

Tesi doctoral presentada per En/Na

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**"Ecophysiological and molecular
characterization of estuarine microbial mats"**

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Departament de Microbiologia



UNIVERSITAT DE BARCELONA



IV. VERTICAL MICROSCALE CHARACTERIZATION OF BACTERIAL DIVERSITY AND PHYSIOLOGICAL STATUS

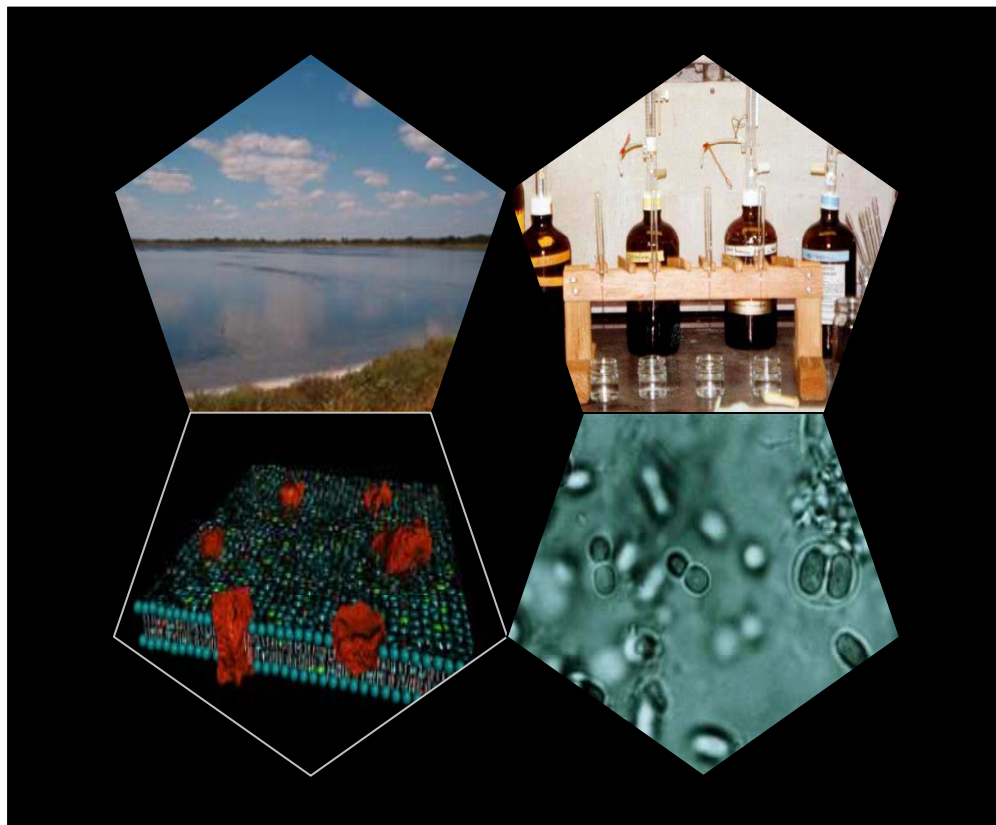


Figure IV. “There must be no barriers for freedom of inquiry. There is no place for dogma in science. The scientist is free, and must be free to ask any question, to doubt any assertion, to seek for any evidence, to correct any errors” Robert Oppenheimer (1904–1967).

From top left to right: Rhone delta (Camargue) microbial mats / Silicic acid chromatography of total lipid extracts / Molecular representation of a phospholipids bilayer / Micrograph of a unicellular cyanobacterium.

- Introduction and objectives of the study

A microbial mat is a model of consortial association. The close spatial relationships between its members have facilitated the establishment of microscale biochemical gradients and ‘microniches’, which leads to a more complete nutrient recycling, the diversification of the microbiota and closer community interactions over a range of temporal and spatial scales (Paerl *et al.*, 2000).

One of the aims in microbial ecology is to understand how microbial communities are patterned with spatial and temporal heterogeneities in the environment (Torsvik *et al.*, 2002). In this sense, population identification is the first step to establish relationships between the whole (community) and its parts (populations). The study of microbial communities has raised questions about their composition, structure, stability and about the activity and function of the individual inhabitants. Nonetheless, the combination of different methodologies allows a more representative picture of the distribution and abundance of microorganisms in complex communities.

White and Findlay (White and Findlay, 1988) developed a community-level approach to characterize the microbial community structure by evaluating shifts in phospholipid fatty acids (PLFA) from environmental samples. Different groups of bacteria are characterized by specific PLFA profiles; therefore a change in the phospholipid pattern would indicate a change in the bacterial composition. This concept has resulted in the identification and quantification of viable biomass and community structure in sediments (Ringelberg *et al.*, 1988; Ibekwe *et al.*, 2001) and in microbial mats (Navarrete *et al.*, 2000). Despite its versatility, PLFA analysis has a limited application to the analysis of gram-negative bacteria (White and Ringelberg, 1997). To overcome this, PLFA studies have been complemented by nucleic acid-based analyses (Macnaughton *et al.*, 1999; Stephen *et al.*, 1999).

Information collected by molecular tools quickly reveals the unsuspected complexity of whole bacterial communities (Ward *et al.*, 1990). The fingerprinting methods are widely adopted and permit the simultaneous analysis of numerous samples (Ferrari and Hollibaugh, 1999). Denaturing gel electrophoresis techniques have been

extensively used to monitor bacterial communities in space and time (Ferris and Ward, 1997; Nübel *et al.*, 1999) or to evaluate the impact of environmental disturbances (Müller *et al.*, 2001).

Recently, DNA-based and lipid analysis have been combined to study the community ecology of microorganisms to provide (i) quantitative estimates of microbial diversity in various environments and (ii) quantitative means of relating microbial community structure to environmental conditions (Fromin *et al.*, 2002; Torsvik and Øverås, 2002; Torsvik *et al.*, 2002). Although microbial community fingerprinting methods include a variety of well-known PCR biases (Wintzingerode *et al.*, 1997; Fromin *et al.*, 2002), these methods have provided comprehensive information on global patterns of microbial diversity and have proved useful for the study of factors that govern microbial diversity, ecology and function in numerous habitats (Casamayor *et al.*, 2002; Ibekwe *et al.*, 2002; Tankéré *et al.*, 2002).

Diversity is an important concept in ecology, often applied in environmental monitoring and conservation management (Hedrick *et al.*, 2000). High diversity may correlate with ecosystem resistance to stresses; in fact, extreme conditions (temperature, pH, salt concentration) can reduce the diversity of affected communities (Strom *et al.*, 1985), and a more diverse physical environment often produces higher community diversity (McArthur *et al.*, 1988). In addition, some theories have been formulated concerning how species diversity is related to ecosystem function (Naeem *et al.*, 1995; Tilman *et al.*, 1996), and how ecosystem stability correlates positively with system diversity. Knowledge of the conditions that affect stability is needed to determine the effects of external parameters on microbial habitats.

The aim of the present report was (i) to assess differences in the metabolic status and microbial diversity of microbial mats by means of a combined lipid-nucleic acid approach, (ii) to identify the microbial members of the system by a nucleic acid-based method, and (iii) to investigate how biotic changes affect the diversification and dynamics of the populations within the mat.

- Material and methods

- Sampling, lipid extraction and separation

Camargue microbial mat samples were taken in April 2002 at two selected times during the day (8:00 am GMT, named A samples; and 3:00 pm GMT, named 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 group 1 to 15; group 16 contained 25 slices 50 µm thick, total depth: 8.75 mm). Duplicate samples were extracted by using the modified Bligh and Dyer method described in chapter 'II. General Material and Methods'. The total lipid extract was fractionated by silicic acid chromatography and the polar lipid fraction was transesterified to fatty acid methyl esters (FAMES) (see 'Polar lipid fraction' section).

- DNA purification and DGGE analysis

Nucleic acid was precipitated from the aqueous phase resulting from the total lipid extraction (see '3. Nucleic acid analysis methods' section of chapter II). PCR amplification of 16S rRNA gene and DGGE was performed as described in Table II.6 and II.7 of the 'Material and Methods' chapter. The separation of the amplified products was performed in DGGE gels with a gradient of 30 to 65% denaturant. Excised DGGE bands were used as a template in a PCR reaction as above, and the purified PCR product was sequenced. Amplification products that failed to generate legible sequence directly were cloned into the pGEM-T® cloning vector according to the protocol mentioned in the 'Enzymatic treatment of DNA and transformation' section in chapter II.

All unique partial rDNA sequences were deposited in the GenBank database under the accession numbers AY525644 to AY525677.

- Statistical analysis of PLFA profiles and DGGE bands

Data analysis of PLFA profiles was performed using the Microsoft® Excel software package. Mean calculations were performed in the duplicate-sample to obtain the PLFA profiles. Divergence index (*D*) (Iwasaki and Hiraishi, 1998; Hiraishi, 1999)

was used to estimate differences between samples by its content in PLFA. D is calculated according to Eq1:

$$D(i, j) = \frac{1}{2} \sum_{k=1}^n |X_{ki} - X_{kj}| \quad [1]$$

where $X_{ki}, X_{kj} \geq 0.01$, $\sum X_{ki} = \sum X_{kj} = 100$, and X_{ki} and X_{kj} indicate the levels (expressed as percent of moles) of certain PLFA k in samples i and j , respectively. The neighbor-joining algorithm (Saitou and Nei, 1987) was used to construct a dendrogram based on D matrix data using the Mega 3.0 software.

Scanned DGGE gels were analyzed with the Scion Image software package for Windows (NIH Image, Scion, USA) as it was previously described in the 'DGGE analysis' section of the 'General Material and Methods' chapter. The Shannon-Weaver index, H' represents the uncertainty in predicting the species of an individual chosen at random. (Shannon and Weaver, 1963), and was calculated using the following function: $H' = -\sum P_i \log P_i$, where P_i is the importance probability of the bands in a gel lane or the mol percent data of each PLFA in a sample. P_i was calculated as follows: $P_i = n_i/N$, where n_i is the band intensity for individual bands or the pmol g^{-1} of certain PLFA and N is the sum of intensities of bands in a lane or the sum of all PLFA in a sample as pmol g^{-1} . The Simpson index (λ ; Simpson, 1949) that indicates the possibility that two individuals chosen at random will belong to the same species (Ludwig and Reynolds, 1988) was also calculated for PLFA and DGGE data.

The PLFA and DGGE data were normalized to a common analytical sensitivity in order to compare their diversity indices (Hedrick *et al.*, 2000). For each sample, the values of the PLFA or DGGE peaks expressed as a percentage of the total were sorted into descending order. The minimum percent reported in each sample was noted and the largest of those was chosen as a cut-off point. The largest minimum percent reported in any sample represents the analysis with the least sensitivity and all samples need to be corrected to this sensitivity in order to establish comparisons between their indices.

- Results

- Viable microbial biomass

Total PLFA (phospholipids fatty acids, as pmol PLFA g⁻¹ dry weight) estimated throughout the vertical profile at 8:00 am GMT and 3:00 pm GMT, is shown in Fig. IV.1. PLFA values ranged from 1×10⁴ to 7×10⁵ pmol g⁻¹ at 8:00 am GMT, and from 2×10³ to 4×10⁵ pmol PLFA g⁻¹ in the 3:00 pm GMT samples. Maximum viable biomass in terms of total PLFA was observed at the top of the mat at 8:00 am GMT. On the contrary, at 3:00 pm GMT, the maximum of viable biomass was found underlying the uppermost layer of the mat. Some differences were detected at different depths in both samples; especially in B samples (3:00 pm GMT), in which there was a maximum of viable cells (total PLFA) concentrated from 2 to 4 mm depth in comparison with the rest of the vertical profile. On the other hand, A samples (8:00 am GMT) showed a maximum of total PLFA between 0.5 to 1.5 mm depth reporting the highest value of both sampling times (approximately 7.5×10⁵ pmol PLFA g⁻¹).

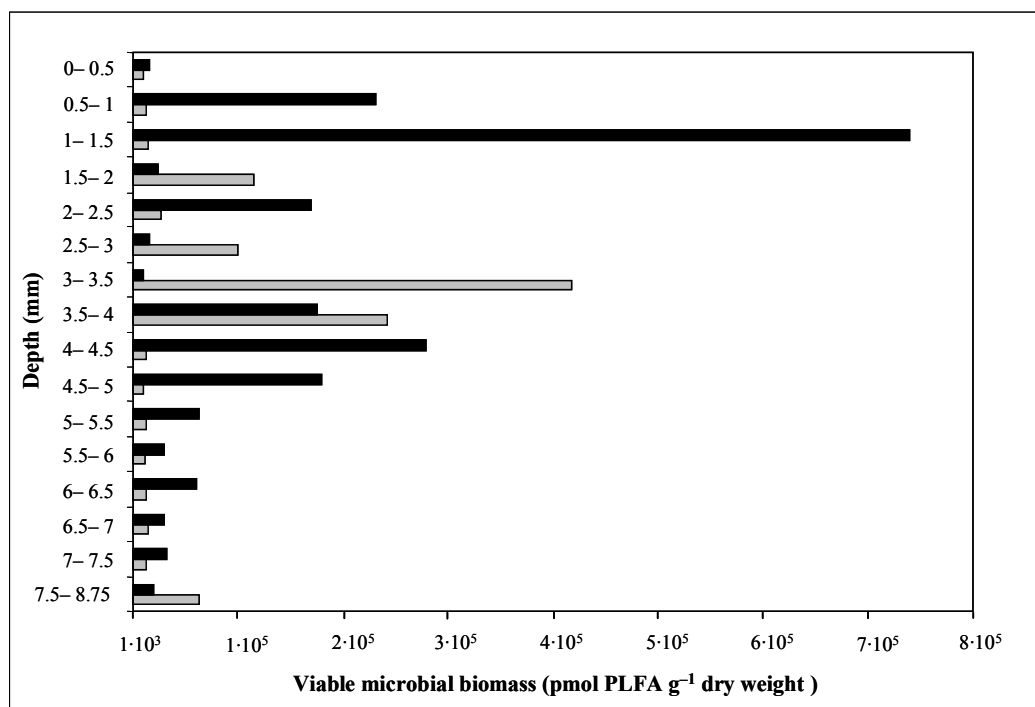


Figure IV.1. Viable microbial biomass as measured by total PLFA as picomoles of PLFA per gram (dry weight).

Black bars: A samples, collected at 8:00 am GMT; Grey bars: B samples, collected at 3:00 pm.

➤ Physiological status

Monoenoic to cyclopropanoic PLFA ratios are presented in Fig. IV.2 A. The slowest growth rate (high *cyclo/ω7c* ratio) throughout the vertical profile was detected in the deepest layers. In the morning (8:00 am), the reduction in the growth rate increased with depth and the highest values of the *cyclo/ω7c* ratio were located from 4 to 6 mm and in the deepest samples (maximum ratio 2.5). However, in the afternoon (3:00 pm) the slowest growth rate was found at the top of the mat and in the middle (around 2 mm and 5.5 mm depth) with a maximum *cyclo/ω7c* ratio of 3.5.

Data concerning metabolic stress are summarized in Fig. IV.2 B. The highest degree of metabolic stress was reported at the topmost layers in the morning (*trans/cis* ratio approx. 1.5). At the rest of the vertical profile, the metabolic stress was similar with values around 0.4. On the other hand, a higher degree of metabolic stress was observed in the middle layers in the afternoon (1–1.5 mm and 2–2.5 mm depth), although a maximum value of *trans/cis* ratio was detected at the bottom of the mat (6–7.5 mm).

➤ Community composition by PLFA analysis

The characterization of the community composition by PLFA analysis indicated some differences between the mat samples taken at 8:00 and 3:00 pm (Fig. IV.3). The microbial community in the morning consisted mainly of gram-negative bacteria (including cyanobacteria) as reported the quantification of monoenoic PLFA (Wilkinson, 1988). The maximum percentages of monoenoic PLFA were located between 2.5–3.5 mm depth (67.2–96.7%). Terminal branched saturated fatty acids (typical of gram-positive bacteria) represented a high proportion of the total PLFA in the middle layers and in the deepest samples (maximum 24.6%). Branched monoenoics and mid-chain branched saturated fatty acids, representative of anaerobic microorganisms (Dowling *et al.*, 1986), were constant along the vertical profile (9.8%). Proportions of polyenoic fatty acids, found in eukaryotes as well as cyanobacteria, were very low in all samples.

In the afternoon (Fig. IV.3 B), the proportion of PLFA of anaerobes was higher (12.4% mean) than at 8:00 am, and increased with depth (maximum values 4–5.5 mm depth). Polyenoics fatty acids reported maximum values between 6–6.5 mm depth (22.6%), and PLFA from gram-negative bacteria were almost constant and predominant in all samples except in the uppermost layers. PLFA found in gram-positive bacteria, were low in all samples except from 2.5 to 4 mm (14.4% mean) and in the deepest sample, but these percentages were lower in comparison with samples taken at 8:00 am.

The 18:1 ω 7c PLFA, marker of gram-negative microorganisms, was the most predominant in almost all sampling depths except from 4–5 mm at 8:00 am (*cy19:0* was dominant). The quantification of PLFAs *cy19:0* and 10Me16:0, markers of anaerobic bacteria and sulfate-reducing bacteria, respectively (Mallet *et al.*, 2004) revealed a high percentage of both FAMES from 1–1.5 mm and 3.5–5.5 mm at 8:00 am, and from 3–3.5 mm and 7.5–8.75 mm at 3:00 pm. Large proportions of branched fatty acids *i15:0* and *i17:0*, which are indicative of gram-positive microorganisms, were important at the top of the mat and in the middle layers in the morning (1–1.5 mm and 4–4.5 mm), in contrast with 3:00 pm samples that reported a lower quantity of these PLFA (mainly observed at 3–3.5 mm).

To compare PLFA patterns from the different depths of the two sampling times the divergence index (*D*) was determined for comparisons of all profiles. The Unweighted pair-group method with arithmetic mean (UPGMA) algorithm was used to create a dendrogram describing pattern similarities (Fig. IV.4). The divergence index (*D*) can be interpreted to reveal the extent of differences in microbial community structures among samples. This analysis showed that depth-related differences appeared to have a greater influence than temporal differences. ‘Cluster 1’ grouped 3:00 pm samples from 4 to 7.5 mm, ‘cluster 2’ grouped 8:00 am samples from 4 to 8.75 mm and 3:00 pm 7.5–8.75 mm sample, and ‘cluster 3’ grouped 8:00 am and 3:00 pm samples from 2.5 to 4 mm. Although the depth-related differences seem to be more important than sampling time differences, in the deepest samples a tendency of clustering according to depth and temporal conditions was observed.

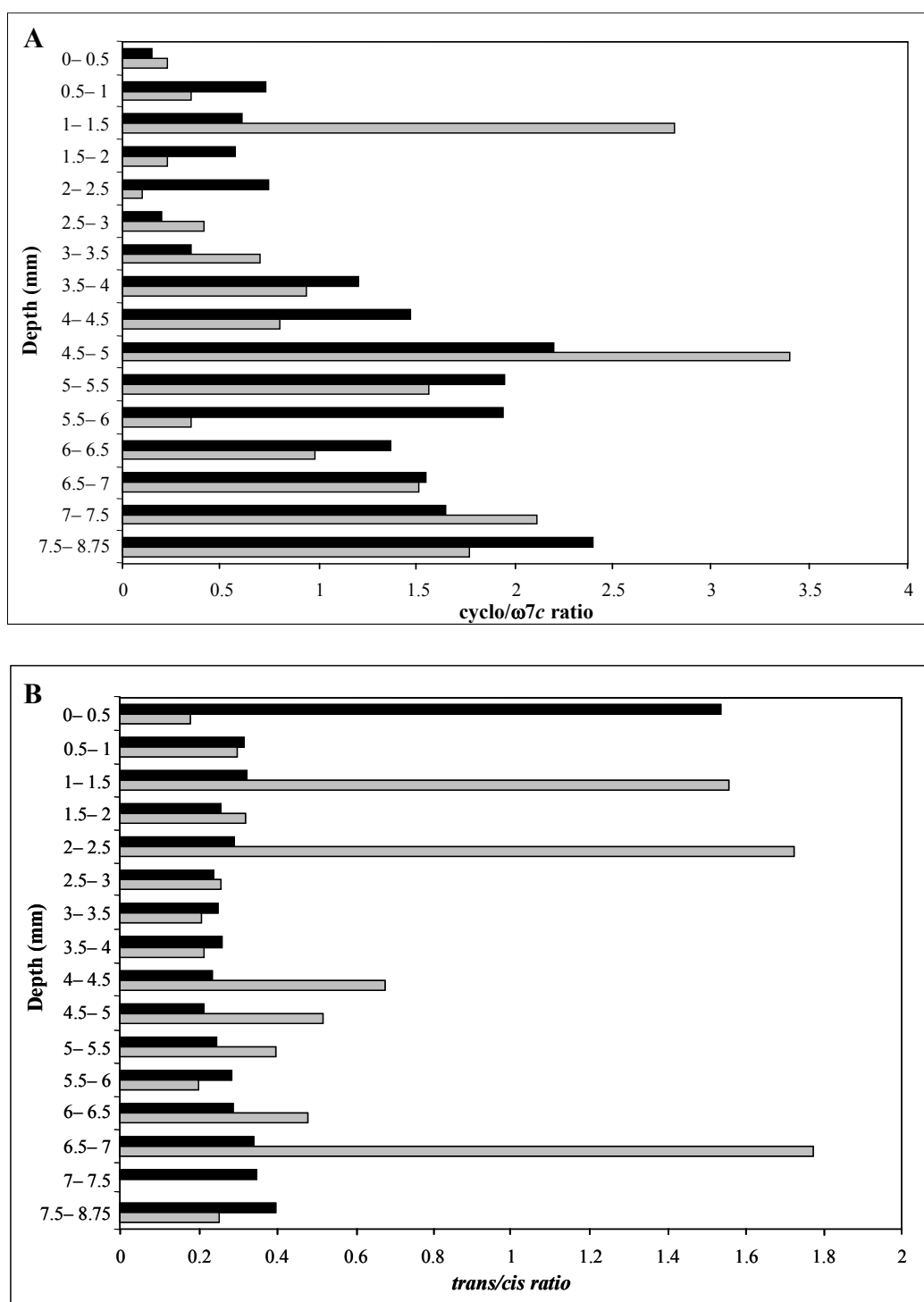


Figure IV.2. (A) Metabolic status or starvation index as the ratio of cyclopropyl to monoenoic PLFAs; (B) Metabolic stress expressed as the *trans/cis* ratio of monoenoic PLFAs.

Black bars: A samples, collected at 8:00 am GMT; Grey bars: B samples, collected at 3:00 pm.

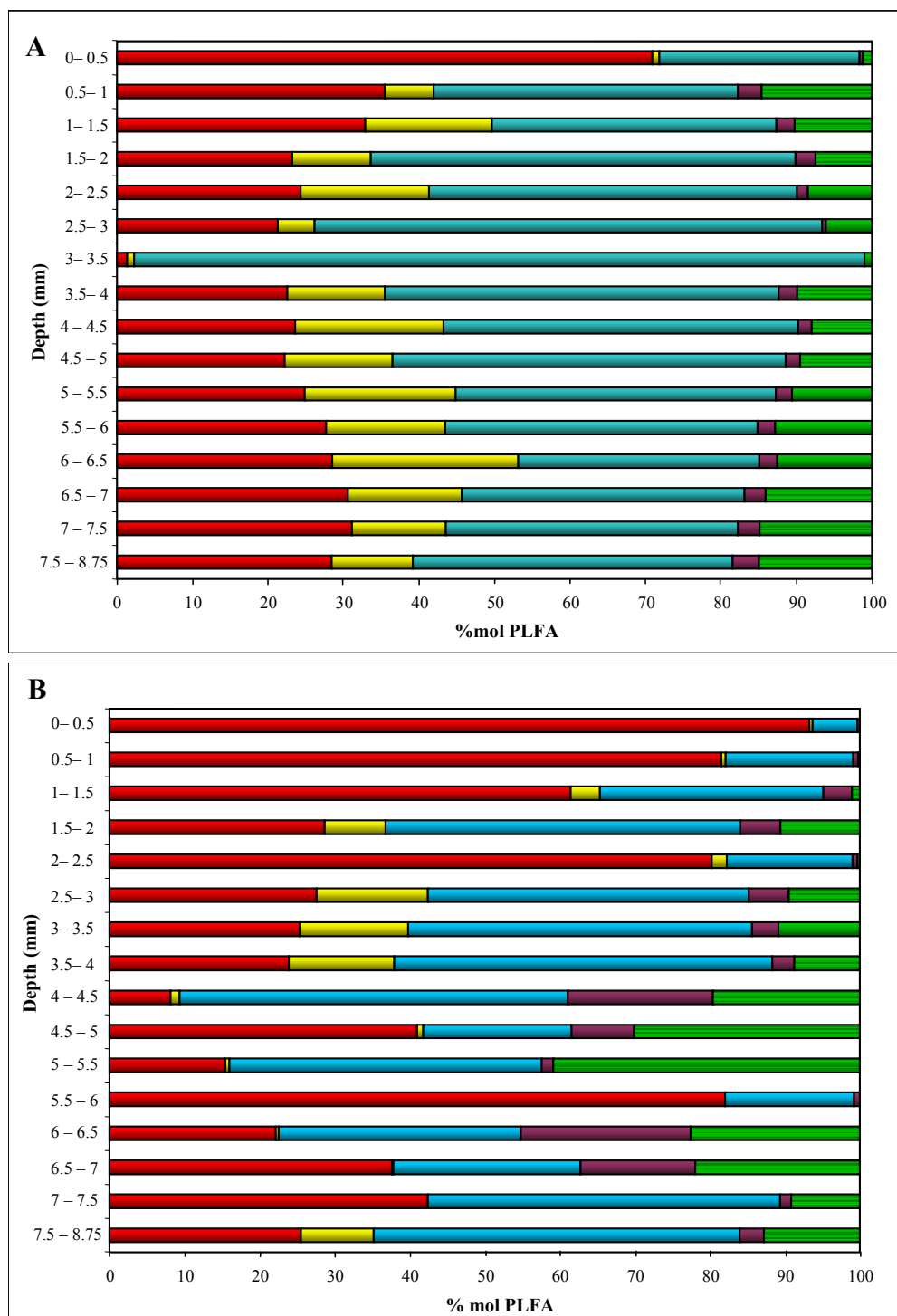


Figure IV.3. Community composition expressed as moles percent PLFA. (A) Samples taken at 8:00 am GMT; (B) Samples taken at 3:00 pm GMT.

Predominant PLFA in microbial groups: Normal saturated (all genera; red bars), terminal branched saturated (gram-positive; yellow bars), monoenoics (gram-negative; blue bars), polyenoics (microeukaryotes, purple bars), and branched monoenoics and mid-branched saturated (anaerobic microorganisms, green bars).

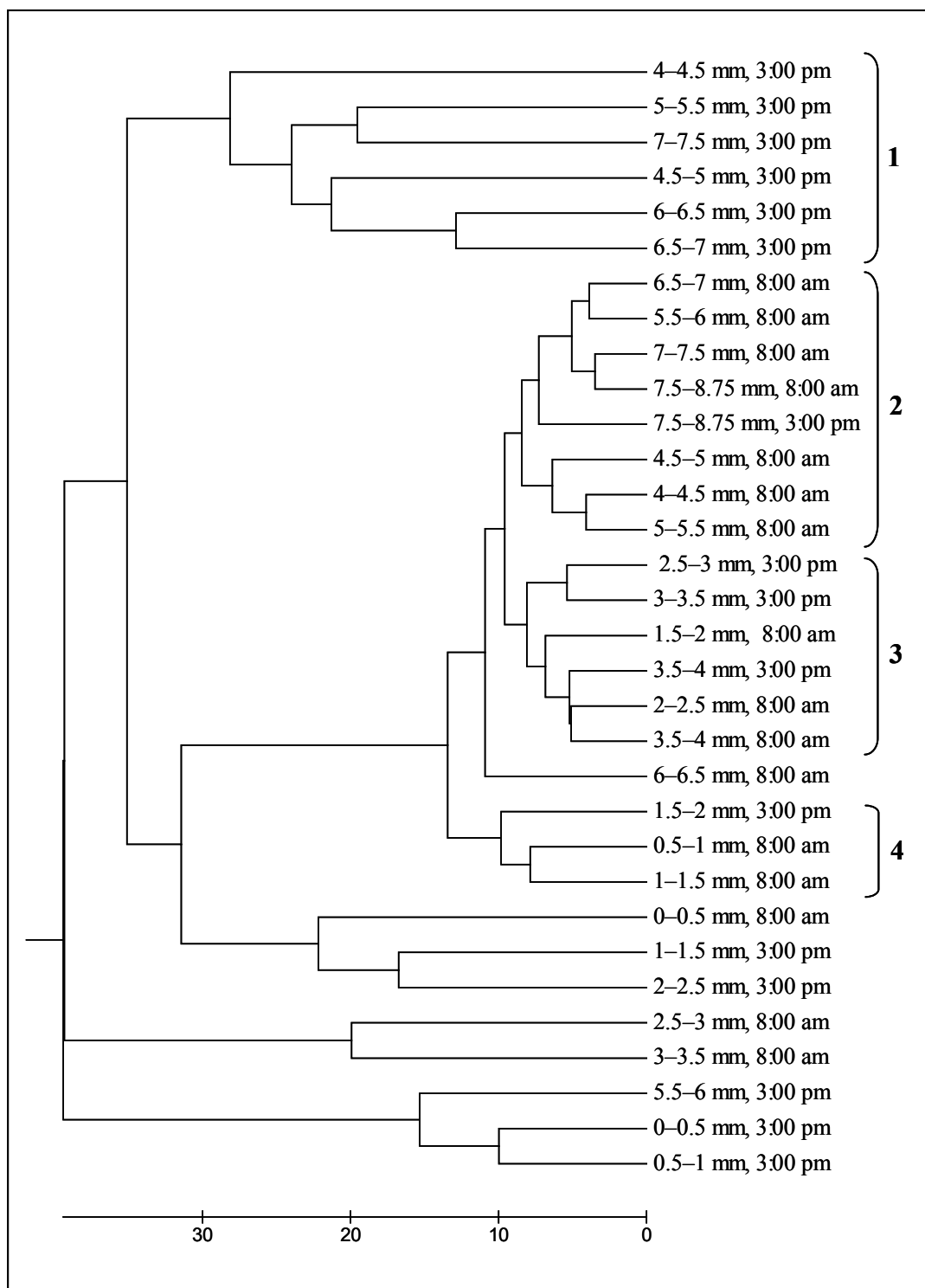


Figure IV.4. Cluster analysis of the PLFA contents.

Dendrogram calculated on the basis of the Divergence index (D) with the clustering algorithm of UPGMA (Unweighted pair-group method with arithmetic mean). Clusters 1 to 4 explained in the text.

➤ PCR-DGGE analysis of the bacterial community structure

PCR-DGGE analysis of bacterial community structure was performed with the DNA recovered from the aqueous phase after total lipid extraction of each sample. Banding patterns for ‘A and B samples’ (taken at 8:00 am and 3:00 pm, and named from number 1 to 16 in increasing vertical depth) are shown in Fig. IV.5 A and B, and the distribution of the bands with depth is indicated in Fig. IV.6. The prominent DGGE bands were sequenced and their phylogenetic affiliations are summarized in Table IV.1 (updated in September 2005).

At the top of the mat (from 0 to 2.5 mm depth), ‘A and B samples’ generated a complex banding pattern with several similarities, for example, the prominent band A2_A that had remained strong until 2 mm depth and then disappeared or weakened, was found in B samples between the same interval (B1_A). Moreover, a novel band A5_A had only appeared from 1.5–3 mm but it could not be found in the ‘B-DGGE gel’ (3:00 pm GMT). Band A5_B was bright between 1–4 mm depth and then was faintly detected in the rest of the vertical profile. Band A3_E was brighter between 0.5–2 mm and its corresponding band in ‘gel B’, B2_B, was found in the same conditions and intensity (high homology with *Marinobacter* sp.). In the topmost layers B1_B band was prominent from 0 to 0.5 mm and weakened to 2 mm depth (as well as B1_D). Moreover, a band equivalent to B1_B was found in the 8:00 am-gel but ranged only between 0–1.5 mm.

On the other hand, band B2_C was brighter from 0 to 1.5 mm and then disappeared. Band A3_D was observed between 0–3 mm depths and, after cloning, two single sequences were recovered (Table IV.1) and they reported a high similarity with uncultured spirochetes and *Bacteroides* species. Apparently, there is not a corresponding band in gel B. Band B5_A remained strong between 1.5–2.5 mm. The derived sequences from these bands suggested dominance of *Flavobacteriaceae* (B1_A, A2_A, B1_D, B5_A), and also the presence of γ -Proteobacteria (B2_B, A3_E), members of the phylum Firmicutes (*Halanaerobiales*, A5_A, A5_B), cyanobacteria (B2_C), members of the *Cytophaga-Flavobacterium-Bacteroides* phylum, and spirochetes (A3_D).

In the middle part of the mat (samples 6 to 10), from 2.5 to 5 mm depth, A and B DGGE gels showed a slight increase of the number of bands. Bands derived from ‘gel

A' remained strong between 2–5.5 mm (A6_B, A6_E, A8_{E-H}) except for bands A6_{D-E}, A9_B, A9_D and A10_A that could be found along the entire vertical profile but seemed to be restricted to certain zones. The sequence derived from band A6_D presented a high similarity (100%) with *Haloanaerobium saccharolyticum* and it was brighter between 1.5 mm and 7 mm. Band A6_D is complementary to B13_A, which is stronger at the bottom of the mat. Band A8_E was bright from 2.5 to 4 mm depth but its complementary band in gel B, B7_E, remained strong almost all the vertical profile. On the other hand, after cloning band A8_H two different clones were obtained at the same ratio. Band A8_H was weak from 2 to 5.5 mm and it seemed to be a correspondence with B7_G, which was stronger from 3 mm to the bottom of the mat. Band A10_A could be found at all depths but there was a vertical decrease in its brightness. Moreover, there was a correspondence with band B13_A that was stronger in the middle and in the deepest parts of the mat. Finally, band B8_C was found at all depths and remained with similar intensity along the vertical profile.

At the bottom of the mat (samples 11 to 16, from 5 to 8.75 mm depth), there was an increase in the band intensity at 3:00 pm compared to 'A samples'. Band A15_B was stronger from 7 to 8.75 mm. This band was cloned and the derived sequences showed a high homology with *Clostridium* sp. (Table IV.1). It is also possible that there was an equivalent band in gel B but bands were too bright in those samples to recover, clone and confirm the sequence.

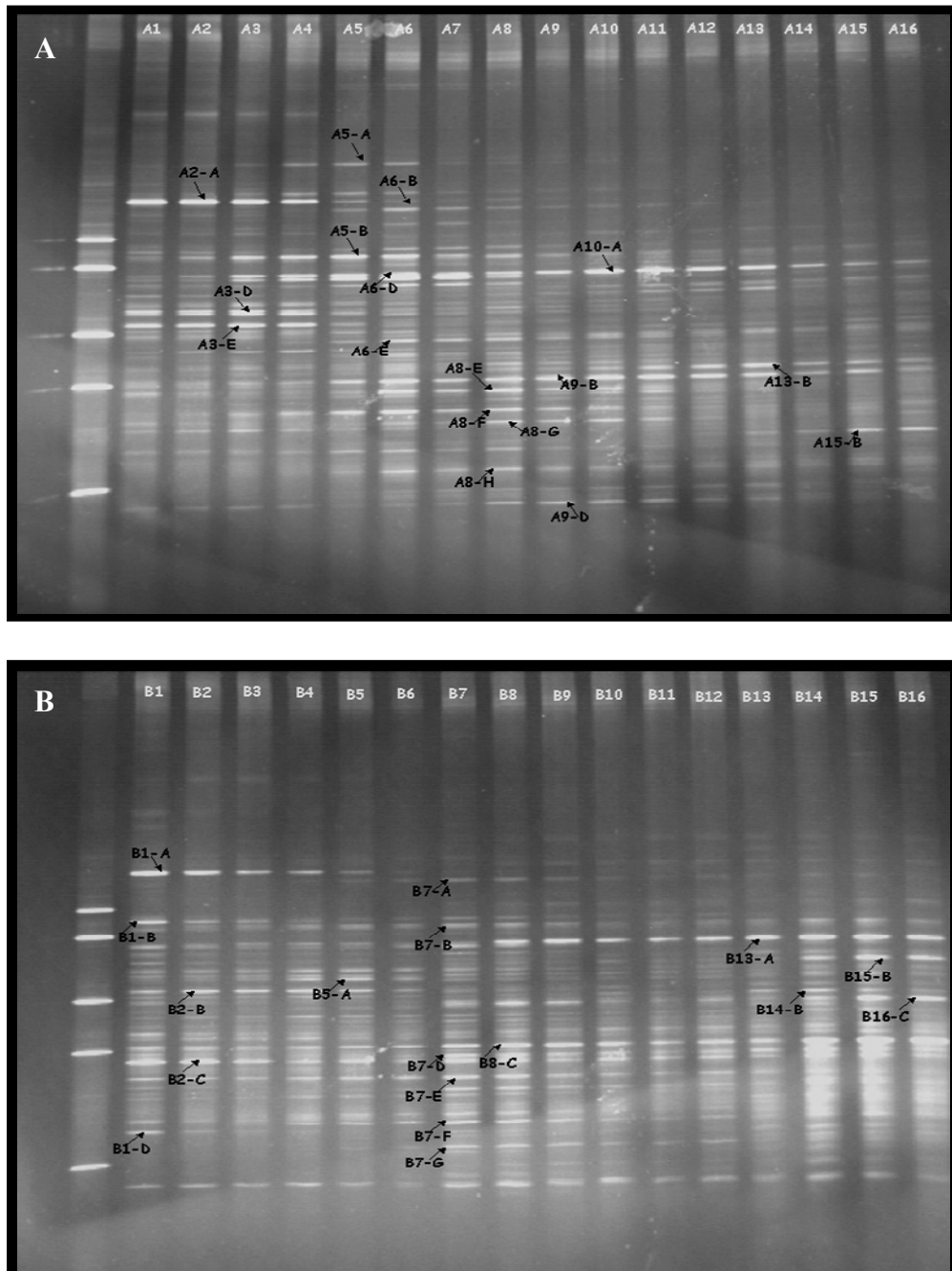


Figure IV.5. DGGE eubacterial community profile of microbial mat samples taken at 8:00 am GMT (A) and at 3:00 pm GMT (B).

Gel lanes are named from 1 to 16 in increasing depth (500 μm thick each). Numbered bands correspond to 16S rDNA sequence types described in Table IV.1.

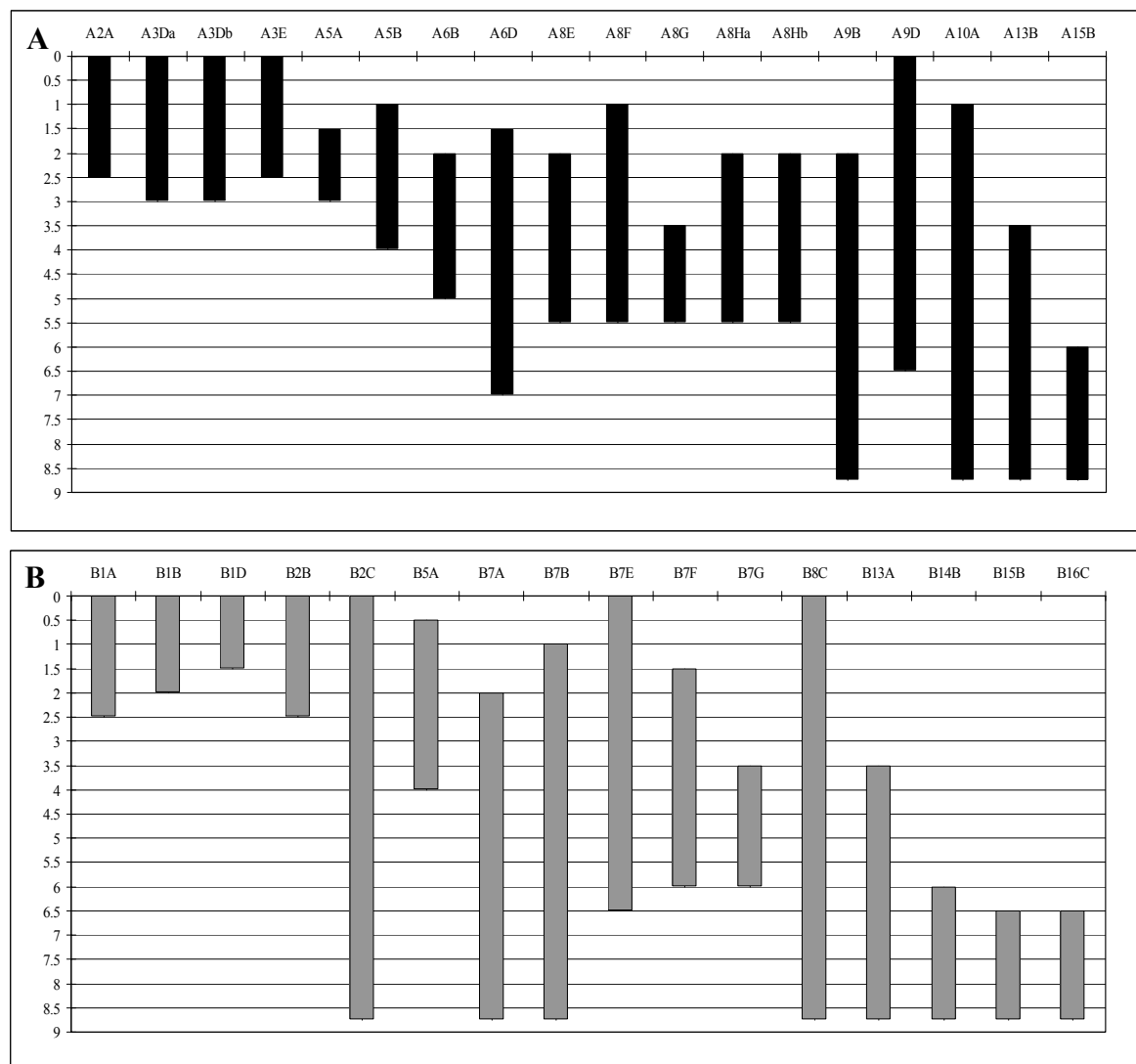


Figure IV.6. DGGE eubacterial community profile of microbial mat samples taken at 8:00 am GMT (A) and at 3:00 pm GMT (B).

Gel lanes are named from 1 to 16 in increasing depth (500 µm thick each). Numbered bands correspond to 16S rDNA sequence types described in Table IV.1.

Table IV.1. Similarity between DGGE bands recovered and closest relatives (similarity in %).

Code	Accession n.	Similarity	Closest relative
A2 _A	AY525644	100	<i>Psychroflexus tropicus</i>
A3 _D	AY525677	95	a)- <i>Spirochaeta halophila</i>
	AY525676	87	b)- Uncultured <i>Bacteroidetes</i> bacterium clone
A3 _E	AY525645	99	Uncultured <i>Marinobacter</i> sp. bacterium clone
A□ _A	AY525647	97	<i>Haloanaerobium saccharolyticum</i>
A□ _B	AY525648	98	<i>Haloanaerobium saccharolyticum</i>
A□ _B	AY525646	92	Uncultured <i>Bacteroidetes</i> bacterium clone
A□ _D	AY525654	100	<i>Haloanaerobium saccharolyticum</i>
A8 _E	AY525658	94	Uncultured <i>Chloroflexi</i> bacterium clone
A8 _F	AY525662	89	<i>Bdellovibrio</i> sp. JS7
A8 _G	AY525665	96	Uncultured <i>Chloroflexi</i> bacterium clone
A8 _H	AY525666	98	a)- Uncultured bacterium clone E2aG04
	AY525667	94	b)- Uncultured <i>Chloroflexi</i> bacterium clone
A9 _B	AY525668	93	Bacteria from anoxic bulk soil (<i>Bacteroidetes</i>)
A9 _D	AY525660	93	Bacteria from anoxic bulk soil (<i>Bacteroidetes</i>)
A10 _A	AY525649	100	<i>Haloanaerobium saccharolyticum</i>
A13 _B	AY525656	97	Uncultured <i>Rhodobacter</i> sp. clone
A1□ _B	AY525675	92	<i>Clostridium</i> sp. ArC6
B1 _A	AY525652	99	<i>Psychroflexus tropicus</i>
B1 _B	AY525651	93	<i>Bizionia myxarmorum</i> (<i>Flavobacteriaceae</i>)
B1 _D	AY525669	96	<i>Psychroflexus tropicus</i>
B2 _B	AY525670	97	Uncultured <i>Marinobacter</i> sp. bacterium clone
B2 _C	AY525671	98	Uncultured cyanobacterium (<i>Microcoleus</i> sp.)
B□ _A	AY525655	88	Uncultured <i>Bacteroidetes</i> bacterium clone
B7 _A	AY525650	93	<i>Bacteroidetes</i> bacterium (<i>Sphingobacteriaceae</i>)
B7 _B	AY525674	98	Uncultured <i>Bacteroidetes</i> bacterium clone
B7 _E	AY525663	92	Uncultured bacterium clone (<i>Chloroflexi</i>)
B7 _F	AY525657	93	Bacteria from anoxic bulk soil (<i>Bacteroidetes</i>)
B7 _G	AY525664	94	Uncultured bacterium clone (<i>Bacteroidetes</i>)
B8 _C	AY525661	94	Uncultured delta proteobacterium clone
B13 _A	AY525653	100	<i>Haloanaerobium saccharolyticum</i>
B14 _B	AY525659	97	Uncultured gamma proteobacterium clone
B1□ _B	AY525673	97	Uncultured <i>Chloroflexi</i> bacterium clone
B1□ _C	AY525672	91	Bacteria from anoxic bulk soil (<i>Bacteroidetes</i>)

➤ Structural diversity of the microbial community

PLFA profiles and DGGE banding patterns can be used as an indication of microbial community diversity (Eichner *et al.*, 1999; Hedrick *et al.*, 2000; Fromin *et al.*, 2002). The Shannon-Weaver index (H') was calculated for the determination of the structural diversity. H' was calculated on the basis of the number and relative intensities of bands in a gel strip, H' (DGGE), and on the basis of the number and moles percent data of PLFAs in a sample, H' (PLFA). The Simpson index (λ) was subtracted from 1 to give a D value that ranged from 0 to 1.

Figure IV.7 illustrates the diversity value H' at both sampling times (8:00 am and 3:00 pm) and along the vertical profile. At 8:00 am, H' (PLFA) remained almost stable all depths with the exception of 2.5–3.5 mm depth, where there was a slightly decrease of the overall diversity. H' (PLFA) values at 8:00 am ranged from 0.27 to 1.33. On the contrary, H' (PLFA) at 3:00 pm reported a decrease of the diversity at 2–2.5 mm and 4.5–6 mm, but the maximum values were observed from 1–4 mm and in the deepest layers. H' (DGGE) at 8:00 am reported similar values throughout the vertical profile with a slightly increase from 3.5–4 mm (values ranged from 1.13–1.38). H' (DGGE) in B samples (3:00 pm), ranged from 1.23–1.43 and as was observed in H' (PLFA) values at 3:00 pm, reported a decrease in the diversity index from 4.5–5.5 mm. H' (PLFA) agreed well with H' (DGGE) in the fact that H' values reported similar profiles in 3:00 pm samples although H' (PLFA) data showed sharper changes throughout the vertical profile. The Shannon-Weaver index was also calculated for PLFA indicative of anaerobic microorganisms (branched monoenoics and mid-branched saturated fatty acids) (Fig. IV.8). Values showed a high diversity of PLFA from anaerobes in 2.5–6 mm depth at 3:00 pm. On the contrary, values observed at 8:00 am indicated a similar diversity in all samples with a slightly increase of H' (PLFA anaerobes) at the topmost layers and in the deepest samples.

Apart from that, D values ($1-\lambda$) reported a similar diversity in both sampling times and depths. Indeed, DGGE data ranged from 0.982–0.994 at 8:00 am and 0.997–0.987 at 3:00 pm, and D (PLFA) values ranged from 0.918–0.935 at 8:00 am and 0.902–0.941 at 3:00 pm.

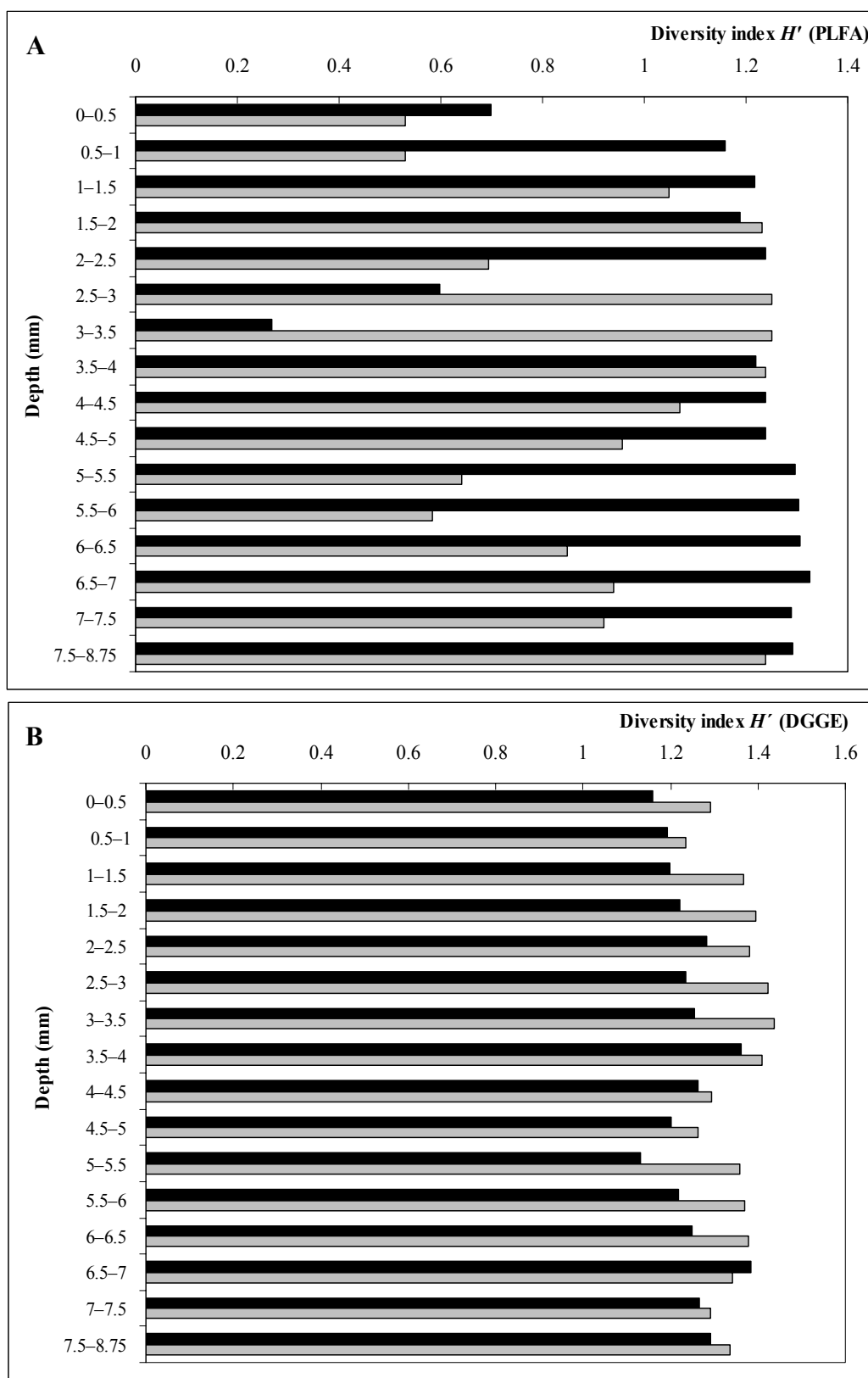


Figure IV.7. Shannon-Weaver index (H') calculated with PLFA data (A), and DGGE data (B).

Black bars: A samples, collected at 8:00 am GMT; Grey bars: B samples, collected at 3:00 pm.

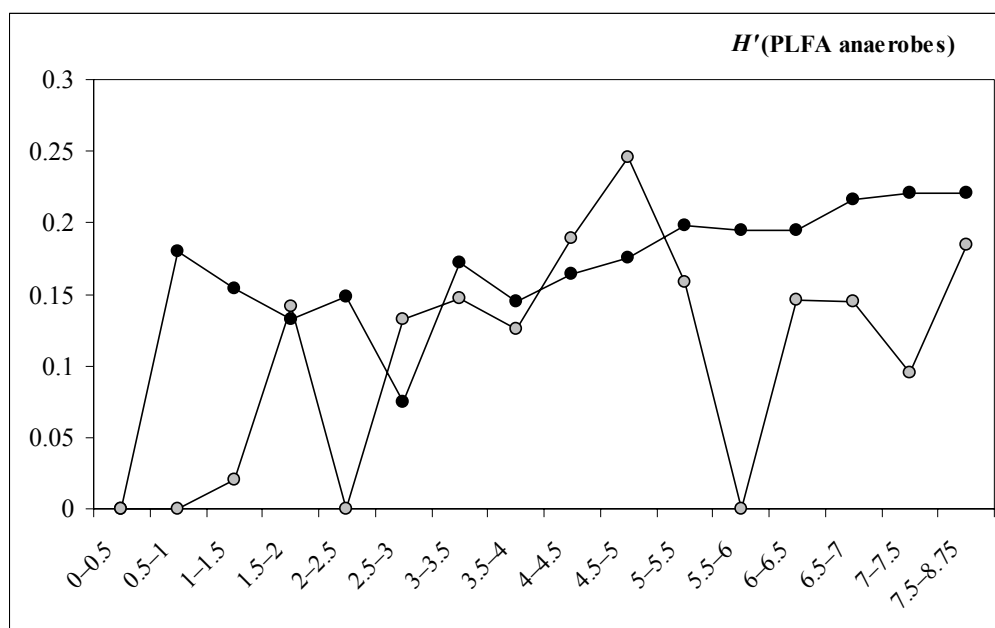


Figure IV.8. Shannon-Weaver index of diversity (H') calculated with PLFA indicative of anaerobic microorganisms.

Black dots: A samples, collected at 8:00 am GMT; Grey dots: B samples, collected at 3:00 pm.

- Discussion and conclusions
 - Signature lipid biomarker analysis

Recent studies performed in artificial cyanobacterial mats (Kühl and Fenchel, 2000), have reported an increase in bacterial biomass in layers of high density of phototrophs, and a structural association between them. This observation, as well as functional evidences obtained in hypersaline mats (Glud *et al.*, 1999), suggested a close coupling of the activity of primary producers (phototrophs) and heterotrophs in microbial mats (Grötzschel and de Beer, 2002). In Camargue mat samples, an explanation for the maximum of viable biomass (Fig. IV.1) observed in the morning could be the migration of cyanobacteria towards the top of the mat in order to avoid the toxic exposure to sulfide (produced by sulfate-reducing bacteria and accumulated in the mat during the night), and purple sulfur bacteria that use the light in the early morning to begin the photosynthetic processes. The maximum of viable biomass found underlying the top of the mat in the afternoon, might be explained by cell lysis at the uppermost layer of the mat due to a high solar irradiance, or because of an increase of aerobic heterotrophs associated in the photic zone.

Cell membranes respond to changes in the environmental conditions modifying their composition (Cossins *et al.*, 1986). An increase in the concentration of cyclopropanoic fatty acids represents the shift to conditions that slow down the growth rate (Sikkema *et al.*, 1995; see chapter 'I. Introduction', 'Lipid biomarkers and the SLB approach' section). The ratio of cyclopropanoic acids respect to their $\omega 7c$ homologues (*cyclo*/ $\omega 7c$ ratio) ranges from 0.05 (logarithmic phase) to 2.5 or higher (stationary phase) in gram-negative bacteria. Another typical modification consists on the increase in the ratio of *trans*- to *cis*-monoenoic PLFA (Heipieper *et al.*, 1992). *Trans/cis* ratios greater than 0.1 have been shown to indicate starvation or environmental stress in bacterial isolates (Guckert *et al.*, 1986) and ratios of 0.05 or lower are found in non-stressed microbial populations. With respect to the physiological status, vertical profiles indicated a slowest growth rate (high *cyclo*/ $\omega 7c$ ratio) in the bottom layers (Fig. IV.2 A). We suggest an increase in the activity of sulfate-reducing bacteria and anaerobic gram-positives especially at 8:00 am because anoxic conditions during the dark trigger

the development of these populations that use the excess of organic matter produced in the daylight hours. The limitation of organic carbon (low carbon/nitrogen rate, C/N) may account for the slowed growth of the sulfate-reducers and fermentative bacteria.

On the other hand, at 3:00 pm (Fig. IV.2 A), the reduction in the growth rate at the top of the mat and in the middle, can be explained by photosynthesis and carbon fixation processes carried out by cyanobacteria and purple sulfur bacteria, since it may be an abundance of organic compounds and a limitation of oxygen of nitrogen (high C/N rate). Previous studies have provided evidence of cross-feeding of heterotrophs by excretion of photosynthates in microbial mats (Fenchel and Kühl, 2000), and the associated heterotrophic bacteria readily recycle the photosynthate. In this case, the activity of phototrophs in the afternoon, might resulted in a higher rate of photosynthate exudation and this could induce the increase in both oxygen consumption and the stimulation of respiratory processes in the mat (Epping and Kühl, 2000). In addition, the close coupling between heterotrophs and phototrophs might favor a situation in which the photosynthesis rate increase even at high oxygen concentrations (Gröttschel and de Beer, 2002). Apart form that, the exposure to sulfide (accumulated during the night by the activity of SRBs) of cyanobacteria provides a suitable explanation for the higher degree of metabolic stress observed at the topmost layer of the mat at 8:00 am (Fig. IV.2 B).

➤ Community composition and diversity analyses

In the morning, the upper layers of the mat reported a major contribution of aerobic heterotrophic bacteria (*Psychroflexus* sp., *Shingobacterium* sp., *Marinobacter* sp.) belonging to the *Cytophaga-Flavobacterium-Bacteroides* group and γ -Proteobacteria. This fact reinforced the hypothesis mentioned above in which the heterotrophic bacteria must have a major role in the mineralization of photosynthates in the photic zone. Likewise, recent studies (Fourçans *et al.*, 2004) performed in Camargue microbial mats indicated the presence of γ -proteobacteria (*Halochromatium*, *Ectothiorhodospira*, *Marinobacter*) in the upper layers by means of terminal restriction length polymorphisms (T-RFLP) patterns.

It is noteworthy the detection of several DGGE bands that showed a high similarity with the *Haloanaerobium* genus. Members of the *Haloanaerobiaceae* family have been isolated from surface saline ecosystems such as the Red Sea (Eder *et al.*, 2001), marine salterns, cyanobacterial mats and hypersaline lakes (Ollivier *et al.*, 1994). Recent studies in anoxic sediments of the salterns of Salin-de-Giraud (Mouné *et al.*, 2003) have also detected a high predominance of anaerobic fermentative bacteria of the genus *Haloanaerobium* and *Orenia salinaria* (Mouné *et al.*, 2000), as well as an important contribution of members of the phylum *Bacteroidetes* that can act as a fermentative bacteria and seem to be adapted to high salinities (Benlloch *et al.*, 2002). In this sense, in the early morning the vertical profile is under anaerobic conditions and the anaerobic fermentative members of the *Halanaerobium* genus would be able to exploit certain 'microniches'. This data matched with the PLFA community composition analysis at 8:00 am (see Fig. IV.3 A), since PLFA of gram-positive bacteria were predominant in the middle and deep layers. Probably, the gram-positive population detected in microbial mats in previous studies (Navarrete *et al.*, 2000), corresponded to members of the *Haloanaerobium* and *Clostridium* genus. Likewise, DGGE results are consistent with the existence of members of the *Clostridium* genus at the bottom of the mat, which has not been reported before in this kind of microbial ecosystems.

Apart from that, PLFA indicative of anaerobic bacteria that were almost constant in the morning would also coincide with the predominant anaerobic conditions at this sampling time. DGGE gel at 8:00 am, reported bands similar to uncultured spirochetes. Uncultured spirochetes have been reported in microbial mats (Guerrero *et al.*, 1993b) and previous studies have detected associations between spirochetes and phototrophic bacteria (Harwood and Canale-Parola, 1984), which suggest they might play a major role in the organic matter recycling. Moreover, Mouné *et al.* (2003) detected spirochaetal-derived sequences in the anoxic sediments of the Salins-de-Giraud; Koizumi *et al.* (2004) also detected a high number of sequences related to spirochetes in meromictic lake sediments of Japan suggesting an active role of this population, together with members of the *Bacteroidetes* group, in the initial degradation of the organic-matter input from the overlying water (Rosselló-Mora *et al.*, 1999).

PLFA representative of gram-negative bacteria were dominant between 2.5–3.5 mm depth at 8:00 am (see Fig. IV.3 A), and this data were concomitant with DGGE band pattern because there was a predominance of bands homologues to green non-sulfur bacteria. On this matter, recent studies on *Chloroflexus* relatives from hypersaline environments (Nübel *et al.*, 2001; 2002; Fourçans *et al.*, 2004) have reported a larger diversity than have been expected. The presented data indicated the relative importance of green non-sulfur bacteria in iron-rich microbial mats (Pierson and Parenteau, 2000).

At 3:00 pm, an unexpected higher proportion of polyenoic fatty acids (indicative of microeukaryotes and cyanobacteria) was detected in the anoxic regions which is consistent with previous studies in microbial mats (Minz *et al.*, 1999). Moreover, the physiological status data did not report a situation of ‘slow growth’ or metabolic stress in the deepest layers, so it seemed that anaerobic bacteria were not affected by a limitation of nutrients. For this reason, we suggest an active role of sulfate-reducing bacteria after the high photosynthetic hours. According to this, it has previously been postulated that the release of photosynthate by phototrophs may stimulate daytime sulfate reduction (Fründ and Cohen, 1992).

The use of clustering techniques, such as the UPGMA, using the divergence index (D) in PLFA data, was applied with the aim of identifying the samples which generate similar patterns (Ibekwe *et al.*, 2001; Boon *et al.*, 2002). This analysis showed that depth-related differences have a greater influence than temporal changes, and may indicate that the PLFAs of the mat populations are not changing in a period of several hours but it may be PLFA profiles related with established microniches.

In this study, we applied the Shannon-Weaver and Simpson indices of diversity to 16S rDNA DGGE and PLFA data from total community. This approach has been successfully used by Eichner *et al.* (1999). Those authors noted that the number and intensity of DGGE bands do not equal the abundance and number of species, due to the 16S rDNA-based phylogeny problems and to bias inherent to PCR amplification of complex template mixtures (Wintzingerode *et al.*, 1997). The H' and D diversity indices reported similar values in all samples which suggested a stable maintenance of a

structurally diverse microbial community. The slightly decrease in the diversity values at 3:00 pm between 4.5 to 5.5 mm depth might indicate a stratification of the community because of the establishment of opposing gradients of oxygen and sulfide (chemocline). The calculations of diversity indices with PLFA and DGGE data can give misleading results and the sensitivity and differences in biomass must be controlled. They measure the most abundant part of the microbial community because they consider that part of the distribution above the analytical sensitivity cut-off point (Hedrick *et al.*, 2000). The major limitations of PLFA data for the measurement of diversity is that many species have similar PLFA profiles and that PLFA profile is reflective of the species that contribute with high proportion to the PLFA detected (as it has been mentioned for DGGE data). For this reason, new statistical approaches have been proposed as an alternative method (Hughes *et al.*, 2001; Fromin *et al.*, 2002).

The results of this work indicate that ‘microniches’ created by differences in physicochemical and biotic factors in microbial mats support the microbial diversity observed. Stable conditions are generally associated with increased diversity, while unpredictable or disturbed environments lead to the outgrowth of dominant, adapted populations (Torsvik *et al.*, 2002; Haack *et al.*, 2004). Indeed, this extremely dynamic community sustains a functional stable ecosystem and the large number of minority populations (not detected by molecular methods) may contribute significantly to this dynamic (Fernández *et al.*, 1999). The ecological success of this kind of ecosystems might be due to the versatility and plasticity of closely related populations that occupy similar microniches in the ecosystem (Casamayor *et al.*, 2002).

In conclusion, the combined analysis based on PLFA and DNA data provides a better understanding of major spatial and temporal shifts in microbial community structure, and support the model of microbial mats as dynamics ecosystems in which vertical migrations and physiological adaptations occur through day-night cycles.

Conclusions

- The quantification of total PLFA reported a higher viable biomass in the top of the mat in the morning, and in the middle layers at 3:00 pm, which can be due to the migration of cyanobacteria towards the top of the mat in order to avoid the toxic exposure to sulfide and to an increase of aerobic heterotrophs associated in the photic zone, respectively.
- Vertical profiles indicate a slowest growth rate in the bottom layers at 8:00 am. This fact can be related with an increase in the activity of sulfate-reducing bacteria and fermentative bacteria with lack of certain nutrients. At 3:00 pm the reduction in the growth rate can be explained by active processes of photosynthesis and carbon fixation carried out by cyanobacteria and purple sulfur bacteria.
- In the morning, the upper layers of the mat reported a major contribution of aerobic heterotrophic bacteria (*Psychroflexus* sp., *Shingobacterium* sp., *Marinobacter* sp.) belonging to the *Cytophaga-Flavobacterium-Bacteroides* group and γ -Proteobacteria.
- Some DGGE bands reported a high similarity with the *Halanaerobium* genus. Important contributions of members of the phylum Bacteroidetes that can also act as fermentative bacteria and seem to be adapted to high salinities have also been assessed. Moreover, the detection of several bands related to *Chloroflexus*-like species suggests an important role of this genus in microbial mats.
- The analysis of the divergence index (D) with PLFA data showed that depth-related differences have a greater influence than temporal changes, and that the PLFA profiles are related with established microniches.
- The diversity indices reported similar values in all samples which suggested a stable maintenance of a structurally diverse microbial community. The slightly decrease in the diversity values at 3:00 pm between 4.5 to 5.5 mm depth might indicate a stratification of the community because of the establishment of opposing gradients of oxygen and sulfide (chemocline).

- Publications and communications
 - **Villanueva L., A. Navarrete, J. Urmeneta, D. C. White, and R. Guerrero.** 2004. Combined phospholipid biomarker-16S rRNA Gene denaturing gradient gel electrophoresis analysis of bacterial diversity and physiological status in an intertidal microbial mat. *Appl. Environ. Microbiol.* **70**:6920–6926

 - **Villanueva L., A. Navarrete, J. Urmeneta, and R. Guerrero.** Combined analysis of signature lipid biomarkers and 16S rDNA-DGGE for the study of the biodiversity and physiological status of microbial mats”. XIX Congress of the Spanish Society for Microbiology. Santiago de Compostela, Spain, September 2003. Poster.

 - **Villanueva L., A. Navarrete, J. Urmeneta, D. C. White, and R. Guerrero.** Daily variations of bacterial diversity and physiological status in an estuarine microbial mat”. 9th International Symposium for Microbial Ecology (ISME-9), Cancún (Mexico), August 2004. Poster.