Tesi doctoral presentada per En/Na

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amb el títol

"Ecophysiological and molecular characterization of estuarine microbial mats"

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BIOLOGIA

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Facultat de Biologia
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II. General Material and Methods

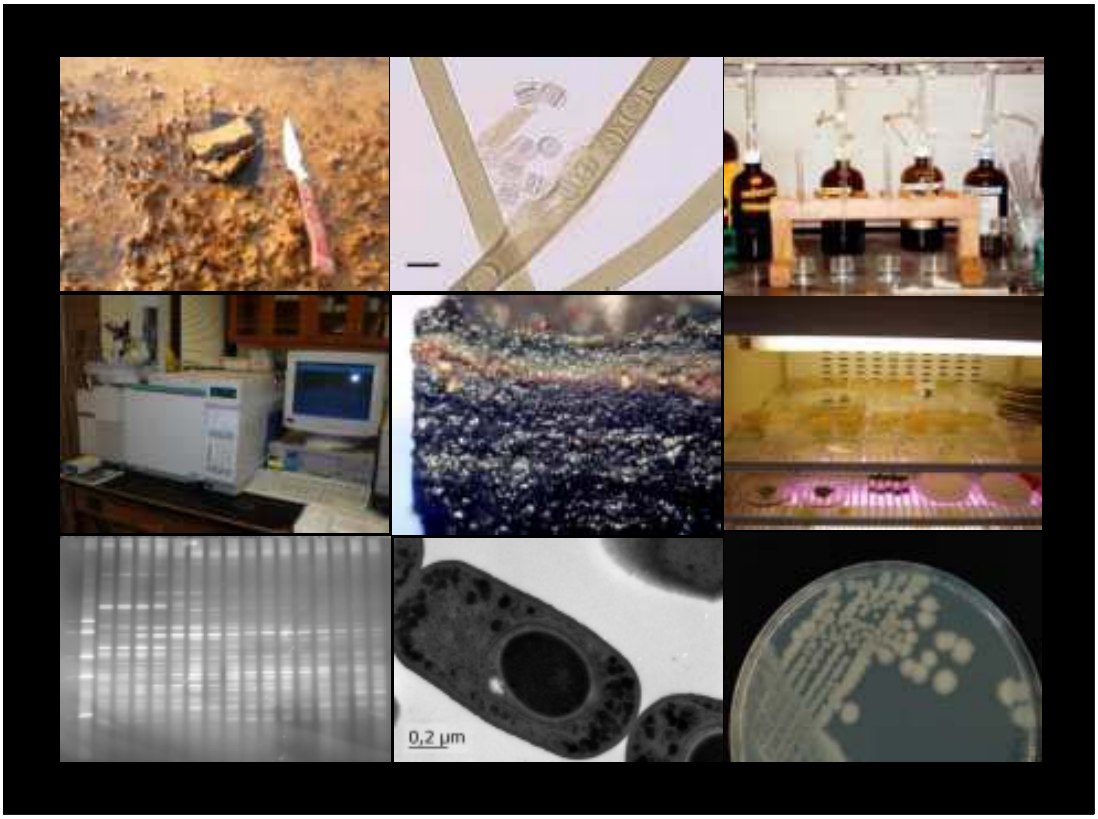

Figure II. "I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale" Marie Curie (1867–1934).

From top left to right: sampling of an Ebro delta microbial mat / Optical micrograph of the cyanobacteria *Lyngbya* sp. / Silicic acid chromatography of total lipid extracts / Gas chromatograph/mass spectrometer (GC/MS) unit / Microbial population layers in a microbial mat / Incubator chamber for cultivation of cyanobacteria / Denaturing gradient gel electrophoresis (DGGE) gel / Transmission electron micrograph (TEM) of an isolated *Clostridium* sp. / Agar plate with growing colonies of a *Bacillus* sp.

NOTE: The general material and methods are explained as it follows; however, specific modifications are detailed in the corresponding chapters.

1. Sampling Sites and physicochemical conditions

- Description of the sampling sites

The studies described in this work have been performed with two kinds of estuarine microbial mats from different locations: Ebro delta microbial mats (Spain), and Camargue microbial mats (Rhone delta, France). Although both microbial ecosystems show similarities, they are exposed to different physicochemical conditions and are formed by different microbial populations. This fact leads us to study these microbial mats as a model of complex microbial communities. The aim of this study is making comparisons between the mentioned ecosystems to increase our knowledge about the microbial evolution and physicochemical relationships of their microbial populations.

Ebro delta microbial mats

The microbial mats studied are located in the Ebro delta, the third largest delta in the Mediterranean Sea (Fig. II.1 and II.2). It is located at the northeastern coast of the Iberian Peninsula (40° 40´ N, 0° 40´ E) and has an area of 320 km^2. It has the characteristic delta shape, and presents two lateral sand bars, El Fangar in the North and La Banya in the South. The studied area is La Banya spit, formed by a narrow sand bar and a peninsula, located south of the main body of the Ebro delta. Sea water can penetrate into the backshore through channel inlets, cutting the steeper coastal barrier of the open sea side of the spit or through the complex drainage channel system of the low-energy beaches in the inner Alfacs Bay. Sea water can stay in the backshore almost permanently, trapped in isolated depressions or lagoons (Mir *et al*., 1991; Rampone *et al*., 1993; Guerrero *et al*., 1993a).

Camargue microbial mats

The estuarine mats studied are located in the salterns of Salins-de-Giraud, which is composed of a succession of water concentration ponds in the Mediterranean littoral zone of the Rhone delta (Southern France). The total surface of the saltern is about 110 km^2 (Caumette *et al*., 1994) (Fig. II.1 and II.2). The sampling site is located at the bottom of a pre-concentration pond and has an area of about 10 km^2. The photosynthetic microbial mats cover a large proportion of this pond, and are constituted of thin cohesive layers from 5 to 10 cm thick (Table II.1, II.2).

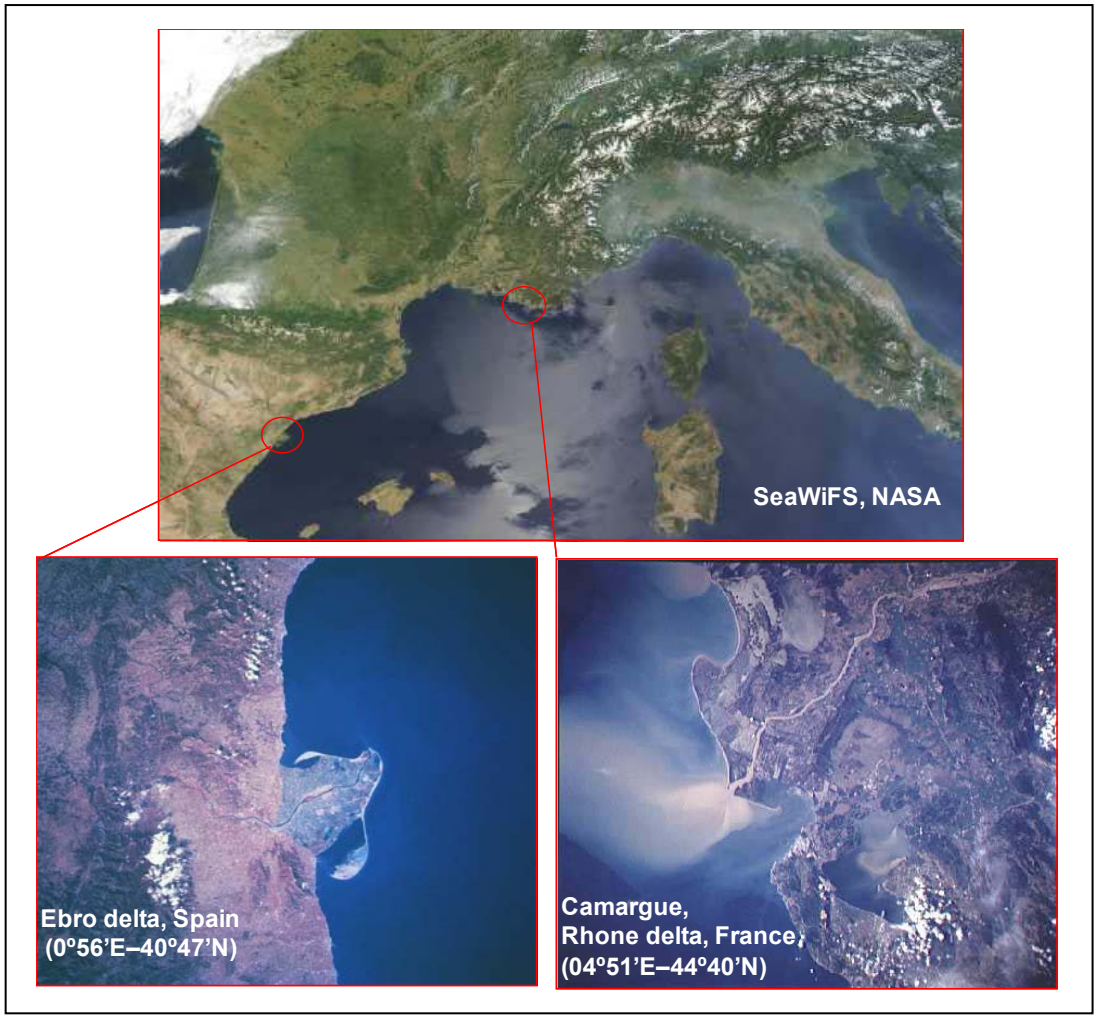

Figure II.1. Geographical location of microbial mats.

Satellite-photographs from the SeaWiFS project (Sea-viewing Wide Field-of-view Sensor; see 'Useful websites' section) from NASA.

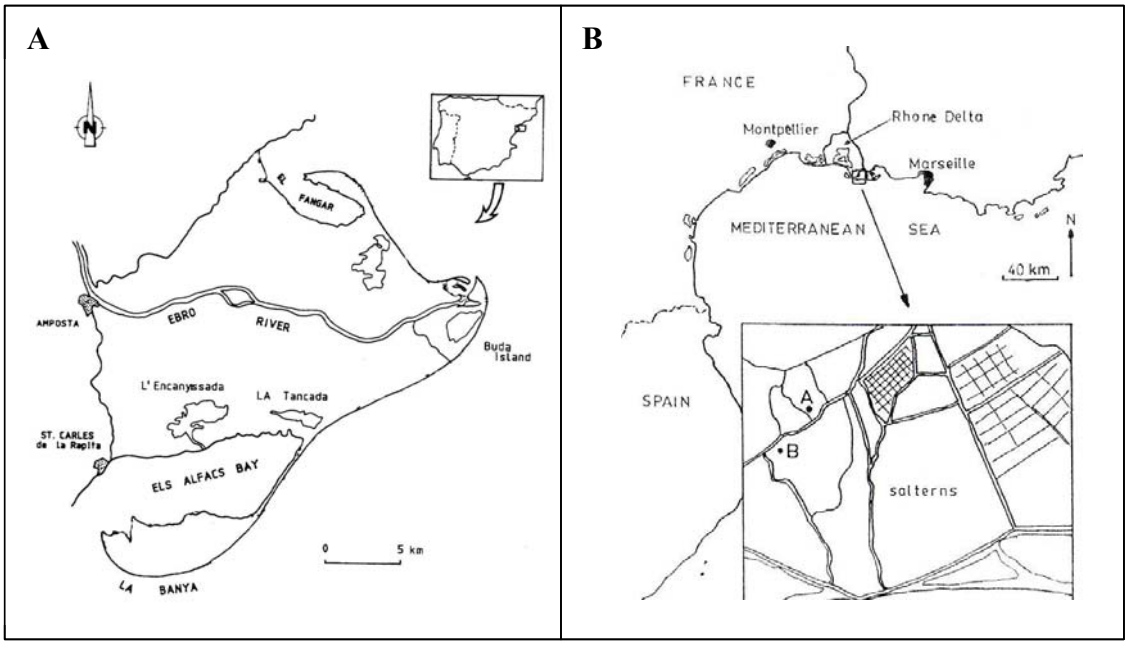

Figure II.2. Sampling sites.

(A) Ebro delta microbial mats (La Banya spit, Ebro delta, North East Spain); (B) Camargue microbial mats (Salins-de-Giraud, Rhone delta, Southern France) (Caumette *et al*., 1994).

Table II.1. Location and physicochemical conditions in Ebro delta and Camargue mats.

Conditions[1]	Ebro delta mats	Camargue mats
Location	40° 47' N, 0° 56' E	44º 40' N, 4º 51'E
Tº ext	3–27ºC	3–25ºC
Annual precipitation	500 mm	750–1000 mm
Mineralogical composition	Quartz, calcite, clay	Clay, gypsum
Salinity	40–70‰	130–200‰
Tº water	12–30ºC	10–34ºC
pH	7.5–9.0	6–7.5
Oxygen production (mmol O_2 m^{-2} h^{-1})	10–30	3.3
Light intensity (µE m^{-2} s^{-1})	500–1500	2000

[1]References: Ebro delta mats: Mir *et al*., 1991; Rampone *et al*., 1993; Guerrero *et al*., 1993a; Urmeneta and Navarrete, 2000; Esteve *et al*., 1994). Camargue mats: Caumette *et al*., 1994; Fourçans *et al*., 2004.

Table II.2. Main microbial members in Ebro delta and Camargue mats.

Group	Ebro delta mats[1]	Camargue mats[2]
Unicellular cyanobacteria	*Synechococcus, Synechocystis, Gloeocapsa, Cyanothece, Chroococcus*	*Aphanothece, Cyanothece, Microcystis, Chroococcus, Gloeocapsa, Microcystis, Synechocystis, Synechococcus*
Filamentous cyanobacteria	*Microcoleus, Lyngbya, Spirulina, Oscillatoria, Pseudoanabaena, LPP group*	*Phormidium, Oscillatoria, Leptolyngbya, Lyngbya, Pleurocapsa, Microcoleus,*
Purple sulfur and non sulfur bacteria	*Thiocapsa, Chromatium, Ectothiorhodospira, Thiospirillum, Thiorhodococcus, Thiorhodovibrio*	*Chromatium, Thiocapsa, Rhodobacter, Ectothiorhodospira, Halorhodospira, Rhodovibrio, Roseobacter*
Green sulfur bacteria	*Prostecochloris, Chlorobium*	*Chlorobium*
Green non sulfur bacteria	*Chloroflexus-like*	*Chloroflexus-like*
Sulfate-reducing bacteria	*Desulfovibrio*	*Desulfovibrio, Desulfobulbus, Desulfobacter*
Sulfur-oxidizing bacteria	*Beggiatoa, Thiomicrospira*	*Beggiatoa, Thiomicrospira*
Heterotrophic bacteria	*Spirilla, spirochetes*	*Halanaerobiales,Bacteroidetes, Cytophaga, Orenia*

[1]References Ebro delta mats: Mir *et al*., 1991; Urmeneta *et al*., 2003; Guerrero *et al*., 1999; Guerrero *et al*., 1993b; Martínez-Alonso *et al*., 2005. [2]References Camargue mats: Caumette *et al*., 1994; Fourçans *et al*., 2004; Diestra *et al*., 2004; van Bleijswijk and Muyzer, 2004; Caumette *et al*., 2004; Hirschler-Rea *et al*., 2003; Guyoneaud *et al*., 2002; Mouné *et al*., 2000.

- Physicochemical conditions

Physicochemical conditions of the overlaying water (1 cm from the sediment–water interface), as well as external conditions (temperature and irradiance), were analyzed in each sampling campaign. A LICOR LI-189 meter was used to measure solar irradiation. Conductivity was measured by a Crison CTD model 523 conductimeter, and pH was measured with a 506 Crison pH/mV-meter.

Sulfide concentration was measured by the Patchmayr colorimetric method (Patchmayr, 1960), according to the following protocol. One ml of the overlying water at each sampling was added *in situ* to a tube which contained 10 ml of a zinc acetate solution (2% zinc acetate w/v, Probus; and 0.02% acetic acid v/v, Panreac). Samples were kept at 4ºC before use. Once in the laboratory, samples were transferred to 50 ml-bottles and distilled water was added to a final volume of 40 ml. Afterwards, 5 ml of *N*,*N*-dimethyl-*p*-phenylenediamine sulfate solution (DPDS; Sigma-Aldrich; 0.2% w/v; DPDS solution: dissolve 2 g of DPDS in 200 ml of distilled water, add 200 ml of 96% sulfuric acid (Panreac) and add distilled water to 1 L final volume) and 0.25 ml of ferric ammonium sulfate dodecahydrate (Probus; 10% w/v; solution: dissolve 10 g of ferric ammonium sulfate in 50 ml of distilled water, add 2 ml of 96% sulfuric acid (v/v), and add distilled water to 1 L final volume) were added to the 50 ml-bottle. The mixture was shaken vigorously, and distilled water was added to the bottle to a final volume of 50 ml. Absorbance at 663 nm was measured in a UV/Vis spectrophotometer (DU 530 Beckman Coulter) after 15 min of incubation at room temperature in the dark. Sulfide concentration was calculated by the following equation:

$$(Abs - Abs_{white}) \times 0.027 \times 50/V_{sample}\ (\text{in mL}) = \text{Sulfide concentration, } [S^{2-}] \text{ in mmol } L^{-1}$$

Note: In this case, the absorbance at 663 nm (Abs) of the 'white' or 'zero' sample was measured in sulfide free artificial seawater. The volume of the original sample (V_{sample}) was 1 ml.

- Sampling conditions and preparation of the samples

Microbial mat samples destined for DNA analyses or FISH (fluorescence *in situ* hybridization) were frozen in liquid nitrogen and stored at –80ºC. Microbial samples were also recovered *in situ* for FISH analysis by scraping the surface of the mat with a sterile razor blade, and fixing the samples as it is detailed in 'Fluorescence *in situ* hydridization' section. Microbial mat samples for diversity studies (isolation and characterization of species) were sampled with sterile Petri dishes and transported on ice to the laboratory in where they were used immediately. Some microbial mat samples were also maintained in the laboratory as microcosms in a controlled culture chamber (Ibercex ASL, Spain) equipped with 18-W fluorescent tubes (Grolux and cool white tubes) maintained at 18–20°C, and under dark-light (14 hours/day at a light intensity of about 150 μE m^{-2} s^{-1}) conditions.

Mat samples for lipid analysis were collected as cores removed from the upper part of the mat with a cork borer (core diameter, 16 mm; thickness, 1 mm). Core samples were wrapped with muffled aluminum foil, frozen in liquid nitrogen and stored at –80ºC. Samples used for depth analyses, were sectioned (50 μm) in a cryomicrotome (2800 Frigocut; Reichert Scientific Instruments) at –17ºC. Combining adjacent slides, 500-μm-thick horizontal mat sections (i.e., cut parallel to the mat surface) were obtained and collected in muffled vials. This mat material or the mat samples as whole cores (1.6 mm diameter, 2.5 cm depth aprox.) were lyophilized in a Virtis apparatus at –55°C and 200 mTorr for 48 h.

2. Lipid analysis methods

- Material and reagents

All solvents used in the lipid analysis were of the purest grade possible (Gas chromatography, GC grade or equivalent; Sigma-Aldrich, Chemical Co, St Louis, MO, USA). All glassware was washed in phosphate-free detergent (Micro® detergent, International Products Corporation, or equivalent), rinsed in tap water ten times, rinsed in Nanopure water (see 'Annex' section) ten times and baked in a muffle furnace (450°C, minimum 4 h) before being used. Items which do not tolerate heating to 450ºC were rinsed with acetone three times, and then allowed to dry. Glass Pasteur pipettes (Fisher Scientific) for transfer purposes, were wrapped in aluminum foil and muffled as above. Chromatography columns were constructed from large volume disposable Pasteur pipettes (Fisher Scientific) packed with glass wool plugs inserted into the bottom of the pipette and fired as above. Silicic acid (100–200 mesh powder; Unisil, Clarkson Chemical Co., Williamsport, PA) has to be activated at 100ºC for a minimum of 1 hour prior to use.

- Total lipid extraction and silicic acid chromatography

Each lyophilized sample was pulverized separately using acetone-cleaned mortars in order to keep background levels of PLFA as low as possible. Then, samples were weighted for giving the results per gram of dry weight of the microbial mat sample. All solvents used in the lipid extraction were of GC grade and were obtained from Sigma-Aldrich.

Samples were extracted according to the modified Bligh and Dyer method (Bligh and Dyer, 1959) described by White and collaborators (White *et al*., 1979), that involves a single-phase organic extraction with chloroform, methanol and phosphate buffer (50 mM, [pH 7.4], see 'Annex' section) in a ratio of 1:2:0.8 (v/v/v). After overnight extraction, chloroform and Nanopure water were added to the extract in equal values to provide a final volume of 1:1:0.9 (for chloroform:methanol:buffer/water), which resulted in a two-phase system. The lower organic phase was transferred with a

pipette into a clean test tube. The solvent was removed from the test tube under constant nitrogen flow using a dry-down with a water bath at a temperature not higher than 37°C. The upper aqueous phase, which contains DNA, was recovered and treated as it is explained in 'Cleaning and concentration of DNA' section.

The total lipid extract was fractionated into neutral, glyco-, and polar lipids by silicic acid chromatography (Guckert *et al*., 1985). Chromatography columns partially blocked with a plug of glass wool (previously damped in chloroform) were loaded with silicic acid slurry (0.5 g silicic acid in ammonium acetate (Merck) 20 mM (w/v) in methanol). The columns were pre-eluted with 5 ml acetone and 5 ml chloroform. The dried total lipid extracts were re-suspended in a minimal volume of chloroform (500 µl) and loaded onto the top of the silicic acid column with a Pasteur pipette. This process was repeated twice more suspending the total lipid extracts with 100 µl chloroform for a quantitative transfer. Once the column was loaded, a series of three solvents of increasing polarity were used to separate the lipid classes: Neutral lipids were eluted with 5 ml chloroform, glycolipids with 5 ml acetone and polar lipids with 10 ml methanol. Lipid classes were collected in separate test tubes set up below the column, and then the solvent was removed from all fractions under a stream of nitrogen. The lipid extracts were stored at –20ºC for further analysis.

- Polar lipid fraction analysis

Sequential fractionation of ester-linked fatty acids, vinyl-ether linked aldehydes, amide-linked moieties, and sphingoid bases

Mayberry and Lane (1993) described a one-tube method for the gas-liquid chromatographic analysis of both ester-linked and amide-linked fatty acids. The method also releases aldehydes, as a result of breaking the acid-labile alk-1-enyl linkage in plasmalogens, and long-chain bases from sphingolipids. The major drawback to the one-tube method is that all the compounds are analyzed together and correct identification can be problematic.

A sequential fractionation method is presented (Fig. II.3) that integrates alkaline trans-esterification for the recovery of ester-linked fatty acids (PLFA, phospholipid fatty acids), with the mild acid release of aldehydes from plasmalogens and the strong acid hydrolysis of other lipid components (e.g., amide-linked fatty acids, alkyl-glycerol ethers, sphingoid bases, and amino acids). Alkaline trans-esterification has been widely used for the release and quantification of ester-linked fatty acids (Guckert *et al*., 1985). However, this approach provides an incomplete description of the lipids since there are several lipid constituents that require acidic conditions for release.

- Step A. Mild-alkaline transesterification for the quantification of FAMEs

The polar lipid fraction recovered from the silicic acid chromatography, was transesterified to fatty acid methyl esters by a mild alkaline methanolysis (Guckert *et al*., 1985). The polar lipid extract was dissolved in 1 ml of 1:1 chloroform:methanol (v/v), and then 1 ml KOH (0.2 M w/v, in methanol) was added. The mixture was vortexed briefly and the samples were incubated at 60°C for 30 minutes. After samples had cooled to room temperature, 2 ml hexane were added and the sample mixed. The samples were neutralized with approximately 200 µl acetic acid (1 N, v/v) in methanol. Later, 2 ml of Nanopure water were added to break the phase and the samples were mixed on a vortex mixer. The phases (upper: organic containing the fatty acid methyl esters, FAMEs; lower: aqueous) were separated by centrifugation (5 min at 2000 rpm). The upper organic phase was transferred to a clean test tube. The lower phase was re-extracted with 2 ml hexane, centrifuged and transferred as above, twice more. The organic phase with the FAMEs was dried under a nitrogen flow and stored at –20ºC until quantification. The lower phases were saved and treated for the sequential release of aldehydes (Step B).

The fatty acid methyl esters were analyzed by Gas chromatography/Mass spectrometry (GC/MS; Fig. II.4), by using 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 (0.32 mm I.D., 0.25 µm film thickness). The injector temperature was maintained at 230°C, and the detector temperature was 280ºC. The column temperature was programmed at 60°C for 2 min and then ramped at

10°C min^{-1} to 150ºC. This was following by ramping at 3ºC min^{-1} to 312ºC. Mass spectra were determined by electron impact at 70 eV. Methyl heneicosanoate (Heneicosanoic acid methyl ester, 21:0; Sigma-Aldrich) was used as the internal standard, and the FAMEs expressed as equivalent peaks against the internal standard.

- Step B. Mild-acid methanolysis for the quantification of vinyl-ethers

This step releases aldehydes, as a result of breaking the acid-labile alk-1-enyl (vinyl-ether) linkage in plasmalogens. Bovine phosphatidyl ethanolamine (BPE; Sigma-Aldrich), with a plasmalogen content of approximately 60%, was used to validate the release and quantification of aldehydes as dimethylacetals (one mole of plasmalogen is considered to be comprised of one mole fatty esters and one mole alk-1-enyl ethers). BPE was diluted in chloroform/methanol (1:1, v/v) at a concentration of 0.5 mg ml^{-1}.

To the lower layers resulting from Step A, 100 µl concentrated HCl (concentrated; Sigma-Aldrich) were added and the tubes were heated at 60°C for 30 min. After the samples cooled to room temperature, each was partitioned three times against 3 ml of hexane. After each partition, the hexane upper layers were aspirated and pooled with the previous washes.

The organic layers containing aldehydes as dimethylacetals (1,1-dimethoxy alkanes) were dried and reconstituted in certain volume of hexane containing the internal standard (Heneicosanoic acid methyl ester, 21:0). The identification and quantification of the dimethylacetals was done by GC/MS with the same conditions explained above. The lower phases were saved and treated for the sequential release of amide-linked fatty acids and other polar lipid moieties (Step C).

- Step C. Strong acid hydrolysis (amide-linked moieties and other compounds)

The remaining lower layers resulting from the Step B, were dried under a stream of nitrogen. Later, 200 µl 6 N HCl and then 1 ml of hexane (overlaying the aqueous phase) were added to the tubes. The samples were heated at 100°C for 24 h in a temperature-controlled heat block.

After cooling to room temperature, 200 µl of Nanopure water and 3 ml of chloroform were added. The tubes were centrifuged for 5 minutes at 2000 rpm and the top aqueous phase was pooled in a clean test tube. The aqueous phase (aprox. 200 µl), was saved for the detection of amino acids by the EZ:faast® Kit (explained below). The remaining was partitioned against 3 ml chloroform twice. After each partition, the chloroform lower layers (organic phase) were aspirated and pooled with the previous washes. The organic phase was dried and saved for the Step D.

- Detection of amino acids in the aqueous phase of the Step C

The aqueous phase derived from the previous step, could be analyzed for components such as inorganic ions, glycerol, amino acids and carbohydrates. In this case, a free amino acid analysis was performed with the EZ:faast® Kit (Phenomenex, USA) for the detection of physiological amino acids in complex mixtures. The procedure consists of a solid phase extraction step, followed by a derivatization, and a liquid/liquid extraction step. Then, the prepared and derivatized samples are analyzed by GC/FID (gas chromatograph with a flame-ionization detector).

The EZ:faast® Kit (Phenomenex, USA), started with the aprox. 200 µl of aqueous phase derived from the Step C. Then, 200 µl of the reagent 2 (provided by the manufacturer) was added to the sample and the protocol was followed as it was recommended by the manufacturer. Apart from the calibration solutions provided by the kit, three calibration standards were prepared with L-Lysine monohydrochloride, L-Serine and L-Ornithine monohydrochloride (Sigma-Aldrich) at 20 nmoles ml^{-1} in Nanopure water. Then, 25 µl and 50 µl of each amino acid and 100 µL of internal standard (Norvaline, reagent 1) were added, and the samples were prepared as above.

The amino acids were analyzed by using Hewlett-Packard 5890 series II gas chromatograph with a flame ionization detector. The GC was equipped with a 60-m non-polar column (0.32 mm I.D., 0.25 µm film thickness). The injector temperature was maintained at 230°C. The GC was programmed with an initial temperature of 75ºC for 3 min, and then ramped at 15°C min^{-1} to 320ºC, and held for 4 min. The oven temperature was maintained at 75ºC, and the volume of sample injected was 2 µl.

- Step D. Methanolysis followed by TMS (trimethylsilyl) derivatization (for sphinganines and Amide-linked hydroxy FA)

The organic phase obtained from the previous step, was re-suspended in 5 ml of chloroform and 250 µl acetic anhydride. Then, 1 ml methanol and 100 µl concentrated HCl (concentrated; Sigma-Aldrich) were added, and the sample were heated at 60°C for 30 min. After cooling at room temperature, 4 ml Nanopure water were added, the tubes were centrifuged for 5 minutes at 2000 rpm, and the lower organic phase was pooled in a clean test tube. The remaining organic phase was partitioned twice more with Nanopure water, and after each partition the chloroform lower layers (organic phase) were aspirated and pooled with the previous washes.

The organic layers, containing sphingoid bases and amide-linked fatty acids, were dried and transferred to a GC vial and dried again. Then, samples were reconstituted in 100 µl of hexane containing the internal standard D-Sphingosine (150 pmol/µl; Sigma-Aldrich), and dried under a stream of nitrogen. The samples were derivatized by adding 100 µl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma-Aldrich) at 60°C for 30 min. After evaporation under a stream of nitrogen, samples were re-dissolved in hexane and analysed by GC-MS. 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; Hewlett-Packard Co, Palo Alto, CA, USA). The temperature program of the column and the conditions were the same as described above for the analysis of FAMEs (Step A).

Alternatively, the detection of sphingoid bases was performed as was described before by Leung and collaborators (Leung *et al*., 1999). The dried total lipid extract was re-dissolved in 1 ml of 3 M HCl (v/v) and heated at 100 ºC for 3 h. The acid digest was cooled to room temperature and adjusted pH 12 by concentrated KOH (6 M; v/v). The digest was extracted three times with 2 ml chloroform, and the chloroform-fractions were pooled in a clean tube and dried under nitrogen. Then, the organic extracts were derivatized with BSTFA+ 1% TMCS (Sigma) as described above.

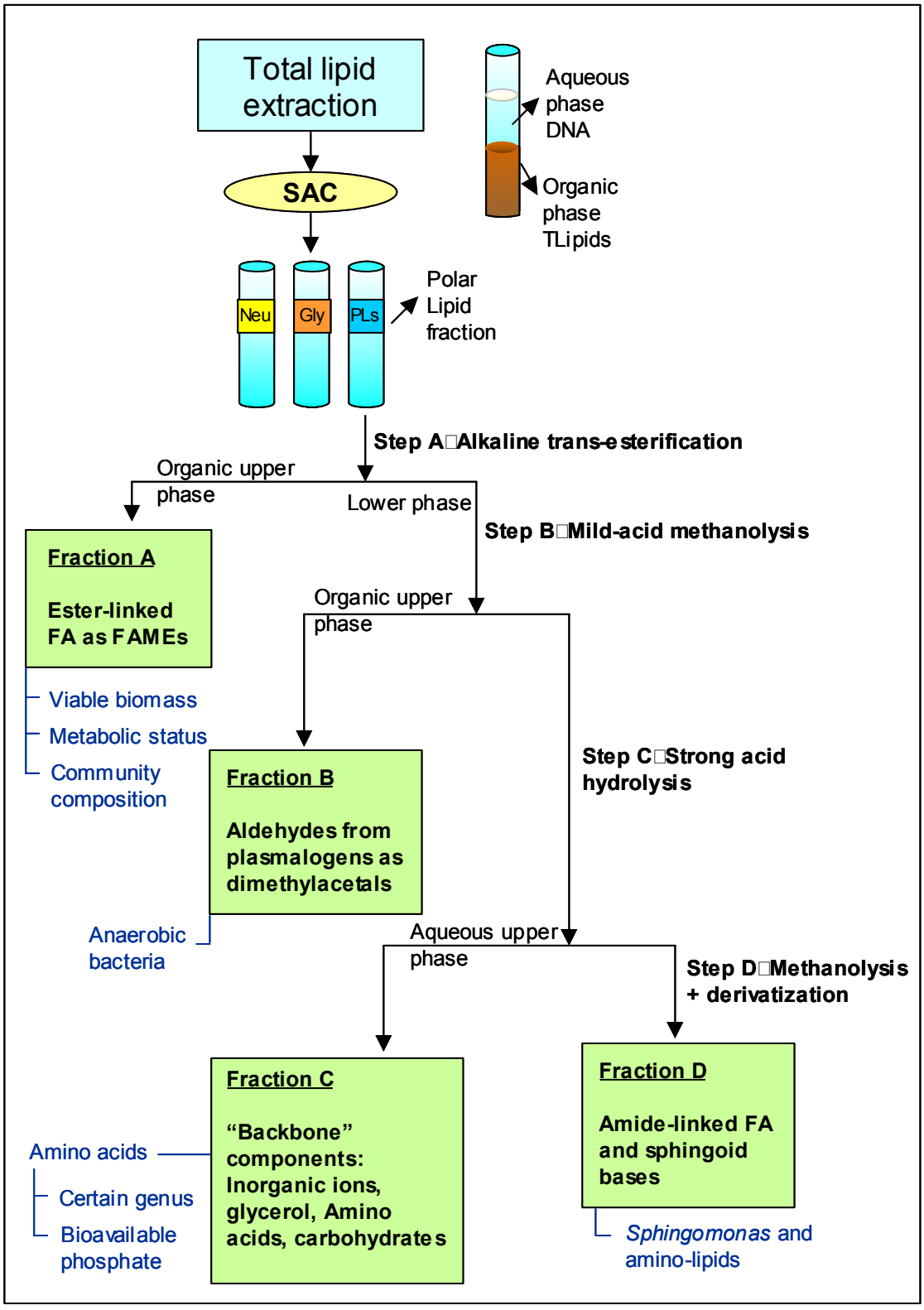

Figure II.3. Sequential fractionation of the polar lipid fraction.

Integrative method for the recovery of ester-linked fatty acids as FAMEs, aldehydes from plasmalogens and other components (e.g., amide-linked fatty acids, sphingoid bases, and amino acids). SAC: Silicic acid chromatography; Neu: neutral lipids; Gly: glycolipids; FA: fatty acids.

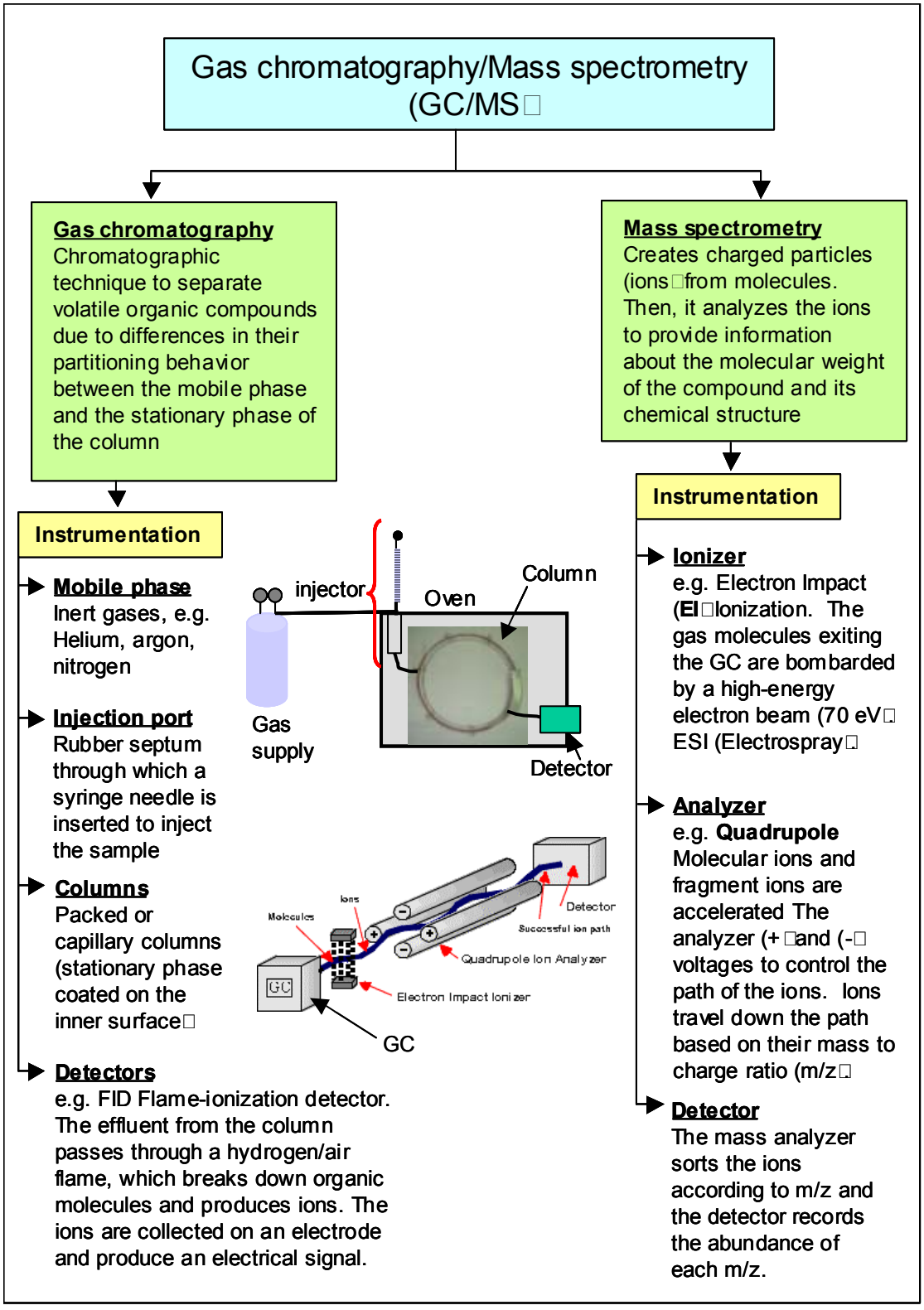

Figure II.4. Gas chromatography/Mass spectrometry.

Upper diagram: GC/FID (Flame-Ionization Detector); Lower diagram: GC/MS with an EI ionizer. More information can be obtained in the 'Useful websites' section.

The samples were analyzed using a GC/MS Shimadzu GCMS-QP2010 with a DB-5 ms GC column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA). The carrier gas (helium) was set at an inlet pressure of 30 p.s.i. and the temperature program of the column was: starting at 100°C, 100–200°C at 10°C min^{-1}, 200°C for 1 min, 200–300°C at 4.5°C min^{-1}, and 300°C for 3 min.

- Glycolipid fraction analysis

➢ Analysis of poly-β-hydroxyalkanoates of the glycolipid fraction by GC/MS

The poly-β-hydroxyalkanoates included in the glycolipid fraction, were fractionated by a mild alkaline methanolysis to release their monomers. In this case β-hydroxybutyrate (3-OH $C_{4:0}$) monomers released from poly-3-hydroxybutyrate and β-hydroxyvalerate (3-OH $C_{5:0}$) monomers released from poly-3-hydroxyvalerate, were derivatized by *N*-tert-butyl-dimethylsilyl-*N*-methyltrifluoroacetamide (MBSTFA, Sigma-Aldrich) as was described before (Elhottová *et al*., 2000) (Fig. II.5).

The *m/z* (mass to charge ratio) spectra of the MBSTFA-derivatized β-hydroxyvalerate (3-OH $C_{5:0}$) monomers, was obtained by performing the protocol with the glycolipid fraction obtained from the chromatography of a total lipid extract of lyophilized cells of *Chromobacterium violaceum* (Sneath, 1956), a bacterial strain that accumulate PHA composed by almost 100% of polyhydroxyvalerate when is grown with sodium valerate as a carbon source (Kolibachuk *et al*., 1999; described in Table II.10), and with *Cupriavidus necator* also grown on sodium valerate (Doi *et al*., 1987).

The glycolipid extracts were dissolved in 1 ml of 15% NaOH in MeOH:water (1:1, v/v) and then heated at 80°C for 30 min. After cooling, the reaction mixture was acidified by 0.34 ml 37% HCl and amended with 0.1 g NaCl. Then, 100 μl of the internal standard solution (β-hydroxyoctanoic acid, 3-OH $C_{8:0}$, DL-β-hydroxycaprylic acid, Sigma-Aldrich, 2 ng μl^{-1} in methanol) was added. The aqueous phase was extracted three times with 1.5 ml dichloromethane and the final extract was evaporated to dryness under nitrogen. The mixture was dissolved in 70 μl of anhydrous acetonitrile (HPLC grade; Sigma-Aldrich) and derivatized by 30 μl of MTBSTFA. The reaction

mixture was subjected to 10 minutes at 25°C, then 10 minutes at 60°C, and finally 60 min at 80°C. After cooling, 100 µl of the solution was transferred to a GC vial and 1 µl was injected directly to the GC/MS under the same conditions and column mentioned above for the analysis of FAMEs (Step A). The injection temperature was 230°C and the helium velocity was 40 cm sec^{-1}. The oven temperature, initially at 60°C, was gradually increased (10°C min^{-1}) to 250°C and held at this temperature for 6 min (run time, 25 min). The MS measurements were performed for parent ions of *m/z* 275 (analyte, MTBSTFA-3-OH butyric acid) and *m/z* 331 (for the internal standard).

- Analysis of poly-β-hydroxyalkanoates by HPLC (High Performance Liquid Chromatography)

Alternatively, the analysis of poly-β-hydroxybutyrate has been performed by HPLC (Brandl *et al*., 1988). This protocol is based on an alkaline hydrolysis of the polymer of β-hydroxybutyrate to get its monomers as crotonic acid that can be detected and quantified (Fig. II.5). The protocol was performed with lyophilized cell extracts of pure cultures of microorganisms that have been growth under conditions of PHB accumulation (see 'Preparation of cultures for SLB analysis and detection of PHA by HPLC' section), or lyophilized samples of microbial mats. Firstly, 2 ml of NaOH 2N (w/v) were added to the lyophilized extracts in glass tubes with teflon-coated caps. The tubes were heated at 100ºC for 45 min. After the tubes had been cooled down at room temperature, 1 ml HCl 5 N was added, and then 1 ml of phosphate buffer 1 M (pH 7), the reaction was let to proceed for several minutes. Then, the tubes were centrifuged at 2000 rpm for 20 min, and the supernatant was transferred to a clean tube and filtrated through a 0.2 µm filter (Albet, Spain). The filtrated extracts were transferred to vials and certain volume was injected directly to the HPLC Hewlett-Packard series II-1040-M equipped with a HPLC Fast analysis column (100 × 7.8 mm; Bio-Rad, Richmond, CA) Hewlett-Packard series 1050. The mobile phase was sulfuric acid 5 mM at a flux of 1 ml min^{-1}. The detection of the peak of crotonate was done at 210 nm. The calibration curve was performed with crotonic acid (Merck) between 0 and 200 mg L^{-1}.

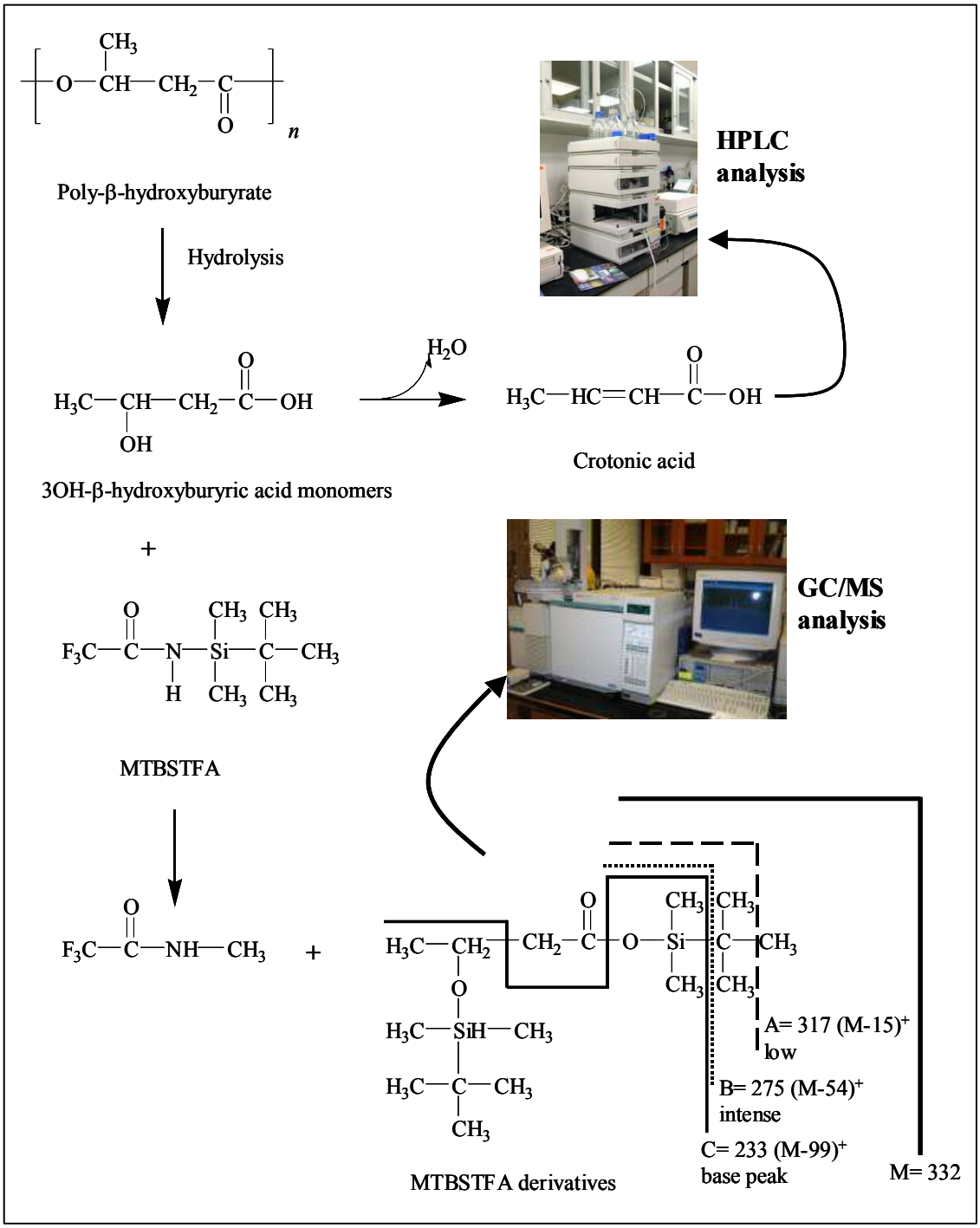

Figure II.5. Detection of poly-β-hydroxyalkanoates by HPLC and GC/MS.

Both methods start with a hydrolysis of the hydroxyalkanoate polymer to get their correspondent monomers. Then, in the HPLC method, the monomers of hydroxybutyric acid are converted to crotonate due to the loss of water (dehydration). In the GC/MS proposed by Elhottová *et al*. (2000), the monomers are derivatized with MTBSTFA which is analyzed by GC/MS and identified by their MS fragmentation spectra.

- Neutral lipid fraction analysis

➢ Analysis of isoprenoid quinones

The isoprenoid quinones included in the neutral lipid fraction (obtained after the silicic acid chromatography) can be identified and quantified directly without further modifications. Classically, quinone analysis has been performed according to the protocols described by Hiraishi and collaborators (Hiraishi *et al*., 1996), which are based on a fractionation of quinones into ubiquinones and menaquinones using a Sep–Pak Vac 3 cc silica gel cartridges (Waters, Milford, MI), and then a quantification by reverse-phase HPLC and photodiode array detection. In addition, the identification of plastoquinone and partially hydrogenated quinone species has been performed by mass spectroscopic detection with atomic pressure chemical ionization.

Lytle and collaborators (Lytle *et al*., 2001) demonstrated the utility of electrospray tandem mass spectrometry (ES-MS/MS) in quantifying ubiquinones. This method took advantage of the formation of the ammonium ion adduct of the quinones, formed from ammonium acetate addition via a pre-column. However, test for ionization of menaquinones using the electrospray source did not yield sufficient amounts of molecular ions, fragment ions, or ammonium adduct ions. Recently, Geyer and collaborators (Geyer *et al*., 2004) have tested other ionization sources for improving the ionization of menaquinones and ubiquinones, such as APCI-MS/MS (atmospheric pressure chemical ionization tandem mass spectrometry). This method has been applied for the identification and quantification of quinones in microbial mat samples and microbial pure cultures. Before explaining the parameters and methods used in this work, a summary of the main characteristics of a LC/MS (liquid chromatograph/mass spectrometer) are detailed as it follows.

LC/MS: Interfacing HPLC (High Performance Liquid Chromatography) and MS (Mass Spectrometry)

Mass spectrometry is one of the most important analytical methods to detect and identify components, previously separated by GC (Gas Chromatography), LC (Liquid Chromatography) or SFC (Supercritical Fluid Chromatography). LC/MS is used in

multiple applications because the liquid chromatography can separate more compounds than gas chromatography that requires volatile substances.

Actually, the large majority of applications are done with electrospray (ESI) and APCI (Atmospheric Pressure Chemical Ionization) ionization (Fig. II.6). Both methods are API (Atmospheric Pressure Ionization) techniques. Ionization takes place at atmospheric pressure and both are considered to be soft ionization methods. An API source always comprises: (i) the probe (ESI or APCI); (ii) the corona electrode (for APCI); (iii) gas flow for mobilization, evaporation and desolvatation; (iv) sampling cone; (v) transfer optics to the MS analyzer.

At atmospheric pressure in the presence of trace amounts of water and nitrogen gas, protonated molecular ions are formed by gas-phase ion-molecule reactions with water cluster ions. In positive ionization mode, the mechanisms are protonation, adduct formation, and charge transfer. In the negative ion mode, electron capture and anion attachment are the primary mechanisms of ion formation. Moreover, adducts and cationized or anionized molecules are often observed with API techniques: these ions can be formed 'accidentally', due for example to the presence of sodium in the mobile phase or intentionally, for more specific or sensitive detection. The UIPAC nomenclature establishes that an adduct ion is an ion formed by interaction of two species, usually and ion and a molecule, and often within the ion source, to form an ion containing all the constituent atoms of one species as well as an additional atom or atoms (e.g. $[M+H]^+$ protonated molecule; $[M–H]^-$ deprotonated molecule; adducts: $[M+Na]^+$; $[M+K]^+$; $[M+HCOO\text{-}]^-$; etc.) (Table II.3). However, a cluster ion is an ion formed by the combination of two or more molecules of a chemical species, often in association with a second species.

Once a sample mixture has been separated by liquid chromatography (LC), and then ionized (by ESI, APCI etc.), goes through the analyzer (mass spectrometer). The analyzers used in these kinds of instruments are quadrupole, ion trap, time of flight, and some other combinations. The quadrupole (Fig. II.7) is the most widely used analyzer due to its ease of use, mass range covered, good linearity, resolution and quality of mass spectra.

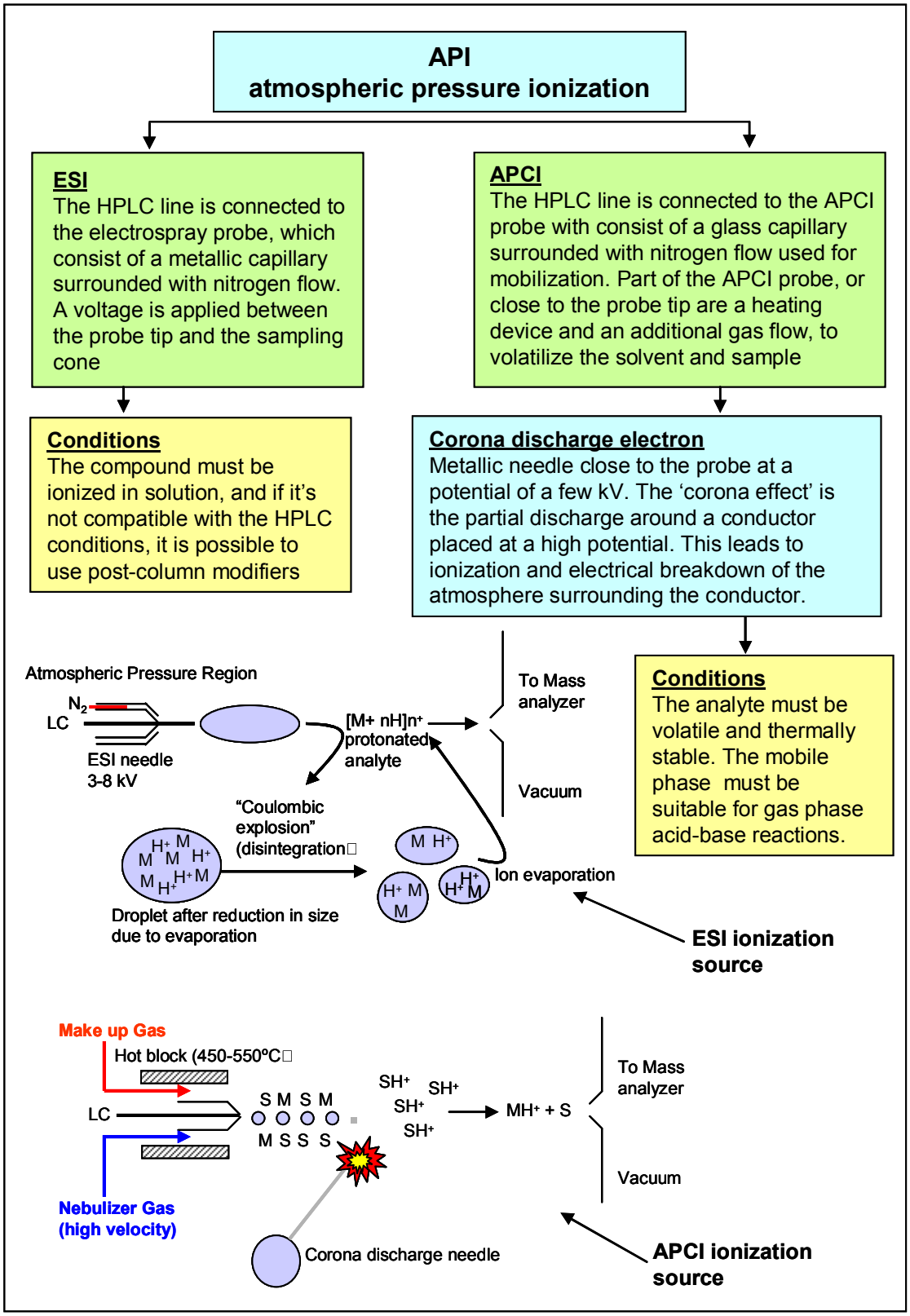

Figure II.6. Atmospheric pressure ionization (API) techniques: ESI and APCI.

In the APCI diagram, solvent molecules (S) are being protonated by the corona (SH^+), and then they react with the analyte molecule (M) to give the protonated form MH^+.

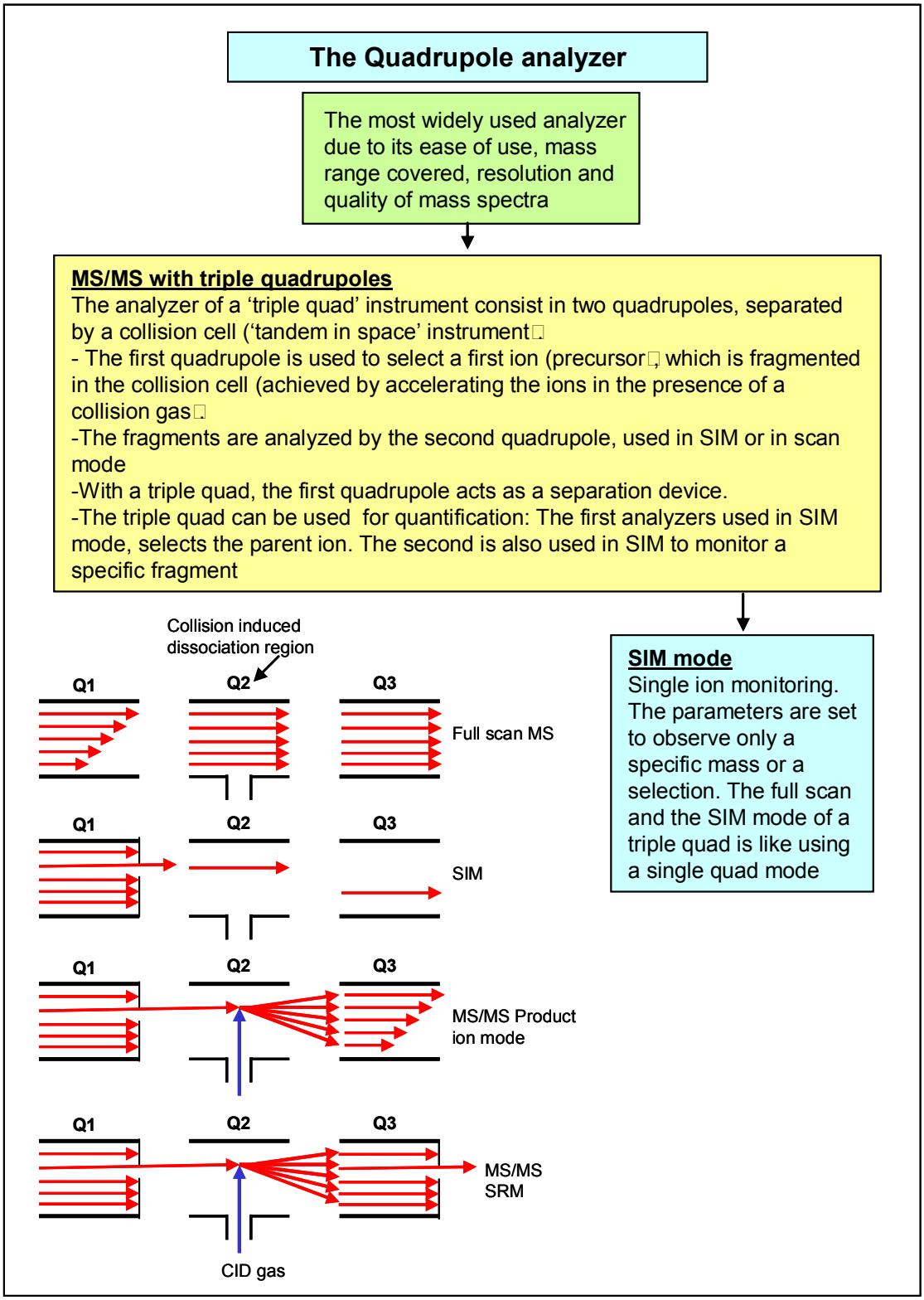

Figure II.7. The Quadrupole analyzer.

Modes of action of a triple quadrupole system.

Experimental design: Analysis of Quinones

Neutral lipid extracts, obtained after the silicic acid chromatography of total lipids, were re-solved in 50–100 µl of methanol prior to measurement. The extracts, which also may contain diglyceride, free fatty acids and other neutral lipids, were in general used without further purification. However, colored samples were cleaned with 0.2 µm spin filters prior to injection on column. Quinones are photosensitive and sensitive to oxygen, and therefore neutral lipid samples were protected from light and air oxygen. Aliquots of quinone standards were used for calibration purposes as described in Geyer *et al*., 2004.

The LC/MS/MS analysis was performed on an Applied Biosystems/MDS SCIEX 4000 QTrap™ LC/MS/MS system (a hybrid of a triple quadrupole and a linear ion trap (LIT) instrument capable of multiple reaction monitoring, MRM), in combination with the APCI ion source interface. A Dash-8 (20 mm × 2.1 mm, 5 µm, Thermo Electron Corp.) high performance liquid chromatography (HPLC) column was used for the chromatographic separation of quinones. A gradient solvent system composed of solvent A (chloroform) and solvent B (methanol) was used with a flow rate of 100 µl min^{-1}. The mobile phase was held isocratically for 10 min with a 20% solvent A and 80% solvent B. The column oven was held at 30ºC.

Conditions for APCI-MS-MS were as follows: nebulizer gas pressure 40 psi, curtain gas 20 (relative value), heater temperature 275ºC, corona discharge needle 4 µA, interface heater ON, and collision gas 5 (relative value, yielding a pressure of $3.5 \cdot 10^{-5}$ Torr in the collision cell). The mass spectrometer was operated in the positive ionization mode. The important compound dependent parameter settings were as listed in Table II.4. The quinones were detected by their base peak obtained from the spectrum of a Q1 scan at the respective mass to charge ratio (*m/z*), and the major ion as $[M+H]^+$, and the major ion in the product spectrum of the protonated molecule was detected in Q3 (Lytle *et al*., 2001; Geyer *et al*., 2004). For example, for ubiquinone-6 (UQ-6) the base peak in the Q1 spectrum was $[M+H]^+$ at *m/z* 591.7. The product ion spectrum of *m/z* 591.7 and of the other ubiquinone protonated molecules showed a base peak at *m/z* 197 (benzylium ion). Accordingly, the menaquinones studied showed a fragment ion of *m/z*

187 as the base peak in Q3, which represent the 2-methylnaphthoquinone core. Moreover, demethylmenaquinone-8 and 9 (DMK-8 and DMK-9) were detected as *m/z* 703.5 and 771.6 in Q1 respectively, and *m/z* 173.1 in Q3 (Fig. II.8). For phylloquinone (K_1) and plastoquinone-9 (PQ-9) the base peaks were *m/z* of 451.2 and 750.0, and the product ions of the protonated molecules were observed at *m/z* 187.1 and 151.1, respectively. A few more mass transitions are included in Table II.4 corresponding to further quinone species we searched for.

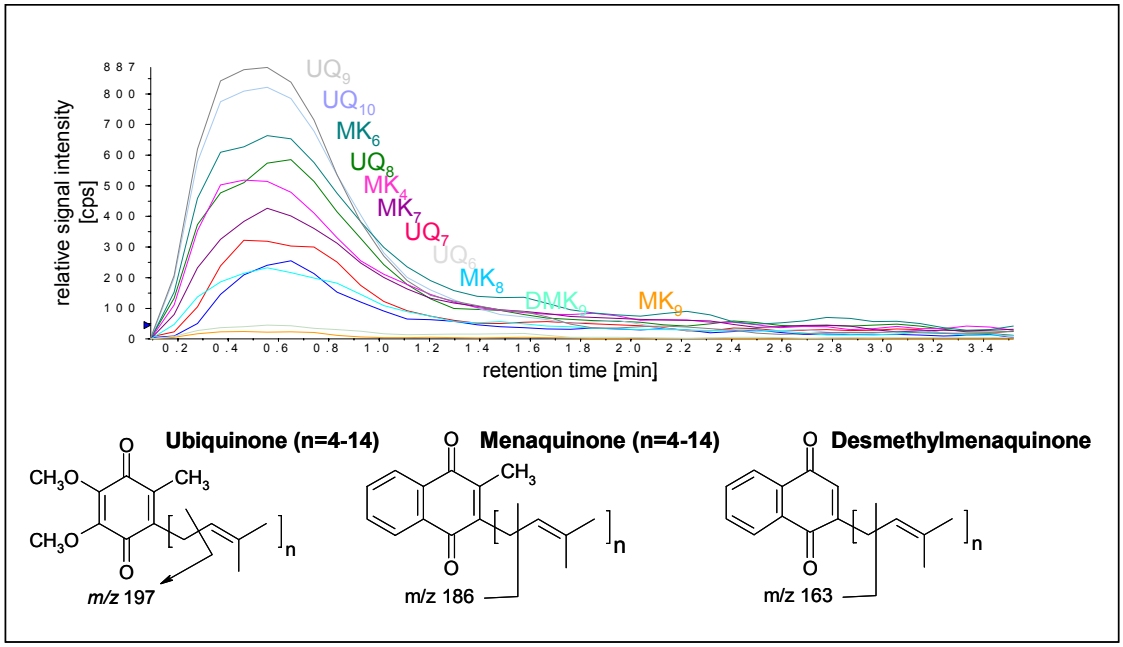

Figure II.8. Detection of quinones by a LC/MS/MS system.

At the top: APCI MS/MS spectrum of a standard mixture of quinones without separation by an HPLC column. FIA analysis (flow-injection analysis).

Table II.3. Nomenclature (McMaster and McMaster, 1998).

Nomenclature
Mass spectrometers operate on the basis of **mass-to-charge ratio (*m/z*)**. **Molecular Ion**: Ion observed at the *molecular mass* that is formed by electron gain or loss (e.g., radical cation formed in electron ionization, M^+). **Protonated Molecule**: Ion that results from addition of a proton to the molecule, $(M+H)^+$ **Molecular Mass**: Mass of the molecule calculated from a specific combination of the isotopes of the elements in the empirical formula of the molecule. Denoted by M. **Molecular Weight**: Sum of the atomic weights of all the composite elements. **Exact Mass**: Mass of a single molecule calculated from the mass of the individual isotopes that compose that molecule.

Table II.4. Mass transition (Q1–Q3) and compound dependent parameters used for quantification of important respiratory quinones by multiple reaction monitoring (MRM).

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP[1] (V)	CE[2] (V)
591.5	197.1	100	76	35
659.6	197.1	100	84	46
727.9	197.1	100	93	48
795.7	197.1	100	102	51
863.9	197.1	100	111	53
445.2	187.1	100	71	31
513.2	187.1	100	76	35
581.4	187.1	100	82	39
649.4	187.1	100	88	43
717.5	187.1	100	94	47
785.6	187.1	100	99	51
853.6	187.1	100	105	55
703.5	173.1	100	94	47
771.6	173.1	100	99	51
451.2	187.1	100	71	31
477.5	187.1	100	76	35
545.6	187.1	100	82	39
613.7	151.1	100	88	43
681.8	151.1	100	94	47
750.0	151.1	100	99	51
818.1	151.1	100	105	55

[1]DP, declustering potential; [2]CE, collision energy.

- Intact polar lipid analysis

Current approaches used for chemical characterization of microbial populations include, (i) phospholipids ester-linked fatty acids (FAMEs) analysis by GC/MS as an established method, and (ii) intact polar lipids profiling (IPLP) using LC/MS/MS as a relative new approach with higher sensitivity and specificity. Both techniques rely on the fact that phospholipids are found in intact membranes of all living cells, but usually not in storage lipids, and are turned over rapidly in dead cells. Thus, their quantification provides an estimation of viable biomass. Recent studies have applied IPLP as a method to determine changes in phospholipids content and composition as an adaptive response (Fang *et al*., 2000) of microbial cultures, or in microbial communities, especially for the

determination of archaeal lipids because of the low variability of their core isoprenoid chains (Hopmans *et al*., 2000; Sturt *et al*., 2004; Gibson *et al*., 2005).

The polar lipid extracts, obtained after the silicic acid chromatography of total lipids, were analyzed by LC/MS/MS performed on an Applied Biosystems/MDS SCIEX 4000 QTrap™ LC/MS/MS system with an electrospray interface. The capillary LC (Agilent 1100 capLC) was equipped with a 8 µl sample loop. A Thermo Dash-8 20 × 2.1 mm (HPLC) column was used for the chromatographic separation of phospholipids. A gradient solvent system composed of solvent A (water) and solvent B (methanol/acetonitrile 90:10 + 0.002% piperidine) was used with a flow rate of 100 µl min^{-1}. At the beginning of the gradient, the mobile phase was 20% of A and 80% of B for 0.5 min. Solvent B was increased to 100% at 15 min. The mobile phase was then held isocratically for 10 min. Solvent B was decreased within 0.5 min to the staring value and the column equilibrated for 5 min. Column oven was held at 40ºC. The mass spectrometer was operated in the negative ionization mode. The nitrogen curtain gas and the ion source gas 1 (nebulizer gas) and 2 (turbo gas at the heater) was set to a pressure of approximately 20, 30 and 60 psi (1.4, 2.0, 4.1 bar), respectively, at a source heater temperature of 450ºC. For negative ionization, the ion transfer voltage (IS) between electrospray needle and vacuum interface was set to –4000 V with the skimmer held at ground potential. The declustering potential (DP, between orifice plate and ground) were set at –80 V. The interfacer heater was turned on at all times.

The mass spectrometer was tuned from 5 to 2000 amu at the same scan speed used for sample analysis (4000 amu s^{-1} for the scan, 250 amu s^{-1} for the enhanced resolution scan, and 1000 amu s^{-1} for the fragmentation scan) using a solution of mixed polypropyleneglycols (PPGs) provided by the manufacturer.

The relative concentration of phospholipids was assessed based on the signal response (cps, counts per second) over the chromatographic area of the individual phospholipids and set in relation to the sample amount. Usually, the reproductibility of LC-MS-MS analysis of phospholipids is better than 87% (n = 5) (Fang *et al*., 2000). Phospholipids were designated as follows: C1:d1/ C2:d2-PL (e.g. C16:0/C18:1-PE), where C1 and C2 are the numbers of carbon atoms in the fatty acyl chains on the *sn*-1

and *sn*-2 positions, respectively; d1 and d2 are the numbers of double bonds of the *sn*-1 and *sn*-2 fatty acyl chains, respectively; and PL is the abbreviation for phospholipids class (Fig. II.9). The identification of the intact polar lipids by their mass spectra were performed by comparison with the mass spectrometry reference tables included in Murphy, 2002.

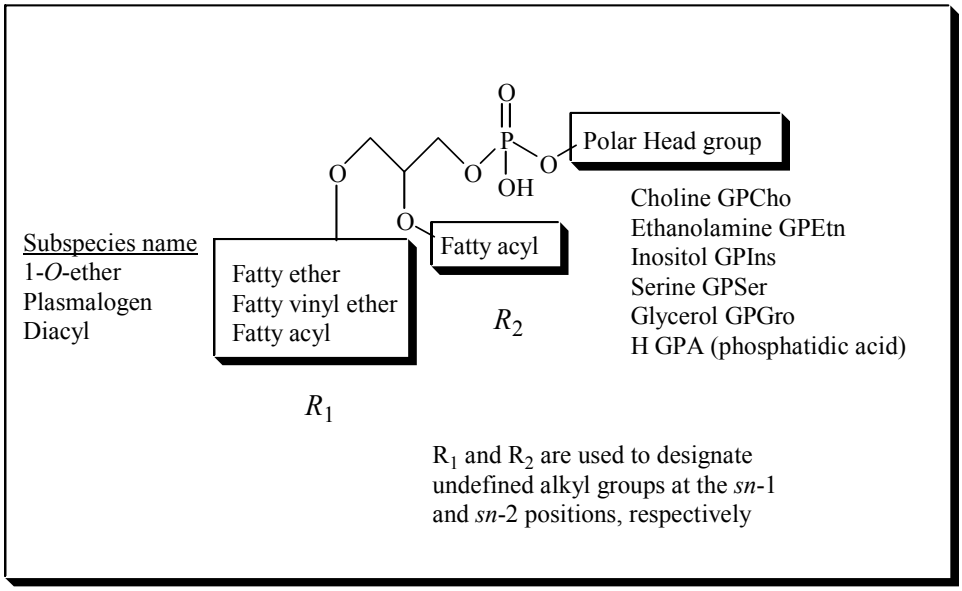

Figure II.9. Structural relationships of glycerophospholipids.

Glycerophospholipids that have ester (acyl), alkyl ether (alkyl) or vinyl ether (alk-1′-enyl) substituents at *sn*-2, and a polar head group at the *sn*-3 position of glycerol (Pulfer and Murphy, 2003).

3. Nucleic acid analysis methods

- DNA isolation, purification and electrophoresis

➢ DNA extraction methods

Boiling of colonies for direct obtaining of DNA

This method allows a direct extraction of DNA from pure culture colonies that is suitable for PCR (Polymerase Chain Reaction) purposes. Alternatively, the final supernatant, with DNA content, that is finally obtained can be purified by ethanol precipitation or phenol:chloroform purification as is explained in 'Cleaning and concentration of DNA' section.

A couple of grown colonies (depending of their size) on agar plates were transferred to a 1.5 ml-tube with 100 µl of TE buffer (see 'Annex' section). The mixture was mixed by vortex and boiled 10 min. Then, the tubes of the corresponding colonies were centrifuged 5 min at 13000×*g*. The supernatant was used as a DNA template for PCR reactions. The boiled cells can be stored at –20ºC.

DNA extraction from polycarbonate filters

This method permits a DNA extraction from filters through which a biological sample has been previously filtered. It has also been proved to be a good method for DNA extraction from filamentous cyanobacterial cultures, or sediment samples (only when it is complemented with a DNA purification method that eliminates the humic substances included in the sample).

The polycarbonate filter was transferred to a 2 ml-tube with 0.5 g of glass beads (150–212 µm, Sigma-Aldrich) and 600 µl of AE buffer (Qiagen GmbH). The mixture was homogenized by vortex 10 min at maximum speed (alternatively, a bed-beater can be used). The sample was centrifuged 4 min at 13000×*g*, and then 500 µl of chloroform were added. The tube was centrifuged again 4 min at 13000×*g*, and the top phase was transferred to a clean tube. Then, 400 µl of phenol:chloroform:isoamyl alcohol

(25:24:1) were added, the sample was mix by vortex and then centrifuged 3 min at 13000×*g*. The supernatant was transferred to a clean tube and $^{1}/_{10}$ vol of ammonium acetate 7.5 M, and 1 vol of isopropanol were added. The sample was stored 1 h at –80ºC or overnight at –20ºC. Later, the sample was centrifuged 20 min at 13000×*g*, and the supernatant was discarded. Finally, the pellet with the DNA was cleaned with ethanol 70 % (v/v) as in the DNA precipitation (see 'Cleaning and concentration of DNA' section).

DNA extraction from soil samples

DNA from microbial mat samples was extracted by using the Power Soil™ DNA isolation kit (Mo Bio Laboratoires, Inc.). This kit is suitable for genomic DNA isolation from environmental samples containing high humic acid content, and it has been proved to be better in DNA isolation and purity for PCR purposes than UltraClean™ soil DNA isolation kit in microbial mat samples (this study).

- Cleaning and concentration of DNA

Phenol:chloroform treatment

It was performed to remove proteins or other contaminants from DNA solutions. DNA was mixed with 1 vol of phenol. After mixing, the sample was centrifuged at 13000×*g* for 2 min at room temperature (RT), and the aqueous phase was recovered. This process was repeated using phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) first, and then chloroform:isoamylalcohol (24:1, v/v) (Sambrook and Russel, 2001).

Ethanol precipitation

It was used to concentrate and clean DNA solutions. DNA was mixed with 2 vol of absolute ethanol at −20ºC and 0.1 vol 3 M sodium acetate (pH 4.8), incubated for 30 min at −20ºC, and centrifuged at 13000×*g* for 20 min at 4ºC. The supernatant was discarded and the pellet was washed twice by addition of 250 µl of 70% (v/v) ethanol at −20ºC, followed by centrifugation at 13000×*g* for 5 min at 4ºC. The supernatant was discarded and the pellet was dried using a speed-vacuum centrifuge (Eppendorf). Dried

DNA was suspended in an appropriate volume of distilled water or TE buffer (Sambrook and Russel, 2001).

Wizard® PCR Preps (Promega) kit

It was used in to carry out direct purifications of DNA from PCR reactions yielding a single amplification product. This kit is based on DNA binding to an ionic exchange resin, followed by resin binding to a column, column washing, and subsequent DNA elution in reduced ionic strength conditions.

Purification of DNA recovered from the aqueous phase of the total lipid extraction

After total lipid extraction, the upper aqueous phase of each sample was recovered to precipitate the DNA suitable for enzymatic amplifications. The DNA was precipitated from the aqueous phase with $^{1}/_{10}$ vol of ammonium acetate (3 M, pH 5) and 1 vol of isopropanol at –20°C for 1 hour. DNA was pelleted by centrifugation at 13000×*g* at 4°C for 15 min and re-dissolved in 500 µL Tris HCl (0.25 M). DNA extract was purified by a glass-milk DNA purification protocol using a Gene Clean™ Spin-kit (Bio-101) as described by the manufacturer.

- DNA electrophoresis

DNA was analyzed using agarose gels as was previously described by Sambrook and Russel (2001). The composition of the solutions used is detailed in Table II.5. DNA samples were prepared for electrophoresis by adding Loading buffer 6×. Then, samples were loaded into an agarose gel (depending on the DNA molecular size, at a concentration between 0.8−2% w/v, in TBE 1× or TAE 1× buffer). Finally, gel running was performed in a horizontal Mini-Sub®Cell GT tray (Bio-Rad) filled with TBE 1× or TAE 1× buffer, using a voltage of 75−120 volts generated by a PAC300 power supply (Bio-Rad).

DNA molecules separated in agarose gels were visualized by gel staining for 15−20 min in distilled water containing 75 µl ml^{-1} ethidium bromide, and subsequent irradiation with UV light (310 nm) using an ImageMaster® VDS (Pharmacia biotech).

DNA size was estimated by comparison of their migration length with that of the following molecular markers: 'λ DNA/*Hind*III' marker (Biotools): bacteriophage λ genome digested with *Hind*III. Range: 23−0.5 kb; and '100 bp Ladder' marker (Biotools): bacteriophage pMLX genome digested with *Eco*147I and *Pvu*I. Range: 1031−80 bp.

Table II.5. Solutions for DNA gel electrophoresis.

Loading buffer 6×	TBE 1×	TAE 1×
0.25% bromophenol blue	90 mM Tris	40 mM Tris
0.25% xylene cyanol	90 mM boric acid	40 mM acetic acid
60% glycerol	2 mM EDTA (pH 8)	1 mM EDTA (pH 8)

Isolation of DNA fragments from agarose gels

Isolation of DNA from agarose gel bands was performed to get DNA suitable for cloning, sequencing, etc. Commercial kit UltraClean™ DNA purification kit (Mo Bio Laboratoires, Inc.) was used for fast purification of DNA following the manufacturer's specifications. This method is based on silica gel binding of the DNA and a subsequent elution by lowering the ionic strength.

- DNA amplification by PCR

PCR (polymerase chain reaction; Sambrook and Russel, 2001) was performed for the *in vitro* synthesis of DNA molecules. Direct PCR consist on the amplification of the central region of a DNA molecule located between two primers (oligonucleotides) whose extension proceeds towards the center of the molecule.

Amplification reactions were performed in an Eppendorf Mastercycler® personal thermocycler, and in a Robocycler™ PCR block (Stratagene, La Jolla, CA) for PCR reactions performed before DGGE (denaturing gradient gel electrophoresis)

analysis. The primers and conditions used are detailed in Table II.6 and II.7. All routine PCR amplifications were performed with *Taq* polymerase from Biotools B&M Labs (Spain; DNA polymerase enzyme without 3´-5´ exonuclease proofreading activity and it generates T-protuberant ends). PCR amplifications for DGGE analysis were performed with Advantage 2 Polymerase mix (Clontech, Palo Alto, CA) which is an optimized PCR enzyme mix for applications that involve longer templates or require high fidelity and PCR performance. The Advantage 2 Polymerase Mix consists of a Titanium™ *Taq* DNA polymerase, and a proofreading polymerase.

Table II.6. Primers used for amplification/sequencing reactions.

Primers
Primers for a partial amplification of 16S rDNA of Bacteria for DGGE (Chapter IV)
- Primer FW: 341F-GC (5´-CGC CCG CCG CGC GCG GCG GGC GG GGC GGG GGC CGG GGG GC CTA CGG GAG GCA GCA G-3´) Tm^1= 85ºC
- Primer BW: 534R (5´-ATT ACC GCG GCT GCT GG-3´) Tm^1= 47ºC
- Amplification: partial 16S rDNA sequence of Bacteria (~193 bp).
- Tm^2= 60ºC
- Reference: Muyzer *et al*., 1993.
Primers for the amplification of 16S rDNA of archaea (Chapter V)
- Primer FW: ARQ21F (5'-TTC CGG TTG ATC CYG CCG GA-3') Tm^1= 74ºC
- Primer BW: ARQ958R (5'-YCC GGC GTT GAM TCC AAT T-3') Tm^1= 67.4ºC
- Amplification: partial 16S rDNA sequence of archaea (~937 bp)
- Tm^2= 55ºC
- Reference: Bano *et al*., 2004.
Universal 16S rDNA primers
- Primer FW: Ty04F (5'-ATG GAG AGT TTG ATC CTG-3') Tm^1= 54.6ºC
- Primer BW: Ty06R (5'-TAC CTT GTT ACG ACT T-3') Tm^1= 44.3ºC
- Amplification: 16S rDNA sequence (~1453 bp).
- Tm^2= 50ºC
- Reference: Baker *et al*., 2003.

Universal primers for the amplification of the 16S rDNA 3' and 5' ends

- Primer FW: 968F (5'-AAC GCG AAG AAC CTT AC-3') Tm^1= 55.2ºC
- Primer BW: 519R (5'-GWA TTA CCG CGG CKG CTG-3') Tm^1= 64.3ºC
- Amplification: 16S rDNA sequence (~1453 bp).
- Tm^2= 50ºC
- Reference: Nübel *et al*., 1996; Lane, 1991.

pGEM-T primers

- Primer FW: T7pGEM-T (5'-TAA TAC GAC TCA CTA TAG GG-3') Tm^1= 43ºC
- Primer BW: SP6pGEM-T (5'-ATT TAG GTG ACA CTA TAG AA -3') Tm^1= 38ºC
- Amplification: amplification of cloned inserts into pGEM®-T Easy vector (Promega) polylinker (Insert length + 177 bp).
- Tm^2= 55ºC
- Reference: this study.

Primers for the amplification of 16S rDNA of the genus *Thiomicrospira* (Chapter VIII)

- Primer FW: Ly02F (5'-GAA TCT ACC CTT TAG TTG-3') Tm^1= 38ºC
- Primer BW: Ly03R (5'-CTT TTT AAT AAG ACC AAC AG-3') Tm^1= 38ºC
- Amplification: partial 16S rDNA sequence of *Thiomicrospira* members (~721 bp)
- Tm^2= 42ºC
- Reference: Brinkhoff and Muyzer, 1997.

Nested primer for the sequencing of Bl-6 clone (Chapter VIII)

- Primer FW: Spiro312F (5'-TCC TAC GGG AGG CAG CAG CT-3') Tm^1= 53ºC
- Tm^2= 50ºC
- Reference: this study.

Notes:

- FW: forward; BW: backward.
- Tm^1= Theoretical melting temperature (at 50 mM Na+) of each primer.
- Tm^2= Melting temperature at which the amplification reaction was performed.
- Primers were purchased from Invitrogen Custom primers or from Sigma-Genosys.

IUPAC nucleotide code:

A= adenosine	R= A or G (purine)	B= C, G, or T	K= G or T
C= cytidine	Y= C or T (pyrimidine)	D= A, G., or T	M= A or C
G= guanosine	N= A, C, G, or T	H= A, C, or T	S= G or C
T= thymidine		V= A, C, or G	W= A or T

Table II.7. DNA amplification conditions.

Reaction composition mixture	Amplification program
Standard PCR reaction - 2 U Taq pol - 5 µl 10× buffer - 2.5 µl $MgCl_2$ (50 mM) - 5 µl dNTP (2 mM each) - 6 µl of each primer (19–21 ng/µl) - 1–100 ng template DNA (or 0.5 to 2 µl of supernatant from the boiling of colonies method) - Bidistilled water up to 50 µl	1 cycle - 5 min at 95ºC 30 cycles - 30 s at 96ºC - 30 s at Tm^2 - 2 min at 72ºC 1 cycle - 2 min 30 s at 72ºC
PCR for DGGE analysis - 1 U of Advantage 2 polymerase mix - 2 µl 10× buffer - 2 µl $MgCl_2$ (2 mM) - 1.5 µl dNTP (1.5 µM each) - 10 pmol of each primer (GC-341F, and 534R) - 0.5 µl Formamide - 1 µl template DNA purified from the aqueous phase of the total lipid extraction) - Bidistilled water up to 25 µl	34 cycles - 20 s at 96ºC - 45 s 60ºC - 45 s 72ºC 1 cycle - 5 min at 72ºC **Note:** amplification temperature Tm^2 depended of the primers used (see Table II.6)

- Enzymatic treatment of DNA and transformation

➢ Enzymatic treatment

Digestion with restriction enzymes

Restriction nucleases and their corresponding buffers were obtained from Roche and used according to the manufacturer's specifications. Double digestions were carried out using the most suitable buffer for both enzymes. However, when the enzymes were low- or non-compatible, double digestions were performed sequentially including a cleaning step by ethanol precipitation between first and second digestion. Digestion reactions were stopped following the manufacturer's specifications (usually by incubation for 20 min at 65−80 ºC).

RNAse treatment

Samples containing RNA, such as digestions of DNA with restriction enzymes, were incubated for 5 min at RT with RNAse A (final concentration: 50 μg ml^{-1}) to remove the RNA and improve the visualization of DNA in agarose gels.

- Ligation of DNA molecules and transformation of *E. coli* with exogenous DNA

Ligation reactions were carried out using T4 DNA ligase included in the commercial kit pGEM®-T Easy vector system (Promega) according to the manufacturer's specifications. The pGEM®-T Easy vector system is a cloning vector with single 3´-T overhangs at the insertion site that improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (Fig. II.10). Transformation of *E. coli DH5α* (Hanahan, 1983; see 'Microorganisms and growth conditions Table. II.10) with exogenous DNA was performed essentially by the method of Cohen *et al*. (1972).

Preparation of E. coli DH5α competent cells

E. coli DH5α cells were grown in LB (Luria-Bertani culture medium; Sambrook and Russel, 2001; see 'Culture media and solutions' Table II.11). The culture was maintained in agitation at 37ºC until an $A_{600\ nm}$ (optical density) was 0.4−0.6 (mid-exponential phase). Then, cells were harvested by centrifugation at 5000×g for 5 min at 4ºC, and suspended in ½ vol. of 50 mM $CaCl_2$ at 4ºC. After 10-min incubation on ice, cell-harvesting and suspension steps were repeated using $^1/_{10}$ vol of 50 mM $CaCl_2$ at 4ºC. The resulting competent cells were maintained for 1−24 h on ice before being used.

Transformation of competent cells

Certain volume of competent cells (100 μl) was mixed with the desired exogenous DNA ligated to the pGEM®-T cloning vector. The resulting mixtures were incubated for 30 min at 4ºC. Then, the samples were incubated at 42ºC for 2 min and transferred to ice. After addition of 0.9 ml LB, the samples were incubated under agitation for 1 h at 37ºC to allow the expression of the plasmid genes involved in

resistance to antibiotics. After that, cells were harvested by centrifugation at 13000×*g* for 2 min at RT, and suspended in 300 µl of fresh LB medium. Then, 25−100 µl of transformed cells were spread on LB agar plates supplemented with the suitable antibiotics and reagents (IPTG, X-gal, etc.) for the selection of the transformed and recombinant cells. Finally, these plates were incubated O/N (overnight) at 37ºC.

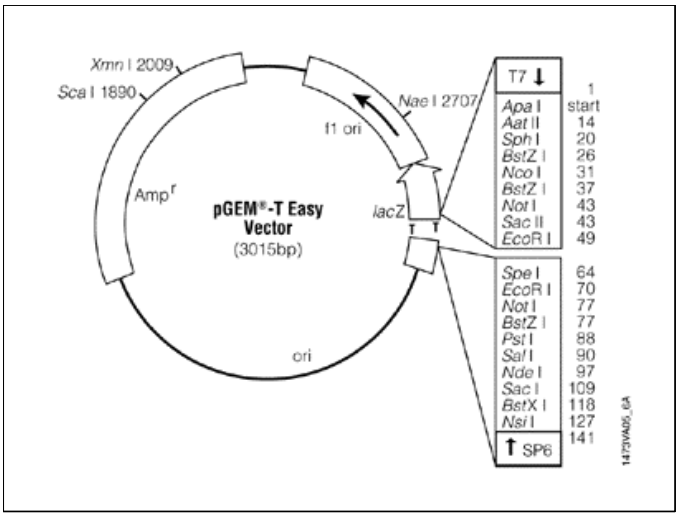

Figure II.10. pGEM®-T vector system.

pGEM®-T vector (Promega) express the amino-terminal fragment of the *lacZ* gene product (β-galactosidase) and display α-complementation in appropriate hosts. Embedded in the coding region of the β-galactosidase gene is a polycloning site. pGEM®-T is used in host cells that code for carboxy-terminal portion of β-galactosidase. Although neither the host-encoded not the plasmid encoded fragments are themselves active, they can associate to form an enzimatically active protein. This type of complementation, in which deletion mutants of the operator-proximal segment of the *lacZ* gene are complemented by β-galactosidase-negative mutants that have the operator-proximal region intact, is called α-complementation. The lac^+ bacteria that result from α-complementation are easily recognized because they form blue colonies in the presence of the cromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). Bacteria carrying recombinant plasmids therefore form white colonies. In the cloning system, is easy to screen recombinant colonies of *E□coli DH5α* (which is amp^s, sensitive to ampicilline due to the lack of the ampicillin resistance gene, as a wild type), because of their white color (disruption of the β-galactosidase gene by insertion of exogenous DNA in polycloning site) and because they grow onto agar plates of LB agar (Luria-Bertani) supplemented with ampicillin, X-Gal and IPTG (Isopropyl-D-thio-galactopiranoside; inductor of protein expression).

- DGGE analysis

DGGE is an electrophoretic separation method based on differences in melting behaviour of double stranded DNA fragments (Fisher and Lermann, 1979). Denaturing gradient gel electrophoresis (DGGE) is one of a group of related methods for mutation detection and screening of DNA fragments. They are all based on the reduction in DNA fragment mobility in a dense medium when part of the double helix unravels. Strand separation can be induced by an increase in temperature (temperature gradient gel electrophoresis, TGGE) or increasing the concentration of chemical denaturants such as formamide or urea (DGGE and constant denaturing gel electrophoresis, CDGE). These methods offer a high probability of detecting any difference between sequences, because molecules with different sequence may have a different melting behavior, and will, therefore, stop migrating at different positions in the gel. Since the applications of these techniques, they have been applied as molecular tools to compare the diversity of microbial communities and to monitor population dynamics (Muyzer and Shamala, 1998).

When analyzed on DGGE, DNA samples (PCR amplicons of 16S rRNA gene) are loaded on the top of a polyacrylamide gel containing denaturing compounds which concentration is linearly in the gel. The gel is afterwards placed in an electric field. During electrophoresis DNA migrates to the positive pole (to the bottom of the gel) and dissociates. The DNA will always start to melt out from one end, because one of the primers used for PCR consists of a GC-clamp. The clamp consists of 40 nucleotides - with G's and C's - and therefore very difficult to melt out. By dissociating, DNA looses mobility due to the change of the geometrical structure and at certain concentration of denaturing compounds it stops. The concentration of denaturing compounds at which a DNA sample will stop migration depends on the sequence of the PCR product. The more G's and C's there are in a PCR product, the higher the percentage denaturing chemicals is needed for melting the product. More information about the DGGE protocol can be found in the ‘Useful websites’ section.

- DGGE conditions

DGGE was performed by using a D-Code 16/16 cm gel system (Bio-Rad, CA) maintained at a constant temperature of 60°C in 0.5×TAE buffer. Gradients were formed between 30% and 65% denaturant (100% denaturant was defined as 7 M urea plus 40% [v/v] formamide) and the gels run at 50 V overnight (Table II.8). Gels were stained in purified water (MilliQ; Millipore) containing ethidium bromide (0.5 mg L^{-1}) and destained twice in 0.5×TAE for 15 min each. Images were captured using an image-analyzing system (Image Master® VDS; Amersham Pharmacia Biotech, Sweden).

- Extraction of DNA from acrylamide gels

The central 1 mm^2 portion of strong DGGE bands were excised using a razor blade and soaked in 50 µl of purified water (MilliQ; Millipore) overnight a 4°C. One µl was removed and used as a template in a PCR-DGGE reaction as above (Table II.6 and 7). The products were checked in a 1% agarose/1×TBE gel, and then purified using the PCR clean-up kit (MoBio, Laboratoires, Inc.) or the Wizard® PCR Preps (Promega) kit.

- Sequencing of purified PCR products and cloning

The PCR products derived from strong DGGE bands, free of minor bands, were sequenced directly using the purified PCR product described above as a template. The sequencing reaction was performed as it is described in the 'DNA sequencing' section. Amplification products that failed to generate legible sequence directly are being cloned into the pGEM-T® easy system II (Promega, USA) cloning vector (see 'Transformation of competent cells' section). Recombinant (white) colonies were boiled as is described in the 'DNA extraction' section, and the aqueous DNA solution was used as DNA template in the PCR reaction with pGEM-T® cloning vector primers T7pGEM-T SP6pGEM-T as is described in Table II.6. The products were analyzed by gel electrophoresis and digested with restriction enzymes to check the presence of different cloned bands by digestion patterns (see 'Enzymatic treatment' section). Sequences that were of high frequency in clone libraries were selected for sequence analysis.

Table II.8. DGGE reagents.

Reagents
Formamide (de-ionized) Add 10 g of mixed bed resin (Sigma) to 100 ml Formamide (Sigma) in an erlentmeyer and stir for 30–60 min. Decant or filter the formamide to separate it from the resin beads. Store the de-ionized formamide in volumes of 35 ml at –20ºC for the preparation of the gel solution. **Acrylamide/bis-acrylamide stock solution (37.5:1; 40 % w/v)** It can be purchased from Bio-Rad or prepare the solution from acrylamide powder (filter the solution and store at 4ºC in a dark bottle). **DGGE acrylamide/bis-acrylamide solutions (for a gradient of 30–65% of denaturant)** 10% acrylamide/ 0% denaturant - 2.5 ml 50×TAE - 62.5 ml acrylamide/bis-acryl 40% - 185 ml H_2O distilled 8% acrylamide/ 0% denaturant - 2.5 ml 50×TAE - 50 ml acrylamide/bis-acryl 40% - 197.5 ml H_2O distilled 10% acrylamide/ 100% denaturant - 2.5 ml 50×TAE - 62.5 ml acrylamide/bis-acryl 40% - 100 ml formamide - 105 g Urea+ H_2O for 250 ml 8% acrylamide/ 100% denaturant - 2.5 ml 50×TAE - 50 ml acrylamide/bis-acryl 40% - 100 ml formamide - 105 g Urea+ H_2O for 250 ml **Note**: These solutions have to be filtered through a 0.45 μm filter and stored at 4ºC **Mixed gel solutions for a DGGE gel** 30% denaturing solution - 7.2 ml of 8% acrylamide/ 100% denaturant solution + 16.8 ml of a 8% acrylamide/ 0% denaturant solution (then, add 32 μl TEMED and 75 μl 10% APS). 65% denaturing solution - 15.6 ml of 10% acrylamide/ 100% denaturant solution + 8.4 ml of 10% acrylamide/ 0% denaturant solution (then, add 32 μl TEMED and 75 μl 10% APS). Stacking gel solution - Solution of 8% acrylamide/ 0% denaturant (10 ml per gel); add 20 μl TEMED and 40 μl 10% APS. **Note**: These solutions have to be degassed for 15 min under vacuum and stored at 4ºC in the dark. TEMED (Bio-Rad) and 10% APS (ammonium persulfate; Fluka) are added before loading the gel **10×Gel loading solution** Bromophenol blue (0.025 g; 0.25% w/v); xylene cyanole (0.025 g; 0.25% w/v); glycerol (5 ml; 100% v/v); water 5 ml

- Extraction of DNA from acrylamide gels

The central 1 mm^2 portion of strong DGGE bands were excised using a razor blade and soaked in 50 µl of purified water (MilliQ; Millipore) overnight a 4°C. One µl was removed and used as a template in a PCR-DGGE reaction as above (Table II.6 and 7). The products were checked in a 1% agarose/1×TBE gel, and then purified using the PCR clean-up kit (MoBio, Laboratoires, Inc.) or the Wizard® PCR Preps (Promega) kit.

- Sequencing of purified PCR products and cloning

The PCR products derived from strong DGGE bands, free of minor bands, were sequenced directly using the purified PCR product described above as a template. The sequencing reaction was performed as it is described in the 'DNA sequencing' section. Amplification products that failed to generate legible sequence directly are being cloned into the pGEM-T® easy system II (Promega, USA) cloning vector (see 'Transformation of competent cells' section). Recombinant (white) colonies were boiled as is described in the 'DNA extraction' section, and the aqueous DNA solution was used as DNA template in the PCR reaction with pGEM-T® cloning vector primers T7pGEM-T SP6pGEM-T as is described in Table II.6. The products were analyzed by gel electrophoresis and digested with restriction enzymes to check the presence of different cloned bands by digestion patterns (see 'Enzymatic treatment' section). Sequences that were of high frequency in clone libraries were selected for sequence analysis.

- Analysis of DGGE profiles

Scanned gels were analyzed with the *Scion Image* software package for Windows (Version Beta 4.0.2 based on NIH Image, Scion, USA). The banding patterns of the samples were aligned according to the alignment information provided by the closest neighboring standard patterns. By aligning the bands of sample tracks from every gel to the standard reference pattern, it becomes possible to compare patterns from different gels with each other (Eichner *et al*., 1999; Sekiguchi *et al*., 2002). After the calibration step, a densiometric curve was calculated for each gel track. The area under the curve corresponding to each band was calculated respect to the total area of the specific track.

- DNA sequencing

Both DNA chains of purified PCR products were sequenced using a BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA) and the analytical system CEQ™8000 (Beckman-Coulter) available at the technical services of the 'Universitat de Barcelona' (Serveis Científico-Tècnics, SCT). This method is based on the Sanger dideoxy-mediated chain termination method (Sanger *et al*., 1977), and consists in the synthesis of DNA molecules in the presence of dideoxynucleotides (dNTPs) that terminate the DNA synthesis and which are labelled with different fluorescent markers. The composition of the reaction mixture and the program used for DNA sequencing, according to the manufacturer's specifications, are detailed in Table II.9. DNA synthesis reaction was carried out in a Eppendorf Mastercycler® personal thermocycler (Eppendorf). Amplified DNA was purified by a modified protocol of ethanol precipitation.

Table II.9. DNA sequencing conditions.

Reaction composition mixture	Amplification program
Standard PCR reaction	
- 4 µl *BigDye*® Terminator v. 3.1 Cycle ready reaction premix (contains thermostable polymerase, buffer, dNTPs and ddNTPs) - 2 µl additional BigDye® buffer (5×) - 3−300 ng purified DNA template - 3.2 pmols primer (FW or BW)	1 cycle - 3 min at 94ºC 25 cycles - 10 s at 96ºC - 5 s at Tm[1] - 4 min at 60ºC Hold at 4ºC **Note**: The Tm was usually 50ºC. Tm[1]= Theoretical melting temperature

- Bioinformatic and phylogenetic analyses

Percent similarity of DNA sequences was analyzed through BLAST (Altschul *et al*., 1997; see 'Useful websites'), and sequence alignments were performed using ClustalW Multalign software (Higgins *et al.,* 1994; or on-line, see 'Useful websites' section) included in the BioEdit Sequence Alignment Editor v.7.0.1 (Hall, 1999).

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (Kumar *et al*., 2004). Phylogenetic trees were calculated by maximum parsimony, neighbour-joining, or maximum-likelihood analyses. To validate the reproducibility of the branching pattern, bootstrap analyses were performed (Felsenstein, 1985). Sequences were checked for chimeric 16S rDNA artifacts, generated during PCR amplification from mixed bacterial populations, with the 'Check_chimera' software of the Ribosomal Database Project (Larsen *et al*., 1993; Cole *et al*., 2003).

Despite of the fact that there are many potential ways to detect chimeric sequences, most detection is done by nearest-neighbor methods. In nearest-neighbor analysis, a newly recovered sequence is split into two subsequences that are then compared with a database of similar sequences. If the sequence can be split in such a way that the phylogenetic affiliation of the parts is inconsistent with the affiliation of the sequence as a whole, a chimera is suspected. The 'Check_chimera' method defines similarity by the number of common oligonucleotides of length k (k-tuples) shared by a sequence. The sequence is broken at 10-base intervals, and the maximum value of the IS (usually defined as the sum of the similarities between the two partial sequences and their nearest neighbors minus the score for the complete sequence compared with its nearest neighbor) over all possible breakpoints is determined.

Note: All sequences were deposited in GenBank database (see 'Useful websites' section).

4. Morphobiochemical characterization of prokaryotes

- Microbiological methods

➢ Sterilization of materials and solutions

Glass and plastic material, culture media, etc., were sterilized by wet-heat and pressure by autoclaving at 1 atmosphere pressure for 20 min at 121ºC (10 min for highly concentrated sugar solutions, and skimmed milk). Thermolabile solutions were sterilized by filtration through sterile 0.22 μm-filters.

➢ Strains, culture media and growth conditions

The microorganisms used in this work, composition of the culture media used for their isolation, growing, and maintenance are detailed in Table II.10, and Table II.11. The maintenance of the monthly cultures was done by incubation of the grown agar plates at 4ºC, except from *Chromobacterium violaceum* (the viability of this strain decrease dramatically at 4ºC in less than two weeks; Efthimion and Corpe, 1969) and sulfate-reducing bacteria that were both incubated at 15ºC. For a longer maintenance, the strains were cryopreserved at –80ºC after being cultured O/N in liquid medium and after addition of sterile glycerol at a final concentration of 20% (v/v) or 7% DMSO (dimethyl sulfoxide; v/v) for *Clostridium* cultures.

In general, liquid cultures were inoculated from plate cultures or previous liquid cultures (using 1:50 or 1:100 dilutions in fresh media) and were incubated under agitation at 200 rpm in a Certomat®R agitator (B. Braun). Anaerobic growth onto plates was performed in anaerobic jars (BBL GasPak system; BBL, Cockeysville, MD) with an anaerobic gas generating kit and an anaerobic indicator (Oxoid). Manipulation of anaerobic microorganisms was done under anaerobic conditions inside an anaerobic chamber (Hands-in-Bag®, Artificial atmosphere chamber, Spilfylter®, JV manufacturing company Inc., Green Bay, WI).

Table II.10. Microorganisms and their growth conditions.

Microorganisms and growth conditions
***E. coli* DH5α** **- Origin/Reference:** *E. coli* DH5α (Hanahan, 1983). **- Maintenance:** monthly cultures on Luria-Bertani (LB) agar plates incubated O/N at 37ºC **- Uses:** host strains for cloning with the pGEM®-T system. - ***E. coli DH5α* recombinant clones:** LB agar plates supplemented with Ampicillin.
Chromobacterium violaceum CECT 494 **- Origin/Reference:** strain from the CECT. **- Maintenance:** monthly cultures on Nutrient agar 1 plates incubated O/N at 25ºC. **- Uses:** Polyhydroxyvalerate accumulation (chapter III).
Cupriavidus necator CECT 4635 (*Alcaligenes eutrophus, Ralstonia* and *Wautersia eutropha*) **- Origin/Reference:** strain from the CECT. **- Maintenance:** monthly cultures on Nutrient agar plates incubated O/N at 30ºC. **- Uses:** Positive control PHA synthesis and PHB analysis (chapter VII).
***Cupriavidus necator* PHB⁻4** DSM 541 (mutant of DSM 428). **- Origin/Reference:** strain from the DSMZ (Schlegel *et al*., 1970). **- Maintenance:** monthly cultures on Nutrient agar plates incubated O/N at 30ºC. **- Uses:** Negative control of poly-β-hydroxybutyrate production (chapter VII).
Thiomicrospira pelophila DSMZ 1534 **- Origin/Reference:** strain from the DSMZ. **- Maintenance:** monthly cultures on *Thiomicrospira* agar plates incubated O/N at 26 ºC. **- Uses:** Control of growth and comparison with isolated *Thiomicrospira* species (chapter VIII).
Pseudomonas oleovorans CECT 4079 **- Origin/Reference:** strain from the CECT. **- Maintenance:** monthly cultures on TSA plates incubated O/N at 30ºC. **- Uses:** Positive control PHA synthesis and inhibition analysis (chapter VII).
Sphingomonas paucimobilis CECT 599, ATCC 29837 **- Origin/Reference:** strain from CECT or ATCC. **- Maintenance:** monthly cultures on Nutrient agar 2 plates incubated O/N at 30ºC. **- Uses:** Sphingolipid composition (chapter III and VII).

Novosphingobium capsulatum CECT 4388 (*Sphingomonas capsulata*)

- Origin/Reference: strain from the CECT.
- Maintenance: monthly cultures on Nutrient agar 2 plates incubated O/N at 30ºC.
- Uses: Sphingolipid composition (chapter VII).

Staphylococcus aureus CECT 240

- Origin/Reference: strain from the CECT or 'Staphylococcal dried cells' from Sigma.
- Maintenance: monthly cultures on TSA plates incubated O/N at 30ºC.
- Uses: Inhibition analysis and validation of SLB approach (chapter III and VII).

Clostridium butyricum ATCC 19398

- Origin/Reference: strain from the ATCC.
- Maintenance: monthly cultures in 'Cooked meat' medium (ATCC).
- Uses: Validation of SLB approach (chapter III).

Pseudomonas putida ATCC 12633

- Origin/Reference: strain from the ATCC (American type culture collection).
- Maintenance: monthly cultures on R2A plates incubated O/N at 30C.
- Uses: Validation of SLB approach (chapter III).

***Pseudoalteromonas* sp. EBD**

- Origin/Reference: Isolated from Ebro delta microbial mats (this study).
- Maintenance: monthly cultures on SWYP or M179 agar plates incubated O/N at 30ºC.
- Uses: New isolate (chapter VII).

***Desulfovibrio* sp. EBD**

- Origin/Reference: Isolated from Ebro delta microbial mats (this study).
- Maintenance: monthly cultures on SRB saline agar plates incubated 2 week at 25ºC.
- Uses: New isolate (chapter VI).

***Clostridium* sp. EBD**

- Origin/Reference: Isolated from Ebro delta microbial mats (this study).
- Maintenance: monthly cultures on SPS saline agar plates incubated O/N at 25ºC.
- Uses: New isolate (chapter VI).

***Sphingomonas* sp. Camargue**

- Origin/Reference: strain isolated from Camargue mats (this study).
- Maintenance: monthly cultures on nutrient agar 2 plates incubated 48 h at 30ºC.
- Uses: New isolate and sphingolipid detection (chapter VII).

***Halomonas* sp. EBD**

- Origin/Reference: strain isolated from Ebro delta mats (del Campo, 2005).
- Maintenance: monthly cultures on M179 or SWYP agar plates incubated O/N at 30ºC.
- Uses: Inhibition analysis (chapter VII).

***Stappia* sp. EBD**

- Origin/Reference: strain isolated from Ebro delta mats (del Campo, 2005).
- Maintenance: monthly cultures on M179 or SWYP agar plates incubated O/N at 30ºC.
- Uses: Inhibition analysis (chapter VII).

Filamentous cyanobacteria

- Origin/Reference: strains isolated from Ebro delta mats (Urmeneta *et al*., 2003).
- Maintenance: fortnightly cultures on MN agar plates incubated at 20ºC in controlled culture chamber equipped with 18-W fluorescent tubes (Grolux and cool white tubes) maintained at under dark-light (14 hours/day at a light intensity of about 150 μE m^{-2} s^{-1}) conditions.
- Uses: Interaction studies between cyanobacteria and *Pseudoalteromonas* sp. (chapter VII).

Note:
CECT: Spanish culture collection (Colección Española de Cultivos Tipo).
DSMZ: German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).
ATCC: American Type Culture Collection.

Table II.11. Culture media.

Culture media
Luria-Bertani broth (LB) (Sambrook and Russel, 2001) • **Properties:** Medium suitable for most chemoheterotrophic microorganisms. • **Composition:** 10 g tryptone, 5 g yeast extract, 10 g NaCl, per L; pH 7. Plus 15 g agar per L for **LB agar**. • **Others:** after autoclaving, it was supplemented with Amp (ampicilline), IPTG and/or X-Gal, when necessary.
Mineral basal medium agar MM (Hareland *et al*., 1975) • **Properties:** Synthetic medium with neither carbon nor energy source. • **Composition:** 100 ml Solution A + 10 ml Solution B + 890 ml H_2O plus 15 g agar per L; pH 7.2. Both solutions are prepared separately using bidistilled water. - Solution A composition: 42.5 g of $K_2HPO_4 \cdot 3H_2O$, 10 g $NaH_2PO_4 \cdot H_2O$, 20 g NH_4Cl, per L. - Solution B composition: 12.3 g Nitrilotriacetic acid salt, 20 g $MgSO_4 \cdot 7H_20$, 1.2 g $FeSO_4 \cdot 7H_2O$, 0.3 g $MnSO_4 \cdot H_2O$, 0.3 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $CoCl_2 \cdot 6H_2O$, per L. • **Others:** - MM + PHB for testing the degrading capacity of microorganims: MM agar plates with an overlay make as follows: Mix 0.5 g Poly-3-hydroxybutyrate (Sigma-Aldrich), 5 ml H_2O and 5 ml 0.1% (v/v) Triton X-100, and sonicate the mixture 2 h. Add to a 100 ml-MM agar and add as an overlay onto MM agar plates.
Nutrient Broth 1 • **Properties:** Medium for chemoheterotrophic microorganisms. • **Composition:** 5 g meat extract, 10 g tryptone, 5 g NaCl, per L; pH 7.2. Plus 15 g agar per L for **Nutrient agar 1**.
Nutrient Broth 2 • **Properties:** Medium for chemoheterotrophic microorganisms. • **Composition:** 1 g meat extract, 5 g tryptone, 2 g yeast extract, per L; pH 7. Plus 15 g agar per L for **Nutrient agar 2**.
Medium 179 (M179) (CECT) • **Properties:** Medium rich in organic compounds for marine microorganisms. • **Composition:** 10 g tryptone, 3 g yeast extract, 24 g NaCl, 0.7 g KCl, 7 g $MgSO_4 \cdot 7H_2O$, 5.3 g $MgCl_2 \cdot 6H_2O$, per L; pH 6.8. Plus 15 g agar per L for **M179 agar**.

Tryptic soy Broth (TSB; Scharlau Microbiology)

- **Properties:** Medium for most chemoheterotrophic microorganisms.
- **Composition:** 15 g casein peptone, 5 g soy peptone, 5 g NaCl, per L. pH 7.3

Plus 15 g agar per L for **TSA agar**.

Sea Water Yeast Peptona (SWYP) medium

- **Properties:** Medium rich in organic compounds for marine microorganisms.
- **Composition:** 5 g tryptone, 3 g yeast extract, 750 ml filtered sea water, 250 ml H_2O, per L; pH 6.8. Plus 15 g agar per L for **SWYP agar**.

Reinforced Clostridial media (RCM; modified from Scharlau Microbiology)

- **Properties:** Medium suitable for the cultivation of anaerobic microorganisms. It is free from inhibitors and contains thioglycolate as reducing agent. Liquid culturing of *Clostridium* sp. EBD (chapter VI).
- **Composition:** 10 g meat extract, 10 g tryptone, 3 g yeast extract, 5 g D(+)-glucose, 1 g starch, 5 g NaCl, 3 g sodium acetate, 0.5 g sodium thioglycolate, 1 ml of 0.1% resarzurine, per L; pH 6.8.

***Thiomicrospira pelophila* medium (medium 142 from DSMZ)**

- **Properties:** Medium for the cultivation of some *Thiomicrospira* species (chapter VIII).
- **Composition:** 25 g NaCl, 1 g $(NH_4)_2SO_4$, 1.5 g $MgSO_4 \cdot 7H_2O$, 0.42 g $CaCl_2 \cdot 2H_2O$, 4 mg bromothymol blue, 0.5 g K_2HPO_4, 5 g $Na_2S_2O_3 \cdot 5H_2O$, 15 µg vitamin B_{12}, 0.20 ml trace element solution, per L.
- **Note:** Adjust pH at 7.2. K_2HPO_4 and $Na_2S_2O_3$ are autoclaved separately each in 10% of the final volume. Sterilize the Vitamin B_{12} by filtration. Adjust the completely medium to pH 7.2 with 0.4% Na_2CO_3 after being sterilized and the mentioned solutions have been added.

Sulfate-reducing bacteria (SRB) agar (Scharlau Microbiology)

- **Properties:** Medium for sulfate-reducing bacteria.
- **Composition:** 1 g yeast extract, 1 g ammonium chloride, 1 g calcium sulfate, 2 g magnesium sulfate, 0.5 g monopotassium phosphate, 0.1 g ascorbic acid, 0.1 g thioglycollic acid, 0.5 g iron sulfate, 3.5 g sodium lactate, per L; pH 6.3.
  Plus 15 g agar per L for **SRB agar**.
- **Others:**

- SRB agar saline: Add 15 g NaCl per L. Isolation of SRB from mats (chapter VI).

SPS agar (Scharlau Microbiology)

- **Properties:** It allows the detection of sulfite-reducing organisms.
- **Composition:** 0.5 g Sodium sulfite, 0.01 g polymixine B sulfate, 0.12 g sodium sulfadiazine, 15 g casein peptone, 10 g yeast extract, 0.5 g ferric citrate, 0.1 g sodium thioglicolate, 0.05 g polysorbate 80, per L; pH 7.0.
  Plus 15 g agar per L for **SPS agar.**
- **Others:** SPS agar saline: Add 15 g NaCl per L. Isolation of *Clostridium* from Ebro delta mats (chapter VI).

MN medium for cyanobacteria (Rippka *et al.*, 1979)

- **Properties:** Mineral medium for cultivation of cyanobacteria.
- **Composition:**

- Solution I: 500 mg $Na_2EDTA{\cdot}2H_2O$, 300 mg Citric acid, 3.8 g $MgSO_4{\cdot}7H_2O$, per 100 ml H_2Od.
- Solution II: 2 g $NaHPO_4{\cdot}H_2O$, per 100 ml H_2Od.
- Solution III: 7.5 g $NaNO_3$, per 100 ml H_2Od.
- Solution IV: 200 mg Na_2CO_3, per 100 ml H_2Od.
- Solution V: 1.8 g $CaCl_2{\cdot}H_2O$, per 100 ml H_2Od.
- Solution VI: 300 mg $FeNH_4(SO_4){\cdot}12H_2O$, some drops of H_2SO_4, per 100 ml H_2Od.

- **Note:**

- All solutions are autoclaved separately and added under sterile conditions as follows: 1 ml Solution I, 1 ml Solution II, 10 ml solution III, 10 ml Solution IV, 1 ml Solution V, 1 ml Solution VI, 1 ml trace element solution, 1 ml Cycloheximide stock solution (see 'Annex'), 2 ml of 1 mM DCMU for inhibitory conditions (see 'Annex'), per L. Plus 15 g agar per L for **MN agar**.

M55 medium (Oeding and Schlegel, 1973; Aragno and Schlegel, 1992)

- **Properties:** Mineral medium with a controlled source of carbon.
- **Composition:**

- Solution A: 9 g $Na_2HPO_4{\cdot}12H_2O$, 1.5 g KH_2PO_4, per 500 ml H_2Od.
- Solution B: 1 g NH_4Cl, 0.2 g $MgSO_4{\cdot}7H_2O$, per 500 ml H_2Od.
- Solution B_2: 0.1 g NH_4Cl, 0.2 g $MgSO_4{\cdot}7H_2O$, per 500 ml H_2Od.
- Solution C: 50 mg Citrate ferric ammonic, 200 mg $CaCl_2{\cdot}2H_2O$, per 100 ml H_2Od.
- Solution D: 50 g Fructose, per 100 ml H_2Od.

- **Note:**

- All solutions are autoclaved separately and added under sterile conditions as follows: 500 ml Solution A, 500 ml Solution B (or B_2 for a nitrogen-limited medium), 10 ml solution C, 10 ml Solution D, 1 ml trace element solution, pH 7–7.2, per L.
- The solution D is the source of carbon and can be replaced for D-Glucose, Sodium gluconate or sodium valerate. Is recommended to prepare sterile stock solutions (10% w/v) Sodium valerate is not commercially available, so it can be obtained by mixing valeric acid (Sigma) and NaOH at equimolar concentration. This medium can be supplemented with Nile Red (see 'Annex') for testing the PHA accumulation capacity.

Mineral marine medium (3M+, modified from Östling *et al.*, 1991)

- **Properties:** Mineral medium for marine microorganisms with a controlled source of carbon.
- **Composition:**

- Eleven Salt Solution (ESS; del Campo, 2005): 17.6 g NaCl, 1.47 g $NaSO_4$, 0.08 g $NaHCO_3$, 0.25 g KCl, 0.04 g KBr, 1.87 g $MgCl_2 \cdot 6H_2O$, 0.41 g $CaCl_2 \cdot 2H_2O$, 0.008 g $SrCl_2 \cdot 6H_2O$, 0.008 g H_3BO_3, 5 mg $NaSiO_3 \cdot 9H_2O$, 4.89 mg $FeCl_3 \cdot 6H_2O$, per L H_2Od. Adjust the pH to 8 and autoclave.

- **Note:**

- After the sterilization of the ES Solution, add 0.51 g NH_4Cl (or 0.05 g NH_4Cl for a nitrogen-limited medium), 0.23 g K_2HPO_4, 8.3 g MOPS, per L of ESS. Autoclave the solution again, add sterile 0.2% D-Glucose and adjust the pH to 8. Plus 15 g agar per L for **3M+ agar**.
- This medium can be supplemented with different sources of carbon (Sodium gluconate, Sodium acetate, etc. at 0.2–3% final concentration). For D-Glucose and Sodium gluconate is recommended to prepare stock solutions concentrated (10% w/v), filter-sterilize, and add them after the medium has been sterilized by autoclaving. Sodium gluconate is recommended to induce the PHA accumulation in microorganisms (Schlegel *et al*., 1970). This medium can be supplemented with Nile Red (see 'Annex' section) for testing the PHA accumulation capacity.

R2A medium (Reasoner and Geldreich, 1985)

- **Properties:** Medium suitable for heterotrophic bacteria from water.
- **Composition:**0.5 g yeast extract, 0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g dextrose, 0.5 g strach, 0.3 g sodium pyruvate, 0.3 K_2HPO_4 (0.05 g for a low-phosphate medium), 0.05 g $MgSO_4$, per L, pH 7.2. Plus 15 g agar per L for **R2A agar**.

Trace element solution

- **Properties:** Provide micronutrients to defined media.
- **Composition:** 286 mg H_3BO_3, 181 mg $MnCl_2 \cdot 4H_2O$, 22 mg $ZnSO_4 \cdot 7H_2O$, 8 mg $CuSO4 \cdot 5H_2O$, 39 mg $Na_2MoO_4 \cdot 2H_2O$, 5 mg $CoCl_2$, 1 mg $NiCl_2$, per 100 ml of H_2Od. pH= 5.5. Autoclave and store at 4ºC.

➢ Preparation of cultures for SLB analysis and detection of PHA by HPLC

Pure cultures of microbial strains can be analyzed by the SLB approach to detect and quantify their FAMEs (fatty acid methyl esters derived from the PLFA, phospholipid fatty acids), respiratory quinones, accumulation of PHA (poly-β-

hydroxyalkanoates), sphingolipids, etc., which are chemotaxonomic characters of a microbial strain and have been widely used for taxonomic classification.

Microbial cultures for SLB analysis were performed in the suitable medium for the microbial strain. The cultures were done as a liquid culture in batch, so that and O/N culture of the specific strain in 5 ml of liquid culture was transferred in 95 ml of fresh media (dilution 1:20). After 24 h of incubation, the culture was centrifuged 15 min at 3000 rpm and the supernatant was discarded. The remaining pellet was washed with sterile 0.85% (w/v) NaCl, centrifuged and the supernatant was discarded. This process was repeated twice in order to eliminate the remaining culture media that can interfere in the lipid analysis (another possibility is include a blank analysis of lipids of a non-inoculated culture media). The washed cell pellets were stored at –80ºC, lyophilized a Virtis apparatus at –55°C and 200 mTorr for 48 h, and homogenized with an acetone-rinsed mortar before starting the total lipid extraction.

The preparation of cultures for the analysis of PHA accumulation by HPLC was done as it follows: Microbial strains were inoculated in 5 ml of liquid culture (M55 for non-marine strains, and 3M+ for marine microorganisms, with certain source of carbon that can induce the accumulation of PHA) and incubated 48 h at their optimal temperature of growth. Then, the cultures were centrifuged and the supernatant was discarded. The pellet was re-suspended in 5 ml of the same culture media but with nitrogen limitation, and the culture was incubated 48 h more under optimal conditions of temperature and agitation (alternatively, the induction of PHA accumulation can be done by ageing of the culture or limitation of other nutrients apart from nitrogen, depending of the microorganisms and it type of metabolism). After that, the culture was centrifuged again, the supernatant was discarded and the pellet was stored at –80ºC before being lyophilized.

- Morphobiochemical characterization

➢ Isolation of microorganisms for microbial mat samples

Screening of sulfate-reducing bacteria (SRB) in mat samples

The manipulations for the isolation of SRB were done in an anaerobic chamber. One cubic centimeter of Ebro delta microbial mat was homogenized in 25 ml of anoxic Ringer ¼ (see 'Annex' section). The mixture was let settle down for 10 min, and then the supernatant was transferred to perform a set of dilutions in anoxic Ringer ¼. Certain volume of the dilutions was spread onto SRB saline plates. The incubation was done in anaerobic jars, and the grown colonies were isolated as pure cultures.

Screening of heterotrophic bacteria in mat samples

One cubic centimeter of Ebro delta or Camargue microbial mats was homogenized in 25 ml of Ringer $^1/_1$ or artificial sea water (see 'Annex' section). The mixture was let settle down for 10 min, and then the supernatant was transferred to perform a set of dilutions in the same isotonic solution. The dilutions were spread onto rich media for marine heterotrophic microorganisms. The incubation was done in aerobic conditions, and the grown colonies were isolated as pure cultures.

For the isolation of *Sphingomonas* species in mat samples, an approach proposed by Vanbroekhoven *et al* (2004) was applied. One cubic centimeter of mat sample (Ebro delta and Camargue mats) was homogenized in 20 ml of Ringer $^1/_1$ and then the protocol proced as above. Dilutions were spread onto MM (mineral medium) agar plates with 0.2% (w/v; final concentration) glucose or fructose, and supplemented with 200 μg ml^{-1} (w/v, final concentration) streptomycin. The yellow-pigmented colonies were identified by molecular and biochemical approaches.

Screening of spore-forming anaerobic bacteria in mat samples

One cubic centimeter of Ebro delta microbial mat was homogenized in 25 ml of Ringer $^1/_1$. The mixture was heated at 80ºC 35 min in order to eliminate the vegetative forms and select spore-forming microorganisms. After the 'heat shock', 5 ml of the

mixture was covered with 45 ml of SPS soft agar. The sample was incubated at 30ºC for 48 h. The grown black-colonies were transferred to SPS agar plates under anaerobic conditions to achieve pure cultures. Once pure culture was obtained, the maintenance was done by a monthly culture of agedly colonies in aerobic conditions (due to the resistance of the spores to aerobic conditions).

- Characterization of the isolates

For the determination of the physiological characteristics of the isolated strains, biochemical, molecular and morphological tests were performed (not all tests were performed for each strain, for details see chapter VII results). Biochemical characteristics are detailed in Table II.12.

Table II.12. Biochemical characteristics.

Biochemical tests
Oxidase and Catalase: Oxidase test was performed by pouring some drops of the oxidase reagent (tetramethyl-*p*-phenylene-diamine). Catalase test was performed as above but by pouring some drops of a hydrogen peroxide solution onto the growing colonies.
Anaerobic growth: Performed by incubation of inoculated agar plates in anaerobic jars.
API 20NE and API 50CH (BioMèrieux): API 20 NE is a standardized set of biochemical tests for the identification of bacillus gram negative non enterobacteriaceae. API 50 CH is a system for the study of the carbohydrate metabolism of microorganisms. The salinity of API 50 CH inoculation medium was amended for the inoculation of marine microorganisms, as it is explained in Donachie *et al*□2004a; Yoon *et al*., 2003; or by mixing 50% API CHB/E medium and 50% saline solution (0.075% $CaCl_2 \cdot 2H_2O$, 1.875% NaCl, 0.375% $MgCl_2$, and 0.017% phenol red, per L, pH 7.4–7.8) for *Pseudoalteromonas* sp. EBD strain.
Gram, motility and structure: Gram staining, and motility of fresh samples checked by phase contrast microscopy (see 'Microscope techniques' section). Ultrastructure, presence and position of flagella and other characteristics were observed by Scanning or transmission electron microscopy (see 'Microscope techniques' section).
Salinity, pH and temperature range of growth: The optimum culture medium for an specific strain was amended at different pH and salinity concentrations, and the incubations were done at different temperatures. Incubations were performed in triplicate and a negative result was considered after 72 h incubation.

Sensitive to antibiotics: An O/N culture of the tested strain was spread onto Müller-Hinton saline agar plates (Scharlau Microbiology; rich medium supplemented with 15g/L NaCl, and with high porosity that allows the diffusion of the antibiotics). This agar diffusion method involved discs impregnated with the following antibiotics (content per disc): Ampicillin 10 µg, Trimetroprim 1.25 µg, Streptomycin 10 µg, Gentamycin 10 µg, Cloramphenicol 30 µg. The inhibitory zone of growth was observed after 48 h incubation.

Activity against the growth of bacteria: was performed using the overlay method on agar plates as described by Rao *et al*. (2005). The strains were grown in 3M+ medium with 0.2% (w/v) trehalose and 0.2% (w/v) glucose as the carbon sources for 48 h, harvested by centrifugation (12000×*g* for 20 min), and resuspended in 3M+ medium containing trehalose at a density of 0.7 g ml^{-1}. Each concentrated cell suspension was incubated without shaking at 28°C for 24 h. Cells were removed by centrifugation (14000×*g* for 1 h), and the concentrated supernatant was sterilized by using a 0.2-µm-pore-size sterile filter (Millipore).
Supernatant samples were assayed for inhibitory activity by the drop test assay. Briefly, 100 µl of an overnight broth culture of the target strain was spread on a SWYP or Nutrient agar saline plate, and the plate was dried at 30°C for 30 min. Drops containing 20 µl of the concentrated supernatant, as well as a control (eleven-salt solution), were placed on the agar surface and incubated overnight at room temperature to allow formation of inhibition zones.

Enzymatic capacities: Apart from the API ZYM (BioMèrieux), which is a standardized set of biochemical tests for the identification of enzymatic capacities, the following tests were performed. The base medium of the degradation plates was the same medium for the optimum growth of the strains.

- DNase activity: Plates of Dnase agar (Scharlab) + 1.5% NaCl. Degradative halos observed after flooding the plate with 1 N HCl (v/v).
- Gelatinase activity: medium plates + 1% gelatine. The degradative halos were observed after flooding the plate with a saturated solution of ammonium sulfate.
- Caseinase/Protease activity: 2× medium agar + an equal quantity of skimmed milk (10 g/L skimmed milk in H_2O, autoclave just 10 min).
- Amylase activity: medium plates + 1% starch. The degradative halos were observed after flooding the plate with lugol solution.
- Cellulase activity: $^1/_{10}$ vol medium agar + 0.9% Carboxymethylcellulose (CMC). The degradative halos can be observed after flooding the plate with a solution of 0.1% Congo Red for 15 minutes and then washing the excess of dye with 1 M NaCl for 15 minutes.
- PHB accumulation: M55 or 3M+ plates with Nile Red (see 'Annex'). *Pseudomonas oleovorans*, *Cupriavidus necator* and mutant $PHB4^-$ were used as + and – control respectively.
- PHB degradation: MM+PHB overlay.
- Lipase activity: medium agar + 1% Tributyrin (TBT; add before autoclaving; Scharlab) and medium agar + 1% olive oil (add a sterile aliquot after autoclaving), both media were supplemented with Rhodamine in order to see the degradative halos in UV (see 'Annex').
- Alginase activity: MM + 1% alginic acid sodium salt (growth with this carbon source).
- Haemolysis: medium agar + 5% Sheep blood (Scharlab).

Detection of oil displacement activity (ODA) of biosurfactants produced by the strain: The method is based on the ability of the biosurfactants to change the contact angle at the oil-water interface (Morikawa *et al*., 1993). A portion of 100 µL of olive oil was placed on the surface of 40 mL of distilled water in a Petri dish to form a thin membrane. Then, 50 µL of the sample solution was placed gently on the centre of the oil membrane. Then, some drops of supernatant obtained from a liquid culture were placed gently on the centre of the oil membrane. Triton X-100 was used a positive control.

Growth on MacConkey plates: Ability to grow onto MacConkey plates (Scharlau Microbiology) supplemented with 1.5% NaCl, for testing the capacity of growth in the presence of bile salts.

Cellular fatty acid composition (FAMEs): FAMEs composition was analyzed as was described in the 'Polar Lipid analysis', step A, and the samples were previously prepared as was described in 'Preparation of cultures for SLB analysis and detection of PHA by HPLC' section.

Respiratory Quinones content: Samples were previously prepared as was described in 'Preparation of cultures for SLB analysis and detection of PHA by HPLC' section. Then, the total lipid extraction and silicic acid fractionation was performed as was described in 'Total lipid extraction and silicic acid chromatography' section. The analysis of quinones was performed as was described in the 'Neutral lipid analysis' section.

16S rDNA sequencing: 16S rDNA sequencing and phylogenetic analysis was performed as was described in the 'Nucleic acid analysis methods' section.

5. Microscope techniques

- Optical and phase contrast microscopy

Optical microscopy observations were carried out with a BX-40 Olympus microscope (Olympus) fitted with achromatic lenses ranging from 20 to 40 enlargements, and a 100× plan-apochromatic fluorite lens. Photomicrographs were taken under bright field with a Nikon D-100 digital camera. Phase-contrast microscopic observations were performed in the same microscope equipped with the objectives UPLFLN 40×PH and UPLFLN 100×0PH, and a U-PCD2 phase contrast condenser (Olympus).

- Scanning (SEM) and Transmission electron microscopy (TEM)

Transmission (TEM) and scanning (SEM) electron microscopy were performed at the technical services (SCT) of the 'Universitat de Barcelona' using microbial cultures grown O/N in their optimal medium and conditions (liquid medium, except from sulfate-reducing cultures that were recovered from agar plates in 600 µl of anoxic Ringer $^1/_1$ in anaerobic conditions).

➢ Fixation and post-fixation of samples

The samples were fixed by mixing 600 µl culture with 400 µl of a fixation solution (100 µl 25% (v/v) glutaraldehyde (Sigma) and 300 µl 0.15 M phosphate buffer, pH 7). The samples were fixed by incubation for at least 1 h at 4ºC. Then, the samples were centrifuged at 10000×*g* for 5 min at 4ºC and the supernatants removed (the pellet was always covered by certain volume of liquid). The washes with fixation solution were repeated twice. After that, the samples were covered with fixation solution and maintained at 4ºC (alternatively, the samples can be covered with distilled water or phosphate buffer to avoid the compacting of the cells, especially when a negative-staining has to be performed).

Alternatively, samples filtered onto polycarbonate filters had been fixed for SEM and TEM microscopy. The fixation process was performed in a glass microanalysis filter holder for 25 mm disc filters (Millipore), so that the filter was always in contact with the fixation and washing solutions. The subsequent preparation of the samples was done in the same way of a liquid culture sample.

Fixed samples were 3-times washed for 10 min in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% osmium tetraoxide (OsO_4) for 1 hour at 4ºC, 3-times washed for 10 min in bidistilled water, and harvested by centrifugation at 10000×*g* for 5 min at 4ºC. The supernatants were then removed, and the samples were prepared for being dehydrated.

Double fixation by steam for SEM microscopy

This protocol was performed with growing bacterial colonies onto agar plates. This process consisted on the fixation of the sample under a steam generated by acroleine (Fluka) and osmium tetraoxide (Sigma) inside a clased chamber for 4–10 days. The humidity of the chamber was removed by placing silicagel inside the compartment. Once the sample was fixed, it was mounted on stubs and sputter-coated with gold-palladium for being visualized on a scanning electron microscope.

- Dehydration and mounting of the samples

TEM samples were dehydrated in the following acetone series (percentages in v/v): 1 × 50% (10 min), 2 × 70% (10 min), 3 × 90% (10 min), 3 × 96% (10 min) and 3 × 100% (15 min). The samples were then infiltrated in an increased series of Spurr resin in acetone, transferred to 100% resin, and polymerized at 55ºC for 24 h. Thin sections were cut on a Ultracut E ultramicrotome (Reichert-Jung) and collected on copper grids. The resulting sections were stained first with 2% (w/v) uranyl acetate for 10–12 min, and then with 1% lead citrate for 3–5 min, and were visualized on a JEM 1010 transmission electron microscope (JEOL).

Post-fixed SEM samples were dehydrated in the following ethanol series (percentages in v/v): 1 × 50% (10 min), 2 × 70% (10 min), 3 × 90% (10 min), 3 × 96% (10 min) and 3 × 100% (15 min). The ethanol was then replaced by liquid carbon dioxide under pressure using a CPD 7501 critical point drier (POLARON). Liquid carbon dioxide was then evaporated raising the temperature. The dried samples were mounted on stubs and sputter-coated with a heavy metal (gold-palladium) to convey electrical conductivity. Finally, the samples were visualized in a S-2300 scanning electron microscope (Hitachi).

- Negative staining for transmission electron microscopy

Specimens that are to be examined in the transmission electron microscope (TEM) usually have to be thin, dry and contain contrast (usually from a heavy metal stain). This preparation method is useful for visualizing suspensions of small particles,

which includes viruses and for the observation of bacterial pure cultures in order to establish the number and position of flagella and other morphotaxonomic characters.

The sample was suspended (or diluted) into water and adsorbed onto a copper-coated or gold-copper formvar film (400 mesh) which is attached to a metal specimen grid. Once the specimen had been adsorbed onto the film surface, the excess sample was blotted off and the grid was covered with a small drop (5 µl) of stain solution (2% phosphotungstic acid in water, pH 6.5 with KOH). This was left on the grid for a few minutes and then blotted off. The sample was dried and examined in the TEM.

- Micromanipulation technique

Skerman micromanipulation system (Skerman, 1968), makes possible manipulation of cells on the surface of the agar media upon which they have to grow. Most ×10 or ×32 long working distance phase contrast objectives can operate through a relatively thick layer of agar in a Petri dish. With such lenses a simple manipulator can be mounted on the objective (Fig. II.11 A) so that it becomes the fixed component in the manipulative process and the object is moved by the mechanical stage. In addition, to produce a manipulator of this type, a microforge (Fig. II.11 B, Fig. II.12) has also been produced for the purpose of fabricating micro-tools.

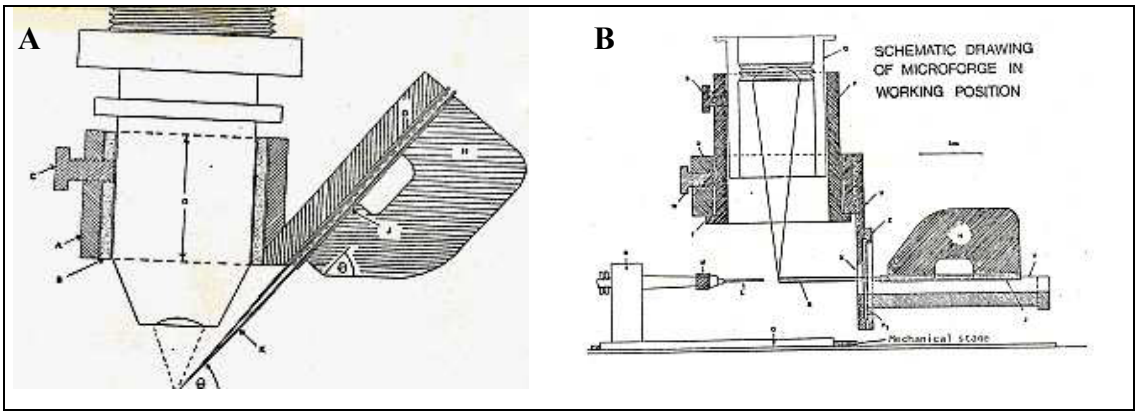

Figure II.11. Skerman micromanipulator: structure.

(A) Lens collar around the confocal objective and the magnetic tool carrier, and (B) schematic drawing of microforge in working position.

Figure II.12. Skerman micromanipulator.

Detail of the magnetic tool-carrier, and an example of micromanipulation onto an agar plate.

- Fluorescence *in situ* hybridization (FISH)

As a technique allowing simultaneous visualization, identification, enumeration and localization of individual microbial cells, fluorescence *in situ* hybridization (FISH) is useful for many applications in all fields of microbiology. FISH not only allows the detection of culturable microorganisms, but also of yet-to-be cultured (so-called unculturable) organisms, and can therefore help in understanding complex microbial communities.

In microbiology the most commonly used target molecule for FISH is 16S rRNA because of its genetic stability, its domain structure with conserved and variable regions, and its high copy number (Woese, 1987). Oligonucleotide probes for each taxonomic level can be designed according to the region of rRNA targeted (Göbel, 1991; Amman *et al*., 1995; Moter and Göbel, 2000).

➢ Design of probes

The objective of probe design is to select an oligonucleotide sequence completely specific (complementary) to a region of the target sequences which has at least one mismatch to the same region in all other (non-target) sequences. A common

rule is to centralize the mismatch or mismatches in the non-target sequences to maximize the destabilizing effect of the mismatch. The steps that were followed for the design of probes are detailed in Table II.13. Designed and tested probes can be also found in 'ProbeBase.net' and online resource for rRNA oligonucleotide probes (see 'Useful websites' section). All probes used in this study were obtained from Sigma-Genosys, and the stock solutions were prepared at 50 ng μl^{-1}.

- Fixation

Prior to hybridization microbial samples were fixed and permeabilized for penetration of the fluorescent probes into the cell. The fixation of pure cell cultures was done as it follows: Cells were harvested during logarithmic growth and an aliquot was fixed with formalin (formaldehyde) (2% v/v, final concentration) for 1–24 h (at –20ºC). Then, cells were centrifuged at 13000×*g* for 5 min, and the supernatant was discarded. Later, 1 ml of PBS (see 'Annex') was added and the pellet was re-suspended (this wash was repeated twice). Finally, cells were centrifuged at 13000×*g* for 5 min, the supernatant was discarded and the pellet was suspended in 1 ml of PBS:ethanol. In this stage, samples can be stored at –20ºC for several months. The fixation method for general samples is detailed in Table II.14.

- Hybridization

Hybridization must be carried out under stringent conditions for proper annealing of the probe to the target sequence. For this step, preheated hybridization buffer is applied to the sample containing the labeled probe. Stringency can be adjusted by varying either the formamide concentration or the hybridization temperature. Formamide decreases the melting temperature by weakening the hydrogen bonds, thus enabling lower temperatures to be used with higher stringency.

Table II.13. Probe design for FISH.

Design of probes for FISH
- 1. Check the accessibility of the 16S rDNA (Fuchs *et al*., 1998). Avoid those target sites this less than 10% of relative accessibility, if possible. **- 2.** The base mismatches between probe and non-target rRNA should not be situated at the 3'- or 5'- ends of the oligonucleotide, but rather at a more central position. **- 3.** Check the non Watson-Crick pairing with the base at the respective target position (a G-T mismatch is weaker is weaker than a G-A mismatch). **- 4.** Check the G+C% content: 50-60% for a 18-24 bp probe. **- 5.** The probe sequence is the reverse complement of the target string, as FISH probes target transcribed rRNA. **- 6.** Confirm probe specificity against all available DNA sequences using the BLAST option of the NCBI database. An exact match will have a score (bits) output twice the value of the number of nucleotides of the submitted probe sequence. Sequences with matches to the probe sequence will have scores less than twice the number of nucleotides of the probe sequence. **- 7.** Also, confirm the probe sequence is reverse complement by checking that "Strand= Plus/Minus" for a number of subject sequences in the BLAST search results. A target string will result in "Strand=Plus/plus" (unless the subject sequence has been submitted to the database as the reverse complement). **- 8.** Confirm that the probe sequence has a melting temperature of ≥57 ºC using the nearest neighbor method (Tm, calculated using 50 mM NaCl and 50 μM oligo). This can be performed on-line using websites such as the Biopolymer calculator or Primer calculator (see 'Useful websites' section). If the Tm is < 57 ºC, it can be raised by increasing the probe length. **- 9.** Name the probe: use a three-letter abbreviation of the target group followed by the nucleotide position at the 3' end of the probe hybridizes to, usually according to standard *E coli* numbering.

Table II.14. Fixation of samples prior to FISH.

Fixation
Formaldehyde fixation for Gram-negatives (9:1:10): 45 μL sample, 5 μL formaldehyde 37%, 50 μL 96% cold ethanol. Keep at –20ºC. **Ethanol fixation for Gram positives** (1:1.2): 250 μL sample, 300 μL 96% cold ethanol. Keep at –20ºC

FISH onto polycarbonate filters

The sample was filtered and stored at –20ºC in a Petri dish until further use. Then, the filter was cut in sections with a razor blade and each section was numbered with a pencil outside the filtered zone. Meanwhile, the 'Hybridization Buffer' was prepared in a 2 ml-eppendorf tube as it follows (Table II.15):

Table II.15. Hybridization buffer.

% Formamide	µL Formamide	NaCl 5M+ Tris HCl 1M+ SDS 10%	H_2O bd (µL)
0	0	360 µL+40 µL+2µL	1598
5	100	360 µL+40 µL+2µL	1498
10	200	360 µL+40 µL+2µL	1398
15	300	360 µL+40 µL+2µL	1298
20	400	360 µL+40 µL+2µL	1198
25	500	360 µL+40 µL+2µL	1098
30	600	360 µL+40 µL+2µL	998
35	700	360 µL+40 µL+2µL	898
40	800	360 µL+40 µL+2µL	798
45	900	360 µL+40 µL+2µL	698
50	1000	360 µL+40 µL+2µL	598
55	1100	360 µL+40 µL+2µL	0

The Hybridization buffer was heated 10 min at 48ºC. In the meantime, the 'Probe solution' was prepared in a 0.5 ml-tube by mixing the following components:

Table II.16. Probe solution.

Nº of probes	Components of the 'Probe solution'
1 probe	2 µL probe + 18 µL Hybridization buffer/section
2 probes	2 µL probe + 2 µL probe + 16 µL Hybridization buffer/section
3 probes	2 µL each probe + 14 µL Hybridization buffer/section
4 probes	2 µL each probe + 12 µL Hybridization buffer/section

A microscope slide was covered with Parafilm®, and then 10 µl of the 'Probe solution' was put onto the slide. The filter section was left onto the drop and then 10 µl of 'Probe solution' was left again onto the filter section. A dark humid chamber was made with a 50 ml-falcon tube with a folded piece of tissue inside, then the glass slide

with the filter section was stored inside the tube and the rest of the 'Hybridization buffer' was poured onto the tissue. The hybridization chamber was immediately transferred to the hybridization oven at the hybridization temperature for 1.5–3 hours (maximum). After that, the 'Washing buffer' was prepared in a 2 mL-eppendorf tube/section, and preheated at 48ºC. The filter was washed in the 'Washing buffer' for 10 min at 48ºC. Then, the excess of washing buffer was removed with distilled water in a Petri dish for 1–2 min, in the dark. Finally, the filter section was dried filter paper and was mounted in Vectashield (Vector Laboratories). The coverslip can be sealed witn nail polish.

Table II.17. Washing solution.

% Formamide	NaCl 5M+ Tris·HCl 1M+ SDS 10%	H_2O bd st (µL)
0	360 µL+40 µL+2 µL	1598
5	252 µL+40 µL+2 µL	1706
10	180 µL+40 µL+2 µL	1778
15	127.2 µL+40 µL+2 µL	1830.8
20	86 µL+40 µL+2 µL	1872
25	59.6 µL+40 µL+2 µL	1898.4
30	40.8 µL+40 µL+2 µL	1917.2
35	28 µL+40 µL+2 µL	1930
40	18.4 µL+40 µL+2 µL	1939.6
45	12 µL+40 µL+2 µL	1946
50	7.2 µL+40 µL+2 µL	1950.8
55	2.1 µL+40 µL+2 µL	1954.5

Counterstaining with DAPI

After the filter section has been mounted in Vectashield (Vector Laboratories), the sample can be counterstaining with DAPI (4´,6-diamino-2-phenylindole dihydrochloride, Sigma). One drop of 1.5 µg ml^{-1} DAPI in Vectashield can be placed onto the filter section and then, the cover slip can be placed again onto the filter section. The observation of the sample was direct, and the fluorescence was detected with an epifluorescence microscope (BX-40, Olympus) equipped with the filter sets UN41007 Cy3, U-MNB, U-MWU for the visualization of Cy3™-labeled probes, FITC-labeled probes and DAPI staining by ultraviolet emission (Olympus). The probes used in this work are detailed in Table II.18.

Table II.18. FISH probes.

FISH Probes
EUB388 - Specificity: Most bacteria (chapter VII and VIII). - Target molecule: 16S rRNA - Position: 338–355; Tm: 55ºC; Formamide: 0–50% - Sequence: 5'-GCT GCC TCC CGT AGG AGT-3' - Labeling: 5' labeling with Fluoresceine-Isothiocyanate (FITC) - Reference: Amann *et al*., 1990.
PSA184 - Specificity: *Pseudoalteromonas*, *Colwelia* (chapter VII). - Target molecule: 16S rRNA - Position: 184–201; Tm: 53ºC; Formamide: 30% - Sequence: 5'-CCC CTT TGG TCC GTA GAC-3' - Labeling: 5' labeling with Fluoresceine-Isothiocyanate (FITC) - Reference: Eilers et al., 2000.
ARC824 - Specificity: Sequence obtained from the spirillum bloom (chapter VIII). - Target molecule: 16S rRNA - Position: 824–844; Tm: 53ºC; Formamide: 0–50% (30%) - Sequence: 5'-GCA ATG TCT AGC ATC ACA AC -3' - Labeling: 5' labeling with Cy3™ - Reference: This study.
UnSpiro465 - Specificity: *Spirochaeta* sp. M6 and P, sequence from spichaetal bloom (chapter VIII). - Target molecule: 16S rRNA - Position: 465–488; Tm: 55.9ºC; Formamide: 0–50% - Sequence: 5'-CTC ATT TCC TAG TAG TTT TAT TCC-3' - Labeling: 5' labeling with Fluoresceine-Isothiocyanate (FITC) - Reference: This study.

	Excitation wavelenght	Emission wavelenght	Color
[1]Cy3™	550 nm	570 nm	Orange/red
FITC	492 nm	528 nm	Green

[1]Cy3: 5,5'-disulfo-1,1-(γ-carbopentynyl)-3,3,3',3'-tetramethylindolocarbocyanin-*N*-hydroxy-succinimide ester.

6. Annex

- Preparation of antibiotic solutions

Ampicillin: prepared at a concentration of 100 mg ml^{-1} in bidistilled water plus the amount of NaOH necessary to achieve its complete dissolution. The final concentration used for pGEM-T plasmid-containing *E. coli* strains was 50 μg ml^{-1}.

Cycloheximide: prepared at a concentration of 50 mg ml^{-1} in 75% methanol.

Streptomycin sulfate: prepared at a concentration of 200 mg ml^{-1} in bidistilled water. Add 1 ml of the stock solution to 1 L of cooling agar to achieve a final concentration of 200 μg ml^{-1}.

Antibiotic solutions were sterilized by filtration and stored at −20 ºC.

- Preparation of specific substances

IPTG (isopropyl-β-D-thiogalactopyranoside): prepared at a concentration of 20 mg ml^{-1} in bidistilled water, sterilized by filtration, and stored at −20 ºC. The final concentration used was 100 μg ml^{-1} for transformation assays.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside): It was prepared at a concentration of 200 mg ml^{-1} in dimethyl formamide, and stored at −20 ºC. The final concentration used in transformation assays was 40 μg ml^{-1}.

Rhodamine B: Rhodamine B was added to culture media supplemented with lipid substrates to detect lipolytic activity by UV illumination (Kouker and Jaeger, 1987). It was prepared at a concentration of 1 mg ml^{-1} (0.1% by w/v) in bidistilled water, sterilized by filtration, and stored at −20 ºC. The final concentration was 2 μg ml^{-1} (0.0002% by w/v).

Nile red: Viable-colony staining method that is based on the direct inclusion of the lipophilic dye Nile red in the agar medium such that the growth of the cells is not affected and the occurrence of PHAs in the colonies can be directly monitored

(Spiekermann *et al*., 1999). Nile red produces a strong orange fluorescence (emission maximum, 598 nm) with an excitation wavelength of 543 nm (maximum) upon binding to poly(3HB) granules in cells. Nile red was prepared at a concentration of 0.1% (w/v) in DMSO (dimethyl sulfoxide) and stored at RT. The final concentration was 0.5 mg Nile red L^{-1} medium (0.5 ml stock solution in 1 L of medium).

DCMU [3-(3',4'-dichlorphenyl)-1,1-dimethylurea]: Since years has been used to inhibit photosystem II with no effect onto photosystem I (Bishop, 1958). DCMU was prepared at a concentration of 1 mM in 1% (v/v) ethanol and then it was sterilized by filtration. The final concentration of DCMU in MN agar plates was 2 μM (2 ml stock solution in 1 L of MN agar). The inhibitory effect of DCMU onto the filamentous cyanobacteria used in the analysis was previously tested by culturing the strains onto MN+DCMU agar plates and testing the absence of growth during 2 weeks under light/dark conditions in a controlled culture chamber (Ibercex ASL, Spain).

- Solutions

Phosphate Buffered Saline (PBS) (Sambrook and Russel, 2001)

- **Properties**: Saline solution for eukaryotic and prokaryotic cells.
- **Composition**: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; pH 7.4.

Ringer ¼ and $^1/_1$ (Scharlau)

- **Properties**: isotonic solution for maintaining the viability of microorganisms without allowing their replication.
- **Composition**: 2.25 g NaCl, 0.105 g KCl, 0.12 g $CaCl_2$, 0.05 g $NaHCO_3$, per L; pH 7.2. [10 g L^{-1} for Ringer $^1/_1$; 2.5 g L^{-1} for Ringer ¼].

Artificial seawater

- **Properties**: Salt solution with a defined composition.
- **Composition**: 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2.6H_2O$, 5.94 g $MgSO_4.7H_2O$ and 1.3 g $CaCl_2.2H_2O$ (Levring, 1946).

TE buffer (10 mM Tris HCl pH 8.0; 1 mM EDTA)

- **Properties**: Solution for a long-maintenance of DNA at –20ºC.
- **Composition**: 1 ml Tris HCl 1 M pH 8.0, 0.2 ml EDTA 0.5 M pH 8.0, H_2Od for a total volume of 100 ml.

Phosphate buffer for lipid extraction purposes

- **Properties**: Lipid-free phosphate buffer
- **Composition**: 8.7 g KH_2PO_4 + 995 ml H_2Od; pH 7.4. Filtrate through a 0.2 µm-Whatman filter (methanol-washed) and recover the filtrate in a muffled vacuum flask. Then, transfer to a 1 L-muffled bottle with 50 ml chloroform. Shake gently and let extract O/N. Place the re-pipetter into the buffer bottle.

Nanopure water

- **Properties**: Lipid-free bidistilled water for lipid extractions.
- **Composition**: Use water HPLC grade or bidistilled water filtrated through a 0.2 µm-filter. Transfer to a 1 L-muffled bottle with 50 ml chloroform. Shake and let extract O/N.

Note: The anoxic solutions were obtained by sterilization at 121ºC (autoclaved), and then closing the solution flasks at high temperature (when the liquid is still bubbling), or alternatively by infusion of nitrogen in the liquid solution.

➢ Staining techniques

Gram-staining and spore-staining were performed as is indicated in Bergey *et al*., 1994.

Nile-Blue staining (Ostle and Holt, 1982)

- **Properties**: Basic oxazine dye that stains PHA granules. Observed at an excitation wavelength of 460 nm.
- **Procedure**: Fixation of the cells onto the slides by heating. Immersion of the slides in 1% (w/v) Nile Blue A in water at 55ºC for 10 min. Wash the excess of Nile Blue A by immersion of the slide in a 8% (v/v) acetic acid solution for 1 min. Wash the slide with tap water and dry. The PHA granules were observed as

orange-fluorescent granules under an epifluorescence microscope (BX-40, Olympus) equipped with the filter set U-MNB (excitation wavelength 400–500 nm; optimum 436 nm).

DAPI staining (Poter and Feig, 1980)

- **Properties**: 4'-6-diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA. Because of this property DAPI is a useful tool in various cytochemical investigations.
- **Procedure**: The microbial sample (preferentially fixed) is added to a filtration ramp with 10 ml of Ringer ¼ 10 µl of DAPI at a concentration of 10 µg ml^{-1} (final concentration 0.01 µg ml^{-1}) is added and the staining is let to proceed for 10 min in the dark. Then, the liquid content of the ramp is filtrated through a dark-stained polycarbonate filter (Millipore) and mounted onto a glass slide and covered with a coverslip.