Physiological Status and Community Composition of Microbial Mats of the Ebro Delta, Spain, by Signature Lipid Biomarkers

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A B S T R A C T

Physiological status of microbial mats of the Ebro Delta (Tarragona, Spain) based on the extraction of lipids considered "signature lipid biomarkers" (SLB) from the cell membranes and walls of microorganisms has been analyzed. Data from a day–night cycle show significant differences in viable cells countings (PLFA cells counts) ranging from 1.5×10^{10} to 5.0×10^{10} cells g^{-1} of sediment. Minimum values were observed at 18:00 and 6:00, when physicochemical conditions change drastically. The diversity of the microbial community was assessed by GC/MS analysis of phospholipid fatty acids (PLFA). The ratio of PLFA, representative of Gram-negative bacteria, comprises 47.8% of the total PLFA of the microbial mat community. The remaining PLFA was representative of Gram-positive (10.0%), anaerobic (5.7%), and eukaryotic microorganisms (5.7%), and other common lipids. Two different approaches were used as a comparative study to assess the physiological status of the microbial mats. Two parameters (cyclopropane fatty acids/ω7c monoenoic fatty acids, and measurement of the *trans/cis* monoenoic PLFA ratio) showed a minimum at midnight, suggesting the highest microbial activity. Higher values were observed at 18:00 and 6:00, coinciding with lower PLFA cell counts.

Introduction

The microbial community is an essential element in the trophodynamics of detrital and benthic ecosystems [10]. Changes in the structure and function relationships in this vital community have proven to be difficult to measure by

methods that require isolation of the microorganisms from their microenvironment. Moreover, these approaches do not allow quantitative measurements [9]. These disruptive methods are suitable to estimate neither the total abundance nor the community composition within environmental samples. Unfortunately, morphology gives no indication of the function or of the state of metabolic activity for most of the major bacterial components of the community. Besides, viable or direct counts of bacteria attached to sediment

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grains are difficult to quantify, and so they could greatly underestimate the cell number and community composition [1, 5].

A microbial mat is a community composed primarily of different populations of bacteria, which form thin horizontal layers. These layers have active growth and can be several millimeters to a few centimeters thick. These layers develop along microgradients established at fluid interfaces (usually water, but occasionally vapor) on solid substrata. A steep redox gradient at the interface is established. Consequently, depletion of oxygen below the mat surface coincides with increases of sulfide (and often methane) with depth. Microbial mats developing in shallow waters are composed primarily of oxygenic cyanobacteria (in the upper layers), purple and green sulfur bacteria (in the middle layers), and sulfate-reducing bacteria (at the bottom). We also observed a population of heterotrophic and fermentative microorganisms and sulfur oxidizers that do not form stable layers and do not have a defined position in the mat. Methanogenic bacteria have also been detected in some microbial mats [26]. Some eukaryotic organisms, such as the amoebamastigote *Paratetramitus jugosus* and the ciliate *Pseudocohnilembus pusillus* [25], are associated with microbial mats. Microbial mats can be found in different locations in the Iberian Peninsula [23]. However, those found in La Banya spit, at the south end of the Ebro Delta [16], are probably the most extensive, stable, well developed, and morphologically varied. Chemical analysis of sediments can indicate function or metabolic activity in prokaryotes, as morphology cannot [3, 33]. Lipids, especially fatty acids, have proven to be of great value in the understanding of phylogenetic and taxonomic classifications [20, 21, 30, 34]. The lipid analysis has been applied to assess microbial communities (bacteria, fungi, protozoa, and metazoa) in mud, soil, rhizosphere, sediments, and bioreactors [32, 34]. The signature lipid biomarkers (SLB) analysis provides a quantitative means of measuring viable microorganisms [1, 11, 13], microbial community composition [13, 34], and community nutritional/physiological status [15, 31]. Extractable phospholipids have been proposed as indicators of microbial abundance. Unlike most other biomarkers, phospholipids are quickly degraded (ranging from minutes to a few hours after cell death) [35]. This rapid degradation of phospholipids to diglycerides makes phospholipid fatty acids excellent biomarkers for viable cells.

Different groups of microorganisms synthesize a variety of PLFAs through various biochemical pathways [36]. This makes PLFAs effective taxonomic markers, useful to define community composition. However, there is an overlap in PLFA composition between different species, and as such, it is not possible to define each species with a unique pattern of PLFA. Only approximately 1–5% of the organisms in the total sedimentary community have actually been isolated and have had their PLFAs analyzed [36].

The aim of this study is to characterize the microbial community of the mats in La Banya spit of the Ebro Delta by determining its microbial composition, physiological status, and day–night variations. Two different approaches to assess the physiological status of the microbial mats have been compared: determination of the formation of cyclopropane fatty acids from their monoenoic homologues, and measurement of the ratio of *trans/cis* monoenoic PLFA.

Methods

Microbial Mats: Site and Sampling

The microbial mats studied are located in the coastal area of the Ebro Delta (Tarragona, Spain) (40°40' N, 0°40' E). The general features of this natural microbial community in terms of species composition, population dynamics, and physicochemical characterization have been described earlier [16, 23]. Sediment samples were taken during the spring of 1997, over a day–night cycle. The upper 3 cm of the mat was sampled (three cores at each time) every 6 hours starting at midday (12:00, 18:00, 24:00, 6:00, and 12:00 of the following day) using a 5 cm inner-diameter methacrylate corer. Samples were frozen in liquid nitrogen, transported to the laboratory within 2 h after collection, and lyophilized in a Virtis apparatus (at −55°C and 200 mtorr).

Physicochemical conditions of the overlying water (1 cm from the sediment–water interface) were analyzed at each sampling time. A CRISON OXI-92 oxymeter was used to measure both temperature and oxygen. A LICOR LI-189 meter was used to measure solar irradiation. Sulfide concentration was measured by the Pachmayr colorimetric method [27], according to the following protocol. One ml of the water sample was added *in situ* to a 125 ml bottle which contains 20 ml of zinc acetate solution (2% w/v) and transported to the laboratory. Once in the laboratory, distilled water was added to around 80 ml. Afterwards 10 ml of dimethylparaphenylene diaminesulfate (DPDS) (0.2% w/v) solution and 0.5 ml of ferric ammonium sulfate (10% w/v) was added to the bottle. The mixture was shaken vigorously, and distilled water was added to a final volume of 100 ml. Absorbance at 663 nm was measured after 15 min of incubation. Sulfide concentration was calculated by the following equation:

$$(Abs - Abs_{white}) \times 0.027 \times 100/V_{sample} \text{ (in ml)}$$
$$= \text{Sulfide concentration (in mM)}$$

Lipid Analysis

Each lyophilized sample was detached to process the main microbial populations of the community. Samples were pulverized separately using sterile tools in order to keep background levels of PLFA as low as possible. All solvents used were GC grade and were obtained from Fisher Scientific (Pittsburgh, PA). In addition, all glassware was thoroughly cleaned and heated in a muffle furnace at 450°C for at least 4 h prior to use. Microbial mat samples (three replicates of 0.3 g of dry weight of each core) were extracted using a modified Bligh and Dyer procedure as described by White and collaborators in 1979 [33]. The total lipids obtained were then fractionated into glyco-, neutral-, and polar lipids [14]. The polar phospholipid fatty acids were then transesterified into methyl esters [14].

Gas chromatography (GC) analysis of fatty acid methyl esters was carried out on a Hewlett-Packard 5890 Series II GC with a flame ionization detector. The GC was programmed from an initial temperature of 60°C to 280°C, at 10°C per min, and then held at this temperature for 3 min. The helium carrier gas flow rate was 1.5 ml min^{-1} at 0.92 kg cm^{-2}. The injection port was at 270°C and the detector at 290°C.

Gas chromatography/mass spectrometry (GC/MS) analysis of fatty acid methyl esters was carried out on a Hewlett-Packard 5890 Series II GC interfaced with a HP 5973 Series mass selective detector (Hewlett-Packard, Wilmington, DE) [35]. Electron impact mass spectrometry was performed at 70 eV. The ion source was held at 200°C, with the electron multiplier voltage at 3,000 V.

Fatty acids were identified by relative retention times, by comparison with authentic standards (Matreya Inc., Pleasant Gap, PA) and by the mass spectral analysis. Fatty acid nomenclature is in the form of "A:BωC" where "A" designates the total number of carbons, "B" the number of double bonds, and "C" the distance of the closest unsaturation from the aliphatic end of the molecule. The suffixes "c" for *cis* and "t" for *trans* refer to geometric isomers. The prefixes "i", "a," and "me" refer to iso and anteiso methyl branching, and midchain methyl branching, respectively, with cyclopropyl rings indicated by "cy" [19].

Cell Number Estimation

A conversion factor was used to transform quantities of PLFA obtained into numbers of cells. It was assumed that there were 2.5 × 10^{12} cells per gram of dry weight, and 10^8 pmol PLFA per gram of dry weight [1]. This yields 2.5 × 10^4 cells per pmol of PLFA. Note that with any conversion factor, the number of cells can vary by up to one order of magnitude [12].

Statistics

Results were analyzed using correlation statistics and analysis of variance (ANOVA). These analyses were applied to determine significant differences between samples through time, and possible relationships between PLFA profiles. All statistical analyses were performed using Microsoft Excel 5.0 for Macintosh (Microsoft Corporation, 1985–1994).

Results
External Conditions

Starting at 12:00, solar light, measured on the sampling point, decreased reaching a minimum value at 24:00 (Fig. 1). Oxygenic photosynthesis decreased as the net oxygen concentration decreased. Temperature also decreased because of the lack of solar light. Sulfide was first detected at 24:00 and was at its maximum (30 μM) at 6:00. Sulfide production during the day was neutralized by the spontaneous reaction with dissolved oxygen and by consumption by anoxygenic photosynthetic bacteria. The following morning temperature and light intensity values rose again to a maximum of 25°C and 2100 μE m^{-2} s^{-1}, respectively. Dissolved oxygen concentration also increased the morning after and sulfide decreased to undetectable limits.

Cell Number Estimates and Microbial Community Composition

Viable cell estimates are given as the number of cells estimated from PLFA data (Fig. 2). They ranged from 1.5 × 10^{10} to 5.0 × 10^{10} cells g^{-1} dry weight. Significant differences were detected at the 6-hour sampling intervals. Maximum cell number values (5.0 × 10^{10} cells g^{-1} dry weight) were observed at 12:00 and 24:00. Minimum values were observed at 6:00 and 18:00 (1.0–1.5 × 10^{10} cells g^{-1} dry weight).

Characterization of the microbial mats using PLFA analysis indicated the presence of a diverse microbial community (Table 1). The community consisted primarily of gram-negative bacteria (including cyanobacteria), as evidenced by the presence of monoenoic PLFAs [37], which comprised 47.8% ± 3.8% of the total PLFA. PLFAs typical of Gram-positive bacteria (terminally branched saturated fatty acids) [24] comprised 10.0% ± 2.6% of the total PLFAs; however, terminally branched saturated fatty acids have also been associated with the presence of gram-negative anaerobic bacteria [37]. Lipids representative of anaerobic microorganisms, including the branched monosaturates and midchain branched saturates [7, 8], comprised 5.7% ± 1.6% of the total PLFAs. Lipids representative of sulfate-reducing bacteria comprised 22.2% ± 3% of the total lipids of anaerobic microorganisms. Although biomarkers indicative of anaerobic microbiota dropped to minimum levels by 24:00, relative proportions of the PLFA biomarkers remained relatively constant during the day–night cycle. Relative proportions of
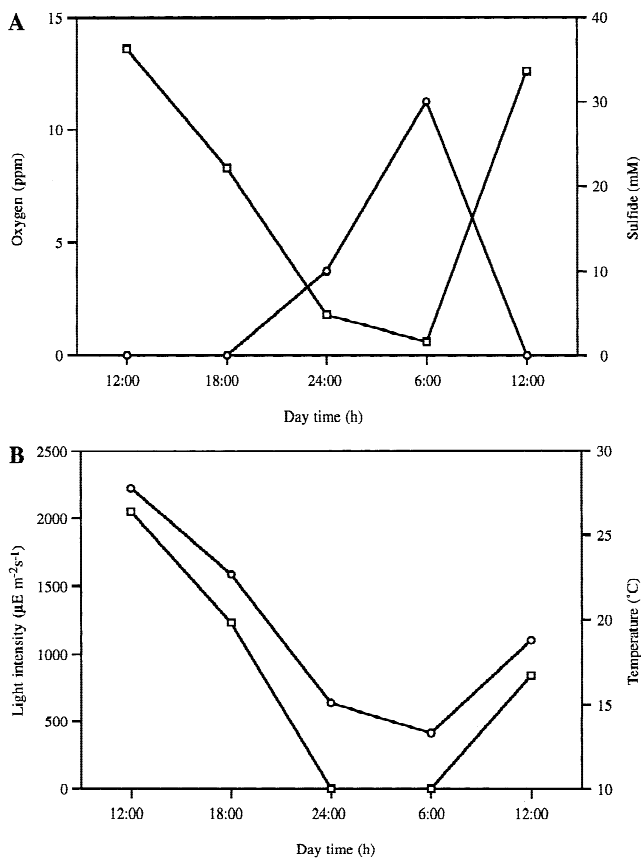
Fig. 1. Physicochemical conditions in the overlying water of microbial mats during day/night cycle. (A) —□— oxygen, —○— hydrogen sulfide; (B) —□— light intensity, —○— temperature.

polysaturated fatty acids, representative of cyanobacteria [4] as well as eukaryotic microorganisms, were constant (5.7% ± 1.5%) at all sampling times except at 24:00 (8.1%). Normal saturated fatty acids (26.4%) are present in both eukaryota and prokaryota and therefore cannot be used in community structure analysis.

Physiological Status of Microbial Mats

The knowledge of specific lipid biosynthetic pathways can provide insight into the physiological or nutritional status (bioavailability of elements, such as carbon and nitrogen) of the microbial community. Certain fatty acids can be used as indicators of environmental stress. Exposures to toxic environments can lead to minicell formations and a relative increase in trans-monoenoic PLFAs when compared with the cis homologues. Other PLFA patterns also change in response to environmental stress. Prolonged exposure to conditions inducing stationary growth phase induce the forma-

tion of cyclopropane PLFA. Ratios of cyclopropane fatty acids to their monoenoic homologues show an increase in response to changes of environmental conditions [15, 18, 31, 35]. The monoenoic fatty acids (16:1ω7c and 18:1ω7c) are converted to cyclopropyl (cy 17:0 and cy 19:0). This change is expressed in two ratios (cy 17:0/16:1ω7c and cy 19:0/18:1ω7c) and represents the shift to conditions which slow down the growth rate. Monoenoic PLFA to cyclopropane PLFA rates of the samples are shown in Fig. 3. The minimum reading was detected in samples taken at 24:00. Maximum values, indicating the slowest growth rates, were observed at 6:00 and 18:00 in the transient phase between day and night. Metabolic stress of the sampled microbial mats, expressed as a trans/cis monoenoic PLFA ratio, is shown in Fig. 4. Again the lowest value was observed at 24:00, which indicates a low level of stress on the microbial community at that time. Ratios ranged from 0.055 to 0.08.

Discussion

External Conditions

Physicochemical conditions within the mat (Fig. 1) were derived from the complex microbial activity in this kind of community. The main microbial metabolic activities detected are oxygenic and anoxic photosynthesis and sulfate reduction. Therefore, in order to gain a better understanding of the functioning of microbial mats, a knowledge of their physiological state is crucial.

Cell Number Estimates and Microbial Community Composition

As in any ecological system, microbial mat dynamics is the result of the influence of the different factors that interact in
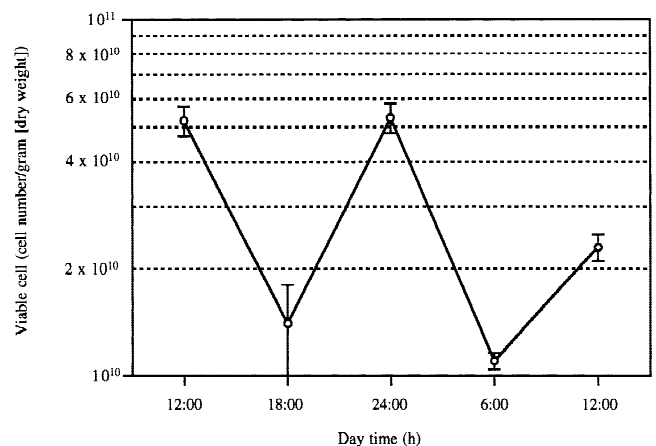

Fig. 2. Viable cell numbers obtained from PLFA direct determination.

Table 1. Community composition approach (percentage of lipid biomarkers during a day/night cycle)

PLFA biomarkers	Group of organisms	Day time (h)				
		12:00	18:00	24:00	6:00	12:00
Normal saturates	All genera	27.08 ± 0.41	23.67 ± 0.14	28.45 ± 0.60	24.49 ± 0.37	28.78 ± 0.24
Monoenoics	Gram negatives	53.36 ± 0.27	47.53 ± 0.57	48.62 ± 0.50	47.04 ± 0.37	42.58 ± 0.68
Terminally branched saturates	Gram positives	6.79 ± 0.21	12.36 ± 0.33	7.91 ± 0.16	10.35 ± 0.55	12.57 ± 0.58
Midchain branched saturates	Anaerobes	2.89 ± 0.06	3.51 ± 0.18	3.59 ± 0.02	5.37 ± 0.17	4.88 ± 0.17
Branched monoenoics	Anaerobes	1.26 ± 0.03	2.09 ± 0.07	1.43 ± 0.04	1.64 ± 0.08	1.84 ± 0.05
Polyenoics	Microeukaryotes	6.25 ± 0.42	4.65 ± 0.10	8.10 ± 0.30	4.24 ± 0.28	5.24 ± 0.30
Other lipids		2.13 ± 0.10	5.55 ± 0.06	1.66 ± 0.13	6.70 ± 0.20	3.63 ± 0.21

its habitat. The development of the mat is ruled by the functioning of the many different microbial populations (diatoms, cyanobacteria, purple and green sulfur bacteria, sulfate reducers, heterotrophs, methanogens, etc.) that co-exist within this complex system. Each of these populations has its own growth and death rates, which depend on the physicochemical conditions of the habitat.

A major problem when measuring viable cells using biochemical techniques is the fact that the results are in micromoles of component per gram of sediment or soil. A simple conversion could be done by determining the biomarker content of monocultured cells and then counting the cells to determine a value per cell [2]. Microbial mat sediment samples were shown to contain sparse prokaryote communities of minicells (observed microscopically). The viable cell number determined by PLFA was equivalent (with a smaller standard deviation) to that estimated by intracellular ATP, cell wall muramic acid, and AODC measurements [1]. It was assumed that there were 2.5×10^{12} cells per gram of dry weight, and that 1 g (dry weight) of cells [1] was equivalent to 10^8 pmol PLFA, which means that 1 pmol of PLFA is equivalent to 2.5×10^4 cells [12].

To determine the community composition by using a signature lipid approach, it is crucial to consider the environment from which the sample was retrieved when interpreting the results. The analysis of SLB by GC/MS provides sufficient information to detect broad subsets of the microbial community that allow quantitative definition of the community. Community composition can be characterized from the pattern and types of PLFA identified in samples [14]. Monoenoic PLFA are found in almost all Gram-negative microorganisms and many types of eukaryotic microorganisms. The ω9 monoenoic PLFAs are formed from the aerobic desaturase pathway common to all cells, whereas the ω7 PLFAs are formed from the anaerobic desaturase pathway, which is often a prokaryotic biochemical pathway

[32]. Branched-chain monoenoic PLFAs are common in the anaerobic *Desulfovibrio*-type sulfate reducing bacteria both in culture and in manipulated sediments [8, 28]. They are also found in certain actinomycetes, which, as a group, contain midchain branched saturated PLFAs, in particular 10Me18:0, with lower amounts of 10 other methyl-branched homologues.

Environments with a large ratio of 10Me16:0 often feature anaerobic Gram-negative *Desulfobacter*-type sulfate reducing bacteria [7, 28]. Polysaturated fatty acids indicate the presence of cyanobacteria and eukaryotic microorganisms [5]. Polyenoic PLFAs have also been sparingly reported in some bacteria [36]. Polyenoic PLFAs with the first unsaturation in the ω6 position are classically considered to be of animal origin, whereas organisms with the first unsaturation in the ω3 position are generally considered to be of either plant or algal origin. Normal saturated PLFA longer than 20 carbons are typical of the microeukaryotes.

Physiological Status of Natural Communities

The monoenoic PLFAs 16:1ω7c and 18:1ω7c are increasingly converted to the cyclopropyl fatty acids cy17:0 and cy19:0, respectively, in Gram-negative bacteria as the microbes move from a logarithmic to a stationary phase of growth. This ratio (growth without cell division) varies from organism to organism and from environment to environment, but usually falls within the range of 0.05 (log phase) to 2.5 or higher (stationary phase) [22]. Slow growth can occur in microbes when carbon source(s) and terminal electron acceptors are present but some essential nutrients are not available. Under these conditions of "unbalanced growth," bacterial can accumulate biopolymers (such as poly-β-hydroxyalkanoates [6]). An increase in cyclopropyl PLFA formation has also been associated with an increased anaerobic metabolism in facultative heterotrophic bacteria in monoculture studies [32].
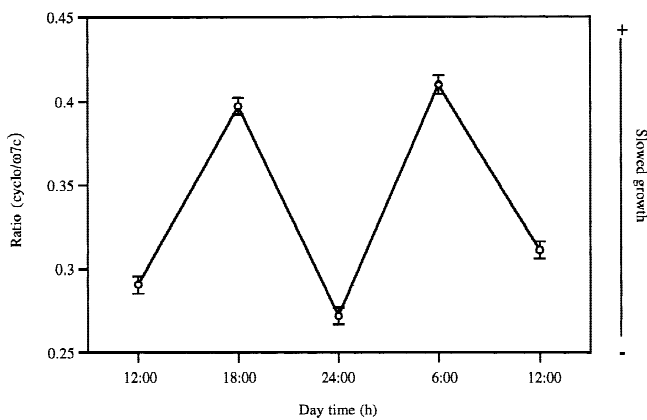
Fig. 3. Metabolic status expressed by relationship between cyclopropyl fatty acids and monoenoic PLFAs.

Bacteria produce *trans*-monounsaturated fatty acids as a result of metabolic stress (e.g., toxicity, starvation) [17]. In addition, *trans/cis* ratios higher than 0.1 indicate starvation in bacterial isolates. This value is usually 0.05 or less in healthy, nonstressed populations.

The 18:00 time point showed an increase of metabolic stress (Fig. 4), and a shift to slow growth was observed. At 6:00 another inflection point in the viable cells (PLFA cell counts) was detected, which coincided with an increase in the metabolic stress and in the anaerobic bacteria proportion (Table 1). During the day, oxygenic and anoxygenic photosynthesis predominates over aerobic respiration and sulfate-reduction activity in the microbial mat ecosystem. Excess carbon assimilates which were not used for growth accumulated in the cells as biopolymers (stress observed at 18:00). At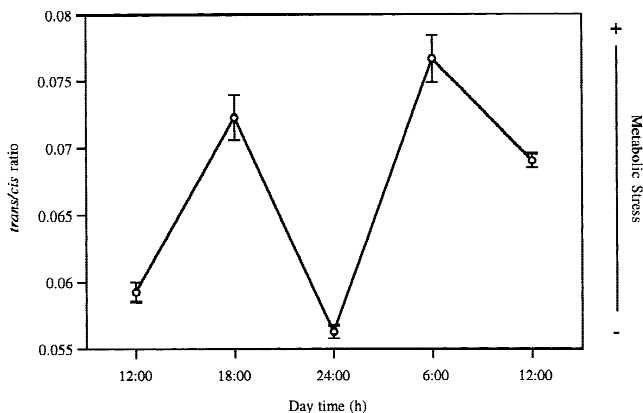 the end of the day, when the light intensity decreases drastically, and also during the night, the respiratory and sulfate reduction activity predominate because the photosynthesis is stopped. Anoxic conditions during the night may also trigger the development of sulfate reducing bacteria which may become very active using the organic matter (reduced products) generated during the day as electron donors for sulfate reduction. In these conditions, sulfide concentration increases and oxygen and readily assimilated organic carbon are quickly depleted inside the mat (stress maximum observed at 6:00).

Sensitive analytical procedures have been developed for the quantitative study of natural microbial communities *in situ*. Although these methods cannot answer all experimental questions regarding natural microbial communities, they do provide a good set of tools for studying microbes and microbial communities. These methods can provide quantitative data on the status and role of these main ecosystem components.

The biochemical assays described in this study estimate the microbial abundance without any need for microbial growth or for the quantitative recovery of microbes from surfaces on which they grow. These methods of signature analysis are proving to be helpful in determining the structure of microbial communities. By correlating measurements of nutritional status with metabolic activity, this type of biochemical analysis can also help to unravel problems associated with the accurate estimation of true *in situ* microbial activity.

The application of sensitive analytical methods to natural microbial communities is just beginning. As methods improve both in sensitivity and in selectivity and the catalogues of signature lipids increase, a deeper insight of the complex microbial communities that drive the biogeochemical cycles of this planet will be possible.

Acknowledgments

Fig. 4. Metabolic status expressed by relationship between *trans/cis* conformations of monoenoic PLFAs.

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