Tesi doctoral presentada per En/Na

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"Ecophysiological and molecular characterization of estuarine microbial mats"

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Facultat de Biologia
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UNIVERSITAT DE BARCELONA

VI. ECOPHYSIOLOGICAL VARIATIONS DURING A CIRCADIAN CYCLE

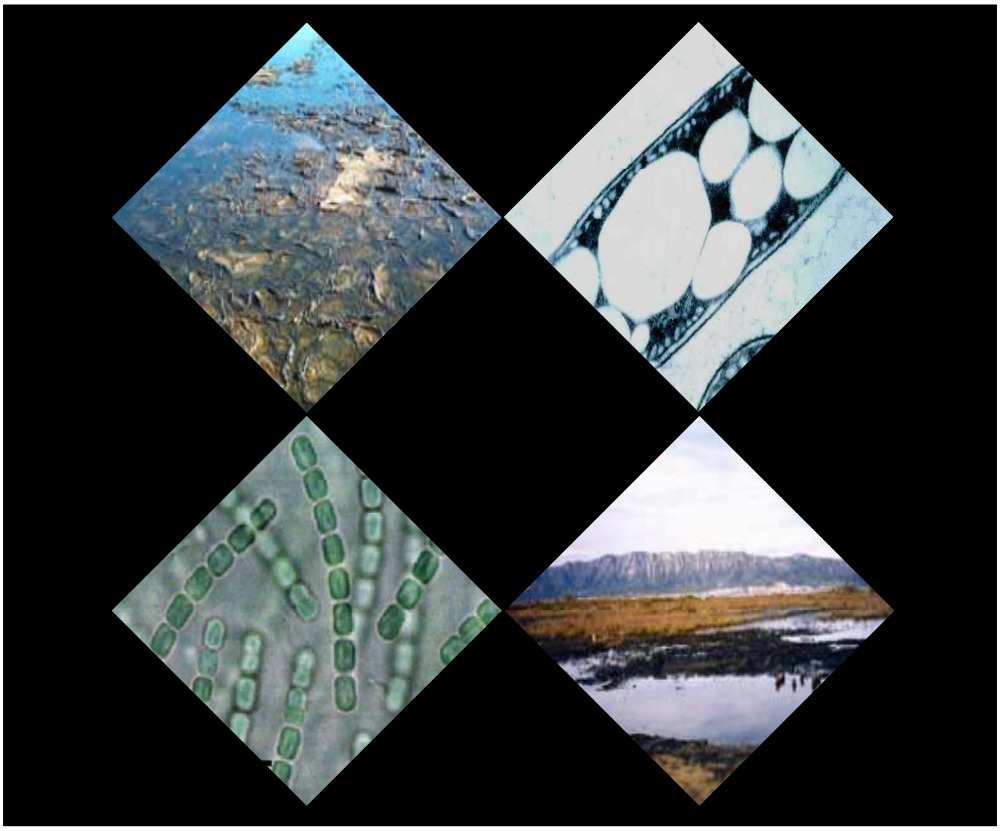


Figure VI. "Results rarely specify their causes unambiguously. If we have no direct evidence of fossils or human chronicles, if we are forced to infer a process only from its modern results, then we are usually stymied or reduced to speculation about probabilities. For many roads lead to almost any Rome" Stephen Jay Gould (1941-2002).

From top left to right: Ebro delta microbial mat / Bacterial accumulation of polyhydroxybutyrate in intracellular granules / Filamentous cyanobacteria isolated from Ebro delta mats / 'La Banya' spit, sampling site of Ebro delta microbial mats.

- Introduction and objectives of the study

The community composition and the effects of environmental stresses on microbial mats are still questions of interests in the study of this kind of ecosystems. One of the aims in microbial mat research is determine if they are stable and predictable environments and follow their dynamics in natural conditions. Studies performed with pure cultures isolated from natural ecosystems complement *in situ* measures but they do not yield insights about diversity, evenness and interactions between microbial populations. For this reason, the application of combined techniques and interdisciplinary studies are needed.

The use of signature lipid biomarker (SLB) analysis in microbial ecology provides valuable information about microbial communities in natural ecosystems, such as microbial abundance, community structure, and nutritional status. The determination of total PLFA provides a quantitative measure of the viable or potentially viable biomass (Vestal *et al*., 1989). The presence of certain groups of microorganims can be inferred by the detection of unique lipids. For example, specific PLFA are prominent in microbial groups or even in certain genera (e.g. specific PLFA of *Desulfovibrio* sp.). In this study, the analysis of other lipids such as plasmalogens-derived dimethylacetals (DMA) found in Clostridia as well as some gram-negative bacteria (Moore *et al*., 1994), and sphingoid bases have provided a more detailed community composition vision.

Microbial physiology can be monitored through the application of signature lipid biomarker (SLB) analysis. In this sense, phospholipid fatty acids (PLFA) are products of biosynthetic pathways and reflect the phenotypic response of microorganisms to environmental conditions. For example, specific patterns of PLFA can indicate physiological stress in certain bacterial populations, e.g. cyclopropyl monoenoics and *trans*/*cis* monoenoic ratios (Guckert *et al*., 1986; Heipieper *et al*., 1992). In addition, other lipid classes can be quantified as indicators of the physiological status of the community, e.g. bacterial poly-β-hydroxyalkanoates (PHA) that are accumulated under situation of unbalanced growth.

Moreover, the analysis of respiratory quinone composition can be used to indicate redox state of the microbial community as well as being a good indicator of microbial biomass and community composition (Hedrick and White, 1986; Hiraishi 1999; Hiraishi *et al*., 2003). The SLB approach can provide a powerful integrating and comprehensive determination of ecological succession and physiological adaptation in microbial mats. Previous studies have applied this approach in microbial mat samples in order to assess their community composition and metabolic state (Navarrete *et al*., 2000; Navarrete *et al*., 2004). However, these studies have been only focused on the analysis of the polar lipid fraction.

This study was undertaken to gain insight on the dynamics of an estuarine microbial mat during a daily cycle, in order to evaluate the responses of metabolic processes at the community level. For this reason, the lipid biomarkers included in the polar lipid, glycolipid and neutral lipid fractions of the SLB approach have been detected and quantified.

The aim of this study was (i) the monitoring of microbial biomass and changes during a circadian cycle in estuarine microbial mats, (ii) to determine situations of starvation, unbalanced growth, stress and redox conditions, (iii) to analyze the microbial community composition by PLFA, quinone content and presence of lipids biomarkers characteristics of certain groups, and (iv) to isolate representatives strains of sulfate-reducing bacteria and anaerobic Gram-positives from mats and analyze the presence of dimethylacetals in their membranes. This study was performed as a result of the previous validation of the sequential fractionation protocol and polyhydroxybutyrate/polyhydroxyvalerate (PHB/PHV) detection performed in the chapter III.

- Material and methods

➢ Sampling and physiochemical conditions

Mat samples were collected in Fall 2002, over a day–night cycle. The upper part of the mat was sampled (two cores at each time every 3 hours starting at midday (12:00, 15:00, 18:00, 21:00, 24:00, 3:00, 6:00, 9:00, and 12:00 B of the following day, GMT, using a 1.5 cm inner-diameter corer. The samples were lyophilized as described in the chapter 'General Material and Methods'. Physicochemical conditions of the overlaying water and solar irradiation were analyzed at each sampling time (conductivity and sulfide concentration.

➢ Lipid analysis

Samples were extracted with 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 neutral, glyco-, and polar lipid fractions were obtained. The polar lipid fraction was treated by the sequential fractionation protocol described in the 'Polar lipid fraction' section. The polar lipid fraction was transesterified to fatty acid methyl esters (FAMEs by mild alkaline methanolysis in the step A. Then, the remaining aqueous phase was treated to release plasmalogen ethers as dimethylacetals (DMAs, step B. The remaining phase obtained from the step B was hydrolyzed under strong acid conditions (step C, and finally the organic phase was treated by methanolysis and then BSTFA-derivatized in order to detect the amide-linked hydroxyl fatty acids and the sphingoid bases (step D. In this study, only the FAMEs, DMAs, and the compounds recovered form the step D were analyzed. The polar lipid fraction of the duplicate samples from the 12:00B sampling time were lost during the analyses; for this reason, only the quinone and PHA results of this point are included in this study.

The poly-β-hydroxyalkanoates (PHB, polyhydroxybutyric acid; PHV polyhydroxyvaleric acid included in the glycolipid fraction were hydrolyzed and MBSTFA derivatized following the protocol described by Elhottová *et al*. (2000 and described in the section 'Glycolipid fraction analysis' of the chapter 'General Material and Methods'. The neutral lipid fraction was examined for respiratory ubiquinone and

menaquinone isoprenologues by liquid chromatography-Tandem mass spectrometry (LC/MS/MS as it was previously described in the 'Neutral lipid fraction analysis' experimental design.

Ubiquinones, menaquinones, demethylmenaquinones, and plastoquinones with *n* isoprene units in their side chain were abbreviated as Q-*n*, MK-*n*, DMK-*n* and PQ-*n* respectively. Phylloquinone was abbreviated as K_1. Reproducibility of quinone analysis was within ±5% (data obtained from $n = 4$ replicates of microbial mat sample cores obtained at the same sampling time. The %mol was defined as picomoles of certain quinone homolog in a sample divided by the picomole sum of all measured quinones set to 100% in that sample. The microbial divergence index of ubiquinones and menaquinones (*MD* ub+mk and the bioenergetic divergence index (*BD* ub+mk (Iwasaki and Hiraishi, 1998; Hiraishi, 1999 were calculated as indicated in the 'chapter V'. *MD* ub+mk values represent the divergence of ubiquinone and menaquinone structural types, and *BD* ub+mk indicates the balance of ubiquinone-mediated aerobic respiration, and menaquinone-mediated anaerobic and aerobic respiration.

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. The detection and quantification of poly-3-hydroxyvaleric acid was previously validated as it was described in the chapter III.

- Screening and characterization of sulfate reducers and anaerobic Gram-positives

The isolation of sulfate-reducing and anaerobic spore-forming bacteria was performed as it was previously described in the section 'Morphobiochemical characterization' of the chapter 'General Material and Methods'. The isolated strains were characterized by means of scanning and transmission electron microscopy, and by sequencing of the 16S rDNA molecule ('Nucleic acid analysis methods'. The total lipid fraction was extracted from lyophilized pure cultures and the polar lipid fraction was obtained after silicic acid chromatography ('Lipid analysis methods'. The polar lipid fraction was treated by the sequential fractionation method to analyze the fatty acid methyl esters (FAMEs, step A, and the dimethylacetals (DMAs derived from plasmalogens (step B. The neutral lipid fraction was analyzed for quinone composition.

- Results

➢ Physicochemical conditions

Starting at 12:00, light intensity (Fig. VI.1 measured at the sampling point decreased reaching a minimum value from 21:00 to 6:00 am. Conductivity values were rather stable (25,000–27,500 microsiemens per cm with slightly fluctuations during the day/night cycle. Sulfide reached its maximum value at 6:00 am (18 μM and it was practically undetectable during the rest of the day (Fig.VI.1 . In fact, sulfide production during the day was neutralized by the spontaneous reaction with dissolved oxygen and by consumption by anoxygenic photosynthetic bacteria (Navarrete *et al*., 2000 .

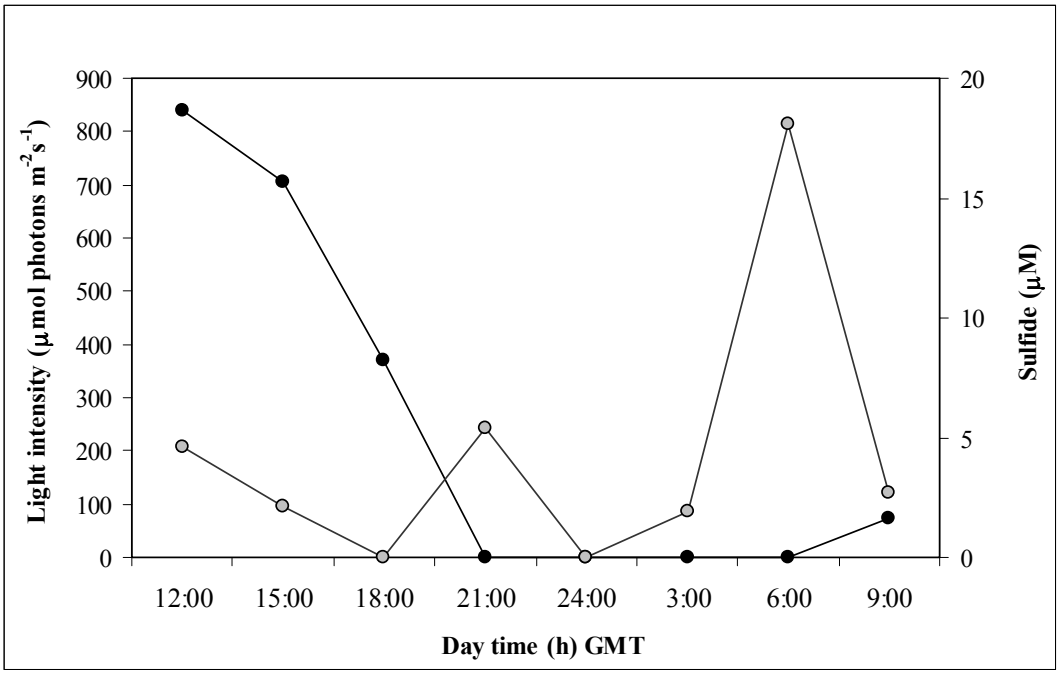


Figure VI.1. Physicochemical conditions in the overlying water of Ebro delta microbial mats.

Black dots: Light intensity given as micromoles of photons per square meter and second; Grey dots: Sulfide concentration in micromolar.

➢ Microbial biomass

Microbial biomass in terms of total PLFA (Fig. VI.2) ranged from 7.7×10^3 to 2.7×10^4 pmol PLFA per gram of dry weight. The minimum value of total PLFA was found at 18:00 (7.7×10^3 pmol g^{-1}) and the maximum value at 15:00. Then, the PLFA values were similar with 8.9×10^3 to 1.3×10^4 pmoles PLFA g^{-1} from 21:00 to 9:00 am of the following day. Microbial biomass as dimethylacetals derived from plasmalogens (found in Clostridia, close relatives and some gram-negative bacteria; Moore *et al*., 1994) were also quantified. The DMA content was higher at 18:00 (3.5×10^3 pmol DMA g^{-1} dry weight). The DMA biomass showed the same trend as the PLFA biomass profile except from a lag time at the maximum peaks (Fig. VI.2).

Hiraishi *et al*. (2003) demonstrate that there is a strong positive correlation between total bacterial counts and total quinones in a broad range of environments. In this study, the quantification of total quinones as pmol g^{-1} reported minimum values at 3:00 (1.3×10^4) and a maximum quantification at 15:00 (4.4×10^4) (Table VI.1).

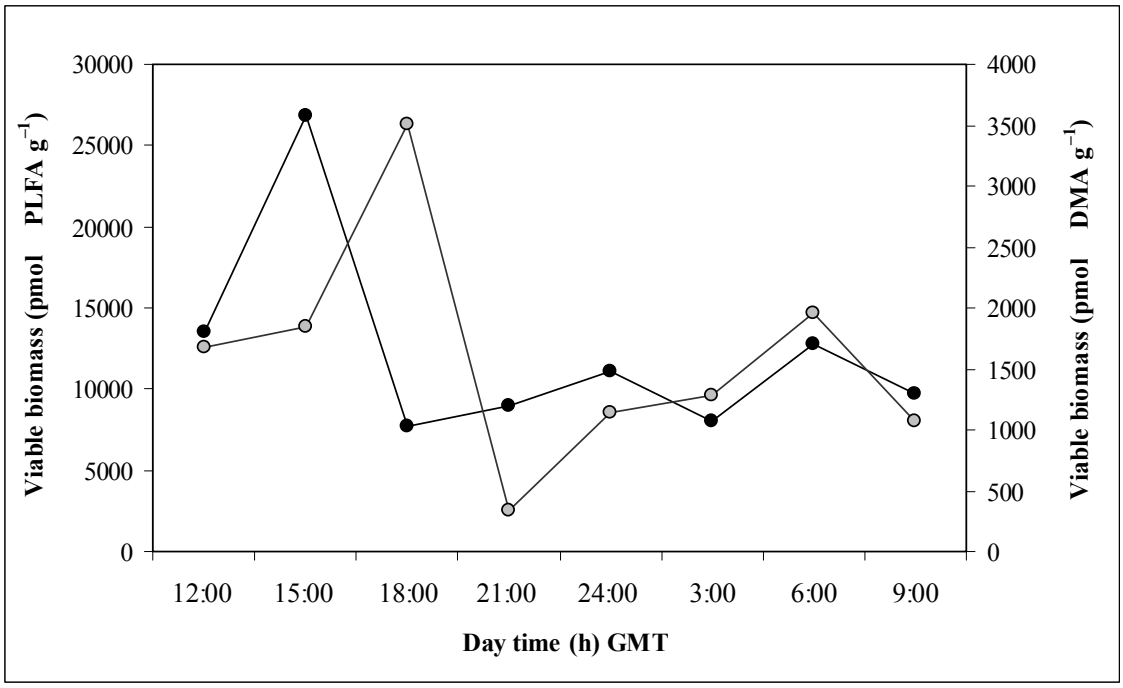


Figure VI.2. Viable microbial biomass in terms of total PLFA and DMAs.

Black dots: picomoles of phospholipids fatty acids (PLFAs) per gram of dry weight; Grey dots: picomoles of dimethylacetals (DMAs) per gram of dry weight.

- Community composition

Figure VI.3 and Table VI.1 indicate the results of the community composition by PLFA. The community consisted mainly of Gram-negative bacteria (including cyanobacteria) at 12:00 and 15:00, as confirmed the presence of a high percentage of monoenoic PLFA (53.4% and 50.6% respectively) (Wilkinson, 1988), which comprised more than 50% of the total PLFA. Maximum values of PLFA indicative of Gram-negative microorganisms were found at 12:00 and minimum values at 18:00 (18.3%). From 12:00 to 15:00 sampling times, terminally branched saturated fatty acids, PLFA typical of Gram-positive bacteria, increased slightly (4.1% to 9.3%) and lipids representative of anaerobic microorganisms (branched monounsaturated and mid-chain branched saturated) decreased (29.4% to 22.2%).

At 18:00, an inversion in the profile dominated by Gram-negative bacteria was observed, showing an increase of PLFA typical of anaerobic bacteria (56.5%) in comparison with the rest of the sampling times. Moreover, at this sampling time, a maximum of DMA, common in strict anaerobes as *Clostridium* sp. and also in soil bacteria grown under anoxic conditions, was detected (Fig. VI.2). Apart from that, lipids representative of microeukaryotes (polyenoic PLFAs) and Gram-positive bacteria (terminally branched saturated PLFA) remained stable from this sampling time. Relative proportions of polyenoic fatty acids (polysaturated), representative of microeukaryotes and cyanobacteria (18:2ω6) were constant at all sampling times. Normal saturated fatty acids, present in both *Eukarya* and *Bacteria*, were constant in all the sampling times except from 12:00 (4%) in which the percentage was lower.

The analysis of specific FAMEs, that are markers of certain microbial groups (Table VI.2), indicated that 16:1ω5c from methanotrophs and *i*15:0/*a*15:0 indicatives of sulfate-reducing bacteria (Findlay and Dobbs, 1993; White *et al*., 1997), reported higher percentages at 15:00. In addition, 18:2ω6 PLFA indicative of cyanobacteria reported high percentages at 12:00, 15:00 and 24:00. The polar lipid samples treated for the quantification of FAMEs also monitor the presence of phytol, a terpenoid lipid (see 'I. Introduction', section 'Classification of lipids') previously reported in microbial mats as the major isoprenoid alcohol (Ward *et al*., 1989). Chlorophyll-*a* from oxygenic

phototrophs (e.g. cyanobacteria) is presumably the most abundant source of phytol, and several studies has proposed the role of denitrifying bacteria in the mineralization of this compound in microbial communities (Rontani *et al.*, 1999).

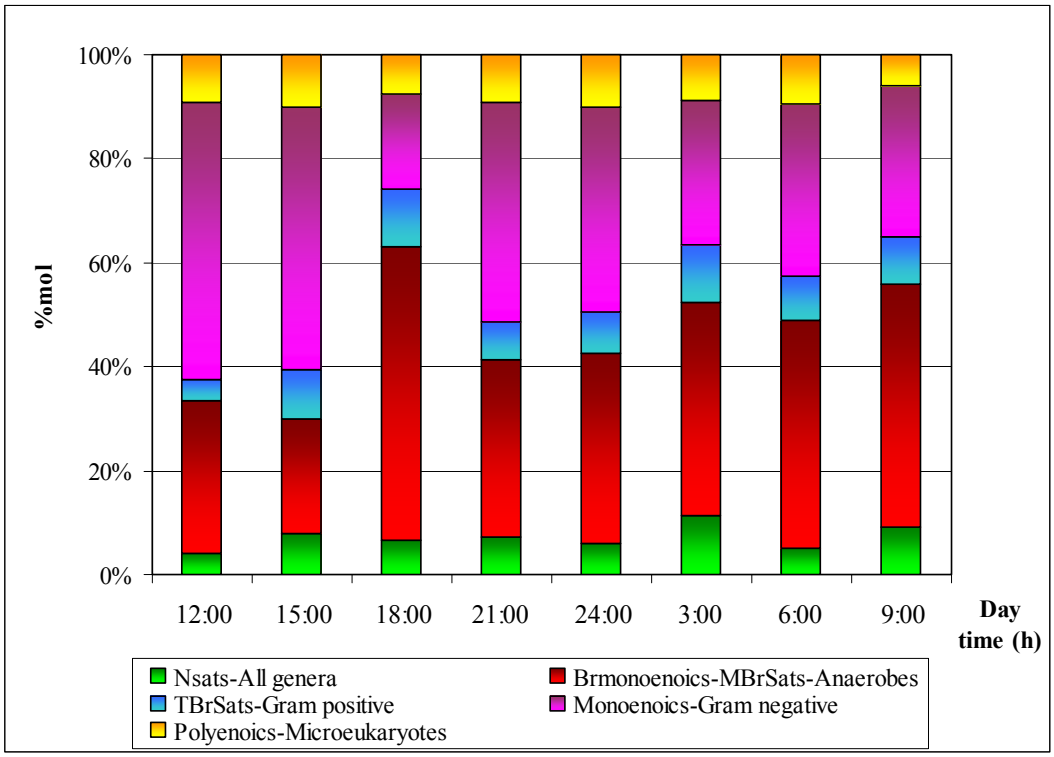


Figure VI.3. Community composition by PLFA analysis (%mol).

Table VI.1. Community composition as picomoles of PLFA per gram of dry weight.

	12:00	15:00	18:00	21:00	24:00	:00	6:00	9:00
Nsats	538.55	2116.85	512.70	655.05	678.18	916.42	644.82	900.95
BrM-MBrSats	3996.37	5957.52	4330.04	3043.75	4012.88	3316.48	5614.96	4509.22
TBrSats	557.71	2490.21	830.58	641.31	893.71	885.64	1073.54	913.97
Monoenoics	1010.60	1447.01	1176.88	566.02	859.01	787.61	1404.47	1133.51
Polyenoics	1236.33	2701.64	581.76	827.18	1099.13	723.72	1216.74	594.54

Nsats: Normal saturated, TBrSats: Terminal branched saturated, Brmonoenoics: branched monoenoics.

Table VI.2. Lipid biomarkers quantification as picomoles per gram of dry weight and %mol.

Biomarker	12:00	15:00	18:00	21:00	24:00	□:00	6:00	9:00
Phytol (pmol g^{-1})	1232.36	1380.39	2811.56	747.34	1346.22	2991.89	2997.51	1923.62
FAME (%mol)								
16:1ω5*c*	0.26	0.49	0.29	0.37	0.20	0.42	0.24	0.24
***i*15:0**	0.81	2.05	1.50	1.22	0.99	2.12	1.23	1.48
***a*15:0**	0.81	1.87	1.63	1.32	0.94	2.34	1.07	1.23
18:2ω6	4.11	4.58	1.21	3.77	4.27	2.23	3.77	1.89

As it has been previously mentioned in 'chapter V', quinone isoprenologs can be used for the classification of microorganisms (Collins and Jones, 1981□. Quinone composition and changes in their profile during the day/night cycle are summarized in Table VI.3 and Fig. VI.4. Among ubiquinones, Q-10 reported the highest percentage at 12:00, 15:00, and 21:00 and then Q-8 was predominant at the rest of the sampling times (between 14–18 %mol□. Q-9 was also important among ubiquinones and showed similar %mol at all times (2–3 %mol□. Based on chemotaxonomic studies, β-Proteobacteria, γ-Proteobacteria, and α-Proteobacteria, were considered as possible major sources of Q-8, Q-9 and Q-10 respectively. Menaquinone-9 reported the highest %mol among quinones (32–57 %mol□and it can be found in members of the *Firmicutes*, *Actinobacteria* and *Bacteroides* groups. MK-7, MK-8 and MK-10 also reported important relative percentages in all samples, and they have been detected in *Actinobacteria*, *Cytophaga-Flavobacteria,* δ-Proteobacteria, *Firmicutes* and *Euryarchaeota* (MK-7 and MK-8□, and in green non-sulfur bacteria (MK-10□; however, MK-4, MK-5 and DMK reported low relative percentages.

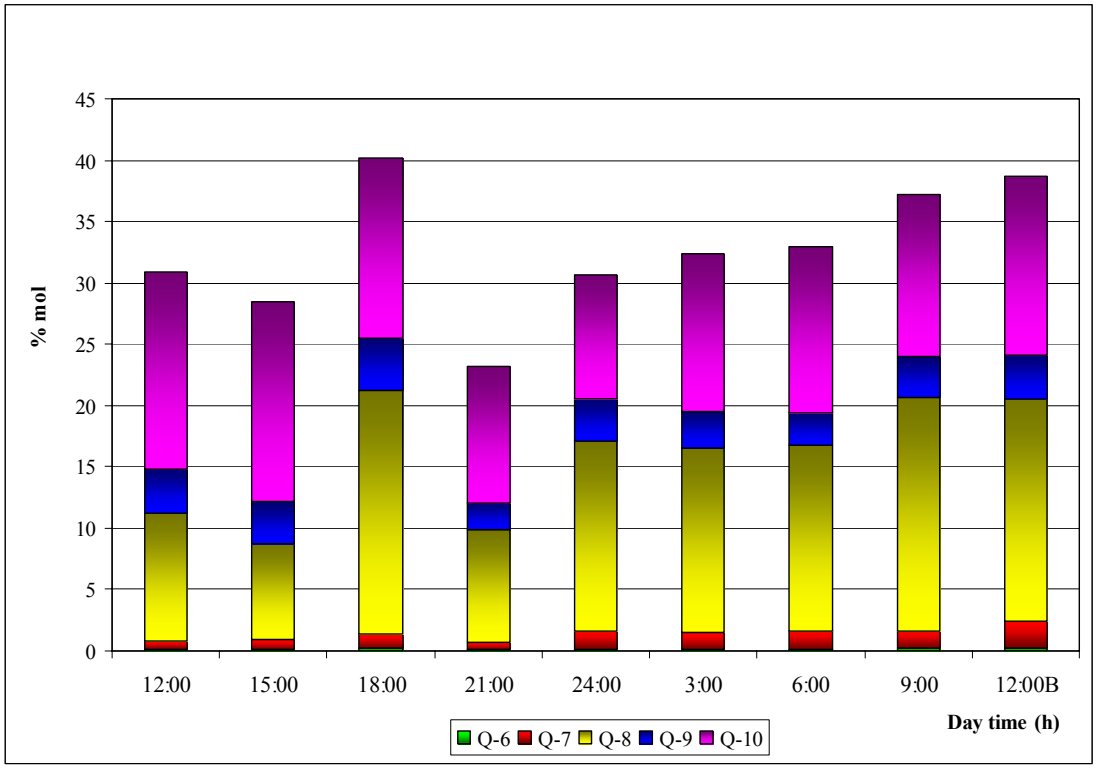
The quantification of the detected quinones is indicated in Table VI.3. Q-8, Q-9, and Q-10 showed an important decrease during the night and then a progressive increase at the beginning of the following day. Apart from that, MK-6, which is found in *Cytophaga-Flavobacterium*, δ- and ε-Proteobacteria, was higher from 12:00–18:00 and then decreased during the night showing the same increase trend as described above. The highest quantification of MK-7 was observed at 15:00 but the rest of the values were similar. In addition, MK-8 showed higher values at 12:00 and 15:00, as

well as MK-9 and MK-10. At 18:00, a decrease of all quinones was reported except from Q-8 that showed a slightly increase. Indeed, a dramatic decrease of MK-9 was observed at this sampling time. Then, a progressive decrease was reported with a minimum value at 9:00, and finally similar values, in comparison with the previous day, were reached at 12:00B. Regarding the total quinone content as picomoles per gram of dry weight, the minimum values were observed during the night (3:00 because of an important decrease of all quinone homologues (except from the photosynthetic quinones from 24:00 to 3:00.

Table VI.3. Quinone content as picomoles per gram of dry weight in Ebro delta mats.

Homolog	12:00	15:00	18:00	21:00	24:00	:00	6:00	9:00	12:00 B
Q-6	43.5	55.6	35.9	18.4	26.0	16.4	26.4	27.8	59.6
Q-7	200.3	290.6	219.2	98.2	248.0	142.3	220.5	213.8	662.8
Q-8	3089.1	3101.6	3595.2	1476.9	2652.3	1600.3	2349.2	2880.9	5549.8
Q-9	1036.8	1370.6	768.1	354.3	585.3	316.6	410.5	501.8	1100.5
Q-10	4753.0	6410.2	2647.7	1805	1736.2	1351.4	2102.5	2008.5	4444.7
MK-4	83.5	112.6	43.7	47.7	36.1	40.2	65.2	56.3	93.2
MK-5	125.1	176.2	88.4	71.2	83.9	51.6	84.0	76.4	130.1
MK-6	683.4	826.9	645.7	367.4	435.2	358.1	555.8	526.7	957.7
MK-7	1617.4	2428.4	1759.6	903.1	1708.5	880.4	1696.2	1457.1	3620.9
MK-8	2118.1	2835.5	1806.8	1104.6	1391.5	877.9	1243.9	1260.8	2671.5
MK-9	15208.3	20900.8	6075.3	9869.4	8327.2	4971.5	6685.0	6141.0	11200.6
MK-10	2243.4	3278.3	1260.7	1106.1	848.1	562.6	908.9	733.4	1566.0
DMK-8	36.3	56.3	30.3	26.5	24.1	25.9	27.1	24.7	35.7
K_1+PQ-9	2404.6	2607.2	2338.1	2030.6	2594.1	2042.6	2211.9	2324.5	2426.1
TQ[1]	33643.1	44451.0	21314.7	19279.5	20696.5	13237.9	18587.2	18233.7	34519.4

[1]TQ: Total quinones.



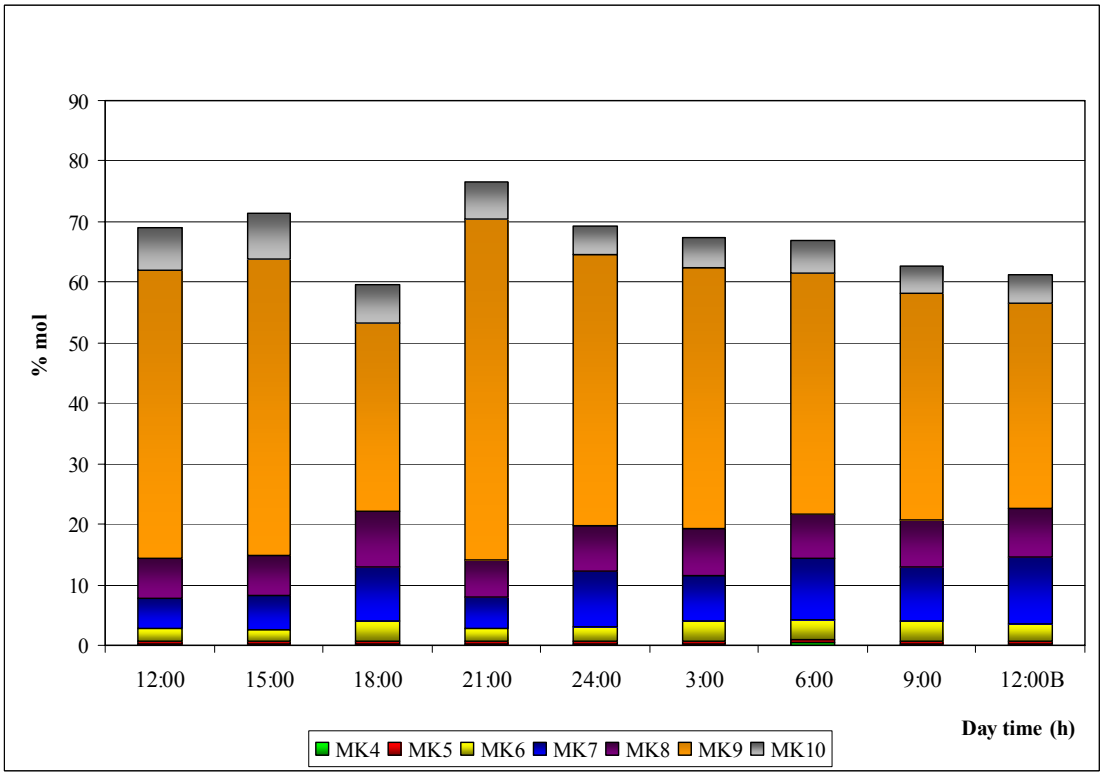


Figure VI.4. Ubiquinone (Q□and menaquinone (MK□composition of Ebro delta mat samples.

Quinone content is given as %mol. Reproducibility of quinone analysis was within ±5%.

Demethylmenaquinones (DMKs), only found in some gram-negative facultative anaerobic rods mainly pathogenic (Collins and Jones, 1981), were almost undetected and only DMK-8 was quantified in the samples. In addition, the photosynthetic quinones (plastoquinone-9 and phylloquinone) showed similar values in all sampling times and represent a considerable amount of the total quinone content. Furthermore, the microbial divergence index of ubiquinones and menaquinones (*MD* ub+mk) was calculated for all samples (see Table VI.8). *MD* ub+mk represents the divergence of ubiquinone and menaquinone structural types detected and it can be used as an indicator of microbial diversity. In this case, the *MD* indices were similar in all samples but the maximum value was detected at 18:00.

Apart from phospholipids fatty acids and respiratory quinones, other lipid classes can be used as biomarkers to estimate the community composition in environmental samples (see chapter 'I. Introduction'). In this study, the sequential protocol was also applied to detect and quantify sphingoid bases and amide-linked hydroxyl fatty acids. Long chain sphingoid bases (LCB) were detected in all samples but, due to the co-elution problems mentioned in 'chapter III', the quantification was not possible. The LCB reported were C18:0 (dihydrosphingosine), C19:0, and C21:1. The strong acid hydrolysis performed in the step C of the sequential protocol, broke the amide-bond and the hydroxy fatty acids amide-linked were released and recovered in the step D. In this case, 3-hydroxy 16:0 (3OH 16:0), 3OH 17:0 and 3OH 18:0 were quantified in Ebro delta samples as detailed in Table VI.4. The lower values were observed at 18:00.

Table VI.4. Hydroxy fatty acids as picomoles per gram of dry weight.

Hydroxy fatty acids	12:00	15:00	18:00	21:00	24:00	:00	6:00	9:00
OH 16:0	5.22	7.40	2.47	4.60	6.27	10.69	8.27	70.89
***i*OH17:0**	5.84	7.99	3.69	6.39	5.12	16.34	11.92	73.53
OH18:0	4.86	5.77	1.99	3.90	7.03	12.10	5.28	75.41

The screening of sulfate-reducing bacteria and anaerobic spore-forming in Ebro delta microbial mats led to the isolation of one strain capable of growing onto 'sulfate-reducing agar' plates and other on 'SPS agar' (Fig. IV.5 and 6□ Pure cultures were obtained and the 16S rDNA molecules were sequenced. The similarities of the isolated strain 16S rDNA molecules with their closest relatives are shown in Table VI.5. In addition, the quinone and cellular fatty acids composition were determined (Table VI.6, 7□ The phenotypic and molecular similarities of the isolated strains allowed their classification as members of the *Desulfovibrio* sp. and *Clostridium* sp. genuses respectively, and we proposed the names *Desulfovibrio* sp. EBD and *Clostridium* sp. EBD.

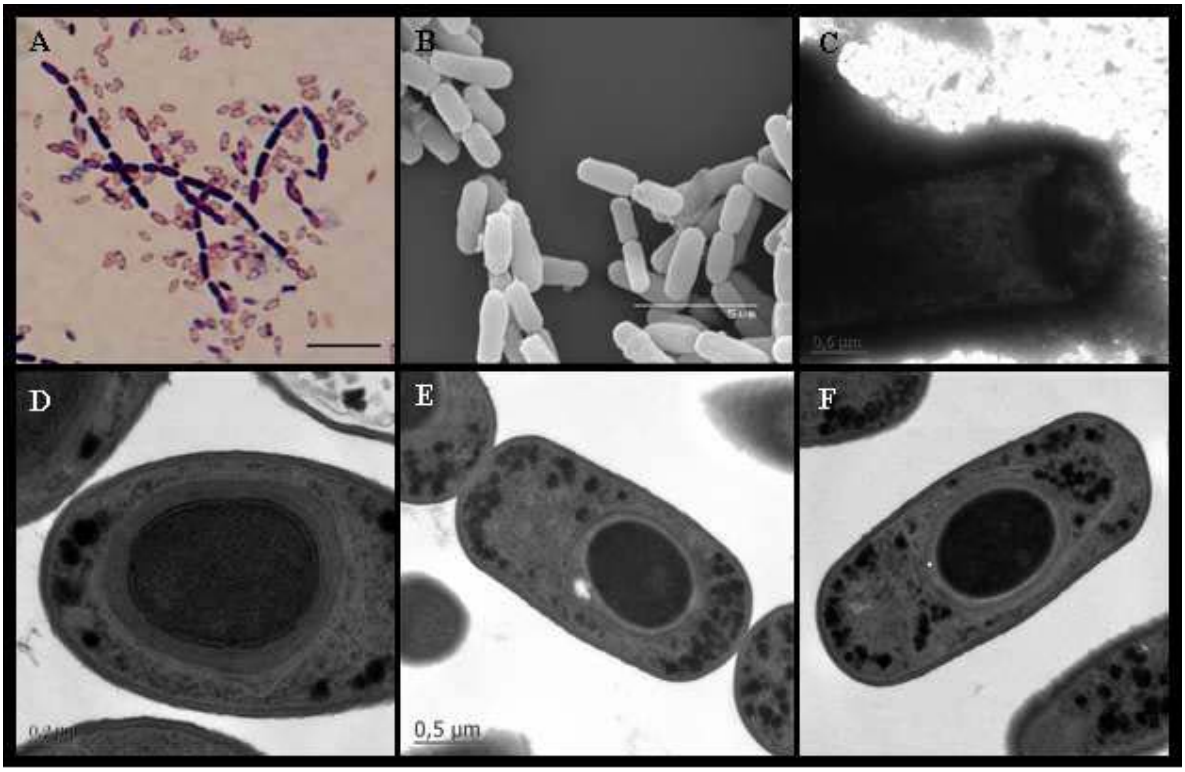


Figure VI.5. Micrographs of the isolated *Clostridium* sp. EBD.

(A□Gram-staining; (B□Scanning electron micrograph showing the association in pairs or in chains; (C□Negatively stained section showing the rest of flagella; (D–F□Transmission electron micrographs showing the central spore and the distended cell wall.

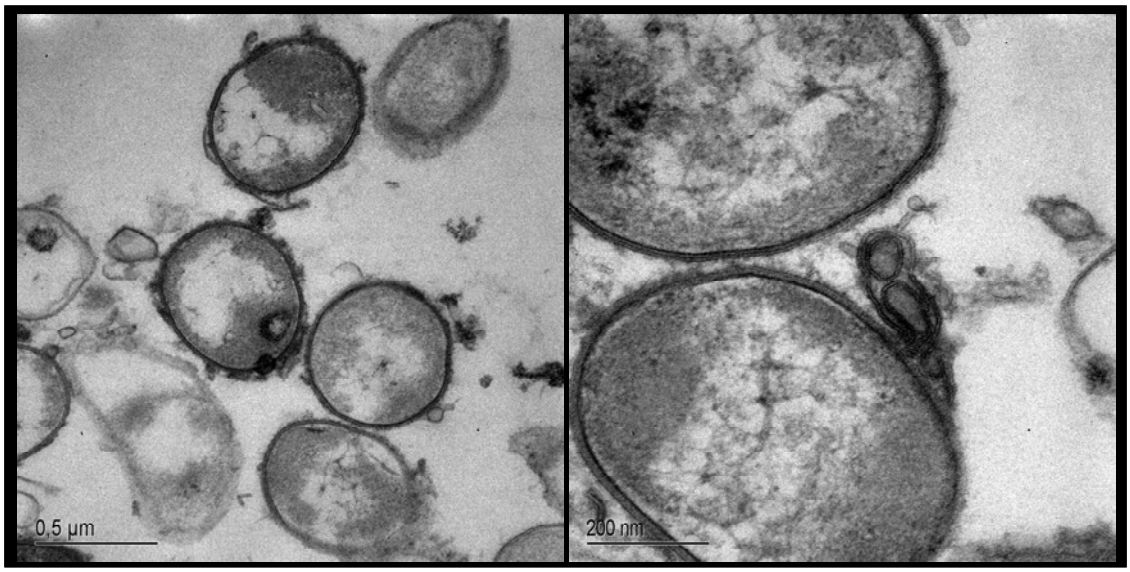


Figure VI.6. Transmission electron micrographs of the isolated *Desulfovibrio* sp. EBD.

Cross-section of *Desulfovibrio* sp. EBD cells and rest of the flagella (right

Table VI.5. Similarity between the 16S rDNA of the isolated strains and closest relatives.

Strain	Accession number	Similarity (%)	Closest relative
***Clostridium* sp. EBD**	DQ218319	99	*Clostridium bifermentans* (AF320283
***Desulfovibrio* sp. EBD**	DQ218320	98	*Desulfovibrio acrylicus* (U32578

Table VI.6. Fatty acid composition of the isolated strains.

Strain	Main fatty acids
***Clostridium* sp. EBD**	14:0, *a*15:0, *i*16:0, 16:1ω9*c*, 16:1ω7*c*, 16:0, 16:1*br*, *i*17:0, *a*17:0, *cy*17:0, 17:0, 18:1ω9*c*, 18:1ω7*c*, 18;1ω7*t*, 18:0, *cy*19:0, 19:1*br*
***Desulfovibrio* sp. EBD**	*cy*17:0, 17:0, 18:1*br*, Me18:0

The quinone content (Table VI.7) indicated a predominance of MK-6 (abundant in δ-Proteobacteria) and also and important contribution of MK-5 in *Desulfovibrio* sp. EBD. However, the predominant quinone in *Clostridium* sp. EBD was MK-9 but its content was four hundred times lower than the picomoles per gram of dry weight of the main quinone in *Desulfovibrio* sp. EBD. Moreover, the presence of dimethylacetals (DMA) derived from plasmalogens was only observed in the *Clostridium* sp. EBD sample as is detailed in Fig. VI.7, and the detected DMA had 16 carbons.

Table VI.7. Quinone content as picomoles per gram of dry weight of the isolated strain.

Strain	Q-6	Q-7	Q-8	Q-9	Q-10	MK-4
***Clostridium* sp. EBD**	70.52	9.50	15.06	74.86	0.48	40.99
***Desulfovibrio* sp. EBD**	36.12	73.02	28.77	900.37	2.02	665.97
Strain	**MK-5**	**MK-6**	**MK-7**	**MK-8**	**MK-9**	**MK-10**
***Clostridium* sp. EBD**	35.16	81.22	48.69	36.01	272.49	29.04
***Desulfovibrio* sp. EBD**	2087.96	106469.13	161.28	68.94	94.50	13.04

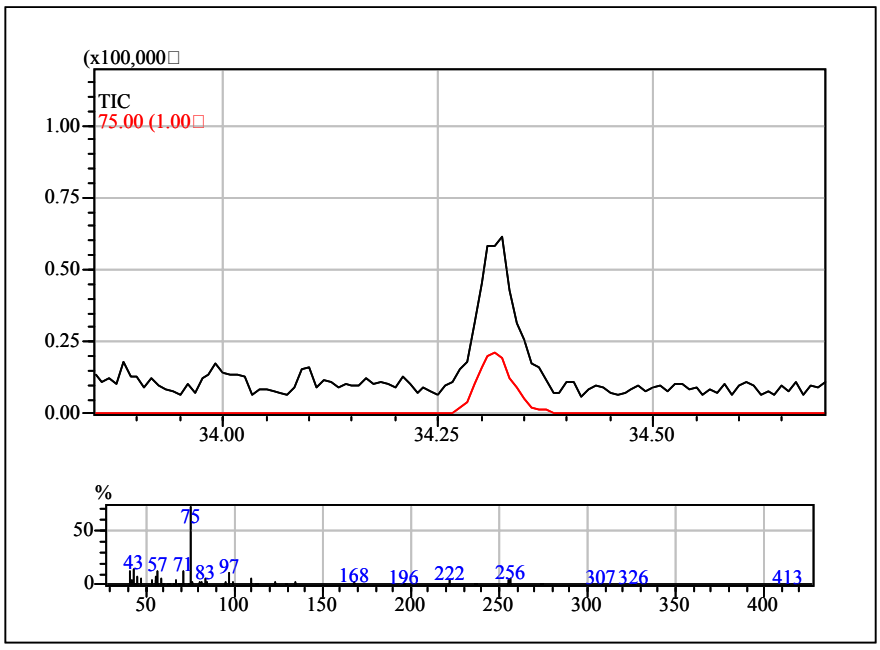


Figure VI.7. Detection of dimethylacetals (DMAs) in *Clostridium* sp. EBD.

Chromatogram and mass spectra of 16DMA (dimethylacetal with 16 carbons) with the base peak at *m/z* 75. The top part refers to the chromatographic separation and then, the corresponding mass spectrum of the peak is shown.

- Physiological and respiratory status

At 18:00 and 3:00, a decrease in the growth rate (high *cyclo*/ω7*c* ratio) and a shift to a higher metabolic stress (high *trans*/*cis* ratio) was observed (Fig. VI.8). Both ratios reported a similar trend during the day/night cycle. The *cyclo*/ω7*c* ratio ranged from 0.25 to 0.55 revealing a situation of exponential to stationary growth phase at each sampling time (*cyclo*/ω7*c* ratio higher than 0.05). As well as in *cyclo*/ω7*c* ratio profiles, the highest values of metabolic stress were located at 18:00 and 3:00. The *trans/cis* values ranged from 0.06 to 0.40 (at 3:00).

Data concerning PHA quantification is indicated in Fig.VI.9. PHV values were higher than PHB in all cases, and the PHV: PHB ratio has a mean value of 7:1. Moreover, the ratios PHB/PLFA and PHV/PLFA (molar ratio, pmol PHA g^{-1} dry weight divided by pmol PLFA g^{-1} dry weight) are indicated in Table VI.8. The ratio PHB/PLFA during the day/night cycle revealed changes in the PHB levels depending on the time of the day. Maximum value of PHB/PLFA ratio was approximately 14 at 18:00, and minimum value was around 5 at 12:00. PHV/PLFA revealed higher values than PHB/PLFA and reported the maximum value at 18:00 and the minimum at 15:00.

As it has been previously mentioned in the 'chapter V', quinone ratios indicate the redox state in the presence of Gram-negative aerobic, facultative or anaerobic bacteria. The UQ/MK ratio near zero indicates long-term exposure to strictly anaerobic conditions. Values below 1 indicate anaerobic conditions with a past history of oxygen availability (Polglase *et al.*, 1966; Geyer *et al.*, 2004), whereas values around 1 and above indicate an aerobic or microaerophilic environment. UQ/MK is considered to be proportional to the ratio of anaerobic to aerobic respiration (Peacock *et al.*, 2004). In this study, UQ/MK values below 1 in all cases were indicative of anaerobic conditions with oxygen availability. Although the values were similar in all the samples, a highest ratio was observed at 18:00 total which can be attributed to a more aerobic character. Besides, the ratio of total quinones to PLFA (Q/PLFA) is proportional to the ratio of respiration/ (respiration + fermentation). In this case, the Q/PLFA ratio ranged from 1.5 to 2.8 and reported a more respiratory activity (Q/PLFA) and a less anaerobic character (UQ/MK) in 12:00, 18:00 and 21:00 samples in comparison with the others.

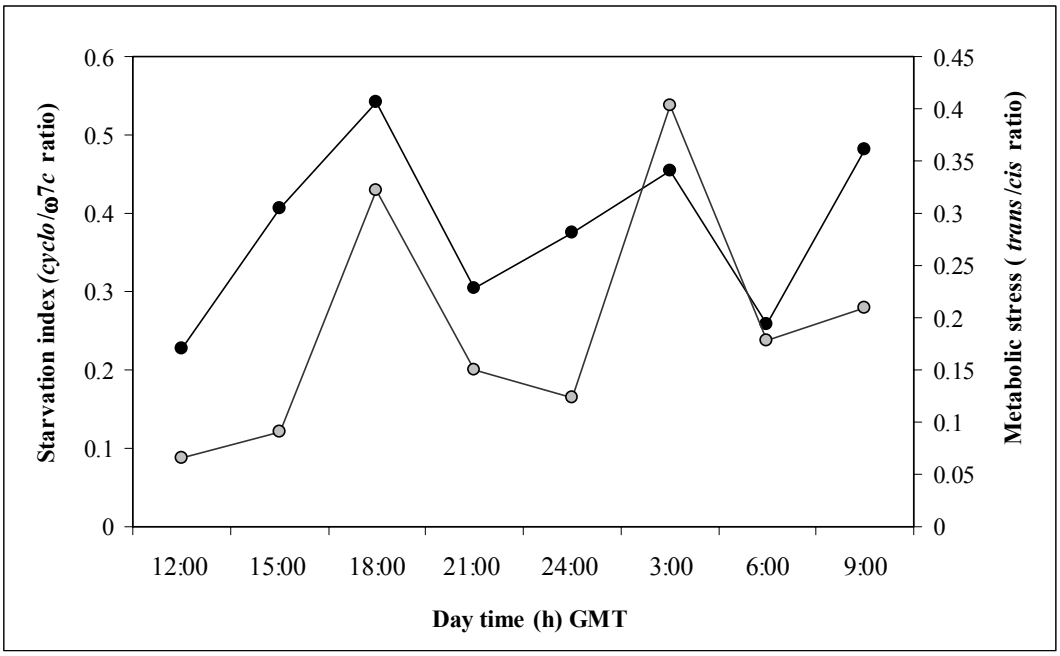


Figure VI.8. Physiological status by PLFA data.

Starvation index (black dots as ratio of cyclopropyl fatty acids to monoenoics (*cyclo*/ω7*c* ratio. Metabolic stress as *trans*/*cis* ratio of monoenoic PLFAs (grey dots.

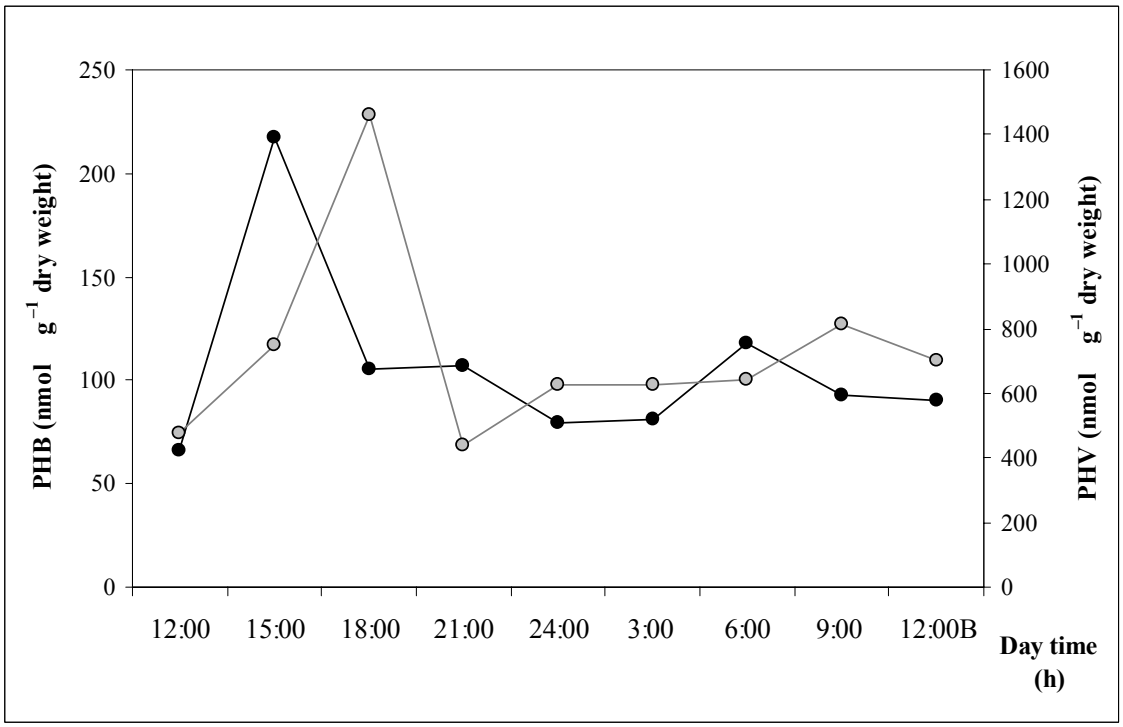


Figure VI.9. Polyhydroxybutyrate (black dots and polyhydroxyvalerate (grey quantification.

Table VI.8. Microbial biomarkers measured in the Ebro delta mat samples.

Sample	12:00	15:00	18:00	21:00	24:00	□:00	6:00	9:00
Biomass[1]								
Total PLFA	13570.44	26882.51	7668.70	8933.01	11045.57	8060.82	12772.44	9708.78
Total DMA	1675.64	1844.18	3513.86	344.08	1141.13	1284.81	1960.99	1073.33
Composition[2]								
Nsats	3.97	7.87	6.69	7.33	6.14	11.37	5.05	9.28
BrM-MBrSats	29.45	22.16	56.46	34.07	36.33	41.14	43.96	46.44
TBrSats	4.11	9.26	10.83	7.18	8.09	10.99	8.41	9.41
Monoenoics	53.36	50.65	18.43	42.16	39.49	27.52	33.06	28.74
Polyenoics	9.11	10.05	7.59	9.26	9.95	8.98	9.53	6.12
***MD* ub+mk**	758.88	754.25	863.32	708.12	789.99	812.52	831.31	833.15
Physiology[3]								
cyclo*/ω7*c	0.23	0.41	0.54	0.31	0.37	0.46	0.26	0.48
trans*/*cis	0.07	0.09	0.32	0.15	0.12	0.40	0.18	0.21
PHB/PLFA	4.87	8.07	13.77	11.96	7.19	10.11	9.26	9.60
PHV/PLFA	35.08	27.87	190.17	48.85	56.65	77.77	50.09	83.54
UQ/MK+DMK	0.41	0.37	0.62	0.28	0.41	0.44	0.45	0.55
***BD* ub+mk**	190.94	188.62	197.22	182.52	190.74	192.18	192.66	195.65
Q/PLFA	2.48	1.65	2.78	2.16	1.87	1.64	1.46	1.88
Conditions								
Sulfide, μM	4.59	2.16	0	5.4	0	1.89	18.1	2.7
Conductivity μS cm^{-1}	27600	26100	25200	26100	26150	27400	27200	27200
Light μE m^{-2} s^{-1}	840	705	370	0	0	0	0	72

[1]Biomass data is given as pmoles g^{-1} and DMA are dimethylacetals; [2]Community composition: NSats Normal saturated-All genera, BrM-MBrSats Branched monoenoics and Mid-chain branched saturated-Anaerobes, TBrSats Terminal branched saturated-Gram positives, Monoenoics-Gram negatives, Polyenoics-Microeukaryotes, *MD* ub+mk microbial divergence index of ubiquinones and menaquinones; [3]Physiological status: *cyclo*/ω7*c* and *trans*/*cis* ratios, PHB/PLFA polyhydroxybutyrate/phospholipids fatty acids in pmol g^{-1}, PHV/PLFA polyhydroxyvalerate/PLFA in pmol g^{-1}, UQ/MK+DMKs calculated with data in %mol, *BD* ub+mk bioenergetic divergence index, Q/PLFA total quinones/PLFA in pmol g^{-1}.

- Discussion and conclusions

Data obtained from microbial mat samples during a circadian cycle revealed temporal-induced changes in the community composition and in the physiological status. Firstly, data concerning viable microbial biomass as PLFA, as well as total quinone content, reported higher values at 15:00. Then, at 18:00 a dramatic decrease in the PLFA content was observed, which was coincident with an increase of dimethylacetals (DMA derived from plasmalogens. Although the DMA content doubled at 18:00, the total DMA quantification during the cycle was only a 13% of the total PLFA quantified. In any case, these results indicate that an analysis restricted to polar lipid fatty acids may underestimate viable biomass as the vinyl ether-containing phospholipids may form a significant proportion of the total phospholipids of microbial mats investigated. The higher content of DMAs detected at 18:00 must be associated with an increase of the activity of plasmalogen-forming anaerobes (low-G+C gram-positive *Clostridium* and some sulfate-reducing bacteria, Moore *et al*., 1994; Rütters *et al.*, 2001.

The microbial community composition was observed by the quantification of PLFA and quinone homologues. The PLFA profile as %mol showed in Fig. VI.3 indicated that the relative percentages of the microbial groups remained stable during the circadian cycle with the exception of the 18:00 sampling time. Due to the decreasing of the total PLFA at this time, the relative percentages indicated a higher predominance of anaerobes (branched monoenoics and mid-branched saturates at the expense of a reduction in the percentage of gram-negative microorganisms (monoenoic PLFAs. However, the quantification of PLFAs as picomoles per gram reported in Table VI.1 indicated an important reduction in the quantification of all the PLFA groups. Indeed, the FAMEs that reported a higher decrease in their content were *cy*19:0 (indicative of anaerobes and 18:1ω7*c* (gram-negatives; data not shown. This fact is also reinforced by the data presented in Table VI.2 in which some PLFA biomarkers of certain bacterial groups monitored a decrease in their %mol at 18:00 in comparison with data obtained at 15:00. Moreover, the higher presence of phytol, as a main product of the degradation of chlorophyll-*a*, at the sampling times with higher decrease of viable microbial biomass (less PLFA content at 18:0 and 3:00 might be associated with a higher degradation of

chlorophyll molecules. Although the ester bond between phytol and the tetrapyrrolic macrocycle can resist hydrolysis, appreciable amounts of free phytol can be detected in recent sediments (Rontani *et al*., 1996; Grossi *et al*., 1998).

The quinone composition observed in the microbial mat samples also provided a vision of the community structure. The quinone content as %mol was similar in all samples with exception to the 18:00 sampling time, in which a higher percentage of Q-8 at the expense of a reduction in the MK-9 percentage was detected. The same vertical composition pattern has been also observed in the PLFA data which can indicate maintenance of the microbial community over a daily cycle. The quinone data as picomoles per gram of dry weight (Table VI.3) indicate an important decrease in all quinones with exception of Q-8 at 18:00. Indeed, this fact can be associated with a slightly increase of γ- and β-Proteobacteria or sulfur-oxidizing bacteria that can use the excess of organic compounds generated during the photosynthetic hours. The quinone content of all the homologues was reduced during the night hours and then reached similar values of those detected in the previous day. As it has been mentioned above, this is consistent with a repeatability of the day/night dynamics in Ebro delta microbial mats

In addition, the dramatic decrease in the MK-9, in comparison with the rest of quinones, can be associated with a decrease of members of the *Firmicutes*, *Actinobacteria* and *Bacteroides* groups. Surprisingly, the increase of the dimethylacetal content at 18:00 did not correspond with an important increase of any of the quinone homologues of the plasmalogen-containing genera. The plasmalogen-containing microorganisms are strict anaerobes mainly *Clostridium*, closer relatives, and several sulfate-reducing bacteria (Rütters *et al*., 2001). In this case, the sulfate-reducing bacteria (δ-Proteobacteria) have MK-6 and 7 in their respiratory chains and their content did not increase at 18:00.

The characterization of members of the *Clostridium* genus in marine environments have been previously performed (Smith, 1970; Mountfort *et al*., 1997; Brisbarre *et al*., 2003; Lauro *et al*., 2004). However, little is known about the presence and role of anaerobic spore-formers in microbial mats. Although recent studies have

characterized *Clostridium* species in Antarctic microbial mats (Spring *et al*., 2003), the isolation of *Clostridium* sp. EBD constitutes the first report of the presence of *Clostridium* species in estuarine microbial mats. Future investigations need to be performed in order to assess the physiological role and their contribution to the pool of Gram-positives members of *Clostridium* species; however, their role should be focused on fermentation processes and recycling of organic compounds in anoxic zones by means of their saccharolytic and proteolytic activities.

An increase in the activity of members of *Clostridium* genus at 18:00 might explain the increase of the DMA content. Although more studies need to be performed about the quinone and dimethylacetals composition in members of the genus *Clostridium*, DMAs were observed in the cell membranes of the isolated *Clostridium* sp. EBD. Moreover, other closer relatives of *Clostridium*, which may contribute to the plasmalogen content, have been detected in microbial mats such as members of the *Haloanaerobium* genus (Zhilina and Zavarzin, 1991). Early studies indicated that members of the genus *Clostridium* lacked respiratory quinones (Gibbons and Engle, 1964). This is in accord with the strictly anaerobic nature of clostridia and with their inability to synthetize heme compounds and cytochromes. However, Gottwald *et al*. (1975) and Yamamoto *et al*. (1998) demonstrated the presence of menaquinones in certain species of the genus *Clostridium*. There is not any previous study reporting the presence of plasmalogens or quinones in members of the *Haloanaerobium* genus; however, they are similar to representatives of the gram-positive bacterial line (ability of form endospores, 16S rDNA). The haloanaerobes have been classified in a separate order of the *Firmicutes* phylum but are considered as gram-negative relatives of the line of gram-positives because of their gram-negative cell-wall structure (Oren *et al*., 1984; Tourova 2000).

Haloanaerobes usually inhabit cyanobacterial mats in hypersaline lagoons (Zhilina *et al*., 1991), as well as in other environments (Ollivier *et al*., 1994). For example, *Haloanaerobium saccharolyticum* (one of the most abundant members detected in Camargue mats in 'chapter IV') has been isolated from *Microcoleus*-dominated mats (Zhilina and Zavarzin, 1991) and reported an important fermentative activity of glycerol and glucosylglycerol produced mainly by cyanobacteria as osmotic

solutes. For this reason, we considered the possibility of an important activity of members of the *Clostridium* genus and closer relatives such as *Haloanaerobium* at 18:00 which could explain the increase in the DMA content and a lack of association with an increase in any quinone homolog.

Although respiratory quinones were detected in *Clostridium* sp. EBD, its predominant MK-9 reported very low values in comparison with the picomoles per gram of dry weight observed in pure bacterial cultures. In this sense, MK-9 is found in *Firmicutes* and we can not exclude the possibility that other members of this genus were reducing their menaquinone content at 18:00 in spite of an increase of plasmalogens in their membranes. Other important fact is that MK-9 is also found in members of the *Bacteroides* genus that are gram-negative anaerobes also found as important members of the Camargue mats (see 'chapter IV'. *Bacteroides* have also a high proportion of sphingolipids in their membranes (Kato *et al*., 1995. Although we could not quantify the sphingoid bases in the mat samples, some bases were detected as well as the hydroxyl fatty acids (OH-FA amide-linked associated to this kind of phospholipids. Data presented in Table VI.4 reported a decrease of OH-FA amide-linked at 18:00, which might indicate a decrease of members of the *Bacteroides* genus and the reduction of part of the MK-9 pool at this time.

The comparison between the quinone content of Ebro delta mat samples and the 'Ebro delta all-core' (sampled at 12:00 analyzed in 'chapter V', revealed that the major quinones were MK-9 and Q-8 in all cases. However, the quinone content was completely different in the first 7.75 mm of Ebro delta mat analyzed in 'chapter V' in which Q-8 was predominant and MK-9 reported low percentages. These results suggest that an important population of anaerobic bacteria with MK-7, 8 and 9 is active in the sediment underlying the first microbial mat unit (approximately from 0 to 7.75 mm as it was previously deduced in 'chapter V'. In connection with this, the total quinone content observed in the Ebro delta samples was a 10% of the total quinone value of the depth profile in 'chapter V' (only 7.75 mm depth and 0.74 g. When a microbial mat sample is analyzed as a 'whole-core' around 2.5 cm depth is processed, homogenized and certain quantity is analyzed. In this case, 0.75 g of each sample was analyzed and the low content of quinones detected in comparison with the first 7.75 mm of the

vertical profile might be indicative of a high turnover of quinones with an important reduction of these molecules in the underlying microbial mat units.

Effects of the day/night changes on the community were also detected by metabolic status biomarkers. The *cyclo*/ω7*c* and *trans*/*cis* ratios indicate a situation of exponential to stationary growth conditions as well as stress conditions during the circadian cycle. At 18:00, an increase of the starvation index was detected, which coincided with an increase in the metabolic stress, a higher proportion of anaerobic bacteria, an increased recovery of DMAs, a higher respiration activity (higher Q/PLFA ratio), a more aerobic character (higher UQ/MK ratio), and a lower proportion of viable cell numbers as total PLFA. This can be explained by high oxygenic and anoxygenic photosynthetic activities during the day that provide photosynthates and osmotic solutes to the heterotrophic populations of the mat. This would be consistent with an excess of assimilated carbon products in comparison with other compounds that changes the carbon/nitrogen ratio and leads to an unbalanced growth situation. Moreover, the higher value of the Q/PLFA ratio at 18:00 indicated a higher respiratory activity in comparison with fermentation activity, which is also consistent with an important activity of aerobic or facultative heterotrophic microorganisms at the expense of an excess of organic compounds.

Apart from that, the excess of carbon assimilated can be accumulated in the cells as biopolymers, which is consistent with the maximum values of metabolic stress before sunset and the maximum PHB/PLFA ratios at the same sampling point (18:00). Ratios greater than 0.2 usually indicate the beginnings of unbalanced growth in at least part of the microbial community (Nickels *et al*., 1979; White *et al*., 1995), therefore data suggested a situation of carbon storage by some of the microbial populations in the mat.

Maximum value of PHA (polyhydroxyalkanoates) detected in the afternoon, is not consistent with a typical PHA production during the night, according to the model suggested by Rothermich *et al*. (2000) that examined *in situ* PHA levels and composition in stratified photosynthetic microbial mats from Great Sippewissett Salt Marsh (MA), and Ebro delta, Spain. In each case, PHA levels increased during the night and decreased during the day. Several of our observations suggest that PHA

accumulation appears to be the result of phototrophic activity because PHA levels rise and fall with the organic carbon supplements in the light-dark cycle, and then heterotrophic microorganisms accumulate PHA from the excess of carbon generated. In addition, data showed that more hydroxyvalerate than hydroxybutyrate is produced in the microbial mats; this is also the case in all reports of PHA occurrence in phototroph-dominated environments (Macarrón, 1998; Mas-Castellà, 1991).

The decrease of the growth rate and the higher metabolic stress detected at 18:00 and 3:00, has been previously reported in Ebro delta microbial mats during a 6h day/night cycle in Spring of 1997 (Navarrete *et al*., 2000) but in that case, the maximum values of *cyclo*/ω7*c* and *trans/cis* ratios were observed at 18:00 and 6:00. In this regard, the minimum viable biomass values reported by Navarrete *et al*. (2000) were found at 18:00 and 6:00, whereas the minimum values of total PLFA of the 3 hours day/night cycle of this study were observed at 18:00 and 3:00. The important reduction in the PLFA content at 18:00 and the increase of cyclopropyl and *trans*-monoenoic fatty acids is clearly correlated with a situation of nutrient deprivation. The loss of PLFAs at this time does not necessarily involve cell death but a decrease in cell volume and a preferential utilization of the *cis*-monoenoic fatty acids as a survival mechanism for the maintenance of membrane integrity during starvation (Guckert *et al*., 1986). In spite of the fact, the reduction of the 28% of the PLFA content in 3 hours might indicate an important turnover of phospholipids in microbial mats that should be studied in future researches.

In general, it seemed to be a good correspondence between and increase of unbalanced growth, higher metabolic stress and decrease of viable cell numbers. The comparison between the presented data and the data summarized in Navarrete *et al*. (2000), reported a similar tendency in microbial mat activities along a day/night cycle that remained stable between seasons and years. Although further studies need to be done in order to assess this hypothesis, these data support the idea that microbial mats are a relative stable complex system in which the development is ruled by different microbial populations and the physicochemical conditions of the surrounding environment.

Conclusions

- Changes in the community composition and in the physiological status of Ebro delta mats during a circadian cycle have been analyzed by the signature lipid approach. Data concerning viable microbial biomass as PLFA, as well as total quinone content, reported higher values at 15:00. However, at 18:00 an important decrease in the PLFA content was observed, which was coincident with an increase of dimethylacetals (DMAs derived from plasmalogens.

- The PLFA profile indicated that the relative percentages of the microbial groups remained stable during the circadian cycle with the exception of the 18:00 sampling time in which a higher predominance of anaerobes at the expense of a reduction in the percentage of gram-negative microorganisms was observed. Moreover, the higher presence of phytol, as a main product of the degradation of chlorophyll-*a*, at 18:00 might be associated with a higher degradation of chlorophyll molecules.

- The quinone percentages were similar in all samples with exception to the 18:00 sampling time, in which a higher percentage of Q-8 at the expense of a reduction in the MK-9 percentage was detected. In addition, the quinone content of all the homologues was reduced during the night hours and then reached similar values of those detected in the previous day, which was consistent with a repeatability of the day/night dynamics in Ebro delta microbial mats.

- The increase of the dimethylacetal content at 18:00 did not correspond with an important increase of any of the quinone homologues of the plasmalogen-containing genera (*Clostridium* and closer relatives The general inability of members of this genus to synthetize quinones might explain the lack of an increase of certain quinones at 18:00.

- *Clostridium* sp. EBD and *Desulfovibrio* sp. EBD have been isolated and characterized from Ebro delta mats. *Desulfovibrio* sp. EBD has MK-6 as a predominant quinone and lack of dimethylacetals in their membranes. However, *Clostridium* sp. EBD (similar to *Clostridium bifermentans* reported a low quinone

content and presence of dimethylacetals in their membranes which supports the idea of an increasing activity of members of this genus at 18:00.

- The reduction of the MK-9 content at 18:00 may be associated with a reduction of members of the *Bacteroides* genus which is also consistent with the observed decrease of hydroxyl fatty acids amide-linked of sphingolipids synthetized by this genus.

- At 18:00, an increase of the starvation index (*cyclo*/ω7*c* was detected, which coincided with an increase in the metabolic stress (*trans*/*cis*, a higher proportion of anaerobic bacteria, an increased recovery of DMAs, a higher respiration activity (higher Q/PLFA ratio, a more aerobic character (higher UQ/MK ratio, and a lower proportion of viable cell numbers as total PLFA. This can be explained by high oxygenic and anoxygenic photosynthetic activities during the day that provide photosynthates and osmotic solutes to the heterotrophic populations of the mat.

- The higher PHB/PLFA and PHV/PLFA at 18:00 suggested a situation of carbon storage and an unbalanced growth. This maximum value of polyhydroxyalkanoates in the afternoon is not consistent with previous studies performed in microbial mats and suggests an important activity of aerobic or facultative heterotrophic microorganisms at the expense of an excess of organic compounds generated by photosynthetic processes.

- Publications and communications

- **Villanueva L., A. Navarrete, J. Urmeneta, R. Geyer, D. C. White, and R. Guerrero.** Increased Metabolic Stress, Slowed Growth, Anaerobic Microniches and Increased Poly-β-hydroxyalkanoate Induced by Photosynthetic Activity during a Circadian Cycle in an Estuarine Microbial Mat. In preparation.

- **Villanueva L., A. Navarrete, J. Urmeneta, R. Guerrero, R. Geyer, and D. C. White.** Increased metabolic stress, slowed growth, anaerobic microniches and increased poly-β-hydroxyalkanotes induced by photosynthetic activity during a circadian cycle in an estuarine microbial mat. 104th ASM General meeting, New Orleans, LA., May 2004. Poster.

- Navarrete A., L. **Villanueva, J. Urmeneta, D. C. White, and R. Guerrero.** Analysis of lipid biomarkers in Ebro delta microbial mats during a circadian cycle: A study of biomass, composition, physiological and redox state. XX Congress of the Spanish Society for Microbiology. Cáceres (Spain), September 2005. Poster.