# VIII. BACTERIAL SUCCESSION IN MICROBIAL MAT SULFUR BLOOMS

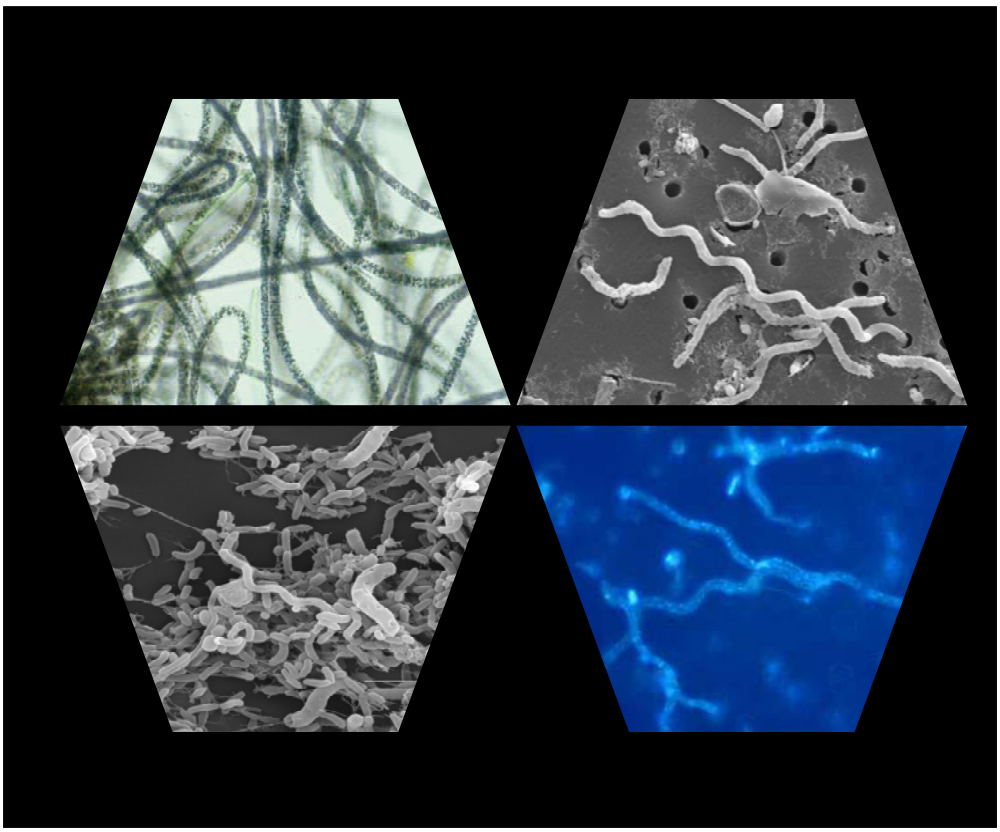

Figure VIII. “It doesn’t matter how beautiful your theory is, it doesn’t matter how smart you are. If it doesn't agree with experiment, it’s wrong” Richard Feynman (1918–1988).

From top left to right: *Beggiatoa* bloom; / Scanning electron micrograph of an spirochaetal bloom / Scanning electron micrograph of an *Spirillum*-like bloom / DAPI staining of an spirochaetal bloom (micrographs by the author).

- Introduction and objectives of the study

Microbial mats are dynamic systems with important changes in oxygen and sulfide gradients during a day/night cycle (Fründ and Cohen, 1992). Indeed, the presence of steep gradients in the concentrations of oxygen and sulfide are characteristic of their vertical profile due to high microbial activity of their former populations. These fluctuations induce harmful conditions for the bacterial populations that may develop strategies to avoid extreme conditions, such as migrations to optimal conditions (García-Pichel *et al*., 1994). For this reason, motility is the major tactic used by bacterial cells to enhance their survival (Barbara and Mitchell, 1996). In this kind of environments, dissolved sulfide is generated by sulfate-reducing bacteria in the black deepest layers of the mats, from where it diffuses upward toward the sediment surface (Jørgensen, 1994b). The dynamic transition zone between oxygen and sulfide is potentially unstable and moves as the environmental conditions change; therefore, many sulfur-oxidizing bacteria, that oxidize reduced sulfur compounds to obtain energy, are motile in order to establish their position in the oxic–anoxic interface (Jørgensen, 1982; Jørgensen and Revsbech, 1983).

The oxic–anoxic interface can move above the sediment surface and positioned in the overlying water column if the sulfide production is high, but in this case only microaerophilic motile bacteria can keep their position in the narrow boundary layer (Jørgensen, 2001). In fact, several studies have been performed in order to characterize the morphology and motility behaviour of the free-swimming microaerophilic bacteria aggregated chemotactically to the oxic–anoxic border (Bernard and Fenchel, 1995; Thar and Kühl, 2002; Thar and Fenchel, 2005). Typical examples of such microorganisms are *Beggiatoa* sp., restricted to solid surfaces by their gliding motility (Nelson, 1992), *Thiovulum* sp., which forms veils at the oxic–anoxic interface by aggregation (La Rivière and Schmidt, 1992), and also the purple sulfur bacterium *Marinochromatium gracile* that form thin layers in the interface under dark conditions (Thar and Kühl, 2001). More recently, free-swimming spirilla and cocci have been also detected in the microbial succession in veils formed in the oxic–anoxic interface (Thar and Kühl,

2002), which have also been reported in mats of colorless sulfur bacteria (Bernard and Fenchel, 1995).

Other sulfur-oxidizing bacteria have been found in interfaces between aerobic water and anaerobic sediment, for example *Thiobacillus*, *Thiomonas*, *Thiomicrospira*, and more recently members of the *Arcobacter* genus (Kuenen and Tuovinen, 1981; Moreira and Amils, 1997; Wirsen *et al*., 2002). The presence of *Thiomicrospira* has been demonstrated in different marine habitats, like intertidal mud flats (Brinkhoff *et al.*, 1999a), deep-sea and shallow-water hydrothermal vent systems, hypersaline microbial mats (Brinkhoff and Muyzer, 1997), and artic sediments (Knittel *et al*., 2005) etc. The main requirement for this presence seems to be existence of reduced sulfur compounds. Apart from the oxic–anoxic interface zones inhabited by sulfur-oxidizing microaerophilic bacteria, other sulfur-rich environments in which syntrophic relationships take place have been described, e.g. '*Thiodendron*' bacterial sulfur mats. These kinds of mats are consortial associations formed by sulfidogenic members and spirochetes that may oxidize reduced sulfur compounds as a protective mechanism (Dubinina *et al*., 2004).

Although previous studies have described microaerophilic sulfur-oxidizing species in oxygen-sulfide gradients, their diversity and phylogenetic affiliation are still unknown (La Rivière *et al*., 1992). All these bacteria share a common ecological niche with changing conditions that promotes morphological and physiological adaptations as well as bacterial tactic responses. Indeed, apart from the gliding motility of *Beggiatoa* and the peritrichous flagella of *Thiovulum*, free-swimming vibrioid-to-spirilloid bacteria have been adapted to the changing conditions of the microaerophilic transition zone, and have been selected for their capacity of form mycelial structures (veils) in order to resist turbulence in this kind of environments (Thar and Kühl, 2002).

The aim of this study was (i) the study of the bacterial succession in sulfur-rich environments of the overlying water of microbial mats, (ii) the morphological and molecular characterization of the microbial members of spirilloid and spirochaetal blooms, and (iii) the design and application of fluorescence *in situ* hybridization (FISH) probes for the detection of bacterial cells previously detected in the blooms.

- Material and methods

➢ Spirochaetal and *Spirillum*-like blooms

Ebro delta mat samples were maintained as a microscosm under conditions similar to those observed in the environment (desiccation and hydration, light/dark conditions, etc). Sulfur-rich blooms, developed in the overlying water of microbial mat samples, were recovered and fixed for electron microscopy, fluorescence *in situ* hybridization (FISH) and DNA purposes as described in the chapter 'General Material and Methods'.

➢ Microscopic observations

The evolution and bacterial succession in sulfur-blooms was followed by phase-contrast, dark-field microcopy or by staining with methylene blue. Bacterial suspensions were stained with DAPI after being filtered through 0.2 and 3 μm-filters.

An aliquot of the spirochaetal-bloom fixed with 2.5% glutaraldehyde was filtered through a 3 μm-filter. The filter was treated for scanning (SEM) and transmission (TEM) electron microscopy as it was explained in the section 'Microscope techniques' ('II. General Material and Methods'). Apart from that, certain volume of the *Spirillum*-like bloom was fixed with 2.5% glutaraldehyde for SEM. In addition, both kind of samples were fixed with formaldehyde and ethanol (see 'Table II.14, 'General Material and Methods'), filtered through 0.2 μm-filters and then hybridized with fluorescent-labeled probes (FISH). The hybridization conditions for the probes ARC824 (labeled with Cy3™) and UnSpiro465 (labeled with fluoresceine-Isothiocyanate FITC) were optimized at different concentrations of formamide.

➢ Isolation and cultivation of *Thiomicrospira/Thiobacillus*

For enrichment cultures, 10 ml of '*Thiomicrospira pelophila* medium' (Table II.11, 'General Material and Methods') was inoculated with 0.1 g of mat sample and incubated at 20ºC in the dark to avoid growth of phototrophic bacteria. After growth was obtained, as indicated by a change in the color of the pH indicator, 0.1 ml was

transferred to '*Thiomicrospira pelophila* medium' agar plates and the isolation of a pure culture was obtained. The isolated strain was compared with *Thiomicrospira pelophila* DSMZ 1534 pure cultures. The isolated colonies onto the agar plates were dehydrated by steam and then photographed by SEM (see protocol in the section 'Microscope techniques', chapter 'General Material and Methods').

- *Thiomicrospira* PCR detection

In order to detect the presence of *Thiomicrospira* members in bloom samples, a genus-specific PCR to amplify a 721 bp-fragment was applied (Brinkhoff and Muyzer, 1997). Primers Ly02F and Ly03R were used in a PCR reaction as explained in the 'Table II.6' of the chapter 'General Material and Methods'.

- 16S rDNA amplification and cloning of spirochaetal and spirillum-like blooms

One ml of spirochaetal bloom sample was filtered through a 3 μm-filter. The filtered was transferred to an eppendorf-tube with 1 ml of bidistilled water. The sample was mixed by vortex and then sonicated. Likewise, the *Spirillum*-like sample was boiled and centrifuged. The supernatants were used as a DNA template in a PCR with the universal 16S rDNA primers Ty04F and Ty06R as is described in 'Table II.6'. The PCR product was purified Wizard® PCR prep. Kit (Promega) and then cloned in pGEM-T. The transformation and selection of recombinant clones was performed as described in the section 'Enzymatic treatment of DNA and transformation'. The positive clones were screened by PCR with pGEM-T primers T7pGEM-T and SP6pGEM-T and the different inserted sequences were detected by restriction enzymatic analysis. The representative clones were sequenced and formation of chimeras was excluded by application of the 'Check_Chimera' program.

The obtained sequences were aligned with Genbank-downloaded sequences by using the Bioedit Sequence Alignment Editor. Evolutionary distance trees (neighbor-joining algorithm and Jukes and Cantor correction) were constructing by using the Mega 3.0 software.

- Results

➢ Occurrence and bacterial succession in sulfur-blooms

After 1–3 days, the surface of the Ebro delta microbial mat microscom developed the typical spaced tufts of *Beggiatoa* sp., especially under situations of high temperatures and irradiance. The observation of those samples under dark-field microscopy revealed the presence of large filaments of *Beggiatoa* members and vibrioid to spirilloid microorganisms. In addition, the *Beggiatoa* filaments had the characteristics light-scattering sulfur inclusions in their cytoplasm, and also large accumulation of sulfur granules were observed outside the cells (Fig. VIII.1). After 4–5 days, the presence of *Beggiatoa* filaments dramatically decreased and a white veil was formed in the underlying water of the mat system. The sulfur-rich veils became thicker if the system was left undisturbed, and dense populations of vibrioid-to-spirilloid bacteria with sulfur-like cytoplasmatic inclusions were observed. The population of those kind bacteria was formed by different morphotypes and the detected population was replaced by a larger size spirilloid-cell pool.

Finally, the population of large-spirilloid bacteria was progressively reduced and large spirochetes were detected. The development of spirochaetal-blooms at this point was not always achieved and in most of the cases the bacterial succession stopped at the stage of spirilloid-bacteria. The spirochaetal blooms were observed under dark and light-field microscopy and several morphotypes were detected. The spirochaetal cells did not show light-scattering inclusions in their cytoplasm and, in most of the cases, *Spirillum*-like cells were also occupying the same microhabitat. In a period of 4–5 days, the large spirochetes reduced their motility and were degraded.

The development of these kind of sulfur-rich bacterial blooms in microbial mats does not tend to happen more than once in a microcosm maintained in the laboratory. This fact is mainly because the deepest layers of a mat sample (black sediment with sulfate-reducing bacteria) are discarded from the microscosm unit.

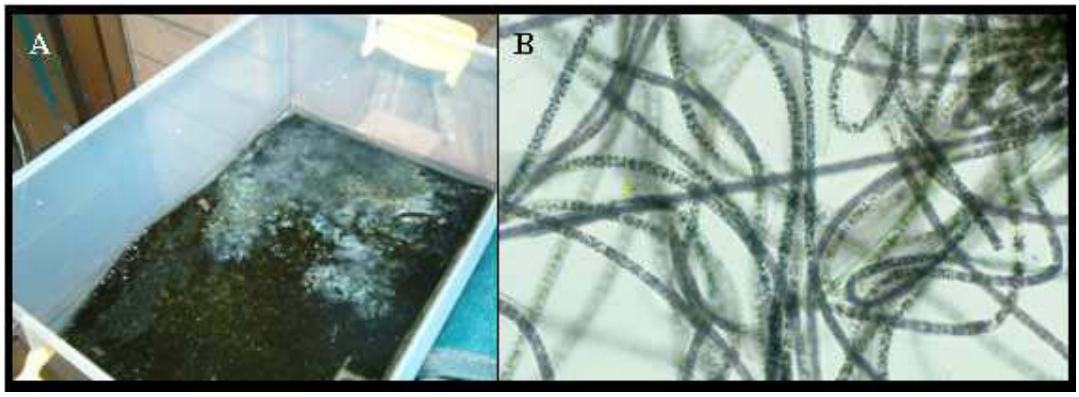


Figure VIII.1. (A) White veils in the overlying water, and (B) *Beggiatoa* filaments.

- *Spirillum*-like bloom

A *Spirillum*-like bloom observed during the bacterial succession in the white veils was recovered and observed under light-field microscopy (Fig. VIII.2). Two different morphotypes were detected, small vibrioid cells 1.5–2.5 µm length and 0.5–0.6 µm wide that became helical as cells lengthen, and helical cells 5–8 µm length and 0.9–1 µm wide. The helical cells showed light-scattering sulfur inclusions and their motility behaviour could be described as a "helical track, run and boomerang" pattern: cells performed helical movements in their fast displacement and sometimes their movement resembled a boomerang. The swimming paths of the cells were directed to sulfur-accumulation zones of cell debris. Occasionally, the swimming direction was disturbed by encounters with other cells, which resulted in deviations but then they recovered the motility behaviour.

The scanning electron micrographs revealed a high amount of the small vibrioid morphology in comparison with the helical cells and a mycelial structure surrounded the analyzed sample (Fig. VIII.3). The helical cells showed deformations and small pits on the surface of the cell wall. Moreover, the cell terminus of the helical morphotype showed one vaulted end with and indentation and residual flagella (Fig. VIII.4). Besides, an ovoid morphotype with 4 µm cell diameter was also identified in the SEM micrographs and some rods were attached to their surface. Some spirochetes around 30–40 µm length and 5–6 coilings were also detected (Fig. VIII.5).

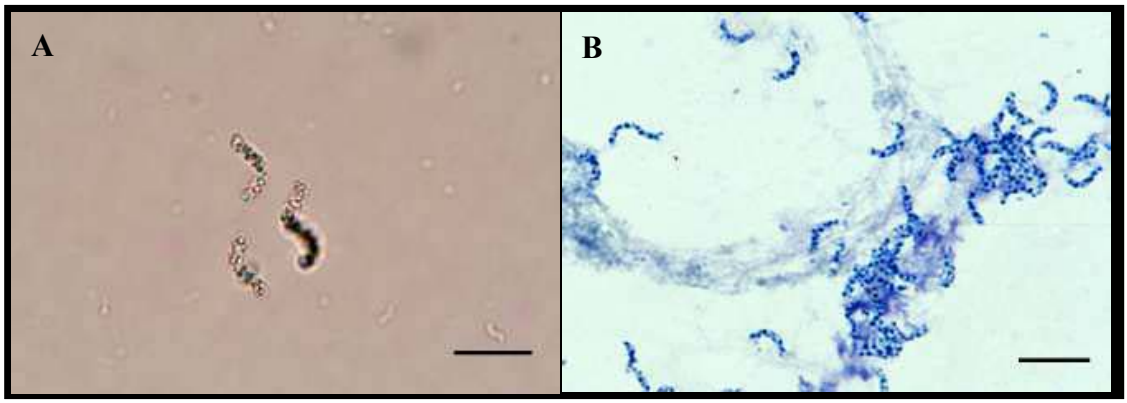


Figure VIII.2. (A) Helical cells showing light-scattering sulfur inclusions, and (B) *Spirillum*-like bloom stained with methylene blue (Bar = 10 µm).

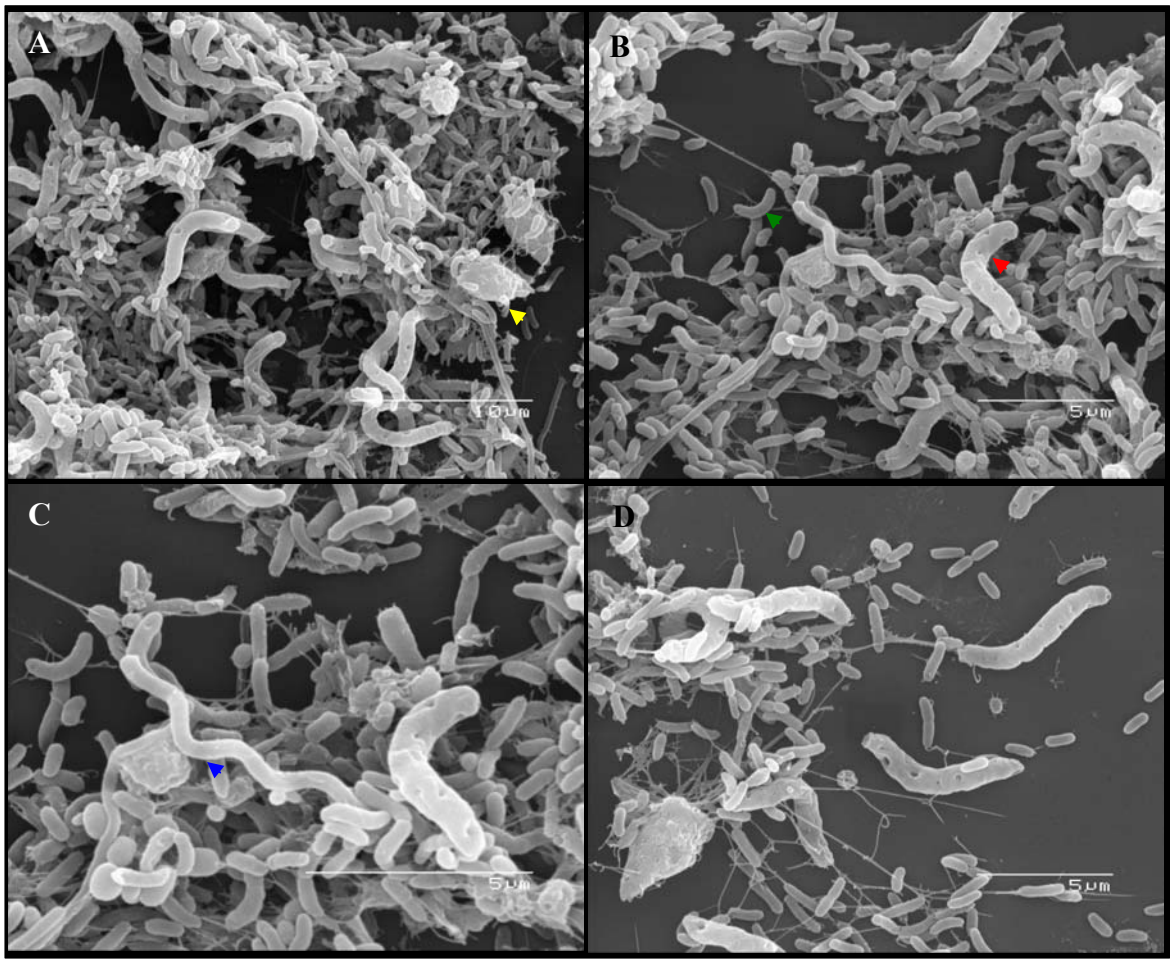


Figure VIII.3. Microbial diversity in the *Spirillum*-like bloom.

From A to D: Abundance of the small vibrioid morphotype (green arrow), helical cells with the distended cell walls (red), ovoid morphotype (yellow), spirochetes (blue), and the mycelial structure.

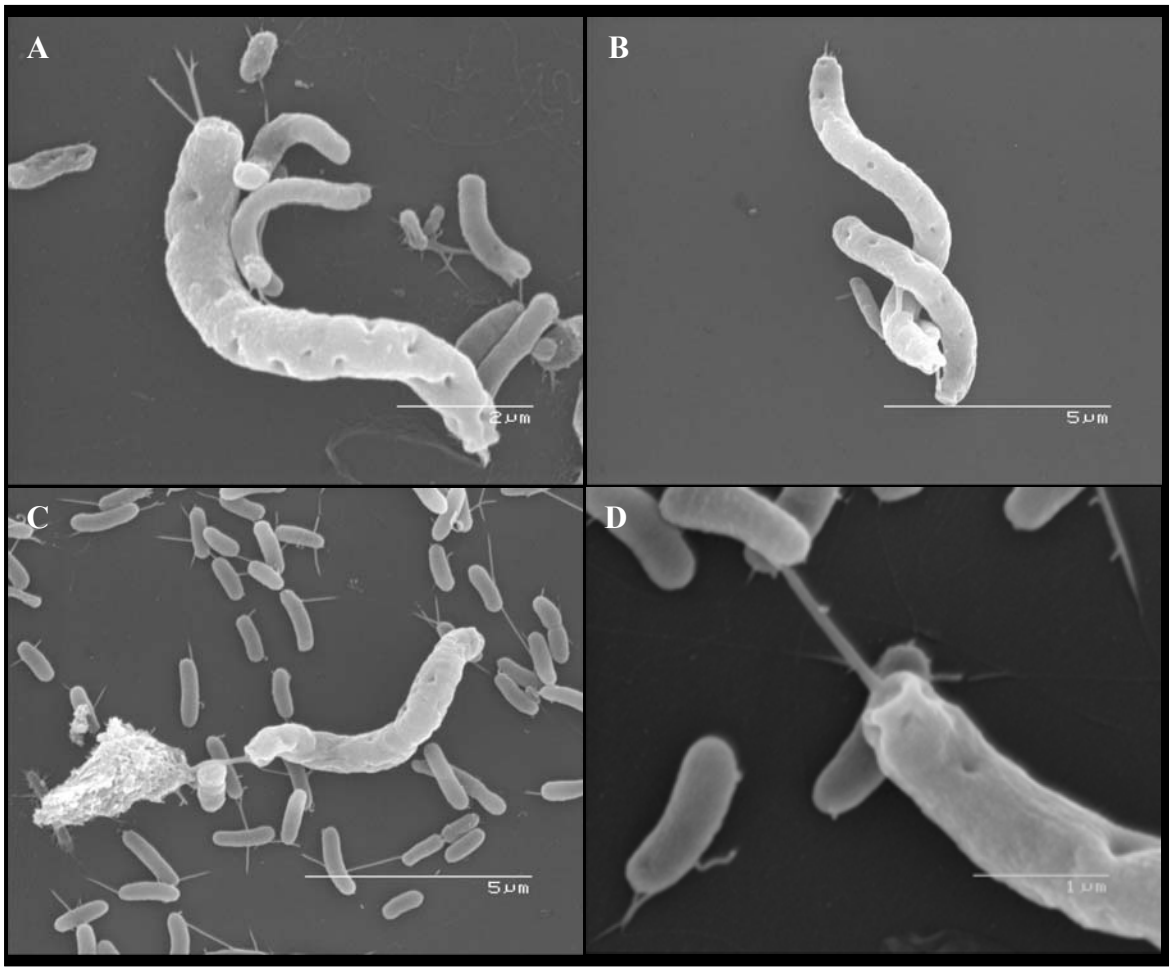


Figure VIII.4. Details of the morphology of the *Spirillum*-like bloom members.

(A) Helical morphotype with the accompanied smaller vibrioid bacteria; (B) Helical morphotype showing the distended cell wall; (C) Helical morphotype and rod-to-vibrioid bacteria; (D) Polar structure of the helical morphotype.

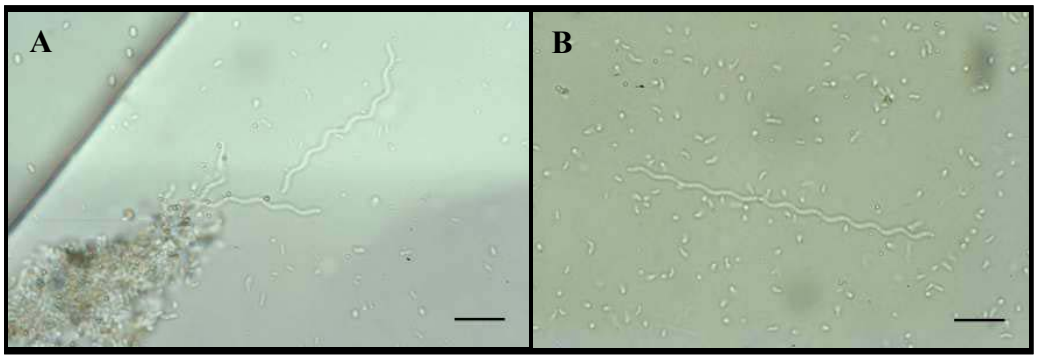


Figure VIII.5. Spirochetal members observed in the *Spirillum*-like bloom (Bar = 10 µm).

An aliquot of the *Spirillum*-like bloom sample was used as a DNA template for the amplification of the 16S rDNA of the bacterial community. The PCR product was cloned and the recombinant clones were sequenced. The obtained sequences were checked in order to exclude chimera formation. The similarities of the representative clones are given in Table VIII.1.

Table VIII.1. Similarity between the cloned sequences and closest relatives (percent %).

| Clone | Similarity (%) | Closest relative |
|---|---|---|
| **Clone LV-6** | 92 | *Candidatus* Arcobacter sulfidicus |
| **Clone LV-10** | 94 | *Thiomicrospira* sp. Tms-MPN/Milos-DII6 (AJ237767) |
| **Clone LV-7** | 90 | Uncultured methanotrophic bacterium (AB161679) |

The sequence of the LV-6 clone revealed a high similarity with uncultured *Arcobacter* bacteria. Therefore, a phylogenetic tree was constructed with the obtained sequence and others belonging to *Arcobacter* species (Fig. VIII.6). The closest relatives of the recovered sequence were *Arcobacter halophilus* and *Arcobacter nitrofigilis*.

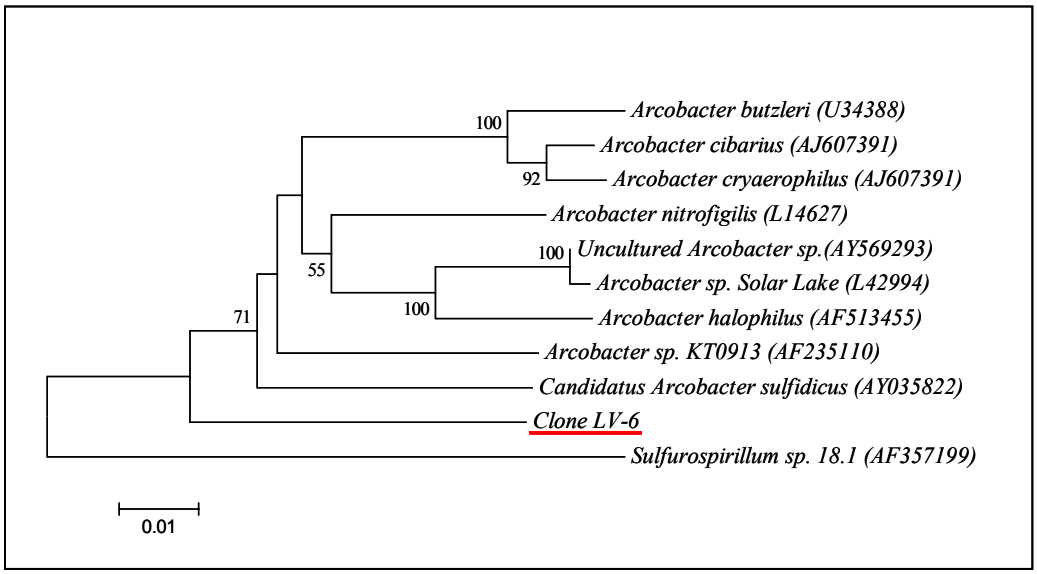


Figure VIII.6. Phylogenetic tree showing the relationship of the LV-6 clone and *Arcobacter* species.

The accession number of the clone LV-6 is DQ218324. Bootstrap values are given on branches. The bar indicates a 1% estimated difference in nucleotide sequences.

An oligonucleotide probe targeting the 16S rRNA sequence of the LV-6 clone was designed by following the steps summarized in the Table II.13 of the chapter 'General Material and Methods'. The retrieved sequence from the LV-6 clone and its closest sequences were aligned (Fig. VIII.7). The ARC824 probe was designed and checked against Genbank sequences and one or more mismatches were observed. The hybridization was optimized at 53ºC of hybridization temperature and 30% formamide.

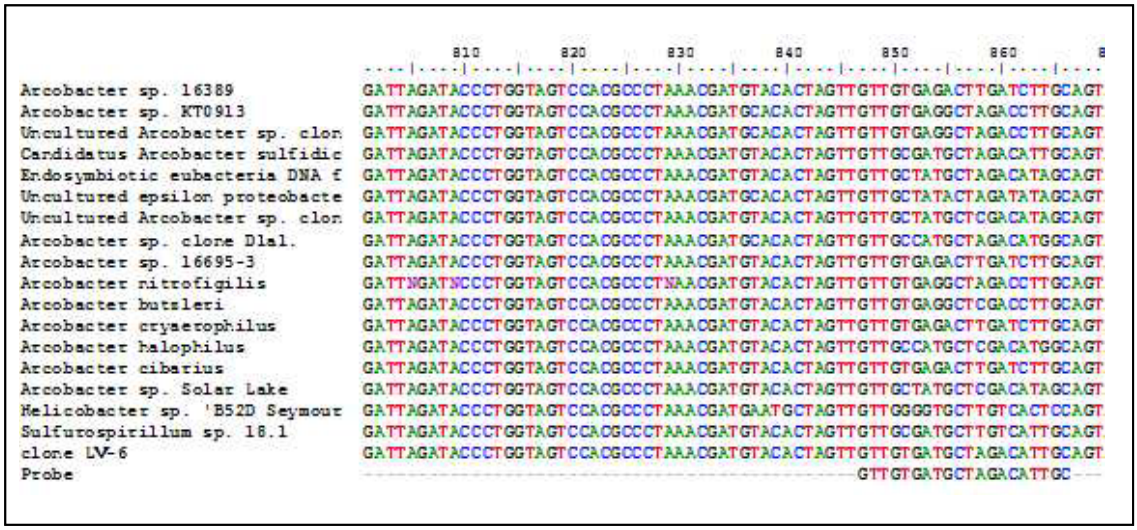


Figure VIII.7. Alignment of the clone LV-6 and closest relative sequences.

The hybridization was performed onto 0.2 µm-filters through which 50 µl of the *Spirillum*-like sample was filtrated. The visualization of the hybridized filter under the epifluorescence microscope revealed that ARC824 probe hybridized and gave positive signals for the small vibrioid bacteria previously described (Fig. VIII.8). The abundance of this morphotype observed in SEM micrographs might explain that the 16S rDNA screening in the *Spirillum*-like bloom provided an important percentage of clones with the 'LV-6 type' sequence.

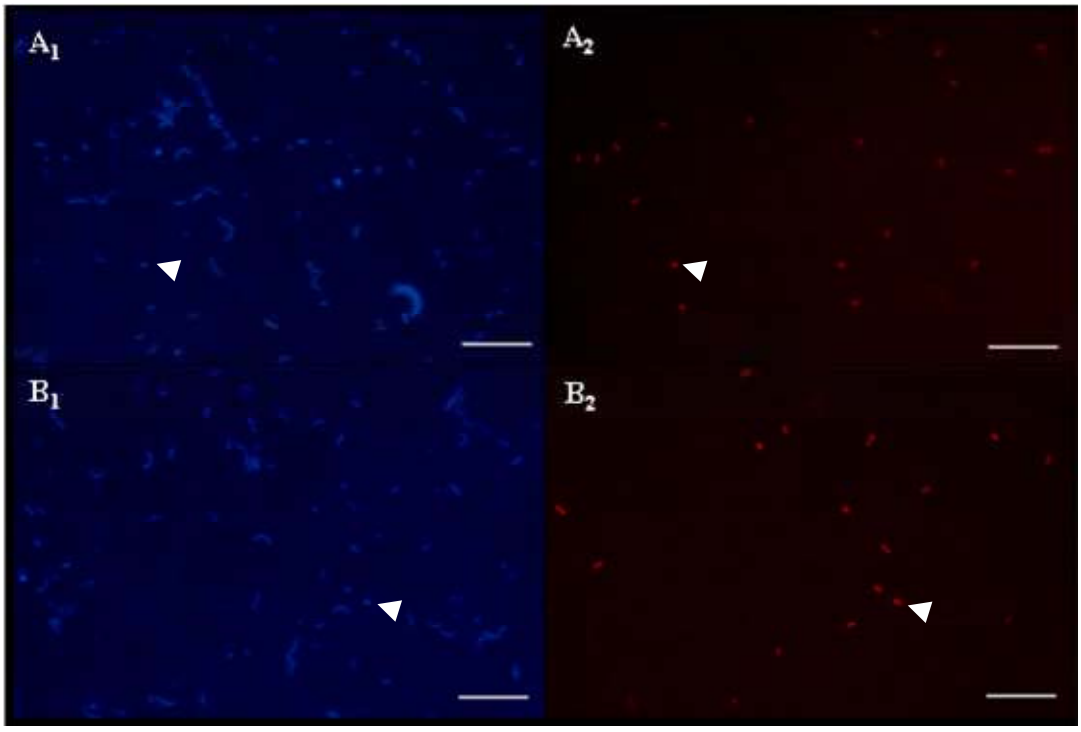


Figure VIII.8. FISH of a *Spirillum*-like bloom sample with the ARC824 probe labeled with Cy3.

$A_1$ and $A_2$ / $B_1$ and $B_2$: DAPI staining and fluorescence signal of the ARC824 probe visualized through UN41007 Cy3 filter, respectively. Arrows point to examples of DAPI-stained cells that gave positive signal in FISH hybridization (Bar = 10 μm).

- Spirochaetal-bloom

During the bacterial succession of a sulfur-rich bloom in microbial mats, a spirochaetal growing population was observed and analyzed by microscopic and molecular techniques. The observation of fresh and methylene blue-stained samples revealed the presence of large spirochaetal-like microorganisms that showed light-scattering granules when they get aged (Fig. VIII.9 A). The swimming speed of the spirochaetal-cells was considerably lower in comparison with the *Spirillum*-like cells. However, a motility behavior towards sulfur-granules accumulated in the aqueous phase was also observed. Moreover, the spirochaetal cells tried to penetrate the sulfur aggregates by means of a 'corkscrew' movement.

The observation of a 0.2 μm-DAPI stained filter revealed a high amount of rod-shaped bacteria and large spirochaetal-like microorganisms (Fig. VIII.9 B). The sample

was filtered through a 3 µm-filter, washed with Ringer ¼ to reduce the amount of rod-shaped bacteria, and finally stained with DAPI (Fig. VIII.9 C–D). An aliquot of the spirochaetal-bloom sample was fixed and visualized under SEM that showed the presence of an important population of pleomorphic bacteria (straight-cells, bent-rods, and spirals cells) of 2–2.5 µm length and 0.5–0.7 µm wide (Fig. VIII.10 A). A PCR with 16S rDNA universal primers and low stringency conditions were performed in order to amplify the main 16S rDNA molecule in the sample. The amplified sequence showed a 95% similarity with *Thiomicrospira kuenenii* (Brinkhoff and Muyzer, 1997). Moreover, the application of the *Thiomicrospira* PCR genus-specific designed by Brinkhoff and Muyzer (1997) gave positive signals in both spirochetal and *Spirillum*-like blooms (Fig. VIII.11). In order to verify the presence of *Thiomicrospira* members in Ebro delta mat and spirochaetal-bloom samples, enrichment cultures and isolation were performed in '*Thiomicropira pelophila*' medium (Fig. VIII.10 B–D). The 16S rDNA of the strains showed a 99% similarity with *Halothiobacillus hydrothermalis.*

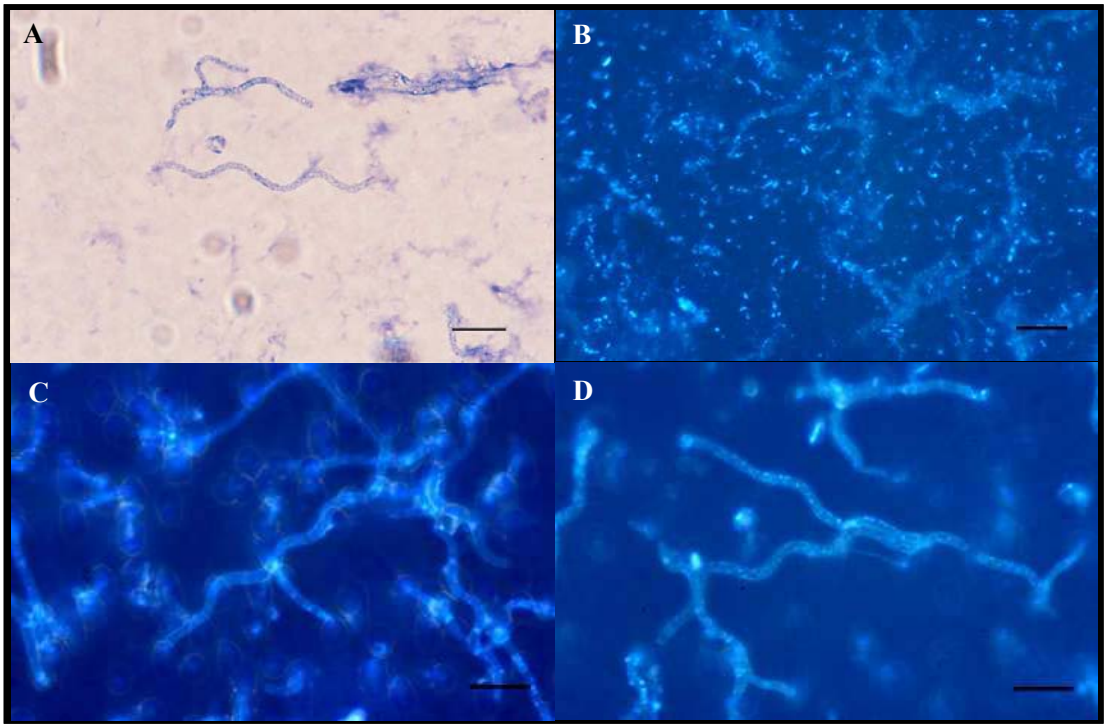


Figure VIII.9. Spirochetal-bloom diversity.

(A) Fresh sample stained with methylene blue; (B) DAPI staining of a 0.2 µm-filtered sample; (C–D) DAPI staining of 3 µm-filtered samples (Bar = 10 µm).

The spirochaetal sample filtered through a 3 µm-filter was treatment for TEM. All the cells visualized bear vacuoles or granules that distended the cell walls and occupied more than 80% of the cytoplasm (Fig. VIII.12 A and D). Furthermore, membranous structures were also detected inside the cells as is shown in Fig. VIII.12 C. Moreover, scanning electron micrographs of the 3 µm-filtered sample showed a homogeneous population of spirochete cells with 40–80 µm length and 1.5–2 µm wide (Fig. VIII.13). In addition, the presence of rod-shaped bacteria was considerably reduced after the filtration and washes; for this reason, the sample was filtered again and used as a DNA template for the 16S rDNA screening. The different recombinant clones were checked by restriction analysis (Fig. VIII.14) and the selected clones were sequenced (Table VIII.2).

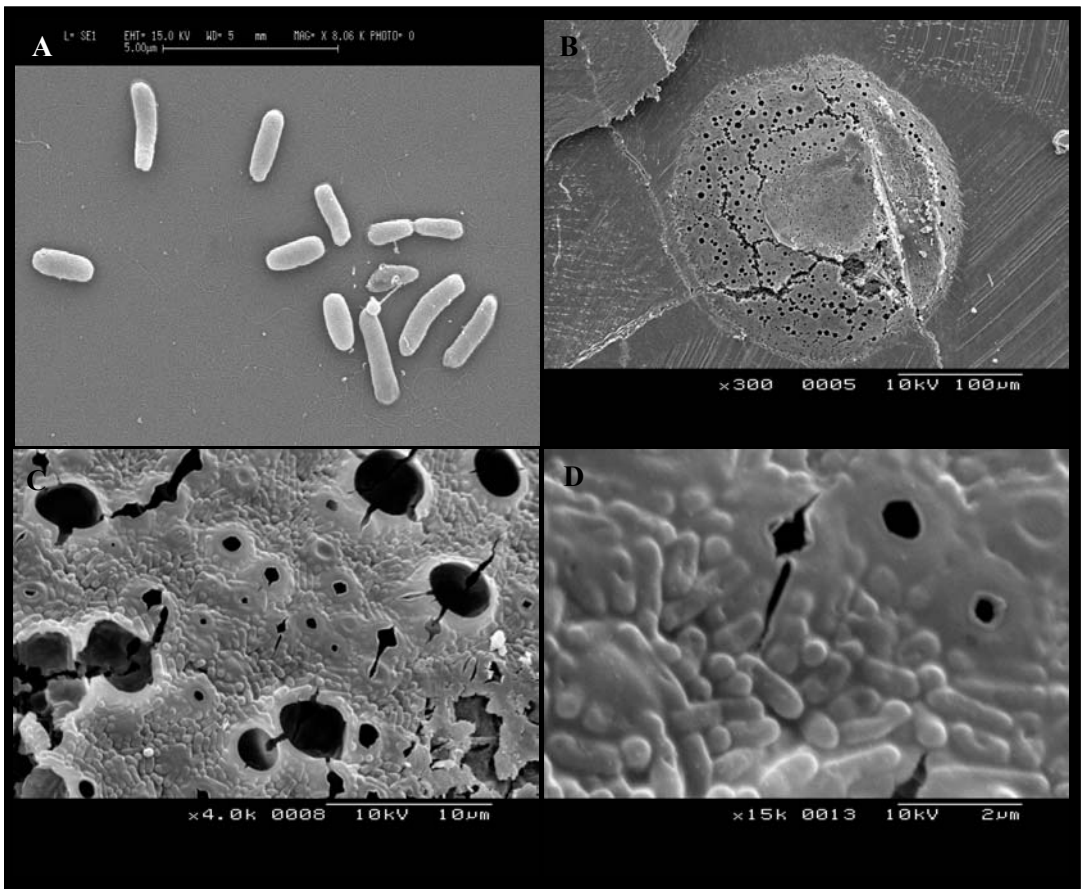


Figure VIII.10. SEM micrographs of rod-shaped bacteria of the spirochaetal-bloom.

(A) Rod-shaped bacteria observed in the sample; (B–D) *Halothiobacillus* sp. colony and bacterial members isolated from the bloom and mat samples.

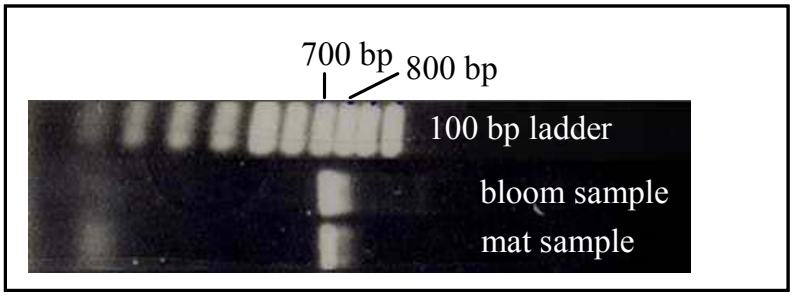


Figure VIII.11. Gel electrophoresis of the *Thiomicrospira* detection-PCR in bloom (A) and mat (B) samples.

The 16S rDNA fragment amplified by Ly02F and Ly03R primers gave a 721 bp-product.

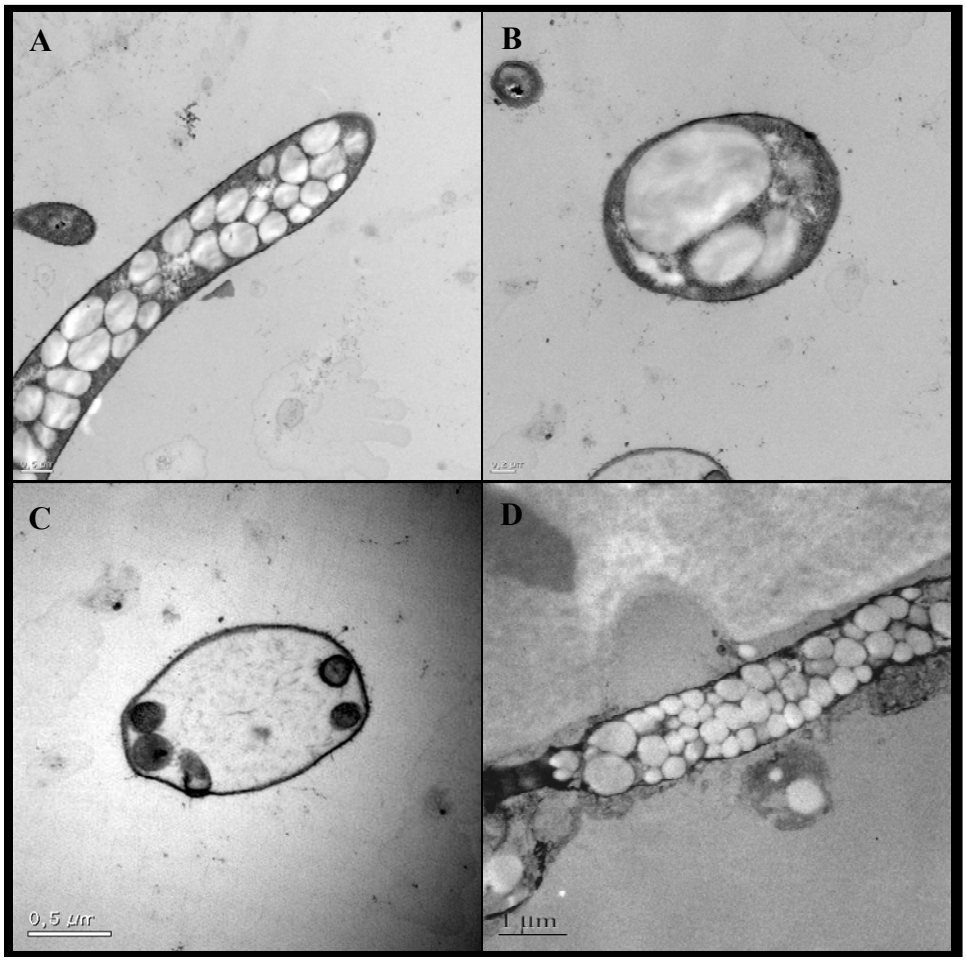


Figure VIII.12. Transmission electron micrographs of the 3 µm-filtered spirochaetal-bloom.

(A–D) Cytoplasmatic granules.

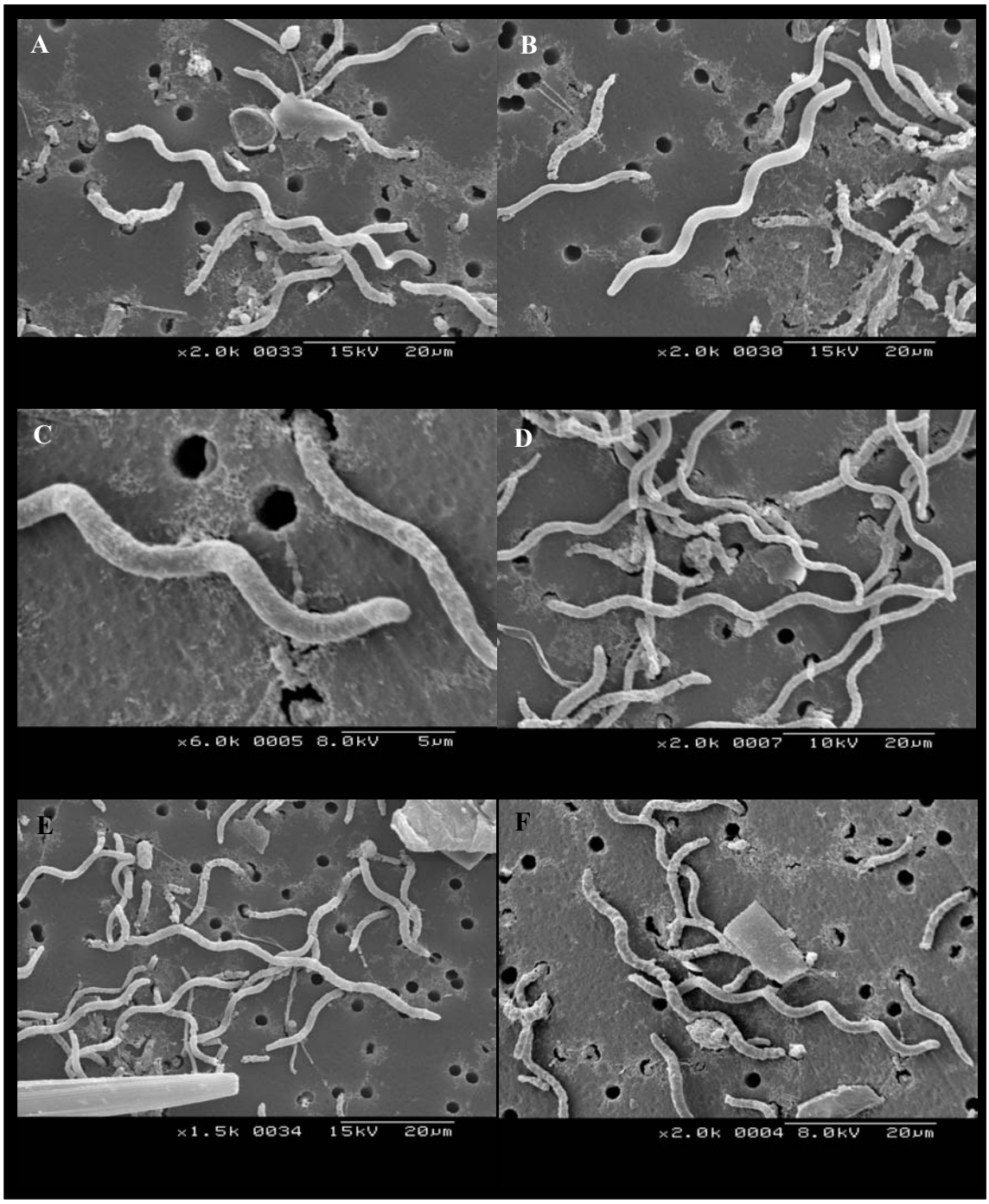


Figure VIII.13. Scanning electron micrographs of the 3 µm-filtered spirochaetal-bloom.

(A–D) Spirochete diversity and details of the cell terminus. Error bar = 15–25%

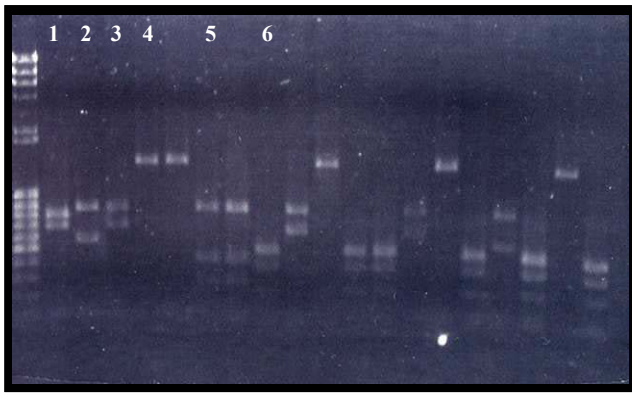


Figure VIII.14. Restriction analysis of the inserted sequences of the recombinant clones.

The different restriction patterns were considered to belong to different 16S rDNA molecules and one representative clone of each pattern was sequenced.

Table VIII.2. Similarity between the cloned sequences and closest relatives (percent %).

| Clone | Similarity (%) | Closest relative |
|---|---|---|
| **Clone Bl-6** (DQ218325)[1] | 99 | *Spirochaeta* sp. M6 (AY337319) |
| **Clone Bl-14** | 97 | Rhodobacteraceae bacterium (*Roseobacter*, AJ810843) |
| **Clone Bl-22** | 94 | *Desulfovibrio salexigens* (M34401) |
| **Clone Bl-28** | 95 | *Clostridium subatlanticum* (AF458779) |
| **Clone Bl-33** | 97 | *Thiomicrospira atlantica* (AJ404731) |
| **Clone Bl-38** | 96 | *Desulfovibrio hydrothermalis* (AF458778) |

[1]Accession number of the GenBank database.

The phylogenetic relationships of the sequenced Bl-6 clone and its closest relatives belonging to the *Spirochaeta* genus, and the similarities between the first *Thiomicrospira* sequence retrieved from the spirochaetal bloom, the 16S rDNA sequence of the isolated *Halothiobacillus* sp., and the clone Bl-33 sequence are indicated in Figs. VIII.15 A–B. Although *Thiomicrospira* and *Thiobacillus* genus are grouped in distinct clusters, their similar physiology and morphology have led to erroneous classification in both genuses (Wood and Kelly, 1993).

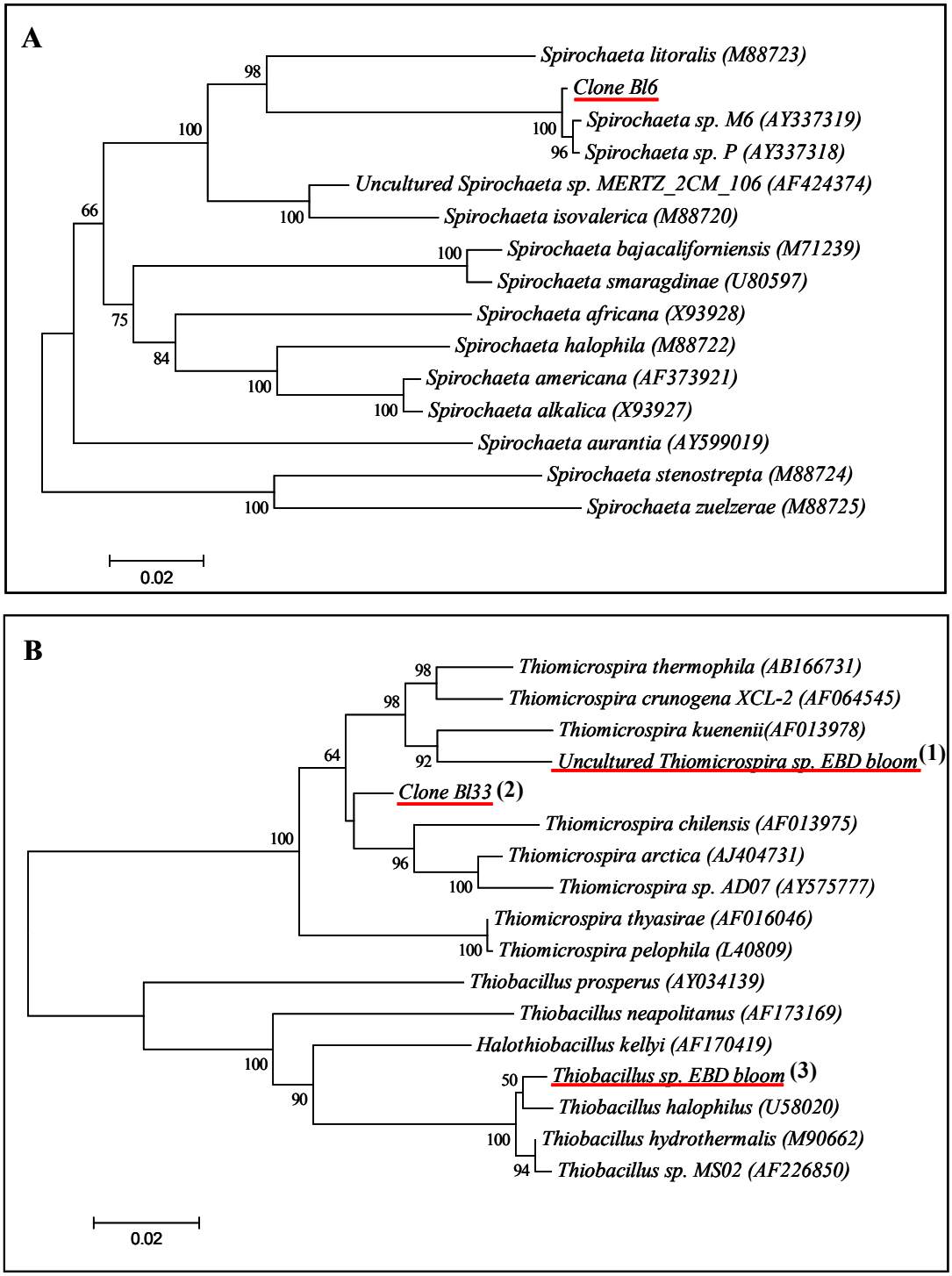


Figure VIII.15 Phylogenetic tree showing the relationship of the obtained sequence and their close relatives.

The trees were constructed using the Neighbor-joining algorithm and the Jukes and Cantor model. Bootstrap values are given on branches. The bar indicates a 2% estimated difference in nucleotide sequences. Accession numbers: [1]DQ218327; [2]DQ218326; [3]DQ218323.

An oligonucleotide probe targeting the 16S rRNA sequence of the Bl-6 clone was designed by following the steps summarized in the Table II.13 of the 'General Material and Methods' chapter. The Bl-6 clone sequence and its closest sequences were aligned (Fig. VIII.16), and the UnSpiro465 probe was designed and checked against Genbank sequences. Probe UnSpiro465 matched in all nucleotides with *Spirochaeta* sp. M6 and P sequences that have a 99% similarity with the Bl-6 clone sequence. This fact may suggest that the mentioned sequences belong to different strains of the same genus and species. The EUB388 universal probe was applied to the spirochaetal samples and no fluorescent signal was obtained, which can be due to an inefficient fixation of the samples. Likewise, the UnSpiro465 probe did not yield positive results.

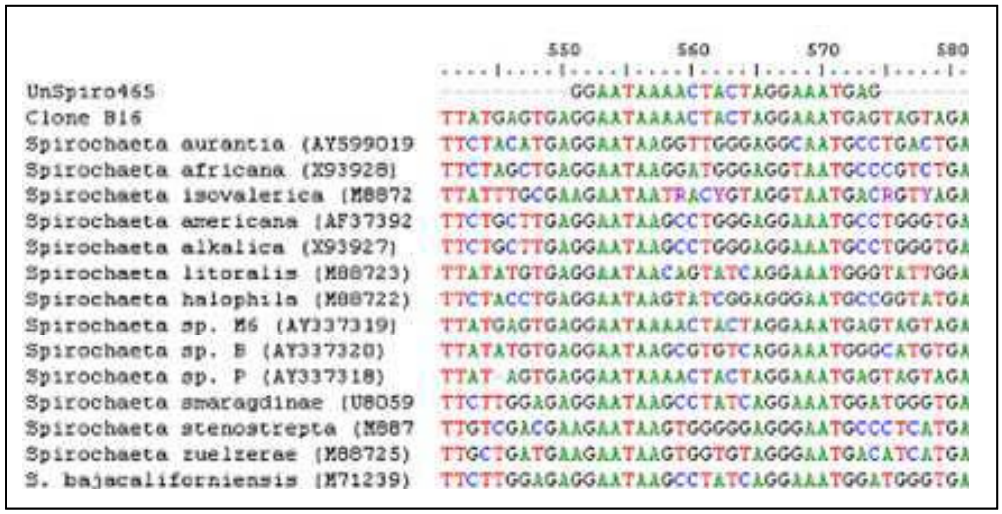


Figure VIII.16. Alignment of the clone Bl-6, UnSpiro465 probe and closest relative sequences.

- Discussion and conclusions

This study about the morphological diversity of free-swimming microaerophilic bacteria has revealed the presence of several morphotypes and a temporal bacterial succession which may be related to the occupation of microniches at different oxygen and sulfide concentrations (Brune *et al*., 2000). Several strategies have found in the oxygen–sulfide interface, e.g. many sulfide-oxidizing bacteria migrate between oxic and sulfidic layers, whereas some species have developed spatial organized structures to enhance oxygen flux (Fenchel and Glud, 1998). Examples of these structures are the veils formed by the aggregation of sulfur-oxidizing bacteria that form a mucous matrix in which they are actively rotating their flagella to create an oxygen gradient (Cogan and Wolgemuth, 2005). The presence of different morphotypes and genus of microaerophylic sulfur-oxidizing bacteria in the analyzed blooms can only be explained by a spatial or a temporal succession depending of the $O_2$ concentration or by sulfur syntrophy established in a consortial association. Indeed, bacterial successions of colorless sulfur bacteria with vibroid-to-spirilloid forms have been previously described in similar environments (Bernard and Fenchel, 1995; Kuever *et al*., 1996; Thar and Fenchel, 2005).

The first steep in the bacterial succession observed in this study was the appearance of *Beggiatoa* sp. filaments in the overlying water of the microbial mat microscoms. This fact has been widely studied and it is due to an increased sulfate reducing activity in the deepest layers of the mat that generates elevated sulfide concentrations diffusing towards the mat–water interface. This process results in an upward shift of the oxygen–sulfide boundary and an upward migration of *Beggiatoa* sp. (Epping *et al*., 2000) and is driven by the sulfate-reducing activity induced under low irradiances in the early hours of a day/night cycle. Due to the fact that an important part of the underlying black mud (sulfate-reducing bacteria) is discarded from the microscoms unit, it is logical that the occurrence of sulfur-oxidizers blooms in the overlying water was limited because of the lack of sulfide sources.

After the growing population of *Beggiatoa* filaments, the veils formed by vibrioid bacteria were observed in the overlying water which indicated that the oxic–

anoxic boundary was still in the water column as a result of the high concentration of sulfide. The formation of these mucous structures has been interpreted as an adaptation mechanism in order to reduce the depth interval where the opposing gradients overlap and as an attachment area to prevent the turbulences in the oxygen–sulfide interface (Thar and Kühl, 2002). The veils were firstly dominated by vibrioid-to-spirilloid cells with different morphotypes. The bigger helical cells showed sulfur inclusions in their cytoplasm and might be related to *Spirillum winogradskyi* and *Aquaspirillum bipunctata* (both formerly belonging to the genus *Thiospira*) that aggregate chemotactically at the interface of opposing oxygen and sulfide gradients (Dubinina *et al*., 1993). Although the helical cells showed a typical morphology, sulfur-granules inclusions, residual presence of flagella and the polar membrane structure also found in the *Aquaspirillum* genus, more studies need to be performed in order to assess the phylogenetic affiliation of the observed morphotype.

The motility behaviour of the helical cells was characterized by a directed swimming direction towards sulfur-depositions accumulated in the aqueous phase. This observation might indicate that the vibrioid-helicoid bacteria exhibited chemotaxis correlated to the oxygen–sulfur gradients or to growth substrates liberated in the degradation of sulfur-accumulating cells. The temporal sensing mechanism exhibited by these microorganisms has been related to the presence of sensor regions attributed to the polar membrane structure (Thar and Kühl, 2003). This multilaminar polar organelle has been observed in members of the '*Epsilonproteobacteria*' (Ritchie *et al*., 1966) and also in spirilloid bacteria observed in microbial mats (*Titanospirillum velox*; Guerrero *et al*., 1999) but a clear function was not still assigned. More recently, this structure has also detected in members of the *Arcobacter* genus and its function has been related to the excretion of sulfur filaments outside the cells (Taylor and Wirsen, 1997; Wirsen *et al*., 2002).

Apart from the bigger helical cells detected in the *Spirillum*-like blooms, and important population of bacteria affiliated with the *Arcobacter* genus was detected by SEM and 16S rDNA screening and confirmed by FISH. The retrieved sequence revealed a 92% similarity with '*Candidatus* Arcobacter sulfidicus' (Wirsen *et al*., 2002) and was closely related to *Arcobacter nitrofigilis* and *Arcobacter halophilus* (Donachie

*et al*., 2005). *Arcobacter* genus has been traditionally associated with human and animal enteric diseases (Vandamme *et al*., 1992) but previous studies revealed its presence in coculture associations with *Desulfovibrio* sp. in the cyanobacterial layer of Solar Lake microbial mats (Teske *et al*., 1996). This association suggested the role of *Arcobacter* species as oxygen-scavenging organisms that allowed the activity and growth of sulfate reducers under aerobic conditions. More recently, members of the *Arcobacter* genus have been detected in coastal environments and in microbial mats of sulfidic cave springs (Fera *et al*., 2004; Engel *et al*., 2003) and their role have been associated with the cycling of carbon and sulfur.

'*Candidatus* Arcobacter sulfidicus' has been described as an autotrophic sulfide-oxidizing bacterium that produces filamentous sulfur. Although filamentous sulfur structures similar to those described for '*Candidatus* Arcobacter sulfidicus' have not been detected in the SEM micrographs of the *Spirillum*-like sample, filamentous mycelial structures were surrounded the small vibrioid cells and we can not discard its relationship with sulfur excreted filaments. However, the low similarity percentage of the cloned sequence with their closest relatives suggested that the organisms identified by FISH may belong to a new species or even to a new genus of the '*Epsilonproteobacteria*'. Therefore, future studies will be focused on the isolation and characterization of these rod-helical cells.

The next steep in the bacterial succession was the occurrence of a large spirochetes bloom. The observation of the sample revealed the presence of a higher population of small-rod bacteria accompanied the spirochaetal cells. The rod-shaped bacteria were identified as members of the *Thiomicrospira* and *Thiobacillus* genus by 16S rDNA and culture techniques. In these habitats, *Thiomicrospira*/*Thiobacillus* species have to compete with other, very specialized bacteria for the electron donor, sulfide, or other reduced-sulfur compounds. For example, in enrichments cultures of the mesophilic *Thiomicrospira chilensis* isolated from a *Thioploca* sulfur mat, a large number of spirillum-like organisms containing sulfur granules were detected (Brinkhoff *et al*., 1999b). These organisms looked very similar to *Aquaspirillum bipunctata* (Dubinina *et al*., 1993a). *Thiomicrospira* seem to play an important role in the re-oxidation of reduced sulfur compounds in marine habitats. However, the diversity and

ecological importance of these bacteria in the sulfur cycle are largely unknown and their identification is difficult and can induce to erroneous conclusions because of their small size and the fact that *Thiomicrospira* can exhibit pleomorphism under stress conditions resulting in spiral morphotypes (Wood and Kelly, 1993).

The presence of large free-swimming spirochetes in microbial mats have been widely studied (Harwood, 1984) and have also been associated with '*Thiodendron*' sulfur bacterial mats (Dubinina *et al*., 1993b). '*Thiodendron latens'* was firstly described as a sulfur mat-forming bacterium and then was identified as a symbiotic association of aerotolerant spirochaetes and anaerobic sulfidogenes (Dubinina *et al*., 1993b). The spirochaete species are the main structural and functional component of these mats and they may accumulate elemental sulfur intracellularly. Indeed, the relationship between the aerotolerant *Spirochaeta* and the *Dethiosulfovibrio* species (or other sulfidogenes) would represent an effective shortcut in the sulfur cycle because spirochetes degrade carbohydrates and provide organic compounds to its sulfidogen partner that prevent toxic hydrogen sulfide gas from percolating into overlying oxygenated waters.

The large spirochaetal-like cells observed in the 3 μm-filtered sample SEM micrographs and the 16S rDNA screening that indicated a high similarity of the Bl-6 cloned sequence with members of the *Spirochaeta* genus, supported the idea of '*Thiodendron*-like' mat structures in the bacterial succession of the oxic–anoxic interface. The closest relative of the Bl-6 cloned sequence, *Spirochaeta* sp. M6 have been described as an aerotolerant spirochete capable of oxidize reduced sulfur compounds as a protective mechanism (Dubinina *et al*., 2004). In this case, sulfur-accumulation granules were not observed inside the spirochetal cells during the first stages of the spirochetal bloom; however, light-scattering inclusions were observed inside the cells when their motility and the sulfide concentration were considerably reduced. This fact can be associated with the detoxification of hydrogen peroxide accompanied by the accumulation of elemental sulfur in cells. Moreover, the cytoplasmatic granules detected inside the spirochaetal cells by TEM were observed in the latter stages of the spirochaetal bloom. Therefore, they may be attributed to stationary reserve compound granules, sulfur granules or resistance structures.

The presence of spirochetes in this kind of environments can be related to a chemotactic response of the spirochaetal cells towards sulfur-rich environments or to growing substrates liberated by sulfur-oxidizing bacteria. In fact, cooperative associations between spirochetes and other groups have been previously described (Harwood, 1984). For example, the large spirochete, *Spirochaeta plicatilis*, has been observed within masses of *Beggiatoa* trichomes (Blakemore and Canale-Parola, 1973). As the level of sulfide generated by biological activities in the mud became low, gradual lysis of the *Beggiatoa* trichomes was observed. This lytic process coincided with a dramatic increase in the number of large spirochetes (Blakemore and Canale-Parola, 1973). Possibly, substances released by the lysing *Beggiatoa* were used as growth substrates for *Spirochaeta plicatilis*. Moreover, other studies have suggested that free-living spirochetes associate and interact with cellulose-degrading bacteria in anaerobic marine environments. It is likely that spirochetes used for growth soluble sugars released from cellulolytic bacteria (Pohlschroeder *et al*., 1994). In this sense, the detection of sequences related to *Clostridium*, *Desulfovibrio* and *Thiomicrospira* genuses in the spirochaetal-bloom may suggest a syntrophic relationship between their members.

The study of spirochetes and the '*Thiodendron* consortium' of free-living spirochetes involved in the sulfur cycle and spirochete motility symbiosis have been suggested as pre-adaptations for chimera evolution. These investigations form part of the search for the modern bacterial co-descendants of the spirochetes which gave rise to the kinetosomes and axonemes (undulipodia) of eukaryotic cells, as hypothesized by the Serial Endosymbiosis Theory (SET; Margulis, 1996; Margulis *et al*., 2000). Microbial mats are ideal environments for bacterial symbiosis and may be modern analogues of environments where the first amitochondriate evolved (Margulis, 1980).

Another important issue of the '*Spirillum*-spirochaetal' sulfur blooms is the programmed bacterial succession and the fact these microorganisms can be recovered long after their removal from the field if the supply of sulfide is maintained. The spirochete and spirillum diversity in intertidal mud environments is greater than previously recognized, and many spirochetes may be capable of evading detection by formation of stages resistant to environmental perturbations. Previous studies have

described this kind of structures in *Spirosymplokos deltaeiberi* (Guerrero *et al*., 1993b; Margulis, 1993) that reported refractile, membranous structures though to be responsible for the survival of spirochetes after stressful conditions. Furthermore, in certain species of *Aquaspirillum* and *Oceanospirillum* coccoid bodies can become predominant in older cultures suggesting a role in resistance to desiccation (Krieg, 1984).

In conclusion, the bacterial tactic responses observed in the 'cline' interfaces are survival advantages that are the result of sensory control of the swimming behaviour towards a more favourable environment, and that can be balanced with other sensory pathways of the whole bacterial group such as the 'quorum sensing' (Armitage, 1999). In addition, the particularities of these opposing gradients interfaces in which the Brownian motion is diminished are responsible for the morphogenesis of the observed bacterial groups, for their sophisticated motility strategies and their high speeds (Luchsinger *et al*., 1999). These bacterial adaptations are interesting questions of study especially for the evolution of motility structures and sensory organelles.

## *Conclusions*

- The occurrence and bacterial succession in sulfur-rich blooms has been studied. The bacterial succession was characterized by a first steep with *Beggiatoa* dominance, followed by *spirillum*-like blooms that formed veils as a protective structure, and finally an optional steep in which large spirochetes were predominant.
- The *Spirillum*-like blooms were formed by bigger helical cells 5–8 μm per 0.9–1 μm that had light-scattering granules in their cytoplasm and displayed a chemotactic behaviour towards sulfur-granules accumulations in the aqueous phase. SEM micrographs revealed a polar structure with indentation and rest of flagella in this morphotype. This polar membrane might be related to the polar organelle observed in '*Epsilonproteobacteria*'.
- The 16S rDNA screening of the *Spirillum*-bloom sample indicated the presence of sequence similar to '*Candidatus* Arcobacter sulfidicus' and *Thiomicrospira* sp.

- The *Spirillum*-like blooms reported a high population of small-rod bacteria that were identified as a member of the *Arcobacter* genus by hybridization with the ARC824 fluorescent probe.

- A spirochaetal-bloom was observed after the dominance of *spirillum*-like cells. The large spirochaetal cells were accompanied by an important amount of rod-bent cells. The isolation of a strain of *Halothiobacillus* sp. and the detection of *Thiomicrospira* by PCR-specific detection and 16S rDNA cloning, suggest the presence of *Thiomicrospira*/*Thiobacillus* members in this kind of bacterial blooms.

- The large spirochetes observed were 50–70 μm length and showed light-scattering granules when they get aged. TEM micrographs of the spirochaetal-bloom revealed big granules in their cytoplasm that can be attributed to sulfur inclusions, reserve material of resistance structures.

- The future application of the designed FISH probes in microbial mats cores may contribute to understand the motility behavior and daily dynamics of spirochetes and *spirillum*-like microorganisms in estuarine microbial mats.

## • Publications

- **Villanueva L.**, A. Navarrete, J. del Campo, J. Urmeneta, and R. Guerrero. Diversity of sulfur-blooms in microbial mats.