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III. VALIDATION OF THE SIGNATURE LIPID BIOMARKER APPROACH IN MICROBIAL MATS

Figure III. "Tell a man that there are 300 billion stars in the universe, and he'll believe you.... Tell him that a bench has wet paint upon it and he'll have to touch it to be sure" Albert Einstein (1879–1955)

From top left to right: Camargue microbial mat / Gas chromatography column / Aqueous and organic phase resulting from a mild alkaline methanolysis of the Polar lipid fraction / Liquid culture of *Pseudomonas putida*.

- Introduction and objectives of the study

The use of signature lipid biomarker analysis in determining the *in situ* viable microbial biomass, community composition, and physiological status has been validated in a series of experiments (White, 1988), and has provided a convenient, quantitative, and comprehensive method for the study of microbial communities.

Lipid biomarker research has concentrated on phospholipids because are essential components of living cells and provide information about different states of microbial communities. In the polar lipid fraction, the phospholipids fatty acids (PLFA) have been widely studied, but other lipids classes such as lipo-amino acids, plasmalogens, acyl ethers and sphingolipids are also present. As it has been mentioned in chapter 'I. Introduction', this lipid classes can be used as a markers of the presence of certain microbial groups. For example, plasmalogens are found in *Clostridia* and other anaerobic bacteria (Moore *et al*., 1994); sphingolipids are found in *Sphingomonas* genus and in *Bacteroides* (Busse *et al*., 1999; Kato *et al*., 1995); lipo-amino acids have been described in several microbial genus (see section '1.4. Amino compound-containing lipids', 'I. Introduction') and the presence of ornithine-lipids have been related with phosphate-limiting conditions (Minnikin and Abdolrahimzadeh, 1974; Taylor *et al*., 1998; Gao *et al*., 2004); and alkyl-ether lipids are present in *Archaea* members as 'archaeol' and 'caldarchaeol' ('1.6. Terpenoid lipids', 'I. Introduction'). Therefore, the sequential fractionation and quantification of different lipids can be used to characterize microorganisms or complex communities (White *et al*., 1997).

Lipid analyses involve the separation into lipid classes and their conversion in a suitable form for gas chromatography analysis. Previous studies have described a one-tube method for gas-liquid chromatography analyses of ester-linked and amide-linked fatty acids (Mayberry and Lane, 1993) by a sequential alkaline/acid hydrolysis. The method also released dimethylacetals derived from plasmalogens and long chain bases from sphingolipids. However, one of the problems of this method was the analysis of all components at the same time that can induce erroneous identifications. The sequential fractionation method presented in this chapter includes a mild alkaline trans-

esterification in order to break the ester bond of the phospholipids fatty acids (PLFA), a second step based on a mild acid methanolysis to break the vinyl ether bonds of plasmalogens and get the dimethylacetals, and a third step that involves a strong acid hydrolysis to break amide and alkyl-ether bonds and liberate hydroxy fatty acids amide-linked, sphingoid bases and aqueous-soluble components.

Apart from the analysis of polar lipids, the glycolipid fraction derived from the silicic acid chromatography of total lipid extracts contains the polyhydroxyalkanoates (PHA) that are indicative of the nutritional status of environmental samples (Findlay and White, 1987). There is a lack of sensitivity and rapid methods for quantifying PHA in environmental samples (Anderson and Dawes, 1990). Traditionally, acidic methanolysis or ethanolysis and analysis of the hydroxyalkanoic esters have been performed (Braunegg *et al.*, 1978; Findlay and White, 1987), but recently, Elhottová *et al*. (2000) have combined the derivatization of 3-hydroxybutyrate and its detection by gas chromatography tandem mass spectrometry.

The aim of the present report was (i) to develop and validate a sequential fractionation protocol to detect lipid classes in polar lipid fractions, (ii) to validate the detection of polyhydroxyvalerate by the method described by Elhottová *et al*. (2000), and (iii) to investigate the variability of microbial mat samples and the applicability of the validated methods in this kind of samples.

- Material and methods

➢ Lipid extraction

Liquid cultures of *Sphingomonas paucimobilis*, *Clostridium butyricum*, *Staphylococcus aureus*, and *Pseudomonas putida* were obtained in the corresponding media listed in Table II.11 of the chapter 'General Material and Methods'. In the case of *Pseudomonas putida*, the cultures were performed in R2A medium with 0.3 g (high-phosphate) or 0.05 g (low-phosphate) K_2HPO_4. The cultures were prepared and lyophilized as previously described in the section '4. Morphobiochemical characterization of prokaryotes' ('General Material and Methods'). *Sphingomonas paucimobilis*, *Clostridium butyricum*, and *Staphylococcus aureus* was chosen because of their content in long chain sphingoid bases (LCB), plasmalogens and lysine amino-lipids, respectively. In addition, *Pseudomonas putida* was cultured under phosphate-limited conditions in order to induce the increase of ornithine amino-lipids (Kawai *et al.*, 1988). Microbial mat samples were sampled at 12:00 am in March and May 2004 (4 replicates each time) as a 'whole-core'. The total lipids were extracted following the modified Bligh and Dyer method and fractionated by silicic acid chromatography as described in the section '2. Lipid analysis methods'.

The polar lipid fraction was treated by the sequential fractionation method modified from Mayberry and Lane (1993) and described in the section 'Polar lipid fraction analysis'. Bovine phosphatidylethanolamine (BPE) with a plasmalogen content of approximately 60% was diluted in chloroform:methanol (1:1) at a concentration of 0.5 mg ml^{-1} and analyzed in the sequential protocol for the identification of dimethylacetals (DMAs) in the step B. The identification of fatty acid methyl esters (FAMEs) were performed in the Step A after a mild alkaline trans-methanolysis. The starvation and stress indices of FAMEs were calculated as it follows: Starvation index: [(*cy*17:0/16:1ω7*c*) + (*cy*19:0/18:1ω7*c*)] and Stress index: [(16:1ω7*t*/16:1ω7*c*) + (18:1ω7*t*/18:1ω7*c*)].

The DMAs derived from plasmalogens were released by a mild acid methanolysis in the Step B. Then, the remaining aqueous phase was treated by a strong acid hydrolysis to break amide-linkages (Step C and the presence of free amino acids released from amino-lipids was investigated by the EZ:faast® Kit (Phenomenex, USA. The remaining organic phase was BSTFA-derivatized (*N,O*-bis(trimethylsilyl trifluoroacetamide in the Step D in order to quantify sphingoid bases and hydroxyl fatty acids amide-linked as previously described in the chapter 'II. General Material and Methods'.

The glycolipid fraction was analyzed by the method described by Elhottová *et al*. (2000 for the detection and quantification of hydroxybutyrate derivatives ('Glycolipid fraction analysis'. Cultures on the strains *Cupriavidus necator* and *Chromobacterium violaceum* grown on sodium valerate were used to validate the detection of derivatized monomers of the polyhydroxyvalerate.

The samples were analyzed using a Hewlett-Packard 5890 series II gas chromatograph interfaced to a Hewlett-Packard 5971 mass selective detector. The GC was equipped with a 60-m non-polar column (HP-1 capillary column. The temperature program and conditions were previously described in chapter 'II. General Material and Methods'. Data analysis of lipid profiles was performed using the Microsoft® Excel software package. Mean values and standard deviations were calculated in the four sample-replicates. Data given as %mol was defined as picomoles (pmol of certain compound in a sample divided by the picomol sum of all measured compounds of the same nature (FAMEs, DMAs etc set to 100% in that sample.

- Results

➢ Sequential protocol of the polar lipid fraction in cultures and microbial mats

To validate the sequential developed method, the recoveries of the fatty acid methyl esters (FAMEs) of mentioned pure culture samples were analyzed in the Step A. Table III.1 summarize the %mol of the main FAMEs detected in the Step A for the pure culture samples. The main fatty acid detected in *Staphylococcus aureus* was *a*15:0 and the lipid membrane composition was dominated by terminal-branched saturated fatty acids. *Sphingomonas paucimobilis* sample was characterized by an important contribution of 16:0. Likewise, *Clostridium butyricum* culture reported a high percentage of 16:0 as well as cyclopropanoic acids with 19 carbons.

The lower aqueous phases resulting from the Step A were treated for the release of dimethylacetals derived for plasmalogens (vinyl-ether linkages) in the Step B. Depending on the concentration of ester-linked fatty acids in the samples, fraction B also contained FAMEs that did not partition into fraction A. In order to evaluate differences in the percentage of recovery of the detected FAMEs between the Step A and B, the %mol of each FAMEs detected in the fraction B was subtracted from the %mol of the same FAME in the fraction A.

Table III.1. Amount of FAMEs (as %mol) observed in the Step A of pure cultures.

FAMEs *S. aureus*[1]	%mol	FAMEs *Sphingom*[2]	%mol	FAMEs *Clost*[3]	%mol
***i*15:0**	10.57	***i*13:0**	4.26	**14:0**	2.63
***a*15:0**	52.83	***i*14:0**	3.94	**16:1ω9*c***	2.39
16:0	3.44	**14:0**	2.84	**16:1ω7*c***	0.52
***i*17:0**	17.45	***i*15:0**	20.68	**16:0**	69.78
***a*17:0**	25.80	***a*15:0**	5.60	**18:1ω9*c***	1.83
18:0	16.15	***i*16:0**	4.12	**18:1ω7*c***	9.44
***i*19:0**	9.20	***a*16:0**	4.29	**18:0**	3.15
***a*19:0**	6.67	**16:1ω7*c***	7.93	***cy*19:0a**	19.98
19:0	1.92	**16:0**	30.90	***cy*19:0b**	55.08
20:0	16.39	***i*17:0**	6.75	**20:1**	1.68

[1]*Staphylococcus aureus* [2]*Sphingomonas paucimobilis* [3]*Clostridium butyricum*.

The amounts of the main FAMEs recovered from the Step A and B of *Pseudomonas putida* 'low-phosphate' and 'high-phosphate' cultures are detailed in Table III.2. The values of %mol in the Step A indicated a change in the predominance of certain FAMEs under different culture conditions of the same strain. *Pseudomonas putida* 'low and high-phosphate' reported an important percentage of 16:1ω9*c*, 16:1ω7*c*, and 16:0. However, *Pseudomonas putida* 'high-phosphate' reported a lower contribution of the 16:1ω9*c* FAME to the total fatty acid content. Table III.2 also include the 'step B %mol' of the main FAMEs detected in the step A, and the standard errors for $n = 3$ replicates. The standard deviation was low for all the fatty acid analyzed with the exception of 16:1ω7*c*, and 16:0 in the step A. The differences in the percentage of recovery of the detected FAMEs between the Step A and B for *Pseudomonas putida* 'low and high-phosphate' are shown in Fig. III.1.

Table III.2. Amount of FAMEs (as %mol observed in the Step A of pure cultures.

Pseudomonas 'low phosphate'[1]	Step A mean of PLFA (%mol)	Step B mean of PLFA (%mol)	*Pseudomonas* 'high phosphate'[2]	Step A mean of PLFA (%mol)	Step B mean of PLFA (%mol)
***i*13:0**	2.08 (0.06	1.35 (0.24	***i*13:0**	3.23 (0.13	1.17 (0.07
***i*15:0**	2.70 (0.06	2.47 (0.06	***i*14:0**	3.23 (0.09	2.20 (0.20
16:1ω9*c*	21.13 (0.70	15.37 (0.12	***i*15:0**	5.83 (0.10	5.32 (0.20
16:1ω7*c*	15.83 (1.84	22.99 (0.09	***a*15:0**	4.05 (0.17	3.65 (0.15
16:0	20.65 (1.05	23.68 (0.16	**16:1ω9*c***	9.28 (0.23	8.08 (0.04
***i*17:0**	1.74 (0.13	1.59 (0.02	**16:1ω7*c***	21.03 (0.42	21.78 (0.02
***cy*17:0**	3.11 (0.09	2.80 (0.03	**16:0**	20.72 (1.24	27.56 (0.26
18:1ω9*c*	20.32 (0.46	18.65 (0.24	***i*17:0**	2.53 (0.11	2.79 (0.04
18:1ω7*c*	4.21 (0.23	4.64 (0.05	**18:1ω9*c***	12.28 (0.29	11.26 (0.25
18:0	1.34 (0.16	1.42 (0.02	**18:1ω7*c***	4.31 (0.13	4.50 (0.11

[1,2]*Pseudomonas putida* 'low and high-phosphate'. Standard deviation is given in parentheses.

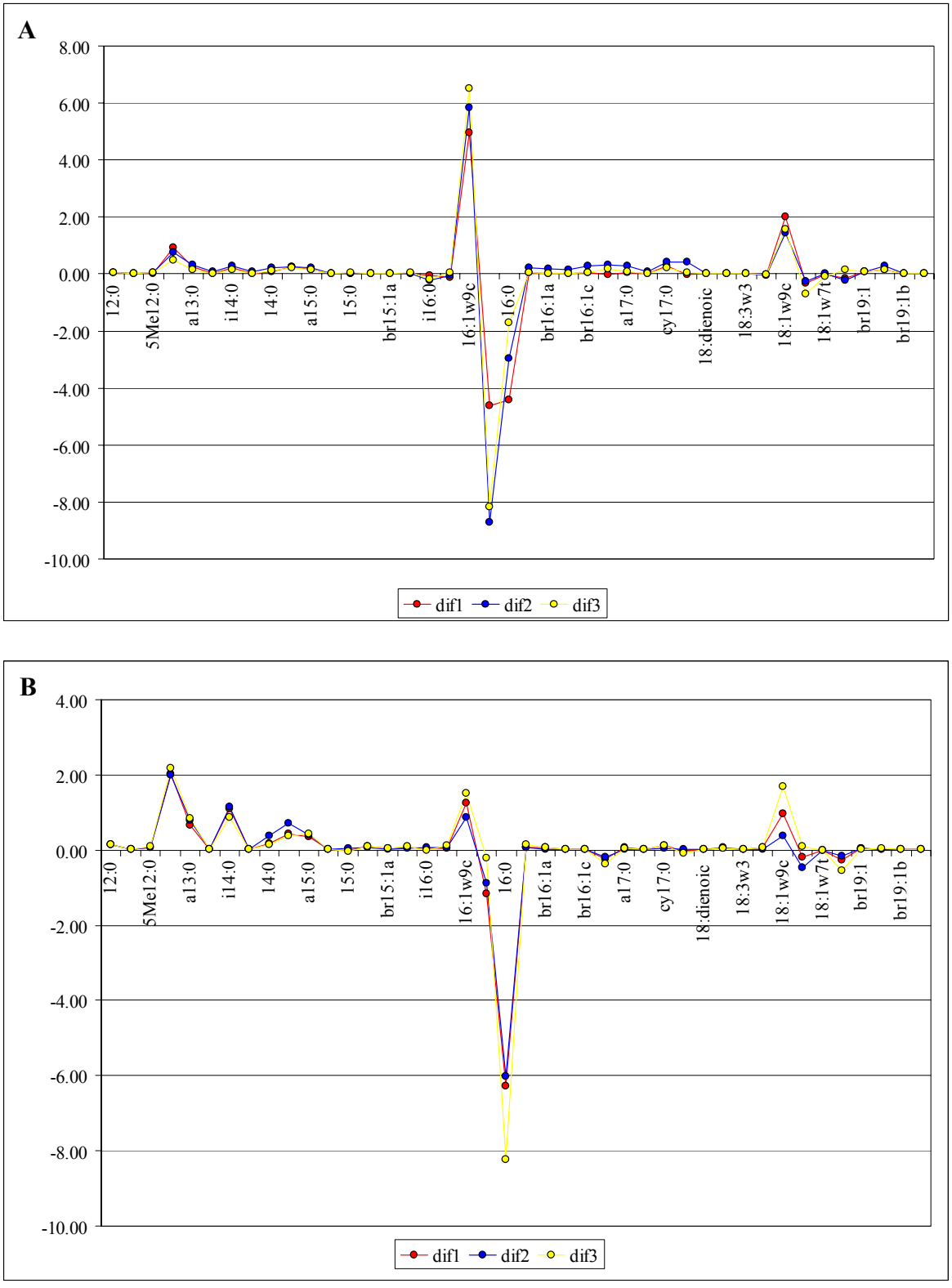

Figure III.1. Differences in the % mol recovery of a detected FAME in the Step A and B.

(A□'low-phosphate'□(B□'high-phosphate'. The differences of the %mol of each fatty acid in the Step A with the corresponding %mol in the Step B are given as 'dif1', 'dif2' and 'dif3' as the subtraction between the 3 replicates.

In Figure III.1, the subtractions above zero corresponded to FAMEs that had a higher percentage of recovery in the Step A in comparison with the Step B. On the contrary, subtraction values below zero indicated a higher recovery in the Step B in comparison with the %mol in the Step A. In this case, 16:1ω9*c* and 18:1ω9*c* reported a higher recovery in the Step A in both culture conditions. On the other hand, 16:1ω7*c* has an important recovery in the Step B of the *Pseudomona*s 'low-phosphate', as well as the 16:0 in the *Pseudomonas* 'high-phosphate' sample.

The FAME content of microbial mat samples were also analyzed in the Step A and B (Table III.3 and 4□and 94 and 91 different fatty acid methyl esters were detected in 'March' and 'May' replicates, respectively. Although the %mol of the detected FAMEs were different between 'March' and 'May' samples, the main fatty acid methyl esters were the monoenoics 16:1ω7*c*, 18:1ω9*c*, 18:1ω7*c* and the normal saturated 16:0. The standard deviation between the $n = 4$ replicates was low in all cases except from the more abundant fatty acids (e.g. 16:0 in 'March' replicates□or FAMEs that tend to co-elute in the same peak under the working conditions (e.g. 18:1ω9*c* and 18:1ω7*c*□.

Table III.3. Main FAMEs (as %mol□in the Step A and B of microbial mat 'March' samples.

Microbial mat FAMEs	Step A Mean	Step B Mean	Microbial mat FAMEs	Step A Mean	Step B Mean
***i*15:0**	3.83 (0.03□	5.16 (0.50□	**18:2ω6**	4.20 (0.07□	3.47 (0.48□
***a*15:0**	2.85 (0.25□	3.19 (0.35□	**18:3ω3**	2.58 (0.20□	2.74 (0.23□
***i*16:0**	2.21 (0.09□	2.39 (0.11□	**18:1ω9*c***	7.88 (0.47□	6.26 (0.37□
16:1ω9*c*	3.08 (0.38□	3.01 (0.25□	**18:1ω7*c***	19.59 (1.09□	16.18 (0.52□
16:1ω7*c*	6.06 (0.44□	6.51 (0.20□	**18:0**	2.69 (0.07□	2.74 (0.14□
16:0	14.30 (1.20□	16.29 (1.01□	***br*19:1**	1.29 (0.15□	2.31 (0.49□
10Me16:0	3.10 (0.04□	2.46 (0.24□	***cy*19:0**	3.43 (0.48□	2.74 (0.24□
***a*17:0**	1.93 (0.08□	1.53 (0.12□	**2Me dimethyl 20:0**	1.25 (0.27□	0.42 (0.03□

Table III.4. Main FAMEs (as %mol) in the Step A and B of microbial mat 'May' samples.

Microbial mat FAMEs	Step A Mean	Step B Mean	Microbial mat FAMEs	Step A Mean	Step B Mean
14:0	1.23 (0.09)	1.13 (0.15)	***i*17:0**	1.03 (0.04)	0.99 (0.03)
***i*15:0**	5.33 (0.35)	5.59 (0.58)	***a*17:0**	1.69 (0.08)	1.51 (0.12)
***a*15:0**	3.42 (0.14)	3.14 (0.21)	**18:3ω3**	3.21 (1.68)	3.16 (0.29)
***i*16:0**	2.20 (0.08)	2.10 (0.08)	**18:1ω9*c***	3.65 (1.22)	7.83 (0.56)
16:1ω9*c*	3.09 (0.48)	2.92 (0.33)	**18:1ω7*c***	26.36 (0.69)	14.70 (1.00)
16:1ω7*c*	5.50 (0.27)	5.69 (0.29)	**18:0**	2.76 (0.11)	2.82 (0.07)
16:0	19.41 (0.37)	18.47 (0.58)	***br*19:1**	1.19 (0.06)	2.32 (0.88)
10Me16:0	2.57 (0.11)	2.22 (0.01)	***cy*19:0**	3.89 (0.27)	3.30 (0.34)

In addition, the starvation and stress indices of FAMEs were calculated with the %mol data obtained in the Step A, Step B and with the re-calculated %mol after the sum of picomoles of each fatty acid in both fractions (Table III.5). In the 'March' samples, both indices reported similar values in all cases; however, 'May' samples reported more differences especially in the starvation index with higher values in the step B. The standard deviation for $n = 4$ was low in all cases. The percentage of recovery of each fatty acid in the Step A and B were also analyzed in microbial mat samples (Fig. III.2).

Table III.5. Starvation and Stress indices of microbial mat samples.

Indices	Step A Mean	Step B Mean	Step A+B[1] Mean
Starvation index MARCH	0.23 (0.02)	0.23 (0.01)	0.23 (0.02)
Stress index MARCH	0.12 (0.02)	0.13 (0.02)	0.12 (0.02)
Starvation index MAY	0.21 (0.01)	0.30 (0.01)	0.22 (0.01)
Stress index MAY	0.14 (0.01)	0.16 (0.01)	0.14 (0.01)

[1]%mol calculated after the sum of picomoles g^{-1} dry weight of each FAME in the Step A and B.

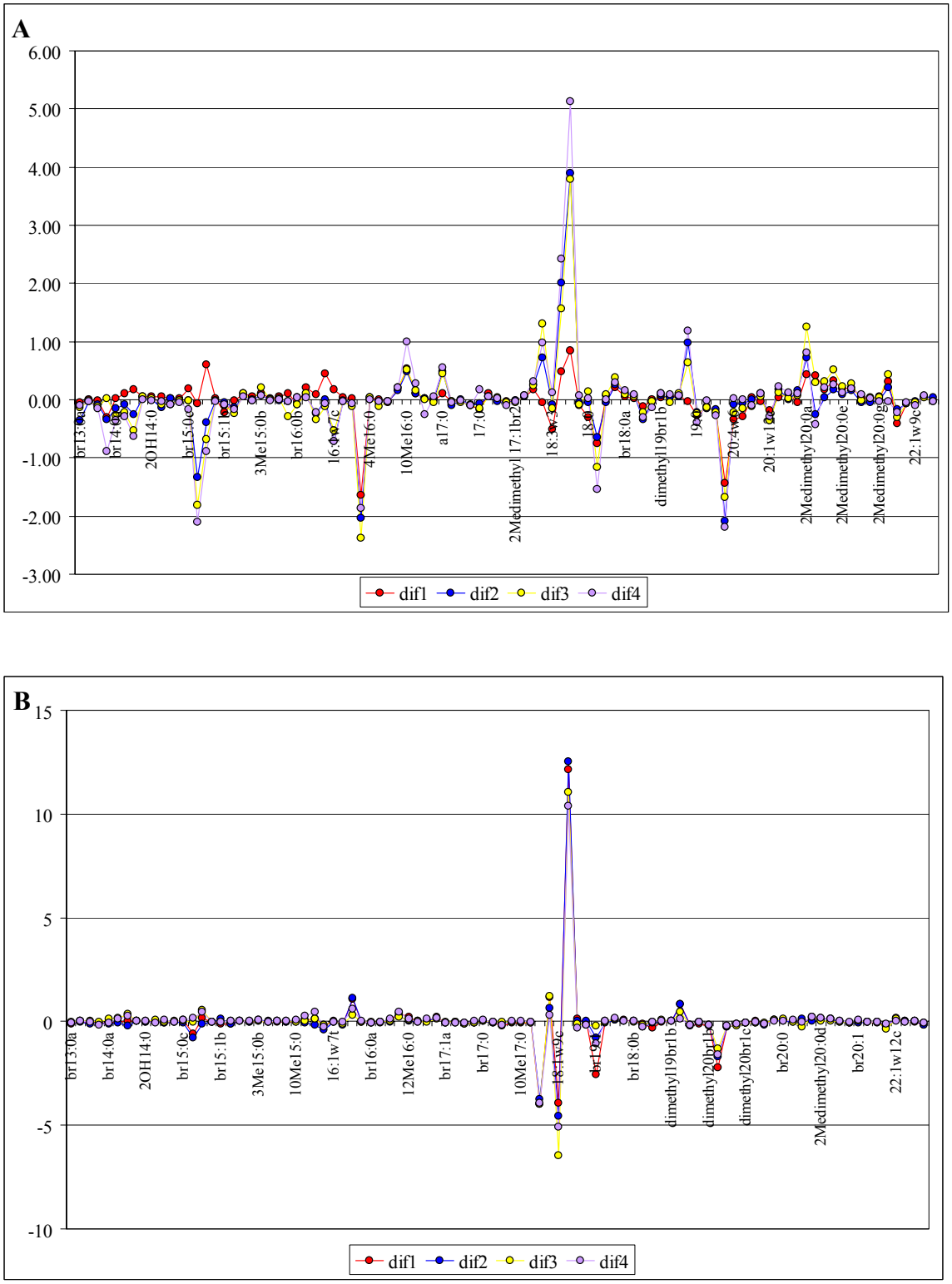

Figure III.2. Differences in the % mol recovery in the Step A and B for microbial mat samples.

(A□'March' samples□(B□'May' samples. The differences of the %mol of each fatty acid in the Step A with the corresponding %mol in the Step B are given as 'dif1', 'dif2', 'dif3' and 'dif4' as the subtraction between the 4 replicates.

Figure III.2 indicated a lower variability in 'May' samples in comparison with 'March' replicates due to the fact that 'May' samples followed a same profile of % recovery in all replicates. In addition, most of the FAMEs detected in 'May' samples reported similar percentages of recovery in both fractions, which was indicated by 'dif' values around zero. The fatty acids that reported a higher recovery in the Step A were 18:1ω7*c* and *cy*19:0 (less evident in both kind of sample. Moreover, the FAMEs with higher recovery in the Step B were 16:0 and 18:1ω9*c* for 'March' and 'May' replicates, respectively.

The Step B of the sequential protocol is based on an acidic hydrolysis to liberate vinyl-ether bonds found in plasmalogens. In the bovine phosphatidylethanolamine (BPE sample, 16:0 DMA, 17:0 DMA, 18:0 DMA and 18:1 DMA were identified. Furthermore, dimethylacetals (DMAs derived from plasmalogens were only found in *Clostridium butyricum* culture and in microbial mat samples. The dimethylacetal content of *Clostridium butyricum* (Table III.6 revealed the presence of DMAs from 16 to 20 carbons and a higher predominance of 19:1 DMAs.

Table III.6. Dimethylacetals derived from plasmalogens in *Clostridium butyricum* culture.

Clostridium DMAs	Mean %mol[1]	*Clostridium* DMAs	Mean %mol
16:1DMAa	7.68 (0.29	**18:1DMAb**	18.20 (0.23
16:1DMAb	1.87 (0.07	**18:1DMAc**	0.48 (0.04
16:1DMAc	0.14 (0.01	**18DMA**	1.72 (0.06
16DMA	3.15 (0.10	**19:1DMAa**	16.15 (0.39
17:1DMA	0.22 (0.03	**19:1DMAb**	27.72 (0.81
18:1DMAa	12.74 (0.06	**20:1DMAa**	0.66 (0.07

[1]Standard deviations of $n = 3$ are given in parentheses.

The %mol of the detected dimethylacetals in the step B (Table III.7) was low in comparison with the rest of FAMEs recovered in that fraction (ca. 1.5–3% of the total picomoles per gram of dry weight) and the most representative dimethylacetal was the 15:0 DMA. Microbial mats recovered in May reported twice the quantity of dimethylacetals observed in 'March' samples, and the higher amount was detected in different dimethylacetals of 15 carbons. Although the %mol values of DMAs were low in all cases, the standard deviation was higher of that observed for FAMEs replicates.

Table III.7. Dimethylacetals derived from plasmalogens in microbial mat samples.

March DMAs	Mean %mol[1]	picomoles g^{-1} dry weight	May DMAs	Mean %mol	picomoles g^{-1} dry weight
14:0 DMAa	0.07 (0.04)	135.65	**14:0 DMAa**	0.05 (0.02)	392.52
14:0 DMAb	0.18 (0.11)	343.75	**14:0 DMAb**	0.10 (0.05)	755.24
15:0 DMAa	0.02 (0.00)	29.73	**15:0 DMAa**	0.01 (0.00)	52.23
15:0 DMAb	0.73 (0.42)	1416.95	**15:0 DMAb**	0.35 (0.19)	2491.46
15:0 DMAc	0.10 (0.05)	196.66	**15:0 DMAc**	0.06 (0.03)	431.99
15:0 DMAd	0.07 (0.03)	135.34	**15:0 DMAd**	0.05 (0.03)	342.12
16:0 DMAa	0.06 (0.02)	114.14	**16:0 DMAa**	0.05 (0.01)	351.97
16:0 DMAb	0.11 (0.04)	222.51	**16:0 DMAb**	0.08 (0.06)	548.90
17:0 DMAa	0.03 (0.01)	54.88	**17:0 DMAa**	0.01 (0.00)	81.14
17:0 DMAb	0.03 (0.01)	56.59	**17:0 DMAb**	0.02 (0.01)	118.52
17:0 DMAc	0.04 (0.02)	86.17	**17:0 DMAc**	0.02 (0.00)	157.08
18:0 DMAa	0.13 (0.05)	255.70	**18:0 DMAa**	0.08 (0.02)	581.69

[1]Standard deviations of $n = 4$ are given in parentheses.

The lower phases resulting from the Step B were treated by a strong acid hydrolysis for the release of amide-linked fatty acids and moieties. In this case, only the fraction C (aqueous phase resulting from the step C with content of amide-linked moieties) of the pure cultures of *Staphyloccocus aureus* and *Pseudomonas putida* 'low and high phosphate' were analyzed by EZ:faast kit (Phenomenex) in order to identify the presence of amino acids released from the amino-lipids of these strains. The solid phase extraction and derivatization of the aqueous fraction failed to detect amino acids in the sample.

The organic phases obtained in the Step C were methanolyzed and derivatized by BSTFA in the Step D in order to identify and quantify the presence of long chain bases and hydroxyl fatty acid-amide linked in *Sphingomonas paucimobilis* and microbial mat samples. For the quantification of both compounds in the same fraction, sphingosine (C18:1) and the standard heneicosanoic acid methyl ester (C21:0) were added before the BSTFA-derivatization. In *Sphingomonas paucimobilis* samples 2OH 14:0 was detected with a mean value of 230.7 nanomoles per gram of dry weight. Microbial mat samples reported the presence of 3-hydroxy 16:0 (3OH 16:0), 3OH 17:0 and 3OH 18:0. The detection and quantification of sphingoid bases (long chain bases, LCB) was difficult because of problems of co-elution in the chromatographic separation of similar structural component with a HP-1 column. Dihydrosphingosine (C18:0) was detected in *Sphingomonas paucimobilis* with a mean value of 61.3 nanomoles per gram of dry weight. The quantification of LCB was not possible in microbial mat samples but C18:0 and C19:0 LCB were detected co-eluted in the same peak with the internal standard sphingosine (C18:1) (Fig. III.3).

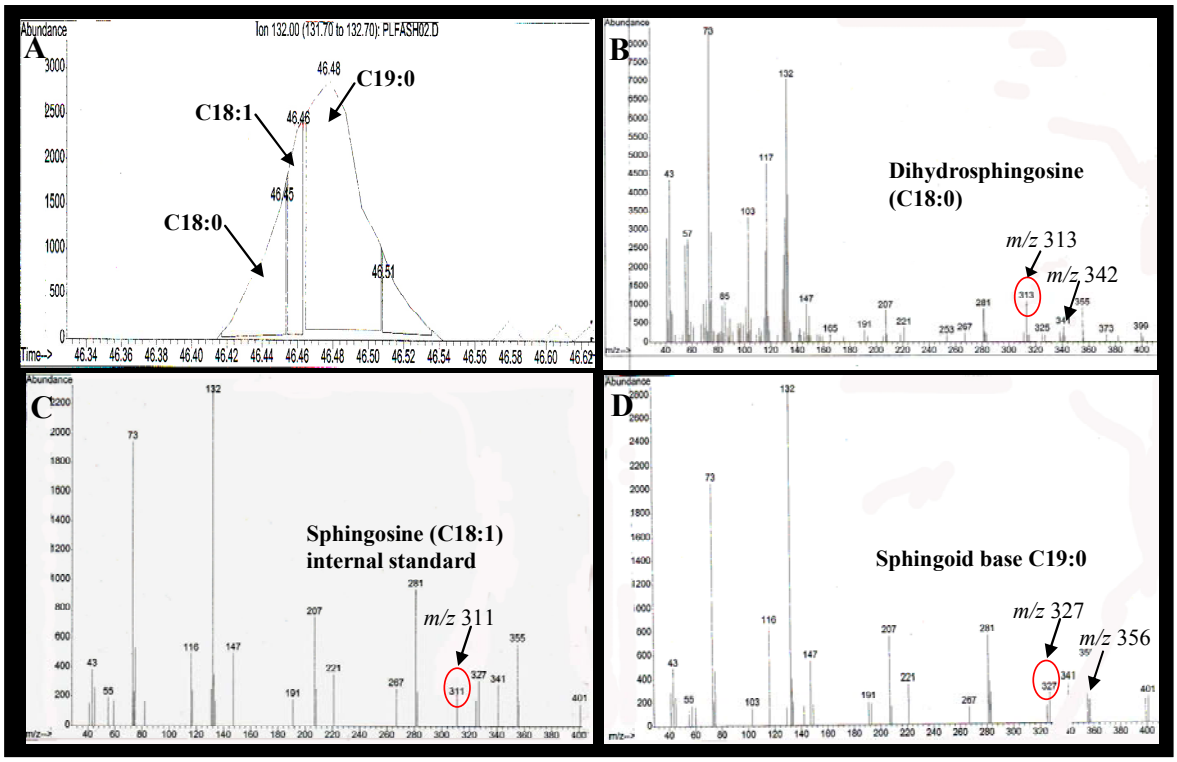

Figure III.3. Detection of Sphingoid bases in the Step D of microbial mat samples.

(A□Gas chromatography peak with the sphingoid bases co-eluted□(B□Dihydrosphingosine (C18:0□mass spectrum with the characteristics *m/z* values at 313 and 342□(C□Sphingosine (C18:1□internal standard with *m/z* = 311□(D□Sphingoid base of 19 carbons (C19:0□with *m/z* values at 327 and 356.

➢ Detection of polyhydroxyalkanoates

The detection of polyhydroxyalkanoates were performed in the glycolipid fraction resulting from the silicic acid chromatography of total lipids. The MBSTFA-derivatized monomers of 3-hydroxybutyric acid were identified by its intense *m/z* fragments $[M–57]^+$ at 275 as was previously described by Elhottová *et al*. (2000□ and was confirmed by the analysis of the glycolipid fraction of *Cupriavidus necator* grown on sodium gluconate as the only source of carbon and energy (Fig. III.4 A□ The fragmentation pattern of the derivatized monomers of 3-hydroxyvaleric acid (polyhydroxyvalerate□were supposed to have a intense *m/z* value of 289 corresponding to $[M–57]^+$ (M: mass of the compound□ and was confirmed by the analysis of the glycolipid fraction of *Cupriavidus necator* grown on sodium valerate by the method

developed by Elhottová *et al.* (2000□ (Fig. III.4 B□ Derivatized monomers of 3-hydroxyoctanoic acid were used as an internal standard.

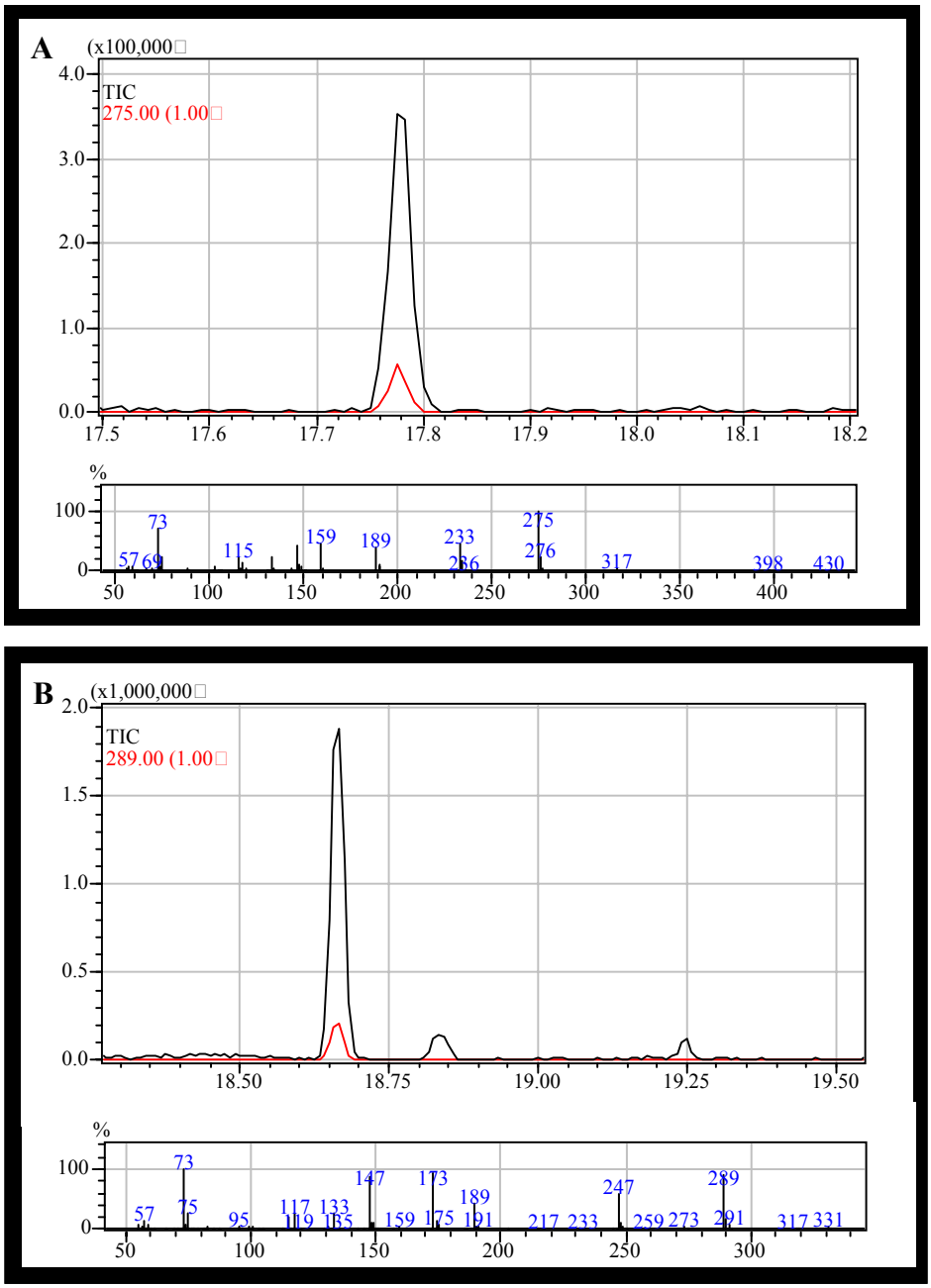

Figure III.4. Detection of MBSFTA-hydroxyalkanoic acids in cultures of *Cupriavidus necator*.

(A□MBSTFA-3-hydroxybutyrate (*m/z* 275□in sodium gluconate culture□(B□MBSTFA-3-hydroxyvalerate (*m/z* 289□ in sodium valerate culture. The top of the figure is the chromatographic separation, and below the fragmentation spectra of the peak is shown.

Once the detection of polyhydroxyvalerate as MBSTFA-hydroxyvaleric monomers was accomplished, the PHB and PHV content in the glycolipid fraction of microbial mats was analyzed (Table III.8). The quantification in 'March' and 'May' replicates showed a higher standard deviation in 'March' samples. Moreover, in both kind of samples the amount of PHV detected was higher and the ratio PHB:PHV was around 1:4.

Table III.8. Quantification of polyhydroxyalkanoates in microbial mat samples.

PHA[1]	March Mean	May Mean
PHB nmol g^{-1} dry weight	37.24 (9.77)	44.03 (3.99)
PHV nmol g^{-1} dry weight	147.97 (42.92)	194.76 (10.70)

[1]PHA: Polyhydroxyalkanoates; PHB and PHV: polyhydroxybutyrate and polyhydroxyvalerate as nanomoles per gram of dry weight. Data given in parentheses are standard deviations of $n = 4$ replicates.

- Discussion and conclusions

The methods presented in this report are an attempt to identify and quantify different lipid classes integrated in the Signature Lipid Biomarker (SLB approach. A sequential protocol of the polar lipid fraction have been presented as a good alternative for the quantification of organic-soluble and amide-linked moieties and for the integration of the data obtained in each fraction. Despite of the good perspectives of the sequential protocol, more studies need to be performed in order to improve the detection of certain compounds as well as the increasing of sensitivity of the analysis.

The sequential protocol was performed in pure culture samples to evaluate the recovery of compounds in each fraction and to determine the standard deviation of the replicates. As the culture replicates came from a single homogenized culture, the differences detected between replicates would be due to the manipulation of the samples and the analytical procedure. Indeed, the values of the detected fatty acid methyl esters (FAMEs in the fraction A reported a low standard deviation among their replicates. In addition to pure bacterial cultures, we performed the sequential fractionation method with microbial mat replicates obtained in different seasons. The detection of FAMEs in microbial mat samples have been previously performed (Navarrete *et al*., 2000, but the main problem of this analysis have been the reproducibility of the analysis in different 'core' samples recovered from the same area of sampling.

Microbial mats communities are characterized by differences in the accumulation of cyanobacteria in the topmost layers and by the distribution of purple sulfur bacteria in 'patches' in the underlying covers. For this reason, mat samples were chosen as a model of complex microbial community with a high number of FAMEs and because we wanted to check the variability between 'cores' sampled at the same time. In fact, the standard deviation of the %mol of fatty acids detected in mat replicates were similar to those observed in the pure cultures, except from the FAMEs with higher %mol values or those that tend to have co-elution problems and their chromatographic areas have to be integrated manually, such as 18:1ω9*c*.

Fatty acid methyl esters (FAMEs were also recovered in the fraction B which means that certain fraction of the organic phase obtained in the Step A remained in the inter-phase of the tube or on the surface of the walls and is then analyzed in the Step B. The amount of the fraction that is not partitioned into the fraction A and their reproducibility depend on the manipulator, and it has been estimated as 3–5% of the total FAMEs recovered in the Step A (data obtained for pure cultures. The relative percentage of the FAMEs recovered in the fraction B were expected to be similar to those observed in the fraction A however, differences in the percentage of recovery were detected.

In *Pseudomonas putida* cultures, 16:1ω9*c* and 18:1ω9*c* reported a higher percentage of recovery in the Step A, and 16:1ω7*c* and 16:0 in the fraction B. On the contrary, in microbial mat samples a higher percentage of recovery of 18:1ω7*c* and *cy*19:0 was observed in the fraction A, and 16:0 and 18:1ω9*c* in the fraction B. There is not a correspondence between the fatty acids with different recoveries between the pure cultures and microbial mat samples, but it seems to be a preferential recovery of monoenoic fatty acids that should be investigated in future studies. However, the preferential recovery of 16:0 in both culture and mat samples suggest a specific reason, maybe related with the chemical structure of this fatty acid or an increased release of this ester-linked FA after the mild acid methanolysis performed in the step B. Although the higher percentage of recovery in the fraction A of *cy*19:0 was not very evident, Mayberry and Lane (1993 have observed that cyclopropane acids are acid-sensitive and undergo degradation particularly in acidic methanol. This fact can explain differences in the recovery of *cy*19:0 that might be partially degraded under the mild acid conditions of the Step B.

The importance of the recovery of FAMEs in the fraction A and B for the calculation of 'Stress and Starvation' indices were also evaluated. Because of the fact that FAMEs recovered in the fraction B represented a low percentage of the total fatty acid content, the indices were not influenced by the missing data of the Step B. Although there is a low quantity of organic phase of the fraction A that remain in the tubes when the Step B start, it may be convenient to sum the picomoles per gram of dry weight data of both fractions and re-calculate the %mol. We have to take into account

that the percentage of organic phase that do not partition into fraction A can be different depending on the manipulator and more efforts should be done in order to standardize this step.

The detection of dimethylacetals (DMAs derived from plasmalogens was achieved under the mild acid conditions in the step B. The standard deviations of the %mol values of DMAs in *Clostridium butyricum* culture were low in all cases however, microbial mat samples reported higher variability that can be related with the detected low values of these compounds. Moreover, the low reproducibility of DMA values can be also due to the fact that DMAs are acid-labile in aqueous solvents and exits the possibility of formation of free aldehydes in the Step B if there is any water in the sample. To avoid this process, the phase resulting from the Step A can be dried and suspended in chloroform:methanol before the acid hydrolysis to avoid the presence of water, and acetic anhydride that acts a water scavenger can be also added. Other process that can induce to differences in the quantifications of plasmalogens is that the common 16:0 and 18:0 DMAs tend to co-elute with *i*16:0 and *i*18:0 FAMEs in a typical GC column (polyethylglycol (Ackman, 1999.

Regarding the detection of amino acid moieties in the Step C, several problems should be solved. The acidic conditions of the strong acid hydrolysis allowed the breakage of the amide-bond and the sphingoid bases and the hydroxy fatty acids (OH FA amide-linked were recovered in the Step D. However, we can not predict the effect of a pH around 1 in the aqueous phase of the fraction C in the amino acid structure. In addition, we can not assure that the solid phase extraction and derivatization of amino acids by the EZ:faast kit (Phenomenex would have failed. For this reason, future studies should be focused on the application of new methods of amino acid detection (as well as other moieties such as carbohydrates or on the neutralization of the acidic pH without interferences in the method.

The Step D of the sequential protocol was characterized by the recovery of sphingoid bases and OH FA amide-linked. Unfortunately, the sphingoid bases tend to co-elute and the quantification was difficult. In order to improve the resolution of these structurally similar compounds, another kind of GC column might be used.

Furthermore, the use of a broader derivatizing agent, e.g. BSTFA + TMCS, can improve the silylation process of amine and amide groups that have a lower reactivity. In addition, other internal standard should be found because the similar structure of Sphingosine (C18:1 to the most common sphingoid bases can induce problems of identification. The evaluation of the sequential protocol have detected several problems that will be solved in future studies, and have also considered the possibility of detection of archaeal ether lipids in the step D after the strong acid hydrolysis capable of breaking the ether bond (White *et al*., 2003 .

In this study, we have validated the detection of MBSTFA-3-hydroxyvalerate by the method described by Elhottová *et al*. (2000 . This fact has proved very useful for the analysis of polyhydroalkanoate (PHA content in the glycolipid fraction of microbial mats, so that previous studies have observed a higher abundance of polyhydroxyvalerate (PHV in these systems (Rothermich *et al*., 2000 . Apart from that, the reproducibility of the PHA detection should be improved and more sensitive methods need to be applied.

This survey is a preliminarily study to evaluate the variability of microbial mat samples and the applicability of the SLB approach. Future studies will be performed in order to evaluate the model of distribution of the lipid data and modelize set of replicates that would support the analysis. In addition, the distribution of fatty acid data of microbial mats from different locations will be analyzed in an attempt of classification of mat systems by means of their FAMEs distribution. For the time being, this preliminarily evaluation allows us to apply the SLB approach in microbial mat samples and formulate hypothesis taken into account the detected variability between mat samples.

Conclusions

- A sequential fractionation method that integrates alkaline trans-methanolysis of FAMEs, the mild acid release of aldehydes from plasmalogens, and the strong acid hydrolysis of other lipid components (amide-linked hydroxyl fatty acids, alkyl-ethers, sphingoid bases, amino-lipids has been evaluated in pure cultures and microbial mat samples.

- Both pure cultures and microbial mat samples reported a low standard deviation between their replicates, except from %mol values of the most representative fatty acid methyl esters.

- Fatty acid methyl esters (FAMEs that did not partition in the fraction A were recovered in the Step B. Although the %mol of the FAMEs in step B were expected to be similar to those recovered in the fraction A, differences in the percentage of recovery were observed.

- *Pseudomonas putida* 'low and high' phosphate cultures reported a higher recovery in the Step A of 16:1ω9*c* and 18:1ω9*c*, and 16:1ω7*c* and 16:0 in the fraction B. On the contrary, in microbial mat samples a higher recovery of 18:1ω7*c* and *cy*19:0 was observed in the fraction A, and 16:0 and 18:1ω9*c* in the fraction B.

- A preferential recovery of monoenoic fatty acids has been suggested. In addition, the higher recovery of *cy*19:0 in the fraction A can be related with the acid-sensitive nature of cyclopropanoic acids that has been previously detected.

- The detection of dimethylacetals in the step B reported a higher variability that can be associated with the acid-labile nature of DMAs in aqueous solvents or by co-elution with FAMEs

- The detection of amino acids and sphingoid bases in the Step C and D should be improved to avoid the acidification problems of the strong acid hydrolysis and the co-elution of sphingoid bases.

- The detection of derivatized monomers of PHV have been validated and applied to the detection of PHV and PHB in microbial mat samples. However, future studies should be focused on more sensitivity methods and increasing the reproducibility of this analysis.

• Publications

- **Villanueva L., A. Navarrete, J. Urmeneta, D. C. White, and R. Guerrero.** 2004. Physiological status and microbial diversity assessment of microbial mats: The Signature Lipid Biomarker Approach. *Ophelia* **58**:165–173