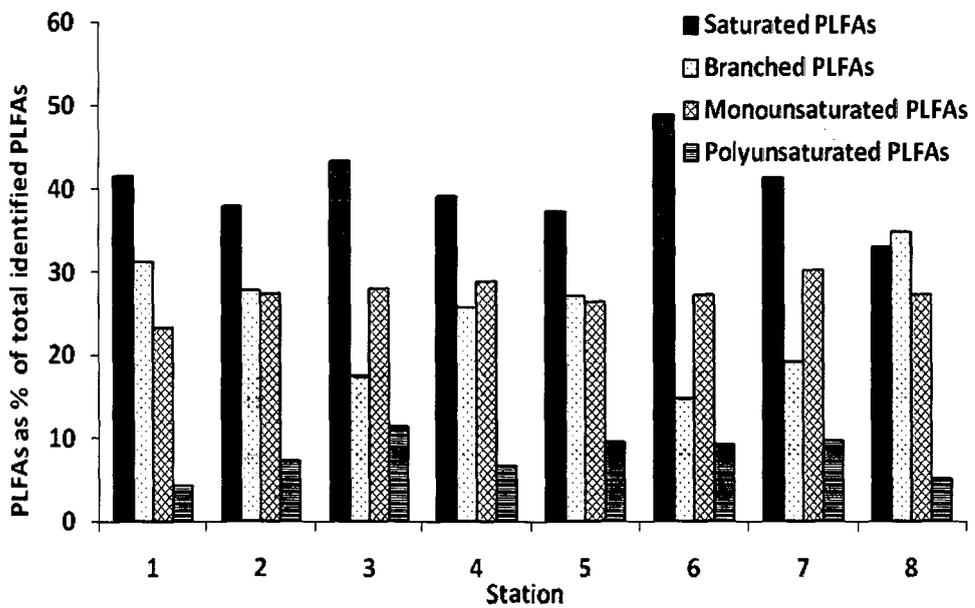


Fig.3.5: Distribution of saturated, branched, monounsaturated and polyunsaturated PLFAs as % of the total identified PLFAs in the sediments of the Zuari estuary, west coast of India.

The major marine microbial PLFAs observed were C14:0, C16:0, C16:1n9c, C18:1n9c, C18:1n9t, i-15:0 and a-15:0 in the Visakhapatnam harbour sediments (Fig. 3.6). Among the saturated PLFAs, C16:0 was the most abundant accounting for 20 % (station #11) to 31 % (station #3) of the total identified PLFAs, except at station #18 (3 %) where the concentrations were relatively less in the harbour sediments (Fig. 3.6). The major PLFAs observed were C14:0, C16:0 and C18:0, C16:1n9c and C18:1 (C18:1n9c and C18:1n11c), i-15:0, a-15:0, C20:3n3 and cyclopropyl-PLFAs (cyclo-9,10-C16) in the Mandovi and Zuari estuaries (Fig. 3.7 and 3.8). C16:0 was also the most abundant PLFA as it accounted for 17.8 to 28.6 % and 20.3 to 31 % of the total identified PLFAs in the Mandovi and Zuari estuary, respectively (Fig. 3.7 and 3.8).

PLFAs such as C14:0, C15:0, C16:0, C17:0, C18:0 and C20:0 were generally abundant among saturated group of PLFAs in the Visakhapatnam harbour sediments (Table 3.3). These PLFAs are ubiquitous in organisms from a wide range of genera (White et al., 1979; Rajendran et al., 1992; Alfaro, et al., 2006). The high proportions of saturated PLFAs infer that a large fraction of viable microbial communities inhabiting the sediments can not be attributed to a certain functional group. Long chain saturated PLFAs (C20:0 – C24:0) were present but their concentrations were low in the sediments of the Visakhapatnam harbour (Table 3.3). Long chain PLFAs (C20:0 – C24:0) were also observed in the Mandovi and the Zuari sediments (Table 3.2 and 3.3). These PLFAs are indicative of inputs from terrestrial plants and mangroves (Rajendran et al., 1992). However, various studies suggests that microalgae (Volkman et al., 1980, 1989; Nichols et al., 1986; Rezanka and Podojil 1986; Dunstan et al., 1992) and perhaps bacteria (Volkman et al., 1988) can also produce these fatty acids, although in small amounts (typically <2%) relative to C14-C20 fatty acids. The source

of long chain saturated PLFAs hence may be algal. However, proof of an algal origin for such fatty acids in contemporary marine sediments has yet to be obtained.

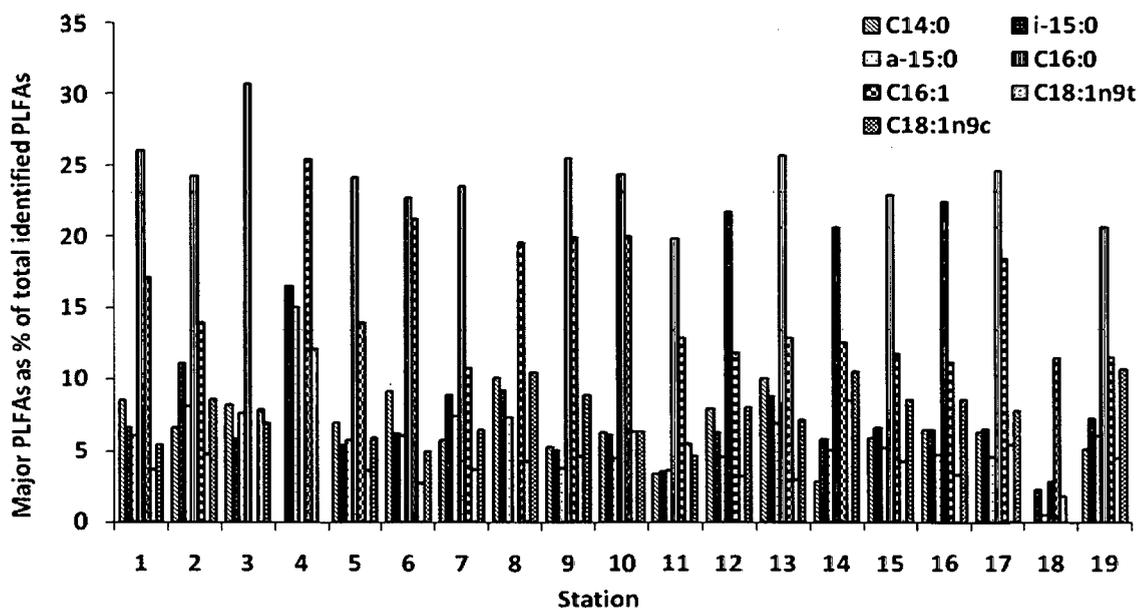


Fig. 3.6: Distribution of major PLFAs as % of the total identified PLFAs in the sediments of the Visakhapatnam harbour, east coast of India.

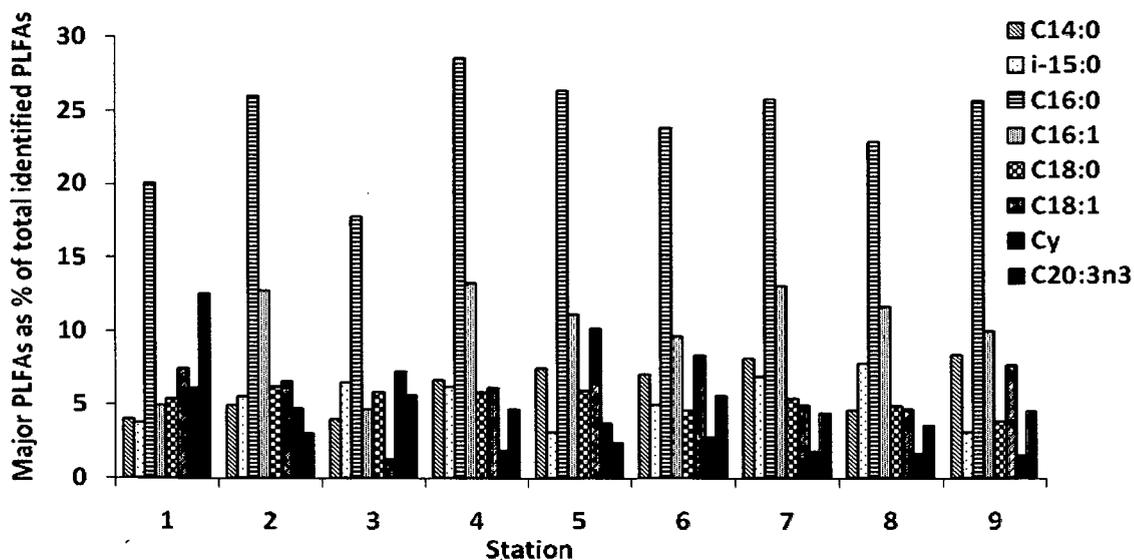


Fig. 3.7: Distribution of major PLFAs as % of the total identified PLFAs in the sediments of the Mandovi estuary, west coast of India.

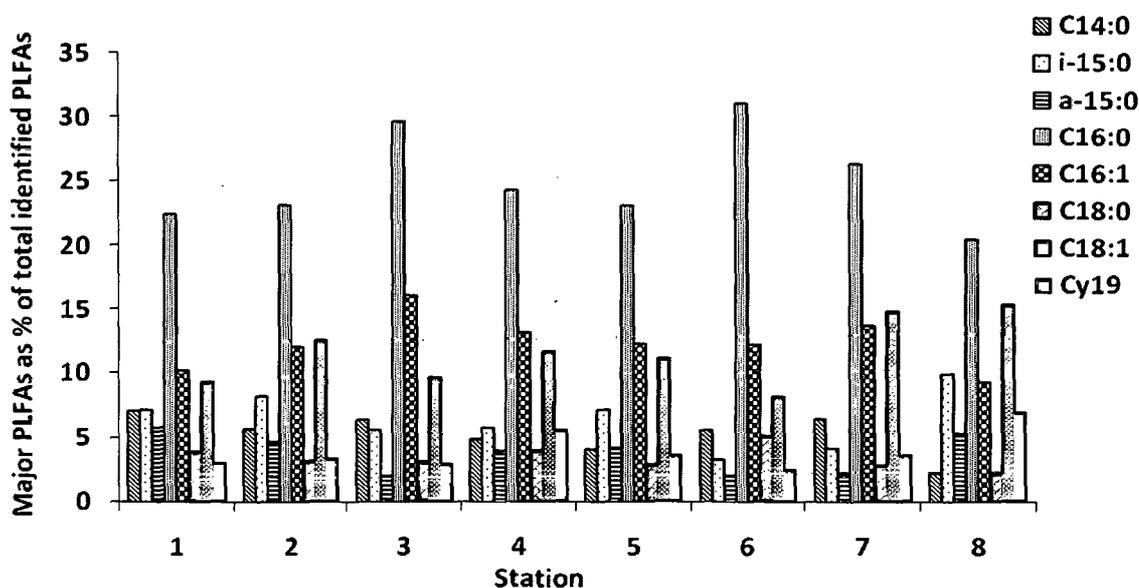


Fig. 3.8: Distribution of major PLFAs as % of the total identified PLFAs in the sediments of the Zuari estuary, west coast of India.

Monounsaturated PLFAs (MUFAs) are mainly produced by bacteria and microalgae (Wakeham, 1995; Rütters et al., 2002; Widenfalk et al., 2008). MUFAs such as C14:1, C15:1, C16:1n9c, C17:1, C18:1 (C18:1n8, C18:1n9t, C18:1n9c), C20:1, C22:1n9 and C24:1 were present in most of the sediment samples of the Visakhapatnam harbour, and Mandovi and Zuari estuaries (Table 3.3, 3.4 and 3.5). Among these, C16:1n9c was the most abundant and accounted for 11 % (station #7) to 25.4 % (station #4), followed by C18:1n9t and C18:1n9c which accounted for 1.8 to 12.2 % and 4.7 to 10.8 % of the total identified PLFAs, respectively in the Visakhapatnam harbour (Fig. 3.6). MUFAs such as C16:1n9c and C18:1 (C18:1n9c+C18:1n11c) were the most dominating PLFAs in both the estuaries (Table 3.4 and 3.5). C16:1 and C18:1 MUFAs accounted for 4.7% (station #3) to 12.8% (station #2) and 1.3% (station #3) to 10.2% (station #5) in the Mandovi estuary, respectively (Fig. 3.7). While C16:1 and C18:1 MUFAs accounted for 9.3% (station #8) to 16% (station #3) and 8.2% (station #6) to 15.3% (station #8), respectively in the Zuari estuary (Fig. 3.8). C16:1 and

C18:1 MUFAs are mostly observed in methanotrophs or thio oxidizing bacteria (Oliver and Colwell 1973; Boon et al. 1996; Ringelberg et al., 1997; Boschker et al., 2005). Although, C16:1n9c is reported in bacteria, in marine environment it can also be introduced through phytoplankton (Muri et al., 2004). PLFA such as C18:1n9c is present in bacteria, cyanobacteria, microalgae and fungi (Wakeham, 1995; Langezaal et al., 2003; Polymenakou et al., 2006).

Zooplankton grazing is an important link between lower and higher trophic levels and fatty acid biomarkers may be employed to determine the importance of zooplankton grazing. Generally, zooplankton feeding predominantly on phytoplankton contains elevated concentrations of long-chain MUFAs such as 20:1 and 22:1 (Parrish et al., 2000). Hence these PLFAs may be used as markers to determine the importance of zooplankton grazing. In the Visakhapatnam harbour sediments, these zooplankton markers were observed at stations #2, #7, #12, #14, #15, #16 and #19 suggesting their role in the trophic food level (Table 3.3). MUFAs such as C20:1 and C22:1 were also observed at various stations in the Mandovi and Zuari estuaries (Table 3.4 and 3.5). This is presumably because of the phytoplankton blooms during the post monsoon season which results in proliferation of zooplankton in both these estuaries (Devassy, 1983). Higher abundance of zooplankton during the sampling period may be responsible for the presence of these PLFAs (Devassy, 1983).

Polyunsaturated fatty acids (PUFAs) C18:2n6c, C18:3n3, C18:3n6, C20:3n3, C20:3n6, C20:4n6, C20:5n3 and C22:6n3 are synthesized mostly by eukaryotes (White et al., 1979; Volkman et al., 1998; Brinis et al., 2004; Widenfalk et al., 2008). They are abundant in planktons, benthic invertebrates and are considered to undergo rapid degradation in the water column compared to MUFAs (Wakeham et al., 1997). Microalgae contain long-chain PUFAs such as C18:2n6, C18:3 (C18:3n6, C18:3n3), 20:5n3 and 22:6n3 among other compounds

(Volkman et al., 1998; Reuss and Poulsen, 2002; Brinis et al., 2004). Cyanobacteria are considered as a major source of C18 PUFAs (Rontani and Volkman, 2005). However green macroalgae also synthesizes C18 PUFAs (Meziane and Tsuchiya, 2000). Similarly, psychrophilic, halophilic, and barophilic marine genera of *γ-Proteobacteria* are able to produce these PUFAs since this group of lipid can play an important role in maintaining optimal membrane fluidity and hence making the organisms better adapted to the deep sea environment (Russell and Nichols, 1999; Nichols and McMeekin, 2002). The contribution of PUFAs to the total identified PLFAs varied from 1.1 (station #2) to 16 % (station #11) in the harbour sediments (Fig. 3.6). The contribution from eukaryotes and *γ-Proteobacteria* to the total PLFA community was minor in the harbour sediments, was evident from the poor abundance of PUFAs. In the Mandovi estuary sediments, C18:3 (C18:3n6, C18:3n3), C20:3n6, C20:3n3, C20:4n6 20:5n3 and 22:6n3 PUFAs were observed, of which C20:3n3 was the most abundant PLFAs (Table 3.4 & 3.5; Fig. 3.7 & 3.8). PLFA C20:5n3 is common in diatoms, whereas 22:6n3 is in dinoflagellates (Colombo et al., 1996; Budge and Parrish, 1998; Meziane and Tsuchiya, 2000). In both the estuaries, diatoms and dinoflagellates were the dominating phytoplankton during the post monsoon season (November) (Devassy, 1983; Matondkar et al., 2007). In the Mandovi estuary, highest relative concentration of PUFAs of phytoplankton origin was observed at station #1 (Fig. 3.7). This station is located at the mouth of the estuary. At this station chlorophyll *a* concentrations are generally high (Matondkar et al., 2007). PUFAs such as C20:5n3 and C22:6n3 were only observed in the Zuari estuary sediments. Similarly, psychrophilic, halophilic, and barophilic marine genera of *γ-Proteobacteria* are reported to produce these PUFAs. However, the Mandovi and Zuari estuaries are coastal and tropical in nature, hence contribution from psychrophilic, halophilic,

and barophilic marine genera of *γ-Proteobacteria* is less likely. Hence the probable sources of PUFAs can be diatoms, dinoflagellates, other algae and cyanobacteria in the Mandovi and Zuari estuaries.

In the Visakhapatnam harbour sediments, branched PLFAs accounted for 2.7 to 45.5 % of the total identified PLFAs. However, the contribution of these PLFAs to total identified PLFAs was higher (23.2 % to 45.5 %) at stations #2, #4, #7, #9, #11 and #13 (Fig. 3.3). Branched PLFAs, especially i-15:0 and a-15:0 were more abundant in the sediments of the Visakhapatnam harbour, together comprising 2.9 to 31.7% of the total identified PLFAs. The branched PLFAs i-15:0, a-15:0, i-16:0 and i-17:0 are well known biomarkers for Gram-positive bacteria, Gram negative anaerobes and sulfate reducing bacteria (O'Leary and Wilkinson, 1988; Wakeham, 1995; Rütters et al., 2002; Harvey et al., 2006; Widenfalk et al., 2008). However, these PLFAs are not detected in algae, phytoplankton, other microorganisms and invertebrates (Tunlid and White, 1992). The presence of these PLFAs in the harbour sediments may be due to the anoxic conditions that prevail at most of the stations due to constant stagnation of the water (Sarma et al., 1982; Kadam and Bhangale, 1993). These branched PLFAs also have been reported from the anoxic environments of the Black Sea and the Arabian Sea (Wakeham, 1995; Wakeham et al., 2002). Iso- and anteiso PLFAs were present in both the estuaries (Table 3.4 and 3.5) and accounted for a substantial fraction of the total PLFAs in the sediments of the Mandovi (6.7 to 20.4 %) and Zuari estuary (10.4 to 23.7 %). Further, 10-methyl branched (10Me16 and 10Me18) and cyclopropyl-PLFAs (cyclo-9,10-C16 and cyclo-9-10-C18) were observed in the sediments of these estuaries. Their relative concentrations varied spatially and ranged between 1.0 to 4.0 % and 0.3 to 7.3 % (10Me16+10Me18), and 1.6 to 7.3 % and 3.0 to 7.0 % (cyclopropyl-PLFAs) of total identified PLFAs in the Mandovi and Zuari estuary sediments, respectively. Methyl branched

and cyclopropyl-PLFAs are generally more abundant in anaerobic environments but are also associated with sulfate-reducing bacteria (Guckert et al., 1985; Rajendran et al., 1992; Wakeham, 1995). 10Me18 is reported in actinomycetes within gram positive bacteria (Kroppenstedt, 1985). Together the terminally branched (iso and anteiso), methyl branched and cyclopropyl PLFAs contributed the highest amount of bacterial biomass (10 to 29 % and 15 to 35 %) in both the estuaries (Fig. 3.4 and 3.5) suggesting gram positive bacteria, anaerobic and sulfate reducing bacteria are important microbial communities in the sediments of both the estuaries. The branched (iso and anteiso), methyl branched and cyclopropyl-PLFAs are generally considered to be synthesized by bacterial communities and are therefore used as biomarkers of bacteria (Jeffries 1972; Volkman et al., 1980).

3.3.3. PLFAs as stress indicators

Tran-monounsaturated PLFA (C18:1n9t) was one of the major PLFAs in the sediments of the harbour (Fig. 6). It appears that this PLFA is produced by bacteria from the corresponding cis- isomer in response to stress due to starvation, toxic chemicals, anaerobic conditions and heavy metal ions etc (Guckert et al., 1986; Findlay et al., 1990; Kieft et al., 1994; Fang et al., 2004). Furthermore, the harbour is subjected to pollution due to municipal sewage, factory discharge, sulfur and petroleum products (Sarma et al., 1982; Kadam and Bhangale, 1993). Presence of these pollutants creates anoxic condition in the harbour which may put stress on benthic microbial communities including bacteria. Thus, the occurrence of trans-monounsaturated PLFAs in the harbour sediments probably indicates the stress induced response of the local bacterial communities. Trans- PLFAs are useful biomarkers of stress in the environment. They were also detected in Mandovi and Zuari estuary sediments. Apart from trans, cyclopropyl-PLFAs were also observed in both the estuarine sediments. The cyclopropyl-PLFAs are also produced due to starvation and anaerobic condition (Guckert et

al., 1986). The occurrence of these PLFAs in the sediments of both estuaries (Table 3.4 and 3.5) indicates the influence of anthropogenic activities, and pollution due to sewage discharge, and petroleum products (Harji et al., 2008).

3.3.4. Principal component analysis (PCA)

In order to further define the PLFA community structure, PLFA data were processed using the principal component analysis (PCA). PCA produced three principal components (PC) in the Visakhapatnam harbour sediments and accounted for 81.2% of the total variability (Table 3.6). PC1 accounted for 57.0% of the variance with most negative loadings (-0.900 to -0.949) on C18:1n9c followed by C17:0, C24:0, C18:1n8, C16:0, C18:0, i-16:0, C14:0, C22:0, C15:0, i-17:0, C20:2 in the Visakhapatnam harbour (Table 3.6). Of these, C18:1n9c, C17:0, C15:0, C18:1n8, i-16:0 and i-17:0 are produced by bacteria (Wakeham et al., 2002), while C18:1n9c is also derived from cyanobacteria and microalgae (Wakeham, 1995; Zhang et al., 2004, Alfaro et al., 2006). Saturated PLFAs C14:0, C16:0 and C18:0 are ubiquitous and hence are not indicators of any organisms (Volkman et al., 1998). PC1 was also influenced by C20:1 and C22:1 and C16:1n9c which indicate input from zooplankton, phytoplankton and bacteria (Parrish et al., 2000; Muri et al., 2004). Bacterial PLFAs i-15:0, a-15:0 and C18:1n9t and the algal PLFAs C20:3n6, C20:4n6, C20:5n3 and C22:6n3 also controlled the PC1.

PC2 accounted for 15.8% of the total variance, and was influenced by PUFAs C20:3n6, C20:4n6, C18:3n3 and C18:3n6 (Table 3.6) produced by eukaryotes such as green algae and red algae. This factor was also controlled by PLFAs such as C20:5n3 and C22:6n3 (Table 3.6) derived from diatoms and dinoflagellates. PC3 showed only 8.4% of the total variance, and was influenced by PLFAs C20:3n3 and C22:2 (Table 3.6) that are present in phytoplankton, algae and other eukaryotes, and C22:1n9 produced by zooplankton.

Table 3.6: Factor loading on the PLFA compounds using principal component analysis in the sediments of the Visakhapatnam harbour, east coast of India.

Variable	PC1	PC2	PC3
C18:1n9c	-0.949	-0.181	-0.2
C17:0	-0.944	-0.053	-0.213
C24:0	-0.943	0.172	0.108
C18:1n8	-0.943	-0.271	-0.102
C16:0	-0.93	-0.276	-0.161
C18:0	-0.924	-0.121	-0.143
i-16:0	-0.923	-0.362	0.019
C14:0	-0.917	-0.229	-0.223
C22:0	-0.911	0.233	0.112
C15:0	-0.909	-0.33	-0.115
i-17:0	-0.903	-0.386	-0.114
C20:2	-0.900	0.313	0.12
C20:1	-0.891	0.354	0.126
C16:1	-0.882	-0.334	-0.247
C18:1n9t	-0.866	-0.368	-0.199
i-15:0	-0.85	-0.455	-0.171
a-15:0	-0.846	-0.487	-0.132
C20:0	-0.821	0.082	-0.09
C18:3n3	-0.809	0.569	-0.032
C22:6n3	-0.796	0.496	0.044
C12:0	-0.794	0.523	-0.218
C20:5n3	-0.781	0.56	0.214
C17:1	-0.765	0.085	0.091
C22:1n9	-0.667	0.138	0.682
C20:3n6	-0.651	0.659	-0.007
C18:2n6c	-0.65	0.282	0.075
C20:4n6	-0.608	0.635	0.056
C18:3n6	-0.602	0.513	-0.013
C21:0	-0.496	0.459	-0.088
C23:0	-0.458	-0.701	0.113
Unknown	-0.449	-0.455	0.193
C14:1	-0.448	-0.107	0.793
C18:2n6t	-0.377	0.056	0.011
C20:3n3	-0.281	-0.352	0.806
C22:2	-0.259	-0.365	0.815
C13:0	-0.251	-0.58	-0.259
C15:1	-0.171	-0.564	0.203

Principal component analysis of the PLFAs in the sediments of the Visakhapatnam harbour was also used to group the stations. Sample scores for individual PLFAs showed three clusters (Fig. 3.9). Stations #2, #7 and #13 were grouped in cluster 1, and stations #12, #15 and #16 in cluster 2. The remaining stations formed the third cluster. Stations belonging to cluster 1 were relatively enriched in iso and anteiso C15:0 and C23:0 PLFAs implying that

these stations were relatively more influenced by the abundance of bacteria (Table 3.3 & 3.6). Second cluster was mostly dominated by PUFAs indicating the importance of marine algae at these stations (Table 3.3 & 3.6). In contrast, cluster 3 had those stations which contained relatively low concentrations of PLFAs (Table 3.3 & 3.6). PCA analysis was useful to fairly classify the stations on the basis of PLFAs. However, in order to get a better picture of station groupings, a large number of samples need to be analyzed.

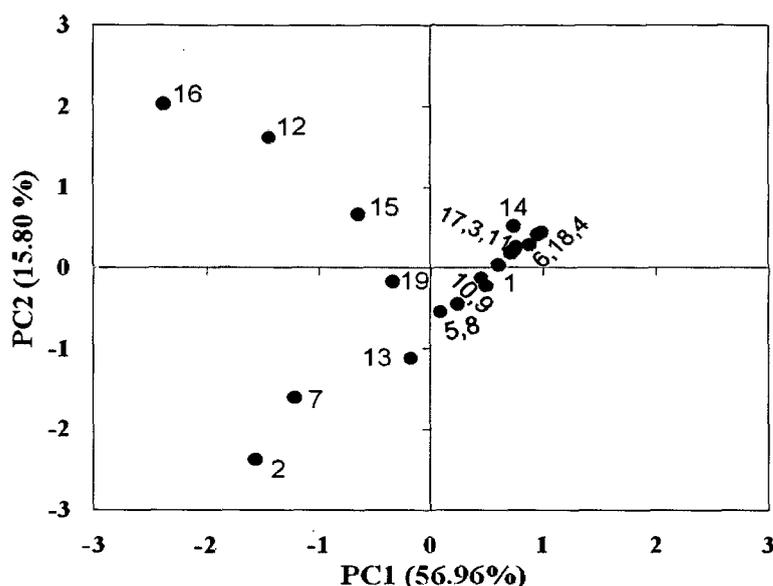


Fig. 3.9. The scores of the first two principal components for the sampling sites in the Visakhapatnam harbour for the PLFA compounds. Numbers indicate stations.

As Mandovi and Zuari estuary covered only few stations, both the sampling sites were combined together for PCA analysis. First three PCs explained 84.5% of the variance in the data set for Mandovi and Zuari estuaries (Table 3.7). PC1 (65.8%) showed high loading (0.983 to -0.702) in the Mandovi and Zuari estuaries on C15:0, C17:0, C16:1n9c, C18:1* (C18:1n9c+C18:1n911c), C18:1n9t, i14:0, i15:0, a15:0, i16:0, i17:0, cy17, cy19, 10Me16, 10Me18, which are produced by bacteria. The iso and anteiso PLFAs, methyl branched and cyclopropyl-PLFAs are synthesized by Gram positive, Gram negative anaerobes and sulfate

reducing bacteria (O'Leary and Wilkinson 1988; Guckert et al., 1985; Rajendran et al., 1992; Wakeham, 1995), while C16:1n9c and C18:1n9c PLFAs are produced by Gram negative bacteria but are also present in phytoplankton and the later PLFA in cyanobacteria as well (Muri et al., 2004; Wakeham, 1995). PC1 was also influenced by C18:3n3, C18:2n6t, C20:2, C20:3n6, C20:4n6, C22:6n3, C20:5n3 PUFAs derived from microalgae and other eukaryotes, C20:1, C22:1 MUFAs produced by zooplankton and C14:0, C16:0 and C18:0 saturated PLFAs which are ubiquitous in origin (Table 3.7). C16:1n9c, C18:1* (C18:1n9c+C18:1n911c) and branched PLFAs were the dominating PLFAs in the sediments (Fig. 3.7 & 3.8). PC2 (12.6%) showed highest loading on C18:3n6, C24:1 and C20:3n3 (Table 3.7). The PUFAs C18:3n6 and C20:3n3 occurs in algae, while C24:1 produced by zooplankton. PC3 showed poor loadings on the PLFAs and contributed only 6.1% to the variability in the data set.

The score plot shows the clusters of the Mandovi and Zuari estuary stations based on the PLFA composition in each stations (Fig. 3.10). 'M' and 'Z' represents Mandovi and Zuari estuary sediment stations, while the number represents the station number in Fig. 3.10. Sample scores for individual PLFAs formed cluster 1 with all the Mandovi estuary stations and three Zuari estuary stations (stations #1Z, #5Z and #8Z) (Fig. 3.10), while the cluster 2 formed with the remaining Zuari estuary stations (stations #2Z, #3Z, #4Z, #5Z and #7Z). The stations #2Z, #3Z, #4Z, #5Z and #7Z of Zuari estuary contained high concentrations of PUFA such as C20:5n3 and C22:6n3 and based on this they appear to be separated from other stations of Zuari and Mandovi estuary. Moreover, none of the Mandovi estuary and the three stations of Zuari estuary (stations #1Z, #5Z and #8Z) contained C20:5n3 and C22:6n3 PLFAs, except a very low amount of C20:5n3 in station #8Z (2ngg^{-1}). Moreover, station #4Z and station #7Z were placed relatively far from other Zuari estuary stations in cluster 2 (Fig. 3.10).

It appears that the stations #4Z and station #7Z contains overall high concentrations of PLFAs (Table 3.5).

Table 3.7. Factor loading on the PLFA compounds using principal component analysis in the sediments of the Mandovi and Zuari estuaries, west coast of India.* C18:1n9c+C18:1n11c

Variable	PC1	PC2	PC3
C15:0	0.983	0.108	-0.041
C20:1	0.981	0.075	0.101
C16:1	0.979	-0.108	-0.090
C18:3n3	0.979	-0.103	-0.010
C16:0	0.976	-0.089	-0.140
C17:0	0.973	0.017	-0.136
i-16:0	0.967	0.121	0.014
i-15:0	0.965	0.077	0.134
Cy-C19:0	0.962	0.042	0.078
C18:1*	0.959	-0.214	-0.118
i-14:0	0.956	0.201	0.149
C18:2n6t	0.954	-0.106	0.157
i-17:0	0.954	-0.014	0.005
a15:0	0.954	0.125	0.207
10MEC16:0	0.944	0.044	0.238
C18:0	0.936	0.215	-0.114
C13:0	0.934	-0.184	0.202
C14:0	0.933	-0.121	-0.250
C20:2	0.923	-0.219	-0.111
C18:1n9t	0.910	0.322	0.047
C20:3n6	0.909	-0.136	0.211
Cy-C17:0	0.888	0.053	0.027
C20:4n6	0.869	-0.455	-0.125
C22:6n3	0.824	-0.502	-0.137
10MeC18:0	0.785	-0.287	-0.368
C22:0	0.781	0.416	0.030
C22:1n9	0.776	-0.357	0.071
C20:0	0.743	0.174	-0.118
C15:1	0.710	0.524	0.337
C20:5n3	0.702	-0.622	-0.284
C24:0	0.689	0.212	-0.530
C21:0	0.686	-0.224	-0.048
C18:1	0.661	0.334	0.584
C16:2N6	0.627	-0.584	-0.307
C18:3n6	0.603	0.726	0.144
C17:1	0.506	0.665	0.280
C22:2	0.405	0.546	-0.371
i-18:0	0.351	0.031	-0.197
C23:0	-0.343	0.329	-0.343
C12:0	-0.268	0.398	-0.509
C24:1	0.242	0.763	-0.397
C20:3n3	0.008	0.804	-0.476

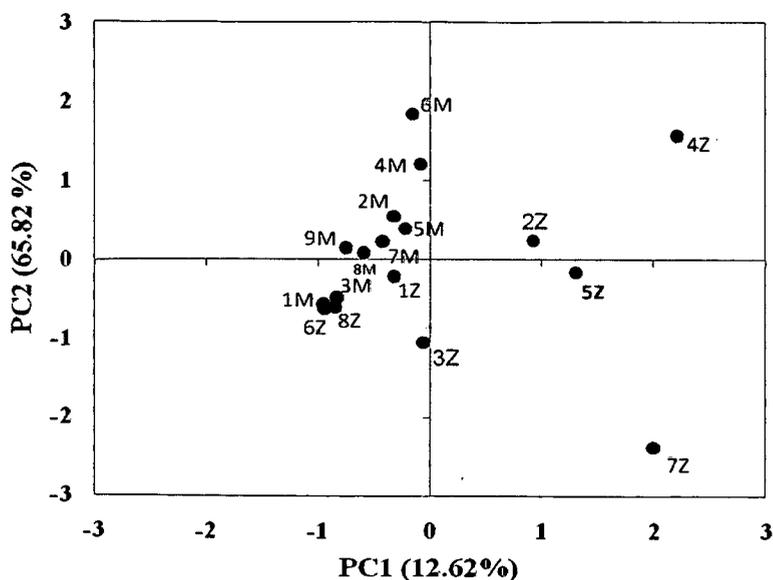


Fig. 3.10. The scores of the first two principal components for the sampling sites in the Mandovi and Zuari estuaries for the PLFA compounds. Numbers indicate stations.

In general the trends of factor loadings on PC1 and PC2 appear to be different due to the differences of OM sources and hence type of microbial communities thriving on such sources. In the Visakhapatnam harbour, anthropogenic inputs appear to be high. In contrast, the Mandovi and the Zuari estuaries contain more terrestrial input and in situ production from phytoplankton.

3.3.4. PLFA based community structure

Relative composition of the PLFA community was calculated using the most abundant PLFAs present in the harbour sediments, except the saturated group which is ubiquitous in origin (Fig. 3.11, 3.12 & 3.13). Gram negative bacteria, cyanobacteria and algae are rich in MUFAs (C16:1n9c, C18:1n8, C18:1n9c and C18:1n11). Branched (i-14:0, i- and a-15:0, i-16:0, i-17:0, i-18:0, 10Me16, 10Me18 and cyclopropyl) PLFAs are abundant in Gram positive bacteria, Gram negative anaerobes and sulfate reducing bacteria. PUFAs (C16:2n6, C18:2n6c, C18:2n6t, C18:3n3, C18:3n6, C20:2, C20:3n3, C20:3n6, C20:4n6, C20:5n3, C22:2 and

C22:6n3) are mostly present in eukaryotes. PLFA community observed in the Visakhapatnam sediments is presented in Fig. 3.11. MUFAs, branched PLFAs and PUFAs accounted for 19 (station #3) to 64 % (station #9), 14 % (station #18) to 61 % (station #4), and from 1 (station #2) to 37 % (station #11) of the total PLFA community in the Visakhapatnam harbour. MUFAs, branched PLFAs and PUFAs accounted for 14% (station #3) to 49% (station #9), 28% (station #9) to 59% (station #3), and from 12% (station #2) to 36% (station #1) of the total PLFA community in the Mandovi estuary (Fig. 3.12). Whereas the MUFAs, branched PLFAs and PUFAs were 38% (station #1) to 51% (station #7), 30% (station #6) to 55% (station #1), and from 7% (station #1) to 21% (station #3) of the total PLFA community in the Zuari estuary (Fig. 3.13). Shift in PLFA composition between the sediment stations indicates the changes in the structure of microbial communities (Frostegård et al., 1993).

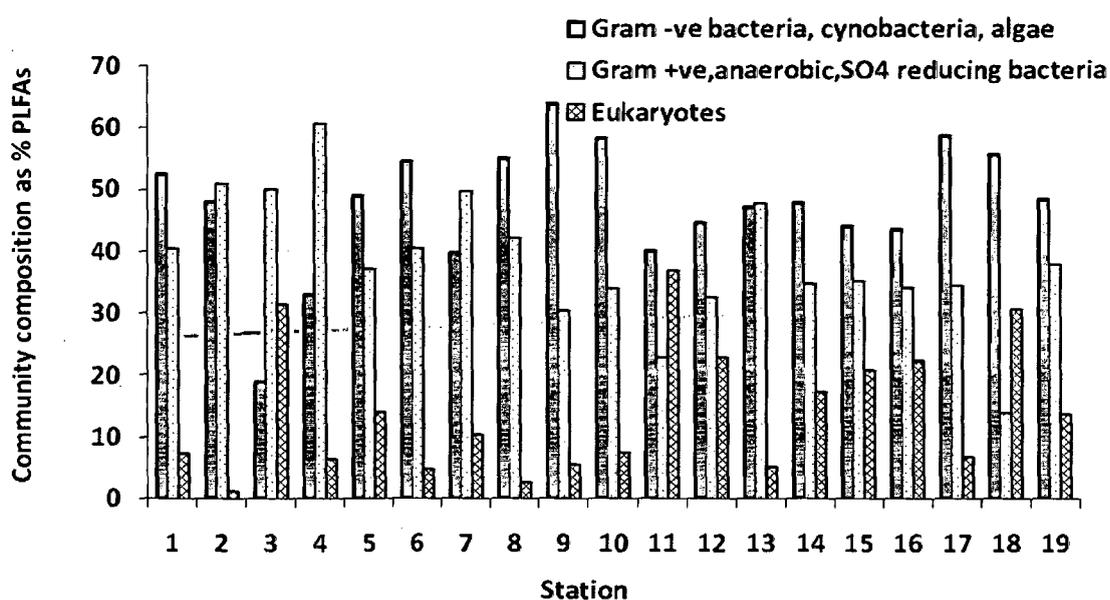


Fig 3.11: Distribution of PLFA community composition as % of the total identified PLFAs excluding saturated PLFAs in sediments of the Visakhapatnam harbour, east coast of India. Sum of PLFAs C16:1n9c, C18:1n8, and C18:1n9c was used to assess the abundance of Gram negative bacteria, cyanobacteria, and algae; sum of i- and a-15:0, i-16:0 and i-17:0 was used to evaluate the abundance of Gram +ve, anaerobic, and SO₄ reducing bacteria; and sum of PUFAs (C18:2n6c, C18:3n3, C18:3n6, C20:3n3, C20:3n6, C20:4n6, C20:5n3 and C22:6n3) was used to assess the abundance of eukaryotes.

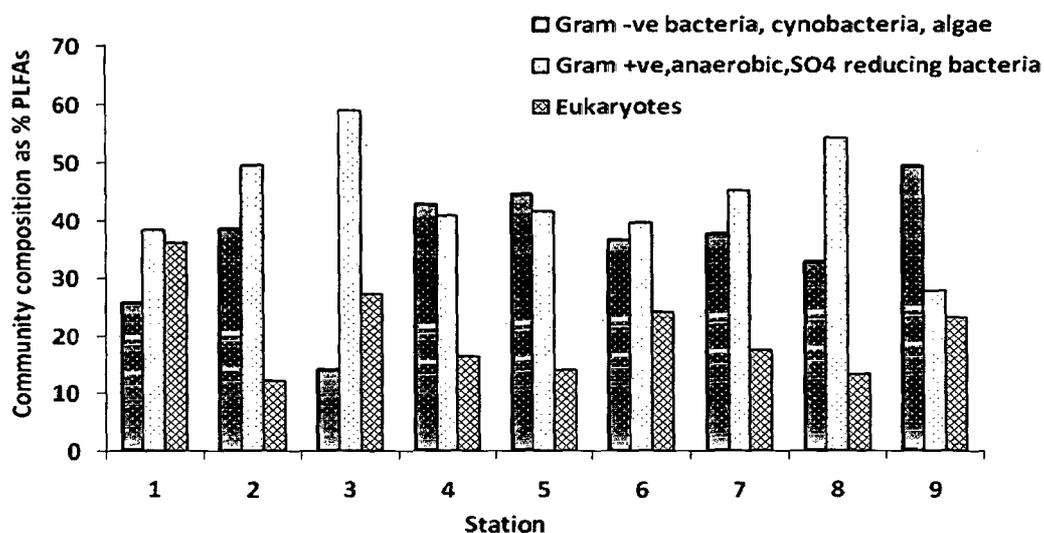


Fig 3.12: Distribution of PLFA community composition as % of the total identified PLFAs excluding saturated PLFAs in sediments of the Mandovi estuary, west coast of India. Sum of PLFAs C16:1n9c and C18:1n9c was used to assess the abundance of Gram negative bacteria, cyanobacteria, and algae; sum of i-14:0, i- and a-15:0, i-16:0, i-17:0, i-18:0, 10Me16, 10Me18 and cy was used to evaluate the abundance of Gram +ve, anaerobic, and SO₄ reducing bacteria; and sum of PUFAs (C18:2n6t, C18:3n3, C18:3n6, C20:2, C20:3n3, C20:3n6, C20:4n6 and C22:2) was used to assess the abundance of eukaryotes.

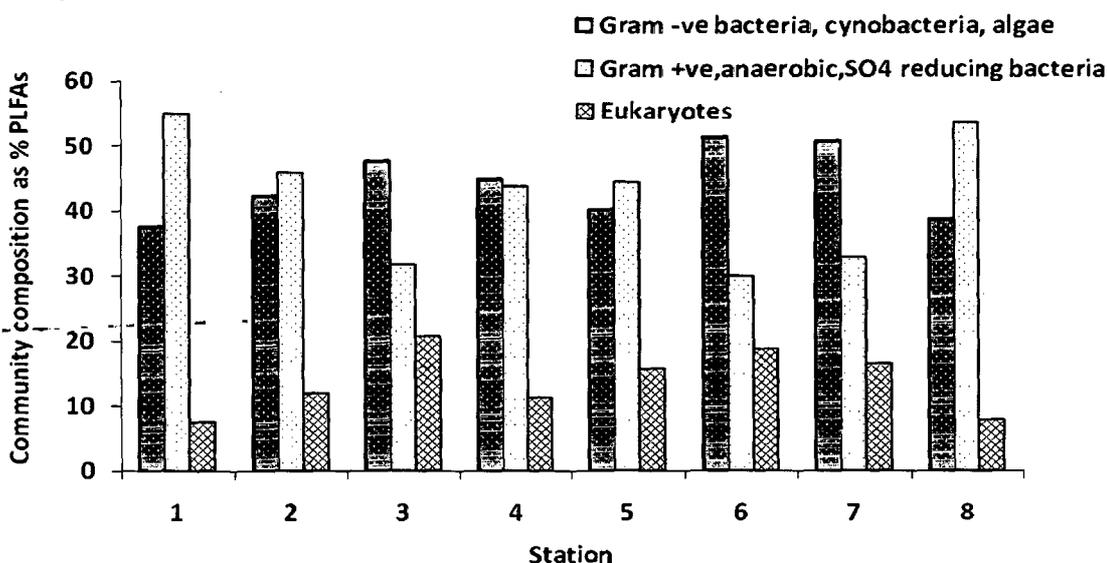


Fig 3.13: Distribution of PLFA community composition as % of the total identified PLFAs excluding saturated PLFAs in sediments of the Zuari estuary, west coast of India. Sum of PLFAs C16:1n9c, C18:1n8, and C18:1n9c was used to assess the abundance of Gram negative bacteria, cyanobacteria, and algae; sum of i- and a-15:0, i-16:0 and i-17:0 was used to evaluate the abundance of Gram +ve, anaerobic, and SO₄ reducing bacteria; and sum of PUFAs (C16:2n6, C18:2n6t, C18:3n3, C18:3n6, C20:2, C20:3n3, C20:3n6, C20:4n6, C20:5n3, C22:2 and C22:6n3) was used to assess the abundance of eukaryotes.

**Distribution and fate of fatty acids in
sediment cores from the Indian Ocean**

4.1. Introduction

Organic matter (OM) in the deep sea sediments is derived from various sources including in situ primary production, terrestrial OM and atmospheric transfer. Nature of OM deposited on the seafloor is influenced by sources of OM, remineralization as well as reworking by organisms in the water column and at the sediment-water interface, concentrations of dissolved oxygen in intermediate and bottom waters, and the sedimentation rate (Budge and Parrish, 1998; Betts and Holland, 1991). Only a small portion (<1%) of the OM produced photosynthetically in the sunlit surface ocean waters by microscopic plants (phytoplankton) sinks through the water column and is ultimately deposited in sediments (Suess, 1980; Martin et al., 1987). Several investigations have shown that the vertical mass flux can be attributed to rapidly sinking, large particles (~50 µm) particles (McCave, 1975; Bishop et al., 1977, 1978, 1980; Honjo, 1978, 1980). Different mineral and organic species, including living and dead organisms, faecal pellets etc are associated with these large particles or aggregates. The flux of the OM from surface waters to deep sediments forms an important source of food to benthic organisms and is an important factor determining the benthic biomass (Maury, 1861; Vinogradov, 1968; Rowe et al., 1991; Gooday, 2002). Mineralization or bacterial degradation is the major transformation process that occurs during transport of particles from surface waters to deep sea sediments (Iturriaga, 1979). The study of the variability of OM deposited on the deep-sea floor sediments and its origin, transformation and degradation is very important for understanding the role of deep sea systems in comprehensive examination of the global carbon cycle.

FAs constitute an important fraction of the lipid pool in living and dead organic material. Their abundance in living organisms, their source specificity with respect to individual compounds and their relative lability make FAs suitable for tracing sources and

diagenetic changes of organic material in water columns and sediments. Numerous studies have used FAs to estimate the relative contributions of terrestrial, algal or planktonic and bacterial FAs to the total FA pool in marine sediments (e.g. Volkman et al., 1980; Prahl et al., 1989; Budge and Parrish, 1998; Zimmerman and Canuel, 2001; Camacho-Ibar et al., 2003). Although most FAs are not unique to one source, major differences in the FA composition of individual or grouped source organisms allow an assignment of predominating sources.

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To understand more fully about the nature and sources of OM and bacterial abundance, the distribution of fatty acids and lipid phosphate (L-PO₄) were studied in two deep sea sediment cores collected from Northern Indian Ocean and Equatorial Indian Ocean. The main objectives of this chapter were 1) to use L-PO₄ to assess the viable bacterial biomass, 2) to evaluate the distribution and fate of fatty acids, and 3) to investigate the sources of OM using fatty acids as biomarkers in these sediment cores.

4.2. Materials and Methods

4.2.1. Description of the sampling areas

Numerous studies are available on the physical, chemical and biological characterization of the waters of the Northern Indian Ocean (Arabian Sea) and Equatorial Indian Ocean (Deuser et al., 1978; Brock et al., 1992; Haake et al., 1993; Azam, et al., 1994; Calvert et al., 1995; Boetius et al., 2000; Naqvi et al., 2002; Wiggert et al., 2005). Northern Indian Ocean is tropical in nature and experience seasonal changes in climate and physical oceanographic processes. The biogeochemistry of the Arabian Sea is strongly influenced by two monsoon periods. Southwest monsoon occurs from late May to early September and the northeast monsoon from December to March (Wiggert et al., 2005). Primary productivity increases dramatically in these two periods due to higher nutrient levels, caused during the southwest-monsoon by coastal upwelling of nutrient-rich waters and during the north-east monsoon by convective mixing together with subsequent advection by large-scale circulation (Naqvi et al., 2002; Kawamiya and Oschlies, 2003). This results in productivity values that are among the highest known for the open ocean. Annual productivity is estimated to be 200 to 400 gCm⁻²yr⁻¹ (Kabanova, 1968; Quasim, 1982). At the end of the algal blooms, detritus sinks through the water column and on to the sea floor (Haake et al., 1993). The sediments of the Arabian Sea therefore receive fluxes of organic material that display strong seasonal variability. Another prominent feature of the Arabian Sea is the midwater Oxygen Minimum Zone (OMZ) with oxygen levels below 0.05 mL/L (van Bennekom et al., 1995), from a water depth of around 100 m down to 1100 m (Wyrki, 1971, 1973; Deuser et al., 1978; Olson et al., 1993; Sinninghe Damsté et al 2002), due to respiration of OM and the input of intermediate waters with low oxygen content, from the Southern Hemisphere (Naqvi, 1987; You and Tomczak, 1993; Warren, 1994). The OMZ coincides roughly with maxima in sedimentary organic carbon, suggesting a link between organic carbon preservation and oxygen availability (Cowie et al., 1999).

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CC2/GCL1 core was 2 meters long. Whole of the CC1/GC3 was analyzed while top 50 cm of CC2/GCL1 was analyzed. The sediment samples in the frozen condition were then transported to the laboratory in an ice box, and stored at -20 °C until lyophilization. The sediments were then freeze dried, homogenized using an agate mortar and pestle to a fine powder and used for the estimation of organic carbon, total lipids, lipid-phosphate and fatty acids.

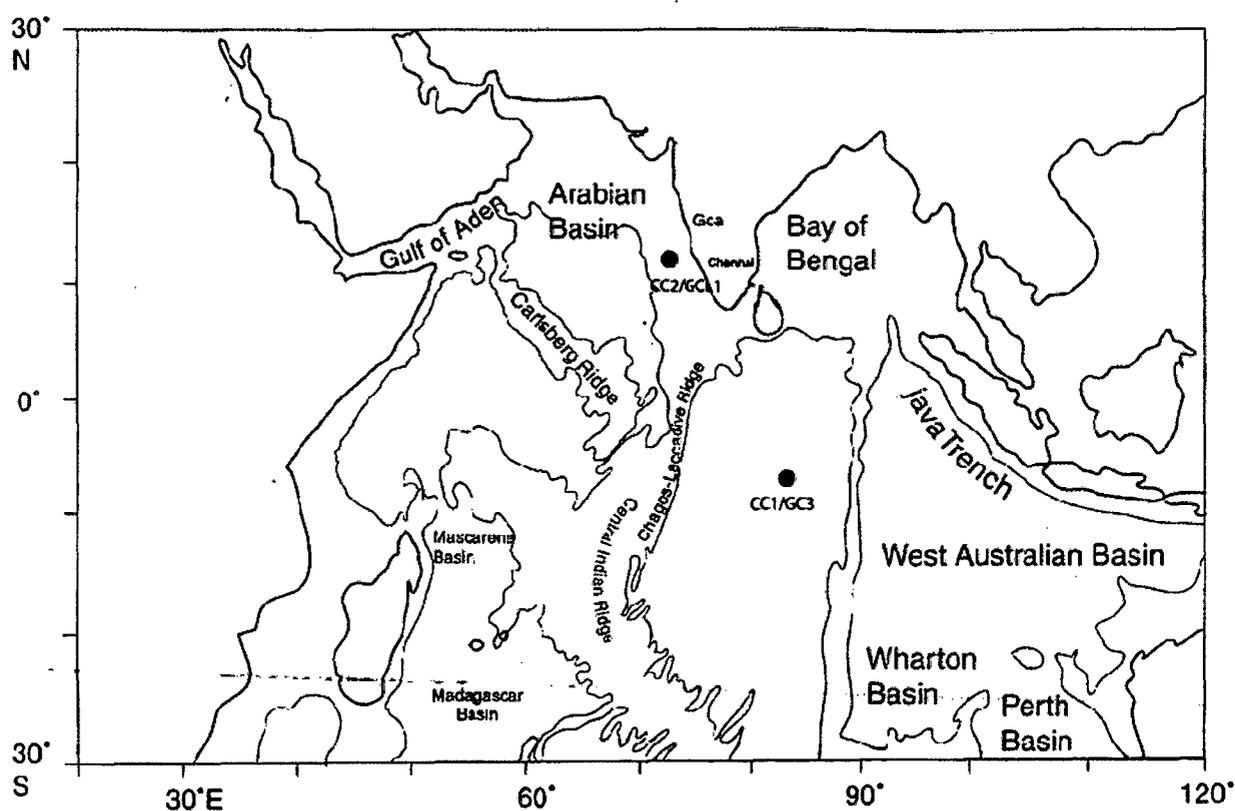


Fig. 4.1. Map showing the locations of the sampling stations of the cores CC1/GC3 in the Equatorial Indian Ocean and CC2/GCL1 in the Northern Indian Ocean.

4.2.3. Cleaning of glassware and purification of solvents

Cleaning of glassware and purification of solvents was done as in chapter 2A.

4.2.4. Estimation of organic carbon (OC)

The OC was determined as given in chapter 3A.

4.2.5. Extraction of lipids

Lipids from the lyophilized sediments (7-15 g for CC1/GC3 and 6-30 g for CC2/GCL1) were extracted following the modified method of Bligh and Dyer (1959) as described by White et al. (1979). In brief, a known quantity of the homogenized sediments was transferred to a clean flask and were ultrasonically extracted with 300ml (100ml x 3) of chloroform-methanol-phosphate buffer (1:2:0.8, v/v/v) in an ice bath for 15 min x 3. The supernatant then transferred to separating funnel and shaken vigorously and kept standing for minimum 24 h at room temperature (28 ± 2 °C) in order to extract the total lipids. Separation of the organic phase was achieved by adding 1:1 ratio of chloroform: water in a separating funnel and shaking it vigorously for 30 min. The lower phase was pooled, filtered through Whatman No. 1 filter paper, and treated with anhydrous sodium sulphate to remove traces of water. The extract was now called as total lipid extracts (TLE). The TLE was rotary evaporated at 30 °C under vacuum to a small volume. Total lipid was estimated by gravimetric method (Zaghden et al., 2005, Harji et al., 2008; Harji et al., 2010) as mentioned in chapter 3A. A known aliquot of TLE was used to estimate lipid-phosphate (White et al., 1979). In short, the TLE was hydrolyzed using perchloric acid (35 %) at 280°C for 3 hrs till colourless solution appears. After cooling 2.4 ml of molybdate (4.4 g ammonium molybdate + 14 ml concentrated H₂SO₄ in 1000ml distilled water (D/W)) and 2.4 ml of ANSA (0.5g 1-amino-2-naphthol-4-sulfonic acid, 30g sodium bisulfite and 2g sodium sulfite, dissolved in 200 ml D/W, the solution diluted 1:12 with D/W before use) were added and boiled at 100 °C for 15 min. Absorbance was read at 830 nm at the room temperature using a spectrophotometer (UV-1700 Pharma Spec UV-VIS, Shimadzu). Glycerol phosphate (Fluka) was used as a standard. Lipid phosphate concentrations were calculated using the factor

obtained with glycerol phosphate standard. The lipid-phosphate method showed a standard deviation of ± 5.3 %, based on replicate analyses.

4.2.6. Separation of FAs

The remaining TLE was transferred into 15 ml amber coloured glass vial. It was dried under nitrogen and saponified with 5 % w/v KOH in 80 % methanol (D'souza, 2004). Saponification was carried out at 80 °C for 2 h. The sample was cooled, 1 ml of milli-Q water was added and extracted with hexane:CHCl₃ (4:1, v/v, 3 times). The upper organic layer contains neutral lipids and the lower aqueous layer contained the total fatty acids.

4.2.7. Preparation of Fatty acid methyl esters

The aqueous layer containing the fatty acids was acidified to pH < 1 using concentrated HCl. The fatty acids were extracted using Hexane:CHCl₃ (4:1, v/v, 3 times). Traces of water were removed using anhydrous sodium sulfate and solvent was evaporated to dryness using nitrogen gas. The fatty acids were methylated by treating with MeOH:CHCl₃:HCl (10:1:1) for 2 h at 80 °C. After cooling, 1 ml of milli-Q water was added and the fatty acid methyl esters (FAME) were extracted with Hexane:CHCl₃ (4:1, v/v, 3 times). The extract was rotary evaporated at 30 °C under reduced pressure and transferred to vials with dichloromethane. The fatty acid methyl esters were analysed by gas chromatography mass spectrometry (GCMS).

4.2.8. Gas chromatography mass spectrometry (GCMS)

GCMS analyses of the fatty acid methyl esters were performed as mentioned in chapter 2A. The quantification of FAMES was done using the data handling system available on the instrument. The identification of individual FAMES was performed by comparing the retention times of FAMES in the sample with those of authentic standards (FAME 37 mix,

Supelco, USA; BAMEs, Supelco and FAME standards, Sigma-Aldrich-Fluka). The peak areas were quantified relative to an internal standard (hexacosanoate methyl ester) added to the sample prior to GCMS- analysis. Peak identification was verified by comparing the EI spectra of sample FAMEs to spectra from standards and published data.

Solvents were passed through the entire analytical procedure and run on GCMS as blanks to check the probable contamination. Sample values have been corrected for the blank levels.

4.2.9. Statistical Analysis

Regression analysis was employed to assess the relationship between various parameters (Sokal & Rohlf, 1981) using the Excell software program loaded on the personal computer.

4.3. Results and discussion

4.3.1 Bulk parameters

Organic carbon (OC) varied from 0.08 % (20-22cm) to 0.36 % (0-2cm) in CC1/GC3 core (Fig. 4.2a), whereas it varied from 1.07 % (20-22cm) to 2.3 % (0-2cm) in CC2/GCL1 core (Fig. 4.2b).—When normalized to sediment dry weight, OC varied from 0.81 to 3.61 mg OC g⁻¹ dw sediment for core CC1/GC3, and 10.7 to 23.2 mg OC g⁻¹ dw sediment for core CC2/GCL1. This shows higher OC values in the sediments of the Northern Indian Ocean. The distribution of OC in these sediment cores was similar to that observed in other marine sediments (e.g., Haddad et al., 1992; Bourbonniere and Meyers, 1996), showing a somewhat erratic trend with a general decrease in concentrations with depth. OC concentrations of 5.5, 33 and 39 mg OC g⁻¹ at the top 0-2 cm depth of the cores VAGO-03, AP-09 and CD-06 collected from the Northern Gulf of California (NGC) was recorded (Camacho-Ibar et al., 2003). The Northern Indian Ocean is a highly productive marine ecosystem with primary

production rates frequently exceeding 200 to 400 $\text{gCm}^{-2}\text{yr}^{-1}$ (Kabanova, 1968; Quasim, 1982), which is equivalent to the global productivity mean in estuaries, coastal lagoons and upwelling ecosystems ($\sim 300 \text{ g C m}^{-2} \text{ year}^{-1}$; Day et al., 1989; Knoppers, 1994). This suggests the occurrence of a large flux of labile organic carbon to the sediments of this ecosystem. Further, due to overlying OMZ (100 to 1100m water depth), high rates of OM accumulation may result from greater primary productivity, high sedimentation rates and increased preservation due to exposure to low oxygen (Sinninghe Damsté, et al., 2002). As compared to the Northern Indian Ocean, the equatorial Indian Ocean (EIO) is biologically less productive. The chlorophyll distribution, with the exception of the coastal boundaries, indicates that the EIO is a 'biological desert' as compared to any other regions of the Northern Indian Ocean (Narvekar & Prasanna Kumar, 2010).

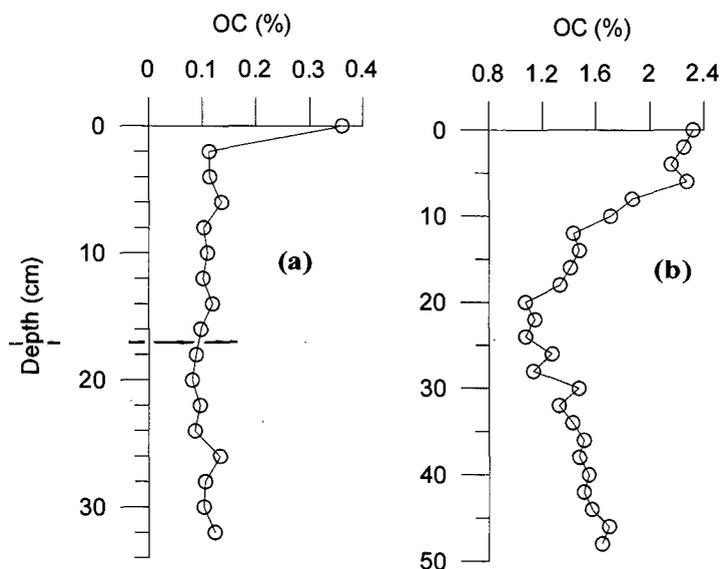


Fig. 4.2 Vertical profiles of organic carbon (OC %) in the sediment core CC1/GC3 and CC2/GCL1 collected from Equatorial Indian Ocean and Northern Indian Ocean

Distribution of the total lipid (TLE), lipid phosphate (L- PO_4) and total fatty acids (TFA) in the cores CC1/GC3, and CC2/GCL is shown in Fig. 4.3 and Fig. 4.4, respectively. In the core CC1/GC3, TLE, L- PO_4 , and TFA varied from 0.07 to 0.52 $\text{mg g}^{-1}\text{dw}$ sediment,

0.27 to 3.45 $\mu\text{g g}^{-1}$ dw sediment, and from 0.65 to 6.42 $\mu\text{g g}^{-1}$ dw sediment, respectively (Fig. 4.3). Similarly, for the core CC2/GCL TLE, L- PO_4 , and TFA varied from 0.12 to 1.41 mg g^{-1} dw sediment, 0.23 to 6.71 $\mu\text{g g}^{-1}$ dw (4-6cm), and from 0.6 to 19.2 $\mu\text{g g}^{-1}$ dw sediment, respectively (Fig. 4.4). TFA levels in cores CC1/GC3 and CC2/GCL1 were similar to those reported for the other deep sea sediments (Saliot et al., 1988). Lower concentrations of TFA were recorded at the deeper sections of CC1/GC3 with subsurface maxima at 4-6 cm and at 18-20 cm sections (Fig. 4.3c), indicating higher abundance of this biomarker in these sections. In the CC2/GCL1 core, maximum concentrations were obtained at 0-2 cm, 10-12 cm, 34-36 cm, 46-48 cm and 48-50 cm depth intervals, whereas lowest values were recorded in 14-16 cm and 26-28 cm of the core (Fig. 4.4c). Maximum concentrations of TFA in the CC2/GCL1 core indicate better preservation of OM. TFA concentrations suggests the Northern Indian Ocean sediments contain higher amounts of FAs ($>2\mu\text{g g}^{-1}$ dw at most sections) compared to the EIO ($<2\mu\text{g g}^{-1}$ dw at most sections). These trends are similar to those observed for the distribution of OC.

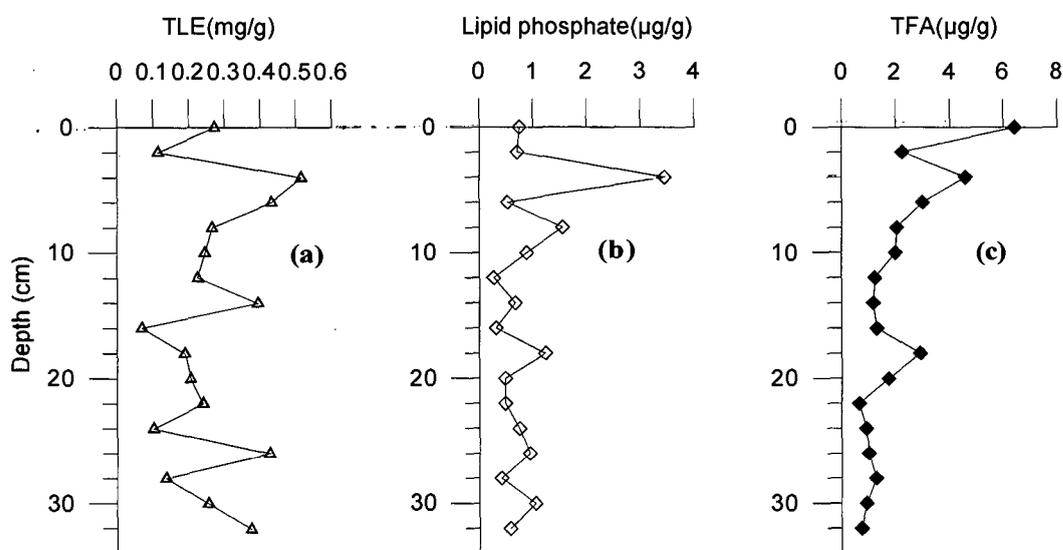


Fig. 4.3. Vertical profiles of total lipid extract (TLE), lipid phosphate and total fatty acids (TFA) in the sediment core CC1/GC3 collected from central Indian Ocean.

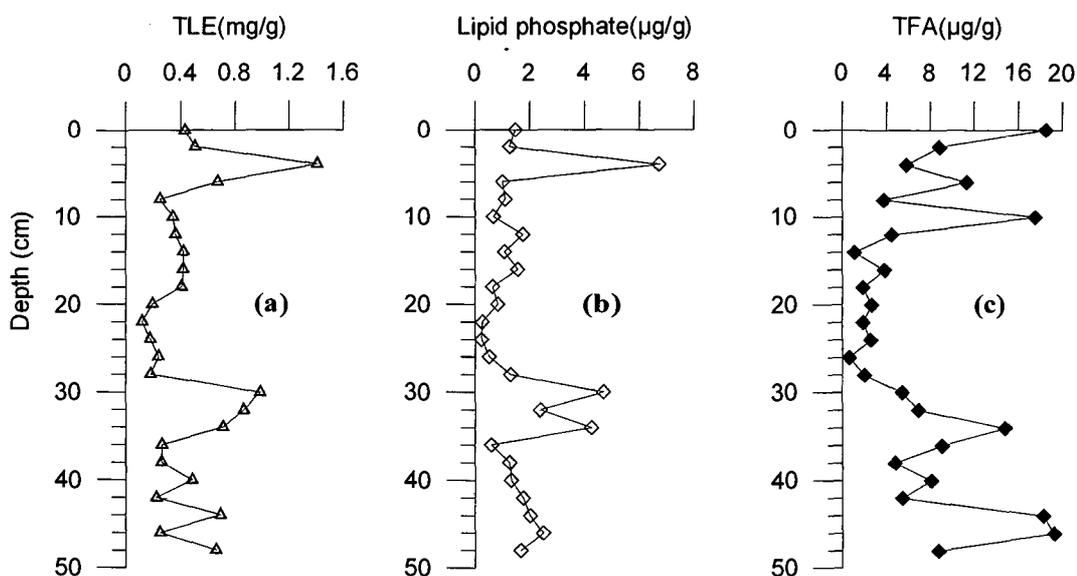


Fig. 4.4. Vertical profiles of total lipid extract (TLE), lipid phosphate and total fatty acids (TFA) in the sediment core CC2/GCL1 collected from northern Indian Ocean.

Differences between the two areas however become apparent when comparing the OC-normalized concentrations. OC normalized TFA values varied from 0.6 (32-34cm) to 4.0 (4-6cm) mg g^{-1} OC in the CC1/GC3 core, whereas in the CC2/GCL1 core the values varied from 0.05 (26-28cm) to 1.2 mg/g OC (44-46cm). These concentrations fall within the range of values reported in the sediments from other oceanic regions. OC normalized values varied from 0.8–1.6 mg g^{-1} OC off Antofagasta surface sediments and 1.8–4.9 mg g mg^{-1} OC off Concepción (Niggeman and Schubert, 2006). Two major factors affect the concentrations of TFA in the surface sediments. During early diagenesis, FAs are degraded preferentially over the bulk organic carbon pool (e.g. Wakeham et al., 1997b; Camacho-Ibar et al., 2003). Moreover, numerous studies provide evidence that deep water depths (>800m) favour low TFA concentrations in sediments, e.g. 1.8 mg g^{-1} OC in Black Sea (Sun and Wakeham, 1994); 2.5 mg g^{-1} OC in the anoxic depocenter and of 0.8 mg g^{-1} OC in the oxic periphery in Santa Monica Basin (Gong and Hollander, 1997). Whereas shallow water depths (<350 m) favour high TFA concentrations in sediments, e.g. 3.2 and 11.5 mg g^{-1} OC in St. Lawrence Estuary

(Colombo et al., 1997), 14 mg g⁻¹ OC in Buzzard Bay (Farrington et al., 1977), 3.4– 22 mg g⁻¹ OC in Cape Lookout Bight (Haddad et al., 1992; Canuel and Martens, 1996), and up to 35.7 mg g⁻¹ OC in Chesapeake Bay (Zimmerman and Canuel, 2001). Another factor influencing concentrations in the sediments is the input of TFA. It has been shown that TFA concentrations in surface sediments reflect seasonal changes in TFA input, e.g. high concentrations coincide with algal bloom events in the overlying surface water (Zimmerman and Canuel, 2001; Gogou and Stephanou, 2004). Both the sampling sites showed in general lower concentrations of TFA mg g⁻¹OC, which is in accordance with the deep sea distribution of TFA. However, this range of OC normalized TFA values in the EIO core (CC1/GC3) were higher compared to the Northern Indian Ocean core (CC/GCL1). From the previous studies it is evident that EIO is a biological desert, which is seen from very low chlorophyll values observed in this area, whereas Northern Indian Ocean is highly productive (Kabanova, 1968; Quasim, 1982). This distribution suggests that TFA are better preserved in the EIO compared to Northern Indian Ocean sediments. As TFA fraction of organic matter is 2–9 times more labile than the whole of the organic carbon pool (Camacho-Ibar et al., 2003), comparatively low values in the Northern Indian Ocean may be due to higher degradation of the TFA in this environment.

4.3.2 Lipid-phosphate based bacterial biomass

The determination of bacterial biomass in complex environmental samples is difficult, as the classical techniques of viable counting most often recover a small and variable proportion of the cells detectable by direct counting (Alexander, 1977; Jenkinson and Ladd, 1981). Hence analysis of cell membrane phospholipids such as lipid-bound phosphate (lipid phosphate) is thus developed and successfully applied to characterize bacterial biomass and its structure (White et al., 1979). Under the conditions expected in natural communities, the

bacteria contain a relatively constant proportion of their biomass as phospholipids (White et al., 1979a; White et al., 1979 b; Balkwill et al., 1988). Phospholipids are not found in storage lipids and have a relatively rapid turnover in sediments so the assay of these lipids gives a measure of the “viable” cellular biomass (White et al., 1979). Bacterial biomass measures based on membrane lipids (estimated as the polar lipid fatty acids and the lipid phosphate), lipopolysaccharide (estimated as the LPS hydroxy fatty acids), cell walls (estimated as the muramic acid), and adenosine triphosphate have previously shown to give identical estimates of cell numbers using conversion factors determined on subsurface microorganism monocultures (White et al., 1979; Balkwill et al., 1988; Findlay et al., 1989). The analysis of L-PO₄ provides a relatively simple and convenient method for estimating bacterial biomass (White et al., 1979; Findlay et al., 1989). This method provides greater sample throughput than the microscopic technique and yields more quantitative recoveries (White et al., 1979; Findlay et al., 1989). It is also seen to agree with AODC counts (Balkwill et al., 1988). L-PO₄ varied between different sections of the sediment cores (Fig. 4.3b and 4.4b). L-PO₄ showed good correlation with TLE ($r = 0.84$, $n = 25$, $p = 0.001$) in CC2/GCL1 core indicating the viable bacterial biomass contributed sufficiently to the TLE, whereas in CC1/GC3 core showed relatively weak relationship ($r = 0.51$, $n=17$, $p = 0.05$).

Relatively higher values of L-PO₄ were observed at 4-6 cm section of both the cores (Fig. 4.3 & Fig. 4.4). However, lower concentrations at 0-2 cm and 2-4 cm of section of these cores indicates the surficial layers may be disturbed due to various activities (bioturbation) occurring at the sediment surface. Higher L-PO₄ concentrations reflect larger viable bacterial biomass (White et al., 1979; Balkwill, et al., 1988; Villanueva et al., 2004). The bacterial biomass was calculated using a conversion factor (50 μmol of lipid phosphate in 2×10^{12} dw cells) by Balkwill et al. (1988). Using this factor bacterial biomass based on lipid-phosphate

4.1. Introduction

Organic matter (OM) in the deep sea sediments is derived from various sources including in situ primary production, terrestrial OM and atmospheric transfer. Nature of OM deposited on the seafloor is influenced by sources of OM, remineralization as well as reworking by organisms in the water column and at the sediment-water interface, concentrations of dissolved oxygen in intermediate and bottom waters, and the sedimentation rate (Budge and Parrish, 1998; Betts and Holland, 1991). Only a small portion (<1%) of the OM produced photosynthetically in the sunlit surface ocean waters by microscopic plants (phytoplankton) sinks through the water column and is ultimately deposited in sediments (Suess, 1980; Martin et al., 1987). Several investigations have shown that the vertical mass flux can be attributed to rapidly sinking, large particles (~50 μm) particles (McCave, 1975; Bishop et al., 1977, 1978, 1980; Honjo, 1978, 1980). Different mineral and organic species, including living and dead organisms, faecal pellets etc are associated with these large particles or aggregates. The flux of the OM from surface waters to deep sediments forms an important source of food to benthic organisms and is an important factor determining the benthic biomass (Maury, 1861; Vinogradov, 1968; Rowe et al., 1991; Gooday, 2002). Mineralization or bacterial degradation is the major transformation process that occurs during transport of particles from surface waters to deep sea sediments (Iturriaga, 1979). The study of the variability of OM deposited on the deep-sea floor sediments and its origin, transformation and degradation is very important for understanding the role of deep sea systems in comprehensive examination of the global carbon cycle.

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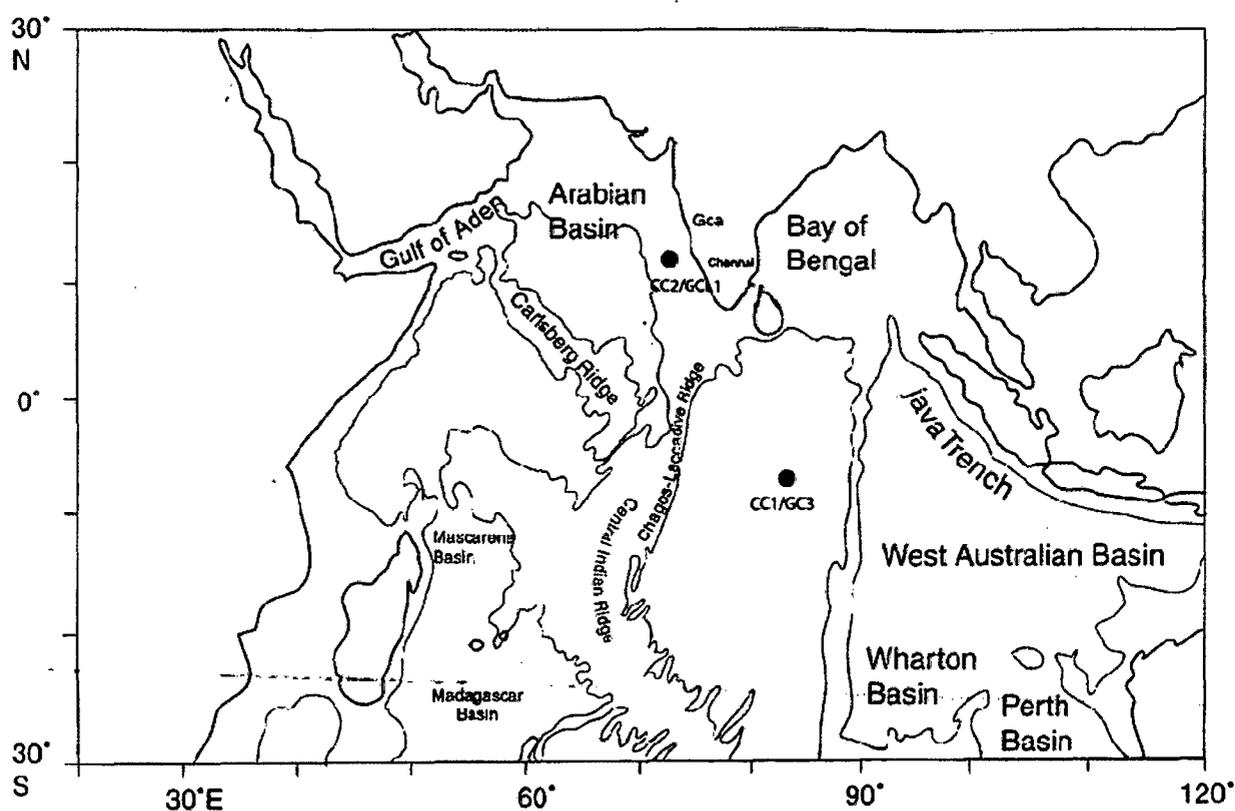


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The OC was determined as given in chapter 3A.

4.2.5. Extraction of lipids

Lipids from the lyophilized sediments (7-15 g for CC1/GC3 and 6-30 g for CC2/GCL1) were extracted following the modified method of Bligh and Dyer (1959) as described by White et al. (1979). In brief, a known quantity of the homogenized sediments was transferred to a clean flask and were ultrasonically extracted with 300ml (100ml x 3) of chloroform-methanol-phosphate buffer (1:2:0.8, v/v/v) in an ice bath for 15 min x 3. The supernatant then transferred to separating funnel and shaken vigorously and kept standing for minimum 24 h at room temperature (28 ± 2 °C) in order to extract the total lipids. Separation of the organic phase was achieved by adding 1:1 ratio of chloroform: water in a separating funnel and shaking it vigorously for 30 min. The lower phase was pooled, filtered through Whatman No. 1 filter paper, and treated with anhydrous sodium sulphate to remove traces of water. The extract was now called as total lipid extracts (TLE). The TLE was rotary evaporated at 30 °C under vacuum to a small volume. Total lipid was estimated by gravimetric method (Zaghden et al., 2005, Harji et al., 2008; Harji et al., 2010) as mentioned in chapter 3A. A known aliquot of TLE was used to estimate lipid-phosphate (White et al., 1979). In short, the TLE was hydrolyzed using perchloric acid (35 %) at 280°C for 3 hrs till colourless solution appears. After cooling 2.4 ml of molybdate (4.4 g ammonium molybdate + 14 ml concentrated H₂SO₄ in 1000ml distilled water (D/W)) and 2.4 ml of ANSA (0.5g 1-amino-2-naphthol-4-sulfonic acid, 30g sodium bisulfite and 2g sodium sulfite, dissolved in 200 ml D/W, the solution diluted 1:12 with D/W before use) were added and boiled at 100 °C for 15 min. Absorbance was read at 830 nm at the room temperature using a spectrophotometer (UV-1700 Pharma Spec UV-VIS, Shimadzu). Glycerol phosphate (Fluka) was used as a standard. Lipid phosphate concentrations were calculated using the factor

obtained with glycerol phosphate standard. The lipid-phosphate method showed a standard deviation of ± 5.3 %, based on replicate analyses.

4.2.6. Separation of FAs

The remaining TLE was transferred into 15 ml amber coloured glass vial. It was dried under nitrogen and saponified with 5 % w/v KOH in 80 % methanol (D'souza, 2004). Saponification was carried out at 80 °C for 2 h. The sample was cooled, 1 ml of milli-Q water was added and extracted with hexane:CHCl₃ (4:1, v/v, 3 times). The upper organic layer contains neutral lipids and the lower aqueous layer contained the total fatty acids.

4.2.7. Preparation of Fatty acid methyl esters

The aqueous layer containing the fatty acids was acidified to pH < 1 using concentrated HCl. The fatty acids were extracted using Hexane:CHCl₃ (4:1, v/v, 3 times). Traces of water were removed using anhydrous sodium sulfate and solvent was evaporated to dryness using nitrogen gas. The fatty acids were methylated by treating with MeOH:CHCl₃:HCl (10:1:1) for 2 h at 80 °C. After cooling, 1 ml of milli-Q water was added and the fatty acid methyl esters (FAME) were extracted with Hexane:CHCl₃ (4:1, v/v, 3 times). The extract was rotary evaporated at 30 °C under reduced pressure and transferred to vials with dichloromethane. The fatty acid methyl esters were analysed by gas chromatography mass spectrometry (GCMS).

4.2.8. Gas chromatography mass spectrometry (GCMS)

GCMS analyses of the fatty acid methyl esters were performed as mentioned in chapter 2A. The quantification of FAMES was done using the data handling system available on the instrument. The identification of individual FAMES was performed by comparing the retention times of FAMES in the sample with those of authentic standards (FAME 37 mix,

Supelco, USA; BAMEs, Supelco and FAME standards, Sigma-Aldrich-Fluka). The peak areas were quantified relative to an internal standard (hexacosanoate methyl ester) added to the sample prior to GCMS- analysis. Peak identification was verified by comparing the EI spectra of sample FAMEs to spectra from standards and published data.

Solvents were passed through the entire analytical procedure and run on GCMS as blanks to check the probable contamination. Sample values have been corrected for the blank levels.

4.2.9. Statistical Analysis

Regression analysis was employed to assess the relationship between various parameters (Sokal & Rohlf, 1981) using the Excell software program loaded on the personal computer.

4.3. Results and discussion

4.3.1 Bulk parameters

Organic carbon (OC) varied from 0.08 % (20-22cm) to 0.36 % (0-2cm) in CC1/GC3 core (Fig. 4.2a), whereas it varied from 1.07 % (20-22cm) to 2.3 % (0-2cm) in CC2/GCL1 core (Fig. 4.2b).—When normalized to sediment dry weight, OC varied from 0.81 to 3.61 mg OC g⁻¹ dw sediment for core CC1/GC3, and 10.7 to 23.2 mg OC g⁻¹ dw sediment for core CC2/GCL1. This shows higher OC values in the sediments of the Northern Indian Ocean. The distribution of OC in these sediment cores was similar to that observed in other marine sediments (e.g., Haddad et al., 1992; Bourbonniere and Meyers, 1996), showing a somewhat erratic trend with a general decrease in concentrations with depth. OC concentrations of 5.5, 33 and 39 mg OC g⁻¹ at the top 0-2 cm depth of the cores VAGO-03, AP-09 and CD-06 collected from the Northern Gulf of California (NGC) was recorded (Camacho-Ibar et al., 2003). The Northern Indian Ocean is a highly productive marine ecosystem with primary

production rates frequently exceeding 200 to 400 $\text{gCm}^{-2}\text{yr}^{-1}$ (Kabanova, 1968; Quasim, 1982), which is equivalent to the global productivity mean in estuaries, coastal lagoons and upwelling ecosystems ($\sim 300 \text{ g C m}^{-2} \text{ year}^{-1}$; Day et al., 1989; Knoppers, 1994). This suggests the occurrence of a large flux of labile organic carbon to the sediments of this ecosystem. Further, due to overlying OMZ (100 to 1100m water depth), high rates of OM accumulation may result from greater primary productivity, high sedimentation rates and increased preservation due to exposure to low oxygen (Sinninghe Damsté, et al., 2002). As compared to the Northern Indian Ocean, the equatorial Indian Ocean (EIO) is biologically less productive. The chlorophyll distribution, with the exception of the coastal boundaries, indicates that the EIO is a 'biological desert' as compared to any other regions of the Northern Indian Ocean (Narvekar & Prasanna Kumar, 2010).

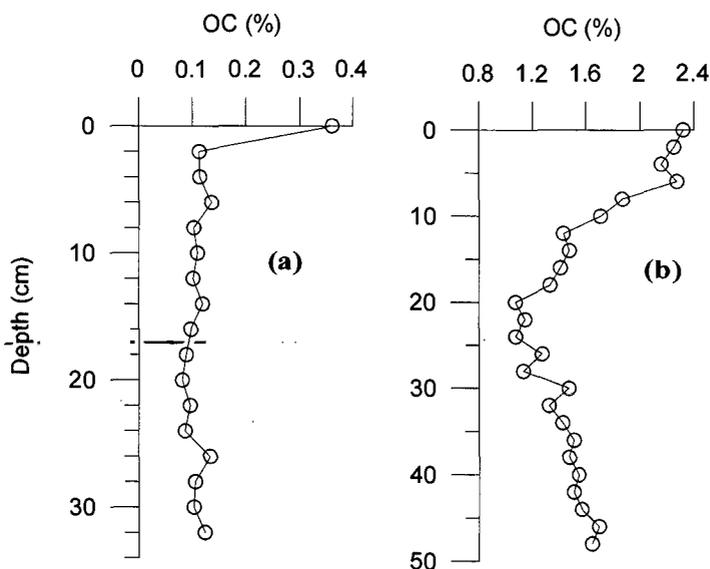


Fig. 4.2 Vertical profiles of organic carbon (OC %) in the sediment core CC1/GC3 and CC2/GCL1 collected from Equatorial Indian Ocean and Northern Indian Ocean

Distribution of the total lipid (TLE), lipid phosphate (L- PO_4) and total fatty acids (TFA) in the cores CC1/GC3, and CC2/GCL is shown in Fig. 4.3 and Fig. 4.4, respectively. In the core CC1/GC3, TLE, L- PO_4 , and TFA varied from 0.07 to 0.52 $\text{mg g}^{-1}\text{dw}$ sediment,

0.27 to 3.45 $\mu\text{g g}^{-1}$ dw sediment, and from 0.65 to 6.42 $\mu\text{g g}^{-1}$ dw sediment, respectively (Fig. 4.3). Similarly, for the core CC2/GCL TLE, L- PO_4 , and TFA varied from 0.12 to 1.41 mg g^{-1} dw sediment, 0.23 to 6.71 $\mu\text{g g}^{-1}$ dw (4-6cm), and from 0.6 to 19.2 $\mu\text{g g}^{-1}$ dw sediment, respectively (Fig. 4.4). TFA levels in cores CC1/GC3 and CC2/GCL1 were similar to those reported for the other deep sea sediments (Saliot et al., 1988). Lower concentrations of TFA were recorded at the deeper sections of CC1/GC3 with subsurface maxima at 4-6 cm and at 18-20 cm sections (Fig. 4.3c), indicating higher abundance of this biomarker in these sections. In the CC2/GCL1 core, maximum concentrations were obtained at 0-2 cm, 10-12 cm, 34-36 cm, 46-48 cm and 48-50 cm depth intervals, whereas lowest values were recorded in 14-16 cm and 26-28 cm of the core (Fig. 4.4c). Maximum concentrations of TFA in the CC2/GCL1 core indicate better preservation of OM. TFA concentrations suggests the Northern Indian Ocean sediments contain higher amounts of FAs ($>2\mu\text{g g}^{-1}$ dw at most sections) compared to the EIO ($<2\mu\text{g g}^{-1}$ dw at most sections). These trends are similar to those observed for the distribution of OC.

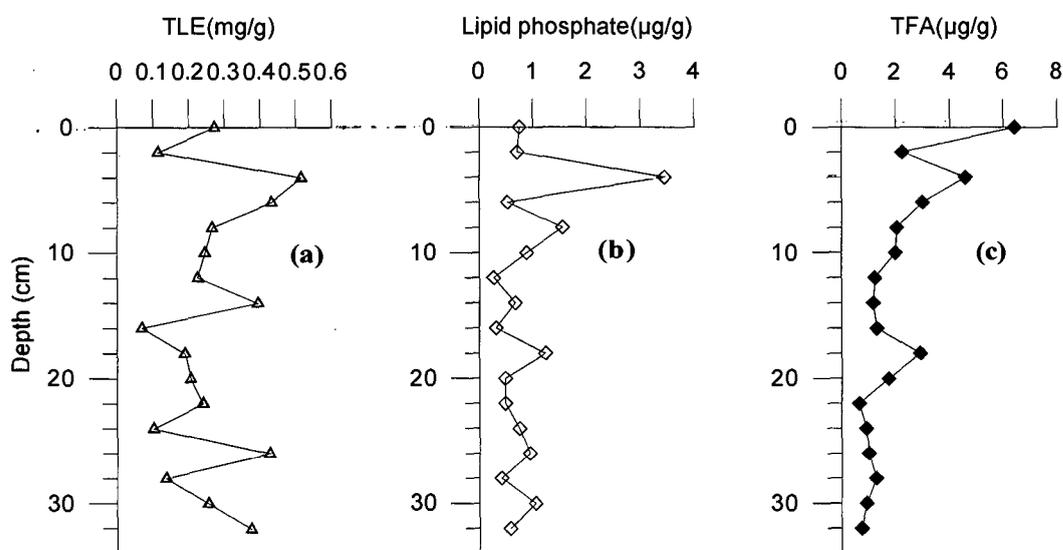


Fig. 4.3. Vertical profiles of total lipid extract (TLE), lipid phosphate and total fatty acids (TFA) in the sediment core CC1/GC3 collected from central Indian Ocean.

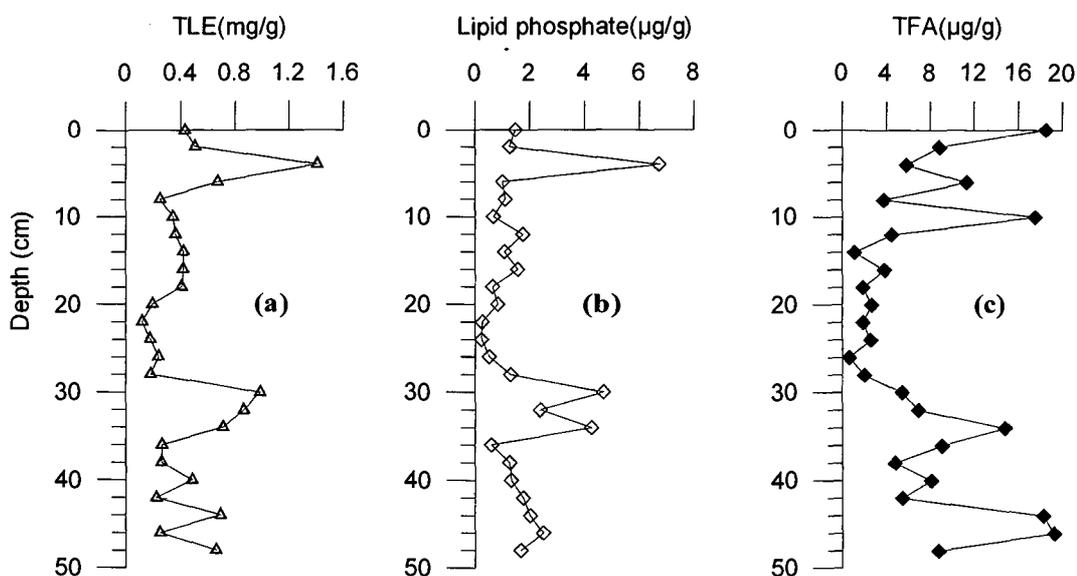


Fig. 4.4. Vertical profiles of total lipid extract (TLE), lipid phosphate and total fatty acids (TFA) in the sediment core CC2/GCL1 collected from northern Indian Ocean.

Differences between the two areas however become apparent when comparing the OC-normalized concentrations. OC normalized TFA values varied from 0.6 (32-34cm) to 4.0 (4-6cm) mg g^{-1} OC in the CC1/GC3 core, whereas in the CC2/GCL1 core the values varied from 0.05 (26-28cm) to 1.2 mg/g OC (44-46cm). These concentrations fall within the range of values reported in the sediments from other oceanic regions. OC normalized values varied from 0.8–1.6 mg g^{-1} OC off Antofagasta surface sediments and 1.8–4.9 mg g mg^{-1} OC off Concepción (Niggeman and Schubert, 2006). Two major factors affect the concentrations of TFA in the surface sediments. During early diagenesis, FAs are degraded preferentially over the bulk organic carbon pool (e.g. Wakeham et al., 1997b; Camacho-Ibar et al., 2003). Moreover, numerous studies provide evidence that deep water depths (>800m) favour low TFA concentrations in sediments, e.g. 1.8 mg g^{-1} OC in Black Sea (Sun and Wakeham, 1994); 2.5 mg g^{-1} OC in the anoxic depocenter and of 0.8 mg g^{-1} OC in the oxic periphery in Santa Monica Basin (Gong and Hollander, 1997). Whereas shallow water depths (<350 m) favour high TFA concentrations in sediments, e.g. 3.2 and 11.5 mg g^{-1} OC in St. Lawrence Estuary

(Colombo et al., 1997), 14 mg g⁻¹ OC in Buzzard Bay (Farrington et al., 1977), 3.4– 22 mg g⁻¹ OC in Cape Lookout Bight (Haddad et al., 1992; Canuel and Martens, 1996), and up to 35.7 mg g⁻¹ OC in Chesapeake Bay (Zimmerman and Canuel, 2001). Another factor influencing concentrations in the sediments is the input of TFA. It has been shown that TFA concentrations in surface sediments reflect seasonal changes in TFA input, e.g. high concentrations coincide with algal bloom events in the overlying surface water (Zimmerman and Canuel, 2001; Gogou and Stephanou, 2004). Both the sampling sites showed in general lower concentrations of TFA mg g⁻¹OC, which is in accordance with the deep sea distribution of TFA. However, this range of OC normalized TFA values in the EIO core (CC1/GC3) were higher compared to the Northern Indian Ocean core (CC/GCL1). From the previous studies it is evident that EIO is a biological desert, which is seen from very low chlorophyll values observed in this area, whereas Northern Indian Ocean is highly productive (Kabanova, 1968; Quasim, 1982). This distribution suggests that TFA are better preserved in the EIO compared to Northern Indian Ocean sediments. As TFA fraction of organic matter is 2–9 times more labile than the whole of the organic carbon pool (Camacho-Ibar et al., 2003), comparatively low values in the Northern Indian Ocean may be due to higher degradation of the TFA in this environment.

4.3.2 Lipid-phosphate based bacterial biomass

The determination of bacterial biomass in complex environmental samples is difficult, as the classical techniques of viable counting most often recover a small and variable proportion of the cells detectable by direct counting (Alexander, 1977; Jenkinson and Ladd, 1981). Hence analysis of cell membrane phospholipids such as lipid-bound phosphate (lipid phosphate) is thus developed and successfully applied to characterize bacterial biomass and its structure (White et al., 1979). Under the conditions expected in natural communities, the

diagenetic changes of organic material in water columns and sediments. Numerous studies have used FAs to estimate the relative contributions of terrestrial, algal or planktonic and bacterial FAs to the total FA pool in marine sediments (e.g. Volkman et al., 1980; Prahl et al., 1989; Budge and Parrish, 1998; Zimmerman and Canuel, 2001; Camacho-Ibar et al., 2003). Although most FAs are not unique to one source, major differences in the FA composition of individual or grouped source organisms allow an assignment of predominating sources.

To determine the microbial biomass in natural environments is one of the greatest challenges to microbial ecologists. In the nature more than 90% of indigenous microorganisms escape cultivation using the traditional culture-dependent techniques (White et al., 1979; DeLong and Pace, 2001). Hence a number of culture independent methods such as lipid phosphate, PLFAs (White et al., 1979; Pinkart et al., 2002; Petsch et al., 2003) and genetic fingerprinting (Polymenakou et al., 2005), have been used to detect viable microbial biomass and changes in the composition of natural microbial communities. Lipid phosphates are used to estimate viable microbial biomass in marine sediments (White et al., 1979; Balkwil et al., 1988; Findlay et al., 1989).

To understand more fully about the nature and sources of OM and bacterial abundance, the distribution of fatty acids and lipid phosphate (L-PO₄) were studied in two deep sea sediment cores collected from Northern Indian Ocean and Equatorial Indian Ocean. The main objectives of this chapter were 1) to use L-PO₄ to assess the viable bacterial biomass, 2) to evaluate the distribution and fate of fatty acids, and 3) to investigate the sources of OM using fatty acids as biomarkers in these sediment cores.

4.2. Materials and Methods

4.2.1. Description of the sampling areas

Numerous studies are available on the physical, chemical and biological characterization of the waters of the Northern Indian Ocean (Arabian Sea) and Equatorial Indian Ocean (Deuser et al., 1978; Brock et al., 1992; Haake et al., 1993; Azam, et al., 1994; Calvert et al., 1995; Boetius et al., 2000; Naqvi et al., 2002; Wiggert et al., 2005). Northern Indian Ocean is tropical in nature and experience seasonal changes in climate and physical oceanographic processes. The biogeochemistry of the Arabian Sea is strongly influenced by two monsoon periods. Southwest monsoon occurs from late May to early September and the northeast monsoon from December to March (Wiggert et al., 2005). Primary productivity increases dramatically in these two periods due to higher nutrient levels, caused during the southwest-monsoon by coastal upwelling of nutrient-rich waters and during the north-east monsoon by convective mixing together with subsequent advection by large-scale circulation (Naqvi et al., 2002; Kawamiya and Oschlies, 2003). This results in productivity values that are among the highest known for the open ocean. Annual productivity is estimated to be 200 to 400 gCm⁻²yr⁻¹ (Kabanova, 1968; Quasim, 1982). At the end of the algal blooms, detritus sinks through the water column and on to the sea floor (Haake et al., 1993). The sediments of the Arabian Sea therefore receive fluxes of organic material that display strong seasonal variability. Another prominent feature of the Arabian Sea is the midwater Oxygen Minimum Zone (OMZ) with oxygen levels below 0.05 mL/L (van Bennekom et al., 1995), from a water depth of around 100 m down to 1100 m (Wyrki, 1971, 1973; Deuser et al., 1978; Olson et al., 1993; Sinninghe Damsté et al 2002), due to respiration of OM and the input of intermediate waters with low oxygen content, from the Southern Hemisphere (Naqvi, 1987; You and Tomczak, 1993; Warren, 1994). The OMZ coincides roughly with maxima in sedimentary organic carbon, suggesting a link between organic carbon preservation and oxygen availability (Cowie et al., 1999).

Equatorial Indian Ocean (EIO) is different from other equatorial regions of the world ocean because of the reversal of the wind system twice a year due to which the surface currents reverse semiannually. Equatorial upwelling does not occur in the Equatorial Indian Ocean as it does in the Pacific and Atlantic, because in those oceans, upwelling occurs as a result of the southeast trade winds which blow across the equator and cause surface divergence. Since in the equatorial Indian Ocean, there is no such wind system in either season (winter and summer) of the year, typical conditions for equatorial upwelling are missing. Chlorophyll pigment concentration shows that EIO away from the coastal boundaries is a 'biological desert' with least chlorophyll concentration compared to any other region in the northern Indian Ocean (Narvekar & Prasanna Kumar, 2010). In the central EIO chlorophyll concentration are almost uniform (0.1-0.2 mg/m³) without much variability..

4.2.2. Sample collection and analysis

One sediment core CC1/GC3 was collected at a station (5.003 °S and 83.052 °E) in the EIO during R.V.A.A. Sidorenko cruise-59 from January 31 to February 24, 2003 (Fig. 4.1). Another core CC2/GCL1 was collected from a station (11.59 °N and 73.30 °E) in the Northern Indian-Ocean during R.V.A.A. Sidorenko, cruise 65 from July 17 to August 16, 2003 (Fig. 4.1). Sediment core CC1/GC3 was retrieved from 4550 m water depth in the EIO while sediment core CC2/GCL1 was collected from 2045 m depth in the Northern Indian Ocean. The cores were collected using a gravity corer equipped with PVC pipes. Cores were sectioned on board into 2 cm intervals and immediately frozen. From the previous observation the OMZ lies in between 100 m down to 1100 m in the Arabian Sea (Naqvi et al., 2000; Sinninghe Damsté et al., 2002). This shows that the core CC2/GCL1 was collected from well below the OMZ in the Northern Indian Ocean. The O₂ levels below 2000 m depth are >2ml/l in the Indian Ocean (Sinninghe Damsté et al., 2002). CC1/GC3 core was 34 cm long and

CC2/GCL1 core was 2 meters long. Whole of the CC1/GC3 was analyzed while top 50 cm of CC2/GCL1 was analyzed. The sediment samples in the frozen condition were then transported to the laboratory in an ice box, and stored at -20 °C until lyophilization. The sediments were then freeze dried, homogenized using an agate mortar and pestle to a fine powder and used for the estimation of organic carbon, total lipids, lipid-phosphate and fatty acids.

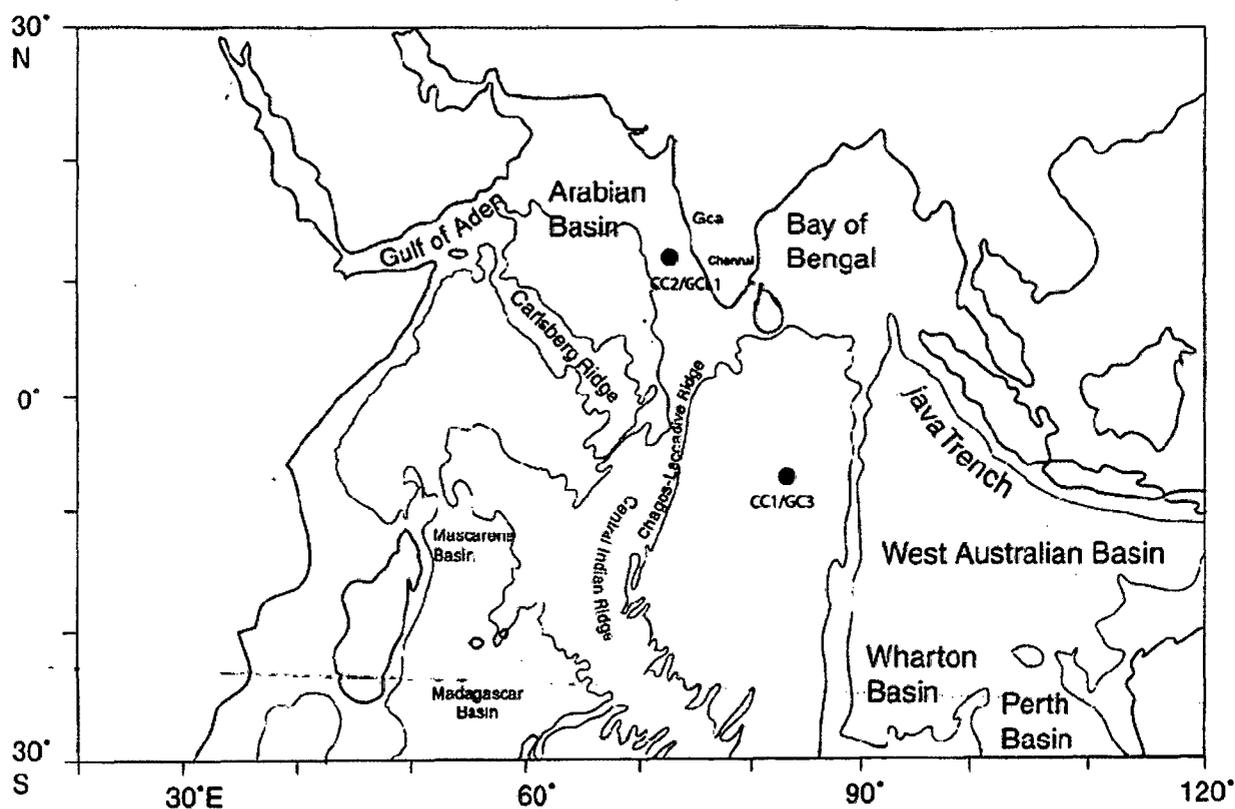


Fig. 4.1. Map showing the locations of the sampling stations of the cores CC1/GC3 in the Equatorial Indian Ocean and CC2/GCL1 in the Northern Indian Ocean.

4.2.3. Cleaning of glassware and purification of solvents

Cleaning of glassware and purification of solvents was done as in chapter 2A.

4.2.4. Estimation of organic carbon (OC)

The OC was determined as given in chapter 3A.

4.2.5. Extraction of lipids

Lipids from the lyophilized sediments (7-15 g for CC1/GC3 and 6-30 g for CC2/GCL1) were extracted following the modified method of Bligh and Dyer (1959) as described by White et al. (1979). In brief, a known quantity of the homogenized sediments was transferred to a clean flask and were ultrasonically extracted with 300ml (100ml x 3) of chloroform-methanol-phosphate buffer (1:2:0.8, v/v/v) in an ice bath for 15 min x 3. The supernatant then transferred to separating funnel and shaken vigorously and kept standing for minimum 24 h at room temperature (28 ± 2 °C) in order to extract the total lipids. Separation of the organic phase was achieved by adding 1:1 ratio of chloroform: water in a separating funnel and shaking it vigorously for 30 min. The lower phase was pooled, filtered through Whatman No. 1 filter paper, and treated with anhydrous sodium sulphate to remove traces of water. The extract was now called as total lipid extracts (TLE). The TLE was rotary evaporated at 30 °C under vacuum to a small volume. Total lipid was estimated by gravimetric method (Zaghden et al., 2005, Harji et al., 2008; Harji et al., 2010) as mentioned in chapter 3A. A known aliquot of TLE was used to estimate lipid-phosphate (White et al., 1979). In short, the TLE was hydrolyzed using perchloric acid (35 %) at 280°C for 3 hrs till colourless solution appears. After cooling 2.4 ml of molybdate (4.4 g ammonium molybdate + 14 ml concentrated H₂SO₄ in 1000ml distilled water (D/W)) and 2.4 ml of ANSA (0.5g 1-amino-2-naphthol-4-sulfonic acid, 30g sodium bisulfite and 2g sodium sulfite, dissolved in 200 ml D/W, the solution diluted 1:12 with D/W before use) were added and boiled at 100 °C for 15 min. Absorbance was read at 830 nm at the room temperature using a spectrophotometer (UV-1700 Pharma Spec UV-VIS, Shimadzu). Glycerol phosphate (Fluka) was used as a standard. Lipid phosphate concentrations were calculated using the factor

obtained with glycerol phosphate standard. The lipid-phosphate method showed a standard deviation of ± 5.3 %, based on replicate analyses.

4.2.6. Separation of FAs

The remaining TLE was transferred into 15 ml amber coloured glass vial. It was dried under nitrogen and saponified with 5 % w/v KOH in 80 % methanol (D'souza, 2004). Saponification was carried out at 80 °C for 2 h. The sample was cooled, 1 ml of milli-Q water was added and extracted with hexane:CHCl₃ (4:1, v/v, 3 times). The upper organic layer contains neutral lipids and the lower aqueous layer contained the total fatty acids.

4.2.7. Preparation of Fatty acid methyl esters

The aqueous layer containing the fatty acids was acidified to pH < 1 using concentrated HCl. The fatty acids were extracted using Hexane:CHCl₃ (4:1, v/v, 3 times). Traces of water were removed using anhydrous sodium sulfate and solvent was evaporated to dryness using nitrogen gas. The fatty acids were methylated by treating with MeOH:CHCl₃:HCl (10:1:1) for 2 h at 80 °C. After cooling, 1 ml of milli-Q water was added and the fatty acid methyl esters (FAME) were extracted with Hexane:CHCl₃ (4:1, v/v, 3 times). The extract was rotary evaporated at 30 °C under reduced pressure and transferred to vials with dichloromethane. The fatty acid methyl esters were analysed by gas chromatography mass spectrometry (GCMS).

4.2.8. Gas chromatography mass spectrometry (GCMS)

GCMS analyses of the fatty acid methyl esters were performed as mentioned in chapter 2A. The quantification of FAMES was done using the data handling system available on the instrument. The identification of individual FAMES was performed by comparing the retention times of FAMES in the sample with those of authentic standards (FAME 37 mix,

Supelco, USA; BAMEs, Supelco and FAME standards, Sigma-Aldrich-Fluka). The peak areas were quantified relative to an internal standard (hexacosanoate methyl ester) added to the sample prior to GCMS- analysis. Peak identification was verified by comparing the EI spectra of sample FAMEs to spectra from standards and published data.

Solvents were passed through the entire analytical procedure and run on GCMS as blanks to check the probable contamination. Sample values have been corrected for the blank levels.

4.2.9. Statistical Analysis

Regression analysis was employed to assess the relationship between various parameters (Sokal & Rohlf, 1981) using the Excell software program loaded on the personal computer.

4.3. Results and discussion

4.3.1 Bulk parameters

Organic carbon (OC) varied from 0.08 % (20-22cm) to 0.36 % (0-2cm) in CC1/GC3 core (Fig. 4.2a), whereas it varied from 1.07 % (20-22cm) to 2.3 % (0-2cm) in CC2/GCL1 core (Fig. 4.2b).—When normalized to sediment dry weight, OC varied from 0.81 to 3.61 mg OC g⁻¹ dw sediment for core CC1/GC3, and 10.7 to 23.2 mg OC g⁻¹ dw sediment for core CC2/GCL1. This shows higher OC values in the sediments of the Northern Indian Ocean. The distribution of OC in these sediment cores was similar to that observed in other marine sediments (e.g., Haddad et al., 1992; Bourbonniere and Meyers, 1996), showing a somewhat erratic trend with a general decrease in concentrations with depth. OC concentrations of 5.5, 33 and 39 mg OC g⁻¹ at the top 0-2 cm depth of the cores VAGO-03, AP-09 and CD-06 collected from the Northern Gulf of California (NGC) was recorded (Camacho-Ibar et al., 2003). The Northern Indian Ocean is a highly productive marine ecosystem with primary

production rates frequently exceeding 200 to 400 $\text{gCm}^{-2}\text{yr}^{-1}$ (Kabanova, 1968; Quasim, 1982), which is equivalent to the global productivity mean in estuaries, coastal lagoons and upwelling ecosystems ($\sim 300 \text{ g C m}^{-2} \text{ year}^{-1}$; Day et al., 1989; Knoppers, 1994). This suggests the occurrence of a large flux of labile organic carbon to the sediments of this ecosystem. Further, due to overlying OMZ (100 to 1100m water depth), high rates of OM accumulation may result from greater primary productivity, high sedimentation rates and increased preservation due to exposure to low oxygen (Sinninghe Damsté, et al., 2002). As compared to the Northern Indian Ocean, the equatorial Indian Ocean (EIO) is biologically less productive. The chlorophyll distribution, with the exception of the coastal boundaries, indicates that the EIO is a 'biological desert' as compared to any other regions of the Northern Indian Ocean (Narvekar & Prasanna Kumar, 2010).

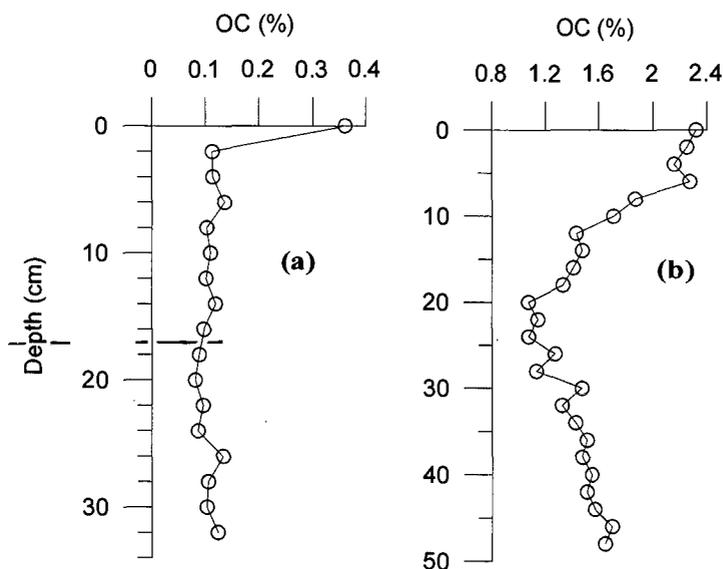


Fig. 4.2 Vertical profiles of organic carbon (OC %) in the sediment core CC1/GC3 and CC2/GCL1 collected from Equatorial Indian Ocean and Northern Indian Ocean

Distribution of the total lipid (TLE), lipid phosphate (L- PO_4) and total fatty acids (TFA) in the cores CC1/GC3, and CC2/GCL is shown in Fig. 4.3 and Fig. 4.4, respectively. In the core CC1/GC3, TLE, L- PO_4 , and TFA varied from 0.07 to 0.52 $\text{mg g}^{-1}\text{dw}$ sediment,

0.27 to 3.45 $\mu\text{g g}^{-1}$ dw sediment, and from 0.65 to 6.42 $\mu\text{g g}^{-1}$ dw sediment, respectively (Fig. 4.3). Similarly, for the core CC2/GCL TLE, L- PO_4 , and TFA varied from 0.12 to 1.41 mg g^{-1} dw sediment, 0.23 to 6.71 $\mu\text{g g}^{-1}$ dw (4-6cm), and from 0.6 to 19.2 $\mu\text{g g}^{-1}$ dw sediment, respectively (Fig. 4.4). TFA levels in cores CC1/GC3 and CC2/GCL1 were similar to those reported for the other deep sea sediments (Saliot et al., 1988). Lower concentrations of TFA were recorded at the deeper sections of CC1/GC3 with subsurface maxima at 4-6 cm and at 18-20 cm sections (Fig. 4.3c), indicating higher abundance of this biomarker in these sections. In the CC2/GCL1 core, maximum concentrations were obtained at 0-2 cm, 10-12 cm, 34-36 cm, 46-48 cm and 48-50 cm depth intervals, whereas lowest values were recorded in 14-16 cm and 26-28 cm of the core (Fig. 4.4c). Maximum concentrations of TFA in the CC2/GCL1 core indicate better preservation of OM. TFA concentrations suggests the Northern Indian Ocean sediments contain higher amounts of FAs ($>2\mu\text{g g}^{-1}$ dw at most sections) compared to the EIO ($<2\mu\text{g g}^{-1}$ dw at most sections). These trends are similar to those observed for the distribution of OC.

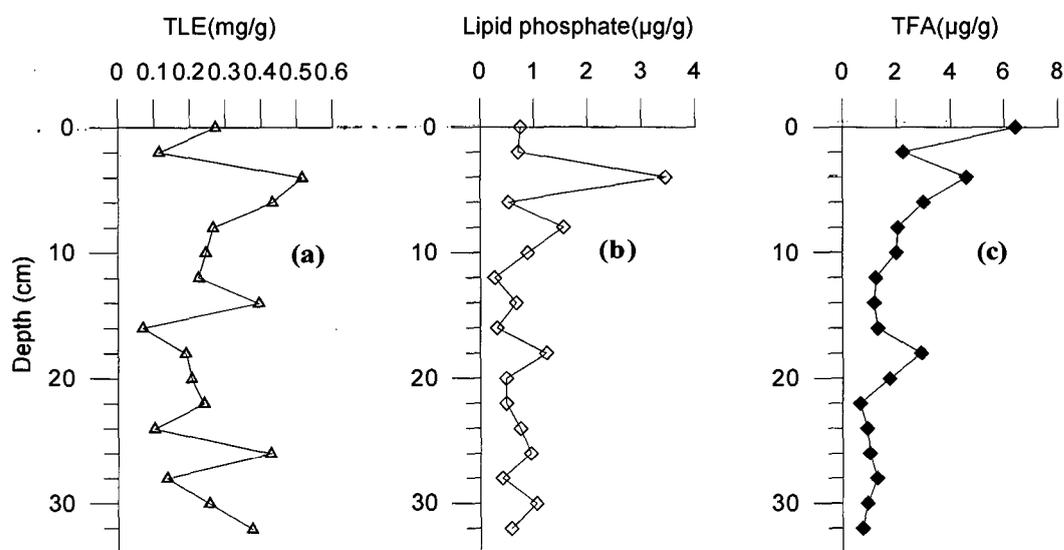


Fig. 4.3. Vertical profiles of total lipid extract (TLE), lipid phosphate and total fatty acids (TFA) in the sediment core CC1/GC3 collected from central Indian Ocean.

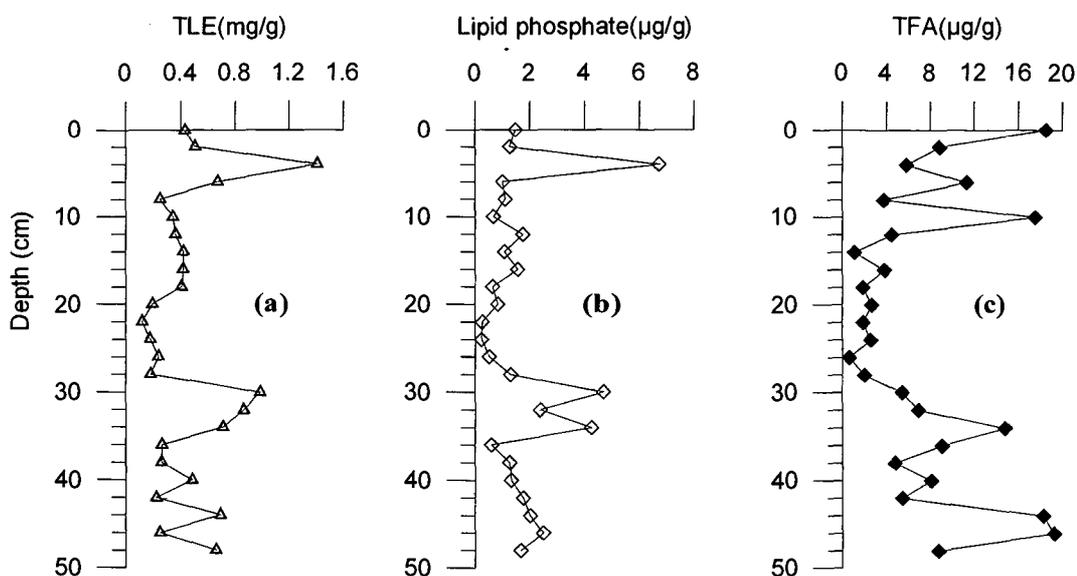


Fig. 4.4. Vertical profiles of total lipid extract (TLE), lipid phosphate and total fatty acids (TFA) in the sediment core CC2/GCL1 collected from northern Indian Ocean.

Differences between the two areas however become apparent when comparing the OC-normalized concentrations. OC normalized TFA values varied from 0.6 (32-34cm) to 4.0 (4-6cm) mg g^{-1} OC in the CC1/GC3 core, whereas in the CC2/GCL1 core the values varied from 0.05 (26-28cm) to 1.2 mg/g OC (44-46cm). These concentrations fall within the range of values reported in the sediments from other oceanic regions. OC normalized values varied from 0.8–1.6 mg g^{-1} OC off Antofagasta surface sediments and 1.8–4.9 mg g mg^{-1} OC off Concepción (Niggeman and Schubert, 2006). Two major factors affect the concentrations of TFA in the surface sediments. During early diagenesis, FAs are degraded preferentially over the bulk organic carbon pool (e.g. Wakeham et al., 1997b; Camacho-Ibar et al., 2003). Moreover, numerous studies provide evidence that deep water depths (>800m) favour low TFA concentrations in sediments, e.g. 1.8 mg g^{-1} OC in Black Sea (Sun and Wakeham, 1994); 2.5 mg g^{-1} OC in the anoxic depocenter and of 0.8 mg g^{-1} OC in the oxic periphery in Santa Monica Basin (Gong and Hollander, 1997). Whereas shallow water depths (<350 m) favour high TFA concentrations in sediments, e.g. 3.2 and 11.5 mg g^{-1} OC in St. Lawrence Estuary

(Colombo et al., 1997), 14 mg g⁻¹ OC in Buzzard Bay (Farrington et al., 1977), 3.4– 22 mg g⁻¹ OC in Cape Lookout Bight (Haddad et al., 1992; Canuel and Martens, 1996), and up to 35.7 mg g⁻¹ OC in Chesapeake Bay (Zimmerman and Canuel, 2001). Another factor influencing concentrations in the sediments is the input of TFA. It has been shown that TFA concentrations in surface sediments reflect seasonal changes in TFA input, e.g. high concentrations coincide with algal bloom events in the overlying surface water (Zimmerman and Canuel, 2001; Gogou and Stephanou, 2004). Both the sampling sites showed in general lower concentrations of TFA mg g⁻¹OC, which is in accordance with the deep sea distribution of TFA. However, this range of OC normalized TFA values in the EIO core (CC1/GC3) were higher compared to the Northern Indian Ocean core (CC/GCL1). From the previous studies it is evident that EIO is a biological desert, which is seen from very low chlorophyll values observed in this area, whereas Northern Indian Ocean is highly productive (Kabanova, 1968; Quasim, 1982). This distribution suggests that TFA are better preserved in the EIO compared to Northern Indian Ocean sediments. As TFA fraction of organic matter is 2–9 times more labile than the whole of the organic carbon pool (Camacho-Ibar et al., 2003), comparatively low values in the Northern Indian Ocean may be due to higher degradation of the TFA in this environment.

4.3.2 Lipid-phosphate based bacterial biomass

The determination of bacterial biomass in complex environmental samples is difficult, as the classical techniques of viable counting most often recover a small and variable proportion of the cells detectable by direct counting (Alexander, 1977; Jenkinson and Ladd, 1981). Hence analysis of cell membrane phospholipids such as lipid-bound phosphate (lipid phosphate) is thus developed and successfully applied to characterize bacterial biomass and its structure (White et al., 1979). Under the conditions expected in natural communities, the

4.1. Introduction

Organic matter (OM) in the deep sea sediments is derived from various sources including in situ primary production, terrestrial OM and atmospheric transfer. Nature of OM deposited on the seafloor is influenced by sources of OM, remineralization as well as reworking by organisms in the water column and at the sediment-water interface, concentrations of dissolved oxygen in intermediate and bottom waters, and the sedimentation rate (Budge and Parrish, 1998; Betts and Holland, 1991). Only a small portion (<1%) of the OM produced photosynthetically in the sunlit surface ocean waters by microscopic plants (phytoplankton) sinks through the water column and is ultimately deposited in sediments (Suess, 1980; Martin et al., 1987). Several investigations have shown that the vertical mass flux can be attributed to rapidly sinking, large particles (~50 µm) particles (McCave, 1975; Bishop et al., 1977, 1978, 1980; Honjo, 1978, 1980). Different mineral and organic species, including living and dead organisms, faecal pellets etc are associated with these large particles or aggregates. The flux of the OM from surface waters to deep sediments forms an important source of food to benthic organisms and is an important factor determining the benthic biomass (Maury, 1861; Vinogradov, 1968; Rowe et al., 1991; Gooday, 2002). Mineralization or bacterial degradation is the major transformation process that occurs during transport of particles from surface waters to deep sea sediments (Iturriaga, 1979). The study of the variability of OM deposited on the deep-sea floor sediments and its origin, transformation and degradation is very important for understanding the role of deep sea systems in comprehensive examination of the global carbon cycle.

FAs constitute an important fraction of the lipid pool in living and dead organic material. Their abundance in living organisms, their source specificity with respect to individual compounds and their relative lability make FAs suitable for tracing sources and

diagenetic changes of organic material in water columns and sediments. Numerous studies have used FAs to estimate the relative contributions of terrestrial, algal or planktonic and bacterial FAs to the total FA pool in marine sediments (e.g. Volkman et al., 1980; Prahl et al., 1989; Budge and Parrish, 1998; Zimmerman and Canuel, 2001; Camacho-Ibar et al., 2003). Although most FAs are not unique to one source, major differences in the FA composition of individual or grouped source organisms allow an assignment of predominating sources.

To determine the microbial biomass in natural environments is one of the greatest challenges to microbial ecologists. In the nature more than 90% of indigenous microorganisms escape cultivation using the traditional culture-dependent techniques (White et al., 1979; DeLong and Pace, 2001). Hence a number of culture independent methods such as lipid phosphate, PLFAs (White et al., 1979; Pinkart et al., 2002; Petsch et al., 2003) and genetic fingerprinting (Polymenakou et al., 2005), have been used to detect viable microbial biomass and changes in the composition of natural microbial communities. Lipid phosphates are used to estimate viable microbial biomass in marine sediments (White et al., 1979; Balkwil et al., 1988; Findlay et al., 1989).

To understand more fully about the nature and sources of OM and bacterial abundance, the distribution of fatty acids and lipid phosphate (L-PO₄) were studied in two deep sea sediment cores collected from Northern Indian Ocean and Equatorial Indian Ocean. The main objectives of this chapter were 1) to use L-PO₄ to assess the viable bacterial biomass, 2) to evaluate the distribution and fate of fatty acids, and 3) to investigate the sources of OM using fatty acids as biomarkers in these sediment cores.

4.2. Materials and Methods

4.2.1. Description of the sampling areas

Numerous studies are available on the physical, chemical and biological characterization of the waters of the Northern Indian Ocean (Arabian Sea) and Equatorial Indian Ocean (Deuser et al., 1978; Brock et al., 1992; Haake et al., 1993; Azam, et al., 1994; Calvert et al., 1995; Boetius et al., 2000; Naqvi et al., 2002; Wiggert et al., 2005). Northern Indian Ocean is tropical in nature and experience seasonal changes in climate and physical oceanographic processes. The biogeochemistry of the Arabian Sea is strongly influenced by two monsoon periods. Southwest monsoon occurs from late May to early September and the northeast monsoon from December to March (Wiggert et al., 2005). Primary productivity increases dramatically in these two periods due to higher nutrient levels, caused during the southwest-monsoon by coastal upwelling of nutrient-rich waters and during the north-east monsoon by convective mixing together with subsequent advection by large-scale circulation (Naqvi et al., 2002; Kawamiya and Oschlies, 2003). This results in productivity values that are among the highest known for the open ocean. Annual productivity is estimated to be 200 to 400 gCm⁻²yr⁻¹ (Kabanova, 1968; Quasim, 1982). At the end of the algal blooms, detritus sinks through the water column and on to the sea floor (Haake et al., 1993). The sediments of the Arabian Sea therefore receive fluxes of organic material that display strong seasonal variability. Another prominent feature of the Arabian Sea is the midwater Oxygen Minimum Zone (OMZ) with oxygen levels below 0.05 mL/L (van Bennekom et al., 1995), from a water depth of around 100 m down to 1100 m (Wyrki, 1971, 1973; Deuser et al., 1978; Olson et al., 1993; Sinninghe Damsté et al 2002), due to respiration of OM and the input of intermediate waters with low oxygen content, from the Southern Hemisphere (Naqvi, 1987; You and Tomczak, 1993; Warren, 1994). The OMZ coincides roughly with maxima in sedimentary organic carbon, suggesting a link between organic carbon preservation and oxygen availability (Cowie et al., 1999).

Equatorial Indian Ocean (EIO) is different from other equatorial regions of the world ocean because of the reversal of the wind system twice a year due to which the surface currents reverse semiannually. Equatorial upwelling does not occur in the Equatorial Indian Ocean as it does in the Pacific and Atlantic, because in those oceans, upwelling occurs as a result of the southeast trade winds which blow across the equator and cause surface divergence. Since in the equatorial Indian Ocean, there is no such wind system in either season (winter and summer) of the year, typical conditions for equatorial upwelling are missing. Chlorophyll pigment concentration shows that EIO away from the coastal boundaries is a 'biological desert' with least chlorophyll concentration compared to any other region in the northern Indian Ocean (Narvekar & Prasanna Kumar, 2010). In the central EIO chlorophyll concentration are almost uniform (0.1-0.2 mg/m³) without much variability..

4.2.2. Sample collection and analysis

One sediment core CC1/GC3 was collected at a station (5.003 °S and 83.052 °E) in the EIO during R.V.A.A. Sidorenko cruise-59 from January 31 to February 24, 2003 (Fig. 4.1). Another core CC2/GCL1 was collected from a station (11.59 °N and 73.30 °E) in the Northern Indian-Ocean during R.V.A.A. Sidorenko, cruise 65 from July 17 to August 16, 2003 (Fig. 4.1). Sediment core CC1/GC3 was retrieved from 4550 m water depth in the EIO while sediment core CC2/GCL1 was collected from 2045 m depth in the Northern Indian Ocean. The cores were collected using a gravity corer equipped with PVC pipes. Cores were sectioned on board into 2 cm intervals and immediately frozen. From the previous observation the OMZ lies in between 100 m down to 1100 m in the Arabian Sea (Naqvi et al., 2000; Sinninghe Damsté et al., 2002). This shows that the core CC2/GCL1 was collected from well below the OMZ in the Northern Indian Ocean. The O₂ levels below 2000 m depth are >2ml/l in the Indian Ocean (Sinninghe Damsté et al., 2002). CC1/GC3 core was 34 cm long and

CC2/GCL1 core was 2 meters long. Whole of the CC1/GC3 was analyzed while top 50 cm of CC2/GCL1 was analyzed. The sediment samples in the frozen condition were then transported to the laboratory in an ice box, and stored at -20 °C until lyophilization. The sediments were then freeze dried, homogenized using an agate mortar and pestle to a fine powder and used for the estimation of organic carbon, total lipids, lipid-phosphate and fatty acids.

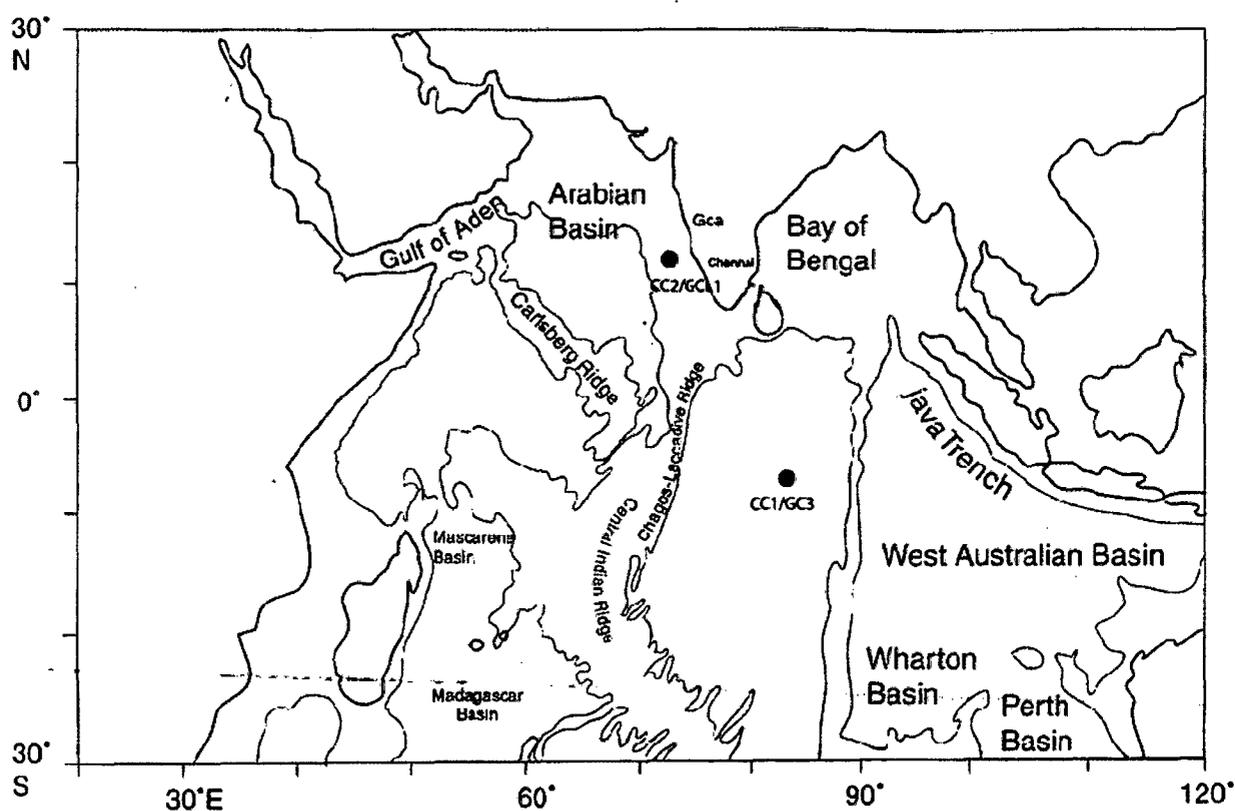


Fig. 4.1. Map showing the locations of the sampling stations of the cores CC1/GC3 in the Equatorial Indian Ocean and CC2/GCL1 in the Northern Indian Ocean.

4.2.3. Cleaning of glassware and purification of solvents

Cleaning of glassware and purification of solvents was done as in chapter 2A.

4.2.4. Estimation of organic carbon (OC)

The OC was determined as given in chapter 3A.

4.2.5. Extraction of lipids

Lipids from the lyophilized sediments (7-15 g for CC1/GC3 and 6-30 g for CC2/GCL1) were extracted following the modified method of Bligh and Dyer (1959) as described by White et al. (1979). In brief, a known quantity of the homogenized sediments was transferred to a clean flask and were ultrasonically extracted with 300ml (100ml x 3) of chloroform-methanol-phosphate buffer (1:2:0.8, v/v/v) in an ice bath for 15 min x 3. The supernatant then transferred to separating funnel and shaken vigorously and kept standing for minimum 24 h at room temperature (28 ± 2 °C) in order to extract the total lipids. Separation of the organic phase was achieved by adding 1:1 ratio of chloroform: water in a separating funnel and shaking it vigorously for 30 min. The lower phase was pooled, filtered through Whatman No. 1 filter paper, and treated with anhydrous sodium sulphate to remove traces of water. The extract was now called as total lipid extracts (TLE). The TLE was rotary evaporated at 30 °C under vacuum to a small volume. Total lipid was estimated by gravimetric method (Zaghden et al., 2005, Harji et al., 2008; Harji et al., 2010) as mentioned in chapter 3A. A known aliquot of TLE was used to estimate lipid-phosphate (White et al., 1979). In short, the TLE was hydrolyzed using perchloric acid (35 %) at 280°C for 3 hrs till colourless solution appears. After cooling 2.4 ml of molybdate (4.4 g ammonium molybdate + 14 ml concentrated H₂SO₄ in 1000ml distilled water (D/W)) and 2.4 ml of ANSA (0.5g 1-amino-2-naphthol-4-sulfonic acid, 30g sodium bisulfite and 2g sodium sulfite, dissolved in 200 ml D/W, the solution diluted 1:12 with D/W before use) were added and boiled at 100 °C for 15 min. Absorbance was read at 830 nm at the room temperature using a spectrophotometer (UV-1700 Pharma Spec UV-VIS, Shimadzu). Glycerol phosphate (Fluka) was used as a standard. Lipid phosphate concentrations were calculated using the factor

obtained with glycerol phosphate standard. The lipid-phosphate method showed a standard deviation of ± 5.3 %, based on replicate analyses.

4.2.6. Separation of FAs

The remaining TLE was transferred into 15 ml amber coloured glass vial. It was dried under nitrogen and saponified with 5 % w/v KOH in 80 % methanol (D'souza, 2004). Saponification was carried out at 80 °C for 2 h. The sample was cooled, 1 ml of milli-Q water was added and extracted with hexane:CHCl₃ (4:1, v/v, 3 times). The upper organic layer contains neutral lipids and the lower aqueous layer contained the total fatty acids.

4.2.7. Preparation of Fatty acid methyl esters

The aqueous layer containing the fatty acids was acidified to pH < 1 using concentrated HCl. The fatty acids were extracted using Hexane:CHCl₃ (4:1, v/v, 3 times). Traces of water were removed using anhydrous sodium sulfate and solvent was evaporated to dryness using nitrogen gas. The fatty acids were methylated by treating with MeOH:CHCl₃:HCl (10:1:1) for 2 h at 80 °C. After cooling, 1 ml of milli-Q water was added and the fatty acid methyl esters (FAME) were extracted with Hexane:CHCl₃ (4:1, v/v, 3 times). The extract was rotary evaporated at 30 °C under reduced pressure and transferred to vials with dichloromethane. The fatty acid methyl esters were analysed by gas chromatography mass spectrometry (GCMS).

4.2.8. Gas chromatography mass spectrometry (GCMS)

GCMS analyses of the fatty acid methyl esters were performed as mentioned in chapter 2A. The quantification of FAMES was done using the data handling system available on the instrument. The identification of individual FAMES was performed by comparing the retention times of FAMES in the sample with those of authentic standards (FAME 37 mix,

Supelco, USA; BAMEs, Supelco and FAME standards, Sigma-Aldrich-Fluka). The peak areas were quantified relative to an internal standard (hexacosanoate methyl ester) added to the sample prior to GCMS- analysis. Peak identification was verified by comparing the EI spectra of sample FAMEs to spectra from standards and published data.

Solvents were passed through the entire analytical procedure and run on GCMS as blanks to check the probable contamination. Sample values have been corrected for the blank levels.

4.2.9. Statistical Analysis

Regression analysis was employed to assess the relationship between various parameters (Sokal & Rohlf, 1981) using the Excell software program loaded on the personal computer.

4.3. Results and discussion

4.3.1 Bulk parameters

Organic carbon (OC) varied from 0.08 % (20-22cm) to 0.36 % (0-2cm) in CC1/GC3 core (Fig. 4.2a), whereas it varied from 1.07 % (20-22cm) to 2.3 % (0-2cm) in CC2/GCL1 core (Fig. 4.2b).—When normalized to sediment dry weight, OC varied from 0.81 to 3.61 mg OC g⁻¹ dw sediment for core CC1/GC3, and 10.7 to 23.2 mg OC g⁻¹ dw sediment for core CC2/GCL1. This shows higher OC values in the sediments of the Northern Indian Ocean. The distribution of OC in these sediment cores was similar to that observed in other marine sediments (e.g., Haddad et al., 1992; Bourbonniere and Meyers, 1996), showing a somewhat erratic trend with a general decrease in concentrations with depth. OC concentrations of 5.5, 33 and 39 mg OC g⁻¹ at the top 0-2 cm depth of the cores VAGO-03, AP-09 and CD-06 collected from the Northern Gulf of California (NGC) was recorded (Camacho-Ibar et al., 2003). The Northern Indian Ocean is a highly productive marine ecosystem with primary

production rates frequently exceeding 200 to 400 $\text{gCm}^{-2}\text{yr}^{-1}$ (Kabanova, 1968; Quasim, 1982), which is equivalent to the global productivity mean in estuaries, coastal lagoons and upwelling ecosystems ($\sim 300 \text{ g C m}^{-2} \text{ year}^{-1}$; Day et al., 1989; Knoppers, 1994). This suggests the occurrence of a large flux of labile organic carbon to the sediments of this ecosystem. Further, due to overlying OMZ (100 to 1100m water depth), high rates of OM accumulation may result from greater primary productivity, high sedimentation rates and increased preservation due to exposure to low oxygen (Sinninghe Damsté, et al., 2002). As compared to the Northern Indian Ocean, the equatorial Indian Ocean (EIO) is biologically less productive. The chlorophyll distribution, with the exception of the coastal boundaries, indicates that the EIO is a 'biological desert' as compared to any other regions of the Northern Indian Ocean (Narvekar & Prasanna Kumar, 2010).

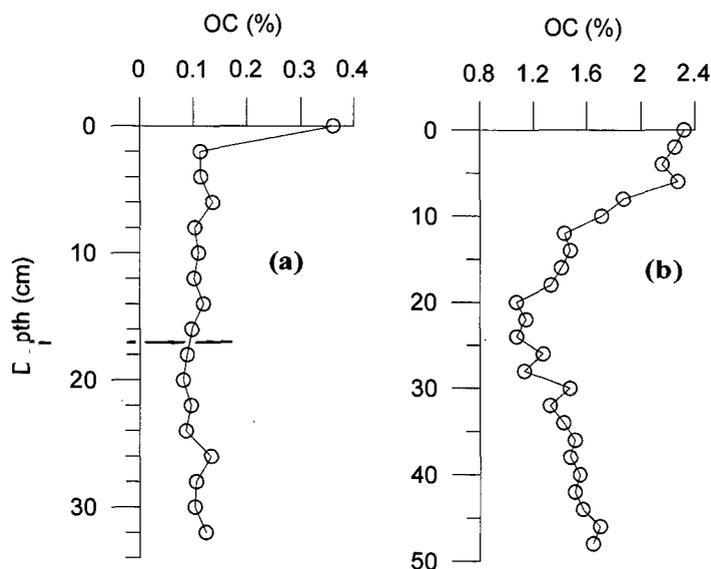


Fig. 4.2 Vertical profiles of organic carbon (OC %) in the sediment core CC1/GC3 and CC2/GCL1 collected from Equatorial Indian Ocean and Northern Indian Ocean

Distribution of the total lipid (TLE), lipid phosphate (L- PO_4) and total fatty acids (TFA) in the cores CC1/GC3, and CC2/GCL is shown in Fig. 4.3 and Fig. 4.4, respectively. In the core CC1/GC3, TLE, L- PO_4 , and TFA varied from 0.07 to 0.52 $\text{mg g}^{-1}\text{dw}$ sediment,

0.27 to 3.45 $\mu\text{g g}^{-1}$ dw sediment, and from 0.65 to 6.42 $\mu\text{g g}^{-1}$ dw sediment, respectively (Fig. 4.3). Similarly, for the core CC2/GCL TLE, L- PO_4 , and TFA varied from 0.12 to 1.41 mg g^{-1} dw sediment, 0.23 to 6.71 $\mu\text{g g}^{-1}$ dw (4-6cm), and from 0.6 to 19.2 $\mu\text{g g}^{-1}$ dw sediment, respectively (Fig. 4.4). TFA levels in cores CC1/GC3 and CC2/GCL1 were similar to those reported for the other deep sea sediments (Saliot et al., 1988). Lower concentrations of TFA were recorded at the deeper sections of CC1/GC3 with subsurface maxima at 4-6 cm and at 18-20 cm sections (Fig. 4.3c), indicating higher abundance of this biomarker in these sections. In the CC2/GCL1 core, maximum concentrations were obtained at 0-2 cm, 10-12 cm, 34-36 cm, 46-48 cm and 48-50 cm depth intervals, whereas lowest values were recorded in 14-16 cm and 26-28 cm of the core (Fig. 4.4c). Maximum concentrations of TFA in the CC2/GCL1 core indicate better preservation of OM. TFA concentrations suggests the Northern Indian Ocean sediments contain higher amounts of FAs ($>2\mu\text{g g}^{-1}$ dw at most sections) compared to the EIO ($<2\mu\text{g g}^{-1}$ dw at most sections). These trends are similar to those observed for the distribution of OC.

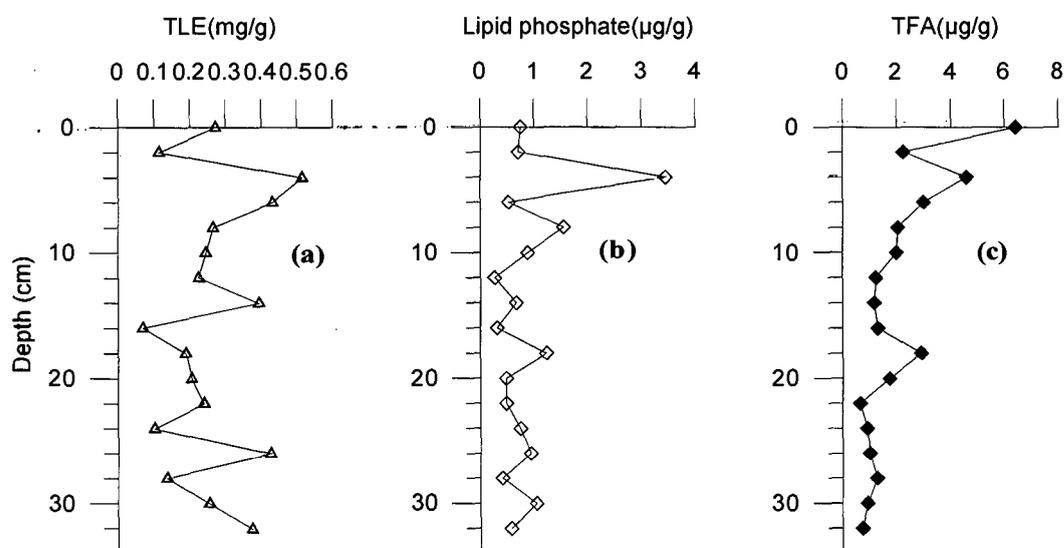


Fig. 4.3. Vertical profiles of total lipid extract (TLE), lipid phosphate and total fatty acids (TFA) in the sediment core CC1/GC3 collected from central Indian Ocean.

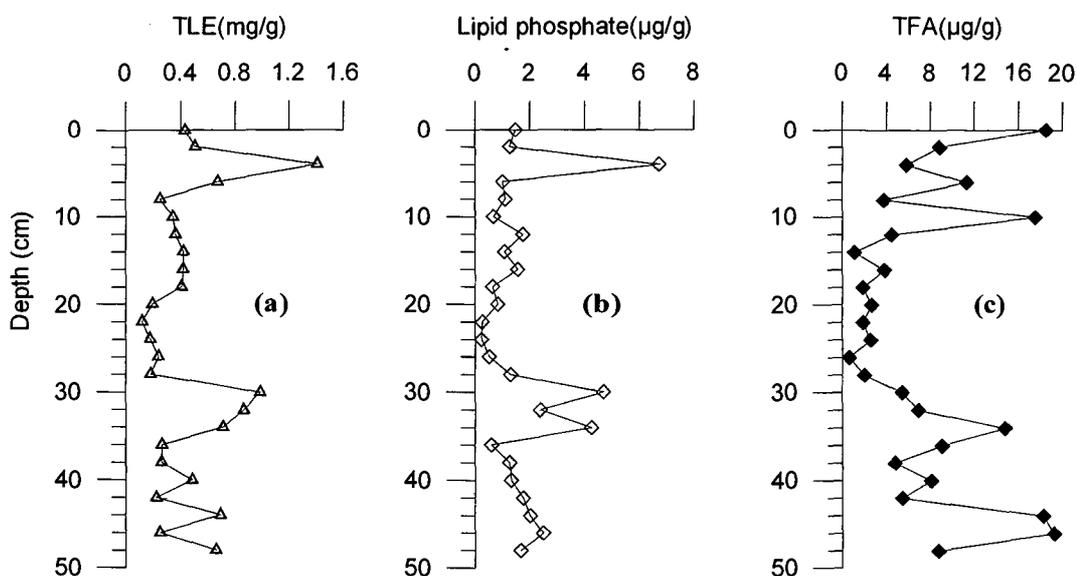


Fig. 4.4. Vertical profiles of total lipid extract (TLE), lipid phosphate and total fatty acids (TFA) in the sediment core CC2/GCL1 collected from northern Indian Ocean.

Differences between the two areas however become apparent when comparing the OC-normalized concentrations. OC normalized TFA values varied from 0.6 (32-34cm) to 4.0 (4-6cm) mg g^{-1} OC in the CC1/GC3 core, whereas in the CC2/GCL1 core the values varied from 0.05 (26-28cm) to 1.2 mg/g OC (44-46cm). These concentrations fall within the range of values reported in the sediments from other oceanic regions. OC normalized values varied from 0.8–1.6 mg g^{-1} OC off Antofagasta surface sediments and 1.8–4.9 mg g mg^{-1} OC off Concepción (Niggeman and Schubert, 2006). Two major factors affect the concentrations of TFA in the surface sediments. During early diagenesis, FAs are degraded preferentially over the bulk organic carbon pool (e.g. Wakeham et al., 1997b; Camacho-Ibar et al., 2003). Moreover, numerous studies provide evidence that deep water depths (>800m) favour low TFA concentrations in sediments, e.g. 1.8 mg g^{-1} OC in Black Sea (Sun and Wakeham, 1994); 2.5 mg g^{-1} OC in the anoxic depocenter and of 0.8 mg g^{-1} OC in the oxic periphery in Santa Monica Basin (Gong and Hollander, 1997). Whereas shallow water depths (<350 m) favour high TFA concentrations in sediments, e.g. 3.2 and 11.5 mg g^{-1} OC in St. Lawrence Estuary

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varied from 0.5 to 6.4×10^8 cells g^{-1} dw in the CC1/GC3 sediment core (Table 4.3), and from 0.4 to 12.4×10^8 cells g^{-1} dw in the CC2/GCL1 sediment core (Table 4.4). This suggests that bacterial biomass was higher in CC2/GCL1 core collected from the northern Indian Ocean.

4.3.3 Major groups of TFAs

FAs are the important components of all cells and hence its distribution provides chemotaxonomic information about organisms in sediment samples (Volkman et al., 1980). Microorganisms contain FAs such as Sat-FAs, UFAs, and Br-FAs. Sat-FAs were the most abundant component of the TFAs in the CC1/GC3 (76.8 to 95%) and CC2/GCL1 (53.1 to 95 %) core sediments. The next abundant FAs were UFAs (4.6 to 21.9 % and 4.8 to 44.1 %) and Br-FAs (undetected to 2.5 % and 0.3 to 4.7 %), in the CC1/GC3 and CC2/GCL1 cores, respectively (Table 4.1 and Table 4.2).

Abundance of Sat-FAs has been reported from coastal (estuaries, rivers, bays, etc), and open ocean environments (Polymenakou et al., 2006). Sat FAs (C14, C16:0 and C18:0) are abundant in the cell membranes of most bacteria and eukaryotes, and thus are considered non-diagnostic (Rajendran et al., 1992; Volkman et al., 1998), however long chain Sat FAs are produced by terrestrial plants (Rajendran et al., 1992). Abundance of Sat FAs indicates bacterial alteration or diagenetic origin of these FAs in sediments. UFAs in marine environment include MUFAs and PUFAs. UFAs are mainly produced by bacteria and microalgae (Oliver & colwell, 1973; Volkman et al., 1998; Rütters et al., 2002; Nichols, 2003) in the marine environment. Br-FAs are produced by bacteria (Wakeham, 1995; Rütters et al., 2002). Methyl branched and cyclopropyl FAs are generally abundant in anaerobic environments (Baird et al., 1985; Guckert et al., 1985; Rajendran et al., 1992). Apart from this, Gram-negative bacteria contain high proportions of normal, even numbered, Sat and Unsat FAs, and odd numbered, cyclopropane acids, whereas Gram-positive bacteria have high

proportions of branched acids and relatively low amounts of straight chain Sat or Unsat acids (Kates, 1964).

Table 4.1: % composition of C16:0, C18:0 and major groups of fatty acids in the CC1/GC3 core

DEPTH	Sat-FA	Br-FA	UFA	C16:0	C18:0
0-2	95.0	0.4	4.6	32.6	56.2
2-4	88.9	2.5	8.1	40.0	40.6
4-6	89.6	1.3	9.0	32.2	50.8
6-8	92.5	0.0	7.5	35.2	52.2
8-10	91.2	1.0	7.8	33.3	52.7
10-12	90.5	0.7	8.8	36.4	48.9
12-14	92.0	0.0	7.1	28.9	58.6
14-16	84.8	2.0	13.2	24.8	48.9
16-18	87.8	2.1	10.1	44.2	33.6
18-20	93.3	0.4	6.3	37.4	50.6
20-22	88.4	1.9	9.3	30.7	51.2
22-24	76.8	1.4	21.9	29.3	40.2
24-26	86.7	1.5	11.8	46.9	32.3
26-28	84.9	0.0	14.2	42.8	33.8
28-30	82.7	2.0	15.3	47.4	22.5
30-32	84.9	0.0	15.1	41.4	35.9
32-34	86.7	0.0	13.3	11.0	57.7

Table 4.2: % composition of C16:0, C18:0 and major groups of fatty acids in the CC2/GCL1 core.

Depth	Sat-FA	Br-FA	Unsat-FA	C16:0	C18:0
0-2	90.4	0.8	8.8	23.6	62.4
2-4	83.3	1.7	15.0	34.2	43.7
4-6	87.1	2.3	10.5	28.7	45.1
6-8	92.0	1.4	6.6	30.9	52.5
8-10	53.1	2.8	44.1	22.8	19.1
10-12	93.2	0.5	6.3	28.2	60.1
12-14	80.0	3.4	16.6	45.1	18.0
14-16	70.6	4.7	24.7	28.2	13.8
16-18	86.5	0.5	13.0	17.2	59.1
18-20	83.0	1.1	15.9	26.4	42.7
20-22	67.2	0.6	32.2	19.0	38.3
22-24	89.4	0.4	10.2	22.9	57.0
24-26	85.4	0.5	14.1	27.2	48.8
26-28	92.0	1.5	6.5	27.0	54.7
28-30	85.5	1.4	13.1	34.6	38.9
30-32	93.3	0.5	6.2	33.0	53.4
32-34	90.5	1.1	8.4	29.1	50.6
34-36	85.5	0.5	13.9	28.5	49.3
36-38	74.7	0.6	24.7	21.1	42.1
38-40	89.0	1.2	9.7	27.0	47.0
40-42	92.6	0.8	6.6	27.6	53.1
42-44	91.1	1.2	7.8	30.6	46.3
44-46	94.9	0.3	4.8	29.1	58.1
46-48	94.8	0.3	4.9	27.1	59.8
48-50	94.1	0.4	5.5	26.3	54.9

4.3.4 Distribution of FAs in CC1/GC3 and CC2/GCL1 core

In the CC1/GC3 core, 29 FAMES (Tables 4.3) and in the CC2/GCL1 core, 43 FAMES were identified (Tables 4.4). It indicated differences in the contribution of biogenic material to the sediment of these two different environments. The distributions of biomarkers in the sediments are extremely complex (Smallwood and Wolff. 2000). Many of the identified compounds are ubiquitous in marine organisms, and it is therefore difficult to distinguish their precise sources.

Total FAs generally decreased with increasing sediment core depth, the relative abundances of individual FAs show changes within the core (Table 4.3 and 4.4). In the CC1/GC3 core, the most abundant fatty acids were C16:0 and C18:0 which varied from 81.2 (32-34 cm section) to 2091 ngg⁻¹ dw sediment (0-2 cm section) and 261.1 (22-24 cm section) to 3603.7 ngg⁻¹ (0-2 cm section) dw sediment, respectively (Table 4.3). Relative contributions of C16:0 (11 to 47.4 %) and C18:0 (22.5 to 58.6 %) to the TFAs was highest in CC1/GC3 core. Other major FAs in CC1/GC3 sediment core include, Sat C14:0, C15:0, C17:0, C20:0, C22:0, C24:0; MUFA C16:1, C18:1n9c, C18:1n9t and C22:1n9c, terminally branched saturated (a-15:0) and PUFA (C18:2n6c) (Table 4.3). Relative proportions of these major FAs accounted for 96.3 % to 100 % in the CC1/GC3 sediment core samples. Similarly, in the CC2/GCL1 core samples, the most abundant fatty acids were C16:0 and C18:0 which varied from 171 (26-28 cm section) to 5306 ng/g (44-46 cm section) dw sediment and 151.5 (14-16 cm section) to 11561.3 ng/g (0-2 cm section) dw sediment (Table 4.4). The relative concentrations of Sat FAs, C16:0 (17.2 to 45.1 %) and 18:0 (13.8 to 62.4 %) also contributed highest to the TFAs in the CC2/GCL1 core (Table 4.2). Other abundant FAs in CC2/GCL1 include, Sat C14:0, C15:0, C17:0, C20:0, C22:0 and C24:0; MUFA C16:1, C18:1n9t, C18:1n9c, C20:1 and C22:1n9c, terminally branched saturated (a-15:0, i-16:0), and PUFA

C18:2n6c (Table 4.4). Sum of these major FAs accounted for 90.5 % to 99.3 % in the CC2/GCL1 sediment core samples. Sat FAs showed even over odd preference throughout the CC1/GC3 and CC2/GCL1 core (Table 4.3 & 4.4). Compared to CC2/GCL1 core, MUFAs (C16:1 and C18:1) and PUFAs were low in concentration, in the CC1/GC3 core, suggesting low amount of OM deposition (Table 4.3). This also can be seen from the low distribution of OC throughout the CC1/GC3 core (Fig. 4.2a).

In the core CC1/GC3 C16:0 and C18:0 FAs were the important and major components of the TFAs and together comprised 69-89% of total FAs. The variation in C16:0 and C18:0 FAs with total FAs are shown in Fig. 4.5. Three maxima were observed for these FAs at 0-2 cm, 4-6 cm and 18-20 cm of CC1/GC3 core sections, respectively. These maxima suggest a comparatively high input of OM during the sedimentation period. Overall the pattern of variation remained same for these FAs. C16:0 and C18:0 FAs showed good relationship with total FAs ($r = 0.98$ and $r = 0.98$; $n = 17$, $p = 0.001$) and OC ($r = 0.70$ and $r = 0.75$; $n = 17$, $p = 0.001$) in CC1/GC3 core sediments. In CC2/GCL1 core sediments, the C16:0 and C18:0 FAs were also major components of TFA, comprising 42-88.3 % of TFAs. As C16:0 and C18:0 FAs are major FA, they showed similar pattern to total FAs (Fig. 4.6). Subsurface maxima were observed for these FAs at 0-2 cm, 6-8 cm, 10-12 cm, 34-36 cm, 44-46 cm and 46-48 cm of CC2/GCL1 core sections, which suggest the maximum OM sedimentation to the deep sea. C16:0 and C18:0 FAs varied co-linearly with total FAs ($r = 0.98$ and $r = 0.98$; $n = 25$, $p = 0.001$) in the CC2/GCL1 core sediments. However C16:0 and C18:0 FAs showed relatively poor relationship with OC ($r = 0.48$ and $r = 0.43$; $n=25$, $p = 0.02$).

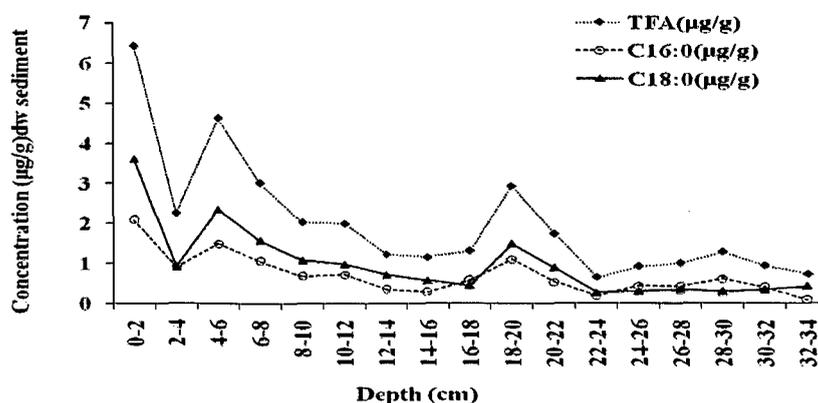


Fig. 4.5. Variation of C16:0, C18:0 and total fatty acids (TFA) in the sediment core CC2/GCL1 collected from northern Indian Ocean

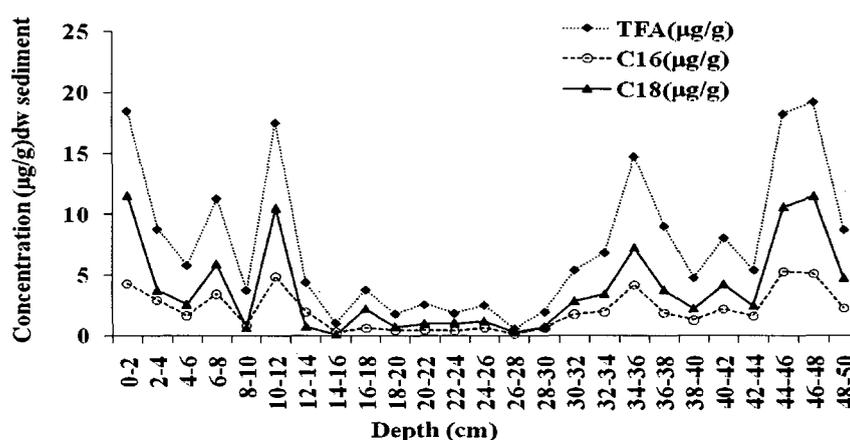


Fig. 4.6. Variation of C16:0, C18:0 and total fatty acids (TFA) in the sediment core CC2/GCL1 collected from northern Indian Ocean

In principle, Sat FAs are more stable in sedimentary environments than Unsat ones (Abelson et al., 1964; Parker and Leo, 1965; Farrington and Quinn, 1971; Johnson and Calder, 1973). The process of biological diagenesis by which deposited FAs are modified in the upper layers of recent sediments is thought to be brought about largely by microbial activity (Eglinton, 1973; Aizenshtat et al., 1973; Johnson & Calder, 1973; Farrington & Quinn, 1973; Matsuda and Koyama, 1977). Conversion of labelled oleic acid in recent sediment to a series of Sat acids has shown that hydrogenation, degradation followed by resynthesis is acting in sediments, presumably due to microorganisms including bacteria (Rhead et al., 1971a, 1971b). This is in agreement with the observation that Unsat acids are

quickly modified in both reducing and oxidizing sediments under aerobic and anaerobic conditions (Eglinton, 1971; Nissenbaum et al. 1972; Johnson & Calder, 1973). Sat C16 FA dominance was previously observed in the deep sea sediments of Oman margin (Smallwood and Wolff, 2000). High abundance of these Sat FAs (C16:0 and C18:0) was thought to be due to microbial transformation from Unsat FAs (Rhead et al., 1971a, 1971b).

Dominating MUFAs were C16:1, C18:1n9t, C18:1n9c and C22:1n9c in the core of EIO and Northern Indian Ocean (Fig. 4.7 & 4.8, and Table 4.3 & 4.4). C16:1 and C18:1 are mostly observed in gram negative bacteria (Oliver and Colwell, 1973; Wilkinson, 1988; Ringelberg et al., 1997). These FAs were present throughout the core sections in EIO and Northern Indian Ocean (Fig. 4.7 & 4.8). Although MUFAs are largely contributed by settling phytoplankton in the water column, other studies suggest the bacterial origin of these FAs. For example, Saliot et al (1982) observed high amounts of Unsat FAs in deep sea particulate matter. However in surface particulate matter the concentrations of Unsat FAs were less. They suggested the enrichment of particles with Unsat FAs was due to bacteriological activity rather than phytoplankton origin, which can transform a large part of the suspended matter, leading to enrichment in unsaturated compounds in the deep sea particulate matter (Volkman et al., 1980). Saliot et al. (1980) also observed numerous bacteria in the interstitial waters of deep sea sediments. This shows that bacteria can be a major source of MUFAs at such depths. Sediments contain active populations of bacteria which are responsible for the degradation and remineralization of organic material (Deming and Baross, 1993). C16:1 acid was reported as the major fatty acid in the extremely peizophilic bacteria (Fang et al., 2002). Hence, the prokaryotic origin of these fatty acids appears to be probable. However these Unsat FAs concentrations decreased with increase in sediment depth, indicating degradation of these fatty acids with increasing sediment core depth. C18:1n9t, and C18:1n9c also varied co-

Table 4.3: Fatty acid composition (ng/g) in the CC1/GC3 core sediments

Depth(cm)	0-2	2-4	4-6	6-8	8-10	10-12	12-14	14-16	16-18	18-20	20-22	22-24	24-26	26-28	28-30	30-32	32-34
NAME	ng/g																
C12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	23.4	0.0	0.0
i14:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	17.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C14:0	57.7	55.4	74.2	8.7	17.5	33.7	0.0	0.0	34.9	12.0	28.4	0.0	0.0	0.0	59.9	0.0	0.0
i15:0	0.0	4.5	0.0	0.0	0.0	0.0	0.0	0.0	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
a15:0	27.8	22.2	28.7	0.0	21.1	13.4	0.0	0.0	12.5	10.6	10.4	0.0	13.5	0.0	11.8	0.0	0.0
C15:0	24.1	30.8	43.7	6.1	4.9	12.6	0.0	0.0	17.1	17.6	19.5	0.0	0.6	0.0	14.0	0.0	0.0
i16:0	0.0	11.1	19.5	0.0	0.0	0.0	0.0	0.0	8.6	0.0	6.3	0.0	0.0	0.0	5.4	0.0	0.0
a16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.4	0.0	0.0
C16:0	2091.0	896.5	1483.4	1053.4	679.8	725.3	356.8	289.2	576.6	1091.4	531.9	190.3	429.6	428.2	604.1	387.1	81.2
I17:0	0.0	5.8	0.0	0.0	0.0	0.0	0.0	6.3	0.0	0.0	7.8	0.0	0.0	0.0	0.0	0.0	0.0
C16:1n7c	0.0	15.6	89.2	57.9	16.4	50.2	19.3	24.7	33.0	48.0	49.6	17.4	22.5	20.1	47.6	20.1	0.0
C16:1n9c	16.2	70.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C17:0	118.8	24.5	48.2	47.2	18.7	13.7	14.5	44.9	20.5	33.0	28.9	18.7	21.6	16.3	23.0	9.1	29.3
a18:0	0.0	12.5	12.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.7	8.9	0.0	0.0	0.0	0.0	0.0
C17:1	0.0	0.0	0.0	0.0	0.0	9.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C18:0	3603.7	910.4	2335.3	1560.7	1075.5	975.4	722.9	570.3	438.5	1475.1	885.5	261.1	295.8	338.5	287.0	335.7	423.8
C18:1n9t	30.1	0.0	25.7	10.2	11.3	5.8	9.3	6.7	9.3	17.4	7.6	8.7	0.0	10.1	7.0	8.8	0.0
C18:1n9c	181.7	56.7	221.4	103.3	69.6	75.7	36.8	66.8	55.6	75.2	72.3	67.4	55.8	67.0	93.3	62.6	23.7
C18:2n6c	24.0	18.9	45.3	27.2	9.2	16.1	22.1	36.3	28.2	27.6	25.1	18.2	21.5	27.7	32.4	31.6	33.8
C20:0	47.1	23.6	47.8	35.4	19.9	22.5	21.8	17.2	19.0	25.5	11.6	8.3	11.5	19.3	12.1	17.3	28.7
C20:1	0.0	0.0	0.0	0.0	30.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C22:0	86.2	26.0	71.2	33.3	23.4	19.2	16.2	25.2	16.1	25.1	11.3	0.0	16.8	25.7	14.5	17.5	28.9
C22:1	41.4	19.6	34.4	15.5	11.8	18.1	0.0	19.6	6.2	15.2	7.1	30.2	8.0	16.9	14.4	18.4	40.1
Me21	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.2	0.0	0.0	0.0	0.0	0.0	0.0
C23:0	21.9	0.0	0.0	0.0	8.8	0.0	0.0	13.4	6.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C22:2	0.0	0.0	0.0	0.0	9.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C24:0	45.7	25.7	19.9	22.4	12.2	1.5	2.1	27.7	16.7	41.2	12.8	19.8	17.9	21.2	16.5	26.7	45.6
C24:1	0.0	0.0	0.0	8.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cy	0.0	0.0	0.0	0.0	0.0	0.0	10.9	0.0	0.0	0.0	0.0	0.0	0.0	9.5	0.0	0.0	0.0
Cell No.X10 ⁸	1.4	1.3	6.4	1.0	2.9	1.6	0.5	1.2	0.6	2.3	0.9	0.9	1.4	1.7	0.8	2.0	1.1
Even/Odd	36.0	35.0	43.9	50.9	56.5	67.6	77.4	15.9	24.9	52.8	30.6	25.6	34.8	51.2	27.5	86.0	20.8
ΣTFA	6417.6	2240.6	4599.9	2990.1	2040.0	1993.1	1232.8	1165.5	1305.4	2915.0	1730.9	648.9	915.1	1000.5	1274.8	935.0	735.0

Table 4.4: Fatty acid composition (ng/g) in the CC2/GCL1 core sediments

Depth	0-2	4-Feb	6-Apr	8-Jun	10-Aug	12-Oct	14-Dec	14-16	16-18	18-20	20-22	22-24	24-26	26-28	28-30	30-32	32-34	34-36	36-38	38-40	40-42	42-44	44-46	46-48	48-50	
NAME	ng/g																									
C12	0	0	0	20.5	7.9	18.5	52.1	1.3	0	0	0	0	0	3.4	1	22.1	3.8	17.9	7.5	7.9	0	0	8.2	0	0	
C13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.5	0	0	0	0	0	0	0	0	0	
14	0	0	0	29.1	0	0	0	0	0	0	0	0	0	1.6	5.2	8.1	9.7	7.4	9.4	4.9	9.4	6.1	0	0		
C14	88.4	139.3	118.2	209.2	80.8	196.2	220.8	49.9	0	19	10.1	3.5	30	9.5	47	87.5	110.2	180.8	89.6	84	94.3	111	147.6	145.9	97.4	
15	31.3	22.2	20.4	12.1	9.7	15.7	17.9	0	0	0	0	0	0	3.6	0	0.7	5.7	4.5	1	5.2	4.5	8.7	4.9	2.2	4.4	
i15	58.5	78.1	74.5	75.3	51.9	31.3	73.6	13.1	4.2	8.8	5.5	1.9	7.5	4	8.5	9.9	22.1	28.2	15.3	14.8	16.2	21.4	19	18.3	11.2	
C14:1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	
C15	54.2	41.8	21.2	60.7	24	37.9	68.7	10.4	6.6	6.9	6.6	1.4	8.1	4.7	13.4	16	24.2	51.6	25.4	22.2	27.2	30.3	32.3	41.3	22.1	
16	29.9	28.6	24.4	26.7	26.5	19.3	50.1	15.3	9.8	9.5	8	5.1	4.6	0	14.7	9.2	20.8	37	24.2	23.9	28.5	17.4	17.7	13.4	12.2	
C16	4361.5	3002	1670.3	3488.9	863.4	4936.2	2008.4	310.5	661.1	489.9	505.5	433	697.1	170.8	698.1	1795.9	2002.6	4217	1912.3	1294.3	2226	1661.9	5306.6	5207.4	2295.9	
17	36.7	0	16.8	9.6	17.9	24.7	8.5	6	5.6	2.4	1.5	0.8	1.4	1.6	3.7	3.5	5.2	0	4.8	6.5	8.5	7.3	0	23.1	7.3	
i17	0	20.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
C16:1n7c	41.8	35.4	19.7	42.6	18.2	25.4	154.7	3.7	0	4.6	6.3	3.7	5.7	5	9.7	0	21.9	0	12.7	6.9	0	0	0	0	23.4	
C16:1n9c	58.1	60.5	59.3	76.9	28.8	16.8	0	7.5	19.3	12.5	13.3	6.7	9.1	0	18.3	12.4	16.3	91	13.6	4.6	21.2	15	61.7	35.1	0	
C16:1n11c	0	26.4	17.2	0	0	0	0	0	0	0	0	0	0	0	0	0	11.7	0	0	0	0	0	0	0	0	
C17	65.2	36.8	37.9	59.8	27.8	39.7	194.4	20.6	36.4	27.3	17	17.4	20.5	14.1	23.9	20	36.2	113.8	128.7	51.6	81.6	153.7	107	395.5	350.3	
18	0	0	0	0	0	0	0	16.9	0	0	0	0	0	0	0	0	10.9	0	0	0	0	0	0	0	0	
C18	11561.3	3828.9	2621.3	5934.9	721.6	10537	799.9	151.5	2267.6	792.9	1017.3	1079.8	1252.3	345.3	783.4	2902.3	3478	7285.1	3808.1	2256	4285.4	2518.7	10619	11497.4	4788.7	
C18:1N9t	151.9	72.2	40.4	75.5	78.8	115.4	62	32.5	34.8	17	20.2	14	20	5.5	18.6	39.8	56.5	94.5	72.5	29.1	59.3	41.9	147.4	159.4	84.9	
C18:1n9c	611.3	588.8	157.4	321.5	871.1	669.5	295.6	73.3	169.9	91.3	366.9	68.7	109.7	15.5	109.8	166	211.7	501.9	967.4	144.7	204.3	127.7	417.4	485.9	199.6	
C18:1n8	190.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
C18:1n11c	0	179.6	97.4	0	0	0	0	0	0	0	0	0	0	0	0	0	25.1	0	0	0	0	19.6	0	0	0	
C19	84.8	30.8	22.8	26.9	13.8	51.4	0	7.9	13.2	7.2	9.9	3.3	7.1	0	6.4	4.6	16	17.6	28.2	9.7	25.6	27	11.3	32.5	16.3	
C18:2n6c	205.9	185.3	54.6	85.7	116.2	133.3	104.6	19.5	33.2	19.4	61.3	20.5	24.5	4.1	18.3	45.8	63.1	151.7	140.7	39.3	54.1	35.7	115.2	126.7	54.4	
C20	260.9	108.1	110.2	142.5	59.4	196.3	73.5	38.8	82.1	48.6	37.2	34.2	41.2	7.7	28.5	60.8	112	184.4	115.3	79.4	131.8	90.9	233.8	243.8	141	
C20:1	39	29.4	16.3	23.5	44.2	35.3	0	12.7	13	11.5	11.4	5.1	20.1	0	6.7	6.5	17.6	160.3	46.2	16.3	18.3	14.6	20.1	10.3	7	
C20:1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7.9	0	0	0	0	0	0	0	0	
C21	7.8	22.2	24.9	30.5	24.4	21.2	86.6	9.7	11.5	8.1	7.6	5.4	5.4	0	4.1	4.2	13.6	16.8	15.2	9.8	14.7	15.4	25.4	17.5	15.6	
C20:2	0	0	0	0	0	31.7	0	0	0	4.2	11.1	4.7	5.9	0	8	12.6	11.6	20.6	19	9.2	18.5	12	28.4	34.9	14.7	
C22	129	67.6	204.6	187.9	79.4	142.8	48.4	38.8	57.7	28.9	26.4	21.9	23.1	9.9	4.3	25.9	103.6	143.3	99.8	66.3	123.7	61	231.3	172.5	96.6	
C22:1	0	0	0	0	13.3	0	0	7.1	11.1	7.1	10.4	3.8	10.5	0	9	2.1	6.6	36.9	41.7	20.7	13.7	9.5	0	0	0	
C22:1n9C	330.7	141.6	150.5	125.5	495.1	73.4	120.9	116	218.2	124	348.7	63	151.3	11.1	62	48.2	121.3	1002.9	891.6	190.5	141.2	146.1	94.9	98	93.8	
C23	0	0	0	0	0	1.3	0	0	11.6	0	0	0	0	0	0	0	3.6	29.1	12.8	7	18.5	3.6	16.9	10.4	17.2	
C22:2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20.5	0	0	0	0	0	0	
C24	117.8	23.9	93.4	91.9	38.4	100.4	8.1	26.4	71.3	22.3	29	23.7	24.8	10.8	7.9	29.8	85.9	182.3	142.1	102	161.8	79.4	293.3	238.9	151.6	
C24:1	0	0	0	0	0	0	0	0	0	4.6	5.8	1.8	4.2	0	2.6	2.3	3.2	0	11.9	5.9	0	0	0	0	0	
C25	0	0	26.6	26	18.4	18	0	14	16.2	10	12	6.9	9.8	0	7.9	7.6	20	27.7	30.2	23.8	31.4	22.8	47.1	36	25.9	
C27	0	0	26.2	35.4	0	0	0	11.1	12.9	7.3	14.1	10.4	10.2	0	6.9	10.4	22.6	36.4	38.1	29.8	53.9	21.1	47.6	29.5	35.1	
C28	0	0	32.5	20.7	14.1	13	0	19.3	36.9	13.1	25.9	13.5	7.4	5.1	16.2	24.3	58.9	75.1	110.6	77.4	115	74.5	125.8	82.6	86.2	
C29	0	0	19.9	15.1	17.4	0	0	13.8	15.5	12.8	13.6	7.2	10.7	0	12.1	10.8	26.7	17.1	45.5	31	37.3	24.5	36	34.5	34.9	
C30	0	0	37	49.9	17.6	24.6	0	28.7	17.1	25.9	29.9	18.3	25.1	0	33.7	24.8	58.6	37.3	84.4	64.2	47.4	46.5	42.6	48.4	42.3	
C31	0	0	0	0	0	0	0	9.1	0	5.1	6.6	3.5	4.9	0	8.8	6	13.7	0	19.9	15.9	0	0	0	0	0	
C32	0	0	0	0	0	0	0	15.8	0	15.5	14.7	9.5	14.7	0	20.3	15.6	38.3	0	46.4	40.4	0	10.7	0	0	0	
Cell No.X10 ¹	2.7	2.4	12.4	1.9	2.1	1.2	3.2	2	2.9	1.2	1.5	0.5	0.4	0.9	2.4	8.7	4.4	7.9	1.1	2.3	2.4	3.2	3.7	4.6	3.1	
Even/Odd	77.9	54.5	27.2	39.9	15	95.4	9.2	7.1	25.8	17.2	19.4	29.5	27.6	29.9	19.7	61.5	34.2	39.7	18.6	20.3	24.8	15.6	52.5	29.5	14.9	
TFA	18517	8770.3	5816	11304.8	3779.8	17526.2	4448.8	1101	3836.6	1857.5	2653.7	1892.8	2567.1	631.7	2015.6	5434.2	6880	14772.7	9050.7	4799.4	8068.8	5439.4	18264.8	19241.3	8730.1	

linearly with total FAs ($r = 0.84$ and $r = 0.79$; $n = 17$, $p = 0.001$) suggesting the common origin of these FAs in CC1/GC3 core.

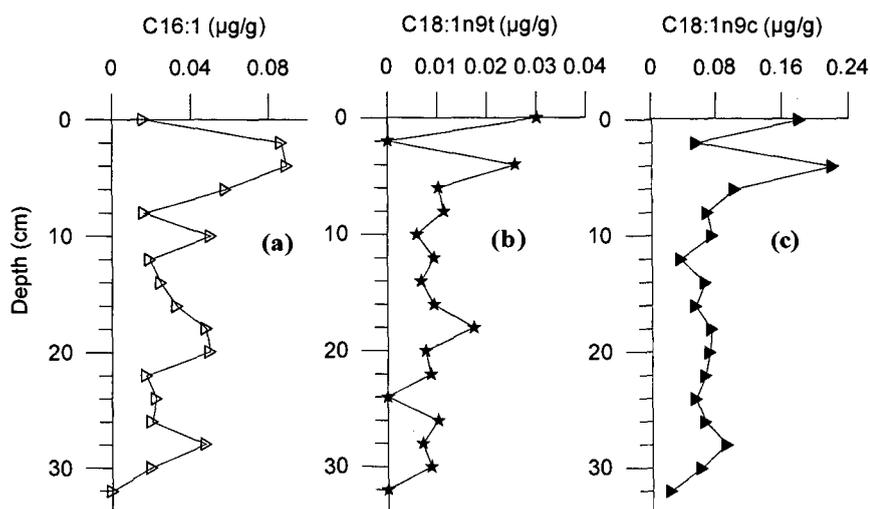


Fig. 4.7. Vertical profiles of C16:1, C18:1n9t and C18:1n9c fatty acids in the CC1/GC3 sediment core collected from Equatorial Indian Ocean.

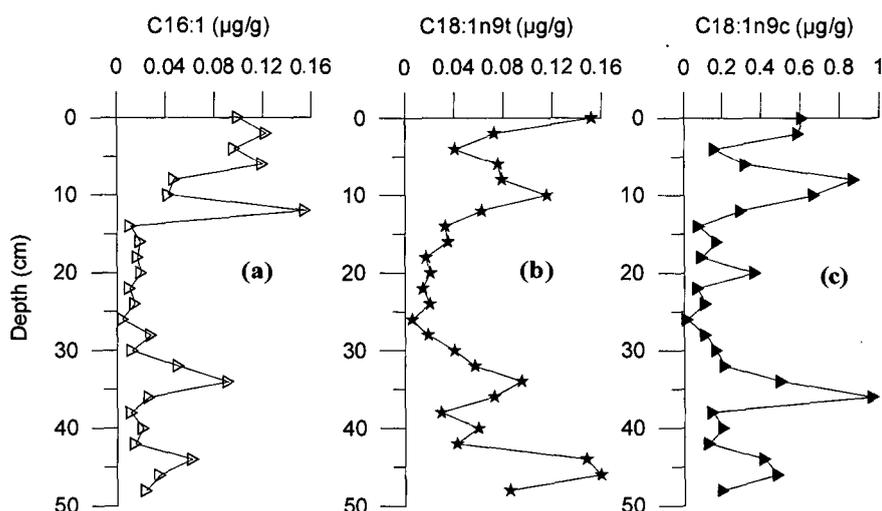


Fig. 4.8. Vertical profiles of C16:1, C18:1n9t and C18:1n9c fatty acids in the CC2/GCL1 sediment core collected from Northern Indian Ocean.

In the CC2/GCL1 core the dominating MUFAs C16:1, C18:1n9t, C18:1n9c showed similar trends throughout the core sections (Fig. 4.8). Compared to CC1/GC3 core (3.2 to 14.4 %) the concentrations of these FAs were relatively higher in CC2/GCL1 core (3.4% to 26.4 % of TFA). They generally showed a crisscross pattern, wherein the concentrations were higher

in the surface sections (0-2 cm to 12-14 cm) and later decreased (14-16 cm to 30-32 cm) and again increased in the deeper sections of the CC2/GCL1 core. The Northern Indian Ocean, where the Arabian Sea is a part of it supports a substantial bacterial community in the water column, which can remineralize a large fraction of primary productivity (Azam et al., 1994). Hence, it is not surprising that the bacterial biomarkers are reflected in higher amounts in the Northern Indian Ocean sediments (Fig. 4.8). Although high primary productivity occurs in the northern Indian Ocean contributing high OC to deep sea sediments, there occurs a relatively higher remineralization of TFA in the northern Indian Ocean. This is also evident from the low values of OC normalized TFA in the CC2/GCL1 compared to CC1/GC3 core. Trans-FAs were observed throughout the cores and the dominating trans FA was C18:1n9t. Its concentration varied as 5.8 (10-12 cm) to 30.1 ng/g dw sediment (0-2cm) in the CC1/GC3 core and 5.5 (26-28cm section) to 159.4 ng/g dw sediment (46-48cm section) in the CC2/GCL1 core. Trans-FAs are indicators of stress in the environment. They are produced from the corresponding cis isomer in response to stress in the bacteria (Guckert et al., 1986; Findlay et al., 1990). The occurrence of these FAs in the sediment sections of the CC2/GCL1 core indicates the influence of high pressure and low temperature. The vertical depth profile also varied as per TFA profile and also showed good relationship with the TFA ($r = 0.94$, $n = 25$, $p = 0.001$), suggesting possible prokaryotic origin of C18:1n9t. The presence of such fatty acids suggests that the microbial community was under environmental stress (Fang et al., 2004). Other FAs varied in concentration within the CC2/GCL1 core. C18:1n9t ($r = 0.94$, $n = 25$, $p = 0.001$), and C16:1 ($r = 0.56$, $n = 22$, $p = 0.01$) also showed good relationship with total FAs in CC2/GCL1 core, suggesting the common origin of these FAs.

Other MUFAs evident in EIO and Northern Indian Ocean sediments were C20:1, C22:1n9c and C24:1. Among these 22:1n9c was one of the major FAs present in the core

sections and showed vertical difference in concentrations (Fig. 4.9a & b and Table 4.3 & 4.4). C20:1 and C24:1 was in few sections in CC1/GC3 core, whereas these FAs were present in most sections of CC2/GCL1 core and showed a similar pattern as C22:1n9c, indicating a common origin of these FAs. Zooplankton feeding predominantly on phytoplankton contains elevated amounts of long-chain MUFAs such as 20:1, 22:1 and 24:1 (Ratnayake and Ackman, 1979; Graeve et al., 1994; Albers et al., 1996; Wakeham et al., 1997a). Zooplankton grazing is an important link between lower and higher trophic levels in the water column and FA biomarkers may be employed to determine the importance of zooplankton sources. Together the relative concentrations of 20:1, 22:1 and C24:1 varied as 0.4 to 5.5 % of TFA in CC1/GC3 core and 0.6 to 14.6 % of TFA in CC2/GCL1 core. Occurrence and abundance of 20:1, 22:1n9c and C24:1 at various core sections indicates zooplanktons contribution to the sediments. Foraminifera were evident in these sediments; hence the other sources of these fatty acids may be foraminifera in both the sedimentary environments.

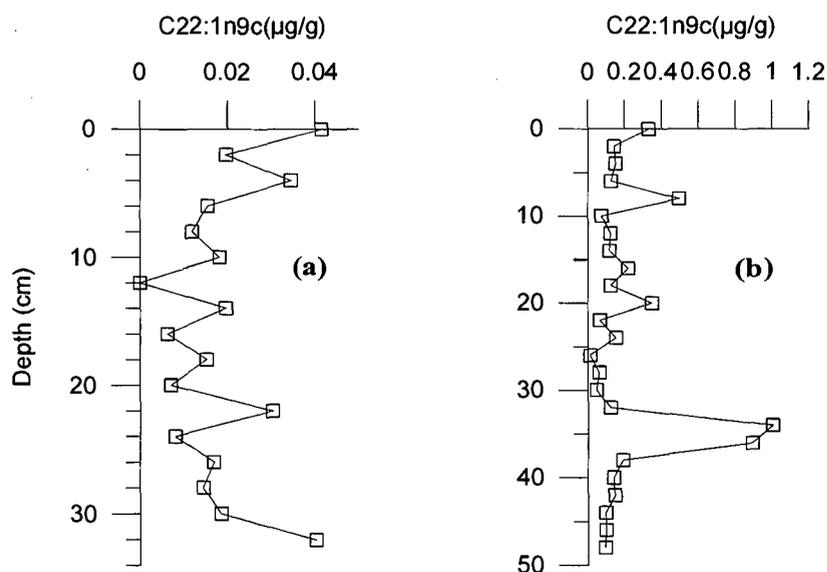


Fig. 4.9. Vertical profiles of C22:1n9c fatty acid in the CC1/GC3 sediment core collected from EIO and CC2/GCL1 sediment core collected from northern Indian Ocean.

Iso- and anteiso FAs such as i14:0, i-15:0, a-15:0, i-16:0, i-17:0 and a18:0 and i18:0 were present in the various core sections (Table 4.3 & 4.4). These compounds accounted for a small fraction (0.4 % to 2.5 %) of the TFAs in the sediment samples of CC1/GC3 core (Table 4.1) and 0.3 % to 4.7 % of the total FAs in the sediment samples of CC2/GCL1 core (Table 4.2). Br-FAs are specific for bacterial population and are not seen in algae, phytoplankton and other microorganisms or invertebrates. Terminally branched saturates (i-14:0, i-15:0, a15:0, i-16:0 and i-17:0) are common biomarkers of gram-positive bacteria, but in some cases, they are also present in gram-negative anaerobes and sulphate reducing bacteria (O'Leary and Wilkinson, 1988; Ringelberg et al., 1997; Rütters et al., 2002). Higher percentages of Br FAs to the total FA has been extensively used as specific tracers of the bacterial contribution in marine and lacustrine sediments (Perry et al., 1979; Li et al., 2007). Further the methyl Br FAs (21-Me-methyl-docosanoate (iso)) and cyclo-FAs (Cyclopropanonanoic acid, 2-[(2-butylcyclopropyl) methyl]-, methyl ester) were observed in CC1/GC3 core but the concentrations were minor as well as were present only in few core sections. Methyl Br and cyclopropyl PLFAs are generally more abundant in anaerobic environments and are common markers of anaerobic gram negative bacterial population (Baird et al., 1985; Guckert et al., 1985; Rajendran et al., 1992). However, these FAs were less abundant in the EIO and were not present in the Northern Indian Ocean. Hence the sediment samples of the CC1/GC3 and CC2/GCL1 core appear to be mostly oxygenated in nature. As iso- and anteiso FAs accounted for a small fraction of the TFAs in the sediment samples of CC1/GC3 core and CC2/GCL1 core, it indicates that gram positive bacterial abundance was low in CC1/GC3 and CC2/GCL1 core sediments.

PUFAs such as C18:2n6c were abundant throughout the CC1/GC3 core samples and fluctuated between 9.2 (8-10 cm section) to 45.3 ngg⁻¹ (4-6 cm section) dw sediment (Fig.

4.10a). Total PUFA concentrations in CC1/GC3 varied as 0.4 to 4.6 % of TFA. Highest relative concentration of C18:2n6c was observed at 4-6 cm section, wherein TLE, L-PO4 and TFA was also high (Fig. 4.2), suggesting C18:2n6c was directly contributed by the microbial community present in the sediment core section of CC1/GC3. PUFAs such as C18:2n6c were also abundant throughout the CC2/GCL1 core samples and varied between 4.1 (26-28 cm) to 205.9 ngg⁻¹ dw sediment (0-2 cm section) (Fig. 4.10b). The concentrations in CC2/GCL1 were very high compared to CC1/GC3. Highest relative concentration of C18:2n6c was observed at 0-2 cm section, where TFA was also highest (Fig. 4.3). Other abundant PUFA observed in CC2/GCL1 core was C20:2 which varied from undetectable to 34.9 ngg⁻¹ dw sediment. Vertical depth profile of C18:2n6c was almost similar to TFA profile in CC2/GCL1 core (Fig. 4.10 & 4.3). PUFAs are abundant in the eukaryotes (planktons, benthic invertebrates, etc) and are considered to undergo rapid degradation in the water column compared to MUFAs (Wakeham et al., 1997). Due to their high energy value, most of the PUFAs of microalgal origin are recycled within the euphotic zone. Since PUFAs are extremely labile (Volkman et al., 1981; Bradshaw and Eglinton, 1993), the eukaryotic derived (mostly phytolankton) PUFAs will not sustain the deep sea pressure and hence, they will be degraded before reaching the deep sea sediments. Hence it appears that C18:2n6c may be contributed by the deep sea microbial communities present in the sediment core section. Although the PUFA association with the euaryotic cells is widely accepted, they are also found with prokaryotic cells such as deep sea bacteria, which are suggested to allow the prokaryotic organisms to maintain membrane fluidity under increasing pressure (Fang et al., 2000). PUFAs are essential nutrients for the normal structure and function of the membranes of benthic animals for the survival and growth (Hazel et al., 1991). The presence of PUFAs at this depth of sediment core explains the prokaryotic origin of fatty acids as the

microorganisms are subjected to high pressure. However, the possibility of the presence of eukaryotic chemosynthetic community can not be ruled out (Li et al., 2007). The FA biomarkers are useful to understand carbon cycling and transfer of material through the food web. Presence of considerable amounts of FAs of dietary importance in various sections of sediment core indicates synthesis of fresh OM of prokaryotic origin within the core sections. PUFAs are essential nutrients for the normal structure and function of the membranes of benthic animals for the survival and growth (Hazel et al., 1991). However the presence of foraminifers within the core sections cannot be excluded for the possibility of their PUFA contribution. C18:2n6c showed good relationship with TFA in CC2/GCL1 core ($r = 0.73$, $n = 25$, $p = 0.001$).

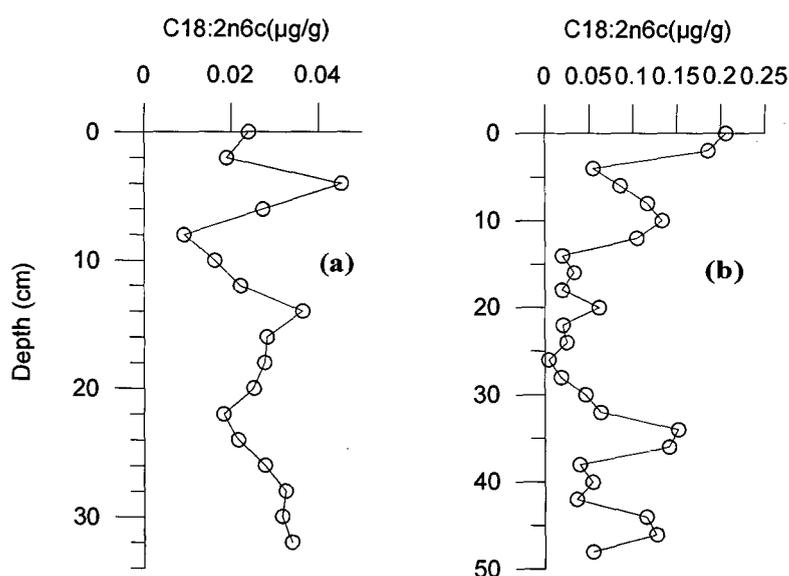


Fig. 4.10. Vertical profiles of C18:2n6c fatty acid in the CC1/GC3 sediment core collected from EIO and CC2/GCL1 sediment core collected from northern Indian Ocean.

In CC1/GC3 core long chain SatFAs (C20:0 – C24:0) were present (Table 4.3). Long chain FAs (C20:0 – C32:0) were also present in the CC2/GCL1 core sediments (Table 4.4). Sat FAs of >C20:0 carbon are often used as terrestrial plant indicators (Rajendran et al., 1992; Harvey, 1994; Santos et al., 1994; Colombo et al., 1997). These long-chain fatty acids are

derived from higher plant waxes and are more abundant in deep than in surface sediments (Rieley et al., 1991; Muri et al., 2004). In the Northern Indian Ocean, long chain fatty acids (C22 to C26) abundance was also observed in Oman Margin core sediments (Smallwood and Wolff, 2000). In most sediment, fatty acids with even-carbon chain are more abundant than those with odd-carbon chain. The core CC1/GC3 was collected from EIO and CC2/GCL1 was collected from northern Indian Ocean. However, past studies have shown that terrestrial derived OM was available in considerable amounts in the open ocean sediments. For example, large plumes of colored dissolved OM stretching from the mouths of large rivers, such as Amazon and Orinoco, to the open ocean are evident in remote sensing imagery (Blough and Del Vecchio, 2002). Refractory fraction of dissolved TOC mixes throughout the ocean water column on time scales of centuries to millennia. Blough and Del Vecchio (2002) suggested that ~25% of the OC in slope sediments is of terrestrial origin, indicating terrestrial organic carbon (TOC) is more abundant in these margin sediments than previously thought. Hence the long chain FA of presumed terrestrial origin is often the best-preserved biomarker. This fits well with the findings of Hoefs et al. (2002) that terrestrial biomarkers often are more refractory and thus become relatively enriched on increasing exposure to oxygen. EIO is very far from the coastal exposure; however the presence of terrestrial FAs suggests the possibility of refractory fraction of dissolved TOC as well as the aeolian transport of terrestrial OM. The preservation of terrestrial FAs may also be enhanced by their inclusion in resistant and microbially inaccessible higher plant matrices (Nishimura and Baker, 1987; Haddad et al., 1992). Hence it is possible that these long chain FAs may be derived from terrestrial OM (Poynter and Eglinton, 1990). Although total input of allochthonous fatty acids to the sediments was minor, these long chain fatty acids were present in both the cores and the

distribution was more prominent (showed C20 to C32 fatty acids) in the CC2/GCL1 core which was collected from a station closer to the land mass (Fig. 4.12).

4.3.5 Fatty acids as biomarkers of various organisms and degradation processes

Although, OM is contributed by phytoplankton from the top photosynthetic zone, the sediments do not show any clear signature of phytoplankton origin (Table 4.3 and 4.4). OM within the upper water column is thought to be efficiently recycled and modified by a complex food web (Passow et al., 1993) before being lost to the sediments. Bacteria are capable of converting the UFAs into saturated compounds. This was evident from the dominance of Sat C16:0 and C18:0 in the sediments of EIO and Northern Indian Ocean and lower percentages of biomarker fatty acids. In order to study the origin and abundance of the various organisms in these two types of environments, specific indicator FAs are grouped and plotted. The MUFAs and PUFAs (16:1, 18:1, 18:2, 20:2 & 22:2) are derived from the bacteria and eukaryotes forms one group, the MUFAs (C20:1, C22:1 and C24:1) are derived from zooplankton and foraminifera forms second group, long chain Sat FAs (>C20:0) are considered to be derived from terrestrial plants forms third group and Br FAs (iso and anteiso branched) are indicators of gram positive bacteria forms fourth group (Fig. 4.11 & 4.12). The relative abundance pattern for the various groups of organisms suggests the bacteria+eukaryotes group dominate the sediments of the cores in the EIO & Northern Indian ocean (Fig. 4.11 & 4.12). Bacteria+eukaryotes group appears to be the most abundant throughout the core sections in CC1/GC3 (Fig. 4.11), while in CC2/GCL1 core, this group seems to dominate the upper core sections (Fig. 4.12). However, the deeper sediment sections (specifically from 38-40 cm onwards) show the abundance of terrestrial FA in the CC2/GCL1 core. Zooplankton marker FAs (C20:1, C22:1 and C24:1) were also abundant in many core sections, which were more prevalent in middle of the core and towards the deeper sections

their abundance decreased in CC2/GCL1 core (Fig. 4.12). In the CC1/GC3 core, zooplankton and gram positive bacterial marker fatty acids were less abundant, whereas the terrestrial biomarkers were second abundant group throughout the core (Fig. 4.11). Distribution of the biomarker FAs suggests the bacteria+eukaryotic FAs were more in the top sections of the CC2/GCL1 core, whereas the terrestrial FAs were accumulated in the deeper sections of the core. However, throughout the cores terrestrial FAs were observed suggesting the refractory portion of OM get preserved better without much alteration in the deep sea environment.

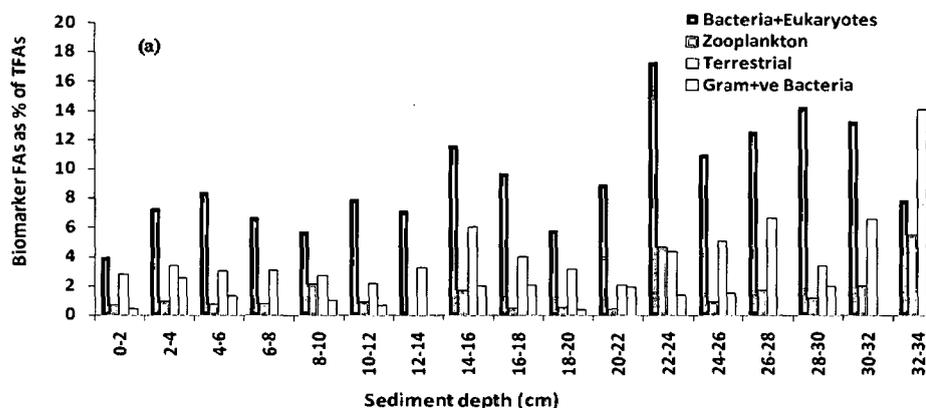


Fig. 4.11. Fatty acids as biomarkers of sources of organic matter in the deep sea sediment core CC1/GC3 collected from Equatorial Indian Ocean. Bacteria+Eukaryotes [MUFAs and PUFAs (16:1, 18:1, 18:2, 20:2 & 22:2)], Zooplankton [MUFAs (C20:1, C22:1 and C24:1)], Terrestrial [long chain sat-FAs (>C20:0)], Gram+ve Bacteria [Br-FAs (iso and anteiso branched)].

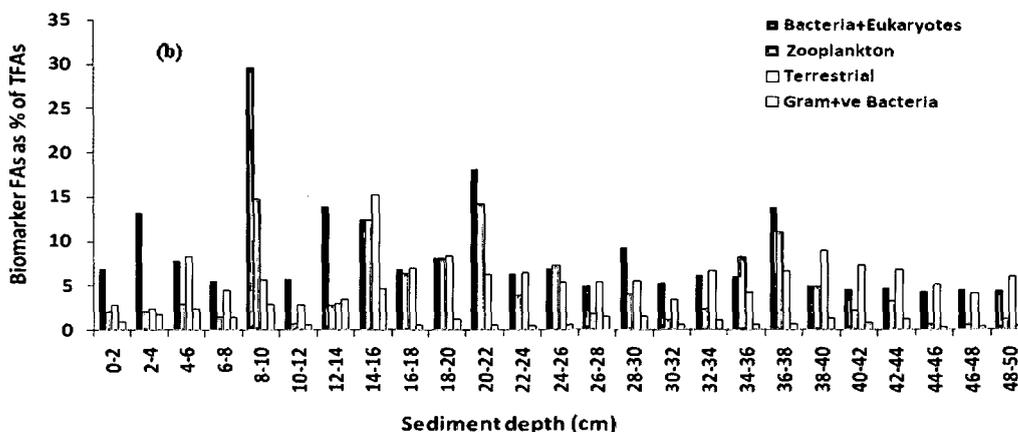


Fig. 4.12. Fatty acids as biomarkers of sources of organic matter in the deep sea sediment core CC2/GCL1 collected from northern India Ocean. Bacteria+Eukaryotes [MUFAs and PUFAs (16:1, 18:1, 18:2, 20:2 & 22:2)], Zooplankton [MUFAs (C20:1, C22:1 and C24:1)], Terrestrial [long chain sat-FAs (>C20:0)], Gram+ve Bacteria [Br-FAs (iso and anteiso branched)].

It has been suggested that TFA fraction of organic matter is 2–9 times more labile than the whole of the organic carbon pool (Camacho-Ibar et al., 2003), which seems to be a common feature of sedimentary organic matter having various components being degraded at different rates (Sun et al., 1997). Long-chain fatty acids (>C_{20:0}) in marine sediments are typically associated with terrestrial inputs of organic matter from higher plants (see review by Meyers, 1997; and references therein). Relatively high abundance of terrestrial fatty acids in deeper sections of these cores (Fig. 4.11 and 4.12), may at least partially reflect the strong selective preservation of terrestrial long-chain fatty acids than Unsaturated fatty acids in these oxic sediments (Gong and Hollander, 1997). Terrestrial OM at the Oman Margin was presumed to be mainly derived from aeolian dust transported to the Arabian Sea (Northern Indian Ocean) *via* the monsoonal winds and transferred to the water column by wet deposition (Ittekkot et al., 1992). Similarly, in the CC2/GCL1 core the presence of terrestrial FAs was due to aeolian transport of terrestrial OM. The patchiness of the sedimentary signal of terrestrial OM was observed (Fig. 4.10 and 4.11), may be due to the variability in strength of the aeolian signal and “swamping” by the sedimentation of autochthonous OM, which may itself be spatially variable (Smallwood and Wolff, 2000). Similarly, the long chain even fatty acids present in the cores must have originated through aeolian transport via the strong monsoonal winds and deposited in the sediments through wet deposition. In the long chain FAs (>C₂₀), even chain FAs were abundant than odd chain. This kind of pattern is usually seen in terrestrial FA. The CC2/GCL1 core was collected from northern Indian Ocean. Hence the possibility of aeolian or shelf origin of refractory portion of terrestrial soil derived OM to the deep sea sediments is possible. CC1/GC3 core was collected from the EIO, wherein the sources of terrestrial although were present but the concentrations were very low.

**Microbial degradation of alkenones:
Implications for paleotemperature
reconstruction.**

5A.1. Introduction

Alkenones are a class of unusual, very long chain mono-, di-, tri- and tetraunsaturated methyl and ethyl ketones synthesized by some haptophyte microalgae (Volkman et al., 1980a, Marlowe et al., 1984, Conte et al., 1994, Volkman et al., 1995). In the open ocean *Emiliania huxleyi* appears to be the dominant source of C₃₇-C₃₉ alkenones (Harvey, 2000), with additional contributions from *Gephyrocapsa oceanica*, and perhaps some other species (Conte et al., 1995a, Volkman et al., 1995). Other haptophytes synthesizing alkenones include *Isochrysis spp.* and *Chrysolita lamellosa* (Marlowe 1984, Marlowe et al., 1984, Rontani et al., 2004) which occur mainly in coastal waters. These compounds are relatively well preserved in sediments. Studies on *E. huxleyi* culture have demonstrated that alkenone biomarkers are attractive geochemical tools for palaeoceanographic studies. As a result, profiles of alkenone unsaturation patterns and the changes in the total alkenone concentrations with depth in dated deep-sea sediment cores (Farrington et al., 1988) are used as paleoceanographic proxies for the reconstruction of sea surface temperatures (SST) (e.g. Brassell et al., 1986, Prahl and Wakeham 1987, Eglinton et al., 1992), assessment of productivity of *E. huxleyi* (Brassell et al., 1986; Prahl and Wakeham, 1987; Prahl et al., 1988), measuring partial pressures of CO₂ (Jasper and Hayes 1990, Jasper et al., 1994) and now, potentially, even sea surface salinity (Engelbrecht and Sachs, 2005; Schouten et al., 2006). Because of this, alkenones are most extensively studied lipid class of compounds in marine sedimentary environments. Recently it was reported that alkenones are metabolic storage lipids that are localized in cytoplasmic vesicles or lipid bodies (Eltgroth et al., 2005).

The proportion of di- to triunsaturated C₃₇ alkenones in cultured cells increases with increasing water temperature (Brassell et al., 1986; Prahl and Wakeham, 1987). On the basis of this finding and of the ubiquity of C₃₇-C₄₀ alkenones in recent and ancient

marine sediments, the ratio $[C_{37:2}] / ([C_{37:2}] + [C_{37:3}])$, commonly referred to as $U_{37}^{K'}$, was proposed as a proxy to estimate SST (Prahl and Wakeham, 1987; Prahl et al., 1988; Müller et al., 1998). This method has become a reference standard for assessment of SST in palaeoceanographic studies.

An underlying assumption in the use of alkenones as a palaeo-temperature proxy is that the temperature signal established during their initial biosynthesis by the alga (Harvey, 2000; Grimalt et al., 2000) is not affected by diagenetic processes or, if there is a change, its extent can be objectively evaluated. However, many studies have now documented significant degradation of alkenones in the water column and in surface sediments (Prahl et al., 1989; Sikes et al., 1991; Freeman and Wakeham, 1992; Conte et al., 1992; Madureira et al., 1995; Hoefs et al., 1998; Prahl et al., 2001; Harada et al., 2003; Sun et al., 2004). If the more unsaturated components are selectively lost or modified, this will alter the $U_{37}^{K'}$ ratio (Harvey, 2000) and so compromise its use as a reliable, absolute measure of SST.

Despite the widespread use of alkenones for paleothermometry, comparatively a few studies have investigated the effects of bacteria on differential degradation of alkenones. Teece et al. (1998) conducted studies on the microbial degradation of *Emiliana huxleyi* cells under oxic and anoxic conditions in order to understand the early diagenesis processes. Although they observed an extensive degradation of the C₃₇ methyl alkenone under all the conditions examined with up to 85% degraded under oxic conditions, the $U_{37}^{K'}$ remained constant. Based on these results, it was concluded by several authors that the $U_{37}^{K'}$ index is unaffected by oxic biodegradation processes.

Recently, Rontani et al. (2005a) isolated bacteria from microbial mats rich in alkenones. These bacteria were able to degrade alkenones very efficiently under aerobic conditions. These authors observed a variable selectivity during the microbial attack on

C₃₇ alkenones resulting in variations of the $U_{37}^{K'}$ index ranging from 0 to + 0.10 that corresponds to an inferred temperature difference of 0 to +3°C. This variability could be attributed to the heterogeneity of the inoculum (microbial mats) and to the very large diversity of the aerobic heterotrophic bacteria present that could attack the alkenone molecules by various different pathways. Thus, it appears unwise to generalize the results obtained from only one experiment (Teece et al., 1998) to the wide spectrum of aerobic bacterial communities found in sediments and seawater.

Analysis of the long-chain alkenone in sediment samples, showed the post-depositional stability of these biomarkers under contrasting redox conditions (Prahl, et al., 1989). In view of this, these compounds are the reliable indicators of sea surface temperature in the ocean. However, the total abundance of these biomarkers in sediments is controlled not only by prymnesiophyte productivity, but also by their degree of exposure to oxidative degradation in the sedimentary process. Such processes may change the $U_{37}^{K'}$ index, affecting the estimations of measured temperature. In order to check this hypothesis, the degradation studies of alkenones by different aerobic bacterial communities isolated from cells of *E. huxleyi* strain TWP1 before and after different treatments of the algal cells with antibiotics were carried out. The following objectives were planned: 1) to study the degradation of alkenones and the corresponding change in unsaturation ratio using aerobic bacterial communities, and 2) to study the metabolic products of the alkenones formed during the microbial degradation of alkenones.

5A.2. Materials and methods

5A.2.1. Algal growth medium

The growth medium used for the culturing of the *Emiliana huxleyi* strain TWP1 was f/2 medium. This was prepared as given in chapter 2C. The f/2 medium was buffered using Tris-HCl (1g/L). The medium was then autoclaved at 120°C (20 min).

5A.2.2. Bacterial growth medium

Bacterial growth medium includes synthetic sea water (SSW), FeSO₄.7H₂O (stock solution of 50mg/50ml D/W + few drops of concentrated HCl to prevent oxidation), K₂HPO₄ (1.86g/100ml D/W). SSW composition includes stock solution A (NaCl (53.5g), MgSO₄ (39.25g), KCl (3.75g), Tris-HCl (30.25g), NH₄Cl (15g) dissolved in 500 ml distilled water (D/W) and stock solution B (CaCl₂ (7.35g) dissolved in 500ml distilled water). To prepare 1000ml bacterial medium, stock solutions were diluted X 10. For this 800 ml of D/W was added to stock solution A and then 100ml of stock solution B was added (1:1). This was done to avoid the precipitation of the medium. Stock solution of FeSO₄.7H₂O and K₂HPO₄ were autoclaved separately at 120°C (20 min). To the diluted SSW (1L), 1g of sodium acetate (as carbon source) was added and autoclaved at 120°C (20 min). The bacterial medium was supplemented with FeSO₄.7H₂O (0.1 mM) and K₂HPO₄ (0.33 mM) just before inoculating with the bacterial communities. For the bacterial degradation studies, sodium acetate was replaced from the medium with the sterilized cells of *Emiliana huxleyi* strain TWP1 (used as carbon source).

5A.2.3. Substrate preparation for the degradation studies of alkenones

Emiliana huxleyi strain TWP1 was used as a substrate for bacterial degradation studies. This microalgal culture was obtained from the Caen Algalbank (France). Stock cultures of *E. huxleyi* strain TWP1 were grown and maintained in 50 ml conical flasks containing 10 ml of f/2 medium at 20°C, using 116 μmol photons m⁻² s⁻¹ cool white fluorescent light (Osram, fluora) under 12 h light:12 h dark regime. Mass culturing of this microalga was done in order to obtain sufficient substrate for the degradation studies. Before the mass culturing of cells, two subsequent transfers of *E. huxleyi* in 10 ml of f/2 medium and grown for 10 days under the same conditions mentioned above was carried out. 10ml culture of *E. huxleyi* was then transferred to a 100 ml f/2 medium and grown

under similar conditions. This 100 ml starter culture of *E. huxleyi* was then transferred to flasks containing 1500 ml of f/2 medium and grown for 10 days as above. In order to obtain sufficient amount of cells as substrate for degradation studies (approximately 200mg dry weight cells of *E. huxleyi*), mass culturing was repeated several times. The cells were harvested using a Beckman J2-21 centrifuge at 5000 rpm for 25 min, concentrated at 15000 rpm using a Beckman GS-15R centrifuge (U.S.A.) for 15 min to remove excess water. All the cells were finally pooled together and then freeze-dried using a Virtis Benchtop 2K lyophilizer, homogenized using a mortar and pestle and stored at -20°C until used. To make the substrate (algal cells) bacteria free, lyophilized and homogenized cells were sterilized before using for degradation studies by bacterial communities.

5A.2.4. Sterilization of the substrate (*E. huxleyi*)

The above lyophilized and homogenized cells of *E. huxleyi* strain TWP1 (10 mg x 16) were weighed in 5 ml screw cap Pyrex vials with Teflon (TFPE) lining. The vials were securely tightened to avoid moisture. These vials were kept in an air tight bottle and autoclaved at 120°C (20 min) so as to sterilize the algal cells.

5A.2.5. Success of the sterilization procedure

Further, the sterilized cells of *E. huxleyi* were also tested to verify the success of the sterilization process. From the sterilized cells two vials were checked for the sterilization success. A vial containing sterilized *E. huxleyi* cells was used to inoculate the bacterial growth media and the contents of the second sterilized vial were added to the f/2 medium. Both flasks were incubated at 20°C for 10 days.

5A.2.6. Success of the stability of the alkenones to sterilization procedure

From the above lyophilized cells of *E. huxleyi* strain TWP1, 10 mg x 2 were weighed in 5 ml screw cap Pyrex vials with Teflon (TFPE) lining and considered as non-

sterilized controls. These two non sterilized controls and two sterilized vials of *E. huxleyi* substrate were extracted for lipids following the procedure of lipid extraction mentioned below. Lipid profiles of these two sterilized vials containing substrate were compared to those of the two non-sterilized controls to check for any changes that might have resulted from the sterilization procedure.

5A.2.7. Enrichment of bacterial community

The bacterial communities used in the degradation studies of alkenones were enriched from the *E. huxleyi* strain TWP1. First a total aerobic bacterial community was enriched from *E. huxleyi* strain TWP1 cells grown in f/2 media at 20°C (labeled TAB - total aerobic bacteria) using 116 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ cool white fluorescent light (Osram, fluora) under 12 h light:12 h dark regime for 10 days without antibiotics. The remaining bacterial communities were obtained by treating the *E. huxleyi* strain TWP1 with various antibiotics. The treatment given is shown in the Table 1. At the Caen Algalbank, these algal cultures were treated with various antibiotics which eliminated specific components of the bacterial community (Table 5A.1). Bacterial communities from these antibiotic treated algal cells were isolated and labeled as ATB1, ATB2 and ATB3 (antibiotic-treated bacteria; Table 5A.1). These antibiotic nontreated and pre-treated *E. huxleyi* cells associated bacterial communities were enriched by successive transfers in bacterial growth medium prepared from synthetic sea water (SSW) (Baumann and Baumann, 1981) supplemented with FeSO_4 (0.1 mM) and K_2HPO_4 (0.33 mM) in the presence of NaAc as carbon source (1 g l^{-1}) under darkness. Subsequently, 5ml of cultures were inoculated with 50 ml of the bacterial medium and incubated at 20 °C for 3 days under aerobic conditions in 250 ml Erlenmeyer flasks and agitated on a reciprocal shaker. Subsequently, two or more transfers of bacterial community (5ml) in the bacterial growth medium (50ml) were

carried out as above to ensure exclusion of all the *E. huxleyi* cells. These communities were then used for the degradation of alkenone studies.

Table 5A.1: Origin of the different bacterial communities used.

Bacterial community	Origin	Ratio (bacterial number/ <i>E. hux.</i> cells) in algal cultures at time of bacterial isolation ^a
TAB	Untreated <i>E. huxleyi</i>	25.1
ATB1	<i>E. huxleyi</i> , treated (24h) with 20% Provasoli's antibiotic concentrate ^b (Sigma).	6.6
ATB2	<i>E. huxleyi</i> , treated (24h) with mixture of penicillin G (100 mg), streptomycin (25 mg) and gentamycin (25 mg) per 10ml.	4.5
ATB3	<i>E. huxleyi</i> , treated (24h) with 20% Provasoli's antibiotic concentrate ^b (Sigma) and transferred x2 in sterile f/2 medium.	8.4

^a Cells counted using epifluorescence technique with fluorochrome [40,6-diamidine-20-phenylindole dihydrochloride (DAPI; Rontani et al., 1999].

^b Mixture of penicillin G (12000 units), chloramphenicol (50 µg), polymyxin B (300 units) and neomycin (60µg) per ml.

5A.2.8. Bacterial community incubations for degradation of alkenones

The above lyophilized, sterilized cells of *E. huxleyi* strain TWP1 were used as substrate for alkenone degradation. Each of the isolated bacterial communities (as mentioned above) (5ml) was incubated (in duplicate) with 10 mg of sterilized *E. huxleyi* cells, in sterile SSW (50ml) supplemented with FeSO₄ (0.1 mM) and K₂HPO₄ (0.33 mM) under continuous shaking conditions at 20°C for 20 days. In order to monitor the degradation of alkenones by the bacterial communities, two sterile controls were also prepared in the same way as the bacterial incubation flasks but without adding the bacterial inoculum prior to incubation. The lipids were extracted (as mentioned below) from these flasks after 20 days to check any probable degradation of alkenones by the bacterial communities.

5A.2.9. Denaturing gradient gel electrophoresis DGGE analyses

Cell lysis of the bacterial communities and DNA extraction were performed as described by Zhou et al. (1996). Briefly, the bacterial communities TAB, ATB1, ATB2

and ATB3 grown for 3 days on SSW supplemented with FeSO_4 (0.1 mM) and K_2HPO_4 (0.33 mM) in the presence of NaAc as carbon source (1 g l^{-1}) under darkness were used for DNA extraction. One ml each of these cultures was centrifuged at 10,000 rpm for 5 min. The cell pellet was suspended with 20 μl of buffer (Tris 20 mM, EDTA 25 mM and lysozyme $1 \mu\text{g/ml}$) and incubated at 37 °C for 15 min. To this 180 μl (1:100 dilution) of sterile ice cold water was added, and extracted with 200 μl (1:1 dilution) of chloroform after vortexing vigorously. The suspension was centrifuged (5 min at 10,000 rpm). Carefully the upper layer was transferred without disturbing the middle layer to another sterilized tube and placed under vacuum to remove residual chloroform. This dried sample was then digested with 1 μl of RNase (10 mg/ml concentration) by incubating at 37 °C for 30 min. The DNA in the solution was diluted to 1:10 (2 μl in 18 μl) in sterile distilled water.

The polymerase chain reaction (PCR) conditions for 16S rRNA genes, including the hot start and a touchdown for primer annealing, were similar to those used by Muyzer et al. (1993). PCR was carried out in a 25 μl reaction mixture containing; enzyme buffer (20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2), 0.25 mM of each deoxyribonucleoside triphosphate, 2 μM of each primer (907RA, GMLF), 0.5 units of *Taq* polymerase (Roche Diagnostics, Mannheim, Germany) and 2 μl of diluted DNA. PCR was performed at a temperature program of 94 °C for 5 min, 94 °C for 1 min, 55 °C for 30 sec, 72 °C for 3 min and final extension was done at 72 °C for 10 min.

The PCR products were analysed on ethidium bromide (1.5%) stained agarose gel electrophoresis prepared in 1 X TBE buffer (10.8 g tris-HCl, pH 8.0, 5.5 g boric acid and 4 ml of 0.5 M Na_2EDTA in 1 L). 1 kb DNA ladder was used for molecular weight determination. Electrophoresis was done at 90 V for 30 min.

DGGE was performed using a D-code Universal Mutation Detection System (Bio-Rad Laboratories Inc). Samples containing approximately equal amounts of PCR products (40 ng DNA) were loaded onto 1-mm-thick, 6 % (wt/vol) polyacrylamide gels with a denaturation gradient from 30 to 50% for 16S rRNA genes (100% of denaturation corresponds to 7 M urea and 40% formamide). Electrophoresis was run at 60°C for 5.5 h at 150 V in 1X TAE buffer (40 mM tris-HCl, 20 mM acetic acid, 1 mM EDTA). Following electrophoresis, the gels were incubated for 30 min in 1X TAE buffer containing ethidium bromide (0.5 µg/ml) and photographed on a UV transilluminator (GelDoc 2000, gel documentation system, Bio-Rad).

5A.2.10. Lipid extraction

After 20 d incubation of the TAB, ATB1, ATB2, and ATB3 communities with sterilized *E. huxleyi* cells, the contents of the each flasks were extracted with chloroform-methanol-water (1:2:0.8, v/v/v) using ultrasonication for 15 min. Recovery of the lipids was estimated by adding an internal standard (C₃₆ *n*-alkane). Chloroform and purified water were added to the combined extracts to give a final chloroform-methanol-water ratio of 1:1:0.9 (v/v/v), to initiate phase separation. The upper aqueous phase was subsequently extracted twice with chloroform. The upper aqueous phase containing salts and water-soluble material was discarded and total solvent-extractable lipids were recovered in the lower chloroform phase, after drying over anhydrous Na₂SO₄, filtration and solvent evaporation under vacuum. The lipid extracts were saponified, reduced and run of GC-EIMS.

5A.2.11. Alkaline hydrolysis

In order to carry out the alkaline hydrolysis, to the total solvent extractable lipids, 25 ml of water, 25 ml of methanol and 2.8 g of KOH were added. The mixture was directly saponified by refluxing at 80°C for 2 h. After cooling, the content of the flask was

acidified with HCl (to pH 1) and the lipids were subsequently extracted with dichloromethane (x3). The combined extracts were dried over anhydrous Na₂SO₄, filtered and evaporated to dryness under vacuum and aliquot were used for the reduction (NaBH₄ or NaBD₄), hydrogenation and osmium tetroxide derivatization. The total lipids, reduced lipids, hydrogenated and osmium tetroxide derivatized lipids were derivatized with BSTFA and pyridine and run on GC-EIMS.

5A.2.12. Alkenone reduction

Aliquot of the total lipid was reduced (20min) in diethyl ether:methanol (3:1, v/v) (5 ml) using excess NaBH₄ or NaBD₄ (10 mg/mg extract). After reduction, 10 ml of a saturated solution of ammonium chloride was added cautiously to destroy excess reagent, the pH was adjusted to 1 with dilute HCl (2N) and the mixture was shaken and extracted with hexane: chloroform (4:1, v/v, x3). The combined extracts were dried over anhydrous Na₂SO₄ and evaporated to dryness under a stream of nitrogen. The reduced lipids were derivatized using BSTFA and pyridine and run on GC-EIMS.

5A.2.13. Hydrogenation

Small aliquot of the total lipids was hydrogenated (under an atmosphere of H₂) in methanol with Pd/CaCO₃ (5% Pd, 10-20 mg/mg of extract) (Aldrich) as a catalyst for 12 h with magnetic stirring. After hydrogenation, the catalyst was removed by filtration and the filtrate was concentrated by rotary evaporation. The lipids then derivatized using BSTFA and pyridine and run on GC-EIMS.

5A.2.14. Osmium tetroxide derivatization

OsO₄ (~2 mg per mg of extract) and an anhydrous pyridine-dioxane solvent mixture (1:8, 5 ml) were added to each total lipid fraction (an aliquot). The resultant solution was homogenized by swirling and then incubated at room temperature (1 hr). Afterwards, a 16% (w/v) suspension of Na₂SO₃ in water-methanol (8.5:2.5, v/v) was

added (6 ml) and the mixture incubated again at room temperature (1.5 hr). The solution was subsequently acidified (pH 3) by dropwise addition of concentrated HCl and extracted with hexane-chloroform (4:1 v/v, 5 ml each, x3). The combined extracts were dried over anhydrous Na₂SO₄, filtered and concentrated by rotary evaporation. The lipids then derivatized using BSTFA and pyridine and run on GC-EIMS.

5A.2.15. Derivatization

The total lipids extracts, reduced extracts, hydrogenated and osmium tetroxide derivatized extracts obtained from degradation studies were evaporated using vacuum evaporator. The dried residues of the total lipids were taken up in 400 µl of a mixture of pyridine and pure N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco) (3:1, v/v) and silylated for 1 h at 50 °C. After evaporation to dryness under nitrogen, the residues were taken up in a mixture of ethyl acetate and BSTFA and analyzed by GC-EIMS.

5A.2.16. Gas chromatography-electron impact mass spectrometry (GC-EIMS) analyses

GC-MS was carried out under electron ionization (EI) conditions with an Agilent 6890 gas chromatograph connected to an Agilent 5973 inert mass spectrometer. The following operating conditions were employed: 30 m x 0.25 mm (i.d.) fused silica capillary column coated with SOLGEL-1 (SGE; 0.25 µm film thickness); oven temperature programmed from 70°C to 130°C at 20°C min⁻¹, from 130°C to 250°C at 5°C min⁻¹ and then from 250°C to 300°C at 3°C min⁻¹; carrier gas (He) maintained at 0.69 bar until the end of the temperature program and then programmed from 0.69 bar to 1.49 bar at 0.04 bar min⁻¹; injector (on column with retention gap) temperature, 50°C; electron energy, 70 eV; source temperature, 190°C; cycle time, 1.99 cycles s⁻¹; scan range m/z 50–800. Quantification of alkenones or alkenols (obtained after NaBH₄ reduction) involved calibration with external standards.

5A.3. Results and discussion

5A.3.1. Sterilization tests

Sterilized cells of *E. huxleyi* strain TWP1 did not grow in f/2 medium showing that the algae cells were indeed dead. Inoculation with bacterial medium also showed no growth of bacteria even after long incubation times. Hence, complete sterilization of *E. huxleyi* cells was achieved. Comparison also showed the relative concentrations of di- and triunsaturated C₃₇ alkenones in sterilized cells were not different from that in non-sterilized control cells. Hence, sterilization was achieved without changing the cellular alkenone profile.

5A.3.2. Degradation of *E. huxleyi* by different bacterial communities

Sterile controls carried out in parallel showed no significant change in the lipid profiles (relative to initial cells) over the course of these experiments. This demonstrates that the changes in the composition of these lipids observed in the present experiments were indeed microbially mediated. After incubation for 20d, a varying degree of aerobic biodegradation of lipids by the four bacterial communities isolated from *E. huxleyi* strain TWP1 cells was observed (Table 5A.2). Fatty acids were strongly degraded in all cases, while the phytol side-chain of chlorophyll and especially the main algal sterol, *epi*-brassicasterol, appeared to be more recalcitrant towards bacterial degradative processes.

Table 5A.2: Lipid degradation (%) after incubation of sterile cells of *E. huxleyi* with different aerobic bacterial communities

	C _{14:0} fatty acid ^a	C _{22:6} fatty acid	Epibrassicasterol	phytol
TAB community	91 ± 2	93 ± 2	17 ± 8	21 ± 10
ATB1 community	57 ± 6	70 ± 5	16 ± 3	30 ± 8
ATB2 community	61 ± 2	93 ± 4	8 ± 1	32 ± 9
ATB3 community	86 ± 4	97 ± 1	- ^b	54 ± 1

^a Calculated relative to sterile controls.

^b No significant degradation.

Though alkenones are generally considered to be much more stable towards degradative processes than most common phytoplanktonic lipids (Harvey, 2000), in the

present study higher biodegradation rates for alkenones was obtained (for three of the four tested communities) (Table 5A.3), than for *epi*-brassicasterol (Table 5A.2). These results are in good agreement with previous observations made during a study of the stability of alkenones in senescing cells of *E. huxleyi* (Rontani et al., 1997) and more recently during a study of aerobic biodegradation of *E. huxleyi* cells by aerobic bacterial communities isolated from microbial mats (Rontani et al., 2005a). Only minor degradation of alkenones was observed with the ATB1 community, while incubation with the ATB2 community resulted in major degradation (Table 5A.3). But, in both cases the degradation of di- and triunsaturated alkenones appeared to be non-selective. In contrast, incubation of *E. huxleyi* strain TWP1 cells with the ATB3 and TAB communities resulted in major and selective degradation of alkenones (Table 5A.3). Based on an established calibration equation (Prahl et al., 1988), increase in $U_{37}^{K'}$ (relative to the control) after incubation for 20d with TAB and ATB3 communities was observed (Table 5A.3), equivalent to an inferred increase in growth temperature of 2°C and 3.3°C, respectively.

Table 5A.3: Alkenone degradation (%) after incubation of sterile cells of *E. huxleyi* with different aerobic bacterial communities

		MeC _{37:3} ^a	MeC _{37:2}	$U_{37}^{K'}$	EtC _{38:3}	EtC _{38:2}	MeC _{38:3}	MeC _{38:2}
Sterile control	Duplicate 1	-	-	0.77	-	-	-	-
	Duplicate 2	-	-	0.76	-	-	-	-
TAB Community ²	Duplicate 1	48.6	24.6	0.83	38.0	23.3	47.7	37.7
	Duplicate 2	49.4	25.7	0.83	39.7	28.2	44.7	36.0
ATB1 community ³	Duplicate 1	8.0	12.0	0.76	5.1	16.3	7.8	18.9
	Duplicate 2	10.0	12.3	0.76	7.6	12.8	7.0	9.1
ATB2 Community ⁴	Duplicate 1	25.0	27.8	0.76	21.1	25.8	40.8	42.4
	Duplicate 2	45.3	45.4	0.77	37.7	37.8	48.8	50.7
ATB3 Community ⁵	Duplicate 1	67.0	23.4	0.88	72.1	21.5	68.8	36.8
	Duplicate 2	61.7	21.2	0.87	77.7	32.7	65.1	38.5

^a Calculated relative to sterile controls.

These results clearly show that the various antibiotic treatments of *E. huxleyi* strain TWP1 cells significantly changed the composition of the bacterial communities associated

with the cells. The changes in bacterial community could be visualized using DGGE analysis (Fig. 5A.1). The DGGE pattern of the TAB community showed a complex structure illustrated by a smear containing several major bands (lane 2). The structure of the bacterial communities associated with *E. huxleyi* maintained in the presence of different antibiotics showed drastic changes. In the case of ATB1 (lane 3) or ATB2 (lane 4) communities the DGGE pattern was dominated by only one band (1), that seemed to be the same for both treatments. In contrast, the DGGE pattern of the ATB3 community (lane 5) exhibited three major bands (1–3), one showing the same electrophoretic mobility as band 1 of ATB1 or ATB2 and another (band 3) corresponding to one of the major bands of TAB.

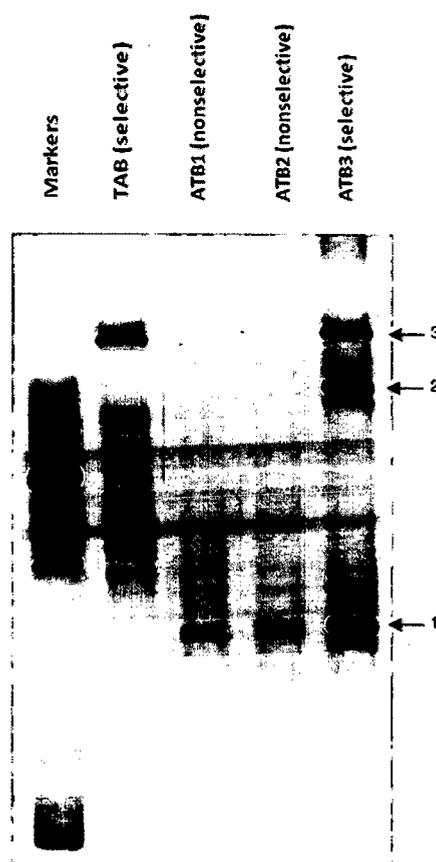


Fig. 5A.1. Negative image of DGGE profiles of the 16S rDNA fragments obtained with primers specific for the domain bacteria and template DNA extracted from TAB, ATB1, ATB2 and ATB3 communities. Markers correspond to a mixture of PCR products amplified from *Clostridium perfringens*, *Marinobacter hydrocarbonoclasticus* sp. cab and *Micrococcus luteus*.

The very distinct results obtained with the four communities strongly suggest the existence of at least two functional classes of aerobic bacteria capable of degrading alkenones, i.e. those able to degrade them either non-selectively or selectively. It is likely that these differences in degradative outcomes are strongly dependent on the particular metabolic pathways used by the two groups of bacteria.

5A.3.3. Metabolic products of alkenone degradation produced by bacterial communities

Analyses of the total lipid extracts obtained after incubation of *E. huxleyi* strain TWP1 cells with the ATB3 community showed the production of small amounts of methyl and ethyl alkenols (ranging from 4.2% to 2.0% of the corresponding alkenone), which were lacking in the sterile controls (Fig. 5A.2). This appears to be the first observation of a probable bacterial production of alkenols from the corresponding alkenones. Alkenols were previously detected in trace amounts in several haptophytes (Rontani et al., 2001) and in microbial mats (Rontani and Volkman, 2005). The sources of these compounds in microbial mats were thought to be either from bacterial reduction of alkenones or direct input by haptophytes. The present observation supports the first assumption. This reductive pathway probably results from non-specific dehydrogenase-hydrogenase activity (Platen and Schink, 1989), which is not necessarily specifically related to alkenone degradation.

NaBH₄ reduction of total lipid extracts obtained after incubation of *E. huxleyi* strain TWP1 with the ATB3 community also showed the presence of three peaks eluting close to the resultant C₃₉ alkenols (Fig. 5A.3). These compounds, which were absent in reduced extracts from the sterile controls, clearly resulted from bacterial degradation of the alkenones. It is interesting to note that they were also present (but in lesser amounts) in the reduced extracts obtained after incubation with the TAB community. On the basis of their EI mass spectra (Fig. 5A.4), the compounds were assigned as isomeric diols and attributed

their formation to the partial NaBH_4 reduction of the corresponding keto epoxides produced after bacterial oxidation of alkenone double bonds. To test this hypothesis, the total lipid extracts were reduced with NaBD_4 instead of NaBH_4 . EI mass spectral data provided unambiguous validation of this hypothesis as shown, for example, by results obtained for peak 1 (Fig 5A.5).

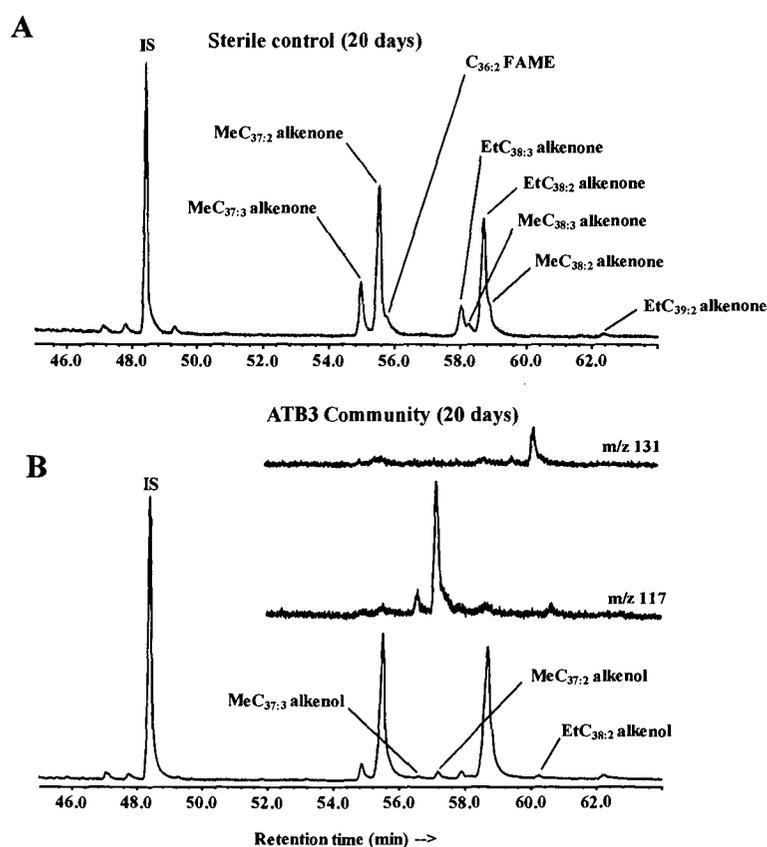


Fig. 5A.2. Partial total ion chromatograms of total lipid extracts obtained after incubation of *E. huxleyi* strain TWP1 in sterile medium (A) and with the ATB3 community (B) for 20 days. The insert shows m/z 117 and 131 chromatograms of the lipid extract obtained after incubation with the ATB3 community.

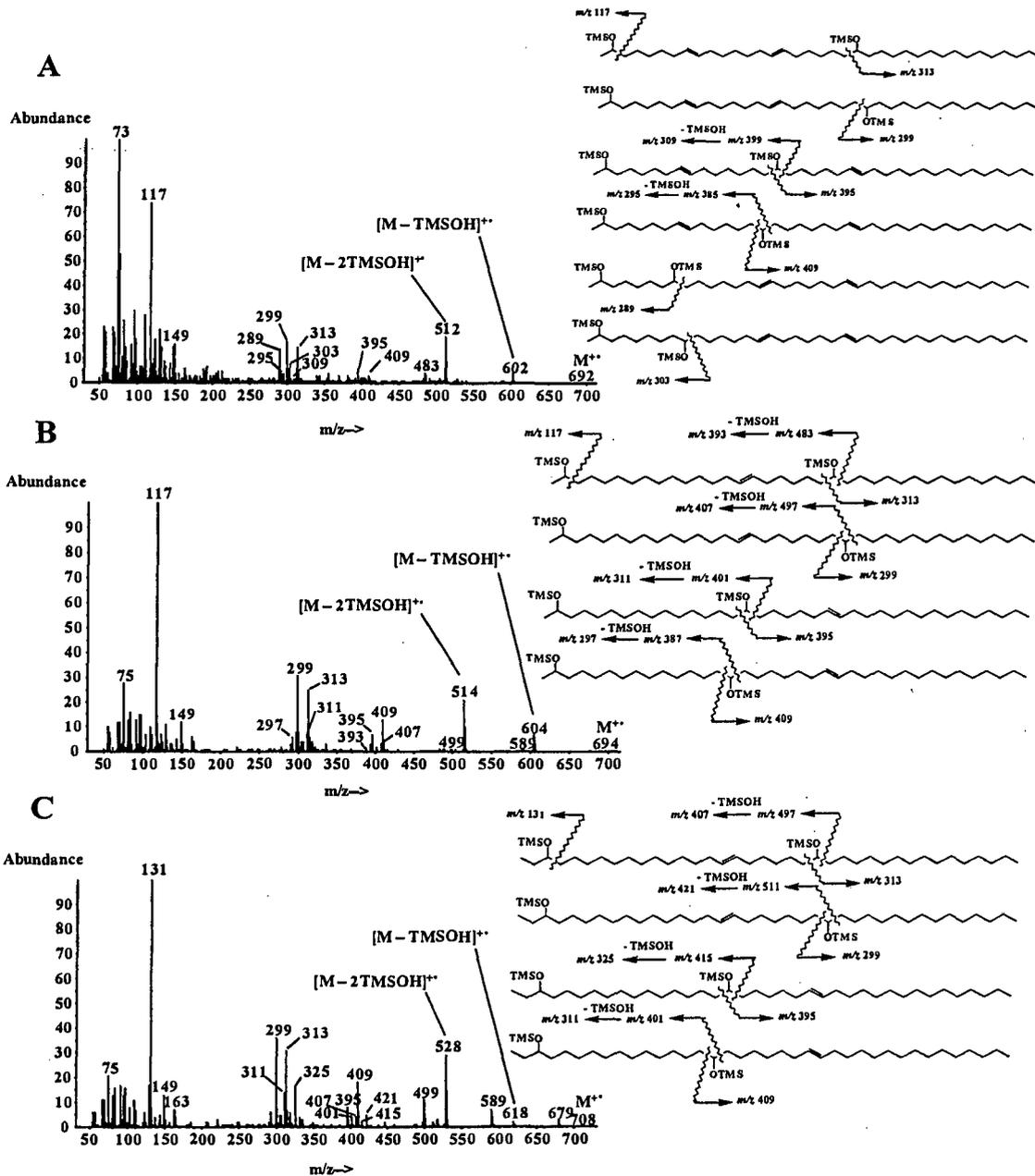


Fig. 5A.4. EI mass spectra of peaks 1 (A), 2 (B) and 3 (C); see insert in Fig. 3.

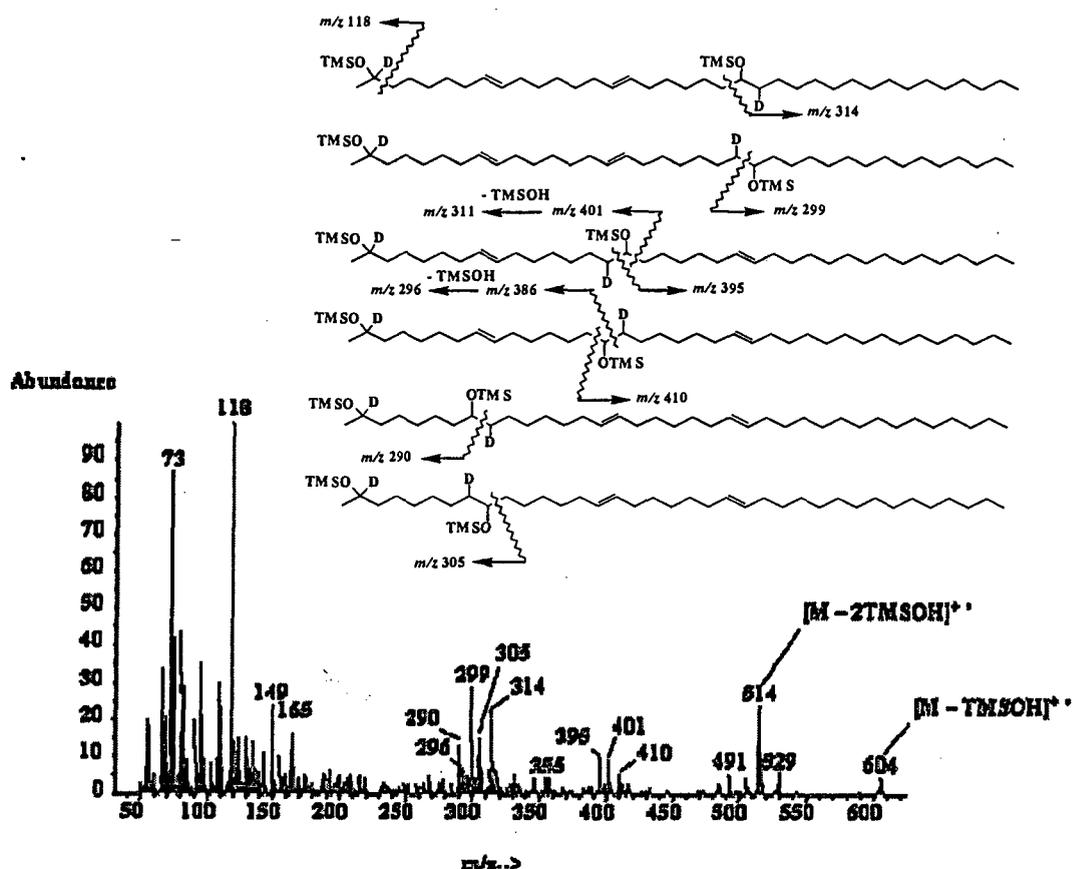


Fig. 5A.5. EI mass spectrum of peak 1 in Fig. 3 obtained after reduction with NaBD₄.

Attack of double bonds by peroxy radicals (autoxidation) can also result in the production of epoxides (Fossey et al., 1995). When the double bonds have allylic hydrogens there is competition between addition of the peroxy radical to the double bond (epoxide formation) and allylic hydrogen abstraction (formation of allylic hydroperoxides). It was previously demonstrated that free radical oxidation of alkenones in solvents (Rontani et al., 2006a) and *E. huxleyi* cells (Rontani et al., 2007) mainly involves allylic hydrogen abstraction. On the basis of these results, the epoxidation of alkenone double bonds observed after bacterial incubation was attributed to bacterial action rather than abiotic chemical processes since such compounds were lacking in sterile controls. Moreover, in NaBH₄-reduced lipid extracts obtained after incubation of *E.*

huxleyi cells with the ATB3 community, large amounts of 11-hydroxyoctadecanoic and 12-hydroxyoctadecanoic acids were also detected (Fig. 5A.6B), which were present only in trace amounts in the controls (Fig. 5A.6A). Reduction with NaBD₄ instead of NaBH₄ demonstrated unambiguously that these compounds resulted from the reduction of 11,12-epoxyoctadecanoic acid. The lack of hydroxyacids resulting from the reduction of the 12,13-epoxy-11-methyloctadecanoic acid, even though the 11-methyloctadec-12-enoic acid was present in similar proportion to *cis*-vaccenic acid in these extracts, clearly demonstrates the specificity of epoxidation processes and attributes the formation of epoxyalkenones unambiguously to enzymatic epoxidation processes.

The selectivity observed during alkenone biodegradation by the ATB3 and TAB communities by enzymatic attack of double bonds must be due to specific components of the bacterial communities. Indeed, these bacteria appear capable of oxidizing all the alkenone double bonds (Fig. 5A.4), but with some selectivity (22%, 32% and 46% oxidation of the ω 15, ω 22 and ω 29 double bonds, respectively). These estimates are based on the abundances of fragment ions resulting from α -cleavage of the TMS ether groups of the fully hydrogenated diols measured at 20 eV (in order to avoid subsequent selective cleavage of these fragment ions) and taking into account the proportion of initial alkenones and the differences of stability of the fragment ions formed. The higher reactivity of the ω 29 double bond towards enzymatic epoxidation processes is in good agreement with the observed preferential degradation of triunsaturated alkenones. The presence of a high proportion of bacteria able to attack alkenone double bonds via such selective processes could thus induce a non-negligible increase in calculated values of the alkenone unsaturation ratio and thereby lead to warmer inferred growth temperatures.

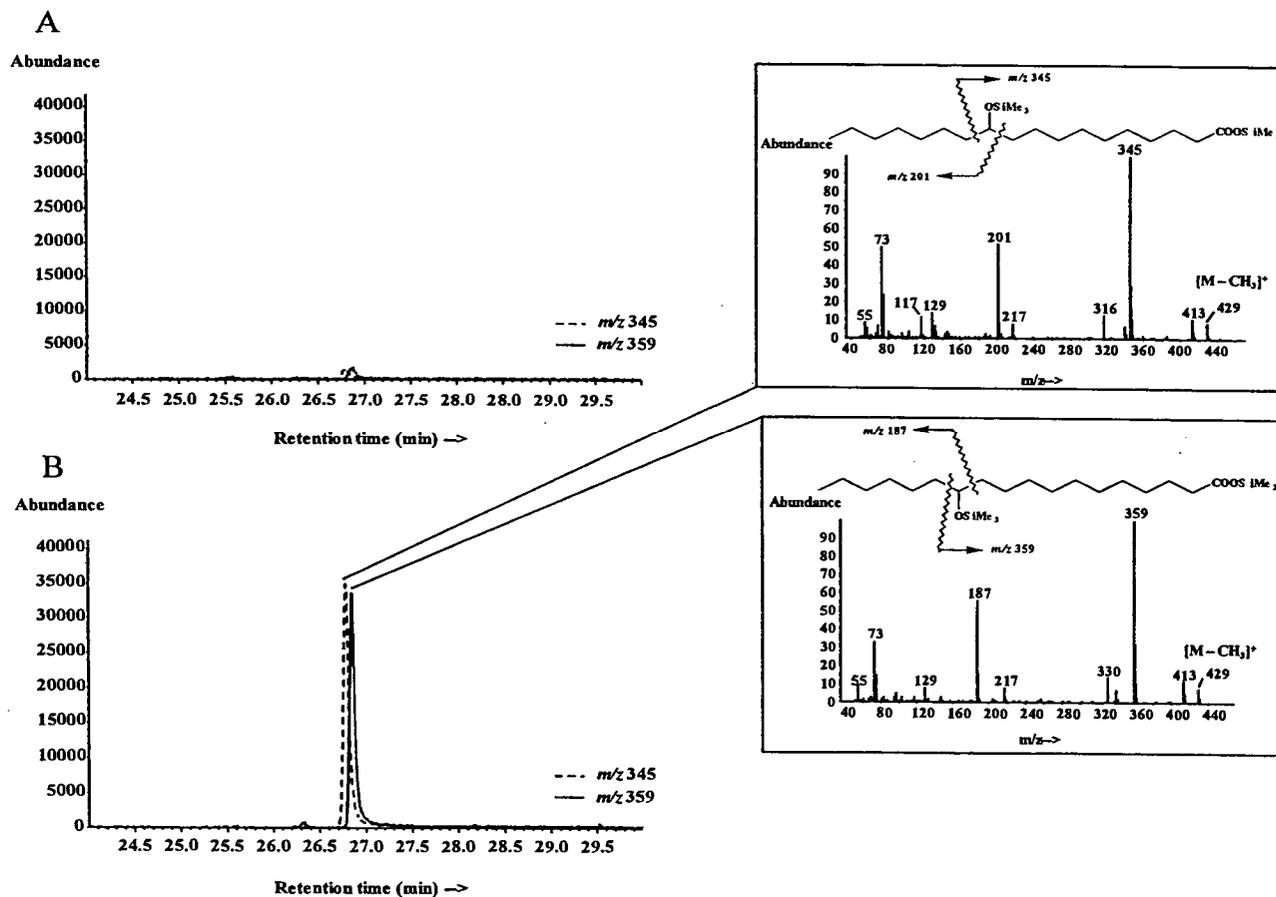


Fig. 5A.6. Partial m/z 345 and 359 chromatograms showing amounts of 11-hydroxyoctadecanoic and 12-hydroxyoctadecanoic acids after incubation of *E. huxleyi* strain TWPI in sterile medium (A) and with the ATB3 community (B) for 20 days.

Epoxidation of double bonds by aerobic bacteria is a well known process induced by cytochrome P-450-dependent monooxygenases. These enzymes can produce epoxides from a broad range of lipophilic substrates such as *n*-alkenes (Klug and Markovetz, 1968; Hartman et al., 1989a; Soltani et al., 2004), terpenes (Duetz et al., 2003), unsaturated fatty acids (for a review see Ratledge, 1994), and styrene (Hartman et al., 1989b). Enzymes from a large number of classes, including dehydrogenases, lyases, carboxylases, glutathione S-transferases, isomerases and hydrolases, are involved in the microbial degradation of epoxides (van der Werf et al., 1998). Among these enzymes, epoxide hydrolases, which catalyze the addition of water to an epoxide to form the corresponding diol, have been extensively studied and seem to be widely distributed in bacteria (e.g. Michaels et al., 1980; van der Werf et al., 1998; Johanson et al., 2005). Thus, the pathways for the bacterial degradation of alkenones via an initial double bond epoxidation were proposed as illustrated in Fig. 5A.7. Processes depicted by these pathways involve hydrolysis of the epoxide to the corresponding diol and subsequent oxidative cleavage. The resulting ketoacid and acid fragments are then totally assimilated by way of classical β -oxidation.

In total lipid extracts obtained after incubation of *E. huxleyi* strain TWP1 cells with the ATB3 community, small amounts of monounsaturated MeC₃₇ and EtC₃₈ alkenones (detected as alkenol derivatives, see Fig. 5A.3) were also detected. Small amounts of monounsaturated alkenones were previously identified in several haptophytes (Rontani et al., 2001) and in Black Sea Unit II samples (Xu et al., 2001; Rontani et al., 2006b). While it is conceivable that these intriguing compounds could be produced by *E. huxleyi* strain TWP1 before sterilization; they were lacking in all the other lipid extracts analyzed (sterile controls and other bacterial incubations) so their formation would seem linked to a bacterial activity specific to the ATB3 community. These compounds could be formed by

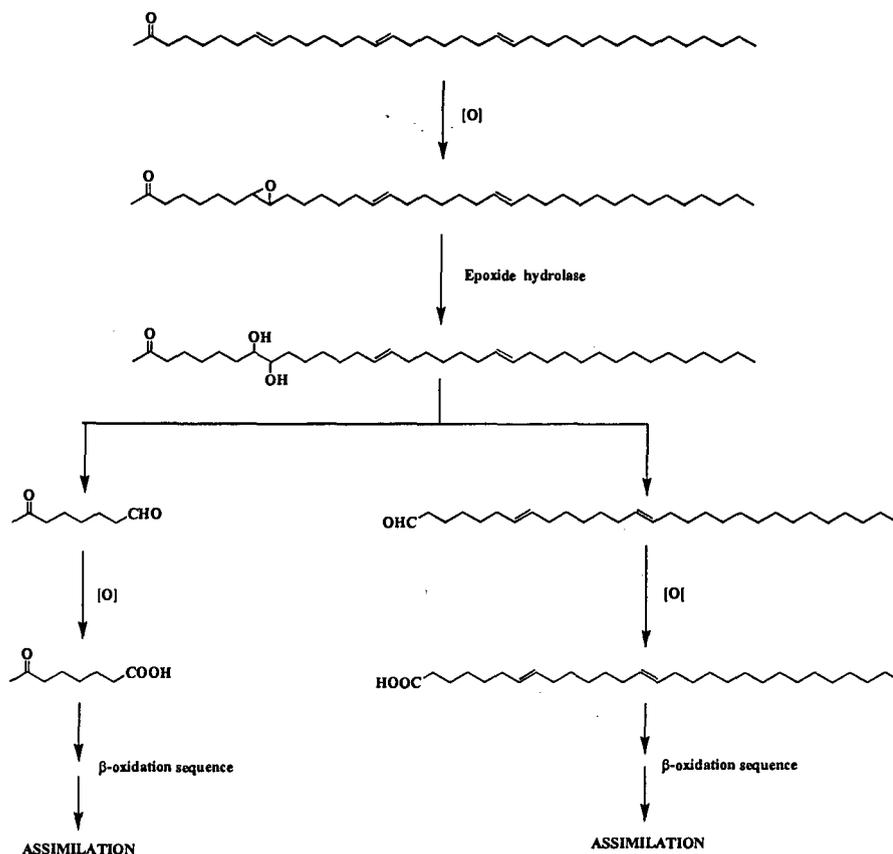


Fig. 5A.7. Proposed pathways for biodegradation of the C₃₇:3 alkenone involving initial epoxidation of ω₉ double bond.

a partial bacterial hydrogenation of diunsaturated alkenones. GC-MS analyses of these extracts after OsO₄ treatment and subsequent silylation confirms this hypothesis. Indeed, while the monounsaturated methyl C₃₇ and ethyl C₃₈ alkenones previously described (Rontani et al., 2001) possessed a double bond in the ω₁₇ position (which does not correspond to that observed in “classical” alkenones), each GC peak for monounsaturated alkenols observed after incubation with the ATB3 community appeared to be composed of two ω₁₅ and ω₂₂ double bond positional isomers (Fig. 5A.8) resulting from the reduction of one double bond of the diunsaturated alkenones. The saturation of fatty acids (biohydrogenation) by mixed cultures of rumen bacteria has long been recognized (for a

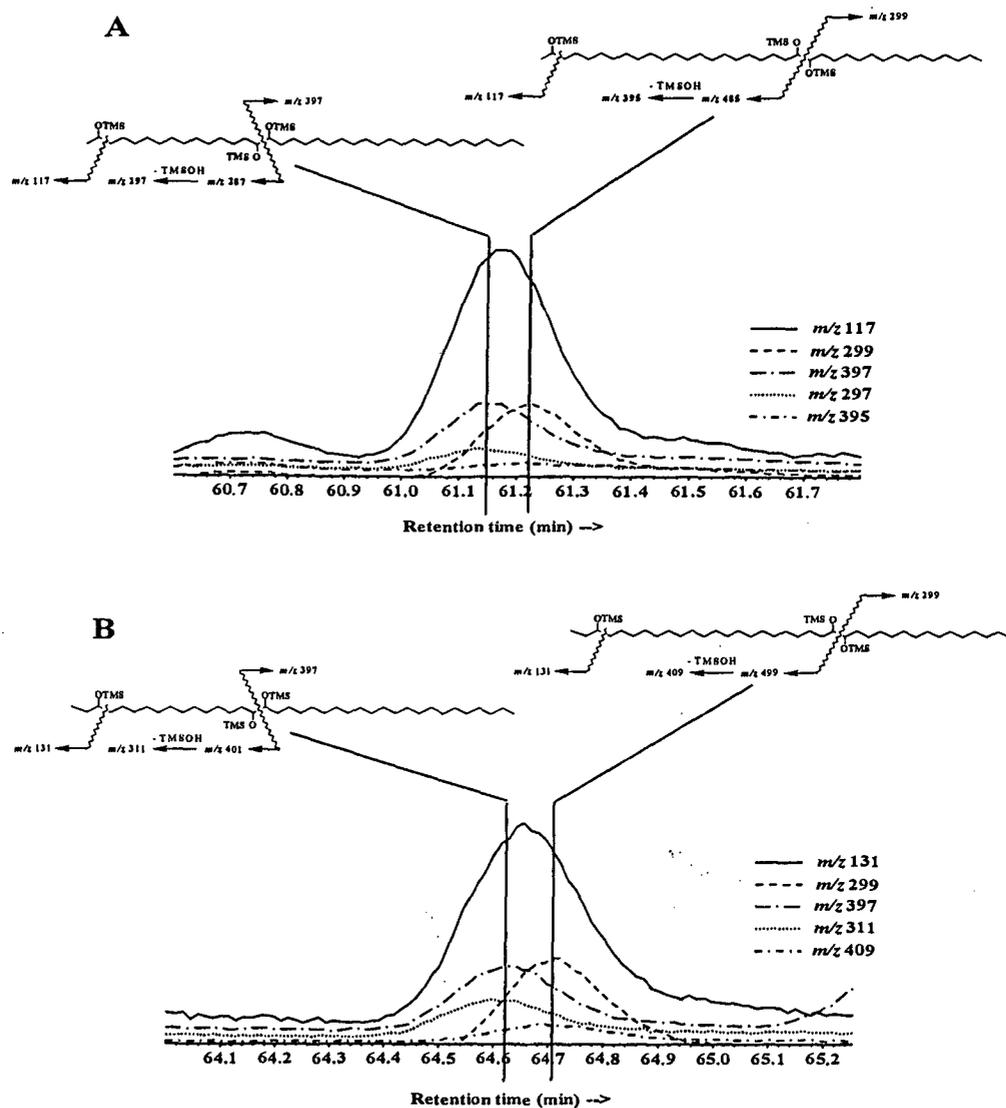


Fig. 5A.8. Partial m/z 117, 299, 397, 297, 395, 131, 311 and 409 chromatograms of silylated triols derived from OsO_4 treatment of (A) MeC37:1 and (B) EtC38:1 alkenols showing the initial presence of two double bond positional isomers.

review see Hammond, 1988), but the processes are generally considered to be restricted to anaerobic bacteria. However, Pereira et al. (2002) and Koritala et al. (1987) observed that bacteria and yeasts, respectively, could convert 18:3(n-3) acid to 18:2(n-6) and 18:1(n-9) acids under aerobic conditions. Although the proportions of monounsaturated alkenones produced were low (2-3 % of the corresponding diunsaturated alkenone), the existence of

alkenone bacterial hydrogenation processes could constitute a problem for paleotemperature reconstruction if this process proves widespread and quantitatively significant in nature.

5A.3.4. Selectivity of aerobic bacterial processes towards alkenones

Aerobic bacterial metabolism of unsaturated aliphatic ketones such as alkenones may be initiated either by the same mechanisms employed in alkanone metabolism or *via* attack at the double bonds. Three main patterns of initial attack of an aliphatic methyl ketone have been recognized (Fig. 5A.9): (i) terminal methyl oxidation (Ratledge, 1978) (Pathway A), (ii) oxidation of the keto terminal methyl and decarboxylation of the resulting α -ketoacid (Gillan et al., 1983) (Pathway B) and (iii) enzymatic oxidation of the ketone group to an ester, analogous to Baeyer-Villiger oxidation with peracids (Britton et al., 1974) followed by hydrolysis of the ester to a primary alcohol and acetic acid (Pathway C). The different compounds formed in each pathway are then assimilated by way of β -oxidation. The previous detection of C_{35:2}, C_{35:3} and C_{35:4} alken-1-ols in alkenone-rich Camargue microbial mats (Rontani and Volkman, 2005) provides support for the degradation of alkenones *via* a bacterial-mediated Baeyer-Villiger sequence (Pathway C) in the natural environment. The positions of the double bonds in the alkyl chain do not constitute a metabolic blockage for β -oxidation owing to the occurrence of 2,3-enoyl-CoA isomerases in bacteria (Ratledge, 1994). Consequently, the presence or absence of an additional double bond in the chain of alkenones has no significant effect on degradation rates. Bacteria degrading alkenones non-selectively, as in the case of the ATB1 and ATB2 communities and in the experiments of Teece et al. (1998), probably metabolized alkenones *via* one of the three proposed pathways (Fig. 5A.9). In contrast, bacteria able to degrade alkenones selectively, present in TAB and ATB3 communities, and probably in the inocula from microbial mats previously used by Rontani et al. (2005a),

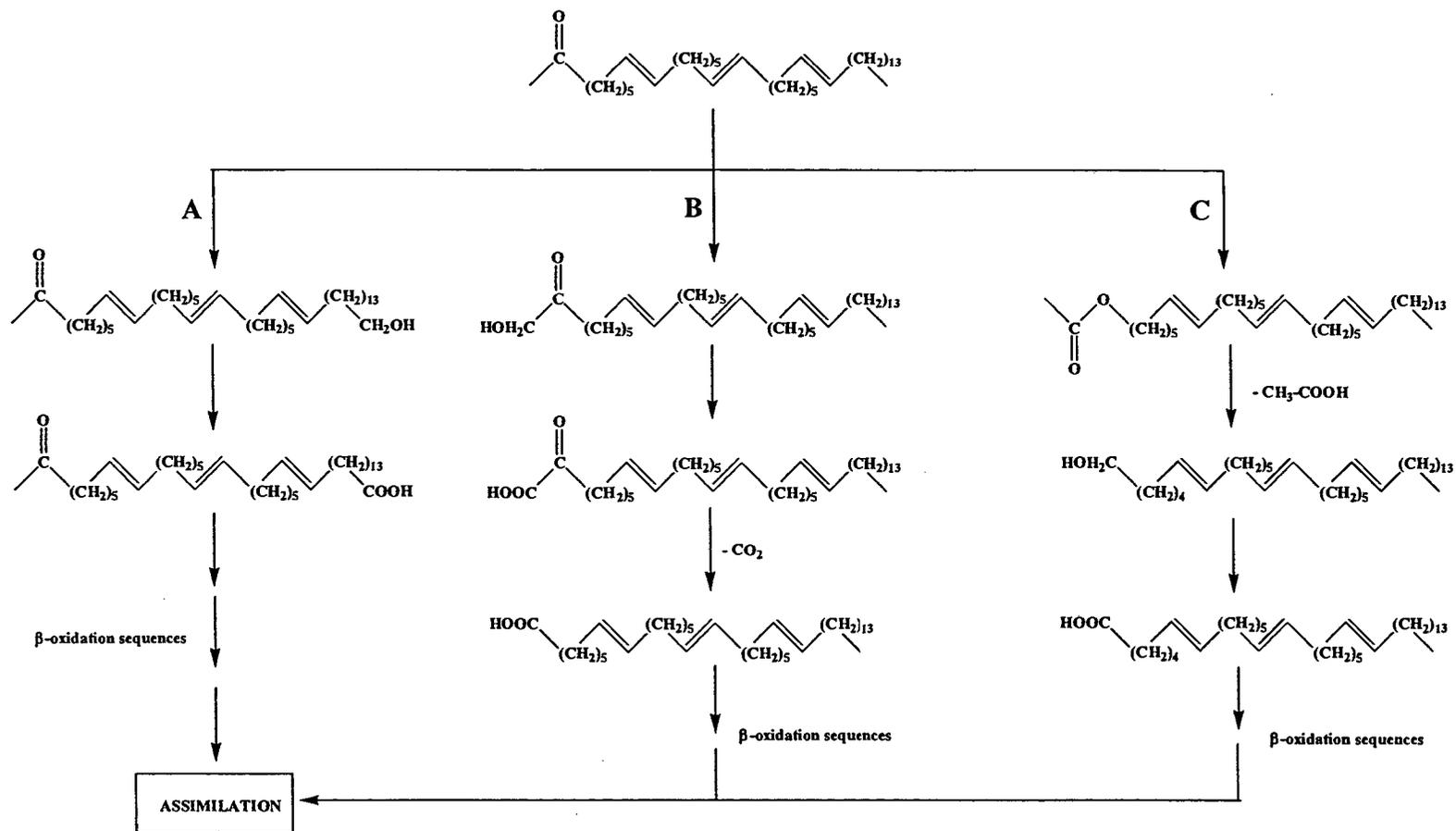


Fig. 5A.9. Metabolic pathways involved during aerobic bacterial degradation of methyl alkenones: attack on terminal methyl and keto groups.

likely do so by attack at the alkenone double bonds. This appears to involve the formation of epoxides (pathway D in Fig. 5A.10) in the case of ATB3 and TAB communities, but the involvement of other processes such as direct oxidation of double bonds by dioxygenases (pathway E in Fig. 5A.10) or addition of water by hydratases (Seo et al., 1981) followed by dehydrogenation and carboxylation (pathway F in Fig. 5A.10) cannot be excluded. In the case of bacteria using one of the pathways (D, E or F) to degrade alkenones, the presence of an additional double bond could significantly increase the degradation rate and thereby control selectivity.

Hydratases can also act under anaerobic conditions (Schink, 1985; Rontani et al., 2002) and thus induce selectivity during the degradation of alkenones by anaerobes. However, alkenones appeared to be degraded non-selectively under methanogenic, sulfate-reducing and denitrifying conditions (Teece et al., 1998; Rontani et al., 2005a). Although the existence of anaerobes able to hydrate alkenone double bonds cannot be totally excluded, it is speculated that anaerobic degradation of alkenones mainly involves attack at the carbonyl group and consequently occurs non-selectively.

5A.3.5. Biogeochemical implications

The results confirm the previous observations of Rontani et al. (2005a) which showed that aerobic bacteria capable of degrading alkenones selectively are not limited to particular environments such as microbial mats and can be actually associated with *E. huxleyi* cells.

Intense aerobic microbial degradative processes have the potential to introduce a bias in palaeotemperature reconstruction, so this factor should be considered when sediments in which there is clear evidence of substantial aerobic microbial degradation of the deposited organic matter are examined. Conceivably, the involvement of selective

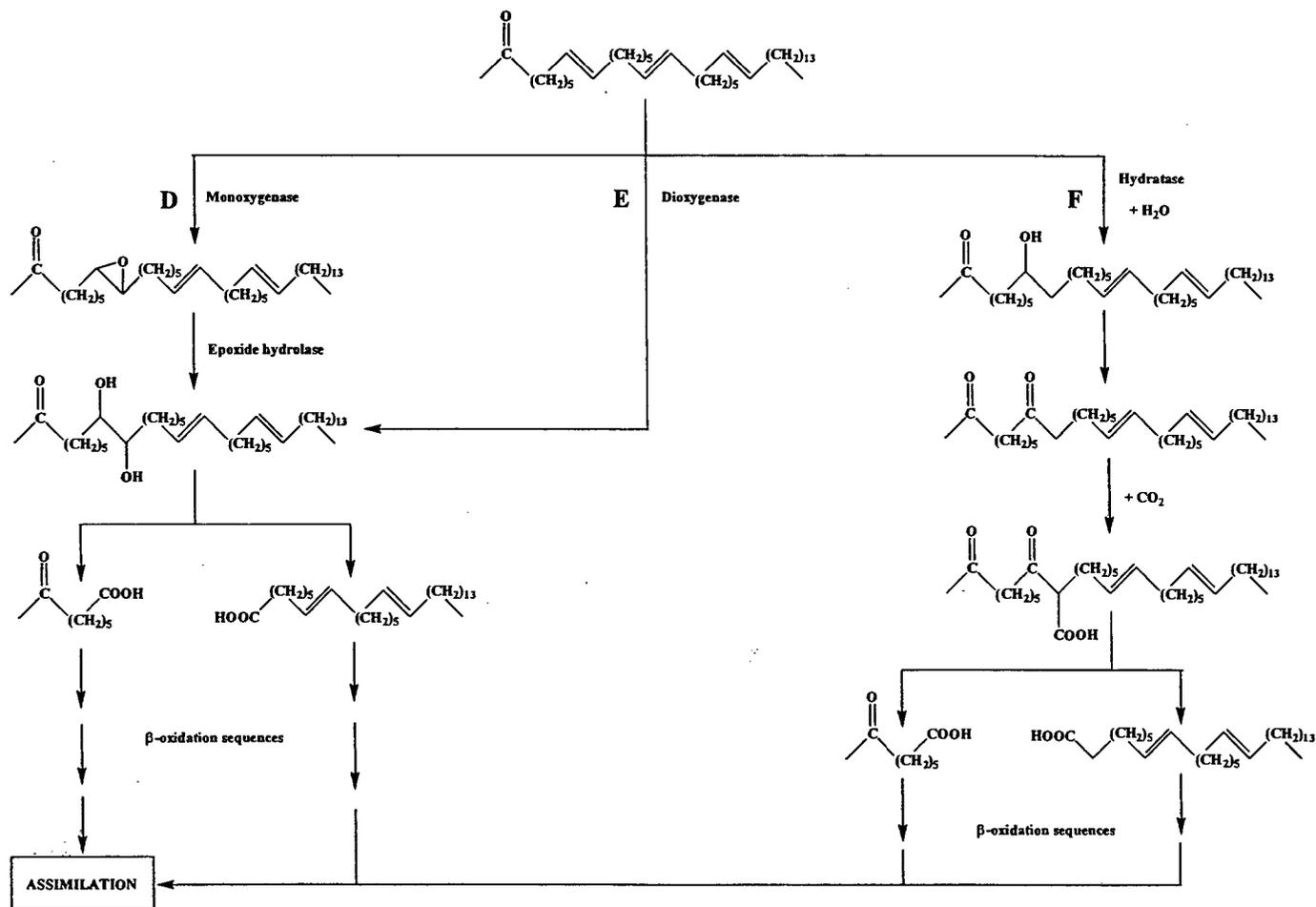


Fig. 5A.10. Metabolic pathways involved during aerobic bacterial degradation of alkenones: attack on double bonds.

aerobic microbial degradative processes could explain the increasing mismatch between values measured in annually averaged sediment trap materials and surface sediment documented by Prahl et al. (1993) along an offshore transect at ~42°N in the northeast Pacific Ocean. Indeed, burial efficiency at the most offshore, open ocean site, where the mismatch was greatest (0.335 vs. 0.447), was very low (~1%) because of high oxygen exposure. The present results are also in good agreement with the observations of Conte et al. (2006). From an extensive compilation of measurements from several sources (n = 629), these authors observed that values for surface sediments were systematically higher than the surface water production temperature. They concluded that the deviations could be attributed to seasonality in production and/or thermocline production as well as differential degradation of C37:3 and C37:2 alkenones.

The epoxy ketones resulting from bacterial epoxidation of alkenone double bonds may prove useful as indicators of in situ aerobic bacterial alteration of the alkenone unsaturation ratio. Their detection is much easier after NaBH₄ reduction to the corresponding diols and subsequent silylation. This treatment also allows for better quantification of alkenones in the form of silylated alkenols (Rontani et al., 2001).

**Evidence of selective bacteria-induced and
auto-oxidative degradation of alkenones
during dark incubation of non-axenic
Emiliana huxleyi cells**

5B.1. Introduction

The alkenones are a class of unusual, C₃₅-C₄₀ mono-, di-, tri- and tetraunsaturated methyl and ethyl ketones that are synthesized by a limited number of haptophyte microalgae (Volkman et al. 1980; 1995; Marlowe et al. 1984, Conte et al. 1994; Rontani et al., 2001; 2004; Prah1 et al., 2006). *Emiliania huxleyi* and *Gephyrocapsa oceanica* appear to be the dominant sources of alkenones in today's oceans and contemporary sediments (Volkman, 2000).

The demonstration that past sea-surface temperatures (SSTs) can be derived from the analysis of alkenones in marine sediments (Brassell et al., 1986; Prah1 and Wakeham, 1987) gave rise to an increased number of studies in which these compounds were used as tools to reconstruct paleotemperatures (e.g., Herbert, 2003). The alkenone method uses the relative abundance of methyl C_{37:2} and C_{37:3} alkenones as defined by the unsaturation index $U_{37}^{K'} = [C_{37:2}] / ([C_{37:2}] + [C_{37:3}])$ (Brassell et al., 1986; Prah1 and Wakeham, 1987), which appeared to be clearly temperature- dependent (for reviews see: Müller et al., 1998; Conte et al., 1998; Prah1 et al., 2000; Volkman, 2000).

However, some authors have suggested that environmental parameters other than temperature may also affect alkenone distributions in the haptophyte cells (e.g. Epstein et al., 1998; Popp et al., 1998; Yamamoto et al., 2000; Versteegh et al., 2001). More recently, Prah1 et al. (2003) showed that the $U_{37}^{K'}$ index dropped by 0.11 units in nutrient-starved cells of *E. huxleyi* strain NEPCC 55a, while continuous exposure to darkness resulted in a significant $U_{37}^{K'}$ increase (+ 0.11). Selective metabolic consumption of alkenones by the algae (as energy reserve substances) as reported in the above experiments may be associated with the observed variations in $U_{37}^{K'}$. However, growth of bacteria associated with live *E. huxleyi* cells, perhaps was enhanced due to cessation of antibiotic production by the algae in dark condition (F. Van Wambeke, unpublished results): Such enhanced

bacterial growth may also have contributed to some extent to the observed selective loss of alkenones in these non-axenic experiments. Isolation of bacterial communities able to degrade C₃₇ alkenones selectively from cells of *E. huxleyi* strain TWP1 (Chapter 5A, Rontani et al., 2008) suggested that bacterial degradation processes could play a role in influencing $U_{37}^{K'}$ (Prahl et al., 2003).

It is shown in the previous experiments (Chapter 5A) that different bacterial communities can be associated with the live *E. huxleyi* cells. Some of these communities were found to be selective towards alkenone degradation. In that experiment, these communities were isolated and then utilized for the degradation of alkenones. However, the effect of bacterial communities associated with the live *E. huxleyi* culture on the alkenone degradation is also important to effectively use the $U_{37}^{K'}$ in paleotemperature assessment. As *E. huxleyi* can grow in wide range of temperature in the marine environment, there is a possibility that it can be associated with different bacterial communities at different times depending on the temperature of growth. If this is true then there is likelihood that associated bacterial communities can exhibit distinct abilities to degrade alkenones selectively. However, to confirm these possibilities, precultures of *E. huxleyi* grown at two different temperatures in order to enrich different bacterial communities which might have the potential to degrade alkenones selectively were used. Moreover, during the dark incubation of the algae, the alkenones are utilized and was linked with the algal consumption; however during dark incubation, the cessation of antibiotic production in the alga can influence the bacterial growth. To prove this assumptions: 1) precultures of non-axenic *E. huxleyi* culture was grown at 15°C and 20°C to enrich different bacterial communities at these two temperatures, 2) the two bacterial communities enriched in *E. huxleyi* cultures were grown at same temperature (15°C) and dark incubated, and 3) degradation was monitored in dark as well as light conditions.

5B.2. Material and methods

5B.2.1. Organism

The non-axenic *Emiliana huxleyi* TWP1 strain was obtained from the Caen Algotank (France). Stock cultures were grown and maintained in 50 ml conical flasks containing 10 ml of sterile f/2 medium at 20°C, using 116 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ cool white fluorescent light (Osram, fluora) under 12 h light:12 h dark regime.

5B.2.2. Enrichment of the bacterial community in non-axenic Emiliana huxleyi

Non-axenic *Emiliana huxleyi* strain TWP1 was enriched for the bacterial communities by growing the alga separately at 15°C and 20°C in 10 ml f/2 media under above growth conditions for 10 days. Subsequently two more transfers in 10 ml of sterile f/2 medium were carried out at both temperatures. A starter culture was carried out by inoculating 10 ml each of the 15°C and 20°C grown culture in 100 ml f/2 medium for 10 days under above conditions.

5B.2.3. Dark incubations

In the case of the Dark incubation 1 (DK1) experiment (December 2006), a starter culture was carried out at 20°C in 100 ml of f/2 medium (Guillard and Ryther, 1964), using 116 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ cool white fluorescent light (Osram, fluora) under a 12 h light:12 h dark regime. After ten days of growth of the microalgae, entire 100 ml was transferred to a flask containing 2000 ml of f/2 medium and the diluted culture was grown at 15°C for eight days. At that time, two aliquots (50 ml each) were removed and the flask was wrapped with aluminium foil and kept in the dark. Sampling was then done in duplicate every two days thereafter in the dark conditions (Table 5B.1). Finally (18th day), the flasks were brought back to the light and two more samplings were done again in duplicate with two days gaps in between (Table 5B.1).

For the Dark incubation 2 (DK2) the 100ml starter culture was grown at 15°C instead of 20°C as above for 10 days and then the culture was incubated in dark for ten days instead of eight. At that time, two aliquots (50 ml each) were removed and the flask was wrapped with aluminium foil and kept in the dark. Sampling was then done in duplicate every two days thereafter in the dark conditions (Table 5B.2). Similarly the culture was brought back to the light after 19 days and one sampling was done after two days gap in the light (Table 5B.2).

5B.2.4. Lipid extraction

The algal samples collected were filtered on precleaned GF/F paper. The filters containing the cells were extracted with chloroform-methanol-water (1:2:0.8, v/v/v) using ultrasonication. Subsequently the lipids containing alkenones were extracted, saponified, reduced and derivatized and run on GC-EIMS as give in chapter 5A.

5B.2.4. Alkaline hydrolysis

Alkaline hydrolysis of the total lipid extract was carried as described in chapter 5A.

5B.2.5. Alkenone reduction

Alkenone reduction procedure is detailed in chapter 5A.

5B.2.6. Derivatization

Derivatization of the lipid compounds was done as given in chapter 5A.

5B.2.7. Gas chromatography-electron impact mass spectrometry (GC-EIMS) analyses

GC-EIMS analyses were carried out with an Agilent 6890 gas chromatograph connected to an Agilent 5973 Inert mass spectrometer, using SOLGEL-1 (SGE; 0.25 µm film thickness) silica capillary column. The detailed procedure is given in chapter 5A.

5B.2.8. Denaturing Gradient Gel Electrophoresis (DGGE) analyses

The two enriched *E. huxleyi* cultures at 15°C and 20°C were used for the DNA extraction. Cell lysis and DNA extraction were performed as described by Zhou et al.

(1996). The PCR conditions for 16S rRNA genes, including the hot start and a touchdown for primer annealing, were similar to those used by Muyzer et al. (1993). The detailed procedure of DNA extraction and PCR preparation is given in chapter 5A.

DGGE was performed as mentioned in chapter 5A.

5B.3. Results and discussion

The alkenone contents of the different samples collected during dark incubations (DK1 and DK2) and light incubations were analyzed after NaBH₄-reduction and subsequent silylation. The silylated alkenols thus formed display better chromatographic characteristics than the corresponding alkenones and have diagnostic EI mass spectra, i.e. strong fragment ions at m/z 117 and 131 due to cleavage α to the functional group, allowing methyl and ethyl alkenols (and hence the parent alkenones) to be readily differentiated by selected ion monitoring (SIM), even at low abundances (Rontani et al., 2001). The results obtained from the light-limited DK1 and DK2 experiments are summarised in Tables 5B.1 and 5B.2, respectively. A significant decrease of alkenone concentration with apparent selective metabolic loss of C₃₇ components was observed during the incubation under darkness in both cases (Tables 5B.1 and 5B.2), which was similar to the trend observed previously by Prahl et al. (2003) and Epstein et al. (2001).

In DK1 experiment, alkenone consumption was accompanied by a dramatic increase in $U_{37}^{K'}$ (+ 0.54) and $U_{38Et}^{K'}$ (+0.45) values (Table 5B.1). This trend was particularly driven by the selective loss of triunsaturated alkenones (Table 5B.1). However, when the cells were returned to light, production of alkenones resumed and after 4 days, $U_{37}^{K'}$ index was similar to that observed before the dark incubation, suggesting that the cultures were not nutrient-starved (Table 5B.1). Prahl et al. (2003) demonstrated that under nutrient (nitrate and phosphate) limited condition, $U_{37}^{K'}$ values decreased significantly. It may be noted that the $U_{38Et}^{K'}$ index in DK1 observed after return to light

Table 5B.1: Summary of the results from the light limited experiment DK1

Incubation conditions	Sampling day	Alkenone Conc.($\mu\text{g ml}^{-1}$)	MeK _{37:3} (%) ^a	MeK _{37:2} (%) ^a	EtK _{38:3} (%) ^a	EtK _{38:2} (%) ^a	MeK _{38:3} (%) ^a	MeK _{38:2} (%) ^a	EtK _{39:3} (%) ^a	EtK _{39:2} (%) ^a	$U_{37}^{K'}$	$U_{38Et}^{K'}$	Epoxide C _{37:2} (%) ^d	Epoxide C _{37:3} (%) ^d	$U_{37}^{K'} - ep U_{37}^{K'}$
Light to dark	8	0.288 ± 0.069 ^b	47.9 ± 0.5	15.0 ± 0.2	17.5 ± 0.2	12.0 ± 0.5	4.5 ± 0.1	1.5 ± 0.1	1.0 ± 0	0.6 ± 0.1	0.25 ± 0.01	0.41 ± 0.02	2.2 ± 0.4	2.2 ± 0.2	+0.01 ± 0.01
Dark	10	0.175 ± 0.082	20.9 ± 2.0	12.2 ± 0.2	32.3 ± 0.5	24.9 ± 2.8	4.5 ± 0.1	1.8 ± 0.1	1.9 ± 0	1.3 ± 0.2	0.37 ± 0.02	0.43 ± 0.03	0.8 ± 0.1	1.7 ± 0.1	+0.15 ± 0.01
Dark	12	0.035 ^c	12.1	15.9	37.4	24.8	3.1	1.7	3.3	1.7	0.57	0.40	1.0	2.4	+0.23
Dark	14	0.030 ± 0.010	7.6 ± 0.5	26.1 ± 0.1	17.7 ± 0.1	39.5 ± 0.2	1.6 ± 0.1	2.7 ± 0.2	2.2 ± 0	2.4 ± 0.1	0.77 ± 0.01	0.69 ± 0	5.6 ± 0.1	2.7 ± 0.1	+0.16 ± 0.01
Dark	16	0.032 ± 0.010	8.8 ± 1.0	33.0 ± 0.5	10.3 ± 2.2	40.4 ± 3.0	1.3 ± 0.3	2.9 ± 0.1	1.2 ± 0.2	2.1 ± 0.1	0.78 ± 0.02	0.80 ± 0.04	- ^e	- ^e	-
Dark to light	18	0.061 ± 0.001	10.4 ± 0.1	38.9 ± 2.6	6.2 ± 0.3	38.4 ± 2.2	1.0 ± 0	2.7 ± 0.1	0.7 ± 0.1	1.7 ± 0.2	0.79 ± 0.01	0.86 ± 0	- ^e	- ^e	-
Light	20	0.181 ± 0.022	44.9 ± 0.9	19.6 ± 2.0	10.7 ± 1.4	15.3 ± 0.7	5.5 ± 0.2	1.8 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	0.30 ± 0.03	0.57 ± 0.02	2.2 ± 0.7	2.2 ± 0.7	+0.02 ± 0.01
Light	22	1.158 ± 0.219	52.6 ± 1.5	18.4 ± 0.9	9.0 ± 0.2	11.3 ± 1.2	5.6 ± 0.8	1.8 ± 0.1	0.6 ± 0	0.6 ± 0	0.26 ± 0.02	0.55 ± 0.02	1.6 ± 0.6	1.4 ± 0.4	+0.03 ± 0.01

^a Concentration as a percentage of total alkenones; ^b Average of duplicates; ^c Only one duplicate analyzed; ^d Percentage relative to the parent alkenone; ^e Not detected.

Table 5B.2: Summary of the results from the light limited experiment DK2

Incubation conditions	Sampling day	Alkenone Conc. ($\mu\text{g ml}^{-1}$)	MeK _{37:3} (%) ^a	MeK _{37:2} (%) ^a	EtK _{38:3} (%) ^a	EtK _{38:2} (%) ^a	MeK _{38:3} (%) ^a	MeK _{38:2} (%) ^a	EtK _{39:3} (%) ^a	EtK _{39:2} (%) ^a	$U_{37}^{K'}$	$U_{38Et}^{K'}$	Epoxide C _{37:2} (%) ^e	Epoxide C _{37:3} (%) ^e	$U_{37}^{K'} - ep U_{37}^{K'}$
Light to dark	10	0.78 ± 0.01 ^b	44.8 ± 0.6	12.7 ± 0.1	21.3 ± 0	12.0 ± 1.1	5.8 ± 0.3	1.2 ± 0	1.5 ± 0.1	0.6 ± 0	0.22 ± 0	0.35 ± 0.03	1.1 ± 0.6	1.0 ± 0.7	-0.02 ± 0.02
Dark	13	0.26 ± 0.06	20.4 ± 0.7	4.4 ± 0.1	47.0 ± 2.3	16.9 ± 1.1	5.3 ± 0.3	1.1 ± 0.1	3.8 ± 0.1	1.1 ± 0.2	0.18 ± 0.01	0.26 ± 0.02	2.6 ± 0.2	1.8 ± 0.3	-0.06 ± 0.03
Dark	15	0.07 ± 0.02	25.2 ± 0.6	7.8 ± 0.1	35.4 ± 1.0	17.6 ± 0.1	5.6 ± 0	1.8 ± 0	4.6 ± 0.1	2.1 ± 0.1	0.23 ± 0.01	0.33 ± 0.01	4.7 ± 0.2	4.1 ± 0.1	-0.02 ± 0.01
Dark	17	0.21 ± 0.03	19.4 ± 2.0	5.0 ± 0.5	46.0 ± 0.2	17.3 ± 1.1	4.9 ± 0	1.0 ± 0.1	5.0 ± 0.1	1.3 ± 0	0.21 ± 0.03	0.27 ± 0.02	1.7 ± 0.3	1.4 ± 0.2	-0.03 ± 0.02
Dark to light	19	0.10 ± 0.03	38.0 ± 0.9	9.3 ± 0.2	23.8 ± 0.9	13.1 ± 0.7	8.4 ± 1.2	2.1 ± 0	3.4 ± 0.1	1.9 ± 0.3	0.20 ± 0.01	0.35 ± 0.01	- ^d	- ^d	-
Light	21	2.80 ± 0.10	55.2 ± 0.2	15.0 ± 0.5	10.2 ± 0.1	9.0 ± 0.2	7.2 ± 0.6	1.8 ± 0.1	0.8 ± 0.1	0.6 ± 0	0.21 ± 0.01	0.46 ± 0.01	1.9 ± 0.8	1.9 ± 0.7	+0.01 ± 0.01

^a Concentration as a percentage of total alkenones; ^b Average of duplicates; ^c Percentage relative to the parent alkenone; ^d Not detected.

remained significantly higher than the initial value (+0.14). However, these results were in contrast with the DK2 experiment. In this case, the decrease of alkenone concentration occurred without significant changes in $U_{37}^{K'}$ values (Table 5B.2).

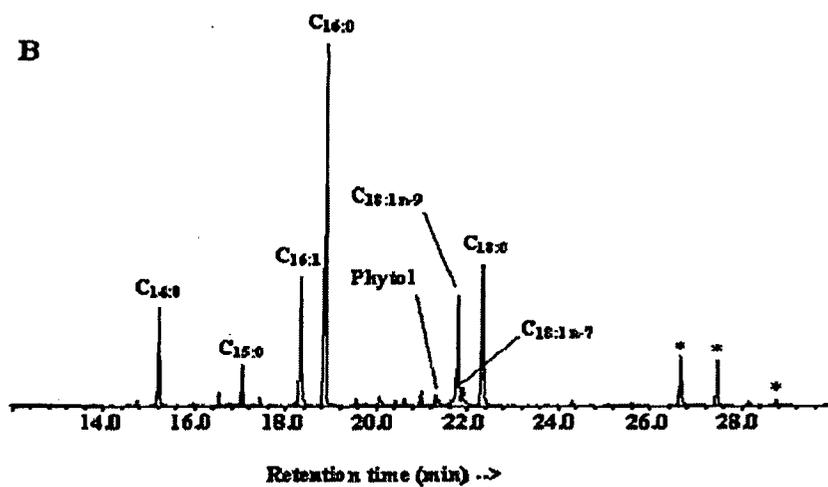
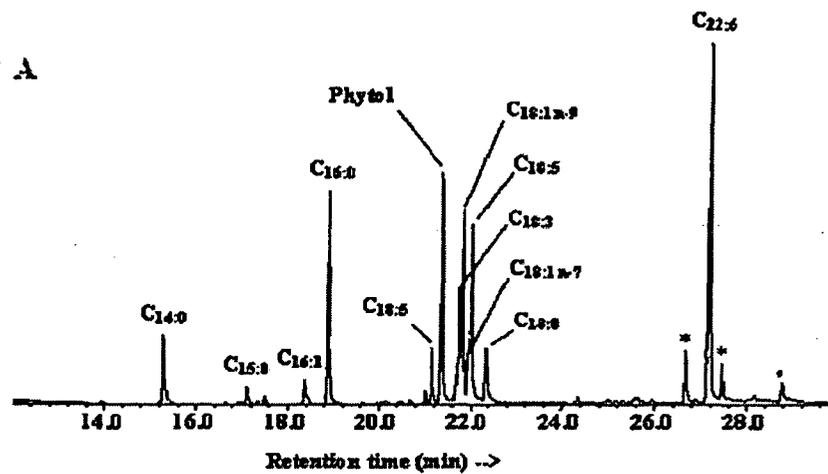
Recently, Eltgroth et al. (2005) observed the presence of lipid bodies containing alkenones, alkenes and alkenoates in *E. huxleyi* cells, which increased in abundance under nutrient limited condition and disappeared under prolonged darkness. They concluded that these compounds were synthesized in chloroplasts and then exported to cytoplasmic lipid bodies for storage. These results of Eltgroth et al. (2005) support the previous suggestion by Epstein et al. (2001) of a possible metabolic role to alkenones in *E. huxleyi* cells. The strong decrease of alkenone concentration observed during incubation under dark condition could be thus attributed to selective metabolic consumption of alkenones by the algae (as energy reserve substances). It is interesting to note that the alkenone consumption during dark incubation of different strains of *E. huxleyi* exhibited various degrees of selectivity (Table 5B.3). These results suggest that the alkenone consumption by *E. huxleyi* seems to be strain-dependent. However, this hypothesis is not supported by: (i) the strongly contrasting results obtained during the two experiments carried out in the present work with the same *E. huxleyi* strain and (ii) the different results previously obtained by Epstein et al. (2001) and Prahel et al. (2003) with *E. huxleyi* strain NEPCC 55a (Table 5B.3). During the DK1 incubation triunsaturated alkenones appeared to be consumed much more quickly than the diunsaturated, while this was not the case in the DK2 experiment. The selectivity observed during this last experiment only affected the chain length distribution of alkenones (the C_{37} being consumed more quickly than the C_{38}) not the unsaturation patterns (Table 5B.2).

Table 5B.3 : Summarizing of the results previously obtained during incubation of different *E. huxleyi* strains under darkness

Strain employed	Initial $U_{37}^{K'}$ index	Variation of $U_{37}^{K'}$ index during incubation	Reference
<i>E. huxleyi</i> strain NEPCC 55a	0.56	+0.11	Prahl et al. (2003)
<i>E. huxleyi</i> strain CCMP 1742	0.54	+0.04	Prahl et al. unpublished results
<i>E. huxleyi</i> strain CCMP 1742	0.43	+0.02	Prahl et al. unpublished results
<i>E. huxleyi</i> strain CCMP 372	0.18	+0.04	Prahl et al. unpublished results
<i>E. huxleyi</i> strain CCMP 373	0.10	-0.04	Epstein et al. (2001)
<i>E. huxleyi</i> strain NEPCC 55a	0.51	+0.04	Epstein et al. (2001)

In order to check such a possibility, the lipid profiles obtained after extraction of samples collected during the two experiments were compared. During the DK2 experiment, lipid profile (Fig. 5B.1A) appeared to be well characteristic of cells of *E. huxleyi* in good physiological state (presence of high proportions of phytol, C_{18:3}, C_{18:5}, oleic and C_{22:6} acids). In contrast, during the DK1 experiment lipid profile was dominated by C_{14:0}, C_{16:0} and C_{18:0} fatty acids with minor presence of phytol and polyunsaturated fatty acids (Fig. 5B.1B). Such a profile (with high proportions of saturated fatty acids) suggests the presence of algal material strongly altered by bacteria during this experiment (Harwood and Russell, 1984).

Bacterial counts carried out at the end of the two dark incubations confirmed a two orders of magnitude higher bacterial abundance at the end of the DK1 experiment (Table 5B.4). These counts also showed a strong increase in bacterial numbers during the incubation under darkness for DK1 (Fig. 5B.2A). This strong bacterial growth could be attributed to the cessation of antibiotic production by the algae under such conditions (F. Van Wambeke, unpublished results). However, an alternate explanation can be offered, i.e. a limitation of bacterial growth in the presence of light in *E. huxleyi* cells (Fig. 5B.3). In senescent phytoplanktonic cells, the production of singlet oxygen (resulting from chlorophyll excitation) exceeds the quenching capacity of the photoprotective system. This excited form of oxygen



* Contaminants

Figure 5B.1. Partial total ion chromatograms showing the fatty acid and phytol content of *E. huxleyi* strain TWP1 collected at the end of the incubation under darkness in the case of the DK2 (A) and DK1 (B) experiments.

Table 5B.4: Bacterial counts carried out at the end of the two dark incubations

Experiment	Bacteria number (ml ⁻¹) ^a before dark incubation	Bacteria number (ml ⁻¹) ^a at the end of dark incubation
DK1	1.6 x 10 ⁷	1.2 x 10 ⁹
DK2	7.6 x 10 ⁵	1.1 x 10 ⁷

^a The cells were counted by the technique of epifluorescence in the presence of fluorochrome (4',6-diamidine-2'-phenylindole dihydrochloride (DAPI)) as previously described (Rontani et al., 1999).

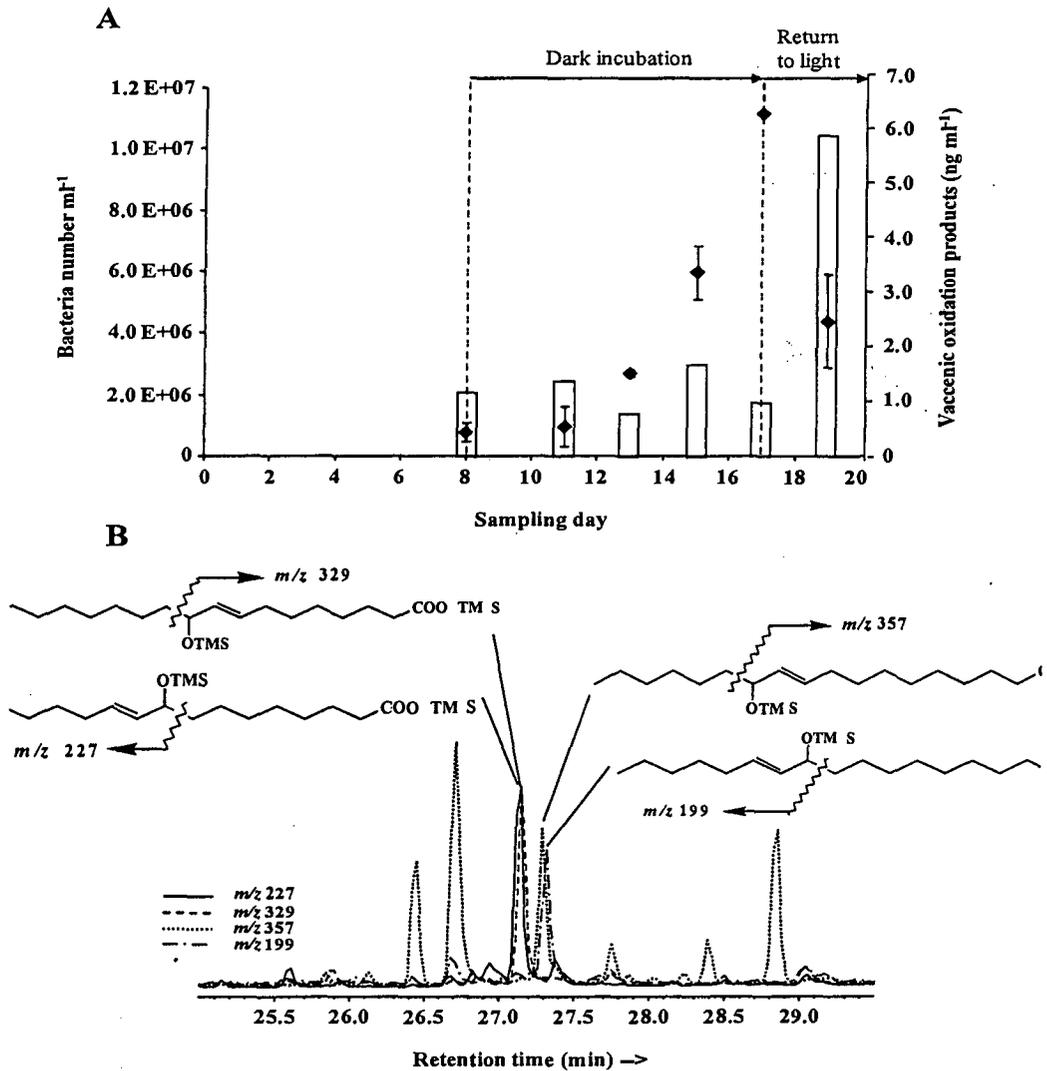


Figure 5B.2. Evolution of bacterial number (Diamonds) and of the amounts of vaccenic acid oxidation products (Open boxes) during the DK1 experiment (A), partial m/z 227, 329, 199 and 357 mass fragmentograms of a silylated NaBH_4 -reduced total lipid extract showing the presence of oleic and vaccenic oxidation products during the DK1 experiment (B).

can migrate outside the chloroplasts and kill the attached bacteria (Rontani et al., 2003) (Fig. 5B.3A). Such possibility was confirmed recently (Christodoulou et al., 2010). Diagnostic degradation patterns for some typical bacterial fatty acids were observed during irradiation of

non-axenic phytodetritus by solar light, while these compounds were unaffected by the same radiations in isolated bacteria (Aboudi et al., 2008). Damages resulting from the transfer of high amounts of singlet oxygen in heterotrophic bacteria may be dramatic due to the lack of adapted photoprotective system in these organisms and could thus limit their growth. The simultaneous increase in the oxidation products of vaccenic acid (a typically bacterial fatty acid, Sicre et al., 1988) due to its interactions with singlet oxygen (11-hydroxyoctadec-12-enoic and 12-hydroxyoctadec-10-enoic acids, Marchand and Rontani, 2001) (Fig. 5B.2) and decrease of bacteria number observed after the return to light (Fig. 5B.2A) well supports this hypothesis.

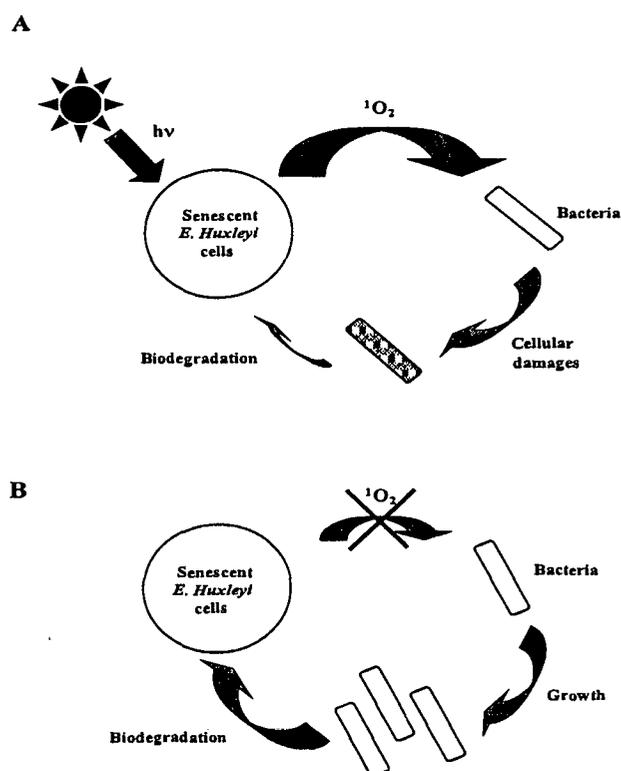


Figure 5B.3. Schematized interactions between senescent cells of *E. huxleyi* and bacteria during lightened (A) and dark (B) incubation.

16S rDNA DGGE analyses were performed at the end of the two dark incubations. The profiles generated (Fig. 5B.4, lanes 3 and 4) clearly showed the presence of two well distinct bacterial communities during DK1 and DK2 experiments. These differences result from the selection of different bacterial communities according to the temperature of the starter cultures (20°C for DK1 and 15°C for DK2).

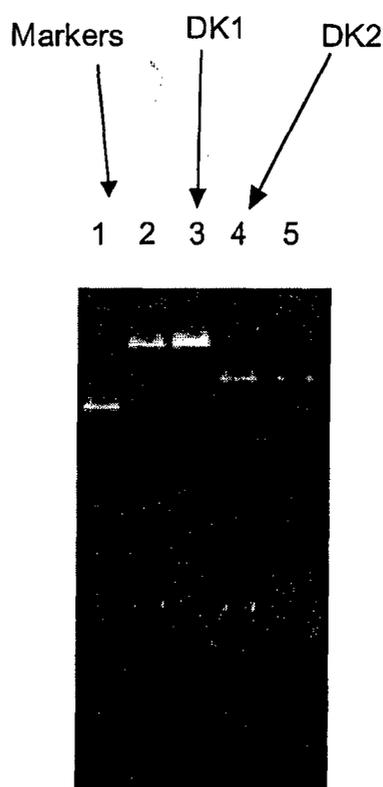


Figure 5B.4. Negative images of DGGE profiles of the 16S rDNA fragments obtained with primers specific for the domain bacteria and template DNA extracted from samples collected at the end of the incubation under darkness during the DK1 and DK2 experiments. Markers correspond to a mixture of PCR products amplified from *Clostridium perfringens*, *Marinobacter hydrocarbonoclasticus* sp. cab and *Micrococcus luteus*.

Different bacterial communities were previously isolated from *E. huxleyi* strain TWP1 cultures (Rontani et al., 2008). These bacterial communities degraded alkenones to varying

degrees ranging from almost none to extensive. Bacterial communities showed an extensive degradation of alkenones with selectivity. It was evident that the alkenone metabolic pathways involving terminal groups are essentially non-selective while those acting on double bonds (notably those producing epoxyalkenones) are selective. Bacterial strain *Dietzia maris* sp. AG1 isolated from non-axenic *E. huxleyi* strain TWP1 cells degraded alkenones selectively by way of epoxidation (Zabeti et al., 2010) support this assumption. Further experiments (Prahl et al., 2009) demonstrated the degree of selectivity of alkenone degradation by bacteria inducing double bond epoxidation strongly depends on the difference between $U_{37}^{K'}$ of the residual alkenones and the unsaturation ratio of the produced epoxyalkenones ($ep U_{37}^{K'} = x [C_{37:2}]/(x [C_{37:2}] + y [C_{37:3}])$), where x and y are the percentage of epoxyketone resulting from the degradation of $C_{37:2}$ and $C_{37:3}$ relative to the respective parent compound. This difference ($U_{37}^{K'} - ep U_{37}^{K'}$) was thus proposed as a useful indicator of aerobic bacterial alteration of the alkenone unsaturation ratio *in situ* (Prahl et al., 2009).

Diols (resulting from $NaBH_4$ reduction of the corresponding epoxyalkenones; Rontani et al., 2008) could be detected in most of the samples investigated (an example is given in Fig. 5B.5). The proportions of epoxyketone products relative to the residual precursor alkenones ranged from 0 to 5% (Tables 5B.1 and 5B.2). The differences observed between DK1 and DK2 dark incubations could be thus attributed to the presence of bacterial communities degrading alkenones with distinct selectivity during these two experiments (Rontani et al., 2008). Quantification of ($U_{37}^{K'} - ep U_{37}^{K'}$) was done in order to check for such a possibility. The results obtained (Tables 5B.1 and 5B.2) clearly showed that the variations of $U_{37}^{K'}$ during the two experiments are well correlated with those of ($U_{37}^{K'} - ep U_{37}^{K'}$). The highest values of

$(U_{37}^{K'} - \text{ep}U_{37}^{K'})$ observed during the dark incubation DK1 (Table 5B.1) suggest the involvement of a strongly selective bacterial degradation of alkenones. In contrast, during the DK2 experiment only very weak values of $(U_{37}^{K'} - \text{ep}U_{37}^{K'})$ were obtained (Table 5B.2) attesting to the weak selectivity of alkenone degradation.

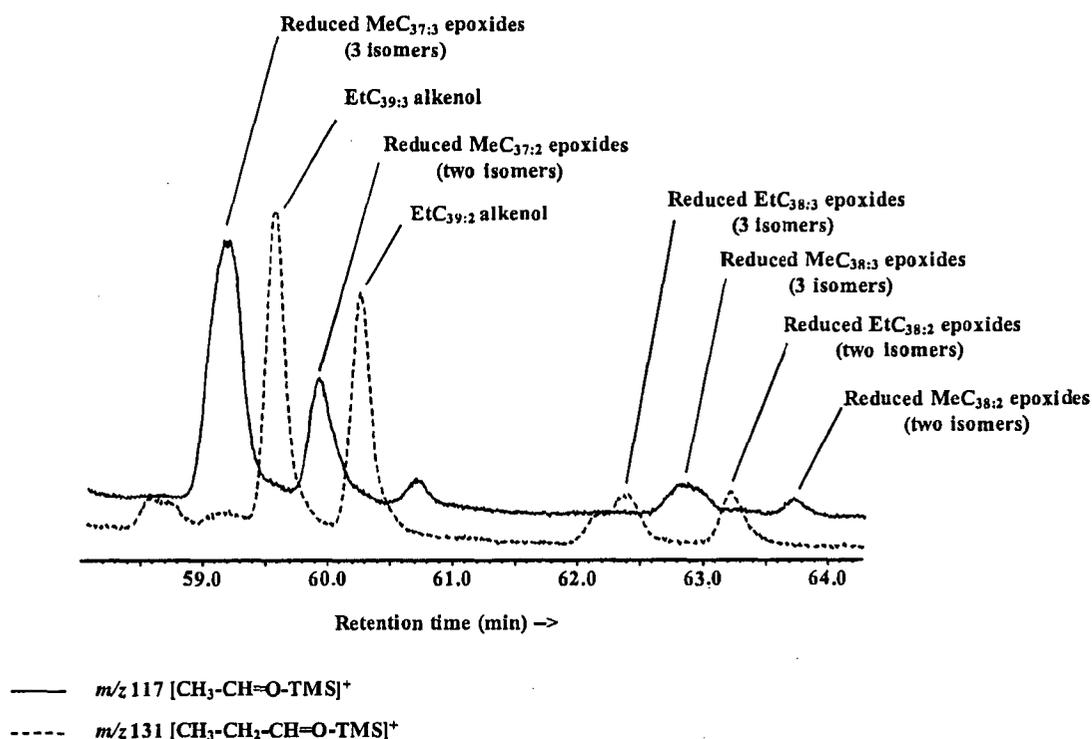


Figure 5B.5. Partial m/z 117 and 131 mass fragmentograms of a silylated NaBH_4 -reduced total lipid extract showing the presence of reduced epoxyalkenones during the DK1 experiment.

In an attempt to empirically constrain the magnitude of selective bacterial degradation-induced warming effect was pursued (Rontani et al., 2008; Prah1 et al., 2009). The plot of the ‘warming’ effect measured after different bacterial incubations of dead *E. huxleyi* cells versus the corresponding $(U_{37}^{K'} - \text{ep}U_{37}^{K'})$ yielded a trend (Prah1 et al., 2009) that is well described by a quadratic equation when the fit is forced through the origin ($y = 110x^2 + 4.84x$, $r^2 = 0.987$). Using this equation as a calibration and assuming that assimilation rates of epoxyketones

resulting from C₃₇ di- and tri-unsaturated alkenones are similar, the bacterial warming effects on the $U_{37}^{K'}$ values obtained during the DK1 incubation were estimated from the measured ($U_{37}^{K'} - epU_{37}^{K'}$) data. Some samples previously obtained by Prah1 et al. (2003) after incubation of the strain *E. huxleyi* strain NEPCC 55a under darkness were re-examined and the epoxyalkenones were subsequently detected. The results of these estimations are summarized in the Table 5B.5. While selective bacterial degradation appeared to explain the totality of the warming effect previously observed by Prah1 et al. (2003) during incubation of *E. huxleyi* strain NEPCC 55a, the correction is only partial in the case of the DK1 samples (Table 5B.5) suggesting the involvement of an additional selective degradation process.

Table 5B.5: Tentative correction of warming effect induced by selective bacterial degradation of alkenones during incubation of *E. huxleyi* cells under darkness.

Experiment	Dark incubation (days)	Measured warming effect (°C) ^a	$U_{37}^{K'}$	$U_{37}^{K'} - epU_{37}^{K'}$	Estimated bacterial warming effect (°C) ^t
DK1	2	1.1	0.25	+0.01	0.1°
DK1	4	3.1	0.37	+0.15	1.1°
DK1	6	4.9	0.57	+0.23	2.2°
DK1	8	5.0	0.77	+0.16	1.2°
DK1	10	5.3	0.78	- ^d	-
Prah1 et al. (2003)	3	0.9	0.59	+0.09	1.3
Prah1 et al. (2003)	4	1.7	0.62	+0.10	1.4
Prah1 et al. (2003)	5	3.2	0.65	+0.16	3.3

^a Determined from the calibration equations of the two strains ($\Delta T = \Delta U_{37}^{K'} / 0.034$ for *E. huxleyi* strain NEPCC 55a (Prah1 et al., 2003) and $\Delta T = \Delta U_{37}^{K'} / 0.102$ for *E. huxleyi* strain TWP1).

^b Determined from the equation: warming effect = $110x^2 + 4.84x$, where $x = U_{37}^{K'} - epU_{37}^{K'}$ (Prah1 et al., 2009).

^c Values corrected according to the calibration equation of the strain *E. huxleyi* strain TWP1.

^d Epoxyalkenones not detected.

By using thermal simulations carried out in solvents, it was previously demonstrated that alkenones are auto-oxidized faster than analogues of other common marine lipids (Rontani et al., 2006). During that work, it was shown that alkenone auto-oxidation rates increased in an approximately linear fashion with the number of double bonds present in the molecule so that auto-oxidation has the potential to significantly affect values of $U_{37}^{K'}$ leading to a warm bias in estimates of palaeo-temperature derived from alkenone ratios in sediments. More recently, the selectivity of auto-oxidative degradation processes on alkenones in cells of *E. huxleyi* strain CS- 57 was confirmed (Rontani et al., 2007). Auto-oxidation of alkenones appears to mainly involve allylic hydrogen abstraction and subsequent oxidation of the allylic radicals thus formed (Rontani et al., 2006). According to these processes, oxidation of each double bond in the alkenones affords four positional isomeric hydroperoxyketones, which had been previously characterized in the form of the corresponding alkenediol trimethylsilyl ether derivatives after NaBH_4 reduction and subsequent silylation (Rontani et al., 2006).

Careful examination of the lipid extracts obtained at the beginning of the two experiments demonstrated high proportions of brassicasterol photooxidation products (Δ^5 -3,7 α/β -dihydroxysterols and Δ^4 -3,6 α/β -dihydroxysterols resulting from NaBH_4 reduction of the corresponding hydroperoxides) in extracts of the DK1 experiment (Fig. 5B.6A), while these compounds were present in smaller amounts in the DK2 experiment (Fig. 5B.6B). These observations attest that at the beginning of the incubation under darkness *E. huxleyi* strain TWP1 cells were in better physiological state during the DK2 experiment than during the DK1. Indeed, in phototrophic organisms photodegradation products, which are induced by chlorophyll, act mainly during the senescence (i.e. when the photosynthetic system is not operative, Nelson, 1993). Probably due to the *trans* configuration of their double bonds,

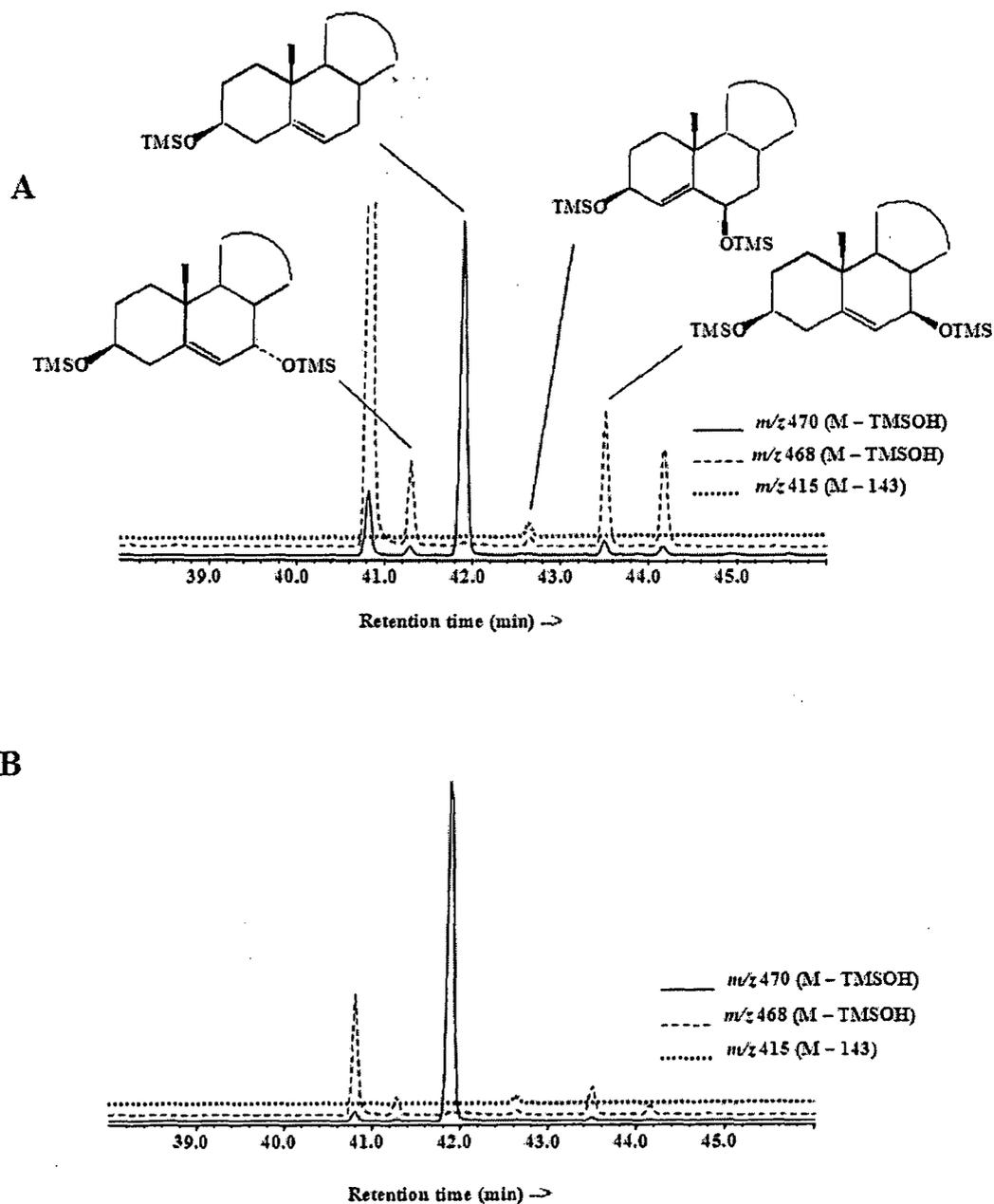


Figure 5B.6. Partial m/z 470, 468 and 415 mass fragmentograms of silylated NaBH_4 -reduced total lipid extracts (before incubation under darkness) showing the presence of brassicasterol and its oxidation products during the DK1 (A) and DK2 (B) experiments.

which are poorly reactive towards singlet oxygen (Hurst et al., 1985), the effects of photooxidation processes are relatively limited on alkenones (Rontani et al., 1997). However, the relatively high photodegradation state of the cells used for the DK1 experiment could be at the origin of auto-oxidative alterations of alkenones. Indeed, homolytic cleavage of the hydroperoxides photochemically-produced could induce free radical oxidation processes (Girotti, 2001).

In order to demonstrate the involvement of such degradative processes on the alkenones of *E. huxleyi* strain TWP1 cells, standard auto-oxidation products were prepared from purified alkenones as previously described (Rontani et al., 2006) and the resultant hydroperoxyketones were reduced (with NaBH₄) to the corresponding alkenediols. Subsequent comparison of retention times and mass spectra with these standard compounds demonstrated unambiguously the presence of significant amounts of specific alkenone auto-oxidation products in *E. huxleyi* strain TWP1 cells at the end of the dark incubation period of the DK1 experiment (Fig. 5B.7). These results confirm that during this experiment auto-oxidation processes also contribute to a significant increase in $U_{37}^{K'}$ values. The involvement of auto-oxidative degradation was attributed to the highest photodegradation state of *E. huxleyi* cells during the DK1 experiment (Fig. 5B.6) and to an intensive bacterial degradation of EDTA (Satroutdinov et al., 2003; Palumbo et al., 1994). Indeed, it is well known that metal ions play an important role in the homolysis of hydroperoxides (Pokorny, 1987; Schaich, 1992). Metal ions may direct the cleavage of hydroperoxides either through alkoxy or peroxy radicals (Fig. 5B.8). In classical culture media (such as f/2) the metal chelator EDTA is present in high amounts and tightly binds free catalytic metal ions and thus renders them unavailable. EDTA thus acts in the culture media as an antioxidant and strongly limits radical

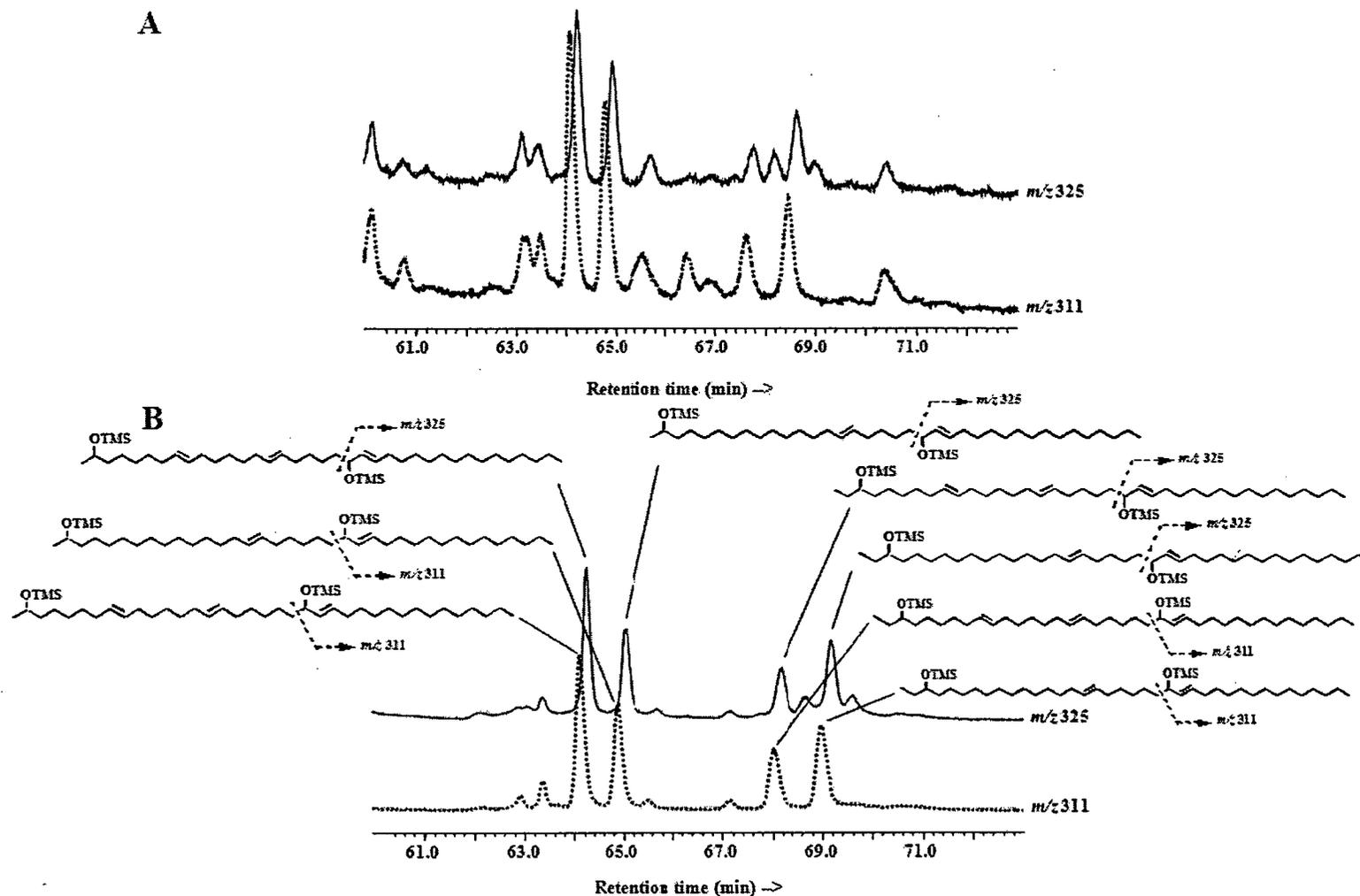


Figure 5B.7. Partial mass fragmentograms of m/z 311 and 325 revealing the presence of silylated C_{37} and C_{38} alkenediols after NaBH_4 -reduction and silylation of A) the total lipid fraction of *E. huxleyi* strain TWPI incubated under darkness (experiment DK1) and B) standard autoxidized alkenones (B).

oxidation processes. In the presence of high amounts of bacteria (as this was the case during the DK1 experiment), biodegradation of EDTA may release metal ions able to catalyse homolytic cleavage of photochemically- produced hydroperoxides thus inducing selective free radical oxidation of alkenones (Fig. 5B.8).

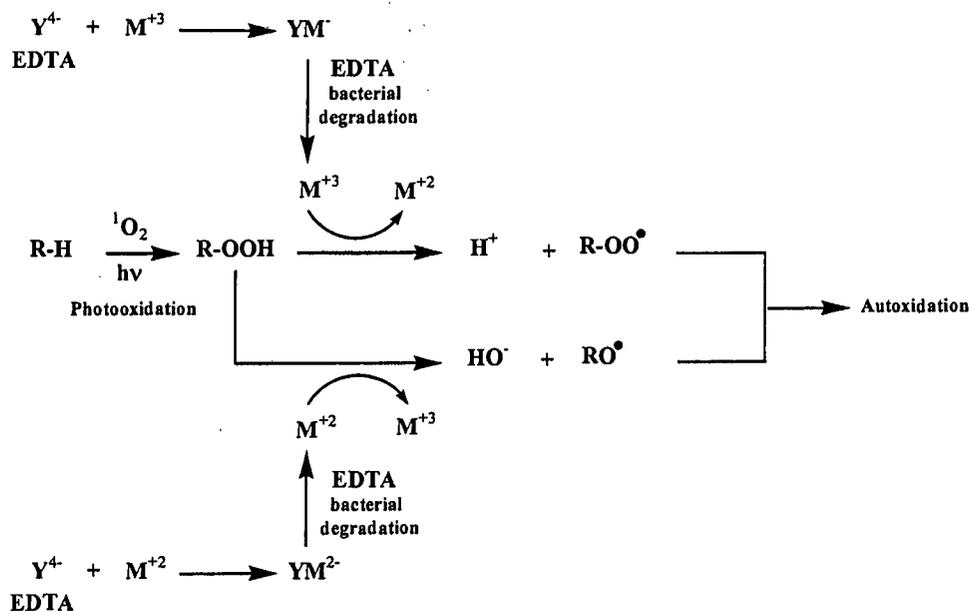


Figure 5B.8. Proposed pathways for the induction of autoxidative degradation processes in non-axenic phytoplanktonic cultures. ($\text{M}^{+3} = \text{Fe}^{+3}$, $\text{M}^{+2} = \text{Cu}^{+2}$, Zn^{+2} , Co^{+2} or Mn^{+2} , $\text{R-H} = \text{unsaturated lipid}$, $^1\text{O}_2 = \text{singlet oxygen}$).

Prolonged incubation of two non-axenic cultures of a same strain of *E. huxleyi* under darkness gave very contrasting results and demonstrated that the strong decrease in alkenone concentration generally observed under such conditions resulted not only from the well known consumption of reserve substances by the algae (Eltgroth et al., 2005), but also from the simultaneous involvement of auto-oxidative and bacterial degradation processes. Free radical oxidation (auto-oxidation) of alkenones, which is induced by homolytic cleavage of photochemically-produced hydroperoxides, affords specific allylic hydroperoxides and is

strongly selective towards di- and triunsaturated alkenones (Rontani et al., 2006; 2007). In contrast, degradation of these compounds by the bacteria associated with the algal cells (attested by the detection of epoxyalkenones, Rontani et al., 2008) may be selective or non-selective according to the bacterial community present. The alteration of alkenone unsaturation index during incubation of *E. huxleyi* cells under darkness thus appears to be strongly dependent on the initial physiological state of the cells (favouring or inhibiting auto-oxidative processes) and on the bacterial communities associated with them. This finding could explain the various degrees of selectivity previously observed during incubation of different strains of *E. huxleyi* under darkness (Table 5B.3).

The results obtained in the present work confirmed the potential of aerobic bacterial degradation and auto-oxidation processes to induce significant increases in $U_{37}^{K'}$ values in *E. huxleyi* cells and thus raising concern about data interpretations of palaeo-temperature reconstructed from the alkenone unsaturation index. While the importance of the warming effect resulting from selective bacterial degradation of alkenones might be estimated on the basis of measured ($U_{37}^{K'} - epU_{37}^{K'}$) data (Prahl et al., 2009), auto-oxidative alteration of $U_{37}^{K'}$ values could not be estimated. Indeed, hydroperoxides resulting from these processes are insufficiently stable to be used as tracers of alkenone free radical oxidation *in situ*.

Chapter 6

Summary

➤ OM in marine environments is derived from several sources including biogenic and terrestrial. Microalgae and bacteria are generally the major sources of OM in oceanic environment. Lipid is one of the major constituent of OM and is comparatively source specific. They are relatively stable compounds for degradation processes in marine environment. Certain lipid compounds such as phospholipid fatty acids (PLFAs) are the major components of microbial cell membranes and are associated with living cells. Hence they are utilized in the measurement of live biomass and community structure of the microorganisms in the natural environment. However, it is observed that PLFAs can be affected by changing environmental conditions such as incubation periods, nutrients temperature and environmental pollutants. Fatty acids on the other hand are derived from the living organisms as well as dead OM in natural environment. Their source specificity with respect to individual compounds and their lability makes FAs suitable for tracing sources and diagenetic changes in organic material in water columns and sediments. A number of studies are available on the lipids from the temperate marine environments; however such studies are less in tropical environment. Moreover, studies on PLFAs concentrations and composition in tropical marine bacteria, diatoms and in the sediments are rarely done in the Indian waters. Alkenone distribution in marine sediment core has been used to assess paleoenvironment. If these compounds have to be used for paleoenvironmental studies, then alkenones biodegradation in water and sediment should not strongly affect temperature signal that was established during alkenone biosynthesis by the alga. Despite the widespread use of alkenones in paleothermometry, comparatively, a few studies have investigated the effect of bacterial degradation on the calculation of SST.

- In this thesis, studies on the phospholipid fatty acids (PLFAs) of the marine bacteria and diatoms were carried out. Various environmental factors such as incubation period, nutrients nitrogen and phosphorus, temperature and environmental toxicant such as tributyl tin (TBT) affecting the PLFAs were studied. Based on the concentrations and composition of the PLFAs the biomass and community structure of microorganisms in the marine environment was determined. The fate of the organic matter (OM) in the marine environment was evaluated using the total fatty acids and the individual fatty acids in the deep sea sediments, where the maximum alteration of OM is possible. Further the most studied microalgal lipid biomarker (alkenones) stability is evaluated by carrying out microbial degradation of alkenones in order to study their implications for paleotemperature reconstruction.
- Total PLFA concentrations changed with respect to the growth condition for the *Bacillus licheniformis*, *B. subtilis* and *Aeromonas hydrophila*. Increase in Nitrogen and phosphorus concentrations resulted in increased production of total PLFAs. Hence, it can be deduced that the PLFA production is dependent upon the nutrient availability in these bacteria. Temperature also played an important role in the total PLFA production, wherein the 30°C grown *B. subtilis* and *A. hydrophila* showed optimum production of total PLFAs. However, *B. licheniformis* showed higher concentrations of total PLFAs at higher temperature (38°C).
- Both the *Bacillus* spp. showed the dominance of iso and anteiso branched PLFAs with minor contribution of saturated PLFAs (Sat), whereas *Aeromonas* showed the predominance of monounsaturated (MUFA) and saturated PLFAs. Different nutrient concentrations did not change the relative % of saturated, branched and unsaturated PLFAs in the *B. licheniformis*, *B. subtilis* and *Aeromonas hydrophila*, although their

total individual concentrations varied. However, temperature influenced the relative % of the saturated and branched PLFAs. In general Iso, saturated and Br17 were higher at low temperature in *B. licheniformis*. In *B. subtilis* iso and Br17 increased and anteiso and Br15 decreased at higher temperature. However saturated PLFAs decreased in both the *Bacillus* cultures, suggesting their minor role in cell membrane fluidity at higher temperature in these cultures. This shows that both the *Bacillus* cultures behave differently to maintain membrane fluidity. Moreover, saturated PLFAs (increased) played very important role in maintaining membrane fluidity at higher temperature, while unsaturated PLFAs (increased) were important at lower temperature in the *A. hydrophila*.

- In *Skeletonema* sp. total PLFAs were high at d 15, while in *Amphora* sp. at d 20 of the incubation period. Higher nitrogen and phosphorus concentrations supported higher PLFA concentrations. *Skeletonema* sp. showed higher PLFAs at low temperature which decreased at higher temperature, while in *Amphora* sp. the opposite was seen. Both the microalgal cultures showed the predominance of C14:0, C16:0, C16:1 and C20:5 PLFAs. Among the PUFAs, C20:5n3 was the most abundant in both the diatoms. Although the concentrations of individual PLFAs changed with the cultivation period, the PLFA composition remained typical of diatoms during all the incubation periods. Decrease in Sat PLFAs, was associated with the increased production of PUFA in *Skeletonema* sp. while increase of Sat PLFAs, resulted in decreased production of PUFA in *Amphora* sp. This suggests that synthesis of PUFA was influenced by the Sat PLFAs in both the microalgae. Nevertheless, the cultures seem to have different requirements of PUFA during various cultivation periods. It was suggested that the increase in saturated fatty acids

in *Amphora* sp. was due to the inhibition of PUFA synthesis during aging. However, the higher relative % of PUFA during various cultivation periods in both the microalgae was also observed. PUFA were the major PLFAs under all the nutrient conditions used. Lower temperature did not induce the higher production of MUFA and PUFA, while Sat PLFAs were influenced at higher temperature.

- Increasing concentrations of tributyltins (TBT) influenced growth of the cells and PLFA concentrations and composition of all the 4 cultures of *Bacillus*. Growth of all the bacterial cultures was delayed and retarded in the presence of TBT. The TBT is a membrane active substance, which alters the concentrations of PLFAs in the membrane lipids. Changes in total PLFAs in response to TBT concentrations suggest the ability of the bacteria to modulate PLFA concentrations. PLFAs are useful in maintaining the fluidity of the cell membrane. *B. subtilis* and *Bacillus* sp. synthesized higher amounts of anteiso, iso and saturated PLFAs at 6.05 $\mu\text{g l}^{-1}$ TBT. However, at higher concentrations of TBT (12.1 $\mu\text{g l}^{-1}$) production of PLFAs by the bacterial cultures was reduced, suggesting higher concentrations are toxic to the cells of the *Bacillus* cultures. The ability of the bacterial cultures to grow with various concentrations of TBT by modulating the PLFA concentration suggests adaptation of these bacterial cultures to the TBT stress.
- $\delta^{13}\text{C}_{\text{oc}}$ signature and two end-members mixing model suggests that the OM was derived from mixed marine and terrestrial sources with relative abundance of latter at most of the stations in the Visakhapatnam harbour. PLFA concentrations varied spatially indicating differences in the living biomass in the harbour sediments. Furthermore, there was no correspondence between OC concentrations or sources and the concentrations or the composition of the total PLFAs. This suggest that the

community of microorganisms deduced by PLFAs was not influenced by concentration and sources of OC. Abundance of saturated PLFAs were observed throughout the stations in the Visakhapatnam harbour. However, they are ubiquitous in organisms and hence are not useful in assessing community structure. Hence a major fraction of PLFAs in these sediments cannot be ascribed to any particular microbial group. The Visakhapatnam harbour also showed the abundance of some specific PLFAs of various organisms such as monounsaturated fatty acids (MUFAs), indicated the abundance of Gram negative bacteria, cyanobacteria and microalgae. The Branched PLFAs (iso and anteiso) suggest the presence of Gram positive bacteria, Gram negative anaerobes and sulfate reducing bacteria. Similarly, PUFAs indicate the presence of eukaryotes. Moreover, the presence of trans-monounsaturated PLFAs in the harbour sediments imply that PLFA community was under stress due to contamination of the sampling sites by sewage and industrial waste, sulfur and petroleum products. Principal component analysis (PCA) based on concentrations of PLFAs was performed in order to segregate the stations which clustered the stations of the Visakhapatnam harbour into three groups. Stations #2, #7 and #13 formed one group and showed the dominance of iso and anteiso C15:0 PLFAs (branched) which are specific for bacteria, suggesting higher contribution of bacterial biomass in these stations. Second cluster was formed with stations #12, #15 and #16 wherein PUFA such as C20:3n6, C18:3n3, C20:4n6, C20:5n3 and C22:6n3 were abundant indicating the importance of marine algae at these stations. Moreover, the third cluster was formed with the stations which had lower concentrations of the PLFAs, suggesting lower microbial biomass at these stations.

Moreover, the PLFAs in the Visakhapatnam harbour sediments suggest the dominance of bacteria along with some contribution from eukaryotes.

- $\delta^{13}\text{C}_{\text{oc}}$ values and two end-members mixing model implies that OM in sediments of the Mandovi and the Zuari estuary was derived from mixed sources consisting of phytoplankton and terrestrial C3 plants. The $\delta^{13}\text{C}_{\text{oc}}$ values indicated that stations near river end and sea end were dominated with mixed sources of marine and terrestrial origin, while the middle estuary was dominated with terrestrial OM in both the estuaries. The concentrations of PLFAs varied spatially and showed good correlation with organic carbon (OC) suggesting that the microbial community was important component of OC. However abundance of PLFA compounds in the sediments indicates that the microbial community was not dependent on the sources of OC and terrestrial and marine carbon sources were equally important in sustaining microbial biomass in both the estuaries. Saturated PLFAs are ubiquitous in nature which indicates that major proportion of PLFAs cannot be attributed to any particular microbial group in these estuaries also. Bacterial biomarkers (C15:0, C17:0, iso, anteiso, 10Me16, 10Me18 and cyclopropyl-PLFAs) associated with the gram positive bacteria, anaerobic bacteria and with sulfate-reducing bacteria were abundant in both the estuaries. MUFA (C16:1 and C18:1) abundance show the influence of gram negative bacteria, cyanobacteria, phytoplankton and other microeukaryotes in the sediments of the Mandovi and Zuari estuary. PUFA (C20:3n3, C20:5n3, C22:6n3) shows the abundance of diatoms, dinoflagellates, other algae and cyanobacteria in the sediments of these estuaries. Presence of trans and cyclo fatty acids in Mandovi and Zuari estuary indicates that the microbial communities were under stress probably due to presence of petroleum products,

heavy metal ions, sewage, anthropogenic wastes and anoxic conditions. PCA formed cluster 1 with all the Mandovi estuary stations and three Zuari estuary stations (stations #1Z, #5Z and #8Z), while the cluster 2 was formed with the remaining Zuari estuary stations (stations #2Z, #3Z, #4Z, #5Z and #7Z). These Zuari stations #2Z, #3Z, #4Z, #5Z and #7Z contained high concentrations of PUFA such as C20:5n3 and C22:6n3 and based on this they appear to be separated from other stations of Zuari and Mandovi estuary. Moreover, none of the Mandovi estuary and the three stations of Zuari estuary (stations #1Z, #5Z and #8Z) contained C20:5n3 and C22:6n3 PLFAs. Moreover, station #4Z and station #7Z were placed relatively far from other Zuari estuary stations in cluster 2 which also contained overall high concentrations of PLFAs.

- The fate of the organic matter (OM) was assessed using fatty acids (FAs). Lipid molecules such as FAs are most useful in studying the fate of OM since they are specific to various organisms and degrade at different rates. For this purpose one sediment core was collected from the Northern Indian Ocean (CC2/GCL1) and the other from the Equatorial Indian Ocean (EIO) (CC1/GC3) and analyzed for FAs using GCMS. Organic carbon (OC), lipid-phosphate and total fatty acids (TFA) were relatively higher in the CC2/GCL1 core as compared to CC1/GC3 core inferring substantially higher OM and microbial biomass in the former core. In both cores, TFA concentration decreased with the increase in downcore depth, suggesting their degradation. Monounsaturated fatty acids (MUFA) abundance was observed in both the sedimentary environments indicating contribution of microbial communities consisting of gram negative bacteria. Low abundance of branched and cyclopropyl FAs suggests the poor abundance of gram positive bacteria, anaerobic and sulfate

reducing bacteria. Moreover, in both the cores dominance of saturated C16:0 and C18:0 was observed. Bacteria and eukaryote group appear to be the most abundant throughout the core sections in CC1/GC3. While these organisms were also dominant in upper core (0-14 cm) sections of the CC2/GCL1 core, whereas at greater depths (38-40cm) FAs of terrestrial OM were more abundant. Zooplankton marker FAs (C20:1, C22:1 and C24:1) were prevalent in middle of the core (14-26 cm) and towards the deeper sections (34-40 cm) of the CC2/GCL1 core. In the CC1/GC3 core, these zooplankton markers were less abundant.

- Studies on *E. huxleyi* culture have demonstrated that alkenone biomarkers are attractive geochemical tools for palaeoceanographic studies. The proportion of di- to triunsaturated C₃₇ alkenones in cultured cells increases with increasing water temperature. On the basis of this finding and of the ubiquity of C₃₇-C₄₀ alkenones in recent and ancient marine sediments, the ratio $\frac{[C_{37:2}]}{([C_{37:2}] + [C_{37:3}])}$, commonly referred to as U_{37}^K , has become a reference standard for assessment of SST in palaeoceanographic studies. For alkenones to be useful as measures of SST in the geological record, it is essential that any effects of alkenone degradation in the water column and in sediments do not affect the temperature signal that was established during their biosynthesis by the alga, or if there is a change its extent can be reasonably estimated. Despite the widespread use of alkenones for paleothermometry (paleotemperature assessment), comparatively few studies have investigated the effects of bacterial degradation of alkenones. Although various other studies have inferred biodegradation of alkenones in different environments, very few have yet examined the details of the biodegradative processes.

➤ Four bacterial communities isolated from *E. huxleyi* strain TWP1 cultures before (TAB- total aerobic bacteria) and after different antibiotic treatments (ATB1, ATB2 and ATB3, antibiotic treated bacteria) were used in the present work to study the biodegradation of alkenones under laboratory-controlled conditions. Incubation of sterilized *E. huxleyi* (as a substrate for alkenones) for 20 days with these bacterial communities resulted in effectively none to extensive degradation of alkenones. Only minor (5.1 to 18.9 %) degradation of alkenones was observed with the ATB1 community, while incubation with the ATB2 community resulted in major (21.1 to 50.7 %) degradation. But, in both cases the degradation of di- and triunsaturated alkenones appeared to be non-selective. In contrast, incubation of *E. huxleyi* strain TWP1 cells with the ATB3 and TAB communities resulted in major (21.2 to 77.7 % and 23.3 to 49.4 %) and selective degradation of alkenones. Because of degradation of alkenones, the observed increases in $U_{37}^{K'}$ are equivalent to a +2°C and +3.3°C change in the inferred temperature when interpreted using a standard calibration equation. The differences are sufficiently large to cause concern about data interpretations of palaeotemperature reconstructed from the alkenone unsaturation index, because some reconstructed temperature differences between the last glacial and interglacial period are only 1–3 °C in magnitude. These results clearly show that the various antibiotic treatments of *E. huxleyi* strain TWP1 cells significantly changed the composition of the bacterial communities associated with the cells. The differences in the bacterial communities were confirmed by different DGGE bands for the four communities. The very distinct results obtained with the four communities strongly suggest the existence of at least two functional classes of

aerobic bacteria capable of degrading alkenones, i.e. those able to degrade them either non-selectively or selectively. It is likely that these differences in degradative outcomes are strongly dependent on the particular metabolic pathways used by the two groups of bacteria. The epoxyketones resulting from bacterial oxidation of alkenone double bonds could be useful indicators of aerobic bacterial alteration of the alkenone unsaturation ratio in situ. The detection of epoxy ketones in some cultures (ATB3) indicates that metabolic pathways involving attack on the terminal groups of the molecule are essentially non-selective, while those acting on alkenone double bonds are selective. The production of alkenols during incubation with ATB3 demonstrated for the first time that bacterial reduction of alkenones can be a potential source of these compounds in the environment. Small amounts of monounsaturated alkenones were also observed in the degradation experiment using this community also raises the possibility of a bacterial reduction of alkenone double bonds.

- In order to determine whether associated bacteria play a role in the selective degradation of alkenones, two starter culture *E. huxleyi* TWP1 were grown at 20°C and 15°C, and these cultures were designated as DK1 and DK2, respectively. These two starter cultures were re-grown at 15°C and then transferred to dark condition. In both cases several days of continuous darkness resulted in a strong decrease (more than 80%) in alkenone concentration. Concerning the selectivity of the degradation between di- and triunsaturated compounds, strongly contrasted results were obtained. Increase in $U_{37}^{K'}$ (+ 0.54) was observed in the case of the DK1 experiment, while no significant increase appeared in the case of DK2. A selective consumption

of these reserve substances by the algae in DK1 and not in DK2 being very unlikely, the very strong alteration of $U_{37}^{K'}$ index observed during the DK1 experiment was attributed to the simultaneous involvement of selective bacterial and auto-oxidative degradation processes. The presence of bacteria able to degrade selectively alkenones in DK1 is well supported by the detection of significant amounts of epoxyalkenones exhibiting an unsaturation ratio (ep $U_{37}^{K'}$) lower than the $U_{37}^{K'}$ ratio of the residual alkenones. The detection of specific auto-oxidation products of alkenones during the DK1 experiment attests that free radical oxidation processes also contributed to the selective degradation of these compounds. Evolution of bacteria number during the incubation (strong increase under darkness and subsequent decrease after return to light) in DK1 was attributed to a transfer of photochemically produced singlet oxygen from senescent *E. huxleyi* cells to the associated bacteria limiting their growth under light/dark regime but not under continuous darkness. Prolonged incubation of two non-axenic cultures of a same strain of *E. huxleyi* under darkness gave very contrasting results and demonstrated that the strong decrease in alkenone concentration generally observed under such conditions resulted not only from the well known consumption of reserve substances by the algae, but also from the simultaneous involvement of autoxidative and bacterial degradation processes. Free radical oxidation (auto-oxidation) of alkenones, which is induced by homolytic cleavage of photochemically-produced hydroperoxides, affords specific allylic hydroperoxides and is strongly selective towards di- and triunsaturated alkenones. In contrast, degradation of these compounds by the bacteria associated to the algal cells (attested by the detection of

epoxyalkenones) may be selective or non-selective according to the bacterial community present. The alteration of alkenone unsaturation index during incubation of *E. huxleyi* cells under darkness thus appears to be strongly dependent on the initial physiological state of the cells (favouring or inhibiting autoxidative processes) and on the bacterial communities associated with them. This finding could explain the various degrees of selectivity previously observed during incubation of different strains of *E. huxleyi* under darkness. The results obtained in the present work confirmed the potential of aerobic bacterial degradation and auto-oxidation processes to induce significant increases in $U_{37}^{K'}$ values in *E. huxleyi* cells and thus raising concern about data interpretations of palaeo-temperature reconstructed from the alkenone unsaturation index. While the importance of the warming effect resulting from selective bacterial degradation of alkenones might be estimated on the basis of measured ($U_{37}^{K'} - e_p U_{37}^{K'}$) data (Prah et al., 2009), autoxidative alteration of $U_{37}^{K'}$ values could not be estimated. Indeed, hydroperoxides resulting from these processes are insufficiently stable to be used as tracers of alkenone free radical oxidation *in situ*.

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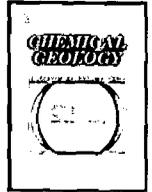
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List of Publications

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3. Jean-François Rontani *, **Ranjita Harji**, Sophie Guasco, Fredrick G. Prahl, John K. Volkman, Narayan B. Bhosle, Patricia Bonin. Degradation of alkenones by aerobic heterotrophic bacteria: Selective or not? *Organic Geochemistry* 39 (2008) 34-51. **IF:2.149.**
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Research paper

Sources of organic matter and microbial community structure in the sediments of the Visakhapatnam harbour, east coast of India

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ABSTRACT

Organic matter in the coastal sediments originates from various terrestrial and marine sources. Within the sampling sites, relative inputs from these sources may vary and can influence the microbial community structure. In order to identify the sources of organic matter, and its influence on microbial community structure in coastal environment, 19 surface sediment samples were collected from various stations in the Visakhapatnam harbour, east coast of India. These samples were analyzed for organic carbon (OC) content, bulk $\delta^{13}C$ signatures, and the concentration and composition of phospholipid fatty acids (PLFAs). Contents of OC, $\delta^{13}C_{oc}$ values, and PLFA concentrations varied spatially and ranged from 0.6 to 7.6%, -29.32 to -23.75% , and 0.30 to $33.30 \mu g g^{-1}$ dw sediment, respectively. The bulk $\delta^{13}C_{oc}$ of sediments reflected mixed carbon sources from marine and terrestrial end members with dominance of the latter at most of the stations. The PLFA community was not influenced by concentration and source of OC. Saturated PLFAs were the most abundant followed by monounsaturated fatty acids (MUFAs), branched PLFAs, and polyunsaturated fatty acids (PUFAs). MUFAs indicate the abundance of Gram negative bacteria, cyanobacteria and microalgae. The Branched PLFAs (iso and anteiso) suggest the presence of Gram positive bacteria, Gram negative anaerobes and sulfate reducing bacteria. Similarly, PUFAs indicate the presence of eukaryotes. Moreover, the presence of trans-monounsaturated PLFAs in the harbour sediments imply that PLFA community was under stress due to contamination of the sampling sites by sewage and industrial waste, sulfur and petroleum products. Principal component analysis (PCA) based on concentrations of PLFAs established three factors that accounted for 81% of the total variance. The first factor contributed 57% of variance, and was mostly influenced by MUFAs and branched PLFAs. The second factor was controlled by PUFAs such as C20:3n6, C18:3n3, C20:4n6, C20:5n3 and C22:6n3, whereas the third factor was influenced by C20:3n3, C22:2 and C22:1n9. PLFA community in the Visakhapatnam harbour sediments was mostly dominated by bacteria along with some contribution from eukaryotes.

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1. Introduction

Organic matter (OM) in the near shore and coastal sediments is derived from various sources including primary production by phytoplankton or benthic microalgae, terrestrial plants, river runoff, municipal sewage and industrial discharge, etc. (Hedges and Keil, 1995; Hedges et al., 1997). Stable carbon isotope ($\delta^{13}C_{oc}$) signatures of the various carbon sources are often different, and despite some overlap between different sources, they are powerful tracers of carbon inputs in various environments (Fry and Sherr, 1984; Prahl et al., 1994; Meyers, 1994; Schelske and Hodell, 1995). Geochemical biomarkers such as *n*-alkanes, alcohols, sterols, alkenones, and fatty acids also are often used to identify the carbon inputs from various organisms in marine environments (Volkman et al., 1992; Tolosa et al., 2003).

However, these geochemical biomarkers and stable carbon isotopes do not differentiate between dead and live organisms.

In marine environment, living biomass consists of a complex mixture of prokaryotes and eukaryotes, whose combined activity determines nearly all of the elemental biogeochemical cycling (Boschker et al., 2001). The nature and sources of OM, nutrient status and pollution may influence the microbial community structure (Guckert et al., 1986; Bååth et al., 1995; Pennanen, 2001). Traditional techniques of isolation and culturing have not been adequate for characterization of microorganisms in environmental samples, especially in evaluating the natural microbial diversity (Fang et al., 2000; Delong and Pace, 2001). Thus to detect changes in bacterial biomass and natural microbial communities culture-independent techniques such as phospholipid fatty acid (PLFA) analysis (Pennanen et al., 1996; Pinkart et al., 2002), and genetic fingerprinting (Polymenakou et al., 2005), are routinely used.

PLFAs are the central component of the microbial cell membranes. They are present in reasonably constant amounts over a wide range of growth conditions and are rapidly hydrolyzed upon cell death (White

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Sources of hydrocarbons in sediments of the Mandovi estuary and the Marmugoa harbour, west coast of India

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ABSTRACT

Surface sediments were collected from various locations of the Mandovi estuary and the Marmugoa harbour. Sediments were analysed for organic carbon (OC), total lipids, *n*-alkanes concentration and composition. Concentrations of OC, total lipids and *n*-alkanes varied spatially and ranged from 1 to 2.5%, 176 to 1413 µg/g dry weight (dw) sediments, and 0.8 to 3.2 µg/g dw sediments of the Mandovi estuary, respectively; and from 0.6 to 2.9%, 233 to 1448 µg/g dw sediments, and 1.6 to 10.7 µg/g dw sediments in the Marmugoa harbour, respectively. Long chain, odd carbon *n*-alkanes (C₂₃–C₃₃) in the Mandovi estuary, whereas short chain, even carbon *n*-alkanes (C₁₁–C₂₁) in the Marmugoa harbour sediments were more abundant. The total HC concentrations, *n*-alkane composition, CPI, UCM and other evaluation indices suggest the dominance of terrestrial hydrocarbons in the estuarine while petroleum derived hydrocarbons in the harbour sediments. This conclusion was further supported by the abundance of hopanes with C₂₉ to C₃₄ α, β compounds and steranes with C₂₇, C₂₈ and C₂₉ compounds in the harbour sediments.

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1. Introduction

Organic matter (OM) in coastal sediments is derived from various sources such as terrigenous, marine, atmospheric and anthropogenic (Gogou et al., 2000; Wang et al., 2006). However, the fate of autochthonous and terrestrially derived OM in estuarine environments is not well understood (Hedges et al., 1997; Wu et al., 2004). The information about processes controlling the delivery of organic matter to coastal environments, and how the signatures of these inputs are reflected in newly deposited sediments is important to our understanding of global biogeochemical cycles.

Lipid molecules such as *n*-alkanes, fatty acids, alcohols and sterols are used to identify the sources of organic matter in marine and terrestrial samples (Volkman et al., 1992; Tolosa et al., 2003; Wu et al., 2004). Of these, the *n*-alkanes are commonly used to characterize organic matter of water, suspended matter and sediments from various environments (Ou et al., 2004; Gao et al., 2007). This is because *n*-alkanes are easy to analyse, and many are source specific. For example, bacteria normally show predominance of even carbon *n*-alkanes, mainly C₁₈ and C₂₀ (Han and Calvin, 1969). Elias et al. (2000) reported that even carbon *n*-alkanes in the C₁₄–C₂₂ range originate from diatoms. On the other hand, planktonic organisms such as cyanobacteria and green, red and brown algae generally produce a simple mixture of odd carbon *n*-alkanes dominated by C₁₅, C₁₇, and C₁₉ with predominant compound being species dependent

(Clarck and Blumer, 1967; Gogou et al., 2000). Whereas, abundance of straight chain odd carbon *n*-alkanes C₂₅, C₂₇, C₂₉ and C₃₁ has been used extensively as an indicator of terrestrial or land derived organic matter (Pearson and Eglinton, 2000; Zhao et al., 2003).

A number of other indices such as concentrations of total hydrocarbons, major *n*-alkanes, the ratios of short chain/long chain *n*-alkanes, total *n*-alkanes/*n*-C₁₆, *n*-C₁₇/Pristane and *n*-C₁₈/Phytane, carbon preference index (CPI) and the presence of an unresolved complex mixture (UCM) also have been used to identify the sources of *n*-alkanes in environmental samples (Bouloubassi et al., 2001; Ou et al., 2004; Gao et al., 2007). Similarly, because of their greater thermodynamic stability, hopanes with the 17α, 21β-configuration, and steranes with 5α, 14α and 17α configuration are useful to identify petroleum derived *n*-alkanes (Zaghden et al., 2005; Gao et al., 2007). Furthermore, δ¹³C analysis is yet another useful technique to identify the sources of *n*-alkanes (Pearson and Eglinton, 2000; Wu et al., 2004; Wang et al., 2006).

There are numerous studies on the physical, chemical and biological characterization of the waters of the Mandovi estuary and the Marmugoa harbour (Selvakumar et al., 1980; Qasim and Gupta, 1981; Shirodkar and Sengupta, 1985; Chanda et al., 1996). There also are studies on the total organic carbon (TOC) and the mineralogy of the surficial sediments of the Mandovi estuary, and total petroleum hydrocarbons in the coastal waters of Goa (Murty et al., 1976; Fondekar et al., 1980; Alagarsamy, 1991). In contrast, little is known about molecular level characterization of OM, especially *n*-alkane concentration and composition in the sediments of the Mandovi estuary and the Marmugoa harbour. Therefore, the aims of the present

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Degradation of alkenones by aerobic heterotrophic bacteria: Selective or not?

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Abstract

Four bacterial communities were isolated from *Emiliania huxleyi* strain TWP1 cultures before and after the algal cells had been treated with different antibiotics. Incubation of *E. huxleyi* with these bacterial communities resulted in dramatically different extents of alkenone degradation, ranging from effectively none to extensive. Selective degradation of the more unsaturated alkenones was observed in experiments using the total bacterial community and one of the communities isolated from antibiotic-treated algal cells. The observed increases in U_{37}^K are equivalent to a +2 °C and +3.3 °C change in the inferred temperature. Our results clearly show that intense aerobic microbial degradative processes have the potential to introduce a significant 'warm' bias in palaeotemperature reconstruction and could explain apparent anomalies in palaeotemperatures inferred from alkenone distributions in strongly oxidizing sedimentary environments. The results show that aerobic bacteria capable of selectively degrading alkenones are not limited to particular environments such as microbial mats and can be actually associated with living *E. huxleyi* cells. The detection of epoxyketones in some cultures indicates that metabolic pathways involving attack at the terminal groups of the molecule are essentially non-selective, while those acting on alkenone double bonds are selective. The epoxyketones resulting from bacterial epoxidation of alkenone double bonds could be useful indicators of aerobic bacterial alteration of the alkenone unsaturation ratio in situ. The production of alkenols during incubation with one of the bacterial communities demonstrated for the first time that bacterial reduction of alkenones can be a potential source of these compounds in the environment. The intriguing production of small amounts of monounsaturated alkenones by one of the bacterial communities also raises the possibility of a bacterial reduction of alkenone double bonds.

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1. Introduction

Alkenones are a class of unusual, very long chain mono-, di-, tri- and tetraunsaturated methyl and ethyl ketones synthesized by a limited number of haptophyte microalgae (Volkman et al., 1980a;

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Biomarkers derived from heterolytic and homolytic cleavage of allylic hydroperoxides resulting from alkenone autoxidation

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Abstract

Laboratory incubation of alkenone mixtures with *tert*-butyl hydroperoxide and di-*tert*-butyl nitroxide (radical initiator) in hexane, as a means to simulate alkenone autoxidation processes, rapidly led to the formation of allylic hydroperoxides, whose presence was recently demonstrated in *Emiliana huxleyi* cells. After incubation in seawater and subsequent reduction with NaBH₄ (to reduce residual hydroperoxides before analysis), these reaction products quickly disappeared and were replaced by complex mixtures of *n*-alcohols, fatty acids, alkyldiols and hydroxyacids. Methyl alkenones produced saturated *n*-alkan-1-ols and fatty acids ranging from C₁₃ to C₁₆ and two series of C₁₃–C₁₆ (ω -1)-hydroxyacids and (1, ω -1)-diols. Ethyl alkenones also afforded C₁₃–C₁₆ saturated *n*-alkan-1-ols and fatty acids, accompanied by the production of C₁₄–C₁₇ (ω -2)-hydroxyacids and (1, ω -2)-diols. Deuterium labelling allowed us to show that most of the *n*-alkan-1-ols, hydroxyacids and alkyldiols resulted from the reduction during the NaBH₄ treatment of the corresponding aldehydes, ketoxyacids and ketoxyaldehydes formed from heterolytic or homolytic cleavages of allylic hydroperoxyl groups resulting from the oxidation of the double bonds of di- and triunsaturated alkenones. Amongst these products, the (ω -1)- and (ω -2)-hydroxyacids formed after NaBH₄ reduction of the (ω -1)- and (ω -2)-ketoxyacids were selected as potential biomarkers for alkenone autoxidation. Re-examination of lipid extracts of post-bloom seawater particulate matter samples from the DYFAMED station in the Ligurian Sea (where strong autoxidative alteration of the lipid distributions had previously been detected) showed the presence of significant amounts of 12-hydroxytetradecanoic, 13-hydroxytetradecanoic, 14-hydroxyhexadecanoic and 15-hydroxyhexadecanoic acids thus providing good evidence that these autoxidative processes occur in natural samples.

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Keywords: Alkenones; Autoxidation products; Heterolytic and homolytic cleavages; Markers; (omega-1) and (omega-2)-hydroxyacids

1. Introduction

Di-, tri- and tetraunsaturated long-chain alkenones (*n*-C₃₇–C₃₉) are biosynthesized by a very few species of

phytoplankton, including the cosmopolitan coccolithophorid *Emiliana huxleyi* and the subtropical *Gephyrocapsa oceanica* in the open ocean (Conte et al., 1994, 1998; Volkman et al., 1980, 1995), and some other members of the Haptophyceae such as *Isochrysis galbana* and *Chrysolita lamellosa* (Marlowe et al., 1984; Patterson et al., 1994; Rontani et al., 2004) in

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