UNIVERSITAT AUTÒNOMA DE BARCELONA

Facultat de Ciències

Departament de Genètica i de Microbiologia

DEVELOPMENT AND CHARACTERIZATION OF SULFIDE-OXIDIZING BIOFILMS

Isabel Ferrera Ceada

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Per

Isabel Ferrera Ceada

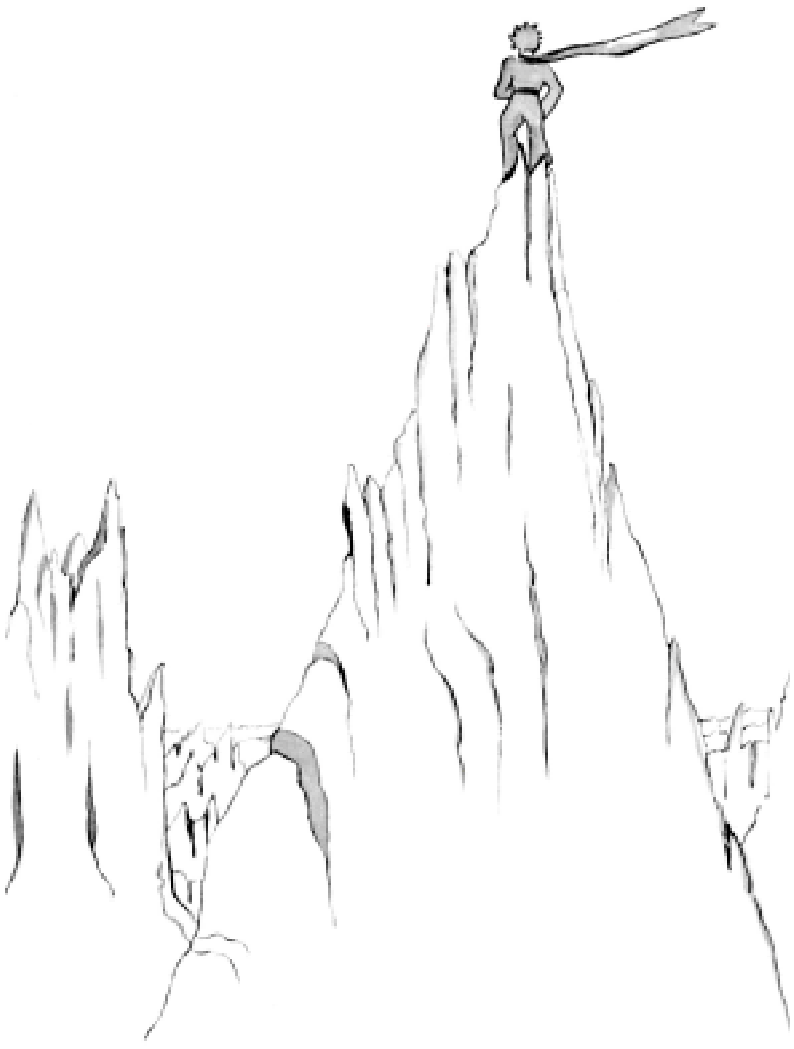
Vist-i-plau dels directors de la tesi

Dr. Jordi Mas Gordi

Dra. Olga Sánchez Martínez

Juny 2004

A tots els que m'heu ajudat a arribar fins aquí però en especial .....



.....als meus pares.

"...I va tornar amb la guineu.
-Adéu – li va dir...
-Adéu – li va dir la guineu -. Aquí tens el meu secret. És molt senzill: només s´hi veu bé amb el cor. L'essencial és invisible als ulls.
-L'essencial és invisible als ulls – va repetir el petit príncep, per recordar-se'n.
-És el temps que has perdut amb la rosa, que la fa tant important.
-És el temps que has perdut amb la rosa...- va fer el petit príncep, per recordar-se'n.
-Els homes han oblidat aquesta veritat – va dir la guineu- tu no l'has d'oblidar...."

"El Petit Príncep"
Antoine de Saint-Exupéry

SUMMARY

This works deals with the development and characterization of complex sulfide-oxidizing biofilms. A bioreactor for biofilm development has been designed. The system is based on a non-aerated illuminated packed-column, which provides a large surface for microbial attachment. The reactor operates as a sulfidostat and the control system allows to maintain a constant concentration of residual sulfide in the micromolar range thus avoiding inhibition of sulfide oxidizers due to excessive sulfide load and ensuring a constant quality in the effluent.

The system was first tested with a pure culture of *Chlorobium limicola* and, later on, with natural samples (freshwater lake sediment and a microbial mat) in order to develop complex biofilms. Biofilms developed vigorously on the column surface and high biomass was achieved in all the experiments. The dynamic behavior of the system was slower than in stirred reactors but more stable in front of sudden environmental changes. The system was able to process highly polluted effluents and to maintain the quality of the output generated even when conditions of light irradiance and sulfide income were suddenly changed.

The biofilms developed were characterized using both, traditional techniques (i.e. microscopy and pigment analysis) and a molecular approach, in particular cloning and sequencing. First, of all, several DNA extraction procedures were evaluated in order to select the most suitable method for performing the diversity analysis of our biofilms. We compared the extraction efficiency (i.e. amount of DNA recovered), as well as the genetic diversity recovered by denaturing gradient gel electrophoresis (DGGE). A DNA extraction based on a mechanical step of bead-beating followed by enzymatic lysis and by phenol-chloroform extraction, was the most appropriate protocol for these biofilms.

Microbial characterization revealed that, in both cases, highly diverse biofilms covering a wide range of phylogenetic and physiologic groups had developed. Both biofilms presented high species richness and a high degree of microdiversity within some species. Some differences were observed in the predominant phylogenetic groups present in each biofilm. We recovered members affiliated to the Alpha and Gamma subclass of the Proteobacteria, the *Cytophaga-Flavobacterium-Bacteroides* group as well as plastids signatures from green

algae in both biofilm libraries. Moreover, in the biofilm developed from the freshwater sample, other clones belonged to the Beta- and Delta-Proteobacteria, the Cyanobacteria and the low G+C Gram-positive whereas we recovered clones belonging to the Epsilon-Proteobacteria and to the Chlorobi only from the marine biofilm. Although members belonging to these phylogenetic groups were different in each case, they represented the same functional groups. Sulfide was oxidized both anaerobically by phototrophic sulfur bacteria and by purple nonsulfur bacteria, and aerobically by colorless sulfur bacteria using the oxygen produced by oxygenic phototrophs, as the system was non-aerated. Other groups, such heterotrophic bacteria, can also contribute to the functioning of the system by recycling organic matter.

In conclusion, we found high diversity at both functional and taxonomic level. Different functional groups represented by different species (heterotrophic, photoautotrophic and chemoautotrophic microorganisms) coexisted in the bioreactor. Moreover, some of the species also showed microdiversity (similarity in 16S rDNA sequences below the species level). Such attributes could be very important for the long-term functioning and versatility of the reactor.

RESUM

En el present treball s'han desenvolupat i caracteritzat biofilms per a la detoxificació d'efluents contaminats amb compostos reduïts de sofre. En primer lloc es va dissenyar un bioreactor basat en una columna il·luminada que aporta una gran i heterogènia superfície d'adhesió per als microorganismes, i en el qual no hi ha aportació externa d'oxigen. El sistema de control, basat en el potencial redox, permet mantenir constant la concentració residual de sulfur d'hidrogen en el rang micromolar, evitant la inhibició dels microorganismes i mantenint al mateix temps la qualitat de l'efluent generat.

S'han realitzat tres experiments per tal de provar el sistema, el primer amb un cultiu pur de *Chlorobium limicola*, i després amb mostres naturals (sediment lacustre i tapet microbià) per tal d'aconseguir biofilms complexos. Els biofilms es desenvolupen ràpidament assolint-se una elevada biomassa en tots els casos. El comportament dinàmic del sistema és més lent que el dels sistemes de biomassa en suspensió, però alhora més estable a les pertorbacions. De fet, el sistema és capaç de mantenir l'oxidació de sulfurs i la qualitat de l'efluent generat fins i tot quan les condicions de llum incident o de concentració de sulfur d'hidrogen a l'entrada del sistema canvien.

S'han caracteritzat els biofilms complexos amb eines clàssiques (microscopi i anàlisi de pigments) i també amb eines moleculars (biblioteques genètiques). En primer lloc, s'han avaluat diferents mètodes d'extracció d'ADN per tal de trobar el millor per a les nostres mostres. S'ha comparat l'eficiència d'extracció quantificant l'ADN obtingut, i la diversitat recuperada en cada mètode amb electroforesi en gels de gradient desnaturalitzant (DGGE). El mètode basat en un trencament mecànic amb microesferes de vidre seguit d'una lisi enzimàtica i una extracció amb fenol és el més apropiat per a l'extracció d'aquests biofilms.

La caracterització del biofilms ha revelat una elevada diversitat microbiana tant a nivell filogenètic com fisiològic. Ambdós biofilms presenten una gran riquesa d'espècies així com un elevat grau de microdiversitat entre alguns grups. S'observen algunes diferències en els grups filogenètics predominants entre els dos biofilms. S'han recuperat membres relacionats amb les subclasses Alpha i Gamma del grup Proteobacteria, amb el grup *Cytophaga-*

Flavobacterium-Bacteroides així com amb cloroplasts d'algues en ambdós biblioteques genètiques. A més, en el biofilm desenvolupat a partir del sediment lacustre, també s'han trobat membres de les subclasses Beta i Delta-Proteobacteria, del grup Cianobacteria i dels bacteris Gram-positius de baix contingut en G+C (Firmicutes). Per contra, la biblioteca realitzada amb el biofilm desenvolupat a partir del tapet microbià conté una elevada proporció de clons relacionats amb les Epsilon-Proteobacteria i amb els Chlorobi. Tot i que els membres trobats pertanyent a tots aquests grups filogenètics són diferents, representen els mateixos grups funcionals. El sulfhídric era oxidat anaeròbicament pels bacteris fototròfics del sofre i els bacteris vermells no del sofre, i aeròbicament pels bacteris quimiolititròfics del sofre utilitzant l'oxigen produït pels organismes fototròfics oxigènics. Altres microorganismes com els bacteris heterotròfics podrien contribuir al funcionament del sistema a través del reciclatge de la matèria orgànica.

En conclusió, trobem una elevada diversitat tant a nivell funcional com taxonòmic en els biofilms desenvolupats. Diferents grups funcionals representats per diferent espècies (heterotròfiques, fotoautotròfiques i quimioautotròfiques) coexisteixen al sistema. A més, també trobem microdiversitat (similitud per sobre del nivell d'espècie en la seqüència del gen 16S ADNr). Aquesta elevada diversitat podria ser molt important per al funcionament a llarg termini del reactor.

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Chapter 1. GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. SULFIDE AS A POLLUTANT

Increase of environmental pollution during the last years has become a common problem in western countries. Industrialization and massive urbanization of some areas have concentrated pollutants derived from human and industrial activities. The most extended pollution is due to wastewater containing high loads of organic matter, and usually also nitrogen and phosphorus compounds. These compounds have to be removed or transformed before being discharged to the environment due to their capacity to introduce drastic alterations into the ecosystems. There are several other pollutants such as heavy metals (mercury) or petroleum compounds (aliphatic and aromatic hydrocarbons) that are toxic to organisms and their release into the environment without previous detoxification must be avoided.

Another group of compounds which contribute massively to pollution are reduced sulfur compounds. They constitute a group of organic (carbon disulfide, dimethyl sulfide, methane thiol, thiophenes among others) and inorganic (hydrogen sulfide, thiosulfate, polysulfides, polythionates) chemical compounds originated both in natural and industrial processes. In natural environments, hydrogen sulfide is the most widespread reduced compound and has a key role in the sulfur cycle (Figure 1.1). Microbial communities inhabiting sulfurous environments have mechanisms of production and recycling of sulfide. The main mechanism of production is through the anaerobic degradation of organic matter by sulfate reducing bacteria (Jørgensen 1982). Anaerobic environments rich in sulfate are found in coastal areas such as seawater pools of salt marshes, closed bays, estuaries, as well as, on sand beaches (microbial mats). These environments are strongly influenced by seawater, where sulfate is the second most abundant anion. Reduced sulfur compounds also can be found in karstic lakes where groundwater contains dissolved calcium sulfate of sedimentary origin. In these environments, aerobic and anaerobic sulfide-oxidizing organisms transform sulfide into sulfur and sulfate among other compounds.

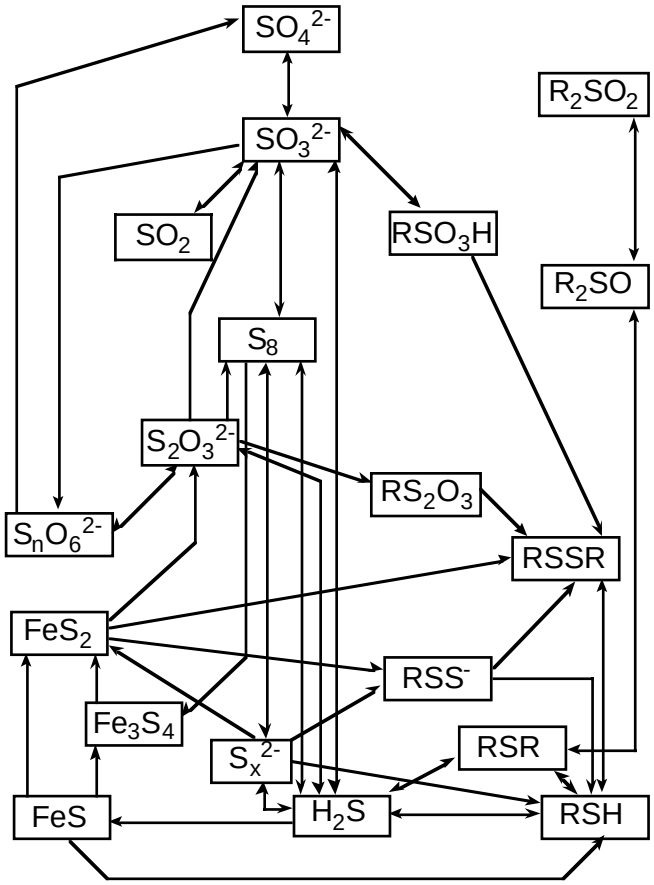

Figure 1.1. Microbial-mediated conversions of sulfur compounds in natural environments (from Visscher 1992)**.**

Sulfide-oxidizing organisms have been studied in many of these environments (reviewed in Van Gemerden and Mas 1995), mostly in microbial mats and sulfurous lakes (Figure 1.2) (e.g. Guerrero et al. 1985; Stal et al.1985; Van Gemerden et al. 1985; Stal and Caumette 1994). Light/dark cycles and oxic/anoxic conditions permit the development of a complex community where aerobic sulfur oxidizers, anaerobic sulfur oxidizers and cyanobacteria coexist (De Wit et al. 1989; De Wit and Van Gemerden 1990). Such sulfide-oxidizing communities act as natural biofilters in the environment because they oxidize reduced sulfur compounds at the oxic/anoxic interface and prevent their diffusion to the upper layer of the water column or to the atmosphere. Despite being subject to changes in environmental conditions, these complex communities are able to cope with these changes and maintain stable oxidation rates (Van Gemerden et al. 1985).

Reduced sulfur compounds are also generated by human processes in different industries such as petrochemical plants, tanneries, viscose rayon manufacturers, the gasification of coal for electricity or by the anaerobic treatment of sulfate containing wastewaters (Rinzema and Lettinga 1988; Kuenen and Robertson 1992). These compounds are usually released as dissolved sulfide in wastewater and in waste gases and constitute an environmental concern.

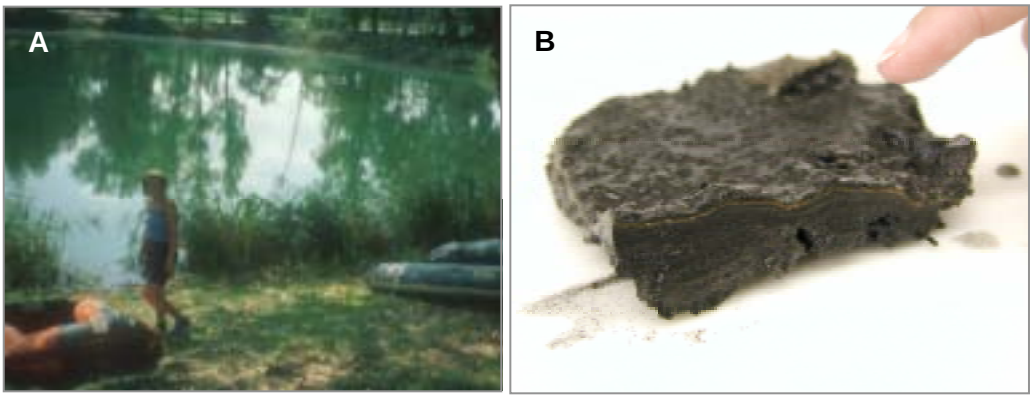

Figure 1.2. Images from the sulfurous Lake Vilar (Banyoles, Spain) (A) and from a Ebro Delta microbial mat sample (B).

Effects of sulfide

There are many chemical forms of reduced sulfur compounds but none has the toxicity of hydrogen sulfide. Sulfide has an unpleasant odor perceptible at concentration of 0.002 ppm. Due to its corrosive properties and high oxygen demand hydrogen sulfide can cause damage at different levels. The corrosive properties of sulfide are apparent in industry where it affects walls of reactors, sewer systems, steel pipelines and combustion engines. Moreover, sulfide reacts with biological molecules and affects human beings causing loss of odor sensitivity, respiratory irritation and, at high concentrations (500ppm), death (Cañete 1994).

Sulfide also causes damage in ecosystems due to its high oxygen demand. When dissolved in water, it reacts rapidly with dissolved oxygen causing depletion of O_2 and death of aerobic organisms. Moreover, emissions to the atmosphere in form of gas contribute to pollution and acid rain.

Methods for sulfide removal

For health and safety reasons, emission of sulfide to the environment is limited by law in many countries. As a consequence, different methods for sulfide removal have been investigated. Physicochemical processes involving direct air stripping, oxidation and chemical precipitation are currently used for the treatment of gas effluents with high content of reduced sulfur compounds (90%). Many different strategies have been developed and the most common for sulfide-containing gases is the Claus process. In this process, sulfide is separated from methane and other compounds and transferred to a Claus plant (Figure 1.3), where it is converted into sulfur. The core of a Claus plant generally consists of a combustion chamber, a waste heat vessel, two or three contact reactors with catalysts, sulfur separators, and sulfur pits. The overall reaction of the Claus process is exothermic, oxygen- and temperature-dependent. The off-gas obtained usually contains a certain amount of remaining sulfur compounds, and since environmental laws limit total sulfur emission, sometimes it is necessary to install additional sulfur recovery units. As a consequence, such processes have high energy or chemical requirements, and therefore, have a high cost. As a general rule, chemical processes are only profitable to treat large flows containing high concentration of sulfurs because they require the construction of expensive tail-gas plants. As an alternative to chemical desulphurization processes, the use of sulfur-oxidizing bacteria has been proposed as a more cost effective way to treat low-H_2S waste streams.

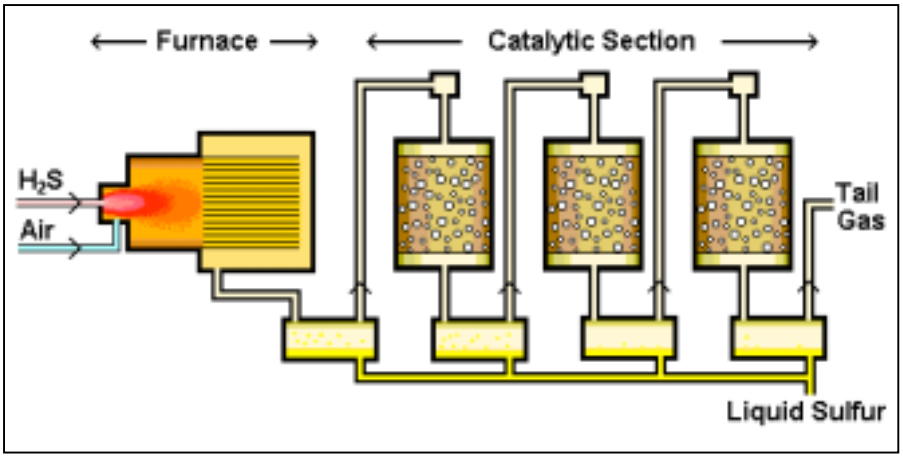

Figure 1.3 Schematic diagram of a Claus plant.

Biological processes operate at ambient temperature and atmospheric pressure, thus avoiding the high costs of heating and pressure generation required by most chemical processes. Some of the biological processes investigated so far involve the use of colorless sulfur bacteria. These microorganisms carry out aerobic oxidation of sulfide through two different reactions:

$$2H_2S + 1/2O_2 \rightarrow 2S^o + 2H_2O$$

$$2H_2S + 4O_2 \rightarrow 2H_2SO_4$$

Aerobic fluidized bed bioreactors have been developed using different species of the genus *Thiobacillus: T. denitrificans, T. thioparus, T. thiooxidans, T. ferrooxidans, T. versutus,* and *T. neopolitanus* (Sublette and Sylvester 1987; Buisman et al. 1989; Tanji et al. 1989; Buisman et al. 1990; Candenhead and Sublette 1990; Cho et al. 1992; Jensen and Webb 1995; Hartikainen et al. 2001).

Phototrophic sulfur bacteria have been proposed as an alternative method for the treatment of sulfide-containing effluents (Cork et al. 1983; Kim et al. 1990) which avoid the requirement of constant oxygen supply. These bacteria perform the anaerobic transformation of sulfide into elemental sulfur and sulfate, using light as energy source according to the following reaction (van Niel 1931):

$$CO_2 + 2H_2S \rightarrow CH_2O + 2S^o + H_2O$$

$$3CO_2 + 2S^o + 5H_2O \rightarrow 3CH_2O + 2H_2SO_4$$

$$4CO_2 + 2H_2S + 4H_2O \rightarrow 4CH_2O + 2H_2SO_4$$

Different genera of purple and green sulfur bacteria perform this reaction. Although illumination is required, the use of photosynthetic microorganisms presents certain advantages: no additives for the oxidation of H_2S are required, energy requirements are

lower, and little or no H_2S escapes to the atmosphere during the treatment making the process odorless.

In particular, the green sulfur bacterium *Chlorobium limicola* has been extensively studied in relation to its possible use in sulfide oxidation processes. This microorganism presents high tolerance to sulfide and low light requirements as the main advantages (Fischer 1988). Initially, Cork et al. (1983) developed a system in which sulfide was removed from acid gases by *Chlorobium limicola* f. *thiosulfatophilum* and elemental sulfur was produced. Acid gas (3% H_2S) was treated in a reactor containing *Chlorobium* and up to 99.9% of the influent H_2S was initially stripped. However, the accumulation of oxidized sulfur compounds in the reactor decreased, and even suppressed, the oxidation of H_2S.

Kobayashi et al. (1983) succeeded in developing a reactor system to remove sulfide from anaerobic waste treatment effluent using also phototrophic green bacteria. They constructed a "phototube" in which biofilms of *Chlorobium limicola* grew, but the percentage of sulfide removed varied between 87-95% depending on the conditions. Despite the fact that a relatively high concentration of residual sulfide remained in the effluent, this study showed that biological anaerobic sulfide removal from waste treatment effluents was a feasible process.

Overall, the biocatalytic removal of H_2S offers the following advantages over conventional physicochemical processes: (i) no chemical additives; (ii) lower energy requirements; (iii) no need for a tail gas plant; (iv) no sulfur waste disposal; (vi) no corrosion problems and (vii) it is a less expensive process. Moreover, biological systems produce elemental sulfur and microbial biomass as end products, which can have further applications (Fischer 1988).

However, biological systems developed up to now present also some drawbacks and, although several of them have been studied at laboratory scale, very few have been tested on a large scale (Jensen and Webb 1995). A common problem in many bioreactors is biomass washout. Most of the systems use suspended cells growing in stirred reactors which can be easily washed out whenever environmental disturbances affect growth rate. For instance, if the oxidation activity decreases, build-up of sulfide can occur inside the reactor. Hydrogen sulfide is toxic at high concentrations even for sulfide oxidizers and exerts an inhibitory effect on growth (K_i=2-4 mM; van Gemerden 1984), which usually causes further

inactivation of the organisms in the reactor. Cells stop growing further, and as a result, the biomass is completely washed out from the reactor.

Another drawback associated to biological systems is their lack of stability. Most sulfide-oxidizing reactors are based on pure cultures or artificial mixes of microorganisms with strict requirements for growth, which make the system unstable and do not guarantee their long-term performance.

Finally, in continuous systems production of an effluent with low levels of sulfide is not guaranteed because this event depends on the physiological state and activity of the cells. For instance, a change in light irradiance may result in a decrease of sulfide-oxidizing activity, and therefore, in an increase in the concentration of sulfide remaining in the effluent.

Utilization of fixed-biomass reactors colonized by complex microbial consortia can be considered as an alternative approach to solve some of the problems mentioned above, as has indeed been shown for other bioremediation processes.

2. BIOFILMS IN BIOREMEDIATION PROCESSES

The ability of aggregates, flocs and biofilms to remain stable in front of environmental changes makes such complex structures good candidates for the development of bioremediation processes. Biofilms are defined as attached accumulations of microbial cells encased in extracellular polymeric substances (EPS) (Characklis and Wilderer 1989). Their thickness ranges from a few cell layers to a few centimeters and they can develop on almost any surface exposed to water. Biofilms are complex structures where the formation of chemically suitable microhabitats and the spatial distribution of different microorganisms permit metabolic synergies among them. Within the biofilm, microorganisms themselves produce and maintain chemical conditions that favor the growth of specific populations that otherwise might not survive.

Microbial biofilm reactors are currently used in wastewater treatment plants, where microorganisms are responsible of carbon and nutrient removal (reviewed by Wagner and Loy 2002). Both the microbial diversity and the structure of these biofilms have been analyzed in detail to understand the main factors influencing the bioremediation process and

to develop new strategies in order to improve removal efficiency. Bacterial diversity in these biofilms is relatively high. Studies carried out in different treatment plants reported a range from 17 to 268 bacterial species (Wagner and Loy 2002). Members of almost all bacterial phylogenetic groups (ε -, ε -, ε -, ε -, and ε-Proteobacteria, *Cytophaga-Flavobacterium-Bacteroides* (CFB), Firmicutes (Low G+C Gram-positives), Actinobacteria (High G+C Gram-positives), Nitrospira, Verrumicrobia, Planctomyces, Chlorobi, Chloroflexi, Fibrobacteres and Fusobacteria) have been reported in wastewater treatment plants. Filamentous bacteria (different members belonging to Actinobacteria, Firmicutes, Chloroflexi, Bacteroidetes, Planctomyces and Proteobacteria) are responsible for organic matter removal. Ammonium and nitrite oxidizers (*Nitrosomonas* and *Nitrobacter*) are responsible for nitrogen removal whereas phosphorous is mainly removed by polyphosphate-accumulating organisms (*Rhodocyclus*).

In addition to organic matter and nutrient removal application, biofilms have been studied for toxic compounds elimination, such as polycyclic aromatic compounds (Massol-Deyá et al. 1997; Radianingtyas et al. 2003) or mercury (Wagner-Döbler 2003). Biofilms systems are especially well suited for the treatment of slowly biodegrading compounds, due to their high microbial biomass and their ability to immobilize compounds by biosorption, bioaccumulation or biomineralization (Gadd 2000). It has also been reported that biofilms are less sensitive to toxicity and more efficient for degrading toxic compounds than suspended biomass systems (Lee 1994). Moreover, microbial diversity increases efficiency of biofilms because it provides a reservoir of species with complementary ecological niches that increase bioreactor performance under changing conditions (Von Canstein et al. 2002).

In this work, a system for the treatment of sulfide, which avoids some of the disadvantages of systems described previously, is proposed. A fixed-biomass reactor based on the use of complex microbial consortia has been developed. The design of the system guarantees the formation of an effluent with low-sulfide concentration even under changing conditions. Natural microbial communities from a sulfurous lake and from microbial mats were used as starters for the development of the complex biofilms. Biofilms were studied in terms of activity but also in terms of diversity, using both classical and molecular approaches.

3. STUDYING MICROBIAL DIVERSITY

In contrast to animals and plants, the morphology of microbes is, in general, too simple to serve as a basis for a sound classification and to allow for a reliable identification. Until recently, microbial identification required the isolation of pure cultures followed by multiple physiological and biochemical tests. The methodology was cumbersome and, as a result, only about 5000 species have been described until now. Comparison of the percentage of cultivable bacteria with total cell counts from different habitats showed enormous discrepancies (summarized by Amann et al. 1995). Therefore, other approaches complementing traditional microbiology were needed to understand microbial diversity and its role in ecosystems. Actually, during the last twenty years, molecular methods have been introduced and are now more and more frequently used to the explore microbial diversity and to analyze the structure of microbial communities without the need of cultivation (reviewed in Head et al. 1998).

In 1977 Woese and Fox published their pioneer paper on microbial phylogeny through the comparison of the rRNA genes, causing a deep revolution among scientists. Through comparative analysis of rRNA sequences they postulated that all organisms belonged to one of three major domains, further named Bacteria, Archaea and Eukarya (Woese et al. 1990) (Figure 1.4). Comparison of rRNA sequences also allowed the establishment of the major divisions within the Bacteria and Archaea domains.

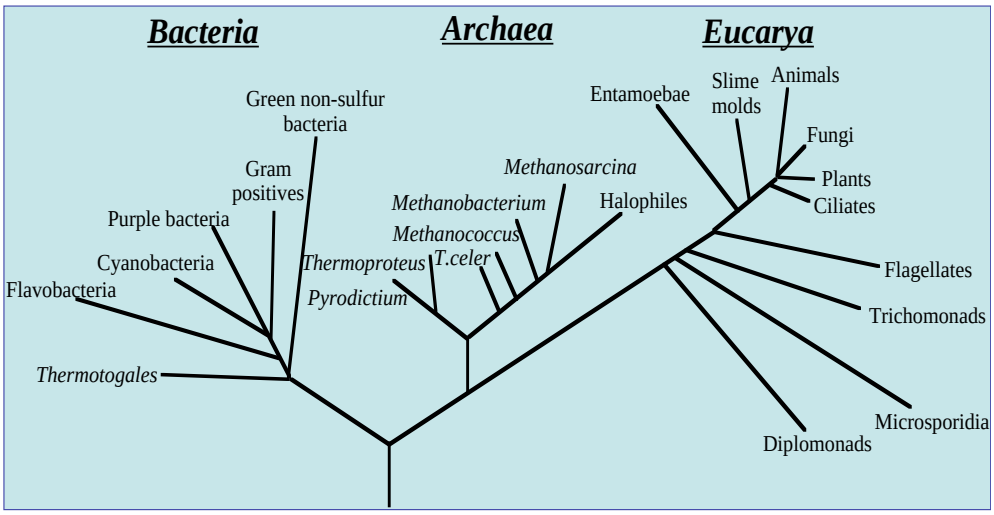

Figure 1.4. Phylogenetic classification of the organisms in three domains: Bacteria, Archaea and Eukarya (from Woese et al. 1990).

The ribosomal RNA (rRNA) has several advantages in front of other biomarkers studied (elongation factors, ATPase subunits, *rec*A) that make it a good molecular clock (Ludwig and Klenk 2001). Ribosomal RNA is a very ancient molecule present in all living species and organelles conserved in structure and function. The rRNA gene sequence changes slowly enough to provide information over the entire evolutionary spectrum. The conservation in secondary structure helps for an accurate sequence alignment (Ludwig and Schleifer 1994). It comprises highly conserved regions interspersed with more variable regions. Such variable regions allow the identification of sequence motifs of increasing phylogenetic resolution. Furthermore, the large size of the molecule minimizes statistical fluctuations. Finally, because rRNA genes are relatively easy to sequence, a large and continuously growing public database is available (www.ncbi.nlm.nih.gov) (Figure 1.5).

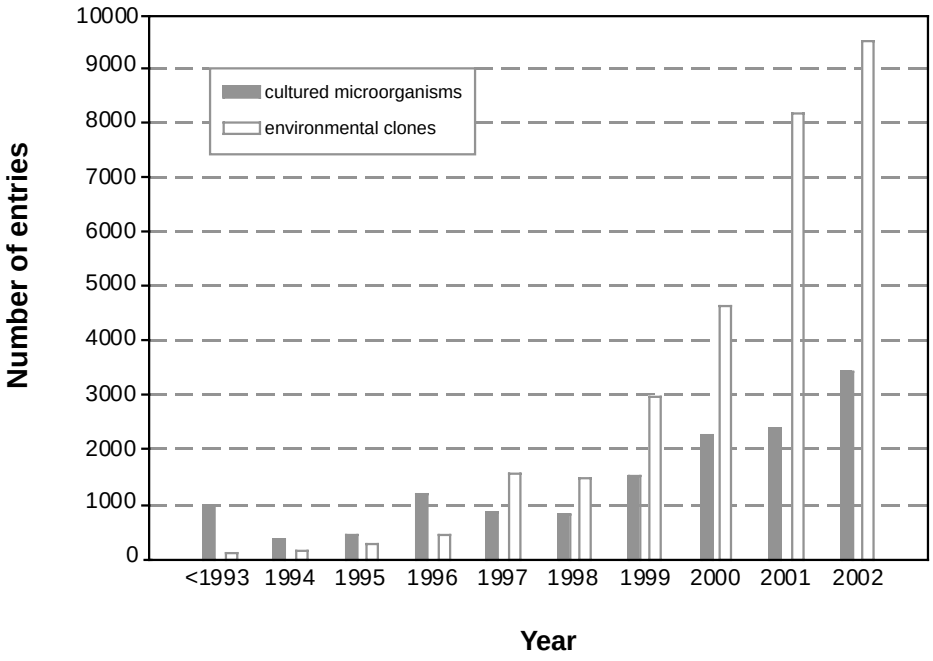

Figure 1.5. Graphs depicting the number of 16S ribosomal RNA gene sequences published in GenBank since 1993. Total number of published 16S rRNA gene sequences from cultivated Bacteria and Archaea (n = 14.434) versus sequences derived from cultivation-independent studies (n = 29.505) as a function of year. All sequences published prior to 1993 are grouped in the first (<1993) column, while the "2002" column includes sequences published through November 19, 2002. As there is currently no standard convention in GenBank for naming or identifying rRNA gene sequences obtained from cultivation-independent studies, the values reported here should be considered indicative rather than absolute. (from Rappé and Giovannoni 2003).

The revolutionary work of Woese and coworkers not only provided a new classification of organisms, it also allowed the development of molecular biology tools for the identification of uncultured microorganisms. The combination of Woese's new phylogeny with molecular biology yield what is now recognized as molecular microbial ecology.

"The rRNA approach"

Microbial community analysis based on the study of 16S rRNA genes circumventing cultivation was called "the rRNA approach". An outline of several procedures commonly used is represented in Figure 1.6. Total DNA is extracted from the complex natural sample and then 16S rRNA genes are amplified by PCR. Amplicons can be either ligated in a vector and cloned in *Escherichia coli* or separated by electrophoresis (e.g. denaturing gradient gel electrophoresis). The 16S rRNA genes are sequenced and compared with databases to yield information about their identity. RNA sequences from clones or from cultured organisms are used to design specific oligonucleotide probes to specifically target the retrieved sequences in the natural sample by whole cell *in situ* hydridization (FISH).

The PCR-clone-sequencing approach "

The development of simple DNA cloning techniques and the polymerase chain reaction (PCR) have allowed high-resolution diversity analysis. The presence of universally conserved regions in the 5' and 3' ends of the 16S rRNA gene (Figure 1.7) allows the amplification of nearly the complete gene from the extracted genome.

The starting point of this procedure is the extraction of high quality DNA to permit the activity of *Taq* polymerase. The extracted DNA is subject to PCR amplification, which can be performed at different taxonomic levels (i.e. domain, family) by using "universal" or "specific primers" (e.g. Cyanobacteria). To construct the clone library, the PCR product is ligated into a vector and used to transform *E. coli* cells. Cells containing the vector are selected and clones are screened to unveil the different 16S rRNA genes present in the library. For the screening, different methods are available. The most used is the extraction of the plasmid, PCR amplification of the insert and digestion of the amplification product with

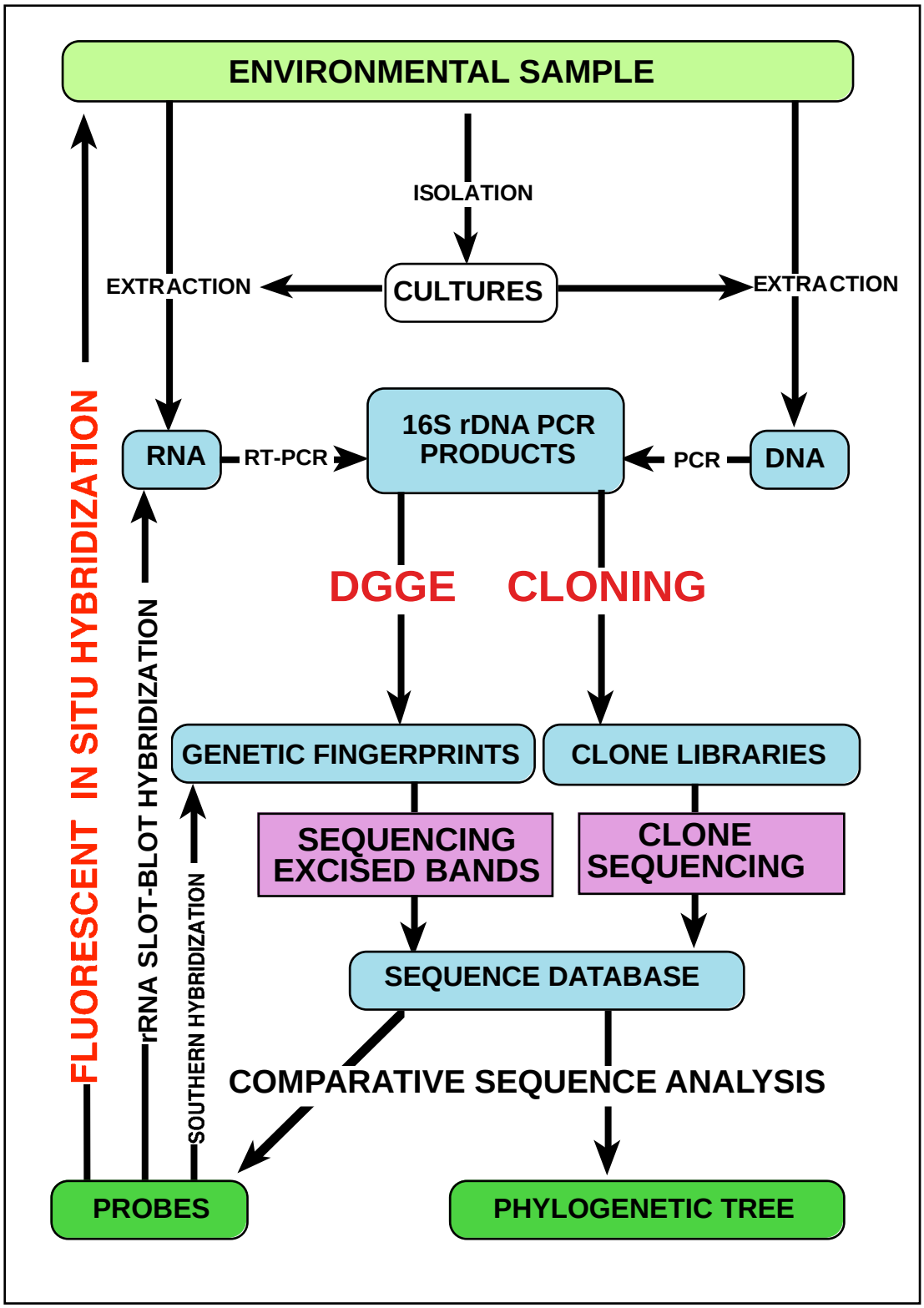

Figure 1.6. Molecular biology techniques commonly used in microbial ecology.

endonucleases. As an alternative to plasmid extraction, direct PCR from colony using sequencing primers flanking the DNA insert is used. Then, the restriction is separated in agarose gels and the pattern of the different clones is compared. Clones with different restriction pattern are selected for sequencing and their sequences are submitted to a database such as GenBank from The National Center for Biotechnology Information, which contains all the available sequences from any gene and any organism. Phylogenetic analysis can be done and the diversity of the microbial populations determined with reference to previously published sequences (Maidak et al. 2000).

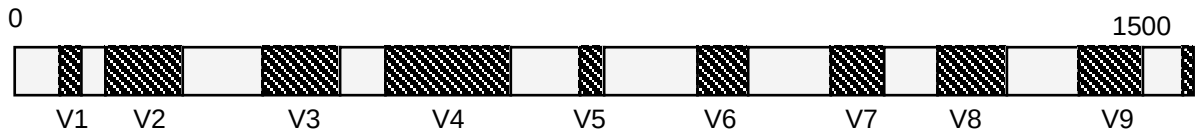

Figure 1.7. Variable regions of the 16S rRNA gene.

Fingerprinting analysis

Although "PCR-clone-sequencing" is a successful approach, it is focused on extensively exploring the microbial diversity in a particular sample. Clone libraries are not appropriate to compare multiple samples because they are time-consuming, labor intensive and expensive. Thus, temporal or spatial dynamics of microbial communities should be studied using other alternatives.

Genetic fingerprinting techniques provide information for the genetic diversity of a microbial community and allow rapid and easy simultaneous comparison of different samples. Several fingerprinting techniques have been developed: lmwRNA (Low molecular weight RNA; Höfle 1988), ARDRA (Amplified ribosomal DNA restriction analysis; Martínez-Murcia et al. 1995), T-RFLP (Terminal restriction length polymorphism; Marsh et al. 2000), DGGE (Denaturing gradient gel electrophoresis; Muyzer et al. 1993), TGGE (Temperature gradient gel electrophoresis; Muyzer and Smalla 1998) or SSCP (Single strand conformation polymorphism; Lee et al. 1996).

The most successful fingerprinting technique until now has been the denaturing gradient gel electrophoresis (DGGE). It was initially described by Fischer and Lerman (1979) to detect punctual DNA mutations, and later was introduced to microbial ecology studies by Muyzer et al. (1993). DGGE is a method in which DNA fragments with the same length but differing in sequence can be separated in high resolution polyacrylamide gels. Separation is based on the decreasing mobility of a partially melted double-strand DNA fragment through a denaturing gradient (urea and formamide) (Figure 1.8). Melting point depends on the DNA sequence, and therefore, fragments differing in sequence will have different migration behavior and will be separated in the gel. Thus, a heterogeneous mixture of PCR amplicons yields a complex band pattern. Band profiles can be visualized under UV light by staining the gel with Ethidium Bromide or SYBR gold among others. Individual bands can be excised, reamplified and sequenced, or challenged with a range of oligonucleotide probes, to give an indication of the composition and diversity of the microbial community. Moreover, fluorescent probes can be designed from DGGE band sequences to target *in situ* specific populations (Casamayor et al. 2002).

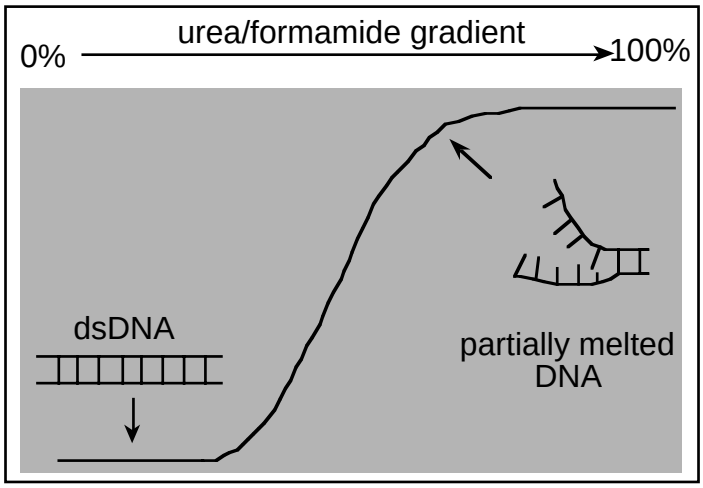

Figure 1.8. Principle of DNA separation in DGGE (Denaturing Gradient Gel Electrophoresis).

DGGE is relatively rapid to perform, and many samples can be run simultaneously (Figure 1.10). It has been successfully applied to study community complexity and temporal and spatial community variations, to monitor the enrichment and isolation of bacteria, to detect microheterogeneity in rRNA encoding genes, to compare different DNA extraction protocols,

to screen clone libraries and to determine PCR and cloning biases (Muyzer and Smalla 1998).

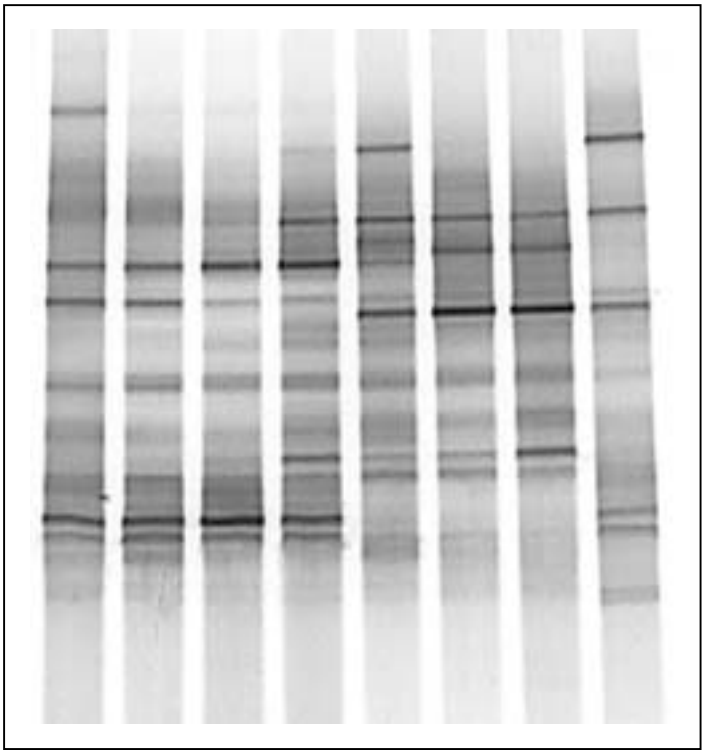

Figure 1.9. Image of DGGE gel containing bacterial 16S rRNA fragments.

Whole cell *in situ* hybridization

Whole cell *in situ* hybridization is based on the use of oligonucleotides probes to detect the rRNA within intact cells. Probes can be designed with different degree of specificity ranging from a group of microorganism to a certain strain. The most common procedure used is the hybridization of whole cells with fluorescent-labeled probes (Fluorescent *in situ* hybridization-FISH) (Figure 1.11). The procedure involves the fixation of the sample usually with paraformaldehyde to permeablize cells and maintain their integrity. Cells attached to a gelatin-coated microscope slide or in solution are immersed in hybridization buffer containing the labeled probe. The sample is then incubated to allow the entrance of the oligonucleotide probe and the binding to its complementary rRNA sequence. After washing, the sample is observed by epifluorescence microscopy. Cells containing the probe can be visualized and

counted. Total cells are dyed with DAPI (4',6'-diamino-2-phenylindole), and through comparison between DAPI-stained cell counts and hybridized cell counts, the contribution of a certain group in the sample can be quantified. Due to the single cell resolution, this method is usually used in quantification analysis as well as in the analysis of the spatial distribution of populations.

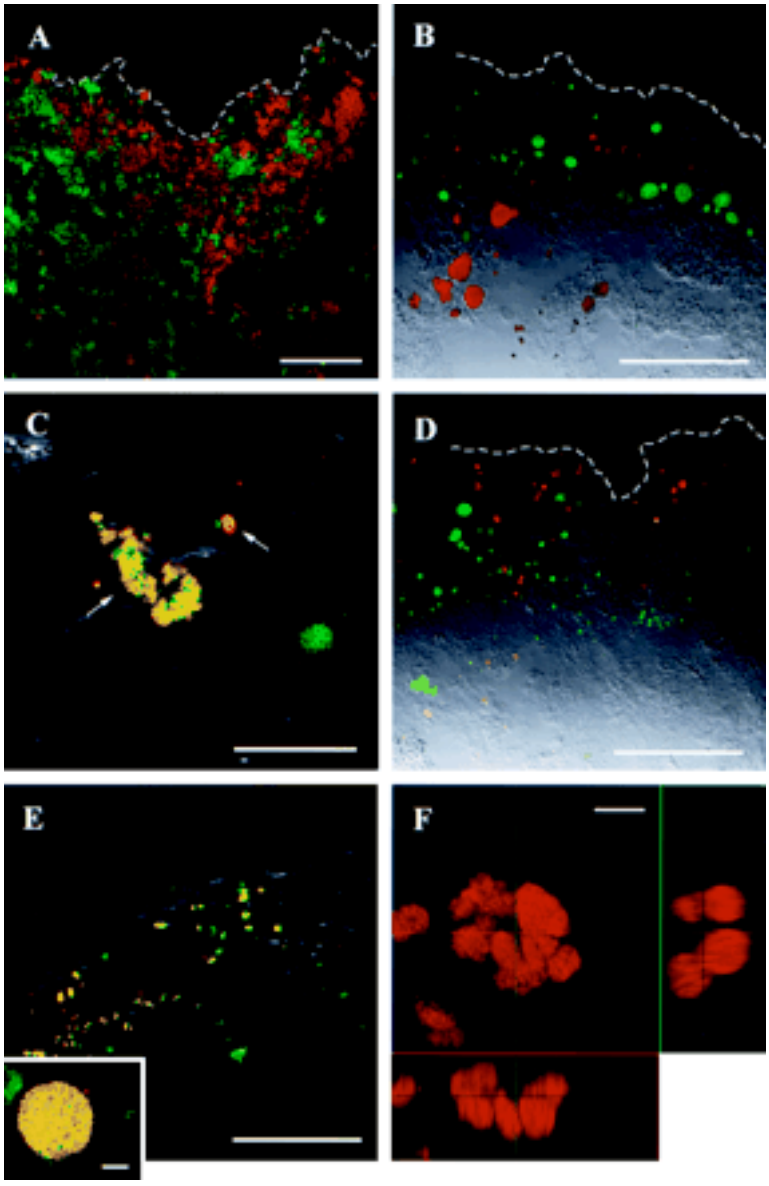

Figure 1.10. Confocal laser-scanning micrographs of vertical thin sections of biofilm as hybridized with different fluorescent oligonucleotide probes. Scale bars are 50 µm (A, B, D, and E), 25 µm (C), and 5 µm (insert in panels E and F), respectively. Dashed lines indicate the surface of the biofilm exposed to the wastewater (from Gieseke et al. 2001).

Limitations of molecular methods

While we have undoubtedly gained much new and valuable knowledge of the microbial world using DNA-based molecular techniques, they are not free of biases and limitations that must be minimized and, at the very least, taken into account when conclusions are drawn. The limitations relate to the extraction of nucleic acids from samples, biases and artifacts associated with enzymatic amplification of the nucleic acids, cloning of PCR products, and sensitivity and target site accessibility in whole-cell hybridization techniques (Von Wintzingerode et al. 1997; Head et al. 1998).

Nucleic acid extraction

A major limitation of most methods, with the exception of hybridization techniques, is the unbiased recovery of DNA from samples. Cells are lysed to extract the DNA, and it is known that some cells are more resistant than others. For instance, Gram-positive cells are more resistant than Gram-negatives to lysis. Although evaluation of the extraction efficiency can be done only in comparative terms because we do not know the total amount of DNA present in a sample, biases can minimized for instance by checking through microscopy the presence of intact cells before and after the extraction. There are many methods of DNA extraction (Fuhrman et al. 1988; Tsai and Olson 1991) but their efficiency depends on the type of sample; it is possible that the same lysis technique may give different results with water, sediment or soil samples. It has been demonstrated that by combining physical and chemical treatments, approximately 96% of cells, even bacterial endospores, are lysed in soil (Moré et al. 1994). It was noted, however, that smaller cells (0.3-1.2 µm) were more resistant to lysis. However, other works have found that even without harsh physical treatments such as bead beating, up to 99.8% lysis can be obtained (Rochelle et al. 1992), although this required long incubations. The selection of the most appropriate method for each sample is a critical factor for the subsequent analyses.

PCR and cloning

Another potential source of biases is the polymerase chain reaction (PCR). Amplification may be selective to certain sequences, introducing bias in the measures of diversity. It is generally accepted that more abundant sequences are preferentially amplified while less

abundant sequences are discriminated (Ward et al. 1992). It has been further suggested that high percent G+C templates are discriminated against due to lower efficiency of strand separation during the denaturation step of the PCR reaction (Reysenbach et al. 1992). Moreover, small differences in the sequence of primer target may result in selective amplification of some sequences particularly when annealing occurs under high stringent conditions. Also, the number of copies of the 16S rRNA gene, which can vary from 1 to 14 (Cole and Girons 1994; Nübel et al. 1996), can affect the results. Although these potential biases can modify the correlation between the number of copies in the PCR and the abundance of a sequence in a sample, other authors have found a good ratio (Suzuki and Giovannoni 1996). Moreover, there are some methodological considerations that can minimize biases and errors. All samples must be amplified in the same PCR reaction and the number of cycles in PCR must be under 35 because according to the kinetic model, when the number of cycles increases there is a tendency for the different amplicons to reach equimolarity (Suzuki et al. 1998). Anyway, the quantitative interpretation of the results needs to be cautious and we can never confidently extrapolate sequence composition of a clone library to a quantitative population composition of environmental samples.

Another problem intrinsic to PCR amplification is the formation of chimeric rDNA products where fragments from two different sequences become fused during the amplification process. Chimeric sequences may be interpreted as additional diversity, but in general, chimeric clones are found at a low percentage (Amann et al. 1995). Fortunately, chimera biases can be minimized with computer algorithms, such as the Check_Chimera option in the Ribosomal Data Project (RDP; Maidak et al. 1996).

Whole cell in situ hybridization

Although whole cell hybridization techniques are not affected by PCR related biases, there are other methodological constraints that must be considered. Permeability of the cell is the first required step to allow the entry of the probe into the cell. Usually, paraformaldehyde is used to permeabilize the membrane but in some cases, such as in Gram-positive bacteria, additional treatments with solvents, acid or enzymes are required (Amann et al. 1995). A good compromise between cell permeability and cell integrity should be found. Even when permeability is achieved, probe hybridization with RNA is not guaranteed. Non-complementarity of probe and target, non-optimal hybridization conditions or non-

accessibility of the probe to the target site can be the reasons (Amann et al. 1995; Fuchs et al. 1998). Furthermore, labeled probes will not yield a signal strong enough to be visualized if cells are not metabolically active, and therefore do not contain a large number of ribosomes. However, this problem can be avoided by using a new technique known as CARD-FISH (Catalyzed reporter deposition and fluorescence *in situ* hybridization; Pernthaler et al. 2002) in which horseradish peroxidase (HRP)-labeled oligonucleotide probes are used to amplify the fluorescent signal.

4. STRUCTURE OF THE THESIS

In this work we design a new bioreactor for the treatment of sulfide-containing effluents and characterize the microbial populations present in the developed biofilms. The system was initially tested with a pure culture of a green sulfur bacterium and later with natural samples from two different environments, i.e., freshwater sediment from sulfurous lake and a marine microbial mat, in order to compare the effect of the inoculum in the final community composition of the biofilms. The work presented here has been divided into the following chapters:

Chapter 2. Description of the methodology used in this work.

Chapter 3. Development of a fixed-biomass reactor to allow the establishment of complex biofilms able to keep a low residual sulfide concentration under changing conditions. The system has been tested with a pure culture and with a complex biofilm developed from a natural sample.

Chapter 4. Evaluation of the 16S rRNA molecular methods used to analyze the microbial diversity present in the biofilm. Different DNA extraction methods were used and their suitability was tested by DGGE (Denaturing Gradient Gel Electrophoresis), cloning and sequencing.

Chapter 5. Development of a sulfide-oxidizing biofilm from sediment of a sulfurous lake. The microbial composition is determined by microscopy, pigment analysis and DNA molecular methods.

Chapter 6. Development of a sulfide-oxidizing biofilm from a microbial mat sample. The microbial composition is determined by microscopy, pigment analysis and DNA molecular methods.

Chapter 7. General discussion of the results.

Chapter 2. MATERIALS AND METHODS

MATERIALS & METHODS

1. BIOREACTOR DESIGN AND OPERATION

Bioreactor design

The system was composed of a stirred vessel and a packed column (Figure 2.1). The stirred vessel (1.8 l volume) was kept in the dark and the content was recycled through the column by means of a peristaltic pump (Watson Marlow 313S) at a constant flow rate of 0.1 $l \cdot min^{-1}$. The column (15.6 mm inner diameter, 275 mm length, 52.5 ml total volume) was packed with glass rings (2 mm diam, 6 mm length), which provided a large surface for microbial attachment (325 cm^2). The glass column was constantly illuminated by two opposed incandescent light bulbs and had a double wall through which water from a refrigerated bath circulated and kept temperature constant at 25ºC. The stirred vessel provided headspace for the insertion of pH and redox sensors, the sampling port, the effluent collector and the inlet of synthetic medium and pH-adjusting solution.

Bioreactor operation and control

The system described above operated as a sulfidostat (Figure 2.2). Bioreactor regulation was based on the system previously described by Sánchez et al. (1996). The control of the process was based on the redox potential, which was mainly determined by the concentration of hydrogen sulfide and, ultimately, by biological activity. In this system, the speed of the peristaltic pump (Watson Marlow 501U) introducing fresh synthetic medium into the reactor was proportional to the oxidation activity of the biofilm and, therefore, hydrogen sulfide concentration was kept at a constant level.

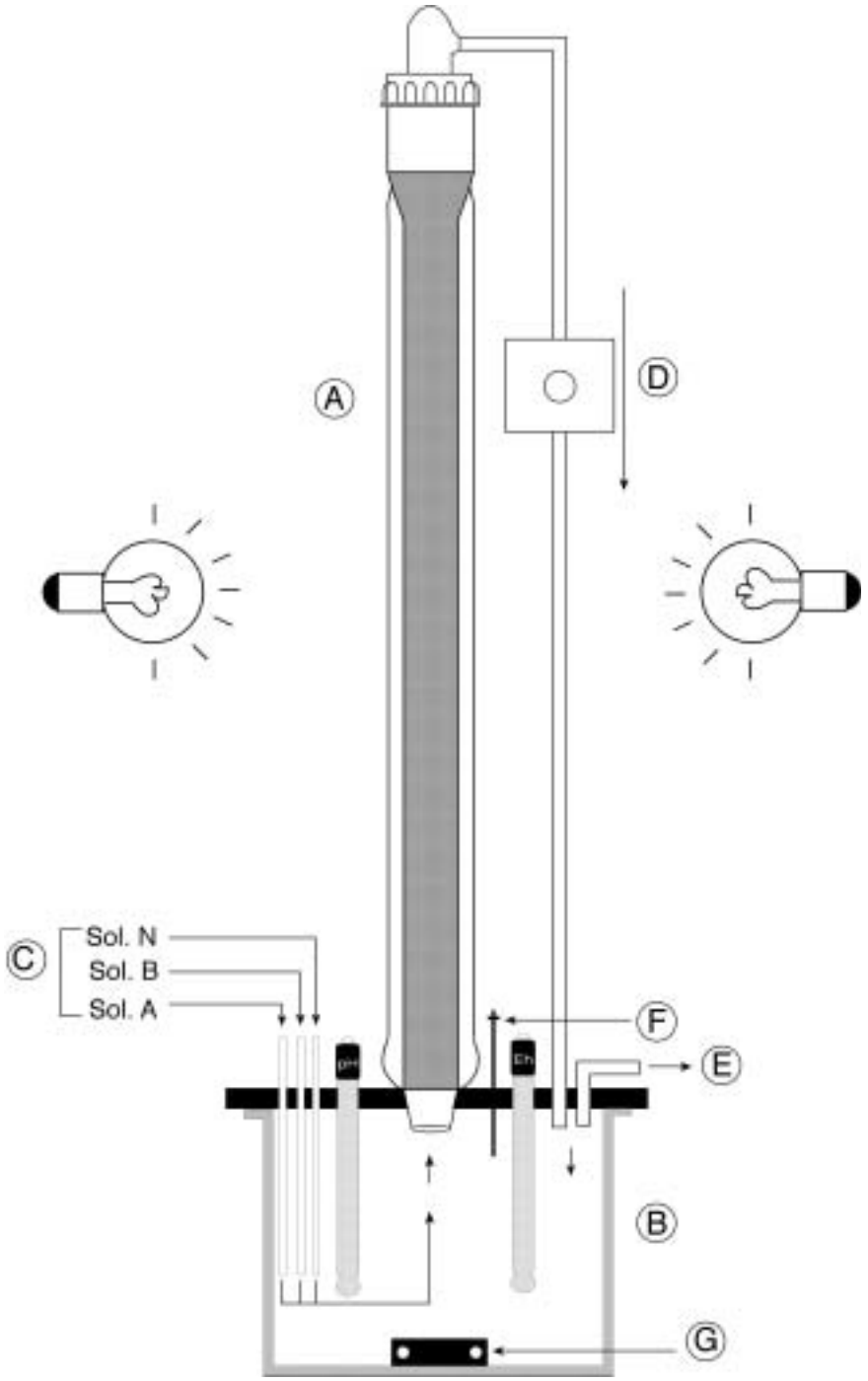

Figure 2.1. Bioreactor design. ***A*** Column, ***B*** stirred vessel, ***C*** medium and acid solutions input, ***D*** recycling pump, ***E*** effluent, ***F*** sampling port, ***G*** magnetic stirrer.

At start up, the reactor was inoculated and the medium was recycled through the column in a closed loop. As a sulfide-oxidizing biofilm developed, the concentration of hydrogen sulfide in the reactor progressively decreased. When this decrease reached a preset value (in our case 50 µM), pumping of the medium into the reactor started at a rate controlled by the redox titrator (Crison 52-61) which was proportional to the rate of sulfide oxidation of the biofilm.

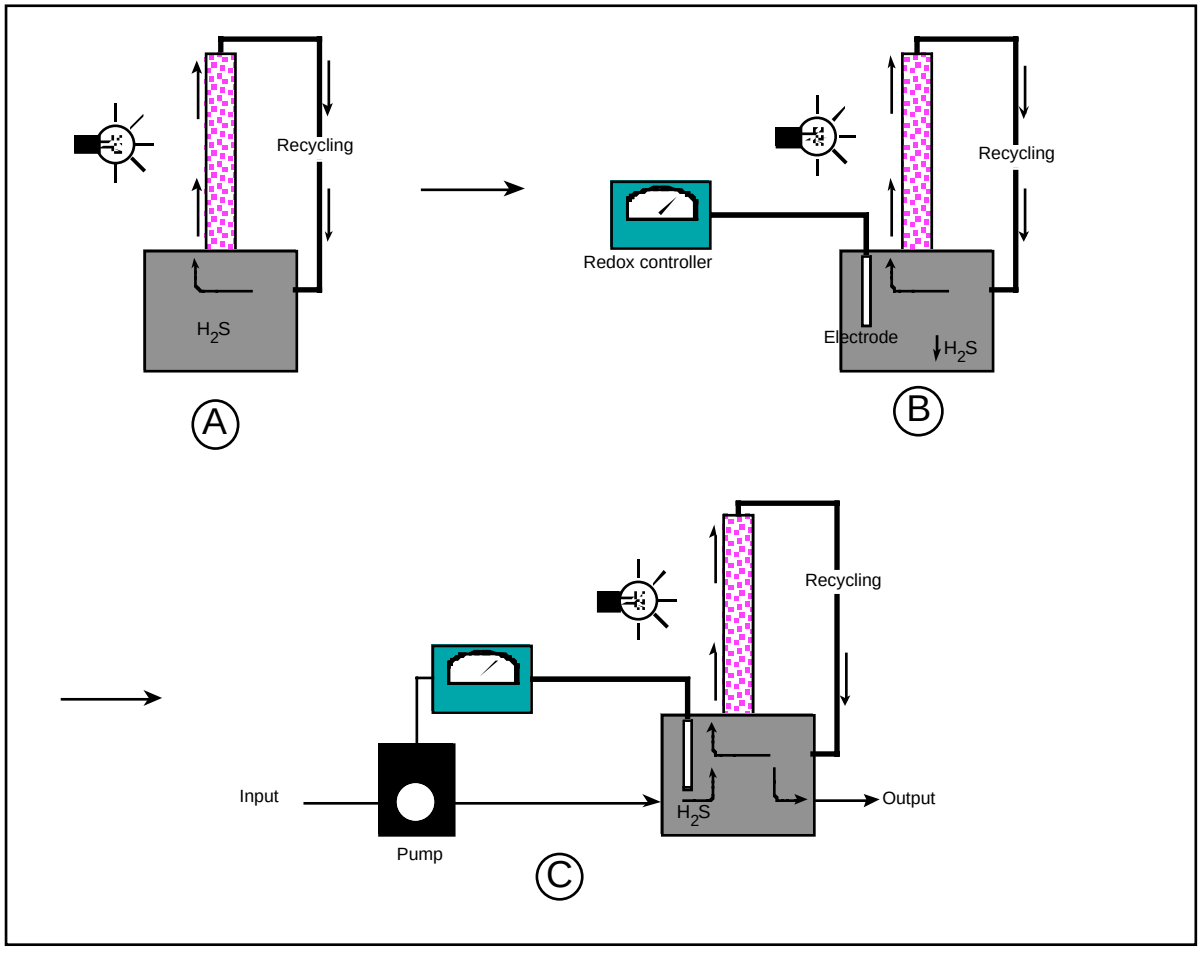

Figure 2.2 Bioreactor operation and control system. (**A**) At start, the medium is recycled in a closed loop. (**B**) A biofilm develops, sulfide concentration decreases and this decrease is detected by a redox electrode. (**C**) When the decrease reached a preset value, redox controller modulates the speed of the pump which introduces medium into the reactor.

2. INOCULA AND GROWTH CONDITIONS

The system was first tested with a pure culture of a phototrophic bacterium and, later on, with natural samples from two different sulfurous environments: Lake Estanya and Ebro Delta microbial mats.

The strain used for the first test was the green sulfur bacterium *Chlorobium limicola* UdG 6008 (kindly provided by Dr. Borrego, University of Girona, Spain).

Lake Estanya (41º 17' N, 2º 04' E) is a karstic monomictic sulfurous lake (Figure 2.3) located in Huesca (Spain) (Guerrero et al. 1987). The lake is stratified during summer and autumn and mixed the rest of the year (Àvila et al. 1984). During the stratification period, blooms of phototrophic sulfur bacteria have been described (Guerrero et al. 1987). Sampling was carried out in February, when the lake was oxygenated and well mixed and sulfide was detected only in the sediment. Sediment was collected, kept at 4ºC in the dark, carried to the lab and used to inoculate the bioreactor the same day.

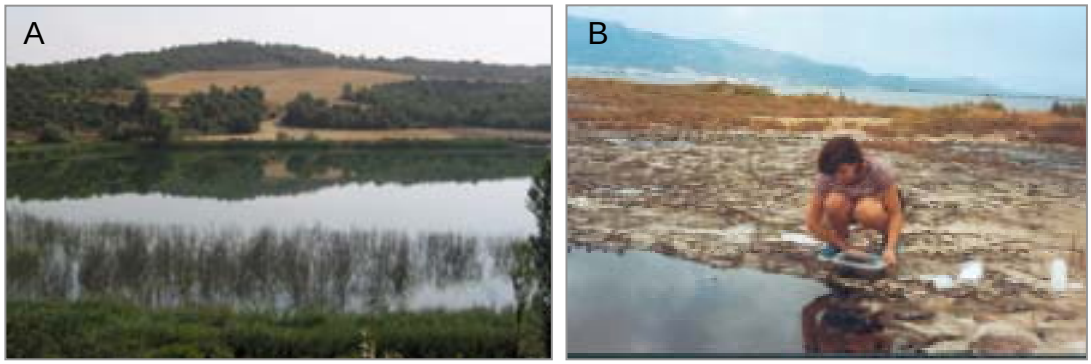

Figure 2.3. Lake Estanya (**A**) and Ebro Delta microbial mats (**B**).

Microbial mats on the sand flats of Ebro Delta are well-studied benthic communities located in Tarragona (Spain) (Mir et al. 1991; Martínez-Alonso 1997). These communities have been analyzed so far using classical methods and recently with DNA-based molecular biology methods (Ramírez-Moreno et al. 2003). These communities are stratified and contain different colored layers dominated by oxygenic phototrophs (diatoms and cyanobacteria),

anoxygenic phototrophs (purple and green sulfur bacteria) and heterotrophs (sulfate reducing bacteria) among others. Microbial mats are exposed to changing conditions of light, temperature or salinity. Moreover, alternation of flooding and desiccation periods occurs constantly. Sampling was carried out in July and the mats were partially inundated. A 15x15 cm piece of mat was excised from the sediment surface (Figure 2.3), kept at 4ºC in the dark, carried to the lab and used to inoculate the bioreactor the same day.

A synthetic medium based on the inorganic sulfide-containing medium described by Van Gemerden and Beeftink (1979) for anaerobic phototrophic bacteria was used in order to simulate a sulfide-polluted effluent. Medium composition is detailed in Table 2.1. This medium contains carbonate as the only carbon source and hydrogen sulfide as electron donor, and it was prepared as two separate solutions. One of them was alkaline (pH 12) and contained carbonate and sodium sulfide. The second solution was acidic (pH 5) and contained the rest of the components. The two solutions were pumped at equal rates into the reactor resulting in a slightly alkaline medium. Final pH adjustment was carried out with an independent tritator (Cole Parmer pH/ORP controller 5652-10) connected to a pump (Masterflex 7554-60), which added a 1 M HCl solution at the rate required to maintain a constant pH of 6.8-7.2

Sulfide load and light irradiance were changed during the experiments in order to study the response of the bioreactor under changing conditions, as well as, its performance during long working periods. Different sulfide loads were established by changing the concentration in the alkaline reservoir solution (S_r). Two opposed 60W incandescent light bulbs provided incident irradiance which was set at 50, 100 or 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ by changing the distance between the column and the light source. Specific S_r and light conditions for each experiment are listed in Table 2.2. The concentration of residual sulfide in the reactor was programmed at 50 µM. In order to establish anoxic conditions all the components of the system were kept under oxygen-free nitrogen at a pressure of 0.1 bar. The system was completely filled to avoid abiotic losses of sulfide to the gas phase. At start up, the reactor was inoculated through the sampling port and the recycling pump was switched on. This was considered time 0 of the experiment.

Table 2.1. Composition of the medium used.

Compound	Amount
NH_4Cl	300 mg
K_2HPO_4	300 mg
$CaCl_2.2H_2O$	225 mg
$MgCl_2.6H_2O$	200 mg
KCl	200 mg
Na_2-EDTA	3 mg
$FeSO_4.7H_2O$	1.1 mg
$CoCl_2.6H_2O$	1.9 mg
$MnCl_2.4H_2O$	0.5 mg
$ZnCl_2$	0.42 mg
$NiCl_2.6H_2O$	0.24 mg
$Na_2MO_4.2H_2O$	0.18 mg
H_3BO_3	3 mg
$CuCl_2.2H_2O$	0.02 mg
Na_2CO_3	2000 mg
$Na_2S.9H_2O$	240 mg (1mM)
Vitamina B_{12}	0.02 mg
NaCl*	37g
pH	6.8-7.2
H_2O	1000 ml

*Only in the experiment carried out with a marine microbial mat sample.

Table 2.2. Conditions of light irradiance and sulfide load for the different experiments.

Inoculum	**Light irradiance $\mu E \cdot m^{-2} \cdot s^{-1}$**	**Sr µM**
Chlorobium	50	1000
	100	1000
	150	1000
	150	2000
	150	3500
Lake Estanya	50	800
	50	2300
	100	2300
Microbial mat	100	1250

3. SAMPLING AND ANALYSIS

Values of redox, pH, pumping rate and sulfide concentration were recorded periodically during the experiment.

Sulfide

Sulfide was measured by the method of Pachmayr (Pachmayr 1960) as described by Trüper and Schlegel (1964). Two-ml samples taken directly from the stirred vessel were fixed with 10 ml of zinc acetate. Then 10 ml of DPDS (N,N-dimethyl-phenilendiamine sulfate) and 1 ml of ammonium ferric sulfate were added, yielding as end-product methylene blue. Water was added up to 25 ml, and the solution was measured by spectrophotometry at 670 nm. Sulfide concentration was calculated as follows:

$$H_2S\ (mM) = A_{670} \cdot 0.027 \cdot \left\{ \frac{V_t}{V_s} \right\} \cdot f_d^{-1}$$

V_t: total volume (chemicals, sample and water)

V_s: volume of sample analyzed

f_d^{-1}: dilution factor

Biofilm sampling

At the end of the experiments we recovered the biofilms for further characterization. The column packing was emptied in a glass beaker, immersed in saline solution (0.9% sodium chloride) and sonicated in an ultrasonic water bath (Bransonic 5, Branson) for a period of three minutes. After sonication, the volume was brought up to a total of 1000 ml and kept in agitation for 1 hour. Part of this volume was split in 10-ml aliquots, which were then centrifuged 20 min at 8000 g (Beckman J2-21). The pellets were stored at –20ºC for further pigment and protein analysis. At the same time, 500 µl-aliquots were centrifuged 5 min at 12000 g (Eppendorf 5415C) for further DNA extraction. The pellets were then stored at –80ºC until analyses were carried out. Other aliquots were diluted and fixed in formaldehyde (2% final concentration) for microscopic counts.

Pigment analysis

Pigments were measured by spectrophotometry in organic solvent extracts as described by Stal and coworkers (1984). Samples were extracted with methanol in the dark and shaken with hexane in a separator funnel. The method allowed the simultaneous estimation of chlorophyll *a*, and bacterioclorophylls *a, c, d, e*. Figure 2.4 shows a scheme of the procedure followed. Concentrations of these compounds were calculated using the absorption coefficients provided by the authors (Table 2.3).

$$\text{Pigment (mg/l)} = \frac{K \cdot 1000 \cdot V_{extract}}{A \cdot V_{sample}}$$

K : Maximum absorbance value

A : Absorption coefficient of each pigment (see Table 2.3)

$\mathbf{V_{sample}}$**:** Volume of sample analyzed

$\mathbf{V_{extract}}$**:** Volume of solvent extract

Table 2.3. Absorption coefficients for the pigments present in the biofilms (from Stal et al. 1984).

Pigment	Absorption coefficients
Bchl *a*	84.1
Bchl *c*	86
Bchl *d*	82.3
Chl *a*	74.5

Protein analysis

Proteins were determined by the method of Lowry (Lowry et al. 1951) after extraction of the cell pellet with methanol in order to remove elemental sulfur that could interfere in the

analysis, and subsequent solubilization of the samples in 1 M sodium hydroxide for 10 min at 100ºC (Herbert et al. 1971).

Microscopy analysis

Fresh samples were directly observed by phase-contrast microscopy in order to identify cells with distinctive morphological traits. Purple sulfur bacteria, colorless sulfur bacteria, and green algae were identified on the basis of their conspicuous morphological features (Canter-Lund and Lund 1995; Brock 1999; Imhoff 2003).

Fixed cells were stained with DAPI (4',6'-diamino-2-phenylindole; Porter and Feig 1980) and counted with an epifluorescence Olympus BH microscope, following previously described statistical recommendations (Kirchman et al. 1982). At least 300 cells were counted in a minimum of 10 different microscopy fields.

Sulfide oxidation rates

Sulfide oxidation rate (S_{ox}) was calculated from the pumping flow rate (F), the concentration of sulfide in the fresh medium (S_r) and the concentration of sulfide in the effluent (S), according to the following equation:

$$\mathbf{S_{ox} = F \cdot (S_r - S)}$$

Specific sulfide oxidation rate (µmols·mg protein^{-1}) was calculated at the end of the experiment from the final sulfide oxidation rate and the total biomass recovered from the column, expressed as proteins.

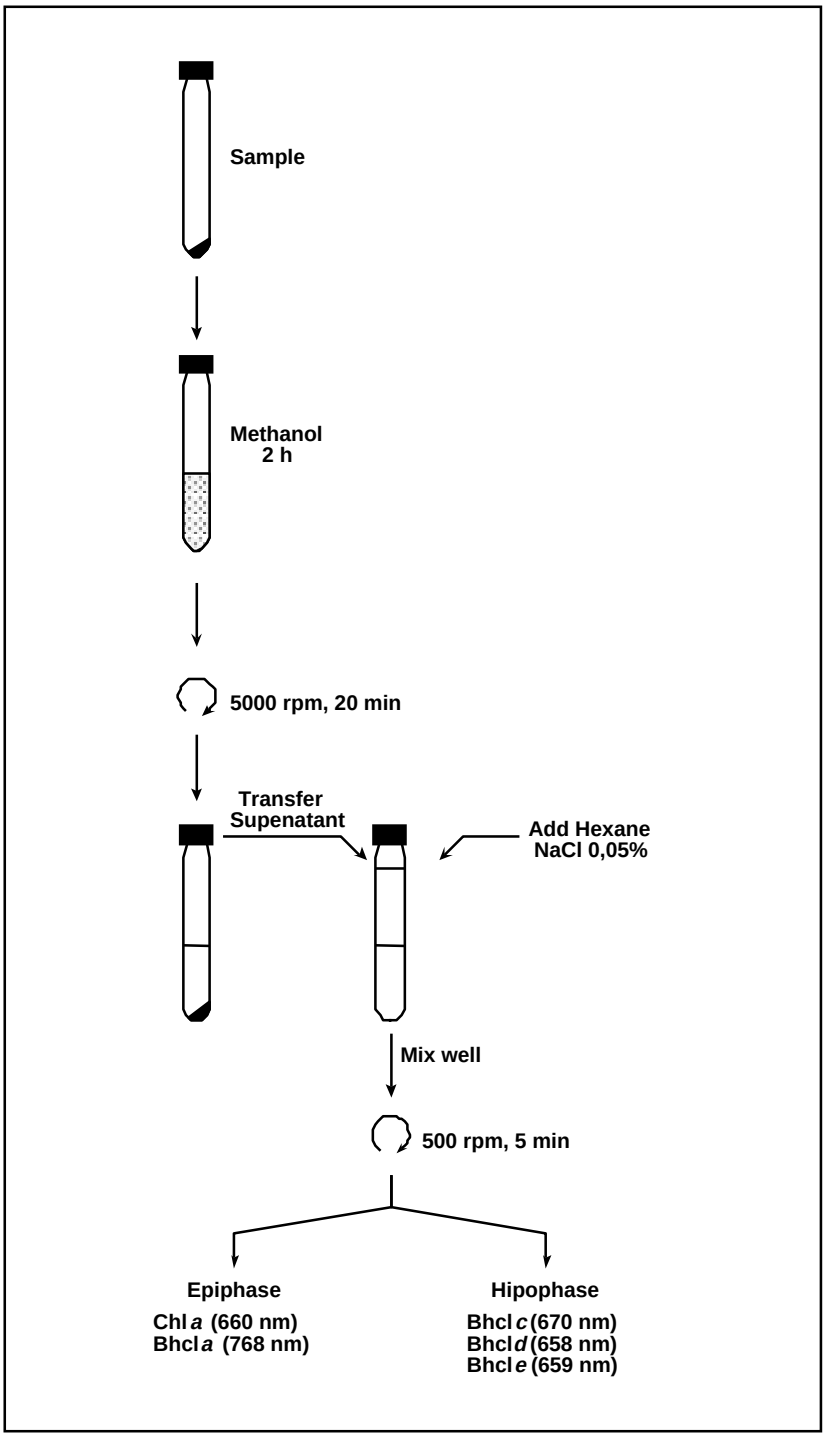

Figure 2.4. Scheme of the procedure followed for the pigment analysis.

4. DNA EXTRACTION AND PCR AMPLIFICATION

DNA extraction methods

Different DNA extraction methods were assayed in this work (see also Chapter 4). Samples collected from the biofilm developed from lake sediment (Chapters 3 and 5) were resuspended in 1 ml of lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose), divided into 100 µl equal samples and each aliquot was tested with a different combination of treatments (Figure 2.5). Biofilm developed from the microbial mat sample was subject to enzymatic lysis (lysozime and proteinase K) after bead-beating followed by phenol-chloroform extraction.

Detailed DNA extraction procedures:

Freeze-Thaw (from Bej et al. 1991):

A sample of biofilm was resuspended in 100 µl of lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose). The mixture was frozen in a cold ethanol bath (-20ºC) for 1 min and then thawed by transferring the tube to a 50ºC water bath for 1 min. The procedure was repeated 6 times and the sample was vortexed vigorously for 15 s every freeze-thaw cycle. After that, the sample was heated at 85ºC for 5 min in a thermal cycler to inactivate proteases and nucleases. The sample was then cooled at room temperature, spun for 10 seconds and directly used as template for PCR or used for subsequent treatments.

Bead-Beating:

A biofilm aliquot was mixed with 500 µl of lysis buffer and with 0.1 g of glass beads (150 µm diameter). The sample was bead-beaten for 3 cycles of 80s and kept 30 s in an ice-bath after each cycle. Afterwards, the lysate was collected by centrifugation (Eppendorf 5417R; 13000 rpm, 1 min) and the supernatant was transferred to a new tube and used to follow with subsequent extraction procedures.

CTAB:

CTAB solution (3% CTAB [cetyltrimethylammonium bromide], 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 8.0) was preheated at 60ºC. The

biofilm sample was added and the solution was gently swirled and incubated 30 min at 60ºC with vortexing every 10 min. Chloroform-isoamyl alcohol (24:1, vol:vol) was finally added. The mixture was centrifuged 10 min at maximum speed and the aqueous phase recovered.

Chemical lysis with lysozyme and proteinase K:

Lysozyme (1 mg·ml^{-1} final concentration) was added to the biofilm resuspended in lysis buffer and the sample was incubated at 37ºC for 45 min in slight movement. Then, sodium dodecyl sulfate (1% final concentration) and proteinase K (0.2 mg·ml^{-1} final concentration) were added and the sample was incubated at 55ºC for 60 min under slight movement. The lysate was used then, to follow with the standard phenol-chloroform extraction method.

Phenol-Chloroform extraction:

Nucleic acids were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, vol:vol:vol). An equal volume of phenol was added to the lysate, carefully mixed and centrifuged 10 min at maximum speed (Eppendorf 5417R; 14000 rpm). Aqueous phase was recovered and the procedure was repeated. Finally, an equal volume of choloroform-isoamyl alcohol (24:1, vol:vol) was added to the recovered aqueous phase in order to remove residual phenol. The mixture was centrifuged and the aqueous phase was recovered for further purification.

TESC method (from Garcia-Pichel et al. 2001):

Biofilm sample was resuspended in lysis buffer and 1 ml of TESC buffer was added (1% CTAB, 1.5 M NaCl, 100 mM EDTA, 100 mM Tris-HCl pH 8.0). The sample was frozen in liquid nitrogen, and then thawed at 65ºC (2 min) in a water bath three times. Then, 5 ml of TESC buffer, sodium dodecyl sulfate (1% final concentration) and proteinase K (0.3 mg·ml^{-1} final concentration) were added and the tube was incubated at 60ºC for 20 min. After that, 5 ml of phenol-chloroform-isoamyl alcohol (25:24:1, by vol) were added and centrifuged 6 min at 3000 g. The aqueous phase was recovered and the phenol phase was re-extracted with 5 ml TESC buffer. Finally, both aqueous phases were pooled and an equal volume of phenol-chloroform-isoamyl alcohol was added and placed in a water bath at 65ºC for 5 min. Extraction at this temperature leads to removal of CTAB together with polysaccharides. After

centrifugation (6 min at 5000 rpm), the aqueous phase was recovered and extracted once with choloroform-isoamyl alcohol (24:1, vol:vol).

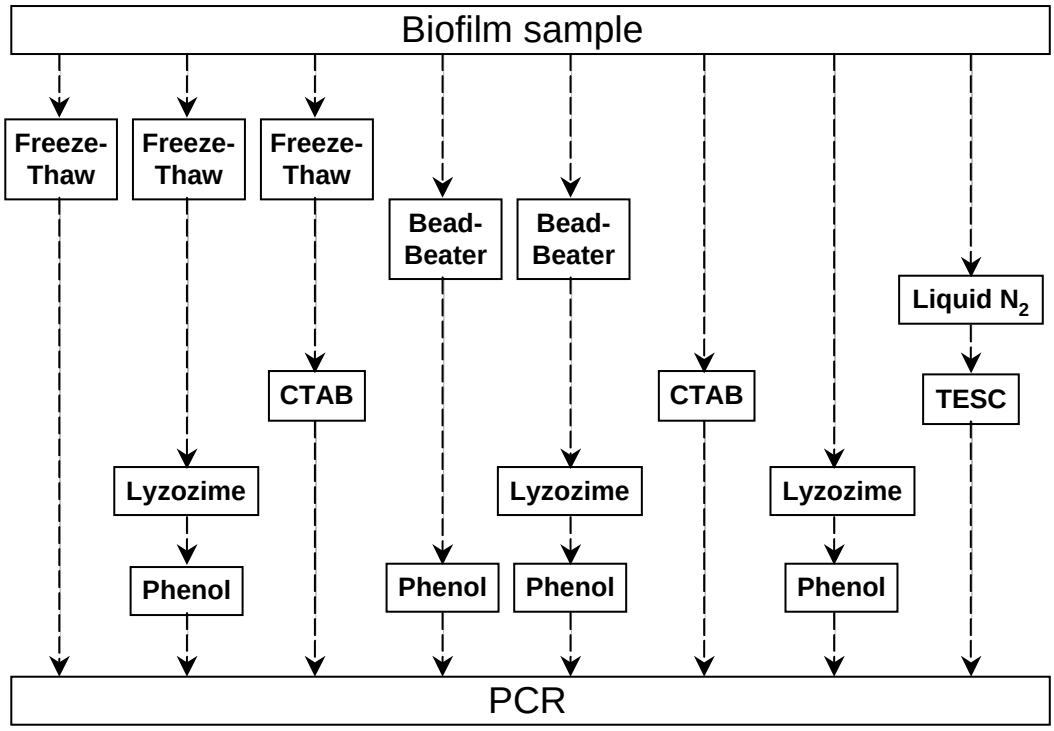

Figure 2.5. Scheme of the different combination of methods used for the extraction of DNA.

DNA purification

Nucleic acids were purified, desalted and concentrated with a Centricon-100 concentrator (Millipore). Only the aqueous phase obtained from the freeze-thaw treatment was directly used for PCR without purification. DNA integrity was checked by agarose gel electrophoresis, and DNA was quantified using a Hoeschst dye fluorescence assay (Paul and Myers 1982).

5. DENATURING GRADIENT GEL ELECTROPHORESIS

Fragments of the bacterial 16S rRNA genes suitable for DGGE analysis were amplified by using the bacterial specific primer 358F with a 40 bp GC-clamp, and the universal primer 907RM (Muyzer et al. 1998) (Table 2.4). We used primers 344F with a 40 bp GC-clamp and 915R (Stahl and Amann 1991; Raskin et al. 1994) to amplify 16S rRNA archaeal genes from biofilm samples but all attempts failed.

Table 2.4. Primers used in this work for amplifying members of the *Bacteria* and *Archaea* domain.

Primer name	Use	Specificity	Sequence (5' -> 3')
358F-GC*	DGGE	Bacteria	CCT ACG GGA GGC AGC AG
907RM	DGGE	Universal	CCG TCA ATT CMT TTG AGT TT
344F-GC*	DGGE	Archaea	ACG GGG YGC AGC AGG CGC GA
915R	DGGE	Archaea	GTG CTC CCC CGC CAA TTC CT

***GC clamp** (5' -> 3'): CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G

Most of bacterial PCR reactions were performed using the program described in Table 2.5. However, by using this cycle, spurious priming occurred during amplification of samples from the experiment carried out with the microbial mat, yielding as a result the appearance of unspecific products. To circumvent this problem, different modifications were tested and finally, the annealing temperature of the PCR cycle was changed. In these PCR reactions, annealing temperature in touchdown cycles decreased from 66 to 56ºC, and in standard cycles it was 56ºC.

To amplify archaeal sequences we used the conditions described in Casamayor et al. (2000). A touchdown protocol for 20 cycles with temperatures ranging from 71 to 61ºC was used; the annealing temperature was reduced 1ºC every two cycles. This procedure was followed by 15 additional cycles at an annealing temperature of 61ºC. Except for the initial

denaturation step (94ºC, 5 min), denaturation and annealing phase steps were 1 min long (the only exception was the final cycle, which was 10 min long).

Table 2.5. PCR cycle used to amplify bacterial 16S rRNA gene fragments suitable for DGGE.

Step		Temp.	Time
Initial denaturation (1x)		94ºC	5 min
Touchdown cycles (20x)			
	Denaturation	94ºC	1 min
	Annealing	65-55ºC*	1 min
	Extension	72ºC	3 min
Standard cycles (10x):			
	Denaturation	94ºC	1 min
	Annealing	55ºC*	1 min
	Extension	72ºC	3 min
Final extension (1x)		72ºC	5 min

*Annealing temperatures were 66-56ºC in touchdown cycles and 56ºC in standard cycles in amplification of biofilm samples from the experiment carried out with the microbial mat.

PCR mixtures contained 1-10 ng of template DNA, each deoxynucleoside triphosphate (dNTPs) at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. BSA (Bovine Serum Albumin) was added to minimize the inhibitory effect of humic substances (Harry et al. 1999). Final concentration of BSA ranged between 150 and 600 µg/ml depending on the purity of the DNA template. Volume of reactions was 50 µl. Finally, PCR products were verified and quantified by agarose gel electrophoresis (1%) with a low DNA mass ladder standard (Invitrogen).

DGGE was run in a D-Code system (Biorad) as previously described (Muyzer et al.1998). A 1 mm thick 6% polyacrylamide gel with a gradient of DNA-denaturant agent was cast by mixing solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and

40% deionized formamide). Different denaturing gradients were tested (20-70%, 30-70% and 40-80%) (Table 2.6). Polyacrylamide polimerization was achieved by adding APS (Ammonium persulfate) and TEMED (N,N,N',N'-Tetramethyl-ethyletilendiamine).

Table 2.6. Volumes used for DGGE gel casting.

	Denaturing concentration					
	0%	**20%**	**30%**	**40%**	**70%**	**80%**
0% (mL)	4	8.2	6.9	5.5	1.4	-
80% (mL)	-	2.8	4.1	5.5	9.6	11
TEMED (µl)	5	10	10	10	10	10
APS (10%) µl	25	60	60	60	60	60

Six hundred ng of PCR product were loaded for each sample and the gel was run at 100 V for 18 h at 60°C in 1xTAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SYBR Gold (Molecular Probes) for 45 min, rinsed with 1xTAE buffer, removed from the glass plate to a UV-transparent gel scoop and visualized in the UV with a digital camera (Kodak DC 120, Invitrogen) or with the Fluor-S MultiImager (Bio-Rad).

DGGE bands were excised from the gel and kept in 20 µl of MilliQ water overnight at 4ºC. Between 1 and 5 µl of the supernatant, depending on DNA concentration, were used for reamplification with the original primer set. PCR product was purified with the High-Resolution Purification kit (Roche) and quantified in agarose gels.

The digitized DGGE image was processed with NIH Image (National Institute of Health, Bethesda, Maryland). The software carried out a density profile through each lane, detected the bands, and calculated the relative contribution of each band to the total band signal in the lane after applying a rolling disk background substraction (rolling ball radious=50). Some of the weakest bands could not be clearly distinguished in the figures presented here, and were only detectable on the computer screen after zooming the image. Bands occupying the same position were identified and a matrix with presence or absence data was built.

6. CLONE LIBRARY CONSTRUCTION AND ANALYSIS

For cloning, almost the entire 16S rRNA bacterial genes were amplified (between positions 27 and 1492, *E. coli* 16S rRNA gene sequence numbering), by using the primers 27F and 1492R (Table 2.7). The PCR cycle is detailed in Table 2.8. The archaeal specific primer 21F and the universal 1492R were used in order to amplify archaeal 16S rRNA genes but PCR reactions did not yield a positive signal in any case. Archaeal reactions were permormed by using the following conditions: an initial denaturation step consisting of 94°C for 3 min, 30 cycles consisting of 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s, and a final elongation step consisting of 72°C for 5 min.

Table 2.7. Primers used in this work for amplifying members of the *Bacteria* and *Archaea* domain.

Primer name	Use	Specificity	Sequence (5' -> 3')
27F	Cloning	Bacteria	AGA GTT TGA TCM TGG CTC AG
21F	Cloning	Archaea	TTC CGG TTG STC CYG CCG GA
1492R	Cloning	Universal	GGT TAC CTT GTT ACG ACT T

PCR mixtures of 50 µl contained 1-10 ng of template DNA, each deoxynucleoside triphosphate (dNTPs) at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. Finally, PCR products were verified and quantified by agarose gel electrophoresis (1%) with a low DNA mass ladder standard (Invitrogen).

The PCR products obtained were ethanol precipitated, and resuspended in 20 µl of sterile water. The PCR product was cloned with the TOPO TA cloning kit (Invitrogen) according to manufacturer's instructions. Four µl of PCR product were mixed with 1 µl of TOPO® vector and with 1 µl of salt solution provided by the manufacturer and incubated for 5 min at 23ºC. The cloning reaction was stopped by placing the tube on ice and, after that, was added to a vial of competent cells of *Escherichia coli* to start transformation. Cells were incubated 30 min on ice and then, heat-shocked for 30 seconds at 42ºC. SOC medium provided by the manufacturer was added and cells were incubated at 37ºC for 1 hour. Transformation

mixture was spread in selective Luria-Bertrani (LB) agar plates containing kanamycin and X-Gal. Putative positive colonies were picked, transferred to a multi-well plate containing LB medium and 7% glycerol, and stored at –80ºC.

Table 2.8. PCR cycle used to amplify the bacterial 16S rRNA gene.

Step		Temp.	Time
Initial denaturation (1x)		94ºC	5 min
Standard cycles (30x):			
	Denaturation	94ºC	1 min
	Annealing	65ºC	1 min
	Extension	72ºC	3 min
Final extension (1x)		72ºC	5 min

Positive clones were grown over night at 37ºC in LB medium. Ten ml of culture were centrifuged and used to extract the recombinant plasmid using the QIAprep spin miniprep kit (QIAgen), following manufacturer's instructions. Purified plasmids were PCR amplified and the product was digested overnight with the restriction endonuclease *Hae*III (Invitrogen) at 37ºC. The product was run in 2.5% low melting point agarose gel at 80V for 3.5 h. A 50-bp ladder (Invitrogen) was also run for comparison between gels and for size band analysis. Gels were analyzed with the NIH Image (National Institute of Health, Bethesda, Maryland) software and all clones with different restriction band pattern were chosen for partial sequencing. Double-stranded plasmids from selected clones were extracted with a QIAprep Kit (QIAgen).

Clone library coverage (**C**) was calculated according to the following equation: $\mathbf{C=1-(}\boldsymbol{n}\mathbf{/}\boldsymbol{N}\mathbf{)}$, where $\boldsymbol{n}$ is the number of unique clones and $\boldsymbol{N}$ is the total number of clones examined (Ravenschlag et al. 1999). To determine whether the clone library was representative of the microbial diversity present in the biofilm, accumulation curves were constructed for the different RFLP patterns and phylotypes. Sequences sharing more than 97% of similarity were grouped as the same phylotype.

7. rDNA SEQUENCING AND PHYLOGENETIC ANALYSIS

Sequencing reactions were performed by QIAgen DNA Sequencing Services (Germany; www.qiagen.com) and "Sistemas Genomicos" Sequencing Services (Valencia, Spain; www.sistemasgenomicos.com). Approximately 10 to 20 ng of PCR product were used for the sequencing reaction of DGGE bands, which were performed using the primer 907RM. For sequencing plasmids, approximately 100 ng of DNA were used and reactions were performed with the primer 27F. Sequences performed in Germany were ran in a ABI PRISM 377 XL lane sequencer, while Spanish services used an ABI PRISM 3100 sequencer.

Sequences were submitted to a BLAST search (Altschul et al. 1997; www.ncbi.nlm.nih.gov/blast/blast.cgi) to get a first indication of the phylogenetic affiliation, and to the Check-Chimera program from RDP (Maidak et al. 2000; www.cme.msu.edu/RDP) to determine potential chimeric artifacts. Sequences were aligned by using the automatic alignment tool of the ARB program package (Ludwig et al. 1998; www.mikro.biologie.tu-muenchen.de). Then partial sequences were inserted into the optimized tree derived from complete sequence data by the ARB program *Quick add using parsimony* tool, which does not affect the initial tree topology. The resulting tree was pruned to save space and only the closest relatives were retained.

Accession numbers

Gene sequences obtained from the biofilms libraries were sent to the EMBL database (www.ebi.ac.uk/embl) and received the following accession numbers:

-Clones from freshwater biofilm: from AJ548890 to AJ548931

-Clones from marine biofilm: from AJ627979 to AJ628015

-DGGE bands excised from the gel comparing the different DNA extraction methods: AJ632076, AJ632077, AJ632078, AJ633126, AJ633127

Chapter 3. A NEW NON-AERATED ILLUMINATED PACKED-COLUMN REACTOR FOR THE DEVELOPMENT OF SULFIDE-OXIDIZING BIOFILMS

This chapter has been accepted for publication in *Applied Microbiology and Biotechnology*

A NEW NON-AERATED ILLUMINATED PACKED-COLUMN REACTOR FOR THE DEVELOPMENT OF SULFIDE-OXIDIZING BIOFILMS

Abstract

This chapter describes an illuminated reactor that allows the spontaneous development of biofilms aimed at the treatment of sulfide-containing streams. The reactor operates as a sulfidostat and is composed of an illuminated packed-column, in which microorganisms are exposed to constant low substrate concentrations, thereby avoiding inhibition due to high sulfide concentrations. The control system allows highly polluted streams to be oxidized by the microbial biofilm while ensuring the quality of the effluent produced. Both monospecies and multispecies biofilms have been developed. Biofilms undergo changes in light irradiance and sulfide load while providing a consistent reduction of the sulfide levels, down to micromolar concentrations. Both types of biofilms developed differ from stirred reactors in that their specific activities are lower, constituting systems with a slow dynamic behavior, and therefore, they are less sensitive to sudden disturbances.

Introduction

Hydrogen sulfide is a toxic compound with an unpleasant smell which can be produced in large amounts during the treatment of organic effluents, in tail-gas from desulphurization plants, or during sulfide-stripping of natural gas. Several chemical treatments have been used for its removal from industrial wastes (Jensen and Webb 1995), but microbiological processes are often considered as a low-cost alternative for sulfide elimination (Janssen et al. 1997). Reactors for this purpose using either aerobic sulfur-oxidizing microorganisms or anoxygenic photosynthetic bacteria have indeed been developed in the past 20 years (Cork et al. 1983; Sublette and Sylvester, 1987; Buisman et al. 1990). Most of the systems proposed use pure cultures growing in suspension in stirred continuous reactors in which, biomass can be easily washed out whenever the specific growth rate decreases due to environmental disturbances, resulting in a net accumulation of hydrogen sulfide. Since this compound exerts an inhibitory effect on sulfide oxidizers (K_i=2-4 mM; van Gemerden 1984), this usually causes further inactivation of the organisms in the reactor, resulting in complete washout of the system.

This problem can be avoided by the utilization of biofilm reactors which, as a rule, provide a more stable performance in front of environmental disturbances (Brown and Gauthier 1993). Early attempts to use biofilms for sulfide removal were carried out by Kobayashi et al. (1983). The authors developed a fixed film system, based on the use of *Chlorobium* sp., able to remove 81-95% of H_2S depending on the retention time. The stability of biofilm reactors can be improved further by using multispecies biofilms, as was shown by von Canstein et al. (2002) in the bioremediation of mercury-polluted effluents.

In this chapter we explore the feasibility of spontaneously developing both monospecies and multispecies biofilms aimed at the treatment of sulfide-containing streams. To this end, we use an experimental setup based on a packed-column reactor operating as a sulfidostat (Chapter 2). Two types of experiments were performed; in the first, the column was inoculated with a pure culture, while in the second we used a complex natural sample. By operating the reactor as a sulfidostat, microorganisms were exposed to constant low concentrations in the micromolar range, thereby avoiding inhibition due to high sulfide concentrations. During the early stages of biofilm development, the feed rate was virtually zero. As the biofilm developed, the system progressively increased the pumping rate,

adjusting it to the sulfide-oxidizing capacity of the biofilm. In both cases, despite fluctuations in environmental conditions, this system managed to keep sulfide concentration inside the reactor below inhibitory levels.

Results

Two experiments were carried out in the bioreactor described in Chapter 2. In one of them, the system was tested using a pure culture of *Chlorobium limicola*. In the other, the development of a complex biofilm was studied by inoculating the system with a sample from the anoxic sediment of a sulfurous lake.

After inoculating the system with a liquid culture of *C. limicola*, colonization of the illuminated column was monitored over time. After 24h, cell attachment was observed as a thin green layer covering the column filling. After 4 days of operation, the whole surface was covered with a thick green biofilm, which persisted until the end of the experiment (24 days). In addition to macroscopic observations, sulfide oxidation rate was also monitored as an index of biofilm development. The results are shown in Figure 3.1. As expected, at start up (t=0) oxidation rate was 0. As time went by and biofilm developed, oxidation rate increased. It reached a stable value 48h after the start of the experiment and remained constant until the next change of conditions.

In the second experiment, the reactor was tested with a natural sample. In this case, the microbial community in the column developed a purple-green color within 8 days (Figure 3.2). Sulfide oxidation rate increased progressively until stable values were reached after approximately eight days (Figure 3.1).

In both experiments, one carried out with *Chlorobium* and the other with a natural inoculum, the biofilms were subject to different conditions of light irradiance and influent sulfide concentration after the system had reached equilibrium. In the *Chlorobium* biofilm, we found that the reactor was able to handle increasing higher concentrations of sulfide in the incoming medium (1000, 2000, 3500 µM) while keeping the concentrations of sulfide in the effluent within the desired range (50 µM).

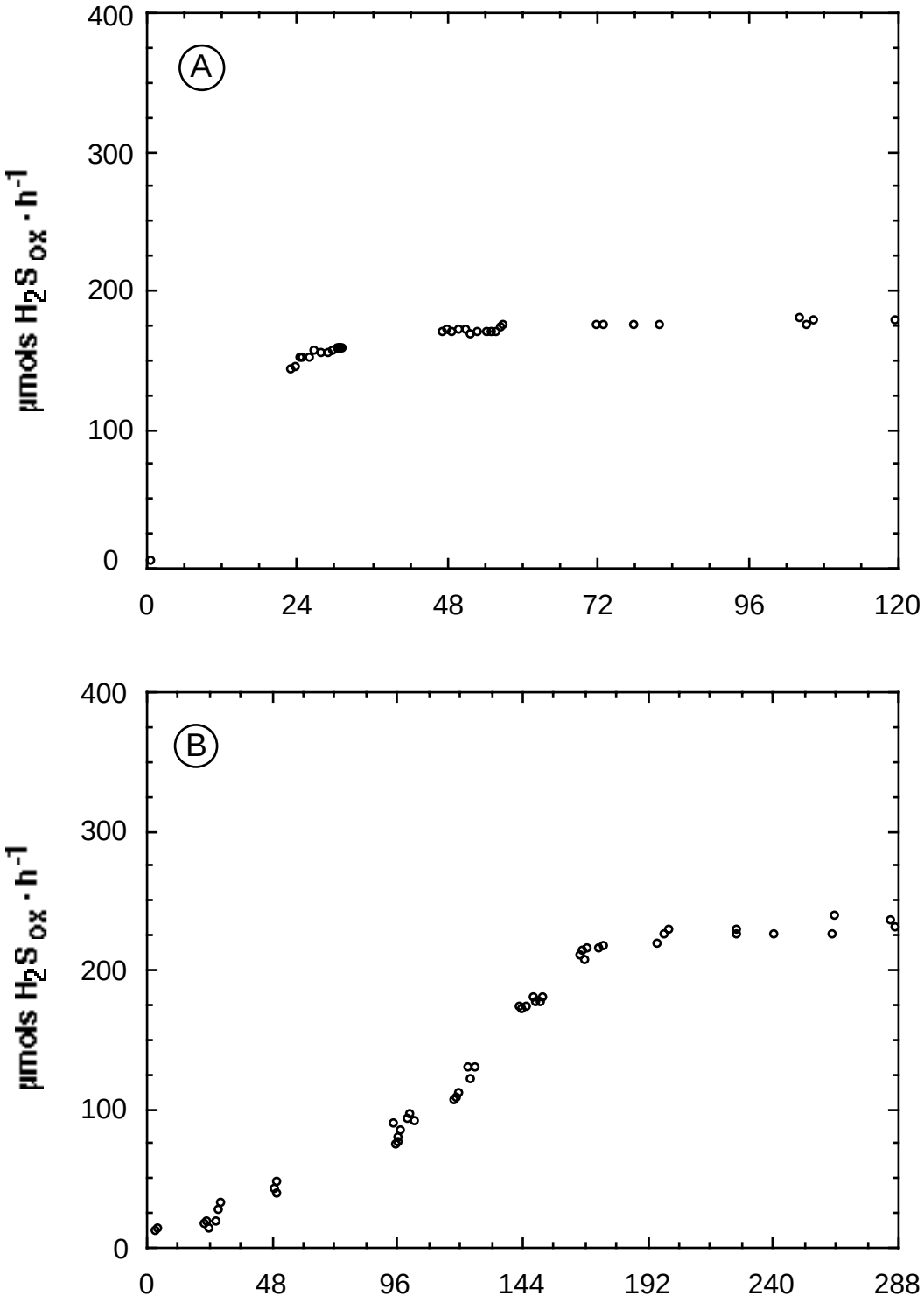

Figure 3.1. Variation of sulfide oxidation rate as a function of time for the *Chlorobium* biofilm (A) and for the biofilm developed from a natural sample (B).

Changing light irradiance (50, 100, 150 $\mu E \cdot m^{-2} \cdot s^{-1}$) did not significantly affect the quality of the effluent, although it had some effect on the rates of sulfide oxidation, which showed a slight decrease (from 180 to 137 $\mu mol \cdot h^{-1}$) at the highest irradiance tested (150 $\mu E \cdot m^{-2} \cdot s^{-1}$), probably because growth of *Chlorobium* (and green sulfur bacteria in general) is better adapted to low-light conditions (Overmann et al. 1992; van Gemerden and Mas 1995). Despite changes in light and incoming sulfide, the ability to reduce the concentration of sulfide and produce stable, low-sulfide effluents was maintained for the length of the experiment (576h).

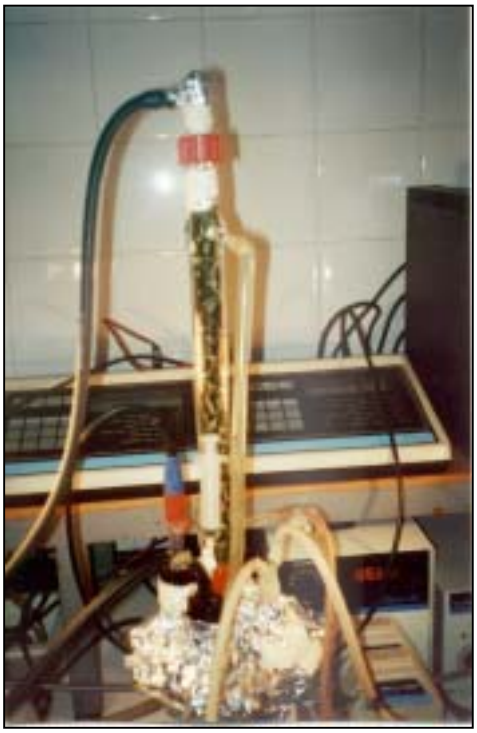

Figure 3.2. Image of the column with the biofilm developed from the lake sediment inoculum.

The biofilm obtained from the inoculum of anoxic sediment responded in a similar manner. In this case, the reactor ran for 32 consecutive days generating and effluent stream with consistently low concentrations of hydrogen sulfide. Increasing the concentration of sulfide in the incoming medium from 800 to 2300 µM, or changing light irradiance from 50 to 100 $\mu E \cdot m^{-2} s^{-1}$, did not result in any significant variations in the sulfide-oxidizing activity, which remained around 200 $\mu mol \cdot h^{-1}$.

After finishing the experiment, the column filling was removed and the biofilms were analyzed. Total counts by epifluorescence microscopy revealed a high density of cells in both cases. The biofilm of *Chlorobium* yielded 1.82×10^{11} cells·cm^{-2} while the density of the complex biofilm was 4.05×10^{11} cells·cm^{-2}. Protein and pigment content are shown in Table 1. Total biomass, expressed as protein, was higher in the biofilm developed from the natural sample than in the pure culture biofilm. However, the specific rate of sulfide oxidation was similar in both cases (see Table 3.1).

Table 3.1. Pigment, protein content and oxidation rates corresponding to *Chlorobium* and Lake Estanya biofilms, and to liquid cultures of *Allochromatium vinosum*. Data have been expressed on a "*per reactor*" basis.

	Pigment	Protein	Oxidation rate	
	mg	mg	µmols $H_2S \cdot h^{-1}$	µmols $H_2S \cdot h^{-1} \cdot mg\ prot^{-1}$
***Chlorobium* biofilm**	61	351	137	0.36
Lake Estanya biofilm	22.4	627	216	0.34
Liquid cultures *Allochromatium vinosum**	52.6-32.8	129.3-405.2	129.3-405.2	2.8-15.9

* Data from Sánchez et al. (1998) corresponding to an equivalent volume of 1.8 l.

The photosynthetic pigments detected were different in each of the biofilms developed. As expected, the biofilm constituted by *Chlorobium limicola* contained bacteriochlorophyll *d*, typical of green sulfur bacteria (Overmann 2000). In the biofilm obtained from the sediment of Lake Estanya, pigment analysis revealed the presence of chlorophyll *a,* characteristic of cyanobacteria (Cohen and Gurevitz 1999) and green algae (Canter-Lund and Lund 1995), as well as bacteriochlorophyll *a* (Imhoff 2003), which is typically found in purple sulfur bacteria. A detailed analysis of the microbial diversity present in this complex biofilm was carried out and results are presented in Chapter 5.

Monitoring the residual sulfide concentration at the reactor outlet showed that despite changes in the sulfide load (S_r) or light irradiance (Figure 3.3), the concentration of residual sulfide remained at 50 µM for the length of the experiment. The percentage of sulfide reduction ranged over 97-99.5% in the different experiments, depending on the sulfide load (S_r).

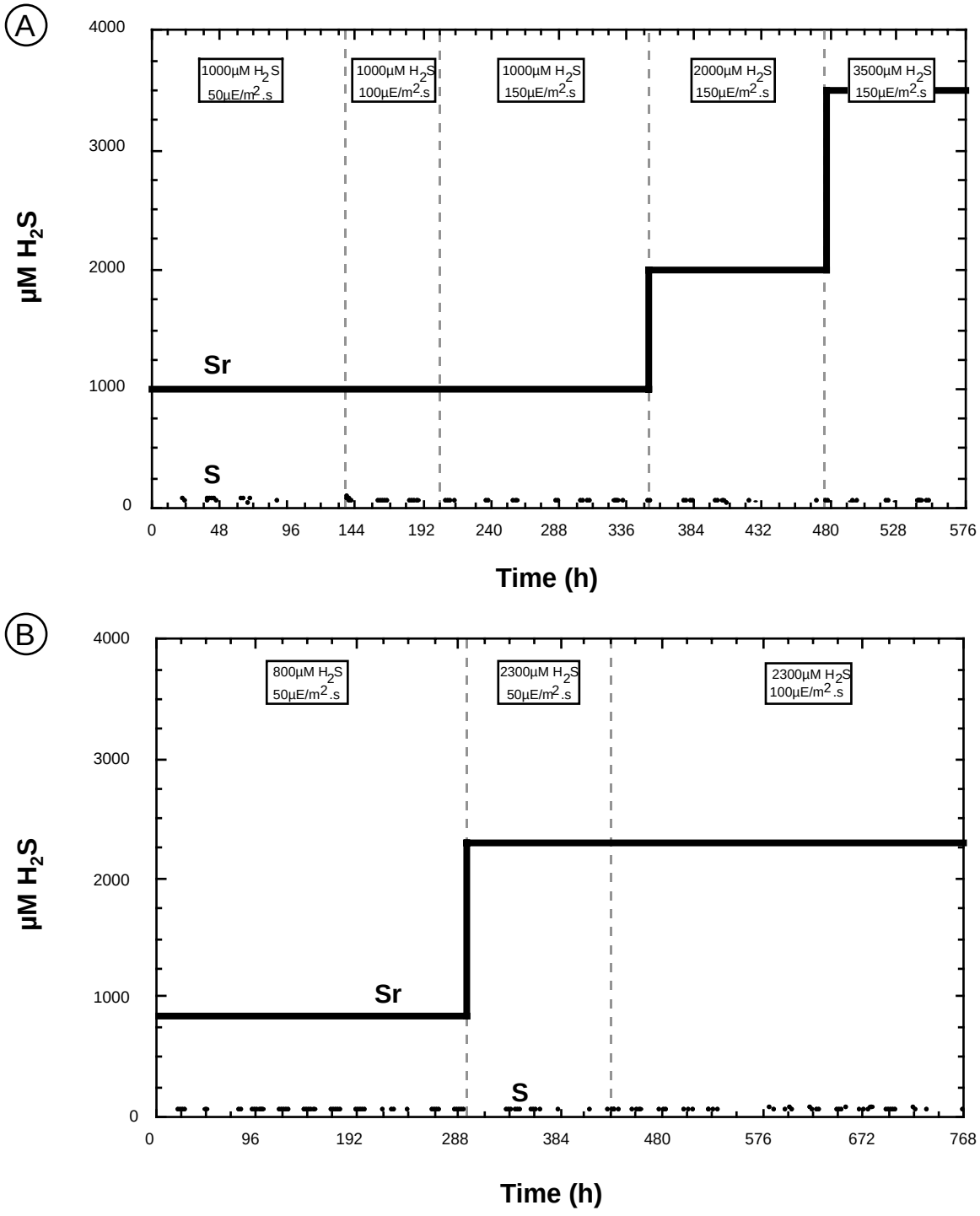

Figure 3.3. Evolution of sulfide in the reservoir (S_r) and in the effluent (S) over time for the *Chlorobium* biofilm (A) and for the biofilm developed from a natural sample (B). Conditions of light irradiance are indicated above each graph.

Discussion

In this work, we propose a biofilm reactor for the treatment of sulfurous effluents, in which a packed column provides a large surface for microbial attachment, no aeration is supplied, and light is the only energy source. Sulfide-oxidizing biofilms developed vigorously on the light-exposed column surface whenever the system was inoculated either with pure cultures or natural samples. In each case, oxidation activity did not undergo important variations, despite changes in the sulfide load or variations in irradiance, and provided a low sulfide output under all conditions.

When the bioreactor was inoculated with a natural sample, a biofilm with a high bacterial diversity and heterogeneity developed, containing aerobic, anaerobic, phototrophic and chemotrophic microorganisms. These results are shown in a Chapter 5.

In order to follow the behavior of the biofilm under different conditions, several light irradiances and concentrations of sulfide in the influent were tested. Changes in irradiance did not result in great alterations of the activity of the biofilm. However, little variations were found in the case of the *Chlorobium* biofilm, probably because the growth of *Chlorobium* (and green sulfur bacteria in general) is adapted to low-light conditions and is inhibited at high irradiances (Overmann et al. 1992). The activity of the system was maintained in spite of modifications in the sulfide load.

When we compare our results with data obtained in continuous stirred reactors (see Table 3.1), we find that, although absolute rates of sulfide oxidation are similar, the specific rates of sulfide oxidation are lower in the microbial biofilm reactor. While the biomass is homogeneously illuminated and active in stirred reactors, biomass is much higher in our reactor, due to the attachment to surfaces and the low impact of washout removal. Also, the amount of light available per unit of growing biomass is lower, therefore resulting in lower specific activities. Besides, many of the organisms detected are heterotrophs (*Cytophaga-Flavobacterium-Bacteroides* group) (Chapter 5). Although these organisms do not contribute to the oxidation of sulfide, they presumably feed on the organic material of the biofilm, contributing to its recycling.

Because biomass is immobilized in biofilms and therefore not subject to washout, high concentrations of slow-growing organisms can be achieved (Schramm and Amann 1998). As a consequence, the dynamic behavior of the system is slower but more stable during sudden environmental changes. Also, the utilization of biofilms allows a taxonomic and metabolic diversity much higher than what is usually found in stirred continuous reactors, in which washout plays a powerful role in selecting a very reduced number of organisms.

Operation as a sulfidostat allows the processing of highly polluted streams, avoiding inhibition of the sulfide oxidizers and maintaining the quality of the effluent produced even when the conditions are suddenly changed. The system is able to perform well at different situations for long periods. Despite not being a highly dynamic system, our biofilm reactor is very stable. Highly polluted streams are oxidized in the bioreactor, maintaining the quality of the effluent produced even when the conditions suddenly change. Due to the stability of the system, this setup could be also applied to the treatment of other types of pollutant.

Chapter 4. COMPARISON OF METHODS FOR DNA EXTRACTION FROM A MICROBIAL BIOFILM

COMPARISON OF METHODS FOR DNA EXTRACTION FROM A MICROBIAL BIOFILM

Abstract

We assessed the efficiency of 8 different DNA extraction procedures combining physical (freeze-thaw), mechanical (bead-beating) and chemical (lyzozyme, proteinase K, CTAB, phenol-chloroform) extraction methods in a sample obtained from an artificial complex microbial biofilm. Methods were compared in terms of extraction efficiency and sequence diversity of 16S rRNA genes revovered. DNA yields were quantified and sequence diversity was evaluated by denaturing gradient gel electrophoresis (DGGE). Big differences were found in DNA yields ranging from 53 to 1841 ng of DNA. The different DGGE fingerprints ranged from 7 to 12 bands. Methods including phenol-chloroform extraction after enzymatic lysis (lysozyme, proteinase K) resulted in the best DNA yields and also provided the highest diversity, while methods including CTAB extraction did not seem suitable for such kind of sample. Methods which presented the best results (enzymatic lysis and phenol extraction with and without bead-beating), were further compared by cloning and sequencing. Clones belonging to members of the Alpha- and Gamma-Proteobacteria, *Cytophaga-Flavobacterium-Bacteroides* group, Cyanobacteria and to the low G+C Gram-positives were recovered from both libraries. However, in the library in which bead-beating was included we also recovered clones belonging to the Beta- and Delta-Proteobacteria as well as plastids signatures. We, therefore, considered phenol-chloroform extraction after bead-beating and enzymatic lysis (lysozyme, proteinase K) as the most suitable method for DNA extraction from such complex biofilm.

Introduction

The analysis of the microbial diversity present in biofilms used in biotechnology is a necessary step to understand the structure of the microbial community and, the chemical and dynamic processes of the populations in the biofilm. Traditionally, studies of microbial diversity were based on microscopy identification and isolation of microbes in pure cultures. However, the high degree of selectivity and bias inherent to culture methods were important limitations. With the development of molecular biology tools and Woese's revolutionary work in phylogeny based on ribosomal RNA genes (Woese and Fox 1977; Woese 1987), the scenario of microbial diversity studies has drastically changed and the analysis of complex microbial communities can be achieved without cultivation.

Molecular methods based on the study of 16S rRNA genes have been established as common tools in many microbial ecology laboratories and are more and more frequently used in microbial community analysis. Despite their enormous potential, these methods are not free of biases and limitations. Most of them, with the exception of some hybridization methods such as FISH (Fluorescent *in situ* hybridization), require a first step of DNA extraction followed by PCR amplification. Unbiased nucleic acids extraction sometimes supposes a limitation (Head et al. 1998). Several procedures are available for the extraction of DNA in complex samples. Methods rely on mechanical or chemical cell-wall disruption such as bead-beating, lysis using enzymes and detergents or a combination of both (e.g. Furhman et al. 1988; van Elsas and Smalla 1995; Nübel et al. 1997). However, their efficiency depends on the composition of the sample. It is known that certain cells are more resistant to chemical treatments than others. For instance, gram-positives are more resistant to cell lysis than gram-negatives are (Head et al. 1998). Therefore, the selection of an appropriate DNA extraction method is a critical factor for the subsequent reliability on the results.

Evaluation of DNA extraction quality is usually based upon the quantity, purity and integrity of the genomic DNA from the community, reflected after electrophoresis in agarose gels. However, a greater quantity of DNA does not necessarily mean higher species richness, and studies comparing extraction methods usually pay no attention to diversity. The 16S rRNA

gene diversity present in different DNA extracts obtained from the same original sample can be easily compared by DNA fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE; Muyzer and Smalla 1998). DGGE comparison is relatively quick, cheap, and gives information of great value. However, only cells with relative abundance higher than 0.5-1% are detected with such technique (Casamayor et al. 2000). The 16S rDNA sequence diversity in a complex sample can be also assessed by cloning and sequencing. Although this approach is more expensive and time-consuming than DGGE, it allows the detection of less predominant organisms because as many clones as desired can be sequenced.

In this work, we assessed the efficiency of 8 different DNA extraction procedures in a complex sample obtained from an artificial microbial biofilm. DNA yields and 16S rRNA gene sequence diversity were evaluated for each treatment. DNA extraction procedures combined different physical (freeze-thaw), mechanical (bead-beating) and chemical (lyzozyme, proteinase K, CTAB, phenol) treatments and 16S rRNA gene sequences were compared by DGGE. The two DNA extracts with the highest yield and also with the highest diversity (as reflected by the number of bands in DGGE fingerprints) were selected for a detailed cloning and sequencing analysis.

Results

Initially, the biofilm sample was resuspended in lysis buffer and the volume was divided into equal aliquots. Each aliquot was subjected to a different DNA extraction procedure. Table 4.1 summarizes the different combination of methods tested (for the detailed procedures see Chapter 2). Physical (freeze-thaw), mechanical (bead-beating) and chemical treatments (lyzozyme, proteinase K, CTAB) were used in different combinations.

Table 4.1. Combination of DNA extraction methods used for comparison.

Method Abbreviation	Procedure as indicated in Chapter 2
F	Freeze-thaw
FEP	Freeze-thaw, enzymatic lysis (lyzozyme and proteinase K) and phenol extraction
FC	Freeze-thaw and CTAB extraction
BP	Bead-beating and phenol extraction
BEP	Bead-beating, enzymatic lysis (lyzozyme and proteinase K) and phenol extraction
C	CTAB extraction
EP	Enzymatic lysis (lyzozyme and proteinase K) and phenol extraction
NKCP	Freezing with liquid N_2, proteinase K, CTAB, and phenol extraction

1. DNA recovery from the biofilm

The aqueous phase obtained after the freeze-thaw treatment (F) was directly used to run PCR without any previous purification step. For the remaining protocols, nucleic acids were purified and quantified prior amplification. We observed large differences in the amount of DNA obtained from each procedure ranging from 53 to 1841 ng of DNA (Figure 4.1). The best procedures, from a quantitative point of view, were those in which enzymatic lysis (with lyzozyme and proteinase K) and phenol extraction were applied. In particular, higher DNA yields were obtained with phenol extraction after freeze-thaw and enzymatic lysis (FEP method), phenol extraction after bead-beating and enzymatic lysis (BEP method), and phenol extraction after enzymatic lysis (EP method), which yielded a total of 1383, 1841 and 1810 ng of DNA respectively. The worst method was CTAB extraction after freeze-thaw (FC method) which only yielded 53 ng of DNA.

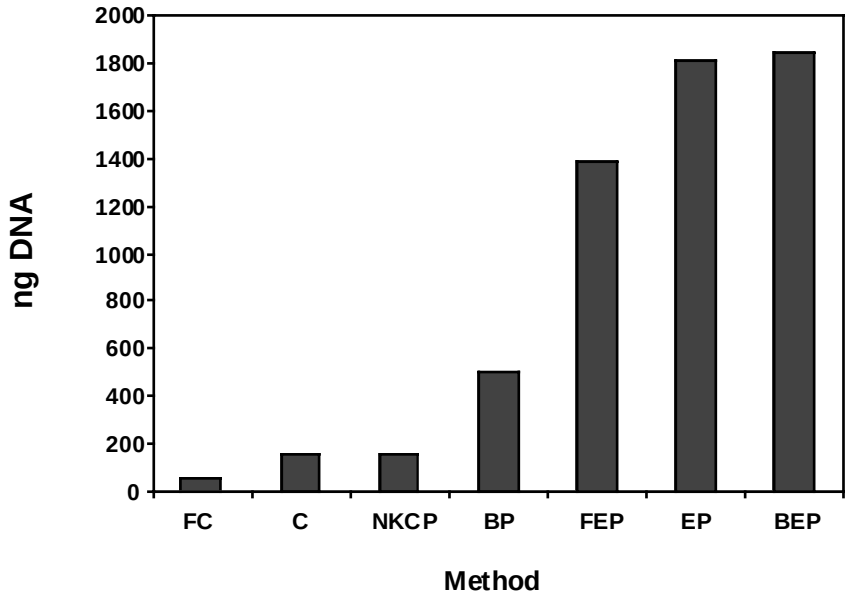

Figure 4.1. DNA yields for the different DNA extraction methods (for name abbreviation see Table 4.1).

2. PCR-DGGE

In order to analyze how the extraction method affected the 16S rRNA genes recovered from each sample, the different PCR products were run in a DGGE gel for comparison (Figure 4.2). The number of bands per lane ranged from 7 to 12. Methods combining enzymatic lysis and phenol-chloroform extraction (FEP, BEP and EP) resulted in a higher number of bands (12 bands), whereas NKCP extraction was the method that gave the lowest number (7 bands). The remaining protocols (FC, BP and C) resulted in a fingerprint of 9 bands. Bands 1, 2, 8, 9, and 12 (for numbering see Figure 4.2) appeared in all lanes while band 4 appeared only in the fingerprints of FEP, BEP and EP methods. The relative band intensity in each lane is represented in Table 4.3. In general, the contribution of each band in the different procedures was similar, and for all methods the predominant bands coincided (Bands 1, 2, 8, 9 and 12).

We excised, reamplified, purified and sequenced 15 DGGE bands corresponding to 9 different positions (see numbers in Fig. 4.2). However, the weakest bands excised did not yield a profitable sequence. Bands that yield usable sequences (Bands 1, 2, 8, 9 and 12) corresponded to bands present in all lanes and accounted for 71 to 92% (mean 81%) of the total band intensity (Table 4.3). Sequences were submitted to a BLAST search to determine

their closest match in public databases. Sequences retrieved affiliated, to the Alpha- and Gamma-Proteobacteria, the CFB group and also to plastids (Table 4.2).

Table 4.2. Sequence similarities of the excised DGGE bands to their closest match in Genbank.

Group	Band	Closest match	Acc nº	Similarity
Plasts	1	uncultured *Scenedesmus*	AJ548895	98.2%
CFB	2	uncultured CFB	AJ548918	94.8%
Alpha-Proteobacteria	8	uncultured *Rhodobacter*	Aj548903	99.8%
Gamma-Proteobacteria	9	uncultured *Thiothrix*	AJ548931	99.4%
	12	*Thiocapsa roseopersicina*	Y12301	98.2%

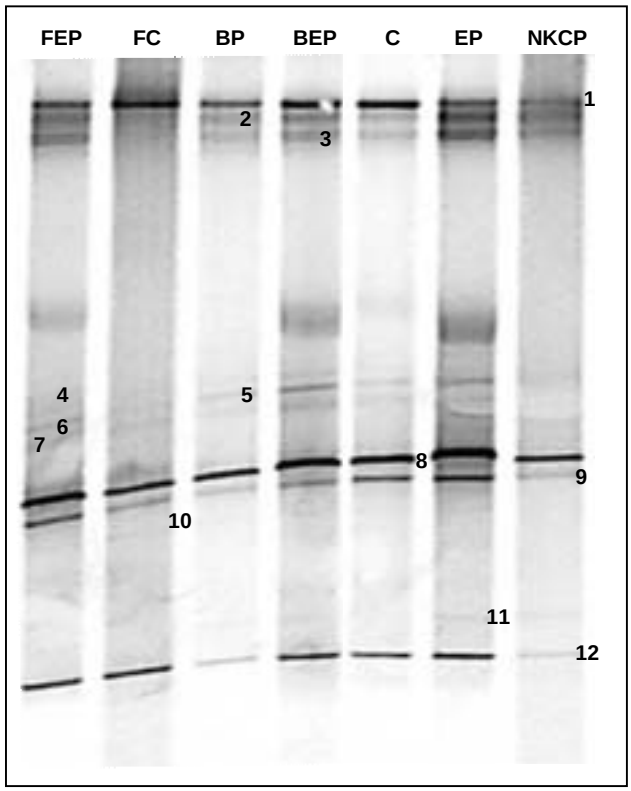

Figure 4.2. Image of DGGE containing bacterial 16S rRNA fragments for the different DNA extraction protocols essayed (for name abbreviation see Table 4.1).

Table 4.3. Relative intensity (%) of bands corresponding to DGGE gel shown in Figure 4.2. Mean values and standard deviations are indicated on the right side of the table (NB= Band position in the gel; n= number of bands). For method abbreviation see Table 4.1.

NB	FEP	FC	BP	BEP	C	EP	NKCP	Mean	SD
1	16.4	24.2	20.8	20.2	23.1	11.7	16.7	**19,0**	±4,4
2	11.0	9.4	13.2	11.5	10.4	14.2	17.7	**12,5**	±2,8
3	9.8	-	9.5	9.7	8.8	13.4	14.6	**11,0**	±2,4
4	1.1	-	-	0.7	-	1.5	-	**1,1**	±0,4
5	2.3	1.7	1.6	1.3	-	1.5	-	**1,7**	±0,4
6	4.2	2.2	6.0	6.0	2.8	5.8	-	**4,5**	±1,7
7	5.5	3.0	5.5	2.5	3.9	2.8	-	**3,9**	±1,3
8	25.6	27.6	28.1	24.4	23.6	23.1	30.9	**26,2**	±2,8
9	11.0	10.2	9.8	10.1	13.6	11.4	13.2	**11,3**	±1,5
10	1.1	1.5	-	1.7	1.5	1.5	-	**1,5**	±0,2
11	1.3	-	-	0.7	-	2.5	2.7	**1,8**	±1,0
12	10.6	20.1	5.4	11.1	1.5	10.6	4.1	**9,1**	±6,1
n	**12**	**9**	**9**	**12**	**9**	**12**	**7**		

The relative intensity of the identified bands is represented in Figure 4.3. Quantitative differences in relative band intensity among the different protocols ranged from 0.2 to 18% for the different bands. Differences among protocols were low for the CFB- (Band 2; 9-18%), the *Rhodobacter*- (Band 8; 23-31%), the *Thiothrix*-like bands (Band 9; 6-14%), and for bands 3, 4, 5, 6, 7, 10 and 11. Signature from *Scenedesmus* plastids (Band 1; 12-30%) and mainly *Thiocapsa* related bands (2-20%) showed higher differences. In general, we can point out that the highest proportion of algae was obtained when combining physical and mechanical with chemical procedures. Contrarily, the contribution of purple sulfur bacteria was higher using lyzozyme, with or without previous treatment.

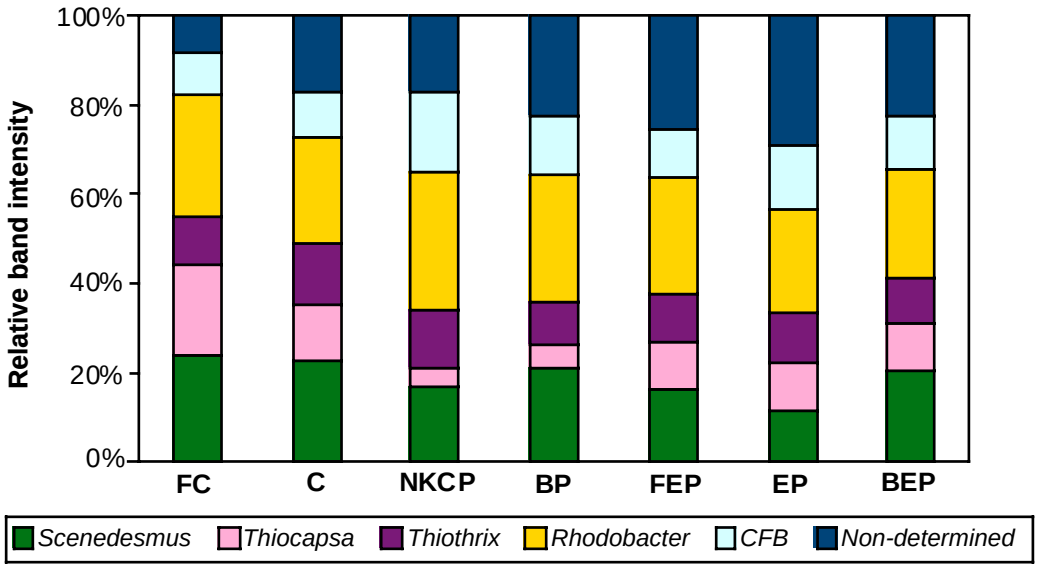

Figure 4.3. Relative DGGE band intensity of identified phylogenetic groups for the different DNA extraction protocols essayed (for name abbreviation see Table 4.1).

3. PCR-Cloning

We chose two of the extraction methods, which resulted in both better DNA yield (≈1800 ng) and better qualitative fingerprint (12 bands), to assess the diversity through cloning and sequencing. Cloning offers lower detection limits than DGGE and therefore, less predominant organisms can be detected. The methods compared (BEP and EP) shared the same chemical treatment but in one of them bead-beating was introduced before the enzymatic lysis. PCR amplification was performed with primers 27F and 1492R and a DNA amplicon of about 1.5 kb was obtained. The PCR product was cloned and 118 positive clones were obtained for BEP library and 109 for EP library. After plasmid extraction, PCR amplification and RFLP digestion, 96 and 82 clones were analyzed respectively. In the BEP library, 42 different RFLP patterns were obtained while in EP library we found 47 patterns. Each RFLP pattern was considered as one operational taxonomic unit (OTU) and a representative clone of each OTU was partially sequenced. The relative abundance of the different phylogenetic groups recovered from the libraries is represented in Table 4.4.

We recovered clones belonging to the Alpha- and Gamma-Proteobacteria, the *Cytophaga-Flavobacterium-Bacteroides* group (CFB), the Cyanobacteria, and to the low G+C Gram-positive bacteria in both libraries. Only small differences were found in the percentage of

these predominant groups between both libraries and moreover, sequences were in agreement with predominant groups in DGGE. Furthermore, sequences affiliated to the Beta- and Delta-Proteobacteria were found in minor abundance only in BEP library. However, plastids from green algae were detected as a predominant group in this library (12.5%), while they were not recovered in EP library.

Table 4.4. Relative abundance of the different phylogenetic groups in the clone libraries performed from the DNA extracted with BEP (Bead-beating, lyzozyme and proteinase K, phenol extraction) and EP (lyzozyme, proteinase K, phenol extraction) methods. (CFB: *Cytophaga-Flavobacterium-Bacteroides*; PNSB: Purple nonsulfur bacteria; PSB: Purple sulfur bacteria; CSB: Colorless sulfur bacteria).

	BEP (%)	EP (%)	Mean	SD
Alpha-Proteobacteria				
PNSB (*Rhodobacter*-like)	32,3	36,4	34,4	± 2,9
Others	1,0	-	-	-
Beta-Proteobacteria	4,2	6,5	5,4	± 1,6
Gamma-Proteobacteria				
PSB (*Thiocapsa*-like)	12,5	18,2	15,4	± 4,0
CSB (*Thiothrix*-like)	11,5	15,6	13,6	± 2,9
Others	1,0	-	-	-
Delta-Proteobacteria	1,0	-	-	-
CFB	20,8	15,6	18,2	± 3,7
Cyanobacteria	1,0	3,9	2,5	± 2,1
Plasts	12,5	-	-	-
Low G+C Gram-positive	2,1	3,9	3,0	± 1,3

Discussion

A prerequisite for an accurate analysis of microbial communities using genetic molecular techniques is obtaining unbiased high-quality environmental DNA. Extracted nucleic acids must be representative of each microbial population present in the community. In the last years, many different DNA extraction protocols have been developed but little effort has been devoted to examine their performance in a certain complex sample. In general, commercial

DNA extraction kits are time saving methodologies but are not recommended for complex samples, such as sediments or biofilms. For these samples, protocols combining chemical and physical methods seem to be the most appropriate. In this work, we investigated the suitability of different DNA extraction methods for studying the bacterial community composition of a complex sulfide-oxidizing biofilm. For this purpose, we compared extraction efficiency (total amount of DNA recovered) and diversity of 16S rRNA genes.

Eight methods combining physical (freeze-thawing), mechanical (bead-beating) and chemical (lyzozime and proteinase K, CTAB, phenol) extraction procedures were essayed. Comparison of DNA yields revealed quantitative differences up to one order of magnitude. Big differences have been also reported in other studies in which different DNA extraction methods from soils and sediments were compared (Stach et al. 2001; Webster et el. 2003). Methods including phenol-chloroform extraction after enzymatic lysis (lysozyme, proteinase K) resulted in the best DNA yields (methods FEP, BEP, EP). Contrarily, CTAB extraction offered the poorest yields. CTAB have been used to extract DNA from complex samples but is usually used for samples rich in cyanobacteria (Nübel et al. 1997 and 1999; Garcia-Pichel et al. 2001; Yeager et al. 2004). However, cyanobacteria were a minor component in our biofilm.

A second requisite, apart from good extraction efficiency, is to obtain DNA with enough quality to allow PCR amplification. In our case, DNA from seven of the methods tested yielded PCR product. However, the fastest and simplest method used (F) in which PCR was carried out directly without previous purification step did not yield amplification product. This method had been described for environmental samples containing low concentration of microorganisms. The high cell concentration and extracellular substances in our biofilm could result in high amount of PCR inhibitors such as pigments and humic acids in the extracts (Tsai and Olson 1991; Rochelle et al. 1992).

Finally, the ability of a treatment to recover as many different 16S rRNA genes as possible is very important. In general, the quality of the DNA is assessed in terms of quantity, as an indication of cell lysis efficiency, and purity that determines whether the DNA can be used in further molecular analysis or not. However, the highest yield does not necessary implies the greatest species richness and, thus, sequence diversity recovered needs to be examined. In this work we compared 16S rRNA gene sequence diversity present in the DNA biofilm

extracts by denaturing gradient gel electrophoresis (DGGE). DGGE fingerprints showed differences in the number of bands from 7 to 12 between the different methods. However, the same predominant bands were present in all methods. Missing bands corresponded to the weakest bands. From a quantitative point of view, the relative intensity of most bands did not differ significantly although some variations were observed (Table 4.3). Again, methods including phenol extraction after enzymatic lysis showed the best suitability in terms of diversity recovered, while those methods in which CTAB extraction was applied resulted in a low number of bands. Thus, in our sample higher quantity implied higher sequence diversity. Despite differences of 1 order of magnitude in the amount of DNA, minor differences were detected in the fingerprints.

Overall, three of the methods tested (FEP, BEP and EP), all including a common step of enzymatic lysis followed with phenol-chloroform extraction, presented the highest DNA yields and the greatest number of bands (12). In particular, BEP and EP methods presented a similar DNA yield and equal number of bands and therefore, apparently any of these methods could be selected as the most appropriate to perform the detailed DNA analysis of the biofilm. However, the absence of clones related to plastids in the method lacking mechanical treatment shows a big bias against algae. The presence of algae was observed as a green layer covering the packing material of the column, and moreover, they were observed microscopically and also recovered by DGGE fingerprint. However, their relative abundance in DGGE was also lower in EP fingerprint. Probably, such difference is due to the lack of mechanical treatment in EP method. Taking into account the conspicuous presence of algae in the biofilm, we can conclude that results from BEP method are more representative of the real community, and in general, it seems to be the best DNA extraction method for this kind of sample.

In our case, a biofilm with high cell concentration, lysis with lyzozyme and proteinase K followed by phenol-chloroform extraction and a step of DNA purification gave the greatest DNA yields. Although differences in yield were very high, they were minor in terms of diversity recovered, which means that the methods tested were equally efficient for most groups. However, for algal DNA extraction, methods including bead-beating showed the best results. In conclusion, we recommend using a complete method with enzymatic lysis and a previous mechanical treatment for the analysis of complex biofilms.

Chapter 5. HIGH-DIVERSITY BIOFILM FOR THE OXIDATION OF SULFIDE-CONTAINING EFFLUENTS

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HIGH-DIVERSITY BIOFILM FOR THE OXIDATION OF SULFIDE-CONTAINING EFFLUENTS

Abstract

In the present work, we describe for the first time the utilization of a complex microbial biofilm for the treatment of sulfide-containing effluents. A non-aerated packed-column reactor was inoculated with anoxic lake sediment and exposed to light. A biofilm developed in the column and showed a stable oxidation performance for several weeks. Microbial species composition was analyzed by microscopy, pigment analysis and by a bacterial 16S rRNA gene clone library. Colorless sulfur bacteria, green algae and purple sulfur bacteria were observed microscopically. Pigment composition confirmed the presence of algae and purple sulfur bacteria. The clone library was dominated by Alpha-Proteobacteria (mostly by representatives of the *Rhodobacter* group), followed by Gamma-Proteobacteria (Chromatiaceae-like and *Thiothrix*-like aerobic sulfur oxidizers) and by the *Cytophaga-Flavobacterium-Bacteroides* group. Plastid signatures from algae were also present and a few clones belonged to both the Beta- (*Rhodoferax* sp. and *Thiobacillus* sp.) and Delta-Proteobacteria (*Desulfocapsa* sp.) and to the low G+C Gram-positive bacteria (Firmicutes group). The coexistence of aerobic, anaerobic, phototrophic and chemotrophic microorganisms in the biofilm, the species richness found within these metabolic groups (42 operational taxonomic units; OTUs), and the microdiversity observed within some species could be very important for the long-term functioning and versatility of the reactor.

Introduction

Sulfide is a toxic and corrosive compound which causes a high oxygen demand and unpleasant odor. Different industries such as petrochemical plants, and the anaerobic treatment of sulfate-containing wastewaters, generate effluents rich in sulfide (Fischer 1988; Buisman et al. 1990). Physicochemical processes are in use for removing sulfide from wastewaters, but their relatively high chemical, catalyst, and disposal costs are important drawbacks of these conventional systems (Jensen and Webb 1995), which are only efficient for wastewaters with high sulfide concentration (Cork et al. 1983). Microbiological processes have been investigated as an alternative to the physicochemical treatments applied for sulfide removal (Jensen and Webb 1995). Aerobic sulfur-oxidizing bacteria, such as different species of the genus *Thiobacillus*, have been used in aerobic reactors (Sublette and Sylvester 1987; Buisman et al. 1990; Janssen et al. 1997). In these reactors, sulfide is transformed to sulfate through an oxygen-dependent process. Anaerobic oxidation by phototrophic sulfur bacteria has been also proposed for the treatment of sulfide-containing effluents (Cork et al. 1983; Kim et al. 1990).

Although several biological processes have been studied at laboratory scale, very few of them have been employed on a large scale (Jensen and Webb 1995). Most bioreactors utilize suspended microbial biomass that can be easily washed out from the system whenever its growth rate is affected by environmental disturbances. As an alternative, reactors using fixed biomass (biofilms) may be more stable. Biofilms are defined as surface-attached accumulations of microbial cells encased in extracellular polymeric substances (Characklis and Wilderer 1989). One of the main advantages of these structures is that cells are immobilized and retained in the reactor, allowing high biomass to be achieved (Schramm and Amann 1998). Microorganisms are not washed out, even if they constitute a minor part of the community or have low specific growth rates. The existence of microenvironments within the biofilm, in which conditions can be completely different from those in the liquid phase, has been related to higher stability and resistance to disturbances or lethal factors (Brown et al. 1988; Brown and Gauthier 1993). Heterogeneous biofilms are also more resistant and their higher taxonomic and physiological diversity results in a better performance of wastewater treatments under changing environmental conditions (Von

Castein et al. 2002). Therefore, biofilm reactors are increasingly used for different wastewater treatments or bioremediation processes (Wagner and Loy 2002).

In the present work, we describe for the first time the use of a complex multispecies biofilm for the treatment of sulfide-containing effluents. After the inoculation of the reactor with a sample of anoxic lake sediment, a complex, metabolically diverse assemblage developed with a highly stable performance. The microbial species composition was determined by microscopy and 16S rDNA cloning and sequencing, revealing the coexistence of aerobic, anaerobic, phototrophic and chemotrophic microorganisms.

Results

After inoculation, the reactor was started-up and the oxidation rate of the biofilm was monitored throughout time. Within 8 days, the column was colonized and the oxidation rate reached equilibrium (200 µmol $H_2S \cdot h^{-1}$). Then, the reactor was subjected to several changes in light irradiance (50,100 $\mu E \cdot m^{-2} \cdot s^{-1}$) and sulfide influent concentration (800, 2300 µM), which did not resulted in significant changes in the sulfide oxidation rate. Details on the sulfide oxidation performance are discussed in Chapter 3. The concentration of sulfide at the outlet was kept at 50 µM, therefore achieving a 98% reduction in sulfide concentration.

After 32 days of operation, the reactor was shut down and we proceeded to characterize the microbial composition of the biofilm.

Microscopic description of microorganisms developing in the biofilm

Daily inspection of the macroscopic aspect of the biofilm showed a complex colorful microbial community developing after 8 days. The predominant colors were purple and green, in agreement with the presence of green algal populations and purple sulfur bacteria observed through the microscope.

Microscopic counts revealed high density of attached cells ($4.05x10^{11}$ cell·cm^{-2}). Different morphotypes were observed such as green algae (4% of total cells belonging to Chlorophyta, 1% to Diatoms), purple sulfur bacteria (PSB; 29% of total cells) and conspicuous cells forming large colorless filaments (*Thiothrix*-like) (Figure 5.1). Such multicellular filaments were counted as a unit and constituted only 3% of total cells. However, since these filaments were multicellular, their contribution in terms of biomass was certainly higher. Among the green algae at least two different morphotypes were observed, one of them identified as *Scenedesmus* sp. Two different PSB-like cells both containing sulfur inclusions but differing in size, were observed: PSB type I (*Thiocapsa*-like spherical cells, 1.5 µm diameter), and type II (*Chromatium*-like oval cells, 4 x 5 µm size). Finally, up to 63% of the cells did not show any characteristic morphological trait and could not be identified by microscopy.

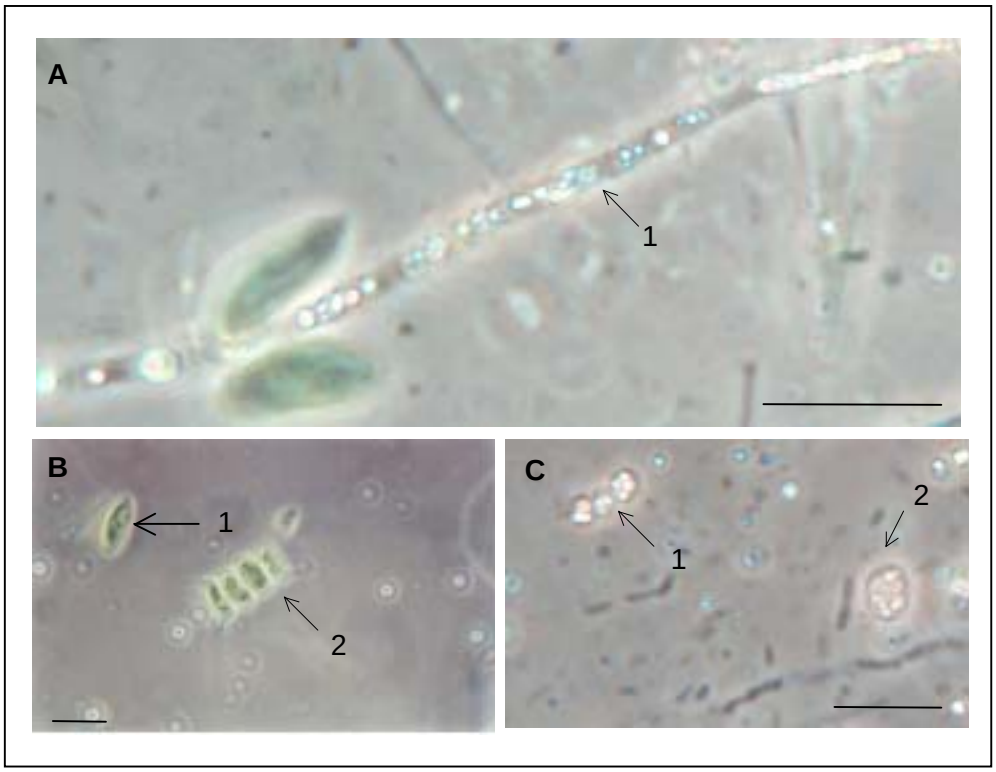

Figure 5.1. Morphotypes observed with the microscope. (A) *Thiothrix*-like cells (1); (B) Non identified Chlorophyta (1) and *Scenedesmus*-like cells (2); (C) Chromatiaceae cells type I (1) and type II (2). Bar=10 µm.

Biomass recovered from the column totalled 627 mg of protein, which corresponded to a density of 1.93 mg protein·cm^{-2} in the column. Pigment analysis indicated presence of Chl *a* (56 µg·cm^{-2}) and Bchl *a* (12 µg·cm^{-2}).

DNA extraction and clone library construction

Ninety-six clones were obtained, which resulted in 42 different RFLP patterns. A representative clone of each pattern was partially sequenced. The retrieved sequences, their frequency in the library and their closest relatives are listed in Table 5.1. Twenty-six clones corresponded to unique RFLP band patterns. The coverage of the library was 73%. After grouping sequences differing in less than 3% the number of phylotypes was 28. The accumulation curve continued increasing after 96 clones screened, probably as a result of the high microdiversity present in the biofilm. However, phylotype accumulation curve showed a decrease in the rate of phyloptype detection after 50 clones screened, indicating that the major part of the diversity in the library was detected.

rDNA sequencing and phylogenetic analyses

Partial 16S rRNA gene sequences (see Table 5.1) were included in a phylogenetic tree (Figure 5.2). Different phylogenetic groups previously identified by microscopy were retrieved, such as green algae and Gamma-Proteobacteria (i.e., Chromatiaceae and *Thiothrix*-like). In addition, Alpha-, Beta- and Delta-Proteobacteria, Cyanobacteria, *Cytophaga-Flavobacterium-Bacteroides* group (CFB) and low G+C Gram-positive bacteria were also detected.

The relative distribution of the recovered clones in the clone library is shown in Figure 5.3. The Alpha-Proteobacteria dominated the clone library (33% of total clones), mostly by representatives of the *Rhodobacter* group (up to 29%), although *Rhodopseudomonas* and *Caulobacter* spp. were also present. The Gamma-Proteobacteria accounted for 25% of total clones (11% belonging to the *Thiothrix*-like aerobic sulfur oxidizers, 13% anaerobic sulfur oxidizers belonging to the Chromatiaceae, and 1% to the *Pseudomonas* group), and for the CFB group, we found up to 21% of the total clones. Chloroplasts from algae were also

abundant in our bacterial clone library (up to 13%) and only a clone belonged to Cyanobacteria (1% of total clones). Finally, a few clones belonged to the Beta- (*Rhodoferax*, *Thiobacillus* and *Azoarcus* spp.) and Delta-Proteobacteria (*Desulfocapsa* sp.), and to the low G+C Gram-positive bacteria (Firmicutes group), being less than 5% of total clones for each case.

Table 1. Phylogenetic affiliation of clones to the closest match and to the closest cultured strain in GenBank (n=sequence length).

Name	Nº clones	n	Closest match (Acc. number)	% similarity	Cultured closest match (Acc. number)	% similarity
δ-Proteobacteria		≥		≥		
BIOEST-7	1	898	*Rhodobacter veldkampii* (D16421)	99,4	The same	99,4
BIOEST-14	16	860	*Rhodobacter veldkampii* (D16421)	99,3	The same	99,3
BIOEST-19	3	877	*Rhodobacter veldkampii* (D16421)	99,2	The same	99,2
BIOEST-15	1	427	*Rhodobacter veldkampii* (D16421)	99,0	The same	99,0
BIOEST-26	1	631	*Rhodobacter veldkampii* (D16421)	99,0	The same	99,0
BIOEST-23	2	913	*Rhodobacter veldkampii* (D16421)	98,5	The same	98,5
BIOEST-16	2	838	*Rhodobacter* sp. TCRI 3 (AB017796)	99,2	The same	99,2
BIOEST-24	1	718	*Rhodobacter azotoformans* (D70847	97,1	The same	97,1
BIOEST-27	1	832	*Rhodobacter azotoformans* (D70847)	96,6	The same	96,6
BIOEST-36	3	834	*Rhodopseudomonas palustris* (AF314063)	99,8	The same	99,8
BIOEST-20	1	770	Uncultured bacterium (AJ295490)	97,6	*Caulobacter fusiformis* (AJ227759)	97,6
ß-Proteobacteria						
BIOEST-17	1	807	*Thiobacillus baregensis* (Y09280)	98,1	The same	98,1
BIOEST-18	1	653	Unidentified eubacterium (Y12371)	99,5	*Rhodoferax antarticus* (AF084947)	97,7
BIOEST-28	1	789	*Azoarcus indigens* (AF011345)	91,5	The same	91,5
BIOEST-37	1	634	Unidentified eubacterium (Y12371)	99,4	*Rhodoferax antarticus* (AF084947)	97,5
δ-Proteobacteria						
BIOEST-40	2	730	Uncultured bacterium (AJ307933)	99,9	*Thiothrix unzii (*AF011345)	99,4
BIOEST-41	2	694	Uncultured bacterium (AJ307933)	99,8	*Thiothrix unzii (*AF011345)	99,4
BIOEST-42	3	813	Uncultured bacterium (AJ307933)	99,7	*Thiothrix unzii (*AF011345)	99,4
BIOEST-34	1	731	Uncultured bacterium (AJ307933)	99,7	*Thiothrix unzii (*AF011345)	99,3
BIOEST-38	1	758	Uncultured bacterium (AJ307933)	99,6	*Thiothrix unzii (*AF011345)	99,2
BIOEST-30	1	858	*Thiothrix* sp. CT3 (AF148516)	97,9	The same	97,9
BIOEST-39	1	661	*Thiothrix nivea* (L40993)	95,9	The same	95,9
BIOEST-32	11	623	*Thiocapsa roseopersicina* (AF112998)	97,4	The same	97,4
BIOEST-33	1	790	*Thiocapsa roseopersicina* (AF112998)	96,5	The same	96,5
BIOEST-31	1	894	*Pseudomonas stutzeri* (AF038653)	99,8	The same	99,8
δ-Proteobacteria						
BIOEST-35	1	663	*Desulfocapsa thiozymogenes* (X95181)	97,9	The same	97,9

CFB Group						
BIOEST-9	3	869	Unkown organism (X85208)	97,9	*Riemerella anatipestifer* (U60101)	93,9
BIOEST-21	1	884	Unkown organism (X85208)	97,7	*Riemerella anatipestifer* (U60101)	93,8
BIOEST-10	1	840	Unkown organism (X85208)	94,1	*Riemerella anatipestifer* (U60101)	93,4
BIOEST-29	2	886	Uncultured bacterium (AJ306736)	93,7	*Riemerella columbina* (AF181448)	89,0
BIOEST-11	2	818	Uncultured bacterium (AJ306736)	93,7	*Riemerella columbina* (AF181448)	87,0
BIOEST-8	2	775	Uncultered Cytophagales(AF268286)	97,9	*Cytophaga* sp. GP1-11 (AJ456975)	92,0
BIOEST-12	5	848	Uncultured bacterium (AF323778)	91,8	*Cytophaga* sp. (AB015264)	87,9
BIOEST-13	2	833	Uncultured bacterium (AJ488099)	90,1	*Cytophaga* sp. (AB015264)	86,2
BIOEST-2	1	821	rhizosphere soil bacterium RSI-24 (AJ252591)	97,9	*Flavobacterium ferrugineum* (M62798)	92,9
BIOEST-3	1	849	Uncultured eubacterium WCHB1-69 (AF050545)	90,5	*Flexibacter canadiensis* (M62793)	87,6
Cyanobacteria/Plasts						
BIOEST-6	10	860	*Scenedesmus obliquus* plast (AF394206)	99,0	The same	99,0
BIOEST-5	1	892	*Scenedesmus obliquus* past (AF394206)	94,1	The same	94,1
BIOEST-4	1	867	*Scenedesmus obliquus* plast (AF394206)	93,2	The same	93,2
BIOEST-1	1	571	*Synechocystis* PCC6308 (AB039001)	94,4	The same	94,4
Low G+C Gram-positive						
BIOEST-22	1	864	Uncultured bacterium (AB062820)	92,2	*Clostridium akagii* (AJ237755)	90,0
BIOEST-25	1	802	Uncultured bacterium (AB062820)	93,1	*Clostridium akagii* (AJ237755)	90,0

Most of the clones were highly related to previously described sequences in the GenBank database. In addition, 73% of the clones had 16S rRNA gene similarities ≥ 95% with cultured species. Prominent among them were members of the Alpha-Proteobacteria, most of them related to cultured strains at the species level (≥97% similarity; Stackebrandt and Goebel 1994). Most of the green algae chloroplasts from the library were closely related to *Scenedesmus obliquus*. Conversely, members of CFB group, Cyanobacteria and low G+C Gram-positive bacteria (Firmicutes) groups were distantly related to cultured species (see Table 5.1). For instance, CFB from the clone library were only between 87 and 93.9% similar to cultivable species. Two of the clones belonging to Beta-Proteobacteria were very similar to *Rhodoferax* spp. (99.5%). However, we also found a Beta-Proteobacteria clone with low affiliation to any relative (the best match was only 91.5% with *Azoarcus indigens*). Therefore, putative new taxa above the genus level developed in the bioreactor and deserve future research.

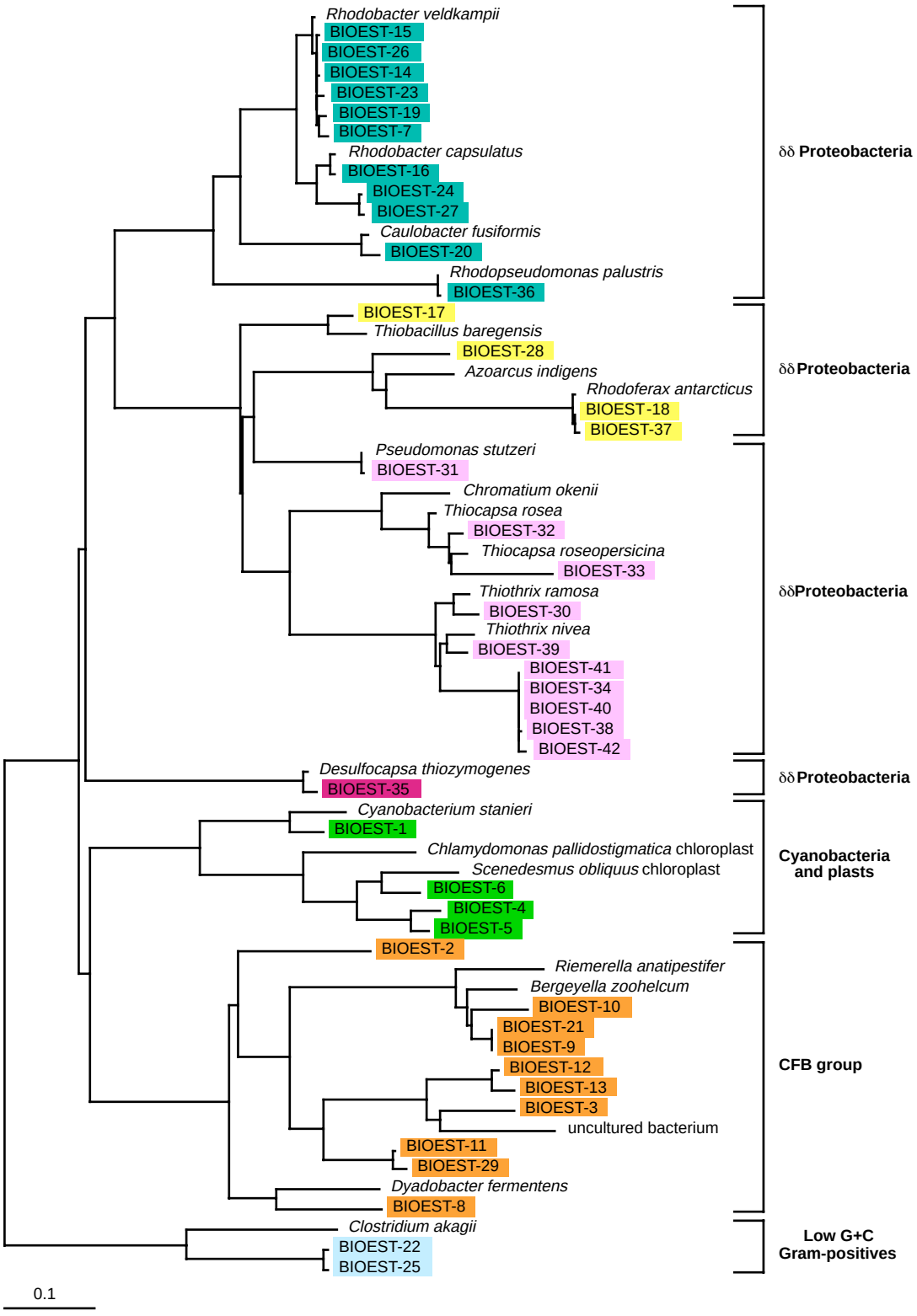

Figure 5.2. Bacterial phylogenetic tree showing the affiliation of the clones retrieved (BIOEST-1 to BIOEST-42). The scale bar indicates 10% estimated sequence similarity.

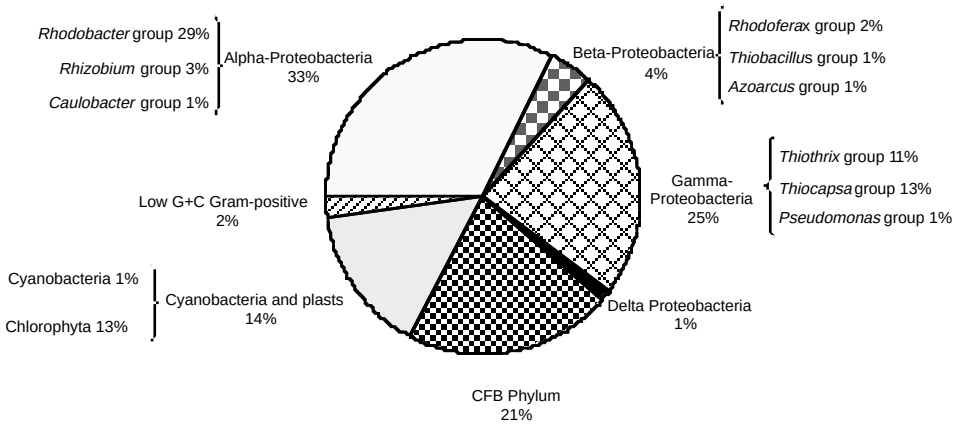

Figure 5.3. Relative abundance of the recovered clones in the library.

The phylogenetic analyses revealed that several of the retrieved sequences showed a considerable degree of microdiversity (Furhman and Campbel 1998), particularly for the *Rhodobacter veldkampii* and *Thiothrix* sp. clusters (see phylogenetic tree, Figure 5.2). Comparisons were carried out between such very similar sequences (Tables 5.2 and 5.3). Six *Rhodobacter veldkampii*-like sequences were on average 99.6% similar to each other. For the five *Thiothrix*-like sequences we found an average of 99.8% similarity among them. A similar phenomenon occurred in other cases, such as clones BIOEST-24 and BIOEST-27 (99.6% similarity to each other) related to *Rhodobacter azotoformans*, BIOEST-18 and BIOEST-37 (99.5%) related to *Rhodoferax fermentans*, and BIOEST-22 and BIOEST-25 (98.6%) related to the Firmicutes group. Finally, CFB sequences BIOEST-9 and BIOEST-21 (99.8%) and BIOEST-11 and BIOEST-29 (99.5%) also showed microdiversity.

Table 5. 2. Similarity matrix for the microdiverse 16S rRNA gene sequences related to *Rhodobacter veldkampii*.

Rhodobacter veldkampii	% Similarity to organism:					
	1	2	3	4	5	6
1. BIOEST-7		99,9	99,8	99,8	99,8	99,0
2. BIOEST-14			99,8	99,7	99,8	99,8
3. BIOEST-15				99,5	99,5	99,8
4. BIOEST-19					99,5	98,7
5. BIOEST-23						99,8
6. BIOEST-26						

Table 5.3. Similarity matrix for the microdiverse 16S rRNA gene sequences related to *Thiothrix* sp.

Thiothrix sp.	% Similarity to organism:				
	1	2	3	4	5
1. BIOEST-34		99.6	99.9	100	99.7
2. BIOEST-38			99.7	99.7	99.6
3. BIOEST-40				100	99.9
4. BIOEST-41					99.9
5. BIOEST-42					

Metabolic traits in the biofilm

Due to the fact that most of the clones were closely related to well characterized cultured strains and that prevailing environmental conditions within the bioreactor were well known, putative general activities and functional roles can be assigned for most of the phylotypes recovered (Figure 5.4). Algae and cyanobacteria (14% of total clones) were grouped as oxygenic photoautotrophs, and Chromatiaceae 16S rRNA gene sequences (13%) as anoxygenic photoautotrophs. *Thiobacillus*-like and *Thiothrix*-like sequences were grouped as aerobic chemoautotrophs (up to 12% of total clones). Clones related to the genera *Rhodobacter*, *Rhodopseudomonas* and *Rhodoferax* were grouped as metabolically versatile because of the wide range of metabolic capabilities described from cultivated species of these genera. These organisms might be able to carry out chemotrophic or phototrophic metabolisms and to use CO_2 or organic matter as carbon source (Imhoff 2001a, 2001b). Cytophagales, Pseudomonadales, Caulobacteriales, Firmicutes and the sulfate reducing bacterium (SRB) *Desulfocapsa* sp. were all grouped as heterotrophic (26% of total clones). Although Cytophagales and Firmicutes clones had their cultured closest relatives above the genus level (95% similarity), we grouped them as heterotrophic prokaryotes because all members of these groups are known to be heterotrophs (Takayuli and Kawamura 2001; Kirchman 2002). The clone BIOEST-28 (whose best match was the Beta-Proteobacterium *Azoarcus* sp., 91.5% similarity) was allocated to the “not determined” metabolic group, due to the fact that a wide range of metabolic capabilities can be found within this phylogenetic lineage.

Surprisingly, sequences from the specialized anaerobic sulfur oxidizers (i.e. the the photosynthetic purple and green sulfur bacteria) were not dominant in the clone library. In fact, 16S rRNA gene sequences from green sulfur bacteria were not detected, in agreement with microscopy and pigment data. Conversely, 16S rRNA gene sequences from the generalist *Rhodobacter* spp. and from the aerobic sulfur oxidizers, together with chloroplast from algae, were recovered from the library in abundance. Most Alpha-Proteobacteria (75% of total Alpha-Proteobacteria) were related to *R. veldkampii* (percentage of similarity ≥ 99%), a species that is able to use both sulfide and elemental sulfur as a photosynthetic electron donors (Imhoff 2001a).

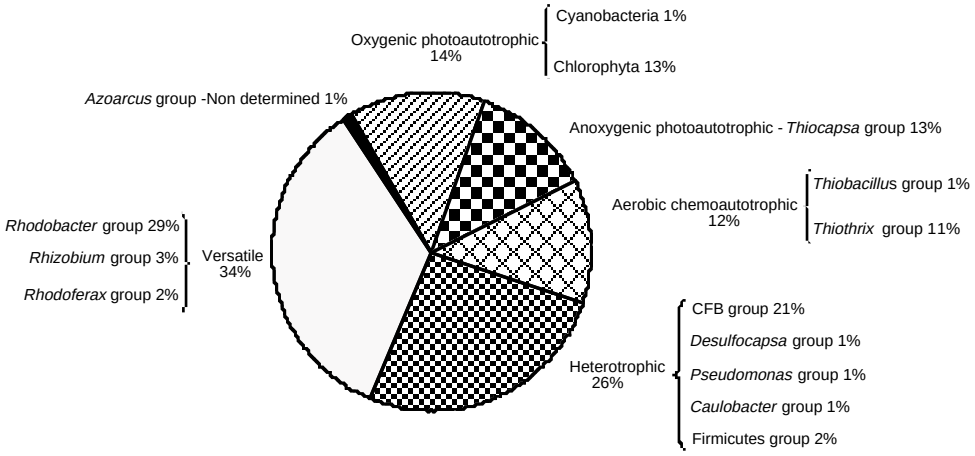

Figure 5.4. General activities and putative functional roles for the phylotypes recovered.

Discussion

In the past, microbial systems developed for sulfide removal from wastewater used two different and mutually exclusive approaches: (i) aerobic sulfide oxidation by thiobacilli (Sublette and Sylvester 1987; Buisman et al. 1990; Janssen et al. 1997) or (ii) anaerobic sulfur oxidation by phototrophic bacteria (Cork et al. 1983; Kobayashi et al. 1983; Kim et al. 1990). These approaches were traditionally based on the use of either pure cultures or mixtures of a few well known bacterial groups growing in liquid culture or in biofilms (Jensen and Webb 1995).

In our fixed-biomass reactor, a biofilm with high cell-density and high genetic and physiological diversity developed after inoculation with anoxic lake sediment. Since we used inorganic mineral medium with carbonate as the only carbon source, hydrogen sulfide as electron donor, a continuous light regime and anaerobic conditions, we expected the development of phototrophic sulfur bacteria. Surprisingly, oxygenic phototrophs and colorless sulfur bacteria were also present together with purple sulfur bacteria. Thus, aerobic and anaerobic sulfide oxidizers coexisted in the reactor. In the past, the systems so far investigated were based on either aerobic or anaerobic oxidation. The microbial assemblage reported here simultaneously combines aerobic and anaerobic sulfide oxidation in a system in which oxygen is internally supplied by oxygenic photosynthesis.

Although some microorganisms could be identified through the microscope, most of the microorganisms could not (up to 63%). Therefore, we used a molecular approach based on 16S rRNA gene characterization in order to identify them. Most sequences retrieved were very similar to cultured organisms. Many different physiological groups were recovered and, furthermore, the coverage of the clone library was relatively high (73%). Although the 16S rRNA gene approach is not free of problems (Von Wintzingerode et al. 1997), for instance PCR amplification could introduce biases in the relative abundance of the different groups, it is very useful to identify most of the populations present in the biofilm. We found a reasonably good qualitative agreement between microscopically observed microorganisms and those recovered in the clone library. From a quantitative point of view, the relative abundance of purple sulfur bacteria in the library and in the microscopic counts differed only by a factor of two. However, higher proportions of green algae and *Thiothrix*-like sequences were retrieved in the library than in direct counts. Overall, such differences are within the range reported by others (Felske et al. 1998; Nübel et al. 1999; Casamayor et al. 2000, 2002) and can also be explained by the heterogeneity of the biofilm and by the presence of aggregates that made it difficult to obtain identical replicates. The scope of this work was to obtain a general assessment of the taxonomic and functional composition of the biofilm. For a more detailed quantification, other techniques such as fluorescent *in situ* hybridization (FISH; Amann et al. 1995) are required. However, this was beyond the scope of our work.

Although our study does not include the analysis of metabolic activities, the presence of organisms belonging to several well known functional groups suggests a complex set of interactions. First, purple sulfur bacteria (*Thiocapsa*-like) and purple nonsulfur bacteria

(*Rhodobacter*-like and *Rhodopseudomonas*-like) can perform anaerobic light-driven oxidation of sulfide. Simultaneously, sulfide can also be oxidized aerobically by colorless sulfur bacteria (*Thiobacillus*-like and *Thiothrix*-like) and by some purple sulfur bacteria (*Thiocapsa*-like; De Wit and Van Gemerden 1990) using the oxygen produced by cyanobacteria and algae. All the primary producers generate organic matter that can be used subsequently by heterotrophs such as Cytophagales, Pseudomonadales, Caulobacteriales, Firmicutes and purple nonsulfur bacteria. Both aerobic oxidation and fermentation of such organic matter could take place. Sulfate reducing bacteria could also use organic matter with sulfate, producing sulfide as end-product. Sulfide production, however, would be a minor process because only a clone related to this group was retrieved. Although only indirect experimental evidences are presented here, the processes outlined above seem plausible and any organism not able to grow would have been washed out of the system. Techniques combining genetic identity and function, such as microautoradiography-FISH and stable isotope probing of nucleic acids (Gray and Head 2001), would help to find out the real contribution of each active population.

The large and heterogeneous attachment surface through the column provided microenvironments where different groups of microorganisms developed. In fact, large metabolic as well as genetic diversity were represented in the clone library. We retrieved new 16S rRNA genes, substantially different from any previously known sequence. Conversely, we observed clusters of closely related sequences below the “species-level” (microdiversity) (Furhman and Campbel 1998). This microheterogeneity in the 16S rRNA gene has been previously reported in clone libraries of Bacteria, Archaea and eukaryotic microorganisms from several natural environments (Field et al. 1997; Massana et al. 2000; Díez et al. 2001). Some of this microdiversity might be explained by *Taq* polymerase errors during PCR amplification (Field et al. 1997) or by rRNA multioperons belonging to a single population (Nübel et al. 1996). However, it is clear that, in many cases the microdiversity has ecological and physiological significance in natural populations (Casamayor et al. 2002). Microdiversity allows niche specialization and therefore the coexistence of ecotypes with different substrate affinities or different light-dependent physiology (West and Scanlan 1999; Casamayor et al. 2002). The meaning of microdiversity in the bioreactor remains to be established, but due to the heterogeneity of our biofilm and the microenvironments developed, niche specialization is a possible explanation.

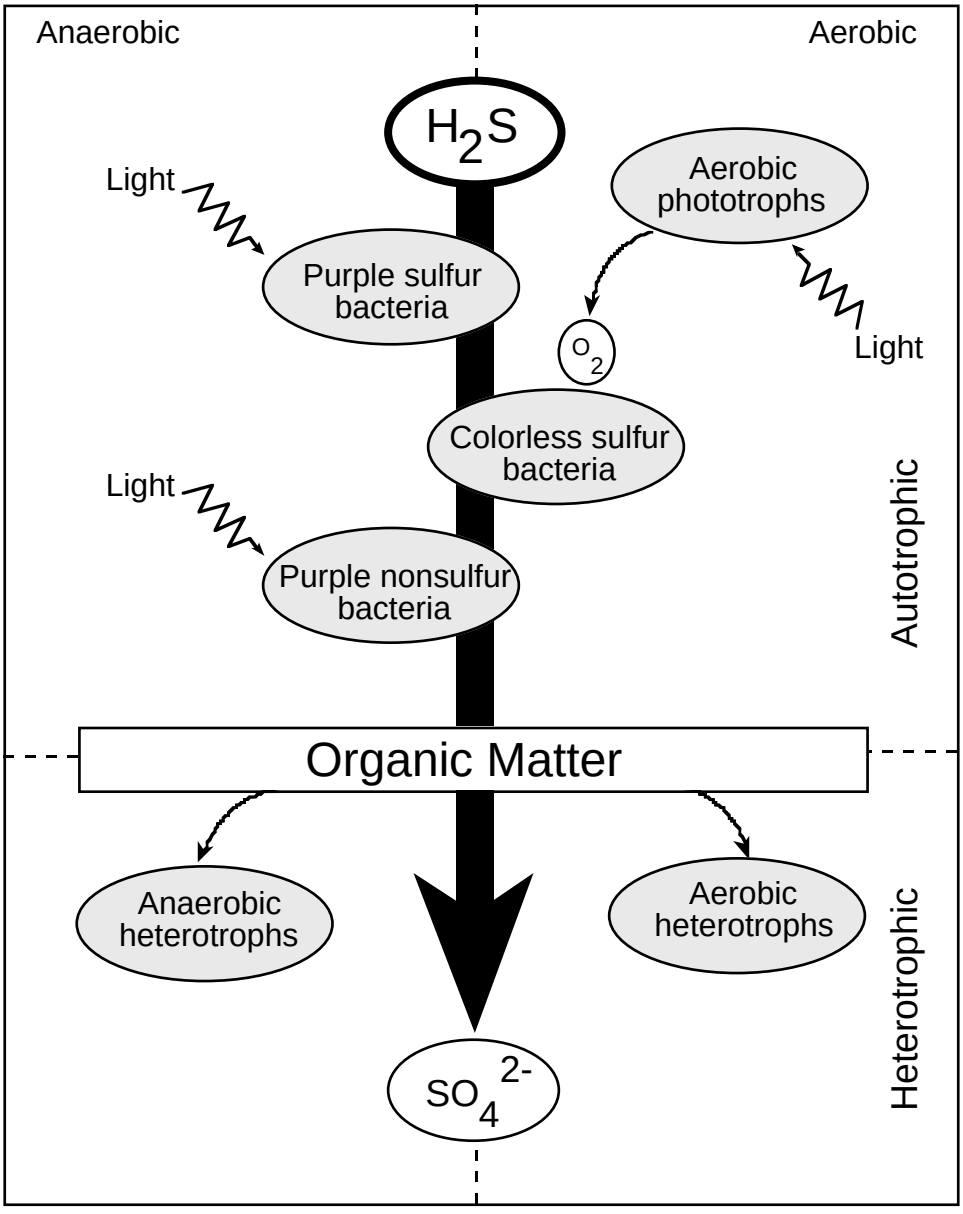

Figure 5.5. Possible interactions in the microbial assemblage. Sulfide would be aerobically and anaerobically oxidized. Autotrophic members would produce organic matter that would be subsequently used by the heterotrophs.

In the present work, we do not attempt to describe a “type” microbial community able to oxidize sulfide, but rather to prove that, using this type of reactor design, we can successfully select complex microbial assemblages carrying out a specific function. In effect, the biofilm mimics to a considerable extent the taxonomic and functional diversity found in stratified sulfurous ecosystems with oxic/anoxic interfaces, such as sulfurous lakes (e.g., Pedrós-Alió and Guerrero 1993; Casamayor et al. 2000). The coexistence of aerobic, anaerobic, phototrophic and chemotrophic microorganisms in the biofilm, and the species richness found within these metabolic groups, together with the microdiversity observed within some species, could be very important for long-term functioning and versatility of the reactor. Heterogeneous, multispecies biofilms composed of different physiological and ecological members should be better adapted to withstand major disruptions in the environmental conditions. The presence of more than one species with the same metabolic profile (e.g., *Thiobacillus* vs. *Thiothrix*, or *Thiocapsa* vs. *Chromatium*) would insure continued functioning in the case that disruptions affected one of the populations but not the others. The complex interactions established are key attributes for the long-term performance of the bioreactor.

Chapter 6. POTENTIAL OF MICROBIAL MATS FOR THE DEVELOPMENT OF COMPLEX SULFIDE-OXIDIZING BIOFILMS

POTENTIAL OF MICROBIAL MATS FOR THE DEVELOPMENT OF COMPLEX SULFIDE-OXIDIZING BIOFILMS

Abstract

A microbial mat sample was used to inoculate an anaerobic bench-scale bioreactor specially designed for the treatment of sulfide-containing effluents. A complex microbial biofilm with sulfide-oxidation activity developed. The microbial composition of the biofilm was studied by pigment, microscopy and 16S rRNA gene analysis. Purple sulfur bacteria and diatoms were observed by microscopy, chlorophyll and bacteriochlorophyll *a* were detected in the pigment analysis and high genetic diversity was found in the 16S rRNA gene library. Specialized anaerobic sulfur oxidizers (i.e., phototrophic purple and green sulfur bacteria) dominated the library. Aerobic phototrophs (diatoms) also developed and the produced oxygen allowed the growth aerobic sulfide oxidizers, such as *Thiomicrospira*-like. Cyanobacteria, which are important organisms in natural microbial mats, did not develop in the reactor but unexpected members from the Epsilon-Proteobacteria developed profusely. Moreover, other minority organisms such as members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) and purple nonsulfur bacteria (*Roseospirillum* sp.) were also present. Results showed that microbial mats were a good source of genetic and metabolic diversity for the development of artificial complex sulfide-oxidizing biofilms.

Introduction

The ability of certain microorganisms to use sulfide has been exploited for the treatment of sulfide-containing wastewaters and gas streams. Particularly, the application of anoxygenic phototrophs (*Chlorobium* sp.) and aerobic chemolitotrophs (*Thiobacillus* sp.) has been studied (Cork et al. 1983; Kobayashi et al. 1983; Sublette and Sylvester 1987; Buisman et al. 1990; Janssen et al. 1997). Most systems described have been based on the use of pure cultures growing in liquid reactors and thus, presented problems of biomass washout or instability in front of environmental disturbances. Recently, we have described a fixed-biomass reactor, which allows the development of complex sulfide-oxidizing biofilms (Chapters 2 and 3). The reactor operates as a sulfidostat and the control system allows highly polluted streams to be oxidized, while microorganisms are exposed to constant low substrate concentrations, thereby avoiding inhibition due to high sulfide concentrations. This reactor was inoculated with anoxic lake sediment and a complex biofilm developed showing a stable oxidation performance. The analysis revealed high genetic and functional diversity within the biofilm, where aerobic, anaerobic, phototrophic and chemotrophic microorganisms coexisted (Chapter 5). Such heterogeneous multispecies biofilm composed of different physiological and ecological members presented a slow dynamic behaviour and therefore, is less sensitive to sudden disturbances.

In this chapter, we explore the possibility of using microbial mats as inoculum for the development of sulfide-oxidizing biofilms. Microbial mats are laminated microbial communities developing in shallow marine sediments and tidal flats. These communities are composed of a wide range of metabolically active groups. Within these structures an intense sulfur cycle develops, with sulfide being produced by sulfate reducing bacteria and being reoxidized by a diverse assembly of anoxigenic phototrophs, chemolititrophs and oxygenic phototrophs (Stal 1991; Caumette et al.1994). These communities have been used as natural models to study carbon and sulfur cycles *in situ*. However, due to their complexity and functional and genetic diversity, they have also been used in applied microbiology, for instance for the degradation of petroleum compounds (Grötzschel et al. 2002). Since microbial mats are benthic structures in which microorganisms spontaneously develop attached to surfaces and to each other, they might be useful as inoculum for the start up of sulfide-oxidizing biofilm reactors.

Thus, we inoculated the sulfide-removal bioreactor previously developed with a microbial mat sample from the Ebro Delta. A complex biofilm developed and microbial species composition was determined by microscopy and 16S rRNA clone library. Our results indicated the existence of a complex, metabolically diverse assemblage where oxygenic (diatoms) and anoxygenic phototrophs (purple and green sulfur bacteria) coexisted with other members of the Alpha-, Gamma- and Epsilon-Proteobacteria, and the *Cytophaga-Flavobacterium-Bacteroides* group.

Results

Description of the inoculum

A microbial mat sample was used to start up the system. The sample was collected in summer from a sand flat at the Ebro Delta, in particular from a site called P3 (40º 40' N, 0º 40' E) described by Mir et al. (1991), which was partially inundated at sampling time. At this site, microbial mats are well developed and vertically stratified (Mir et al. 1991; Esteve et al. 1992; Guerrero et al. 1993). In the uppermost layer diatoms are abundant, the second layer is composed mainly of the filamentous cyanobacterium *Microcoleus* and underneath anoxygenic phototrophic bacteria, especially purple sulfur bacteria, can be found. Several species of non-phototrophic bacteria, i.e, spirochetes and unidentified small rods and cocci, have been observed in such mats (Mir et al. 1991; Margullis et al. 1993). Pigment analysis of the inoculum showed the presence of chlorophyll (Chl) and bacteriochlorophyll (Bchl) *a*. Observations of the sample by phase-contrast microscopy showed the presence in the inoculum of cyanobacteria, diatoms and purple sulfur bacteria among others.

Biofilm development

The bioreactor column was inoculated at day 0 and its macroscopic development was inspected and registered daily with a digital camera. Images showed that cell attachment occurred slowly, and until 10 days after inoculation the whole column was not completely

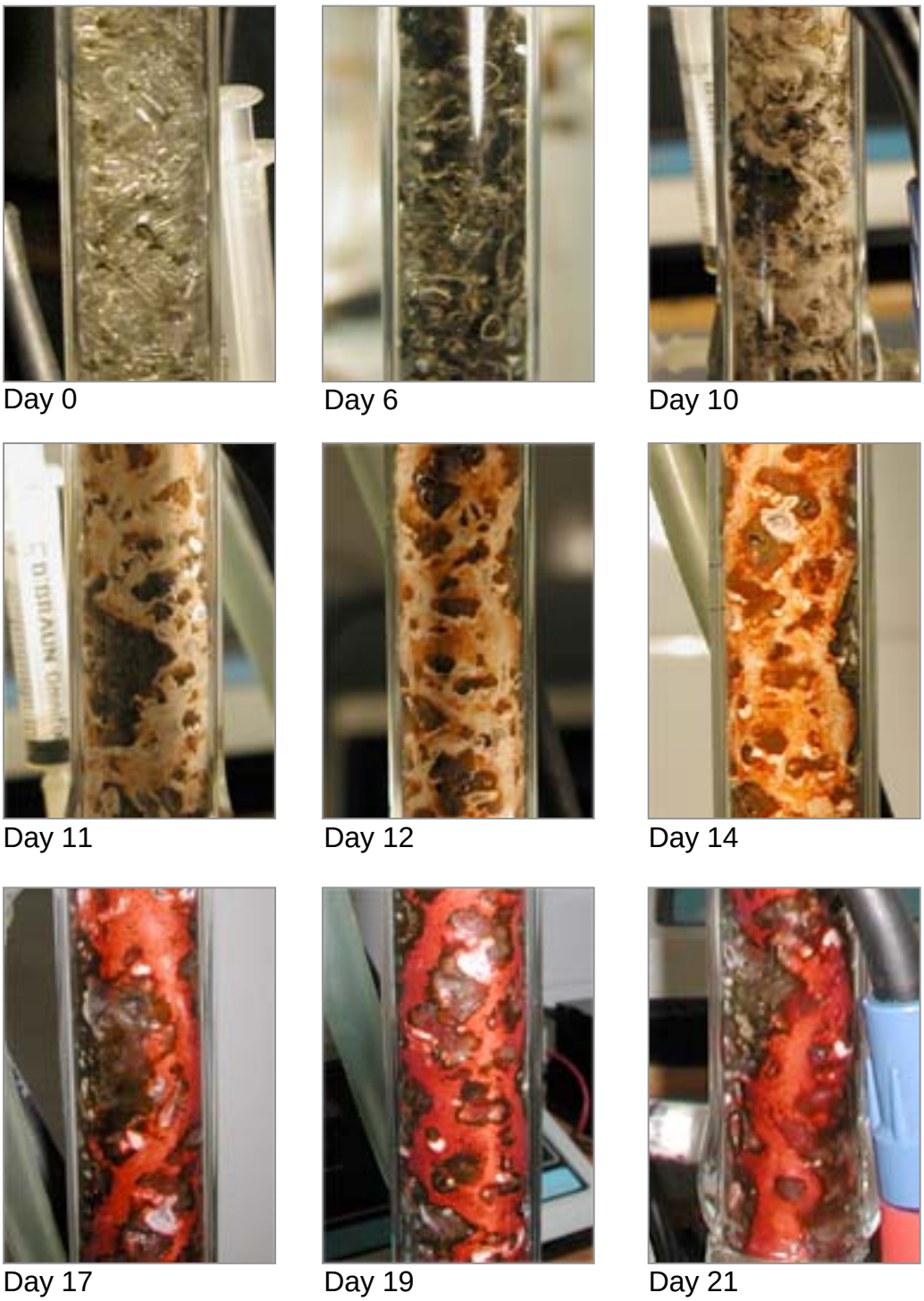

Figure 6.1. Images showing changes in the macroscopic aspect of the column throughout time.

colonized (Figure 6.1). The appearance of the column changed progressively, and finally, a complex colorful biofilm developed and maintained visible characteristics from day 17 on. Predominant colors were red and brown-green, in agreement with the presence of purple sulfur bacteria and diatoms further observed with the microscope.

Monitoring of the oxidation rate agreed with the temporal development of the biofilm (Figure 6.2). The initial concentration of sulfide in the reactor decreased progressively down to 50 µM during the first six days. During this period, no fresh medium was pumped into the reactor, and therefore, no effluent was produced. From then on, pump speed increased gradually and sulfide oxidation rate reached 158 $\mu mol \cdot h^{-1}$. Under these conditions the percentage of sulfide concentration reduction was 96%. After 21 days running, and once the oxidation rate was stabilized, the experiment was finished and the microbial community forming the biofilm was analyzed.

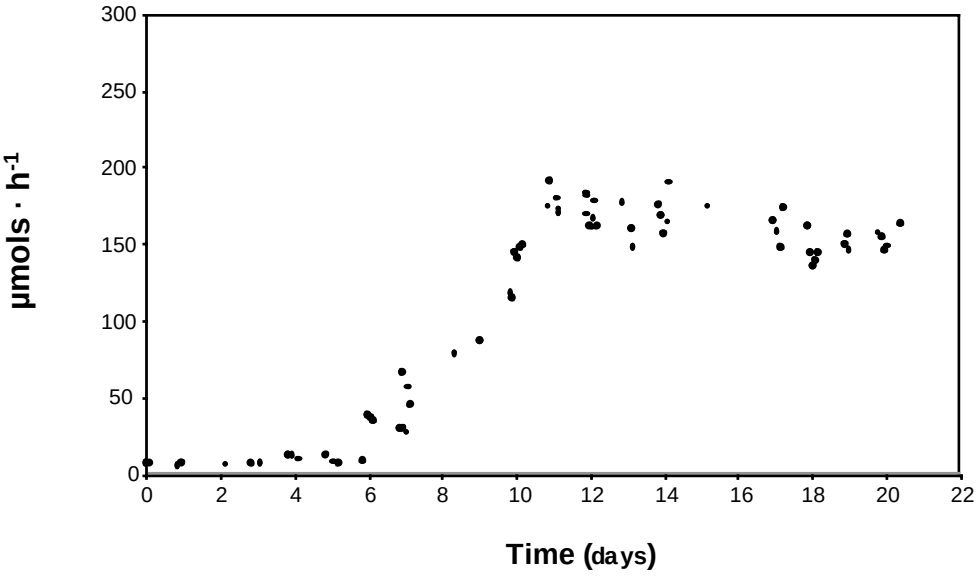

Figure 6.2. Sulfide oxidation rate (µmols of sulfide oxidized per hour) of the biofilm as a function of time.

Characterization of the biofilm. Biofilm was removed (see Materials and Methods) and observations by phase-contrast and epifluorescence microscopy showed the presence of different morphotypes, such as diatoms and purple sulfur bacteria. However, many cells did not show any characteristic morphological trait, and therefore, could not be identified by microscopy. Biomass recovered from the column totaled 431 mg of protein that corresponded to a density of 1.33 mg protein·cm^{-2}. Pigment analysis indicated presence of Chl *a* (10.8 µg·cm^{-2}) and Bchl *a* (21.5 µg·cm^{-2}).

Clone library construction. Amplification with archaeal primers yielded no positive signal even though two sets of primers were used and the procedure was repeated several times using different DNA template concentrations in the reaction. In all the attempts, DNA from *Sulfolobus* used as positive control yielded a positive signal. On the contrary, the bacterial specific set of primers (27F/1492R) yielded a PCR product which was used to construct the clone library. After the clone restriction analysis, sequencing and the Check-Chimera analysis, 37 different sequences were obtained. The retrieved sequences, their frequency in the library and their closest relatives are listed in Table 6.1. Sequences sharing more than 97% similarity were grouped and then, the number of phylotypes decreased to 26 (Figure 6.3). Twenty-three clones corresponded to unique RFLP band pattern. The coverage of the library was 86.5%. Although the diversity in the biofilm was probably not fully covered, curves indicate that most diversity in the library was recovered.

rDNA sequencing and phylogenetic analysis. Partial 16S rRNA gene sequences recovered were included in two phylogenetic trees (Figures 6.4 and 6.5). Different phylogenetic groups previously identified by microscopy were retrieved, such as green algae (diatoms) and ε-Proteobacteria (i.e., Chromatiaceae). In addition, ε - and ε-Proteobacteria and *Cytophaga-Flavobacterium-Bacteroides* (CFB) group were also detected.

The relative distribution of the clones recovered in the library is shown in Figure 6.6. Gamma-Proteobacteria dominated the library (52% of total clones), mostly by representatives of *Chromatium* and relatives (up to 46%). Epsilon-Proteobacteria were also dominant in the library (22% of total clones) while Alpha-Proteobacteria were less abundant (8%). The

remaining clones belonged to chloroplasts from algae (4%), Chlorobi (5%) and the CFB (8%). Finally, 1% of clones were not affiliated with a significant percentage to any phylogentetic group and were grouped as non-identified.

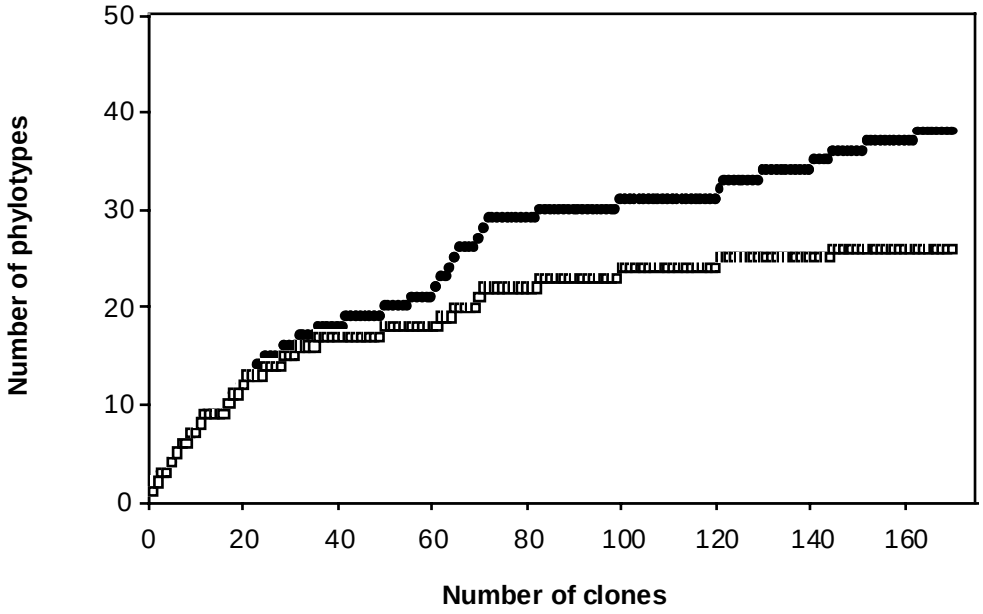

Figure 6.3. Accumulation curves of diversity in the bacterial clone library. Black circles represent the sequential detection of cumulative phylotypes following RFLP patterns. White squares represent phylotype accumulation after grouping sequences sharing more than 97% similarity.

Most of the clones were highly related to previously described sequences in the GenBank database. In particular, 63% of the clones had more than 97% of similarity to cultured species. All the purple sulfur bacteria related clones were affiliated to *Marichromatium purpuratum* while other Gamma-Proteobacteria present but less abundant were affiliated to cultured strains of the genera *Thiomicrospira* and *Vibrio*. All clones affiliated to the Chlorobiaceae were highly related to *Prosthecochloris aestuarii*. Among the ε subclass most clones were related to the so-called phototrophic purple ε-Proteobacteria, in particular to *Roseospirillum parvum,* which was isolated from a marine microbial mat (Glaeser and Overmann 1999). Chloroplasts from algae were also present in the bacterial clone library (4%), and in agreement with microscopy observations, sequences were related to a diatom (97.4% of similarity). Sequences belonging to the *Cytophaga-Flavobacterium-Bacteroides*

Table 6.2. Phylogenetic affiliation of clones to the closest match in Genbank (n:frequency; nt:sequence length).

Group	name	n	nt	Closest match	Acc nº	Similarity
Alpha-proteobacteria						
	2B4	1	692	*Rhodovulum adriaticum*	D16418	100
	1E5	1	674	*Erythrobacter litoralis*	AF465836	99,6
	1G1	1	669	*Roseospirillum parvum*	AJ011919	99,5
	2E12	2	681	*Roseospirillum parvum*	AJ011919	99,5
	2H6	1	686	*Roseospirillum parvum*	AJ011919	99,5
	1B9	8	693	*Roseospirillum parvum*	AJ011919	99,2
Gamma-Proteobacteria						
	1F4	1	648	*Marichromatium purpuratum*	AF294031	99,7
	3A9	1	700	*Marichromatium purpuratum*	AF294031	99,7
	2G6	3	715	*Marichromatium purpuratum*	AF294031	99,6
	1A10	3	621	*Marichromatium purpuratum*	AF294030	99,5
	2G3	1	709	*Marichromatium purpuratum*	AF294031	99,4
	1B2	69	695	*Marichromatium purpuratum*	AF294031	98,6
	1G4	1	690	*Thiorhodococcus drewsii*	AF525306	99,4
	2C9	2	697	*Thiomicrospira chilensis*	AF013975	99,6
	2E11	1	675	*Thiomicrospira kuenenii*	AF013978	95,4
	1C3	1	667	*Vibrio* sp.	AF064637	99,0
	2E10	2	651	*Vibrio* sp.	AY374383	98,9
	1D3	1	673	Uncultured gamma proteobacterium	AF453551	94,7
	1F10	1	656	uncultured gamma proteoacterium	AF453551	94,7
	1G5	1	611	*Escarpia spicata* endosymbiont	AF165908	92,0
Epsilon-proteobacteria						
	1B6	1	654	Uncultured *Sulfurospirillum*	AF513952	97,2
	1C6	26	639	Uncultured epsilon proteobacterium	AJ441205	96,9
	1F5	1	656	Uncultured epsilon proteobacterium	AJ441205	96,0
	1B8	1	630	Uncultured epsilon proteobacterium	AJ575993	95,2
	2A6	5	642	Uncultured epsilon proteobacterium	AJ575993	95,2
	1D10	4	660	Uncultured epsilon proteobacterium	AJ575993	91,9
CFB						
	1A6	1	648	*Marinilabilia salmonicolor*	M62423	96,0
	1G6	1	688	*Marinilabilia salmonicolor*	M62422	94,8
	1A5	1	672	*Marinilabilia salmonicolor*	M62422	94,5
	1A4	2	645	Uncultured Bacteroidetes	AJ347762	94,3
	1D5	6	690	uncultured bacterium	AJ548901	91,3
	2A10	1	643	Bacteria from anoxic bulk	AJ229236	90,5
	2F2	1	616	Bacteria from anoxic bulk	AJ229236	90,1
Chlorobiales						
	1B5	8	650	Chlorobiaceae bacterium	AF513460	98,6
Plasts						
	1D12	7	697	*Haslea salstonica*	AF514854	98,2
ND-Bacteria						
	2G8	1	663	Uncultured bacterium	AF371929	88,8
	1F8	1	676	uncultured bacterium clone SJA-101	AJ009480	88,3

cluster accounted for 8% of the clones and some were related to the marine species *Marinilabilia salmonicolor*. Most of sequences belonging to the Epsilon-Proteobacteria were affiliated to “uncultured organisms” and only a sequence matched to *Sulfurospirillum* sp.

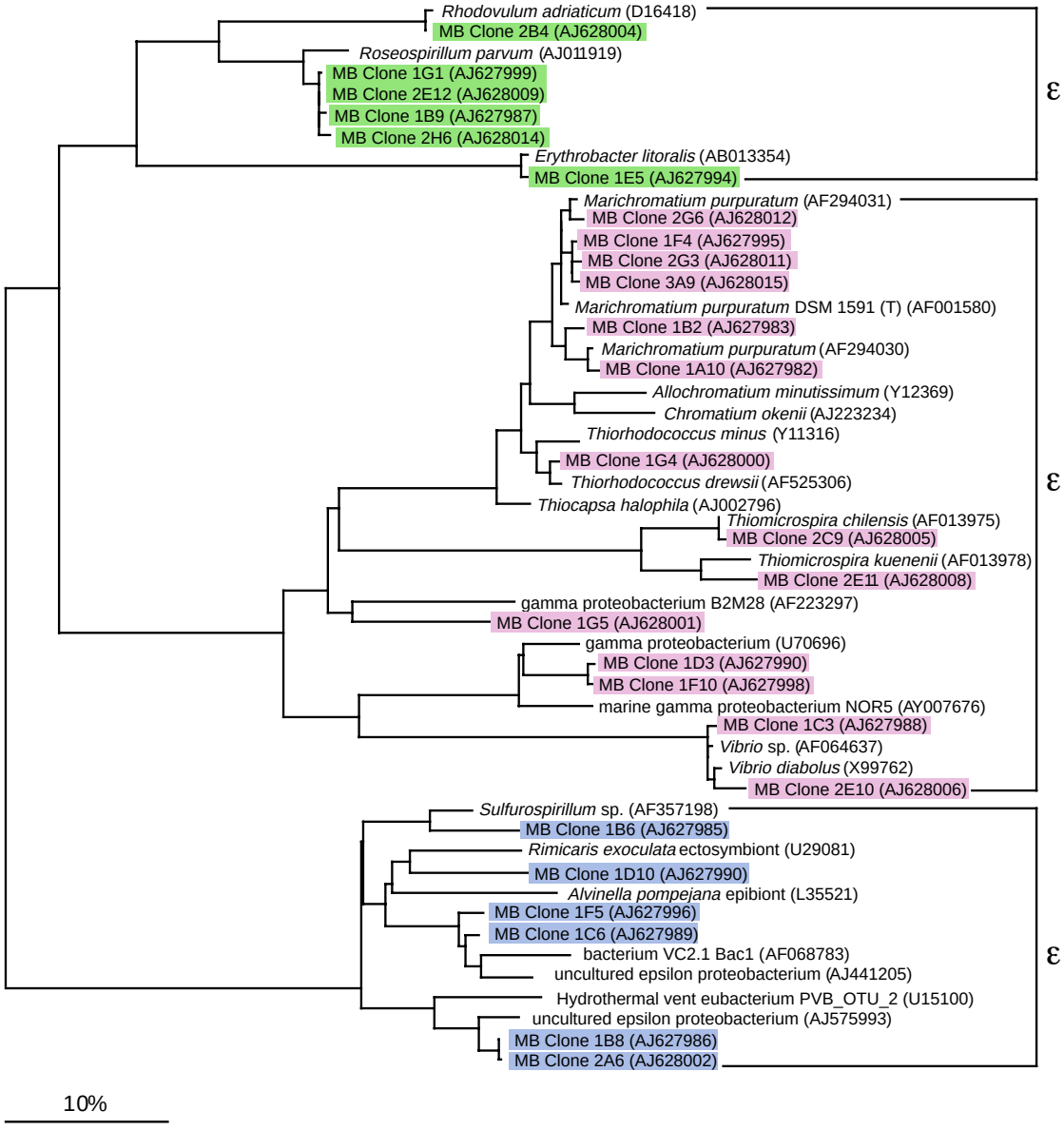

Figure 6.4. Phylogenetic tree including sequences recovered from the biofilm that affiliated to Proteobacteria.

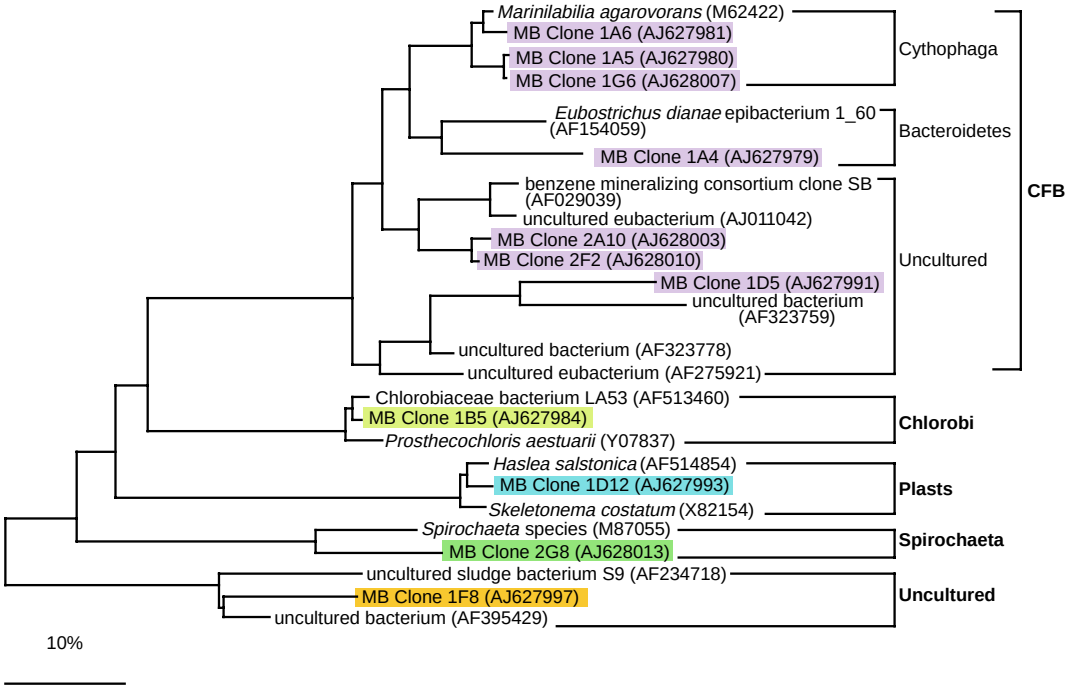

Figure 6.5. Phylogenetic tree including sequences recovered from the biofilm that affiliated to the CFB group, Chlorobi, Plasts and other bacteria.

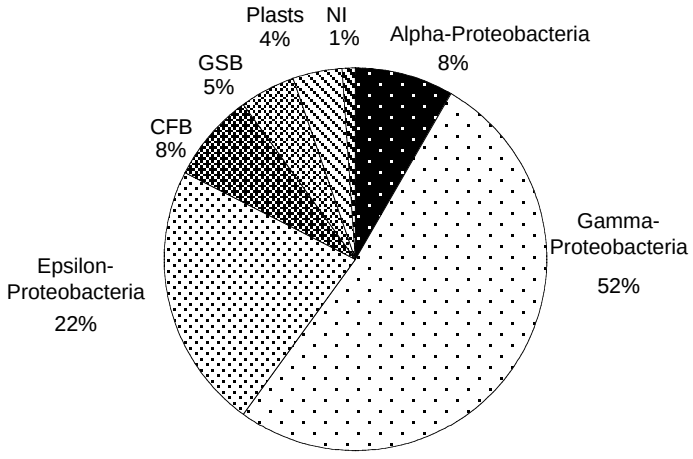

Figure 6.6. Relative abundance of the phylogenetic groups recovered in the library (GSB: Green sulfur bacteria; CFB: *Cytophaga-Flavobacterium-Bacteroides*; NI: Non identified).

Phylogenetic analyses also revealed that some of the retrieved sequences showed a considerable degree of microdiversity (Furhman and Campbell 1998), especially for some clones related to *Roseospirillum parvum* and *Marichromatium purpuratum* (see phylogenetic tree, Figure 6.4). Similarity matrices between such similar sequences were carried out with an ARB tool; four *Roseospirillum parvum*-like sequences were on average 99.6% similar to each other and three *Marichromatium purpuratum*-like sequences were on average 99.3% similar among them.

Discussion

A complex biofilm aimed at the oxidation of sulfide-containing effluents was developed after the inoculation of a bench-scale reactor with a natural sample from the Ebro Delta microbial mats. Different phylogenetic and physiologic groups developed in the bioreactor and were characterized by using traditional techniques and a molecular approach, in particular cloning and sequencing. Some organisms could be identified by microscopy (purple sulfur bacteria and diatoms) but most of them could not, and were identified by sequencing their 16S rRNA genes. The quantitative results of the clone library could present biases due to problems related to PCR-based techniques (e.g., selective amplification; Reysenbach et al. 1992; Farrelly et al. 1995; von Wintzingerode et al. 1997). However, although the relative abundance of each group in the library might differ from their real contribution, in our study, the presence of some groups recovered by cloning was supported by microscopy and pigments analysis (i.e., purple sulfur bacteria and green algae). Relative abundance of Chromatiaceae (PSB) represented a 47% and in the case of plastids, the percentage was 4%. Purple sulfur bacteria were predominant both in the molecular and classical analysis carried out, and certainly, the purple-red aspect of the column gave a good impression of which was the predominant group (Figure 6.1). On the contrary, pigments from GSB were not detected while they were recovered by cloning. This may be due to the fact that the concentration of such bacteriocholorophylls was below the detection level or because of the heterogeneity of the aliquots.

In the clone library, a high number of different sequences were recovered and most of them (63%) had 16S rRNA gene similarities over the species level with cultured species (>97%; Stackebrandt and Goebel 1994). Then, because they are closely related to well-

characterized cultured strains and because the prevailing environmental conditions within the bioreactor were well known, we can tentatively assign putative general activities for the microorganisms behind such 16S clone rRNA sequences (Figure 6.7).

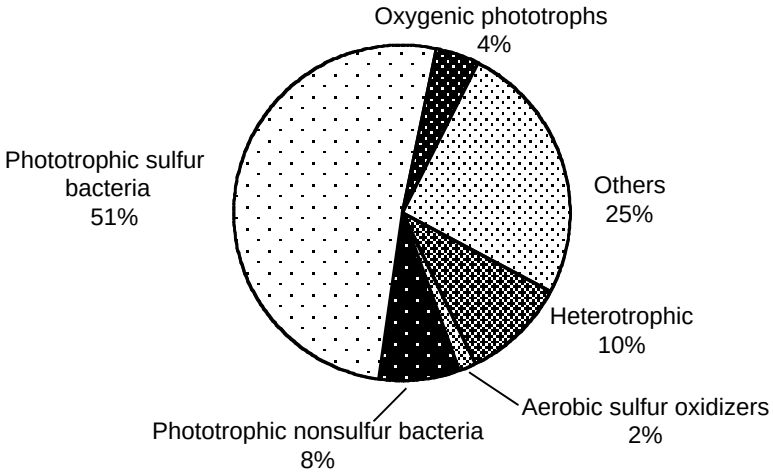

Figure 6.7. Microbial guilds recovered from the marine biofilm clone library.

As expected, most of the microorganisms were involved in the sulfur cycle. Specialized anaerobic sulfur oxidizers carrying out anoxygenic photosynthesis, such as purple (PSB) and green (GSB) sulfur bacteria, dominated the biofilm. Both are microorganisms typically found in benthic-illuminated environments containing sulfide, such as microbial mats (van Gemerden and Mas 1995). In particular, the PSB developed in the bioreactor was affiliated to *Marichromatium purpuratum* at the species level (>99% similarity in 16S rDNA). Usually, this species is found in association with sponges (Imhoff and Trüper 1976) rather than in suspension, and this ability may facilitate its profuse growth attached to the column filling material. Although some species of green sulfur bacteria (GSB) have been isolated from microbial mats, usually purple bacteria dominate mats to the exclusion of green species, and therefore, they are rarely abundant in such ecosystems. Surprisingly, we enriched green sulfur bacteria highly related to *Prosthecochloris aestuarii* in our bioreactor.

Apart from sulfur bacteria, diatoms were another important phototrophic group present in the biofilm. In the bioreactor, since there was no aeration, they would represent a source of oxygen produced by photosynthesis, which could be used by aerobic organisms. Diatoms

usually can be found in the upper layer of microbial mats together with cyanobacteria. In Ebro Delta microbial mats, the filamentous cyanobacteria *Microcoleus* and *Lyngbya* have been reported as the most abundant oxygenic phototrophs (Mir at al. 1991; Guerrero et al. 1993; Martínez-Alonso 1997). Thus, their development in the column was expected but instead algae developed. From an applied point of view, both organisms would have the same function in the biofilm, which is oxygen production through oxygenic photosynthesis, and therefore, to facilitate the development of other aerobes. Thus, although taxonomically the structure of the biofilm is not identical to a microbial mat, the photrophic metabolisms were represented in the biofilm, in particular by aerobic phototrophs (diatoms) and anaerobic phototrophs (PSB and GSB). Moreover, other metabolisms were also represented contributing to biofilm heterogeneity and complexity.

Other Gamma-Proteobacteria less abundant were affiliated with cultured strains of the genus *Thiomicrospira*, a colorless sulfur bacteria which have shown ability to chemolitotrophically oxidize reduced sulfur compounds (Robertson and Kuenen 1999), and of the genus *Vibrio,* which have been described in marine environments as heterotrophic bacteria (Farmer and Hickman-Brenner 1999). Among the Alpha-Proteobacteria, most clones were related to the purple nonsulfur bacteria, in particular to *Roseospirillum parvum*. This group presents high metabolic versatility and in laboratory experiments, can carry anoxygenic photosynthesis using both CO_2 or organic matter as carbon source and organic matter or reduced sulfur compounds as electron donors (Imhoff 2001a). In nature, these bacteria are found in those anoxic waters and sediments that receive sufficient light to allow phototrophic development, and often accompanying the purple sulfur bacteria. While freshwater isolates have very low tolerance to sulfide, most marine species resist higher concentrations, and they even use sulfide and thiosulfate as photosynthetic electron donors (Imhoff 2001a)**.** Obviously, in our bioreactor, such microorganisms tolerated sulfide, and might have grown autotrophically using sulfide as electron donor.

Cytophaga-Flavobacterium-Bacteroides-related organisms also developed in the biofilm. These bacteria are ubiquitous, abundant in several natural organic-rich habitats and probably, play a major role in the turnover of organic matter in nature (Cottrell and Kirchman 2000; Kirchman 2002). Within this group aerobic, microaerophilic and anaerobic members are found and most of cultured CFB are chemoorganotrophs. In the past, their presence in sediments was not investigated because of the lack of appropriate techniques for their

identification. However recent studies have found out the importance of CFB in microbial mat ecosystems (Mouné et al. 2003). Most 16S rRNA sequences from the CFB cluster found in databases belong to uncultured organisms and few sequences are available of cultured strains. Therefore, sequences recovered are usually affiliated to "uncultured". Surprisingly some our sequences are related to the marine specie *Marinilabilia salmonicolor* (synonym *Cytophaga salmonicolor*), which as most of *Cytophaga* species is able to grow aerobically or under microaerophily, and this species in particular, can perform fermentation producing CO_2 and H_2 (Reychenbach 1999).

However, the most surprising finding was that a significant part of the sequences were related to uncultured Epsilon-Proteobacteria. This phylogenetic group is the smallest and more recently recognized line of descent within the Proteobacteria (Kersters et al. 2003). Several recent studies have demonstrated the presence and dominance of thermophilic Epsilon-Proteobacteria both free-living and associated with metazoans (Polz and Cavanaugh 1995; Longnecke and Reysenbach 2001; Alain et al. 2002) or in thermophilic microbial mats (Moyer et al. 1995) at deep-sea hydrothermal vents, where they play an important role in carbon and sulfur cycling. Moreover, ε-*Proteobacteria* have also been detected and/or isolated from deep subsurface sediments, oil fields, activated sludge, marine snow, and in the oxic/anoxic chemocline from the Black Sea (Campbell et al. 2001; Kodama and Watanabe 2003; Vetriani et al. 2003). Although these organisms have never been reported as important members in mesophilic microbial mats, recently Mouné et al. (2003) recovered a few clones in a library carried out from the anoxic sediment from hypersaline microbial mats. Chemoautotrophic growth using sulfide, elemental sulfur, thiosulfate, and hydrogen as electron donors under anaerobic or microaerophil conditions has been described within this phylogenetic group (Kodama and Watanabe 2003). Surprisingly, members of this subclass of the Proteobacteria found optimal environmental conditions and flourished in our biofilm. Our sequences were related to hydrothermal deep-sea bacteria. Unfortunately, we have only a few clones to extract conclusions on their metabolic capacities and thus, they were grouped as “other” functional role in Figure 6.7. However, taking into account the environmental conditions within the reactor, they may be involved in sulfide oxidation. The use of such bioreactor could be very useful for further metabolic studies or even for the isolation of these organisms.

Summarizing, a complex biofilm aimed at the treatment of sulfide-containing effluents was obtained by using a microbial mat as inoculum. High genetic diversity was found and revealed the coexistence of metabolically diverse groups. The large and heterogeneous attachment surface of the column allowed the existence of microenvironments that can explain the coexistence of strict anaerobes (GSB) and oxygen producers (diatoms) in the system, as well as the presence of closely related sequences below the “species-level” (microdiversity; Furhman and Campbell 1998). Cell immobilization in the reactor allowed the presence of organisms constituting a minor part of the original community, which would have been washed out in liquid systems. This contributed to the diversity and complexity of the assemblage, in which a wide array of metabolic pathways was available, therefore, contributing to the stability of the community. Because of such complexity, this biofilm is comparable to a natural community, and we can conclude that microbial mats are a good source of genetic and metabolic diversity for the development of sulfide-oxidizing biofilms.

Chapter 7. GENERAL DISCUSSION

GENERAL DISCUSSION

The work presented here deals with the development and characterization of sulfide-oxidizing biofilms. First of all, a bioreactor for biofilm development was designed. Biofilm systems are more stable than stirred reactors because biomass is immobilized and therefore, not subject to washout. The system was based on a packed-column, which provided a large surface for microbial attachment. In this bioreactor, no aeration was supplied and light was the only initial energy source, to force the development of specialized sulfide-oxidizing organisms. The reactor operated as a sulfidostat and the control system kept the residual sulfide concentration constant in the micromolar range (**Chapter 2**). Thus, highly polluted streams could be efficiently processed avoiding inhibition of sulfide-oxidizing bacteria while maintaining the quality of the effluent.

The system was tested using first a pure culture of *Chlorobium limicola,* then, a natural sample from the sediment of a freshwater lake and, finally, a marine microbial mat. Biofilms developed vigorously on the light-exposed column surface and high microbial biomass was achieved in all experiments. As a consequence, although the dynamic behavior of the system was slower than in stirred reactors, it was more stable in front of sudden environmental changes. Actually, oxidation activity did not undergo important variations despite changes in sulfide load and in incident irradiance. Under all conditions tested, the output generated contained a low sulfide concentration (50 µM) therefore, avoiding the undesirable effects of high levels of sulfide (**Chapter 3**).

Another goal of this work was the development of complex biofilms. The utilization of immobilized biomass yielded much higher taxonomic and metabolic diversity than what is usually found in stirred continuous reactors (in which washout plays a powerful role in selecting a very reduced number of organisms). We used samples from two contrasted natural sulfide-containing environments to induce the development of complex microbial biofilms. Microbial characterization was carried out combining traditional techniques (i.e.

microscopy and pigment analysis) and a molecular approach, in particular 16S rRNA gene cloning and sequencing. Such technique is very useful to describe the genetic diversity present in a sample without the need of culturing. However, a previous unbiased DNA extraction step is required. Thus, nucleic acids extracted must be representative of the total diversity present in the community. Because of the complex composition of our samples, we tested several protocols in order to select the most suitable for the diversity survey. The efficiency of the different methods of extraction was compared by direct total DNA quantification while the genetic diversity recovered was assessed by denaturing gradient gel electrophoresis (DGGE) (**Chapter 4**). Large differences were found in DNA yields within one order of magnitude, with methods combining enzymatic lysis with lyzozyme and proteinase K, and phenol-chloroform extraction offering the best results. Despite large differences in extraction efficiency, differences observed in DGGE fingerprints were minor, and the most abundant groups were recovered with all methods. However, differences in the weakest bands were observed, and again methods combining chemical lysis and phenol-chloroform extraction yielded the best results. Two of the extraction methods were further compared in detail by cloning and sequencing and, we found minor qualitative and quantitative (relative abundances) differences for most of the groups recovered (see Chapter 4 for details). DNA extraction method including a mechanical step of bead-beating in addition to enzymatic lysis and phenol-chloroform extraction was the most appropriate protocol for the studied biofilms.

In order to test whether we could obtain well-developed complex sulfide-oxidizing bioiflms in our reactor, we carried out two experiments using samples from two contrasted natural communities: freshwater lake sediment and a marine microbial mat. In both cases, highly diverse biofilms, covering a wide range of phylogenetic and physiological microbial groups, developed in the bioreactor (Chapters 5 and 6). Both biofilms presented high species richness and many different groups were represented. However, differences were observed among the most abundant phylogenetic groups present in both cases (Table 7.1). For the Proteobacteria, we retrieved clones affiliated to the Alpha and Gamma subdivisions in both biofilms. Conversely, minor members belonging to the Beta- and Delta-Proteobacteria were present only in the freshwater biofilm, whereas we recovered many clones belonging to the Epsilon-Proteobacteria only from the marine biofilm. Members of the *Cythophaga-Flavobacterium-Bacteroides* group were abundant in both biofilms, most of them belonging to

Table 7.1. Phylogenetic groups recovered from the complex biofilms developed. The closest match in Genbank are listed. Numbers indicate the percentage of clones retrieved in each library. See Chapters 5 and 6 for details.

Phylogenetic groups	Sediment lake-Biofilm	Microbial mat-Biofilm
Alpha-Proteobacteria	**33%** *Rhodobacter veldkampii* *Rhodobacter azotoformans* *Rhodopseudomonas palustris* *Caulobacter fusiformis*	**8%** *Rhodovulum adriaticum* *Erythrobacter litoralis* *Roseospirillum parvum*
Beta-Proteobacteria	**4%** *Rhodoferax antarticus* *Azoarcus indigens* *Thiobacillus baregensis*	**-**
Gamma-Proteobacteria	**25%** *Thiothrix unzii* *Thiothrix nivea* *Thiocapsa roseopersicina* *Pseudomonas stutzeri*	**52%** *Marichromatium purpuratum* *Thiorhodococcus drewsii* *Thiomicrospira chilensis* *Thiomicrospira kuenenii* *Vibrio* sp. Uncultured gamma proteobacteria
Delta-Proteobacteria	**1%** *Desulfocapsa thiozymogenes*	**-**
Epsilon-Proteobacteria	**-**	**22%** Uncultured epsilon proteobacteria
Chlorobi	**-**	**5%** *Prosthecochloris aestuarii*
CFB group	**21%** Uncultured CFB	**8%** Uncultured CFB *Marinilabilia salmonicolor*
Cyanobacteria/Plasts	**14%** *Scenedesmus obliquus* *Synechocystis* sp.	**4%** *Haslea salstonica*
Low G+C Gram-positive	**2%** Uncultured bacteria	**-**
Others	**-**	**1%** Uncultured bacteria

uncultured species. Chloroplasts from algae appeared in both libraries too but a clone belonging to Cyanobacteria was recovered only in the freshwater biofilm. Clones affiliated to the low G+C Gram-positive bacteria (Firmicutes) were specific from the freshwater biofilm while Chlorobi-affiliated clones were specific from the marine biofilm. However, the development of Chlorobiaceae in the freshwater biofilm would have been limited by the

presence of oxygen produced by algae that were very abundant (**Chapter 5**). In addition, we cannot discard that minor components could appear after analyzing a larger number of clones.

Although some phylogenetic groups were present in both biofilms, we found totally different species composition. Such differences agree with the fact that each inoculum used came from a different origin (i.e., freshwater and marine), and we used the medium supplemented with 3.7% of sodium chloride in the microbial mat experiment (see “Inoculum and growth conditions” in the Materials and Methods section).

Some of the differences found are in accordance with what is usually found in microbial assemblages from natural environment. For instance, Glöckner et al. (1999) compared the bacterioplankton from many lakes and oceans and found that the Beta subclass of the Proteobacteria were abundant in freshwater environments while they were essentially absent in the marine samples analyzed. On the contrary, most of environmental Epsilon-Proteobacteria have been isolated from marine environments, in particular from deep sea (Polz and Cavanaugh 1995; Longnecke and Reysenbach 2001; Alain et al. 2002). Moreover, the species of purple sulfur bacteria found in marine and freshwater environments are usually different (Imhoff 2003), as it occurred in our biofilms, in which *Marichromatium purpuratum*-like dominated the marine biofilm while *Thiocapsa roseopersicina*-like was the species present in the freshwater biofilm. The first species was isolated from a marine sponge (Imhoff and Trüper 1976) and its optimal growth is at 5% NaCl, while *Tca. roseopersicina* has not salt requirements for growth (Imhoff 2003).

Macroscopically both biofilms looked also different. The predominant color in the freshwater biofilm was green, in agreement with algae observed microscopically and represented by an important percentage of clones in the library. Oxygen production by algae was likely to occur and would allow the development of aerobic oxidizers while, probably, inhibited the development of strict anaerobic sulfur oxidizers (green sulfur bacteria). On the contrary, in the marine biofilm the predominant color were red in accordance with the dominance of anaerobic purple sulfur bacterium observed by microscopy and recovered in the library (46% of clones). Only few algae were observed microscopically and recovered in the clone library, and accordingly, aerobic oxidizers represented a low percentage of the library. Probably, due to the low oxygen production, green sulfur bacteria developed in this biofilm.

Despite differences found in the species composition and in the macroscopic aspect, biofilms were very similar under a functional perspective. The same physiologic groups developed in both biofilms, represented by different species in each case (Table 7.2). Aerobic, anaerobic, phototrophic, chemotrophic, autotropic and heterotrophic organisms were recovered in both experiments. Oxygen production by algae occurred in both cases, and in minor proportion probably, by cyanobacteria in the freshwater biofilm. However, in the freshwater case, more oxygen must be produced because algae were more abundant, as shown by microscopy observations and pigment content (Chlorophyll *a* content in the freshwater was 5 times higher than in the marine biofilm). Probably, they developed profusely in this case because the initial concentration of sulfide was lower than in the marine experiment.

Table 7.2. Physiologic groups recovered from the complex biofilms developed. The closest match in Genbank are listed. Numbers indicate the percentage of clones retrieved in the libraries. See Chapters 5 and 6 for details.

Microbial Guild	Freshwater biofilm	Marine biofilm
Oxygenic phototrophs	**14%**	**4%**
	Scenedesmus obliquus	*Haslea salstonica*
	Synechocystis sp.	
Phototrophic sulfur bacteria	**13%**	**51%**
Purple sulfur bacteria	*Thiocapsa roseopersicina*	*Marichromatium purpuratum*
		Thiorhodococcus drewsii
Green sulfur bacteria	-	*Prosthecochloris aestuarii*
Aerobic sulfur oxidizers	**12%**	**2%**
	Thiothrix unzii	*Thiomicrospira chilensis*
	Thiothrix nivea	*Thiomicrospira kuenenii*
	Thiobacillus baregensis	
Phototrophic non-sulfur Bacteria	**34%**	**8%**
	Rhodobacter veldkampii	*Roseospirillum parvum*
	Rhodobacter azotoformans	*Rhodovulum adriaticum*
	Rhodopseudomonas palustris	
	Rhodoferax antarticus	
Heterotrophic	**26%**	**10%**
	CFB members	CFB members
	Desulfocapsa thiozymogenes	*Vibrio* sp.
	Pseudomonas stutzeri	*Erythrobacter litoralis*
	Caulobacter fusiformis	
	Firmicutes members	
Others	**1%**	**25%**
	Azoarcus indigens	Uncultured β proteobacteria
		Uncultured β proteobacteria
		Uncultured bacteria

Anaerobic sulfur oxidizers were represented by members of the purple sulfur bacteria in both biofilms and also, by green sulfur bacteria in the marine sample. These organisms carry out anaerobic light-driven oxidation of sulfide. The relative abundance of this functional group was higher in the marine case, probably, because the lower dominance of algae. Aerobic sulfur oxidizers (also known as colorless sulfur bacteria), represented by *Thiobacillus*-like and *Thiothrix*-like species in the freshwater biofilm, were abundant while only a few clones related to the genus *Thiomicrospira* were found in the marine biofilm. Again, the higher algae contribution would have produced higher oxygen concentration that would favor aerobic oxidizers in the freshwater biofilm.

In general, most of sulfide was oxidized by a combination of aerobic and anaerobic processes in the freshwater biofilm, while the most important process in the marine biofilm was the anaerobic oxidation carried out by specialized anaerobic sulfur oxidizers. Although absolute abundances of each group has not been measured (e.g., using FISH counts with specific probes) and we have only relative abundances from the clone libraries, these results are in reasonable agreement with microscopic counts carried out for some conspicuous cells (e.g., algae, Chromatiaceae) and with the macroscopic aspect of the biofilms.

Apart from the specialists, oxidation of sulfide can be also performed by purple nonsulfur bacteria (some phototrophic species of the β- and β- Proteobacteria). This group was present in both cases, but especially abundant in the freshwater biofilm. Most groups cited are primary producers and the organic matter generated by them, could be subsequently used by heterotrophs, represented by organisms such as members of the *Cytophaga-Flavobacterium-Bacteroides* group among others, which were important in both biofilms. Finally, we recovered many clones belonging to uncultured species of the β-Proteobacteria of unknown functional role (“other” in Table 7.2) in the marine microbial mat biofilm. In fact, most of β-Proteobacteria isolated from natural environments are related to the sulfur cycle. Thus, we cannot discard that they were involved too in sulfide oxidation.

We observed microdiversity (Furhman and Campbel 1998) within some species in both biofilms. This microheterogeneity in the 16S rRNA gene has been previously reported in clone libraries of Bacteria, Archaea and eukaryotic microorganisms from several natural environments (Field et al. 1997; Díez et al. 2001; Massana et al. 2000). Some of this microdiversity might be explained by biases inherent to PCR-depended techniques and to

the presence of multioperons for the 16S rRNA gene (Nübel et al. 1996; Field et al. 1997) but it is clear that in many cases, the microdiversity has ecological and physiological significance in natural populations (Casamayor et al. 2002 and references therein). Microdiversity allows niche specialization of different ecotypes from the same species and allows coexistence of ecotypes with different substrate affinities or different light-dependent physiology (West and Scanlan 1999; Casamayor et al. 2002). The meaning of microdiversity in the bioreactor remains to be established, but due to the heterogeneity of our biofilm and the microenvironments developed, niche specialization is a possible explanation.

Summarizing, very complex biofilms aimed at the treatment of sulfide-containing effluents were obtained by using both, a freshwater lake sediment and a microbial mat as inoculum. A high genetic diversity was found, together with the coexistence of metabolically diverse groups, which can be explained by the formation of different microenvironments through the large and heterogeneous attachment surface. Clusters of closely related sequences below the “species-level” were observed, but in addition, we retrieved new 16S rRNA genes, substantially different from any previously known sequence. Many sequences were affiliated to uncultured organisms, especially for the CFB group and for the Epsilon-Proteobacteria. These results indicated that the bioreactor provides a growth environment different enough from the conditions traditionally used for isolating new organisms. Over the years, investigations of bacterial phisiology have been carried out in the laboratory using pure cultures in liquid suspensions or agar plates, and under fairly uniform conditions. Most of the microorganisms retrieved are not those abundant in nature, and therefore, these methods have weakly contributed to our understanding of the ecology of microorganisms. Now, the challenge for microbial ecophysiologist is to mimic in the laboratory the microbial scenarios as they occur in the environment, and to use methods and tools that allow detailed studies of relevant features under controlled laboratory conditions (Tolker-Nielssen and Molin 2000). Bioreactor systems and DNA-based molecular tools open a new perspective for microbial ecophysiology studies in the XXI century.

CONCLUSIONS

CONCLUSIONS

1. The new designed non-aerated, illuminated bench-scale bioreactor allows the development of stable and complex sulfide-oxidizing biofilms. High microbial biomass developed vigorously in all experiments.

2. Sulfide oxidation activity did not undergo important variations despite changes in sulfide load and in incident irradiance, indicating a high stability in front of sudden changes.

3. The redox control system kept the residual sulfide concentration constant in the micromolar range (50 µM). Effluents containing high sulfide concentration were processed avoiding the inhibition of sulfide-oxidizing bacteria, while maintaining the quality of the effluent even under changing environmental conditions.

4. Comparison of different methods for the DNA extraction of the biofilm revealed quantitative differences in the nucleic acid yields of one order of magnitude. However, qualitative differences were minor. The same predominant phylotypes were recovered with all methods tested, and only differences in minor groups were observed. The method based on bead-beating followed by enzymatic lysis and phenol-chloroform extraction was the most appropriate for these biofilms.

5. High microbial diversity was found in both the freshwater and the marine biofilms, covering a wide range of metabolic and taxonomic groups. Although the species composition was different for each case, functional groups were equivalent.

6. Coexistence in the bioreactor of apparently incompatible groups, such as oxygen producers and strict anaerobes, was probably caused by the presence of different microenvironments within the column.

7. Oxidation of sulfide in the freshwater biofilm was carried out mainly by both specialized sulfur-oxidizing bacteria (purple sulfur bacteria) and aerobic oxidizers (colorless sulfur bacteria), which used the oxygen internally supplied by oxygenic phototrophs (green algae and cyanobacteria).

8. In the marine biofilm, although a few aerobic oxidizers were present, oxidation of sulfide was mainly performed by purple and green sulfur bacteria, and probably also by members of the Epsilon-Proteobacteria.

9. In addition to the high species richness, we found in both biofilms a high degree of diversity below the species level (microdiversity) in the 16S rRNA gene. The heterogeneity of the biofilms and the microenvironments developed suggests niche specialization as a possible explanation.

10. Many sequences affiliated to uncultured organisms were recovered, especially for the CFB group and for the Epsilon-Proteobacteria. These results indicate that the bioreactor provided a growth environment different enough from the conditions traditionally used for culturing new organisms in the laboratory, and suggest that our reactor could be very useful for studying the physiology and ecology of such microbes.

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Agraïments

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