Microbial Ecology

Monitoring Diel Variations of Physiological Status and Bacterial Diversity in an Estuarine Microbial Mat: An Integrated Biomarker Analysis

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Abstract

Microbial mats are highly productive microbial systems and a source of not-yet characterized microorganisms and metabolic strategies. In this article, we introduced a lipid biomarker/microbial isolation approach to detect short-term variations of microbial diversity, physiological and redox status, and also characterize lipid biomarkers from specific microbial groups that can be further monitored. Phospholipid fractions (PLFA) were examined for plasmalogens, indicative of certain anaerobes. The glycolipid fraction was processed for polyhydroxyalkanoates (PHA) and the neutral lipid fraction was used to evaluate respiratory quinone content. Data demonstrate an increase in the metabolic stress, unbalanced growth, proportion of anaerobic bacteria and respiratory rate after the maximal photosynthetic activity. Higher accumulation of polyhydroxyalkanoates at the same sampling point also suggested a situation of carbon storage by heterotrophs closely related to photosynthetic microorganisms. Besides, the characterization of lipid biomarkers (plasmalogens, sphingolipids) from specific microbial groups provided clues about the dynamics and diversity of less-characterized mat members. In this case, lipid analyses were complemented by the isolation and characterization of anaerobic spore formers and sulfate reducers to obtain insight into their affiliation and lipid composition. The results revealed that temporal shifts in lipid biomarkers are indicative of an intense change in the physiology, redox condition, and community composition along the diel cycle, and support the hypothesis that interactions between heterotrophs and primary producers play an important role in the carbon flow in microbial mats.

Introduction

Microbial mats are stratified microbial systems characterized by cyclic seasonal fluctuations of flooding and desiccation, and by diel fluctuations of temperature, light, pH, oxygen, sulfide, and nutrients. Phototrophic mats are dense communities composed by phototrophs (cyanobacteria, diatoms, purple and green (non)sulfur bacteria), anaerobic bacteria (sulfate-reducing bacteria, methanogens, fermenters) and aerobic bacteria (heterotrophs and chemolitoautotrophs). In this kind of systems, mat-building organisms respond to changes in steep vertical physicochemical gradients and orientate themselves to find optimal conditions [6], which results in visible lamination of the resident microbial populations [10]. The community composition of microbial mats and the effects of environmental stress on these ecosystems remain active areas of research. In fact, one of the aims of microbial mat research is to determine whether mats comprise a stable and predictable environment and to follow their metabolic and growth-related dynamics under natural conditions. Studies carried out with pure cultures isolated from natural ecosystems have complemented *in situ* analyses, but have not yielded insight into the diversity and interactions between microbial populations.

In this study, we investigated the dynamics of an estuarine microbial mat (Ebro delta, Spain) during a diel cycle. We used a combined signature lipid biomarker

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(SLB) analysis and microbial isolation to assess physiological status and redox conditions of the mat [41], and also to get insight on less characterized microbial members. With this approach, the presence of certain groups of microorganisms can be inferred by the detection of unique lipids because specific phospholipids fatty acids (PLFA) are prominent in microbial groups or sometimes certain genera. Other lipid classes serve as quantitative indicators of the physiological status of a microbial community. One such class is bacterial poly-β-hydroxyalkanoates (PHA), which accumulate under conditions of unbalanced growth. The analysis of respiratory quinone profile can be used to determine the redox state of the microbial community, and provide a reliable indicator of microbial biomass and community composition [17, 19, 20].

In this case, the analysis of plasmalogen-derived dimethylacetals (DMA), and sphingoid bases has also provided additional knowledge of community composition. The presence of plasmalogen-containing microorganisms (*Clostridia*, closer relatives, and several sulfate-reducing bacteria) [31, 38] was shown to clearly correlate with the anoxic conditions of the mat system [36]. To further our understanding of microbial ecosystems, lipid analysis was complemented by the isolation and characterization of specific members of the community. In the present study, we characterized anaerobic spore formers and sulfate reducers (well known as important members of microbial mats), as little is known about their affiliation and lipid composition. Members of the genus *Clostridium* present in marine environments have been characterized [5, 26, 32, 39, 40]. However, the presence and role of anaerobic spore formers in estuarine microbial mats have not been well-studied, although their saccharolytic and proteolytic activities suggest involvement in fermentation processes and in the recycling of organic compounds in anoxic zones [23, 35]. In connection with SLB analysis, previous studies indicated the presence of menaquinones in certain species of the genus *Clostridium*. The relevance of this finding is that fermentative bacteria classically do not require quinones because they do not translocate electrons across the cell membranes to gain energy [12, 46].

Apart from that, the dynamics of PHA turnover has been evaluated as a physiological strategy to overcome environmental changes, and also to increase our knowledge about microbial mats as a source of new PHA producer and degrader species with potential biotechnological applications [3].

Although the SLB approach has been previously used to examine the community composition and metabolic state of microbial mat samples [33, 34, 42], those studies focused on the polar lipid fraction. In this article, we combined expanded SLB approaches to include neutral and glycolipid fractions, and thus gain insight deeper into microbial community dynamics. This was done by: (1) monitoring microbial biomass under starvation, stress, and different redox conditions; (2) characterizing the microbial community composition based on PLFAs, quinone content, and other specific lipids biomarkers; and (3) isolating representative strains of sulfate-reducing bacteria and anaerobic Gram-positive bacteria from mats and determining the presence of lipid biomarkers in their bacterial membranes.

Materials and Methods

Sampling and lipid extraction. Samples were obtained from microbial mats located in the Ebro delta, on the northeastern coast of Spain (40°47′N, 0°56′E; for a more detailed description, see [16, 30]). Samples were collected in the fall of 2002 (October), over a day/night cycle. The upper part of the mat was sampled every 3 h starting at midday (12:00, 15:00, 18:00, 21:00, 24:00, 3:00, 6:00, 9:00, and 12:00 h of the following day, GMT+01:00), using a 1.5-cm inner-diameter corer. Conductivity and sulfide concentration of the overlaying water and the amount of solar irradiation were analyzed at each sampling time, as described by Navarrete *et al.* [33]. Samples were extracted according to the method of Bligh and Dyer [4], as modified by White *et al.* [43]. Total lipid extracts were fractionated into neutral, glycolipids, and polar lipids by silicic acid chromatography [14].

Lipid analysis. The polar lipid fraction was transesterified to fatty acid methyl esters (FAME) by mild alkaline methanolysis to protect cyclopropane PLFA, followed by mild acid methanolysis to release plasmalogen ethers as DMA, according to the sequential method of Mayberry and Lane [28]. FAME and DMA were detected and quantified as described by Peacock *et al.* [36]. The remaining phase obtained from mild acid methanolysis of the DMAs was hydrolyzed under strong acid conditions, after which the organic phase was treated by methanolysis and derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) to detect amide-linked hydroxyl fatty acids and sphingoid bases from sphingolipids [27, 29]. Polyhydroxyalkanoates (PHB, polyhydroxybutyric acid; PHV, polyhydroxyvaleric acid) contained in the glycolipid fraction were hydrolyzed and derivatized with *N-tert*-butyl-dimethylsilyl-*N*-methyltrifluoroacetamide (MBSTFA) following the protocol described by Elhottová *et al.* [9]. The spectra of the MBSTFA-derivatized β-hydroxyvalerate monomers were obtained by analyzing the glycolipid fraction of *Cupriavidus necator* cultures grown on sodium valerate as carbon source [8]. The neutral lipid fraction was examined for respiratory ubiquinone (Q), menaquinones (MK), and demethylmenaquinone (DMK) isoprenologues (-*n*) by liquid chromatography tandem mass spectrometry (LC-MS/MS), as described by Geyer *et al.* [11]. The microbial divergence

index of ubiquinones and menaquinones (*MD* ub + mk) and the bioenergetic divergence index (*BD* ub + mk) were calculated according to a previously published method [19, 21].

Statistical analysis. Analysis of variance (ANOVA) and standard deviation were used to determine differences among lipid biomarker data in sample replicates (2 sampling events × 4 replicate mat cores). ANOVAs were performed with Statgraphics Plus 5.1 for Windows (StatPoint, Inc., Virginia).

Screening and characterization of sulfate-reducing and anaerobic Gram-positive bacteria. Sulfate-reducing and anaerobic spore-forming bacteria present in mat samples were screened by manipulation under anoxic conditions and culture on SRB agar plates (sulfate-reducing bacteria [SRB] agar, Scharlau Microbiolgy, Spain; amended with 15 g NaCl L^{-1}], or after heat shock (80°C, 10 min) and culture on SPS agar (Scharlau Microbiology, Spain, amended with 15 g NaCl L^{-1}). The isolated strains were characterized by scanning and transmission electron microscopy and by 16S rRNA sequencing [2]. Total lipids were extracted from lyophilized cultures and the polar and neutral lipid fractions were analyzed for FAME, DMA, and quinone composition as described above.

Results

To reveal temporal changes in the microbial community of a complex estuarine mat, biomarkers were assessed for microbial biomass, physiological status, and general community composition during a diel cycle (24 h). Mat samples and physicochemical data were obtained every 3 h starting at noon of a normal sunny day in fall.

Physicochemical conditions. Starting at noon, the light intensity at the sampling site decreased, reaching a minimum value between 21:00 and 6:00 h. Conductivity values were relatively stable (25,000–27,500 μS cm^{-1}) with slight fluctuations during the day/night cycle. Sulfide measured in the overlying water reached its maximum value at 6:00 h (18 μM) and was below the limit of detection of the applied method during the rest of the day.

Microbial biomass and community composition Microbial biomass, as measured by polar lipid fatty acids (PLFA; Table 1), ranged from 7.7×10^3 to 2.7×10^4 pmol PLFA per gram of dry weight. The minimum value of total PLFA occurred at 18:00 h and the maximum value at 15:00 h. From 21:00 h to 9:00 h of the following day, PLFA values varied slightly (ca. 7%). DMA derived from plasmalogens, indicating rather anaerobically microorganisms, were higher at 18:00 h and showed the same trend as the PLFA biomass profile except for a time lag between the maximum peaks of each (Table 1). In addition, total quinones, measured as picomole per gram (pmol g^{-1}), reached a minimum at 3:00 h and a maximum at 15:00 h (Table 3).

Table 2 shows diel changes of the community composition based on PLFA analysis. The community consisted mainly of Gram-negative bacteria (β-and γ-Proteobacteria, *Firmicutes*, and Bacteroides groups) at 12:00 h and 15:00 h, as indicated by the presence of a high percentage of monoenoic PLFA [45]. Minimum values of PLFA species, indicative of Gram-negative bac-

Table 1. Physiological and redox state measurements in mat samples

Sample	12:00	15:00	18:00	21:00	24:00	3:00	6:00	9:00
Indices[1]								
cyclo/ω7c	0.2	0.4	0.5	0.3	0.4	0.5	0.3	0.5
trans/*cis*	0.07	0.09	0.3	0.1	0.1	0.4	0.2	0.2
UQ/MK+DMK	0.4	0.4	0.6	0.3	0.4	0.4	0.4	0.5
BD ub+mk	191	189	197	182	191	192	193	196
Q/PLFA	2.5	1.6	2.8	2.2	1.9	1.6	1.5	1.9
MD ub+mk	759	754	863	708	790	812	831	833
PHB/PLFA	5	8	14	12	7	10	9	10
PHV/PLFA	35	28	190	49	57	78	50	83
Values[2]								
PLFA pmol g^{-1}	13,570	26,883	7,669	8,933	11,046	8,061	12,772	9,709
DMA pmol g^{-1}	1,676	1,844	3,514	344	1,141	1,285	1,961	1,073
PHB nmol g^{-1}	66	217	106	107	79	81	118	93
PHV nmol g^{-1}	476	749	1,458	436	626	627	640	811

[1] *cyclo*/ω7*c* and *trans*/*cis* ratios, PHB/PLFA polyhydroxybutyrate/phospholipid fatty acids in pmol g^{-1}, PHV/PLFA polyhydroxyvalerate/PLFA in pmol g^{-1}, UQ/MK+DMKs ubiquinone to menaquinone ratio calculated with percent mole data, *BD* ub+mk bioenergetic divergence index, Q/PLFA total quinones/PLFA in pmol g^{-1}.

[2] PLFA phospholipid fatty acids; DMA dimethylacetals; PHB polyhydroxybutyrate; PHV polyhydroxyvalerate. Decimal position avoided. Standard deviation for PLFA, DMA, and PHA (polyhydroxyalkanoates) between replicates was less than 10%. ANOVA determined among lipid biomarker data between mat core replicates gave *p* values around 1 (significantly similar).

*The polar lipid fraction of the 12:00B sampling time was lost during the analyses; thus, only the quinone and PHA results of this sample were included in the study.

Table 2. Community composition by PLFA analysis (% mol)

PLFA biomarkers[1]	12:00	15:00	18:00	21:00	24:00	3:00	6:00	9:00
Nsat (All genera)	3.97	7.87	6.69	7.33	6.14	11.37	5.05	9.28
BrMon-MBrSat (Anaerobes)	29.45	22.16	56.46	34.07	36.33	41.14	43.96	46.44
TBrSat (Gram positives)	4.11	9.26	10.83	7.18	8.09	10.99	8.41	9.41
Monoenoics (Gram-negatives)	53.36	50.65	18.43	42.16	39.49	27.52	33.06	28.74
Polyenoics (Microeukaryotes)	9.11	10.05	7.59	9.26	9.95	8.98	9.53	6.12

Standard deviation between samples ±10% (data obtained from $n = 4$ replicates of microbial mat sample cores).
[1]NSat = Normal saturates; BrMon-MBrSat = Branched monoenoics and Medium-branched saturates; TBrSat = Terminal branched saturates.

teria, were found at 18:00 h. From 12:00 to 15:00 h, terminally branched saturated fatty acids, typical of Gram-positive bacteria, increased slightly whereas lipids representative of anaerobic microorganisms (branched monounsaturated and mid-chain branched saturated fatty acids) decreased. At 18:00 h, there was an inversion in the profile dominated by Gram-negative bacteria such that PLFA typical of anaerobic bacteria were higher at this sampling time. At all sampling times, the abundance of lipids representative of microeukaryotes (polyenoic PLFAs) and Gram-positive bacteria (terminally branched saturated PLFA) remained stable.

As confirmed in previous studies [7, 19], quinone isoprenologues act as suitable biomarkers for the classification of microorganisms. Quinone composition and changes in the quinone profile during the day/night cycle are summarized in Table 3. Among the ubiquinones, Q-10 predominated at 12:00, 15:00, and 21:00 h, whereas Q-8 was the major ubiquinone at all other sampling times. Furthermore, Q-9 was also an important ubiquinone and its levels (measured as percent mole [%mol], picomoles of certain quinone homolog in a sample divided by the picomole sum of all measured quinones set to 100%; data not shown) remained relatively constant at all sampling times. 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. Likewise, menaquinone-9 (MK-9), common in members of the *Firmicutes*, *Actinobacteria*, and *Bacteroides* groups, as well as Rhodospirillaceae, was the most abundant (percent mole) among all the quinones. The relative percentages of MK-7, MK-8, and MK-10 were also high in all samples. These menaquinones have been detected in *Actinobacteria*, *Cytophaga-Flavobacteria*, δ-Proteobacteria, *Firmicutes*, and *Euryarchaeota* (MK-7 and MK-8), and in green nonsulfur bacteria (MK-10). By contrast, levels of MK-4, MK-5, and DMK were relatively low in all sampling points. Q-8, Q-9, and Q-10 significantly decreased during the night and then progressively increased again at the beginning of the following day. A similar trend was observed for MK-6, which is found in *Cytophaga-Flavobacterium*, δ-Proteobacteria, and ε-Proteobacteria. The levels of this menaquinone were higher from 12:00 to 18:00 h and decreased during the night. Relative MK-7 levels were highest at 15:00 h. In general, at 18:00 h, the amount of all ubiquinones decreased, except Q-8, which slightly increased. Furthermore, the amounts of quinones related to photosynthetic bacteria (plastoquinone-9 and

Table 3. Quinone content as picomoles per gram of dry weight in mat samples

Homolog	12:00	15:00	18:00	21:00	24:00	3:00	6:00	9:00	12:00B[1]
Q-6	43	56	36	18	26	16	26	28	60
Q-7	200	291	219	98	248	142	220	214	663
Q-8	3,089	3,102	3,595	1,477	2,652	1,600	2,349	2,881	5,550
Q-9	1,037	1,371	768	354	585	317	410	502	1,100
Q-10	4,753	6,410	2,648	1,805	1,736	1,351	2,102	2,008	4,445
MK-4	83	113	44	48	36	40	65	56	93
MK-5	125	176	88	71	84	52	84	76	130
MK-6	683	827	646	367	435	358	556	527	958
MK-7	1,617	2,428	1,760	903	1,708	880	1,696	1,457	3,621
MK-8	2,118	2,835	1,807	1,105	1,391	878	1,244	1,261	2,671
MK-9	15,208	20,901	6,075	9,869	8,327	4,971	6,685	6,141	11,201
MK-10	2,243	3,278	1,261	1,106	848	563	909	733	1,566
DMK-8	36	56	30	26	24	26	27	25	36
K_1+PQ-9	2,405	2,607	2,338	2,031	2,594	2,043	2,212	2,324	2,426
TQ[2]	33,643	44,451	21,315	19,279	20,696	13,238	18,587	18,234	34,519

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).
[1]12:00B = 12:00 A.M. sampling point of the following day
[2]TQ = Total quinones

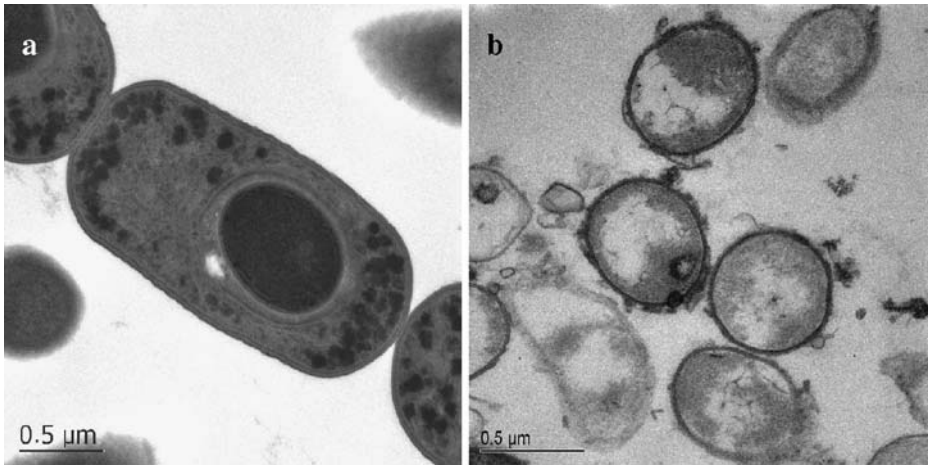

Figure 1. Micrographs of the isolated *Clostridium* sp. EBD (Ebro Delta) (a) and *Desulfovibrio* sp. EBD (Ebro delta) (b). (a) Scanning transmission micrograph showing the central spore and the distended cell wall; (b) Transmission electron micrograph showing cross-sectioned cells of the *Desulfovibrio* strain.

phylloquinone) remained similar at all sampling times and represented a considerable amount of the total quinone content (ca. 5–10%).

In addition to phospholipid fatty acids and respiratory quinones, other lipid classes can be used as biomarkers to estimate the community composition of environmental samples. In this study, a sequential protocol was applied to also detect and quantify sphingoid bases and amide-linked hydroxyl fatty acids derived from sphingolipids. The most abundant sphingoid bases were C18:0 (dihydrosphingosine), C19:0, and C21:1. Furthermore, 3-hydroxy 16:0 (3-OH 16:0), 3-OH 17:0, and 3-OH 18:0 were present in mat samples. The lowest values occurred at 18:00 h (2–3.7 pmol g^{-1}) and the higher ones at 9:00 h (70.9–75.4 pmol g^{-1}).

Screening for sulfate-reducing bacteria and anaerobic spore-forming bacteria in microbial mat samples led to the isolation of one strain representative of each group (Fig. 1). Pure cultures were sequentially isolated and their 16S rRNA was sequenced. The similarities of the 16S rRNA from the isolated strains and their quinone and cellular fatty acid compositions are shown in Table 4. The phenotypic and molecular properties of the isolated strains were consistent with their classification as members of the *Desulfovibrio* sp. and *Clostridium* sp. genera, respectively. Thus, the strains were tentatively named *Desulfovibrio* sp. EBD (Ebro delta) and *Clostridium* sp. EBD (Ebro delta). Analysis of the quinone content (Table 4) indicated a predominance of MK-6 (abundant in δ-Proteobacteria) and also an important contribution of MK-5 in *Desulfovibrio* sp. EBD. Although MK-9 was detected in *Clostridium* sp. EBD, its abundance was 400-fold less than that of MK-6, the main quinone in *Desulfovibrio* sp. EBD. Dimethylacetals derived from plasmalogens were observed only in *Clostridium* sp. EBD and the detected DMA contained 16 carbons (Fig. 2).

Physiological and respiratory status. As shown in Table 1, the growth rate (high *cyclo*/ω7*c* FA ratio) decreased at 18:00 and 3:00 h and there was a shift to a higher level of metabolic stress (high *trans*/*cis* FA ratio, [18]). The *cyclo*/ω7*c* ratio indicated exponential to stationary growth of the mat bacteria at each sampling time. The highest levels of metabolic stress occurred at 18:00 and 3:00 h, as reflected by the *cyclo*/ω7*c* ratio profiles. In addition, PHA quantification demonstrated that PHV values were higher than PHB at all sampling times. Likewise, the ratio PHB/PLFA during the day/night cycle reflected changes in the PHB levels depending on the time of day. The PHV/PLFA ratio was higher than the PHB/PLFA ratio; the maximum value was measured at 18:00 h and the minimum at 15:00 h (Table 1). Moreover, the ubiquinone/menaquinone (UQ/MK) ratio was similar in all samples; however, the highest ratio occurred at 18:00 h, which could be attributed to the more aerobic character of the microbial community at that time. The UQ/MK ratio near zero indicates long-term exposure to strictly anaerobic conditions. Values below 1 indicate

Table 4. Similarity of 16S rRNA of the isolated strains, main fatty acids, and quinones detected

Strain	Similarity (%)	Closest relative	Main fatty acids	Main quinone[2]
Clostridium sp. EBD(DQ218319)[1]	99	*Clostridium bifermentans* (AF320283)	14:0, *a*15:0, *i*16:0, 16:1ω9*c*, 16:1ω7*c*, 16:0, *i*17:0, *a*17:0, *cy*17:0, 17:0, 18:1ω9*c*, 18:1ω7*c*, 18:0, *cy*19:0	MK-9 (272)
Desulfovibrio sp. EBD(DQ218320)[1]	98	*Desulfovibrio acrylicus* (U32578)	*cy*17:0, 17:0, 18:1*br*, Me18:0	MK-6(1.06×10^5)

[1]Accession number of the 16S rRNA sequences
[2]In parentheses, quantification of the main quinone as picomoles per gram of dry weight

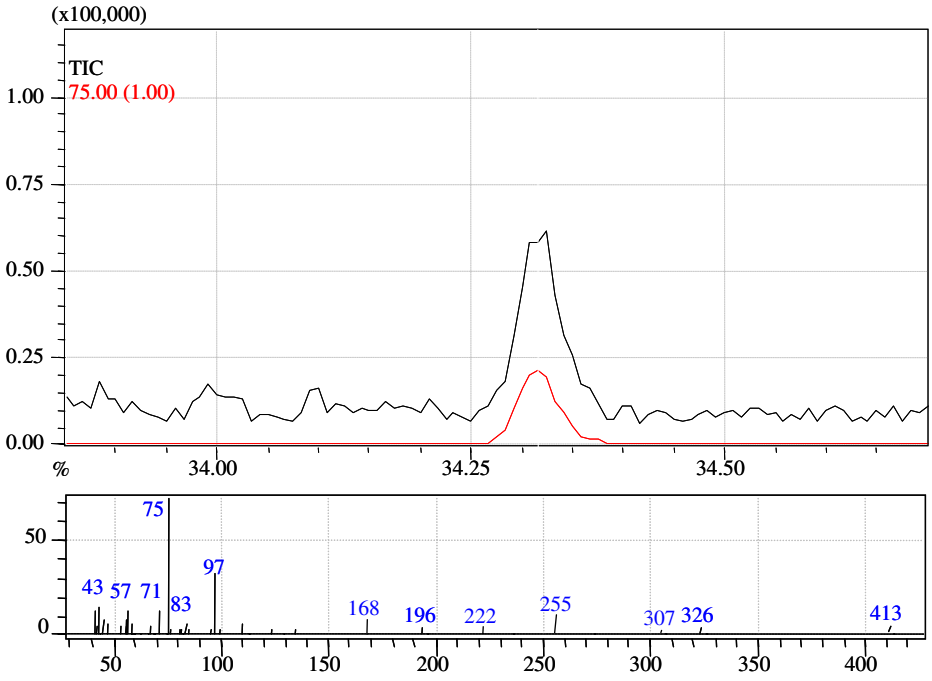

Figure 2. Detection of dimethylacetals (DMAs) in *Clostridium* sp. EBD (Ebro Delta). Chromatogram and mass spectra of 16DMA (dimethylacetal with 16 carbons) with the base peak at *m/z* 75. The top part refers to the chromatographic separation and then, the corresponding mass spectrum of the peak is shown.

anaerobic conditions with a past history of oxygen availability [11], 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 [36]. Besides, the ratio of total quinones to PLFA (Q/PLFA) is proportional to the ratio of respiration/(respiration + fermentation). The ratio of total quinones to PLFA (Q/PLFA) indicated a high level of respiratory activity and a less anaerobic character (UQ/MK) of the 12:00, 18:00, and 21:00 h-samples. Microbial divergence indices (Table 1) were similar in all samples, but maximum divergence was detected at 18:00 h.

Discussion

These results give clues about the repeatability of the day/night trends in Ebro delta microbial mats according to the PLFA and quinone data observed. However, future studies of the daily dynamics in microbial mats over consecutive days and seasons should be performed to conclude this hypothesis. In this study, physiological status and community composition remained stable with the exception of the 18:00 h sampling point. At this time, an increase of the starvation index was detected, which coincided with an increase in the metabolic stress, an increased recovery of dimethylacetals, a higher respiration activity, a more aerobic character, and a lower proportion of viable cell numbers as total PLFA. Moreover, the reduction in the total PLFA content in this sampling point was selective, which can be attributed to differences in PLFA structure, resulting in a higher proportion of PLFA representative of anaerobic bacteria. The changes observed at this sampling point 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 [13, 22]. Moreover, higher values of PHA at this time also suggested a situation of carbon storage and an unbalanced growth. These data are not consistent with previous studies performed in microbial mats [37]. In fact, several of our observations suggest that PHA accumulation is the result of phototrophic activity because PHA levels rise and fall with the organic carbon supplements in the light/dark cycle, suggesting that aerobic or facultative heterotrophic microorganisms accumulate PHA from the excess carbon that is generated and excreted by photosynthetic microorganisms. This fact suggests a situation of excess of assimilated carbon products and changes in the carbon/nitrogen ratio, which leads to unbalanced growth during late daytime. Moreover, the higher quinone-to-PLFA (Q/PLFA) ratio revealed a higher respiratory activity in comparison with fermentation processes, and also higher presence of aerobic or facultative heterotrophs.

The important reduction in PLFA content and the increase in cyclopropyl and *trans*-monoenoic fatty acids clearly correlated with nutrient deprivation. However, the loss of PLFA does not necessarily involve cell death, but may instead reflect a decrease in cell volume and preferential utilization of the *cis*-monoenoic fatty acids as a survival mechanism for the maintenance of membrane integrity during starvation [15]. Nonetheless, future studies should take into account that a dramatic reduction

of the PLFA content over a short time period could be caused by an important turnover of phospholipids in microbial mats. These results also indicate that an analysis in which only polar lipid fatty acids are measured may underestimate viable biomass because vinyl-ether-containing phospholipids may comprise a significant proportion of the total phospholipids in microbial mats. The higher DMA content was most likely associated with an increase in the abundance and activity of plasmalogen-forming anaerobes.

As part of this study, we isolated *Clostridium* sp. Ebro delta (EBD). To our knowledge, this is one of the first isolations of *Clostridium* species in estuarine microbial mats. Several strict anaerobes of the Clostridia class generate fermentation products that are used by sulfate-reducing bacteria, methanogens, and other fermentative bacteria in microbial mat systems [1, 35]. Thus, further studies should focus on the importance of anaerobic spore formers in mat decomposition and carbon flow and potential syntrophic associations with other spore-forming members. Our results emphasize the importance of the presence of spore formers in mats and their ability to persist as dormant cells when environmental conditions become unfavorable [25]. Significant amounts of DMA were observed in the cell membranes of *Clostridium* sp. EBD. Members of this genus and its closer relatives may have been responsible for the increase in DMA content and the lack of a corresponding increase in quinone homologues, as their fermenting activity do not require quinones. Although respiratory quinones were detected in *Clostridium* sp. EBD, the main quinone, menaquinone-9 (MK-9), was present in very low amounts. Menaquinone in Clostridia was unexpected but found before by other authors [12]. The dramatic decrease in MK-9 content during the diel cycle may have been associated with a temporary decrease in the proportion of members of the *Firmicutes*, *Actinobacteria*, and *Bacteroides* groups. It is surprising to note that the increase in the DMA content at 18:00 h did not correspond to a significant increase in any of the quinone homologues characteristic of plasmalogen-containing genera commonly detected in estuarine microbial mats. Thus, common sulfate-reducing bacteria (δ-Proteobacteria) have menaquinone-6 and 7 as predominant isoprenologues in their respiratory chains, but the content of these compounds did not increase at that sampling time. Menaquinone-9 is also found in other *Firmicutes* groups and in Rhodospirillaceae; thus, the possibility that other microorganisms in the samples reduced their menaquinone content cannot be ruled out. In addition, this menaquinone is found in members of the *Bacteroides* genus, which harbor a high proportion of sphingolipids in their membranes [24]. The presented data revealed a decrease of hydroxyl fatty acids amide-linked to sphingolipids before sunset, suggesting a decrease in the proportion of members of the *Bacteroides* genus and reduction of part of the menaquinone-9 pool.

In general, there was good correspondence of the increase in unbalanced growth and higher metabolic stress with the decrease in total PLFA. A comparison between the presented data and the data summarized in Navarrete *et al.* [33] reveals a similar tendency in microbial mat activities following the maximum photosynthetic activity (maximum stress and minimum phospholipid content) and also during the night period (recovery of PLFA content and balanced growth). However, differences in data at the beginning of the day remain unclear and might be attributed to changes between seasons related with temperature, salinity, and irradiance [44]. These findings support the idea that microbial mats are a relatively stable, complex system in which development is governed and maintained by different microbial populations and the physicochemical conditions of the surrounding environment.

Our results complete and integrate previous studies giving some clues about short-term effects of physicochemical gradients on the physiological status, diversity, and redox state of microbial communities. In this study, the combination of lipid biomarker analysis with the isolation and lipid characterization of mat-building microorganisms provides further knowledge about the presence and strategies of important heterotrophs (such as sulfate-reducing bacteria, Clostridia, Bacteroides, etc), and a whole vision of the application of new monitoring approaches to get insight on the dynamics and composition of microbial mats.

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