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"Bacterial and archaeal community composition along the water column and in the sediment of Averno lake as revealed by 16S rRNA and [FeFe]-hydrogenases genes-based approaches"

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Abbreviations

ARISA – automated ribosomal intergenic spacer analysis

BCC – bacterioplankton community composition

DGGE – Denaturing Gradient Gel Electrophoresis

DG-DGGE – double gradient denaturing gradient gel electrophoresis

DO – dissolved oxygen

Eh – redox potential

FHGs – [FeFe]-hydrogenase genes

FISH – fluorescence in situ hybridization

H' – Shannon Weaver index

Hmd – methylentetrahydromethanopterin dehydrogenase

HPB – H_2-producing bacteria

IGS – ribosomal intergenic spacers

ITS – Internal transcribed spacers

LSU rRNA – large subunit of ribosomal RNA

OTU – operational taxonomic unit

PCA – principal component analysis

PCR – Polymerase Chain Reaction

PPFM – pink-pigmented facultatively methylotrophic

R – richness index

rDNA – ribosomal DNA

RDP – Ribosomal Database Project

RISA – ribosomal intergenic spacer analysis

rRNA – ribosomal RNA

S – Simpson index

SSCP – single strand conformation polymorphism

SSU rRNA – small subunit of ribosomal RNA

T – temperature

T-RF – terminal restriction fragment

T-RFLP – Terminal restriction fragment length polymorphism

V – hypervariable region of 16S rRNA gene

PART 1

BACKGROUND, OBJECTIVES AND OVERVIEW OF THE THESIS

Background

Prokaryotes are not only the most abundant organisms in lake habitats, but they are also key players in biogeochemical processes (e.g. the metabolization of dissolved organic carbon or nitrogen cycling). Furthermore, these microorganisms have a relevant role in the processes controlling the water quality, because they are involved in the biodegradation of pollutants deriving from urban, industrial and agricultural activities. Detailed knowledge of the diversity, specific functions and ecology of prokaryotes dwelling in lakes is an essential prerequisite for the sustainable management of these freshwater resources. Moreover, since the academic and industrial interest in the discover of novel microorganisms that could produce undescribed secondary metabolites or that could have a potential biotechnological application is on the rise, the knowledge of the microbial diversity of lakes, representing a rich source of bacteria with different metabolic pathways, could provide important information in that regard.

During the last two decades, methods based on direct Polymerase Chain Reaction (PCR) and analysis of ribosomal RNA (rRNA) genes were developed and allowed a more comprensive analysis of microbial communities in comparison with cultivation based techniques. Among the techniques which no longer require the isolation and cultivation of bacteria, Denaturing Gradient Gel Electrophoresis (DGGE) analysis of 16S rRNA gene fragments has been widely used to assess the diversity of complex microbial communities in sediments and water column of lakes. Recent microbial ecology studies have begun to explore the links between diversity and function or identity and activity of microbial assemblages using not only rRNA-based primers, but also primers specific for determined functional groups, such as ammonia-oxidizing bacteria, sulphate-reducing bacteria, denitrifiers, methanotrophs and methanogens. Recently, primers specific for [FeFe]-hydrogenase genes have been developed to identify hydrogen producing bacteria occurring in anaerobic H_2 biofermentation systems, but no studies have been performed to detect them in natural samples.

Aims of the work

In this respect, the aims of this thesis were:

- to investigate the in situ distribution, abundance, and diversity of prokaryotes along the water column of Lake Averno, in relation to the depth gradient of physicochemical properties;
- to identify predominant populations constituting the microbial community of Averno lake superficial sediment and to detect hydrogen-producing bacteria naturally occurring in this habitat.

Overview of the thesis

PART 2 of the thesis presents an overview of the literature related to the content of this work. **Chapter I** starts with a general introduction to the microbial ecology of lakes. This chapter provides general information about the microbial functional groups involved in the main metabolic processes occurring in lakes, giving a particular attention to the hydrogen-producing bacterial community. **Chapter II** deals with the molecular tools mainly used to investigate lake bacterial community composition with particular emphasis on DGGE and cloning techniques and the use of functional genes as markers to study microbial diversity. Moreover, it gives a short overview on the use of [FeFe]-hydrogenases gene to investigate the diversity of H_2-producers communities.

PART 3 of the thesis presents the experimental work performed in this Ph.D. study. **Chapter III** describes the methods used to investigate the depth-related changes in entire microbial communities of the Lake Averno water column. In the same chapter, the obtained molecular results are shown and discussed in relation to the depth gradient of physicochemical properties of the lake. **Chapter IV** is a description of the overall microbial community found in superficial sediments of Lake Averno using the molecular methods described in Chapter III with the addition of a cloning step of DGGE bands, necessary to overcome the limit of the technique for the complex microbial community of sediment. Moreover, this chapter describes the composition of H_2–producing bacteria using as molecular target the gene encoding [FeFe]-hydrogenase enzyme. Therefore, this chapter reviews the conclusions drawn from results obtained developing a new set of PCR primers

targeting the functional *Clostridium* iron-only [FeFe]-hydrogenase genes, known as the most indispensable element for hydrogen-evolving microorganisms.

PART 2

OVERVIEW OF THE LITERATURE

Chapter I: Lake microbial ecology

I.1. General introduction

Prokaryotes are a important component of lake ecosystem because of their abundance, their species diversity and the multiplicity of their metabolic activities (Miskin et al., 1998; Wetzel, 2001; Wei et al., 2008). They play a key role in the decomposition of organic matter, in the biogeochemical cycles of the main elements (carbon, nitrogen, phosphorus, etc) and of trace elements (iron, nickel, mercury, etc), as well as in biodegradation of pollutants deriving from anthropic activities. Therefore, a detailed knowledge of the diversity, specific functions and ecology of microorganisms inhabiting lakes is needed for the sustainable management of these ecosystems. Several lakes have been studied as model systems to analyze the inter-relationships between microorganisms and their living and non living environments (Clark and Walsby, 1978; Gorlenko et al., 1977; Kuznezow, 1977). In particular, stratified lakes, characterized by zonation and gradient forming processes (Overbeck, 1972; Pfennig, 1979; Sorokin, 1970), represent an important topic in the microbial ecology.

In a stratified lake (Fig. 1), the warming of the surface layers yields a less dense upper layer (epilimnion) of circulating, aerated water and a dense, cold, stagnant bottom layer (hypolimnion). The intermediate layer (metalimnion) is characterized by a temperature gradient (the thermocline) and, after oxygen deprivation and anaerobic decomposition of organic matter in the hypolimnion, by a chemical gradient (chemocline) also.

The upper part of the epilimnion is the zone of primary production by the oxygenic phototrophic organisms, such as photosynthetic higher plants, algae, diatoms, flagellates, green algae, and cyanobacteria. These primary producers are accompanied by bacteria, protozoa, and metazoa that consume photosynthetized biomass, which results in secondary production and release of cellulosic detritus (Fenchel and Jøorgensen, 1977). Part of the biomass usually sinks to the hypolimnion and to the bottom of the lake where it is degraded. Degradation is accompanied by oxygen consumption, resulting in a decrease in oxygen concentration that leads to anoxic conditions in the bottom layer. Continued anaerobic degradation results in the production of organic fermentation products as well as hydrogen, methane, hydrogen sulfide, and carbon dioxide, which diffuse upwards forming concentration gradients. Methane, produced by methanogenic bacteria using H_2 + CO_2, formate and acetate as main substrates is released from the sediment and discharged in the form of gas bubbles.

Part of the methane is dissolved in the water as it moves upwards and is oxidized by methane-utilizing aerobic bacteria. The quick removal of oxygen from the hypolimnion is primarily due to the quick dispersal of methane and the growth of methane-oxidizing bacteria. Soluble fermentation products, hydrogen gas included, are used not only to produce methane but also to reduce sulfate to hydrogen sulfide. In the water column, the activity of sulfate reduction has two maxima: one in the hypolimnion bottom layers and one below the chemocline (Sorokin, 1970). While the reduction power for the first process originates from the sediment, sulfate reduction below the chemocline depends on the presence of purple and green sulfur bacteria that release sulfate during their anoxygenic photosynthesis. Simultaneously, nitrate is reduced to nitrite, which transiently accumulates and forms a distinct layer, and then to nitrogen.

The metalimnion is a layer of high biological activity. Because of its richness in inorganic nutrients as compared to the epilimnion, it is inhabited by a few aerobic photosynthetic prokaryotes that can tolerate hydrogen sulfide and anaerobic conditions. Because of the oxidation of hydrogen sulfide by the purple sulfur bacteria in the light and diffusion of oxygen from the epilimnion, the chemocline moves downward several meters during the daytime. Diurnal vertical fluctuations of the redox discontinuity layer obviously represent a major selective factor for the organisms occupying this habitat.

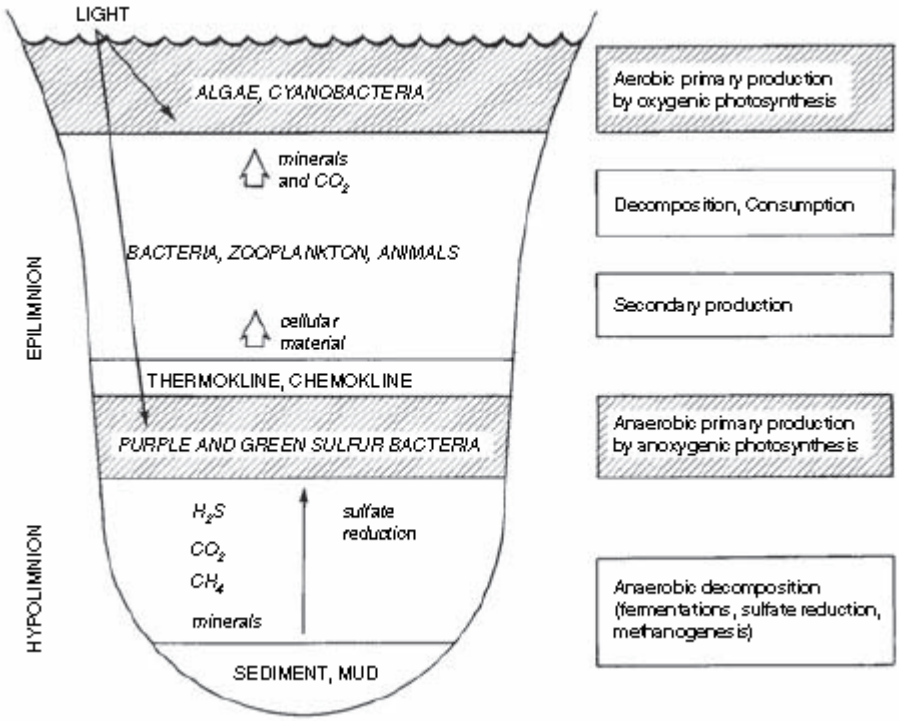

Figure 1. Diagram of production, consumption, and decomposition in an aquatic ecosystem with a chemocline (from Schlegel and Jannasch, 2006).

The groups of bacteria involved in the above mentioned metabolic processes of lake are included into different taxonomic groups commonly retrieved in plankton and sediment of lakes.

Bacterioplankton community composition has been studied extensively, and research has shown that it is highly variable among different lakes (Lindstrom et al., 2000; Yannarell et al., 2005). The prevalent taxa detected belong to *β-Proteobacteria, Actinobacteria, α-Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia, OP-10,* and *Planctomycetes* (Zwart et al., 2002; Wei et al., 2008). Generally, *β-Proteobacteria* are the most dominant bacterial taxa detected in freshwater (Cottrell et al., 2005; Van der Gucht et al., 2005). In some freshwater systems, dominance by *β-Proteobacteria* is rivaled by *Actinobacteria* (Allgaier & Grossart, 2006; Newton et al., 2006) and *Bacteroidetes* (Schauer et al., 2005; Eiler & Bertilsson, 2007). Less is known about *Archaea* in the water column of freshwater habitat although several studies have investigated these communities (Casamayor, 2001; Percent et al., 2008). From previous culture-dependent studies, members of the *Archaea* found in freshwater plankton were restricted to methanogens and sulfur-metabolizing thermophiles (Aravalli et al., 1998) of the anoxic layer. On the contrary, using culture-independent molecular techniques, sequences related to methanogens were found in the anaerobic sulfide-rich waters of Lake Ciso´ and Lake Vilar as well as from the aerobic depths (Casamayor et al., 2000).

The composition of microbial communities in freshwater sediments, may be largely influenced by interactions with surrounding aquatic and terrestrial habitats. In this respect it is of great interest to compare the composition of bacterial populations in surface sediments and the overlying water column, in order to reveal what sort of bacteria thriving in freshwater sediments are indeed restricted to this habitat. For example, sequences affiliated to the *β*-subclass of *Proteobacteria* were frequently recovered both from freshwater sediments and the overlying water body, while some sequences of *δ-Proteobacteria* were retrieved only from sediments, indicating that the occurrence of several representatives of this group is restricted to benthic environments. Defining or quantitatively describing the lake microbial community diversity is often difficult and incomplete, expecially for sediment that probably represents one of the most complex microbial habitat on earth, containing thousands of bacterial species in one gram (Urakawa et al., 2000). It has also been observed that the presence of *Eubacteria* and *Archaea* that are not affiliated to known phylogenetic groups is quite high in freshwater sediments (Spring et al., 2000).

In the following paragraphs the prokaryotes involved in the main metabolic processes occurring in lakes are described.

I.2. Phototrophic prokaryotes

The capacity for chlorophyll-based photosynthetic energy conversion is found in five of the 36 currently recognized bacterial lineages (Hugenholtz et al., 1998) that include the *Chloroflexus* subgroup, the green sulfur bacteria, the *Proteobacteria*, the *Cyanobacteria*, and the *Heliobacteriaceae*. With the exception of the *Cyanobacteria*, phototrophic bacteria perform anoxygenic photosynthesis, which is not accompanied by photochemical cleavage of water and does not lead to the formation of molecular oxygen.

Among the phototrophic prokaryotes, *Chloroflexus* subgroup and *Heliobacteriaceae* species have been mainly retrieved from geothermal springs and soil, respectively (Overmann and Garcia-Pichel, 2006; Madigan and Ormerod, 1995). More widespread occurrence in freshwater lakes was observed for the other groups. Van Gemerden and Mas, (1995) reported on the presence of phototrophic sulfur bacteria in 63 different lakes and 7 sediment ecosystems. The phototrophic sulfur bacteria (green and purple sulfur bacteria) grow preferentially by photolithoautotrophic oxidation of reduced sulfur compounds. Therefore, they are limited to those environments where light reaches anoxic, sulfide-containing bottom layers. Because light and sulfide occur in opposing gradients, growth of phototrophic sulfur bacteria is confined to a narrow zone of overlap and is only possible if the chemical gradient of sulfide is stabilized against vertical mixing.

Green sulfur bacteria (*Chlorobiaceae* family) represent a coherent and isolated group within the domain *Bacteria*. They are strict photolithotrophs and contain chlorosomes. During the oxidation of sulfide, elemental sulfur is deposited extracellularly. Another typical feature of this group is the very limited physiological flexibility.

Purple sulfur bacteria belong to the γ-subclass of *Proteobacteria*. This group includes two families of phototrophic species, the *Chromatiaceae* and *Ectothiorhodospiraceae*, typically found in freshwater environments and in hypersaline waters, respectively. *Chromatiaceae* accumulate sulfur globules within the cells, whereas members of the *Ectothiorhodospiraceae* deposit elemental sulfur extracellularly. The purple sulfur bacteria contain bacteriochlorophylls *a* and *b*, and all components of the photosynthetic apparatus are located in the intracellular membrane.

Purple nonsulfur bacteria, belonging to the *α- and β-Proteobacteria* groups, do not form separate phylogenetic clusters but are highly intermixed with various other phenotypes. Characteristically, members of these two groups exhibit a high metabolic versatility and are capable of photoorganotrophic, photolithoautotrophic and chemoorganotrophic growth. Photosynthetic pigments are bacteriochlorophyll *a* or *b* and a variety of carotenoids. Light-harvesting complexes, reaction centers, and the components of the electron transport chain are located in intracellular membrane systems of species-specific architecture. Purple nonsulfur bacteria include members of *Rhodospirillales* (e.g. *Rhodospirillum)* and *Rhizobiales*, (e.g. *Rhodopseudomonas palustris)* orders and members of *Rhodobacteraceae* (e.g. *Rhodobacter*), *Rhodocyclaceae* (e.g. *Rhodocyclus*) and *Comamonadaceae* (e.g. *Rhodoferax*) families. In contrast to the phototrophic members of the *γ-Proteobacteria* that form dense layers in pelagic environment, purple nonsulfur bacteria of the *α*- and *β*-subclasses of *Proteobacteria* do not accumulate under natural conditions (Biebl and Drews, 1969; Swoager and Lindstrom, 1971; Steenbergen and Korthals, 1982). However, they have been isolated from a wide variety of lacustrine environments (Imhoff and Trüper, 1989).

Oxygenic photosynthesis is only found in members of *Cyanobacteria.* The *Cyanobacteria* (= oxyphotobacteria) are defined by their ability to carry out oxygenic photosynthesis (water-oxidizing, oxygen-evolving, plant-like photosynthesis) based on the coordinated work of two photosystems. All of them are able to grow using CO_2 as the sole source of carbon, which they fix using primarily the reductive pentose phosphate pathway. Their chemoorganotrophic potential typically is restricted to the mobilization of reserve polymers (mainly starch but also polyhydroxyalkanoates) during dark periods, although some strains are known to grow chemoorganotrophically in the dark at the expense of external sugars.

Cyanobacteria as a group exhibit the widest range of habitats of all phototrophic prokaryotes due to the ubiquity of water, their preferred electron donor for the reduction of CO_2. *Cyanobacteria* are found in the plankton of coastal and open oceans and in freshwater and saline inland lakes. They thrive in the benthos of marine, intertidal, lacustrine and fluvial waters as well as in a large variety of terrestrial habitats (soils, rocks, trees).

I.3. Aerobic methylotrophic prokaryotes

Methylotrophic bacteria are those organisms with the ability to utilize (as their sole source of carbon and energy) reduced carbon substrates with no carbon-carbon bonds. By this definition the group includes bacteria that can grow on substrates such as methane, methanol, methylated amines, halogenated methanes and methylated sulfur species. Methylotrophic bacteria are quite widespread in nature, being found in a variety of aquatic and terrestrial habitats (King, 1992). They appear to play an important role in the cycling of carbon in specific habitats (King, 1992), and they comprise the principal biological sink for methane and other methylated greenhouse gases, highlighting an important role in global warming (King, 1992; Oremland and Culbertson, 1992). Although many anaerobic methylotrophic bacteria are known, especially among the methanogens, this paragraph will cover only the aerobic and facultatively anaerobic methylotrophs (for convenience, termed “aerobic methylotrophs”). Table 1 lists the major groups of aerobic methylotrophs with examples of the genera that have been described to contain methylotrophs.

Group	Major assimilation pathway	N_2 fixing	Phylogenetic position[a]	References
Obligate methylotrophs				
Type I methanotrophs				
Methylomonas	RuMP	Yes	γ-Proteobacteria	Anthony, 1982
Methylobacter	RuMP	Yes	γ-Proteobacteria	Anthony, 1982
Methylococcus	RuMP	Yes	γ-Proteobacteria	Anthony, 1982
Methylomicrobium	RuMP	No	γ-Proteobacteria	Bowman et al., 1995
Methylosphaera	RuMP	No	γ-Proteobacteria	Bowman et al., 1997
Methylocaldum	RuMP	No	γ-Proteobacteria	Bodrossy et al., 1997
Type II methanotrophs				
Methylosinus	Serine	Yes	α-Proteobacteria	Anthony, 1982
Methylocystis	Serine	Yes	α-Proteobacteria	Anthony, 1982
Methylocella	Serine	Yes	α-Proteobacteria	Dedysh et al., 2000
Restricted facultative methylotrophs				
Methanol utilizers				
Hyphomicrobium	Serine	No	α-Proteobacteria	Harder and Attwood, 1978; Stackebrandt et al., 1988
Methylophilus	RuMP	No	β-Proteobacteria	Jenkins and Jones, 1987
Methylobacillus	RuMP	No	β-Proteobacteria	Bratina et al., 1992
Methylophaga	RuMP	No	γ-Proteobacteria	Janvier and Grimont, 1995
Facultative methylotrophs				
Methylobacterium (*Pseudomonas*)	Serine	No	α-Proteobacteria	Green and Bousfield, 1983
Aminobacter	Serine	No	α-Proteobacteria	Urakami et al., 1992
Methylorhabdus	Serine	No	α-Proteobacteria	Doronina et al., 1995
Methylopila	Serine	No	α-Proteobacteria	Doronina et al., 1998
Methylosulfonomonas	Serine	No	α-Proteobacteria	Holmes et al., 1997
Marinosulfonomonas	Serine	No	α-Proteobacteria	Holmes et al., 1997
Paracoccus	CBB	No	α-Proteobacteria	Anthony, 1982
Xanthobacter	CBB	Yes	α-Proteobacteria	Jenni et al., 1987
Ancylobacter (*Microcyclus*)	CBB	Yes	α-Proteobacteria	Raj, 1989
Thiobacillus	CBB	No	α-Proteobacteria	Chandra and Shethna, 1977
Rhodopseudomonas	CBB	No	α-Proteobacteria	Anthony, 1982
Rhodobacter	CBB	No	α-Proteobacteria	Anthony, 1982
Acetobacter	RuMP	ND	γ-Proteobacteria	Yamada et al., 1997
Bacillus	RuMP	ND	Gram-positive (low G+C)	Dijkhuizen et al., 1988
Mycobacterium	RuMP	ND	Gram-positive (high G+C)	Reed and Dugan, 1987
Arthrobacter	RuMP	ND	Gram-positive (high G+C)	Levering et al., 1981
Amycolatopsis (*Nocardia*)	RuMP	ND	Gram-positive (high G+C)	De Boer et al., 1990

Abbreviations: RuMP, ribulose monophosphate; CBB, Calvin-Benson-Bassham; and ND, no data.
[a]Many phylogenetic affiliations can be found at the Ribosomal Database Project (http://www.cme.msu.edu/RDP/html/index.html) or National Center for Biotechnology Information websites and in Bratina et al. (1992).

Table 1. Characteristics of aerobic methylotrophic bacteria (from Lidstrom, 2006).

Aerobic methylotrophic bacteria are phylogenetically diverse, with representatives found among the *Proteobacteria* as well as the high and low G+C Gram-positive bacteria (Table 1). Two functional groups of methylotrophs may be distinguished: those capable of growth on methane and methanol, called "methanotrophs," and those capable of growth on methanol and/or other methylated compounds but not on methane.

Methanotrophs are found in most environments in which both methane and O_2 are present, and have been isolated from a variety of environments including those with extremes values of pH and temperature (Hanson and Hanson, 1996; Bodrossy et al., 1997; Bowman et al., 1997; Dedysh et al., 2000). They are an integral part of the natural ecosystem, consuming much of the methane that is biogenically (through methanogenesis) and non-biogenically

(e.g., from hydrocarbon seeps, natural gas fields and coal mines) derived. This interception of methane helps maintain a balance of atmospheric methane. Methanotrophs can utilize methane as they possess an enzyme called methane monooxygenase (MMO). There are two major groups of methanotrophs named Type I and II. Type I methanotrophs belong in the γ subdivision of the *Proteobacteria* and are split into two more groups (Type I and Type X). The biology of Type I and Type II methanotrophs differs in phylogeny, chemotaxonomy, internal ultrastructure, carbon assimilation pathways, and certain other biochemical aspects. These differences are summarized in Table 1. The Type I methanotrophs are included in the *Methylococcaceae* family and are organized in six genera: *Methylococcus* (the type genus), *Methylocaldum*, *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylosphaera*. The first two genera are also referred to as Type X methanotrophs, and this group is distinguished by certain physiological, biochemical and phylogenetic characteristics. The Type II methanotrophs are grouped in a family called the *Methylocystaceae* with *Methylocystis* (type genus) and *Methylosinus* as the member genera assigned to the subdivision of the α-*Proteobacteria* (Bratina et al., 1992; Brusseau et al., 1994). Methanotrophs make up a high proportion of the total bacterial biomass in many freshwater aquatic environments, mostly within surface sediments (Boon et al., 1996; Ross et al., 1997). Indirect immunofluoresence studies indicate that both Type I and Type II methanotrophs are common in surface sediments and in the water column of various fresh and brackish water environments (Reed and Dugan, 1978; Saralov et al., 1984; Ambramochkina et al., 1987; Malashenko et al., 1987; Galchenko et al., 1988; Galchenko, 1994).

The bacteria capable of growth on methanol and other methylated compounds but not on methane are more diverse than those capable of growing on methane. So far, all of the Gram-positive and α-proteobacterial strains are facultative methylotrophs, whereas the β-proteobacterial and γ-proteobacterial strains are either obligate methylotrophs or restricted facultative methylotrophs (Anthony, 1982).

Many but not all of the methylotrophs also can use N_2 as a nitrogen source and, therefore, are considered to be diazotrophs (Table 1). In addition, several of the methylotrophs also affect the nitrogen cycle by carrying out transformations of ammonia and nitrate (Anthony, 1982). Some methylotrophs are known that can use methylated sulfur species, and these appear to play an important role in sulfur cycling (DeBont et al., 1981; Kelly and Murrell, 1999). A number of methylotrophs can grow on halogenated methanes (Leisinger and Braus-Stromeyer, 1995) and have the potential to play an important role in the detoxification of these pollutants.

Because the natural diversity of methylotrophs is still under investigation, the current clustering of phylogenetic and physiological groups may not hold up, as new strains are identified and characterized.

Among the known non-methane utilizing Methylotrophs, the genus *Methylobacterium*, composed of a variety of pink-pigmented facultatively methylotrophic (PPFM) bacteria, is widely distributed in a variety of habitats (Green and Bousfield, 1981; Green and Bousfield, 1983), including also stratified lake systems in which some dissolved oxygen exists (Hanson, 1980), where they occupy a special niche.

I.4. The methanogenic bacteria

The methanogenic bacteria are strictly anaerobic *Archaea* that produce methane as the major product of their energy metabolism. They obtain energy for their growth from the conversion of a limited number of substrates to methane gas. The major substrates are H_2 + CO_2, formate, and acetate. In addition, some other C-1 compounds such as methanol, trimethylamine, and dimethylsulfide and some alcohols such as isopropanol, isobutanol, cyclopentanol and ethanol are substrates for some methanogens. All of these substrates are converted stoichiometrically to methane. In this regard, the metabolism of the methanogens is strikingly different from that of the so-called “minimethane” producers, which are other anaerobic microorganisms that produce very small amounts of methane as a consequence of side reactions of their normal metabolism.

The list of substrates for growth of methanogens may be divided into three groups. In the first group, the energy substrate (electron donor) is H_2, formate, or certain alcohols and the electron acceptor is CO_2, which is reduced to methane. The ability to utilize H_2 as an electron donor for CO_2 reduction is almost universal among methanogens. Likewise, many methanogens also utilize formate, but the ability to utilize alcohols is less common (Bleicher et al., 1989; Zellner and Winter, 1987). Some methanogens also utilize carbon monoxide as an electron donor, but growth is very slow (Daniels et al., 1977). In the sediments of lakes, only about one-third of the methane is formed from CO_2 reduction. However, this reaction is very important for maintaining the very low concentrations of H_2 and formate typical of these anaerobic habitats and facilitating the process of interspecies electron transfer.

In the second group, the energy substrate is one of a variety of methyl-containing C-1 compounds, which can serve as substrates for a few taxa of methanogens. Usually these

compounds are disproportionated. Some molecules of the substrate are oxidized to CO_2. The electron acceptors are the remaining methyl groups, which are reduced directly to methane.

In the third group, acetate is the major source of methane, but the ability to catabolize this substrate is limited to species of *Methanosarcina* and *Methanosaeta ("Methanothrix").* Methane synthesis proceeds through an aceticlastic reaction, in which the methyl carbon of acetate is reduced to methane and the carboxyl carbon is oxidized to CO_2. Methanogenesis from acetate is common in anoxic freshwater sediments where the catabolism of acetate by other anaerobes is limited by the availability of alternate electron acceptors such as sulfate or nitrate.

A summary of the major genera of methanoarchaea is given in Table 2.

Order, family and genus	Morphology	Major energy substrates[b]	Temperature optimum (°C)	Cell wall[c]
Order Methanobacteriales				
Family Methanobacteriaceae				
Genus *Methanobacterium*	Rod	H_2, (formate, alcohols)	37–45	Pseudomurein
Methanothermobacter	Rod	H_2, (formate)	55–65	Pseudomurein
Methanobrevibacter	Short rod	H_2, (formate)	37–40	Pseudomurein
Methanosphaera[d]	Coccus	H_2 + methanol	37	Pseudomurein
Family Methanothermaceae				
Genus *Methanothermus*	Rod	H_2	80–88	Pseudomurein + protein
Order Methanococcales				
Family Methanococcaceae				
Genus *Methanococcus*	Coccus	H_2, formate	35–40	Protein
Methanothermococcus	Coccus	H_2, formate	60–65	Protein
Family Methanocaldococcaceae				
Genus *Methanocaldococcus*	Coccus	H_2	80–85	Protein
Methanotorris	Coccus	H_2	88	Protein
Order Methanomicrobiales				
Family Methanomicrobiaceae				
Genus *Methanomicrobium*	Rod	H_2, formate	40	Protein
Methanoculleus	Irregular coccus	H_2, formate (alcohols)	20–55	Glycoprotein
Methanofollis	Irregular coccus	H_2, formate (alcohols)	37–40	Glycoprotein
Methanogenium	Irregular coccus	H_2, formate (alcohols)	15–37	Protein
Methanolacinia	Rod	H_2 (alcohols)	40	Glycoprotein
Methanoplanus	Plate or disc	H_2, formate (alcohols)	32–40	Glycoprotein
Family Methanospirillaceae				
Methanospirillum	Spirillum	H_2, formate (alcohols)	30–37	Protein + sheath
Family Methanocorpusculaceae				
Genus *Methanocorpusculum*	Small coccus	H_2, formate (alcohols)	30–40	Glycoprotein
Methanocalculus[d]	Irregular coccus	H_2, formate	30–40	ND
Order methanosarcinales				
Family Methanosarcinaceae				
Genus *Methanosarcina*	Coccus, packets	Methanol, $MeNH_2$, (H_2, Ac, DMS)	35–60	Protein + HPS
Methanococcoides	Coccus	Methanol, $MeNH_2$	23–35	Protein
Methanohalophilus	Irregular coccus	Methanol, $MeNH_2$	35–40	Protein
Methanohalobium	Flat polygons	Methanol, $MeNH_2$	40–55	ND
Methanolobus	Irregular coccus	Methanol, $MeNH_2$ (DMS)	37	Glycoprotein
Methanomethylovorans	coccus, packets	Methanol, $MeNH_2$ DMS, MT	34–37	ND
Methanomicrococcus	Flat polygons	H_2 + Methanol, H_2 + $MeNH_2$	39	ND
Methanosalsum	Irregular coccus	Methanol, MeH_2, DMS	35–45	ND
Family Methanosaetaceae				
Genus *Methanosaeta* (*Methanothrix*)	Rod	Ac	35–60	Protein + sheath
Order Methanopyrales				
Family Methanopyraceae				
Genus *Methanopyrus*	Rod	H_2	98	Pseudomurein

Abbreviations: $MeNH_2$, methylamines (monomethylamine, dimethylamine, and trimethylamine); DMS, dimethylsulfide; MT, methanethiol; Ac is acetate; HPS, heteropolysaccharide; and ND, not determined.

[a] All of the methanoarchaea are members of the phylum Euryarchaeota.

[b] Major energy substrates for methane synthesis. Alcohols are some or all of ethanol, isopropanol, isobutanol and cyclopentanol. Parentheses means utilized by some but not all species or strains.

[c] Cell wall components.

[d] Placement in higher taxon is tentative.

Table 2. Taxonomy of the methane-producing *Archaea* [a]. From Hedderich and Whitman, 2006

Methanogenic bacteria are abundant in habitats where electron acceptors such as O_2, NO_3^-, Fe^{3+} and SO_4^{2-} are limiting. Methanogens are generally absent from the water column of unstratified lakes and rivers because convection currents rapidly aerate the deep waters. However, the diffusion of O_2 between the layers of stratified lakes is often too slow to maintain oxic conditions in the lower layers. Similarly, the physical structure of sediments limits dispersive mechanisms, so deeper sediments are often anoxic and harbor methanogens.

In anoxic environments, the presence of NO_3^-, Fe^{3+} and SO_4^{2-} inhibits methanogenesis by allowing other organisms to outcompete methanogens for reduced substrates. For instance, in

the presence of sulfate, sulfate-reducing bacteria utilize H_2 at concentrations lower than the minimum concentration which can be utilized by methanogens (Kristjansson et al., 1982; Lovley, 1985). Presumably, the ability of the sulfate reducing bacteria to outcompete the methanogens is a direct consequence of the more-positive reduction potential of SO_4^{2-} compared to that of CO_2. In environments with sufficient quantities of sulfate, hydrogen sulfide is the predominant reduced product, and the major fate of biodegradable organic carbon is oxidation to CO_2. If sulfate becomes limiting, methane replaces hydrogen sulfide as the reduced product, and the organic carbon is disproportionated to CO_2 and methane.

Sulfidogenesis normally dominates in estuarine, marine, and hypersaline sediments, where sulfate diffuses from overlying water, whereas methanogenesis is the dominant process in anaerobic environments that contain large amounts of easily degradable organic matter such as the anoxic compartment of lakes.

I.5. Sulfate- and sulfur-reducing prokaryotes

Sulfate-reducing bacteria gain energy for growth by coupling the oxidation of organic compounds or molecular hydrogen (H_2) to the reduction of sulfate (SO_4^{2-}) to sulfide (H_2S, HS^-). Hence, sulfate-reducing bacteria are easily recognized by the production of high sulfide concentrations (with non-limiting electron donor and sulfate, usually in the range of several millimolar) concomitantly with growth and the strict dependence of this process on the presence of free sulfate. This process is also termed “dissimilatory sulfate reduction,” to allow clear differentiation from assimilatory sulfate reduction. Assimilatory sulfate reduction generates reduced sulfur for biosynthesis (e.g., of cysteine) and is a widespread biochemical capacity in prokaryotes and plants. Assimilatory sulfate reduction does not lead to the excretion of sulfide. The assimilated reduced sulfur is released as sulfide only through decay (putrefaction) of the biomass. The amounts of sulfide produced by dissimilatory sulfate reduction with a given amount of biomass is by orders of magnitude higher than the amount of sulfide liberated from the organic sulfur during putrefaction of the same amount of biomass.

The production of high concentrations of H_2S often indicates the activity and presence of sulfate-reducing microorganisms in natural habitats. The presence of H_2S is obvious by its characteristic smell, black precipitation of ferrous sulfide when iron minerals are present, and white patches of elemental sulfur as an oxidation product formed in contact with air. Such

signs for the activity of sulfate reducers are often encountered if organic substances accumulate in the presence of sulfate under anoxic conditions. Growth conditions for sulfate-reducing microorganisms prevail in sediments of virtually all aquatic habitats, which may be cold, moderate or geothermally heated up to approximately 105°C. But also flooded soils such as rice paddies and technical aqueous systems (as for instance sludge digestors, oil tanks or vats in the paper-making industry) may offer suitable growth conditions for sulfate-reducing microorganisms. From such habitats, in particular marine sediments, a great variety of sulfate-reducing microorganisms has been isolated.

Bacterial sulfate reducers fall into three major branches, the δ-subclass of *Proteobacteria* with more than twenty-five genera, the Gram-positive bacteria with the genera *Desulfotomaculum* and *Desulfosporosinus*, and branches formed by *Thermodesulfobacterium* and *Thermodesulfovibrio*. Sulfate reducers in the latter branch are thermophilic, whereas the two other branches comprise psychrophilic, mesophilic as well as thermophilic species.

Recently, also archaeal sulfate reducers have been detected. However, in comparison to bacterial species, fewer archaeal sulfate reducers are known. Moreover, they are less ubiquituous than their bacterial counterparts. Indeed, archaeal sulfate reducers appear to be restricted to habitats like hydrothermal vents, hot springs and deep, warm oil reservoirs.

Currently recognized genera of sulfate-reducing prokaryotes and their main morphological and physiological properties are summarized in Table 3.

Genus	Morphology	Optimum temperature (°C)	Desulfoviridin[a]	Electron acceptors for growth (other than SO_4^{2-})	Oxidation of organic electron donors[b]	Electron donors[c]: H_2	Acetate	Propionate	Higher fatty acids	Ethanol	Lactate	Succinate, fumarate, and/or malate	Fructose, and/or glucose	Phenyl-substituted organic acids	Others utilized by some species
Bacteria[d]															
Desulfovibrio	Vibrio	30–38	+	SO_3^{2-}, $S_2O_3^{2-}$, Fumarate	i	+	–	–	–	+	+	±	±	–	Methanol, glycerol, glycine, alanine, choline, furfural
Desulfomicrobium	Oval or rod	28–37	–	SO_3^{2-}, $S_2O_3^{2-}$	i	+	–	–	–	±	+	+	–	–	–
Desulfobulbus	Oval	28–39	–	SO_3^{2-}, $S_2O_3^{2-}$, NO_3^-	i	+	–	+	–	+	+	–	–	–	–
Desulfobacter	Oval or vibrio	28–32	–	SO_3^{2-}, $S_2O_3^{2-}$	CAC	±	+	–	–	±	[e]	–	–	–	–
Desulfobacterium	Oval	20–35	–	$S_2O_3^{2-}$	CO	±	(+)	(±)	±	±	±	±	–	±	Methanol, glutarate, glutamate, phenol, aniline, nicotinate, indole
Desulfococcus	Sphere	28–36	+	SO_3^{2-}, $S_2O_3^{2-}$	CO	–	(+)	+	+	+	+	–	–	+	Acetone
Desulfosarcina	Oval (forms aggregates)	33	–	SO_3^{2-}, $S_2O_3^{2-}$	CO	+	(+)	+	+	+	+	±	–	+	–
Desulfomonile	Rod	37	+	$S_2O_3^{2-}$, 3-Cl-benzoate	c	+	–[f]	ND	ND	–	–	–	–	+	3- or 4-Anisate
Desulfonema	Multicellular filaments	30–32	±	SO_3^{2-}, $S_2O_3^{2-}$	c	±	(+)	+	+	–	±	+	–	±	–
Desulfobotulus	Vibrio	34	–	SO_3^{2-}	i	–	–	–	+	–	+	–	–	–	–
Desulfoarculus	Vibrio	35–39	–	SO_3^{2-}, $S_2O_3^{2-}$	CO	–	(+)	(+)	+	–	–	–	–	–	–
Desulfotomaculum	Straight or curved rod, sporulates	30–38 50–65[h]	–	$S_2O_3^{2-}$ Fumarate	i or CO	±	±	±	±	+	±	±	±	±	Methanol, alanine
Desulfosporosinus	Straight or curved rod, sporulates	30–37	–	$S_2O_3^{2-}$	i	+	–	–	ND	+	+	ND	–	+	3,4,5-Trimethoxybenzoate
Thermodesulfovibrio	Vibrio	65	–	SO_3^{2-}, $S_2O_3^{2-}$	i	+	–	–	–	–	+	–	–	–	–
Thermodesulfobacterium	Rod	65–70	–	$S_2O_3^{2-}$	i	+	–	–	–	–	+	–	–	ND	–
Thermodesulforhabdus	Rod	60	–	SO_3^{2-}	c	–	+	–	+	+	+	+	ND	–	–
Desulfacinum	Oval	60	–	SO_3^{2-}, $S_2O_3^{2-}$	ND	+	+	+	+	+	+	+	–	–	Hexanol
Desulforhopalus	Oval	18–19	–	SO_3^{2-}, $S_2O_3^{2-}$	i	+	–	+	ND	+	+	–	–	–	–
Desulforhabdus	Rod	37	–	SO_3^{2-}, $S_2O_3^{2-}$	c	+	+	+	ND	+	+	–	–	–	–
Desulfonatronovibrio	Vibrio	37	–	SO_3^{2-}, $S_2O_3^{2-}$	ND	+	–	–	ND	–	–	–	–	ND	–
Desulfonatronum	Vibrio	37–40	–	SO_3^{2-}, $S_2O_3^{2-}$	i	+	–	–	ND	+	–	–	–	ND	–
Desulfohalobium	Rod	37–40	–	SO_3^{2-}, $S_2O_3^{2-}$, S^0	i	+	–	–	ND	+	+	–	–	ND	–
Desulfofustis	Rod	28	–	SO_3^{2-}, S^0	i	+	+	+	+	+	+	+	+	+	Glycolate, betaine, choline, triethanolamine, indole
Desulfocella	Vibrio	34	–	–	i	–	–	–	+	–	–	–	–	–	1-Alanine, 2-methylbutyrate
Desulfocapsa	Rod	20–30	–	–	i	–	–	–	ND	+	–	–	–	–	–
Desulfobacca	Oval	37	–	SO_3^{2-}, $S_2O_3^{2-}$	c	–	+	–	–	–	–	–	–	–	–
Desulfuromusa	Rod	30	ND	S^0, NO_3^- Fumarate DMSO Fe(III)-citrate	c	ND	+	+	±	–	+	+	–	ND	–
Desulfospira	Curved rod	26–30	–	SO_3^{2-}, $S_2O_3^{2-}$, S^0	c	+	–	–	+	–	+	+	–	±	Betaine, proline
Desulfobacula	Rod	28	ND	ND	c	–	+	–	–	+	–	+	ND	+	Toluene, *p*-cresol, benzaldehyde, benzoate, phenylacetate, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate
Desulfofrigus	Rod	10	–	SO_3^{2-}, $S_2O_3^{2-}$ Fe(III)-citrate	c	±	+	–	+	+	+	+	–	–	–
Desulfofaba	Rod	7	–	SO_3^{2-}, $S_2O_3^{2-}$	i	–	–	+	–	+	+	+	–	–	–
Desulfotalea	Rod	10	–	SO_3^{2-}, $S_2O_3^{2-}$ Fe(III)-citrate	i	+	–	–	–	+	+	+	–	–	–
Archaea[d]															
Archaeoglobus	Sphere	82–83	–		CO	+	–[g]	ND	ND	ND	+	ND	+	ND	Starch, peptides

[a] Symbols: +, present; ±, present or absent; –, absent.
[b] Symbols: c, complete to CO_2 via unknown pathway; CAC, complete oxidation via citric acid cycle; CO, complete oxidation via carbon monoxide dehydrogenase/C_1 pathway; i, incomplete oxidation to acetate as an end product.
[c] Symbols: +, utilized; (+), poorly utilized; ± , utilized or not utilized; (±) poorly of not utilized; –, not utilized; ND, not determined or not reported.
[d] References: *Desulfovibrio* (Postgate, 1984b), *Desulfomicrobium* (Rozanova et al., 1988), *Desulfobulbus* (Widdel and Pfennig, 1982), *Desulfobacter* (Widdel and Pfennig, 1981b; Widdel, 1987), *Desulfobacterium* (Brysch et al., 1987), *Desulfococcus* (Widdel, 1980), *Desulfosarcina* (Widdel, 1980), *Desulfomonile* (DeWeerd et al., 1990), *Desulfonema* (Widdel et al., 1983), *Desulfobotulus* (Widdel, 1980), *Desulfoarculus* (Widdel, 1980), *Desulfotomaculum* (Widdel and Pfennig, 1977; Widdel and Pfennig, 1981b), *Desulfosporosinus* (Stackebrandt et al., 1997; Campbell and Postgate, 1965; Klemps et al., 1985), *Thermodesulfovibrio* (Henry et al., 1994), *Thermodesulfobacterium* (Zeikus et al., 1983), *Archaeoglobus* (Burggraf et al., 1990; Stetter et al., 1987; Stetter, 1988), *Thermodesulforhabdus* (Beeder et al., 1995), *Desulfacinum* (Rees et al., 1995), *Desulforhopalus* (Isaksen and Teske, 1996), *Desulforhabdus* (Oude Elferink et al., 1995), *Desulfonatronovibrio* (Zhilina et al., 1997), *Desulfonatronum* (Pikuta et al., 1998), *Desulfohalobium* (Ollivier et al., 1991), *Desulfofustis* (Friedrich et al., 1996), *Desulfocella* (Brandt et al., 1999), *Desulfocapsa* (Janssen et al., 1996), *Desulfobacca* (Oude Elferink et al., 1999), *Desulfuromusa* (Liesack and Finster, 1994), *Desulfospira* (Finster et al., 1997a), *Desulfobacula* (Rabus et al., 1993), *Desulfofrigus* (Knoblauch et al., 1999b), *Desulfofaba* (Knoblauch et al., 1999b), and *Desulfotalea* (Knoblauch et al., 1999b).
[e] Utilized by a few unnamed strains but not by the validly published species.
[f] May be utilized with thiosulfate as electron acceptor.
[g] For further description see Daumas et al., 1988; Min and Zinder, 1990; Nazina et al., 1988; and Widdel, 1988.
[h] Thermophilic species.

Table 3. Morphological and physiological properties of the genera of sulfate-reducing *Bacteria* and *Archaea*. From Rabus et al., 2006.

In addition to sulfate-reducing microorganisms, a variety of prokaryotes exists that reduce elemental sulfur but not sulfate. Among the lower oxidation states, the elemental sulfur is probably the most widespread sulfur species in sediments and geological deposits. Many chemical and biological oxidation processes of H_2S do not directly lead to sulfate (the highest oxidation state) but rather to elemental sulfur, which therefore may accumulate. Prokaryotes

that reduce elemental sulfur are widespread among different phylogenetic groups of *Bacteria* and *Archaea*.

Bacterial sulfur reducers may be mesophilic or moderately thermophilic, whereas archaeal sulfur reducers are all extremely thermophilic. Typical habitats of the hyperthermophilic sulfur reducers are solfataric fields, hot springs and hydrothermal systems in the deep sea, whereas mesophilic bacterial sulfur reducers can be isolated from almost every freshwater or marine sediment, or even from wet soil. Moreover, bacterial sulfur reducers have been found both in oxic and anoxic environment. Indeed, in contrast to dissimilatory sulfate reduction, the capacity for sulfur reduction has also been observed in bacteria that grow with O_2 and which are, therefore, facultative anaerobes. However, many sulfur-reducing microorganisms are strictly anaerobic.

An overview of the morphological and physiological properties of *Bacteria* and *Archaea* capable of S_0-respiration is provided in Table 4.

				Electron donors				Electron acceptors						
Species	Morphology	Optimum temperature (°C)	Oxidation of organic electron donors[a]	H_2	Acetate	Lactate and/or pyruvate	Others utilized by some species	Sulfur	Thiosulfate	Sulfite	Sulfate	Fumarate	Nitrate	O_2
Bacteria[a]														
Desulfuromonas acetoxidans	Rod	30	c	–	+	±	Ethanol, propionate, succinate, glutamate	+	–	–	–	±	±	–
Desulfurella acetivorans	Rod	52–57	c	–	+	–	–	+	–	–	–	–	–	–
Desulfovibrio gigas	Vibrio	30–36	i	ND[b]	–	+	–	+	+	+	+	+	–	–
Desulfomicrobium species	Rod	28–37	i	ND	–	+	–	+	+	+	+	–	–	–
Dethiosulfovibrio peptidovorans	Vibrio	42	i	–	–	–	Peptides, amino acids	+	+	–	–	–	–	–
Desulfitobacterium chlororespirans[c]	Rod	37	i	+	–	+	Butyrate	+	+	+	–	–	–	–
Sulfurospirillum deleyianum	Curved spiral	25–30	ND	+	–	+	Succinate, fumarate, malate aspartate, oxaloacetate[d]	+	+	+	–	+	+	+
Wolinella succinogenes and similar spirilloid types	Spirillum or vibrio	30–37	i	+	–	+	Formate	+	+	+	–	+	+	+[e]
Pseudomonas mendocina subsp.	Rod	35–36	ND	+	ND	ND	Succinate, malate, glutamate	+	+	ND	–	ND	+	+
Geobacter sulfurreducens	Rod	35	ND	+	+	+	Ethanol, butyrate, succinate	+	–	–	–	+	–	–
Pelobacter carbinolicus	Rod	35	i	–	–	–	2,3-butandiol, acetoin,ethylene glycol	+	–	–	–	–	–	–
Sulfurosvirillum arcuchonense	Curved rods	26	i	+	+	+	glutarate. glutamate	+	–	–	–	–	–	+
Hippea marttima	Rod	52–54	c	+	+	–	Ethanol, stearate, palmitate	+	–	–	–	–	–	–
Desulfurobacterium thermolithotrophum	Rod	70	–[f]	+	–	ND		+	+	+	–	ND	–	–
Aquifex pyrophilus	Rod	85	–	+	–	–	(With O_2: sulfur, thiosulfate)	+	ND	ND	ND	ND	+	+
Ammonifex degensii	Rod	70	ND	+	–	+	formate	ND	–	–	+	–	+	–
Archaea[a]														
Acidianus infernus	Lobed coccus	90	–[f]	+	–	–	(With O_2: sulfur)	+	–	–	–	ND	–	+
Sulfolobus ambivalens	Lobed coccus	88	–[f]	+	–	–	(With O_2: sulfur)	+	–	–	–	ND	–	+
Pyrobaculum islandicum	Long rod	100	ND	+	–	–	Yeast extract	+	+	+	–	ND	–	–
Pyrodictium occultum	Disc with fibers	105	–[f]	+	–	–	–	+	–	–	–	ND	–	–
Thermoproteus tenax	Rod	80	c	+	–	–	Yeast extract	+	+	+	–	ND	–	–
Stygiolobus azoricus	Lobed	80	–[f]	+	–	ND		+	ND	–	–	ND	ND	–
Stetteria hydrogenophila	Coccus (irregular)	95	–[f]	+	–	–	Yeast extract	+	+	–	ND	–	–	ND
Thermodiscus marttimus	Disk	85	–[f]	+				+						

Symbols: c, complete oxidation (under anoxic conditions); i, incomplete oxidation; +, utilized; ±, utilized or not utilized; –, not utilized; ND, not determined or not reported.

[a]For further description and literature: *Desulfuromonas acetoxidans*, *Desulfovibrio gigas* and *Desulfomicrobium* species (Pfennig and Biebl 1976, 1981; Biebl and Pfennig, 1977; and Widdel, 1988; some data were personal communication from R. Bache and N. Pfennig); *Desulfurella acetivorans* (Bonch-Osmolovskaya et al., 1990; Schmitz et al., 1990); *Dethiosulfovibrio peptidovorans* (Magot et al., 1997); *Desulfitobacterium chlororespirans* (Sanford et al., 1996); *Sulfurospirillum deleyianum* (Wolfe and Pfennig, 1977; Schumacher et al., 1992); *Wolinella succinogenes* (Wolin et al., 1961; Macy et al., 1986); *Pseudomonas mendocina* subsp. (Balashova, 1985); *Geobacter sulfurreducens* (Caccavo et al., 1994); *Pelobacter carbinolicus* (Schink, 1984; Lovley et al., 1995); *Sulfurospirillum arcuchonense* (Finster et al., 1997b; Stolz et al., 1999); *Hippea marittima* (Miroshnichenko et al., 1999); *Desulfurobacterium thermolithotrophum* (L'Haridon et al., 1998); *Aquifex pyrophilus* (Huber et al., 1992); *Ammonifex degensii* (Huber et al., 1996); *Acidianus infernus* (Segerer et al., 1985, 1986; Stetter et al., 1990); *Sulfolobus ambivalens* (Zillig et al., 1985, 1986 [the 1986 paper is about *Desulfurolobus ambivalens*]); *Pyrobaculum islandicum* (Huber et al., 1987; Selig and Schönheit, 1994); *Pyrodictium occultum* (Fischer et al., 1983; Stetter et al., 1983); *Thermoproteus tenax* (Zillig et al., 1981; Fischer et al., 1983; Schäfer et al., 1986; Stetter et al., 1990; Selig and Schönheit, 1994); *Stygiolobus azoricus* (Segerer et al., 1991); *Stetteria hydrogenophila* (Jochimsen et al., 1997); *Thermodiscus marttimus* (Fischer et al., 1993).

[b]Not tested, but likely to be utilized.

[c]*Desulfitobacterium chlororespirans* can also grow on lactate coupled to reductive dehalogenation of 3-chloro-4-hydroxybenzoate.

[d]Utilized during fermentative metabolism.

[e]Low partial pressure (microaerobic conditions).

[f]Obligate lithoautotrophs that do not oxidize organic compounds.

Table 4. Morphological and physiological properties of *Bacteria* and *Archaea* capable of respiratory reduction of elemental sulfur. From Rabus et al., 2006.

I.6. The Colorless Sulfur Bacteria

The name “colorless sulfur bacteria” has been used since the time of Winogradsky to designate prokaryotes that are either able, or believed to be able, to use reduced sulfur compounds (e.g., sulfide, sulfur and organic sulfides) as sources of energy for growth. Today, it is known that this group comprises a very heterogeneous collection of bacteria, many of which have little or no taxonomic relationship to each other. The colorless sulfur bacteria play an essential role in the oxidative side of the sulfur cycle. Like all of the element cycles, the sulfur cycle has an oxidative and a reductive side, which, in most ecosystems, are in balance. However, this balance does not always exist, and accumulations of intermediates such as sulfur, iron sulfides, and hydrogen sulfide are often found. On the reductive side, sulfate (and sometimes elemental sulfur) functions as an electron acceptor in the metabolic pathways used by a wide range of anaerobic bacteria, leading to the production of sulfide. Conversely, on the oxidative side of the cycle, reduced sulfur compounds serve as electron donors for anaerobic, phototrophic bacteria or provide growth energy for the extremely diverse group of (generally) respiratory colorless sulfur bacteria. Common oxidation products of sulfide are elemental sulfur and sulfate. The adjective “colorless” is used because of the lack of photopigments in these bacteria, although it should be noted that colonies and dense cultures can actually be pink or brown because of their high cytochrome content.

Table 5 lists the genera that have traditionally been regarded as colorless sulfur bacteria, as well as genera containing species originally not classified as such that have now been shown to be able to obtain energy from the oxidation of reduced sulphur compounds.

Genus	pH requirement		Thermal requirement		Anaerobic growth		
	Neutrophilic	Acidophilic	Mesophilic	Thermophilic	Denitrifier	S^0/Fe^{3+} as electron acceptor	Symbiont
A. Traditional colorless sulfur bacteria							
Thiobacillus	+[a]	+	+	+	+	V	+
Thiomicrospira	+	−	+	−	+	−	?[b]
Thiosphaera	+	−	+	−	+	−	−
Sulfolobus	−	+	−	+[c]	−	−	−
Acidianus	−	+	−	+[c]	−	+	−
Thermothrix	+	−	−	+	+	−	−
Thiovulum[d]	+	−	+	−	−	−	−
Beggiatoa	+	−	+	−	−	+	−
Thiothrix	+	−	+	−	−	−	−
Thioploca[d]	+	−	+	−	−	−	−
Thiodendron[d]	+	−	+	−	−	−	−
Thiobacterium	+	−	+	−	−	+	−
Macromonas	+	−	+	−	−	+	−
Achromatium[d]	+	−	+	−	−	+	−
Thiospira[d]	+	−	+	−	−	−	−
B. Other bacteria capable of growth on reduced sulfur compounds							
Paracoccus	+	−	+	−	+	−	−
Hyphomicrobium	+	−	+	−	−	−	−
Alcaligenes	+	−	+	−	+	−	−
Pseudomonas	+	−	+	−	+	−	−
Hydrogenobacter	+	−	−	+	−	−	├

+, example known to exist; −, example unknown; V, variable.
[a] 16S rRNA analysis indicates a possible relationship.
[b] Hyperthermophilic archaebacterium.
[c] Axenic cultures are not available.

Table 5. Genera of the colorless bacteria traditionally recognized as being capable of growth on reduced sulfur compounds and their environmental parameters. From Robertson and Kuenen, 2006.

In addition to inorganic sulfur compounds, some species can also gain energy from the oxidation of other inorganic compounds such as hydrogen or ferrous iron. As well as differences in substrate range, there are also some variations in electron acceptor usage. Although most colorless sulfur-oxidizing bacteria require oxygen, a few are able to grow anaerobically using nitrogen oxides (e.g., nitrate) as their terminal electron acceptor during denitrification. One or two species (of the genus *Acidianus*) are capable of anaerobic metabolism by the reduction of sulfur (Segerer and Stetter, 1989), during which organic compounds or hydrogen serve as electron donors. *Thiobacillus ferrooxidans* is known to be able to reduce ferric iron under anaerobic conditions (Sugio et al., 1985). A somewhat exotic example of a sulfate reducer that might also be considered to be a colorless sulfur bacterium is *Desulfovibrio sulfodismutans*, which can grow anaerobically by the disproportionation of thiosulfate to sulfate and sulfide (Bak and Pfennig, 1987).

The heterogeneous group of colorless sulfur bacteria occurs in almost every life-supporting environment where reduced sulfur compounds are present.

In natural habitats, the reduced sulfur compounds available tend to be either sulfides (including metallic ores) or sulfur. Due to the activities of sulfate-reducing bacteria, especially

in anoxic sediments, hydrogen sulfide is very commonly available. One of the main factors that bacteria growing on hydrogen sulfide have to contend with is the chemical reaction between sulfide and oxygen. Therefore, the colorless sulfur bacteria are frequently found in the gradients at the interface between anoxic, sulfide-containing areas and aerobic waters and sediments where, at very low oxygen and sulfide concentrations, they can effectively compete with the spontaneous chemical oxidation reaction.

I.7. The denitrifying prokaryotes

The denitrifying prokaryotes reduce nitrate to gaseous nitrogen oxides, principally nitrogen gas.

The initial step in this process is the reduction of nitrate to nitrite, as occurs in ammonification. In the next step, nitrite is reduced to nitric oxide, a gaseous nitrogen oxide. This conversion of a fixed, non-gaseous form of nitrogen to gaseous forms has led this reaction to be termed “denitrification” because biologically preferred forms of nitrogen are lost.

In prokaryotes, reduction of nitrate is a respiratory process. That is, it is coupled to ATP synthesis via electron transport chains. However, with one or two exceptions, denitrifiers can also respire with oxygen as the terminal electron acceptor and, because it is usually available at higher concentrations, oxygen is typically the preferred electron acceptor. When oxygen becomes limiting, the capacity to utilize nitrate as a terminal oxidant allows denitrifying bacteria to continue respiration using an alternative electron acceptor.

Because each nitrogen oxide reduction has a positive redox couple, every step in denitrification need not be carried out to achieve a net conservation of energy. In fact, it is quite common to isolate bacteria that express only portions of the denitrification electron transport chain. Those prokaryotes that contain partial denitrification chains will be included in this paragraph and a list of prokaryotic genera suggested to include dentrifying strains is shown in Table 6.

Archaea
Haloarcula
Halobacterium
Haloferax
Ferroglobus
Pyrobaculum

Bacteria
Gram-negative

Aquifex
Flexibacter (formerly *Cytophaga*)
Empedobacter
Flavobacterium
Sphingobacterium
Synechocystis sp. PCC 6803

Purple Bacteria
α subdivision

Agrobacterium
Aquaspirillum
Azospirillum
Blastobacter
Bradyrhizobium
Gluconobacter
Hyphomicrobium
Magnetospirillum
Nitrobacter
Paracoccus
Pseudomonas (G-179)
Rhizobium
Rhodobacter
Rhodoplanes
Rhodopseudomonas
Roseobacter
Sinorhizobium (formerly *Rhizobium*)
Thiobacillus

β subdivision

Achromobacter
Acidovorax
Alcaligenes
Azoarcus
Brachymonas
Burkholderia
Chromobacterium
Comamonas
Eikenella
Hydrogenophage
Janthinobacterium
Kingella
Microvirgula
Neisseria
Nitrosomonas
Ochrobactrum
Oligella
Ralstonia (formerly *Alcaligenes*)
Rubrivivax
Thauera
Thermothrix
Thiobacillus
Vogesella
Zoogloea

γ subdivision

Acinetobacter
Alteromonas
Azomonas
Beggiatoa
Deleya
Halomonas
Marinobacter
Moraxella
Pseudoalteromonas
Pseudomonas
Rugamonas
Shewanella
Thiopioca
Thiomargarita
Xanthomonas

δ subdivision

None

ε subdivision

Wolinella
Campylobacter
Thiomicrospira

Others
Gram-positive

Bacillus
Corynebacterium
Frankia
Dactylosporangium
Dermatophilus
Gemella
Jonesia (formerly *Listeria*)
Kineosporia
Micromonospora
Microtetraspora
Nocardia
Pilimelia
Propionibacterium
Saccharomonospora
Saccharothrix
Spirrilospora
Streptomyces
Streptosporangium

Table 6. Listing of microbial genera that are suggested to include denitrifiers. From Shapleigh, 2006.

Denitrification ability is widespread among *Eubacteria*, and almost exclusively in those strains that are capable of aerobic growth. Rarely it occurs in anaerobic bacteria. The majority of currently characterized denitrifiers are found in α β γ and ε subdivisions of the *Proteobacteria* group. The δ subdivision, which contains a number of strict anaerobes, has not been found to contain any strains that denitrify, as yet.

The α subdivision of the *Proteobacteria* contains a number of well characterized denitrifiers including *Paracoccus denitrificans*, various *Rhodobacter* strains, and several rhizobia. Important denitrifiers in the α subdivision include *Hyphomicrobium*, budding bacteria often found in waste-water treatment facilities (Fesefeldt, 1998). Denitrification among the rhizobia, which are known for their roles as nitrogen-fixing symbionts, is also noteworthy because these strains have the seemingly contradictory capacity for both nitrogen fixation and denitrification (O'Hara, 1985). This ability to both fix and "unfix" nitrogen is fairly widespread among denitrifiers: *Rhodobacter*, *Hyphomicrobium*, *Frankia*, *Azospirrilum*, *Azoarcus* and some pseudomonad strains can both fix nitrogen and reduce nitrates to nitrogen gas. Some members of the genus *Nitrobacter*, which are nitrifying *α-Proteobacteria*, produce nitric oxide from nitrite. Even though a putative nitrite reductase has been purified from *Nitrobacter vulgaris* (Ahlers, 1990), other studies did not reveal production of nitric oxide or nitrous oxide in cultures of *Nitrobacter* species (Baumgartner, 1991; Goreau, 1980). Further studies are required to determine if these nitrifying bacteria are also denitrifiers.

Although a number of members of the *β-Proteobacteria* have the capability to denitrify, they are not as well studied, on the whole, as members of the α or γ subdivisions. Among these, a number of aromatic compound-degrading denitrifiers have been isolated, and many belong to the genera *Azoarcus* and *Thauera* (Fries, 1994; Springer, 1998; van Schie, 1998; Anders, 1995). Moreover it has been well documented that several species of *Neisseria* can denitrify (in fact, one species is named *Neisseria denitrificans*). Also some species of the genus *Nitrosomonas*, that are included into the *β-Proteobacteria* subdivision, have been described for their ability to oxidize ammonia to nitrite. The finding of a copper-containing nitrite reductase from *Nitrosomonas europaea* (Miller, 1985; Dispirito, 1985) strongly indicates that *Nitrosomonas* species are nitrifying denitrifiers. However, the role of denitrification in *Nitrosomonas*, whether for detoxification or for energy conservation, has not been established as yet.

Denitrification in members of *γ-Proteobacteria* subclass has been well studied in *Pseudomonas stutzeri* and *Pseudomonas aeruginosa*. Also *Beggiatoa* and *Thioploca* are presumed to be denitrifiers. These species have the unique capacity to accumulate nitrate in

internal vacuoles (McHatton, 1996; Fossing, 1995). Then they use this accumulated nitrate as an oxidant and sulfide in the surrounding environment as a reductant. The use of reduced sulfur compounds as electron donors is known to occur in several denitrifiers including *Aquifex* (Huber, 1992), *Paracoccus* (Friedrich, 1981), and *Thiobacillus* (Schedel, 1980).

A few denitrifiers have been found in the ε subdivision of the *Proteobacteria*. Some exhibit truncated denitrification chains. For example, both *Wolinella succinogenes* and some *Campylobacter* species have the capacity to grow with nitrous oxide as sole terminal oxidant (Yoshinari, 1980; Payne, 1982). However, neither bacterium is able to reduce nitrite to nitrous oxide. *Thiomicrospira denitrificans*, which is closely related to the *Campylobacter* group (Muyzer, 1995), can reduce nitrite to gaseous end products (Timmer-ten Hoor, 1975). Also, *T. denitrificans* can use sulfur compounds as reductants.

Among the Gram-negative bacteria that are not *proteobacteria*, denitrifying strains are found in the genera *Aquifex* (Huber, 1992), *Flexibacter* (Jones, 1990), and *Flavobacterium* (Coyne, 1989). Even though the majority of denitrifiers are Gram-negative, denitrifying bacteria are well represented among Gram-positive bacteria. For example, there have been a number of denitrifying *Bacillus* species described. Other Gram-positive bacteria capable of denitrification include strains of *Propionibacterium* (Swartzlander, 1993) and *Jonesia denitrificans* (originally *Listeria denitrificans*) (Rocourt, 1987). These strains are somewhat unusual because they seem to be denitrifying strains in groups of bacteria that are primarily non-denitrifiers. A high number of denitrifying strains were found in *Frankia* genus (Lensi, 1990). Recently, it has been shown that a number of actinomycetes, including *Streptomyces*, *Dermatophilus* and *Nocardia*, are capable of nitrous oxide evolution from nitrate or nitrite (Shoun, 1998).

Only a few *Archaea* capable of denitrification have been isolated. Most of the denitrifying *Archaea* are halophilic bacteria capable of aerobic respiration but more recently, also denitrifying non-halophilic *Archaea* such as the hyperthermophile *Pyrobaculum aerophilum* (Volkl, 1993) have been described.

I.8. Dinitrogen-fixing prokaryotes

Dinitrogen fixation, the biocatalytic conversion of gaseous nitrogen (N_2) to ammonium, is an exclusive property of prokaryotes. The enzyme catalyzing this reaction is the nitrogenase.

Nitrogen fixation (N_2 fixation) is the most important way by which N_2 present in the atmosphere enters biological systems. Many diazotrophs (*di* = two, *azo*te = nitrogen; *trophs* = eaters: dinitrogen fixers) are found to be associated with the roots of plants where they exchange fixed nitrogen for the products of photosynthesis. Biological N_2 fixation is the main source of nitrogen in soil, marine environments such as oligotrophic oceanic waters (where dissolved fixed-nitrogen content is extremely low; Zehr et al., 1998; Staal et al., 2003), subtropical and tropical open ocean habitats (Karl et al., 2002), and hydrothermal vent ecosystems (Mehta et al., 2003). N_2-fixing prokaryotes inhabit a wide range of environments including soils, seas, oceans, insects, cow rumena, human intestines, and feces (Bergersen and Hipsley, 1970). Nevertheless, the presence of a N_2-fixing bacterium is not evidence for the occurrence of N_2 fixation. This is the case of many lakes, such as Mono Lake, where no significant nitrogen fixation rate was detected, although potential nitrogen fixers were present (Steward et al., 2004). Also the N_2 fixation rates measured in lake Cadagno were moderate, but the detection of nitrogenase gene and of its expression in both oxic and anoxic waters, indicated that N_2 fixation might be occurring throughout the water column (Halm et al., 2009). Anyway, at the present time, little is known about N_2 fixation occurring in lakes.

Diazotrophs are encountered in *Bacteria* and in some groups of *Archaea*. Nowadays, only 6 out of 53 currently identified major lineages or phyla within the domain *Bacteria* (*Proteobacteria*, *Cyanobacteria*, *Chlorobi* (green nonsulfur), *Spirochetes* and the Gram-positives (*Firmicutes* and *Actinobacteria*) have nitrogen-fixing members. The N_2-fixing capability is unevenly distributed throughout these prokaryotic taxa, and N_2-fixing bacteria are in restricted clusters among species of non-N_2-fixing bacteria.

Among *Eubacteria*, N_2-fixing species seem to be dominant in *Rhodospirillaceae* (Madigan et al., 1984) whereas only a subset of cyanobacterial species are able to fix N_2. *Gluconacetobacter diazotrophicus* and a couple of other N_2-fixing species are the only diazotrophs in a larger group comprising *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* (Fuentes-Ramírez et al., 2001). Similarly, among aerobic endospore-forming *Firmicutes* (Gram-positive bacteria), N_2 fixers are encountered mainly in a discrete group (defined by cluster analysis from 16S rRNA gene sequences) corresponding to *Paenibacillus* (Achouak et

al., 1999). Among the *Actinomycetes*, N_2-fixing *Frankia*, represented by a diversity of phenotypes from different habitats, are grouped on the basis of their 16S rRNA gene sequences (Normand et al., 1996).

In *Archaea*, N_2-fixing organisms are found in the methanogen group and in the halophile group within the *Euryarchaeota* but not in the sulfur-dependent *Crenarchaeota* (Young, 1992).

I.9. The inorganic nitrogen compounds oxidizing prokaryotes

Nitrifying bacteria are separated into two groups, the ammonia- and the nitrite oxidizers, on the basis of their ability to lithotrophically oxidize ammonia to nitrite and nitrite to nitrate, respectively. These two groups possess very different key enzyme systems: among the main enzymes involved in oxidation of ammonia and nitrite, the enzyme ammonia monooxygenase (AMO) catalyzes the conversion of ammonia to hydroxylamine, the first intermediate of ammonia oxidation, whereas the enzyme nitrite oxidoreductase (NO_2-OR) catalyzes the oxidation of nitrite to nitrate.

Nitrifying bacteria are present in oxic and even anoxic environments. They are widely distributed in freshwater, seawater and soils. Nitrifiers also could be enriched or isolated from extreme habitats like heating systems with temperatures of up to 47 °C (Ehrich et al., 1995) and permafrost soils up to a depth of 60 m at a temperature of down to -12 °C. Although the pH optimum for cell growth is 7.6–7.8, nitrifiers were frequently detected in environments with suboptimal pH (e.g., acid tea soils and forest soils at pH values below 4) but also in highly alkaline soda lakes showing pH values of 9.7–10.5 (Sorokin et al., 2001).

Lithotrophic nitrifiers are Gram-negative bacteria and conventionally have been placed in the family *Nitrobacteriaceae* (Buchanan, 1917; Watson, 1971; Watson et al., 1989). However, phylogenetically the lithoautotrophic ammonia oxidizers, characterized by the prefix *Nitroso-*, and nitrite oxidizers, characterized by the prefix *Nitro-*, are not closely related (Teske et al., 1996; Purkhold et al., 2000).

Comparative 16S rRNA sequence analysis demonstrated that all recognized ammonia oxidizers are either members of the *β*- or *γ*-subclass of *Proteobacteria* (Fig. 2). The genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* form a closely related monophyletic assemblage within the *β*-subclass of *Proteobacteria* (Head et al., 1993; Woese et al., 1984; Teske et al., 1994; Utåker et al., 1995; Pommerening-Röser et al., 1996; Purkhold et al., 2000), whereas the genus *Nitrosococcus*

constitutes a separate branch within the γ-subclass of *Proteobacteria* (Woese et al. 1985; Purkhold et al., 2000). Among the nitrite oxidizers, the genera *Nitrobacter*, *Nitrococcus* and *Nitrospina* were assigned to the α-, γ-, and δ subclass of *Proteobacteria*, respectively (Orso et al., 1994; Teske et al., 1994). Nitrite oxidizers of the genus *Nitrospira* are affiliated with the recently described *Nitrospira*-phylum, which represents an independent line of descent within the domain *Bacteria* (Ehrich et al., 1995).

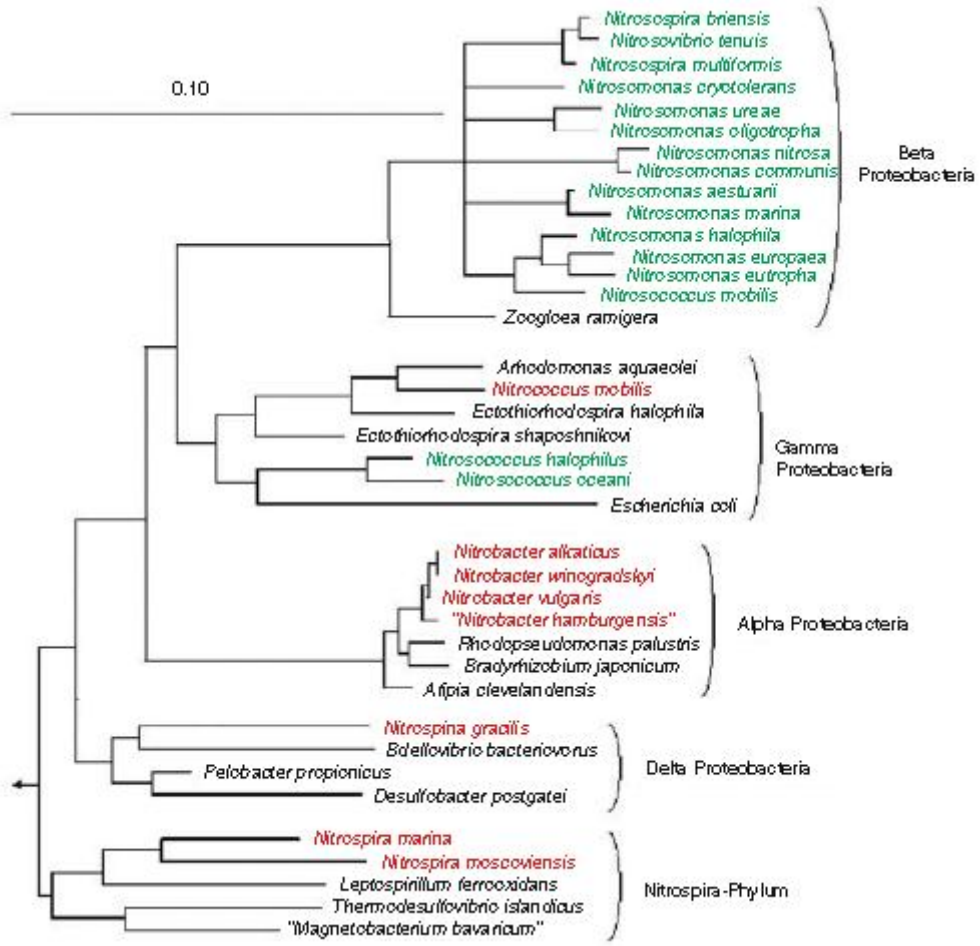

Figure 2. 16S rRNA-based tree reflecting the phylogenetic relationship of ammonia- and nitrite-oxidizing bacteria. The consensus tree is based on the results of a maximum likelihood analysis of the 16S rRNA primary structure data from the nitrifying bacteria shown in the tree and a selection of reference sequences. Only homologous positions that share identical residues in at least 50% of all available almost complete bacterial 16S rRNA sequences were included for tree reconstruction. In the tree, ammonia oxidizers are labeled green, and nitrite oxidizers are depicted in red. It should be noted that the assignment of the genus *Nitrospina* to the δ-*Proteobacteria* is tentative and might change if additional reference sequences become available. Multifurcations connect lineages for which no unambiguous branching order could be retrieved using different treeing methods. Bar represents 10% estimated sequence divergence. From Bock and Wagner (2006)

In addition to lithotrophic nitrifiers, various heterotrophic bacteria, (Focht and Verstraete, 1977; Killham, 1986; Papen et al., 1989) are capable of oxidizing ammonia to nitrate. However, in contrast to lithotrophic nitrification, heterotrophic nitrification is not coupled to energy generation. Consequently, heterotrophic nitrifiers are dependent on the oxidation of

organic substrates (Focht and Verstraete, 1977; Kuenen and Robertson, 1987). During heterotrophic nitrification, ammonia or reduced nitrogen from organic compounds (e.g., the amino group of amino acids) is co-oxidized to hydroxylamine, gaseous nitrogen oxides, nitrite or nitrate. For example, methane-oxidizing bacteria were shown to co-oxidize ammonia to nitrite (Anthony, 1982; Yoshinari, 1985; O'Neil and Wilkinson, 1977; Zahn et al., 1994; Bergmann et al., 2000).

The environmental importance of heterotrophic nitrifiers is controversial in the literature. Generally it is assumed that in most environments, the biological conversion of reduced forms of nitrogen to nitrite and nitrate is catalyzed mainly by the lithoautotrophic ammonia- and nitrite-oxidizing bacteria and not by heterotrophic nitrifiers. This reflects the fact that the nitrification rates of heterotrophic nitrifiers are small compared to those of autotroph ic nitrifiers (Robertson and Kuenen, 1988).

I.10. The hydrogen-metabolizing prokaryotes

Hydrogen is an important and widespread metabolite among prokaryotes living in oxic as well as in anoxic habitats. The physiological role of H_2 in microbes is dual. Firstly, H_2 is a growth substrate, i.e., a source of energy and reductant. Prokaryotes of different metabolic types, such as methanogens, anoxygenic phototrophs, and aerobic knallgas bacteria, use H_2. Secondly, H_2 production is a means of dispersing excess reductant from fermentative metabolism. Hydrogen is among the fermentation products of both facultative fermenters, such as *Escherichia coli*, and obligate fermenters such as *Clostridium pasteurianum*.

The list of H_2-metabolizing prokaryotes (Tables 7 and 8) shows as H_2 metabolism is not limited to specialized microbes grouped in a few taxonomic units.

Group	Order	Species[a]	E[b]	References
	Aquificales	*Aquifex aeolicus*	G	Deckert et al., 1998
		Aquifex pyrophilus	P	Huber et al., 1992
		Hydrogenobacter hydrogenophilus[c]	P	Kryukov et al., 1983
		Hydrogenobaculum acidophilum[d]	P	Shima and Suzuki, 1993
		Hydrogenobacter thermophilus	P	Kawasumi et al., 1984
				Shiba et al., 1984
		Hydrogenothermus marinus	P	Stöhr et al., 2001
		Thermocrinus ruber	P	Huber et al., 1998
Cyanobacteria	Chlorococcales	*Synechococcus*[e] PCC6301	P	Peschek, 1979
				Howarth and Codd, 1985
			A	Schmitz et al., 1995
				Schmitz and Bothe, 1996
		Synechococcus PCC6307	P	Howarth and Codd, 1985
		Synechocystis PCC6803	P	Howarth and Codd, 1985
		Synechocystis PCC6714	P	Howarth and Codd, 1985
		Synechocystis[f] PCC6308	P	Serebryakova et al., 1996
		Cyanothece PCC7822	P	van der Oost et al., 1989
		Microcystis PCC7820	P	Howarth and Codd, 1985
		Microcystis PCC7806	P	Moezelaar and Stal, 1994
	Nostocales	*Nostoc* sp. PCC73102	P	Lindblad and Sellstedt, 1990
		Nostoc sp.[g] PCC7937	PA	Mikheeva et al., 1995
				Serebryakova et al., 1994
			I	Serebryakova et al., 1996
		Nostoc muscorum PCC7120	PA	Houchins and Burris, 1981a
			I	Houchins and Burris, 1981b
		Anabaena cylindrica[h] PCC7122	PA	Bothe et al., 1977
				Lambert and Smith, 1980
			I	Ewart and Smith, 1989
	Stigonematales	*Fischerella muscicola*[i] PCC73103	A	Lambert and Smith, 1980
	Oscillatoriales	*Oscillatoria chalybea*	P	Bader and Abdel-Basset, 1999
		Oscillatoria limnosa	P	Heyer et al., 1989
		Microcoleus chthonoplastes	P	Moezelaar et al., 1996
Flexibacteria		*Chloroflexus aurantiacus*	PA	Holo and Sirevåg, 1986
Firmicutes	Actinomycetales	*Frankia* sp.	A	Sellstedt, 1989; Murry and Lopez, 1989
		Streptomyces thermoautotrophicus	P	Gadkari et al., 1990
	Bacillales	*Bacillus schlegelii*	P	Schenk and Aragno, 1979
		Bacillus tusciae	P	Bonjour and Aragno, 1984
	Clostridiales	*Clostridium pasteurianum*	P	Nakos and Mortenson, 1971
			I	Chen and Mortenson, 1974
				Chen and Blanchard, 1978
		Clostridium acetobutylicum	P	Gray and Gest, 1965
			I	Vasconcelos et al., 1994
		Clostridium kluyveri	P	Bornstein and Barker, 1948
		Clostridium tetanomorphum	P	Woods and Clifton, 1938
		Clostridium aceticum	P	Braun et al., 1981
				Braun and Gottschalk, 1981
		Clostridium mayombei	P	Kane et al., 1991
		Clostridium magnum	P	Bomar et al., 1991
		Clostridium scatologenes	PA	Küsel et al., 2000
		Eubacterium limosum	P	Sharak-Genthner et al., 1981
		Carboxydothermus hydrogenoformans	P	Svetlichny et al., 1991
			I	Soboh et al., 2002
		Ruminococcus albus	P	Miller and Wolin, 1973
		Ruminococcus hydrogenotrophicus	P	Bernalier et al., 1996
		Ruminococcus productus	P	Lorowitz and Bryant, 1984
				Bernalier et al., 1996
		Ruminococcus hansenii	P	Bernalier et al., 1996
		Ruminococcus schinkii	P	Rieu-Lesme et al., 1996
		Syntrophomonas wolfei	PA	McInerney et al., 1979
				McInerney et al., 1981a
		Megasphaera elsdenii	I	van Dijk et al., 1979
				Atta and Meyer, 2000
		Desulfitobacterium dehalogenans	P	Utkin et al., 1994

(Continued)

Group	Order	Species[a]	E[b]	References
		Rhodococcus opacus	P	Aggag and Schlegel, 1973
			I	
		Mycobacterium gordonae	P	Park and DeCicco, 1974
		Sporomusa termitida	P	Breznak et al., 1988
		Sporomusa sphaeroides	PA	Dobrindt and Blaut, 1996
		Sporomusa sylvacetica	P	Kuhner et al., 1997
		Acetonema longum	P	Kane and Brezhnak, 1991
		Desulfotomaculum orientis	P	Klemps et al.,1985
			A	Cypionka and Dilling, 1986
		Acetobacterium woodii	P	Balch et al., 1977
				Ljungdahl and Wood, 1982
			I	Ragsdale and Ljungdahl, 1984
		Acetobacterium bakii	P	Kotsyurbenko et al., 1995
		Acetobacterium paludosum	P	Kotsyurbenko et al., 1995
		Acetobacterium fimetarum	P	Kotsyurbenko et al., 1995
	Thermoanaero-bacteriales	*Thermoanaerobacter kivui*	PA	Leigh et al., 1981
				Daniel et al., 1990
				Pusheva et al., 1991
		Moorella thermoacetica[j]	PA	Fontaine et al., 1942
				Drake, 1982
				Daniel et al., 1990
			P	Pezacka and Wood, 1984
				Martin et al., 1983;
				Kerby and Zeikus, 1983
		Moorella thermoautotrophica	PA	Clark et al., 1982
				Wiegel et al., 1981
		Thermacetogenium phaeum	P	Hattori et al., 2000
	Halanaerobiales	*Halanaerobium alcaliphilum*	P	Tsai et al., 1995
		Acethalobium arabaticum	P	Zhilina and Zavarzin, 1990
	Bacterioidales	*Acetomicrobium flavidum*	P	Soutschek et al., 1984
			I	Mura et al., 1996
Proteobacteria	α Class	*Renobacter vacuolatum*	P	Malik and Schlegel, 1981
		Aquaspirillum autotrophicum SA32	P	Aragno and Schlegel, 1978
		Bradyrhizobium japonicum	P	Hanus et al., 1979
				Emerich et al., 1979
			A	McCrae et al., 1978
			I	Harker et al., 1984
		Paracoccus pantotrophus[k]	P	Kühnemund, 1971
			A	Schneider and Schlegel, 1977
			I	Knüttel et al., 1989
				Sim and Vignais, 1978
		Methylosinus trichosporium OB3b	A	Chen and Yoch, 1987
		Rhizobium leguminosarum	P	Dixon, 1968
				Nelson and Salminen, 1982
		Rhodobacter capsulatus	P	Yen and Marrs, 1977
				Madigan and Gest, 1978
				Madigan and Gest, 1979
			I	Colbeau et al., 1983
		Rhodobacter sphaeroides	P	Uffen and Wolf, 1970
		Rhodospirillum rubrum	P	Ormerod and Gest, 1962
				Gest, 1954
				Gorrell and Uffen, 1978;
				Voelskow and Schön, 1980
				Uffen, 1981
			I	Adams and Hall, 1979
		Rhodopseudomonas palustris	P	Qadri and Hoare, 1968
				Uffen and Wolf, 1970
		Thiorhodococcus minus	P	Guyoneaud et al., 1997
		Thiocystis violacea	P	Winogradsky, 1988
		Rhodomicrobium vannieli	P	Duchow and Douglas, 1949
		Xanthobacter flavus 301	P	Malik and Claus, 1979
		Xanthobacter autotrophicus	P	Schneider et al., 1973
				Baumgarten et al., 1974

Group	Order	Species[a]	E[b]	References
			I	Schink, 1982
			A	Eberhardt, 1969
				Schneider and Schlegel, 1977
		Oligotropha carboxidovorans	P	Meyer and Schlegel, 1978
			I	Santiago and Meyer, 1997
		Azospirillum lipoferum	P	Malik and Schlegel, 1981
		Ancylobacter aquaticus	P	Malik and Schlegel, 1981
	β Class	*Acidovorax facilis*	P	Willems et al., 1990
		Acidovorax delafieldii	P	Willems et al., 1990
		Variovorax paradoxus	P	Davis et al. 1970
			A	Schneider and Schlegel, 1977
		Achromobacter ruhlandii	P	Packer and Vishniac, 1955
				Aragno and Schlegel, 1977
		Alcaligenes latus H-4	P	Palleroni and Palleroni, 1978
			I	Pinkwart et al., 1983
		Alcaligenes hydrogenophilus	P	Ohi et al., 1979
		Hydrogenophaga flava	P	Willems et al., 1989
		Hydrogenophaga pseudoflava	P	Willems et al., 1989
		Hydrogenophaga palleroni	P	Davis et al., 1970
			P	Willems et al., 1989
		Hydrogenophaga taeniospiralis	P	Lalucat et al., 1982
				Willems et al., 1989
		Ralstonia eutropha H16	P	Eberhardt, 1966
			I	Schneider and Schlegel, 1976
				Schink and Schlegel, 1979;
				Bernhard et al., 2001
		Ralstonia metallidurans CH34	PA	Mergeay et al., 1985
		Hydrogenophilus hirschii	P	Stöhr et al., 2001
		Hydrogenophilus thermoluteolus	P	Hayashi et al., 1999
		Rubrivivax gelatinosus[l]	P	Wertlieb and Vishniac, 1967
				Uffen, 1976
		Thiobacillus plumbophilus	P	Drobner et al., 1992
		Pseudomonas saccharophila	PA	Bone, 1960
				Bone et al., 1963
				Podzuweit et al., 1983
	γ Class	*Azotobacter chroococcum*	P	Lee and Wilson, 1943
			I	van der Werf and Yates, 1978
		Azotobacter vinelandii	P	Hyndman et al., 1953
				Wong and Maier, 1985
			I	Seefeldt and Arp, 1986
		Derxia gummosa	P	Pedrosa et al., 1980
		Acidithiobacillus ferrooxidans	P	Drobner et al., 1990
				Fischer et al., 1996
		Escherichia coli	P	Stephenson and Stickland, 1931
				Peck and Gest, 1957
			A	Krasna, 1980
				Krasna, 1984
			I	Adams and Hall, 1979
				Ballantine and Boxer, 1985
				Sawers and Boxer, 1986
		Salmonella typhimurium	A	Krasna, 1980
		Citrobacter freundii	A	Krasna, 1980
		Allochromatium vinosum	I	Gitlitz and Krasna, 1975
		Shewanella putrefaciens	P	Lovley et al., 1989
		Thiocapsa roseopersicina BBS	P	Bogorov, 1974
				Gogotov et al., 1974
			I	Zorin and Gogotov, 1975
				Gogotov et al., 1976
		Hydrogenovibrio marinus	P	Nishihara et al., 1990
				Nishihara et al., 1991
			I	Nishihara et al., 1997
		Methylococcus capsulatus Bath	P	Stanley and Dalton, 1982
			A	Hanczár et al., 2002

Group	Order	Species[a]	E[b]	References
		Pseudomonas hydrogenovora	P	Kodama, T. et al., 1975 Igarashi, Y. et al., 1980
	δ Class	*Desulfomicrobium norvegicum*[m]	P	Genthner et al., 1997
		Desulfomicrobium baculatum[n]	P	Rozanova et al., 1988 Fauque et al., 1991
		Desulfomicrobium apsheronum	P	Rozanova et al., 1988
		Desulfobacterium autotrophicum	P	Brysch et al., 1987
		Desulfovibrio vulgaris	P	Hatchikian et al., 1976 Badziong et al., 1978 Traore et al., 1983
			I	Yagi, 1970 van der Westen et al., 1978
		Desulfovibrio fructosovorans	P	Malki et al., 1997
		Desulfovibrio desulfuricans	P	Vosjan, 1975 Tsuji and Yagi, 1980
		Desulfovibrio gigas	P	Hatchikian et al., 1976 Hatchikian et al., 1978
		Desulfovibrio profundus	P	Bale et al., 1997
		Desulfovibrio senezii	P	Tsu et al., 1998
		Desulfobacter hydrogenophilus	P	Widdel, 1987
		Desulfobulbus propionicus	P	Laanbroek et al., 1982a
		Desulfonema limicola	P	Widdel et al., 1983
		Desulfuromonas acetoxidans	IA	Brugna et al., 1999
		Desulfurella multipotens	P	Miroshnichenko et al., 1994
		Desulfurella kamchatkensis	P	Miroshnichenko et al., 1998
		Hippea maritima	P	Miroshnichenko et al., 1999
		Pelobacter acetylenicus	P	Schink, 1985
		Syntrophus aciditrophicus	P	Jackson et al., 1999
		Syntrophus buswellii	PA	Mountfort et al., 1984 Wallrabenstein and Schink, 1994
		Syntrophobacter wolinii	P	Boone and Bryant, 1980 Wallrabenstein and Schink, 1994
		Syntrophobacter pfennigii	PA	Wallrabenstein et al., 1995
		Smithella propionica	P	Liu et al., 1999
	ε Class	*Helicobacter pylori*	A	Maier et al., 1996a
		Nautilia lithotrophica	P	Miroshnichenko et al., 2002
		Wolinella succinogenes	P	Wolin et al., 1961
			I	Aspen and Wolin, 1966 Dross et al., 1992
		Campylobacter jejuni	PA	Laanbroek et al., 1982b Goodman and Hoffman, 1983
		Campylobacter sputorum	P	Schumacher et al., 1992
		Sulfurospirillum deleyianum	P	Schumacher et al., 1992
	Thermotogales	*Thermotoga maritima*	P	Huber et al., 1986
			I	Juszczak et al., 1991
		Thermotoga neapolitana	P	Jannasch et al., 1988
		Thermotoga thermarum	P	Windberger et al., 1989
		Fervidobacterium islandicum	P	Huber et al., 1990

Abbreviations: E, evidence; I, hydrogenase has been isolated; P, physiological evidence for hydrogenase activity; G, genetic evidence; and A, biochemical assay.
[a]Green: H_2 producer; brown: H_2 consumer; and blue: both.
[b]Type of evidence.
[c]Formerly *Calderobacterium hydrogenophilum*.
[d]Formerly *Hydrogenobacter acidophilus*.
[e]Formerly *Anacystis nidulans* (= SAG 1402-1).
[f]CCAP 1430/1, or CALU 743.
[g]CCAP 1403/13A, or ATCC 29413.
[h]CCAP 1403/2A, SAG 1403-2, or ATCC 27899.
[i]CCAP 1427/1.
[j]Formerly *Clostridium thermoaceticum*.
[k]Formerly *Paracoccus denitrificans*.
[l]Formerly *Rhodocyclus gelatinosus*.
[m]Formerly *Desulfovibrio desulfuricans* Norway 4.
[n]Formerly *Desulfovibrio baculatus*.

Table 7. H_2-metabolizing *Bacteria*. From Schwartz and Friedrich, 2006

Group	Order	Species[a]	E[b]	References
Crenarchaeota	Desulfurococcales	*Pyrodictium brockii*	PA	Stetter et al., 1983
				Pihl et al., 1989
			I	Pihl and Maier, 1991
		Pyrodictium occultum	P	Stetter et al., 1983
				Fischer et al., 1983
		Pyrodictium abyssi	P	Pley et al., 1991
		Thermodiscus maritimus	P	Fischer et al., 1983
		Pyrolobus fumarii	P	Blöchl et al., 1997
		Hyperthermus butylicus	P	Zillig et al., 1990
		Ignicoccus islandicus	P	Huber et al., 2000
		Ignicoccus pacificus	P	Huber et al., 2000
		Stetteria hydrogenophila	P	Jochimsen et al., 1997
	Thermoproteales	*Thermoproteus tenax*	P	Zillig et al., 1981
				Fischer et al., 1983
		Thermoproteus neutrophilus	P	Fischer et al., 1983
		Pyrobaculum islandicum	P	Huber et al., 1987
		Pyrobaculum aerophilum	P	Völkl et al., 1993
	Sulfolobales	*Sulfolobus solfataricus*	P	Brock et al., 1972
		Sulfolobus acidocaldarius	P	Brock et al., 1972
		Metallosphaera sedula	P	Huber et al., 1989
		Acidianus brierleyi	P	Segerer et al., 1986
		Acidianus infernus	P	Segerer et al., 1986
		Stygiolobus azoricus	P	Segerer et al., 1991
Euryarchaeota	Archaeglobales	*Archaeoglobus fulgidus*	P	Stetter, 1988
		Archaeoglobus profundus	P	Burggraf et al., 1990
		Archaeoglobus lithotrophicus	P	Stetter et al., 1993
	Methanosarcinales	*Methanosarcina barkeri* Fusaro	I	Fiebig and Friedrich, 1989
			G	Künkel et al., 1998
				Vaupel and Thauer, 1998
				Meuer et al., 1999
		Methanosarcina barkeri DSM 800	P	Weimer and Zeikus, 1978
			I	Fauque et al., 1984
		Methanosarcina mazei Gö1	I	Mah, 1980
				Deppenmeier et al., 1992
			G	Deppenmeier et al., 1995
				Deppenmeier et al., 2002
	Methanomicrobiales	*Methanospirillum hungatei*	P	Ferry et al., 1974
			I	Sprott et al., 1987
		Methanocalculus halotolerans	P	Ollivier et al., 1998
		Methanomicrobium mobile	P	Paynter and Hungate, 1968
		Methanocorpusculum parvum	P	Zellner et al., 1989
		Methanocorpusculum sinense	P	Zellner et al., 1989
		Methanocorpusculum bavaricum	P	Zellner et al., 1989
		Methanogenium organophilum	P	Widdel et al., 1988
		Methanogenium marinigri	P	Romesser et al., 1979
		Methanogenium cariaci	P	Romesser et al., 1979
		Methanogenium frigidum	P	Franzmann et al., 1997
		Methanoplanus limicola	P	Wildgruber et al., 1982
		Methanoplanus endosymbiosus	P	van Bruggen et al., 1986
	Methanobacteriales	*Methanothermobacter thermoautotrophicus*[c] ΔH	P	Zeikus and Wolfe, 1972
				Jacobson et al., 1982
			I	Kojima et al., 1983
				Fox et al., 1987
			G	Alex et al., 1990
				Smith et al., 1997
		Methanothermobacter marburgensis[d] DSM 2133	PA	Zirngibl et al., 1990
				Afting et al., 1998
			I	Setzke et al., 1994
			G	Tersteegen and Hedderich, 1999
		Methanobacterium formicicum sp.	P	van Bruggen et al., 1984
		Methanobacterium formicicum MF	IA	Jin et al., 1983

Group	Order	Species[a]	E[b]	References
		Methanobacterium formicicum JF-1	IA	Baron and Ferry, 1989a; Baron and Ferry, 1989b; Baron et al., 1989 Baron et al., 1989
		Methanobacterium alcaliphilum	P	Blotevogel et al., 1985 Worakit et al., 1986
		Methanothermus fervidus	P	Stetter et al., 1981
		Methanothermus sociabilis	P	Lauerer et al., 1986
		Methanosphaera stadtmanae	P	Miller and Wolin, 1985
		Methanobrecibacter arboriphilicus	P	Zeikus and Henning, 1975; Zehnder and Wuhrmann, 1977
	Methanococcales	*Methanococcus jannashii*	P	Jones et al., 1983a
			I	Shah, 1990
			G	Halboth and Klein, 1992 Bult et al., 1996
		Methanothermococcus thermolithotrophicus	P	Huber et al., 1982 Belay et al., 1986
		Methanococcus vanielli	I	Yamazaki, 1982
		Methanococcus voltae	I	Muth et al.,1987
		Methanococcus maripaludis	P	Jones et al., 1983b
		Methanococcus igneus	P	Burggraf et al., 1990
	Methanopyrales	*Methanopyrus kandleri*	P	Kurr et al., 1991
			G	Slesarev et al., 2002
	Thermococcales	*Pyrococcus furiosus*	P	Fiala and Stetter, 1986
			I	Bryant and Adams, 1989 Ma et al., 1993 Sapra et al. 2000; Silva et al., 2000
		Pyrococcus abyssi	P	Erauso et al., 1993
		Pyrococcus woesei	P	Zillig et al., 1987
		Thermococcus litoralis	P	Neuner et al., 1990
			I	Rákhely et al., 1999
		Thermococcus stetteri	P	Miroshnichenko et al., 1989 Pusheva et al., 1991
			I	Zorin et al., 1996
		Thermococcus celer	P	Zillig et al., 1983

Abbreviations: E, evidence; I, hydrogenase has been isolated; P, physiological evidence for hydrogenase activity; G, genetic evidence; and A, biochemical assay.
[a]Green: H_2 producer; brown: H_2 consumer; and blue: both.
[b]Letter codes for type of evidence.
[c]Formerly *Methanobacterium thermoautotrophicum* ΔH.
[d]Formerly *Methanobacterium thermoautotrophicum* Marburg.

Table 8. H_2-metabolizing *Archaea*. From Schwartz and Friedrich, 2006

The common denominator of the disparate taxonomic and physiological groups treated in this paragraph is the presence of hydrogenase, the main enzyme responsible both for hydrogen evolution and consumption. These reactions are not catalyzed by hydrogenases alone. Nitrogenase, the enzyme which catalyzes the production of NH_3 from atmospheric N_2, produces H_2 as a byproduct of N_2 fixation and therefore also N_2-fixing prokaryotes give an important contribution to the global H_2 flux.

Both biogenic and abiogenic H_2 production can support growth of H_2-utilizing prokaryotes. Many H_2-utilizing species profit from fermentative H_2 production in anoxic sediments. The two most important groups of H_2-consumers in such biotopes are the methanogenic *Archaea* and the sulfate-reducing *Bacteria*. It has been known for some time

that methanogens and sulfate reducers compete for H_2 (Winfrey and Zeikus, 1977; Winfrey et al., 1977; Abram and Nedwell, 1978; Oremland and Polcin, 1982; Lovley et al., 1982; Lovley and Klug, 1983). Sulfate reducers outcompete the methanogens in the presence of sulfate, because the former have a higher affinity for H_2 and a higher growth yield (Kristjansson et al., 1982; Schönheit et al., 1982). The key factor determining which of the two terminal degradation processes—sulfidogenesis or methanogenesis— prevails in a given habitat is SO_4^{2-} concentration. In anoxic marine sediments, where there is an abundant supply of SO_4^{2-}, sulfate reduction is the dominant process consuming most of the available H_2 and acetate. The sulfate level in lakes varies depending on their trophic state, but in general is lower than in seawater. The thickness of the zone of sulfate reduction varies accordingly. In eutrophic lakes the sulfate concentration in the sediment drops sharply. Here the zone of sulfate reduction is only a few centimeters thick. In lakes with lower nutrient contents the zone of sulfate reduction extends deeper into the sediment (Lovley and Klug, 1983). Competition for H_2 does not mean that sulfate reduction and methanogenesis are mutually exclusive processes. Various methanogens can exploit substrates, e.g. methylamine, that are not utilized by sulfate reducers (Oremland and Polcin, 1982; Winfrey and Ward, 1983). Therefore, the two groups of organisms can coexist in the same biotope, as has been shown for instance for estuarine sediments. In sediment from an oligotrophic lake, it was reported that the total flux of electrons and carbon routed through sulfate reduction is between 30 and 81% of the total terminal metabolism (Lovley and Klug, 1983). Sulfate reduction is also an important process in extreme environments, such as the anaerobic sediments of soda lakes. Among the specialized, H_2-utilizing, sulfate-reducing bacteria found in such sediments are the alkaliphilic lithoheterotroph *Desulfonatronovibrio hydrogenovorans* and the alkaliphilic lithoautotroph *Desulfonatronum lacustre* (Zhilina et al., 1997; Pikuta et al., 1998).

H_2-evolving prokaryotes will be treated more exhaustively in the next paragraph.

I.10.1. Hydrogen-producing bacteria and hydrogenases

The decomposition of organic matter via fermentation is one of the major biotic energy-yielding processes in anaerobic habitats. Various types of fermentation result in the formation of H_2 as a terminal product and hence constitute a substantial contribution to the global H_2 balance. Both obligate and facultative anaerobic bacteria produce H_2. These microorganisms are limited to anoxic zones rich in organic substance, such as marine and lacustrine sediments. These habitats are fed by a constant influx of organic material derived from photosynthetic

primary producers and from the ensuing food chains. The upper, oxic layer of the sediment varies in depth both in marine and freshwater sediments. Below the upper, oxic layer is the zone of anoxic decomposition. In this layer, the H_2 produced neither accumulates nor does it escape in significant quantities to the oxic zone. If H_2 were to accumulate, the fermentative metabolic processes would soon come to a halt, since these are inhibited by relatively low concentrations of H_2 in the environment. The inhibitory concentrations vary for the different fermentative reactions, depending on their energetics. Fermentation of fatty acids such as butyrate and propionate to acetate, H_2 and CO_2, for instance, is more endergonic than the fermentation of ethanol to acetate and H_2 and the former process ceases at a much lower concentration of external H_2 than the latter.

Hydrogen is generated also as a byproduct of N_2 fixation in both oxic and anoxic environments. Cyanobacteria and prochlorophytes are probably the most widespread diazotrophs on earth. These organisms inhabit the upper, oxic zones of oceans and lakes. The anoxygenic photosynthetic bacteria, which occupy deeper zones depleted for O_2, also engage in N_2 fixation. These and other diazotrophs contain uptake hydrogenases and, hence, are capable of exploiting at least a part of the H_2 generated by nitrogenase. However, hydrogenase-free strains are widespread in nature and produce large quantities of H_2.

Bacteria that possess the capability to produce hydrogen include strict anaerobes (*Clostridia*, and species belonging to the groups of methylotrophs, rumen bacteria, methanogenic bacteria and *Archaea*), facultative anaerobes (*Escherichia coli*, *Enterobacter*, *Citrobacter*), and even aerobes (*Alcaligenes, Bacillus*). A comprehensive review on the hydrogen-producing characteristics of these bacteria was published (Nandi and Sengupta, 1998). Among the hydrogen-producing bacteria, *Clostridium* sp. and *Enterobacter* are the most widely studied. Species of genus *Clostridium* are gram-positive, rod-shaped, strict anaerobes and endospore formers (Holt et al., 1994; Nandi and Sengupta, 1998), whereas *Enterobacter* species are gram-negative, rod-shaped, and facultative anaerobes (Holt et al., 1994). The hydrogen producing characteristics of these two genera and three thermophilic species were reviewed by de Vrije and Claassen (2003). Species of *Clostridium* and *Enterobacter* genera that have been studied for H_2 production include *Clostridium sp*. (Taguchi et al., 1992, 1993, 1994, 1995), *Clostridium parapatrificum* (Evvyernie et al., 2000, 2001), *Clostridium butyricum* (Heyndrickx et al., 1986, 1990), *Enterobacter aerogenes* (Tanisho et al., 1983, 1987, 1989; Tanisho and Ishiwata, 1994; Rachman et al., 1998) and *Enterobacter cloacae* (Kumar and Das, 2000, 2001; Kumar et al., 2000). The thermophiles

include *Thermotoga maritime* (Schr¨oder et al., 1994), *Thermotoga elfii* (de Vrije et al., 2002), and *Caldicellulosiruptor saccharolyticus* (van Niel et al., 2002).

Hydrogenases, the enzymes that catalyze the reversible oxidation of molecular H_2, are widespread in prokaryotic and lower eukaryotic biological systems (Stephenson and Stickland, 1931; Vignais et al., 2001). Hydrogenases are diverse in their protein structure and in the type of electron carrier they use (e.g. ferredoxins, rubredoxins and quinines etc.).

Early classification schemes, which had mainly biochemical data to go on, grouped hydrogenases on the basis of metal content or redox cofactors (Fauque et al., 1988; Przybyla et al., 1992). A rapidly growing base of nucleotide sequence data was used to attempt a classification on the basis of comparisons of deduced amino-acid sequences (Voordouw, 1992). In a similar study, Wu and Mandrand (1993) went a step further. These authors generated multiple alignments for full-length amino acid sequences and performed cluster analysis on the pairwise alignment scores. On the basis of the resulting dendrograms, 30 hydrogenases were grouped in six classes. Recently, Vignais and coworkers have refined and extended this classification system, including a greatly expanded database (Vignais et al., 2001; Vignais and Billoud, 2007). They carried out cluster analyses using both complete amino acid sequences and segments corresponding to functional domains.

The results of these important studies have led to the identification of three phylogenetically distinct classes of proteins, the [NiFe]-hydrogenases, the [FeFe]-hydrogenases, and the iron-sulfur-free hydrogenases, initially called metalfree and now renamed [Fe]-hydrogenases.

I.10.1.1. [NiFe]-hydrogenases

The revised system of Vignais and coworkers subdivides the [NiFe]-hydrogenases into four groups (Vignais et al., 2001):

- Group 1. Energy-transducing hydrogenases:

Enzymes which couple the oxidation of H_2 to electron-transport phosphorylation. This group includes both membrane-bound hydrogenases attached to the periplasmic side of the cytoplasmic membrane and nonmembrane-bound, periplasmic hydrogenases. The group breaks down into two subclusters. One contains the membrane-bound hydrogenases of the *Proteobacteria*, and the other, archaeal membrane-bound hydrogenases.

- Group 2. Sensory hydrogenases:

These soluble proteins are components of signal-transmitting circuits governing the expression of hydrogenase genes. Specialized hydrogenases of this type have to date been identified in *Bradyrhizobium japonicum*, and strains of *Ralstonia* and *Rhodobacter*. The group includes a second subcluster containing cyanobacterial uptake hydrogenases.

- Group 3. Multimeric cytoplasmic hydrogenases:

These are enzymes of complex subunit composition, which interact with soluble cofactors. The four subclusters contain F_{420}- reducing hydrogenases of methanogenic *Archaea*, F_{420}-nonreducing hydrogenases of *Methanothermobacter* and *Methanococcus* strains, sulfhydrogenases of thermophilic *Archaea*, and cytoplasmic NAD-reducing hydrogenases of *Ralstonia eutropha, Rhodococcus. opacus* and *Cyanobacteria*.

- Group 4. *Escherichia coli* hydrogenase 3 and relatives:

Escherichia coli hydrogenase 3 is an H_2-evolving enzyme, which is part of the multisubunit formate-hydrogen lyase complex. Similar enzymes have been identified in various *Bacteria* and *Archaea* including *Rhodospirillum rubrum, Carboxydothermus hydrogenoformans, Methanosarcina barkeri, Methanobacterium thermoautotrophicus* and *Pyrococcus furiosus.*

I.10.1.2. Iron-sulfur cluster-free hydrogenase (Hmd)

Another class of hydrogen-activating enzymes functions as H_2-forming methylenetetrahydromethanopterindehydrogenase (Hmd). Originaly discovered in *Methanothermobacter marburgensis*, Hmd enzymes have been found in other methanogenic *Archaea* including *Methanococcus voltae* and *Methanopyrus kandleri* (Thauer et al., 1996).

I.10.1.3. [FeFe]-hydrogenases

Unlike [NiFe]-H_2ases, composed of at least two subunits, many [FeFe]-H_2ases are monomeric and consist of the catalytic subunit only (although dimeric, trimeric and tetrameric enzymes are also known (Vignais et al., 2001)). The smallest [FeFe]-H_2ases (ca 45-48 kDa) have been found in green algae (Happe and Naber, 1993; Happe et al, 1994). The catalytic subunit of [FeFe]-H_2ases vary considerably in size. Besides the conserved domains of approximately 350 residues containing the active site (H-cluster, Adams, 1990), they often comprise additional domains, which accommodate Fe-S clusters. The H-cluster consists of a

binuclear [FeFe] center bound to a [4Fe-4S] cluster by a bridging cysteine and attached to the protein by four cysteine ligands. Non-protein ligands, CN^- and CO, are attached to both iron atoms (Peters et al., 1998; Peters, 1999; Nicolet et al., 1999).

[FeFe]-H_2ase is found in anaerobic prokaryotes, such as clostridia and sulfate reducers (Adams, 1990; Atta and Meyer, 2000 and Vignais et al., 2001) and in eukaryotes (Florin et al., 2001, Forestier et al., 2003). Several hydrogenases have been sequenced and characterized from *Clostridium* species, including *C. pasteurianum* (Meyer and Gagnon, 1991), *C. acetobutylicum* (Santangelo et al., 1995; Gorwa et al., 1996), *C. perfringens* (Kaji et al., 1999), and *C. paraputrificum* (Morimoto et al., 2005). [FeFe]-H_2ases are the only type of H_2ase to have been found in eukaryotes, and they are located exclusively in membrane-limited organelles, i.e. in chloroplasts or in hydrogenosomes. While [NiFe]-H_2ases is mainly involved in H_2 consumption, [FeFe]-H_2ases are usually involved in H_2 production. However, the periplasmic [FeFe]-H_2ase of *Desulfovibrio vulgaris* has been demonstrated to function as an uptake H_2ase (Pohorelic et al., 2002). Recently, components of an [FeFe]-H_2ase have been found associated with formate dehydrogenases from *Eubacterium acidaminophilum* (Graentzdoerffer et al., 2003).

The genetic information for [FeFe]-H_2ases is contained, judging by the available sequence data, in simple transcriptional units consisting of 1–3 genes. The [FeFe]-H_2ase of *D. vulgaris*, the first hydrogenase ever to be cloned and sequenced, is encoded by a pair of genes (*hydAB* genes) for the large and small subunits (Voordouw and Brenner, 1985). The structure of the *hydAB* operon of the closely related strain *Desulfomicrobium norvegicum* (formerly *Desulfovibrio desulfuricans* strain Norway) is the same (Hatchikian et al., 1999). Owing to the lack of transcript data on the one hand and to the paucity of sequence data on the other, it cannot be ruled out that additional genes belong to the same transcriptional unit or that hydrogenase accessory genes are present at other sites. The monomeric hydrogenase (CpI) of *C. pasteurianum* is encoded by a solitary gene which appears to be a fusion of the paired genes which normally code for the two hydrogenase subunits (Meyer and Gagnon, 1991). The same applies to the monomeric hydrogenase of *Megasphera elsdenii* (Atta and Meyer, 2000). The set of genes encoding the [FeFe]-hydrogenase of the hyperthermophile *T. maritima* is a singular case. Three genes, designated "*hydC*," "*-B*" and "*-A*," determine the γ, β, and α subunits of the trimeric enzyme (Verhagen et al., 1999).

References

Abram, J. W., and Nedwell, D. B. 1978. Inhibition of methanogenesis by sulphate reducing bacteria competing for transferred hydrogen. *Arch. Microbiol*. 117:89–92.

Achouak, W., Normand, P., Heulin, T. 1999. Comparative phylogeny of rrs and nifH genes in the *Bacillaceae*. *Int. J. Syst. Bacteriol*. 49:961–967.

Adams, M. W. 1990. The structure and mechanism of iron-hydrogenases. *Biochim. Biophys. Acta* 1020:115–145.

Ahlers, B., Konig, W., and Bock, E. 1990. Nitrite reductase activity in Nitrobacter vulgaris. FEMS *Microbiol. Lett.* 67:121–126.

Allgaier, M., & Grossart, H. P. 2006. Diversity and seasonal dynamics of *Actinobacteria* populations in four lakes in northeastern Germany. *Appl Environ Microb* 72: 3489–3497.

Ambramochkina, F. N., Bezrukova, L. V., Koshelev, A. V., Galchenko, V. F., Ivanov, . V.. 1987. Microbial oxidation of methane in a body of freshwater. *Microbiologiya* (English Translation) 56:375–382.

Anders, H. J., Kaetzke, A., Kaempfer, P., Ludwig, W., and Fuchs, G. 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thauera*, as *Thauera aromatica sp. nov*., and *Azoarcus*, as *Azoarcus evansii sp. nov*., respectively, members of the *beta* subclass of the *Proteobacteria. Int. J. System. Bacteriol*. 45:327–333.

Anthony, C. 1982. The Biochemistry of Methanotrophs. Academic Press. London, United Kingdom. Yoshinari, T. 1985. Nitrite and nitrous oxide production by *Methylosinus trichosporium. Can. J. Microbiol*. 31:139–144.

Anthony, C. 1982. The Biochemistry of Methylotrophs. *Academic Press Ltd*., London.

Aravalli, R. N., Q. X. She, and R. A. Garrett. 1998. *Archaea* and the new age of microorganisms. *Trends Ecol. Evol.* 13:190–194.

Atta, M., and Meyer, J. 2000. Characterization of the gene encoding the [FeFe]-hydrogenase from *Megasphaera elsdenii. Biochim. Biophys. Acta* 1476:368–371.

Bak, F., Pfennig, N. 1987. Chemolithotrophic growth of *Desulfovibrio sulfodismutans sp.nov*. by disproportionation of inorganic sulfur compounds. *Archives of Microbiology* 147:184–189.

Baumgartner, M., Sameluck, F., Bock, E., and Conrad, R. 1991. Production of nitric oxide by ammonium-oxidizing bacteria colonizing building stones. FEMS *Microbiol. Lett.* 85:95–100.

Bergersen, F. J., and Hipsley, E. H. 1970. The presence of N_2- fixing bacteria in the intestines of man and animals. *J. Gen. Microbiol.* 60:61–65.

Bergmann, D. J., Zahn, J. A., DiSpirito, A. A. 2000. Primary structure of cytochrome c' of *Methylococcus capsulatus* Bath: Evidence of a phylogenetic link between P460 and c¢-type cytochromes. *Arch. Microbiol*. 173:29–34.

Biebl, H., Drews, G. 1969. Das in vivo Spektrum als taxonomisches Merkmal bei Untersuchungen zur Verbreitung der Athiorhodaceae. *Zbl Bakt Abt* II 123:425–452.

Bleicher, K., Zellner, G., Winter, J. 1989. Growth of methanogens on cyclopentanol/CO2 and specificity of alcohol dehydrogenase. FEMS *Microbiol. Lett*. 59:307–312.

Bock, E., Wagner, M. 2006. Oxidation of Inorganic Nitrogen Compounds as an Energy Source. In: *Prokaryotes* (2006) 2:457–495

Bodrossy, L., Holmes, E. M., Holmes, A. J., Kovacs, K. L., Murrell, J. C. 1997. Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, *Methylocaldum gen. nov. Arch. Microbiol.* 168:493–503.

Boon, P. I., Virtue, P., Nichols, P. D. 1996. Microbial consortia in wetland sediments—a biomarker analysis of the effects of hydrological regime, vegetation and season on benthic microbes. *Mar. Fresh. Res*. 47:27–41.

Bowman, J. P., McCammon, S. A., Skerratt, J. H. 1997. *Methylosphaera hansonii* gen. nov., sp. nov., a psychrophilic, group I methanotroph from Antarctic marinesalinity, meromictic lakes. *Microbiology* 143:1451–1459.

Bratina, B. J., Brusseau, G. A., Hanson, R. S. 1992. Use of 16S rRNA analysis to investigate phylogeny of methylotrophic bacteria. *Int. J. Syst. Bacteriol*. 42:645–648.

Brusseau, G. A., Bulygina, E., Hanson, R. S. 1994. Phylogenetic analysis and development of probes differentiating methylotrophic bacteria. *Appl. Environ. Microbiol*. 60:626–636.

Buchanan, R. E. 1917. Studies on the nomenclature and classification of bacteria. *J. Bacteriol*. 2:347–350.

Casamayor, E. O., Muyzer, G., Pedro´s-Alio´, C. 2001. Composition and temporal dynamics of planktonic archaeal assemblages from anaerobic sulfurous environments studied by 16S rDNA denaturing gradient gel electrophoresis and sequencing. *Aquat. Microb. Ecol*. 25, 237–246.

Casamayor, E. O., Schaefer, H., Bañeras, L., Pedro´s-Alio´ s, C., Muyzer, G. 2000. Identification of spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol*. 66, 499–508.

Clark, A. E., Walsby, A. E. 1978. The development and vertical distribution of populations of gas-vacuolate bacteria in a eutrophic, monomictic lake. *Archives of Microbiology* 118:229–233.

Cottrell, M.T., Waidner, L. A, Yu, L. Y. & Kirchman, D. L. 2005. Bacterial diversity of metagenomic and PCR libraries from the Delaware River. *Environ Microbiol* 7: 1883–1895.

Coyne, M. S., Arunakumari, A., Averill, B. A., and Tiedje, J. M. 1989. Immunological identification and distribution of dissimilatory heme cd1 and nonheme copper nitrite reductases in denitrifying bacteria. *Appl. Environ. Microbiol.* 55:2924–2931.

Daniels, L., G. Fuchs, G., Thauer, R. K., Zeikus., J. G. 1977. Carbon monoxide oxidation by methanogenic bacteria. *J. Bacteriol.* 132:118–126.

de Vrije, T., and Claassen, P. A. M. 2003. Dark hydrogen fermentations. In: Bio-methane and Bio-hydrogen, Reith, J. H., Wijffels, R. H., and Barten, H., eds. Dutch biological hydrogen foundation, Smiet Offset, The Hague, the Netherlands, 103–123.

de Vrije, T., de Haas, G.G., Tan, G.B., Keijsers, E.R.P., Claassen, P.A.M. 2002. Preatment of Miscanthus for hydrogen production by *Thermotoga elfii*. *Int. J. Hydrogen Energy* 27(11/12), 1381–1390.

DeBont, J. A. M., VanDijken, J. P., Harder, W. 1981. Dimethylsulphoxide and dimethylsulphide as a carbon, sulphur and energy source for growth of *Hyphomicrobium. J. Gen. Microbiol*. 127:315–323.

Dedysh, S. N., Liesack, W., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Bares, A. M., Panikov, N. S., Tiedje, J. M. 2000. *Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int. J. Syst. Evol. Microbiol*. 50:955–969.

Dispirito, A. A., Taaffe, L. R., Lipscomb J. D., and Hooper, A. B. 1985. A blue copper oxidase from *Nitrosomonas europaea. Biochim. Biophys. Acta* 827:320–326.

Ehrich, S., Behrens, D., Lebedeva, E., Ludwig, W., Bock, E. 1995. A new obligately chemolithoautotrophic, nitrite oxidizing bacterium, *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. *Arch. Microbiol*. 164:16–23.

Eiler, A., & Bertilsson, S. 2007. *Flavobacteria* blooms in four eutrophic lakes: linking population dynamics of freshwater bacterioplankton to resource availability. *Appl Environ Microb* 73: 3511–3518.

Evvyernie, D., Morimoto, K., Karita, S., Kimura, T., Sakka, K., Ohmiya, K. 2001. Conversion of chitinous wastes to hydrogen gas by *Clostridium paraputrificum* M-21, *J. Biosci. Bioeng*. 91, 339–343.

Evvyernie, D., Yamazaki, S., Morimoto, K., Karita, S., Kimura, T., Sakka, K., Ohmiya, K. 2000. Identification and characterization of *Clostridium paraputrificum* M-21, a chitinolytic, mesophilic and hydrogen-producing bacterium, *J. Biosci. Bioeng.* 89, 596–601.

Fauque, G., H. D. Jr Peck, J. J. Moura, B. H. Huynh, Y. Berlier, D. V. DerVartanian, M. Teixeira, A. E. Przybyla, P. A. Lespinat, I. Moura, Le Gall., J. 1988. The three classes of hydrogenases from sulfate-reducing bacteria of the genus *Desulfovibrio*. FEMS *Microbiol. Rev.* 4:299–344.

Fenchel, T. M., Jørgensen, B. B. 1977. Detritus food chains in aquatic ecosystems: The role of bacteria. Advances in *Microbiol Ecology* 1:1–58.

Fesefeldt, A., Kloos, K., Bothe, H., Lemmer, H., and Gliesche, C. G. 1998. Distribution of denitrification and nitrogen fixation genes in *Hyphomicrobium spp*. and other budding bacteria. *Can. J. Microbiol*. 44:181– 186.

Florin, L., A. Tsokoglou, Happe, T. 2001. A novel type of iron hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetic electron transport chain. *J. Biol. Chem*. 276:6125–6132.

Focht, D. D., and Verstraete, W. 1977. Biochemical ecology of nitrification and denitrification. *Adv. Microbial Ecol.* 1:135–214.

Forestier, M., King, P., Zhang, L., Posewitz, M., Schwarzer, S., Happe, T., Ghirardi, M.L., Seibert, M. 2003. Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. *Eur. J. Biochem.* 270, 2750-2758.

Fossing, H., Gallardo, V. A., Jorgensen, B. B., Huttel, M., Nielsen, L. P., Schulz, H., Canfield, D. E., Forster, S., Glud, R. N., Gunderson, J. K., Kuver, J., Ramsing, N. B., Teske, A., Thamdrup, B., and Ulloa, O. 1995. Concentration and transport of nitrate by the mat-forming sulphur bacterium *Thioploca. Nature* 374:713–715.

Friedrich, C., and Mitrenga, G. 1981. Oxidation of thiosulfate by *Paracoccus denitrificans* and other hydrogen bacteria. FEMS *Microbiol. Ecol.* 10:209–212.

Fries, M., Zhou, J., Chee, S. J., and Tiedje, J. M. 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60:2802–2810.

Fuentes-Ramírez, L. E., Bustillos-Cristales, R., Tapia-Hernandez, A., Jimenez-Salgado, T., Wang, E. T., Martinez-Romero, E., Caballero-Mellado, J. 2001. Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae sp.* nov. and *Gluconacetobacter azotocaptans sp.* nov., associated with coffee plants. *Int. J. Syst. Evol. Microbiol*. 51:1305–1314.

Galchenko, V. F. 1994. Sulfate reduction, methane production and methane oxidation in various water bodies of the Bunger Hills Osasis of Antarctica. *Microbiologiya* (English Translation) 63:388–396.

Galchenko, V. F., Ambramochkina, F. N., Bezrukova, L. V, Sokolova, E. N., Ivanov, M. V. 1988. Species composition of aerobic methanotrophic microflora in the Black Sea. *Microbiologiya* (English Translation) 57:248–253.

Goreau, T. J., Kaplan, W. A., Wofsy, S. C., Mcelroy, M. B., Valois, F. W., and Watson, S. W. 1980. Production of nitrite and nitrous oxide by nitrifying bacteria at reduced concentrations of oxygen. *Appl. Environ. Microbiol*. 40:526–532.

Gorlenko, W. M., Dubinina, G. A., Kuznezow, S. J. 1977. Ecology of aquatic microorganisms. [In Russian.] Moscow, Nauka.

Gorwa, M.F., Croux, C., Soucaille, P., 1996. Molecular characterization and transcriptional analysis of the putative hydrogenase gene of *Clostridium acetobutylicum* ATCC 824. *J. Bacteriol*. 178, 2668–2675.

Graentzdoerffer, A., Rauh, D., Pich, A., and Andreesen, J.R. 2003. Molecular and biochemical characterization of two tungsten- and selenium-containing formate dehydrogenases from *Eubacterium acidaminophilum* that are associated with components of an iron-only hydrogenase. *Arch. Microbiol.* 179, 116-130.

Green, P. N., and Bousfield, I. J. 1981. The taxonomy of pinkpigmented facultatively methylotrophic bacteria. In: H. Dalton (Ed.) Microbial Growth on C1-compounds. Heyden and Son. London, 285–293.

Green, P. N., and Bousfield, I. J. 1983. Emendation of Methylobacterium Patt, Cole, and Hanson 1976; Methylobacterium rhodinum (Heumann 1962) comb. nov. corrig.; *Methylobacterium radiotolerans* (Ito and Iizuka 1971) comb. nov. corrig.; and *Methylobacterium mesophilicum* (Austin and Goodfellow 1979) comb. nov. *Int. J. Syst. Bacteriol.* 33:875–877.

Halm, H., Musat, N., Lam, P., Langlois, R., Musat, F., Peduzzi, ., Lavik, G., Schubert, C. J., Sinha, B., LaRoche, J., Kuypers, M. M. 2009. Co-occurrence of denitrification and nitrogen fixation in a meromictic lake, Lake Cadagno (Switzerland). *Environ Microbiol*. 11(8):1945-58.

Hanson, R. S. 1980. Ecology and diversity of methylotrophic organisms. *Adv. Appl. Microbiol*. 6:3–39.

Hanson, R. S., and Hanson, T. E. 1996. Methanotrophic bacteria. *Microbiol. Rev*. 60:439–471.

Happe, T., and Naber, J. D. 1993. Isolation, characterization and N-terminal amino acid sequence of hydrogenase from the green alga *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 214:475–481.

Happe, T., B. Mosler, Naber, J. D. 1994. Induction, localization and metal content of hydrogenase in the green alga *Chlamydomonas reinhardtii. Eur. J. Biochem.* 222:769–774.

Hatchikian, E. C., V. Magro, N. Forget, Y. Nicolet, Fontecilla-Camps, J. C. 1999. Carboxy-terminal processing of the large subunit of [FeFe] hydrogenase from *Desulfovibrio desulfuricans* ATCC 7757. *J. Bacteriol.* 181:2947–2952.

Head, I. M., Hiorns, W. D., Embley, T. M., McCarthy, A. J., Saunders, J. R. 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. *J. Gen. Microbiol*. 139:1147–1153.

Hedderich, R., Whitman, W.B. 2006. Physiology and Biochemistry of the Methane-Producing *Archaea*. In: *Prokaryotes* 2:1050–1079

Heyndrickx, M., Vansteenbeeck, A., Devos, P., Deley, J. 1986. Hydrogen gas production from continuous fermentation of glucose in a minimal medium with *Clostridium butyricum* LMG 1213tl, *Syst. Appl. Microbiol*. 8(3), 239–244.

Heyndrickx. M., Devos, P., Deley, J. 1990. H_2 production from chemostat fermentation of glucose by *Clostridium butyricum* and *Clostridium pasterianum* in ammoniumlimitation and phosphate limitation, *Biotechnol. Lett*. 12(10), 731–736.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., Williams, S. T. 1994. Bergey's Manual of Determinative Bacteriology, 9th ed. Williams & Willkins, Baltimore, MD.

Huber, R., Wilharm, T., Huber, D., Trincone, A., Burggraf, S., Koenig, H., Rachel, R., Rockinger, I., Fricke, H., and Stetter, K. O. 1992. *Aquifex pyrophilus*, new genus new species, represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. *Sys. Appl. Microbiol.* 15:340–351.

Hugenholtz, P., Pitulle, C., Hershberger, K. L., Pace, N. R. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. *J Bacteriol* 180:366–376.

Imhoff, J. F., Trüper, H. G. 1989. Purple nonsulfur bacteria. In: Staley JT, Bryant MP, Pfennig N, Holt JG (Eds.) Bergey's Manual of Systematic Bacteriology. Williamsand Wilkins. Baltimore, 1658–1682.

Jones, A. M., Adkins, A. M., Knowles, R., and Rayat, G. R. 1990. Idenitification of a denitrifying gliding bacterium isolated from soil and able to reduce nitrous oxide in the presence of sulfide and acetylene as *Flexibacter canadensis*. *Can. J. Microbiol*. 36:765–770.

Kaji, M., Taniguchi, Y., Matsushita, O., Katayama, S., Miyata, S., Morita, S., Okabe, A., 1999. The hydA gene encoding the H_2- evolving hydrogenase of *Clostridium perfringens*: molecular characterization and expression of the gene. FEMS *Microbiol. Lett.* 181, 329–336.

Karl, D., Michaels, A., Bergman, B., Capone, D., Carpenter, E., Letelier, R., Lipschultz, F., Paerl, H., Sigman, D., Stal, L. 2002. Dinitrogen fixation in the world's oceans. *Biogeochemistry* 57:47–98.

Kelly, D. P., and Murrell, J. C. 1999. Microbial metabolism of methanesulfonic acid. *Arch. Microbiol*. 172:341–348.

Killham, K. 1986. Heterotrophic nitrification. In: J. I. Prosser (Ed.) Nitrification. IRL Press. Oxford, UK. 117–126.

King, G. M. 1992. Ecological aspects of methane oxidation, a key determinant of global methane dynamics. *Adv. Microb. Ecol.* 12:431–474.

Kristjansson, J. K., Schönheit, P., Thauer, R. K. 1982. Different Ks-values for hydrogen of methanogenic bacteria and sulfate reducing bacteria—an explanation for the apparent inhibition of methanogenesis by sulfate. *Arch. Microbiol*. 131:278–282.

Kristjansson, J. K., Schönheit, P., Thauer, R. K.. 1982. Different K, values for hydrogen of methanogenic bacteria and sulfate reducing bacteria. *Arch. Microbiol*. 131:278–282.

Kuenen, J. G., and Robertson, L. A. 1987. Ecology of nitrification and denitrificationM. In: J. A. Cole and S. Ferguson (Eds.) The Nitrogen and sulfur cycles. Cambridge University Press. Cambridge, UK. 162–218.

Kumar, N., and Das, D. 2000. Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08, *Process Biochem.* 35(9), 589–593.

Kumar, N., and Das, D. 2001. Continuous hydrogen production by immobilized *Enterobacter cloacae* IIT-BT 08 using lignocellulosic materials as solid matrixes, *Enzyme Microb. Technol*. 29(4/5), 280–287.

Kumar, N., Monga, P.S., Biswas, A.K., Das, D. 2000. Modeling and simulation of clean fuel production by *Enterobacter cloacae* IIT-BT 08, *Int. J. Hydrogen Energy* 25(10), 945–952.

Kuznezow, S. I. 1977. Trends in the development of ecological microbiology. 1–48. Droop, M. R., and Jannasch, H. W. (ed.) *Advances in aquatic microbiology*. London, Academic Press

Leisinger, T., and Braus-Stromeyer, S. A. 1995. Bacterial growth with chlorinated methanes. *Environ. Health Perspect.* 103:33–36.

Lensi, R., Beaupied, H., and Moiroud, A. 1990. Denitrifying activity in the actinorhizae. *Acta Oecol*. 11:391–398.

Lidstrom, M. E. 2006. Aerobic Methylotrophic Prokaryotes. In: *Prokaryotes* 2:618–634

Lindstrom, E. S. 2000 Bacterioplankton community composition in five lakes differing in trophic status and humic content. *Microb Ecol* 40:104–13.

Lovley, D. R. 1985. Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Appl. Environ. Microbiol*. 49:1530–1531.

Lovley, D. R., and Klug, M. J. 1983. Sulfate reducers can outcompete methanogens at fresh-water sulfate concentrations. *Appl. Environ. Microbiol.* 45:187–192.

Lovley, D. R., Dwyer, D. F.,. Klug, M. J. 1982. Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. *Appl. Environ. Microbiol*. 43:1373–1379.

Madigan, M. T., Ormerod, J. G. 1995. Taxonomy, physiology and ecology of Heliobacteria. In: Blankenship RE, Madigan MT, Bauer CE (Eds.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publisher. Dordrecht, The Netherlands. 17–30.

Madigan, M., Cox, S. S., Stegeman, R. A. 1984. Nitrogen fixation and nitrogenase activities in members of the family *Rhodospirillaceae. J. Bacteriol.* 157:73–78.

Malashenko, Y. R., Kaier, Y., Budkova, E. N., Isagulova, Y., Berger, U., Krishtab, T. P., Chernyshenko, D. V., Romanovskaya., V. A. 1987. Methane-oxidizing microflora in bodies of fresh and salt water. *Microbiologiya* (English Translation) 56:115–120.

McHatton, S. C., Barry, J. P., Jannasch, H. W., and Nelson, D. C. 1996. High nitrate concentrations in vacuolate, autotrophic marine *Beggiatoa* spp. *Appl. Env. Microbiol*. 62:954–958.

Mehta, M. P., Butterfield, D. A., Baross, J. A. 2003. Phylogenetic diversity of nitrogenase (nifH) genes in deep sea and hydrothermal vent environments of the Juan de Fuca Ridge. *Appl. Environ. Microbiol*. 69:960–970.

Meyer, J., Gagnon, J., 1991. Primary structure of hydrogenase I from Clostridium pasteurianum. *Biochemistry* 30, 9697–9704.

Miller, D. J., and Nicholas, D. J. D. 1985. Characterization of a soluble cytochrome oxidase-nitrite reductase from Nitrosomonas europaea. *J. Gen. Microbiol*. 131:2851– 2854.

Miskin, I., Rhodes, G., Lawlor, K., Saunders, J. R., and Pickup, R. W. 1998. Bacteria in post-glacial freshwater sediments. *Microbiology* 144 (Pt 9): 2427-2439.

Morimoto, K., Kimura, T., Sakka, K., Ohmiya, K., 2005. Overexpression of a hydrogenase gene in *Clostridium paraputrificum* to enhance hydrogen gas production. FEMS *Microbiol. Lett*. 246, 229–234.

Muyzer, G., Teske, A., Wirsen, C. O., and Jannasch, H. W. 1995. Phylogenetic relationship of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol*. 164:165– 172.

Nandi, R., and Sengupta, S. 1998. Microbial production of hydrogen—An overview, *Crit. Rev. Microbiol*. 24(1), 61–84

Newton, R.J., Kent, A. D., Triplett, E. W. & McMahon, K. D., 2006. Microbial community dynamics in a humic lake: differential persistence of common freshwater phylotypes. *Environ Microbiol* 8: 956–970.

Nicolet, Y., C. Piras, P. Legrand, C. E. Hatchikian, Fontecilla-Camps, J. C. 1999. *Desulfovibrio desulfuricans* iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center. Struct. Fold. Des. 7:13–23.

Normand, P., Orso, S., Cournoyer, B., Jeannin, P., Chapelon, C., Dawson, J., Evtushenko, L., Misra, A. K. 1996. Molecular phylogeny of the genus Frankia and related genera and emendation of the family Frankiaceae. *Int. J. Syst. Bacteriol.* 46:1–9.

O'Hara, G. W., and Daniel, R. M. 1985. Bacterial denitrification: a review. *Soil. Biol. Biochem*. 17:1–9.

O'Neil, J. G., and Wilkinson, J. F. 1977. Oxidation of ammonia by methane-oxidizing bacteria and the effect of ammonia on methane oxidation. *J. Gen. Microbiol*. 100:407– 412.

Oremland, R. S., and Culbertson, C. W. 1992. Importance of methane-oxidizing bacteria in the methane budget as revealed by the use of a specific inhibitor. *Nature* 356:421–423.

Oremland, R. S., and Polcin, S. 1982. Methanogenesis and sulfate reduction: competetitive and non competitive substrates in an estuarine environment. *Appl. Environ. Microbiol*. 44:1270–1276.

Orso, S., Gouy, M., Navarro, E., Normand, P. 1994. Molecular phylogenetic analysis of *Nitrobacter spp. Int. J. Syst. Bacteriol.* 44:83–86.

Overbeck, J. 1972. Zur Struktur und Funktion des aquatischen Ökosystems. Berichte der Deutschen Botanischen Gesellschaft 85:553–579.

Overmann, J., Garcia-Pichel, F. 2006. The Phototrophic Way of Life. In: *Prokaryotes* 2:32–85

Papen, H., von Berg, R., Hinkel, I., Thoene, B., Rennenberg, H. 1989. Heterotrophic nitrification by Alcaligenes faecalis: NO^{2-}, NO^{3-}, N_2O, and NO production in exponentially growing cultures. *Appl. Environ. Microbiol*. 55:2068–2072.

Payne, W. J., Grant, M. A., Shapleigh, J. P., and Hoffman, P. 1982. Nitrogen oxide reduction in *Wolinella succinogenes* and *Campylobacter* species. *J. Bacteriol.* 152:915– 918.

Percent, S. F., Frischer, M. .E., Vescio, P. A., Duffy, E. B., Milano, V., McLellan, M., Stevens, B. M., Boylen, C. W., Nierzwicki-Bauer, S. A. 2008. Bacterial community structure of acid-impacted lakes: what controls diversity? *Appl Environ Microbiol* 74: 1856-1868.

Peters, J. W. 1999. Structure and mechanism of iron-only hydrogenases. *Curr. Opin. Struct. Biol*. 9:670–676.

Peters, J. W., W. N. Lanzilotta, B. J. Lemon, Seefeldt, L. C. 1998. X-ray crystal structure of the Fe-only hydrogenase (Cpl) from *Clostridium pasteurianum* to 1.8 angstrom resolution. *Science* 282:1853–1858.

Pfennig, N. 1979. General physiology and ecology of photosynthetic bacteria. 3–18. Sistrom, W. R., and Clayton, R. (ed.) Photosynthetic bacteria. New York. Plenum.

Pikuta, E. V., Zhilin, T. N., Zavarzin, G. A., Kostrikina, N. A., Osipov, G. A., Rainey, F. A. 1998. *Desulfonatronum lacustre* gen. nov., sp. nov.: a new alkaliphilic sulfatereducing bacterium utilizing ethanol [in Russian]. *Mikrobiologiya* 67:123–131.

Pohorelic, B. K. J., J. K. Voordouw, E. Lojou, A. Dolla, J. Harder, and G. Voordouw. 2002. Effects of deletion of genes encoding Fe-only hydrogenase of *Desulfovibrio vulgaris* Hildenborough on hydrogen and lactate metabolism. *J. Bacteriol.* 184:679–686.

Pommerening-Röser, A., Rath, G., Koops, H.-P. 1996. Phylogenetic diversity within the genus *Nitrosomonas. Syst. Appl. Microbiol.* 19:344–351.

Przybyla, A. E., J. Robbins, N. Menon,. Peck, H. D. 1992. Structure-function relationships among the nickel-containing hydrogenases. FEMS *Microbiol. Rev*. 8:109–135.

Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M. C., Koops, H. P., Wagner, M. 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. *Appl. Environ. Microbiol*. 66: 5368–5382.

Rabus, R. Hansen, T.A., Widdel, F. 2006. Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes. In: *Prokaryotes* 2:659–768

Rachman, M.A., Nakashinmada, Y., Kakizono, T., Nishio, N. 1998. Hydrogen production with high yield and high evolution rate by self-flocculated cells of Enterobacter aerogenes in a packed-bed reactor, *Appl. Microbiol. Biotechnol*. 49(4), 450–454.

Reed, W. M., and Dugan, P. R. 1978. Distribution of *Methylomonas methanica* and *Methylosinus trichosporium* in Cleveland Harbor as determined by an indirect fluorescent antibody-membrane filter technique. *Appl. environ. Microbiol.* 35:422–430.

Robertson, L. A., and Kuenen, J. G. 1988. Heterotrophic nitrification in *Thiosphaera pantotropha*—oxygen uptake and enzyme studies. *J. Gen. Microbiol.* 134:857– 863.

Robertson, L. A., Kuenen, J. G. 2006 The Colorless Sulfur Bacteria. In: *Prokaryotes* 2:985–1011

Rocourt, J., Wehmeyer, U., and Stackebrandt, E. 1987. Transfer of *Listeria denitrificans* to a new genus, *Jonesia* gen. nov., as *Jonesia denitrificans* comb. nov. *Int. J. Syst. Bacteriol*. 37:266–270.

Ross, J. L., Boon, P. I., Ford, P., Hart, B. T. 1997. Detection and quantification with 16S rRNA probes of planktonic methylotrophic bacteria in a floodplain lake. *Microb. Ecol.* 34:97–108.

Santangelo, J.D., Durre, P.,Woods, D.R., 1995. Characterization and expression of the hydrogenase-encoding gene from *Clostridium acetobutylicum* P262. *Microbiology* 141, 171–180.

Saralov, A. I., Krylova, I. N., Saralova, E. E., Kuznetsov, S. I. 1984. Distribution and species composition of methane-oxidizing bacteria in lake waters. *Microbiologiya* (English Translation) 53:695–700.

Schauer, M., Kamenik, C., & Hahn, M. W. 2005. Ecological differentiation within a cosmopolitan group of planktonic freshwater bacteria (SOL cluster, Saprospiraceae, Bacteroidetes). *Appl Environ Microb* 71: 5900–5907.

Schedel, M., and Truper, H. 1980. Anaerobic oxidation of thiosulfate and elemental sulfur in *Thiobacillus denitrificans. Arch. Microbiol.* 124:205–210.

Schlegel, H. G., Jannasch H.W. 2006. Prokaryotes and their habitats. In: *Prokaryotes* 1:137–184

Schönheit, P., Kristjansson, J. K., Thauer, R. K. 1982. Kinetic mechanism for the ability of sulfate reducers to out-compete methanogens for acetate. *Arch. Microbiol.* 132:285–288.

Schröder, C., Selig, M., Sch¨onheit, P. 1994 Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritime*: Involvement of the Embden-Meyerhof pathway, *Arch. Microbiol.* 161(6), 460–470.

Schwartz, E., Friedrich, B. 2006. The H_2-Metabolizing Prokaryotes In: *Prokaryotes* 2:496–563

Segerer, A., Stetter, K. O. 1989. The genus *Acidianus*, 2251–2253. Staley, J. (ed.) Bergey's manual of systematic bacteriology, vol. 3. Williams and Wilkins. Baltimore.

Shapleigh, J.P. 2006. The Denitrifying Prokaryotes. In: *Prokaryotes* 2:769–792

Shoun, H., Kano, M., Baba, I., Takaya, N., and Matsuo, M. 1998. Denitrification of actinomycetes and purification of dissimilatory nitrite reductase and azurin from *Streptomyces thioluteus. J. Bacteriol.* 180:4413–4415.

Sorokin, D. Y., Tourova, T., Schmid, M., Wagner, M., Koop, H. P., Kuenen, J. G., Jetten, M. 2001. Isolation and properties of obligately chemolithoautotrophic and extremely alkalitolerant ammonia oxidizing bacteria from Mongolian soda lakes. *Arch. Microbiol.* 176:170– 177.

Sorokin, Y. I. 1970. Interrelations between sulphur and carbon turnover in meromictic lakes. Archiv für Hydrobiologie 66:391–446.

Spring, S., Schulze, R., Overmann, J., Schleifer, K. 2000. Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: molecular and cultivation studies. FEMS *Microbiology Reviews*, 24: 573-590

Springer, N., Ludwig, W., Philipp, B., and Schink, B. 1998. *Azoarcus anaerobius* sp. nov., a resorcinol-degrading, strictly anaerobic, denitrifying bacterium. *Int. J. Sys. Bacteriol.* 48:953–956.

Staal, M., Meysman, F. J. R., Stal, J. J. 2003. Temperature excludes N_2-fixing heterocystous cyanobacteria in the tropical oceans. *Nature* 425:504–507.

Steenbergen, C. L. M., Korthals, H. J. 1982. Distribution of phototrophic microorganisms in the anaerobic and microaerophilic strata of Lake Vechten (The Netherlands). *Limnol Oceanogr* 27:883–895.

Stephenson, M., and Stickland, L. H. 1931. Hydrogenase: a bacterial enzyme activating molecular hydrogen. I: The properties of the enzyme. *Biochem. J.* 25:205–214.

Steward, G. F., Jenkins, B. D., Ward, B. B., and. Zehr, J. P. 2004. Development and testing of a DNA macroarray to assess nitrogenase (nifH) gene diversity. *Appl. Environ. Microbiol.* 70:1455–1465.

Sugio, T., Domatsu, C., Munaka, O., Tano, T., Imai, K. 1985. Role of a ferric iron reducing system in sulfur oxidation of *Thiobacillus ferrooxidans. Applied and Environmental Microbiology* 49:1401–1406.

Swartzlander, J. H., Rehberger, T. G., and Hibberd, C. A. 1993. In vivo denitrification by a selected strain of *Propionibacterium acidipropionici* in beef cattle. *J. Anim. Sci*. 71:277–277.

Swoager, W. C., Lindstrom, E. S. 1971. Isolation and counting of *Athiorhodaceae* with membrane filters. *Appl Microbiol* 22:683–687.

Taguchi, F., Chang, J. D., Mizukami, N., Saito-Taki, T., Hasegawa, K., and Morimoto, M. 1993. Isolation of a hydrogen-producing bacterium, *Clostridium beijerinckii* strain AM21B, *Can. J. Microbiol.* 39, 726–730

Taguchi, F., Chang, J. D., Takiguchi, S., Morimoto, M. 1992. Efficient hydrogen production from starch by a bacterium isolated from termites, *J. Ferment. Bioeng.* 73, 244–245.

Taguchi, F., Mizukami, N., Hasegawa, K., and Saito-Taki, T. 1994. Microbial conversion of arabinose and xylose to hydrogen by a newly isolated *Clostridium* sp. no. 2. *Can. J. Microbiol.* 40, 228–233.

Taguchi, F., Mizukami, N., Saito-Taki, T., and Hasegawa, K. 1995. Hydrogen production from continuous fermentation of xylose during growth of *Clostridium* sp. strain no. 2, *Can. J. Microbiol.* 41, 536–540.

Tanisho, S., and Ishiwata, Y. 1994. Continuous hydrogen production from molasses by the bacterium *Enterobacter aerogenes. Int. J. Hydrogen Energy* 19(10), 807–812.

Tanisho, S., Kamiya, N., and Wakao, N. 1989. Hydrogen evolution of Enterobacter aerogenes depending on culture pH: Mechanism of hydrogen evolution from NADH by means of membrane-bound hydrogenase, *Biochim. Biophys. Acta* 973, 1–6.

Tanisho, S., Suzuki, Y., Wakao, N. 1987. Fermentative hydrogen evolution by *Enterobacter aerogenes* strain E. 82005. *Int. J. Hydrogen Energy* 12(9), 623–627.

Tanisho, S., Wakao, S., Kosako, Y. 1983. Biological hydrogen production by *Enterobacter aerogenes. J. Chem. Eng. Jpn*. 15, 529–530.

Teske, A., Alm, E., Regan, J. M., Toze, S., Rittmann, B. E., Stahl, D. A. 1994. Evolutionary relationship among ammonia- and nitrite oxidizing bacteria. *J. Bacteriol.* 176: 6623–6630.

Teske, A., Wawer, C., Muyzer, G., Ramsing, N. B. 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most probable- number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl. Environ. Microbiol.* 62: 1405–1415.

Thauer, R. K., A. R. Klein, Hartmann, G. C. 1996. Reactions with molecular hydrogen in microorganisms: evidence for a purely organic hydrogenation catalyst. *Chem. Rev*. 96:3031–3042.

Timmer-ten Hoor, A. 1975. A new type of thiosulphate oxidizing, nitrate reducing microorganism: *Thiomicrospira denitrificans* sp.nov. *Neth. J. Sea Res.* 9:344– 350.

Urakawa, H., Yoshida, T., Nishimura, M. and Ohwada, K. 2000. Characterization of depth-related population variation in microbial communities of a coastal marine sediment using 16S rDNA-based approaches and quinone profiling. *Environ. Microbiol.* 2, 542- 554.

Utåker, J. B., Bakken, L., Jiang, Q. Q., Nes, I. F. 1995. Phylogenetic analysis of seven new isolates of ammonia oxidizing bacteria based on 16S rRNA gene sequences. *Syst. Appl. Microbiol*. 18:549–559.

Van der Gucht, K., Vandekerckhove, T., Vloemans, N., Cousin, S., Muylaert, K., Sabbe, K., Gillis, M., Declerk, S., De Meester, L., Vyverman, W. 2005. Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. FEMS *Microbiol Ecol* 53: 205–220

van Gemerden, H., Mas, J. 1995. Ecology of phototrophic sulfur bacteria. In: Blankenship RE, Madigan MT, Bauer CE (Eds.) Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers. Dordrecht, The Netherlands. 49–85.

van Niel, E.W.J., Budde, M.A.W., de Haas, G.G., van der Wal, F.J., Claassen, P.A.M., Stams, A.J.M. 2002. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and Thermotoga *elfii*. *Int. J. Hydrogen Energy*. 27(11/12), 1391–1398.

van Schie, P. M., and Young, L. Y. 1998. Isolation and characterization of phenol-degrading denitrifying bacteria. *Appl. Env. Microbiol*. 64:2432–2438.

Verhagen, M. F., T. O'Rourke,. Adams, M. W. 1999. The hyperthermophilic bacterium, *Thermotoga maritima*, contains an unusually complex iron-hydrogenase: amino acid sequence analyses versus biochemical characterization. *Biochim. Biophys. Acta* 1412:212–229.

Vignais P.M., Billoud B. 2007. Occurrence, Classification, and Biological function of Hydrogenases: An Overview. Chem. Rev. 107: 4206-4272

Vignais, P. M., B. Billoud, Meyer, J. 2001. Classification and phylogeny of hydrogenases. FEMS Microbiol. Rev. 25:455–501.

Volkl, P., Huber, R., Drobner, E., Rachel, R., Burggraf, S., Trincone, A., and Stetter, K. O. 1993. *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. *Appl. Environ. Microbiol*. 59:2918–2926.

Voordouw, G. 1992. Evolution of hydrogenase genes. *Adv. Inorg. Chem.* 38:397–423.

Voordouw, G., and S. Brenner. 1985. Nucleotide sequence of the gene encoding the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough). *Eur. J. Biochem.* 148:515–520.

Watson, S. W. 1971. Taxonomic considerations of the family *Nitrobacteraceae Buchanan*: Requests for opinions. *Int. J. Syst. Bacteriol.* 21:254–270.

Watson, S. W., Bock, E., Harms, H., Koops, H. P., Hooper, A. B. 1989. Nitrifying bacteria. In: R. G. E. Murray, D. J. Brenner, M. P Bryant, J. G. Holt, N. R. Krieg, J. W. Moulder, N. Pfennig, P. H. A. Sneath, J. T. Staley, and S. Williams (Eds.) Bergey's Manual of Systematic Bacteriology. 3. Williams and Wilkins. Baltimore, MD. 1808–1834.

Wei, C., Bao, S., Zhu, X., Huang, X. 2008. Spatio-temporal variations of the bacterioplankton community composition in Chaohu Lake, China. *Progress in Natural Science* 18, 1115–1122

Wetzel, R. G. 2001. Bacterioplankton. In: Limnology: lake and river ecosystems. Wetzel R.G. (ed). New York: *Academic*, pp. 489–525.

Winfrey, M. R., and Ward, D. M. 1983. Substrates for sulfate reduction and methane production in intertidal sediments. *Appl Environ. Microbiol*. 45:193–199.

Winfrey, M. R., and Zeikus, J. G. 1977. Effect of sulfate on carbon and electron flow during microbial methanogenesis in freshwater sediments. *Appl. Environ. Microbiol.* 33:275–281.

Winfrey, M. R., Nelson, D. R, Klevickis, S. C., Zeikus, J. G. 1977. Association of hydrogen metabolism with methanogenesis in Lake Mendota sediments. *Appl. Environ. Microbiol.* 33:312–318.

Woese, C. R., Weisburg, W. G., Hahn, C. M., Paster, B. J., Zablen, L. B., Lewis, B. J., Macke, T. J., Ludwig, W., Stackebrandt, E. 1985. The phylogeny of purple bacteria: The *gamma* subdivision. *Syst. Appl. Microbiol.* 6:25–33.

Woese, C. R., Weisburg, W. G., Paster, B. J., Hahn, C. M., Tanner, R. S., Krieg, N. R., Koops, H.-P., Harms, H., Stackebrandt, E. 1984. The phylogeny of purple bacteria: The *beta* subdivision. *Syst. Appl. Microbiol*. 5:327–336.

Wu, L. F., and Mandrand, M. A. 1993. Microbial hydrogenases: primary structure, classification, signatures and phylogeny. FEMS *Microbiol. Rev*. 10:243–269.

Yannarell, A. C., Triplett, E. W. 2005. Geographic and environmental sources of variation in lake bacterial community composition. *Appl Environ Microbiol* 71:227–39.

Yoshinari, T. 1980. N_2O reduction by *Vibrio succinogenes*. *Appl. Env. Microbiol*. 39:81–84.

Young, J. P. W. 1992. Phylogenetic classification of nitrogenfixing organisms. In: G. Stacey, R. H. Burris, and H. J. Evans (Eds.) Biological Nitrogen Fixation. Chapman and Hall. New York, NY. 43–86.

Zahn, J. A., Duncan, C., DiSpirito, A. A. 1994. Oxidation of hydroxylamine by cytochrome P-460 of the obligate methylotroph Methylococcus capsulatus. *Bath. J. Bacteriol*. 176:5879–5887.

Zehr, J. P., Mellon, M. T., Zani, S. 1998. New nitrogenfixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (nifH) genes. *Appl. Environ. Microbiol*. 64:3444–3450.

Zellner, G., Alten, C., Stackebrandt, E., Conway de Macario, E., Winter, J.. 1987. Isolation and characterization of Methanocorpusculum parvum, gen. nov., spec. nov., a new tungsten requiring, coccoid methanogen. *Arch. Microbiol.* 147:13–20.

Zhilina, T. N., Zavarzin, G. A., Rainey, F. A., Pikuta, E. N., Osipov, G. A., Kostrikina, N. A. 1997. *Desulfonatronovibrio hydrogenovorans* gen. nov., sp. nov., an alkaliphilic, sulfate-reducing bacterium. *Int. J. Syst. Bacteriol.* 47:144–149.

Zwart, G., Crump, B. C., Kamst-van Agterveld, M. P., Ferry, H., Suk-Kyun, H. 2002. Typical freshwater bacteria: an analysis of available 16S rRNA sequences from plankton of lakes and rivers. *Aquat. Microb. Ecol*. 28, 141–155.

Chapter II: Molecular approaches to the assessment of biodiversity in lake microbial communities

I.I.1. General introduction

Traditionally, the occurrence of microorganisms in a given environment has been studied by a pure cultures–based approach. However, several studies performed on different environments estimated that more than 99% of organisms observed microscopically are not cultivated on traditional culture media, demonstrating that microbial diversity is much greater than that previously anticipated, and that culture techniques are insufficient for exploring the enormous "reservoir" of hidden diversity in natural habitats (Amann, 1995; Muyzer, 1999). In the last years, the increased development and routine application of molecular-based techniques has made possible a more accurate evaluation of the biodiversity of microbial communities. Since its introduction in the mid-1980s, PCR has become a fundamental aspect of molecular ecology, and several PCR-based techniques have been developed to study microbial communities. These methods involve an initial PCR amplification step achieved by means of primers that are specific for the organisms of interest. The second step involves the detection of sequence variations in the PCR fragments either by a cloning/sequencing analysis, which provides a complete characterization of the fragments, or by an electrophoretic analysis, which provides a visual separation of the mixture of fragments according to sequence polymorphism (denaturing or temperature gradient gel electrophoresis, single strand conformation polymorphism) or length polymorphism (terminal-restriction fragment length polymorphism, automated ribosomal intergenic spacer analysis) (Fig. 1).

The PCR-based techniques most widely used for assessing the genetic diversity of microbial communities will be discussed in this chapter to illustrate the principles, advantages and shortcomings of each of them.

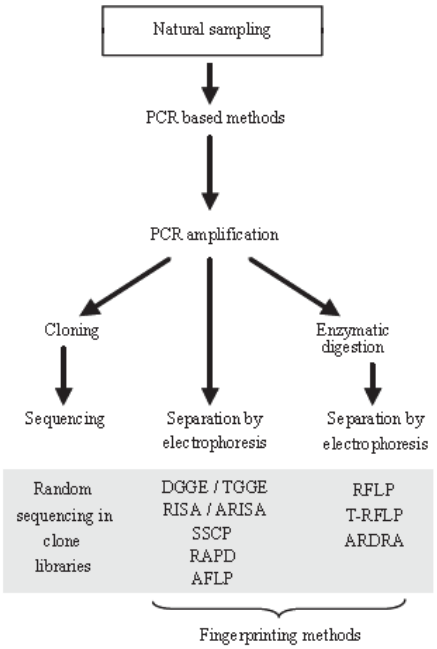

Figure 1. Diagram of the different molecular approaches for assessing the genetic diversity of natural microbial communities (Dorigo et al., 2005)

I.I.2. Bacterial community diversity based on 16S rDNA

At first, phylogenetic identification has been performed by comparing the primary structures of macromolecules such as cytochromes and ferredoxins (Zuckerkandl & Pauling, 1965; Ambler et al., 1979). Subsequently, the bacterial rRNA operon became the most frequently used molecular marker to assess the diversity of microbial communities.

Ribosomal RNA, that together with proteins forms the ribosomes, plays a structural function and also a role in ribosomal binding of mRNA and tRNAs. Three types of rRNA are classified by their sedimentation rates during ultracentrifugation as 23S, 16S and 5S. They have lengths of approximately 3300, 1650 and 120 nucleotides, respectively. The rRNAs genes are typically organized as a cotranscribed *rrn* operon (Fig. 2). One or more copies of this operon may be present in the genome.

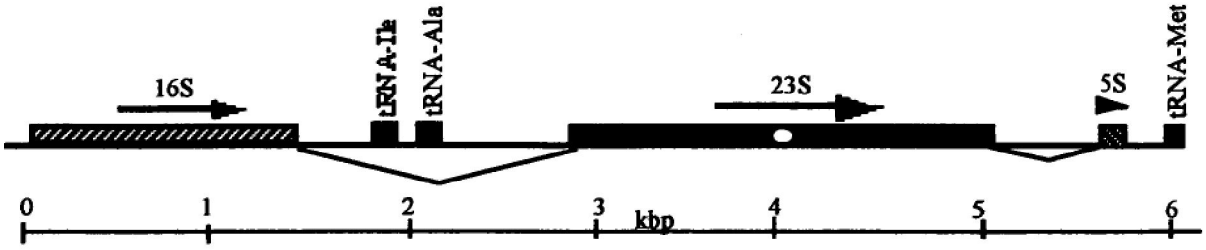

Figure 2. Diagram of the ~6 kbp rRNA operon in prokaryotes (on *A. tumefaciens* MAFF301001 example). Ribosomal RNA genes and tRNA genes are illustrated as shaded boxes drawn to scale in kbp. Internal transcribed spacers (ITS) are shown with brackets. An arrow above each rRNA gene reveals the direction of transcription (Bautista-Zapanta et al., 2002).

Initially, the analysis of microbial diversity relied on direct extraction, purification, and sequencing of 5S rRNA molecules from environmental samples (Lane et al. 1985; Stahl et al. 1984, 1985). Even though these studies offered new insights, the information content of the 120-nucleotide 5S rRNA is quite small, and its low number of independently varying nucleotide positions limits its usefulness in the study of distant phylogenetic relationships (Olsen et al. 1986). By contrast, the 16S rRNA (small-subunit SSU) is large enough for phylogenetic inference. Though the 23S (large-subunit LSU) contains about twice as much information as the 16S and should provide more possibilities to pinpoint phylogeny, it is the SSU that has become the established phylogenetic reference because of its ease to sequence.

16S rRNA gene offers also other advantages. First of all, it is ancient and at the same time stable over long evolutionary times, i.e. during $40\text{-}50 \times 10^6$ years the primary structure will change by 1% (Stackebrandt and Pukall, 1999). Moreover, all bacteria (and all living organisms) harbour this gene in which the presence of both conserved and highly variable regions permits the discrimination of taxa at different taxonomic levels (Amann, 1995). Finally a comprehensive sequence data set (approximately 30,000 entries in 2002) is available in widely accessible databases (Maidak et al., 2001; Stoesser et al., 2002; Van de Peer et al., 2000), and the number of entries is permanently increasing. It is also widely accepted to apply the 16S rRNA technology as an integrated part of a polyphasic approach for new descriptions of bacterial species or higher taxa (Ludwig et al., 1998; Stackebrandt & Goebel, 1994).

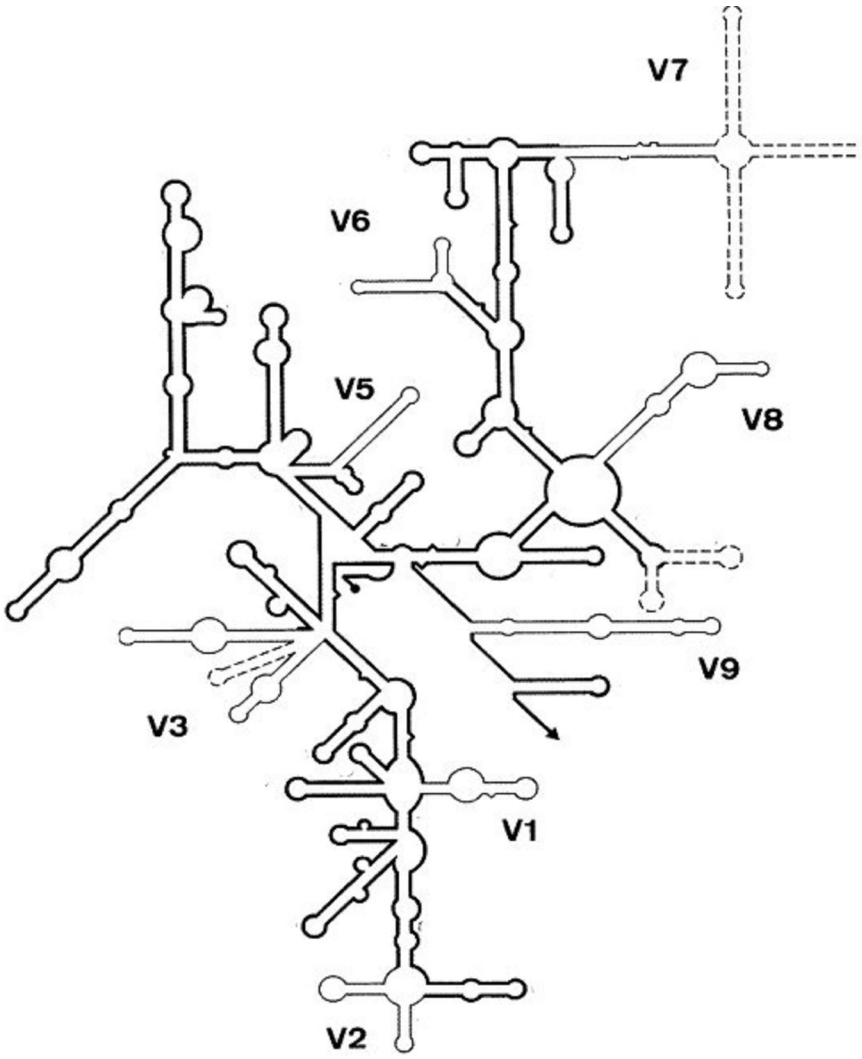

Figure 3. The secondary structure model of prokaryotic 16S rRNA molecule. The 5'-terminus is symbolized by a filled circle and the 3'-terminus by an arrowhead. Helices are numbered in the order of occurrence from 5'- to 3'-terminus. Helices bearing a single number are common to the prokaryotic model. Relatively conserved areas are drawn in bold lines, areas of sequence – and length variability in thin lines. Eight variable areas (V), numbered V1 to V9, are distinguished, V4 being absent in prokaryotic rRNAs. Helices drawn in broken lines are present in a small number of known structures only (Neefs et al., 1990).

Microheterogeneities in the nine hypervariable regions of the 16S rRNA molecule (Fig. 3) may account for some of the variability, particularly among very closely related genes (Britschgi and Giovannoni, 1991). The heterogeneity that sometimes occurs among the rRNA operons of a single organism may also be a problem, because significant 16S ribosomal DNA (rDNA) interoperon differences have been detected in different bacterial species (Amann and Ludwig, 2000). The intragenomic heterogeneity can influence 16S rRNA gene tree topology, phylogenetic resolution and operational taxonomic unit (OTU) estimates at the species level or below (Case et al., 2007). In virtually all species, the sequences of multicopy rRNA genes are identical or nearly identical and the homogeneity is thought to be governed by concerted evolution, which may originate from stringent selective pressure on the primary sequences of

rRNA molecules to maintain their precise interactions with components of the complex protein-synthesizing machinery (Ueda et al., 1999). Several recent studies using rDNA sequencing have reported the existence of divergent 16S rDNA sequences within a single organism. These studies clearly showed the presence of sequence heterogeneity between *rrn* operons in single genomes, but it has not been elucidated whether such intra-rRNA heterogeneity is peculiar or general. To clarify this, the extent of such sequence heterogeneity should be systematically studied (Ueda et al., 1999). It is generally considered that mutations, such as random base anomalies, depend on a number of factors, including mis-incorporation and misrepair by DNA polymerase and the number of PCR cycles used during DNA replication or sequencing (Speksnijder et al., 2001). In case of estimating the natural microbial diversity, it is useful to know, that the 16S rRNA gene may be too well-conserved to discriminate between closely related populations. Indeed, different species may have almost identical 16S rDNA sequences. For example, studies on *Burkholderia* species demonstrated that analysis of PCR-amplified 16S rDNA is limited in its ability to differentiate the *B. cepacia* complex, since nucleotide sequence variation within this gene is not sufficient to identify all current genomovars. Therefore, other genes, such as recA (Mahenthiralingam et al., 2000), have been used in bacterial taxonomy to circumvent this problem. In some cases, the 23S rRNA may be also useful (Case et al., 2007).

I.I.3. Single-strand conformation polymorphism

Single-strand conformation polymorphism (SSCP) analysis detects sequence variations among amplified DNA fragments, which are usually 16S rRNA gene sequences. SSCP was originally described by Orita et al. (1989), and was first used to assess the diversity of natural microbial communities by Lee et al. (1996). At low temperatures, single-stranded DNA will adopt a three-dimensional conformation determined by the intramolecular interactions that influence their electrophoretic mobility in a non-denaturing polyacrylamide gel. PCR fragments of the same size, but with different nucleotide sequences will be separated due to their differing electrophoretic mobility. Differences in mobility are detected on autoradiograms (radioactive detection) by silver staining of the gel or using fluorescently labeled primers that are subsequently detected by an automated DNA sequencer (non-radioactive detection) (Widjojoatmodjo et al., 1995).

There are a few publications that report studies on microbial diversity in lake ecosystems using the SSCP method. Lee et al. (1996) studied the differences between bacterial populations in an oligotrophic lake and in a eutrophic pond, using SSCP pattern analyses of 16S rRNA genes. More recently, Wenderoth and Abraham (2005) compared the microbial communities of 14 acidic lakes by their 16S rDNA-based SSCP fingerprints. Moreover, SSCP analysis, applied to the study of the C23O gene, proved to be a powerful tool for assessing the diversity of specific populations involved in the aromatic catabolism of contaminated sites (Junca and Pieper, 2004).

The detection of sequence variation using SSCP is generally good, but the detection sensitivity tends to decrease as the fragment length increases (Hayashi and Yandell, 1993). Phylogenetic assignation of the bands is possible by sequencing, if the fragment separation is carried out in gel. However, it has been demonstrated that a single band may consist of several sequences, and that the electrophoretic conditions may therefore affect the resolution of genetic profiles and thus the estimated diversity (Schmalenberger and Tebbe, 2003).

I.I.4. Terminal restriction fragment length polymorphism

Terminal-restriction fragment length polymorphism (T-RFLP) analysis is a fingerprinting technique based on the restriction digest of double-stranded fluorescently end-labeled PCR fragments (Liu et al., 1997; Marsh, 1999). One primer is labeled at the 50 terminus with a fluorescent dye. As a general rule, a single species will contribute a single terminal fragment of a given size, although several species may have terminal fragments of identical size. T-RFLP is a high-throughput, reproducible method that can be used to carry out both qualitative and quantitative analyses of specific members of a microbial community. Usually the 16S rRNA is the target gene. The fragments are separated by gel electrophoresis in non-denaturing polyacrylamide gels or by capillary electrophoresis, and distinguished by laser induced fluorescent detection. These fluorescence data are converted into electropherograms, in which the peaks represent fragments differing in size, and the areas under the peaks indicate the relative proportions of the fragments. The advantage of this technique is its ability to detect even the rarest members of a microbial community. In addition, phylogenetic assignments can be inferred from the sizes of the terminal restriction fragment (T-RF) using web-based resources that predict T-RF sizes for known bacteria (Kent et al., 2003).

T-RFLP technique has been often used to compare the dynamics both between and within microbial populations in lake ecosystems (Nusslein et al., 2002; Konstantinidis et al., 2003; Kritzberg et al., 2006; Schwartz et al., 2007). The limitations and pitfalls that have been identified for this technique include the formation of pseudoterminal restriction fragments, which can result in the overestimation of microbial diversity (Egert and Friedrich 2003). The choice of the primers and restriction enzymes also seems to be very important to obtain an accurate evaluation of the microbial diversity (Engebretson and Moyer, 2003; Lueders and Friedrich, 2003). In addition, it has been reported that T-RFLP is very useful for estimating diversity in communities characterized by low-to-intermediate species richness, but is not suitable for complex microbial populations (Engebretson and Moyer, 2003).

I.I.5. Ribosomal intergenic spacer analysis and automated ribosomal intergenic spacer analysis

Ribosomal intergenic spacer analysis (RISA) was developed by Borneman and Triplett (1997) and was firstly applied to study the microbial diversity in soils. The method involves PCR amplification of the intergenic region between the 16S and 23S rRNA genes. This region is extremely variable in size (ranging from 50 bp to more than 1.5 kb) and nucleotide sequence. The polymorphism revealed by RISA is linked to the length heterogeneity. Amplification products different in length are separated on polyacrylamide gels on the basis of their size and visualized by silver staining. This tool has been used successfully to assess community fingerprints with each band corresponding to at least one organism. As in other fingerprinting techniques, these gel bands can be isolated and sequenced. A sequence database exists for ribosomal intergenic spacers (IGS) region of the rRNA operon *rrl*, thus, theoretically, the sequenced bands can be taxonomically identified. However, this database is not as large or comprehensive as the 16S rDNA database. Therefore, species identification from RISA bands is much less likely than with other methods that use 16S rDNA. Other potential problems with RISA arise from PCR biases. The preferential amplification of shorter sequences is of particular concern, since IGS amplicons cover such a broad range of sizes (Fisher and Triplett, 1999). Biases imposed by secondary structures in the rDNA genes flanking the amplified region may also pose a problem. The main advantage of RISA is its rapidity and simplicity compared with other rDNA fingerprinting methods. It requires no denaturing gradients or long electrophoresis times and no restriction digestion or the associated optimization procedures.

An automated version of RISA, named ARISA, was developed in order to assess bacterial community diversity more rapidly and more efficiently (Fisher and Triplett, 1999). PCR amplification of the 16S–23S region is performed using a fluorescently labeled, forward primer, which makes it possible to detect the amplicons by automated capillary electrophoresis. The total number of distinct fluorescent peaks in the ARISA data within a given sample is taken as an estimate of species diversity, and the sizes of the fragments can be compared to those present in the GenBank database.

Recently, ARISA was successfully applied to investigate the influence of geographic and environmental variables on the diversity of bacterial communities in different lakes (Fisher et al., 2000; Graham et al., 2004; Yannarell and Triplett, 2005).

I.I.6. Denaturing gradient gel electrophoresys

I.I.6.1. Theoretical and practical aspects of PCR-DGGE fingerprinting method

Denaturing gradient gel electrophoresis is a method that separates PCR-amplified DNA fragments, according to their melting properties, on the basis of their differential mobility through a DNA-denaturing gel. DGGE has been introduced into microbial ecology by Muyzer et colleagues (1993), and is frequently used to assess the diversity of microbial communities, and to monitor their dynamics (Green et al., 2009).

In this method, PCR-amplified DNA from different taxonomic microbial groups is run on a special polyacrylamide gel, which has embedded a gradient of DNA-denaturing compounds, usually urea and formamide. As DNA passes through a concentration gradient (i.e., from low to high) of denaturant, it comes under increasing pressure to separate into single strands. The DNA is unable to denature completely because of the presence of a GC clamp, which is included in one of the primers for the PCR reaction. However, DNA becomes increasingly denatured as it passes through the gel and its mobility decreases. The DNA comes to rest when it is almost fully denatured. The position along the gradient at which this occurs is determined primarily by the relative proportions of G+C and A+T in a given amplicon, since G-C bonds are more difficult to denature than A–T bonds. Thus, differences in sequence between amplicons, based on differences in G-C content, will result in DNA migration to different positions in the gel. Properly calibrated, DGGE is sensitive enough to detect even single base-pair differences between amplicons (Miller et al. 1999). Each isolated fragment or

"melting domain" represents a predominant population of the environmental sample. Nucleic acid fragments from individual domains, isolated on gradient gels, can be eluted from excised DGGE bands, reamplified and sequenced to identify individual constituents of the DNA or RNA extracted from environmental samples (Muyzer and de Waal, 1994).

Innovations in the use of DGGE include the use of double-gradient DGGE (i.e. the combined application of a gradient of acrylamide and a gradient of denaturants to obtain a better resolution), the use of terminally labeled fluorescent PCR products and of fluorescent intra-lane standards to detect community members, and the use of functional genes as molecular markers to discriminate between closely related but ecologically different populations (Muyzer, 1999).

I.I.6.2. Microbial community diversity analysis using DGGE banding patterns

DGGE fingerprint interpretation assumes that each band represents a single species and that the band intensity is directly related to the species abundance (Nubel et al., 1999).

'Species' is the most commonly used unit in biodiversity studies, but in Microbiology it is not easy to define (Mayden, 1997). Microbial taxonomists have equated species with OTUs (Achtman and Wagner, 2008), usually defined as the number of distinct 16S ribosomal RNA sequences at a certain cut-off level of sequence diversity.

The variations between DGGE profiles are described visually on a single gel by the disappearance, appearance or the changes in the intensity of specific bands. Banding pattern similarity can be compared using dendrograms, showing the degree of intra-group similarity (Stamper et al., 2003). The number of bands visible in the DGGE gels provides an estimate of richness, and the relative band intensity allowes to calculate the proportional abundance ("evenness") of each population. Several software packages are employed to estimate the relative band densities necessary to calculate diversity indices (Fromin et al., 2002; Stamper et al., 2003). Most microbial diversity indices are based on indices developed for plant and animal studies, for example the Shannon Weaver (*H'*) and Simpson (*S*) indices. These indices incorporate aspects of both species richness and species evenness, weighting individual classes by their relative abundances, and are the most common diversity indices used by microbial ecologists.

DGGE has been used in a huge number of studies of eubacterial, archaeal and eukaryotic populations in freshwater and coastal waters (Øvreas et al., 1997; van Hannen et al., 1998; El

Fantroussi et al., 1999; Casamayor et al., 2000, 2001, 2002; Lindstrom, 2000; Riemann and Middelboe, 2002; Zwart et al., 2002; Lyautey et al., 2003; Schauer et al., 2003), allowing to obtain a considerable amount of information about the dynamics of aquatic microbial communities diversity also in relation to spatio-temporal variations.

I.I.6.3. Advantages, limitations, and complementarity

DGGE is perhaps the most commonly used method to study biodiversity of natural microbial communities, appearing in approximately the 15% of the articles that deal with this topic (Spiegelman et al., 2005). DGGE technique allows rapidly the screening of multiple samples to study the diversity of microbial communities in relation to temporal and geographical differences and the phylogenetic affiliation of the community members (Muyzer and Smalla, 1998; Muyzer et al., 1993). It also allows to obtain taxonomic information by excising, re-amplifying and sequencing specific DNA fragments or by hybridization analysis with taxon-specific oligonucleotides probes (Heuer et al., 1999; Riemann and Winding, 2001). Furthermore, this technique is reliable, reproducible, rapid and inexpensive. In spite of the advantages of DGGE, there are a few problems associated with the specialized electrophoretic system needed for this method and with the potential number of bands that can be produced from complex environmental samples. Indeed, before a community sample can be properly subjected to DGGE analysis, careful calibration must be done to ensure the optimal gradient and electrophoretic duration (Muyzer et al. 1993; Muyzer and Smalla 1998). Moreover, a DGGE experiment is limited to DNA fragments typically below 500 bp in size (Myers et al. 1985*a*, 1985*b*). Since a common complementary technique involves the isolation of DGGE bands for sequencing (for taxonomic identification of bands of interest), this size limit restricts the amount of sequence available for identification or for oligonucleotide probe design (Muyzer and Smalla 1998). Another limitation of DGGE concerns the rather large quantities of DNA required for effective resolution, often in the order of 500 ng of PCR product for complex environmental samples (Nakagawa and Fukui 2002).

Although DGGE is a useful method for visualizing the major members of a microbial community, some limitations exist also in this regard. The brightest bands in a DGGE profile are often assumed to represent the dominant members of the community (Forney et al. 2004). However, the biases associated with PCR could cause relative under- or over-representation of a given taxon in the DGGE profile. Consequently, quantitative inferences about species richness must be confined to general statements about species predominance (Bonin et al.

2002; Nakagawa and Fukui 2002; Stephen et al. 1999). On the other side of the spectrum of abundance, the limit of resolution of this method seems to be about 1% of the community population, that is, only DNA from organisms comprising 1% or more of the community sample can be visualized (Muyzer et al. 1993; Murray et al. 1998). In addition, this method can be difficult to apply to extremely complex communities that produce hundreds of bands on a DGGE profile, which become difficult to visualize individually. Some ambiguities could exist in associating a single band in the community DGGE profile with a single microbial species due to the possibility that multiple amplicons co-migrate to the same location in the gel (Nübel et al. 1997). However, the opposite problem of multiple bands for a single species, due to the existence of multiple copies of rRNA genes in a single organism, introduces an unavoidable element of ambiguity in DGGE (Nübel et al. 1997).

In spite of the described limitations of this technique, several studies suggest that the results of DGGE are readily amenable to statistical analysis, provided there is sufficient standardization of analytical procedures (Fromin et al., 2002). This could provide not only statistical validation of observational conclusions, but also allow researchers to approach questions of causality by testing the correlation of DGGE profiles with environmental variables.

I.I.7. Random sequencing in clone libraries

Species identification of individual community members by random sequencing in clone libraries involves cloning of the PCR products and the subsequent sequencing of the obtained clones. Sequence analysis allows to identify the dominant species present in the PCR products. Comparison of these sequences with those available in sequence databases (Genebank^{TM} or EMBL) yields information about the identity or relatedness of the sequences to known species. The most incisive way to infer phylogenetic relationship from molecular sequence data is to construct phylogenetic trees (Olsen et al., 1986; Woese, 1987). The sequence information can be used to compare species richness or diversity in different samples. This approach has been widely used to study bacterial diversity in different lakes, revealing that planktonic bacteria of predominant bacterial divisions are distributed world-wide (Eiler and Bertilsson, 2004; Glockner et al., 2000; Hiorns et al., 1997) and that freshwater systems have specific bacterial communities which are distinct from those occurring in soil and in sediments (Zwart et al., 2002)

I.I.7.1. Advantages and limitations

The main limitations and drawbacks of the PCR-cloning and sequencing approach are essentially technical. It is time-consuming since as many as a few thousand clones must be analyzed in order to cover the phylogenetic richness hidden in a prokaryotic gene library (Loy et al., 2006). As a result, wide application of gene library surveys has generally been limited in the past (Borneman and Triplett, 1997; Dunbar et al., 1999), irrespective of the continuous advances in high-throughput sequencing that made the application of clone libraries easier but more expansive. Therefore, clone libraries are generally constructed in parallel to fingerprinting techniques like DGGE, T-RFLP or SSCP, that describe the complexity of the community and allow well-informed decisions on the number of clones to be sequenced (Duthoit et al., 2003; Handschur et al., 2005; Delbe` s et al., 2007; Juste´ et al., 2008). Alternatively, restriction digestion of the clones may be helpful for initial screening of the clone library and distinguishing different restriction types which then can be sequenced (Lagace´ et al., 2004; Kim and Chun, 2005). Another limit of this technique could be bias due to the PCR (i.e. choice of primers, annealing temperature and numbers of cycles, inhibition of the enzyme by humic compounds, formation of chimaeric PCR products) or the cloning strategy used, both of which could have a direct impact on the sampling of the cloned DNA sequences and their identification. Consequently, the analysis of diversity of bacterial communities may not be totally reliable. However, despite the numerous biases that can occur in the PCR cloning method, many studies showed that PCR of DNA fragments, followed by cloning assisted sequence analysis, allowed a fine identification of bacteria as well as an estimation of their relatedness to known species (Kowalchuk et al., 1997; Ranjard et al., 2000).

I.I.8. Use of functional genes to target populations involved in specific metabolic processes

Most of the molecular techniques uses 16S rDNA gene to assess biodiversity of microbial communities. It is obvious that the phylogenetic properties of 16S rDNA gene, as well as the large amount of sequences available, offer a considerable advantage, but there are also disadvantages. For example, the heterogeneity of 16S rDNA gene between multiple copies within one bacterial species (Klappenbach et al., 2001; Shimizu et al., 2001) can lead to a wrong interpretation of molecular results so that, caution and thoroughness are extremely

important if this gene is used to draw ecological conclusions concerning diversity and abundance (Zhou et al., 2002).

Different microbial communities may be composed of quite different groups of species performing the same processes. When a specific microbial process is of interest, the functional diversity in a given environment may be monitored by assessing the diversity of so-called 'functional genes', encoding key enzymes in the process of interest, and the identification of predominant gene polymorphisms. Therefore, functional genes can be used in microbial diversity studies, especially to investigate the relationships between microbial community structure and microbial functioning. Their use requires that the gene sequence be conserved (to allow group-specific probes or primer design) and, ideally, contain phylogenetic information. Several functional genes have proven amenable for studying bacterial functional processes in different environments. Some examples include the genes involved in the nitrogen fixation (*nifH*), denitrification (*nirS, nirK, nosZ, narG*), nitrification (*amoA*), methanogenesis (*pmoA, mmoX*), and methanotrophy (Affourtit et al., 2001; Steward et al., 2004; Smith et al., 2007; Cebron et al., 2004; Banning et al., 2005; Kalyuzhnaya et al., 2005). For example, this approach was applied to study changes in the distribution of different diazotrophs along a salinity and nitrate concentration gradient (Affourtit et al., 2001; Steward et al., 2004), using PCR amplification, cloning, and sequencing of *nifH* gene.

PCR-DGGE using functional genes as molecular target is also suited to investigate the temporal and spatial distribution of bacterial populations with specific metabolic activity (Jeffrey et al., 1996; Paul, 1996). This approach was used to detect particulate methane monooxygenase (*pmoA*) and methanol dehydrogenase (*mxaF*) of methanotrophs or to target the dissimilatory (bi) sulfite reductase β subunit-encoding gene (*dsrB*) to describe sulfate-reducing prokaryotes diversity (Henckel et al., 1999; Lin et al., 2005; Geets et al., 2006; Miletto, 2007). The diversity of *Nitrobacter* species, based on the functional gene *nxrA* encoding the catalytic subunit of the nitrite oxidoreductase in nitrite-oxidizing bacteria, and the differential expression of the [NiFe]-hydrogenase gene from different *Desulfovibrio* populations, were also investigated by PCR-DGGE method (Wertz et al., 2008; Wawer et al., 1997).

I.I.8.1. Use of [FeFe]-hydrogenases gene to investigate the diversity of H_2-producers communities

Many molecular techniques (DGGE, cloning, T-RFLP) were used to determine microbial composition of H_2-producers communities (Fang et al., 2002; Ueno et al., 2001a; Ueno et al., 2001b; Sung et al., 2002), using 16S rDNA as target gene. However, this approach is not sufficient to reveal detailed information on the contribution of specific microbial populations to particular metabolic activities, such as the H_2 production, because of the great biodiversity of the studied ecosystems and the consequent complexity of the obtained results. Thus, an efficient strategy to overcome this limit is the direct amplification of genes encoding [Fe]-H_2ases (*hyd)* that offers a more complete view of the bacterial populations associated with H_2 production. Indeed, it was demonstrated that not all H_2-producing bacteria (HPB) identified by *hyd* sequences had been previously described by studies based on the 16S rRNA gene (Xing et al., 2008).

Many HPB belong to the genus *Clostridium* (Chang et al., 2006). The hydrogenase of several *Clostridium* species have been sequenced and characterized, including *C. pasteurianum* (Meyer and Gagnon, 1991), *C. acetobutylicum* (Santangelo et al., 1995; Gorwa et al., 1996), *C.perfringens* (Kaji et al., 1999) and *C. paraputrificum* (Morimoto et al., 2005). On the basis of these sequences available in the Gen Bank database, primers specific for genes encoding [Fe]-H_2ases were designed to investigate the diversity of HPB. Subsequently, hydrogenase-gene-targeted molecular biology approaches such as RT-PCR, fluorescence in situ hybridization (FISH) and DGGE analysis have been used to investigate the diversity of consortia in anaerobic H_2 biofermentation systems (Fang et al., 2006; Chang et al., 2006).

References

Achtman M. and Wagner M. 2008. Microbial diversity and the genetic nature of microbial species, *Nat. Rev. Microbiol.*, 6, 431-440.

Affourtit, J., Zehr, J., Paerl, H. 2001. Distribution of nitrogen-fixing microorganisms along the Neuse River Estuary, North Carolina. *Microbial Ecology*. 41: 114-123

Amann, R. and Ludwig, W. 2000. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS *Microbiology Reviews*, 24: 555-565

Amann, R. I. 1995. *In situ* identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. *Molecular Microbial Ecology*. Manual 3.3.6: 1-15

Ambler, R. P., Daniel, M., Hermoso, J., Meyer, T. E., Bartsch, R. G, Kamen, M. D. 1979. Cytrochrome c2 sequence variation among the recognised species of purple nonsulfur photosynthetic bacteria. *Nature* 278, 259-660.

Banning, N., Brock, F., Fry, J. C., Parkes, R. J., Hornibrook, E.R.C., Weightman, A. J. 2005. Investigation of the methanogen population structure and activity in a brackish lake sediment. *Environmental Microbiology*. 7: 947-960.

Bautista-Zapanta, J., Suzuki, K., Yoshida, K. 2002. Characterization of four ribosomal RNA operons in the genome of *Agrobacterium tumefaciens* MAFF301001. *Oxford University Press Nucleic Acids Research Supplement*, 2: 91-92

Bonin, P. C., Michotey, V. D., Mouzdahir, A., and Rontani, J.-F. 2002. Anaerobic biodegradation of squalene: Using DGGE to monitor the isolation of denitrifying bacteria taken from enrichment cultures. FEMS *Microbiol. Ecol*. 42: 37–49.

Borneman, J., Triplett, E. W. 1997. Molecular microbial diversity in soils from Eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol*. 63, 2647–2653.

Britschgi, T. B. and Giovannoni, S. 1991. Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene cloning and sequencing. *Applied and Environmental Microbiology*, 57(6): 1707-1713

Casamayor, E. O, Schäfer, H, Baneras, L, Pedros-Alio, C, Muyzer, G. 2000. Identification of spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol*. 66, 499–508.

Casamayor, E. O., Massana, R., Benlloch, S., Øvreas, L., Diez, B., Goddard, V. J., Gasol, J. M., Joint, I., Rodriguez-Valera, F., Pedros-Alio, C., 2002. Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond saltern. *Environ. Microbiol*. 4, 338–348.

Casamayor, E.O., Muyzer, G., Pedros-Alio, C., 2001. Composition and temporal dynamics of planktonic archaeal assemblages from anaerobic sulfurous environments studied by 16S rDNA denaturing gradient gel electrophoresis and sequencing. *Aquat. Microb. Ecol*. 25, 237–246.

Case, R.J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F., Kjelleberg, S. 2007. Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. *Applied and Environmental Microbiology*, 73(1): 278-288

Cebron, A., Coci, M., Garnier, J., Laanbroek, H. J. 2004. Denaturing gradient gel electrophoretic analysis of ammonia-oxidizing bacterial community structure in the lower Seine river: Impact of Paris wastewater effluents. *Applied Environmental Microbiology*. 70: 6726-6737.

Chang, J. J., Chen, W. E., Shih, S. Y., Yu, S. J., Lay, J. J., Wen, F. S., Huang, C. C. 2006. Molecular detection of the clostridia in an anaerobic biohydrogen fermentation system by hydrogenase mRNA-targeted reverse transcription-PCR, *Appl Microbiol Biotechnol* 70 (5), pp. 598–604.

Delbès, C., Ali-Mandjee, L., Montel, M. C. 2007. Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analyses. *Appl. Environ. Microbiol*. 73, 1882–1891.

Dorigo, U., Volatier L., Humbert, J. F. 2005. Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Res.* 39:2207–2218.

Dunbar, J., Takala, S., Barns, S. M., Davis, J. A., Kuske, C. R. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Appl. Environ. Microbiol*. 65, 1662–1669.

Duthoit, F., Godon, J. J., Montel, M. C. 2003. Bacterial community dynamics during production of registered designation of origin salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. *Appl. Environ. Microbiol*. 69, 3840–3848.

Egert, M., Friedrich, M.W. 2003. Formation of pseudoterminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Appl. Environ. Microbiol.* 69, 2555–2562.

Eiler, A., Bertilsson, S., 2004. Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environ. Microbiol*. 6, 1228–1243.

El Fantroussi, S., Verschuere, L., Verstraete, W., Top, E. M. 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Appl. Environ. Microbiol*. 65, 982–988.

Engebretson, J.J., Moyer, C.L. 2003. Fidelity of select restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism. *Appl. Environ. Microbiol.* 69, 4823–4829.

Fang, H. H. P., Zhang, T. Li, C. 2006. Characterization of Fe-hydrogenase genes diversity and hydrogen-producing population in an acidophilic sludge. *J. Biotechnol*. 126, 357 – 364.

Fisher, M. M., Klug, J. L., Lauster, G., Newton, M., Triplett, E. W. 2000. Effects of resources and trophic interactions on freshwater bacterioplankton diversity. *Microb. Ecol*. 40, 125–138.

Fisher, M. M., Triplett, E. W. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl. Environ. Microbiol.* 65, 4630–4636.

Forney, L. J., Zhou, X., Brown, C. J. 2004. Molecular microbial ecology: land of the one-eyed king. *Curr. Opin. Microbiol.* 7: 210–220.

Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Aragno, M., Rossi, P. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ. Microbiol*. 4, 634–643.

Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., and Aragno, M. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ. Microbiol*. 11: 634–643.

Geets, J., Borrernans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, D., Vanbroekhoven, K. 2006. DsrB gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria, *J. Microbiol. Methods* 66, pp. 194–205

Glöckner, F.O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A., Amann, R. 2000 Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of *Actinobacteria*, *Appl Environ Microbiol* 66 pp. 5053–5065.

Graham, J. M., Kent, A. D., Lauster, G. H., Yannarell, A.C., Graham, L.E., Triplett, E. W. 2004. Seasonal dynamics of phytoplankton and planktonic protozoan communities in a Northern temperate humic lake: diversity in a dinoflagellate dominated system. *Microb. Ecol*. 48, 528–540.

Green, S. J., Leigh, M. B. and Neufeld, J. D. 2009. Denaturing gradient gel electrophoresis (DGGE) for microbial community analysis. Pages 4137-4158 in: Timmis, K.N. (Ed) Handbook of Hydrocarbon and Lipid Microbiology. Springer (Heidelberg, Germany).

Handschur, M., Pinar, G., Gallist, B., Lubitz, W., Haslberger, A. G. 2005. Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. *Food Chem. Toxicol*. 43, 1595–1605.

Hayashi, K., Yandell, D. W. 1993. How sensitive is PCR-SSCP. *Hum. Mutat*. 2, 338–346.

Henckel, T., Friedrich, M.,. Conrad, R. 1999. Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase, *Applied and Environmental Microbiology* 65 pp. 1980–1990.

Heuer, H., Hartung, K., Wieland, G., Kramer, I., Smalla, K. 1999 Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. *Applied and Environmental Microbiology*, 65(3): 1045-1049

Hiorns, W. D., Methe´, B.A., Nierzwicki-Bauer, S. A., Zehr, J. P., 1997. Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequences. *Appl. Environ. Microbiol.* 63, 2957–2960.

Jeffrey W. H., Nazaret S., Barkay T. 1996 Detection of the *merA* gene and its expression in the environment. *Microb. Ecol*. 32: 293–303

Junca, H., Pieper, D.H., 2004. Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. *Environ. Microbiol.* 6, 95–110.

Juste´, A., Lievens, B., Klingeberg, M., Michiels, C.W., Marsh, T.L., Willems, K.A. 2008. Predominance of Tetragenococcus halophilus as the cause for sugar thick juice degradation. *Food Microbiol*. 25, 413–421.

Kaji, M., Taniguchi, Y., Matsushita, O., Katayama, S., Miyata, S., Morita, S., Okabe, A. 1999. The hydA gene encoding the H(2)-evolving hydrogenase of *Clostridium perfringens*: molecular characterization and expression of the gene, *FEMS Microbiol. Lett.* 181 pp. 329–336.

Kalyuzhnaya, M. G., Bowerman, S., Nercessian, O., Lidstrom, M. E., Chistoserdova, L. 2005. Highly divergent genes for Methanopterinlinked C1 transfer reactions in Lake Washington, assessed via metagenomic analysis and mRNA detection. *Applied Environmental Microbiology*. 71: 8846-8854

Kent, A. D., Smith, D. J., Benson, B. J., Triplett, E. W. 2003. Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. *Appl. Environ. Microbiol*. 69, 6768–6776.

Kim, M., Chun, J. 2005. Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis. *Int. J. Food Microbiol*. 103, 91–96.

Klappenbach, J. A., Saxman, P. R.,. Cole J. R., Schmidt, T. M. 2001. *rrndb*: The ribosomal RNA operon copy number database. *Nucleic Acids Res* 29 pp. 181–184.

Konstantinidis, K. T., Isaacs, N., Fett, J., Simpson, S., Long, D. T., Marsh, T.L., 2003. Microbial diversity and resistance to copper in metal-contaminated lake sediment. *Microb. Ecol*. 45, 191–202.

Kowalchuk, G. A., Stephen, J. R., Boer, W. D., Prosser, J. I., Embley, T. M., Woldendorp, J. W. 1997. Analysis of ammoniaoxidizing bacteria of the β subdivision of the class *proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl. Environ. Microbiol*. 63: 1489–1497.

Kritzberg, E. S., Langenheder, S., Lindström, E. S. 2006. Influence of dissolved organic matter source on lake bacterioplankton structure and function — implications for seasonal dynamics of community composition. FEMS *Microbiology Ecology* 56, 406–417.

Lagace´, L., Pitre, M., Jacques, M., Roy, D., 2004. Identification of the bacterial community of maple sap by using amplified ribosomal DNA (rDNA) restriction analysis and rDNA sequencing. *Appl. Environ. Microbiol*. 70, 2052–2060.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D.A., Sogin, M. L., Pace, N. R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci*. U.S.A. 82: 6955–6959.

Lee, D.H., Zo, Y.G., Kim, S.J. 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. *Appl. Environ. Microbiol*. 62, 3112–3120.

Lin, J. L., Radajewski, S., Eshinimaev, B. T., Trotsenko, Y. A., McDonald, I. R., Murrell, J. C. 2004. Molecular diversity of methanotrophs in Transbaikal soda lake sediments and identification of potential active populations by stable isotope probing. *Environmental Microbiology*, 6, 1049–1060

Lindstrom, E.S. 2000. Bacterioplankton community composition in five lakes differing in trophic status and humic content. *Microb. Ecol*. 40, 104–113.

Liu, W.-T., Marsh, T.L., Cheng, H., Forney, L. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol*. 63, 4516–4522.

Loy, A., Taylor, M. W., Bodrossy, L., Wagner, M. 2006. Applications of nucleic acid microarrays in soil microbial ecology. In: Cooper, J.E., Rao, J.R. (Eds.), Molecular Approaches to Soil, Rhizosphere and Plant Microorganism Analysis, vol. 1. CABI Publishing, Wallingford, Oxfordshire, UK, pp. 18–39.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, m., Neumaier, J., Bachleitner, M. & Schleifer, K. H. 1998. Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* 19, 554-568.

Lueders, T., Friedrich, M.W. 2003. Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. *Appl. Environ. Microbiol*. 69, 320–326.

Lyautey, E., Teissier, S., Charcossey, J.-Y., Rols, J.-L., Garabetian, F., 2003. Bacterial diversity of epilithic biofilm assemblages of an anthropised river section, assessed by DGGE analysis of a 16S rDNA fragment. *Aquat. Microb. Ecol*. 33, 217–224.

Mahenthiralingam E, Bischof J, Byrne SK, Radomski C, Davies JE, Av-Gay Y, Vandamme P. 2000. DNA-based diagnostic approaches for identification of *Burkholderia cepacia complex, Burkholderia vietnamiensis, Burkholderia multivorans, Burkholderia stabilis,* and *Burkholderia cepacia genomovars* I and III. *J Clin Microbiol* 38: 3165–3173.

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T., Jr., Saxman, P. R., Farris, R. J., Garrity, G. M., Olsen, G. J., Schmidt, T. M., Tiedje, J. M. 2001. The RDP-II (Ribosomal Database Project). *Nucleic Acids Research* 29, 173-174.

Marsh, T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol*. 2, 323–327.

Mayden, R. L. 1997. A hierarchy of species concepts: the denouement in the saga of the species problem, In Species: the Units of Biodiversity (Claridge, M.F. et al., eds), pp. 381–425, Chapman and Hall

Meyer, J., and Gagnon, J. 1991. Primary structure of hydrogenase I from *Clostridium pasteurianum*, *Biochemistry* 30, pp. 9697–9704.

Miletto, M., Bodelier, P.L.E., Laanbroek H. J. 2007. Improved PCR-DGGE for high resolution diversity screening of complex sulfate-reducing prokaryotic communities in soils and sediments. *J Microbiol Methods* 70: 103–111

Miller, K. M., Ming, T. J., Schulze, A. D. and Withler, R. E. 1999. Denaturing gradient gel electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. *Biotechniques*. 27(5):1016-8, 1020-2.

Morimoto, K., Kimura, T., Sakka, K., Ohmiya, K. 2005. Overexpression of a hydrogenase gene in *Clostridium paraputrificum* to enhance hydrogen gas production, *FEMS Microbiol. Lett.* 246 pp. 229–234

Murray, A. E., Preston, C. M., Massana, R., Taylor, L. T., Blakis, A., Wu, K., and DeLong, E. F. 1998. Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. *Appl. Environ. Microbiol*. 64: 2585– 2595.

Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology*, 2: 317-322

Muyzer, G. and de Waal, E. C. 1994. Determination of the genetic diversity of microbial communities using DGGE analysis of PCR-amplified 16S rDNA. In: Microbial Mats-structure, Development and Environmental Significance (Stal, L.J. and Caumette, P. Eds.), NATO ASI Series G35, pp. 207–214. Springer, Berlin.

Muyzer, G. and Smalla, K. 1998 Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek, 73: 127–141

Muyzer, G., De Waal, E. C., Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol*. 59:695- 700.

Myers, R. M., Fischer, S. G., Lerman, L.S., and Maniatis, T. 1985*a* Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res*. 13: 3131–3145.

Myers, R. M., Fischer, S. G., Maniatis, T., and Lerman, L. S. 1985*b*. Modification of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* 13: 3111–3129.

Nakagawa, T., and Fukui, M. 2002. Phylogenic characterization of microbial mats and streamers from a Japanese alkaline hot spring with a thermal gradient. *J. Gen. Appl. Microbiol*. 48: 211–222.

Neefs, J-M., Van De Peer, Y., Hendriks, L., De Wachter, R. 1990. Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Resarch*, 18: 2237-2317

Nübel, U., Garcia-Pichel, F., and Muyzer, G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol*. 63: 3327–3332.

Nübel, U., Garcia-Pichel, F., Kühl, M., Muyzer, G. 1999. Quantifying microbial diversity: morphotypes 16S rRNA genes and carotenoids of oxygenic phototrophs in microbial mats. *Appl. Environ. Microbiol*. 65, 422–430.

Nusslein, B., Chin, K.J., Eckert, W., Conrad, R. 2002. Evidence for anaerobic syntrophic acetate oxidation during methane production in the profundal sediment of subtropical Lake Kinneret (Israel). *Environ. Microbiol*. 3, 460–470.

Olsen, G. J., Lane, D.J., Giovannoni, S. J., Pace, N. R., Stahl, D.A. 1986. Microbial ecology and evolution: a ribosomal RNA approach. *Annu. Rev. Microbiol*. 40: 337–365.

Orita, M., Suzuki, Y., Sekiya, Y., Hayashi, K. 1989. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5, 874–879.

Øvreas, L., Jensen, S., Daae, F. L., Torsvik, V. 1998. Microbial community changes in a perturbed agricultural soil investigated by molecular and physiological approaches. *Appl. Environ. Microbiol*. 64, 2739–2742.

Paul J. H. 1996. Carbon cycling: molecular regulation of photosynthetic carbon fixation. *Microb. Ecol*. 32: 231–245

Ranjard, L., Poly, F., Nazaret, S. 2000. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. *Res Microbiol* 151:167–177

Riemann, L., Middelboe, M. 2002. Stability of bacterial and viral community composition in Danish coastal waters as depicted by DNA fingerprinting techniques. *Aquat. Microb. Ecol*. 27, 219–232.

Riemann, L., Winding, A. 2001. Community dynamics of free-living and particle-associated bacterial assemblages during a freshwater phytoplankton bloom. *Microb. Ecol*. 42, 274–285.

Santangelo, J. D., Durre, P.,. Woods, D. R. 1995. Characterization and expression of the hydrogenase-encoding gene from *Clostridium acetobutylicum* P262, *Microbiology* 141 pp. 171–180.

Schauer, M., Balague´, V., Pedros-Alio, C., Massana, R. 2003. Seasonal changes in the taxonomic composition of bacterioplankton in a coastal oligotrophic system. *Aquat. Microb. Ecol*. 31, 163–174.

Schmalenberger, A., Tebbe, C. C. 2003. Bacterial diversity in maize rhizospheres: conclusions on the use of genetic profiles based on PCR-amplified partial small subunit rRNA genes in ecological studies. *Mol. Ecol*. 12, 251–262.

Schwarz, J. I. K., Eckert, W., Conrad, R. 2007. Community structure of *Archaea* and *Bacteria* in a profundal lake sediment Lake Kinneret (Israel). *Systematic and Applied Microbiology* 30:239–254.

Shimizu, T., Ohshima, S., Ohtani, K., Hoshino, K., Honjo, K., Hayashi, H. 2001. Sequence heterogeneity of the ten rRNA operons in *Clostridium perfringens*. *Syst Appl Microbiol* 24 pp. 149–156.

Smith, C. J., Nedwell, D. B., Dong, L. F., Osborn, A. M. 2007. Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. *Applied Environmental Microbiology*. 73: 3612-3622.

Speksnijder, A.G.C.L., Kowalchuk, A., De Jong, S., Kline, E., Stephen, J.R., Laanbroek, H. J. 2001. Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences. *Applied and Environmental Microbiology*, 67(1): 469-472

Spiegelman, D. Whissell, G., Greer, C. W. A survey of the methods for the characterization of microbial consortia and communities. *Can J Microbiol* 2005;51:355–86.

Stackebrandt, E. and Goebel, B. M. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology* 44, 846-849.

Stackebrandt, E., Pukall, R. 1999. Response to Althoff et al. (Mar Biol 130: 529-536). Deriving taxonomic decisions from 16S rDNA: a case study. *Marine Biology*, 133: 159-161

Stahl, D. A., Lane, D. J., Olsen, G. J., Pace, N. R. 1984. Analysis of hydrothermal vent-associated symbionts by ribosomal RNA sequences. *Science* (Washington, D.C.), 224: 409.

Stahl, D. A., Lane, D. J., Olsen, G. J., Pace, N. R. 1985. Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. *Appl. Environ. Microbiol*. 49: 1379–1384.

Stamper, D. M., Walch, M., Jacobs, R. N. 2003. Bacterial population changes in a membrane bioreactor for graywater treatment monitored by denaturing gradient gel electophoretic analysis of 16S rRNA gene fragments. *Appl. Environ. Microbiol.* 69:852-860.

Stephen, J. R., Chang, Y.-J., Gan, Y. D., Peacock, A., Pfiffner, S. M., White, D. C., Barcelona, M. J., and Macnaughton, S. J. 1999. Microbial characterization of a JP-4 fuel-contaminated site using a combined lipid biomarker/polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE)-based approach. *Environ. Microbiol.* 1: 231–241.

Steward, G. F., Zehr, J. P., Jellison, R., Montoya, J. P., Hollibaugh, J. T. 2004. Vertical distribution of nitrogen-fixing phylotypes in a meromictic, hypersaline lake. *Microbial Ecology*. 47: 30-40.

Stoesser, G., Baker, W., van den Broek, A., Camon, E., Garcia-Pastor, M., Kanz, C., Kulikova, T., Leinonen, R., Lin, Q., Lombard, V., Lopez, R., Redaschi, N., Stoehr, P., Tuli, M. A., Tzouvara, K., Vaughan, R. 2002. The EMBL Nucleotide Sequence Database. *Nucleic Acids Research* 30, 21-26.

Ueda, K., Seki, T., Kudo, T., Yoshida, T., Kataok, M. 1999. Two Distinct Mechanisms Cause Heterogeneity of 16S rRNA. *Journal of Bacteriology*, 181:78–82

Van de Peer, Y., De Rijk, P., Wuyts, J., Winkelmans, T., De Wachter, R. 2000. The European small subunit ribosomal RNA database. *Nucleic Acids Research* 28, 175-176.

van Hannen, E. J., von Agterveld, M. P., Gons, H. J., Laanbroek, H. J. 1998. Revealing genetic diversity of eukaryotic microorganisms in aquatic environments by denaturing gradient gel electrophoresis. *J. Phycol*. 34, 206–213.

Wawer, C., Jetten, M. M., Muyzer, G., Genetic diversity and expression of the [NiFe] hydrogenase large-subunit gene of *Desulfovibrio* spp. in environmental samples. *Appl. Environ. Microbiol.* 63 (1997), pp. 4360–4369.

Wenderoth D. F. and. Abraham W-R. 2005. Microbial indicator groups in acidic mining lakes. *Environ. Microbiol 7 (1), 133–139*

Wertz, S., Poly, F., Le Roux, X., Degrange, V. 2008 Development and application of a PCR-denaturing gradient gel electrophoresis tool to study the diversity of Nitrobacter-like nxrA sequences in soil. FEMS *Microbiol Ecol* 63:261–271

Widjojoatmodjo M N, Fluit A C, Verhoef J. 1995. Molecular identification of bacteria by fluorescence-based PCR–single-strand conformation polymorphism analysis of the 16S rRNA gene. *J Clin Microbiol*. 33:2601–2606

Woese, C.R., 1987. Bacterial evolution. *Microbiol. Rev*. 51, 221–271.

Xing, D. F., Ren, N. Q., Wang, A. J., Zhao, Q. L., Feng, Y. J., Ma, F. 2008. Continuous hydrogen production of auto-aggregative *Ethanoligenens harbinense* YUAN-3 under non-sterile condition, *Int. J. Hydrogen Energ.* 33 pp. 1489–1495

Yannarell, A. C., Triplett, E. W. 2005. Geographic and environmental sources of variation in lake bacterial community composition. *Appl. Environ. Microbiol*. 71, 227–239.

Zhou, J., Xia, B., Treves, D. S., Wu, L.-Y., Marsh, T. L., O'Neill, R. V., Palumbo, A. V., Tiedje, J.M. 2002. Spatial and resource factors influencing high microbial diversity in soil. *Appl Environ Microbiol* 68 pp. 326–334

Zuckerkandl, E. and Pauling, L. 1965. Molecules as documents of evolutionary history. *Journal of Theoretical Biology* 8, 357-366.

Zwart, G., Crump, B. C., Kamst-van Agterveld, M. P., Ferry, H., Suk-Kyun, H. 2002. Typical freshwater bacteria: an analysis of available 16S rRNA sequences from plankton of lakes and rivers. *Aquat. Microb. Ecol*. 28, 141–155.

PART 3

EXPERIMENTAL WORK

Chapter III: Vertical distribution of bacterioplankton in Lake Averno in relation to water chemistry

III.1. Introduction

Prokaryotes are key organisms in lakes as they play an important role in the biogeochemical cycling of elements such as carbon and nitrogen (Wetzel, 2001). Furthermore, these microorganisms play a relevant role in processes controlling the water quality of lacustrine ecosystems since they are involved in biodegradation of pollutants deriving from urban, industrial and agricultural activities (Briée et al., 2007). Investigations on the relationships between the diversity of microbial community and environmental factors offer useful information which lead to a better understanding of the process of biogeochemical cycling of nutrient elements and offer the possibility to predict ecosystem responses to environmental changes (Fuhrman et al., 2006). In fact, bacterioplankton community composition (BCC) is clearly affected by certain environmental factors, such as pH, water temperature, water chemistry, nutrient condition, geographical and seasonal variations (Lindstrom et al., 2005; Hahn, 2006; Zeng et al., 2009).

Several studies have investigated the depth-related changes in entire microbial communities in lakes water column (Øvreas et al., 1997; Bosshard et al., 2000; Hollibaugh et al., 2001; Humayoun et al., 2003; De Wever et al., 2005; Jiang et al., 2008; Dimitriu et al., 2008;). However, only a few studies have related these changes to the depth gradient of physicochemical properties taking into account a considerable number of environmental factors (Salcher et al., 2008; Wei et al., 2008; Zeng et al., 2009) and using multivariate methods to correlate them with BCC composition (Wei et al., 2008).

In the present study, the *in situ* distribution, abundance, and diversity of prokaryotes along the water column of Lake Averno, a volcanic lake close to Naples (Southern Italy), were investigated. Furthermore, the relationships between changes in BCC and some environmental parameters were explored to identify which factors have a significant impact on the BCC vertical distribution. The microbial ecology of Lake Averno is of interest because it is an almost water closed basin, polluted by waste waters from nearby communities and agricultural activities, in which water overturn occurred occasionally in the past, causing an increase in toxic levels of H_2S with consequently a fish kill event (Caliro et al., 2008). Previously reported data concerning depth-related changes in the physico-chemical parameters of Lake Averno water column, indicate that it generally comprises two distinct

water masses, an oxic epilimnion and an anoxic hypolimnion with evident zonation of the microbial processes along the entire water column. Breaking of the vertical/thermal stratification occurs in the winter periods when temperatures of epilimnetic waters remain below 7 °C for a long time (Caliro et al., 2008). However, no investigations have been carried out on the BCC of this lake. In the present study, the diversity of bacterial and archaeal communities along the stratified water column has been studied by means of DGGE technique. The correlation between the presence of specific taxonomic groups and some physico-chemical parameters, such as temperature (T), pH, redox potential (Eh) and water chemistry has been investigated using the principal component analysis (PCA). Moreover, the isolation and sequencing of DGGE bands offered information concerning the phylogenetic characterization of *Eubacteria* and *Archaea* occurring naturally in Lake Averno.

III.2. Material and methods

III.2.1. Site and sampling

Lake Averno fills the mouth of one of several volcanic craters in the Phlegrean Field region, about 15 Km West of Naples, in Southern Italy (40° 50' N, 14° 04' E, 1.10 m above sea level). It is almost elliptic (100 m long and 700 m wide) covering a surface of 0.54 $Km^{2,}$ and is 34 m deep in the centre, with a total volume of approximately $6x10^6$ m^3. A canal, almost 1 km long, links the lake to the sea. The chemical and isotopic composition of its waters suggests that it originates from mixing of shallow waters with a Na-Cl hydrothermal component coupled with an active evaporation process. The lake is normally stratified, with an oxic epilimnion (from the surface to 6 m depth) and anoxic hypolimnion (from 6 m to 33 m). The water overturn is an unusual phenomenon, occurring in the winter periods, when temperatures of epilimnetic waters remain below 7°C for a long time (Caliro et al., 2008). Over the last few years, the surrounding area has been subjected to considerable human impact, such as construction works, recreation facilities and agriculture. In addition, Lake Averno has been affected, for a long time, by intermittent organic enrichment from sewage discharges (Improta et al., 2004).

Water samples (250 ml) were collected from various depths (1, 3, 5, 6, 9, 15, 21, 27, 32, 33 m below the surface) at the site of maximal depth. Sampling was carried out in May 2006, using a Niskin water sampler. The samples collected were kept in a cooler until processed

(within 6 h from sampling). Samples were filtered through 0.22 µm pore-size membranes (diameter 50 mm, Millipore) to recover the microbial biomass for subsequent DNA extraction. Filtres were placed in cryovials and stored at -80°C until processed.

III.2.2. Physico-chemical analyses of water column

All the physicochemical analyses of water column were performed by the group of Environmental Biology and Nature Conservation of ENEA.

The temperature, conducibility, Eh and pH were measured by means of a multiprobe logger (Hydrolab CTD). The nominal precisions are as follows: depth ± 0.05 m; Eh ± 20 mV; T ± 0.01°C; pH ± 0.05 pH-units. The samples for nutrients and SO_4^{2-} were immediately transferred to 500 ml flasks and taken to the laboratory in cool boxes, filtered through a 0.22 µm cellulose acetate membrane (GSW-Millipore) and stored at -18°C until processed. Nutrient concentrations were measured with a spectrophotometric technique, using the automated colourimetric analysis Easychem Plus (Systea Scientific LCC). In particular, orthophosphates were determined by ascorbic acid reduction, nitrites by the colourimetric method of sulphanylamide diazotization, nitrates by a cadmium-reducing column and ammonia by the phenate method (A.P.H.A., 2005). SO_4^{2-} was assessed by ion chromatography. For sulphide analysis, oxygen-free 125 ml serum bottles, sealed with butyl rubber stoppers and containing 0.4 ml 2M zinc acetate solution, were filled completely with water samples and stored at 4°C. Sulphide was assessed by the iodimetric method (A.P.H.A., 2005). Water sampling and analysis for the methane, CO_2, $HCO_3^-$and SO_4^{2-}determination were performed using a method described by Caliro et al., (2008).

III.2.3. DNA extraction and PCR amplification

Total DNA was extracted from each filtre containing bacterial cells using the phenol/chloroform/isoamyl alcohol extraction and sodium acetate-ethanol precipitation methods described by Vetriani et al., 2003. The pellet of nucleic acid was resuspended in sterile deionized water, to a final volume of 200 µl.

Crude extracts were further purified using the Wizard DNA clean-up system (Promega) according to the supplier's instructions. Quantity and purity of DNA were checked fluorospectrometrically by NanoDrop (NanoDrop Technologies).

A nested PCR approach was used to amplify eubacterial and archaeal 16S rRNA gene fragments from total DNA samples. For members of the *Eubacteria* domain, a first PCR amplification was performed with 20 ng of DNA using the forward primer 27F and the reverse primer 1495R (Table 4) as described by Di Cello et al., (1997). Dilutions 1:100 (2 µl) of PCR products of 1450 bp were then used as template for nested PCR using the forward primer 63F-GC-clamp and the reverse primer 518R (Table 1) to produce 495 bp fragments suitable for DGGE analysis (El Fantroussi et al., 1999). Both PCRs were performed in Qiagen *Taq* buffer (10X) containing 1.5 mM $MgCl_2$, with 150 ng of each primer, 250 µM (each) deoxynucleoside triphosphates, and 0.5 U of *Taq* DNA polymerase (Qiagen) in 25 µl reaction volume. Cycling parameters for PCR with primers 27F-1495R and with primers 63F-GC and 518R were as those previously described by Di Cello et al. (1997) and El Fantroussi et al. (1999), respectively.

Amplification of the almost-complete archaeal 16S rRNA gene was carried out with 20 ng of total DNA using the primers S-D-Arch-0025-a-S-17) and S-*-Univ-1517-a-A-21 (Table 1) (Vetriani et al., 1999). In contrast to the original procedure, a touchdown thermal program was used, with an initial denaturation step (95°C, 2 min), followed by 30 cycles of touchdown amplification [denaturation (94°C, 30 s), annealing (56°C, 30 s with 0.5°C decrease each cycle to 45°C) and extension (72°C, 30 s)], 5 cycles of amplification [denaturation (94°C, 30 s), annealing (45°C, 30 s) and extension (72°C, 30 s)], and a final extension (7°C, 7 min). A dilution 1:100 of this PCR product (1500 bp) was used as template (2 µl) for the amplification of a 456-bp fragment using the primers A934F and 1390R-GC (Table 1) and the touchdown thermal program previously described by Wu et al. (2001). Both archaeal PCRs were performed in Qiagen *Taq* buffer (10X) containing 1.5 mM $MgCl_2$, with 0.1 µM (each) of primers, 200 µM (each) of deoxynucleoside triphosphates and 2.5 U of *Taq* DNA polymerase (Qiagen) in 25 µl reaction volume.

Primer [a]	Sequence (5' to 3')	Specifity
27F	GAGAGTTTGATCCTGGCTCAG	Most *Bacteria*
1492R	CTACGGCTACCTTGTTACGA	Most *Bacteria*
63F-GC [b]	AGGCCTAACACATGCAAGTC	Most *Bacteria*
518R	ATTACCGCGGCTGCTGG	Most *Bacteria*
S-D-Arch-0025-a-S-17	CTGGTTGATCCTGCCAG	Most *Archaea*
S-*-Univ-1517-a-A-21	ACGGCTACCTTGTTACGACTT	Most *Archaea*
A934F	AGGAATTGGCGGGGGAGCA	Most *Archaea*
1390R-GC [c]	ACGGGCGGTGTGTGCAA	Most *Archaea*

Table 1. Primers used in this study.

a. Target gene of all primers used in this study is 16S rRNA
b. This primer has the following GC clamp at its 5'end:
5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG3' (Øvreas et al.,1997)
c. This primer has the following GC clamp at its 5'end: 5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGC3' (Wu et al., 2001)

III.2.4. DGGE

Eubacterial fragments were resolved by double gradient denaturing gradient gel electrophoresis (DG-DGGE) as described by Cremonesi et al., (1997), in a DCode universal mutation detection system (Bio-Rad). A 6%-12% polyacrylamide (acrylamide: N,N-methylenebisacrylamide, 37.5:1) gel with denaturing gradient ranging from 30% to 60% was used. For the domain *Archaea*, PCR products were screened by DGGE as reported by Muyzer et al., (1993). PCR samples were loaded onto 6% polyacrylamide gel prepared with a denaturant gradient ranging from 40% to 60%. A 100% denaturing solution contained 7 M urea and 40% deionized formamide. Approximately 700 ng of the purified PCR products were loaded in each well. The gels were run for 16 h at 75 mV in 1X TAE buffer at 60°C, stained with 50 μg/ml ethidium bromide for 30 min, destained in water and photographed with the UVIpro Platinum Gel Documentation System (GAS7500/7510).

III.2.5. Cluster analysis and diversity indices

The statistical comparison of the different DGGE patterns, run on the same gel, was made with Quantity One software package (Bio-Rad). The similarity between the band patterns was calculated using the Dice coefficient and, the clustering analysis was performed with the unweighted pair-group method using arithmetic averages (UPGMA) for dendrogram construction. Relative signal intensities of detected bands, in each gel track, were determined using the Quantity One software package (Bio-Rad) and calculated from the peak area of the densitometric curves.

The number of DGGE bands present in each sample was used to measure the richness index (*R*). The Shannon-Weaver index of general diversity (*H'*) was calculated using the following equation (Shannon and Weaver, 1963; Andreoni et al., 2004):

$$H' = -\sum P_i \log P_i$$

The Simpson index of dominance, *S* (Simpson, 1949) was also calculated using the following function:

$$S = \sum P_i^2$$

For both indices P_i was the relative signal intensity of the bands in a track.

Correlations between physical and chemical parameters and all the above-mentioned indexes of biodiversity were performed using PCA with the Unscrambler 9.8 software package (PCA, Unscrambler® version 9.8, CAMO 2008). PCA was also used to define the relationships between bacterial shifts, as determined by DGGE, and environmental variables. All DGGE bands were treated as "passified variables" in order to study their correlation with environmental variables, and to avoid their possible influence as category variables on the PCA model (Martens and Martens, 2001).

III.2.6. Extraction, sequencing and phylogenetic analysis of DGGE bands

Prominent DGGE bands were excised from the gels with a sterile scalpel, transferred into 50 µL sterile water and incubated overnight at 4 °C to allow diffusion of the DNA. Eluted DNA from each band was reamplified as described above and submitted again to a DGGE run to confirm electrophoretic mobility.

Sequencing reactions were prepared from PCR products amplified with unclamped primers using an Applied Biosystem Big Dye® Terminator sequencing kit version 3.1, according to the manufacturer's instructions and analysed using a 3730 DNA Analyzer Applied Biosystem apparatus. Each sequence was submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU) to detect the presence of possible chimeric artifacts. Sequence similarity searches were performed using the BLAST network service of the NCBI database and Seqmatch tool of the RDP (http://www.ncbi.nlm.nih.gov/BLAST/ and http://rdp.cme.msu.edu/, respectively). For the phylogenetic analysis, identification of 16S rRNA gene sequences was performed using the RDP Classification Algorithm (http://rdp.cme.msu.edu/classifier/classifier.jsp). Phylogenetic trees showing the relationships between sequences of DGGE bands and reference sequences obtained by BLAST analysis were constructed using Neighbour Joining with Kimura 2 parameter distances in MEGA software version 4 (Tamura et al. 2007).

Nucleotide accession number

The sequences generated in this study have been deposited in the Gen Bank database under accession numbers. GU224058 to GU224089

III.3. Results

III. 3.1. Physico-chemical parameters of the lake

Results of the physico-chemical analysis of the Lake Averno water column are reported in Table 2. The vertical profiles of T, pH and dissolved oxygen (DO) indicated that this lake, in May 2006, was made up of two distinct water masses. The DO concentration decreased rapidly from 3 to 9 m depth. However, complete hypolimnetic anoxia was not reached at the bottom of the lake. The interface between the oxic and anoxic layer is characterized by strong variations in redox potential (Eh), T and pH. The nitrate concentration was approximately 1 μM in the entire water column with the exeption of a maximum value at 5 m, where the vertical gradient of DO decreased, and at 9 m, where the vertical gradient of Eh increased slightly. Nitrite concentration was detectable only at a depth of 9 and 33 m. Ammonia concentration increased towards the bottom with the highest value being 643 μM at 33 m and it displayed a concave up distribution between 15 and 5 m. Inorganic phosphorus showed an identical profile of ammonia with the highest value at 33 m and a step gradient between 15 m and 9 m. Methane concentration showed a progressive increase from the upper water column to the bottom. Sulphide concentration increased with depth reaching a maximum value of 1507.7 μM at 33 m, whereas sulphate concentrations showed constant values with the exception of a slight decrease at 3 m and a sharp decrease at 32 and 33 m. HCO_3^- also showed a slight increase, from 1 to 6 m, in the epilimnion, a constant concentration from 6 to 22 m, and a sharp increase at 33 m. pCO_2 values varied between 0.22 and 4.61 mbar in the epilimnion and between 8.18 and 10.5 mbar, from 9 to 32 m, with the exception of a value of 14.6 mbar measured at a depth of 15 m. The maximum pCO_2 value (45.69 mbar) was observed at 33 m.

	Samples									
	1 m	3 m	5 m	6 m	9 m	15 m	21 m	27 m	32 m	33 m
T (°C)	26.0	22.5	14.6	11.9	9.8	9.0	8.8	8.7	8.8	9.1
pH	9.19	9.15	8.01	7.70	7.73	7.59	7.56	7.55	7.54	7.00
Eh (mV)	117.2	128.9	178.9	-184.9	-165.8	-298.0	-300.0	-300.00	-301.8	-305.2
DO (mM)	352.8	531.2	98.3	14.24	10.05	7.89	7.11	6.65	5.94	4.69
E.cond.(mS/sec)	2.84	2.85	2.96	3.00	3.02	3.08	3.08	3.08	3.08	3.48
NO_2^- (µM)	0.0	0.0	0.0	0.0	3.0±0.03	0.0	0.0	0.0	0.5±0.02	1.5±0.05
NO_3^{2-}(µM)	1.0±0.02	0.8±0.01	1.5±0.01	0.8±0.01	3.8±0.03	1.1±0.02	0.8±0.02	0.9±0.03	0.8±0.02	1.2±0.03
NO_2^-+NO_3^{2-}	1.0	0.8	1.5	0.8	6.8	1.1	0.8	0.9	1.3	2.7
NH_4^+ (µM)	3.2±0.01	3.1±0.11	6.3±0.22	16.5±0.27	77.0±1.01	171.0±16.5	160.6±8.18	179.0±2.39	328.4±12.8	643.1±15.1
N_{OX}/N_{RID}	0.31	0.26	0.23	0.05	0.09	0.01	0.00	0.01	0.00	0.00
PO_4^{3-} (µM)	0.3±0.01	0.3±0.01	0.4±0.02	0.4±0.01	0.4±0.03	3.3±0.01	3.4±0.01	3.4±0.01	9.2±0.01	25.7±0.23
H_2S(µM)	11.3	92.8	59.9	5.75	5.81	76.9	65.5	79.8	347.8	1507.7
SO_4 (mM)	1.8	1.7	1.8	1.8	1.8	1.8	1.8	1.9	1.4	0.3
HCO_3 (mM)	4.3	4.3	5.4	6.1	6.3	6.4	6.5	6.5	7.2	11.2
CH_4 (µM)	0.37	0.54	0.9	1.46	2.08	17.4	22.6	31.3	304.2	2958.8
pCO_2 (mb)	0.22	0.21	1.72	4.61	8.18	14.6	6.29	6.60	10.5	45.7

Table 2. Physico-chemical properties along Lake Averno water column

III.3.2. DGGE fingerprints and PCA

The eubacterial and archaeal communities recovered at various depths showed different DGGE profiles based on the number and distance of migration of the PCR products (Fig. 1A and Fig. 1B). In the DGGE profile, these correspond to 16S rRNA fragments that differ in nucleotide sequence. Thus, the bands reflect distinct numerically dominant microbial populations in the community.

For both domains, the microbial communities were divided into two main groups as a function of depth: eubacterial patterns of the upper samples (1 to 9 m) clustered together and were separated from those of the zone from 15 to 33 m, archaeal profiles of the anoxic zone (6 to 33 m) were grouped into a cluster distinct from that comprising samples from 1, 3 and 5 m depths (Fig. 1A and Fig. 1B). The averages of all similarity coefficients among eubacterial and archaeal communities were 0.66 ± 0.16 and 0.45 ± 0.33, respectively.

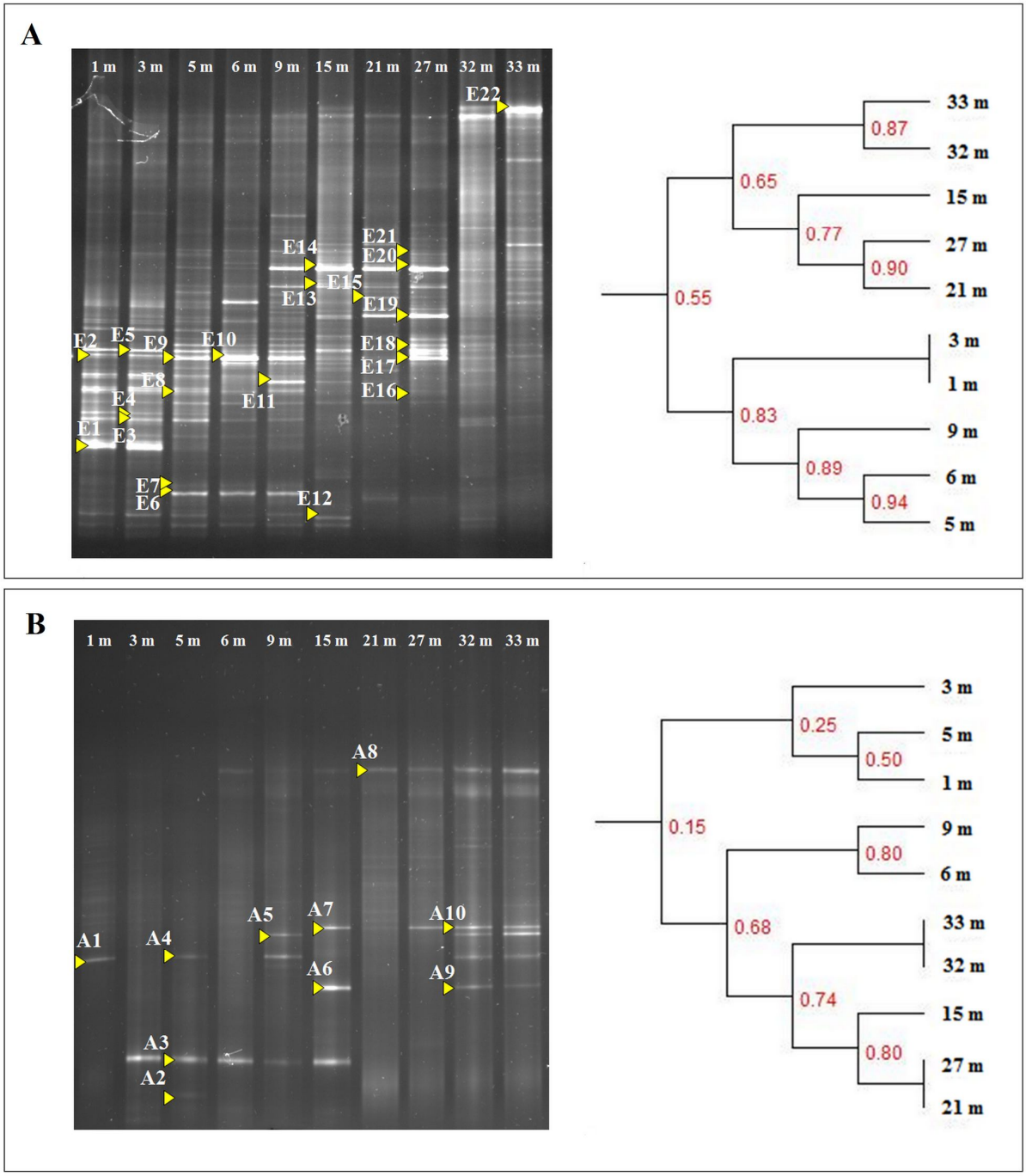

Figure 1. DGGE fingerprints of 16S rDNA fragments and cluster analysis by UPGMA of eubacterial (A) and archaeal (B) communities from different depths of Lake Averno water column. Arrows indicate sequenced bands.

The diversity indices from all the DGGE patterns were also calculated (Table 3). The values of the richness index (*R*) ranged from 29 to 39 in the eubacterial communities, whereas lower values, from 1 to 7 were observed in the archaeal communities. Dominance (*S*) and the general index of biodiversity (*H'*) were calculated on the basis of the number and relative intensities of bands on the gel track. *Eubacteria* appeared to show the highest microbial diversity ($1.38<H'<1.56$) associated with the lowest concentration of dominance ($0.031<S<0.051$). Conversely, *Archaea* exhibited the lowest diversity indexes ($0<H'<0.82$) associated with high dominance indexes ($0.15<S<1$) (Table 3). In the *Eubacteria* banding patterns, *H'* remains relatively constant throughout the water column, while for *Archaea* it tends to increase with the depth (Fig. 2).

Sample	Richness index (*R*)		Shannon diversity (*H'*)		Simpson index (*S*)	
	Eubacteria	*Archaea*	*Eubacteria*	*Archaea*	*Eubacteria*	*Archaea*
1 m	29	1	1.38	0	0.051	1
3 m	29	1	1.41	0	0.044	1
5 m	36	3	1.52	0.36	0.032	0.51
6 m	34	4	1.5	0.34	0.034	0.6
9 m	39	6	1.56	0.66	0.031	0.25
15 m	31	6	1.43	0.54	0.042	0.32
21 m	29	3	1.4	0.44	0.046	0.39
27 m	29	4	1.38	0.5	0.048	0.37
32 m	35	7	1.51	0.82	0.033	0.15
33 m	34	7	1.48	0.78	0.037	0.18

Table 3. Diversity indexes calculated from eubacterial and archaeal DGGE fingerprints shown in Fig. 1A and B.

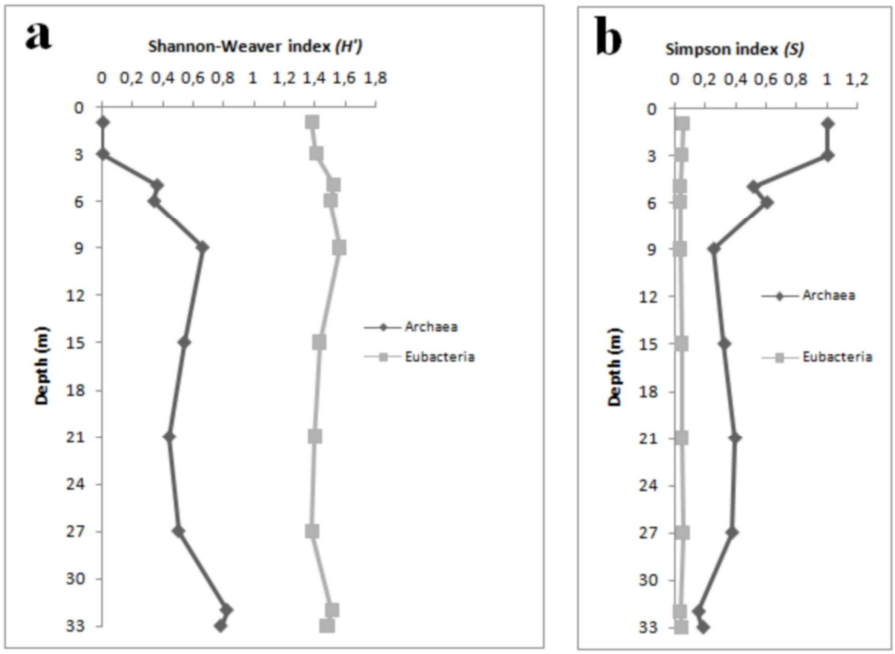

Figure. 2. Vertical changes of Shannon-Weaver index of diversity (a) and Simpson index of dominance (b) based on the number and relative intensities of the bands identified by DGGE of PCR-amplified 16S rRNA.

PCA was used to correlate diversity indices with environmental parameters. The first two principal components (PC1 and PC2) were able to explain 83–11% and 81–12% of the variance for the eubacterial (Fig. 3A) and archaeal (Fig. 3B) samples, respectively. The indices *R*, *S* and *H'* of *Eubacteria* did not significantly influence on the first 2 components. *H'* and *R* were positively correlated but showed a negative correlation with *S*. *H'* and *R* measured from archaeal banding patterns were positively correlated with the PC1, while *S* was negatively correlated. *S* was positively correlated with the DO, T and pH, influencing primarly the distribution of samples D1 and D3, while *H'* and *R* were strictly correlated with depth, Eh, HCO_3^-, $NH4^+$, pCO_2 and Nox/Nred, influencing the distribution of samples D32 and D33 in the score plot.

The results of PCA applied to environmental variables and to the presence/absence of eubacterial and archaeal bands within 16SrDNA patterns are shown in Figs. 4A and 4B, respectively. Due to the use of passified variables (DGGE bands), PC1 and PC2 explained 83% and 11% of the total variance, for both eubacterial and archaeal domains. PCA separated the samples into 4 distinct groups, according to the physico-chemical characteristics of the water column. Significant correlations between some groups of eubacterial and archaeal populations and some environmental factors were found, reflecting the separation of the upper layers (1 m, 3 m, 5 m, corresponding to D1-D3-D5) and deeper samples (32 m, 33 m corresponding to D32-D33) in two main individual clusters. PCA revealed that eubacterial upper samples are separated from the others due to their positive relationship with the physico-chemical parameters such as T, DO and pH, which, in turn, showed the same correlation loading (on PC1) of some specific eubacterial DGGE bands of the oxic layer (1 to 9 m) (Fig. 4A). On the contrary, the complete absence of a relationship between these physico-chemical parameters and the archaeal populations revealed that samples D1, D3, and D5 were correlated mainly on the basis of the similarity of their physico-chemical characteristics (Fig 4B).

Deeper samples (D32 and D33) were correlated with chemical parameters such as pCO_2 and concentration of CH_4 and H_2S, as well as with specific eubacterial and archaeal DGGE bands (Figs 4A and 4B).

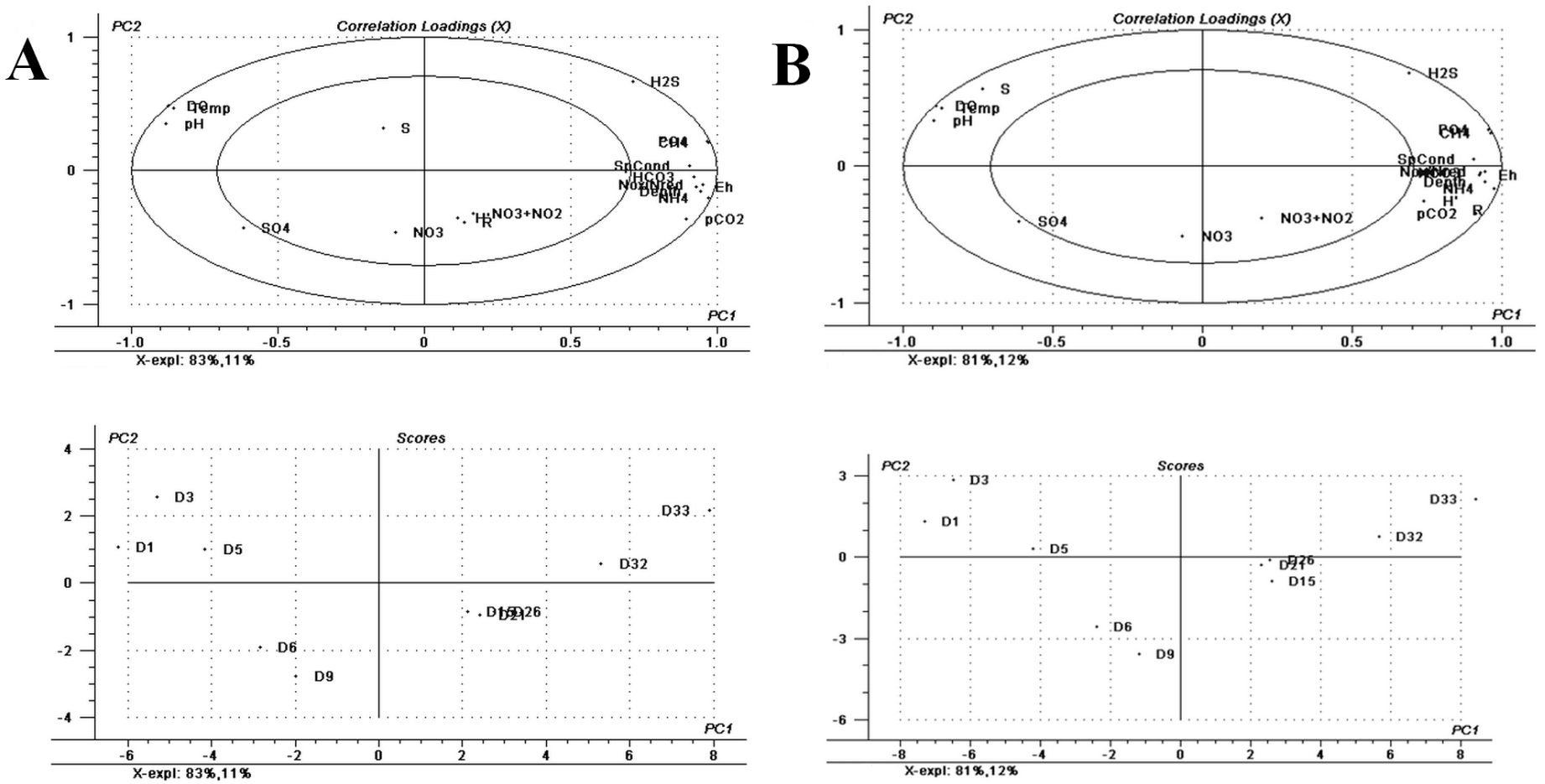

Figure 3. PCA results (for the first two principal components) of diversity indexes based on DGGE profiles and environmental parameters, showing Score plot and Correlation loadings of the *Eubacteria* (A) and *Archaea* (B) domains..

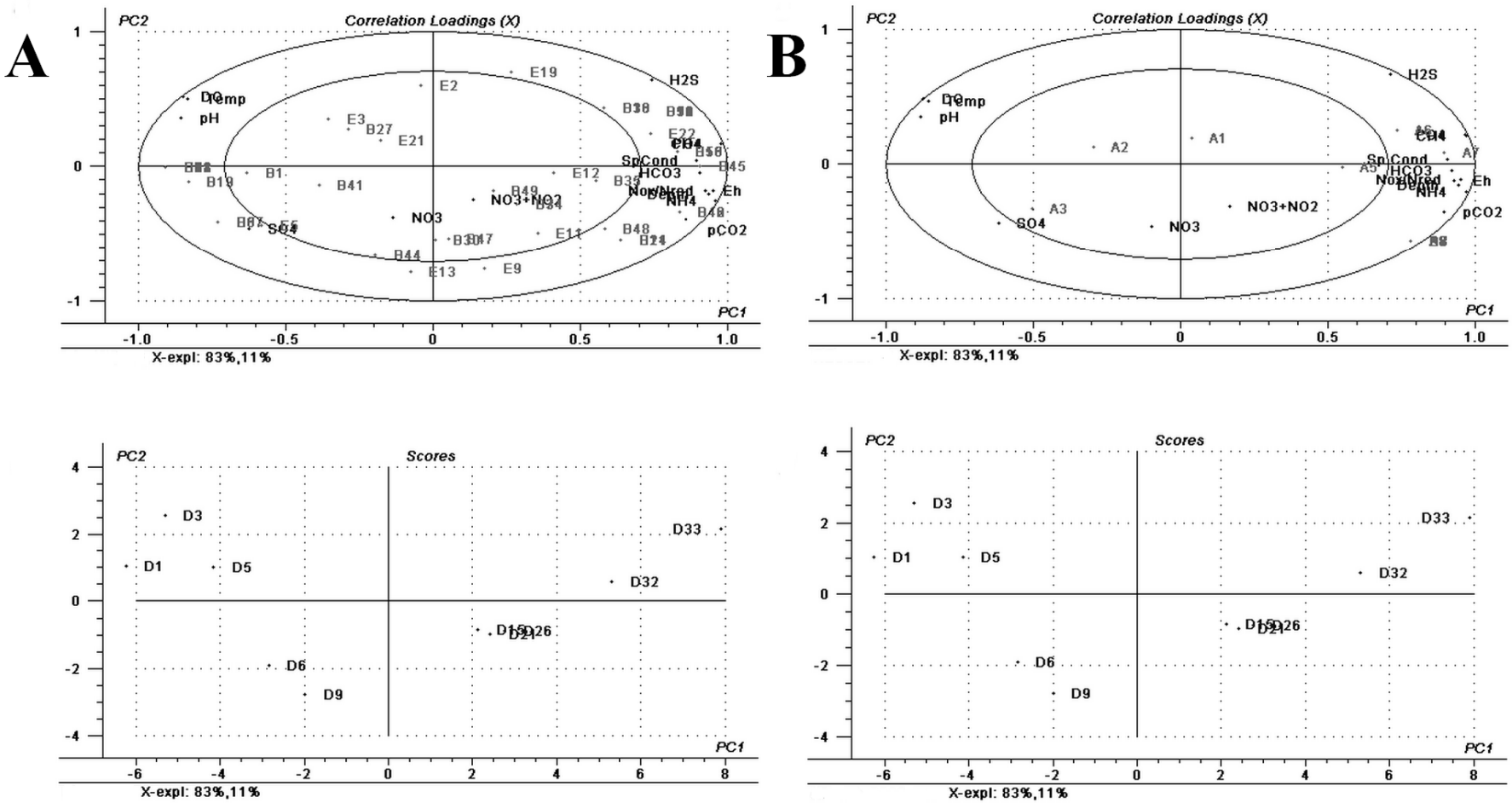

Figure 4. PCA results of the DGGE bands (in grey) and environmental parameters, showing Score plot and Correlation loadings of the *Eubacteria* (A) and *Archaea* (B) domains. DGGE bands were treated as passified variables. Eubacterial and archaeal sequenced DGGE bands are indicated by the letters E and A, respectively, whereas bands not sequenced are indicated by the letter B for both domains.

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III.3.3. Phylogenetic analysis

Of 44 excised eubacterial bands, 22 (from E1 to E22 in Fig. 1A) and 10 out of 22 archaeal bands (from A1 to A10 in Fig. 1B) produced useful and reliable sequences without ambiguous positions, showing close similarity (sequence identity ≥ 95%) to 16S rDNA sequences present in the NCBI databases, in most cases belonging to uncultured relatives (Table 4). These sequences, corresponding for each lane to an average of 40% and 85% of the total intensity of eubacterial and archaeal bands, respectively, have been included in the phylogenetic trees (Fig. 5 and Fig. 6) whereas all the other bands present in the gel were assigned to an "unidentified" class (Fig. 7A and 7B). Eubacterial sequenced bands were assigned to five phylogenetic groups (Fig. 5): sequences related to *δ Proteobacteria* (bands E6 and E7) were present in the upper part of the Lake (from 1 m to 9 m), while *Bacteroidetes* (band E22) and *Firmicutes* (band E12) were found only in the deepest part of the water column at a of depth 15 m. *β-proteobacteria* (bands E2, E5, E11, E13, E14, E15 E20 and E21) and *α Proteobacteria* (bands E1, E3, E4, E8, E9, E10, E16, E17, E18, and E19) were homogeneously distributed throughout the water column. All the archaeal DGGE bands analysed were affiliated to euryarchaeotal sequences. Within this phylum, phylogenetic analysis showed two clusters of methanogens, belonging to *Methanomicrobiales* (bands A1, A4, A5, A7, A10) and *Methanosarcinales* (bands A2, A3, A6, A8, A9) orders (Fig. 6).

Vertical changes in archaeal communities were observed in DNA-based DGGE analyses: bands A6 and A8 (affiliated with *Methanomethylovorans* and *Methanotrix*, respectively), and A7 (not affiliated with any known genus), were found only in the anaerobic depths analysed, while bands A1, A2 and A3 (with close similarity to the uncultured relatives, not identified at genus level) disappeared in the deepest part of the water column.

	Bands	Closest relative[a]	Accession no.	Identity	Band phylogenetic affiliations[b]		
					Order	**Family**	***Genus***
Eubacteria	E1	*Roseomonas fridaquae* strain CW67	EU290160	98%	*Rhodospirillales* [100%]	*Acetobacteraceae* [99%]	*Roseomonas* [58%]
	E2	Uncultured bacterium clone S23_830	EF572731	97%	*Burkholderiales* [100%]	*Incertae sedis* [100%]	*Ideonella* [70%]
	E3	Unidentified bacterium clone K2-S-3	AY344379	100%	*Rhodobacterales* [100%]	*Rhodobacteraceae* 100%]	*Rhodobacter* [49%]
	E4	Unidentified bacterium clone K2-S-3	AY344379	100%	*Rhodobacterales* [100%]	*Rhodobacteraceae* 100%]	*Rhodobacter* [74%]
	E5	Uncultured bacterium clone S23_830	EF572731	97%	*Burkholderiales* [100%]	*Incertae sedis* [100%]	*Ideonella* [67%]
	E6	Uncultured bacterium clone T106D	AM158388	99%	*Myxococcales* [78%]	*Nannocystaceae* [27%]	*Plesiocystis* [21%]
	E7	Uncultured bacterium clone T106D	AM158388	99%	*Myxococcales* [87%]	*Polyangiaceae* [44%]	*Sorangium* [23%]
	E8	Uncultured bacterium clone FRC-FBR-69d-10.31-96	DQ646440	99%	*Rhodobacterales* [100%]	*Rhodobacteraceae* [99%]	*Paracoccus* [78%]
	E9	Uncultured bacterium clone 3C003151	EU801779	100%	*Rhodobacterales* [100%]	*Rhodobacteraceae* [100%]	*Rhodobacter* [90%]
	E10	Uncultured bacterium clone 3C003151	EU801779	100%	*Rhodobacterales* [100%]	*Rhodobacteraceae* [100%]	*Rhodobacter* [95%]
	E11	Uncultured bacterium clone X-132	AM905635	99%	*Burkholderiales* [100%]	*Comamonadaceae* [100%]	*Malikia* [69%]
	E12	Uncultured bacterium clone E07	EF590062	98%	*Clostridiales* [96%]	*Ruminococcaceae* [96%]	*R. Incertae Sedis* [41%]
	E13	Uncultured bacterium clone RB7C2	AF407381	99%	*Burkholderiales* [100%]	*Comamonadaceae* [100%]	*Rhodoferax* [100%]
	E14	Uncultured bacterium clone RB7C2	AF407381	99%	*Burkholderiales* [100%]	*Comamonadaceae* [100%]	*Rhodoferax* [100%]
	E15	Uncultured bacterium clone d123	AF422672	100%	*Burkholderiales* [100%]	*Comamonadaceae* [100%]	*Simplicispira* [96%]
	E16	Uncultured *Rhodobacteraceae* TDNPBbc97_72_2_135	FJ516819	99%	*Rhodobacterales* 100%]	*Rhodobacteraceae* [100%]	*Rhodobacter* [69%]
	E17	Uncultured bacterium clone 3C003151	EU801779	99%	*Rhodobacterales*[100%]	*Rhodobacteraceae* [100%]	*Rhodobacter* [89%]
	E18	Uncultured bacterium clone 3C003151	EU801779	98%	*Rhodobacterales* 100%]	*Rhodobacteraceae* [100%]	*Rhodobacter* [96%]
	E19	Uncultured bacterium clone TH_a72	EU272948	99%	*Sphingomonadales*[100%]	*Sphingomonadaceae* [100%]	*Novosphingobium* [92%]
	E20	*Rhodoferax antarcticus* strain Fryx1	AY609198	98%	*Burkholderiales* [100%]	*Comamonadaceae* [100%]	*Rhodoferax* [100%]
	E21	Uncultured bacterium clone LE201B11	FJ694328	99%	*Burkholderiales* [100%]	*Comamonadaceae* [100%]	*Rhodoferax* [100%]
	E22	Uncultured bacterium D242_27F_BAC2_013	AB447719	97%	*Bacteroidales* [100%]	*Porphyromonadaceae* [100%]	*Paludibacter* [100%]
Archaea	A1	Uncultured archaeon clone F5	EU910621	99%	*Methanomicrobiales*[100%]	*Methanomicrobiaceae* [89%]	*Methanoculleus* [48%]
	A2	Uncultured archaeon clone L7A6	EF644786	100%	*Methanosarcinales* [94%]	*Methanosaetaceae* [54%]	*Methanothrix* [54%]
	A3	Uncultured archaeon clone L7A4	EF644784	100%	*Methanosarcinales* [99%]	*Methanosarcinaceae* [66%]	*Methanolobus* [26%]
	A4	Uncultured euryarchaeote clone F5	EU910621	99%	*Methanomicrobiales* [100%]	*Methanomicrobiaceae* [92%]	*Methanoculleus* [47%]
	A5	Uncultured euryarchaeote clone F18	EU910620	99%	*Methanomicrobiales* [100%]	*Methanomicrobiaceae* [99%]	*Methanoculleus* [80%]
	A6	*Methanomethylovorans* sp. Z1	EF174501	100%	*Methanosarcinales* [100%]	*Methanosarcinaceae* [100%]	*Methanomethylovorans* [100%]
	A7	Uncultured *Methanospirillaceae* ME-17	AB288683	96%	*Methanomicrobiales* 100%]	*Methanomicrobiaceae* [81%]	*Methanoculleus* [34%]
	A8	Uncultured bacterium clone mbII-a9	AB426163	100%	*Methanosarcinales* [100%]	*Methanosaetaceae* [100%]	*Methanothrix* [100%]
	A9	*Methanomethylovorans* sp. Z1	EF174501	99%	*Methanosarcinales* [100%]	*Methanosarcinacea*e [100%]	*Methanomethylovorans* [100%]
	A10	Uncultured euryarchaeote clone F5	EU910621	96%	*Methanomicrobiales* [100%]	*Methanomicrobiaceae* [77%]	*Methanofollis* [31%]

Table 4.

a. Sequence similarities between rRNA sequences of DGGE bands and those of the closest relatives retrieved from NCBI database

b. Identification performed using the RDP Classification Algorithm. Bootstrap confidence values are given in brackets (classification is well supported for confidence > 80%).

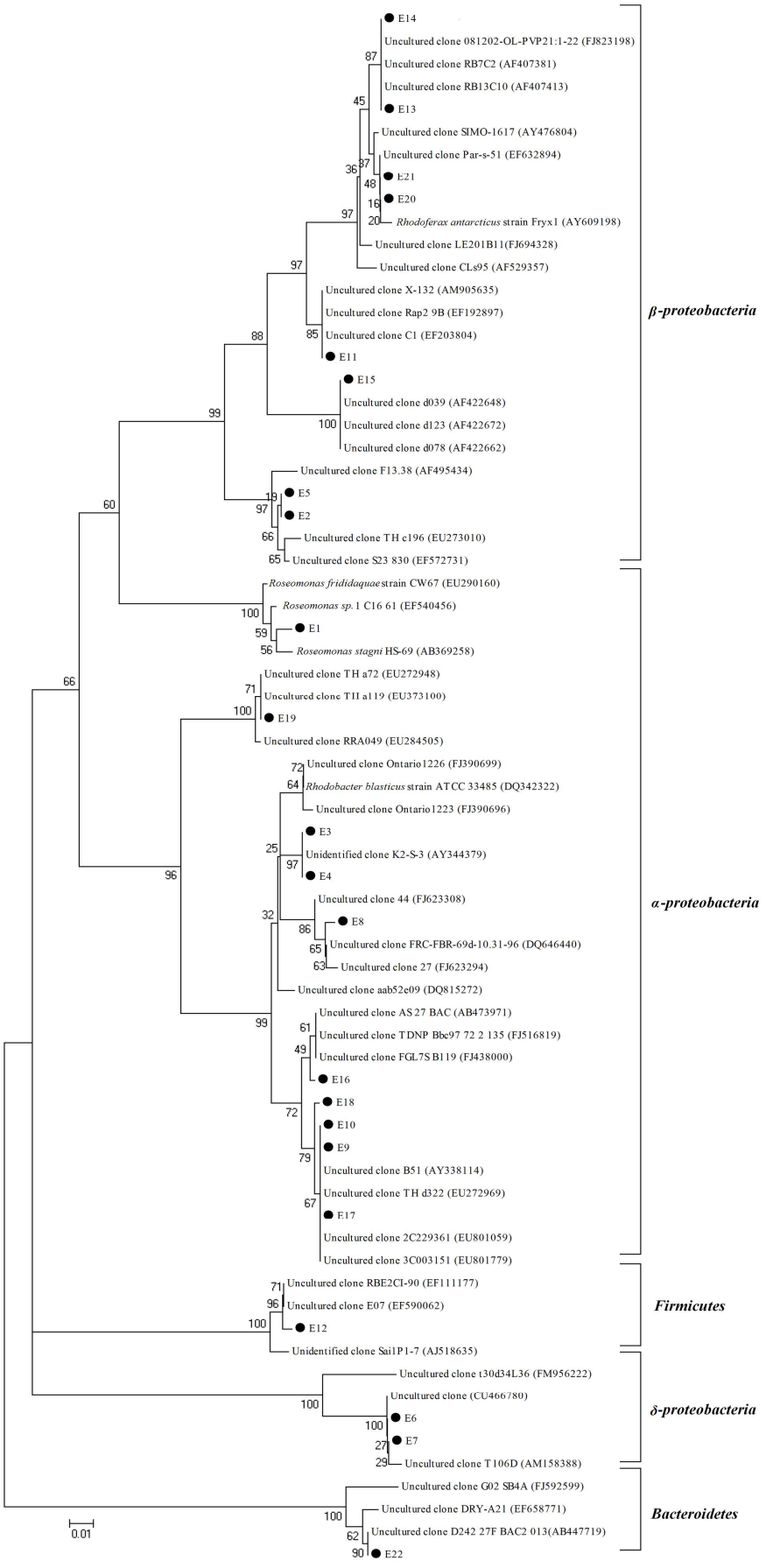

Figure 5. Phylogenetic tree based on the comparative analysis of the eubacterial 16S rDNA sequences from excised DGGE bands (see Fig. 1A) and their closest relatives. Black circles indicate the sequences obtained in this study..

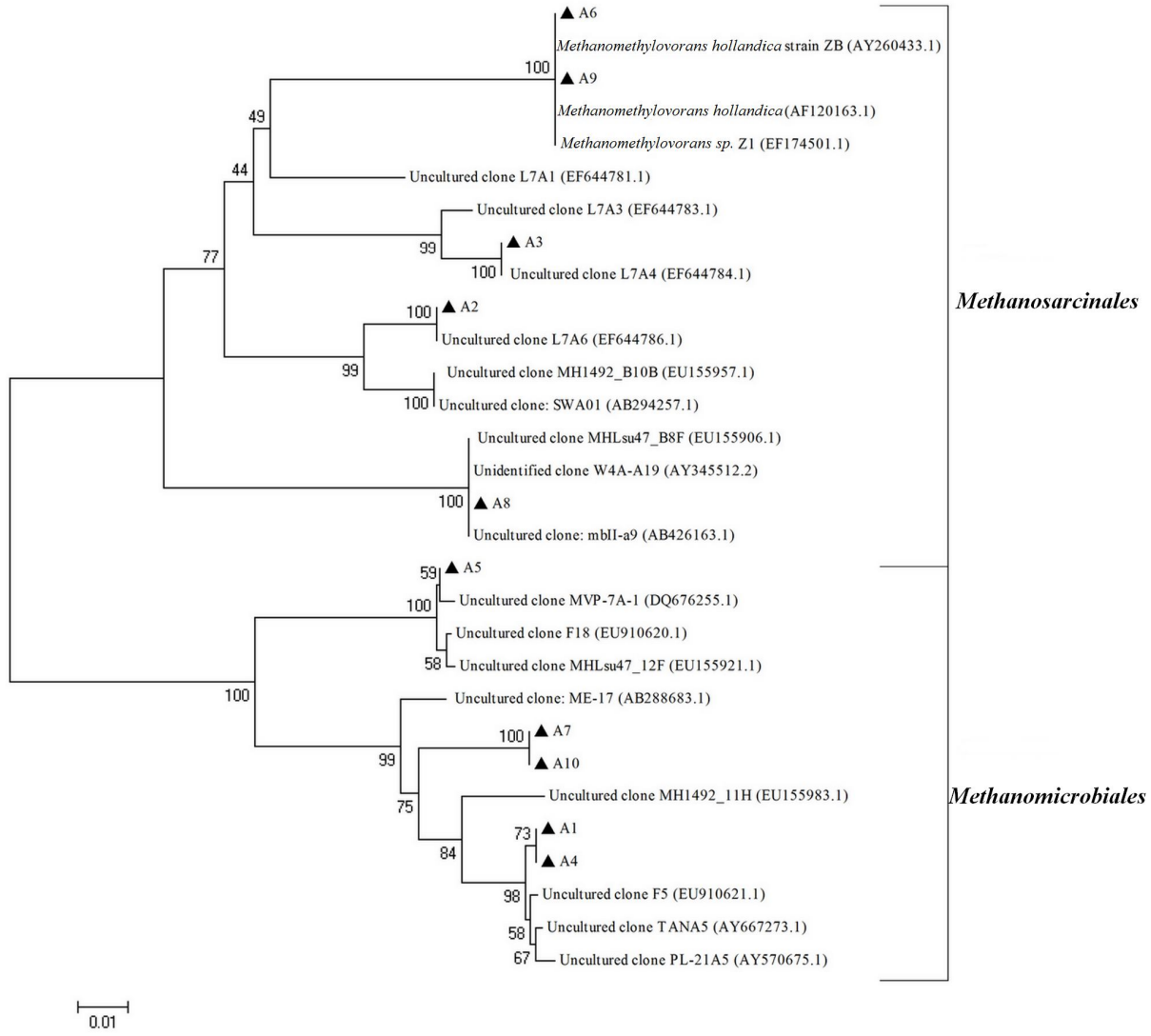

Figure 6. Phylogenetic tree based on the comparative analysis of the archaeal 16S rDNA sequences from excised DGGE bands (see Fig. 1B) and their closest relatives. Black triangles indicate the sequences obtained in this study.

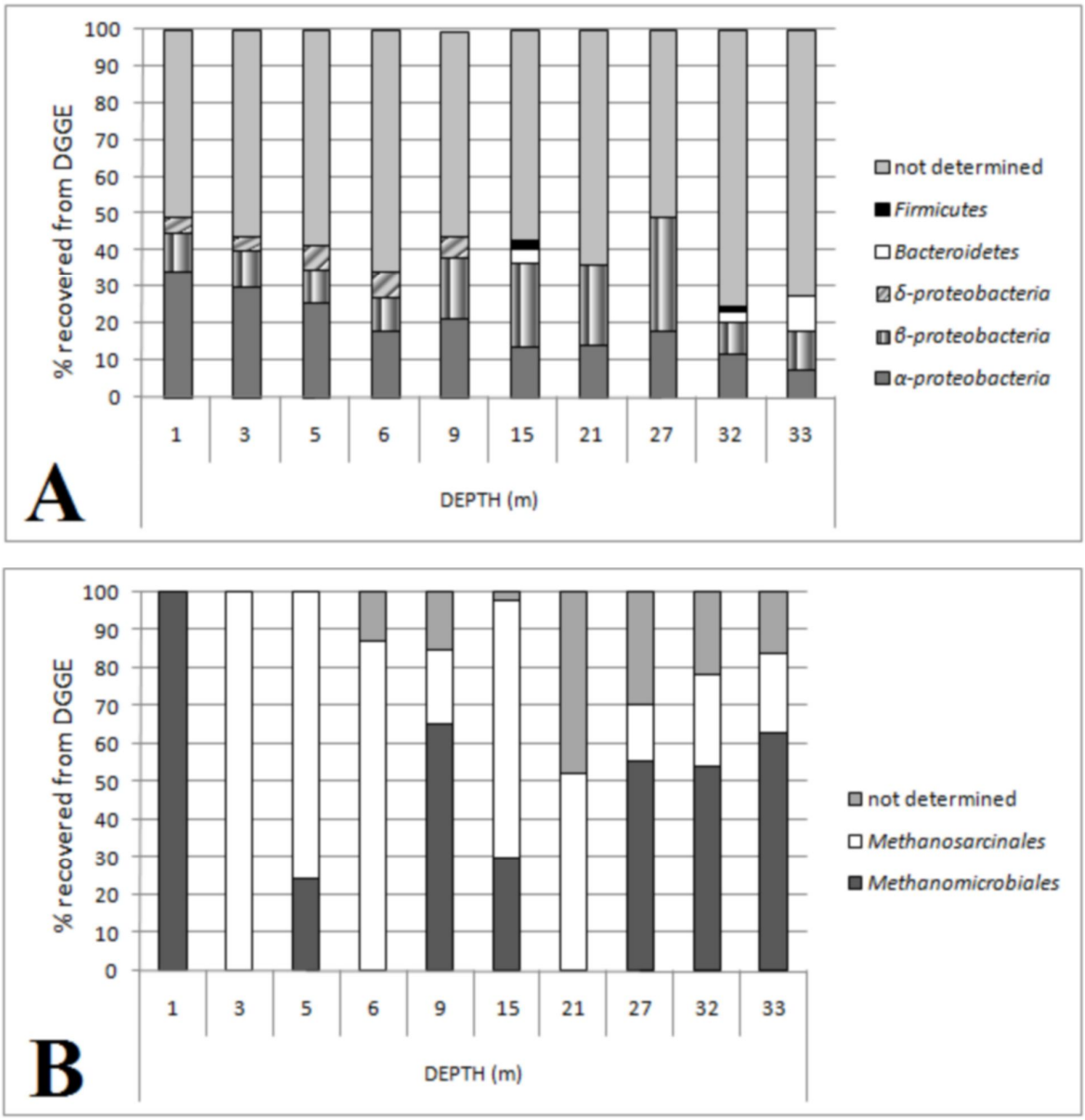

Figure 7. Relative abundance of eubacterial (A) and archaeal (B) groups at different depths of Lake Averno water column. Quantitative data were obtained from band intensities in the gels (see Fig. 1A and B)

III.4. Discussion

Cluster analysis of the DGGE fingerprints of *Eubacteria* and *Archaea* showed that the microbial composition shifts with depth. These results are in agreement with those of previous studies carried out in other stratified lakes showing vertical heterogeneity of the microbial assemblages (Konopka et al., 1999; Casamayor et al., 2000; Dominik and Höfle, 2002; Koizumi et al., 2004). Similarity values in the eubacterial DGGE profiles, indicate that some populations are not restricted to either aerobic or anaerobic locations, whereas, the low similarity values between archaeal communities of the oxic and anoxic zone, indicate that different archaeal populations occur at different depths.

Several diversity indices were calculated from DGGE banding patterns and the correlation between these and the physico-chemical parameters of water samples was investigated by means of PCA to establish whether the shifts of microbial assemblages along the water column could be related to vertical changes of the environmental variables. The lack of a significant correlation between the eubacterial diversity indices and the physico-chemical parameters, suggests that eubacterial diversity could depend primarily on inter-specific interactions and/or competition. On the contrary, the significant correlations between physical parameters and the archaeal diversity indices suggests that some defined parameters could influence the diversity of the archaeal community and/or vice versa.

PCA was also used to correlate the presence/absence of eubacterial and archaeal bands to environmental variables. The significant taxonomic units-environment correlations obtained formed two main clusters corresponding to those found by UPGMA, thus, demonstrating that, as reported by Zeng et al. (2009), sampling sites with similar water qualities, also have similar BCC. Indeed, for both eubacterial and archaeal domains, samples from the upper part of the Lake (1-5 m) were clearly separated from those of the deeper part (32-33 m) revealing that variations in microbial composition can be attributed in part, to environmental variables. In particular, DO, pH, and T, as already reported by other authors (Lindström et al., 2005; Martinez-Alonso et al., 2008; Zeng et al., 2009), emerged as significant environmental factors affecting the composition of eubacterial superficial microbial communities, while the correlation between H_2S and CH_4 with the deeper samples, could be related to the presence of specific archaeal and bacterial populations living in the anaerobic compartment which are probably involved in the sulphate reduction and methanogenetic processes (Caliro et al., 2008).

Previous studies demonstrated that *Proteobacteria* and *Bacteroidetes* are the most abundant bacterial groups in eutrophic freshwater ecosystems (Jasper et al., 2001; Trusova and Gladyshev, 2002) where they are considered to play important roles for global biogeochemical cycling. In our study, most of the sequenced eubacterial DGGE bands retrieved from the various sampling depths, were affiliated with the *α*-and *β-Proteobacteria* and grouped in several different phylogenetic clusters, thus confirming the ecological success and the extreme metabolic diversity of these phyla in freshwater ecosystems. In contrast to Jasper et al. (2001) and Kondo et al. (2009), who reported that members of the phylum *Bacteroidetes* were the dominant bacterioplankton populations in eutrophic and meromictic lakes, we found only one sequence, restricted to a deep location, belonging to this group. This sequence was related to the novel genus *Paludibacter*, a strictly anaerobic, chemo-organotrophic bacterium that uses various sugars and produces useful bioactive compounds, such as acetate and propionate (Ueki et al., 2006). We cannot exclude, however, the presence of other *Bacteroidetes* species, in this lake, below the DGGE threshold limit (0.5-1% of the total targeted gene pool, Casamayor et al., 2000), since several faint bands could not be recovered from the gels and sequenced. We did not detect members of *Actinobacteria*, which are common in freshwater ecosystems (Kolmonen et al., 2004; Hahn, 2006; Martinez-Alonso et al., 2008). This absence could be explained by the fact that this group is relatively more prevalent in oligo-trophic lakes than in eutrophic lakes (Kolmonen et al., 2004; Zeng et al., 2009). We retrieved members of the *Firmicutes* and *δ Proteobacteria* groups, that rarely occur in freshwater samples (De Wever et al., 2005). Sequences belonging to *δ Proteobacteria* sub-phylum were recovered from the upper part of Lake Averno and, according to PCA results, they were gathered in the group of bands which were positively correlated with DO. The low bootstrap confidence values obtained for their classification at the family level, by the RDP Classification Algorithm, suggested that they could belong to previously uncharacterized taxonomic units. Although most of the *δ Proteobacteria* present in lakes are expected to be sulphate reducers restricted to the anoxic layer (Karr et al., 2005, Lehours et al., 2005), we were unable to identify this functional microbial group in sequences belonging to this sub-phylum. However, PCA results revealed a strong positive correlation between a group of 10 faint, not sequenced bands, present only at 32 and 33 m of depth, and a high concentration of H_2S, thus suggesting involvement of these populations in sulfate reduction processes.

The archaeal sequenced bands retrieved from the various sampling depths were related to methanogen sequences belonging to the Orders of *Methanosarcinales* and the

Methanomicrobiales. This finding is consistent with the results of earlier studies on lake water samples (Øvreas et al., 1997; Lehours et al., 2005; Lehours et al., 2007)., Lehours et al., (2005), in particular, observed that methane concentrations in the anoxic zone of Lake Pavin correlated with the presence of methanogens belonging to *Methanosarcinales*. In agreement with this finding, two of our sequences, that belong to this order and were identified as *Methanomethylovorans* (bootstrap confidence value of 100%), clustered positively with the presence of methane, as revealed by PCA. However, in our study, methanogen-related sequences were found not only in the anaerobic methane-rich waters, but also in the aerobic layer. Although it is possible that communities from different zones could have become mixed due to sedimentation of organisms in large aggregates of cells, the buoyancy of cells with gas vesicles or transportation with gas bubbles, the presence and relative abundance of archaeal groups found in the surface waters of Lake Averno make this explanation unlikely. Moreover, although the finding of these microorganisms, in the oxic layer, could be explained by the presence of transient anoxic microzones (De Long, 1992) or endosymbiotic niches (Vogels et al., 1980), the low bootstrap values for the phylogenetic placement, at genus level, of these sequences and the fact that they exhibit a strong similarity only with sequences of uncultured organisms of unknown physiology and metabolism, support the hypothesis that they could represent unknown mesophilic aerobic members of *Archaea*. In contrast with the considerable contribution of *Archaea*, to the methanogenesis process, occurring through decomposition of organic matter, neither sequencing of DGGE bands, nor taxonomic units-environment correlations, demonstrated by PCA, revealed the presence of *Archaea* involved in the reduction of sulphate.

In summary, the results of the present study offer the first description of the bacterial and archaeal communities in the Lake Averno. Sequencing of the predominant bands, in the DGGE gels, revealed the presence of bacterial groups commonly found in freshwater ecosystems, but also the presence of members not included in the typical freshwater cluster. Moreover, our results suggest the presence of archaeal and eubacterial populations not previously characterized. Furthermore, the use of PCA demonstrated the relationships between BCC and environmental factors providing information regarding the presence of microorganisms involved in important microbial processes.

References

Andreoni, V., Cavalca, L., Rao, M. A., Nocerino, G., Bernasconi, S., Dell'Amico, et al. 2004. Bacteria, communities and enzyme activities of PAHs polluted soils. *Chemosphere* 57: 401-412.

APHA 2005. Standard methods for the examination of water and wastewater. 21st ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.

Bosshard, P. P., Santini, Y., Grüter, D., Stettler, R., Bachofen, R. 2000. Bacterial diversity and community composition in the chemocline of the meromictic alpine Lake Cadagno as revealed by 16S rDNA analysis. *FEMS Microbiol Ecol* 31: 173-182.

Briée, C., Moreira, D., López-García, P. 2007. Archaeal and bacterial community composition of sediment and plankton from a suboxic freshwater pond. *Res Microbiol* 158: 213-227.

Caliro, S., Chiodini, G., Izzo, G., Minopoli, C., Signorini, A., Avino, R., Granieri, D. 2008. Geochemical and biochemical evidence of lake overturn and fish kill at Lake Averno, Italy. *J Volcanol Geother Res* 178: 305-316.

Casamayor, E.O., Schafer H., Bañeras, L., Pedrós-Alió, C., Muyzer, G. 2000. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 66: 499-508.

Cremonesi, L., Firpo, S., Ferrari, M., Righetti, P.G., Gelfi, C. 1997. Double-gradient DGGE for optimized detection of DNA point mutations. *Biotechniques* 22: 326-330.

De Long, F. 1992. *Archaea* in coastal marine environments. *Proc Natl Acad Sci* 89: 5685-5689.

De Wever, A., Muylaert, K., Van der Gucht, K., Pirlot, S., Cocquyt, C., Descy, J-P. et al. 2005. Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Appl Environ Microbiol* 71: 5029-5037.

Di Cello, F., Bevivino, A., Chiarini, L., Fani, R., Paffetti, D., Tabacchioni, S., Dalmastri, C. 1997. Biodiversity of a *Burkholderia cepacia* population isolated from maize rhizosphere at different plant growth stages. *Appl Environ Microbiol* 63: 4485-4493.

Dimitriu, P. A., Pinkart, H. C., Peyton, B. M., .Mormile, M. R. 2008. Spatial and temporal patterns in the microbial diversity of a meromictic soda lake in Washington State. *Appl Environ Microbiol* 74: 4877-4888.

Dominik, K., Höfle, M. G. 2002. Changes in bacterioplankton community structure and activity with depth in a eutrophic lake as revealed by 5S rRNA analysis. *Appl Environ Microbiol* 68: 3606-3613.

El Fantroussi, S., Verschuere, L., Verstraete, W., Top, E. M. 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Appl Environ Microbiol* 65: 982-988.

Fuhrman, J. A., Hewson, I., Schwalbach, M. S., Steele, J. A., Brown, M.V. Naeem, S. 2006. Annually reoccurring bacterial communities are predictable from ocean conditions. *Proc Natl Acad Sci* 103: 13104-13109.

Hahn, M. W. 2006. The microbial diversity of inland waters. *Curr Opin Biotechnol* 17: 256-261.

Hollibaugh, J. T., Wong, P. S., Bano, N., Pak, S. K., Prager, E.M., Orrego, C. 2001. Stratification of microbial assemblages in Mono Lake, California, and response to a mixing event. *Hydrobiologia* 466: 45-60.

Humayoun, S. B., Bano, N., Hollibaugh, J. T. 2003. Depth distribution of microbial diversity in Mono Lake, a meromictic soda lake in California. *Appl Environ Microbiol* 69: 1030-1042.

Improta, C., Andini, S., Ferrara, L. 2004. Chemical and ecotoxicological characterization of Averno lake. *Bull Environ Contam Toxicol* 72: 472-481.

Jasper, E., Nauhaus, K., Cypionka, H., Overmann J. 2001. Multitude and temporal variability of ecological niches as indicated by the diversity of cultivate bacterioplankton. *FEMS Microbiol Ecol* 36: 153-164.

Jiang, H., Dong, H., Yu, B., Ye, O., Shen, J., Rowe, H., Zhang, C. 2008. Dominance of putative marine benthic *Archaea* in Qinghai lake, north-western China. *Environ Microbiol* 10: 2355-2367.

Karr, E. A., Sattley, W. M., Rice, M. R., Jung, D. O., Madigan, M. T., Achenbach, L. A. 2005. Diversity and distribution of sulfate-reducing bacteria in permanently frozen Lake Fryxell, Dry Valleys, Antarctica. *Applied Environ Microbiol* 71: 6353-6359.

Koizumi, Y., Takii, S., Fukui, M. 2004. Depth-related change in archaeal community structure in a freshwater lake sediment as determined with denaturing gradient gel electrophoresis of amplified 16S rRNA genes and reversely transcribed rRNA fragments. *FEMS Microbiol Ecol* 48: 285-292.

Kolmonen, E., Sivonen, K., Rapala, J., Haukka, K. 2004. Diversity of cyanobacteria and heterotrophic bacteria in cyanobacterial blooms in Lake Joutikas, Finland. *Aquat Microb Ecol* 36: 201-211.

Kondo, R., Nakagawa, A., Mochizuki, L., Osawa, K., Fujioka, Y., Butani J. 2009. Dominant bacterioplankton populations in the meromictic Lake Suigetsu as determined by denaturing gradient gel electrophoresis of 16S rRNA gene fragments. *Limnology* 10: 63-69.

Konopka, A., Bercot, T., Nakatsu, C. 1999. Bacterioplankton community diversity in a series of thermally stratified lakes. *Microb Ecol* 38:126-135.

Lehours, A-C., Bardot, C., Thenot, A., Debroas, D., Fonty, G. 2005 Anaerobic microbial communities in Lake Pavin, a unique meromictic lake in France. *Appl Environ Microbiol* 71: 7389-7400.

Lehours, A-C., Evans, P., Bardot, C., Joblin, K., Gerard, F. 2007. Phylogenetic diversity of *Archaea* and *Bacteria* in the anoxic zone of a meromictic lake (Lake Pavin, France) *Appl Environ Microbiol* 73: 2016-2019.

Lindström, E.S., Kamst-Van Agterveld, M.P, Zwart, G. 2005. Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. *Appl Environ Microbiol* 71: 8201-8206.

Martens H, Martens M. 2001. *Multivariate analysis of quality: an introduction.* Chichester, U.K.: John Wiley and Sons.

Martínez-Alonso, M., Méndez-Álvarez, S., Ramírez-Moreno, S., González-Toril., E., Amils, R., Gaju, N. 2008. Spatial heterogeneity of bacterial populations in monomictic Lake Estanya (Huesca, Spain) *Microb Ecol* 55: 737-750.

Muyzer, G., Dewall, E. C., Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturant gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59: 695-700.

Øvreas, L., Forney, L., Daae, F. L., Torsvik, V. 1997. Distribution of bacterioplankton in meromictic Lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol* 63: 3367-3373.

Salcher, M. M., Pernthaler, J., Zeder, M., Psenner, R., Posch, T. 2008. Spatio-temporal niche separation of planktonic *Betaproteobacteria* in an oligo-mesotrophic lake. *Environ Microbiol* 10: 2074-2086.

Shannon, C. E., Weaver, W. 1963. The *mathematical theory of communication.* Urbana, IL: University of Illinois, Press. pp.117.

Simpson, E. H. 1949. Measurement of diversity. *Nature* 163: 688.

Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol.* 24: 1596-1599.

Trusova, M. Y., Gladyshev, M. I. 2002. Phylogenetic diversity of winter bacterioplankton of eutrophic Siberian reservoirs as revealed by 16S rRNA gene sequences. *Microb Ecol* 44: 252-259.

Ueki, A., Akasaka, H., Suzuki, D., Ueki, K. 2006. *Paludibacter propionicigenes* gen. nov., sp. nov., a novel strictly anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. *Int J Syst Evol Microbiol* 56: 39-44.

Vetriani, C., Jannasch, H. W., MacGregor B.J., Stahl, D.A., Reysenbach, A-L. 1999. Population structure and phylogenetic characterization of marine benthic *Archaea* in deep-sea sediments. *Appl Environ Microbiol* 65: 4375-4384.

Vetriani, C., Tran, H. V., Kerkhof, L. J. 2003. Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black sea. *Appl Environ Microbiol* 69: 6481-6488.

Vogels, G. D., Hoppe, W. F., Stumm, C. K. 1980. Association of methanogenic bacteria with rumen ciliates. *Appl Environ Microbiol* 40: 608-612.

Wei, C., Bao, S., Zhu, X., Huang, X. 2008. Spatio-temporal variations of the bacterioplankton community composition in Chaohu lake, China. *Prog Nat Sci* 18: 1115-1122.

Wetzel R. G. 2001. Bacterioplankton. In: *Limnology: lake and river ecosystems.* Wetzel R.G. (ed). New York: Academic Press, pp. 489-525.

Wu, J-H., Liu, W-T., Tseng, I-C., Cheng, S-S. 2001. Characterization of microbial consortia in a terephthalate-degrading anaerobic granular sludge system. *Microbiology* 147: 373-382.

Zeng, J. Yang, L., Du, H., Xiao, L., Jiang, L., Wu, J., Wang, X. 2009. Bacterioplankton community structure in a eutrophic lake in relation to water chemistry. *World J Microbiol Biotechnol* 25: 763-772.

Chapter IV: Microbial community composition of surface sediment from Lake Averno revealed by 16S rDNA and [FeFe]-hydrogenase genes-based approaches

IV.1. Introduction

Lake sediment represents one of the most complex microbial habitats on Earth being a heterogeneous ecosystem which gives rise to many different environmental niches even on a millimeter scale (Spring et al., 2000). One of its most important characteristic is that it is not spatially separated from adjacent habitats, but is part of complex ecosystems. Therefore, it harbors highly complex microbial communities with regard to species composition and metabolic activity, that are largely infuenced by interactions with surrounding aquatic and terrestrial habitats.

Prokaryotes play a key role in metabolic processes in the surface layers of lake sediment, such as methanogenesis (Chan et al., 2005) sulphate reduction (Li et al., 1999) and ammonia oxidation (Hastings et al., 1998), influencing the nutrient cycles of freshwater lakes. They also contribute to the biodegradation of pollutants deriving from urban, industrial and agricultural activities. Consequently, investigating the microbial communities and their function in freshwater sediment will greatly enhance our understanding of lake ecosystem (Tamaki et al., 2005). However, the biogeochemical processes associated with microorganisms living in sedimentary environments are poorly understood partially because the majority of sediment dwelling microorganisms are uncultivated and most are phylogenetically distinct from those in better characterized terrestrial environments (Nelson et al., 2007). Therefore, much remains to be learned about the diversity, distribution, and function of sediment microbial communities. Moreover, the identification of unknown prokaryotes could provide information about the presence in sediment of microorganisms useful for biotechnological applications. From this point of view, as hydrogen-evolving microorganisms have been attracting great interest due to their potential in biological hydrogen production by utilizing renewable biomass, and since Kawagoshi et collegues (2005) demonstrated that lake sediment represents an efficient inoculum for an appreciable hydrogen production, it is useful to assess the diversity of H_2 producing bacteria naturally occurring in this habitat. Lake Averno sediment, anoxic and abundant in organic matter (Caliro et al., 2008), could represent an important source of microorganisms of such biotechnological interest. Up to now, no investigation have been conducted to determine the diversity of prokaryotes inhabiting this ecosystem.

The objective of this study was to characterize the overall microbial community in the superficial layer of the sediment of Lake Averno by 16S rDNA-based DGGE and to investigate the diversity of hydrogen-producing populations naturally occurring in this habitat by PCR amplification, cloning and sequencing of [FeFe]-hydrogenase gene.

IV.2. Material and methods

IV.2.1. Sample collection

In May 2006, upper sediment (0-10 cm) was sampled aseptically with an orizontal ENEA core sampler at the site of maximal depth of Lake Averno (33 m). The physico-chemical characteristics of this lake have been described in chapter III. After collection, the sample was cooled on ice for transport back to the laboratory where it was stored at -80°C until microbial analysis.

IV.2.2. DNA extraction

Five grams of sediment sample were used to obtain total DNA by phenol/chloroform extraction and isopropanol precipitation methods according to the procedure described by Zhou et al., 1996. The pellet of nucleic acid was resuspended in sterile deionized water, to give a final volume of 500µl.

Crude DNA extract was further purified using the Wizard DNA clean-up system (Promega, WI, USA) according to the supplier's instructions. Quantity and purity of DNA were checked fluorospectrometrically by NanoDrop (NanoDrop Technologies, USA).

IV.2.3. PCR amplification of the 16S rRNA gene and DGGE

PCR amplification of the eubacterial and archaeal 16S rRNA genes and DGGE procedures were performed as described in the paragraphs III.2.3 and III.2.4

IV.2.4. Recovery, cloning and sequencing of excised DGGE bands

Individual bands were cut from eubacterial and archaeal DGGE gels using a razor blade, placed in 50 µL of sterile distilled water, and incubated overnight at 4 °C to allow diffusion of the DNA.

Eluted DNA from each band was reamplified and run on a new DGGE gel to confirm its melting behavior. Sequencing reactions were prepared from PCR products amplified with unclamped primers using Applied Biosystem Big Dye® Terminator sequencing kit version 3.1, according to the manufacturer's instructions and analyzed using a 3730 DNA Analyzer Applied Biosystem apparatus. The obtained sequences were checked for the presence of PCR-amplified chimeric sequences by the check_chimera program of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU).

Eubacterial bands whose sequences did not reveal a specific and unique bacterial population were cloned. Two microlitres of a 1:10 dilution of their PCR product were cloned using the StrataClone^{TM} PCR Cloning Kit (Stratagene) according to the manufacturer's instructions. Ten clones were selected randomly from each band and sequenced.

The 16S rRNA gene sequences obtained in the present study have been submitted to Gen-Bank database and are being processed to obtain the accession numbers.

IV.2.5. Phylogenetic analysis of sequenced DGGE bands

The analysis of the partial eubacterial and archaeal 16S rRNA gene sequences and the construction of Neighbor-Joining phylogenetic trees, showing the relationship between sequences of DGGE bands and those of their closest relatives retrieved by BLAST analysis, were performed as described in the paragraph III.2.6.

IV.2.6. Primer design and PCR hydrogenase gene amplification

A new primer, named HydA1-F, was designed by the alignment of six DNA sequences of clostridial [FeFe]-hydrogenase genes (FHGs) (accession numbers AB016775, U09760, AB159510, M81737, AF148212, and AY827554 in the GenBank database) previously used by Fang and collegues (2006) to construct the primer set HydA-F and HydA-R. This primer

was used together with the primer HydA-R (Fang et al., 2006) to amplify the FHG from sediment sample total DNA.

PCR amplification of the FHG from total DNA of the sediment sample was carried out in Qiagen *Taq* buffer (10X) containing 1.5 mM $MgCl_2$, 1.5 µM each primer (HydA1-F and HydA-R), 200 µM each deoxyribonucleoside triphosphate, 1 U of *Taq* DNA polymerase (Qiagen), 20 ng of the DNA extract, and sterile deionized water to reach the final volume of 20 µl.

Cycling parameters were as previously described by Fang et al. (2006).

IV.2.7. Cloning, sequencing and phylogenetic analysis of FHG fragments

The PCR-amplified products of the FHG were used to construct a clone library using the StrataClone™ PCR Cloning Kit (Stratagene) according to the supplier's instructions. Clones were selected randomly and sequenced. Nucleotide and amino acidic sequences of FHG fragments obtained from this study were aligned with those of their closest relatives and with the six sequences of clostridial FHGs used to design primer HydA1-F, for the construction of respective phylogenetic trees using Neighbour Joining with Kimura 2 parameter distances in MEGA software version 4 (Tamura et al. 2007).

IV.3. Results and discussion

IV.3.1. Eubacterial DGGE profile and sequence analysis of excised bands

Eubacterial DGGE analyses of the 16S rDNA fragments obtained by PCR from lake sediment generated a complex fingerprint, reflecting the high biodiversity of the microbial community (Fig. 1). Among the only five clearly visible and distinct bands (indicated by letters) that were excided and directly sequenced (Fig. 1), only band A revealed a unique OTU showing a similarity of 100% to the sequence of an uncultured bacterium clone isolated from Baltic Sea sediment. The other bands resulted in a co-migration of heterogeneous DNAs on DGGE. For each one of these bands, a clone library was constructed from which 10 clones were randomly picked and sequenced.

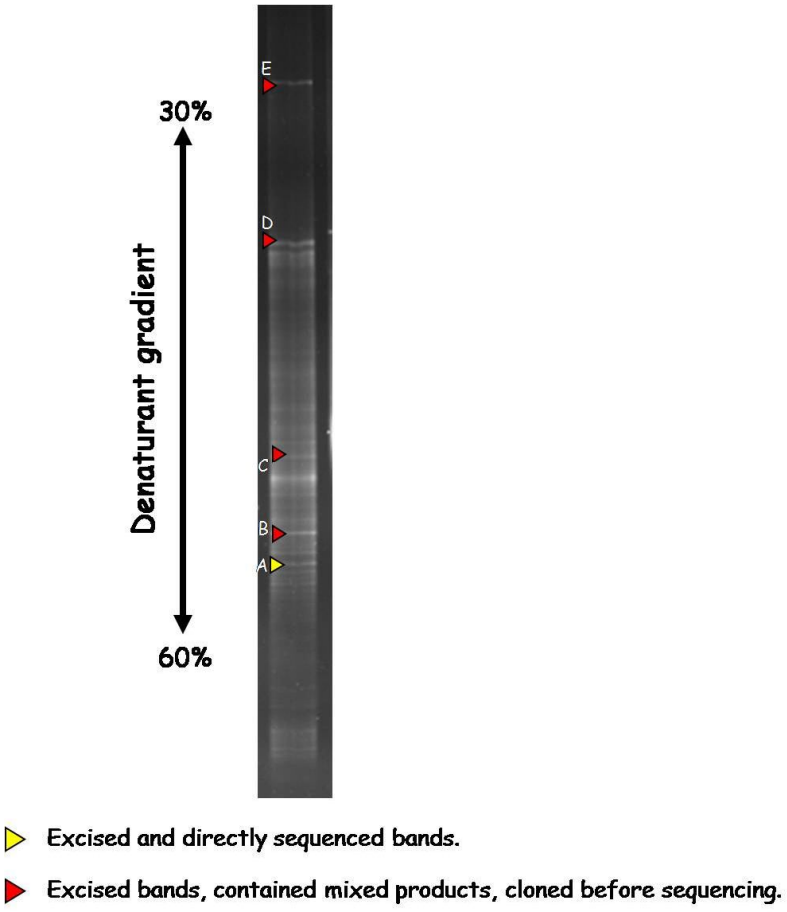

Figure 1. DGGE analysis of the eubacterial community of Lake Averno sediment.

Sequences obtained from band A and clones are shown in Table 1, and the phylogenetic tree showing their relationship with sequences of closest relatives is shown in Fig. 2.

Most of the sequences showed low values of similarity (<97%) with known sequences indicating that they could represent new species or genera (Stackebrandt and Goebel, 1994). This result was confirmed by the RDP phylogenetic classification of these sequences, at the Order, Family or Genus level, that was not well supported by high bootstrap confidence values (Table 1).

Phylogenetic analysis showed that 35 of 41 eubacterial sequences were affiliated with 7 known taxa of *Bacteria*. Most sequences were allocated into the *Proteobacteria* phylum (58.5% of the total number of clones), in which the dominant organisms were placed in the δ, and γ classes (29.5, and 22%, respectively), whereas β and α classes account only for 4.8 and 2.4%, respectively. The second most dominant group, represented by 12.2% of the total number of clones, was classified into the phylum *Chlorobi*. The other groups of the clone library were determined to be, in order of abundance, in the *Firmicutes* and *Acidobacteria* phyla. The remaining six clone sequences (B14, C42, D1, D14, D28 and E4) could not be

clearly affiliated to any described bacterial division, because of the low bootstrap values obtained for their classification.

Proteobacteria represent the most predominant phylum in freshwater sediments (Miskin et al., 1999; Spring et al., 2000; Wise et al., 1997; Tamaki et al., 2005; Wobus, 2003). In particular, δ *Proteobacteria* has been indicated as the representative bacterial group in benthic environments (Spring et al., 2000, Tamaki et al., 2005; Miskin et al., 1999), since it was more frequently recovered from sediments than from water samples, in which α, β, and γ *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* are the dominant groups (Hiorns et al., 1995; Lindstrom and Leskinen, 2002; Spring et al., 2000). Moreover, in our study, as well as in previous reports (Purdy et al., 1997; Li et al., 1999; Spring et al., 2000), the most abundant group within the δ *Proteobacteria* was allocated to strictly anaerobic organisms such as syntrophic bacteria (*Smithella*) capable of using butyrate, propionate, malate, and fumarate in syntrophic association with H_2- or formate-utilizing microorganisms. On the contrary, we did not find the other frequently detected δ *Proteobacteria* group of sulfate reducers belonging to *Desulfococcus*, *Desulfomonile*, and *Desulfonema* genera. We cannot exclude, however, the presence of these species in this lake, since several faint or not well separated bands could not be recovered from the gels and sequenced.

The presence of phototrophic green sulphur bacteria (*Chlorobiaceae*) and purple non-sulfur bacteria (*Rhodospirillales*) in the dark sediment was unexpected. This could be explained by the sedimentation of these bacteria from the bacterioplankton. In addition, it has been argued that genomic DNA of dead cells can be very stable, surviving for a long periods of time (Spring et al., 2000). However, *Chlorobiaceae* has also been observed in the sediment of Lake Kaiike (Koizumi et al., 2004) and Lake Kinneret (Schwarz et al., 2007). Sierevag and Ormerod (1977) showed that *Chlorobium thiosulfatophilum* is able to survive in the dark by obtaining energy from the degradation of intracellular polyglucose as a storage material (Schwarz et al., 2007).

Most of the retrieved *Firmicutes* clones (B32, C2, E9 and E27) showed low sequence similarity (<97%) to known sequences and may represent new taxa.

In conclusion, these results indicate that the eubacterial community in the freshwater sediment of Lake Averno is remarkably diverse and it could be also composed of unknown bacterial species.

Band /Clone	Closest relative[a]	Accession no.[a]	Identity[a]	Phylogenetic affiliation[b]		
				Order	Family	Genus
A	Uncultured bacterium 307c2	EF459997	100%	*Chromatiales* [100%]	*Chromatiaceae* [100%]	*Lamprocystis* [46%]
B1	Uncultured bacterium AV9-8	AM1818114	87%	*Burkholderiales* [95%]	*Incertae sedis* [30%]	*Xylophilus* [21%]
B2	Uncultured bacterium ORSFAM_h05	EF393287	98%	*Syntrophobacterales* [100%]	*Syntrophaceae* [100%]	*Smithella* [89%]
B7	Uncultured bacterium 628_H15	EU644370	91%	*Chromatiales* [81%]	*Chromatiaceae* [75%]	*Rhabdochromatium* [41%]
B12	Uncultured bacterium C5Lks29	AM086120	92%	*Syntrophobacterales* [25%]	*Syntrophaceae* [22%]	*Smithella* [19%]
B13	Uncultured bacterium C5Lks2	AM086120	92%	*Syntrophobacterales* [31%]	*Syntrophaceae* [29%]	*Smithella* [26%]
B14	Uncultured bacterium cs14	DQ088239	97%	*Cystobacterineae* [29%]	*Cystobacteraceae* [29%]	*Archangium* [25%]
B20	Uncultured bacterium ORSFAM_h05	EF393287	97%	*Syntrophobacterales* [98%]	*Syntrophaceae* [98%]	*Smithella* [79%]
B26	Uncultured bacterium R1_4	AJ876724	99%	*Oceanospirillales* [74%]	*Oceanospirillaceae* [72%]	*Thalassolituus* [51%]
B32	Uncultured bacterium SHD-209	AJ278163	92%	*Clostridiales* [99%]	*Ruminococcaceae* [97%]	*Acetanaerobacterium* [23%]
B43	Uncultured bacterium Pav-026	DQ642339	97%	*Syntrophobacterales* [99%]	*Syntrophaceae* [99%]	*Smithella* [73%]
C2	Uncultured bacterium 35-56	DQ833493	98%	*Clostridiales* [88%]	*Ruminococcaceae* [42%]	*Acetivibrio* [35%]
C10	*Chlorobium limicola* DSM 245	CP001097	96%	*Chlorobiales* [100%]	*Chlorobiaceae* [100%]	*Chlorobium* [79%]
C24	*Chlorobium limicola* DSM 245	CP001097	99%	*Chlorobiales* [100%]	*Chlorobiaceae* [100%]	*Chlorobium* [100%]
C27	Uncultured bacterium LaC15L8	EF667819	87%	*Syntrophobacterales* [27%]	*Syntrophaceae* [27%]	*Smithella* [21%]
C32	Uncultured bacterium Pav-026	DQ642339	97%	*Syntrophobacterales* [99%]	*Syntrophaceae* [99%]	*Smithella* [78%]
C35	*Chlorobium limicola* DSM 245	CP001097	100%	*Chlorobiales* [100%]	*Chlorobiaceae* [100%]	*Chlorobium* [93%]
C39	*Chlorobium limicola* DSM 245	CP001097	100%	*Chlorobiales* [100%]	*Chlorobiaceae* [100%]	*Chlorobium* [92%]
C40	Uncultured bacterium WS159JS0307	EU707315	93%	*Alteromonadales* [37%]	*Incertae sedis* [37%]	*Saccharophagus* [15%]
C42	Uncultured bacterium Amb16S1280	EF018810	87%	*Syntrophobacterales* [27%]	*Syntrophobacteraceae* [27%]	*Desulforhabdus* [22%]
C45	*Chlorobium limicola* DSM 245	CP001097	94%	*Chlorobiales* [56%]	*Chlorobiaceae* [56%]	*Chlorobium* [48%]
D1	Uncultured bacterium d6-25	AM409986	96%	*Syntrophobacterales* [28%]	*Syntrophobacteraceae* [24%]	*Desulforhabdus* [16%]
D6	Uncultured bacterium 307c2	EF459997	99%	*Chromatiales* [100%]	*Chromatiaceae* [100%]	*Thiocystis* [26%]
D10	Uncultured bacterium STU13	EU700155	97%	*Burkholderiales* [98%]	*Incertae sedis* [38%]	*Schlegelella* [15%]
D14	Uncultured bacterium 2N1-21	EU160002	89%	*Syntrophobacterales* [57%]	*Syntrophaceae* [57%]	*Smithella* [57%]
D20	Uncultured bacterium NGD32	EF614052	97%	*Acidobacteriales* [100%]	*Acidobacteriaceae* [100%]	*Gp6* [100%]
D25	Uncultured bacterium HM64	AM909864	91%	*Syntrophobacterales* [97%]	*Syntrophaceae* [97%]	*Smithella* [96%]
D28	Uncultured bacterium C5Lks1	AM086102	90%	*Acidithiobacillales* [15%]	*Thermithiobacillaceae* [15%]	*Thermithiobacillus* [15%]
D30	Uncultured bacterium ORSFAM_h05	EF 393287	97%	*Syntrophobacterales* [99%]	*Syntrophaceae* [99%]	*Smithella* [86%]
D44	Uncultured bacterium 109b1	EF459899	93%	*Syntrophobacterales* [42%]	*Syntrophaceae* [42%]	*Smithella* [16%]
D46	Uncultured *Acidobacteria* BGC.0081	EF457415	94%	*Acidobacteriales* [100%]	*Acidobacteriaceae* [100%]	*Gp13* [100%]
E1	Uncultured bacterium 63F518R-27	EU703582	100%	*Enterobacteriales* [100%]	*Enterobacteriaceae* [100%]	*Shigella* [87%]
E4	Uncultured bacterium Pav-121	DQ785307	95%	*Nitrospirales* [32%]	*Nitrospiraceae* [32%]	*Magnetobacterium* [32%]
E6	Uncultured bacterium EB11	EU334526	96%	*Syntrophobacterales* [99%]	*Syntrophaceae* [99%]	*Smithella* [97%]
E9	Uncultured bacterium RA13C8	AF407407	95%	*Clostridiales* [98%]	*Ruminococcaceae* [95%]	*Ruminococcaceae Incertae Sedis* [42%]
E13	Uncultured bacterium LiUU-5-144	AY509400	93%	*Rhodospirillales* [81%]	*Acetobacteraceae* [81%]	*Paracraurococcus* [40%]
E19	Uncultured bacterium Kas202B	EF203209	95%	*Methylococcales* [39%]	*Methylococcaceae* [39%]	*Methylococcus* [23%]
E21	Uncultured bacterium anSA06	EF034815	94%	*Chromatiales* [87%]	*Ectothiorhodospiraceae* [42%]	*Alkalilimnicola* [26%]
E23	Uncultured bacterium ORSFAM_h05	EF393287	97%	*Syntrophobacterales* [98%]	*Syntrophaceae* [98%]	*Smithella* [73%]
E25	Uncultured bacterium anSA06	EF034815	94%	*Chromatiales* [88%]	*Chromatiaceae* [63%]	*Rhabdochromatium*[40%]
E27	Uncultured bacterium 234b2	EF459933	90%	*Clostridiales* [83%]	*Ruminococcaceae* [77%]	*Acetivibrio* [69%]

Table 1.

a. Sequence similarities between rRNA sequences of DGGE bands/clones and those of the closest relatives retrieved from NCBI database

b. Identification performed using the RDP Classification Algorithm. Bootstrap confidence values are given in brackets (classification is well supported for confidence > 80%).

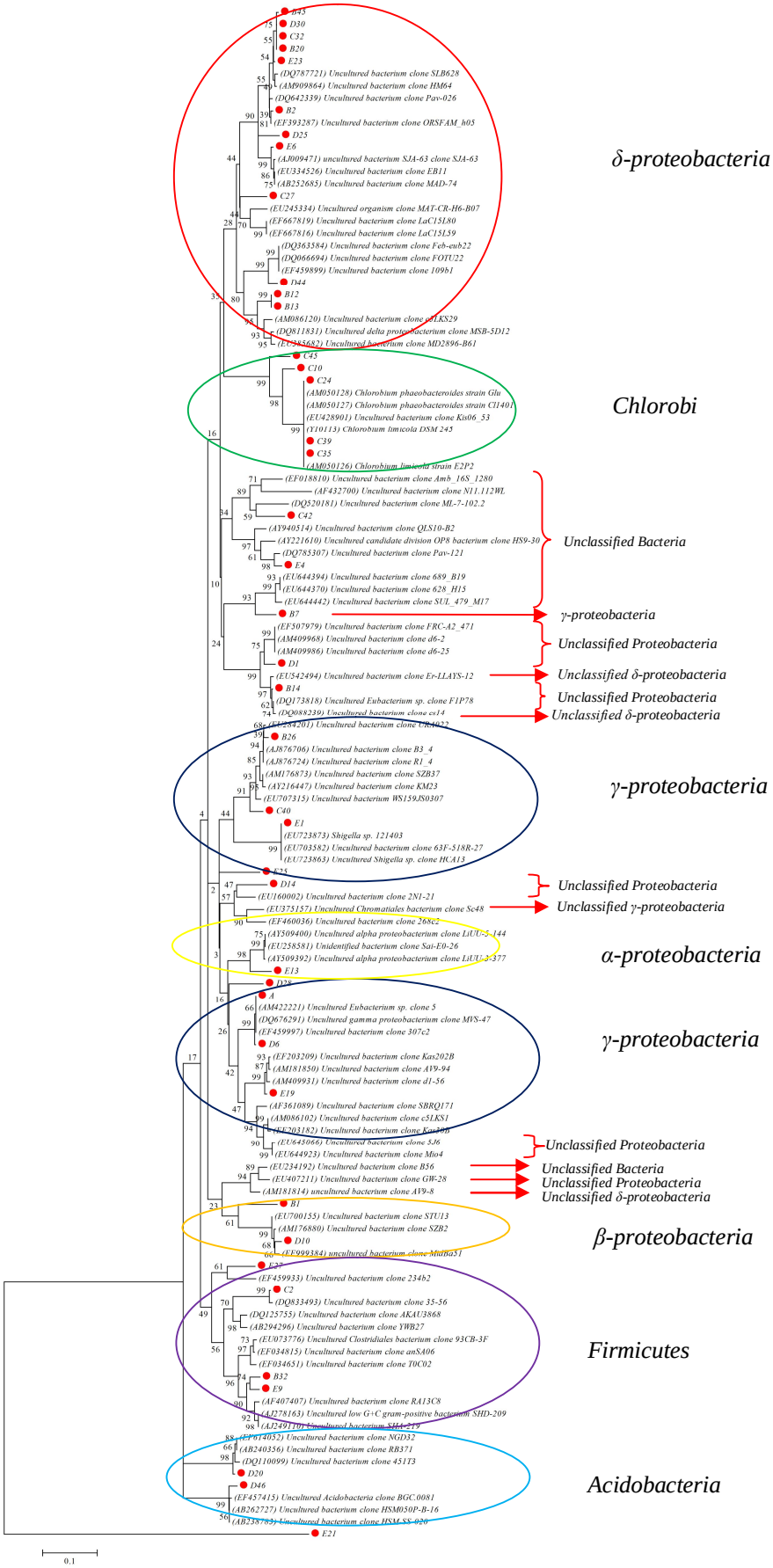

Figure 2. Neighbor-joining tree showing relationship between eubacterial 16S rRNA gene sequences from sediment (red circles) and reference sequences obtained through BLAST analysis. Strains out of the circles, with less than 80% confidence, are displayed under an "unclassified" taxon.

IV.3.2. Archaeal DGGE profile and sequence analysis of excised bands

DGGE profile of sediment archaeal community revealed a distinct profile with six well distint bands that were excised from the gel (Fig. 3).

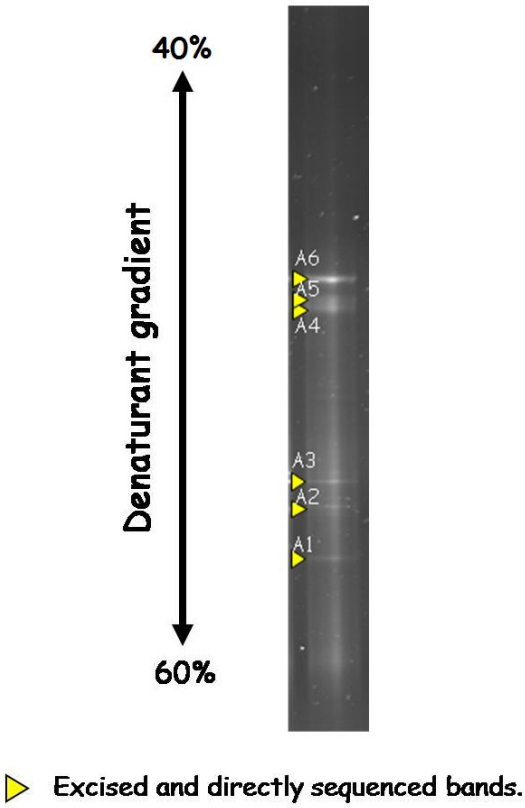

Figure 3. DGGE analysis of the archaeal community of Lake Averno sediment.

Archaeal PCR amplification of the DNA eluted from bands A5 and A6 failed, whereas the other bands that were successfully sequenced showed high similarity (sequence identity 99-100%) to sequences belonging to uncultured relatives present in the NCBI databases (Table 2). With the exception of band A3, the RDP phylogenetic classification of these sequences, at Genus level, was well supported by the bootstrap confidence values.

Band	Closest relative[a] (accession number)	Identity[a]	Phylogenetic affiliation (b)		
			Order	Family	Genus
A1	Uncultured clone SD69 (EU283044)	99%	*Methanomicrobiales* [100%]	*Methanomicrobiaceae* [100%]	*Methanoculleus* [84%]
A2	Uncultured clone MHLsu47_1B (EU155933)	100%	*Methanomicrobiales* [100%]	*Methanomicrobiaceae* [100%]	*Methanoculleus* [83%]
A3	Uncultured clone MVP-7A-1 (DQ676255)	99%	*Methanomicrobiales* [100%]	*Methanomicrobiaceae* [99%]	*Methanoculleu s*[77%]
A4	Uncultured *Archaea* mbII-a9 (AB426163)	100%	*Methanosarcinales* [100%]	*Methanosaetaceae* [100%]	*Methanothrix* [100%]

Table. 2.
a. Sequence similarities between 16S rRNA gene sequences of bands and those of the closest relatives in the NCBI database
b. Identification performed with RDP Classification Algorithm. Bootstrap confidence values are given between brackets (classification is well supported for confidence > 80%).

Previous studies suggested that *Archaea* may account for only a minor fraction (approximately 1%) of the total prokaryotic community of the sediment (0–10 cm) (Sharz et al., 2007). This is in agreement with our study, in which we observed a less archaeal diversity in comparison with results obtained for the eubacterial domain. Comparative sequence analysis revealed that all the detected archaeal 16S rRNA genes were affiliated to euryarchaeotal group and clustered into the *Methanomicrobiales* (bands A1, A2, A3) and *Methanosarcinales* (band A6) divisions (Fig. 4)

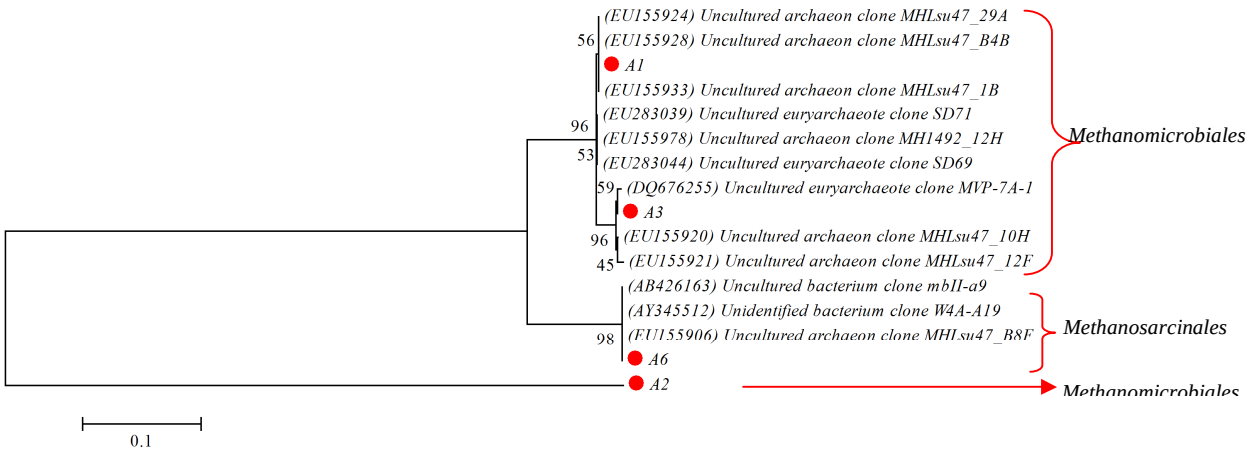

Figure 4. Neighbor-joining tree showing relationship between archaeal 16S rRNA gene sequences from sediment (red circles) and sequences of their closest relatives obtained through BLAST analysis.

These sequences were linked to known hydrogenotrophic methanogens (methane production from H_2 and CO_2) of the *Methanomicrobiaceae* family and to the acetoclastic (methane production from acetate) *Methanosaetaceae* (Table 2). This dominance of *Methanosaetaceae* and *Methanomicrobiales* among the total sedimentary archaeal community

has also been shown for other meso- to eutrophic freshwater lakes, e.g. Lake Biwa in Japan (Koizumi et al., 2003), Lake Soyang in South Korea (Go et al., 2000), Lake Dagow in Germany (Glissman et al., 2004). Although the different detection methods used, all these studies indicated that *Methanomicrobiales* and *Methanosaetaceae* are the most widespread *Archaea* in freshwater sediments. This could suggests that *Methanomicrobiales* and *Methanosaetaceae* are efficient syntrophic partners (removal of hydrogen and acetate) in the complete degradation of organic biomass in freshwater sediments.

IV.3.3. Diversity of FHGs in sediment sample and phylogenetic analysis

The new primer HydA1-F (5'-CATGTAATAGTTGCTATGGC-3', Tm 56.3), enabled to amplify a longer nucleotide fragment of the clostridial FHG (~650 bp), in comparison to that previously obtained with the set of primers HydA-F/HydA-R (Fang et al., 2006). Sequences analysis of PCR products fully confirmed the specificity of the used primer pairs.

The nucleotide sequences of the 33 selected clones were compared with all available [FeFe]-hydrogenase sequences in the GenBank database, showing low similarity values (< 87%) in most cases, except for clones HydA-10, HydA-21, HydA-28 and HydA-32 (99-100% of identity) (Table 3). Similar low identity values were obtained by the comparison between the amino acid sequences, deduced from the corresponding DNA sequences of these [FeFe]-hydrogenases, and their closest relatives (Table 4)

Clone	Closest relative	Accession no.	Identity
HydA1	*Clostridium paraputrificum*	AB159510	82%
HydA4	*Clostridium paraputrificum*	AB159510	81%
HydA12	*Clostridium paraputrificum*	AB159510	82%
HydA15	*Clostridium paraputrificum*	AB159510	82%
HydA17	*Clostridium paraputrificum*	AB159510	82%
HydA19	*Clostridium paraputrificum*	AB159510	80%
HydA22	*Clostridium paraputrificum*	AB159510	82%
HydA16	*Clostridium butyricum* strain FBR1	EU689097	80%
HydA23	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA33	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA34	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA35	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA44	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA46	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA84	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA91	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA103	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA104	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA105	*Clostridium butyricum* strain FBR1	EU689097	81%
HydA10	*Clostridium perfrigens* str. 13	BA000016	99%
HydA21	*Clostridium perfrigens* ATCC 13124	CP000246	100%
HydA28	*Clostridium perfrigens* ATCC 13124	CP000246	99%
HydA8	*Clostridium acetobutylicum* ATCC 824	AE001437	86%
HydA27	*Clostridium acetobutylicum* ATCC 824	AE001437	86%
HydA32	*Clostridium perfrigens* SM101	CP000312	99%
HydA24	Uncultured *Clostridium sp.* PCR-KE3-2	AY660727	89%
HydA26	*Clostridium butyricum* strain DSM10702	EF627973	82%
HydA2	*Clostridium sp.* IBUN 13A	GQ180215	82%
HydA5	*Clostridium sp.* IBUN 13A	GQ180215	80%
HydA6	*Clostridium sp.* IBUN 13A	GQ180215	80%
HydA31	*Clostridium sp.* IBUN 13A	GQ180215	80%
HydA3	*Clostridium saccharobutylicum* P626	U09760	80%
HydA13	*Clostridium botulinum* B str. Eklund 17B	CP001056	80%

Table 3. Similarities between FHG nucleotide sequences of bands and those of the closest relatives in the NCBI database

Clone	Closest relative	Accession no.	Identity
HydA1	*Clostridium paraputrificum*	BAD29951	82%
HydA4	*Clostridium paraputrificum*	BAD29951	81%
HydA12	*Clostridium paraputrificum*	BAD29951	82%
HydA15	*Clostridium paraputrificum*	BAD29951	82%
HydA17	*Clostridium paraputrificum*	BAD29951	82%
HydA19	*Clostridium paraputrificum*	BAD29951	81%
HydA22	*Clostridium paraputrificum*	BAD29951	82%
HydA16	*Clostridium cellulovorans* 743B	ZP_04803630	83%
HydA33	*Clostridium cellulovorans* 743B	ZP_04803630	83%
HydA46	*Clostridium cellulovorans* 743B	ZP_04803630	81%
HydA84	*Clostridium cellulovorans* 743B	ZP_04803630	82%
HydA103	*Clostridium cellulovorans* 743B	ZP_04803630	81%
HydA104	*Clostridium cellulovorans* 743B	ZP_04803630	81%
HydA105	*Clostridium cellulovorans* 743B	ZP_04803630	81%
HydA10	*Clostridium perfrigens* CPE str. F4969	ZP_02638882	97%
HydA28	*Clostridium perfrigens* CPE str. F4969	ZP_02638882	98%
HydA21	*Clostridium perfrigens* E str. JGS1987	ZP_02631180	100%
HydA8	*Clostridium acetobutylicum* ATCC 824	NP_346675	90%
HydA27	*Clostridium acetobutylicum* ATCC 824	NP_346675	90%
HydA32	*Clostridium perfrigens* SM101	YP_699606	97%
HydA24	*Clostridium pasteurianum*	P29166	83%
HydA26	*Clostridium sp.* 7_2_43FAA	ZP_05132432	88%
HydA2	*Clostridium sp.* 7_2_43FAA	ZP_05132432	88%
HydA5	*Clostridium sp.* 7_2_43FAA	ZP_05132432	86%
HydA6	*Clostridium sp.* 7_2_43FAA	ZP_05132432	86%
HydA23	*Clostridium sp.* 7_2_43FAA	ZP_05132432	86%
HydA31	*Clostridium sp.* 7_2_43FAA	ZP_05132432	85%
HydA34	*Clostridium sp.* 7_2_43FAA	ZP_05132432	82%
HydA35	*Clostridium sp.* 7_2_43FAA	ZP_05132432	82%
HydA44	*Clostridium sp.* 7_2_43FAA	ZP_05132432	83%
HydA91	*Clostridium sp.* 7_2_43FAA	ZP_05132432	83%
HydA3	*Clostridium butyricum*	AB042543	81%
HydA13	*Clostridium sp.* 7_2_43FAA	ZP_05132432	87%

Table 4. Similarities between FHG amino acid sequences of bands and those of the closest relatives in the NCBI database

Table 5 shows the calculated similarities of amino acid sequences between the thirty-three new identified [FeFe]-hydrogenase fragments and the six known *Clostridium* species used to design primer HydA1-F. A similar table was also compiled for the similarity analysis of DNA sequences (Table 6). Results show that clones found in this study were rather similar to each other, with similarity values ranging from 69 to 100% and from 19 to 99.7% based on amino acid and nucleotide sequences, respectively. These values are bigger than the similarities found among the amino acid sequences (33-75%) and among the nucleotide sequences (8-66%) of the 6 known hydrogenase genes alone. The highest similarity value (100%) among amino acid sequences was found among HydA-1, HydA-12 and HydA-22 clones. The clone sequences obtained in this study have low similarities to *C. saccharoperbutylacetonicum* and *C. thermocellum*, i.e. 37-58% based on amino acid sequences and 7-18% based on DNA sequences. Instead, these clones were more closely related to *C. pasteurianum*, *C. paraputrificum*, *C. saccharobutylicum* and *C. perfringens*, with values of similarity ranging from 73 to 100% and from 13 to 97.8% based on amino acid and nucleotide sequences, respectively.

	HydA-1	HydA-2	HydA-3	HydA-4	HydA-5	HydA-6	HydA-8	HydA-10	HydA-12	HydA-13	HydA-15	HydA-16	HydA-17	HydA-19	HydA-21	HydA-22	HydA-23	HydA-24	HydA-26	HydA-27	HydA-28	HydA-31	HydA-32	HydA-33	HydA-34	hydA-35	hydA-44	HydA-46	HydA-84	HydA-91	HydA-103	HydA-104	HydA-105	1	2	3	4	5
HydA-1																																						
HydA-2	83,16																																					
HydA-3	76,67	80,00																																				
HydA-4	99,49	82,65	76,11																																			
HydA-5	84,10	93,33	78,89	83,59																																		
HydA-6	84,18	93,37	78,89	83,67	98,97																																	
HydA-8	79,19	80,10	76,11	78,57	91,28	77,55																																
HydA-10	77,17	80,43	78,57	76,63	79,78	79,89	72,83																															
HydA-12	100,00	83,16	76,67	98,49	84,10	84,18	79,08	77,17																														
HydA-13	80,56	87,22	85,37	80,00	83,80	84,44	76,11	82,22	80,56																													
HydA-15	98,36	81,42	74,25	97,81	82,42	82,51	77,60	77,60	98,36	80,56																												
HydA-16	83,42	93,01	81,11	82,01	91,40	91,40	79,03	79,31	83,07	82,35	82,08																											
HydA-17	99,49	83,16	76,67	98,49	84,10	84,18	79,08	77,17	98,99	80,56	97,81	83,07																										
HydA-19	91,98	76,92	75,98	90,77	77,84	77,44	72,82	71,74	91,28	74,44	89,62	81,08	90,77																									
HydA-21	79,59	82,14	80,00	79,08	81,54	81,63	79,49	97,83	79,59	82,22	78,14	81,18	79,59	74,36																								
HydA-22	100,00	82,70	76,67	98,40	83,78	83,78	78,38	76,30	99,47	79,88	98,26	83,51	98,94	96,74	78,92																							
HydA-23	79,70	89,29	75,56	78,89	90,77	90,82	75,00	81,52	78,89	85,56	83,61	85,71	79,40	74,36	79,08	78,19																						
HydA-24	78,89	82,22	78,05	78,33	80,45	80,56	75,00	77,78	78,89	79,44	79,89	80,00	78,89	72,22	77,78	78,11	81,11																					
HydA-26	83,16	98,98	80,00	82,65	93,85	93,88	80,10	80,43	83,16	87,22	81,42	92,47	83,16	76,92	82,14	82,70	89,80	81,67																				
HydA-27	80,10	80,10	76,11	79,59	77,44	77,55	98,98	72,83	80,10	75,56	78,69	79,57	80,10	73,33	79,49	78,92	75,00	74,44	80,10																			
HydA-28	74,32	77,05	76,11	73,77	76,50	76,50	69,40	96,49	74,32	81,44	77,06	77,05	74,32	75,27	93,44	74,32	80,00	76,65	77,05	69,40																		
HydA-31	84,78	93,44	79,44	83,33	99,45	99,45	77,05	79,53	83,96	83,23	82,94	91,94	84,41	83,52	81,42	84,95	89,25	80,24	93,99	77,60	77,05																	
HydA-32	77,72	80,73	78,33	76,41	80,21	80,21	73,44	95,56	77,04	80,11	77,65	80,42	77,44	74,87	96,88	77,66	76,92	78,98	80,73	73,44	91,26	80,21																
HydA-33	84,34	93,37	81,11	82,91	91,79	91,84	80,20	79,89	83,92	83,33	82,51	99,47	83,92	76,92	81,63	84,04	86,43	81,11	92,86	80,10	77,05	92,47	80,00															
HydA-34	83,33	92,86	81,11	82,41	91,28	91,33	80,20	79,89	82,41	83,33	81,97	98,41	82,91	76,41	81,63	81,91	86,93	80,56	92,35	80,10	77,05	90,32	79,49	98,02														
hydA-35	83,33	92,86	80,56	82,41	91,28	91,33	79,70	79,35	82,41	82,78	81,97	98,41	82,91	76,41	81,12	81,91	86,93	80,56	92,35	79,59	76,50	90,32	78,97	97,52	98,51													
hydA-44	83,84	93,37	81,11	82,91	91,79	91,84	79,70	79,89	83,92	83,33	82,51	99,47	83,92	76,92	81,63	84,04	86,43	81,11	92,86	80,10	77,05	92,47	80,00	99,01	97,03	96,06												
HydA-46	84,34	93,37	81,11	82,91	91,79	91,84	80,20	79,89	83,50	83,33	82,51	99,47	83,92	76,92	81,63	84,04	86,43	81,11	92,86	80,10	77,05	92,51	80,10	99,50	97,52	98,03	98,03											
HydA-84	83,33	92,86	80,56	82,91	91,28	91,33	79,70	79,35	82,41	82,78	81,97	98,41	82,91	76,41	81,12	81,91	86,43	80,56	92,35	79,59	76,50	90,32	78,97	98,02	98,51	98,52	97,04	97,04										
HydA-91	83,84	93,37	81,11	82,91	91,79	91,84	80,20	79,89	82,91	83,33	82,51	98,94	83,42	76,92	81,63	82,45	87,44	81,11	92,86	80,10	77,05	90,86	79,49	98,02	99,01	99,51	96,55	98,52	98,03									
HydA-103	83,84	93,37	81,11	83,42	91,89	91,84	79,70	79,89	83,92	83,33	82,51	98,94	84,42	76,92	81,63	83,51	86,93	81,11	92,86	80,10	77,05	91,94	79,49	98,51	97,52	97,03	99,50	98,02	97,52	97,52								
HydA-104	82,83	92,86	80,56	81,91	91,28	91,33	79,19	79,35	82,50	82,78	81,97	99,47	82,91	76,41	81,12	82,98	85,93	80,56	92,35	79,59	76,50	91,44	80,10	98,02	97,03	96,06	99,01	97,06	97,04	96,55	98,51							
HydA-105	83,33	93,37	81,11	83,42	91,79	91,84	79,70	79,89	82,91	83,33	82,51	98,94	83,42	76,92	81,63	82,45	86,93	81,11	92,86	80,10	77,05	90,86	79,49	97,52	98,02	97,04	98,52	96,55	98,52	97,54	99,01	98,52						
1	79,26	82,14	80	77,89	81,54	81,63	74,62	97,83	78,5	82,22	78,14	81,48	78,89	74,36	100	78,72	77,89	77,78	82,14	74,49	93,44	80,75	96,43	81,68	81,19	79,8	80,79	80,39	80,79	80,3	80,2	80,39	82,16					
2	79,80	81,63	77,22	78,39	83,08	83,16	74,11	80,43	79,00	80,56	78,69	80,95	78,89	73,33	82,14	79,79	77,89	81,11	81,63	73,98	75,96	82,35	79,59	81,19	80,2	79,31	80,3	79,9	80,3	79,8	79,7	79,9	79,64	71,08				
3	80,30	84,18	80	78,89	82,56	82,65	80,71	79,89	79,50	83,33	80,33	82,01	79,90	74,87	80,61	79,26	77,89	83,89	84,18	80,10	75,96	81,82	79,59	82,67	81,68	80,79	81,77	81,37	81,77	81,28	81,19	81,37	80,99	68,87	67,3			
4	82,83	82,14	79,44	81,41	82,56	82,65	79,7	80,43	82	80	81,97	80,95	82,41	76,92	82,14	82,45	76,38	78,89	82,14	79,59	75,96	81,82	80,1	81,19	80,2	79,31	80,3	79,9	80,3	79,8	79,8	79,9	79,38	71,25	75,09	69,57		
5	57,07	54,08	52,22	55,78	54,36	55,1	52,79	57,61	56,00	54,44	56,83	53,44	56,28	52,31	58,16	54,79	51,26	53,89	54,08	52,55	53,01	52,41	55,61	55,45	55,45	54,19	54,68	54,41	55,17	54,68	53,96	54,41	54,18	46,43	47,91	44,71	47,24	
6	43,43	44,9	41,11	42,71	43,59	43,88	42,12	43,55	43	44,44	44,26	43,92	42,71	37,95	43,37	41,49	42,21	43,89	44,90	41,33	37,16	41,71	40,82	45,54	45,54	44,83	44,83	44,61	45,32	44,83	44,55	44,61	43,98	35,37	35,42	33,62	35,94	44,04

Clostridium species: 1. *C. perfringens*; 2. *C. saccharobutylicum;* 3. *C. pasteurianum;* 4. *C. paraputrificum;* 5. C. *thermocellum; 6. C. saccharoperbutylacetonicum*

Table 5. Similarity comparison of amino acid sequences of FHG fragments obtained in this study and those of *Clostridium* species used for the primer design.

	HydA-1	HydA-2	HydA-3	HydA-4	HydA-5	HydA-6	HydA-8	HydA-10	HydA-12	HydA-13	HydA-15	HydA-16	HydA-17	HydA-19	HydA-21	HydA-22	HydA-23	HydA-24	HydA-26	HydA-27	HydA-28	HydA-31	HydA-32	HydA-33	HydA-34	hydA-35	HydA-44	HydA-46	HydA-84	HydA-91	HydA-103	HydA-104	HydA-105	1	2	3	4	5
HydA-1																																							
HydA-2	80,3																																						
HydA-3	70,4	72,3																																					
HydA-4	97,8	78,8	69,1																																				
HydA-5	81,4	85,7	72,8	79,9																																			
HydA-6	81,9	85,5	72,4	80,4	98,8																																		
HydA-8	75,1	77,4	70,9	73,4	74	74,5																																	
HydA-10	74,6	73	67,6	73,1	73,6	74,7	70,2																																
HydA-12	98	78,9	69,2	99,2	80,2	80,7	73,6	73,3																															
HydA-13	72,4	73,6	68,3	71,3	70,9	71,8	69,4	74,6	71,1																														
HydA-15	93,3	74,9	65,5	91,5	76,3	77,3	69,4	79,5	91,7	77,2																													
HydA-16	75,6	80,1	76,3	76,7	83,2	82,5	70,8	67,6	76,5	66,9	70,3																												
HydA-17	98	79,2	69,2	99,2	80,1	80,6	73,6	73,5	99,7	71,2	91,7	76,5																											
HydA-19	93,3	78,5	68,9	91,2	79,4	80,2	74,4	75,3	91,4	72,1	88,6	73,8	91,4																										
HydA-21	80,4	79	72,1	78,9	79,2	79,7	75,6	92,9	79	70	75	73,2	79,2	79,8																									
HydA-22	93	75,7	73,5	93,9	76,9	76,2	69,9	69,1	94	67,3	86,9	81,2	94	86,9	74,8																								
HydA-23	81,1	85	71,2	82,1	90,2	90,4	77	73,7	82,3	70,7	75,8	84,2	82,1	79,6	79	77,9																							
HydA-24	72,2	74,5	66,3	71,1	73,5	74,6	67,8	77,8	71	79,9	76,6	69,2	71,3	72,1	73,3	67,5	73,5																						
HydA-26	80,8	93,4	73,1	79,3	86,7	87,3	78,1	74,9	79,4	75	75,9	81,3	79,9	80	79,9	75,5	87,3	75																					
HydA-27	75,3	77,5	71,3	73,9	74,1	74,6	99,3	70,5	74	69,5	69,9	71	74	74,8	75,9	70,1	76,9	68,1	78,2																				
HydA-28	73,6	72,7	75,8	74,5	73,3	72,6	69,1	84,4	74,8	63,9	68,2	77	75	73	91,4	79,2	74,5	66,1	72,9	69,6																		
HydA-31	75,6	79,4	76,3	76,4	92,1	91,4	68,2	67,6	76,9	65,2	18,7	87,7	76,8	73,4	73,1	81,3	86,4	67,6	80,1	68,4	79,6																		
HydA-32	76,4	77,3	72,2	77,2	77,3	76,7	72,2	86,5	77,5	67,1	71,4	75,3	77,5	75,6	93,2	76,5	78,3	70,3	77,1	72,5	91,5	76,2																	
HydA-33	79,7	82,9	71,4	80,6	85,9	86,4	74,8	71,5	80,4	69,7	73,8	92,8	80,4	77,4	77	76,3	88,6	73,2	84,8	74,6	72,1	82	75,3															
HydA-34	79,1	82,3	71,2	80,2	85,5	85,9	74,4	71,1	80,2	69,3	73,4	92,5	80,3	77	76,6	75,9	88,3	72,8	84,2	74,2	72	81,9	75,5	99,3															
hydA-35	78,9	82	70,8	80	85	85,5	73,9	70,8	80	69,3	73,2	92	80	76,8	76,3	75,6	87,8	72,3	84	73,7	71,6	81,5	75,3	98,9	92,5														
hydA-44	79,3	82,7	71,3	80,4	85,8	86,3	74,4	71,4	80,2	69,6	73,7	92,6	80,3	77	76,9	76,1	88,1	73,1	84,7	74,5	72	81,9	75,2	99,5	98,9	98,9													
HydA-46	79,2	82,3	70,9	80,2	85,3	85,8	74,3	71	80,1	69,2	73,3	92,2	80,2	76,9	76,5	75,8	88,1	72,7	84,3	74,1	71,9	81,8	75,1	99,3	99	99,3	99,3												
HydA-84	79	82,2	70,9	80,3	85,2	85,7	74	70,9	80,1	69,5	73,3	92	80,1	76,9	76,4	75,6	87,8	72,4	84,1	73,8	71,6	81,4	75,1	98,9	98,9	99,3	98,5	99											
HydA-91	78,9	82,2	70,8	80	85,2	85,7	74,2	70,9	80	69,1	73,2	92,4	80	76,8	76,4	75,6	88	72,6	84,1	74	71,8	81,6	75,3	99,2	99,2	99,5	99,3	99,7	99,2										
HydA-103	79	82,5	71	80,4	85,5	86	74,1	71,2	80,6	69,3	73,5	92,3	80,6	76,7	76,7	75,9	88	72,8	84,4	74,3	72	81,9	75,2	99,2	98,9	98,7	99,3	99,2	98,5	99									
HydA-104	78,5	82,2	70,8	79,8	85,2	85,7	73,9	70,9	79,8	69,1	73,2	92,2	79,8	76,5	76,4	75,4	87,5	72,6	84,1	74	71,6	81,4	75,1	98,7	98,5	98,9	99,2	99,2	98,7	99,4	98,9							
HydA-105	78,5	82,2	70,2	80,1	85,2	85,7	73,9	70,9	79,9	69,1	73,2	92,2	80,1	76,5	76,4	75,4	87,5	72,6	84,1	74	71,6	81,4	75,1	98,7	98,7	99,2	99	99,2	99,2	99,3	99	99,2							
1	74,9	14,6	13,4	15,1	14,5	14,6	14	17	15,1	13	13,8	14	15,1	14,7	18,3	14,2	15	13,6	14,8	14	17,3	13,9	17,6	15,1	15,1	15,2	15,1	15,2	15,1	15,2	15,1	15,2	15,1						
2	20,9	20	18,2	97,8	19,8	19,9	19,4	18,9	21,2	18,3	19,5	19,5	21,2	20,3	20,4	20,1	20,6	18,7	20,4	19,3	19,1	18,9	20,2	21,1	21,1	21,1	21	21,1	21	21,2	21	21,1	21,1	49,7				
3	17,2	17,1	15,8	7,6	16,7	16,9	16,7	16,6	17,8	15,9	15,9	15,9	17,8	16,8	18	15,8	17,3	16,2	16,9	16,7	16,6	15,7	17,5	17,3	17,2	17,3	17,3	17,3	17,3	17,3	17,2	17,4	17,4	43,6	48,5			
4	7,4	7,6	7	7,6	7,4	7,4	7,6	7,3	7,5	7,1	7	7,7	7,6	7,2	7,6	7,3	8,1	7	7,8	7,6	7,4	7,1	7,5	8	8,1	8,1	8	8,1	8	8,1	8,1	8,2	8,2	42,1	31,3	26,2		
5	20	19,9	17,9	20,3	20	20,1	19,6	18,8	20,2	18,4	18,7	19,4	20,3	19,8	20,1	19	20,6	18,9	20,3	19,5	19,1	89,6	20	20,9	20,9	21	20,9	21	20,9	21,1	20,9	21	20,9	37,2	49,3	38	25,9	
6	21,3	21,2	19,7	21,5	20,8	20,9	19,7	19,9	21,5	18,7	19,9	19,7	21,5	20,9	21,2	20,4	21,5	19,6	21,2	19,7	20	19,9	20,7	21,3	21,3	21,3	21,3	21,4	21,3	21,4	21,2	21,3	21,2	50,9	66	52,1	30,7	48,4

Clostridium species: 1. *C. perfringens*; 2. *C. paraputrificum*; 3. C. *thermocellum;* 4. *C. saccharoperbutylacetonicum;* 5. *C. pasteurianum; 6. C. saccharobutylicum*

Table 6. Similarity comparison of nucleotide sequences of FHG fragments obtained in this study and those of *Clostridium* species used for the primer design.

Phylogenetic relationships of the new identified FHG fragments and the six above mentioned known species, according to their DNA and amino acid sequences, are illustrated in Fig. 5 and Fig. 6, respectively. Some general features are found from these trees. As evidenced by the high bootstrap values, the thirty-three clones were classified into six main groups that formed distinct branches within the *Clostridium* genus, being not related to any known reference sequence, with the exception of the clones, belonged to Cluster V, closely affiliated to *Clostridium perfringens*.

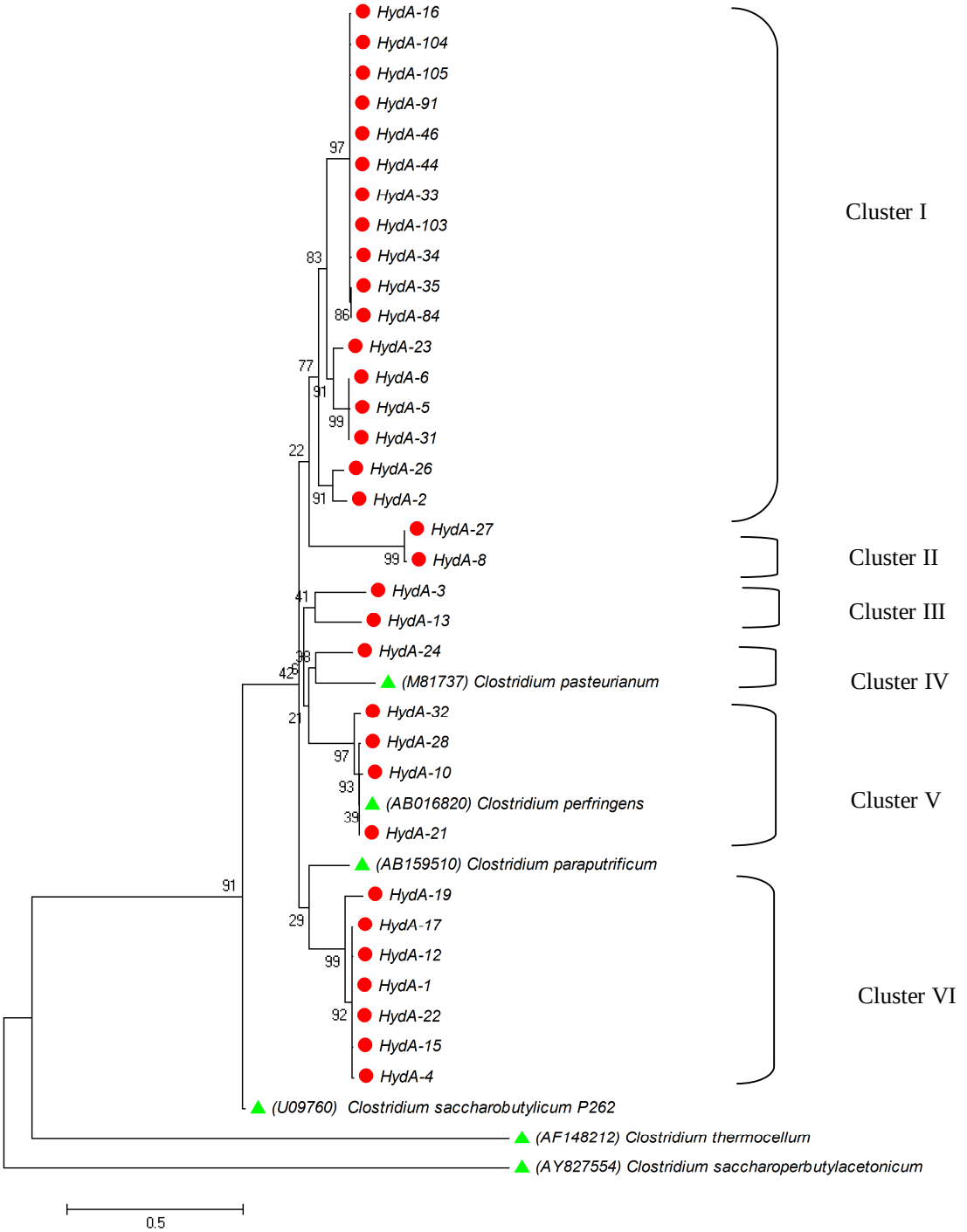

Figure 5. Neighbor-joining tree showing relationship between nucleotide FHG gene sequences from sediment (red circles) and those of *Clostridium* species used for the primer design (green triangles)**.**

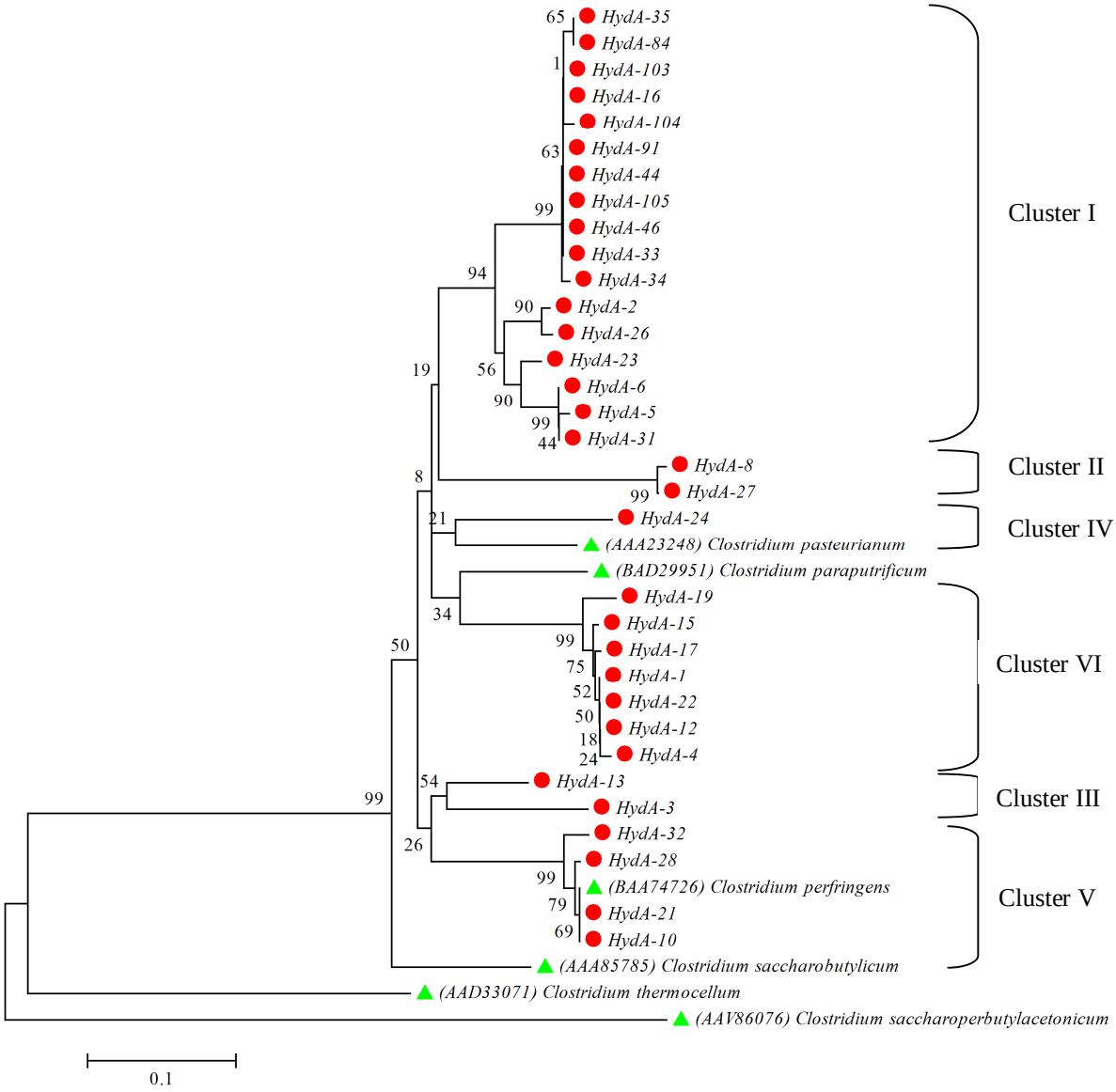

Figure 6. Neighbor-joining tree showing relationship between amino acid FHG gene sequences from sediment (red circles) and those of *Clostridium* species used for the primer design (green triangles)

Phylogenetic trees, showing the relationships between thes new identified FHG fragments and their closest relatives, with the addition of the six *Clostridium* species used to design primer, were also constructed on the basis of the DNA and amino acid sequences (Fig. 7 and Fig. 8).

HydA nucleotide sequences of the clones retrieved in this study fell into the same six main groups previously evidenced (Fig. 5). Clones of cluster II, cluster IV and Cluster V, were affiliated, with high values of bootstrap, to *Clostridium acetobutylicum, Clostridium sp* and *Clostridium perfringens,* respectively. Clones of cluster VI were weakly related to *Clostridium paraputrificum* (low bootstrap value), whereas cluster I and cluster III were not related to the reference sequences and formed distint branches. Similar groups were obtained

from the tree based on the amino acid sequences, with the exception of cluster IV, in which clone HydA-24 was not related to any amino acid reference sequence (Fig. 8)

In conclusion, these results evidenced not only the presence of well known hydrogen-producing bacteria such as *Clostridium acetobutylicum*, *Clostridium sp.*, *Clostridium perfringens* and *Clostridium paraputrificum*, but also the existence of new species of putative hydrogen-producer in the natural environment of lake sediment.

Figure 7. Neighbor-joining tree showing relationship between FHG gene sequences from sediment (red circles) and reference sequences obtained through blastn analysis**,** with the addition of the six *Clostridium* species used to design primer (green triangles).

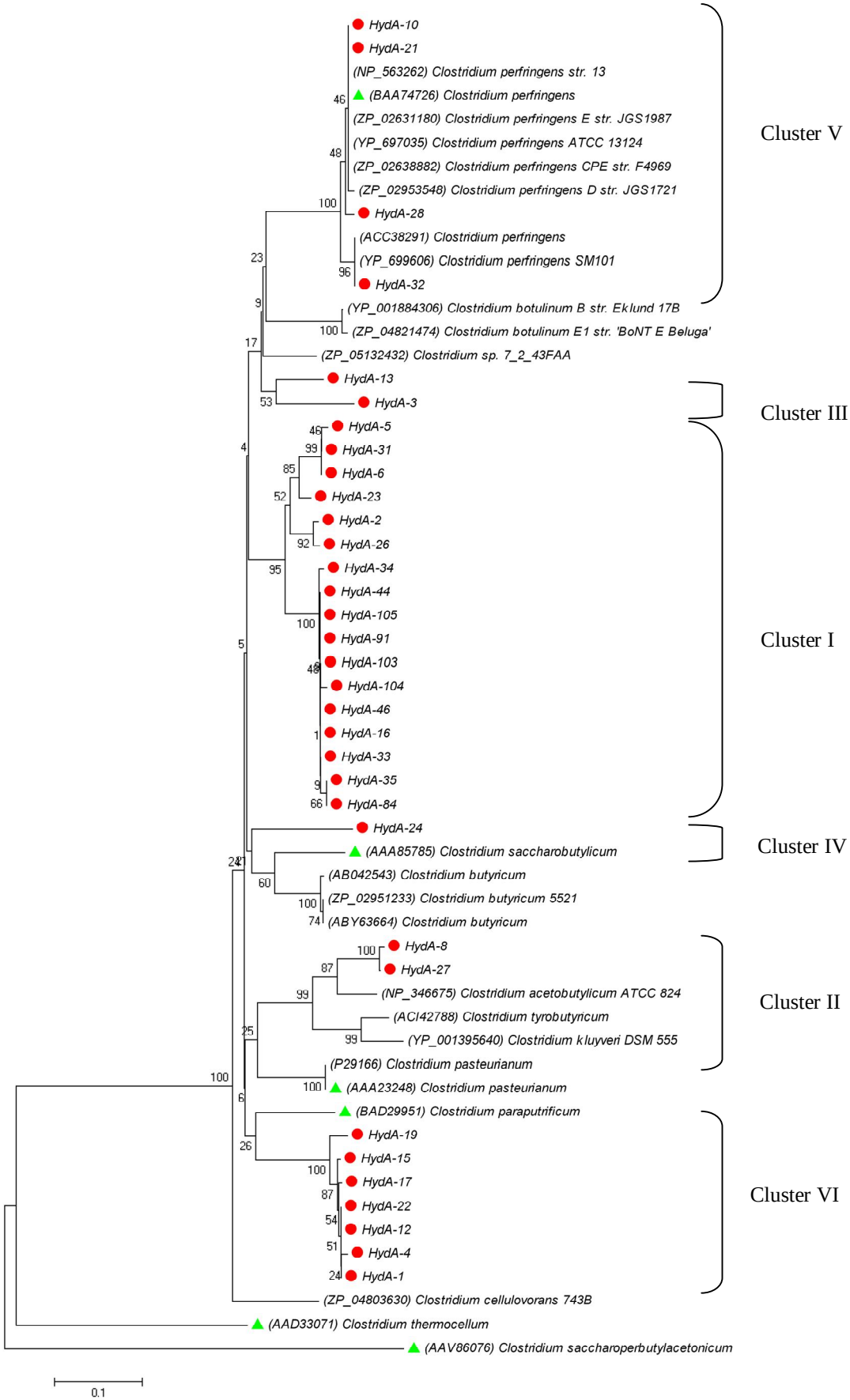

Figure 8. Neighbor-joining tree showing relationship between FHG gene sequences from sediment (red circles) and reference sequences obtained obtained through blastx analysis**,** with the addition of the six *Clostridium* species used to design primer (green triangles)

References

Caliro, S., Chiodini, G., Izzo, G., Minopoli, C., Signorini, A., Avino, R., Granieri, D. 2008 Geochemical and biochemical evidence of lake overturn and fish kill at Lake Averno, Italy. *J Volcanol Geother Res* 178: 305-316.

Chan, O. C., P. Claus, P. Casper, A. Ulrich, T. Lueders, Conrad, R. 2005. Vertical distribution of structure and function of the methanogenic archaeal community in Lake Dagow sediment. *Environ. Microbiol.* 7, 1139-1149.

Fang, H H P; Zhang T.; Li C. 2006. Characterization of Fe-hydrogenase genes diversity and hydrogen-producing population in an acidophilic sludge. *Journal of biotechnology*. 126(3):357-64.

Glissmann, K., Chin, K. J., Casper, P., Conrad, R. Methanogenic pathway and archaeal community structure in the sediment of eutrophic Lake Dagow: effect of temperature, *Microbial. Ecol.* 48 (2004) 389–399.

Go, Y. S., Han, S. K., Lee, I. G., Ahn, T. Y. 2000 Diversity of the domain *Archaea* as determined by 16S rRNA gene analysis in the sediments of Lake Soyang, *Arch. Hydrobiol.* 149, 459–466.

Hastings, R. C., Saunders, J. R., Hall, G. H., Pickup, R. W., McCarthy, A. J. 1998. Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. *Appl. Environ. Microbiol.* 64, 3674-3682

Hiorns, W. D., Hastings, R. C., Head, I. M., McCarthy, A. J., Saunders, J. R., Pickup, R. W., Hall G. H. 1995. Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. *Microbiology* 141:2793–2800.

Kawagoshi Y., Hino N., Fujimoto A., Nakao M., Fujita Y., Sugimura S., Furukawa K. 2005. Effect of inoculum conditioning on hydrogen fermentation and pH effect on bacterial community relevant to hydrogen production. *Journal of Bioscience and Bioengineering.* Vol. 100; pp. 524-530

Koizumi, Y., Kojima, H., Fukui, M. 2004. Dominant microbial composition and its vertical distribution in saline meromictic Lake Kaiike (Japan) as revealed by quantitative oligonucleotide probe membrane hybridisation, *Appl. Environ. Microbiol*. 70 4930–4940.

Koizumi, Y., Takii, S., Nishino, M., Nakajima, T. 2003. Vertical distributions of sulfate-reducing bacteria and methane-producing *Archaea* quantified by oligonucleotide probe hybridization in the profundal sediment of a mesotrophic lake, FEMS *Microbiol. Ecol.* 44 101–108.

Li, J. H., Purdy, K. J., Takii, S., Hayashi, H. 1999. Seasonal changes in ribosomal RNA of sulfate-reducing bacteria and sulfate reducing activity in a freshwater lake sediment. FEMS *Microbiol. Ecol.* 28:31–39.

Lindstrom, E. S., Leskinen, E. 2002. Do neighboring lakes share common taxa of bacterioplankton? Comparison of 16S rDNA fingerprints and sequences from three geographic regions. *Microb. Ecol.* 44:1–9.

Miskin, I. P., Farrimond, P., Head, M. 1999. Identification of novel bacterial lineages as active members of microbial populations in a freshwater sediment using a rapid RNA extraction procedure and RT-PCR. *Microbiology* 145:1977–1987.

Nelson D. M., Ohene-Adjei S., Hu F. S., Cann I. K O, Mackie R. I. 2007. Bacterial diversity and distribution in the holocene sediments of a northern temperate lake. *Microbiol. Ecol.* 54(2):252-63.

Purdy, K. J., Nedwell, D. B., Embley, T. M., Takii, S. 1997. Use of 16S rRNA-targeted oligonucleotide probes to investigate the occurrence and selection of sulfate-reducing bacteria in response to nutrient addition to sediment slurry microcosms from a Japanese estuary. FEMS *Microbiol. Ecol*. 24:221–234.

Schwarz, J. I. K., Eckert, W., Conrad, R. 2007 Community structure of *Archaea* and *Bacteria* in a profundal sediment, Lake Kinneret (Israel). *Syst. Appl. Microbiol*. 30, 239-254.

Spring, S., Schulze, R., Overmann, J., Schleifer, K. 2000. Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: molecular and cultivation studies. FEMS *Microbiology Reviews*, 24: 573-590

Stackebrandt, E., Goebel, B. M. 1994. A place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 44, 846–849.

Tamaki, H., Sekiguchi, Y., Hanada, S., Nakamura, K., Nomura, N., Matsumura, M., Kamagata, Y. 2005. Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based techniques. *Appl. Environ. Microbiol.* 71, 2162-2169.

Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol.* 24: 1596-1599.

Wise, M. G., McArthur, J. V. Shimkets, L. J. 1997. Bacterial diversity of a Carolina Bay as determined by 16S rRNA gene analysis: confirmation of novel taxa. *Appl. Environ. Microbiol*. 63:1505–1514.

Wobus, C., Bleul, S., Maassen, C., Scheerer, M., Schuppler, E., Jacobs, Roske, I., 2003. Microbial diversity and functional characterization of sediments from reservoirs of different trophic state, FEMS *Microbiol. Ecol.* 46 331–347.

Zhou, J.-Z., Bruns, M. A. Tiedje, J. M. 1996. DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol.* 62:461–468.

Summary

The diversity of lake microbial communities has been investigated for many years using methods based on isolation of microorganisms on culture media. However, as the proportion of cells from environmental samples which can be cultured is estimated to be 0.1% or at most 10% of the total population, this method provides partial data concerning the composition of these communities. Recent advances in the field of molecular biology (extraction of nucleic acids, polymerase chain reaction (PCR) amplification, fingerprinting methods) allowed the development of techniques which no longer require the isolation and cultivation of bacteria.

Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments has been widely used to assess the diversity of complex microbial communities. Several cultivation-independent studies of microbial diversity in sediments and water column of lakes have been conducted, but the importance and role of microorganisms in these ecosystems require further investigation, especially with respect to their potentially important partecipation in the significant biogeochemical cycling of elements such as carbon and nitrogen. For this purpose it could be also useful to characterize the bacterial population with specific metabolic activities, such as nitrification/denitrification, sulfate reduction, methane production and hydrogen production, on the basis of their functional genes.

In particular, in the last years, hydrogen-evolving microorganisms have attracted much attention due to their potential in biological hydrogen production from renewable biomass. Since it was demonstrated that lake sediment represents an efficient inoculum for an appreciable hydrogen production (Kawagoshi et al., 2005), it is useful to assess the the diversity of H_2 producing bacteria naturally occurring in this habitat.

In the present study PCR-DGGE technique was employed to assess the microbial diversity along the water column of Lake Averno in relation to environmental parameters, and to investigate the bacterial community composition of its superficial sediment.

Moreover, the diversity of H_2 producing bacteria from lake sediment was assessed by PCR amplification and subsequent cloning and sequencing of the FHG fragments.

Results obtained in this work have been presented in the following:

Abstracts & Poster presentations:

- **P. PAGANIN,** S. LAL, L. CHIARINI, C. DALMASTRI, A. BEVIVINO, A. SIGNORINI, C. VARRONE, G. IZZO, S. TABACCHIONI, 2007 **"***Correlation between DGGE profiles of microbial communities and environmental parameters of Averno lake by PCA"*, FISV Federazione italiana Scienze della vita IX Annual Congress 2007, Riva del Garda, 26-29 September.

- S. LAL, **P. PAGANIN,** L. CHIARINI, A. BEVIVINO, C. DALMASTRI, S. TABACCHIONI, 2007 **"***Eutrophic lake sediments host a community of H_2 producing strains of Paenibacillus polymyxa"*, FISV Federazione italiana Scienze della vita IX Annual Congress 2007, Riva del Garda, 26-29 September.

- **P. PAGANIN,** L. CHIARINI, A. BEVIVINO, C. DALMASTRI, G. IZZO, S. TABACCHIONI, 2008 *"Archaeal and bacterial community composition of plankton and sediment from Averno lake."*, FISV Federazione italiana Scienze della vita X Annual Congress 2008, Riva del Garda, 24-27 September.

- **P. PAGANIN ,** V. D'ANGELO, L. CHIARINI, S. TABACCHIONI, 2009 *"Characterization of hydrogen-producing population and Fe-hydrogenase gene diversity in Averno lake sediments"*, IDROBIO International Symposium, Accademia Nazionale dei lincei, 2-3 November.

- S. ROMANO, **P. PAGANIN**, S. TABACCHIONI, L. CHIARINI, 2009 *"Hydrogen production: metabolic and molecular characterization of bacterial consortia selected from lake sediments"* IDROBIO International Symposium, Accademia Nazionale dei lincei, 2-3 November.

Oral presentations:

- **P. PAGANIN**, L. CHIARINI, C. DALMASTRI, A. BEVIVINO, A. SIGNORINI, G. IZZO, S. TABACCHIONI, 2007**"***Microbial profiling of the water column of lake Averno, a volcanic eutrophic lake in Italy"*, "Bertinoro Meeting di Microbiologia Ambientale"BMMA 2007, Centro Residenziale Universitario di Bertinoro (CEUB) 18-19 Maggio 2007

Publications:

- **P. PAGANIN**, L. CHIARINI, A. BEVIVINO, C. DALMASTRI, G. IZZO, A. SIGNORINI, C. VARRONE, S. TABACCHIONI, 2010 **"***Vertical distribution of bacterioplankton in Lake Averno in relation to water chemistry"*, Environmental Microbiology (SUBMITTED).

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