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Combined Phospholipid Biomarker-16S rRNA Gene Denaturing Gradient Gel Electrophoresis Analysis of Bacterial Diversity and Physiological Status in an Intertidal Microbial Mat

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A combined lipid biomarker-16S rRNA gene denaturing gradient gel electrophoresis analysis was used to monitor changes in the physiological status, biomass, and microbial composition of a microbial mat. In the morning hours, an increase in the biomass of layers containing a high density of phototrophs and a decrease in the growth rate in the deep layers were observed. The combined approach also revealed differences in major groups of microorganisms, including green nonsulfur, gram-positive, and heterotrophic bacteria.

Photosynthetic microbial mats are sedimentary structures composed of different populations of bacteria distributed in multilayered communities (2, 10, 21, 22). The use of signature lipid biomarker analysis in microbial ecology studies provides an estimate of the viable microbial biomass (25) and physiological status of the microbial community, since phospholipid fatty acids (PLFAs) reflect the phenotypic response of microorganisms to environmental conditions. Despite its versatility, PLFA analysis has limited application to the analysis of gram-negative bacteria (28). To overcome this, PLFA studies have been complemented by nucleic acid-based analyses (15, 24). In the present report, differences in the metabolic status and microbial diversity of estuarine microbial mats were monitored by means of a combined lipid-nucleic acid approach.

Mat samples were taken from the Camargue (Rhone Delta, France) in April 2002 at two times during the day, 8:00 a.m. Greenwich mean time (GMT) (A samples) and 3:00 p.m. GMT (B samples). Each sample was cut by microtomy into layers 50 μm thick, and 10 cuts were grouped to form each sample group (from sample groups 1 to 15; group 16 contained 25 slices 50 μm thick). Samples were extracted with the single-phase chloroform-methanol-buffer system of Bligh and Dyer (1), as modified by White et al. (26, 27). The total lipid extract was fractionated by silicic acid chromatography, and the polar lipid fraction was transesterified to fatty acid methyl esters (8, 18). Nucleic acid was precipitated from the PLFA aqueous phase of the total lipid extraction (13). PCR amplification of 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) were carried out as described by Muyzer et al. (17). Excised DGGE bands served as templates in PCRs as noted above, and the purified PCR products were sequenced. Amplification products that failed to directly generate legible sequences were cloned into the pGEM-T Easy System II (Promega, Madison, Wis.) cloning vector according to the manufacturer's instructions.

Maximum viable biomass as measured by total PLFAs (Fig. 1) accumulated at the top of the mat early in the day; however, in the afternoon, the highest levels of biomass were found underlying the uppermost layers.

An increase in the concentration of cyclopropanoic fatty acids represents the shift to conditions that slow down the growth rate (23). In addition, gram-negative communities make *trans*-monoenoic fatty acids in response to changes in their environment (9, 11). In the morning hours, the reduction in the growth rate (the ratio of cyclopropyl fatty acids to monoenoic PLFAs) increased with depth (Fig. 2A), whereas in the afternoon, the slowest growth was detected at the top and in the middle of the mat. Data concerning metabolic stress (Fig. 2B) indicated maximum stress at the topmost layers in the morning; however, the ratio of *trans* to *cis* monoenoic PLFAs increased at the bottom of the mat in the afternoon.

In the morning (Fig. 3A), the microbial community consisted mainly of gram-negative bacteria, as indicated by the presence of monoenoic PLFAs (30). Terminal branched saturated fatty acids (characteristic of gram-positive bacteria) comprised a high proportion of the total PLFA in the middle layers of the biomat and from samples at the deepest layers (maximum 24.6%). Branched monoenoics and mid-chain branched saturated fatty acids, representative of anaerobic microorganisms (3), were constant along the vertical profile. In the afternoon (Fig. 3B), the proportion of anaerobic microorganisms was higher and increased with depth. The prominent DGGE bands were sequenced, and their corresponding vertical positions and phylogenetic affiliations are shown in Fig. 4 and Table 1, respectively.

Determining the viable biomass of a microbial community provides an estimate of the amount of active microorganisms and their capacity for metabolic transformation (25). The maximum viable biomass (Fig. 1) observed in the morning hours

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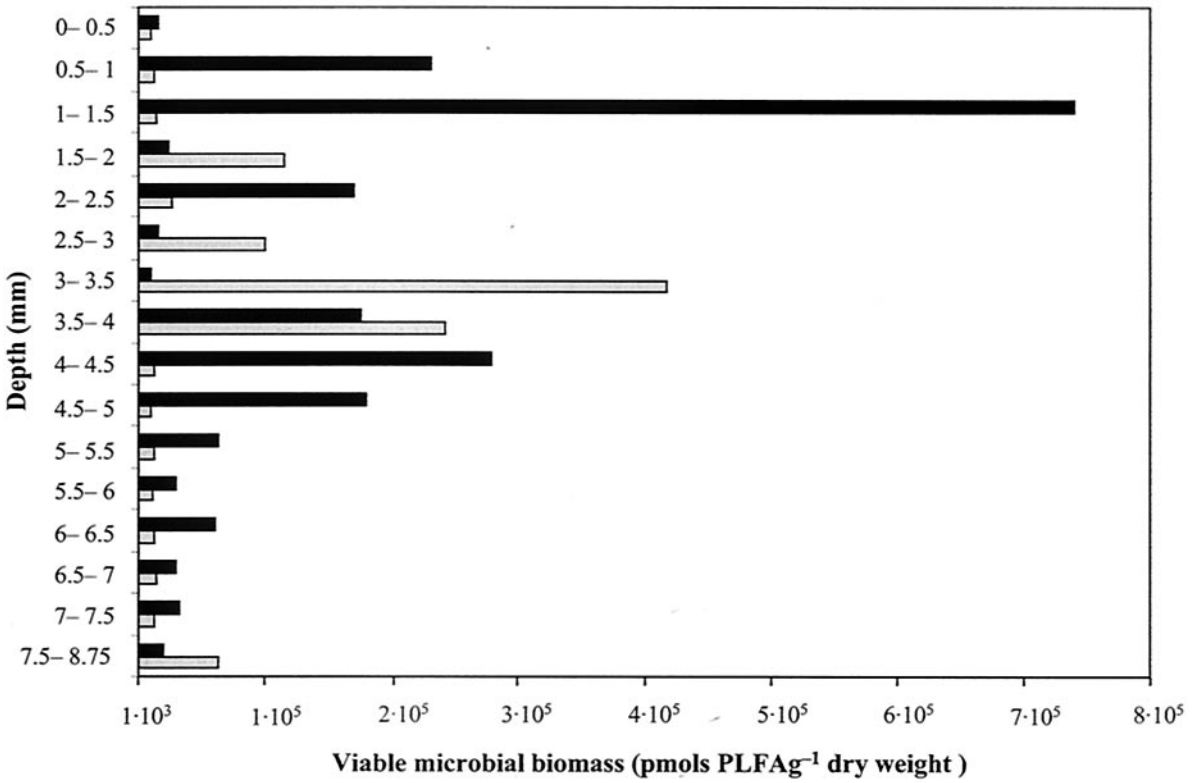

FIG. 1. Viable microbial biomass as measured by total PLFA, expressed as picomoles of PLFAs per gram (dry weight). Determination of the total PLFA provides a quantitative measure of the viable or potentially viable biomass. Viable microorganisms have an intact membrane that contains phospholipids (and PLFAs) which are turned over rapidly after cell death by means of cellular enzymes that hydrolyze the phosphate group from phospholipids, resulting in formation of diacylglycerols. ■, A samples, collected at 8:00 a.m. GMT; ▨, B samples, collected at 3:00 p.m. GMT.

might be explained by the migration (i) of cyanobacteria towards the top of the mat in order to avoid toxic exposure to sulfide (produced by sulfate-reducing bacteria and accumulated during the night) and (ii) of purple sulfur bacteria, which use the light in the early morning to begin photosynthetic processes. In fact, recent studies have reported an increase in bacterial biomass in layers containing a high density of phototrophs (6, 7, 14). The slow growth rate at the bottom of the mat (Fig. 2A) might be due to an increase in the activity of sulfate-reducing bacteria, especially at 8:00 a.m. In fact, during the night, anoxic conditions trigger the development of this population, which uses organic matter and produces sulfide. Limiting amounts of organic carbon (low C/N rate) may account for the slow growth of the sulfate reducers. The reduction in the growth rate at the top and in the middle of the mat at 3:00 p.m. (Fig. 2A) can be explained by photosynthesis and carbon fixation carried out by cyanobacteria and purple sulfur bacteria, resulting in the generation of abundant organic compounds and limiting amounts of nitrogen (high C/N rate). The exposure of cyanobacteria to sulfide (accumulated during the night) most likely accounts for the high degree of metabolic stress observed at the topmost layer in the morning. In this layer, a major contribution of aerobic heterotrophic bacteria during the morning hours has been reported. Indeed, previous studies provided evidence of cross-feeding of heterotrophs by excretion of photosynthates in microbial mats (4, 5, 12).

It is noteworthy that the DGGE band pattern showed high similarity to that of genus *Halanaerobium*. These data are consistent with the PLFA community composition analysis at 8:00 a.m. (Fig. 3A), since PLFAs of gram-positive bacteria were predominant in middle and deep layers of the microbial mat. PLFAs representative of gram-negative bacteria were dominant between a depth of 2.5 and 3.5 mm at 8:00 a.m. (Fig. 3A). These results are in agreement with the DGGE pattern, which showed a predominance of bands related to green nonsulfur bacteria (19, 20). A higher proportion of polyenoic fatty acids (indicative of microeukaryotes and cyanobacteria) in the afternoon was consistent with results of previous studies (16) in which an increase in the eukaryotic population in the anoxic region was reported. Finally, the detection at both sampling times of bands homologous to those from gamma-proteobacteria and purple nonsulfur bacteria supports recent findings for Orkney Island microbial mats (29).

The data provide a complete picture of the response of microbial mats to physicochemical variables influenced by time and depth, since ecological processes affect the cycling of nutrients at a community level. Changes in metabolic status can be attributed mainly to the activity of phototrophs, which in-

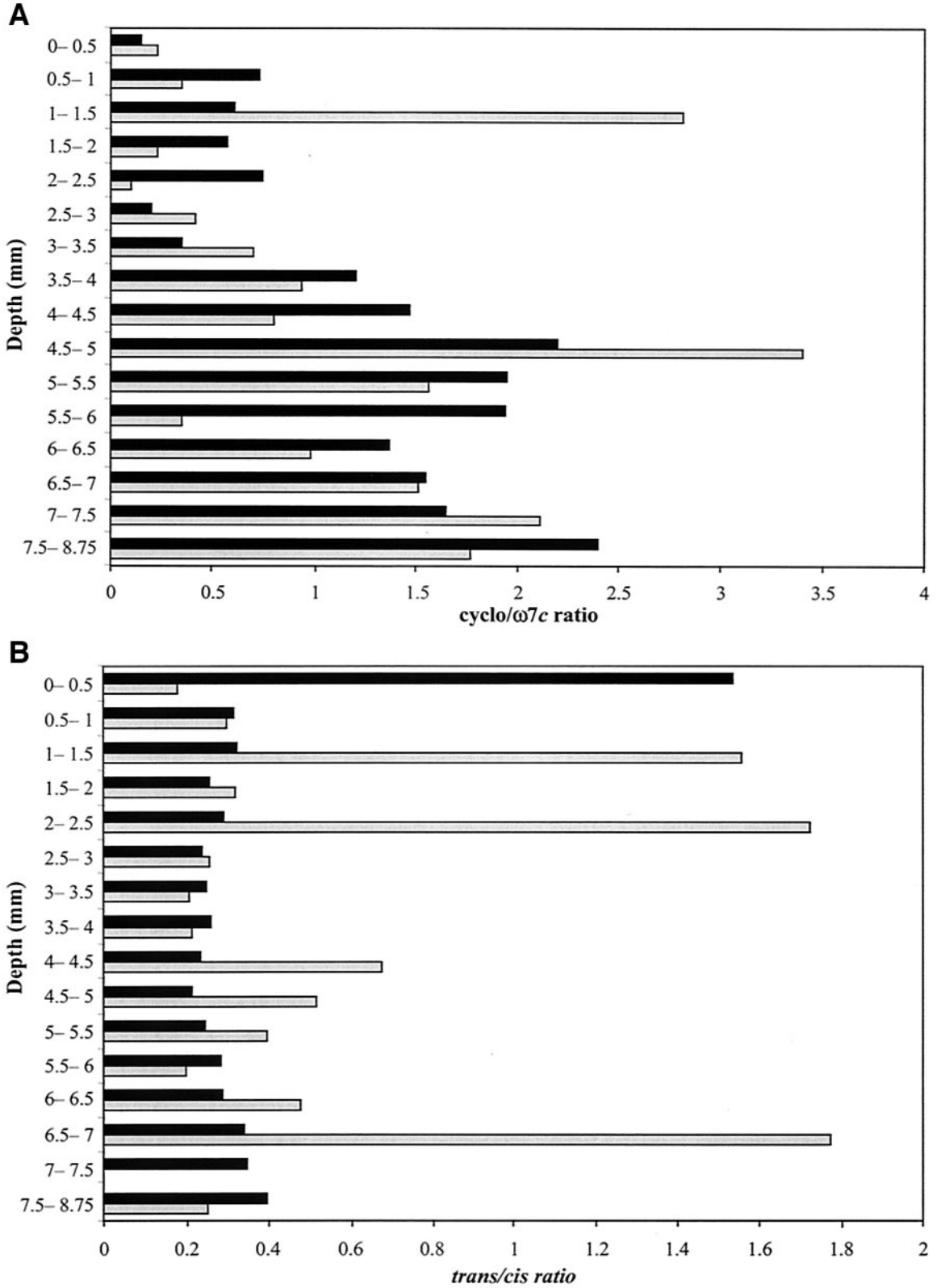

FIG. 2. (A) Metabolic status or starvation index as the ratio of cyclopropyl fatty acids to monoenoic PLFAs (cyclo/ω7*c* ratio). An increase in the concentration of cyclopropanoic fatty acids represents the shift to conditions that slow down the growth rate. This ratio ranges from 0.05 (exponential phase) to 2.5 or higher (stationary phase) in gram-negative bacteria. (B) Metabolic stress expressed as the *trans*/*cis* ratio of monoenoic PLFAs. Gram-negative bacterial communities make *trans*-monoenoic fatty acids in response to changes in their environment (exposure to solvent, toxic metals, or starvation). *Trans*/*cis* ratios greater than 0.1 indicate starvation in bacterial isolates, while ratios of 0.05 or lower are found in nonstressed microbial populations. ■, A samples, collected at 8:00 a.m. GMT; ▨, B samples, collected at 3:00 p.m. GMT.

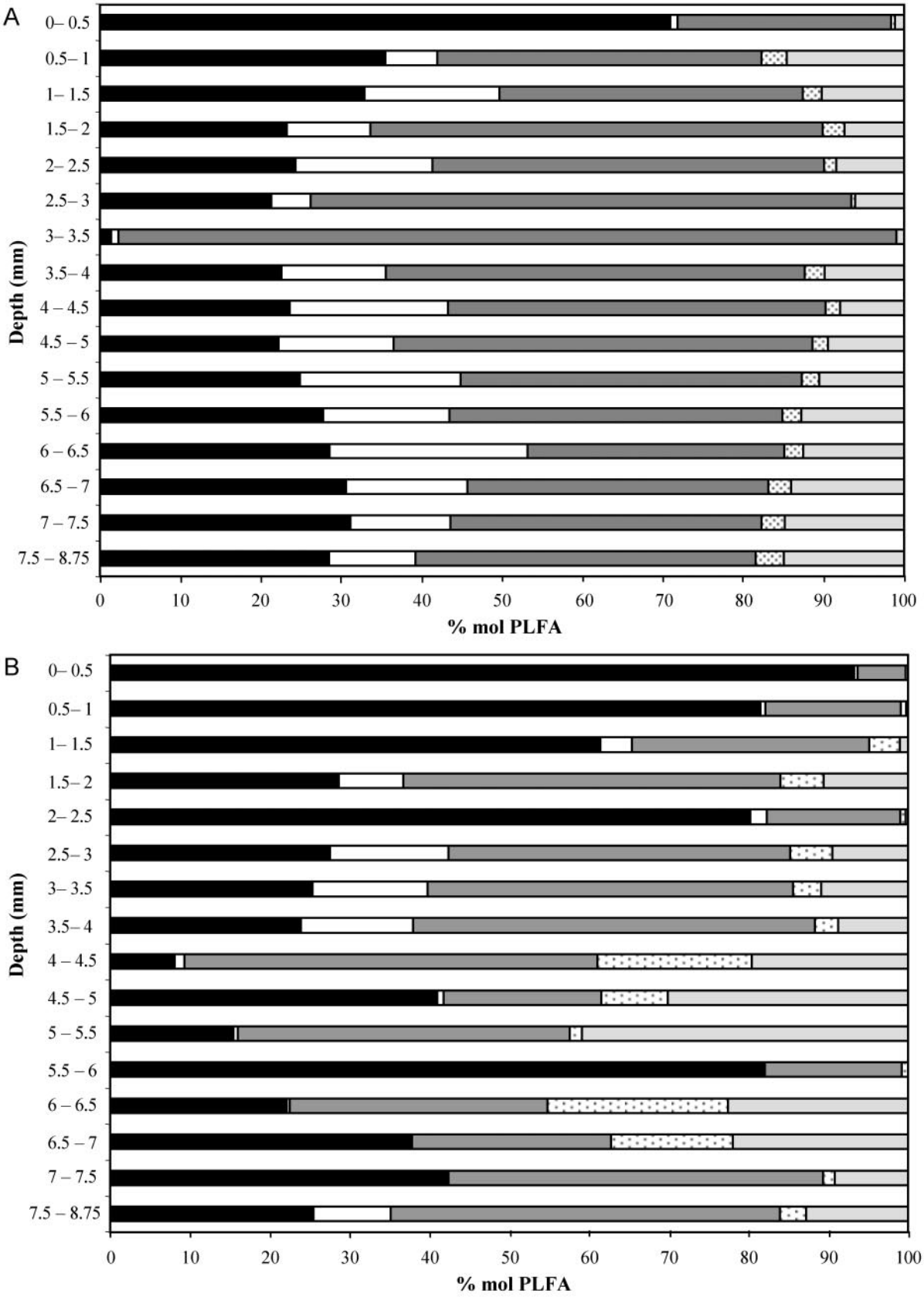

FIG. 3. Community composition expressed as moles percent PLFA. (A) Samples taken at 8:00 a.m. GMT; (B) samples taken at 3:00 p.m. GMT. The presence of certain groups of microorganisms can be inferred by the detection of unique lipids. For example, specific PLFAs are prominent in microbial groups as follows: as normal saturated PLFAs (all genera) (black bars), as terminal branched saturated PLFAs (gram-positive bacteria) (white bars), as monoenoic PLFAs (gram-negative bacteria) (dark-gray bars), as polyenoic PLFAs (microeukaryotes) (dotted bars), and as branched monoenoic and mid-branched saturated PLFAs (anaerobic microorganisms) (light-gray bars).

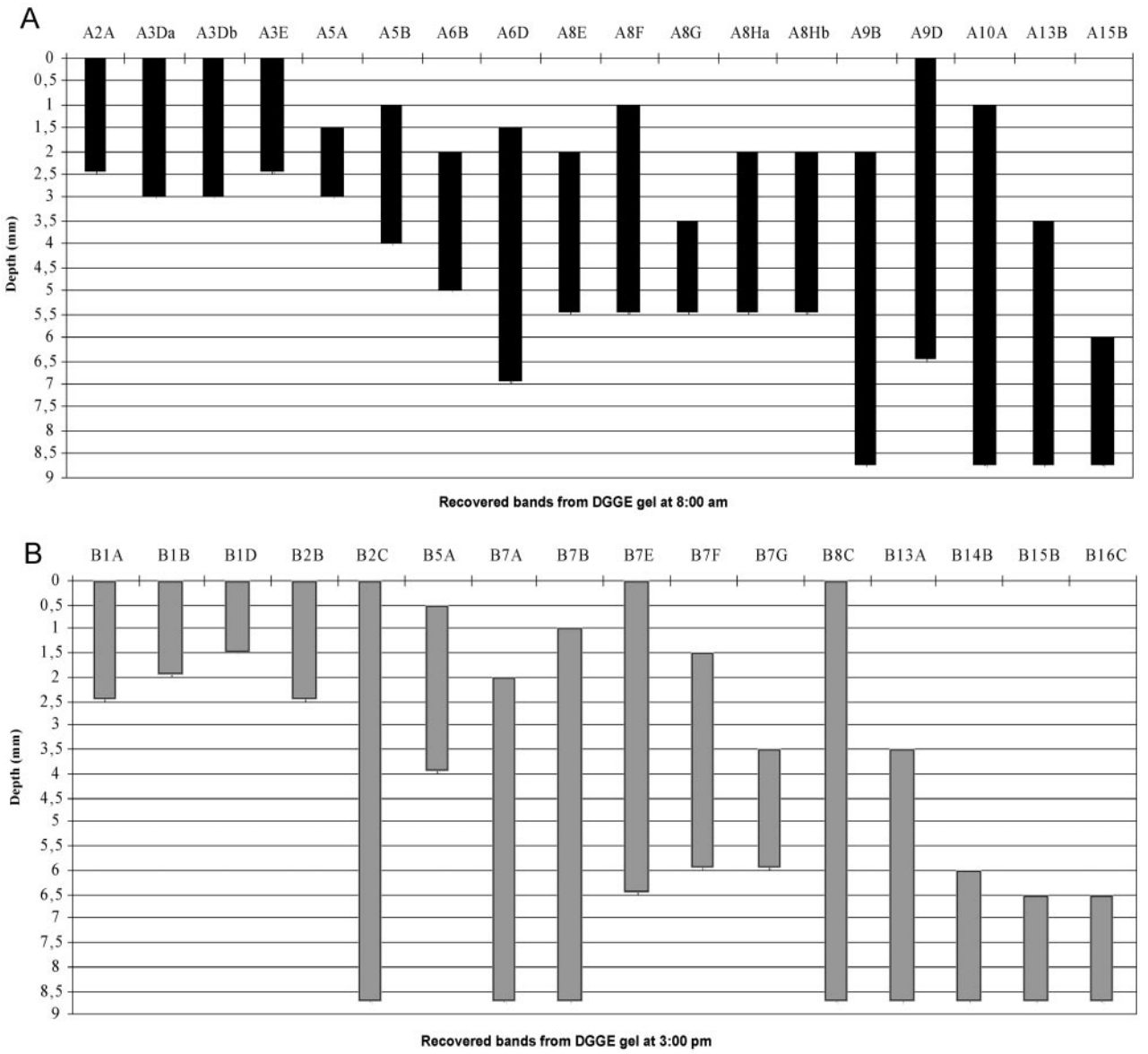

FIG. 4. DGGE analysis of the microbial mat community at two sampling times. Amplified products were separated on a gradient of 30 to 65% denaturant. Labeled bands were excised from the gel, reamplified, and sequenced. Bands that failed to generate legible sequences were cloned. The vertical distribution of DGGE bands recovered from gel A at 8:00 a.m. (A) and from gel B at 3:00 p.m. (B) is shown. For example, band $A2_A$ was obtained from a sample taken at a depth of 0 to 2.5 mm.

duce the exudation of photosynthates, the cross-feeding of associated heterotrophic bacteria, and the growth of anaerobic microorganisms. Combining phenotypic analyses based on PLFA with DNA analyses provides greater insight into the dynamic shifts in metabolism in microbial mat communities than that obtained from nucleic acid studies alone.

The DGGE and PLFA data strengthen the idea of the model of microbial mats as complex and dynamic ecosystems in which vertical migrations and physiological adaptations occur over intervals of hours and progress through day-night cycles.

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TABLE 1. Similarity between DNA recovered from DGGE gels and closest relatives

Band code	GenBank accession no.	Similarity (%)	Closest relative
$A2_A$	AY525644	100	*Psychroflexus tropicus*
$A3_D$	AY525677	95	Uncultured *Spirochaetaceae* bacterium
	AY525676	87	Uncultured bacterium clone C11-F10
$A3_E$	AY525645	99	Gamma-proteobacterium GWS-SE-H242b
$A5_A$	AY525647	97	*Halanaerobium saccharolyticum*
$A5_B$	AY525648	98	*Halanaerobium saccharolyticum*
$A6_B$	AY525646	93	Uncultured bacterium clone s22
$A6_D$	AY525654	100	*Halanaerobium saccharolyticum*
$A8_E$	AY525658	94	Uncultured green nonsulfur bacterium
$A8_F$	AY525662	89	*Bdellovibrio* sp. strain JS7
$A8_G$	AY525665	95	Uncultured green nonsulfur bacterium
$A8_H$	AY525666	98	Uncultured bacterium clone ZB56
	AY525667	94	Uncultured *Chloroflexi* bacterium clone
$A9_B$	AY525668	93	Bacteria from anoxic bulk soil
$A9_D$	AY525660	93	Bacteria from anoxic bulk soil
$A10_A$	AY525649	100	*Halanaerobium saccharolyticum*
$A13_B$	AY525656	97	Uncultured *Rhodobacter* sp.
$A15_B$	AY525675	92	*Peptoniphilus harei*
$B1_A$	AY525652	99	*Psychroflexus tropicus*
$B1_B$	AY525651	93	*Psychroflexus tropicus*
$B1_D$	AY525669	96	*Psychroflexus tropicus*
$B2_B$	AY525670	97	Gamma-proteobacterium GWS-SE-H242b
$B2_C$	AY525671	98	Uncultured cyanobacterium (*Microcoleus* sp.)
$B5_A$	AY525655	88	Uncultured bacterium clone C11-F10
$B7_A$	AY525650	93	Uncultured bacterium GR-Sh2-12
$B7_B$	AY525674	98	Uncultured *Bacteroidetes* bacterium clone
$B7_E$	AY525663	91	Uncultured bacterium clone ZB69
$B7_F$	AY525657	93	Bacteria from anoxic bulk soil
$B7_G$	AY525664	94	Uncultured bacterium clone s22
$B8_C$	AY525661	94	Uncultured delta-proteobacterium clone
$B13_A$	AY525653	100	*Halanaerobium saccharolyticum*
$B14_B$	AY525659	95	Uncultured gamma-proteobacterium clone
$B15_B$	AY525673	95	Uncultured green nonsulfur bacterium clone
$B16_C$	AY525672	91	Bacteria from anoxic bulk soil

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