Thèse/Université de Bretagne Occidentale
*sous le sceau de L'université Européenne de Bretagne*

# Subseafloor archaeal communities: from the surface to a deep hot biosphere?

Pour obtenir le grade de

**Docteur de l'Université de Bretagne Occidentale**
Discipline: Microbiologie

présentée par
M. Erwan G. Roussel

Laboratoire de Microbiologie des environnements extrêmes
UMR6197 UBO CNRS IFREMER
École Doctorale des Sciences de la Mer

Composition du jury :

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John Baross, Professeur, Université de Washington
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Marie-Anne Cambon-Bonavita, Chargée de recherches, IFREMER Brest
Daniel Prieur, Professeur, Université de Bretagne Occidentale

Soutenue le 17 octobre 2008, devant la commission d'examen

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# Les communautés *Archaea* du plancher océanique : de la surface à une biosphère profonde et chaude ?

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# Remerciements

Cette thèse est présentée en vue d'obtenir le titre de Docteur en Sciences de la Vie de l'Université de Bretagne Occidentale (spécialité Microbiologie). Cette thèse a été financée par une allocation du Ministère de l'Education Nationale, de la Recherche et de la Technologie. Ont participé également à la réalisation de ce projet l'IFREMER et la Région Bretagne.

J'exprime ma reconnaissance aux membres du jury qui me font l'honneur d'examiner ce travail. Merci au Professeur John Baross, de l'Université de Washington, et au Professeur John Parkes, de l'Université de Cardiff, d'avoir accepté spontanément d'être les rapporteurs de ce travail de recherche.
Mes remerciements s'adressent également au Professeur Georges Barbier, au Docteur Jean-Jacques Godon et au Docteur Christian Mustin pour leur participation à ce jury de thèse.

Ma reconnaissance va également au Professeur Daniel Prieur pour m'avoir accueilli au sein de son équipe de recherche. Merci d'avoir su m'orienter avec pertinence tout au long de cette thèse. Merci encore d'avoir su créer les opportunités qui m'ont permis de la mener à bien.

J'adresse mes vifs remerciements au Docteur Marie-Anne Cambon-Bonavita pour son soutien et son optimisme sans faille en toutes circonstances. Merci Marie-Anne de m'avoir fait découvrir la biologie moléculaire, et aussi pour ta disponibilité de tous les instants. Merci enfin de m'avoir permis de participer à ma première campagne océanographique.

I am very deeply indebted to Professor John Parkes for his invaluable help and knowledge, and for the opportunities of working in his lab in Cardiff. Thank you also for your decisive support.

J'exprime ma reconnaissance au Docteur Joël Querrellou qui m'a accueilli dans son laboratoire durant mon DEA et qui m'a permis de travailler sur de précieux échantillons.

Mes remerciements s'adressent également aux géologues et géochimistes avec qui j'ai eu le plaisir de collaborer. Je remercie Yves Fouquet pour ses analyses et ses discussions scientifiques. Je remercie également Jean-Luc Charlou d'avoir partagé avec moi ses solides connaissances et sa rigueur scientifique, ainsi que pour son aide à bord du Marion-Dusfresne et de l'Atalante. Je remercie aussi vivement Eric Chaumillon pour sa curiosité et son ouverture d'esprit qui nous ont permis de mener à bien notre collaboration. Merci également à Jonathan Allard, Carine Chaduteau, Jean-Pierre Donval et Cécile Konn pour leur participation à ce travail.

Ma reconnaissance va également aux chefs de mission des campagnes océanographiques auxquelles j'ai eu la chance de participer (EXOMAR, MATACOR, AUSFAIR, ZoNéCo12 et Mobidyc5). Je tiens particulièrement à remercier Yvon Balut d'avoir donné à un jeune doctorant l'opportunité de travailler sur des sédiments de l'autre bout du monde. Merci aussi à tous les membres d'équipage, ainsi qu'à l'équipe du ROV, pour m'avoir fait partager intensément la vie

à bord. Je remercie également Magalie Zbinden et Bruce Shillito pour leur efficacité et leur décontraction.

Je remercie Morgan Perennou et Sarah Romac pour leur aide durant le séquençage sur la plateforme de Roscoff.

Je tiens à remercier Laurent Toffin qui a initié la thématique de recherche sur les sédiments marins profonds à travers le programme "DeepBug".
Merci à Stéphane L'Harridon et à Christian Jeanthon pour leurs remarques et leurs discussions scientifiques.
Merci à Valérie Cueff-Gauchard pour son aide et sa précieuse organisation.
Je remercie aussi les stagiaires et toutes les personnes qui m'ont aidé techniquement : Solène Grayo, Eloïse Delage, Floriane Duval, Vincent Lecomte et Laëtitia Hervé.

Je remercie également Claire Geslin de m'avoir apporté son expérience d'enseignante.

I would like also to thank Barry Cragg and Gordon Webster for the help they provided in the study of the very deep ODP 210 samples.

J'en profite pour remercier tout le Laboratoire de Microbiologie des Environnements Extrêmes d'avoir supporté avec patience mes "TOC liés à la stérilité". Un grand merci à Christine Lamy pour son efficacité sans faille et sa bonne humeur permanente.
Je tiens aussi à remercier pour la qualité de leur travail les doctorants qui m'ont précédé, plus particulièrement : Olivier Nercessian et Hélène Moussard.

Bon courage pour la suite à toute l'équipe sub-surface.

Merci à tous les thésards du labo pour leur soutien et leur bonne humeur : Lucile, Sébastien, Cassandre, Mathieu G1, Zeng, Nathalie, Mathieu G2... Bon courage pour la suite.
Un grand merci à Raja pour son amitié et son intarissable source d'information... Bon courage.
Merci aussi à Leila d'avoir participé à l'activité nocturne de l'IUEM.
Je remercie aussi à cette occasion la barrière de l'IFREMER de nous avoir toujours soutenue...
Je remercie particulièrement Adeline pour ses discussions scientifiques et un peu moins scientifiques...
Merci aussi au petit groupe virus : Aurore et Pauline, c'est à votre tour maintenant, bon courage... A Olivier et à Charlotte également, bon courage pour la suite. Merci aussi à Benoît et Vivi pour la découverte de mon frère jumeau... Merci aussi à toi « Ô » Valentin, notre explorateur au long cours.

Merci aussi aux compagnons de route : Fanny, Carine, Laëtitia, Camille, Caroline, Karima, Christophe.

Merci aussi à Vitaly Zinkevich (Portsmouth) et Carlos Blanco (Rennes), scientifiques passionnants et passionnés.

A mes parents, André et Margaret, je vous suis infiniment reconnaissant de m'avoir soutenu, et aidé tout au long de mon parcours, et aussi pour vos patientes relectures.

Merci à ma "hotline", mon frère Tangi, pour avoir patiemment toujours su résoudre tous mes problèmes. Bon courage pour ta thèse.

Enfin merci infiniment à Anne-Laure sans qui ce travail aurait été difficilement réalisable. Merci pour ta curiosité, ta patience, ta persévérance, tes discussions scientifiques et ton appui de tous les instants. Merci pour tout.

*To Gran and Grandad.*

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# ABBREVIATIONS

| | |
|---|---|
| **AAG** | Ancient Archaeal Group |
| **ANME** | ANaerobic MEthane oxidizers |
| **AOA** | Ammonia Oxidizing Archaea |
| **AOM** | Anaerobic Oxidation of Methane |
| **AMO** | Ammonium MonoOxygenase |
| **anammox** | anaerobic ammonium oxidation |
| **BET** | Ethidium bromide |
| **bp** | base pair |
| **CARD–FISH** | CAtalysed Reported Deposition –Fluorescence *in situ* Hybridization |
| **CFB** | Cytophaga-Flavobacterium-Bacteroides |
| **CP** | Critical Point |
| **DGGE** | Denaturing Gradiant Gel Electrophoresis |
| **DHVE** | Deep-Sea Hydrothermal Vent Euryarcachaeotal group |
| **DNA** | DesoxyriboNucleique acid |
| **DSDP** | Deep Sea Drilling Project |
| **DSR** | Dissimilatory (bi)Sulphite Reductase |
| **DSV** | Deep Submergence Vehicule |
| **EPR** | East Pacific Rise |
| **FISH** | Fluorescent *in situ* Hybridisation |
| **GNS** | Green Non Sulfur |
| **HMMV** | Hakon Mosby Mud Volcano |
| **IODP** | Integrated Ocean Drilling Program |
| **JS1** | Japan Sea 1 group |
| **LCHF** | Lost City Hydrothermal Field |
| **MBG-B** | Marine Benthic Group B |
| **MBG-D** | Marine Benthic Group D |
| **mbsf** | meters below sea floor |
| **MCG** | Miscellaneous Crenarchaeotal Group |
| **mcr** | methyl-coenzyme M reductase |
| **MG-1** | Marine Group 1 |
| **MHVG** | Marine Hydrothermal Vent Group |
| **MAR** | Mid-Atlantic Ridge |
| **MMO** | Methane MonoOxygenase |
| **MOR** | Mid-Ocean Ridge |
| **ODP** | Ocean Drilling Program |
| **OM** | Organic Matter |
| **OP8** | Opal Pool 8 group |
| **PCR** | Polymerase Chain Reaction |
| **RCB** | Rotary Core Barrel |
| **RFLP** | Restriction Fragment Length Polymorphism |
| **RNA** | RiboNucleique Acid |
| **ROV** | Remote Operated Vehicle |
| **rRNA** | ribosomal RiboNucleique Acid |
| **RT-PCR** | Reverse Transcription-PCR |
| **SAGMEG** | South African Gold Mine Euryarchaeotic Group |
| **SIP** | Stable Isotope Probing |
| **SMTZ** | Sulfate-Reducing and Methanogenesis Transition Zone |
| **SRB** | Sulfate Reducing Bacteria |
| **SSCP** | Single Strand Conformation Polymorphism |
| **TEAP** | Terminal Electron Accepting Process |
| **TGGE** | Temperature Gradiant Gel Electrophoresis |
| **T-RFLP** | Terminal-Restriction Fragment Length Polymorphism |
| **VBNC** | Viable But Not Culturable |
| **WRC** | Whole Round Core |
| **XCB** | eXtended Core Barrel |

# Introduction

# INTRODUCTION

At the end of the nineteenth century, the first oceanographic cruise of HMS "*Challenger*" [1] opened the way to studies of deep marine sediments (Figure 1). At the same time, Jules Verne was writing *Journey to the Centre of the Earth* (Verne, 1864). In 1875, an Austrian geochemist Eduard Suess introduced the biosphere concept, which was later developed by Vernadsky (Vernadsky, 1926). In the early twenties, the search for deeply buried life forms was followed by the discovery of sulphate reducing *Bacteria* in terrestrial oilfield waters by Bastin et Greer (Bastin *et al.*, 1926). However, Moritta and Zobell, in 1955, suggested that life was impossible below 7.47 metres below sea-floor (Morita and Zobell, 1955). At the end of the sixties began the first research program exploring the Earth's history and structure as recorded in seafloor sediments and rocks (Deep Sea Drilling Project, DSDP). This program was latter followed by the Ocean Drilling Program (ODP) and the Integrated Ocean Drilling Program (IODP). The discovery of hydrothermal vent ecosystems also at the end of seventies deeply modified the concept of energy sources available for life in dark deep-sea ecosystems. Moreover, hydrothermal vents could even constitute a window to a deep hot biosphere (Deming and Baross, 1993*a*).

To date, prokaryotes from the sub-seafloor have been detected as deep as 842 meters below seafloor (mbsf) (Wellsbury *et al.*, 2002) and in hydrothermal fluids well over 100°C (Takai *et al.*, 2004). As the sediment cover can extend to more than 10 km below the surface seafloor, this deep biosphere can extend probably deeper. Therefore, the sub-seafloor biosphere may contain two thirds of Earth's total microbial biomass and encompass 10 % of the Earth global biomass (Parkes *et al.*, 2000).

The microorganisms in deeply buried sediments were shown to be metabolically active, and play an important role in global biogeochemical cycles (Bird *et al.*, 2001; Parkes *et al.*, 2000; D'Hondt *et al.*, 2004; D'Hondt *et al.*, 2002; Parkes *et al.*, 2005, for e.g.). As the dark and

1. Corfield, R. (**2003**) *The Silent Landscape: The Scientific Voyage of HMS Challenger*; Joseph Henry Press.

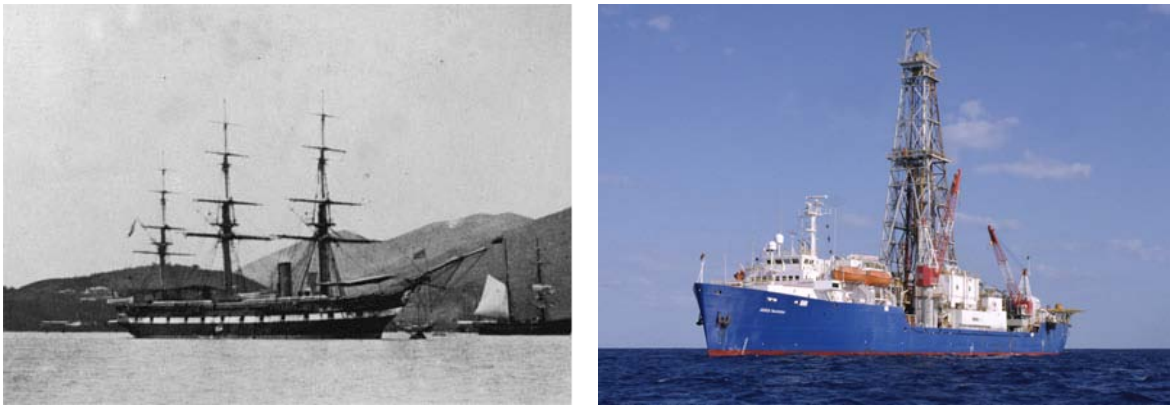

FIG. 1: HMS Challenger (left), and the drill ship JOIDES Resolution mainstay of the Ocean Drilling Program and Integrated Ocean Drilling Program (right).

anoxic sediments provide extremely low energy flux available to individual cells, a majority of the microorganisms are probably lithoautotrophs (Jorgensen and Boetius, 2007). Moreover, most deep-biosphere microorganisms detected so far have been extremely resistant to cultivation, which might be expected considering their incredibly slow *in situ* growth rates. The few isolates that were obtained are associated with sulfate and methane cycles, the two main energy sources in deep sediments. However, more than 99 % of the deep sediment microorganisms are still uncultivated (Amann *et al.*, 1995; Rappe and Giovannoni, 2003). Most of the studies avoided this cultivation bias by using molecular approaches. Therefore, we have only a limited understanding of the metabolic process driving the prokaryotic communities. Hence, here are some of the key questions we focus on in the present study:

- At what depth does the deep biosphere start?
- What are the main environmental factors selecting the prokaryotic diversity?
- How deep does the sub-seafloor biosphere extend?
- Is there a deep hot biosphere fuelled by compounds synthesized abiotically?

In order to try to answer these questions, the present work is divided in three chapters. The first chapter describes the general bibliographical context and objectives. The second chapter displays the results obtained during this research, and is composed of five studies organised in a progression from a shallow coastal subsurface to a deep hot biosphere located at the Mid-Ocean Ridges (Figure 2). The first study describes the methodological considerations and limitations associated to the analysis of sub-seafloor microbial communities. The second study describes the archaeal diversity associated with shallow coastal methane bearing sediments in the Marennes-Oléron Bay (France; see 2 of Figure 2). In the third study we examine the boundary between

surface and subsurface communities associated with deep marine sediments (see 3 of Figure 2). Archaeal indicators of deep hot sub-seafloor ecosystems were also investigated, as some sites were characterized by a strongly faulted cold seafloor. Going deeper, the fourth study presents results that extend the known sub-seafloor biosphere to at least 1,626 mbsf (see 4 of Figure 2). Getting hotter, finally we investigate the archaeal community potential associated with hydrothermal fluids from three ultramafic sites of the Atlantic Ocean (see 5 of Figure 2).

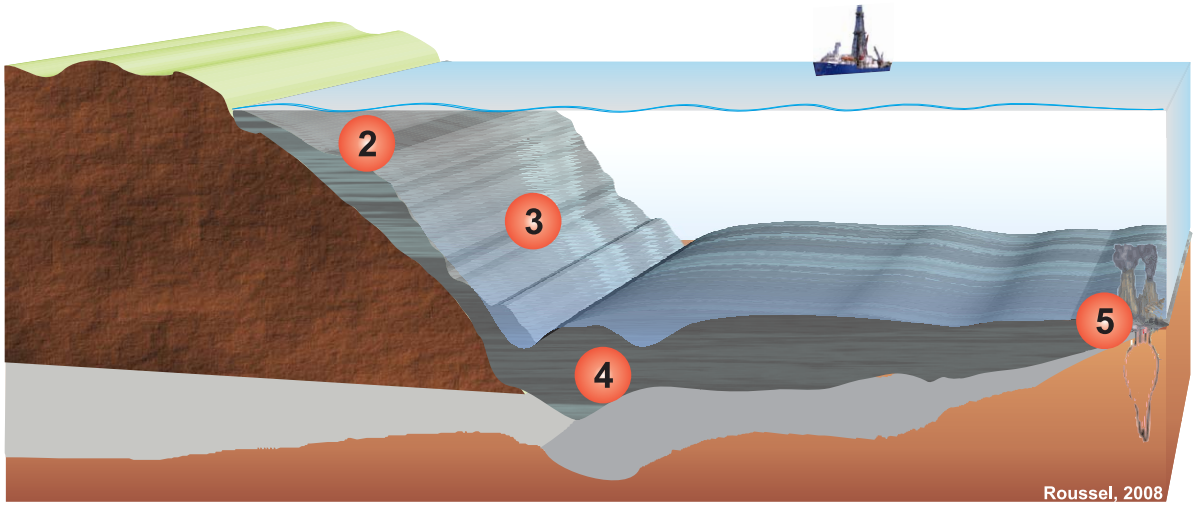


FIG. 2: Cross-section representation of the organisation of the second chapter of the study. Each number indicates the location on of the each study

In the last chapter, we discuss the results presented in the previous chapters, and give further interpretation. This part provides also the final concluding remarks and perspective for future research on the sub-seafloor biosphere.

*Les premières campagnes océanographiques entreprises par le HMS "Challenger", en 1872, permirent d'atteindre des sédiments situés à 8200 m sous la surface de la mer (Figure 1). A la même époque, Jules Verne imaginait d'incroyables êtres vivants au centre de la terre (Verne, 1864). En 1875, Eduard Suess, un géochimiste Autrichien, énonçait la définition de la biosphère, définition remaniée en 1926 par Vernadsky (Vernadsky, 1926). Les premières recherches de vie en sub-surface débutent dans les années 1920 avec E. Bastin et F. Greer qui étudient des bactéries sulfato-réductrices isolées d'échantillons d'eaux souterraines à des centaines de mètres sous la surface (Bastin* et al.*, 1926). En 1955, C. Zobell et R. Morita étudient la biosphère marine, et en raison de leur incapacité à cultiver des microorganismes en dessous de 7,47 mètres sous la surface du sédiment (mbsf), ils définissent sa limite à cette profondeur (Morita and Zobell, 1955). Plus tard, dans les années 1970, commencent les campagnes de forage du "Deep Sea Drilling Project" (DSDP), plus tard appelé "Ocean Drilling Program" (ODP), puis "Integrated Ocean Drilling Program" (IODP). Ces expéditions ont pour but d'étudier l'histoire de la Terre à travers les sédiments et les roches des fonds océaniques. La découverte des sources hydrothermales sous-marines, en 1977, modifia profondément le concept de la vie sur Terre, car bien que ces écosystèmes soit totalement dépourvus de lumière solaire, ils abritent pourtant une biomasse considérable. Les cheminées hydrothermales pourraient même vraisemblablement constituer des "fenêtres" sur une biosphère profonde et chaude (Deming and Baross, 1993*a*).*
*Actuellement, la présence de vie microbienne a été mise en évidence dans des sédiments marins atteignant une profondeur de 842 mbsf (Wellsbury* et al.*, 2002), et dans des fluides hydrothermaux dont la température est bien supérieure à 100°C (Takai* et al.*, 2004). Sachant que la couche sédimentaire peut atteindre jusqu'à 10 km d'épaisseur, il est probable que cette biosphère profonde soit très étendue. Ainsi le plancher océanique pourrait contenir les deux tiers de la biomasse microbienne, représentant 10 % du carbone organique de la planète Terre (Parkes* et al.*, 2000).*
*Les microorganismes profondément enfouis dans le sédiment sont actifs, jouant ainsi un rôle important dans les cycles biogéochimiques (Bird* et al.*, 2001; Parkes* et al.*, 2000; D'Hondt* et al.*, 2004; D'Hondt* et al.*, 2002; Parkes* et al.*, 2005, e.g.). Les temps de génération de ces microorganismes, probablement extrêmement élevés, les rendent difficilement cultivables. La majorité des procaryotes cultivés sont impliqués dans le cycle de sulfate et du méthane, les deux principales sources d'énergies disponibles dans les sédiments marins profonds. Néanmoins, plus de 99 % des microorganismes des sédiments marins profonds demeurent non-cultivés (Amann* et al.*, 1995; Rappe and Giovannoni, 2003). L'approche moléculaire permet souvent de contourner ces biais liés à l'utilisation de l'approche culturale. La connaissance des processus métaboliques de ces communautés microbiennes restent ainsi encore très limité. Au cours de cette étude, nous nous concentrerons sur les questions suivantes :*

- *A quelle profondeur commence la biosphère profonde ?*
- *Quelles sont les facteurs environnementaux qui déterminent la diversité des procaryotes ?*

- *Jusqu'à quelle profondeur sous le plancher océanique s'étend la biosphère ?*
- *Existe-t-il une biosphère profonde et chaude alimentée par des composés abiotiques ?*

*Les travaux présentés dans ce manuscrit ont pour objectif de fournir des éléments de réponse aux quatre questions ci-dessus, et dans ce but, il est organisé en trois chapitres. Le premier chapitre expose l'état actuel des connaissances, le contexte et les objectifs. Les résultats du chapitre deux sont présentés en cinq études organisées suivant une progression allant de l'étude de sédiments côtiers jusqu'aux systèmes hydrothermaux situés le long d'une ride océanique (Figure 2). La première étude détaille l'aspect méthodologique ainsi que ses limitations liées à l'analyse moléculaire des communautés microbiennes de sub-surface. La seconde étude présente l'analyse de la diversité archéenne associée à des sédiments côtiers riches en méthane situés dans la baie de Marennes-Oléron (France; voir 2 Figure 2). La troisième étude cherche à définir la limite qui existe entre les communautés archéennes de surface et celles de sub-surface dans les sédiments marins profonds. Des indices potentiels concernant l'existence d'une biosphère profonde et chaude ont aussi été recherchés, étant donné que certaines des zones étudiées étaient caractérisées par une fracturation importante de la couche sédimentaire (voir 3 Figure 2). L'analyse de couches sédimentaires plus profondes est présentée dans la quatrième étude, étendant ainsi la profondeur de la biosphère à au moins 1626 mètres sous la surface du sédiment (voir 4 Figure 2). Enfin, la cinquième étude analyse les communautés archéennes de sub-surface associées aux fluides hydrothermaux de trois sites ultramafiques de l'océan Atlantique (voir 5 Figure 2).*
*Dans le dernier chapitre, nous discuterons les résultats présentés dans le chapitre précédent, et nous présenterons les conclusions de ce travail de recherche tout en essayant d'ouvrir de nouvelles pistes pour l'étude de la biosphère profonde.*

# Chapter I – The sub-seafloor ecosystems: general context

# CHAPTER 1

# THE SUB-SEAFLOOR ECOSYSTEMS: GENERAL CONTEXT

**Contents**



The biosphere can be defined as all the compartments of the lithosphere, atmosphere, hydrosphere, biosphere and where life dwells. The Earth's ecosphere is divided into ecosystems that owe their cohesion to interaction (Patten *et al.*, 1997). Over 70 % of the Earth's surface is covered by Ocean, therefore encompassing a wide range of microbial habitats that are controlled by small- to large-scale geological processes.

Fifty two solid tectonic plates of ocean crust and continents move along the Earth's surface at various speeds (Figure 1.1). The tectonic processes, that give motion to the plates, are driven by the mantle convection movements. The plate boundaries can be classified into 7 types: continental convergence zone, continental transform fault, continental rift, oceanic spreading ridge, oceanic transform fault, oceanic convergent boundary, subduction zone (Bird, 2003).

One of them, the oceanic spreading, occurs along the Mid-Ocean Ridge (MOR), where new basaltic crust is produced and mountain chains are created at depths of 2,000–4,000 m below the ocean surface, as a result of the convection to the surface of magma from the hot mantle.

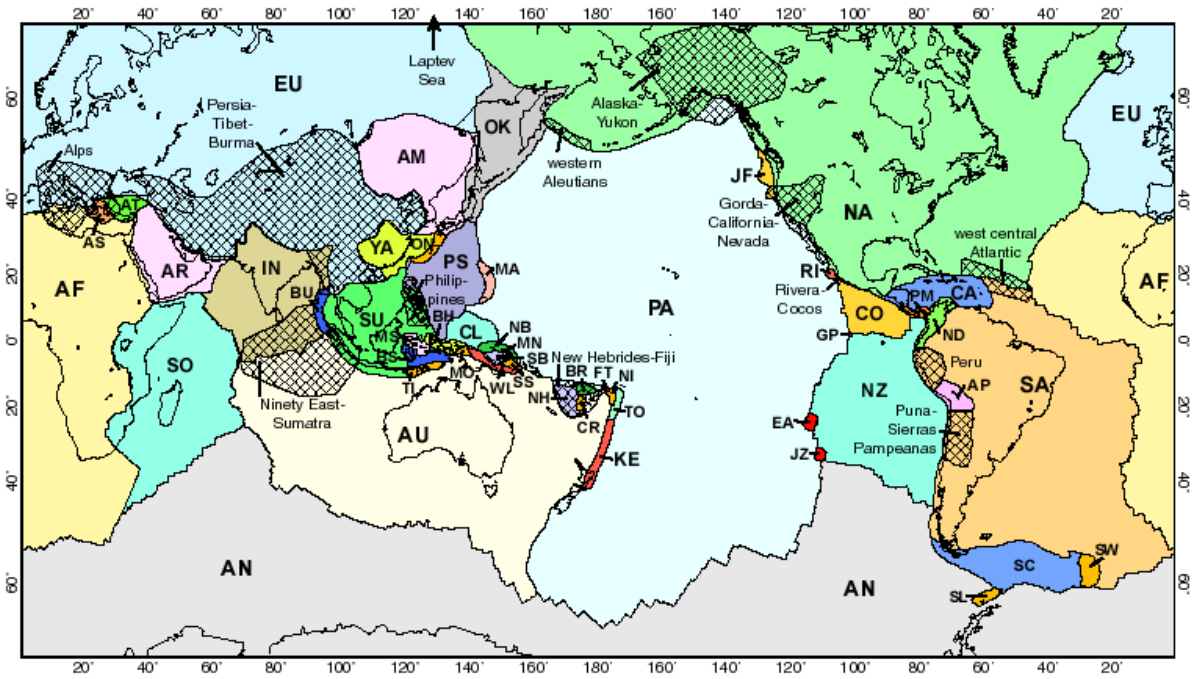


FIG. 1.1: The 52 tectonic plate-model is shown with contrasting colors (Bird, 2003). The 13 cross-hatched areas are not expected to be accurate. AF Africa, AM Amur, AN Antarctica, AP Altiplano, AR Arabia, AS Aegean Sea, AT Anatolia, AU Australia, BH Birds Head, BR Balmoral Reef, BS Banda Sea, BU Burma, CA Caribbean, CL Caroline, CO Cocos, CR Conway Reef, EA Easter, EU Eurasia, FT Futuna, GP Galapagos, IN India, JF Juan de Fuca, JZ Juan Fernandez, KE Kermadec, MA Mariana, MN Manus, MO Maoke, MS Molucca Sea, NA North America, NB North Bismarck, ND North Andes,NH New Hebrides, NI Niuafo'ou, NZ Nazca, OK Okhotsk,ON Okinawa,PA Pacific, PM Panama, PS Philippine Sea, RI Rivera, SA South America,SB South Bismarck, SC Scotia, SL Shetland, SO Somalia, SS Solomon Sea, SU Sunda, SW Sandwich, TI Timor, TO Tonga, WL Woodlark, YA Yangtze.

At these sites, hydrothermal systems can also occur, where high temperature fluids are expelled (350–400°C; Figure 1.2) (Kelley *et al.*, 2002).

As the ocean crust moves slowly away from the MOR, sinking particulate debris from the water column accumulate and form the sediment cover. The open-ocean sediments are mainly the result of the deposition of aeolian dust and small carbonate skeletons of plankton organisms, whereas terrigenous material eroded from the land constitutes the main fraction of sediments closer to the continents. Ultimately, the seabed is recycled by the mantle at the subduction zones. The compressed and geothermally altered sediment expels fluid and gases forming gas-hydrate deposits, gas chimneys, mud volcanoes and diverse seep systems. However, similar geosystems are also abundant at passive margins.

The following section will describe the geological, geochemical, microbiological context associated with the sub-seafloor marine sediments and deep hydrothermal ecosystems.

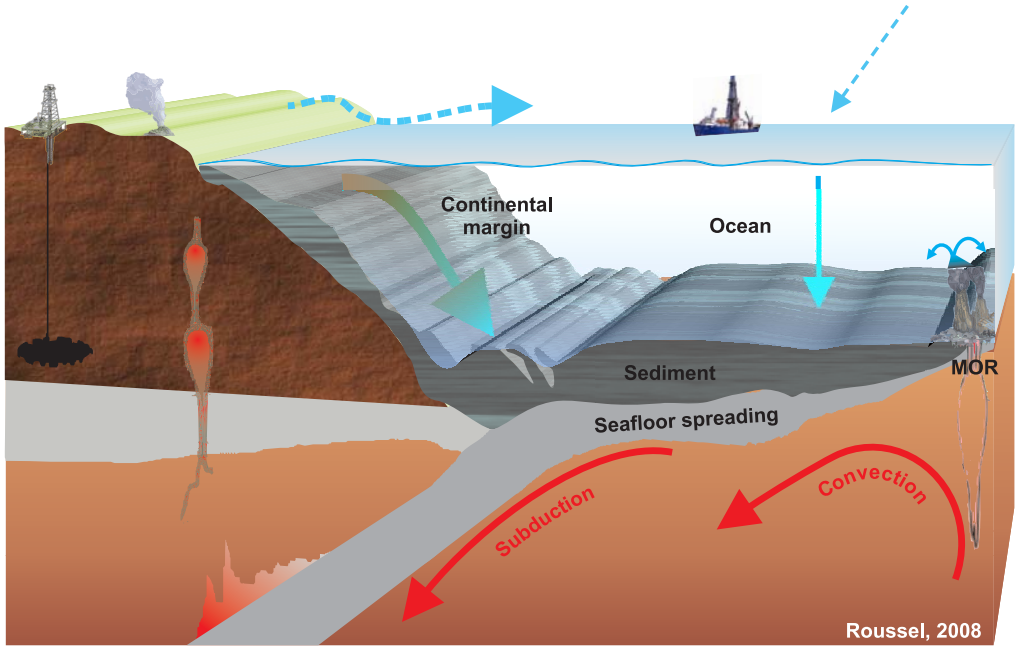


FIG. 1.2: Cross-section of the seafloor and seabed structures and possible interaction between the tectonic plate structures. The blue arrows indicate the sediment processes (Not to scale).

## 1.1 Marine sediments

### 1.1.1 Geological and Geochemical context

#### 1.1.1.1 Coastal, continental margins and open ocean sediments

Sediment can be defined as unconsolidated organic and inorganic particles that accumulate on the ocean floor. Ocean sediments are heterogeneous in their composition, and display a considerable degree of geographical variations. The classification of marine sediments is based on grain size or origin of matter. The sediments can originate from various sources such as weathering and erosion of continents, volcanic eruptions, biological activity, chemical processes and to a lesser extent impacts of extra-terrestrial objects. Hence, they are classified as terrigenous, biogenic, authigenic, volcanogenic or cosmogenous (Schulz and Zabel, 2006). The sediment cover thickness is 500 m on average over the oceans but can locally reach over 10 km (Figure 1.3).

Coastal sediments and the upper slope of continental margins are mainly composed of terrigenous sediments. Terrigenous sediments are derived from the erosion of rocks on land. Consisting of sand, mud, and silt carried to sea by rivers, their composition is usually related to their source rocks (Weltje and von Eynatten, 2004). Sediment grain size commonly depends on the energy of the depositional environment, with more energy leading to larger grain sizes. The terrigenous detrital material varies progressively from coarse-grained sands and silts along the

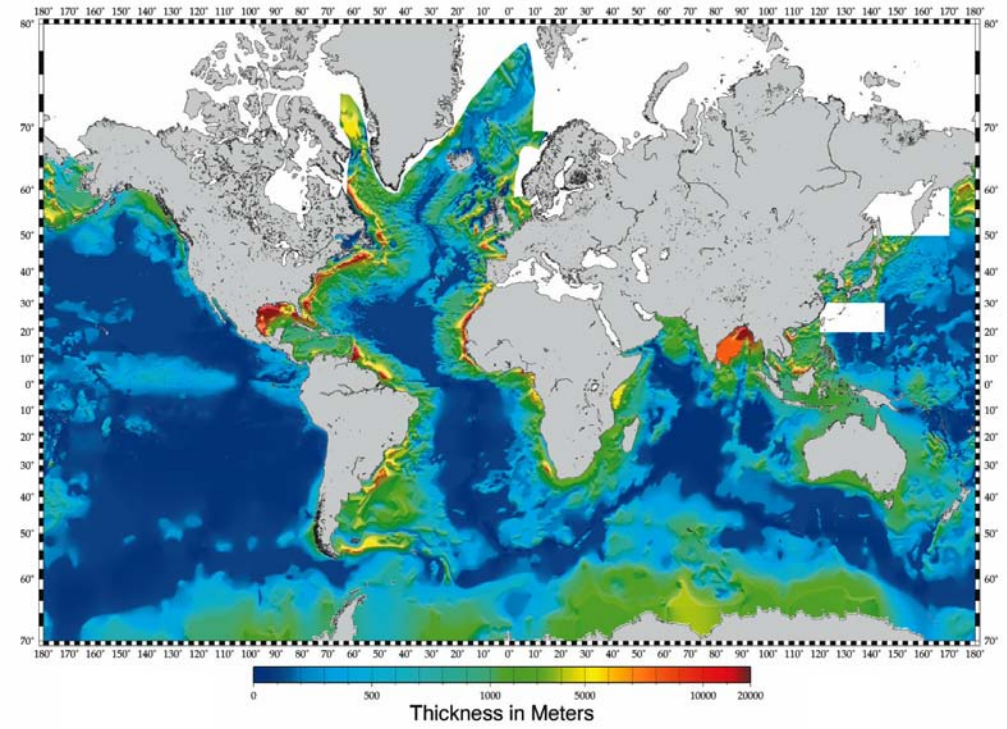


FIG. 1.3: Total sediment thickness of the World's Ocean and marginal seas (from http://www.ngdc.noaa.gov/mgg/sedthick/sedthick.html).

coast to progressively increasingly finer grained particles. Coastal and passive margin sediments are very dynamic environments as they are frequently subjected to sediment resuspension and are usually characterised by high sedimentation rates that bury large amounts of organic carbon. Hence, deltas and estuaries commonly constitute appropriate settings for the generation of shallow biogenic gases such as methane or hydrogen sulphide, due to the rapid accumulation of sediments with high organic matter concentrations (Fleischer *et al.*, 2001). Free gas is known to modify the geotechnical properties of the sediment by increasing the seabed compressibility (Sills and Wheeler, 1992). The geoacoustic properties of the seabed are also modified by the presence of gas bubble, and can be revealed by acoustic turbidities (Abegg and Anderson, 1997).

At continental margins, similar processes such as fluid flows can be observed. The fluid circulation is linked to sediment compaction or to tectonical compression. Several different geological structures can result from the fluid circulation as described in figure 1.4 (Berndt, 2005). One of the most remarkable examples is the Hakon Mosby Mud Volcano (HMMV) located on the south-west Barents Sea shelf, where gas and fluids are expelled by active mud volcanism (Feseker *et al.*, 2008; Niemann *et al.*, 2006). Gas hydrate structures can also be found in margins where pressure and temperature conditions combine to make it stable (high pressure or low temperature) (Kvenvolden, 1993). Gas hydrates are ice-like crystals that are

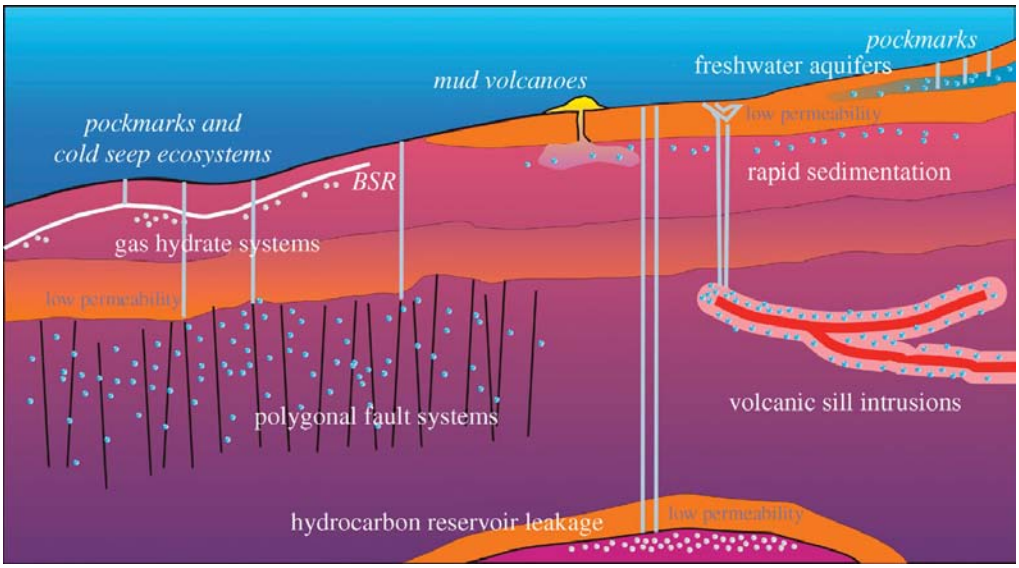


FIG. 1.4: Sketch of the different flow systems on continental margins, from (Berndt, 2005).

stable at high pressures and low temperatures, and which contain large amounts of biogenic methane and $CO_2$.

Unlike margin sediments, the open-ocean sediments are mainly composed of carbonate ooze, a biogenic detritus produced by pelagic organisms. The pelagic organisms are heterotrophic and autotrophic eukaryotes that produce calcareous ($CaCO_3$) or siliceous ($SiO_2$) shells. These sediments can also comprise lithogenous particles such as aeolian dusts or volcanic ashes (Schulz and Zabel, 2006). Most of the organic carbon buried in sediment originates from deltas and the upper slopes of margins (84 %), as the nutrient supply from land stimulates the coastal primary production, whereas the pelagic sediments contain less than 5 % of organic carbon (Hedges and Keil, 1995; Baturin, 2006). Hence, marine sediments reveal also two types of subsurface ecosystems: an open-ocean province, and an ocean-margin one (D'Hondt *et al.*, 2002).

#### 1.1.1.2 Sediment diagenesis

Between 5 to 10 billion tons of particulate organic matter (OM) is constantly sinking in the world oceans and accumulating as sediments, therefore encompassing between 25 and 50 % of the surface ocean primary production (Deming and Baross, 1993*b*). Over ∼99 % of this matter is recycled by the water/sediment interface microbial activity, but over geological time the remainder accumulates, representing the largest global reservoir of organic carbon (Wakeham and Canuel, 2006; Hedges and Keil, 1995; Hedges and Keil, 1999).

**1.1.1.2.1 Diagenesis: a general scheme.** After deposition, the sediment undergoes diagenesis, a series of combined physical, chemical and biological processes that greatly modify composition and structure. At shallow levels, compaction reduces the porosity and is one of the main physical processes. However, at depth, the increasing temperature and pressure transform the unconsolidated sediment into rock by the process of lithification. Decomposition and preservation of organic material in marine sediments control the cycling of biogeochemically important elements over a wide range of time scales.

**Depolymerisation**. After deposition of dead OM on the sediment surface, the degree of decomposition depends on a number of factors. As the organic matter is composed of complex molecules such as proteins, nucleic acids and lipids, it cannot be directly utilized by prokaryotic cells. Therefore, the depolymerisation of the organic matter is performed by extracellular enzymes produced by microorganisms that reduce the biopolymers to monomers or end products such as carbon dioxide. The depolymerisation could be partially controlled by the association of the organic matter with mineral particles, that could protect the OM from the hydrolytic enzymes (Wakeham and Canuel, 2006, for review) (Figure 1.5). The OM-mineral association could therefore contribute to the control of the amount of buried OM. Most of the preserved biomacromolecules such as lignins, tannins, cutans, suberans, cutins and suberins come from vascular plants (De Leeuw and Largeau, 1993).

**Zonation**. Sedimentation rate is another factor that controls the OM burial (Pedersen and Calvert, 1990). As the aerobic respiration rates exceed the anaerobic respiration rates in high organic accretion zones, a larger fraction of OM is transferred to the anoxic zone. This explains why up to 40 % of the OM input is preserved in continental margins.
Biological processes are also responsible for changes of the sediment pore water and mineralogical composition. The terminal electron accepting process (TEAP) controls the composition of dissolved $O_2$, $NO_3^-$, $SO_4^{2-}$, Mn, Fe and $CO_2$. The different redox reactions occur according to the availability and the free energy yield of the specific reaction (Hoehler, 2007)(Figure 1.6). The change in free energy during a reaction ($\Delta G^0$) is determined under standard conditions at 25°C, with all products and reactants initially at 1 molar. Hence, a specific redox reaction is evidenced as a biogeochemical and mineralogical zone in the sediment reflecting the dominant microbial metabolic process at that depth. As a consequence the sediment pore water and solid phase profiles are organized into successive zones (Fenchel and Jorgensen, 1977).

At the oxic sediment-water interface, aerobic respiration is the first known metabolic pathway ($\Delta G^0$ = -854 kJ/reaction). This reaction is mainly performed by the heterotrophic *Bacteria* that depolymerise the OM to soluble substrates. The $O_2$ is usually rapidly consumed in the first centimetres, depending on the available OM and the overlaying water dioxygen concentration. The nitrogen cycle in marine sediment is relatively complex, as several metabolic activities can

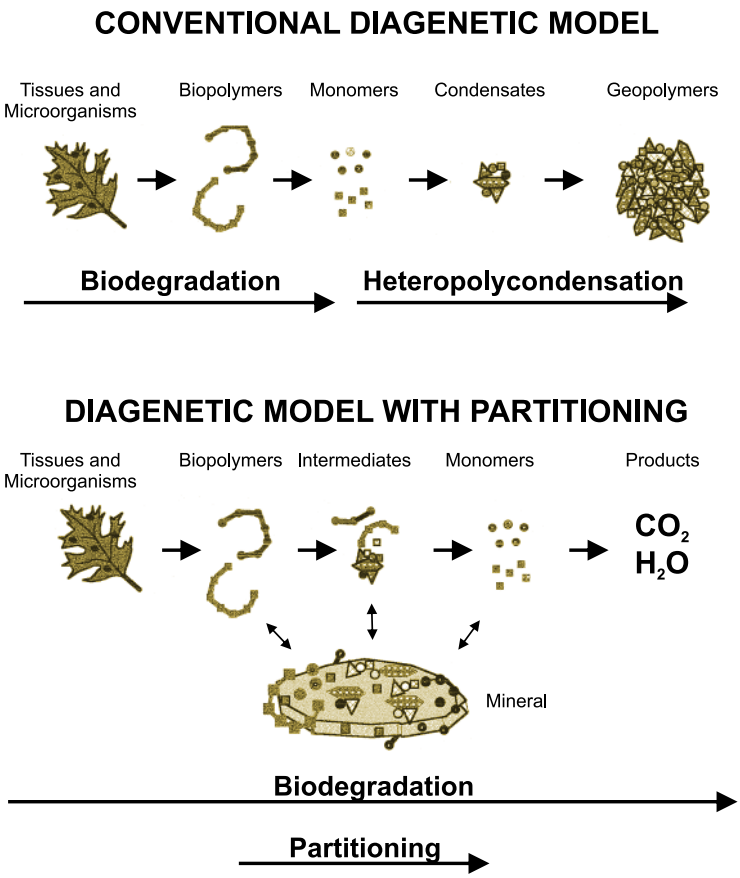


FIG. 1.5: Illustration of the conventional biodegradation/repolymerization model alternate to the biodegradation/sorption model, from (Wakeham and Canuel, 2006).

be involved in the nitrification/denitrification processes (Figure 1.7). The nitrification process aerobically produces nitrates that can be utilized in the underlying anoxic zone by denitrifing *Bacteria*. Moreover, the recently discovered anaerobic ammonium oxidation could play a key role in stable marine systems as a nitrogen sink (Kuenen, 2008, for review).

Metal reduction is also an important process in anoxic sediment. Iron and manganese reduction, also shown to be a dissimilatory process (Lovley and Phillips, 1988; Myers and Nealson, 1988), are probably overlooked processes in marine sediments (Lovley and Coates, 2000*a*). For example, manganese reduction accounts for up to 80 % of the organic matter remineralisation in the Black sea. Moreover, it was suggested that humic substances could be catalysed by Fe(III) (Lovley and Coates, 2000*b*). Other metals such as U(VI), Co(III), Cr(VI), and metalloids such as As(V), Se(VI) can be reduced by microbial activity (Newman *et al.*, 1998; Stolz and Oremland, 1999; Coates *et al.*, 1998). It was also recently suggested that radiolysis of water may provide a continuous flux of an electron donor (molecular hydrogen) to subsurface microbial communities (Blair *et al.*, 2007).

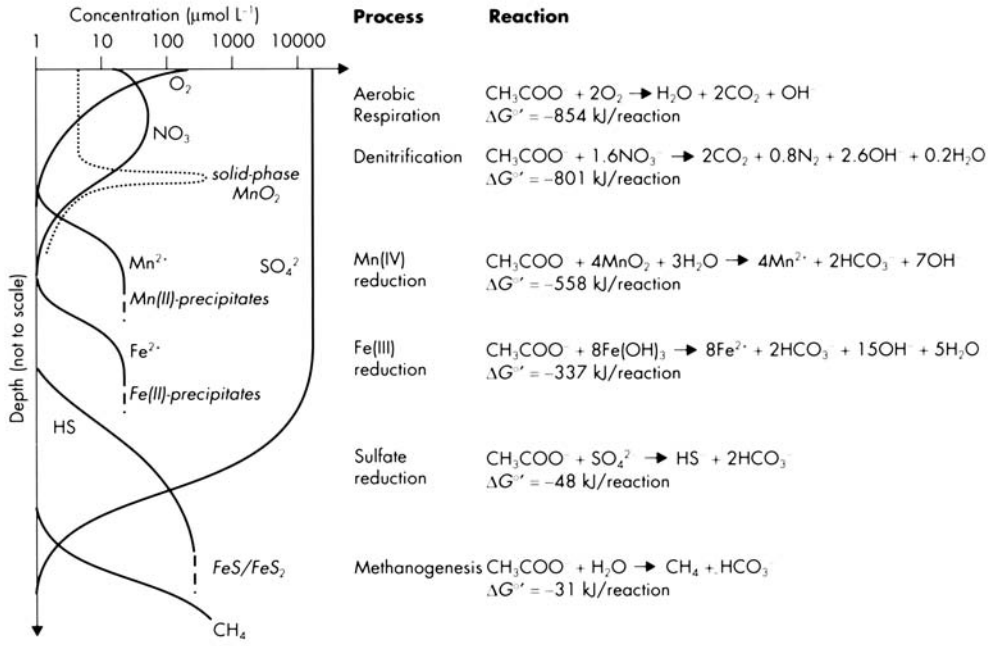


FIG. 1.6: Idealized pore water and solid-phase profiles based on successive utilization of terminal electron acceptors during organic matter decomposition in marine sediments; adapted from (Froelich *et al.*, 1979; Konhauser, 2006).

However, in deeper anoxic sediments, sulfate reduction and methanogenesis represent the principal metabolic processes in the deep sub-seafloor (D'Hondt *et al.*, 2004; D'Hondt *et al.*, 2002; Parkes *et al.*, 2005). As shown in figure 1.6, acetate is a key element required in all the listed reactions and accounts for 60 % of sulfate reduction reactions (Wellsbury and Parkes, 1995). Sulfate-reducers outcompete the acetate utilizing methanogens, as sulfate reduction is energetically more favourable (Muyzer and Stams, 2008, for review). Methanogenesis can use several different pathways (Schink, 1997, for review), one uses hydrogen and carbon dioxide, the others use acetate or C1 carbon compounds. Methanogenesis dominates the deeper sediment layers as sulfate is depleted (Reeburgh, 2007, for review). Hence, acetoclastic methanogenesis could be the main pathway for methane production in deep marine sediments. Moreover, methane production is more limited in sulfate rich open-ocean sediments than in the ocean-margin province where sulfate is limited to shallow sediments (D'Hondt *et al.*, 2002). The methanogens utilizing C1 compounds usually dominate the marine methanogens within the zone of sulfate reduction zone, since sulfate reducing *Bacteria* (SRB) do not compete for the same substrates (Purdy *et al.*, 2003).

Anaerobic oxidation of methane (AOM) was shown to occur in limited zone between the sulfate-reducing and methanogenesis transition zones (SMTZ) in non methane seep zones, where sulfate and methane are both available (Valentine, 2002; Iversen and Jorgensen, 1985, for review).

During AOM, methane is oxidized with sulfate as electron acceptor via the following net reac-

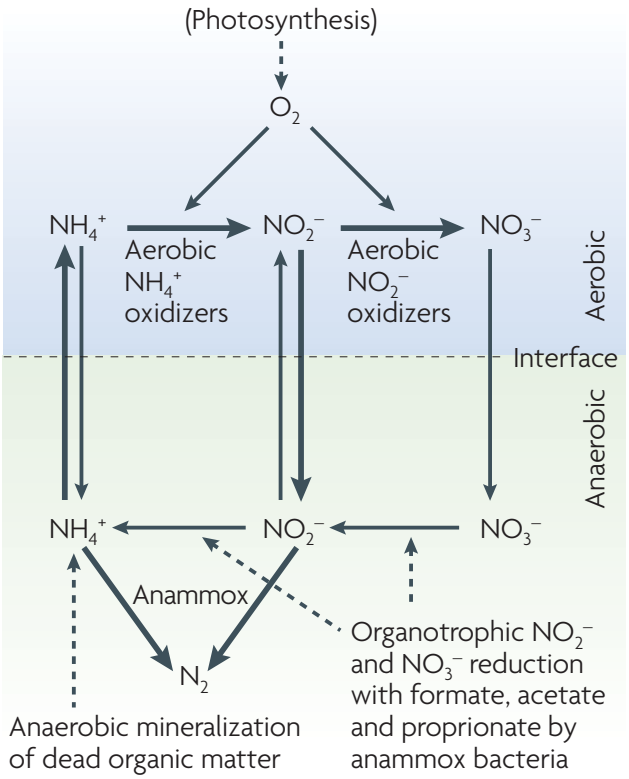


FIG. 1.7: Nitrogen cycle between aerobic and anaerobic nitrifiers (Kuenen, 2008).

tion (Nauhaus *et al.*, 2002*a*):

$$CH_4 + SO_4^{2-} \rightarrow HCO_{3-} + HS^- + H_2O \ (\Delta\ G^0 = 25\ \text{kJ/reaction})$$

Hence, the presence of methane, only below the sulfate penetration zone, could therefore be due to the anaerobic oxidation of methane at the SMTZ that would limit the methane fluxes in the sediment column (Martens *et al.*, 1999). Unlike AOM, aerobic $CH_4$ oxidation tends to dissolve carbonates as $CO_2$ is a weak acid. Hence, methane oxidation represents an important inorganic carbon sink, as anaerobic oxidation causes the precipitation of carbonates (Suess *et al.*, 1999; Thiel *et al.*, 1999).

Interestingly, it was recently shown that deep sediment layers, close to the basaltic basement, were supplied with oxygen and nitrate. The succession of the redox-zones that extend from the basement interface were therefore mirrored by the similar succession observed as previously described at the sediment surface (Figure 1.8) (D'Hondt *et al.*, 2004). However, at this deep sediment basement interface, respiration is probably limited by electron donor availability. The oxic seawater fluid fluxes, presumably circulating in specific open-ocean systems through the highly fractured basaltic crust, may therefore fuel different microbial communities in comparison to the sediment surface (Engelen *et al.*, 2008).

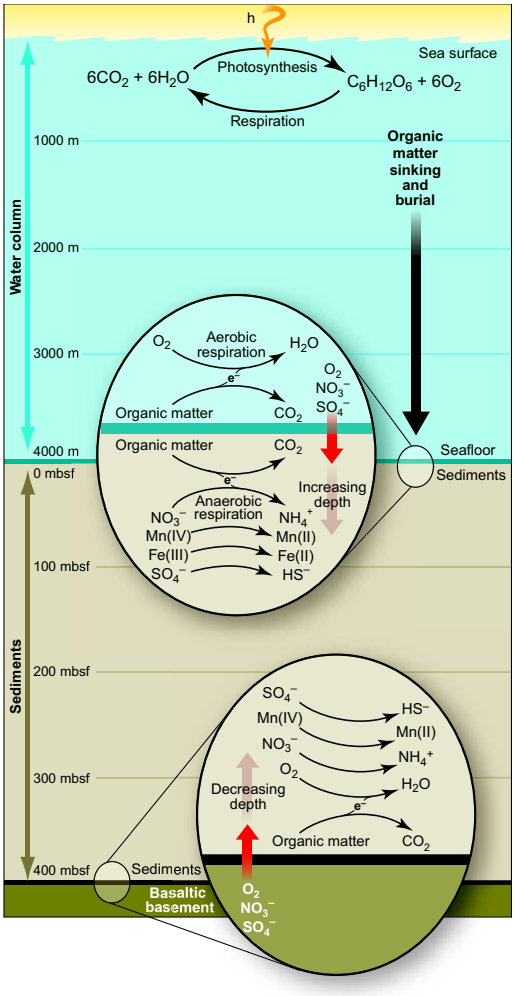


FIG. 1.8: Observed processes in the upper sediments of the seafloor compared to the deep basaltic basement of the open-ocean, from (DeLong, 2004).

**1.1.1.2.2 Deep sediment energy and carbon sources.** One of the limiting factors of prokaryotic life at depth is bioavailable energy sources such as labile organic carbon (Parkes *et al.*, 2000). Even though the overall subsurface sediments contain large amounts of organic matter, as seen in the previous section, they are thought to be resistant to biodegradation and to have a restricted bioavailability (Hedges and Keil, 1995; Burdige, 2007), and are therefore probably inadequate to provide sufficient energy for sub-surface prokaryotes. It has been suggested that labile organic matter could control the decomposition of refractory OM (Hee *et al.*, 2001; Turnewitsch *et al.*, 2007). This insoluble OM is also referred to as kerogen. Moreover, the physical constraints at depth, such as temperature and pressure, control the abiotic kerogen maturation reactions such as condensation, cracking and aromatisation. These diagenesis and catagenesis processes convert the different recalcitrant kerogens into volatile products such as

oil and gas (Quigley and Mackenzie, 1988; De Leeuw and Largeau, 1993). However, recent findings also suggest that ethane and propane could be biologically produced in cold, deeply buried marine sediments (Hinrichs *et al.*, 2006).
Although, sulfate reducers were isolated from oil wells since 1930, it is only recently that biological mediated oil degradation aroused interest (Head *et al.*, 2003, for review), as crude-oil could be principally biologically degraded by hydrogenotrophic methanogenesis (Head *et al.*, 2003; Parkes, 1999; Aitken *et al.*, 2004; Jones *et al.*, 2008). However, several metabolic pathways can be used to convert oil into methane depending on environmental conditions (Dolfing *et al.*, 2008, for review).
Recent studies also showed that long term heating experiments of sediments stimulated bacterial activity by a considerable production of $H_2$, $CH_4$ and other hydrocarbons (Parkes *et al.*, 2007; Horsfield *et al.*, 2006). Congruently, a previous study showed that acetate generation in deep marine sediment could be associated to temperature increases during burial, supporting the activity of anaerobic acetoclastic *Bacteria* (Wellsbury *et al.*, 1997). Mineral diagenesis coupled with increasing temperature (Surdam and Crossey, 1985) might also produce microbial substrates.
Hence formation of $H_2$ at high temperature could indicate an abiotic reaction involving ferrous silicate minerals (Stevens and Mckinley, 2000). It has been assumed that hydrogen and methane could therefore play a key role in deep subsurface ecosystems, suggesting that deep sub-surface microbial communities could carry out their own primary production (Kotelnikova, 2002; Pedersen, 2000; Stevens, 1997). Temperature elevation with increasing depth increases the reactivity/bioavailability of buried organic matter and could therefore fuel the deep biosphere communities (Parkes *et al.*, 2007; Horsfield *et al.*, 2006; Hoehler, 2004).

### 1.1.2 Deep sedimentary subsurface ecosystems

#### 1.1.2.1 Definition of deep sedimentary subsurface ecosystems

The term "deep sub-surface" was firstly used 20 years ago in a special issue of the Geomicrobiology Journal. The several studies published in this issue had different definitions of localisation of the deep subsurface. For instance, it was used to describe microbial communities found in terrestrial samples as deep as 500 meters (Fliermans and Balkwill, 1989; Phelps *et al.*, 1989; Sargent and Fliermans, 1989). It was vaguely defined as located between 100 and a 1,000 metres below land surface (Ghiorse and Wobber, 1989). However, Fredrickson and colleagues considered that the term could be applied to sediments buried to depths approximately greater than 10 m (Fredrickson *et al.*, 1989).
Althougth, Oremland and colleagues reported bacterial activity in deep marine sediments in 1982 (Oremland *et al.*, 1982), it's only later on that Parkes and Cragg began to systematically enumerate microorganisms in deep marine sediment cores (Parkes *et al.*, 2000, for review). It

was therefore observed that the depth of the deep subsurface was linked to the physicochemical conditions associated to the studied environment (Lovley and Chapelle, 1995). The deep subsurface nomenclature was recently defined by microbial ecological criteria as "sediment layers with distinct microbial communities that lack a microbial imprint of water column communities" (Teske and Sorensen, 2008). Therefore, the deep subsurface starts where the water column prokaryotes are replaced by the subsurface communities, and the boundary can be defined as the shallow surface. This definition can therefore be applied to any sediment, including coastal sediments.

#### 1.1.2.2 Abundance of the Prokaryotes: $\frac{2}{3}$ of Biosphere

The very large number of prokaryotic cells near the sediment surface (1-5 $\times$ $10^9$ cells/$cm^3$), drastically decreases with increasing depth and temperature, down to $10^6$ cells per $cm^3$ at the 1,000 m subsurface. However, local variations occur according to the adaptation of the microbial communities to specific environment. The total cell counts were mainly performed using acridine orange direct counts, and contamination tests, using perfluorocarbon tracers in the drilling fluid and fluorescent microbeads, confirmed that the cells counted were indigenous to the deep biosphere. The depth distribution of prokaryotic cells in marine sediment follows a regression equation established by Parkes and colleagues (Parkes *et al.*, 2000) (Figure 1.9):

$$\log_{10} \text{cell number} = 8{,}06 - 0{,}715 \log_{10} \text{depth (mbsf)}$$

It was therefore hypothesized that 55 % - 85 % of the prokaryotic biomass of the Earth could be buried in marine sediments (Whitman *et al.*, 1998). These assumptions were consistent with a recent study that calculated that the cellular carbon in the global marine subsurface could reach 90 Pg (Lipp *et al.*, 2008).
The prokaryotic cell density is significantly correlated with depth but much less with sediment age or porosity (Parkes *et al.*, 2000). However, the *Bacteria*-sediment mechanical interaction was analysed in order to predict the possible fate of *Bacteria* at depth (Figure 1.10). These results show several mechanical factors affecting the interaction with bacterial cells such as porosity, grain size and depth (Rebata-Landa and Santamarina, 2006). However, near-surface sediments prokaryotes could be sufficiently motile to avoid burial, conversialy to deeper sediment layers, where prokaryotes could be mostly be trapped in the sediment (Parkes *et al.*, 2000; Fenchel, 2008).

Temperature appears to be the major limiting factor at depth, therefore the known upper temperature limit for prokaryotes (113-122°C) could define the depth limit for living prokaryotes in marine sediments. Thus, the sub-seafloor biosphere depth limit has probably only been reached at sites with high thermal gradient such as hydrothermal zones (Parkes *et al.*, 2000). However, local variations in prokaryotic cell densities occur according to the adaptation of

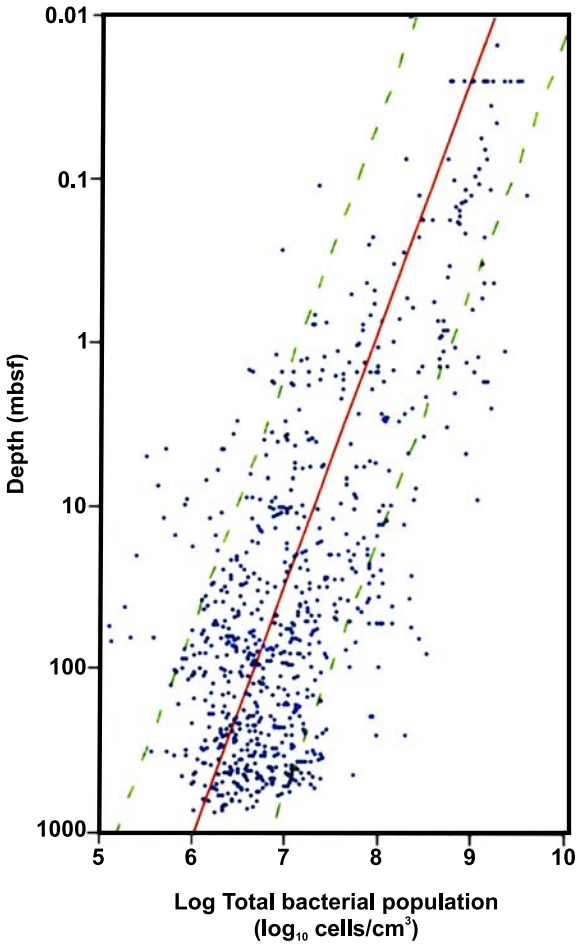


FIG. 1.9: Depth distribution of subsurface prokaryotic populations of ODP sediments obtained by acridine orange counts. The red line represents the general regression equation, with upper and lower prediction limits (95 %) shown by dashed lines, from (Parkes *et al.*, 2000).

microbial communities to specific environmental variations such as brine incursions or the advection of thermogenic compounds. For instance local increases in cell density can occur at the SMTZ and are correlated with high activity rates (Parkes *et al.*, 2005, for e.g.).

#### 1.1.2.3 *Archaea* or *Bacteria*: Which domain is dominant at depth?

As suggested in the previous section, there are some remarkable systematic links between the prokaryotic cell abundance and sediment environmental factors. However, the fluorochrome acridine orange, routinely applied in microscopic cell counting, binds unspecifically to nucleic acids (DNA or RNA) and therefore does not provide discriminants between dead, dormant or active cells (Kepner and Pratt, 1994).
To date, RNA-based and phospholipid fatty acid-based approaches are probably the most

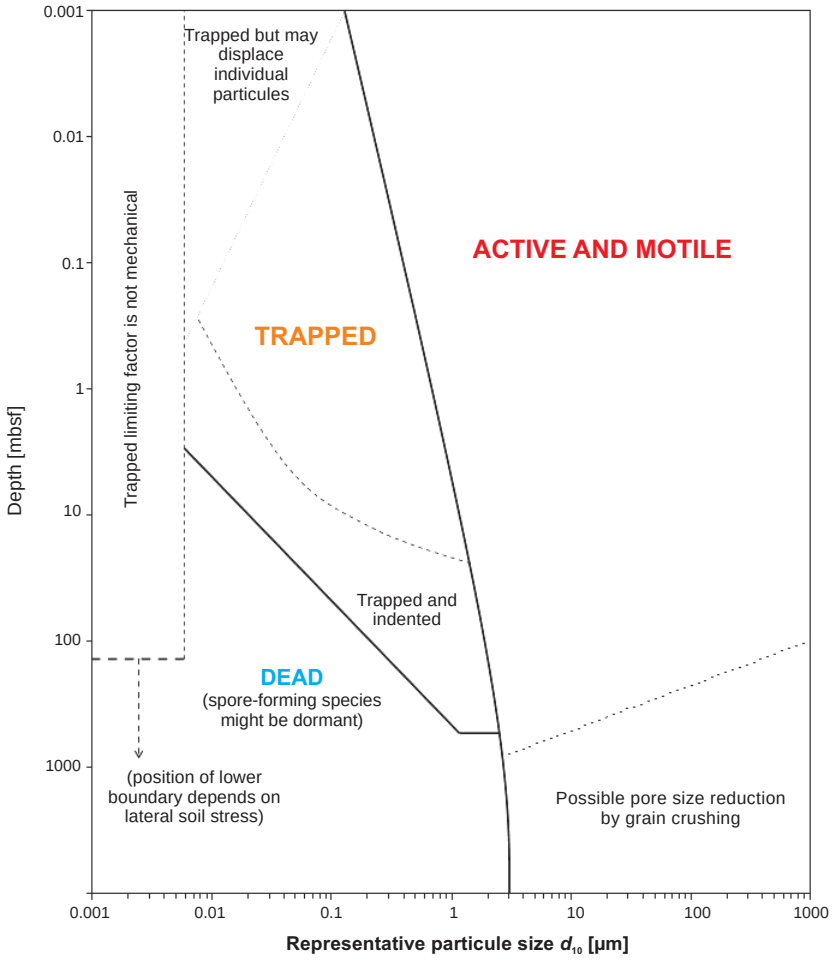


FIG. 1.10: Bacterial predicted fate according to their mechanical interaction with the sediment, adapted from (Rebata-Landa and Santamarina, 2006).

revealing culture–independent techniques to investigate the living prokaryotic fraction, as these molecules have a rapid turn-over (Kemp *et al.*, 1993; Parkes and Taylor, 1983, for e.g.). Several recent studies of sediments at the Peru margin revealed living prokaryotic cells (Biddle *et al.*, 2006; Lipp *et al.*, 2008; Mauclaire *et al.*, 2004; Schippers and Neretin, 2006; Schippers *et al.*, 2005; Teske, 2005). However, it was difficult to resolve whether *Archaea* or *Bacteria* were more abundant and whether they were being detected with equal efficiency. Hence the disparate views are probably the result of methodological differences, probably caused by variations in levels of cellular activity. However, using fluorescent *in situ* hybridisation (FISH), quantitative PCR and phospholipid fatty acids, most of these studies indicated that *Archaea* could constitute up to 98 % of total prokaryotic cells, therefore representing the major fraction of the living marine sediment subsurface biomass (Biddle *et al.*, 2006; Lipp *et al.*, 2008; Mauclaire *et al.*, 2004; Biddle *et al.*, 2008). It was also shown that *Crenarchaeota* could encompass most of the

*Archaea* detected in the Peru margin sub-seafloor sediments (Biddle *et al.*, 2008). Although *Archaea* probably constitute the most abundant domain, it does not necessarily imply that they are the most active.

#### 1.1.2.4 Microbial diversity in deep marine sediments

**1.1.2.4.1 Cultivation of deep biosphere microorganisms.** To date, over 27 of the 52 bacterial phyla and at least 20 of the over 50 archaeal phyla harbour cultivated relative (Keller and Zengler, 2004; Hugenholtz, 2002; Rappe and Giovannoni, 2003; Schleper *et al.*, 2005). Deep sea sediments are even more resistant to cultivation as less than 0.25 % of the deep sediment microbial diversity has been cultured (Table 1.1) (Amann *et al.*, 1995; Rappe and Giovannoni, 2003).

| Habitat | Culturability (%) |
|---|---|
| Seawater | 0.001–0.1 |
| Freshwater | 0.25 |
| Mesotrophic lake | 0.1–1 |
| Unpolluted estuarine waters | 0.1–3 |
| Activated sludge | 1–15 |
| Sediments | 0.25 75 |
| Soil | 0.3 153 |

Table 1.1: Culturability determined as a percentage of culturable *Bacteria* in comparison with total cell counts. Culturable *Bacteria* are measured as CFU, modified from (Amann *et al.*, 1995).

Until now, the number of pure cultures does not probably exceed a few hundred (Jorgensen and Boetius, 2007). Most of these show metabolic capabilities that match the main deep subsurface measured activities, such as sulfate-reduction, methanogenesis (Parkes *et al.*, 1995; D'Hondt *et al.*, 2004; Toffin *et al.*, 2004, for e.g.). *Desulfovibrio profondus*, a sulphate reducer isolated in deep sediments ($>$ 80 mbsf), reveals a wide growth temperature up to 65°C (Bale *et al.*, 1997). Several methanogen isolates such as *Methanoculleus submarines*, *Methanococcus aeolicus*, confirm the presence of living methanogenic *Archaea* in deep subsurface sediments. Although rarely detected by molecular approaches, their occurrence is inferred by geochemical measurements (Kendall *et al.*, 2006; Mikucki *et al.*, 2003). However, the most commonly cultured phylotypes are *Proteobacteria*, *Actinobacteria*, *Bacterioidetes* and the spore forming *Bacillus* genera (Toffin *et al.*, 2004; D'Hondt *et al.*, 2004; Kopke *et al.*, 2005). The *Rhizobium* genus seems also to be widespread in subsurface sediments (D'Hondt *et al.*, 2004).
At least in the upper boundary of subsurface sediments, fast growing-*Bacteria* dominate the enrichment cultures (Toffin *et al.*, 2004). Moreover, piezotolerant *Bacteria* were also isolated from subsurface sediments, matching with the high environmental hydrostatic pressures (Toffin

*et al.*, 2005, for e.g.). However, to date, most of the phylotypes detected with independent culture approaches remain uncultured. The limited available carbon and energy sources in deep subsurface sediments result in very slow prokaryotic metabolic rates and generation times, ranging from years to thousands of years (Schippers *et al.*, 2005; Parkes *et al.*, 2000). Up to now the current cultural technique may not be adapted to the growth of such slow metabolisms, thus explaining the failure to culture typical subsurface prokaryotes. Molecular approaches based on nucleic acid or phospholipid fatty acid analysis are the outstanding tools for exploring the deep subsurface sediment microbial diversity.

#### 1.1.2.4.2 General diversity distribution assessed by culture independent approaches.

The 16S rRNA gene-based molecular techniques applied on deep subsurface marine sediments revealed a relatively constrained prokaryotic diversity that usually decreased with depth (Inagaki *et al.*, 2006*b*; Inagaki *et al.*, 2003*a*; Parkes *et al.*, 2005; Sorensen *et al.*, 2004; Sorensen and Teske, 2006). The sediment surface contains more ubiquist microorganisms than the deeper sediment section (Inagaki *et al.*, 2006*b*; Inagaki *et al.*, 2003*a*; Sorensen *et al.*, 2004; Webster *et al.*, 2004; Sorensen and Teske, 2006). The prokaryotic diversity can also vary according to the sediment composition (pelagic, terrestrial, volcanic) (Inagaki *et al.*, 2006*a*, for e.g.).

***Bacteria***

Most of the bacterial 16S rRNA genes detected in deep subsurface sediments are affiliated to putative heterotrophic prokaryotic groups (Biddle *et al.*, 2006). The dominant phylotypes detected with 16S rRNA gene bacterial clone libraries are generally affiliated to *Chloroflexi* (Green Non Sulfur, GNS) and to *Proteobacteria*, mainly *γ-Proteobacteria* (Inagaki *et al.*, 2006*a*; Kormas *et al.*, 2003; Parkes *et al.*, 2005; Webster *et al.*, 2006)(Figure 1.11). GNS seem constrained to specific geochemical environments, such as methane bearing systems, whereas *γ-Proteobacteria* have a broader distribution (Inagaki *et al.*, 2003*a*; Parkes *et al.*, 2005). The bacterial group *Chloroflexi* has also been shown to be a major part of the subsurface microbial community in both the metagenome and qPCR analysis (Biddle *et al.*, 2008). The potential metabolic diversity of this group is unknown, and although many of the genes from the subsurface are very similar to those known from *Dehalococcoides ethenogenes*, the ribosomal sequences are still quite distinct, making metabolic assignment based on this cultivated representative unreliable. The *Proteobacteria* orders, *Cytophaga-Flavobacterium-Bacteroides* (CFB) and *Planctomycetes* phylums were also retrieved but seem to have a more limited distribution depending on the sediment origin (Inagaki *et al.*, 2006*b*; Inagaki *et al.*, 2003*a*; Kormas *et al.*, 2003; Marchesi *et al.*, 2001; Newberry *et al.*, 2004; Parkes *et al.*, 2007; Webster *et al.*, 2003). However, although sulfate reduction is thought to be one of the major metabolic activity evidence for sulfate-reducing *Bacteria* are very scarce even by using metagenomic approaches (Biddle *et al.*, 2008).
Although most of the cultured phylotypes are also retrieved with molecular approaches, sev-

eral lineages with no cultured relative were detected, such as the Opal Pool 8 group (OP8) (Kormas *et al.*, 2003; Parkes *et al.*, 2007) and the recently described Japan Sea 1 group (JS1) (Webster *et al.*, 2007, for e.g.). The JS1 are usually predominating subsurface sediment clone libraries and are exclusively detected in anoxic sedimentary environments, such as deep marine sediments, methane hydrate bearing sediments (Inagaki *et al.*, 2006*b*), marine and terrestrial volcanoes (Alain *et al.*, 2006; Niemann *et al.*, 2006), near-surface hydrothermal marine sediments (Teske *et al.*, 2002), near-surface and coastal sediments (Webster *et al.*, 2004), benzene degrading (Phelps *et al.*, 1998) and acetate-utilizing sulphate-reducing sediment enrichments (Webster *et al.*, 2006). The JS1 are usually retrieved from muddy, subsurface sediments with reduced sulphate and organic carbon concentrations, whereas *Chloroflexi* may out-compete JS1 in shallow, sandy, subsurface sediments (Webster *et al.*, 2007). The members of these two groups are probably typical subsurface sediment communities.

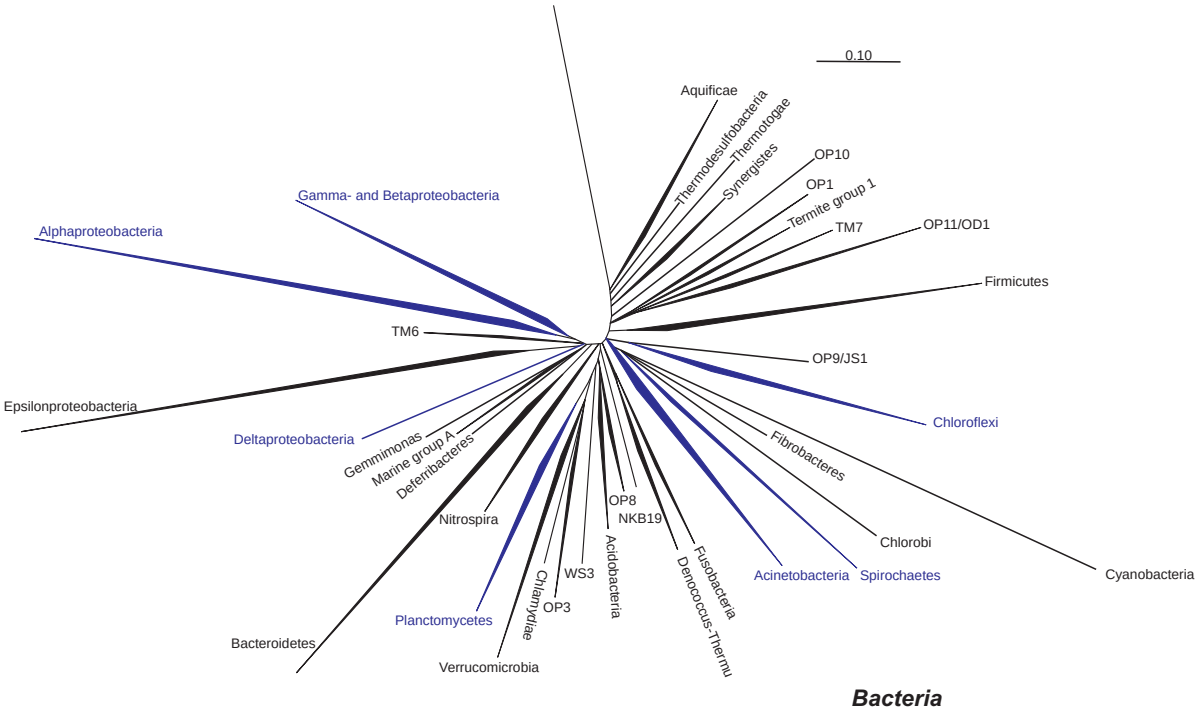


FIG. 1.11: Phylogenetic tree of the most frequently bacterial lineages retrieved from deep subsurface sediments (in blue).

### *Archaea*

The archaeal diversity is relatively reduced compared to the bacterial diversity and is mostly dominated by members of the *Crenarchaeota* phylum (Biddle *et al.*, 2008, for e.g.). Using quantitative PCR, clone libraries and metagenomics, most of these studies indicated that the Miscellaneous Crenarchaeotal Group (MCG) and the Marine Benthic Groups B (MBG-B, synonymous of Deep-Sea Archaeal Group) dominate the subsurface sediments (Biddle *et al.*, 2008;

Biddle *et al.*, 2006; Inagaki *et al.*, 2003*a*; Parkes *et al.*, 2005; Sorensen *et al.*, 2004; Sorensen and Teske, 2006). 16S rRNA gene related to Marine Benthic Group D (MBG-D) are also frequently retrieved from these environments (Newberry *et al.*, 2004; Parkes *et al.*, 2007; Sorensen and Teske, 2006). The Marine Group I *Archaea* mostly dominate the surface of marine sediments and is the major seawater archaeal phylotype (Inagaki *et al.*, 2001; Newberry *et al.*, 2004; Sorensen *et al.*, 2004). The *Euryarchaeota* are mainly represented by the South African Gold Mine Euryarchaeotic Group (SAGMEG), even though it was firstly retrieved in African gold mines (Inagaki *et al.*, 2006*b*; Parkes *et al.*, 2007; Sorensen *et al.*, 2004; Sorensen and Teske, 2006). Members of the Ancient Archaeal Group (AAG), the Marine Hydrothermal Vent Group (MHVG) and Deep-Sea Hydrothermal Vent Euryarcachaeotal Group 6 (DHVE-6) also occur in these deep anoxic environments (Teske and Sorensen, 2008, for review)(Figure 1.12).

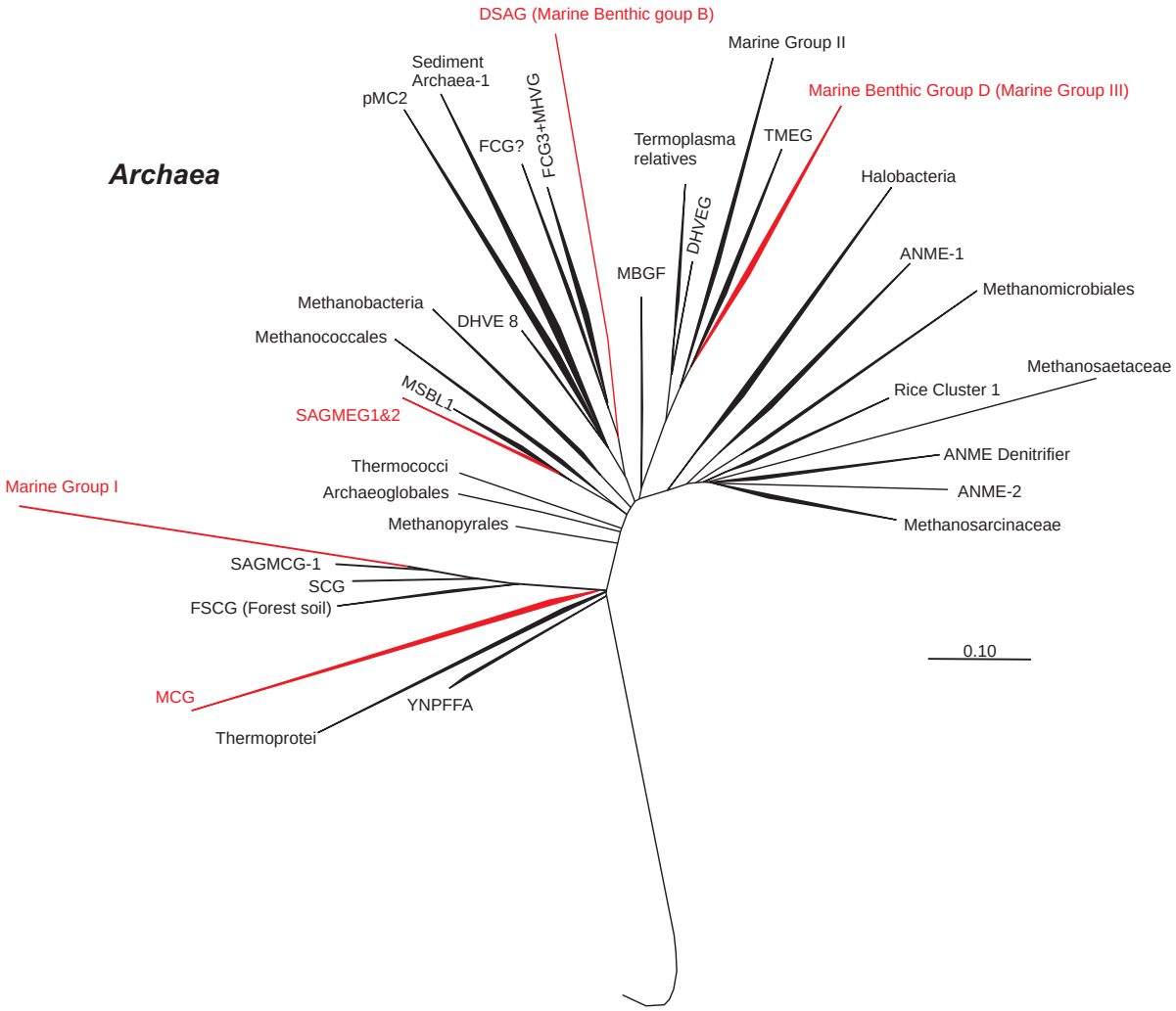


FIG. 1.12: Phylogenetic tree of the most frequently eury- and crenarchaeal lineages retrieved from deep subsurface sediments (in red).

Although methane cycling is reported to be one of the main prokaryotic activities in deep subsurface sediments, to date very few 16S rRNA genes related to methanogens or methanotrophs have been detected. Methanogenesis is mediated by methanogenic *Archaea* and only occurs in anoxic environments (Wolfe, 1971). However, studies investigating the functional genes for methanogenesis (Methyl-coenzyme M reductase, *mcr*) confirmed the occurrence of archaeal methanogens (Marchesi *et al.*, 2001; Newberry *et al.*, 2004; Parkes *et al.*, 2007; Parkes *et al.*, 2005; Biddle *et al.*, 2008). The analysis of the *mcr* gene mainly in subsurface biogenic methane bearing sediments, revealed sequences related to $H_2/CO_2$ utilizing *Methanobacteriales*, and to methylotrophic *Methanosarcinales*. The failure of molecular genetics to detect methanogens in sub-seafloor sediments may rely on too great molecular divergence of these prokaryotes with the presently described methanogens to be accurately interpreted (Biddle *et al.*, 2008).
One revealing example could be the MBG-B that were shown to benefit directly or indirectly from AOM as they dominate and are metabolically active within the SMTZ and other methane anaerobic methane bearing sediments (Biddle *et al.*, 2008; Biddle *et al.*, 2006; Sorensen and Teske, 2006)(Figure 1.13).

However, sequences related to anaerobic methane oxidizers (ANME) (Boetius *et al.*, 2000; Hinrichs *et al.*, 1999) were retrieved only very rarely in deep subsurface sediments (Parkes *et al.*, 2007). Four archaeal clades (ANME-1, ANME-2, ANME-3 and ANME-4) oxidize methane under anaerobic conditions, either as single cells, or associated to *Bacteria* some of which can be sulfate-reducers (Boetius *et al.*, 2000; Niemann *et al.*, 2006; Orphan *et al.*, 2002; Orphan *et al.*, 2001*a*; Raghoebarsing *et al.*, 2006). ANME could possibly reverse methanogenesis using a modified MCR (Boetius *et al.*, 2000; Hallam *et al.*, 2003; Hallam *et al.*, 2004). However, these microbial methane cycling communities have bee recently revealed in non seep or gas hydrate coastal sediments (Parkes *et al.*, 2007; Treude *et al.*, 2005*b*; Thomsen *et al.*, 2001). As most of the methane cycling prokaryotes may still remain overlooked in deep subsurface sediments coastal sediment could help to define the biogeochemical habitats of these communities in the deep biosphere.

**1.1.2.4.3 (Hyper)thermophiles in deep cold subsurface marine sediments: myth or reality?** 16S rRNA gene sequences related to (hyper)thermophilic members of the *Thermococcales* and *Methanococcales* genera have recently been retrieved from cold (1-12°C) deep subsurface marine sediments (Inagaki *et al.*, 2006*b*; Inagaki *et al.*, 2001; Kormas *et al.*, 2003). Kormas and colleagues suggested that these *Euryarchaeota* were probably not metabolically active in the environment where they were detected, suggesting they could be relics of hydrothermal deposits or could have been introduced by deep fluid advection (Kormas *et al.*, 2003). The persistence of DNA in marine sediments over geological times is still controversial (Damste and Coolen, 2006). For instance, Inagaki and colleagues claimed to have detected

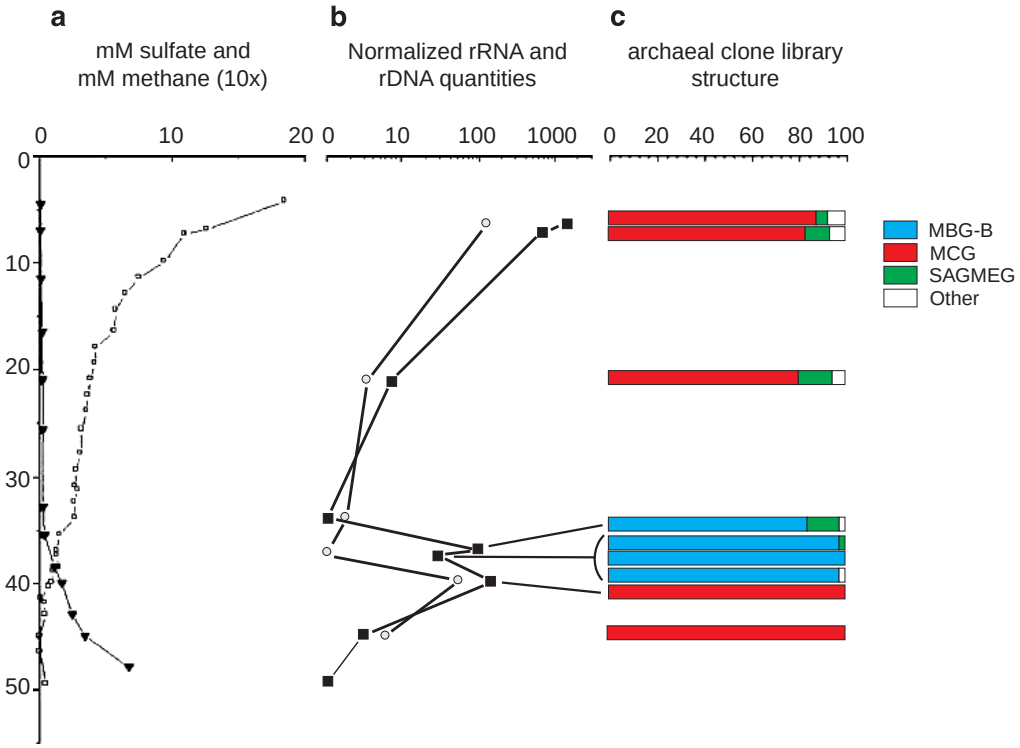


FIG. 1.13: Stratified archaeal communities in Peru Margin subsurface sediments of ODP Site 1227, detected by reverse transcription and sequencing of extracted 16S rRNA. (a) Sulfate (empty circles) and methane (black triangles) porewater profiles. The methane concentrations are multiplied by factor 10. (b) Relative amounts of 16S rRNA and 16S rRNA genes (rDNA) in different sediment depths. The 16S rRNA genes (rDNA) were quantified by Q-PCR (grey circles). (c) Phylogenetic composition of archaeal clone libraries from reverse-transcribed 16S rRNA from each depth. Figure modified from (Teske and Sorensen, 2008).

fossilized DNA from sulphate-reducing *Bacteria* conserved in marine sediments more than 2 million years old (Inagaki *et al.*, 2005). Interestingly Sørensen and colleagues suggested that the finding of putative (hyper)thermophile in cold marine sediments could probably be the consequence of a disproportionate number of archaeal sequences from hot vent environments in the database leading to a misleading interpretation (Sorensen *et al.*, 2004). The presence of putative thermophiles in "cold" marine sediments is a revealing question as they could represent markers of deep hot sub-seafloor ecosystems.

## 1.2 Deep hydrothermal systems: a window to a deep hot biosphere?

The discovery of hydrothermal vents ecosystems along the Galapagos Rift at the end of the seventies by the American submersible "*Alvin*" drastically modified the concept of the energy

sources available for life (Corliss *et al.*, 1979). Their discovery confirmed the early claims about the importance of hydrothermal circulation for cooling the young oceanic lithosphere (Lister, 1972) and for facilitating chemical exchanges between the oceanic crust and oceans (Craig *et al.*, 1975). It was estimated that the entire volume of the ocean circulates through the crust approximately once every million years (Edwards *et al.*, 2005). Hence, the constant fluid flow within the oceanic crust has important consequences for the global heat budget, the chemical evolution of the Earth, and the deep sub-seafloor ecosystems (Bach *et al.*, 2004, for review). Volcanic heat and sporadically exothermic reactions drive circulation of highly reduced fluids from which chemosynthetic organisms can gain metabolic energy. The permeable igneous oceanic crust, combined to theses large available chemical energy sources, could therefore constitute ideal settings for extensive subsurface ecosystems at mid-ocean ridges.

### 1.2.1 Geological and Geochemical context

The seafloor spreading occurs along the 60,000 km of the Mid-Ocean Ridge (MOR), where new basaltic crust is produced and mountain chains are created at depths of 2,000–4,000 m, as a result of the convection to the surface of the magma from the hot mantle. The exact morphology of any spreading system is governed by the interplay among melt supply rate, spreading rate, and the effectiveness of hydrothermal cooling so that, along a given spreading centre, slow-, intermediate-, and fast-spread morphologies may develop (Figure 1.14). At hydrothermal vent sites along the oceanic spreading centres, hot hydrothermal fluids resulting from the cooling process are expelled. Hydrothermal systems can also occur in Back-Arc Basins that are associated with the subduction zones.

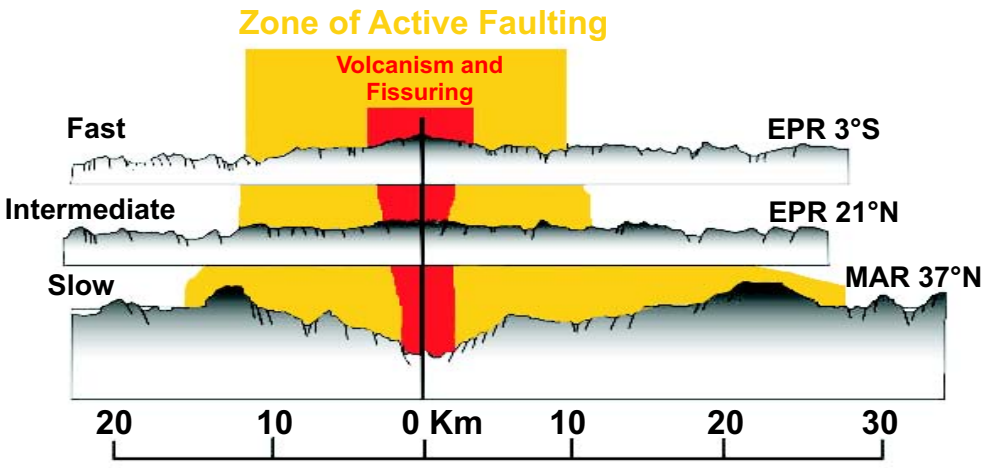


FIG. 1.14: Cross-axis profiles of general seafloor spreading process. EPR: East Pacific Rise; MAR: Mid-Atlantic Ridge, modified from (Kelley *et al.*, 2002; Macdonald *et al.*, 1992).

#### 1.2.1.1 Hydrothermal activity

Hot or molten rock (magma) beneath the ocean floor is the engine that drives hydrothermal vents as the temperature gradient is over a few hundred degrees per kilometre. The dense cold seawater (∼2°C) infiltrates through cracks down to a few kilometres in the seafloor and is progressively heated deep below the ocean crust to as high as 400°C (Kelley *et al.*, 2002, for review)(Figure 1.15). The wide pH range (∼2.8 to ∼11) and salinity (0.1 to 2 times the seawater salinity) of the hydrothermal fluids vary according to the hydrothermal site. To reach its critical point, the circulating fluids require high temperatures and pressure conditions (CP = 298 bars and 407°C for seawater) (Bischoff and Rosenbauer, 1988). Hence, over the CP, chloride and metals forming seawater-like fluids will separate into the low-salinity vapour and brine phases. As hydrothermal vent habitats usually exceed 50°C, the seawater that seeps in the seafloor is depleted in $O_2$, $Mg^{2+}$, $NO_3^-$ and $SO_4^{2-}$ and recharged with other components (Lilley *et al.*, 1993). Chemical alteration of the basement rocks (basalts, peridotites, gabbros) by the fluid changes the composition of both the aging ocean crust and seawater (Bach *et al.*, 2004, for review). The discharged high-temperature anaerobic fluids deliver base metals such as $Mn^{2+}$, $Fe^{2+}$, $Cu^{2+}$, $Zn^{2+}$, and dissolved gases such as $CO_2$, $CH_4$, $H_2$, CO and $H_2S$ (von Damm, 1995). The mechanisms that control the pH of the hydrothermal fluids, resulting in usually very acidic (pH ∼3) fluids, are not completely understood but in some cases they can be very alkaline (pH ∼11). The hot fluid then rises through the crust to the surface and is expelled at the vent openings (0.4-3 $m.s^{-1}$) or flows slowly through warm diffusers (0.1-0.5 $m.s^{-1}$). Unlike the large vent openings where the fluid temperature can exceed 350°C, the small diffusers expel fluids up to 100°C as a result of a fluid dilution over 70 %. As a consequence of the lower temperatures the diffusers are characterised by a different mineral precipitation. Hence, deep-sea hydrothermal vent deposits are formed by mineral precipitation occurring as hot, reduced, and metal enriched fluids mix with the cold seawater at the seafloor. The walls of these structures form permeable mineral matrices, and thus allow a gradual mixing between flowing hydrothermal fluids and surrounding seawater (Haymon, 1983; Fouquet *et al.*, 1993*b*). Therefore, steep physical gradients and multiple chemical disequilibria are characteristic of these environments. The nature and composition of these fluids vary according to the geochemical, mineralogical, and physical characteristics, and to the spreading centre speed.

#### 1.2.1.2 Basaltic hosted-systems

**1.2.1.2.1 Fast and intermediate spreading systems.** In fast and intermediate spreading systems diking events and volcanic eruptions exceed the effects of tectonism, creating a higher topology above the seafloor than the slow spreading model (Figure 1.14). For example, the 3°S on the East Pacific Rise is a typical fast spreading system with a spreading speed up to 150 mm per year, and can rise up to 100 m above the surrounding seafloor. This shallowest portion, directly overlying the magma chamber, corresponds to the most volcanically and

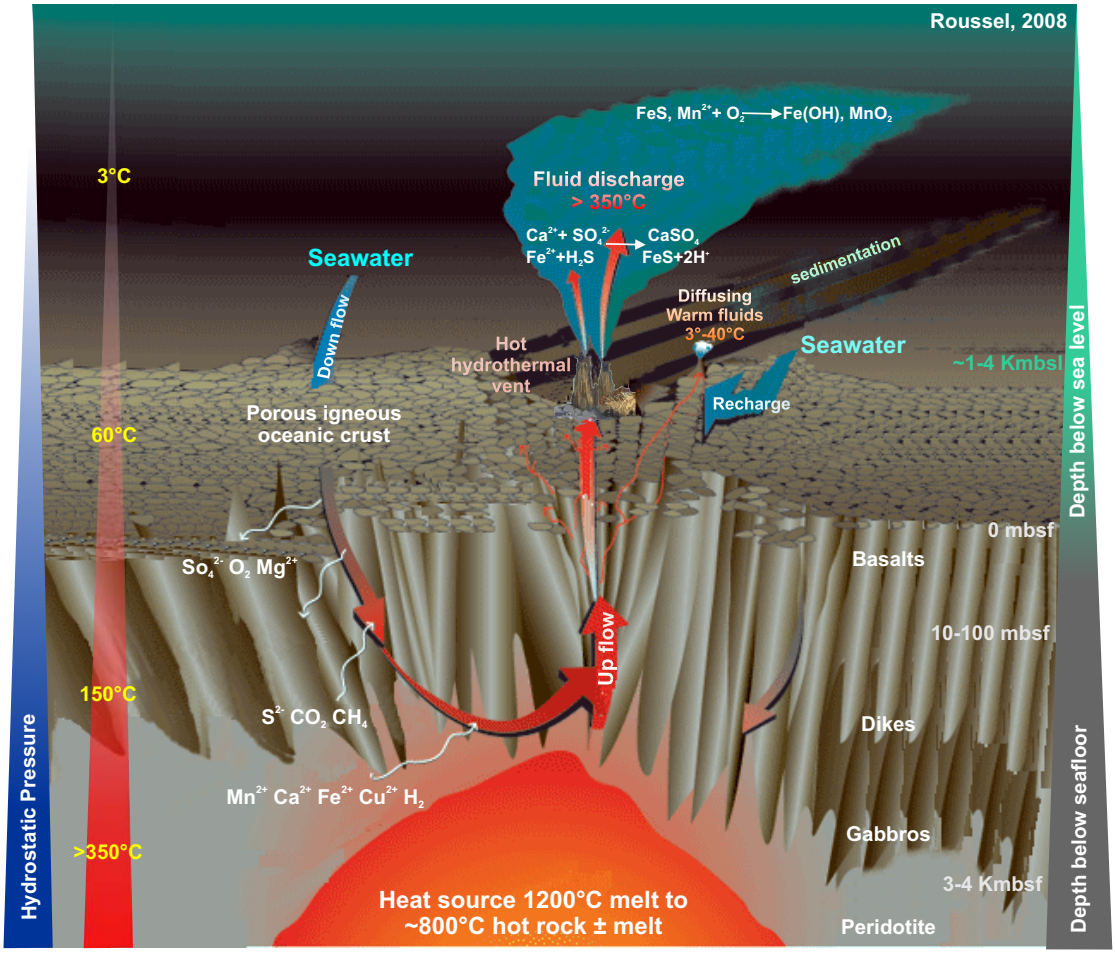


FIG. 1.15: Chemical and physical characteristics of the hydrothermal general circulation of fluids in the oceanic crust (modified from Woods Hole Oceanographic Institution according to (Jannasch, 1995; Kelley *et al.*, 2002).

hydrothermally active part of the ridge. Hydrothermal fluids circulating through basalt are highly enriched in $H_2S$, and oxidation of sulphur compounds is potentially the main source of metabolic energy (McCollom and Shock, 1997). However, local and brief (up to several months) elevated concentrations of $H_2$ and $CH_4$ can be measured in fast and intermediate basaltic spreading systems, as a result of intense water-rock interaction following a volcanic event (Kelley *et al.*, 2002, for review)(Figure 1.16).

**1.2.1.2.2 Slow spreading systems.** By contrast, in slow-spreading systems where tectonic processes dominate, the crust is believed to be built by a series of small intrusions as melt supply is intermittent. These systems such as the Mid-Atlantic Ridge are characterized by a spreading speed up to 40 mm per year and have a deep fault-bounded axial rift valley

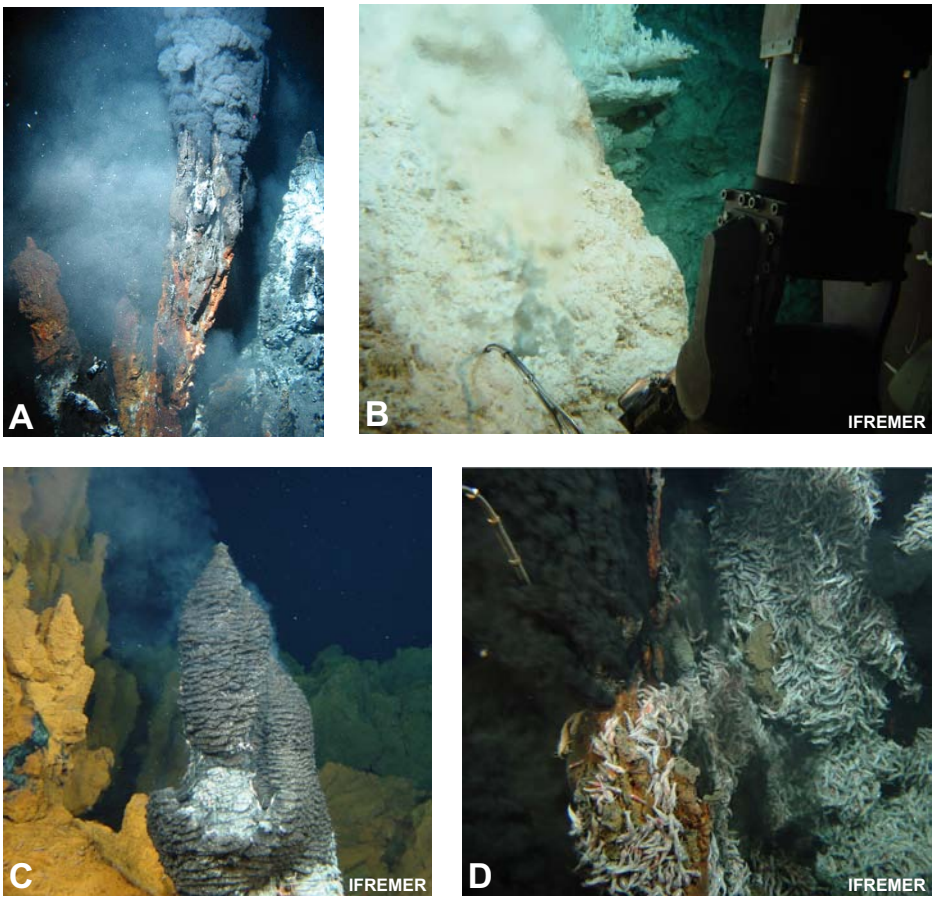


FIG. 1.16: A. Photograph of hydrothermal vent at 9°N East Pacific Rise (http://epr2004.sr.unh.edu/). B. Photograph of fluid sampling at Lost City hydrothermal field (EXOMAR 2005, IFREMER). C. Photograph of the "Beehive" , a warm diffuser at Rainbow hydrothermal field (EXOMAR 2005, IFREMER). D. Photograph of fluid sampling at TAG hydrothermal field (EXOMAR 2005, IFREMER).

populated with sporadic volcanic edifices. The discontinuous magma-tectonic processes result in a variety local deviations of 1 to 30 km in the linearity of the spreading axis. The hydrothermal fluid composition and its mineralogical and geological characteristics in such hydrothermal environments can vary drastically from basaltic-hosted to ultramafic-hosted systems. Unlike ultramafic-hosted systems, basaltic-hosted systems slow spreading have a hydrothermal fluid composition relatively similar to the fast and intermediate spreading system, characterized by a relatively low $H_2$, $CH_4$, iron oxides concentrations and relatively high concentrations of sulphur compounds.

#### 1.2.1.3 Ultramafic-hosted systems

Since the discovery of the first hydrothermal environments, studies of deep sea hydrothermal systems have mainly focused on basaltic hosted systems (Kelley *et al.*, 2002, for review). However, there has been an increasing interest in hydrothermal systems hosted in ultramafic rocks as their characteristics differ from the typical basaltic-hosted systems (Charlou *et al.*, 2002; Fruh-Green *et al.*, 2003; Kelley *et al.*, 2005). The ultramafic-hosted systems correspond to small zones where the lithospheric mantle is exposed, and are thought to be driven by the reaction of the seawater with the ultramafic rocks such as peridotite. Hence, as these hydrothermal systems are generally less frequently affected by strong events such as eruptions, it has been suggested that ultramafic-hosted systems on slow-spreading ridges could be more stable than fast-spreading basalt-hosted systems (Schmidt *et al.*, 2007).
Ultramafic rocks, such as peridotite and dunite, are primarily composed of two minerals, olivine and pyroxene, and occurrences of ultramafic rocks at the seafloor represent blocks of the mantle that have been exposed by tectonic processes. Ferrous Fe-bearing minerals such as olivine and pyroxene are unstable in the presence of water at low temperatures. Hence, exposures of ultramafic rocks to circulating seawater undergo aqueous alteration named serpentinization leading to the formation of mineral assemblage such as antigorite, lizardite, and chrysotile. Several studies (Wetzel and Shock, 2000; Berndt *et al.*, 1996) indicate that high $H_2$ and $CH_4$ concentrations are produced by peridotite–seawater reactions via the following reaction:

$$\text{Olivine} + H_2O + \text{C (or } CO_2) \rightarrow \text{Magnetite} + \text{Serpentine} + \text{Brucite} + H_2 + CH_4$$

Hence, serpentinization of ultramafic rocks results in substantially different mineralogical alteration and vent fluid compositions from those occuring during hydrothermal alteration of basaltic rocks. Hydrothermal alteration of basalt generates much lower amounts of $H_2$, $CH_4$ and iron oxides than serpentinization of ultramafic rocks, even though the Fe content of basalt is typically much higher (Table 1.2).

To date several ultramafic-hosted sites have been explored such as Rainbow, Logatchev (Schmidt *et al.*, 2007; Charlou *et al.*, 2002). However, the off-axis Lost City Hydrothermal Field (LCHF), recently discovered on the Atlantis massif of the Mid-Atlantic Ridge, is outstanding by its mineralogical and geochemical characteristics. This field of tall, (30-60 m), and active carbonate-magnesium hydroxide chimneys appears to be the surface expression of warm and very alkaline fluids ($< 90°C$; pH $< 11$) emanating from the underlying fault zones (Kelley *et al.*, 2001; Kelley *et al.*, 2005). Although $H_2$ concentrations are in the range of the usual concentrations found at ultramafic-hosted sites, due to the very alkaline environment, no $CO_2$ was detected. Hence the energy and carbon source for metabolic activity of LCHF impacts directly the associated ecosystem.
Serpentinization also provides unique perspectives on heat-generating process. However, a recent study indicates that significant changes in temperature are not likely to occur at Rainbow

| | Seawater | Rainbow MAR | Logatchev MAR | Lost City off-axis | TAG MAR | 21°N OBS EPR |
|---|---|---|---|---|---|---|
| Host Rock | | ultramafic | ultramafic | ultramafic | basalt | basalt |
| T (°C) | 2 | 365 | 352 | 40–91 | 321 | 350 |
| pH | 7.8 | 2.8 | 3.3 | 9.0 to ~11 | 2.8 | 4.3 |
| $O_{2(aq)}$ | 0.100 | 0 | 0 | 0 | 0 | 0 |
| $H_{2(aq)}$ | 0.0000004 | 16 | 12 | 1-15 | 0.15 | 1.7 |
| $SO_4^{2-}$ | 27.9 | 0 | 0 | 1-4 | 0 | 0 |
| $H_2S_{(aq)}$ | 0 | 1.2 | 0.8 | — | 6.7 | 7.3 |
| $CH_{4(aq)}$ | 0.0000003 | 2.5 | 2.1 | — | 0.124 | 0.07 |
| CO | — | 0.005 | — | — | — | — |
| $\Sigma CO_2$ | 2.3 | 16 | 10.1 | — | 2.9 | 5.72 |
| $Na^+$ | 464 | 553 | 430 | — | 584 | 432 |
| $Cl^-$ | 546 | 750 | 515 | 550 | 659 | 490 |
| $Br^-$ | 0.84 | 1.18 | 0.82 | — | 0.88 | 0.8 |
| $NO_3^-$ | 0.03 | — | — | — | — | — |
| $Ca^{2+}$ | 10.2 | 66.6 | 27.3 | to 30 | 26 | 15.6 |
| $Mg^{2+}$ | 52.7 | 0 | 0 | 0 | 0 | 0 |
| $K^+$ | 9.8 | 20.4 | 21.9 | — | 18 | 23.2 |
| $SiO_{2(aq)}$ | 0.16 | 6.9 | 8.2 | — | 22.0 | 17.6 |
| Fe | 0.0000015 | 24.1 | 2.5 | — | 1.64 | 1.66 |
| $Mn^{2+}$ | 0 | 2.25 | 0.33 | — | 1.0 | 0.96 |
| $Cu^{2+}$ | 0.000007 | 0.16 | 0.05 | — | 0.15 | 0.035 |
| $Zn^{2+}$ | 0.00001 | 0.185 | 0.03 | — | 0.046 | 0.10 |

Table 1.2: Hydrothermal fluid composition from ultramafic-hosted, basaltic-hosted and seawater, from (McCollom, 2007).

and Lost City as a result of the exothermic serpentinization (Allen and Seyfried, 2004). Hence, Rainbow high temperatures are linked to magmatic processes, and Lost City heat could be the result of the rise of deep lithospheric circulation. However, cooler (7-9°C) hydrothermal fluids such as at Saldanha hydrothermal field located at the top of a serpentinized massif (Dias and Barriga, 2006), suggest that heat is acquired through serpentinization process. Although once thought to be anomalous, ultramafic-hosted hydrothermal environments are increasingly recognized to be widespread on the seafloor (Fruh-Green *et al.*, 2004; Bach *et al.*, 2002).

### 1.2.2 Sub-seafloor ecosystems at Mid-Ocean Ridges and Back-Arc Basins

As the hydrothermal sub-seafloor ecosystems do not directly benefit from the solar radiation, chemolithoautotrophic microorganisms are the primary producers since they can use the "dark energy", a wide range of chemical compounds derived from hydrothermalism (Bach *et al.*, 2006, for e.g.). However, some of chemosynthetic life is not completely independent of photosynthesis as it depends on access to seawater dissolved oxygen and on nitrate as electron acceptors that are ultimately derived from photosynthesis. Chemoautotrophic microorganisms can gain energy by oxidizing hydrogen, methane, hydrogen sulphide, ammonia, iron (II) and manganese (II), all of which occur in vent fluids as seen previously. According to the infiltration and dilution of seawater, $CO_2$, $SO_4^{2-}$, S, Fe (III), $NO_3^-$ or $O_2$ can be available as oxidants.

#### 1.2.2.1 A wide range of subsurface habitats encompassing a large biomass

The steep thermal and geochemical gradients, combined with the wide range of mineralogical substrates define a wide range of possible habitats for prokaryotic communities. These habitats extend from the oceanic crust hydrothermal subsurface biosphere at mid-ocean ridges and ridge flanks to the hydrothermal sediments (Figure 1.17). In hydrothermal environments, the steep thermal gradients are one of the major limiting factors at depth and therefore favour the presence of thermophilic microorganisms. At present, the temperature limit for growth of the two most hyperthermophilic microorganisms in pure culture stands at 113 and 122°C; even higher temperatures may be tolerated during short-term survival (Blochl *et al.*, 1997; Kashefi and Lovley, 2003; Takai *et al.*, 2008). Hence, the high geothermal gradients in the oceanic crust at Mid-oceanic ridges suggest that the habitable zone in the subsurface may increase with distance from the ridge centre, possibly reaching a few hundred metres at the furthest (Alt, 1995). The deep hydrothermal subsurface also includes the extensive zones where seawater is entrained into porous, freshly formed ocean crust and sediment deposits. As the upper crust has an estimated volume of $10^{18}$ $m^3$ and as prokaryotic communities colonize large delimited zones, it was estimated that theses prokaryotic communities could represent 1 % of the Earth's biomass (Fisk *et al.*, 1998).

**1.2.2.1.1 Oceanic crust at mid-ocean ridges.** Several studies have revealed that abundant prokaryotic communities could colonize the basaltic oceanic crust (Cowen, 2004, for review). The analyses of crustal fluids from deep zone of the oceanic crust ($<$ 300 mbsf) showed lower cell densities than the seafloor-exposed basalts ($\sim 8 \times 10^4$ cells per mL), and mostly encompassed nitrogen and sulfate cycling *Bacteria* (Cowen *et al.*, 2003). Low prokaryotic cell densities were also previously observed in deep sea basalts and showed evidence of microbial weathering (Fisk *et al.*, 1998). The prokaryotic cell abundances on seafloor-exposed basalts are 3–4 orders of magnitude greater ($\sim 10^6$ to $10^9$ cells per mL) than in overlying deep sea water ($\sim 10^4$ cell per ml), suggesting that the product of the basalt alteration could at least partially fuel these communities (Santelli *et al.*, 2008). Moreover, massive prokaryotic output from fissures and vents created during diking and eruption characterised the early stages of several eruptive events studied (Ehrhardt *et al.*, 2007; Holden *et al.*, 1998; Huber *et al.*, 2002*b*; Summit and Baross, 1998; Juniper *et al.*, 1995), supporting the existence of large subsurface prokaryotic communities in these zones. Cells and nucleic acids were detected in the superheated hydrothermal fluids although the endmember fluids remained sterile, suggesting that subsurface prokaryote communities could grow in protected niches and could be entrained in a dynamic subsurface flow (Takai *et al.*, 2004). Hence, the evidence would more likely represent relics from these subsurface communities than viable cells (Deming and Baross, 1993*a*).

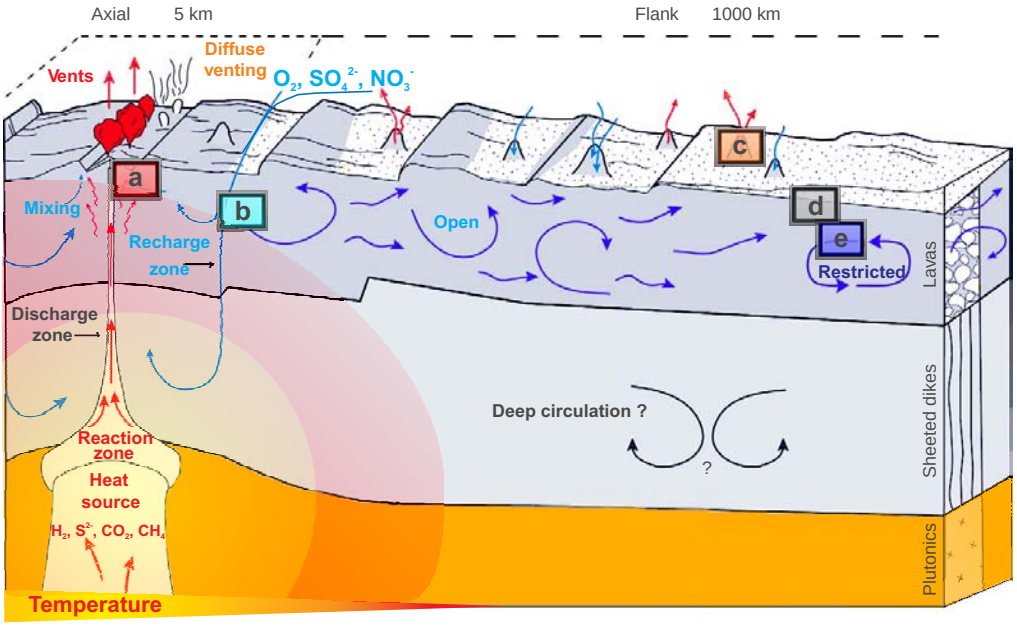


FIG. 1.17: Schematic representation of fluid flow regimes within the ocean crust. (a) At mid-ocean ridge axes, hot, reducing hydrothermal solutions mix with cold oxygenated seawater. Prominent metabolic reactions for lithoautotrophs include oxidation of sulphide, Fe(II), methane and hydrogen, as well as in the anoxic subsurface autotrophic sulfate reduction and methanogenesis. (b) In recharge zones and young ridge flanks, oxygenated seawater has ready access to fresh rock surfaces. Likely lithoautotrophic reactions include aerobic and anaerobic oxidation of Fe(II) and sulphide. (c) Off-axis discharge is focused where seamounts penetrate the sediment cover. Possible reactions are oxidation of Fe(II), $H_2$ and sulphide. (d) Sedimentary organic matter might sustain heterotrophic respiration and fermentation. (e) More-restricted circulation might allow for the abiotic generation of $H_2$ by hydrolysis on ferrous oxides in basalt; this $H_2$ could support Fe and $SO_4^{2-}$ reducers as well as methanogens in ridge flanks; modified from (Edwards *et al.*, 2005).

**1.2.2.1.2 Hydrothermal sediments.** Unlike the unsedimented zones, where hydrothermal diluted endmember fluids emerge directly into the water column, fluids in sediment-covered vents rise through the accumulated sediment layers. Thus, chemical transformations of the sediments by the hydrothermal fluids occur in these environments extending up to several hundred metres below the seafloor. Several Pacific hydrothermal sites where these processes occur, (e.g. Guaymas Basin), have been shown to contain large prokaryotic populations. For example, at the Bent Massive Sulphide site (Juan de Fuca hydrothermal vent field), prokaryotic cell counts significantly decrease with increasing depth and temperature (Figure 1.18) (Parkes *et al.*, 2000). However, prokaryotic communities are still at significant densities over a temperature of 80°C, suggesting that these communities are partially composed of hyperthermophiles or seawater representatives infiltrated from shallower sediments. Moreover, the high primary production, sedimentation rates and temperature gradients occurring at the Guaymas Basin

sediments fuel the large thermophilic communities contained in the first cmbsf (Amend and Teske, 2005, for review). In a recent study of Pacific ridge-flank sediments, cell counts and methane oxidation coupled to sulfate reduction were shown to increase near basement, suggesting that crustal fluids may fuel deep subsurface marine sediments (Engelen *et al.*, 2008). Hence, zones with less steep temperature gradient could represent interesting models to study and understand certain aspects of a deeply buried hot biosphere.

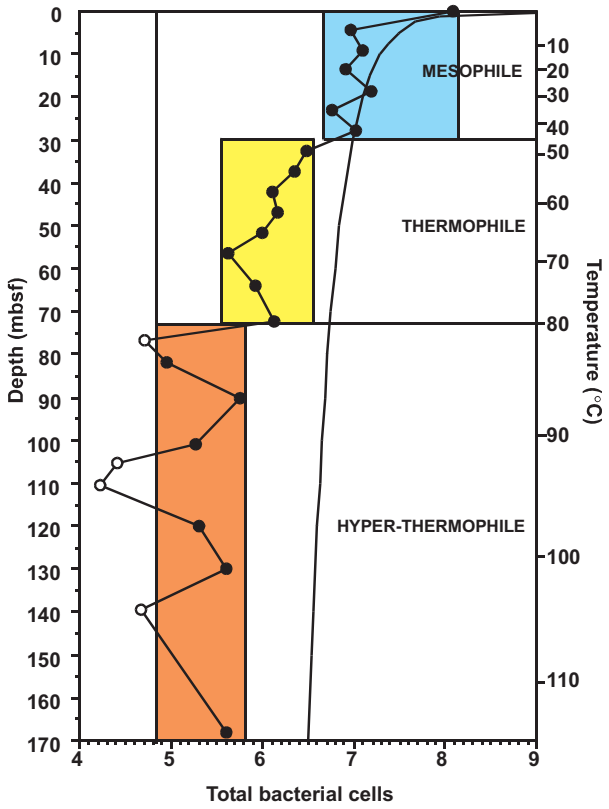


FIG. 1.18: Prokaryotic cell distribution in the Juan de Fuca hydrothermal vent fields (ODP, Leg 169) according to depth and temperature. The solid curve represents the general regression model (see 1.1.2.2). The solid vertical line represents the significant detection limit, from (Parkes *et al.*, 2000).

#### 1.2.2.2 General phylogenetic and metabolic diversity

As the subsurface habitats at Mid-ocean ridges encompass a wide range of physical (e.g. pH, temperature, pressure) and geochemical (e.g. concentrations of $O_2$, $H_2$, $SO_4^{2-}$) habitats, the archaeal and bacterial metabolic and phylogenetic diversities are characterized by an equally large number of extremophiles. Therefore, hyperthermophilic to mesophilic prokaryotes will occur depending on the distance from the heat source and the rise of fluids. Even if heterotrophic prokaryotes are widespread, most studies show that autotrophes fuelled by the huge amounts of

$CO_2$ dominate the subsurface hydrothermal ridges (Nakagawa and Takai, 2008; Edwards *et al.*, 2005; Amend and Teske, 2005). The aerobic or anaerobic chemolithoautotrophic metabolisms described in table 1.3 are mostly distributed depending on the seawater dilution (Figure 1.17). The subsurface basaltic crust in ridge flanks at moderate temperatures (∼10-60°C) is dominated by indigenous *Bacteria* such as *Epsilonproteobacteria* and GNS (Huber *et al.*, 2006). Moreover the sulfate-reducer *Ammonifex* and the methanogen *Methanothermococcus* were shown probably to dominate deeper crustal fluids (Nakagawa *et al.*, 2006).

| Reaction | Energy available (kJ .mol$^{-1}$ reaction)[a] | Energy per electron transferred (kJ .mol$^{-1}$) | Location of reaction (see FIG. 1.17) |
|---|---|---|---|
| **Aerobic lithoautotrophic metabolism** | | | |
| Sulfide oxidation ($HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$) | 750 | 94 | a, b, c |
| Methanotrophy ($CH_4 + 2O_2 \rightarrow HCO_3^- + H^+ + H_2O$) | 750 | 94 | a, c |
| Iron (II) oxidation ($Fe^{2+} + 1/4O_{2,aq} + H^+ \rightarrow Fe^{3+} + 1/2\ H_2O$) | 65 | 65 | a, b ,c |
| Mn (II) oxidation ($Mn^{2+} + 1/2O_{2,aq} + H_2O \rightarrow MnO_{2,s} + 2H^+$) | 50 | 25 | a, b ,c |
| Hydrogen oxidation ($H_2 + 1/2O_{2,aq} \rightarrow H_2O$) | 230 | 115 | a, b |
| **Anaerobic lithoautotrophic metabolism** | | | |
| Methanogenesis ($HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O$) | 130 | 16 | e ; a if anoxic |
| Sulfate reduction ($SO_4^{2-} + H^+ + 4H_2 \rightarrow HS^- + 4H_2O$) | 170 | 21 | e ; a anoxic |
| Anaerobic iron oxidation ($10Fe^{2+} + 2NO_3^- + 12H^+ \rightarrow 10Fe^{3+} + N_2 + 6H_2O$) | 100 | 10 | b, e |
| **Heterotrophic metabolism** | | | |
| Respiration ($CH_2O + O_2 \rightarrow CO_2 + 2H_2O$) | 500 | 125 | d |
| Fermentation ($CH_2O \rightarrow 1/3C_2H_6O + 1/3CO_2$) | 50 | | d |

Table 1.3: Possible chemical reactions that are major sources of metabolic energy for lithoautotrophs in subsurface and deep-sea ecosystems, with representative heterotrophic reactions for comparison; modified from (Edwards *et al.*, 2005).

An increasing number of thermophilic prokaryotes are cultivated from hydrothermal environments (Huber *et al.*, 2002*a*; Reysenbach *et al.*, 2006; Wagner and Wiegel, 2008; Miroshnichenko and Bonch-Osmolovskaya, 2006). Although usually isolated from hydrothermal chimney fragments, these cultured prokaryotes could probably occur in a moderate to hot hydrothermal subsurface according to the seawater fluxes, and encompass majority of hydrogen oxidizers such as some members of the *Ignicoccus, Geogemma, Persephonella, Desulfurobacterium* genera and most members of the *Epsilonproteobacteria* class and *Methanococcales, Methanopyrales* and *Aquificales* orders. Recently, a methagenomic approach confirmed the widespread presence and dominance of *Epsilonproteobacteria* in hydrothermal vent fluids (Huber *et al.*, 2007).
Besides hydrogen, sulfur and sulfate are also thought to be the other major sources of metabolic energy for prokaryotic communities (McCollom and Shock, 1997). Hence, autotrophic sulfate-reducers such as the *Archaeoglobus* and *Thermodesulfatator* genera, and heterotrophic sulfate-reducers such as the *Desulfovibrio* and *Desulfonauticus* genera are commonly found at hydrothermal environments. Besides hydrogen and sulphur metabolizing prokaryotes, various metals, such as iron, are thought to constitute an important energy source for hydrothermal subsurface communities (Kashefi *et al.*, 2004). However, only a few metal cycling prokaryotes have been isolated from hydrothermal environments, such as Fe(III) reducers like *Geoglobus ahangari*, *Geogemma barossii*, *Paleococcus ferrophilus*. Although only negligible amounts of

energy are available from oxidation of $Fe^{2+}$ or $Mn^{2+}$ compounds or $Fe^{3+}$ reduction according to their availability, these metabolic processes have probably been overlooked.
The hyperthermophilic *Archaea, Pyrolobus fumarii*, and strain 121 hold the global record for growth at high temperatures (113 and 121°C, respectively) (Blochl *et al.*, 1997; Kashefi and Lovley, 2003). Moreover, Takai and colleagues have recently cultured *Methanopyrus kandleri* strain 116°C up to 122°C using a new technique for cultivation of chemolithoautotrophs under high hydrostatic pressures (Takai *et al.*, 2008). This temperature range seems to represent the upper limit for life. Even so, at more moderate temperatures, diverse mesophilic *alpha-* and *Gammaproteobacteria* are widespread, including the chemosynthetic endosymbionts of various tubeworms and bivalves.
However, in addition to cultivation efforts, molecular methods based on molecular phylogenetic identification have revealed several new lineages (Takai *et al.*, 2006; Kormas *et al.*, 2006; Takai and Horikoshi, 1999; Nercessian, 2003; Moussard *et al.*, 2006*a*, for review). Metagenomic approaches and functional gene analyses have successfully characterized the metabolic and physical properties of these communities (Moussard *et al.*, 2006*b*; Moussard *et al.*, 2006*c*; Nercessian *et al.*, 2005*a*; Dhillon *et al.*, 2005). A heterotrophic member of the ubiquitous, abundant and apparently endemic deep-sea hydrothermal vent euryarchaeota group DHVE2 was recently cultivated. As predicted in previous molecular investigations DHVE2 are thermophilic *Euryarchaeota* (Moussard *et al.*, 2006*b*), with an optimum growth at a low pH (4.2–4.8), and utilizing sulphur and iron as electron acceptors. Hence, the archaeal community could play a key role in hydrothermal subsurface communities.

#### 1.2.2.3 (Hyper)thermophilic *Archaea*: hot biosphere communities?

*Archaea* encompass up to 33-50 % of the total microbial community in deep-sea hydrothermal environments (Harmsen *et al.*, 1997; Takai *et al.*, 2001*a*; Nercessian *et al.*, 2003) and could even represent up to 99.9 % on some chimney fragments (Takai *et al.*, 2004). These archaeal communities could be fuelled by inorganic compounds (Amend and Shock, 2001). Most of the deep-sea hydrothermal vent *Archaea* are dominated by the *Euryarchaea* phylum (Pagé *et al.*, 2008; Takai *et al.*, 2004, for e.g.), often encompassing cultured *Thermococcales* and *Methanococcales*.
Although microbial communities occupy both aerobic and anaerobic habitats, anaerobic hyperthermophilic *Archaea* were reported to be associated with the hottest parts of these environments, and were therefore likely to be entrained by hydrothermal fluids from subsurface ecosystems (Deming and Baross, 1993*a*; Kelley *et al.*, 2002; Takai *et al.*, 2004). Moreover, thermophilic *Archaea* such as *Thermococcus, Pyrococcus, Pyrodictium*, and *Archaeoglobus* have been isolated directly from vent plumes and volcanic fragments associated with submarine eruptions and diking events (Huber *et al.*, 1990; Summit and Baross, 1998). Strains of the hyperthermophilic genera *Thermococcus* and *Methanococcus* were also cultured from warm

(3–30°C) hydrothermal vent effluents but were not hot enough to allow the growth of these organisms. These requested higher optimum temperatures than the temperature of the fluids from which they were sampled. Hence, they probably inhabited the higher temperature subsurface and were expelled to the surface by hydrothermal circulation (Holden *et al.*, 1998). Moreover, hyperthermophilic *Archaea* of the genera *Thermococcus* and *Pyrococcus* isolated from low-temperature diffuse fluids exiting the basaltic crust showed distinct phylogenetical and physiological characteristics indicating that these strains were distinct from previous isolates obtained from surficial vent samples (Summit and Baross, 2001*b*). Overall these investigations of the subsurface hydrothermal archaeal communities tend to show that specific phylotypes inhabit these ecosystems, suggesting that such phylotypes as the *Thermoccocales* could represent indicators of sub-seafloor ecosystems (Kelley *et al.*, 2002).

#### 1.2.2.4 Hydrogen based ecosystem at hydrothermal vents?

The essential consideration in understanding any ecosystem is to define the relative contributions of each energy source possibly fuelling primary productivity, and therefore the conversion of carbon dioxide into biomass. At temperatures over 70°C, photosynthesis is not known to occur, but thermophilic microbial ecosystems develop well beyond that temperature. Although some studies are controversial, several terrestrial (hyper)thermophilic chemoautolitrophic communities were shown recently to sustain probably by using $H_2$ as unique energy source (Anderson *et al.*, 1998; Chapelle *et al.*, 2002; Spear *et al.*, 2005; Stevens and Mc Kinley, 1995; Stevens and Mckinley, 2000).

As seen in the "geological and geochemical" section, sulphur cycling could be, together with methanogenesis, the dominant metabolic process in deep sea hydrothermal systems. However, some hydrothermal environments can release high concentrations of $H_2$ and $CO_2$ and low sulphide and sulphate concentrations. According to geochemical cultivation-dependent and independent methods, Takai and colleagues suggested that a hydrogen-driven ecosystem could occur beneath an active deep-sea hydrothermal field in the Central Indian Ridge, and was primarily consisted of *Methanococcales* and *Thermococcales* (Takai *et al.*, 2004). Interestingly, several groups of *Archaea*, including a member of the *Thermococcus* genus, were recently shown to grow autotrophically on carbon monoxide, producing $H_2$ (Sokolova *et al.*, 2004). It was suggested that the high hydrogen content released by ultramafic-hosted hydrothermal system could fuel the ecosystems at these sites (Charlou *et al.*, 2002; Takai *et al.*, 2004). Moreover the prokaryotic communities in the Logatchev vent fluids, a ultramafic-hosted hydrothermal field located on the Mid-Atlantic Ridge, were mainly composed of putative hydrogen oxidizers such *Methanococcales* and *Epsilonproteobacteria* (Perner *et al.*, 2007). Although these biological evidences seem to suggest the possible occurrence of hydrogen-driven ecosystems, further approaches are necessary to confirm these assumptions (Nealson *et al.*, 2005; Nealson, 2005).

| Chemolithoautotrophic energy source | Ultramafic-hosted system (Rainbow) | | Basalt-hosted system (21¡N EPR)[a] | |
|---|---|---|---|---|
| | Maximum available energy[a] | Biomass potential[b] | Maximum available energy[a] | Biomass potential[b] |
| Hydrogen oxidation | 3.7 | 97 | 0.39 | 11 |
| Methanotrophy | 2.1 | 14 | 0.05 | 0.3 |
| Iron (II) oxidation | 1.3 | 9 | 0.05 | 0.3 |
| Sulfide oxidation | 0.9 | 6 | 3.2 | 21 |
| Methanogenesis | 0.5 | 13 | 0.03 | 0.7 |
| Sulfate reduction | 0.9 | 24 | 0.04 | 1.1 |
| Nitrate reduction | 0.6 | 16 | — | — |
| Anaerobic methane oxidation | 0.3 | 8 | 0.003 | 0.4 |
| Anaerobic iron oxidation | 0.3 | 2 | — | — |

Table 1.4: Geochemical energy sources and potential biomass yields resulting from mixing of seawater with hydrothermal fluids through the walls of vent chimneys; from (McCollom, 2007). [a]Maximum chemical energy available in kJ per kg hydrothermal fluid. [b]Biomass potential in mg dry wt of biomass per kg hydrothermal fluid. Calculations assume that hydrogen oxidation, methanogenesis, sulfate reduction, nitrate reduction, and anaerobic methane oxidation require 38 kJ per g dry wt biomass and all other reactions require 152 kJ per g dry wt biomass.

As for the Yellowstone geothermal ecosystem, geochemical numerical models showed that the large concentrations of $H_2$ and $CH_4$ in fluids at the Rainbow ultramafic hydrothermal system could fuel the whole ecosystem at this site (McCollom, 2007). Hence, hydrogen oxidation, methanotrophy, sulfate reduction, and methanogenesis, are predicted to represent the predominant metabolic processes at Rainbow (Table 1.4). Moreover, anaerobic metabolic reactions should dominate higher-temperature environments supporting up to ∼20-30 mg biomass by anaerobes. This study indicates that ultramafic-hosted systems are probably capable of supplying about twice as much chemical energy as analogous deep-sea hydrothermal systems hosted in basaltic rocks, suggesting that hydrogen-driven ecosystems at ultramafic-hosted hydrothermal systems such as Rainbow are reasonable inferences, which could follow the model described by figure 1.19.

## 1.3 Approaches for studying microbial diversity in sub-seafloor environments

The study of the diversity of microorganisms in their environment is one of the important tasks to understand a microbial ecosystem. Many methods and techniques were developed to achieve this objective. On account of their location, deep subsurface marine environmental samples are typically difficult to obtain. Moreover, these samples are usually characterized by low biomass and metabolic rates, making difficult to analyse the associated ecosystem. Firstly, we will describe some of the tools that can be used in order to retrieve sediment and hydrothermal fluid samples. Secondly we will briefly introduce the culture dependent methods. A selection

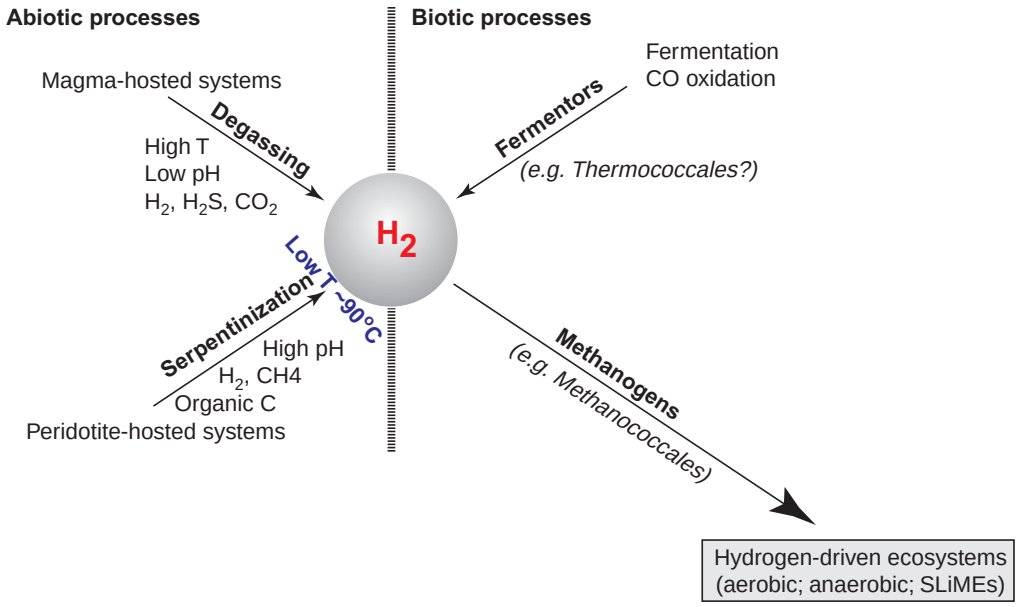


FIG. 1.19: Possible interaction between biotic and abiotic processes in a Hydrogen-driven ecosystem; modified from (Nealson *et al.*, 2005).

of culture independent tools developed and applied to investigate microbial metabolic activity and diversity in subsurface environments will be presented, exclusively focusing on molecular genetic approaches.

### 1.3.1 Sampling tools

A central goal in ecology is to understand the spatial scaling of biodiversity. Patterns in the spatial distribution of organisms provide important clues about the underlying mechanisms that structure ecological communities (Green and Bohannan, 2006; Martiny *et al.*, 2006; Green *et al.*, 2008, for review). Hence, the sampling approach is crucial in order to minimize artefacts due to undersampling or to the choice of unrepresentative samples.

#### 1.3.1.1 Sediment sampling

**1.3.1.1.1 Corers.** There is a range of sampling devices commonly used for sediment sampling, each designed to provide a certain type of sample over a specific ground type. The surface of seafloor sediment can accurately be sampled using a push-core device (Figure 1.20). At great depth, this tool has to be manipulated by a submersible, either a Remote Operated Vehicule (ROV) or a deep submergence vehicule (DSV). Although, the sediment depth accessible with this tool is limited to a few centimetres (∼30 cm), the structure of the surface sediments layers are preserved. It is therefore possible to investigate precisely the succession of

the microbial metallic activities in sediment surface. To access to more deeply buried sediment layers, a vibra-corer can be launched from a oceanographic vessel. Although vibra-coring is limited to relatively shallow depths as the vibration unit is linked to the ship by a power cord (Figure 1.20), this technique can retrieve sediment cores of several metres (∼5 m) from locations where gravity cores cannot be used. However, gravity cores can access to deep subsurface sediments from deep water depths, for example the CALYPSO giant gravity piston corer IPEV able to recover sediment cores up to 70 m long (Figure 1.20).

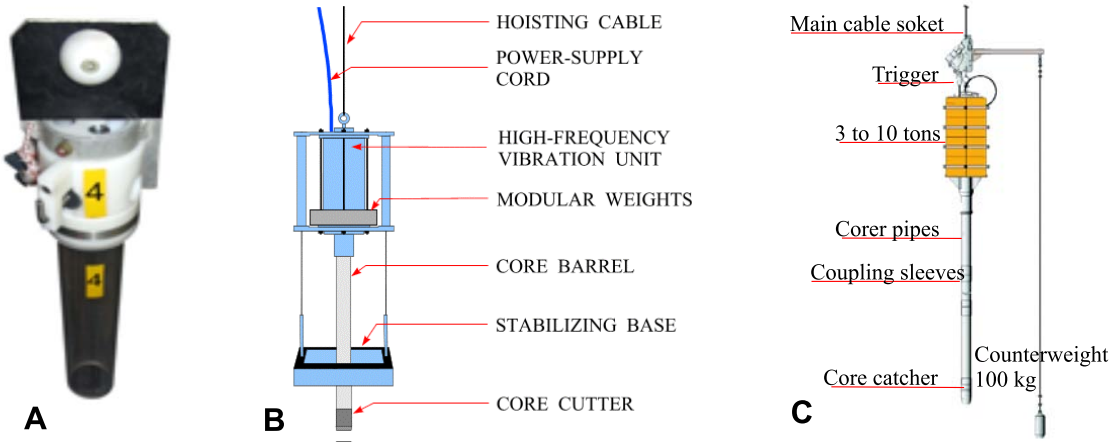


FIG. 1.20: Three sediment sampling corers. A. Photographe of a push-core (IFREMER). B. Schematic representation of a vibra-corer. C. Schematic representation of the CALYPSO giant corer (IPEV).

**1.3.1.1.2 Drilling devices and projects.** Drilling is an alternative to access to sediment below the depth range of corers. Pieces of drill tubes are fixed together and lowered from the derrick of the drilling vessel. The rotating drill bit at the end of the drill pipe, grinding rock at the base of the hole, is spaced (∼6 cm) so as to preserve a cylinder of rock that is pushed up into the core tube. Several types of drill bits can be used according to the seafloor nature: the Advanced Piston Corer (APC) is used in soft ooze and sediments, the Rotary Core Barrel (RCB) is used in medium to hard crystalline sediments, the Extended Core Barrel (XCB) is used in firm sediments, the Advanced Diamond Core Barrel (ADCB) is used in hard sedimentary or igneous formations and the newly designed Pressure Core Sampler (PCS) is used in sediments and is able to maintain the sediment at *in situ* pressure. The "JOIDES Resolution" is an American riserless vessel that uses seawater as primary drilling fluid which is pumped down through the drillpipe (Figure 1.21). The seawater cleans and cools the drill bit and lifts cuttings out of the hole, piling them in a cone around the hole.
Another drilling technology is used by "Chikyu" a Japanese riser vessel that includes an outer casing that surrounds the drill pipe to provide return-circulation of the drilling fluid to maintain

pressure balance within the borehole. A blowout preventer (BOP) protects the vessel from gas and oil. This technology is necessary for drilling several thousand meters into the Earth.

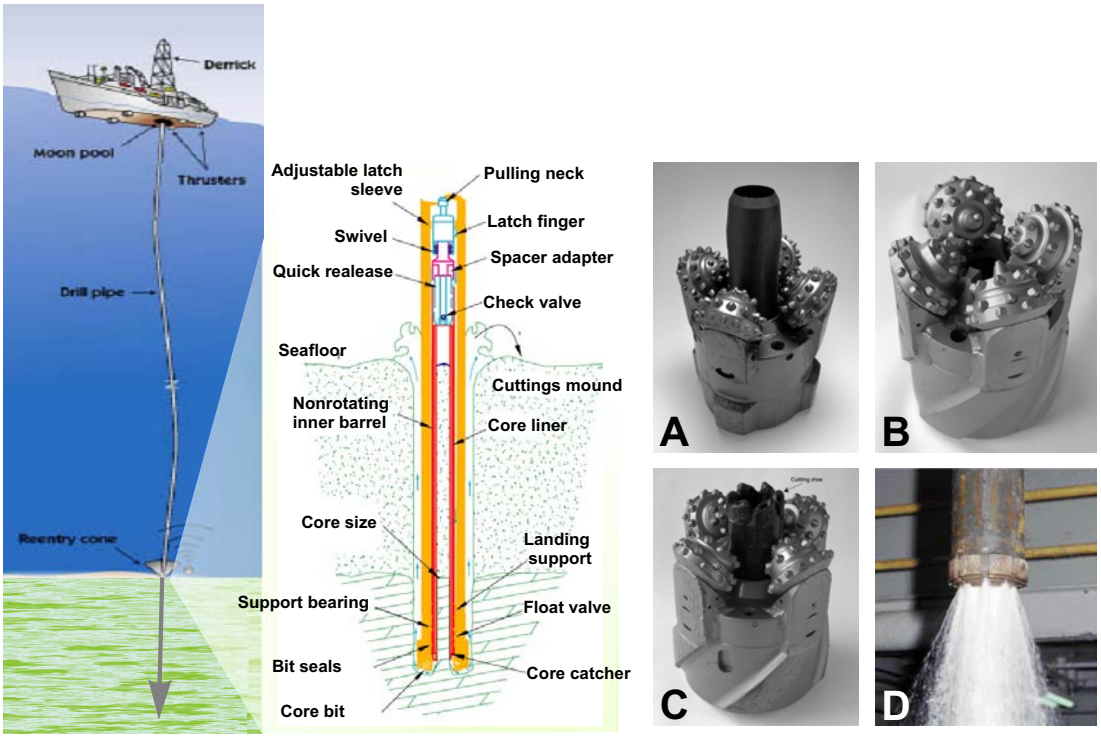


FIG. 1.21: General principle of the drilling riserless vessel "JOIDES Resolution" drilling with a RCB bit. A. APC bit, B. RCB bit, C. XCB bit, D. ADCB.

The understanding of a deep subsurface microbial ecosystem is inherently based on the quality of the studied samples. Hence, one of the major concerns is to evaluate potential contaminations of the sediment cores by exogenous microorganisms. These microorganisms can be introduced from the drilling fluid into the recovered core material during coring (Griffin *et al.*, 1997; McKinley and Colwell, 1996). Two tracer methods are used to quantify the amount of contamination. These methods were modified from land-based drilling operations for use on the "JOIDES Resolution". Tracer experiments involve the delivery of both chemical and particulate tracers in the drilling fluid during drilling and their quantification in the ODP cores (Lever *et al.*, 2006; Smith *et al.*, 2000*b*; Smith *et al.*, 2000*a*). Although the particulate tracer are microspheres that mimic the size of microorganisms and can be visualized by epifluorescence microscopy, their surface properties are different, and they may cause a difference in the migration into pores, cracks and attachment to mineral surfaces. Chemical tracers are composed of Perfluorocarbon (PFT) that can be detected by gas chromatography analysis. The contamination level depends on the sediment structure and depth. For example the fractured basaltic rocks are more likely to be contaminated by drilling fluids. Shallow water sediments

are usually less contaminated than those in cores from deepwater sites as their retrieval time is shorter. However, contamination is usually negligible as it is estimated that the sediment cores encompass less than 50 contaminating prokaryotes per gramme (House *et al.*, 2003). However, to lower the risk of contamination, the sediment cores are systematically subsampled by removing the potentially contaminated outer surface of the core. The subsampling of the sediments core can be performed by using 5 ml plastic syringes with the luer end removed (Parkes *et al.*, 1995), or by using a subcoring rig if the sediment is too hard as described by Wellsbury and colleagues (Wellsbury *et al.*, 2001).
The Integrated Ocean Drilling Program (IODP) is an international marine research program that explores the Earth's history and structure as recorded in seafloor sediments and rocks, and monitors sub-seafloor environments. IODP builds upon the earlier Deep Sea Drilling Project (DSDP) and Ocean Drilling Program (ODP). Although these previous scientific drilling programs recovered cores in water depths up to 8.2 kilometers and achieve substrate penetration up to 2 kilometres, the multiplatform structure of the IODP, launched in 2003, will be able, to investigate below 7,000 mbsf with the support of a new drilling vessel the "Chikyu". Japan, one of the three major partners in this international program, is conducting sea trials for the "Chikyu". Its special capabilities will allow it to probe high-pressure environments such as subduction zones, which are typically the source of major earthquakes. With funding from the National Science Foundation, the U.S.A are in the process of procuring and converting a drill ship to replace the "JOIDES Resolution", the workhorse of the ODP. The IODP's third member, the European Consortium for Ocean Research Drilling, is providing platforms for expeditions with special requirements such as operation in the Arctic pack ice and in extremely shallow waters.

#### 1.3.1.2 Hydrothermal fluids sampling

As the expelled hot fluid (up to 365°C) from the hydrothermal vent undergoes intense dilution and mixing with the ambient seawater, it is difficult to gain access to the potential endogenous microbial communities associated with this hydrothermal fluid. Two strategies were therefore developed in order to reach these microbial communities avoiding seawater contamination. Titanium syringes can sample up to 750 mL of endmember hydrothermal fluid using a ROV or DSV (Figure 1.22). Although the fluids sampling titanium syringes are suitable for the geochemical analysis of the dissolved ionic constituents, they release pressure as the vehicle rises and in so doing, release much of the dissolved gas. Hence gas tight samplers are used to sample volatiles. Titanium syringes have been previously successfully utilized for sampling hydrothermal fluids for microbiological analyses (Straube *et al.*, 1990, for e.g.). As the biomass associated with the hydrothermal fluid is very low, an alternate solution consists in using a colonizer device containing either organic, inorganic, or porous mineral substrates (Figure 1.22). The microbial components are "trapped" in the colonizer device, increasing the amount of

biomass available for microbial analyses. The systems are interesting as they can be equipped with probes that can measure physical parameter such as temperature and pH. Several studies showed promissing results using this technique (Pagé *et al.*, 2008; Takai *et al.*, 2004; Nercessian *et al.*, 2003, for e.g.).

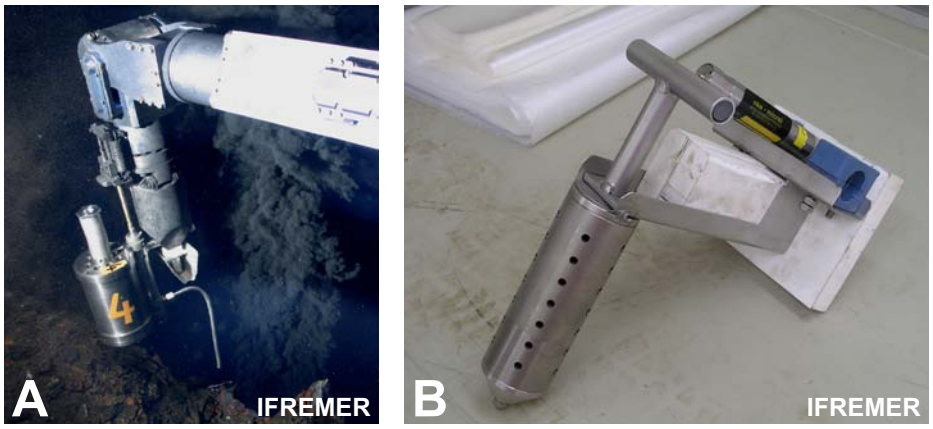


FIG. 1.22: A. Photograph of a titanium syringe (IFREMER). B. Photograph of a colonizer device (IFREMER).

### 1.3.2 Cultural approaches: how to culture the uncultured

Microbiologists have a difficult task when describing the microbial diversity, and have therefore developed several approaches since Antonie van Leeuwenhoek first provided evidence for the existence of prokaryotes three centuries ago. Unlike most biologists, who can initially use morphological traits to distinguish new species, microbiologists need to rely on physiological or phylogenetic traits, although the definition of the microbial species is much debated (Cohan, 2002; Rossello-Mora and Amann, 2001, for review). Hence, in order to have a comprehensive understanding of microbial physiology, or to access metabolic pathways, cultivation of microorganisms is required.

Diversity has traditionally been assessed by enrichment and cultivation techniques developed by Pasteur, Koch, Beijerinck and Winogradsky. Although most of the cells obtained from the environment and visualized by microscopy are viable, recreating accurately the growth requirements of microorganisms by traditionally culturing methods is complicated, frequently resulting in a culture-biased view of diversity. Until now, only 27 of the 52 bacterial phyla and at least 20 of the over 50 archaeal phyla harbour cultivated relatives (Keller and Zengler, 2004; Hugenholtz, 2002; Rappe and Giovannoni, 2003; Schleper *et al.*, 2005)(Figures 1.23 and 1.24). As previously seen in section 1.1.2.4.1, deep sea sediments are even more resistant to cultivation as less than 0.25 % of the deep sediment microbial diversity has been cultured (Table 1.1)(Torsvik *et al.*, 2002; Amann *et al.*, 1995; Rappe and Giovannoni, 2003).

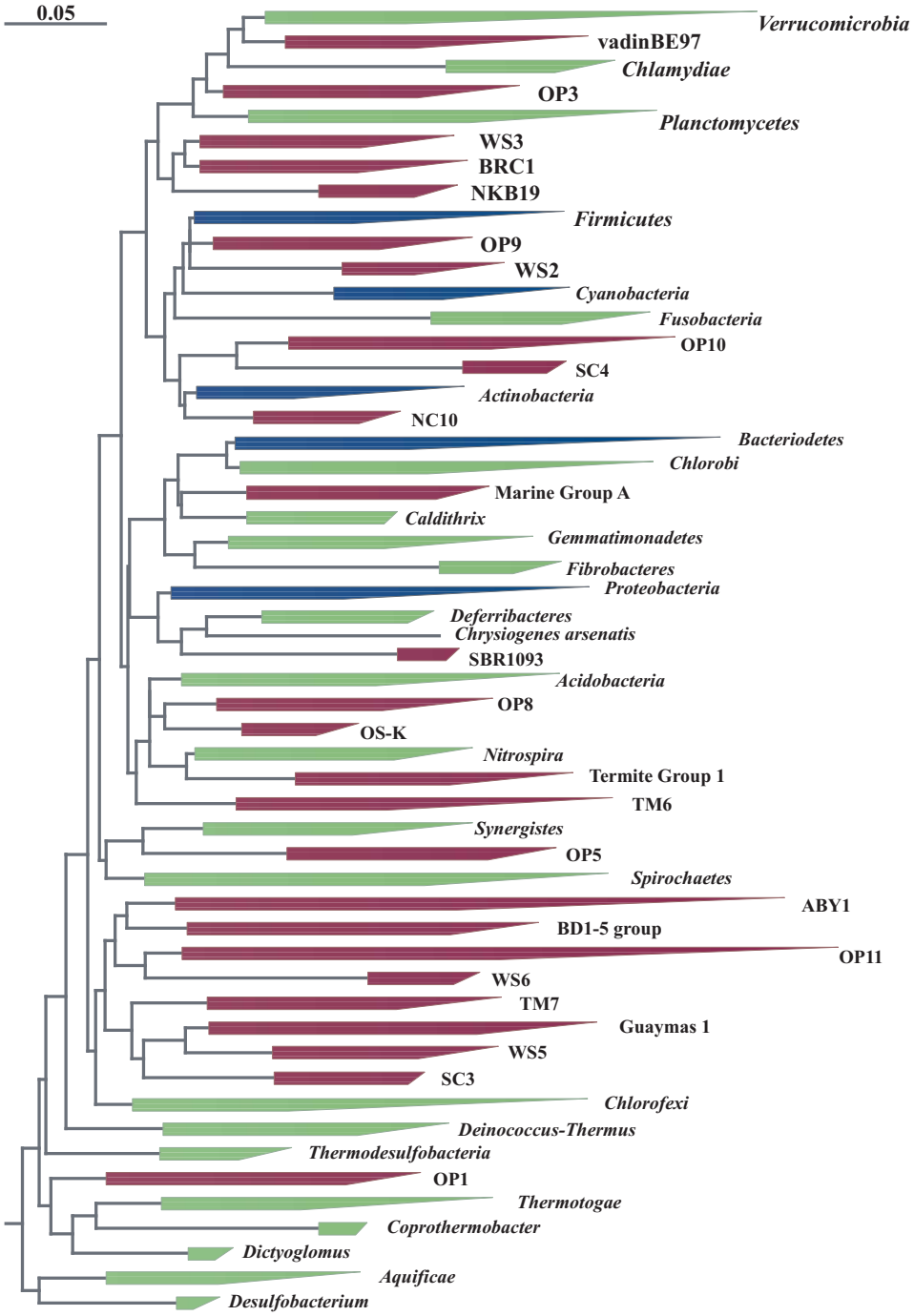


FIG. 1.23: Phylogenetic tree illustrating the major lineages (phyla) of the domain *Bacteria*. Wedges shown in blue are the 12 original phyla, as described by Woese (Woese, 1987), in green are the 14 phyla with cultivated representatives recognized since 1987, and in red are the 26 candidate phyla that contain no known cultivated representatives, from (Rappe and Giovannoni, 2003).


FIG. 1.24: The phylogenetic diversity of the domain of *Archaea*. Triangles in light colours represent branches with exclusively uncultivated species, dark triangles show branches with cultivated species. The size of the triangle is proportional to the number of sequences analysed. The red hexagon in the centre indicates rooting to species in the domain *Bacteria*. The phylogenetic tree is based on comparisons of 16S rRNA gene sequences from the *Euryarcheota*, *Crenarcheota* and *Korarchaeota* phyla together with Nanoarchaeum equitans (dotted lines indicate uncertain phylogenetic positions), clone pOWA133 and the ancient archaeal group (AAG), which are not classified within these phyla; modified from (Schleper *et al.*, 2005).

The "great plate count anomaly" is probably a consequence of one or several combined reasons (Staley and Konopka, 1985). For example, the growth of at least $10^5$ cells in a colony on plate-count medium is required for visualizing colonies by eye alone. Commonly used plate-growth media selects fast-growing microorganisms which grow to high density, are resistant to high concentrations of nutrients and able to grow in isolation. Hence, slow growing or unadapted microorganisms are probably overlooked (Keller and Zengler, 2004), and often designed as non-culturable or viable but not culturable (VBNC) (Gauthier, 2000, for e.g.). Isolation techniques were therefore improved with the use of new technologies, such as the optical tweezers. This micromanipulation technique is based on the use of a Nd-YAG laser (1,064 nm wave length)

which separates cells under a computer controlled microscope. Optical tweezers were used to attempt the isolation of members of the *Nanoarchaeota* phylum (Jahn *et al.*, 2008, for e.g.).
Recreating accurately the physical and chemical properties of the environment by traditional culturing methods can also be a factor leading to the failure to cultivate most microorganisms in pure culture (Torsvik *et al.*, 2002, for e.g.). For example, trying to culture microbes from deep-sea hydrothermal vents is quite challenging, as developing appropriate media and recreating the extreme physical conditions (pH, temperature, pressure) in the laboratory are technically difficult. The cultivation of microorganisms under high pressure has been achieved in only a few cases (Toffin *et al.*, 2005; Bale *et al.*, 1997, for e.g.).
These constraints can also apply to subsurface microorganisms to which we should add their possibly very slow generation times. For instance, some members of the ANME can have a generation time up to 40 days in a continuous-flow bioreactor (Girguis *et al.*, 2005). Hence, Janssen and colleagues isolated new species of *Acidobacteria, Actinobacteria* and *Proteobacteria* by extending the incubation times and reducing the amount of nutrients delivered in the media (Janssen *et al.*, 2002). As seen previously, accessing an adequate source of carbon could be a limiting factor for subsurface microorganisms. Therefore by reducing the amount or/and the nature of the carbon source, it is possible to enrich different subsurface species (Toffin *et al.*, 2004).
The possible interactions between different microorganisms such as symbiosis, mutualism, or parasitism, could also limit the growth of these microorganisms in artificial conditions. For example members of the ANME group and the *Nanoarchaea* phylum have still not been isolated although they are cultured, probably due to their metabolic interaction with an other microorganism (Huber *et al.*, 2002*a*; Nauhaus *et al.*, 2007).

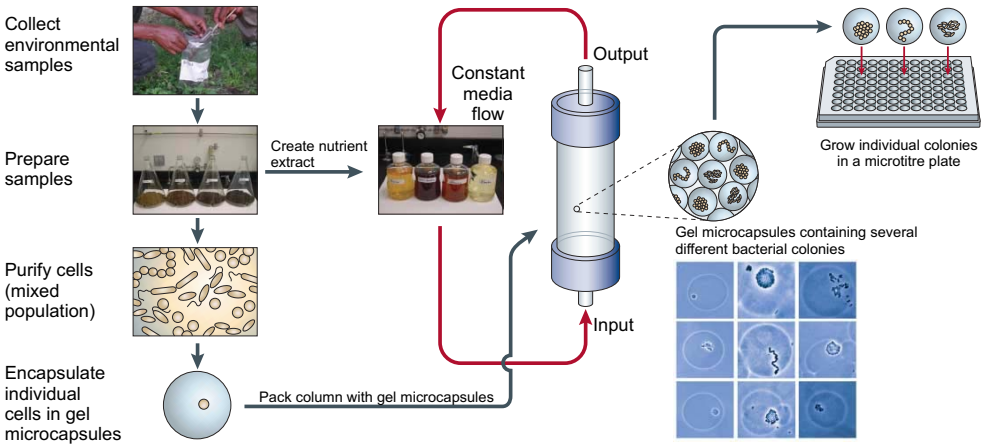


FIG. 1.25: Flow chart of the high-throughput Zengler cultivation process. The process is based on encapsulation of single cells in microcapsules for parallel microbial cultivation under low nutrient flux, from (Keller and Zengler, 2004).

However, although many different cultivation methods and media have been developed to try to mimic the environmental conditions, such as using low substrate concentrations, supplying sterile sediment extracts, adding particles, maintaining low temperatures and monitoring cryptic growth using sensitive radiotracer techniques, their environmental relevance remains undetermined. Therefore, new promising cultivation methods have been developed to increase the number of culturable prokaryotic species (Ingham *et al.*, 2007; Zengler *et al.*, 2002; Martens-Habbena, 2005; Bollmann *et al.*, 2007; Godfroy *et al.*, 2006, for e.g.). For example, Zengler and colleagues have recently designed a high-throughput cultivation technique (Zengler *et al.*, 2002). This technique combines encapsulation of cells in gel microdroplets for massively parallel microbial cultivation under low nutrient flux conditions, followed by flow cytometry to detect microdroplets containing microcolonies.
However, although the cultural techniques are quickly evolving, most of the phylotypes detected with independent culture approaches remain uncultured. Molecular approaches based on nucleic acid or phospholipid fatty acid analysis are the revealing tools for exploring the deep subsurface sediment microbial diversity.

### 1.3.3 Molecular approaches and their limits

As seen in the previous section, the insight into the subsurface microbial diversity can be quite limited when using cultural dependant techniques. Hence, over the past two decades, several cultural independent methods were developed, mainly based on the use of molecular markers that are excellent tools for determining evolutionary relationships between microorganisms (Zuckerland and Pauling, 1965). The molecular identification and characterization of microorganisms can therefore be assessed by using several different molecular methods as described in the figure 1.26. This section will give an overview of molecular techniques used to describe microbial diversity and metabolic activity.

#### 1.3.3.1 Molecular phylogenetics

Phylogenetics is the science of estimating the evolutionary past of organisms; in the case of molecular phylogeny the comparison is based on DNA or protein sequences. Although the represention of these hypotheses as trees dates back to Darwin, the numerical calculation of trees using quantitative methods is relatively recent, and their application to molecular data even more. We will therefore describe the molecular bases of phylogenetic analysis before the tools that can be used to investigate the Tree of life.

**1.3.3.1.1 Ribosomal RNA: a universal marker?** Although many earlier studies proposed nucleic acid or protein sequences as phylogenetic markers, molecular phylogenetics really

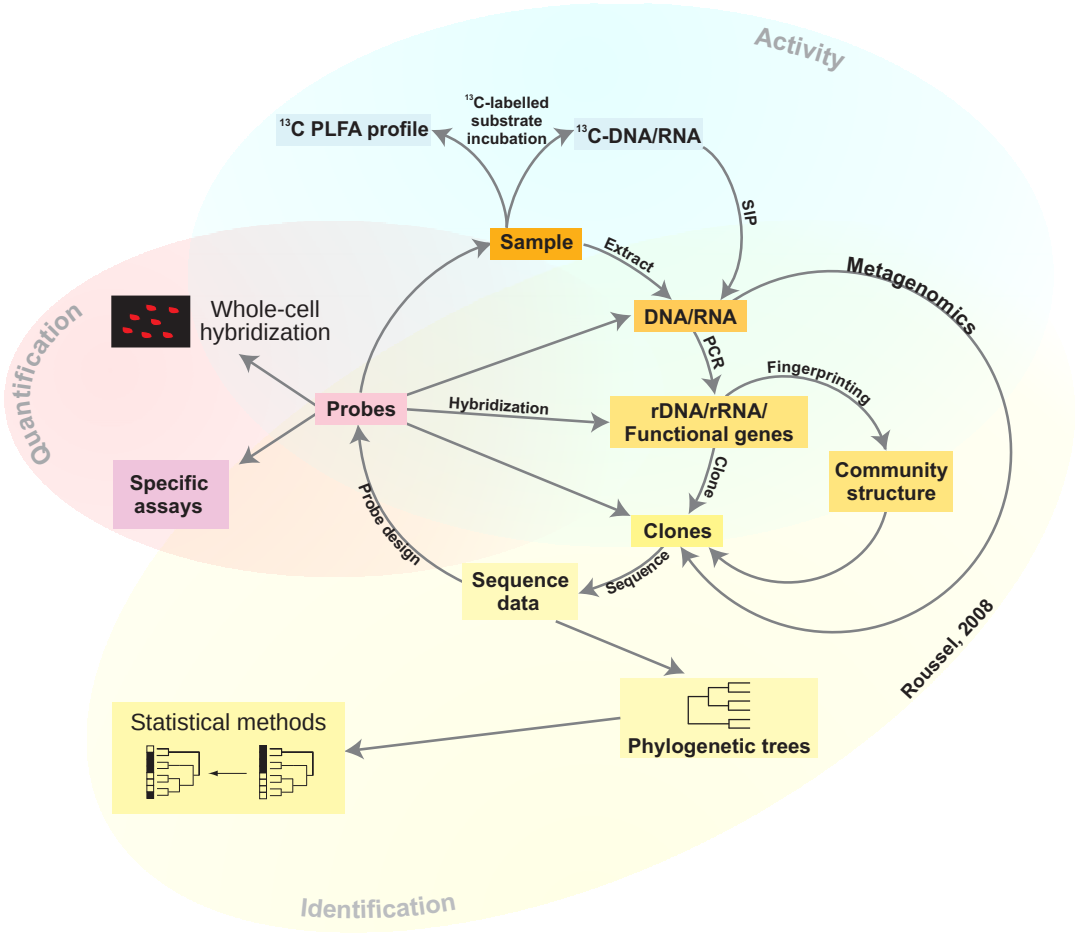


FIG. 1.26: Culture-independent methods detect and identify microorganisms and their metabolic activity on the basis of their nucleic acids and lipids; modified from (Walker and Pace, 2007).

began only with the publication in 1977 of the tree of life by Woese and Fox (Woese and Fox, 1977). This pioneering work on comparative analysis of small-subunit ribosomal RNAs (16S and 18S rRNAs) provided an objective framework for determining evolutionary relationships between organisms and thereby "quantifying" diversity as sequence divergence on a phylogenetic tree. This major breakthrough was made possible by the advances in DNA sequencing that occurred at the time (Sanger *et al.*, 1977).

Ribosomes are macromolecular multifunctional complexes composed of both RNA and proteins that synthesize proteins from amino acids with remarkable speed and accuracy, their function being fundamentally confined to ribosomal RNA (rRNA) (Green and Noller, 1997). rRNA of prokaryotes is composed of 3 components that are 23, 16, and 5S in size. The Ribosomal 16S/18S RNA gene was chosen, as it meets all the requirements to be a molecular marker for prokaryotes and eukaryotes:

- rRNA is found in all the living eukaryotes and prokaryotes.
- its function was conserved through the past 3.8 billions of years of evolutionary divergence.
- the primary sequence has conserved and variable regions correlated to the taxonomic levels (Figures 1.27 and 1.28)
- the 1,540 base pair of the sequence gives enough information to build significant phylogenetic assumptions.

However, biased interpretation can be made from 16S rRNA gene analysis. For instance, thermophilic prokaryotes can be artificially related due to the high G+C content of their rRNA (Weisburg *et al.*, 1989). Moreover, a low interspecies polymorphism can occur in some taxonomic groups, as a consequence of a high level of conservation (Schloss and Handelsman, 2004). The *Pseudomonas* genus has a very well-conserved 16S rRNA gene (∼99 %), making this marker difficult to use to assign a phylogentical position (Guasp *et al.*, 2000, for e.g.). Moreover, biases can be created by the existence of multiple heterogeneous copies of the 16S rRNA gene within a genome (Crosby and Criddle, 2003; Dahllof *et al.*, 2000). Hence, it is important to choose the most relevant molecular marker for the specific microorganisms being studied.

**1.3.3.1.2 Other phylogenetic markers.** Since the systematic use of 16S rRNA gene as the "gold standard" for microbial phylogeny, several other molecules were tested and used according to their properties. The genetic relatedness between different bacterial taxons was investigated by multilocus sequence typing based on simultaneous analysis of several housekeeping genes (Holmes *et al.*, 2004; Rubin *et al.*, 2005), such as the *rpoB, gyrB*, elongation factor Tu (EF-Tu), phosphoglycerate kinase (*pgk*), and heat shock protein (*dnaK*) genes, as well as the 16S–23S rRNA intergenic transcribed spacer region (Kamla *et al.*, 1996; Kim *et al.*, 2003; Wolf *et al.*, 2004; Drancourt and Raoult, 2005; Lepage *et al.*, 2004).

**1.3.3.1.3 Phylogenetic analysis and the tree of life.** According to the previous section, phylogenetic representation can be built from the comparison of rRNA gene sequences (Baldauf, 2003, for basic review). The heart of the matter is the multiple sequence alignment. Until 1989 these were all assembled by hand because the exhaustive alignment of more than six or eight sequences was computationally unfeasible. Nowadays, most multiple sequence alignments are constructed by progressive sequence alignment using computer programs such as MAFFT (Multiple Alignment using Fast Fourier Transform), MUSCLE (MUltiple Sequence Comparison by Log-Expectation), T-Coffee, Kalign and many others, the most accurate program usually depending on the 16S rRNA gene conservation level of the sequences analysed.

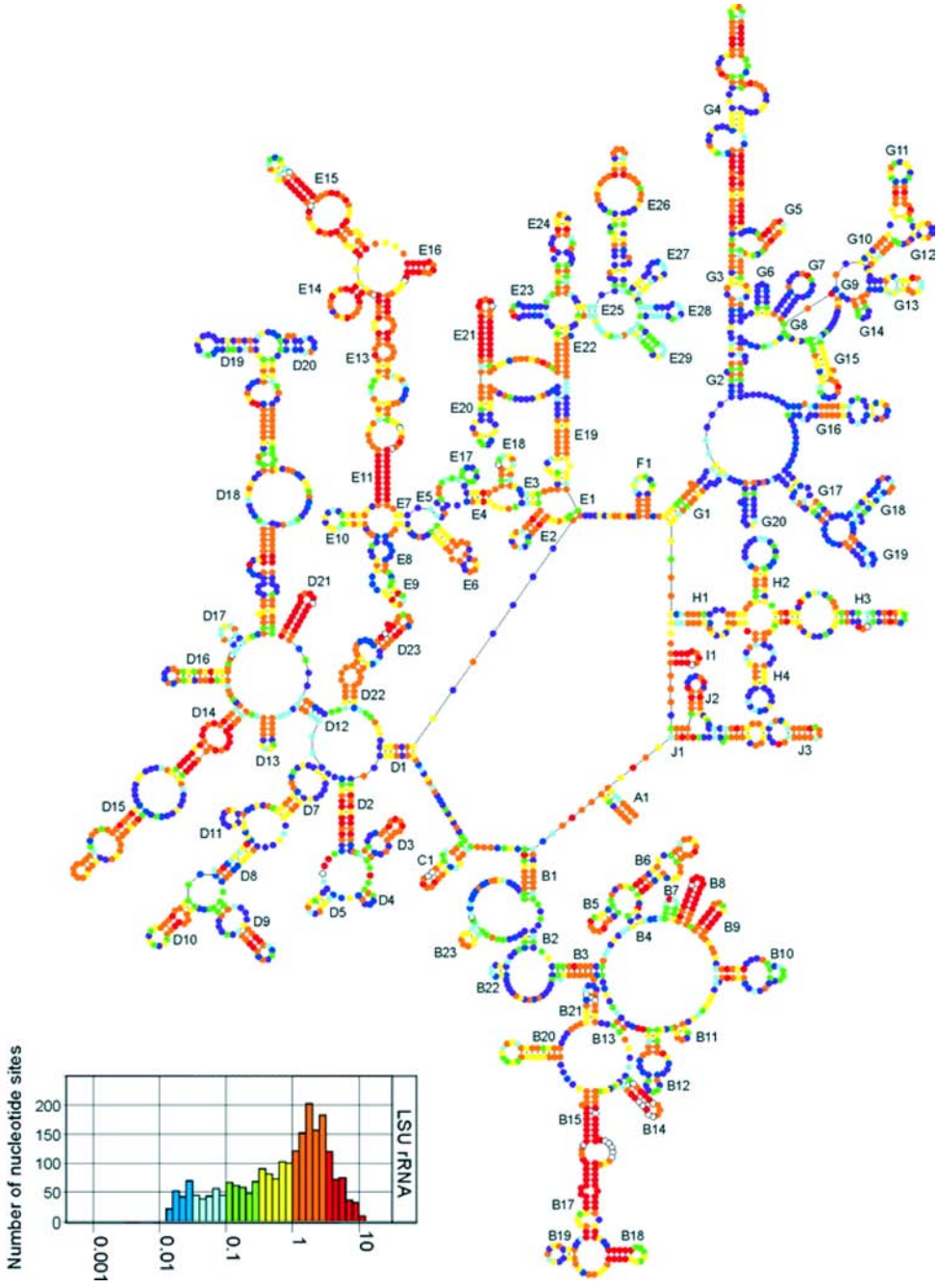


FIG. 1.27: Variability maps of bacterial LSU rRNA superposed on the secondary structure models of the *T.thermophilus* molecules. Sites are subdivided into seven groups according to their relative substitution rate, coloured purple (lowest rate) to red (highest rate). The rate was not measured for sites occupied in $< 25$ % of the sequence alignment, which are shown as hollow dots. The histogram inset is the substitution rate spectra for the molecule (Wuyts *et al.*, 2001).

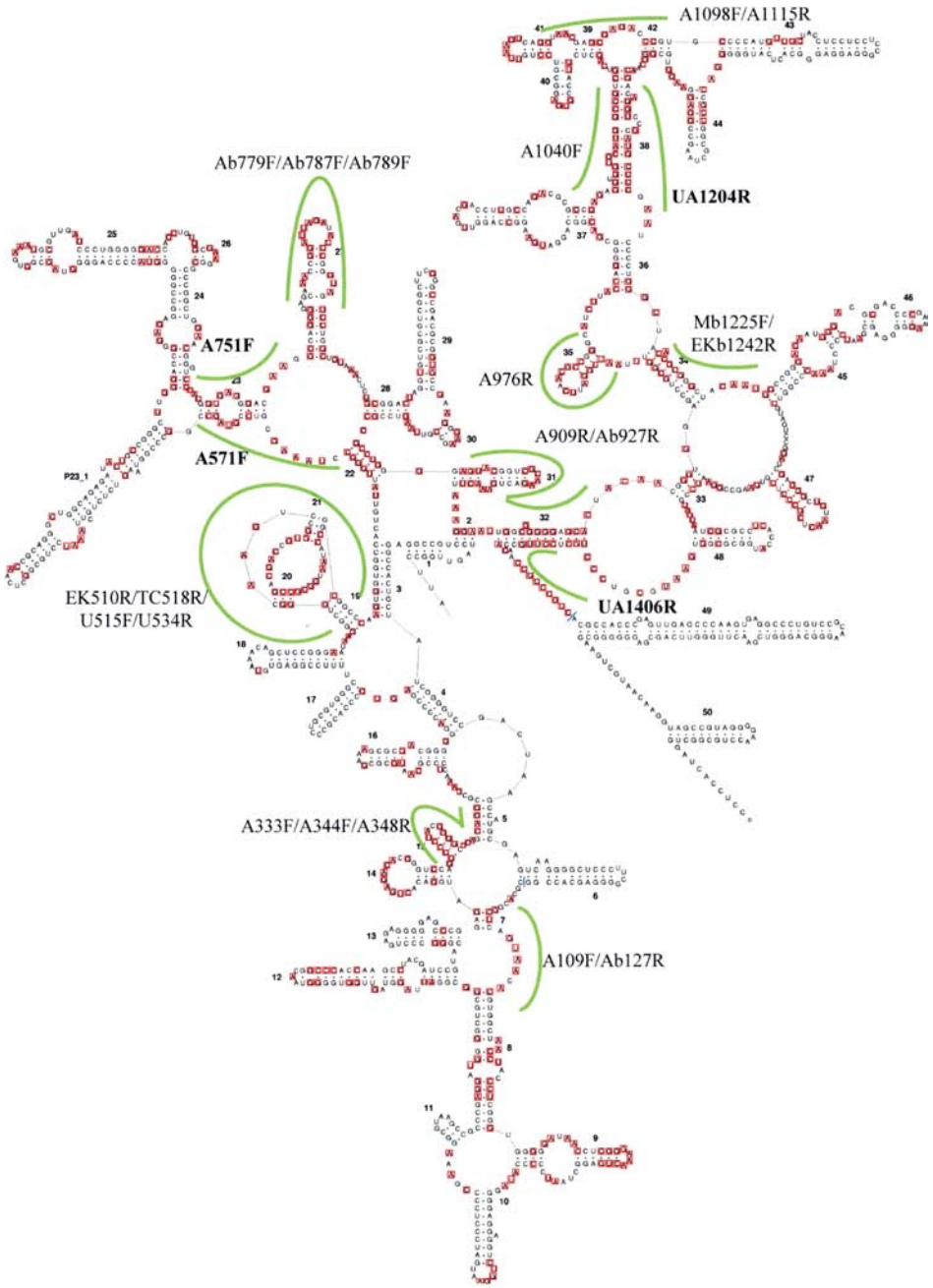


FIG. 1.28: Secondary structure map of *M. jannaschii* annotated with *Archaea*-specific priming sites (green). Nucleotides (105–1406; *E. coli* numbering) conserved between representatives of the *Euryarchaeota, Crenarchaeota, Korarchaeota* and *Nanoarchaeota* are highlighted in red, from (Baker and Cowan, 2004).

Although, ClustalW2 gives usually the best results, the alignment should systematically be adjusted "by eye" to minimize insertion/deletion events using a program such as SeaView, BioEdit, ARB, GCG package or MEGA4 (Larkin *et al.*, 2007; Galtier *et al.*, 1996; Tamura *et al.*, 2007; Hall, 1999; Ludwig *et al.*, 2004). The only way to root a tree is with an "outgroup", an external point of reference that is not a natural member of the group of interest (i.e. the "ingroup"). The choice of the outgoup is essential, as the phylogenetic distance between the outgroup and the ingroup can modify the scale and topology of the phylogenetic tree.
The second step is constructing a phylogenetic tree from the multiple sequence alignment. It is necessary to decide whether the molecular tree should be built from protein-coding genes or from DNA sequences that will provide more (or even to much) variability du to codon bias (Gustafsson *et al.*, 2004, for review). Another important consideration is to select the positions of the alignment that will be chosen for the tree computing in order to exclude the most ambiguous. This operation can eventually be managed by programs such as Gblock (Castresana, 2000). The method for calculating phylogenetic trees can either be classified as distance-matrix methods such as UPGMA, neighbor-joining, Fitch-Magoliash, or as discrete data methods such as parsimony, maximum likelihood and Bayesian methods. The distance matrix methods are quite simple, whereas discrete data methods are more elaborate as they infer a hypothesis for each column in the alignment. Hence the tree topology is usually inferred by comparing the results of both classes of methods. Numerous programs can perform these calculations such as ARB, PhyloWin, PAUP, PHYLIP and many others (Galtier *et al.*, 1996; Ludwig *et al.*, 2004; Swofford, 2003; Felsenstein, 2005). The significance of each branch can be inferred by several methods such the bootstrap value or jacknife.
By using simpler methods still based on the same idea, Woese and colleagues found that cellular life can be divided into three primary lineages (domains), one eukaryotic (*Eucarya*, also called *Eukaryota*) and two prokaryotic (*Bacteria* and *Archaea*), and he also defined 11 major lineages (phyla or divisions) within the bacterial domain on the basis of 16S rRNA gene sequences obtained from cultivated organisms. This new "Tree of Life" based on non phenotypic characteristics was a major breakthrough. The leading reference source in prokaryotic taxonomy, Bergey's Manual of Systematic Bacteriology, has adopted a rRNA framework to classify prokaryotes, replacing the previous ad hoc scheme that was based on traditional phenotypic characterization.

#### 1.3.3.2 Identification of the prokaryotic communities

Over the past three decades of molecular-phylogenetic studies, researchers have compiled an increasingly relatively robust map of evolutionary diversification over the universal Tree of life. In the mid-eighties, Norman Pace gave a strong impulsion to the application of molecular-phylogenetic methods to study natural microbial ecosystems without the traditional cultivation, it resulted in a broad description of the diversity of microbial ecosystems and the discovery of

many unexpected evolutionary lineages (Pace *et al.*, 1985). On the whole the method usually follows the same process (Yellow part of figure 1.26): the nucleic acids (DNA or RNA) extracted from a sample are sequenced after PCR-fingerprinting analysis, PCR-cloning or metagenomic techniques. However, it is important to keep in mind that these techniques are to some degree inherently biased too.

**1.3.3.2.1 Nucleic acid extraction, Polymerase chain reaction and their biases.** The validity of using molecular techniques in environmental studies depends on obtaining representative extracts of nucleic acids from entire microbial communities. Successful direct DNA extractions from subsurface environmental samples have to overcome several pitfalls linked to the usually low biomass, high concentration of PCR inhibitors, contamination, absorption of the extracted nucleic acids by the sample matrix and microorganisms refractory to cell lysis (Miller *et al.*, 1999; von Wintzingerode *et al.*, 1997; Webster *et al.*, 2003; Barton *et al.*, 2006; Roose-Amsaleg *et al.*, 2001, for e.g.). According to the samples, the solution to these problems has to combine the most adapted cell lysis and purification methods, always insuring the highest extracted nucleic acid yield and quality. If the nucleic acid yield is not sufficient, it is always possible to pool and concentrate multiple DNA/RNA extractions (Webster *et al.*, 2003, for e.g.). If the quality or the purity of the DNA/RNA is not sufficient, it is also possible to performe futher purification in order to remove PCR inhibitors (such as humic acids) (Juniper *et al.*, 2001, for e.g.).
The next step is usually to amplify a specific DNA fragment such as 16S rRNA gene or a functional gene utilizing Polymerase Chain Reaction (PCR). One of the major limitations of PCR is that only phylotypes containing nearly matching priming sites may be detected. The application of primers matching only part of the archaeal population will therefore directly affect results by under-representing or excluding groups of microorganisms (5-71 %) that have frequent mismatches (Teske and Sorensen, 2008, for e.g.). The yield and quality of the PCR product are strongly dependent on the number of PCR cycles, the hybridization and denaturation temperatures, and the ratio between reactant and the DNA template. A nested-PCR can be performed using internal primers if the yield or specificity of the PCR product is still unsufficient after a first PCR.
Although, the nucleic acid extraction coupled with PCR is a powerful tool to access to the microbial uncultured diversity of low biomass subsurface environments, the biases associated with these techniques are numerous and are fully detailed in the following references: (von Wintzingerode *et al.*, 1997; Polz and Cavanaugh, 1998; Suzuki and Giovannoni, 1996; Wilson, 1997). A more detailed description of these techniques will be given in the first study of the chapter II.

**1.3.3.2.2 Genotypic Microbial Community Profiling.** Several genotypic methods can be used to analyse the diversity of microbial communities associated with a subsurface sample. Most of the techniques are based on the segregation of PCR-amplified sequences of a specific molecular marker such as 16S rRNA. However, molecular genotypic methods can be time consuming, expensive and sometimes labour-intensive, and therefore we summarized their advantages and disadvantages in table 1.5. We will briefly describe the most appropriate techniques and their suitability according to the targeted objective.

**DGGE and TGGE**

Although previously used for the study of polymorphism and gene mutation (Fischer and Lerman, 1979; Myers *et al.*, 1987), Denaturing Gradient Gel Electrophoresis (DGGE) was first applied to the analysis of whole bacterial communities in 1993 by Muyzer (Muyzer *et al.*, 1993). Denaturing gel electrophoresis allows the separation of small PCR-products, usually up to 400 bp, such as partial 16S rRNA gene. The separation of the DNA fragments is achieved on a denaturant acrylamide gel as a function of their different melting behaviour, primarily determined by the GC content and distribution of the DNA fragment (Myers *et al.*, 1985). The direction of electrophoresis is perpendicular to the denaturing gradient. Such a gradient is obtained using either denaturing chemicals (urea and formamide) for denaturing gradient gel electrophoresis (DGGE), or heat for Thermal Gradient Gel Electrophoresis (TGGE) and Temporal Thermal Gel Electrophoresis (TTGE). As they progress through the gel and are subjected to increasingly strong denaturing conditions, PCR products reach a point where strand separation of double-stranded DNA occurs. The higher the intrinsic melting point, the stronger the denaturant condition must be to achieve strand separation. The physical shape of the molecules also directly affects their mobility during electrophoresis.

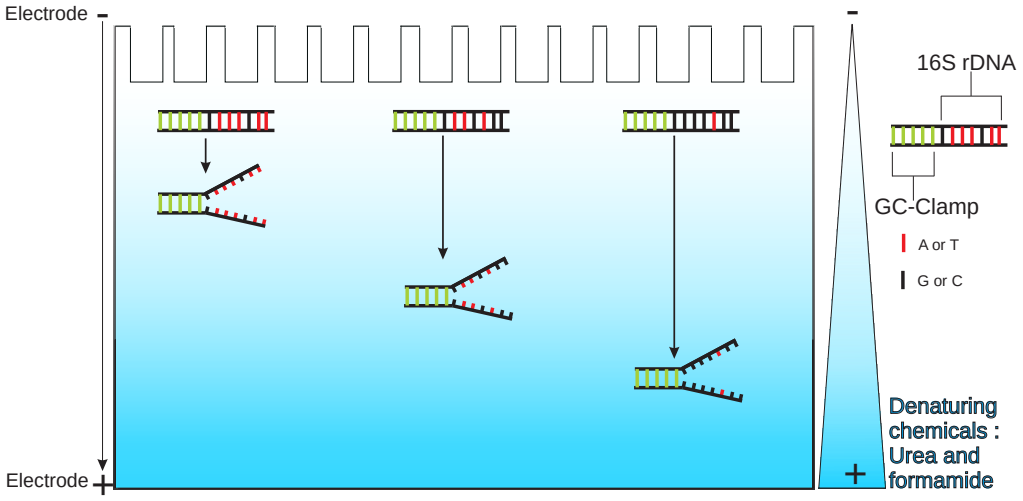


FIG. 1.29: Schematic representation of the migration of a 16S rDNA fragment using DGGE.

A complete strand separation of the fragment is avoided by the GC-rich clamp. This clamp,

| Genotypic methods | Advantages | Disadvantages | Suitability |
|---|---|---|---|
| **Cloning and sequencing** | • High phylogenetic resolution<br>• Allows species identification/determination of closest phylogenetic neighbor | • Although the sequencing can be automated, the cloning is time-consuming | • When phylogenetic assignment is relevant and high-throughput sequencing service is available |
| **ARDRA** | • No special equipment needed | • Needs several restrictions for adequate genotypic resolution<br>• Time- and labor-intensive | • For low resolution comparisons of simple communities or as a screening tool for identification of clones of interest for sequencing |
| **ARISA** | • Allows high resolution of subtle differences<br>• Compatible with RFLP and sequencing for further downstream analysis | • A single organism can contribute more than one signal because of interoperonic length variation | • For high resolution of subtle species differences |
| **T-RFLP** | • High sensitivity<br>• High throughput and short run times<br>• Potentially direct phylogenetic assignment of signals<br>• Allows good between-runs comparability | • Incomplete restriction digestion can result in overestimation of diversity<br>• Multiple restrictions are needed for precise analysis<br>• Complicated profiles make phylogenetic assignments very challenging<br>• Restriction digestion can result in pseudo-T-RFs | • The high sensitivity allows application to communities with higher species richness<br>• The good comparability between runs makes it suitable for study of time courses and for large sample numbers |
| **DGGE/TGGE** | • Bands of interest can be excised from gel for sequencing<br>• Affordability | • Limited sensitivity<br>• Primer GC clamp decreases yield and favors primer dimers<br>• Handling of gels needs experience<br>• Difficult comparability between gels because of gel variability | • For communities with a limited number of abundant members |
| **SSCP** | • No clamped primers or restriction digestion required<br>• Bands of interest can be excised from gel for sequencing<br>• Compatible with automated high-throughput analysis | • High rate of reannealing of single strands with high DNA concentrations<br>• Multiple bands per species possible | • When high sensitivity is desired without the need of restriction digestion or GC clamp |
| **DHPLC** | • High throughput and short run times<br>• High sensitivity when using fluorescenrT labels<br>• No sample manipulation necessary | • Separation parameters have to be optimized for different samples | • Promising for automated and fast analysis after initial optimization but more validation for its application in microbial ecology is need |

Table 1.5: Summunrized advantages and disadvantages of different genotypic techniques; modified from (Nocker *et al.*, 2007).

usually composed of 40 G and C nucleotides, has a very high melting point and holds the strands together and this modifies the secondary structure of the fragment strongly retarding the migration in the gel in comparison with unmelted molecules. This GC-clamp is added at the 5'-end by PCR amplification of the products (Figure 1.29). Although the handling of DGGE and TGGE techniques requires experience, they can analyse a great number of samples at once, and is therefore one of the fastest and cheapest methods to evaluate the microbial diversity. Moreover, the 16S rDNA fragments can be cut out of the gels and be sequenced in order to partially identify the associated microorganisms. Hence, the DGGE technique was applied using 16S rDNA fragments to the analysis of prokaryotic communities in several studies of subsurface and hydrothermal environments (Pagé *et al.*, 2008; Parkes *et al.*, 2005; Webster *et al.*, 2003).

**Cloning and Sequencing**

Cloning of direct PCR-amplified sequences is the first step for subsequent species identification by sequencing. Although, most studies of microbial diversity subsurface environments use PCR product for cloning, direct cloning of total DNA attracts increasing interest. This technique known as metagenomic approach will be discussed in the following section.

The analysis of microbial communities by cloning of direct PCR-amplified products is the method offering the highest phylogenetic resolution, as fragments as long as the entire 16S rRNA can be analysed, providing a more accurate identification or determination of similarity to already known species through the use of extensive and rapidly growing sequence databases. Hence, cloning and sequencing is the basis for construction of phylogenetic trees and for other comparative studies. In order to increase ligation efficiency, some cloning methods use the overhanging 3'-A added by the Taq polymerase at the end of the PCR product (Marchuk *et al.*, 1991). Although, the phylogenetic diversity which can be described by this method is limited to the number of clones sequenced, the phylogenetic resolution level can be chosen convesevely to the fingerprinting techniques. As the sequencing costs decrease and as a high degree of automation is possible with 96- and 384-well plate technology, it is now possible to reduce the screening task and to increase the number of sequenced clones, therefore increasing the ability to detect rare organisms (Nocker *et al.*, 2007; Tiedje *et al.*, 1999). To date the majority of the molecular studies of subsurface samples used cloning and sequencing approaches (Inagaki *et al.*, 2006*b*; Kormas *et al.*, 2003; Sorensen *et al.*, 2004; Takai *et al.*, 2004, for e.g.).

**T-RFLP**

Although, Terminal Restriction Fragment Length Polymorphism (T-RFLP) was developed at the end of the nineties (Avaniss Aghajani *et al.*, 1996), its use for the study of subsurface samples increased ever since (Takai *et al.*, 2001*b*; Inagaki *et al.*, 2002; Moser *et al.*, 2003; Parkes *et al.*, 2007, for e.g.).

PCR product are labelled with a fluorescent dye attached to the 5'-end before being digested by a restriction enzyme. Hence, the polymorphism is based solely on one parameter, the fragment length, as the restricted PCR products are separated using acrylamide sequencing

gels or sequencing capillaries. This accurate technique can be calibrated using a size standard labelled with a different fluorophore allowing the precise assignment of fragment lengths with single base pair resolution. The whole approach can easily be automated using increasingly powerful sequencing technologies. Moreover, as the T-RFLP polymorphism is based solely on fragment length it allows direct reference to sequence databases such as the rapidly expanding RDP (http://rdp.cme.msu.edu/) for putative identification (Cole *et al.*, 2007).

**DHPLC**

Although, Denaturing High-Performance Liquid Chromatography (DHPLC) was originally developed for gene mapping, mutation detection, and identification of clinical isolates (Wagner *et al.*, 1999, for e.g.), it was recently successfully adapted for analysis of environmental communities (Barlaan *et al.*, 2005, for e.g.). A heterogeneous mixture of 16S PCR products is separated using high-performance liquid chromatography (HPLC) technology and is based on temperature and chemical denaturation in order to achieve separation of PCR products of similar size. DNA is injected into an oven-based HPLC cartridge in a solution containing triethylammonium acetate (TEAA) and acetonitrile. The TEAA dissociates in a solution forming the positively charged TEA+ that has both a hydrophobic and a hydrophilic end. The hydrophobic end binds to the hydrophobic beads in the cartridge whereas the positive charge forms ionic bonds to the negatively charged phosphate backbone of the double-stranded DNA. The TEA+ molecules thus serve as a bridge to bind DNA to the cartridge material. The strength of DNA binding depends on the fragment length and the content and position of G and C nucleotides. Differential elution of the bound DNA mixture is achieved by an increase in temperature and an increasing gradient of acetonitrile which weaken the bridging capabilities of the TEA+ ions. This promising method requires only a few minutes to establish the microbial genotypic profile and can be coupled to an automatic sequencer for identification. This method possibly offers an alternative to the pyrosequencing systems in the genotypic high-throughput technologies.

#### 1.3.3.2.3 Metagenomics: identification and metabolic characterisation all in one?

Metagenomics was a term first used by Handelsman in 1998 to define a habitat-based investigation of mixed microbial populations at the DNA level (Handelsman *et al.*, 1998). An alternative to the sequencing of specifically amplified conserved genes such as 16S rRNA consists in the sequencing of randomly cloned community DNA, such as the "whole-genome shotgun cloning" or the "pyrosequencing" approaches (Riesenfeld *et al.*, 2004; Steele and Streit, 2005, for review). Hence the metagenomic approaches were only made possible consequently to the major technological advances made by sequencing (speed and cost).

The whole-genome shotgun cloning approach is based on the sequencing of relatively small pieces of cloned genomic DNA produced by physical shearing and size fractionation. DNA is extracted directly from the environment and cloned into cosmid, fosmid or BAC vectors producing large insert libraries. Theoretically, a metagenomic library contains clones representing

the entire genetic complement of a single habitat, although this is dependent on the efficiency of DNA extraction and cloning methods. The information held within a metagenomic library can be used to determine community diversity and activity, the presence of specific microorganisms or biosynthetic pathways as well as simply to search for the presence of individual genes This approach was successfully used to elucidate the metagenome of a natural acidophilic biofilm (Tyson *et al.*, 2004). However, this approach is difficult to perform on subsurface sample, as they are usually characterised by low biomass and high concentration of inhibitors, and would therefore require a high concentration or amplification of the whole DNA extracted.

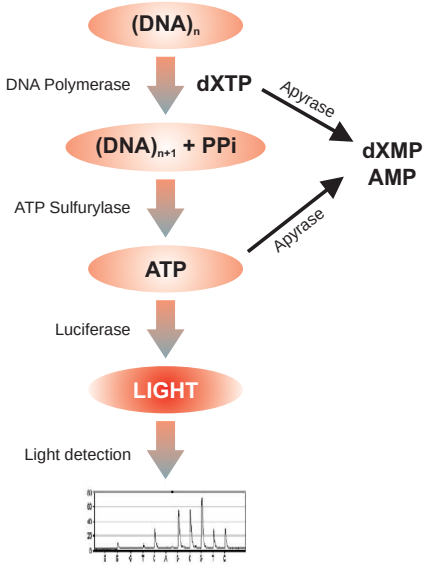


FIG. 1.30: The general principle of Pyrosequencing reaction system. A polymerase catalyzes incorporation of nucleotide(s) into a nucleic acid chain. As a result of the incorporation, a PPi molecule(s) is released and subsequently converted to ATP, by ATP sulfurylase. Light is produced in the luciferase reaction during which a luciferin molecule is oxidized. A photon detector device then detects the light and raw data will be available for further analysis. dXTP and ATP will be degraded by apyrase allowing iterative addition of nucleotides; modified from (Ronaghi and Elahi, 2002).

A new alternative to whole-genome shotgun cloning is the even more recently adapted technique to microbial ecology called "pyrosequencing" (Handelsman *et al.*, 1998, for review)(figure 1.30). Pyrosequencing is a real-time DNA sequencing technique generating short reads rapidly and inexpensively (Ronaghi *et al.*, 1996; Ronaghi *et al.*, 1998). This technology has the potential advantage of accuracy, ease-of-use, high flexibility and is now emerging as a popular platform for microbial typing. Pyrosequencing uses coupled enzymatic reactions to detect inorganic pyrophosphate (PPi) released as a result of nucleotide incorporation by DNA polymerase. The released PPi is converted to ATP by ATP sulfurylase, which provides the energy for luciferase to oxidize luciferin and generate light. Unincorporated nucleotides are degraded by apyrase

prior to addition of the next nucleotide allowing iterative addition of nucleotides. Since the added nucleotide is known, the sequence of the template can be determined. The method was adapted to environmental samples by Sogin and colleagues who PCR-amplified the V6 hypervariable region of ribosomal RNA (Sogin *et al.*, 2006). Although ribosomal sequence tags do not permit an accurate phylogenic placement (Neufeld *et al.*, 2008, for review), their short sequences allow extensive sampling of the microbial community (Roesch *et al.*, 2007, for e.g.). An alternate procedure is to perform whole-genome amplification using a polymerase achieving rolling circle amplification, such as the Φ29 (Lipp *et al.*, 2008; Kwon and Cox, 2004; Dean *et al.*, 2001; Raghunathan *et al.*, 2005). Several interesting studies successfully using these approaches described the subsurface environmental prokaryotic ecosystems (Biddle *et al.*, 2008; Huber *et al.*, 2007). The high-throughput sequencing technologies give access to the "rare biosphere" that will undoubtedly serve as a target for decades of research into the adaptations and advantages of sustaining a low-abundance lifestyle.

#### 1.3.3.3 Quantitative approaches

**1.3.3.3.1 Microscopic techniques.** Since the introduction of Nuclepore filters and staining by DAPI (4',6-diamidino-2-phenylindole) more than 30 years ago (Porter and Feig, 1980; Hobbie *et al.*, 1977; Zimmermann and Meyer-Reil, 1974), epifluorescence microscopy total counts has become a standard procedure to enumerate prokaryotes in subsurface sediments (Parkes *et al.*, 1994; Parkes *et al.*, 2000, for e.g.). The method has ever since been improved in order to increase specificity and accuracy. As DAPI-stained cells fade rapidly it was successfully replaced by Acridine orange, an other nucleic acid binding stain, for enumeration of estuarine particle-associated *Bacteria* (Crump *et al.*, 1998). A standard protocol using Acridine orange for total cell count in deep subsurface sediments was published by Cragg and colleagues in 2000 (Cragg *et al.*, 2000). However, Acridine orange was shown to display high background fluorescence (Figure 1.31) (Martens-Habbena, 2005) and owing to its superior specificity and fluorescence, SYBR Green I is now rapidly replacing these stains (Lunau *et al.*, 2005; Kallmeyer *et al.*, 2008; Danovaro *et al.*, 2001, for e.g.).

Another problem concerns samples with high amounts of particle- and surface-associated *Bacteria*, because these *Bacteria* are unevenly distributed and a certain fraction may be obscured by opaque particles and escape enumeration. Separation of cells from the sediment matrix can help overcome these difficulties (Frischer *et al.*, 2000; Velji and Albright, 1986; Lunau *et al.*, 2005, for e.g.). A new procedure developed by Kallmeyer and colleagues allows an effective and unspecific detachment (over 65 % recovery) of cells from deep subsurface sediments, has a much lower minimum detection limit, and could also be adapted to molecular techniques , such as nucleic acid extractions (Kallmeyer *et al.*, 2008).

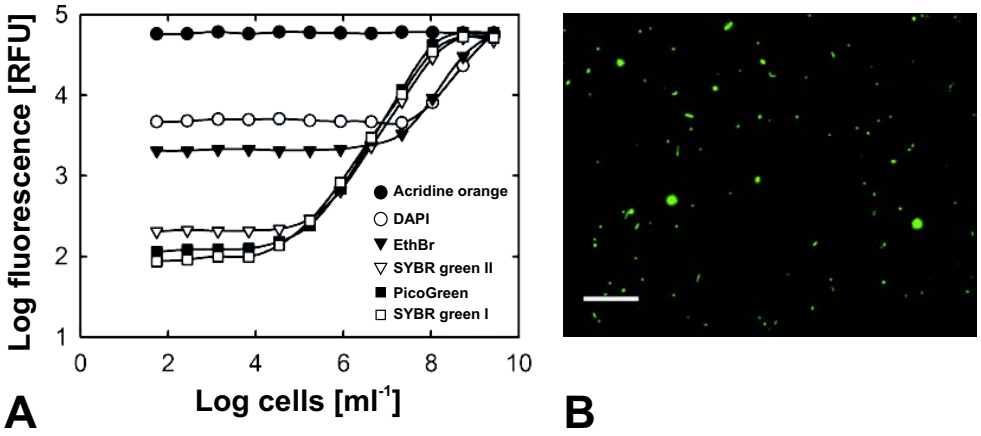


FIG. 1.31: A. Comparison of six nucleic acid dyes for the detection of bacterial cells in a microplate assay. Each data point represents the mean of four (PicoGreen) or eight replicates. Standard deviations were omitted for clarity, from (Martens-Habbena, 2005). B. Photograph of total cell count using SYBR green I and the new cell extraction procedure developed by Kallmeyer and colleagues (Kallmeyer *et al.*, 2008).

**1.3.3.3.2 Fluorescent *in situ* hybridisation and alternatives.** *In situ* hybridization is a commonly used method for the detection and quantification of phylogenetically defined microbial communities in marine environmental samples (Amann and Fuchs, 2008; Wagner *et al.*, 2003, for review). Although initially based in the late eighties on the use of rRNA radio-labelled probes (Giovannoni *et al.*, 1988), DeLong and colleagues developed the use of fluorescent labels as they are more specific and allow the use of multiple different probes on the same sample (Delong *et al.*, 1989). This method was designed as fluorescence *in situ* hybridization (FISH) and gradually optimised for the analysis of subsurface prokaryotic communities.
Fluorescently labelled oligonucleotide probes targeting the relatively conserved rRNA can be designed with specificities that range from the species level to the level of phyla or even domains. Although the general principle is simple (Figure 1.32), an extensive optimisation can be necessary to obtain a specific signal according to the sample characteristics and to the targeted microbial communities. The first step is to fix and permeabilize the cell membrane of the microbial cells by treatment with chemical fixatives, such as paraformaldehyde (PFM) and ethanol. Then the sample is incubated with probes that are generally 15 to 25 nucleotides in length and are labelled covalently at the 5'end with a fluorescent dye, such as CY3 or CY5. The labelled oligonucleotide will diffuse to its intracellular targets and form specific hybrids. The correct hybridisation of the probes will strongly depend on the stringent conditions applied, such as temperature and formamide concentration, but also on the probe design and the cellular ribosome content. After stringent washing, specifically stained cells are detected via epifluorescence microscopy or flow cytometry.

Several hundred rRNA-targeted oligonucleotide probes suitable for FISH have been de-

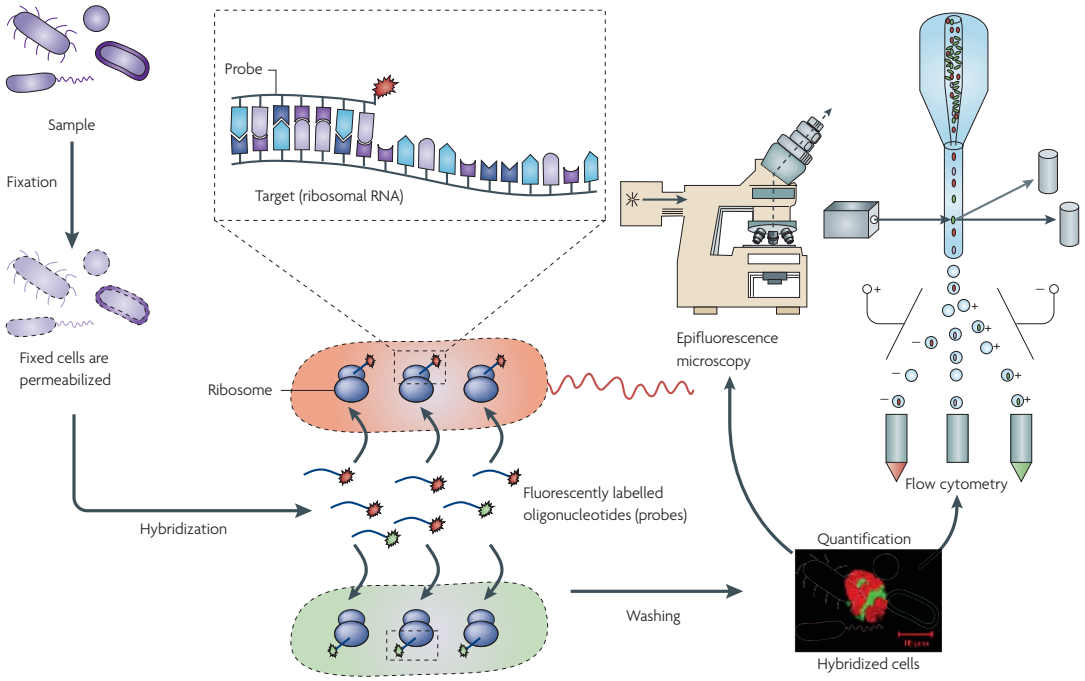


FIG. 1.32: The basic steps of FISH using rRNA-targeted oligonucleotide probes, modified from (Amann and Fuchs, 2008).

scribed and published on an online database (http://www.microbial-ecology.net/probebase). Although these *in situ* hybridization techniques have been widely applied to describe the prokaryotic communities in hydrothermal and sediment surface environments (Harmsen *et al.*, 1997; Nercessian *et al.*, 2003; Takai *et al.*, 2004; Treude *et al.*, 2005*b*, for e.g.), FISH fails to detect accurately prokaryotes in deep subsurface sediments, probably as a result of the low content of RNA.

A more sensitive technique can be used to enhance the signal using an enzyme. This in situ hybridization technique based on FISH is designed as CAtalysed Reported Deposition FISH (CARD-FISH). Here, unlike FISH, the probes are labelled horseradish peroxidase enzyme (40 kDa). Hence, as the labelled probe is much larger than with a fluorescent label, some impermeable archaeal cells are recalcitrant to hybridisation (Teske, 2005).The hybridization involves a single oligonucleotide that is covalently crosslinked to the very stale horseradish peroxidase. Amplification of the signal is achieved by the radicalization of multiple tyramide molecules by a single horseradish peroxidase (Figure 1.33). The activated tyramides subsequently bind permanently to the cells, thereby conferring a strong and stable fluorescent signal. Although, sometimes controversial, the study of deep subsurface sediments using CARD-FISH, could be a powerful tool to visualize the main embers of the "rare biosphere" (Schippers and Neretin,

2006; Schippers *et al.*, 2005; Teske, 2005; Santelli *et al.*, 2008).

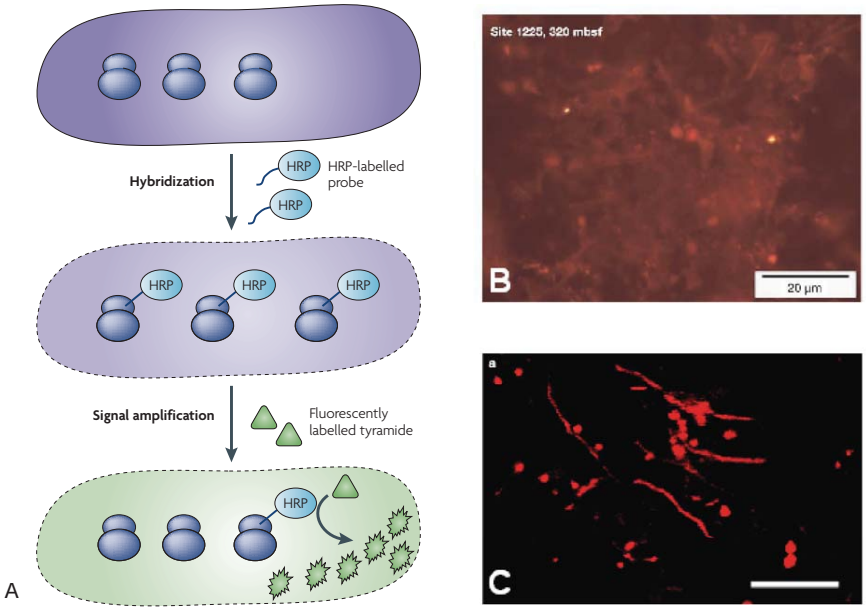


FIG. 1.33: A. The general principle of CARD–FISH (catalysed reported deposition–fluorescence *in situ* hybridization), from (Amann and Fuchs, 2008). B. Confocal laser scanning micrographs of CARD–FISH hybridised cells associated with deep subsurface sediments (Schippers *et al.*, 2005). C. Confocal laser scanning micrographs of CARD–FISH hybridised cells associated with ocean crust (Santelli *et al.*, 2008).

#### 1.3.3.3.3 Other quantitative approaches. Quantitative PCR

By adding ethidium bromide to a PCR amplification, Higuchi and colleagues showed that it was possible to access to the kinetics of the amplification reaction and therefore to calculate the initial amount of template amplified (Higuchi *et al.*, 1992). The technique was gradually optimised by the use of different nucleic acid binding stains, such as SYBR green (Figure 1.34). Another alternative and more specific approach consists in using a TaqManTM probe to hybridise with a region within the targeted amplicon allowing detection and quantification of the initial template concentration (Livak *et al.*, 1995; Lie and Petropoulos, 1998; Takai and Horikoshi, 2000, for e.g.)(Figure 1.34). This system is relatively sensitive for detection and quantification of small amounts of the targeted amplicon. Moreover, it can be used for large numbers of samples and specimens due to the advantages of PCR such as 96-well technology, therefore making it less laborious to perform on a large number of samples taken from a sediment core, or for time series investigations. The use of quantitative PCR (also designed as real time PCR) is beginning to be used frequently to quantify subsurface prokaryotic communities (Biddle *et al.*, 2008; Lipp *et al.*, 2008; Schippers and Neretin, 2006; Schippers *et al.*, 2005; Takai

and Horikoshi, 2000).

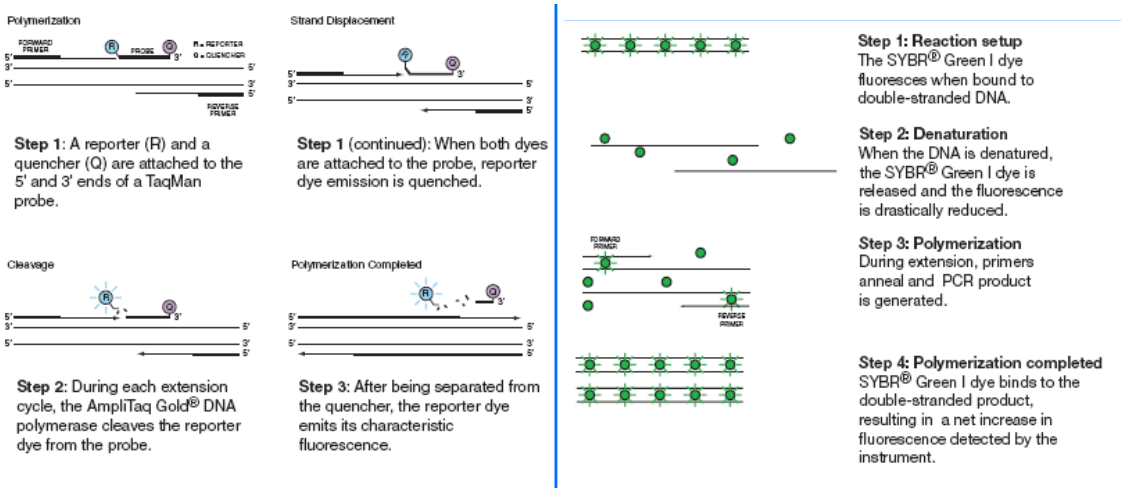


FIG. 1.34: The general principle of quantitative PCR (modified from "Relative Quantification Getting Started Guide", Applied Biosystems).

**Phospholipid-based fatty acids (PLFAs)**

As phospholipid-based fatty acids (PLFAs) are rapidly degraded after cell death, (weeks to months), it was suggested that they could be significantly measured in order to determine the living bacterial biomass (Lipp *et al.*, 2008; Zink *et al.*, 2008). This concept was also extended to the analysis of intact phospho- and glycolipids from archaeal communities. The analysis of intact phospholipids are performed by high-performance liquid chromatography (HPLC)-electrospray ionisation (ESI) mass spectrometry (MS), whereas the PLFAs are analysed with chromatography (GC)-MS. Hence, IPLs could provide a robust basis for estimating biomass and broadly constraining the phylogenetic affiliation of the major contributors to living biomass in deep subsurface sediments, as Lipp and colleagues showed by the analysis of deep subsurface sediments from several locations (Lipp *et al.*, 2008).

#### 1.3.3.4 Metabolic and functional approaches

The key facts to understand a microbial ecosystem are the links between the biotic and abiotic process. Although molecular phylogenetic and cultural approaches can identify and potentially characterize the microbial communities associated to subsurface environment, they do not address the metabolic activities in situ. Hence, several approaches were developed in order to identify and characterize *in situ* the active members of the microbial communities.

**1.3.3.4.1 Isotope Probing: linking microbial identity to function.** The isotope based techniques are some of the most revealing approaches that make it possible to study the function and activity of microorganisms in their environment. Stable isotope probing (SIP) involves principally incubating an environmental sample with substrate highly enriched in a stable isotope, after which the "labelled" biomarkers (lipids or nucleic acid) can be extracted from the sample and analysed, indicating whether the substrate was incorporated by the microorganisms, therefore identifying the active members of the microbial community (Dumont and Murrell, 2005; Boschker and Middelburg, 2002, for review)(Figure 1.35). Although SIP has only been carried out using $^{13}$C, there are possibilities for some applications to use other stable isotopes, such as $^{15}$N.

SIP was first applied in the analysis of phospholipid fatty acids (PLFA) extracted from environmental samples and were analysed by isotope-ratio mass spectrometry (IRMS). As several microorganisms have signature PLFA, it is often possible to identify these microorganisms when they have incorporated the $^{13}$C-substrate (Pombo *et al.*, 2005, for e.g.). The disadvantage of this technique is that nothing is known about the PLFA patterns of microorganisms for which there are no cultivated representatives. Hence the use of labelled nucleic acids as biomarkers has the potential to identify a wider range of prokaryotes as 16S rRNA gene is the most informative universal biomarker. Therefore, several DNA- and RNA-based SIP studies are able to identify the active methane cycling communities (Radajewski *et al.*, 2002; Lu and Conrad, 2005, for e.g.).

Interestingly, a recent study combined for the first time both approaches at once. Gordon Webster and colleagues identified the sediment uncultured JS1 group as actively metabolising glucose and acetate under anaerobic sulfate-reducing conditions (Webster *et al.*, 2006). However, it is essential to check whether it is the $^{13}$C-substrate that has been incorporated by the identified microorganism, and not a by-product from different microorganism that would have incorporated the $^{13}$C-substrate.

Another variant of isotope probing is FISH–microautoradiography that utilizes radioactive tracers, such as $^{14}$C, to monitor the incorporation of substrate (Figure 1.35). Orphan and colleagues exploited the extremely low natural abundance of $^{13}$C in methane compared with other carbon compounds to demonstrate, using FISH combined with secondary ion mass spectrometry, that methane is oxidized in anoxic cold seep sediments by a group of *Archaea* that are associated with the *Methanosarcinales* (Orphan *et al.*, 2001*a*). These *Archaea* mediate the anaerobic oxidation of methane in a consortium with sulphate-reducing *Bacteria*.

**1.3.3.4.2 RNA: who is metabolically active?** However, up to 90 % of the total DNA in shallow marine sediments is extra cellular (Dell'Anno and Danovaro, 2005; Danovaro *et al.*, 1999), hence DNA-based molecular approaches do not distinguish between living and dead microbial communities (Damste and Coolen, 2006; Dell'Anno and Corinaldesi, 2004). For

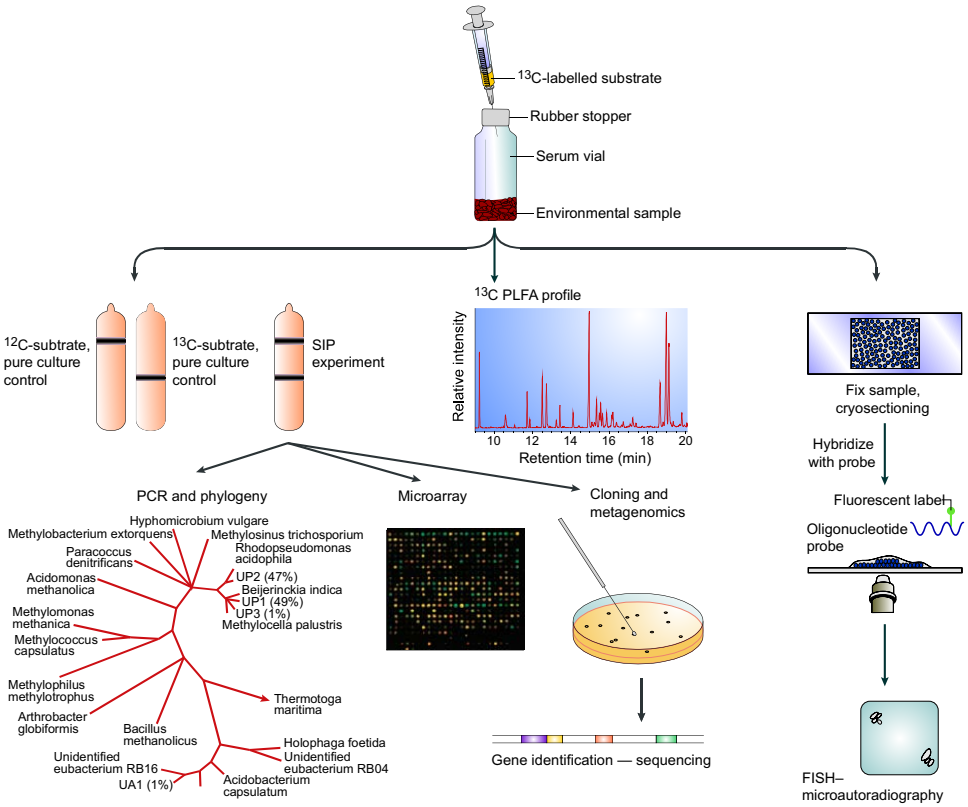


FIG. 1.35: DNA-based isotope probing, $^{13}$C-phospholipid fatty acids (PLFA) analyses and cultivation-independent identification of microorganisms using radioisotopes, modified from (Dumont and Murrell, 2005).

example, the occurrence of eukaryotic DNA in deep marine sediments, suggests that DNA originating from the surface can be preserved in deeply buried sediments (Schippers and Neretin, 2006). It is therefore crucial to target the metabolically active communities in order to correlate their phylogeny with variable environmental factors (Biddle *et al.*, 2006; Sorensen and Teske, 2006; Lloyd *et al.*, 2006; Treude *et al.*, 2005*a*). Although fluorescent *in situ* hybridization has commonly been used to identify metabolically active microbial communities, this approach does not provide an overall picture of the active microbial communities. In order to target the active cells from subsurface sediments (Inagaki *et al.*, 2005), extractable archaeal rRNA can be used, as rRNA has a rapid turnover (Danovaro *et al.*, 1999; Kramer and Singleton, 1993; Kerkhof and Ward, 1993; Kemp *et al.*, 1993). However, as the reverse transcriptase is a poorly processive enzyme, the amplification of RNA can be be relatively difficult in low biomass sediments (Weller *et al.*, 1991). Only a few studies have used rRNA-derived clone libraries to investigate the diversity of active methane cycling communities in subsurface sediments (Biddle *et al.*, 2006; Sorensen and Teske, 2006).

| Process | Enzyme | Genes |
|---|---|---|
| $N_2$ fixation | Nitrogenase | nif |
| Denitrification | Nitrite reductase | nirK, nirS |
| Nitrification<br>$NH_4$ oxidation | Ammonium monooxygenase<br>Hydroxylamine oxidoreductase | amo<br>hao |
| $CO_2$ fixation | RUBP carboxylase-oxygenase | cbbL, cbbM |
| Methanogenesis | Methyl-coenzyme M reductase | mcr |
| Methane oxidation | Methane mono-oxygenase | mmo |
| Sulfate reduction | Dissimilatory sulfite reductase | dsr |
| Sulfur oxidation | Sulfite oxidoreductase | sor |

Table 1.6: Examples of microbial genes and enzymes mediating biogeochemical processes.

**1.3.3.4.3 Functional genes.** Microorganisms mediate key steps in biogeochemical cycles through the production of particular enzymes which are encoded by functional genes (Table 1.6). Describing the presence, abundance and expression of these particular genes, and identifying the organisms in which they occur would help to understand, by culture dependant approaches, how microbial communities interact with their environment. The first key step is to identify functional groups within microbial communities, and the enzymes which mediate particular biogeochemical processes. Hence, the presence of a functional gene in a specific genome will determine the functional group of the associated microorganism. According to the dominant metabolic activities previously described in subsurface sediments, we will briefly describe some of the most representative functional genes (*mcrA, dsrA, amoA*).

Methanogenesis is mediated by methanogenic *Archaea* and only occurs in anoxic environments (Wolfe, 1971). In all known methanogens, the methyl-coenzyme M reductase (MCR) enters the final step in methane synthesis (Ellermann *et al.*, 1988). It is composed of three subunits ($\alpha, \beta, \gamma$) and coded by *mcrA, mcrB* and *mcrG* respectively. The 16S rRNA gene tree topology is very similar to the *mcrA* topology, as the *mcrA* gene is remarkably conserved (Luton *et al.*, 2002). The *mcrA* gene can therefore be used to target and describe the methanogen diversity by using molecular techniques (Springer *et al.*, 1995; Luton *et al.*, 2002). Several studies have previously investigated the *mcrA* gene diversity in subsurface marine sediments and hydrothermal environments (Newberry *et al.*, 2004; Webster *et al.*, 2006; Nercessian *et al.*, 2005*a*; Dhillon *et al.*, 2005; Nunoura *et al.*, 2006, for e.g.).

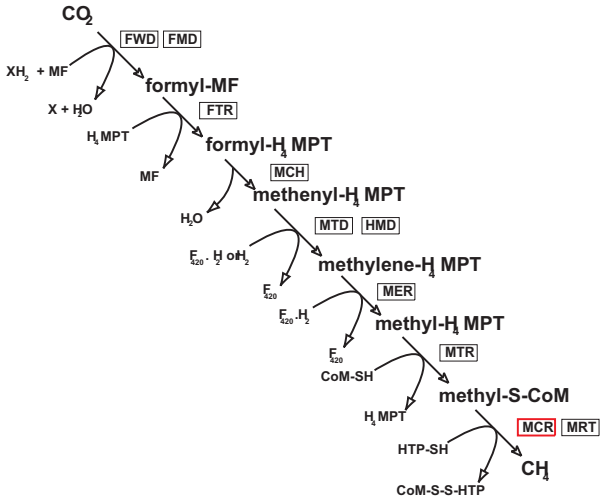


FIG. 1.36: Biochemical pathway of $H_2$-dependent reduction of $CO_2$ to $CH_4$. FWD, W-containing formylmethanofuran dehydrogenase; FMD, M-containing formylmethanofuran dehydrogenase; FTR, formylmethanofuran tetrahydromethanopterin formyl transferase; MCH, $N_5N_{10}$-methenyltetrahydromethanopterin cyclohydrolase; MTD, coenzyme F420-reducing $N_5N_{10}$-methylenetetrahydropterin dehydrogenase; HMD, $H_2$-forming $N_5N_{10}$-methylenetetrahydropterin dehydrogenase; MER, $N_5N_{10}$-methylenetetrahydropterin reductase; MTR, $N_5$-methyltetrahydromethanopterin :methyl transferase; MCR, methyl coenzyme M reductase I; MRT, methyl coenzyme M reductase II.

The ability to use sulfate as a terminal electron acceptor is characteristic of several bacterial lineages and of one thermophilic genus of *Archaea*. Dissimilatory (bi)sulphite reductase (DSR) is the central enzyme in the dissimilatory sulphate reduction and catalyses the final 6 e- transfer to bisulphite to form sulphide (Figure 1.37). Dissimilatory (bi)sulphite reductase gene sequences of the alpha and beta subunits of the *dsr* gene, dsrAB, have shown to be a promising and specific approach for investigating the diversity of sulphate-reducing *Bacteria* (Wagner *et al.*, 1998). Although detected in hydrothermal environments, *dsr* genes were rarely retrieved from deep subsurface sediments (Biddle *et al.*, 2008).

The *amoA* gene encodes the catalytic $\alpha$-subunit of ammonia monooxygenase, the enzyme responsible for catalyzing the rate-limiting step in bacterial ammonia oxidation. Because *amoA* is well conserved and required by all ammonia oxidising *Bacteria*, this gene has been used extensively as a molecular marker for cultivation-independent studies of these communities. Moreover, despite their different physiological roles in these *Bacteria*, genes encoding particulate methane monooxygenase (*pmo*) and ammonia monooxygenase share high sequence identity, as the analysis of the predicted amino acid sequences of these genes revealed strong conservation of both primary and secondary structure (Figure 1.38)(Holmes *et al.*, 1995).The possibility that some *Archaea* carry an *amoA* was recently confirmed (Schleper *et al.*, 2005). A definitive link between this novel *amoA* and archaeal ammonia oxidation was only recently established

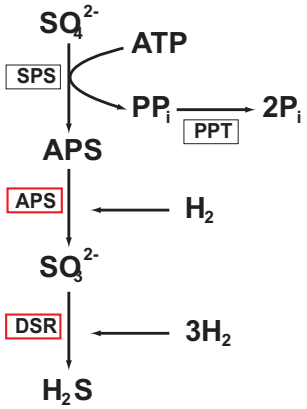


FIG. 1.37: Biochemical pathway of sulphate reduction to hydrogen sulphide in *Archaeoglobus fulgidus*. SPS, ATP sulphurylase; PPT, pyrophosphatase; APS, adenylylsulphate reductase; DSR, sulphite reductase.

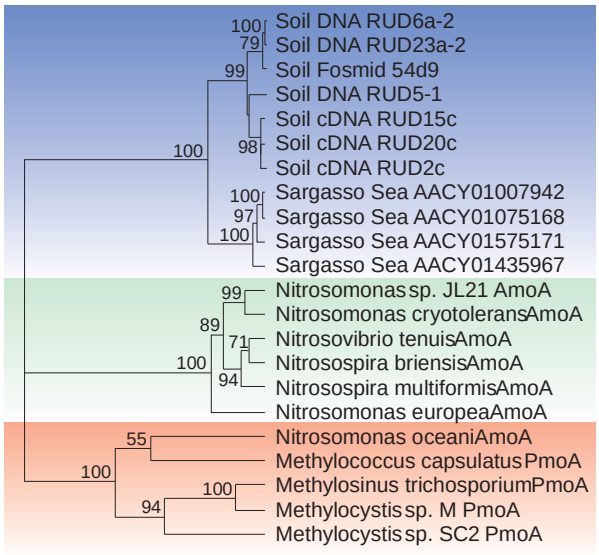


FIG. 1.38: An *amoA/pmoA* phylogeny that was reconstructed using 200 aligned amino-acidpositions of AmoA (ammonia monooxygenases) and PmoA (particulate methane monooxygenases subunit)(Schleper *et al.*, 2005).

by cultivation of an ammonia-oxidizing member of the marine group 1 *Crenarchaeota*, the first cultivated representative from this dominant archaeal lineage. The ammonia oxidizing *Archaea* (AOA) distribution in the marine environment, and their relative importance in the global N cycle, remain unknown.

## 1.4 Objectives

As seen in the previous sections, although many studies describe the prokaryotic ecosystems associated to the sub-seafloor, knowledge on physiology and distribution of these prokaryotes remains limited. As most deep-biosphere microorganisms detected so far have been extremely resistant to cultivation, most of the studies avoided this cultivation bias by using molecular approaches. Therefore, we only have a limited understanding of the metabolic process driving the prokaryotic communities. Although combined molecular based approaches could at least partially give access to the phylogenetic and metabolic diversity of these prokaryotes, the answers to the following questions remain still unclear:

- At what depth does the deep biosphere start?
- What are the main environmental factors selecting the prokaryotic diversity?
- How deep does the sub-seafloor biosphere extend?
- Is there a deep hot biosphere fuelled by compounds synthesized abiotically?

In order to try to answer these questions, the second chapter displays the results obtained during this thesis; it is composed of five studies presented as scientific papers either in preparation, submitted, accepted or published. The five studies are organised in a progression, discussing results from a shallow coastal subsurface to a deep hot biosphere located at the Mid-Ocean Ridges (Figure 1.39):

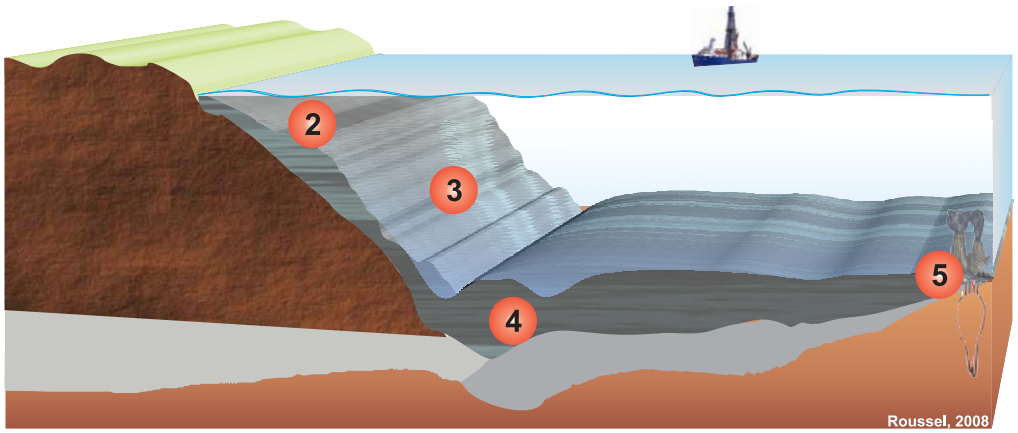


FIG. 1.39: Cross-section representation of the organisation of the second chapter of this thesis. Each number indicates the location of each study.

**Study 1** This study describes the methodological considerations and limitations associated to the analysis of sub-seafloor microbial communities. The strategy was to optimise the

DNA extractions and the DGGE technique in order to analyse the prokaryotic diversity from low biomass subsurface samples and deal with potential contaminations.

**Study 2** This study describes the archaeal diversity associated with shallow coastal methane bearing sediments in the Marennes-Oléron Bay (France) (see 2 of Figure 1.39). The analysis of the archaeal diversity and community structures was performed using denaturant gradient gel electrophoresis (DGGE), and cloning PCR-amplified 16S rRNA and mcr functional genes from total DNA and RNA extracts.

**Study 3** In this study we examine the boundary between surface and subsurface communities associated with deep marine sediments (see 3 of Figure 1.39). Archaeal indicators of deep hot sub-seafloor ecosystems were also investigated, as some sites were characterized by a strongly faulted cold seafloor. The analysis of the archaeal diversity and community structures was performed on surface and subsurface sediments from the New-Caledonia and Fairway Basins, with different terrestrial influences, using denaturant gradient gel electrophoresis (DGGE) and cloning PCR-amplified taxonomic and functional genes.

**Study 4** This study presents results that extend and characterise the known sub-seafloor biosphere (see 4 of Figure 1.39).

**Study 5** This study investigates the archaeal community potential associated with hydrothermal fluids from three ultramafic sites of the Atlantic Ocean (see 5 of Figure 1.39). The molecular genetic diversity of three ultramafic-hosted hydrothermal sites, Rainbow, Lost City and Ashadze, was analysed, using 16S rRNA gene and functional genes of methanogens, methanotrophs and sulfate-reducers associated. As these hydrothermal fluids are highly enriched in methane and hydrogen, it is crucial to identify specific prokaryotic communities possibly associated with potential subsurface chemolithoautrophic ecosystems.

# Chapter II – Results: from the coast to the Mid-ocean ridges

# CHAPTER 2

# RESULTS: FROM THE COAST TO THE MID-OCEAN RIDGES

**Contents**



## 2.1 Methodological considerations and limitations

E. Roussel, E. Delage, S. Grayo, F. Duval, M.-A. Cambon-Bonnavita

It is technically challenging to study prokaryotic communities from chemically complex geological samples, such as low biomass deep marine sediments, using molecular genetic approaches. Moreover, other potential problems, when working with low biomass samples and DNA from unknown prokaryotic populations, result from the introduction of artefact 16S rDNA from contamination in the DNA extraction and PCR reagents. The present study aims to firstly optimise the DNA extractions and to deal with the contamination issues. In a second section, the DGGE, a finger printing technique, was optimised in order to obtain different levels of resolution for the description of the prokaryotic diversity. Moreover, a new method, Co-Migration DGGE (CM-DGGE) was used to access the active archaeal diversity of low biomass deep marine sediments.

*L'étude des communautés microbiennes associées à des environnements chimiques et géologiques complexes, comme les sédiments de faible biomasse, est techniquement difficile. De plus, le*

*risque de contamination est élevé lors de l'étude de l'ADN d'échantillons de faible biomasse. Les sources de contamination peuvent être nombreuses, mais sont principalement liées à l'extraction d'ADN et aux réactifs de PCR. L'étude suivante a pour objectif d'optimiser la technique permettant l'extraction d'ADN, et aussi de maîtriser les risques de contamination. La seconde partie de cette étude sera consacrée à l'optimisation de la DGGE, une technique d'empreinte moléculaire, afin d'obtenir différents niveaux de résolution. Une nouvelle méthode, la Co-Migration DGGE (CM-DGGE), a été mise au point afin de décrire et de comparer la diversité archéenne issue de l'extraction d'ARN de sédiments marins.*

## Optimisation of the DNA extraction on samples with a low biomass

The study of the 16S rDNA molecular diversity of low biomass sediment samples, such as those retrieved during the ODP leg 210 (see study 4), requires the optimisation of the molecular techniques. Hence, the first step was to enhance the yield and quality of DNA extracted. However, increasing the yield of extracted DNA also increases the risk of contamination. It was therefore necessary to deal with these contamination issues as well.

### Materiel and methods

As seen in a previous section, the extraction of nucleic acids is divided in two main steps, cell lysis and nucleic acid purification. Nucleic acid extraction methods suffer from multiple inefficiencies in the individual component steps, including incomplete cell lysis, DNA adsorption to the sample surfaces, co-extraction of enzymatic inhibitors from samples, and loss, degradation, or damage of DNA. Thus, studies of DNA extraction techniques have indicated that these techniques can introduce biases of their own (Miller *et al.*, 1999, for e.g.). To determine therefore the optimum combination, two cell lysis and two purification procedures were tested (Table 2.1). All the extractions and amplifications were performed in triplicates.

A physical disruption technique, bead mill homogenization (also known as bead-beating), is used by the FastDNA® Spin Kit for soil (MP Biomedicals™). Several studies have established that bead mill homogenization yields more DNA than other physical disruption methods such as freeze-thaw disruption yields (Webster *et al.*, 2003; More *et al.*, 1994; Leff *et al.*, 1995; Miller *et al.*, 1999). However, the drawbacks to bead mill homogenization include the fact that larger amounts of contaminating humic acids are recovered, and the fact that, in some instances, the DNA is sheared (Leff *et al.*, 1995; Webster *et al.*, 2003). The chemical lysis procedures used in the methods previously described also vary, but their lysis mixtures can be categorized into mixtures that contain detergent (either sodium dodecyl sulfate [SDS] or Sarkosyl), mixtures that contain NaCl, and mixtures that contain various buffers (usually Tris or phosphate, pH

| Lysis[b] | | Bead beating | | | | SDS/ Sarkozyl/ proteinase K | |
|---|---|---|---|---|---|---|---|
| Decontamination[a] | | Yes | | No | | No | |
| DNA purification and DNA elution[c] | | FastDNA® Spin kit for Soil | Phenol-chlorophorm/isopropanol/ethanol/Dna-free water | FastDNA® Spin kit for Soil | Phenol-chlorophorm/isopropanol/ethanol/Dna-free water | FastDNA® Spin kit for Soil | Phenol-chlorophorm/isopropanol/ethanol/Dna-free water |
| Cell density of the sample (cells/mL) | $5 \times 10^8$ | + | − | + | − | + | − |
| | $7 \times 10^7$ | + | − | + | − | − | − |
| | $10^5$ | − | − | − | − | − | − |
| Negative control | | + | − | + | ± | ± | ± |

Table 2.1: Comparison of the combination of different DNA extraction methods assessed by PCR-amplification on sediments with different cell densities from the West African Equatorial margin (BioZaire1) site CRAVO and from the Newfoundland margin (ODP leg 210) site 1276 at 1147 mbsf. ±: poor PCR reproductibility.

7 to 8). The modifications of the basic chemical lysis techniques include high-temperature (60°C to boiling) incubation, a phenol or chloroform extraction step, and the incorporation of chelating agents (EDTA and Chelex 100) to inhibit nucleases and disperse soil particles. In the present study, we chose to compare bead mill homogenization to chemical lysis.

- The bead mill homogenization was performed using the first step of the FastDNA® Spin Kit for soil (MP Biomedicals™) as following: 978 $\mu$L of Sodium phosphate buffer, 122 $\mu$L of MT buffer, 200 $\mu$g of poly-adenylic acid potassium salt (poly A) and 1 g of sediment sample was added to the Lysing Matrix E tube and homogenized in the FastPrep® Instrument for 30 seconds at a speed setting of 5.5. The poly A was shown to enhance the yield of DNA extracted from the sediment, probably by avoiding the adsorption of the extracted DNA on the sediment (Figure 2.1). After centrifugation for 30s at 14,000 g, the supernatant was used for purification (see next steps). During the extraction, the tubes were kept on ice.

- The chemical lysis was performed by homogenising 500 $\mu$L of TNE [1] and 1 gM of sediment for 5×30s and cooled on ice. Cells were lysed at room temperature for 15 min with 10 $\mu$L of RNAse (2 mg.mL$^{-1}$) and 1 mg.mL$^{-1}$ of lysozyme. Then, 1 % SDS and Sarcosyl were added to the lysis suspensions and the tubes were homogenised. The tube was then incubated over 20 min at 55°C with 50 $\mu$L of proteinase K (20 mg.mL$^{-1}$).

1. TNE composition: Tris-HCl 100 mM [pH 8.0], NaCl 100 mM, EDTA 50 mM [pH 8.0]

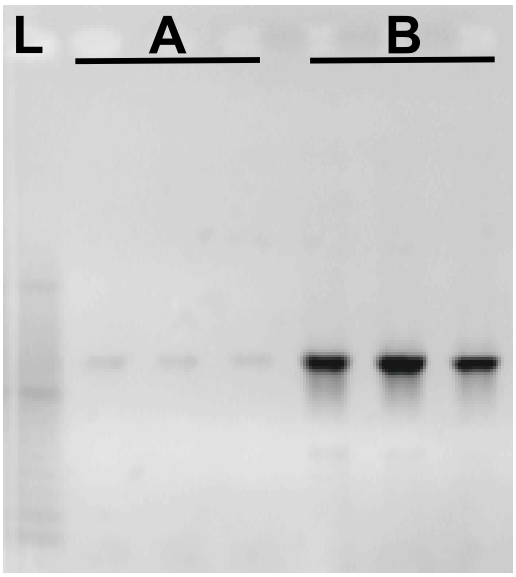


FIG. 2.1: PCR amplification of 16S rRNA gene from DNA extraction performed with the FastDNA® Spin Kit for soil from cultures with cell density of $10^7$ cells.mL$^{-1}$. DNA extraction performed: [A] without adjunction of Poly A. [B] with adjunction of Poly A.

The second step, consisted in comparing the purification method from the FastDNA® Spin Kit for soil (MP Biomedicals™) to a purification method using phenol–chloroform–isoamyl alcohol mixture.

- The FastDNA® Spin Kit for soil (MP Biomedicals™) method was performed by mixing 250 $\mu$L of PPS (Protein Precipitation Solution) with the product of the cell lyses, followed by a 5 min centrifugation at 14,000 g. The supernatant was first incubated 30 min at 37°C (∼50 rpm) with 1 mL of Binding Matrix Suspension and then left 30 min (no homogenisation). The following procedure was according to the manufacturer's instructions, except that after addition of elution solution (DES) the column was incubated 1 hour at 37°C before centrifugation (5 min at 14,000 g).

- The method using pre-warmed (55°C) phenol–chloroform–isoamyl alcohol was performed as described by Teske and colleagues (Teske *et al.*, 2002).

To avoid contaminations, all manipulations were carried out in a PCR cabinet exclusively dedicated to the present study (Biocap™ RNA/DNA, erlab®), using Biopur® 1.5 mL Safe-Lock micro test tubes (Eppendorf™), Rnase/Dnase Free Water (MP Biomedicals™) and UV-treated ($>$ 60 min) plasticware and pipettes. Moreover, a decontamination method was performed at different levels on the FastDNA® Spin Kit for soil (MP Biomedicals™), in order to identify the potential origins of contaminants. For further precaution, some studies (Study 4) were

performed in a differente laboratory, with a completely set of plasticware, pipettes and a new dedicated PCR cabinet.

- Lysing Matrix E tube was washed twice using NaOH (1N) and a 5 min centrifugation at 1,200 g. The tube was then rinsed twice with Rnase/Dnase Free Water (MP Biomedicals™) and dried using 2 mL of Ethanol (95°) and centrifuged 5 min at 1,200 g. Further drying was achieved by leaving the tube in the PCR cabinet for 2 hours under UV treatment.

- All the solutions were filtered using a modified protocol from Mohammadi and colleagues by utilizing a YM-100 Microcon (Amicon/Millipore™) centrifugal device (Mohammadi *et al.*, 2005).

In order to compare the efficiency and quality of the different DNA extraction methods, the study was performed on 3 different sediment samples characterized by different prokaryotic cell abundances (Table 2.1). The two sediment samples which had $5\times10^8$ and $10^7$ cells.cm$^{-3}$ were retrieved from the West African Equatorial margin (BioZaire1) site CRAVO. The sample which had $10^5$ cells.cm$^{-3}$ was retrieved from the Newfoundland margin (ODP leg 210) site 1,276 at 1,147 mbsf.
As the final objective was to optimise the DNA extraction in order to obtain PCR products that could be cloned, the evaluation of the quality and yield of the DNA extracted was assessed by PCR amplification of bacterial 16S rRNA gene. Bacterial 16S rRNA gene amplification was conducted by PCR with combination of primers E8f (5'- AGA GTT TGA TCA TGG CTC AG-3') and U1407r (5'-GAC GGG CGG TGW GTR CAA-3'). PCR cycles for the first round (E8f/U1407r) were as follows: 1 cycle of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1.5 min at 53°C and 2 min at 72°C and 1 cycle of 6 min at 72°C. The PCR products were visualized on a 0.8 % (w/v) agarose gel stained with ethidium bromide. In order to identify potential contaminants, negative controls were also carried out with DNA extractions performed with no sample.
All visible PCR amplification products were cloned using the TOPO TA Cloning kit (Invitrogen) following the manufacturer's instructions. Positive transformants were screened by PCR amplification of the insert using the vector-specific M13 primers. Plasmid extraction, purification and sequencing of the insert, were carried out by the sequencing OUEST-Genopole platform® of Roscoff Marine laboratory (France).

### Comparison of the extraction methods

According to the various DNA extraction methods used, the PCR amplifications showed differences in the levels of amplification of bacterial 16S rRNA gene (Table 2.1). However, the decontamination procedures did not negatively affect the PCR amplification.
Although the chemical lysis is efficient on sediment samples with high cell densities, the bead

mill homogenization gives higher yield of extracted DNA. As mentioned above, this result confirms previous studies showing that bead mill homogenization usually yields more DNA than other methods (Webster *et al.*, 2003; More *et al.*, 1994; Leff *et al.*, 1995; Miller *et al.*, 1999). The chemicals used for the chemical lysis are probably not suitable for the lysis of cells from deep marine sediments.
The phenol–chloroform–isoamyl alcohol purification method is clearly not suitable for the extraction of DNA from marine sediments, as no PCR amplification product was detected. As the FastDNA® Spin Kit for soil purification method showed positive results for samples with cell densities over $7\times10^7$, we suggest that the FastDNA® Spin Kit for soil may be more efficient in removing inhibitor compounds and that it gives higher yields of DNA. The purification efficiency of the FastDNA® Spin Kit for soil is probably related to the high specificity of the binding matrice for DNA.
Overall, as previously shown by Webster and colleagues, the FastDNA® Spin Kit for soil is the most reliable method to extract DNA from deep marine sediments (Webster *et al.*, 2003). Moreover, it was also shown that the FastDNA® Spin Kit for soil is the most reliable method to extract DNA from *Thermococcales* cultures (unpublished data). However, no PCR amplification was obtained from the sample with the lower cell density ($10^5$).

### Contamination issues

Contamination is one of the major issues in studying environmental samples. For example, Tanner and colleagues noted the presence of highly similar sequences ($>$ 99 %) in clone libraries that were obtained from many physically and chemically distinct environments (Tanner *et al.*, 1998). Moreover, some commercialised molecular kits and reagents were shown to be contaminated (Evans *et al.*, 2003; van der Zee *et al.*, 2002; Mohammadi *et al.*, 2005; Tanner *et al.*, 1998).
Although all PCR negative controls showed no PCR amplification products, it is interesting to note that several DNA extraction negative controls showed PCR amplification of bacterial 16S rRNA products. The level of contamination is probably quite low as no PCR amplification product was detected for the samples with the lower cell densities. This suggests that the sediment could adsorb most of the contaminants, explaining why no PCR amplification product is detected when low biomass sediment is added.
Two approaches are possible to deal with the bacterial contamination issue:

- Reduce the level of bacterial contamination (Mohammadi *et al.*, 2005; Rys and Persing, 1993; Meier *et al.*, 1993)
- Identify the bacterial contaminants (Barton *et al.*, 2006)

Despite the substantial decontamination procedures, the negative controls still showed evidence of contamination. Hence, clone libraries were constructed from negative controls, and

120 clones were sequenced in order to identify the bacterial contaminants. As several different extraction methods were used, it was possible to identify the origin of each contaminating phylotype.
The bacterial contaminants showed a broad diversity (Table 2.2), and were manly affiliated to *Proteobacteria, Firmicutes, Cytophaga-Flavobacterium-Bacteroides* (CFB) and *Actinobacteria*. Most of the contaminants (*Ralstonia*, *Herbaspirilum, Caulobacter, Escherichia, Acinobacter, Tuber borchii* symbiont and uncultured *Bacteroides*) seemed to originate from the PCR reagents. For example, *Escherichia coli* has previously been detected in Taq polymerase solutions (Tanner *et al.*, 1998). The sequences affiliated to *Bradyrhizobium, Parracoccus, Clostridium, Acidovorax* and *Stenotrophomonas* were detected from the FastDNA® Spin Kit for soil, and the sequences affiliated to *Methylobacterium* and *Algidimarina* seem to be extracted from the phenol–chloroform–isoamyl alcohol mixture. However, the origin of the sequences affiliated to *Pseudomonas tolaasii, Staphylococcus* and *Streptococcus* was uncertain, and could be therefore related to aerial contamination.

### Conclusion and recommendations

The present study shows that the FastDNA® Spin Kit for soil (MP Biomedicals™), performed with modifications (Webster *et al.*, 2003, and this study), is the most efficient method to extract DNA from deep marine sediments. However, an inherent low bacterial contamination can be detected from FastDNA® Spin Kit for soil as also for some of the reagents used for DNA extraction. Although this contamination does not interfere with the analysis of the bacterial diversity of samples which are characterized by high biomass, this becomes a critical issue on low biomass environments. Hence, we recommend the use of the contamination database from the present study, in order to subtract potential contamination from clone libraries constructed from low biomass samples (database and paper in preparation). This approach can also be adapted to DGGE as recommended by Webster and colleagues (Webster *et al.*, 2003).
Moreover, the same strategy was used in order to detect a potential archaeal contamination. No archaeal contamination was ever detected. However, as a further precaution, "blank" extractions (negative control) were pooled, purified and concentrated in an YM-100 Microcon (Amicon/Millipore™) centrifugal device and nested PCR were performed systematically for all studies, in order to exclude the possibility of archaeal contamination (also following the exact method used to process the environmental samples). No archaeal contamination or cross-contamination (from different samples) was ever detected in any of the following studies.

## CM-DGGE: who is there and who is active?

As seen in the first chapter DGGE is a suitable technique for comparing the diversity of fairly large numbers of samples. Although relatively affordable compared to DHPLC, T-RFLP or SSCP, DGGE requires a significant optimisation and calibration before it can be accurately

| Taxonomic group | genera of the contaminant | Origin of the contaminants |
|---|---|---|
| α Proteobacteria | *Acidovorax.sp* | FastDNA® Spin Kit for soil |
| | *Ralstonia sp.* | PCR reagent |
| | *Herbaspirilum sp.* | PCR reagent |
| β Proteobacteria | *Caulobacter sp.* | PCR reagent |
| | *Methylobacterium sp.* | phenol–chloroform–isoamyl alcohol mixture |
| | *Bradyrhizobium sp.* | FastDNA® Spin Kit for soil |
| | *Paracoccus sp.* | FastDNA® Spin Kit for soil |
| γ Proteobacteria | *Escherichia sp.* | PCR reagent |
| | *Acinetobacter sp.* | PCR reagent |
| | *Pseudomonas tolaasii* | phenol–chloroform–isoamyl alcohol mixture |
| | *Stenotrophomonas sp.* | FastDNA® Spin Kit for soil |
| Firmicutes | *Staphylococcus sp.* | phenol–chloroform–isoamyl alcohol mixture |
| | *Streptococcus sp.* | phenol–chloroform–isoamyl alcohol mixture |
| | *Clostridium sp.* | FastDNA® Spin Kit for soil |
| δ Proteobacteria | *Algidimarina sp.* | phenol–chloroform–isoamyl alcohol mixture |
| CFB | *Tuber borchii symbiont* | PCR reagent |
| | *uncultured Bacteroidetes bacterium* | PCR reagent |

Table 2.2: Contaminants identified from the the FastDNA® Spin Kit for soil and reagents used for extraction and PCR amplification.

used to compare the prokaryotic diversity of large number of samples. Moreover, as SSCP and T-RFLP, DGGE has a limited resolution when analysing microbial communities characterized by a high phylogenetic diversity (Loisel *et al.*, 2006; Smalla *et al.*, 2007). Hence, a preliminary optimisation of the DGGE conditions was necessary before each of the following studies (study 2, 3 and 5). This optimisation includes:

- The choice of the PCR-DGGE primers
- The optimisation of the PCR conditions
- The optimisation of gel conditions (concentration of denaturant and acrylamide)

These optimisations lead often to the same conclusions. For example, the PCR-DGGE analysis with optimised gel conditions of the archaeal 16S rRNA gene using the A344f-915r primer set showed a lower archaeal diversity than when using Parch519r-SA1+2f primer set (for technical precision see study 3). However, the Parch519r-SA1+2f primer set was limited in describing the 16S rRNA gene archaeal diversity of phylotypes characterized by a high GC content.
Moreover, DGGE is relatively limited, when searching for rare phylotypes, as a result of the low signal to noise ratio of the ethidium bromide used to stain the gels. Hence, the sensitivity of the DGGE technique can be enhanced by the use of SYBR Gold® (Molecular Probes). However, Neufeld and Mohn recently showed that the use of primers labelled with fluorophores (Cy5 or Cy3) showed the best signal to noise ratios. These observations were also confirmed by the present study. Figure 2.2 clearly shows the higher level of signal to noise ratios of the PCR-DGGE products using a labelled primer (Figure 2.2B) set compared to the same product revealed with SYBR Gold® (Figure 2.1, lane 1). The absence of differences in the intensity between lane 2, 3 and 4 of figure 2.2A, stained with SYBR Gold®, compared to figure 2.2B (labelled primers), is the consequence of the slight overlap between the emission wave lengths. Moreover, the fluorophore (Cy3 or Cy5) did not modify the migration of the PCR product in the gel (Figure 2.2A, lane 1 compared to 2, 3 and 4).

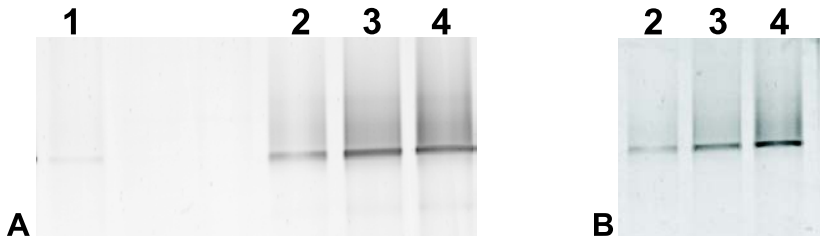


FIG. 2.2: DGGE gel either visualized with SYBR Gold® (A) or using a Cy3 labelled primer set (A344f-A915r*)(B). Lane 1: unlabelled primers (25 $\mu$L of PCR product was loaded). Lane 2, 3 and 4: Labelled primer set, and increasing amount of PCR product load on the gel (5, 10 and 20 $\mu$L respectively).

Therefore, the PCR product using different labels could be pooled on the same lane and would be still distinguished. Muyzer and co-workers proposed using this method to enable running intralane standards, providing improved sample-to-sample comparisons (Muyzer, 1999). Here, we propose to use this approach to enable running RNA- and DNA- derived PCR products from the same sample in order to compare the diversity (for technical details see study 3). Hence, the active fraction of the prokaryotic community in deep-sea sediments could be compared to DNA- derived diversity by this new molecular approach based on DGGE, named Co-Migration DGGE (CM-DGGE).

## 2.2 Archaeal Methane Cycling Communities Associated with Gassy Subsurface Sediments of Marennes-Oléron Bay (France)

Geomicrobiology Journal, vol. 26, 2009 (in press)

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As the prokaryotes from deep sub-surface sediments remain recalcitrant to growth when using conventional culturing methods, their physiology and interaction with the *in situ* environmental factors remain largely unknown. Methane cycling *Archaea* are likely to be one of the key prokaryote communities in deep sub-surface sediments. Since methane cycling *Archaea* are commonly retrieved from deep sub-seafloor and methane-seep sediments, the study of coastal gassy sediments could therefore help to define the biogeochemical habitats of deep biosphere communities. Although none of these groups have been obtained in pure culture, few studies have attempted to investigate the effect of environmental factors on AOM activities of different ANME communities (Nauhaus *et al.*, 2007; Nauhaus *et al.*, 2002*b*; Valentine and Reeburgh, 2000).

It is essential to target the metabolically active communities in order to correlate their phylogeny with variable environmental factors (Biddle *et al.*, 2006; Sorensen and Teske, 2006; Lloyd *et al.*, 2006; Treude *et al.*, 2005*a*). However, DNA-based molecular approaches do not distinguish between living and dead microorganisms (Damste and Coolen, 2006; Dell'Anno and Corinaldesi, 2004). In order to target the active cells from sub-surface sediments, extractable archaeal rRNA can be used, as rRNA has a rapid turnover rate (Danovaro *et al.*, 1999; Kramer and Singleton, 1993; Kerkhof and Ward, 1993; Kemp *et al.*, 1993). Only a few studies have used rRNA-derived clone libraries to investigate the diversity of active methane cycling communities in sub-surface sediments (Biddle *et al.*, 2006; Sorensen and Teske, 2006). Moreover, few studies, to date, have found microbial methane cycling communities in non seep or gas hydrate coastal sediments (Parkes *et al.*, 2007; Treude *et al.*, 2005*b*; Thomsen *et al.*, 2001). This is the reason why, in this study we conducted an integrated seismic, sedimentologic, biogeochemical and molecular genetic survey of the sub-surface gassy sediments of the Marennes-Oléron Bay.

*Les connaissances du métabolisme et de la physiologie des communautés microbiennes de sub-surface restent ainsi encore très limitées, du fait de la difficulté à les cultiver. Les Archaea impliquées dans le cycle du méthane jouent probablement un rôle majeur parmi les communautés procaryotes des sédiments marins de sub-surface. La présence d'Archaea impliquées dans le cycle du méthane étant fréquente dans les sédiments marins profonds et dans les zones de fluides froids, l'étude de sédiments gazeux et côtiers pourrait permettre de mieux définir les habitats des communautés microbiennes de la biosphère profonde. Bien qu'aucun isolat d'ANME ne soit disponible, quelques études ont tenté de déterminer les effets de paramètres environnementaux sur l'oxydation anaérobie du méthane réalisée par ces communautés (Nauhaus* et al., *2007; Nauhaus* et al., *2002*b*; Valentine and Reeburgh, 2000).*
*Afin de corréler la diversité phylogénétique avec les facteurs environnementaux, il est essentiel de déterminer quelles sont les communautés métaboliquement actives (Biddle* et al., *2006; Sorensen and Teske, 2006; Lloyd* et al., *2006; Treude* et al., *2005*a*). Les approches moléculaires basées sur l'ADN ne permettent pas de distinguer les cellules mortes des cellules vivantes (Damste and Coolen, 2006; Dell'Anno and Corinaldesi, 2004). Ainsi, afin de décrire la fraction active des communautés archéennes, il est possible d'utiliser des approches basées sur l'ARN (Danovaro* et al., *1999; Kramer and Singleton, 1993; Kerkhof and Ward, 1993; Kemp* et al., *1993). En effet, l'ARN à une demi-vie inférieure à celle de l'ADN. Quelques études seulement ont utilisé l'ARN pour caractériser les communautés actives des sédiments marins de sub-surface (Biddle* et al., *2006; Sorensen and Teske, 2006). De plus, des procaryotes impliqués dans le cycle du méthane n'ont été retrouvés que très rarement en dehors des zones de fluides froids et d'hydrates de gaz (Parkes* et al., *2007; Treude* et al., *2005*b*; Thomsen* et al., *2001). Cette étude pluridisciplinaire utilise des techniques de sismique, de sédimentologie, de biogéochimie et de biologie moléculaire, afin de caractériser les sédiments de sub-surface de la baie de Marennes-Oléron.*

# ARCHAEAL METHANE CYCLING COMMUNITIES ASSOCIATED WITH GASSY SUBSURFACE SEDIMENTS OF MARENNES-OLÉRON BAY (FRANCE)

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The authors thank the officers and crew of RV Côtes de la Manche (CNRS/INSU). We also acknowledge anonymous referees who provided comments and suggestions which improved the manuscript. This work was supported by the Université de Bretagne Occidentale, the Centre National de la Recherche Scientifique and the Région Bretagne. We are also grateful to Ouest-Génopole for the use of their facilities. E. Roussel was supported by a grant from the Ministère de la Recherche.

## Abstract

In Marennes-Oléron Bay, a macro-tidal bay located on the French Atlantic coast, kilometer-scale acoustic turbidity reveals an accumulation of free gas in the sediment. Large concentrations of organic matter and rapid sedimentation rates provide ideal settings for biogenic methane cycling. We integrate seismic, sedimentologic, biogeochemical and molecular genetic approaches to determine whether microbial methane cycling is involved in this process. Here we show that the acoustic turbidity upper boundary matched with X-ray facies displaying fissures with the highest methane concentrations, demonstrating the existence of methane bubbles in the sediment. 16S rRNA and *mcrA* gene clone libraries were dominated by sequences affiliated to the three known ANME lineages and to putative methanogens. Sequences related to the marine benthic group B (MBG-B) and miscellaneous crenarchaeotal group (MCG) were also detected. However, the highest methane concentration facies was the only section where active *Archaea* were detected, using reverse-transcribed rRNA, indicating that these communities were involved either directly or indirectly in the methane cycling process. Moreover, three metabolically active novel uncultivated lineages, related to putative methane cycling *Archaea*, could be specifically associated to these methane bearing sediments. As methane cycling *Archaea* are commonly retrieved from deep subseafloor and methane seep sediment, the study of coastal gassy sediments, could therefore help to define the biogeochemical habitats of deep biosphere communities.

## Introduction

Over 85 % of the annual emissions of methane are biogenically produced by methanogenesis in various environments (Reeburgh, 2007; Valentine *et al.*, 2004), such as marine sediments which contain the largest global reservoir of methane (Kvenvolden, 1988; Hovland *et al.*, 1993). Deltas and estuaries commonly constitute appropriate settings for methane production due to the rapid accumulation of sediments with high organic matter concentrations (Fleischer *et al.*, 2001). Recent studies on Marennes-Oléron Bay, a macro-tidal bay located on the French Atlantic coast, have revealed a kilometer-scale acoustic turbidity (Bertin and Chaumillon, 2005). The acoustic turbidity, interpreted as an accumulation of free gas in the sediment, is correlated with a high sediment gain area in excess of 3 m since 1824 and could be related to anthropic processes (Bertin and Chaumillon, 2005; Bertin *et al.*, 2005). The lack of thermogenic activity in the bay, and the high primary production could indicate the generation of biogenic methane on decade to century time-scales (Bertin and Chaumillon, 2005). The present study aims at determining whether the acoustic turbidity in an estuarine environment associated with high sedimentation rate is a consequence of methane accumulation and whether active methane cycling microbial communities are involved in this process.

Methanogenesis is mediated by methanogenic *Archaea* and only occurs in anoxic environments

(Wolfe, 1971). In all known methanogens, the methyl-coenzyme M reductase (MCR) enters the final step in methane synthesis (Ellermann *et al.*, 1988). Therefore, the *mcrA* gene can be used to target methanogens by using molecular techniques (Springer *et al.*, 1995; Luton *et al.*, 2002). However, over 80 % of the biogenic methane that rises from anoxic marine sediment is consumed by anaerobic methane oxidization (AOM) (Orphan *et al.*, 2001*a*), therefore representing 5 to 20 % of the net modern atmospheric methane flux (Valentine and Reeburgh, 2000). Molecular techniques have recently shown that AOM is mediated by anaerobic methane oxidizers (ANME) (Boetius *et al.*, 2000; Hinrichs *et al.*, 1999). Three archaeal clades (ANME-1, ANME-2, ANME-3) oxidize methane under anaerobic conditions either as single cells or associated to sulfate-reducing *Bacteria* (SRB) (Boetius *et al.*, 2000; Niemann *et al.*, 2006; Orphan *et al.*, 2002). ANME could reverse methanogenesis using a modified MCR (Hallam *et al.*, 2003; Hallam *et al.*, 2004). Although, none of these groups have been obtained in pure culture, a few studies have attempted to investigate the effect of environmental factors on AOM activities of different ANME communities (Nauhaus *et al.*, 2007; Nauhaus *et al.*, 2002*b*; Valentine and Reeburgh, 2000).

It is therefore crucial to target the metabolically active communities in order to correlate their phylogeny with variable environmental factors (Biddle *et al.*, 2006; Sorensen and Teske, 2006; Lloyd *et al.*, 2006; Treude *et al.*, 2005*a*). However, DNA-based molecular approaches do not distinguish between living and dead microorganisms (Damste and Coolen, 2006; Dell'Anno and Corinaldesi, 2004). Alternatively, fluorescent in situ hybridization has commonly been used to identify metabolically active microorganisms, but this approach does not provide an overall picture of the active microbial communities. In order to target the active cells from subsurface sediments (Inagaki *et al.*, 2005), extractable archaeal rRNA can be used, as rRNA has a rapid turnover rate (Danovaro *et al.*, 1999; Kramer and Singleton, 1993; Kerkhof and Ward, 1993; Kemp *et al.*, 1993). Only a few studies have used rRNA-derived clone libraries to investigate the diversity of active methane cycling communities in subsurface sediments (Biddle *et al.*, 2006; Sorensen and Teske, 2006). Moreover, few studies, to date, have found microbial methane cycling communities in non seep or gas hydrate coastal sediments (Parkes *et al.*, 2007; Treude *et al.*, 2005*b*; Thomsen *et al.*, 2001). Hence, in this study we conducted an integrated seismic, sedimentologic, biogeochemical and molecular genetic survey of the subsurface gassy sediments of the Marennes-Oléron Bay.

## Materiel and methods

### Site description and sampling

The Marennes-Oléron Bay is located on the French Atlantic coast, immediately north of the Gironde Estuary. This bay is a 150 $km^2$ wide semi-enclosed environment connected to the Atlantic Ocean through the Pertuis d'Antioche to the North and to the Maumusson inlet to the South. Intertidal areas represent 60 % of the bay. Two small rivers, the Charente and the

Seudre, flow into the Marennes-Oléron Bay. Tides affecting the study area are semi-diurnal and range from less than 2 m to more than 6 m. Swells are strongly attenuated by the narrow entrances of the bay, but wind-waves can produce high turbidity in the water column due to wind-driven resuspension.
The Marennes-Oléron Bay, the first oyster farming area in Europe, is an area of high primary production (466 $g.m^{-2}.yr^{-1}$) (Bertin and Chaumillon, 2005). Most of the suspended sediments supplied to the bay are derived from the Gironde Estuary (Froidefond *et al.*, 1998). The sediment budget, computed by subtracting the 1824 profile from the 2004 bathymetric digital elevation model, shows a volume difference of $+120.10^6$ $m^3$ for the last 181 years (Bertin and Chaumillon, 2006). This positive sediment budget shows high sedimentation rates, in agreement with 210 Pb measurement results made on the eastern mudflat of the bay (Gouleau *et al.*, 2000). Very high resolution seismic profiles were recorded in the central Marennes-Oléron Bay during the MOBIDYC5 cruise (2006, CNRS-INSU on board the RV Côte de la Manche). Two cores, M5VC42 and M5VC43 (45°57'N, 01°10'W) were collected on the seismic profile m4b31 within the acoustic turbidity area (Figure 2.3a). M5VC42 was used for sedimentary analysis and M5VC43 for biogeochemical and molecular genetic approaches.

### Sedimentary analysis

The 4 m long M5VC42 core was cut into 1.5 m slabs that were opened transversely, directly photographed, and described according to the Munsell colour chart. Slabs were X-radiographed using the Scopix system (Migeon *et al.*, 1999). Subsamples for grain-size analyses were performed only where X-ray images showed noticeable changes. Grain-size analyses were carried out using a Malvern Mastersizer diffractometer. Correlation between sedimentologic and seismic data was done on the basis of a depth-to-time conversion of the core datasets, according to the relationships between P-wave velocities and sediment grain size (Hamilton, 1972).

### Geochemical analysis

Methane analyses were performed on core M5VC43 at 30, 60, 90, 120, 150, 180, 210, 240 and 300 centimeters below the seafloor (cmbsf) using the headspace technique (Kolb, 1999). The cores were immediately sub-sampled on board using 5 mL syringes (luer end removed) and added to headspace vials (20 mL) filled with a $NaCl/HgCl_2$ work solution. Methane concentrations were determined using a HP 7694 automatic headspace sampler connected to a HP 5890 gas chromatograph equipped with FID and TCD detectors. The 2-sigma uncertainty was better than 4 %. However, methane concentration measurements have to be considered as minimum values as gas escapes occur during core handling. Results were expressed as microlitre per litre wet sediment ($\mu$L/L wet sediment).
Carbon isotope ratio analyses were carried out on sediment samples collected at different levels: 30, 60, 90, 120, 150, 180, 210, 240 and 300 cmbsf in M5VC42. Inorganic carbon was removed

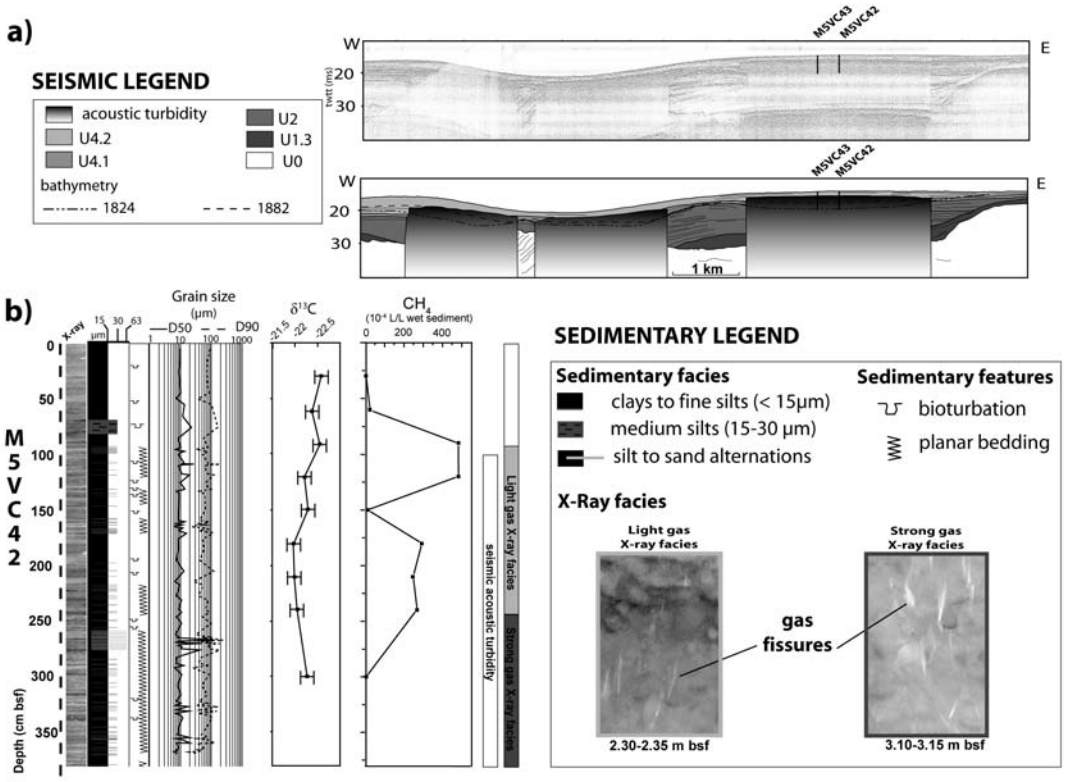


FIG. 2.3: (a) VHR seismic profile m4b31 and its interpretation showing the geometry of the sediment infilling of the northern Marennes-Oléron Bay. Locations of the cores VC42 and VC43 are indicated. Seismic unit U0 corresponds to the bedrock, and seismic units U1.3, U2, U4.1 and U4.2 correspond to the soft sediment incised valley-fill. Locations of the acoustic turbidity areas are indicated. (b) Detailed description of the core VC42. From left to right: elevation in meters (NGF), X-ray image, sedimentary facies, sedimentary features, median grain-size in micrometers, variations in $\delta^{13}$C and methane ($CH_4$) concentration. Correlation with acoustic turbidity and X-ray facies are also indicated.

by acidification (HCl 2M ; 1 mL/100 mg DW). To evaporate excess acid, samples were dried overnight at 60°C under a hood. They were then mixed to 1 mL MilliQ water, freeze-dried and finely grounded using a ball mill. Analyses were performed by CF-IRMS using an Isoprime mass spectrometer (Micromass, UK). Results were reported in the standard $\delta^{13}$C notation relative to the Vienna PeeDee Belemnite standard (VPDB) where $\delta^{13}C = [(R_{sample}/R_{PDB})\text{-}1] \times 10^3$, with $R = {}^{13}C/{}^{12}C$. Sample analytical precision was ±0.1 ‰.

## DNA extractions and PCR amplification

DNA was extracted from 5×1 g of uncontaminated frozen sample, core M5VC43 at 0, 60, 90, 125, 185 and 305 cmbsf, following a modified FastDNA® Spin Kit for Soil (Bio101 Systems, MP Biomedicals™) protocol (Webster *et al.*, 2003). Replicate extractions were pooled, purified

and concentrated in an YM-100 Microcon (Amicon/Millipore™) centrifugal device to give a final volume of 100 $\mu$L.
For the amplification, all manipulations were carried out in a PCR cabinet, using Rnase/Dnase Free Water (MP Biomedicals™) and performed using a GeneAmp PCR system 9700® (Applied Biosystems™). All PCR mixtures (50 $\mu$L) contained 5 $\mu$L of DNA template, 1X Taq DNA polymerase buffer (MP Biomedicals™), 1 $\mu$L of dNTP (10 mM of each dATP, dCTP, dGTP and dTTP), 10 $\mu$M of each primer and 0.5 $\mu$L of Taq DNA polymerase (MP Biomedicals™). Negative controls were also carried out with DNA extractions performed with no sample. For all negative controls, no PCR products were observed. Inhibition of PCR amplification by soluble contaminants in the DNA extracts was also tested as described elsewhere (Juniper *et al.*, 2001). For all DNA extracts, no PCR inhibition was observed.
Archaeal 16S rRNA gene amplification was conducted by nested PCR with combination of primers A8f (5'-CGG TTG ATC CTG CCG GA-3') and A1492r (5'-GGC TAC CTT GTT ACG ACT T-3') in the first round (Casamayor *et al.*, 2000), with A344f (5'-AYG GGG YGC ASC AGG SG-3') and A915r (5'-GTG CTC CCC CGC CAA TTC CT-3') in the second round (DeLong, 1992; Eden *et al.*, 1991). PCR cycles for the first round (A8f/A1492r) were as follows: 1 cycle of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1.5 min at 51°C and 2 min at 72°C and 1 cycle of 6 min at 72°C. PCR cycles for the second round (A344f/A915r) were: 1 cycle of 3 min at 94°C, 30 cycles of 1 min at 94°C, 1.5 min at 57°C and 2 min at 72°C, and 1 cycle of 5 min at 72°C. Five independent PCR products from the first round were pooled and purified (QIAquick PCR purification Kit; Qiagen™) and used as template for the second round. This nested PCR was necessary to obtain visible PCR products on a 0.8 % (w/v) agarose gel stained with ethidium bromide. A portion of the *mcrA* gene was amplified using the ME primers (Hales *et al.*, 1996). To obtain visible amplification products, two rounds with the following reaction conditions were performed: 1 cycle of 5 min at 94°C, 30 cycles of 45 s at 94°C, 1.5 min at 50°C and 2 min at 72°C, and 1 cycle of 10 min at 72°C. An aliquot (5 $\mu$L) of 3 pooled PCR products of the primary amplification was used as templates for the second amplification round.

### RNA extractions and RT-PCR amplification

Total RNA was extracted from each uncontaminated frozen sample (5×1 g) of the M5VC43 core (0, 60, 90, 125, 185 and 305 cmbsf) using the FastRNA® Pro soil direct Kit (Bio101 Systems, MP Biomedicals™) following the manufacturer's instructions. To digest trace amounts of DNA, the extraction products were pooled and 150 $\mu$L were incubated 1 hour at 37°C with 1X of TURBO DNase® buffer and 18U of TURBO DNase® (Ambion™). The digestion was stopped by adding EDTA to a final concentration of 15 mM and heating 10 min at 65°C. The product was finally concentrated and purified with the RNeasy minikit (Qiagen™) according to the manufacturer's instructions to give a final volume of 100 $\mu$L.
The purified RNA product was immediately serially diluted (1 to 50 times) and reverse transcribed using the OneStep RT-PCR kit (Qiagen™), according to the manufacturer's instructions,

with combination of 16S rRNA primers for *Archaea* with A8f (5'-CGG TTG ATC CTG CCG GA-3') and A1492r (5'-GGC TAC CTT GTT ACG ACT T-3') and the following touchdown PCR protocol: 30 min at 50°C, 15 min at 95°C followed by 20 cycles of 1 min at 94°C, 1 min at 58°C (decreasing 0.5°C every cycle), 4 min at 72°C, then followed by 20 cycles of 1 min at 94°C, 1 min at 51°C, 4 min at 72°C and a final amplification step of 10 min at 72°C. To obtain visible products, a nested PCR was performed as described for the 16S rRNA gene amplification. Nested PCR assays, using the 16S rRNA primers for *Archaea*, without the reverse transcription step, showed no DNA contamination.

### PCR-DGGE analysis

In order to characterize the general archaeal community depth structure, a 16S rRNA gene PCR-DGGE analysis was undertaken. Nested PCR was performed as described for the archaeal 16S rRNA gene amplification using primers Saf and PARCH 519R as previously described (Nicol *et al.*, 2003). PCR products were analyzed by DGGE using a DCode Universal Mutation Detection System® (BioRad™) on a 1 mm thick (16×16 cm) 8 % (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 40 %, 37, 5:1, BioRad™). The gel had a denaturant gradient between 30 and 60 % and was prepared with 1X TAE buffer (pH 8, 40 mM Tris Base, 20 mM acetic acid, 1 mM EDTA, MP Biomedicals™) and poured "Gradient maker" (Hoefer SG30®). Electrophoresis was carried in 1X TAE buffer at 60°C for 330 min at 200 V (initially at 80 V for 10 min). The gel was stained with SYBRGold® nucleic acid gel stain for 45 min, washed for 10 min with 1X TAE buffer and scanned using a Phospho fluorimager Typhoon 9400® (Amersham Biosciences™).

### Cloning and sequencing

According to archaeal DGGE profiles, clone libraries were constructed: a) three DNA-derived 16SrRNA gene (DNA65, DNA125 and DNA185), b) two RNA-derived 16S rRNA gene (RNA95 and RNA125), and c) six DNA-derived *mcrA* gene (mcr0, mcr60, mcr90, mcr125, mcr185 and mcr305). The number indicates the depth of each sample (cmbsf). To minimize PCR bias (Polz and Cavanaugh, 1998), five independent PCR products were pooled and purified (QIAquick PCR purification Kit; Qiagen™) and cloned into *Escherichia coli* (XL10-Gold®; Stratagene™) using the pGEM-T Easy vector system I (Promega™) following the manufacturer's instructions. Positive transformants were screened by PCR amplification of the insert using the vector-specific M13 primers. Plasmid extraction, purification and sequencing of the insert, were carried out by the sequencing OUEST-Genopole platform® of Roscoff Marine laboratory (France).

### Phylogenetic analysis

All the 16S rRNA base pair sequences (∼553 bases) were checked for chimeras, using the CHIMERA-CHECK version 2.7 algorithm from the RDP-II (Cole *et al.*, 2003). Out of a

total of 376 sequences (including those from the 16S rRNA gene and *mcrA* gene), 2 were found to be chimeras and were excluded from the phylogenetic analyses. The phylogenetic placement was carried out using NCBI BLAST search program within GenBank (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.*, 1990). The 16S rRNA sequences were then edited in the BioEdit 7.0.5.3 program (Hall, 1999) and aligned using CLUSTALW (Thompson *et al.*, 1994). The phylogenetic trees were constructed by the PHYLO-WIN program (http://pbil.univ-lyon1.fr/) (Galtier *et al.*, 1996) with neighbour-joining method (Saitou and Nei, 1987) and Jukes and Cantor correction. The nonchimeric *mcrA* sequences (∼770 bases) were translated into amino acids using BioEdit and then aligned using CLUSTALW. The PHYLO-WIN program with neighbor-joining algorithm and PAM distance (Dayhoff *et al.*, 1978) was then used for phylogenetic tree construction. For the 16S rRNA and *mcrA* gene phylogenetic reconstruction, the robustness of inferred topology was tested by bootstrap resampling (500), values over 50 % are shown on the trees. The richness from the clone libraries was estimated, with the rarefaction curves at 99 %, 97 % and 95 % sequence identity levels, using the DOTUR program (Schloss and Handelsman, 2005). Operational taxonomic units (OTUs), using a 97 % sequence similarity, were generated with the SON program (Schloss and Handelsman, 2006) and the percentage of coverage (Cx) of the clone libraries was calculated by Good's method (Good, 1953) as described by Singleton and colleagues (Singleton *et al.*, 2001). Statistical estimators, the significance of population differentiation among clone libraries ($F_{ST}$) (Martin, 2002), and the exact tests of population genetic differentiation (Raymond and Rousset, 1995), were calculated using Arlequin 3.11 (Excoffier *et al.*, 2005).

### Nucleotide sequence accession numbers

The sequences are available from GenBank database under the following accession numbers: 16S rRNA gene and rRNA (AM942119 to AM942178) and *mcrA* gene (AM942079 to AM942118).

## Results and discussion

### High methane concentration in the gassy sediments

X-ray images revealed that physical structures were dominated by millimeter to centimeter-thick planar bedding corresponding to strong and low X-ray attenuations. X-ray also showed that sediment was slightly bioturbated (Figure 2.3b). Three major X-ray facies were identified. From the top of the core to 95 cmbsf, F1 X-ray facies consisted of poorly laminated sediments. From 95 to 245 cmbsf, F2 X-ray facies consisted of laminated sediments showing sparse sub-vertical and elongated fissures (about 1 cm long and less than 1 mm wide). The transition from F1 to F2 X-ray facies was close to the acoustic turbidity upper boundary observed on m4b31 seismic profile at about 100 cmbsf (Figure 2.3). From 245 to 380 cmbsf, F3 X-ray facies consisted of laminated sediments showing several sub-vertical and elongated fissures (about 1

cm long and more than 1 mm wide). No such fissures were observed in cores collected outside acoustic turbidity areas (Billeaud *et al.*, 2005). Grain size analyses showed that sediments were mainly composed of black clay to fine silts ($< 15$ $\mu$m) alternating with dark greenish (5Y 4/1) coarse silt (30 to 63 $\mu$m) and sparse very fine sand (63 to 125 $\mu$m) layers. These grain-size alternation layers were often correlated with X-ray attenuation. However the transitions between F1-F2 and F2-F3 X-ray facies did not match with any grain size change.
The acoustic turbidity layer (Figure 2.3a), evidenced by seismic profiles, was correlated with the highest sedimentation rates of the bay (Bertin and Chaumillon, 2005). Moreover, the acoustic turbidity upper boundary matched with the top of F2 X-ray facies displaying fissures and with the highest methane concentrations (Figure 2.3 and 2.4). In contrast, other studies have found the acoustic turbidity upper boundary below the methane peak concentration (Abegg and Anderson, 1997; Thiessen *et al.*, 2006). Hence, both acoustic turbidity and elongated fissures demonstrated the existence of methane bubbles in the sediment (Abegg and Anderson, 1997). The occurrence of gas bubbles suggests that the sediment pore-fluids were saturated with methane. In oversaturated cores, it is well-known that most (up to 95 %) of the methane escapes during core recovery due to the significant decrease of methane solubility related to pressure and degassing (Hensen *et al.*, 2007). This gas loss during ex situ sampling could explain the variation in methane concentration below 120 cmbsf because the bubble repartition and the degassing were probably not homogeneous in the sediment core.

However, the general methane concentration profile in sediment pore-fluids (Figure 2.4b) was similar to the ones found in coastal marine sediments or tidal flats (Parkes *et al.*, 2007; Wilms *et al.*, 2007; Thomsen *et al.*, 2001). At 30 cmbsf, the $CH_4$ concentration was relatively low ($\sim$4 $\mu$L/L wet sediment), probably reflecting the regional background. At 60 cmbsf the $CH_4$ concentration increased with depth, reaching a maximum peak value of 483 $\mu$L/L wet sediment between 90 and 120 cmbsf, which matched the acoustic turbidity upper boundary, and which generally corresponds to the sulfate methane transition zone (SMTZ) (Iversen and Jorgensen, 1985; Thomsen *et al.*, 2001). However, this hypothesis would need to be confirmed by sulfate analyses.
Values of $\delta^{13}$C (-22.0 and -22.6 ‰) were intermediate between values in shallower depths (15-30 cmbsf) of the intertidal mudflats lining the bay (Figure 2.3b): -20.2 ‰ on the western side (Oléron Island), -23.0 ‰on the continental side. Inputs from the continental side thus seem to have more influence on the organic matter origin in the studied cores, assuming negligible changes occurred during diagenesis (Sackett, 1964). On the continental side of the bay, organic matter was mainly of microalgal origin in the surficial layers and consisted of a mixture of sedimented estuarine phytoplankton (-22.7 ‰), of a larger fraction of microphytobenthos (-16 ‰), and of $^{13}$C-depleted terrestrial material (-28 ‰) (Riera and Richard, 1996), while in deeper layers this later became more abundant. A slight and irregular decrease in the organic carbon content was observed with increasing depth. In average 0.83 % C was measured

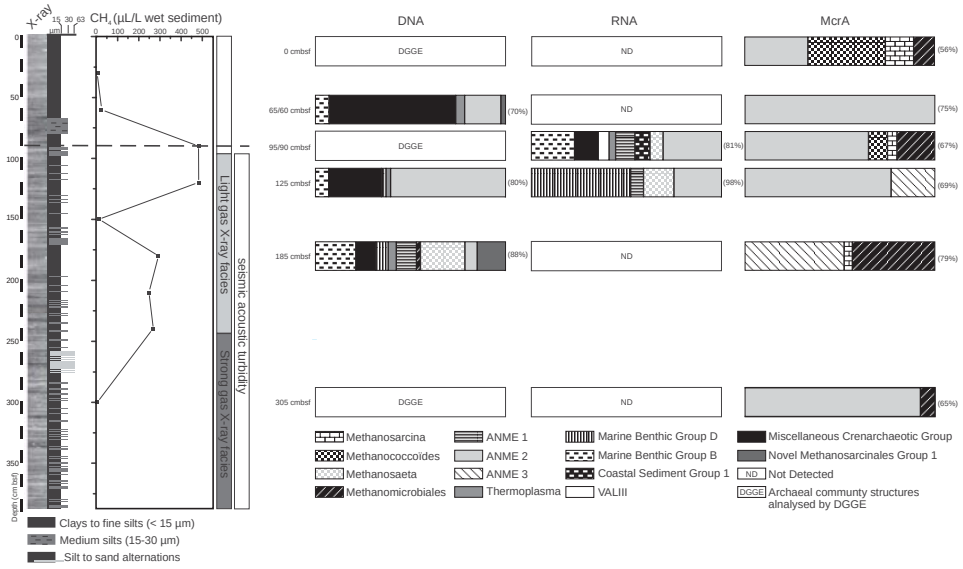


FIG. 2.4: Depth distribution of the archaeal phylogenetic community structures based on 16S rRNA, crRNA and *mcrA* gene compared to X-ray image, sedimentary facies and methane ($CH_4$) concentration. The percentage of coverage of each clone library examined is indicated in brackets. The phylogenetic affiliation of each clone sequence was determined by similarity analysis. The relative abundance of each phylotypes in the clone library was calculated and represented in a column diagram. DNA designs the DNA-derived libraries; RNA, the RNA-libraries; McrA, the *mcrA* gene libraries.

(dry sediment collected in M5VC42 core) which was about twice the 0.46 % C detected in a core collected outside the acoustic turbidity. These relatively large amounts of organic matter combined with the highest sedimentation rates of the bay (Bertin and Chaumillon, 2005) could create ideal settings for active methanogenic microbial communities producing high amounts of biogenic methane (Valentine, 2002).

## Archaeal diversity depth distribution

**Analysis of the clone libraries.** Eleven different clone libraries were constructed, representing a total of 376 sequences. All the DNA-derived 16S rRNA PCR products from all depths were screened by DGGE prior cloning, in order to select the most representative phylogenetic diversities of the core (Webster *et al.*, 2003). Hence, three depths were chosen to construct DNA-derived clone libraries. The whole 16S rRNA sequences, derived from RNA and DNA, were assigned to 48 OTUs based on a 95 % genus level of phylotype differentiation (Schloss and Handelsman, 2004). After technical optimisation, archaeal amplifiable DNA was only retrieved by nested PCR from all depths and 17, 11 and 14 OTUs were respectively assigned to DNA65, DNA125 and DNA185 clone libraries. Amplifiable RNA was detected only from 95 cmbsf and 125 cmbsf depths, which are correlated with the highest methane concentrations. The

two RNA-derived clone libraries from these depths were respectively assigned with 27 OTUs and 5 OTUs. The coverage values for the 16S rRNA clone libraries ranged from 70 to 98 % (Figure 2.4). Rarefaction curves were strongly curvilinear for all the libraries attesting for a sufficient sampling effort (data not shown).
The molecular techniques (PCR and cloning), used to build clone libraries, are known to be inherently biased (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). However, the ratios between the 16S rRNA gene and rRNA per cell have been reported to be proportional to the metabolic activity of the cells, the rRNA content per cell increasing with metabolic activity (Danovaro *et al.*, 1999; Kramer and Singleton, 1993; Kerkhof and Ward, 1993; Kemp *et al.*, 1993). As ANME-1 and *Methanosaeta* lineages were detected in RNA125 library, but absent in DNA125 library, we hypothesized that the sediments at 125 cmbsf could harbor low cell concentrations of very active ANME-1 and *Methanosaeta* lineages. Moreover, insignificant $F_{ST}$ and P tests ($P < 0.01$) suggested that the sequences from the DNA185 library, compared to the RNA95 and RNA125 libraries, were from similar lineage distributions and were indistinguishable from the combined communities (Martin, 2002). DNA derived libraries in marine sediments, built from short 16S rRNA fragments, could result partially from the DNA amplification of dead, quiescent cells (Dell'Anno and Danovaro, 2005; Corinaldesi *et al.*, 2005). For this reason, we suggest that the DNA derived from the different lineages detected at depth was also the result of the accumulation, during the sedimentation process, of the undegraded DNA from dead, quiescent and active communities from the shallower depths. Hence, the ANME-1 and *Methanosaeata* lineages detected in the DNA185 library could be the consequence of an accumulation of DNA from dead or quiescent cells from the shallowest depths (95-125 cmbsf) where they were found active.
The 16S rRNA clone libraries were, on the whole, dominated by sequences related to *Euryarchaeota* (65 %), except for DNA65 where *Crenarchaeota* were a majority (74 %; Figure 2.4). The archaeal phylogenetic diversity was quite high, forming a total of 11 different lineages: ANME-1, ANME-2, *Methanosaeta, Methanomicrobiales, Thermoplasmales*, Marine Benthic Group D (MBG-D), Miscellaneous Crenarchaeotal Group (MCG), Marine Benthic Group B (MBG-B), and 3 novel lineages (Figure 2.5 and 2.6). Unexpectedly, no sequences related to the Marine Group 1 (MG-1) *Archaea*, an ubiquitous *Archaea* in marine sea beds were detected. Furthermore, the general phylogenetic diversity of the shallow gassy sediments of Marennes-Oléron Bay was similar to the one retrieved from methane bearing environments such as cold seeps and gas hydrates (Kendall *et al.*, 2007; Knittel *et al.*, 2005), mainly characterized by a large proportion of methanogens and methane oxidizers. A majority of sequences were related to uncultured environmental clones from these environments (highest similarity to pure culture, 98 %).

**Archaeal methane oxidizing communities.** ANME *Archaea* mediate AOM (Boetius *et al.*, 2000; Hinrichs *et al.*, 1999), and dominated the 16S rRNA (34 %) and the *mcrA* libraries (74 %)

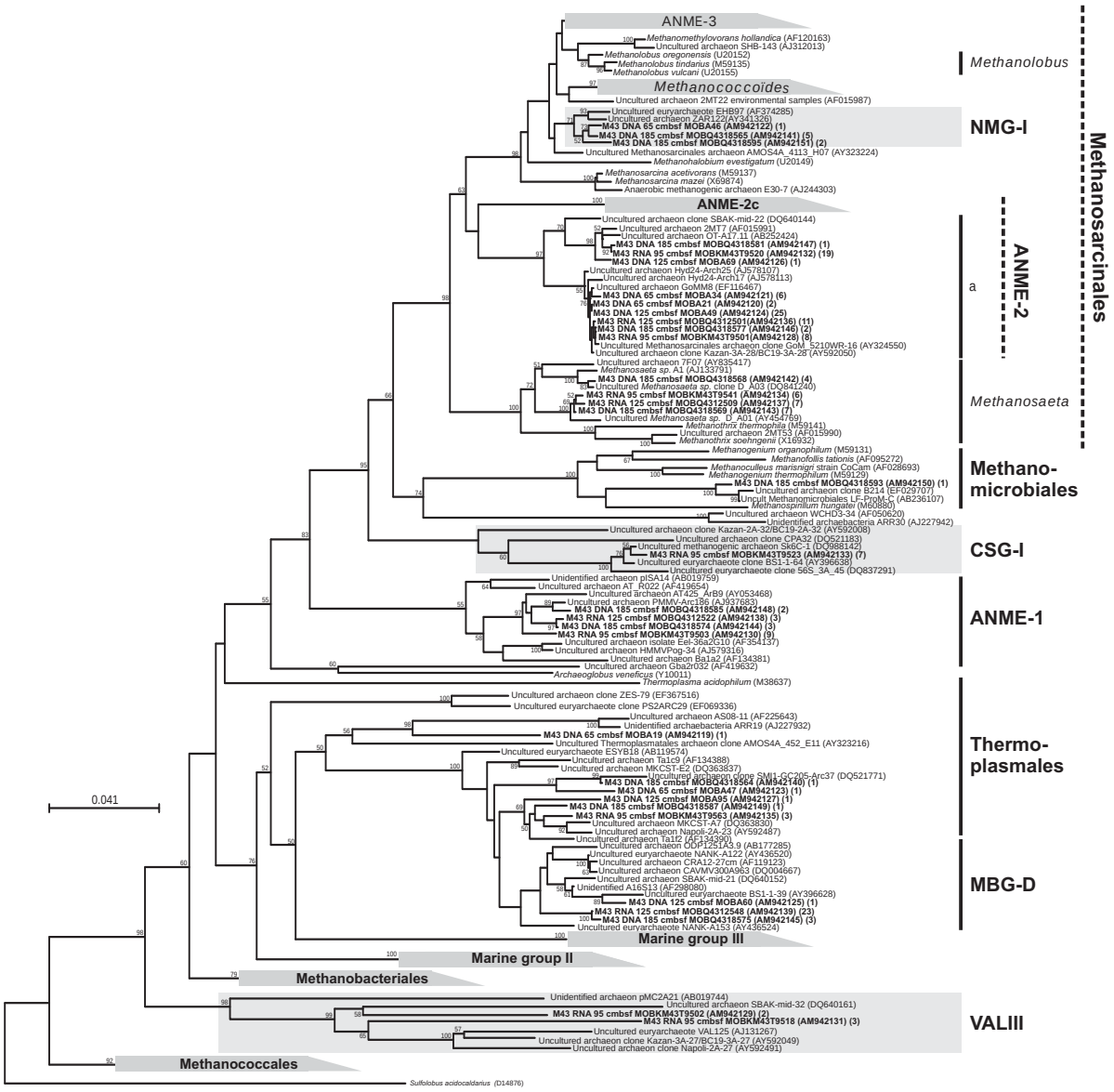


FIG. 2.5: Phylogenetic tree representing the *Euryarchaeota* 16S rRNA gene sequences DNA- and RNA derived, designed by DNA and RNA respectively. Each phylotype is represented by one sequence with $> 97$ % similarity grouping. The tree was constructed using the neighbour-joining method with Jukes and Cantor correction. Bootstrap values $< 50$ % are not shown. CSG-1: Coastal Sediment Group 1, MBG-D: Marine Benthic Group D, NMG-1: Novel Methanosarcinales group 1, VALIII: VAL III cluster (Jurgens *et al.*, 2000).

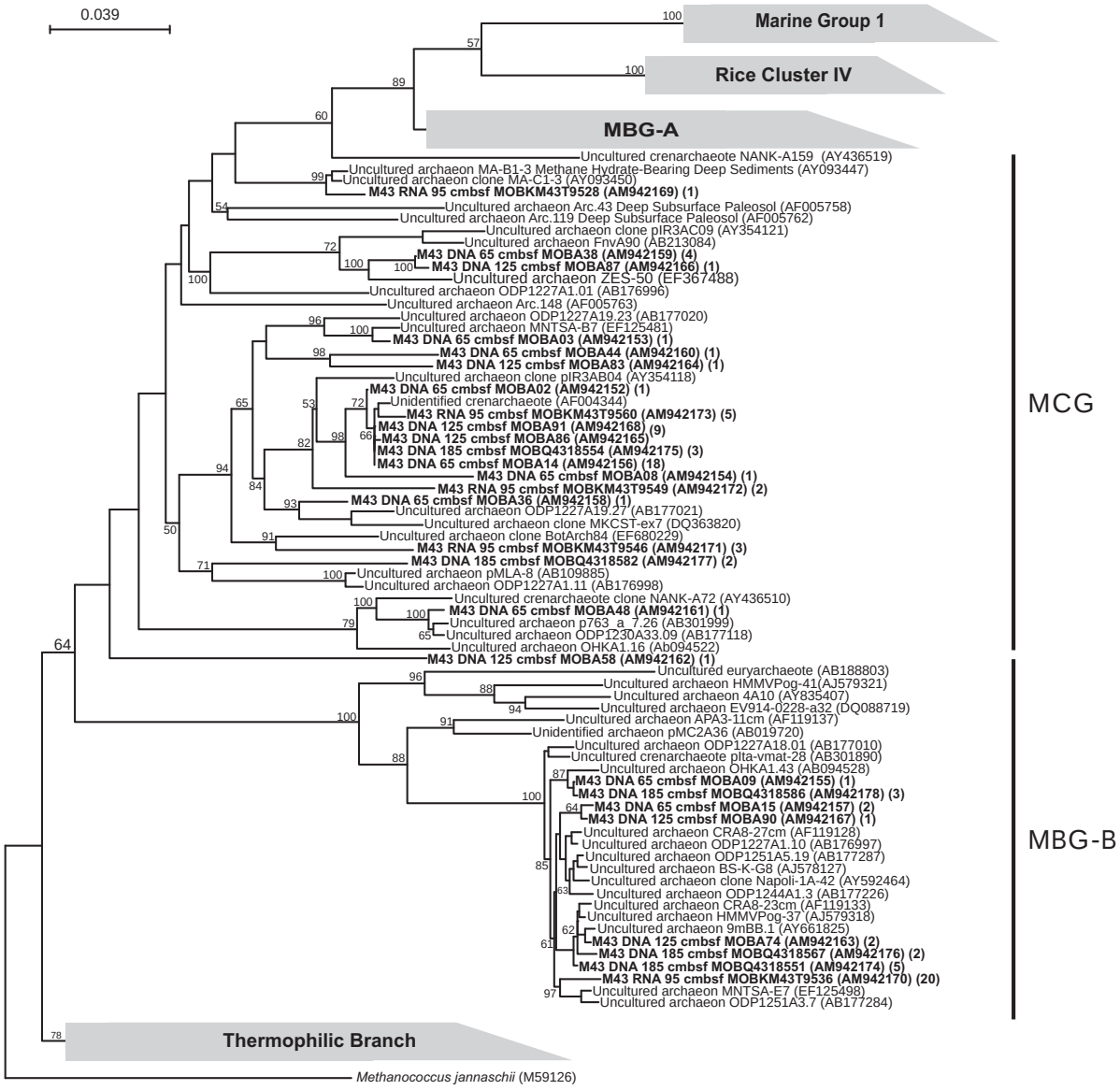


FIG. 2.6: Phylogenetic tree representing the *Crenarchaeota* 16S rRNA gene sequences DNA- and RNA derived, designed by DNA and RNA respectively. Each phylotype is represented by one sequence with $> 97$ % similarity grouping. The tree was constructed using the neighbour-joining method with Jukes and Cantor correction. Bootstrap values $< 50$ % are not shown. MCG: Miscellaneous Crenarchaeotal Group, MBG-B: marine benthic group B, MBG-A: Marine Benthic Group A.

(Figure 2.4). Sequences related to ANME-2a, representing 28 % of the clones in the libraries, were most abundant in the DNA125 (61 %). These sequences were shown to form 2 clusters (Figure 2.5); the first cluster is affiliated to sequences from cold seep environments (Heijs *et al.*, 2007). The second cluster is related to sequences from salt marshes (unpublished) and $CO_2$-hydrate-bearing sediments (Inagaki *et al.*, 2006*a*). Moreover, the 6 *mcrA* libraries (n = 112, coverage = 69 %) were also dominated by sequences related to ANME-2a (61 %), except at 185 cmbsf where they were replaced by ANME-3 (52 %) (Figure 2.4). The sequences related to ANME-2 formed also two clusters within the McrA group e (Figure 2.7). However, ANME-2a sequences from DNA-derived libraries were also present at all depths, whereas ANME-1 were detected only in the deeper parts of the core, (below 95 cmbsf) as previously described in other environments (Knittel *et al.*, 2005; Nunoura *et al.*, 2006). The depth-dependant distribution of ANME-1 tends to support the hypothesis that they are more sensitive to oxygen and to lower temperatures than AMNE 2 (Knittel *et al.*, 2005; Nauhaus *et al.*, 2005). Active ANME-1 were also detected at the highest methane concentrations suggesting that ANME-1 are present at high methane flow rates (Girguis *et al.*, 2005)(Figure 2.4). Moreover, Orcutt and colleagues have recently proposed that ANME-1 could also be involved in methanogenesis depending on the environmental conditions (Orcutt *et al.*, 2005). Although the operon coding for the MCR-I, which includes McrA subunit, is found in all known methanogens (Reeve *et al.*, 1997), ANME-1 and ANME-3 were detected only by one type of primer couple (Figure 2.4), whereas ANME-2a were found among the *mcrA* and 16S rRNA libraries. The absence of *mcrA* sequences related to ANME-1, detected at the same depth 16S rRNA clone libraries, could be the consequence of the lower percentages of coverage of the mcrA libraries or due to a higher number of copies per genome of 16S rRNA gene than *mcrA* (Nunoura *et al.*, 2006).
Moreover, RNA-derived libraries (RNA95 and RNA125) were also dominated by sequences related to these ANME lineages (36 %; Figure 2.4), suggesting that these *Archaea* were also metabolically active. These *Archaea* were detected between 95 and 125 cmbsf, where the methane concentration was the highest. The archaeal communities associated with the other depths were either below the detection limit or less metabolically active. Therefore, AOM could represent one of the major archaeal microbial activities in these sediments.

**Archaeal methanogenic communities.** Sequences affiliated to archaeal methanogenic lineages represented an important component of the total libraries (26 %) and were composed of *Methanococcoïdes, Methanosarcina, Methanosaeta* and *Methanomicrobiales* (Figure 2.4). However, putative methanogens represented less than 10 % of the 16S rRNA libraries and were found only below the highest methane concentration zone (95 cmbsf). A large proportion of these sequences (9 %) were related to the *Methanosaeta* lineage. Only one sequence is related to the *Methanomicrobiales* lineage. In *mcrA* libraries, the depth distribution of the methylotrophic *Methanococcoïdes* and *Methanosarcina* decreased with depth and seems to be therefore restricted to the shallowest part of the core. The methanogens utilizing C1 compounds usually

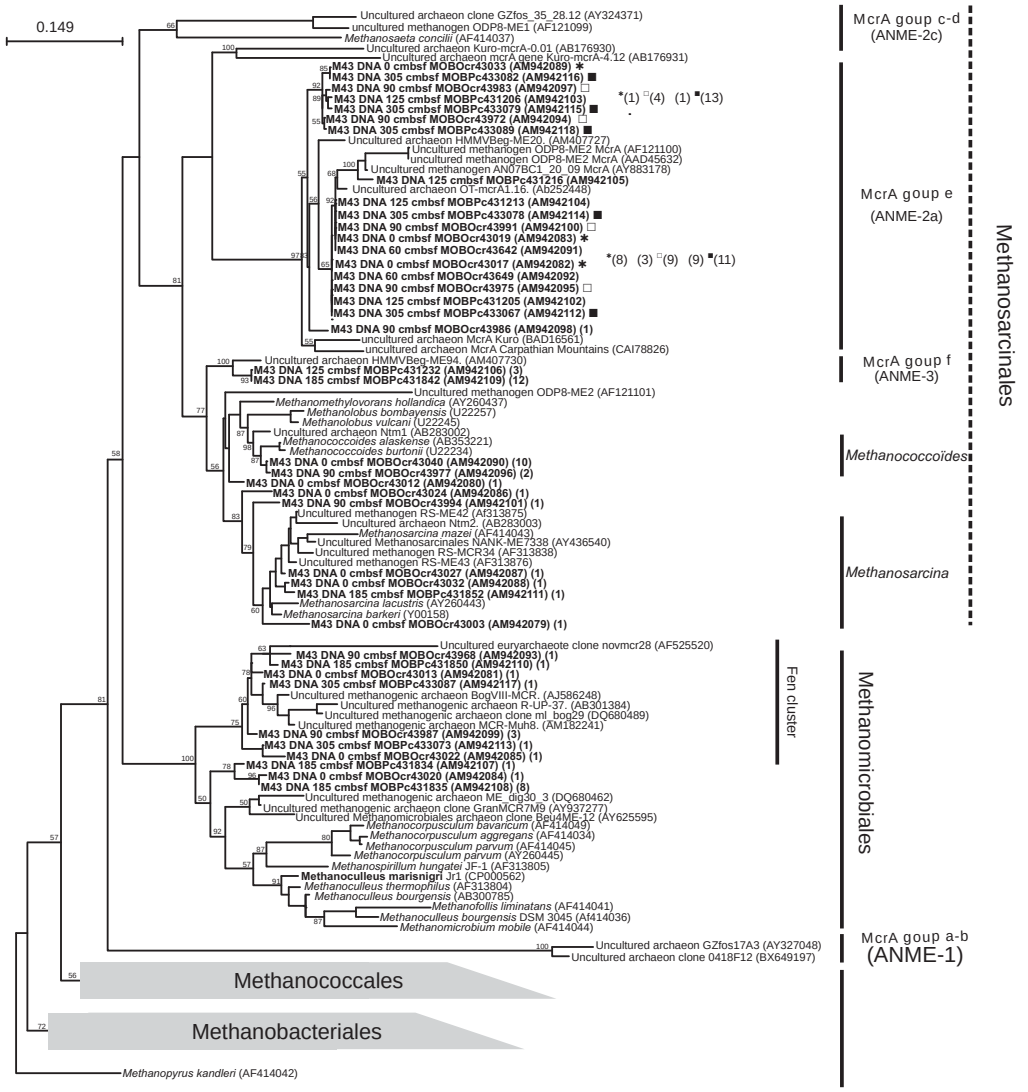


FIG. 2.7: Phylogenetic tree based on translated, partial amino acid sequences of *mcrA* gene ($<$ 260 amino acids). The tree was constructed using the neighbour-joining method using PAM distance (Dayhoff *et al.*, 1978). The robustness of inferred topology was tested by the bootstrap. Bootstrap values $<$ 50 % are not shown.

dominate the marine methanogens within the zone of sulfate reduction since sulfate reducing *Bacteria* (SRB) do not compete for the same substrates (Purdy *et al.*, 2003). Conversely sequences related to *Methanosaeta*, an acetoclastic methanogens (Ma *et al.*, 2006), were detected only below 95 cmsf (Figure 2.4), given that SRBs out-compete acetoclastic methanogens in the zone of sulfate reduction. Nine sequences related to the $H_2/CO_2$-utilizing *Methanomicrobiales* (Zellner *et al.*, 1999) were detected at all depths even though they were found in higher proportions in the libraries at 185 cmbsf and branched in the Fen cluster (Juottonen *et al.*, 2006), a group of environmental clones from peatlands (Figure 2.4 and 2.7).

**Subsurface sediment Archaeal communities.** The *Crenarchaeota* sequences were related to the Marine Benthic Group B (MBG-B) and Miscellaneous Crenarchaeotic Group (MCG) lineages (Figure 2.6), representing 35 % of the 16S rRNA clone libraries, and were also detected among the core (Figure 2.4). The proportion of MBG-B sequences in the libraries increases with depth (Figure 2.4) and forms two distinct clusters with environmental clones (Figure 2.6) . The MBG-B *Archaea*, a deep-branching lineage within *Crenarchaeota*, were found to be most active where the methane concentration was the highest (95 cmbsf). MBG-B, also synonymous of Deep Sea Archaeal Group (DSAG), have been previously detected in a wide range of anoxic marine environments (Takai and Horikoshi, 1999; Lloyd *et al.*, 2006; Biddle *et al.*, 2006; Sorensen and Teske, 2006). Recent studies have also revealed that MBG-B were metabolically active within the SMTZ, suggesting that MBG-B were directly or indirectly linked to AOM (Biddle *et al.*, 2006; Inagaki *et al.*, 2006*b*; Sorensen and Teske, 2006; Teske and Sorensen, 2008). MCG sequences dominated the shallowest library (DNA65) (67 %) and were also found, in smaller proportions, in all libraries (Figure 2.4) . The MCG, an ubiquitous lineage in marine sediments, were also active at 95 cmbsf, and are thought to be heterotrophic anaerobes utilizing complex organic substrates (Teske and Sorensen, 2008).
Sequences related to uncultured *Thermoplasmales* and MBG-D lineages were also found in small proportions in the DNA-retrieved libraries (7 %), at all depths. However, sequences related to MBG-D dominated (52 %) the RNA-derived library below 95 cmsf. This lineage within the *Thermoplasmatales* is also frequently found in methane seep sediments (Knittel *et al.*, 2005; Orphan *et al.*, 2001*a*) and deep marine sediments (Parkes *et al.*, 2005; Sorensen and Teske, 2006).

**Novel archaeal lineages.** In the present study, three potential new lineages with no closely related culture isolates were detected in high methane concentration zones. The first novel lineage, formed an independent cluster within the methane cycling *Methanosarcinaceae* family. This group of 8 sequences were closely related to environmental clones from low salinity methane-harboring environment such as a sulfide rich spring (Elshahed *et al.*, 2004) and estuary sediments (Purdy *et al.*, 2002). This new potential cluster, distantly related to the methylotrophic *Methanolobus oregonensis* (96 % similarity), was named Novel *Methanosarci-*

*nales* group 1 (NMG-1).

The detection by RNA-based approaches of active novel lineages in methane-bearing environments led to the discovery of the ANME lineages (Niemann *et al.*, 2006; Hinrichs *et al.*, 1999; Orphan *et al.*, 2001*b*). Interestingly, the RNA-derived sequences usually fell with the same phylotypes as the DNA-derived sequences, except for 2 groups of sequences that formed novel lineages (Figure 2.5). These two novel lineages were found metabolically active with a majority of the methane cycling communities (AMNE, MBG-B and *Methanosaeta*), where the sediments harbored the highest methane concentrations. Moreover the second novel lineage, designed as the Coastal Sediment group-1 (CSG-1), deeply branching within *Euryarchaeota* phylum, was distantly related to the *Mehanosarcinales* family (Figure 2.5). The CSG-1 only grouped with a few environmental clones, and was distantly affiliated to the new methylotrophic methanogen *Methermicoccus shengliensis* (highest similarity to pure culture, 86 %). The KM43T9523 sequence, which groups with the CSG-1 novel lineage, was closely related (98 % similarity) to the environmental clones from tidal flats (Kim *et al.*, 2005) and coastal sediments (Parkes *et al.*, 2007) where methane cycling *Archaea* also occurred (Figure 2.5). The third cluster groups with the VAL III sequences, a novel lineage distantly related to *Methanobacteriales* and *Methanococcales* (Jurgens *et al.*, 2000). The KM43T9502 and KM43T9518 sequences (highest similarity to cultured relative, 76 %) were related to environmental clones (85 % similarity) from mud volcanoes and deep marine sediments where methane also occurred (Heijs *et al.*, 2007; Sorensen and Teske, 2006). As all three lineages were related to methanogens or environmental clones found in methane-bearing environments, and as two were only found active at the highest methane concentrations, we suggest these lineages could be methane cycling *Archaea*.

## Summary

In Marennes-Oléron Bay, the areas with the highest sedimentation rates and the highest organic matter concentrations harbored kilometer-scale acoustic turbidity zones related to the presence of free methane. In these gassy sediments, the archaeal diversity was dominated by metabolically active methane cycling communities (ANME, methanosarcinales, methanomicrobiales and MBG-B) congruently with the highest methane concentrations. Moreover, metabolically active novel uncultivated lineages, associated to putative methane cycling *Archaea*, could be specifically associated to methane-bearing sediment habitats. As these lineages are also typical deep subseafloor and methane seep sediment communities, the study of coastal gassy sediments submitted to high environmental and anthropic variables, which modify the microbial niches, would help to define the biogeochemical habitats of the deep biosphere communities.

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## 2.3 Archaeal communities associated with shallow to deep sub-seafloor sediments of the New-Caledonia Basin

# Environmental Microbiology, (in revision)

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Owing to technical limitations, such as fine scale sampling in the range from the first centimetres of sediments to several metres, the sequential distribution of prokaryotic communities in deep marine sediment is poorly investigated, and their interaction with the Geosphere remain unclear. Moreover, in previous studies, putative thermophilic *Archaea* were suggested to represent indicators of hot subseafloor ecosystems.

The CALYPSO giant gravity piston corer (IPEV) is able to recover sediment cores up to 70 m long without removing the first centimetres of the surface sediments. Hence, during the ZoNéCo 12 scientific cruise, sediment cores from several different sites were recovered from the New-Caledonia and Fairway Basins. The current study investigates some of the spatial and abiotic variables that could possibly control the archaeal community distribution and activities from the surface to deep sub-seafloor marine sediments. According to their geological context, six cores were analysed using an integrated seismic, sedimentologic, biogeochemical and molecular genetic survey. Moreover, in order to search for putative indicators of deep hot subseafloor ecosystems, sediment cores retrieved from highly faulted zones were also analysed. The analysis of the archaeal diversity and community structures was performed on surface and subsurface sediments, with different terrestrial influences, using denaturant gradient gel electrophoresis (DGGE) and cloning PCR-amplified taxonomic and functional genes. The active fraction of the archaeal community in deep-sea sediments was also assessed by a new molecular approach based on DGGE.

*La distribution séquentielle des communautés microbiennes dans les sédiments allant de la surface à la sub-surface ainsi que leur interaction avec la géosphère sont très mal connues principalement pour des raisons techniques. De plus, quelques études ont suggéré que certaines Archaea thermophiles pourraient être des représentants d'écosystèmes de sub-surface profonds et chauds.*

*Le carottier géant CALYPSO (IPEV) permet d'obtenir des carottes sédimentaires de plus de 70 mètres de long tout en ne perturbant que faiblement les sédiments de surface. Durant la campagne océanographique ZoNéCo12, plusieurs carottes sédimentaires des bassins de Nouvelle-Calédonie et Fairway, ont pu être prélevées dans différentes zones. Cette étude a permis l'analyse de facteurs biotiques et abiotiques contrôlant la distribution des communautés archéennes de la surface jusqu'à la sub-surface de sédiments marins profonds. En fonction de leur contexte géologique, six carottes de sédiment ont été analysées via des techniques de sismique, de sédimentologie, de biogéochimie et de biologie moléculaire. Des représentants potentiels d'un écosystème de sub-surface profond et chaud ont aussi été recherchés dans des zones de couches sédimentaires fortement fracturées. L'analyse de la diversité archéenne a été réalisée grâce à des techniques d'empreintes moléculaires (DGGE) et de PCR-Clonage sur des sédiments de surface et de sub-surface présentant une composition variable en apports terrigènes. La fraction active des communautés archéennes a aussi été déterminée grâce à une nouvelle approche basée sur le principe de la DGGE.*

# ARCHAEAL COMMUNITIES ASSOCIATED WITH SHALLOW TO DEEP SUB-SEAFLOOR SEDIMENTS OF THE NEW-CALEDONIA BASIN

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## Abstract

The distribution of the archaeal communities in cold deep sub-seafloor open-ocean sediments (0-36 mbsf) from the New Caledonia Basin (Oceanographic cruise ZoNéCo 12, February 2006) was investigated using DNA- and RNA-derived 16S rRNA clone libraries, functional genes, and denaturing gradient gel electrophoresis (DGGE). A new method, Co-Migration DGGE (CM-DGGE) was used to access the active archaeal diversity of low biomass deep marine sediments. Here we show that active Marine Group 1 (MG-1) *Archaea*, involved in the nitrogen cycle through ammonium oxidation, dominated the suboxic surface archaeal community (0 to 1.5 mbsf). However, the anoxic sub-surface methane-poor sediments (below 1.5 mbsf) were dominated by less active archaeal communities probably involved in the methane cycle (*Methanosarcinales*, ANME-2 and DSAG/MBG-B). Moreover, this study provides the first evidence of living *Thermococcales* in deep cold sub-seafloor sediments. These putative thermophilic microorganisms formed a new cluster within the *Thermococcales* (TG-1) and could have been disseminated by rafting terrestrial matter originating from thermal vents of the New Caledonia atoll. Furthermore, 16S rDNA sequences retrieved from a strongly faulted cold seafloor, probably associated to fluid migration, formed the "Deep Sub-Seafloor *Thermococcales* Group" (DSSTG) a unique divergent *Thermococcales* cluster closely affiliated (99.6 %) to the deepest (1,626 mbsf) microorganisms ever detected in deep marine sediments. This new cluster associated to deep sub-surface sediments may represent the evidence of a deep hot biosphere community.

## Introduction

The sub-seafloor biosphere may comprise as much as two thirds of Earth's total prokaryotic biomass (Whitman *et al.*, 1998) and extends to at least 1,626 meters below the seafloor (mbsf) (Roussel *et al.*, 2008). Ubiquitous microbial communities present in the sub-seafloor play an important role in global biochemical cycles (D'Hondt *et al.*, 2004; D'Hondt *et al.*, 2002). The prokaryotic cell density drastically decrease with depth and decreasing available energy supply due to reduction in efficient electron acceptors and bioavailable organic carbon sources (Parkes *et al.*, 2000; Schippers *et al.*, 2005). However, local increases in cell density occur in response to the adaptation of microbial communities to specific geochemical environments (D'Hondt *et al.*, 2004; Sorensen and Teske, 2006; Parkes *et al.*, 2005).

The boundary between surface and subsurface could be defined as a change of the microbial community composition, shifting from surface (e.g. the water column) to deep subsurface communities (Teske and Sorensen, 2008). Moreover, microbial metabolic processes in marine sediments are stratified according to sequential consumption of the electron acceptors diffusing into sediments from the overlying seawater. Oxygen, the main electron acceptor in surface sediment, is rapidly depleted, followed by nitrate and manganese (Froelich *et al.*, 1979). How-

ever, in deeper anoxic sediments, sulfate reduction and methanogenesis represent the principal metabolic processes in the deep sub-seafloor (D'Hondt *et al.*, 2004; D'Hondt *et al.*, 2002; Parkes *et al.*, 2005). Organic rich coastal sediments, under terrestrial influence, harbour higher microbial densities and activities compared to open-ocean sediments (D'Hondt *et al.*, 2004). Hence, there appears to be a correlation between the origin of the organic matter and the biochemical processes, such as methanogensis (Sivan *et al.*, 2007). Active archaeal communities, involved in biochemical cycles such as methane cycling at depth, could represent the dominant microbial community of the deep marine subsurface (Biddle *et al.*, 2006; Sorensen and Teske, 2006; Mauclaire *et al.*, 2004).

The distribution and metabolisms of the sub-seafloor microbial communities are mostly understood through culture-independent techniques (Biddle *et al.*, 2006; Fry *et al.*, 2006; Newberry *et al.*, 2004; Parkes *et al.*, 2005; Sorensen and Teske, 2006, for e.g.), since most of these prokaryotes do not have a closely related cultured relative. In shallow marine sediments, DNA-based molecular approaches are strongly biased as up to 90 % of the total DNA is extra cellular (Dell'Anno and Danovaro, 2005; Danovaro *et al.*, 1999), resulting in an inability to distinguish between living and dead microbial communities (Damste and Coolen, 2006; Dell'Anno and Corinaldesi, 2004). In order to target metabolically active communities, to correlate their phylogeny with variable environmental factors (Biddle *et al.*, 2006; Sorensen and Teske, 2006; Lloyd *et al.*, 2006; Treude *et al.*, 2005*a*), fluorescent *in situ* hybridization has commonly been used. However this approach does not provide an overall picture of the active microbial communities. As rRNA has a rapid turnover (Danovaro *et al.*, 1999; Kramer and Singleton, 1993; Kerkhof and Ward, 1993; Kemp *et al.*, 1993), extractable archaeal rRNA can be used to target the active cells from subsurface sediments (Inagaki *et al.*, 2005).

The current study investigates some of the spatial and abiotic variables that could possibly control the archaeal community distribution and activities in deep-sea sediments. The analysis of the archaeal diversity and community structures was performed on surface and subsurface sediments from the New-Caledonia and Fairway Basins, with different terrestrial influences, using denaturant gradient gel electrophoresis (DGGE) and cloning PCR-amplified taxonomic and functional genes. The active fraction of the archaeal community in deep-sea sediments was also assessed by a new molecular approach based on DGGE.

## Results

### Site description and mineralogical composition of sediments

The sediments were collected from six sites in the New Caledonia and Fairway basins (Figure 2.8). Site MD06-3018 sediments, mainly situated in an open-ocean context, contained homogeneous sediment facies composed of foraminiferal clay (Foucher *et al.*, 2006). Conversely site MD06-3019 sediments, located in a canyon deriving a substantial amount of terrigenous matter from New Caledonia, comprised a succession of terrigenous sequences composed of dark

carbonate clay interspaced with sands (Foucher *et al.*, 2006). MD06-3019 sediments had higher contents of minerals with a probable detrital origin such as quartz, chlorite, kaolinite, montmorillonnite, halite and albite, than MD06-3018 sediments, which displayed higher contents of calcite (Table S1). Probable authigenic minerals (aragonite and clinoenstatite) were only detected in shallowest sediments of MD06-3019 (Table S1). Sites MD06-3022, MD06-3026, MD06-3027 and MD06-3028 sediments were located in an open-ocean context in the Fairway Basin. Sites MD06-3026 and MD06-3027 represented reference zones, conversely to MD06-3022 and MD06-3028 where the sedimentary sequence was pierced by a diapir (Figure S1) and presented an extensive faulting of the sedimentary cover which could be the consequence of gas migration through the sediment cover (Auzende *et al.*, 2000).

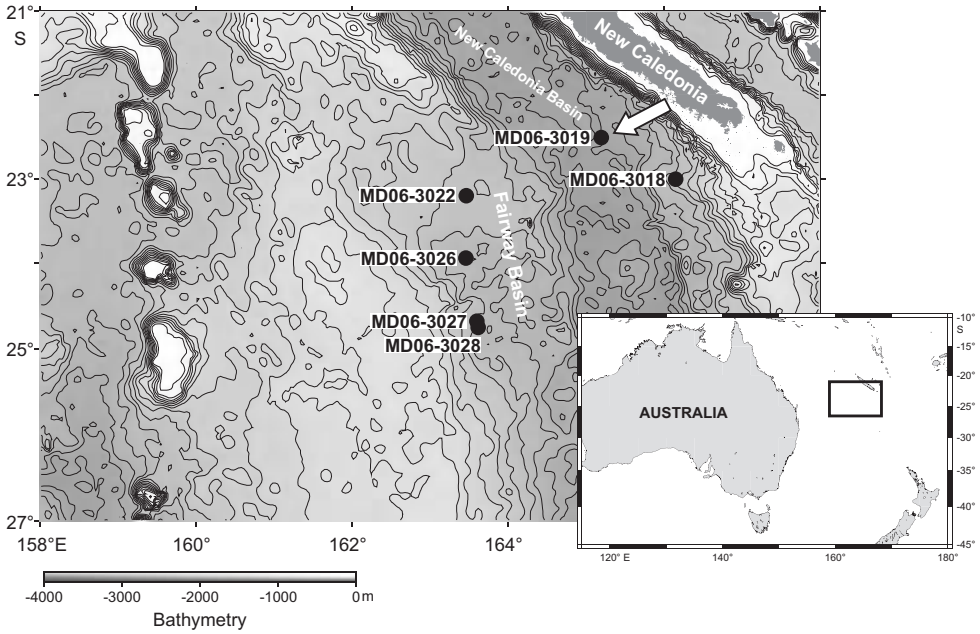



FIG. 2.8: Bathymetric map of the location of ZoNéCo 12 sites. The white arrow represents substantial amounts of terrigenous matter derived from New Caledonia.

## Geochemistry and total prokaryotic count depth profiles

The *in situ* sediment temperatures at all sites were in a range between 2 to 3°C (Foucher *et al.*, 2006). At site MD06-3019, sodium ($r = 0.919$; $P < 0.0001$) and chloride ($r = 0.774$; $P < 0.0001$) concentrations increased, whereas sulphate ($r = -0.913$; $P < 0.0001$) and calcium ($r = -0.931$; $P < 0.0001$) concentrations decreased with increasing depth (Figure 2.9). Methane was the only volatile hydrocarbon detected. Though concentrations at all sites were very low, the methane concentrations at sites MD06-3022 ($r = 0.996$; $P < 0.001$), MD06-3026 ($r = 0.881$; $P < 0.05$) and MD06-3028 ($r = 0.999$; $P < 0.0001$) were correlated with increasing depth (Figure 2.9). On

the whole, the prokaryotic cell counts were within the limits of the Parke's regression equation, even if the distribution at site MD06-3019 was more heterogeneous than at the open-ocean sites (Figure 2.9). The prokaryotic abundances at the open-ocean sites (MD06-3022, MD06-3026 and MD06-3028) were $\sim$ 3.5 times lower than at the site under terrestrial influence (MD06-3019). No correlation was found between prokaryotic counts and biogeochemical data except at site MD06-3028 where prokaryotic counts were correlated with depth (r = -0.814; $P < 0.05$).

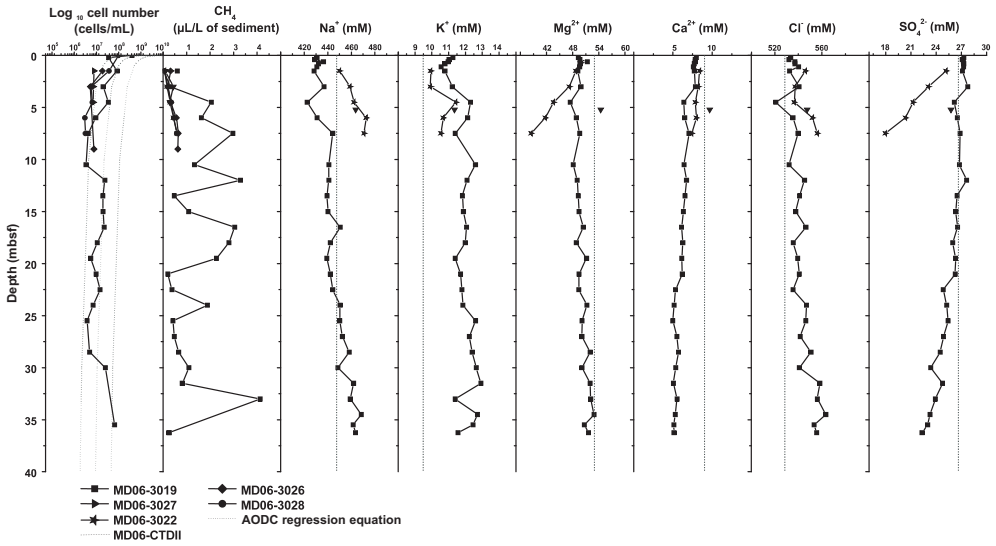



FIG. 2.9: Geochemical and total prokaryote cell depth profiles of the sediments from the Fairway and New Caledonia Basin sites.

## Archaeal community structure and diversity depth distributions

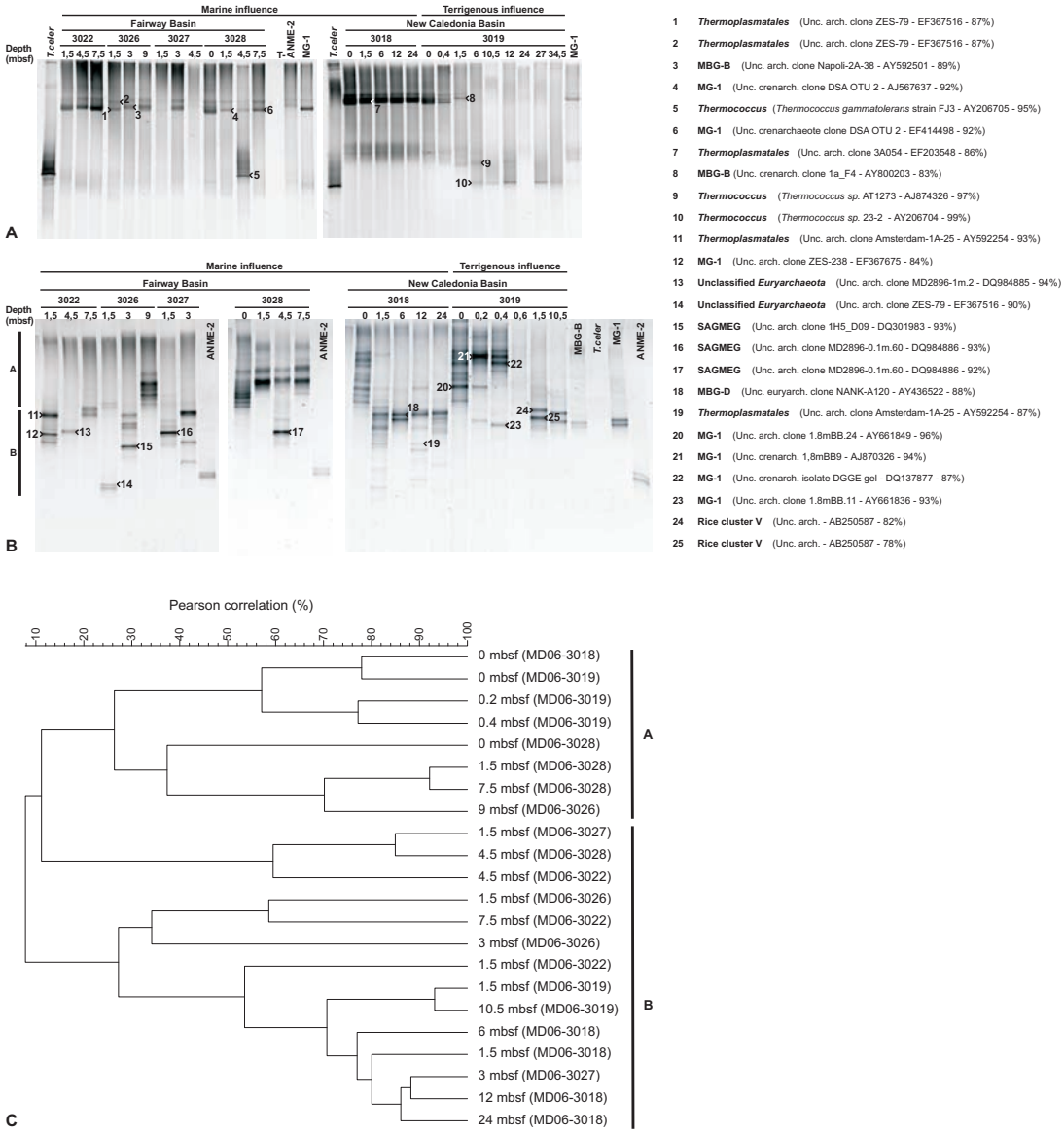
Archaeal community structures of all samples were assessed by PCR-DGGE analysis of 16S rRNA gene fragments. As the degree of separation between DGGE fragments decreases with size due to the melting of multiple melting domains in the larger fragments (Myers and Cox, 1988; Sheffield *et al.*, 1989), two sets of primers (A344fGC-A915r and Saf-PARCH 519r) and gel conditions were optimised in order to obtain different scales of band discrimination (unpublished data). Hence, A344fGC-A915r was characterized by low band discrimination, and was used to compare archaeal communities with a high GC content difference (Figure 2.10A), whereas Saf-PARCH 519r was used to discriminate communities with a similar GC content (Figure 2.10B). However, the PCR surveys of archaeal communities using the Saf-PARCH 519r primer set were probably less biased than with A344fGC-A915r primer set, as there is a lower number of primer mismatches with specific subsurface and hydrothermal vent archaeal sequences (Teske and Sorensen, 2008).

DGGE patterns, using A344fGC-A915r, between 0 to 1.5 mbsf at the open-ocean sites (MD06-

3018, MD06-3022, MD06-3026, MD06-3027 and MD06-3028), and site MD06-3019, displayed similar fragments with low GC content affiliated to *Thermoplasmatales*, MBG-B and MG-1 (Figure 2.10A). A transition in the community structure was observed at site MD06-3019 at 0.6 mbsf (Figure 2.16), between a 16S rRNA archaeal community with low GC content and another with higher GC content, related to the *Thermococcales* order (Figure 2.10A and 2.16). However, *Thermococcales* were the unique detected phylotype below 1.5 mbsf at site MD06-3019 (Figure 2.10A).
The resulting dendrograms of DGGE patterns, using Saf-PARCH 519r, displayed two major clusters (Figure 2.10C). The Cluster A depths ranged from 0 to 1.5 mbsf, except for two samples (7.5 and 9 mbsf). Cluster A fragments were exclusively affiliated to MG-1, and were characterized by a lower GC content than cluster B (Figure 2.10B), which was composed of fragments related to 6 different phylotypes (Rice cluster V, *Thermoplasmatales*, MBG-D, SAGMEG, unclassified *Euryarchaeota*).

### Statistical analyses of the clone libraries

Twenty five different clone libraries were constructed, representing a total of 771 sequences. All the DNA-derived 16S rRNA PCR products from all depths were screened by DGGE prior cloning, in order to select the depths with the most representative archaeal phylogenetic distribution of each core (Webster *et al.*, 2003). Whole 16S rRNA sequences, derived from RNA and DNA, were assigned to 71 OTUs based on a 95 % genus level of phylotype differentiation (Schloss and Handelsman, 2004). Amplifiable DNA was obtained from all depths, and 1 to 13 OTUs were respectively assigned per clone library. The DNA-derived clone libraries were named DNAxTy, where x represents the core number and y the depth of the clone library. Amplifiable RNA was only obtained above 1.5 mbsf and was not detected at any other depth (Figure 2.12 and 2.16). Thus, two RNA-derived clone libraries, named RNA18T150 (MD06-3018, 1.5 mbsf) and RNA19T150 (MD06-3019, 1.5 mbsf), were respectively assigned with 27 OTUs and 5 OTUs. The coverage values for the 16S rRNA clone libraries ranged from 18 to 100 % (Figure 2.11). Rarefaction curves were strongly curvilinear for all clone libraries attesting adequate sampling, excepted for DNA19T150 and DNA19T4 as a result of strong intra-lineage diversities (see supplementary material, Figure S2). Differences between all DNA-derived clone library diversity indices ($F_{ST}$ and the exact test method) were statistically significant ($P < 0.01$) except between 13 clone libraries distributed among 3 groups, as described in figure 2.11. Differences between all the DNA-derived and the RNA-derived clone library diversity indices ($F_{ST}$ and the exact test method) were statistically significant ($P < 0.01$) except between clone libraries DNA19T0, DNA19T2, DNA19T4, RNA18T150 and RNA19T150




1 ***Thermoplasmatales*** (Unc. arch. clone ZES-79 - EF367516 - 87%)
2 ***Thermoplasmatales*** (Unc. arch. clone ZES-79 - EF367516 - 87%)
3 **MBG-B** (Unc. arch. clone Napoli-2A-38 - AY592501 - 89%)
4 **MG-1** (Unc. crenarch. clone DSA OTU 2 - AJ567637 - 92%)
5 ***Thermococcus*** (*Thermococcus gammatolerans* strain FJ3 - AY206705 - 95%)
6 **MG-1** (Unc. crenarchaeote clone DSA OTU 2 - EF414498 - 92%)
7 ***Thermoplasmatales*** (Unc. arch. clone 3A054 - EF203548 - 86%)
8 **MBG-B** (Unc. crenarch. clone 1a_F4 - AY800203 - 83%)
9 ***Thermococcus*** (*Thermococcus sp.* AT1273 - AJ874326 - 97%)
10 ***Thermococcus*** (*Thermococcus sp.* 23-2 - AY206704 - 99%)
11 ***Thermoplasmatales*** (Unc. arch. clone Amsterdam-1A-25 - AY592254 - 93%)
12 **MG-1** (Unc. arch. clone ZES-238 - EF367675 - 84%)
13 **Unclassified *Euryarchaeota*** (Unc. arch. clone MD2896-1m.2 - DQ984885 - 94%)
14 **Unclassified *Euryarchaeota*** (Unc. arch. clone ZES-79 - EF367516 - 90%)
15 **SAGMEG** (Unc. arch. clone 1H5_D09 - DQ301983 - 93%)
16 **SAGMEG** (Unc. arch. clone MD2896-0.1m.60 - DQ984886 - 93%)
17 **SAGMEG** (Unc. arch. clone MD2896-0.1m.60 - DQ984886 - 92%)
18 **MBG-D** (Unc. euryarch. clone NANK-A120 - AY436522 - 88%)
19 ***Thermoplasmatales*** (Unc. arch. clone Amsterdam-1A-25 - AY592254 - 87%)
20 **MG-1** (Unc. arch. clone 1.8mBB.24 - AY661849 - 96%)
21 **MG-1** (Unc. crenarch. 1,8mBB9 - AJ870326 - 94%)
22 **MG-1** (Unc. crenarch. isolate DGGE gel - DQ137877 - 87%)
23 **MG-1** (Unc. arch. clone 1.8mBB.11 - AY661836 - 93%)
24 **Rice cluster V** (Unc. arch. - AB250587 - 82%)
25 **Rice cluster V** (Unc. arch. - AB250587 - 78%)

FIG. 2.10: Denaturant gradient gel electrophoresis (DGGE) analysis of archaeal 16S rRNA genes of the cores from the Fairway and New Caledonia Basin sites. The numbered bands were excised and sequenced. The lineage and the sequence similarity of the closest match by BLASTN search are given on the right. (A) PCR products were amplified with the A344fGC-A915r primer set and electrophoresis was performed using a gradient of 40–70 % denaturant. (B) PCR products were amplified with the Saf-PARCH 519r primer set and electrophoresis was performed using a gradient of 30–60 % denaturant. (C) Cluster analysis of DGGE band patterns. The dendrogram was calculated by Pearson correlation and UPGMA.

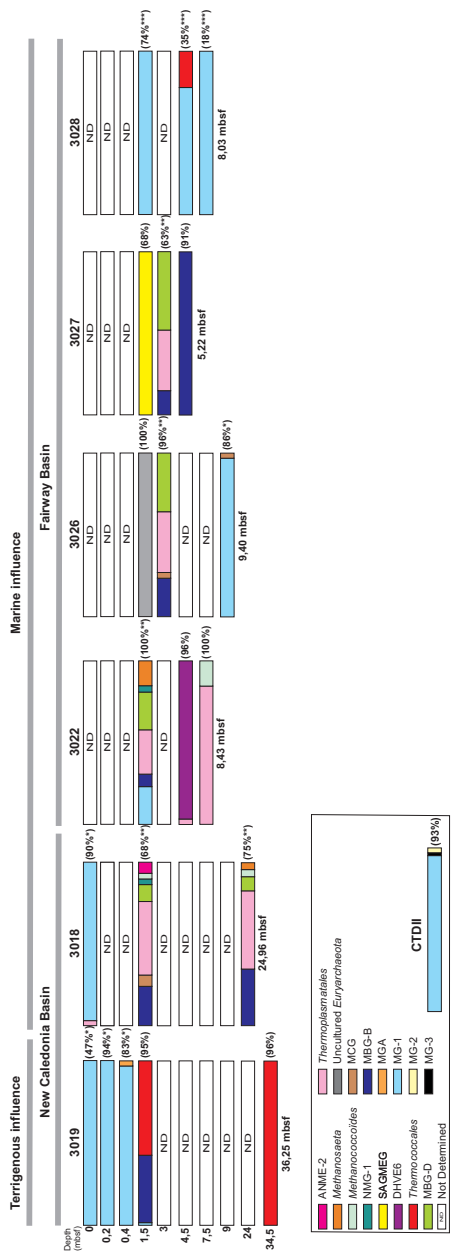



FIG. 2.11: Depth distribution of the archaeal phylogenetic community structures based on 16S rRNA gene from the Fairway and New Caledonia Basin sites. The phylogenetic affiliation of each clone sequence was determined by similarity analysis. The relative abundance of each phylotype was calculated and represented in a column diagram. The percentage of coverage of each clone library examined is indicated in brackets. The asterisks indicate groups of clone libraries with insignificant ($P < 0.01$) differences between all the diversity indices ($F_{ST}$ and the exact test method). ANME-2: anaerobic methane oxidizers, NMG-1: Novel *Methanosarcinales* group 1, SAGMEG: South African Gold Mine Euryarchaeotic Group, DHVE6: Deep-sea Hydrothermal Vent 6, MBG-D: Marine Benthic Group D, MCG: Miscellaneous Crenarchaeotal Group, MBG-B: marine benthic group B, MBG-A: Marine Benthic Group A, MG-1 (2, 3): Marine Group 1 (2, 3), CTDII: seawater clone library.

### Archaeal 16S rRNA gene diversity based on clone libraries

The seawater archaeal diversity, in the CTDII clone library, was exclusively composed of sequences related to marine groups, such as MG-1 (95 %), MG-2 (3 %) and MG-3 (2 %) (Figure 2.11). Only 2 sequences (DNA19T150), out of 673 sequences from sediment samples, matched with sequences from the seawater clone library (Figure 2.13), which suggests that seawater contamination was negligible.

The general archaeal phylogenetic diversity, in sediment-derived clone libraries (Figure 2.11), was similar to that usually obtained from subsurface sediments and methane-rich environments (Bidle *et al.*, 1999; Inagaki *et al.*, 2006*a*; Inagaki *et al.*, 2003*a*; Sorensen *et al.*, 2004; Sorensen and Teske, 2006; Newberry *et al.*, 2004; Parkes *et al.*, 2005). A majority of clones were related to uncultured environmental clones from these environments (highest similarity to pure culture, 99 %). The sediment-derived clone library diversities were very heterogeneous, either strongly dominated by sequences related to *Crenarchaeota* or by sequences related to *Euryarchaeota* (Figure 2.11).

Overall, euryarchaeal lineages represented less than 1 % in the clone libraries from surface sediments (0 to 1.5 mbsf), whereas below they represent 59 %. The whole *Euryarchaeota* phylogenetic diversity was high, representing a total of 10 different lineages (Figure 2.11): *Methanococcoides*, Novel *Methanosarcinales* group 1 (NMG-1), *Methanosaeta*, ANME-2, *Thermoplasmatales*, Marine Benthic Group D (MBG-D), Deep-sea Hydrothermal Vent 6, South African Gold Mine Euryarchaeotic Group (SAGMEG), *Thermococcales* and Uncultured *Euryarchaeota* (see supplementary material, Figure S3). Sequences related to the very ubiquitous *Thermoplasmatales* and MBG-D lineages (Teske and Sorensen, 2008, for a review) were found in 44 % of the libraries, representing 26 % of the clones in the libraries below 1.5 mbsf (Figure 2.11).

However, putative methane cycling communities represented less than 4 % of the libraries below 1.5 mbsf (Figure 2.11). A large proportion of these sequences (44 %) were related to the *Methanococcoides* lineage, a methylotrophic methanogen (Singh *et al.*, 2005). The *Methanosaeta* lineage, an acetoclastic methanogen (Ma *et al.*, 2006), was also detected. Two sequences were related to ANME-2, a putative anaerobic methane oxidizer (Boetius *et al.*, 2000; Hinrichs *et al.*, 1999) rarely detected in deep subsurface sediments (Roussel *et al.*, 2008). Interestingly, two sequences were related to Novel *Methanosarcinales* group 1 (NMG-1) (Roussel *et al.*, 2009), a new phylotype in the *Methanosarcinaceae* family (see supplementary material, Fig. S3). These sequences grouped with environmental clones (highest similarity to pure culture, 96 % to *Methanolobus oregonensis*) from a sulfide rich spring (Elshahed *et al.*, 2004) and from estuary sediments (Purdy *et al.*, 2002; Roussel *et al.*, 2009).

However, at site MD06-3019 below 1.5 mbsf, *Thermococales*, a putative (hyper) thermophylic *Euryarchaeota*, was the unique lineage detected by DGGE and clone library analysis (Figure 2.10 and 2.11). These sequences were shown to form a unique cluster (Figure 2.15), named

*Thermococcales* Group 1 (TG-1), within the genera *Thermococcus*. TG-1 16S rRNA sequences contain a high GC content (66 %), and were related to sequences retrieved from sulphide rich hydrothermal environments (Summit and Baross, 2001*b*). A second cluster within the *Thermococcales* comprised sequences detected at the strongly faulted site (MD06-3028). These sequences grouped in a new cluster (Figure 2.15) with sequences from deep hot subseafloor sediments (1,626 mbsf) (Roussel *et al.*, 2008), and was therefore named Deep Sub-Seafloor *Thermococcales* Group (DSSTG).
Crenarchaeal lineages related to Marine Group I (MG-1), an ubiquitous putative ammonia-oxidizing *Archaea* in marine sediments (Teske and Sorensen, 2008, for a review), dominated all the clone libraries and DGGE community structures above 1.5 mbsf (Figure 2.10 and 2.11). The MG-1 diversity was high (Figure 2.13), covering more than 5 subclades ($\alpha$, $\beta$, $\varepsilon$, $\xi$ and $\eta$) and related to sequences from deep marine sediments (Sorensen *et al.*, 2004; Newberry *et al.*, 2004). Moreover, the subseafloor MG-1 lineages were also detected at the sites MD06-3026 and 3028 below 1.5 mbsf (Figure 2.11). However, the other crenarchaeal sequences, detected below 1.5 mbsf, clustered into the Miscellaneous Crenarchaeotic Group (MCG) ($< 1$ %) and the Marine Benthic Group B (MBG-B, also called DSAG) lineages (15 %) (Figure 2.10 and 2.11, see supplementary material, Figure S4). MCG *Archaea* are ubiquitous in subsurface environments and are thought to be heterotrophic anaerobes utilizing complex organic substrates (Teske and Sorensen, 2008), whereas the MBG-B *Archaea* are probably linked to anaerobic methane oxidization (AOM) (Biddle *et al.*, 2006; Sorensen and Teske, 2006; Teske and Sorensen, 2008), and limited to marine environments such as hydrothermal vents (Takai and Horikoshi, 1999), cold seeps (Lloyd *et al.*, 2006) and subsurface sediments (Biddle *et al.*, 2006; Sorensen and Teske, 2006).

### The Metabolically active *Archaea* community structure.

The active fraction of the archaeal community was assessed and compared to the DNA-derived archaeal community by a new molecular approach based on DGGE and named Co-Migration DGGE (CM-DGGE, see experimental procedures). By using the same primer sets and gel conditions as for the previous DGGE analyses, we showed that the archaeal communities were most active between 0 to 1.5 mbsf, as no complementary ribosomal DNA (crDNA) was detected below 1.5 mbsf (Figure 2.12 and 2.16). Congruently, the two RNA-derived libraries (RNA18T150 and RNA19T150) were dominated by sequences related to the same MG-1 sequences as the DNA-derived libraries (Figure 2.13) demonstrating that these MG-1 communities were active. Interestingly, a crDNA fragment related to the *Thermococcales* order was detected only at site MD06-3019 0.6 mbsf by CM-DGGE, indicating the presence of living *Thermococcales* at this depth (Figure 2.16).

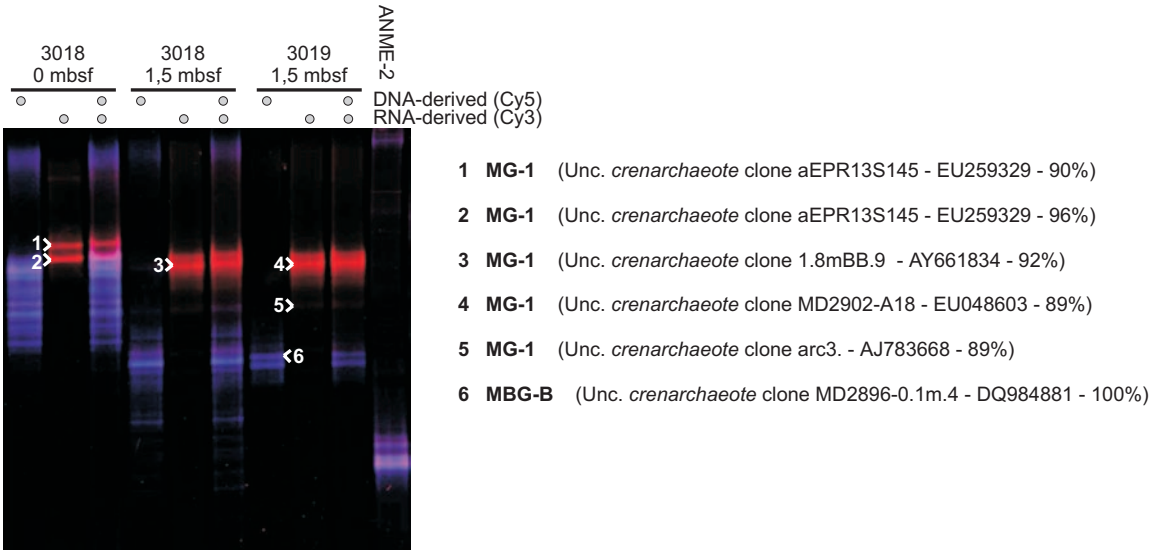



FIG. 2.12: Co-migration denaturant gradient gel electrophoresis (CM-DGGE) analysis of archaeal 16S rRNA genes DNA-derived (blue) and RNA-derived (red) from MD06-3018 and MD06-3019. The numbered bands were excised and sequenced. The lineage and the sequence similarity of the closest match by BLASTN search are given on the right. PCR products were amplified with the Saf-PARCH 519r*Cy5 (blue) or Saf-PARCH 519r*Cy3 (red) primer set and electrophoresis was performed using a gradient of 30–60 % denaturant.

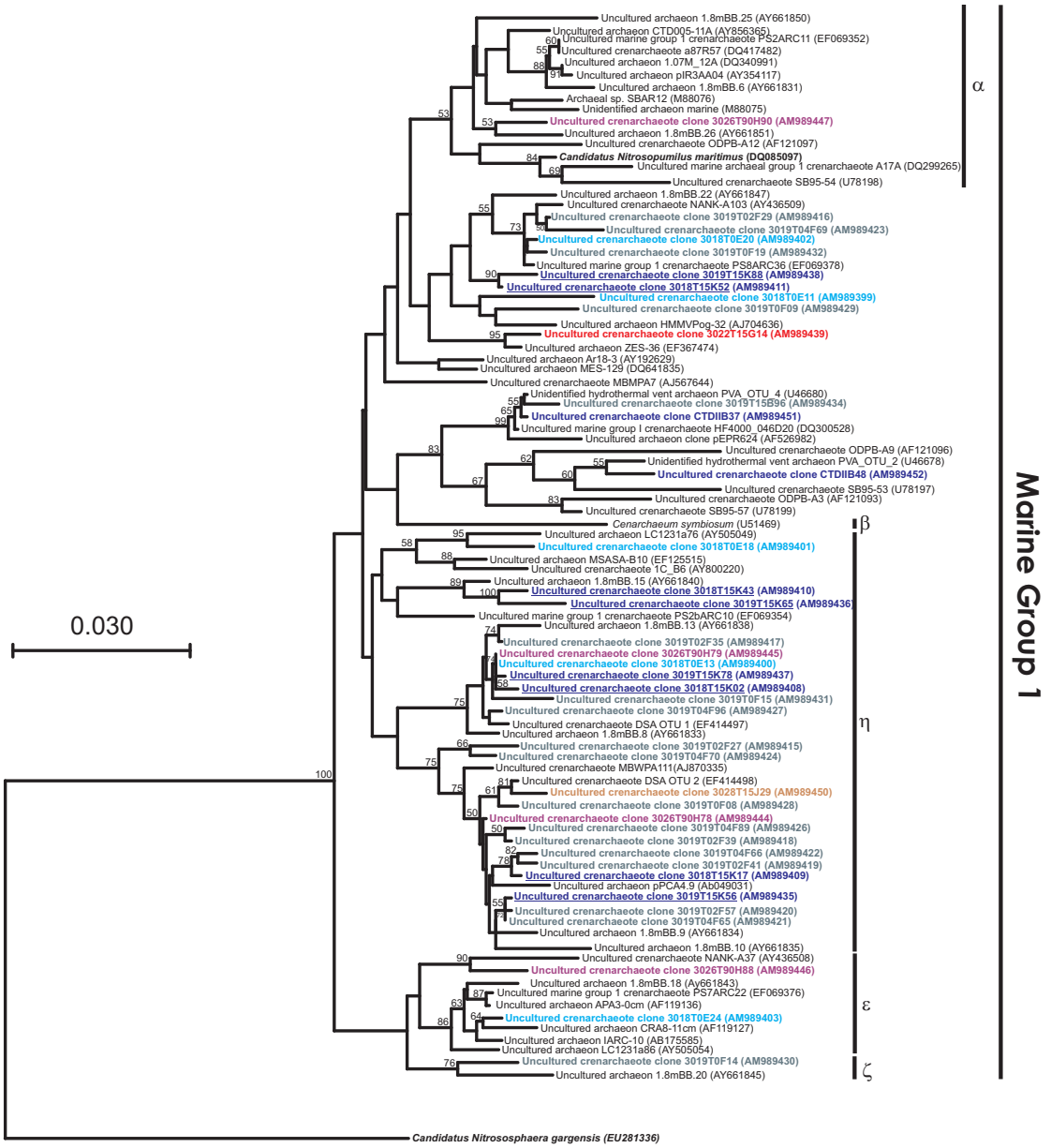



FIG. 2.13: Phylogenetic tree representing the Marine Group 1 (MG-1) 16S rRNA gene sequences DNA- and RNA derived. RNA-derived sequences are underlined. Each phylotype from each clone library is represented by one sequence with $> 97$ % similarity grouping. The tree was constructed using the neighbour-joining method with Jukes and Cantor correction. Bootstrap values $< 50$ % are not shown.

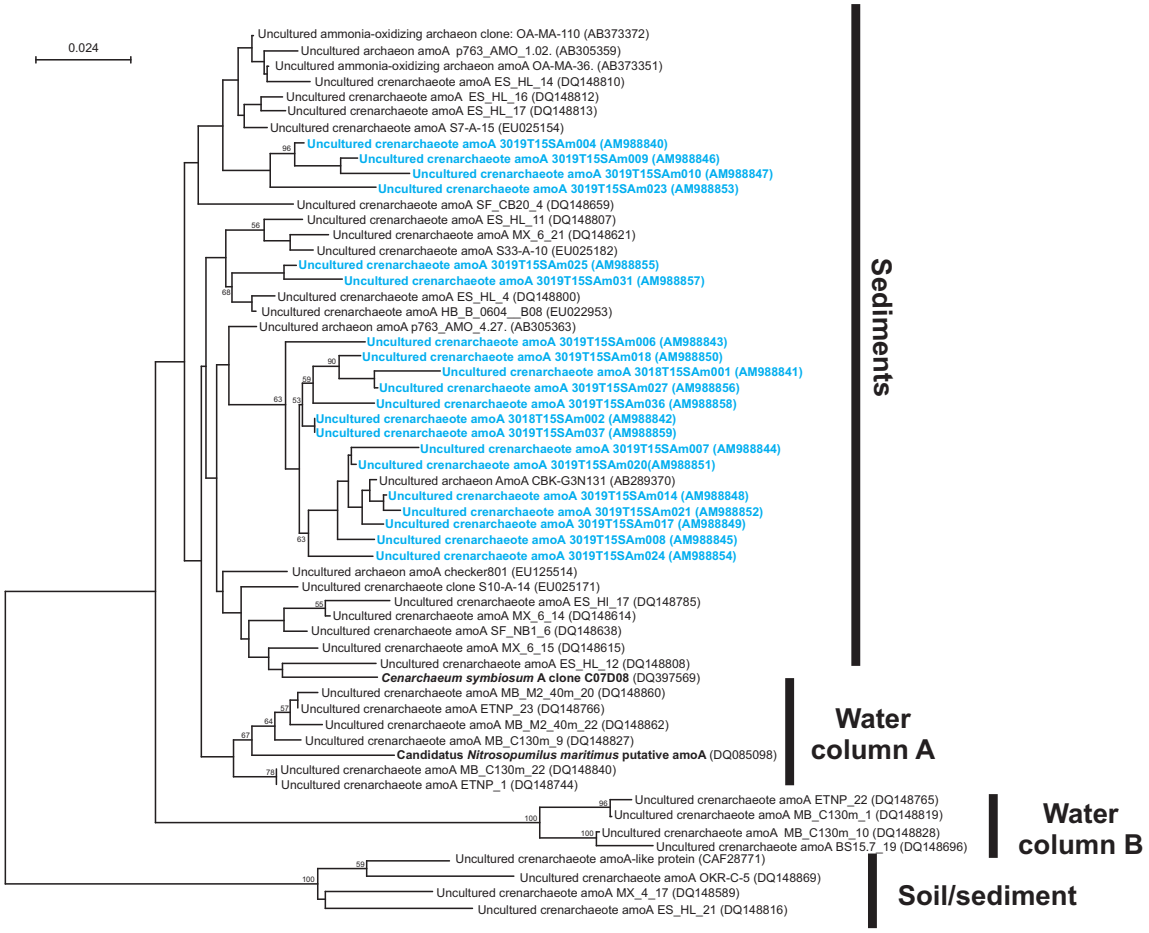



FIG. 2.14: Phylogenetic tree based on translated, partial amino acid sequences of *amoA* gene ($<$ 212 amino acids). The tree was constructed using the neighbour-joining method using PAM distance (Dayhoff *et al.*, 1978). The robustness of inferred topology was tested by the bootstrap. Bootstrap values $<$ 50 % are not shown.

## Diversity based on *amoA* gene clone libraries

As MG-1 are putative ammonia-oxidizing *Archaea*, amplifications of the *amoA* gene, using archaeal *amoA* primers (Francis *et al.*, 2005), were performed in order to confirm the occurrence of ammonia-oxidizing *Archaea* (AOA) in deep marine sediments at sites MD06-3018 and 3019. Archaeal *amoA* gene was only detected above 1.5 mbsf. To investigate the *amoA* gene diversity, two archaeal *amoA* libraries (n = 40, coverage = 83 %) were analysed. These libraries were exclusively composed of 635-bp length sequences related to uncultured *Crenarchaeota* (Figure 2.14). These sequences formed 15 OTUs (based on a 2 % cutoff) grouping in a distinct phylogenetic group of sequences from sediments (Francis *et al.*, 2005). However, no *amoA* sequences related to water column *Archaea* were detected (Figure 2.14).

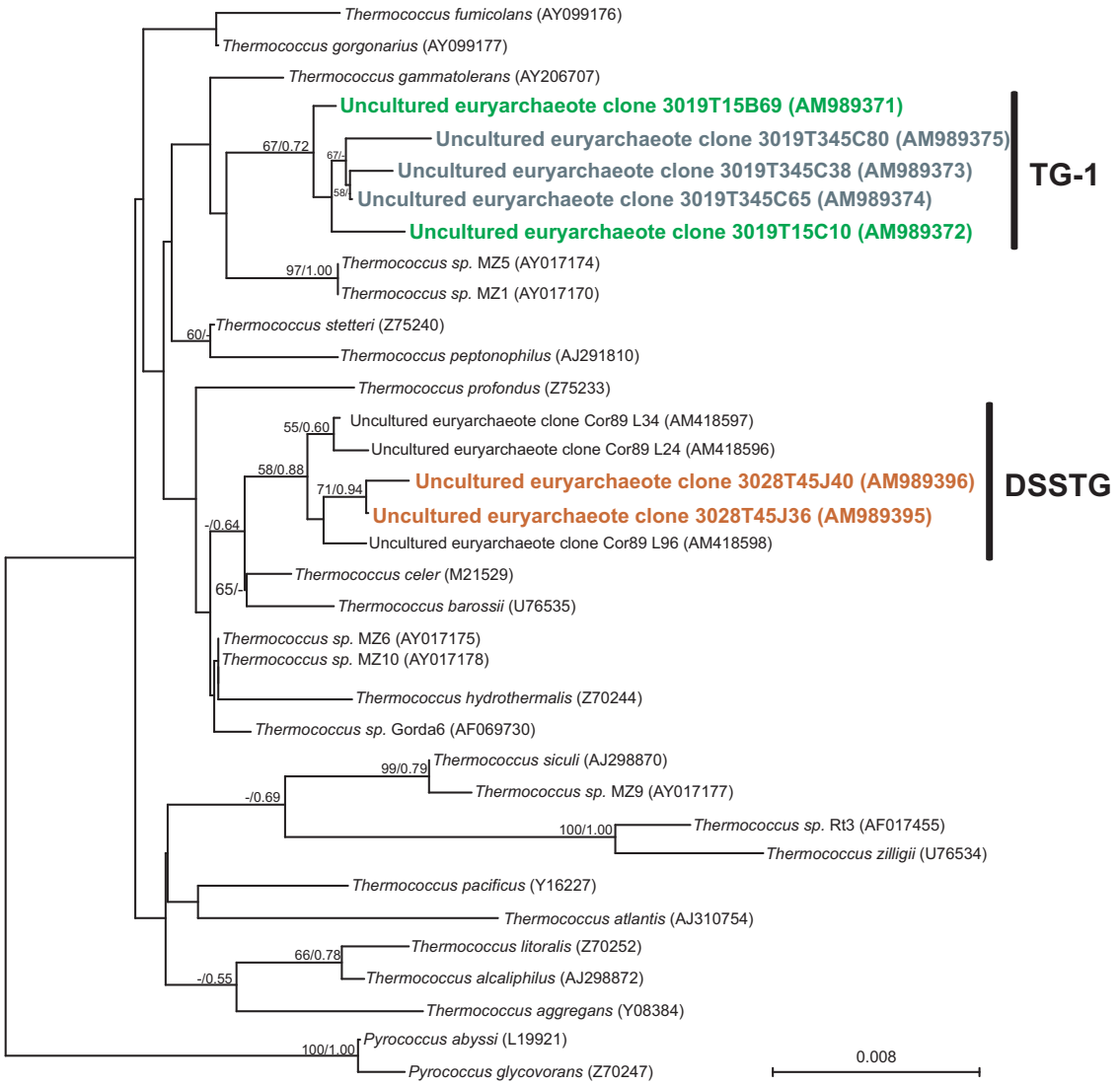



FIG. 2.15: Phylogenetic tree representing the *Thermococcus* 16S rRNA gene sequences from the Fairway and New Caledonia Basin sites. Tree topology was inferred by neighbour-joining analysis on 550 bases with Jukes and Cantor correction. Bootstrap support values over 50 % (1,000 replicates) and bayesian posterior probabilities are indicated at nodes. Closely related sequence clusters are represented by single sequences. TG-1: *Thermococcales* Group 1, DSSTG: Deep Sub-Seafloor *Thermococcales* Group.

# Discussion

## CM-DGGE: who is there and who is active?

One of the most revealing questions, in sub-seafloor studies, is discriminating and identifying active and living from the dead or quiescent microbial communities. Nucleic acid-based molecular analyses of deep sub-seafloor microbial communities are limited, as sediment samples are usually characterized by low biomass and limited material (Webster *et al.*, 2003). Fingerprinting techniques, such as DGGE, are recommended for describing the microbial community structures, as a large number of samples can be analysed (Smalla *et al.*, 2007, for a review). However, as DGGE analyses can be biased by gel variations (Ferrari and Hollibaugh, 1999; Nunan *et al.*, 2005, for e.g.), running intra-lane standards enhances the sample-to-sample comparisons (Muyzer, 1999; Neufeld and Mohn, 2005). Here, we developed a new fingerprinting approach (CM-DGGE), adapting the use of different terminally labelled fluorescent PCR products, to compare simultaneously the DNA-derived microbial community structure to the active fraction based on crDNA, in deep-sea sediments. As the use of labelled primers improves the sensitivity and specificity of DGGE fingerprint detection (Neufeld and Mohn, 2005), it is possible to use crDNA to detect minor archaeal activities, such as the TG-1 at site MD06-3019 (Figure 2.16). Moreover, this technique is also time saving and minimizes the problems of gel handling and staining.

## Prokaryote abundance and terrigenous influence

The low methane content and cell numbers, in the Fairway Basin sediments, are consistent with the general depth distribution of methane and prokaryotic cells in open-ocean sub-seafloor sediments, presumably reflecting the lower inputs of organic carbon compared with the site under terrigenous influence (D'Hondt *et al.*, 2004; Wellsbury *et al.*, 2002; D'Hondt *et al.*, 2002). Thus, the depth profile of prokaryotic cell numbers at site MD06-3019 was on the whole $\sim$3.5 times higher than at the open-ocean sites, and did not show a clear depth trend, fluctuating between the lowest and highest prediction limits. Moreover, the low cell number between 7.5 mbsf and 10.5 mbsf, was correlated with the occurrence of driftwood in the sediment. Therefore, as the core lithology comprised a succession of terrigenous sequences, the heterogeneous prokaryotic cell number distribution and methane depth profile could be related to the strong erosion events of New Caledonia.

## Archaeal diversity and activity: from sediment surface to subsurface

The archaeal diversity of the seawater clone library was significantly different from all sediment clone libraries ($P < 0.01$). Moreover, as the studied sediment did not contain any 16S rRNA or *amoA* genes related to seawater phylotypes, seawater contamination of the sediments could be excluded, therefore suggesting that the detected *Archaea* are deep marine sediment communi-

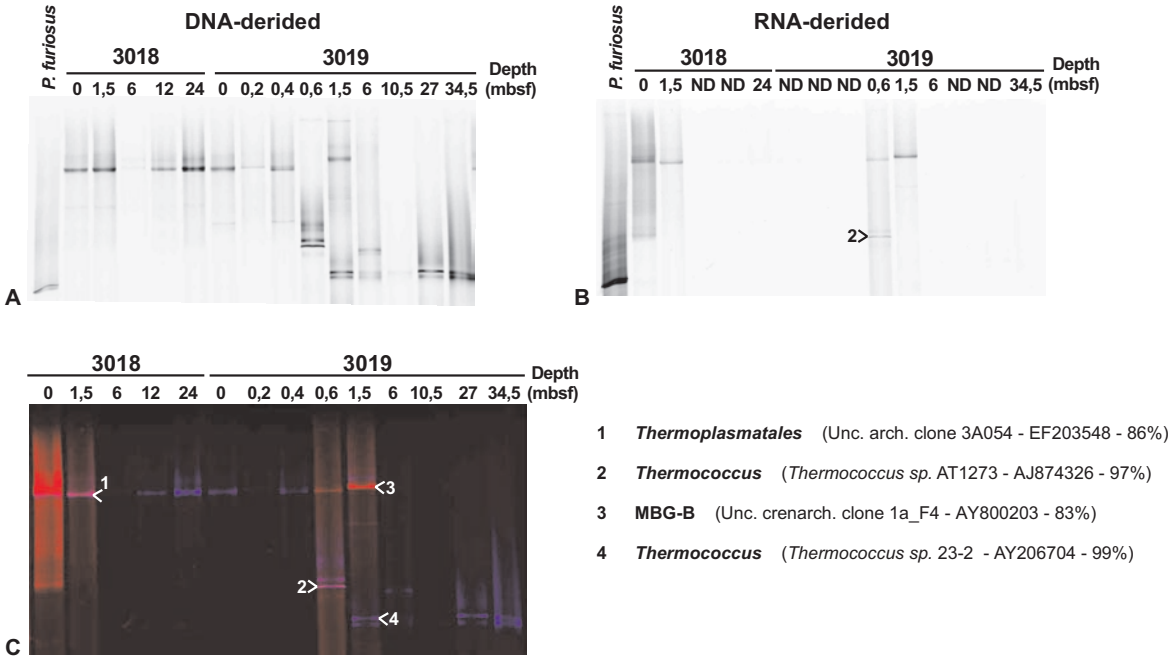



FIG. 2.16: Co-migration denaturant gradient gel electrophoresis (CM-DGGE) analysis of archaeal 16S rRNA genes DNA- (blue) and RNA-derived (red) of the cores from MD06-3018 and MD06-3019. The numbered bands were excised and sequenced. The lineage and the sequence similarity of the closest match by BLASTN search are given on the right. PCR products were amplified with the A344fGC-A915r*Cy5 (blue) or A344fGC-A915r*Cy3 (red) primer set and electrophoresis was performed using a gradient of 40–70 % denaturant. (A) DNA-derived (A344fGC-A915r*Cy5). (B) RNA-derived (A344fGC-A915r*Cy3). (C) both DNA- and RNA-derived.

ties. However, both cluster analysis of DGGE band patterns and the distribution of archaeal lineages in clone libraries showed distinct phylogenetical archaeal communities either restricted to a surface or to subsurface sediment horizon.

The surface archaeal community habitat, exclusively dominated by MG-1, was restricted to the sediment horizon between 0 to 1.5 mbsf. MG-1 *Archaea*, identified as aerobic autotrophic ammonia oxidizers (Konneke *et al.*, 2005; Francis *et al.*, 2005; Hallam *et al.*, 2006), are commonly found in seawater and marine sediments, forming several phylogenetic clusters with currently two cultured relatives (Konneke *et al.*, 2005; Preston *et al.*, 1996). Moreover, based on the analysis of the first sequenced genome of a cultured relative (*Crenarchaeum symbiosum*), the MG-1 were recently proposed as a novel archaeal phylum named *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008). The MG-1 phylotypes retrieved in the anoxic sediments were different from those found in the oxic seawater, suggesting that specific MG-1 subclades could be adapted to suboxic conditions (Teske and Sorensen, 2008). Moreover, the CM-DGGE analysis and RNA-derived clone libraries suggest that the MG-1 *Archaea* were the most active phylotype above

1.5 mbsf. Although the molecular techniques (PCR and cloning), used to build clone libraries, are known to be inherently biased (Suzuki and Giovannoni, 1996), significant differences in lineage distribution were detected between the RNA and DNA derived libraries at the same depth (RNA18T150, RNA19T150, DNA18T150 and DNA19T150). The ratios between the 16S rRNA gene and rRNA per cell have been reported to be proportional to the metabolic activity of the cells (Dell'Anno *et al.*, 1998), the rRNA content per cell increasing with metabolic activity. As the MG-1 lineage was detected in RNA18T150 and RNA19T150 libraries, whereas absent in DNA18T150 and DNA19T150 libraries, we hypothesized that the sediments at 1.5 mbsf could have low cell concentrations of very active MG-1 lineage. Moreover, *amoA* genes related to the sediment cluster were only detected at the depths with MG-1. Altogether, these evidences strongly suggest that ammonia-oxidizing is the main archaeal activity at the sediment surface. However, MG-1 *Archaea* were also detected below 1.5 mbsf at strongly faulted sites (MD-3026 and 3028). These sub-surface MG-1 communities were phylogenetically related to the MG-1 *Archaea* found at the sediment surface horizon, and *amoA* genes related to MG-1 were only detected at depths containing MG-1. We can therefore hypothesize that these communities could be fuelled by ammonium rich fluids originating either from seawater intrusion or from fluid advection from depth. However, no RNA was detected from these depths, suggesting that sub-surface MG-1 are less active or less abundant than the surface communities.

Conversely, the sub-surface archaeal community, composed of typical sub-seafloor lineages (MBG-B, MBG-D, MCG and SAGMEG) (Teske and Sorensen, 2008, for a review), was restricted to depths below 1.5 mbsf. The archaeal diversity of some sediment layers at different sites were restricted to only one lineage (Uncultured *Euryarchaeota*, DHVE6, MBG-B, MG-1 and SAGMEG), suggesting that these lineages were selected by specific geochemical conditions. However, no crDNA was retrieved below 1.5 mbsf, probably below the detection limit, suggesting that these less active deep subseafloor archaeal communities are adapted to these low energy and organic carbon availability environments (D'Hondt *et al.*, 2002).

Sulphate reduction and methane cycling activities widely occur in deep marine sediments (Biddle *et al.*, 2006; D'Hondt *et al.*, 2004; D'Hondt *et al.*, 2002; Parkes *et al.*, 2005; Sorensen and Teske, 2006). Sulfate concentrations decreased with depth at all sites, suggesting the occurrence of sulfate-reducing prokaryotes. However, no genes encoding for the dissimilatory sulfate reductase (*dsr*) were detected (data not shown), probably resulting from a too low number of sulphate-reducing *Bacteria*. Remarkably, 16S rDNA sequences related to methanogenic and methanotrophic *Archaea* are also very rarely detected in deep marine sediments (Parkes *et al.*, 2005). However, even though no methyl-coenzyme M reductase (*mcr*) genes were detected (data not shown), putative methanotrophs (ANME-2), acetoclastic methanogens (*Methanosaeta*) and methylotrophic methanogens (*Methanococcoides*), were detected at two open-ocean sites. Moreover, MBG-B *Archaea*, a lineage possibly involved in AOM, are numerous in clone libraries below 1.5 mbsf. Altogether, these archaeal communities suggest that low methane cycling rates could comprise a significant proportion of the archaeal activities at these sites.

However, contamination by exogenous DNA was of particular concern (see Experimental Procedures), and as all the contamination controls were negative, the detection of rare sub-seafloor sediment lineages, such as *Methanosarcinales* and *Thermococcales*, was probably related to methodological implications. PCR-based surveys are biased, as only the most abundant lineages with very similar matching priming sites are detected (Teske and Sorensen, 2008). Thus, the several DNA extractions followed by pooling and concentration of several PCR and nested PCR products, combined to the use of a primer (A344f) containing degeneracies (Teske and Sorensen, 2008), may reduce PCR biases and facilitate the detection of these rare deep subseafloor lineages.

### Living *Thermoccocales*: a reality (Damste and Coolen, 2006)

Although sites MD06-3018 and MD06-3019 were geographically close ($<$ 100 km), they showed drastic differences in lithology and archaeal diversity. The MD06-3018 sediments were mainly characterized by a homogeneous distribution of carbonate clay, and had a high diversity of archaeal lineages commonly found in marine sediments. Conversely, MD06-3019 sediments were characterized by a heterogeneous distribution of sand and clay. Consistently, *Archaea* belonging to the Rice Cluster V lineage, probably a non methanogenic *Archaea* found in anoxic rice paddy soils (Grosskopf *et al.*, 1998), were detected in these terrigenous sediments. Interestingly, the low archaeal diversity of MD06-3019 sediments below 1.5 mbsf was dominated by *Thermococcales*, a putative thermophilic *Euryarchaeota* commonly found at hydrothermal vent sites and representing an excellent indicator of subseafloor ecosystems (Kelley *et al.*, 2002). Sequences affiliated to putative *Thermococcales* have been previously detected in other cold (1-12°C) deep marine sediments (Inagaki *et al.*, 2006*b*; Inagaki *et al.*, 2001; Kormas *et al.*, 2003). Their occurrence was usually interpreted as a deposition consequent to fluid migration, or as buried microbial relicts, representing "fossil DNA" (Inagaki *et al.*, 2006*b*; Inagaki *et al.*, 2001; Kormas *et al.*, 2003). Even though the minimum temperature required for the growth of a *Thermococcus* is 40°C (Miroshnichenko *et al.*, 2001), *Thermococcales* can survive over long periods in cold (4°C), oxygenated samples (Jannasch *et al.*, 1992), possibly allowing a wide dissemination in marine environments. Furthermore, at site MD06-3019, the CM-DGGE analysis detected living *Thermococcales* at 0.6 mbsf, which was correlated with the occurrence of authigenic minerals, such as aragonite. All the sequences affiliated to the *Thermococcales* order found at MD06-3019 grouped in a unique cluster (TG-1), therefore suggesting a same origin. Recently, an extensive active alkaline hydrothermal field was revealed in the south-west lagoon of New Caledonia (Pelletier *et al.*, 2006). As the MD06-3019 site shows no signs of fluid migration and as it is located in a canyon deriving substantial amounts of terrigenous matter from the lagoon, we hypothesized that the TG-1 could have rafted from New-Caledonia on biotic (wood) or terrigenous substrates (Thiel and Gutow, 2005; Thiel and Haye, 2006), progressively becoming less active as organic matter became recalcitrant with burial (Wellsbury *et al.*, 2002). An other distinct *Thermococcus* cluster was detected at the strongly faulted site (MD06-3028,

Figure S1), whereas absent at the non-faulted adjacent site MD06-3027. Yet, the *Thermococcales* detected were characterized by low activity as no rRNA phylotypes were detected by RT-PCR. However, the DNA-derived 16S rRNA gene sequences detected at site MD06-3028 grouped with sequences from very deep and hot subseafloor sediments (1,626 mbsf) (Roussel *et al.*, 2008), suggesting that these communities could have been introduced by a vertical fluid migration. Hence, the DSSTG may represent a unique "deep hot biosphere community".
In conclusion, the deep marine sediment archaeal distribution clearly differs from the seawater and depends on spatial and geochemical variables as previously shown (Fry *et al.*, 2006; Parkes *et al.*, 2005; Sorensen and Teske, 2006). This study also shows the transition between active surface archaeal communities, a component of which is capable of ammonium oxidation and less active deep typical sub-seafloor lineages. Moreover, the occurrence of living putative thermophiles in cold marine sediments suggests that the dispersion of the hydrothermal archaeal communities could either occur by rafting through the surface seawater or by fluid migration through the sub-seafloor.

## Experimental procedures

### Site description and sampling

Six piston cores (MD06-3018, MD06-3019, MD06-3022, MD06-3026, MD06-3027, and MD06-3028) were collected from the New Caledonia and Fairway basins (Figure 2.8) during Marion Dufresne Cruise ZoNéCo 12 in 2006, using a Calypso piston corer. Site MD06-3018 (23°00.19'S, 166°08.97'E; 2,470 meters water depth; core length 24.96 m), located on the New Caledonian Basin 50 km from the New Caledonia coast, is mainly in an open-ocean context (Foucher *et al.*, 2006). Site MD06-3019 (22°30.64'S, 165°11.75'E; 3,522 meters water depth; core length 36.25 m), 80 km from the coast, is located in a canyon deriving a substantial amount of terrigenous matter (Foucher *et al.*, 2006). Sites MD06-3022 (23°12.11'S, 163°27.94'E; 2,294 meters water depth; core length 8.43 m), MD06-3026 (23°56.26'S, 163°27.72'E; 2,717 meters water depth; core length 9.40 m), MD06-3027 (24°40.61'S, 163°36.14'E; 2,720 meters water depth; core length 5.22 m), and MD06-3028 (24°45.20'S, 163°36.95'E; 2,716 meters water depth; core length 8.03 m) are located in an open-ocean context in the Fairway Basin.
The cores were aseptically sub-sampled on board, every 20 cm on the first 100 cm, and then every 150 cm, using 5 mL syringes (luer end removed). The samples were then immediately stored anaerobically at –80°C for molecular analysis and at 4°C for prokaryotic enumeration and for enrichment cultures. As a contamination control, seawater from the water column was collected (MD06-CTD2; 24°45.22'S, 163°36.95'E, 2,690 meters water depth) using a CTD rosette and immediately stored at –80°C.

### Total prokaryotic cell enumeration

Total prokaryotic counts were determined, with an epifluorescence microscope (BX60, Olympus™), by acridine orange staining, on subsamples stored at 4°C under anaerobic conditions in the dark (< 5 days), as previously described (Cragg *et al.*, 2000).

### Mineralogical composition of sediments

The mineralogy of 8 sediment samples from cores MD06-3018 and MD06-3019 was determined by X-ray diffraction (XRD) analysis using a Bruker D8 Advance equipped with a Cu X Ray tube and a Vantec detector. Samples were not dried before analysis and diffraction patterns were obtained between 5° and 70°. Mineral determination and semi-quantitative estimations were performed with the EUA program.

### Geochemical analysis

Methane analysis were performed on cores MD06-3019, MD06-3022, MD06-3026, MD06-3027 and MD06-3028 at the end of each core segment (1.5 m long) using the headspace technique. The cores were immediately sub-sampled on board using 5 mL syringes (luer end removed) and added to headspace vials (20 mL) filled with a $NaCl/HgCl_2$ work solution. Methane concentrations were determined using a HP 7694 automatic headspace sampler connected to a HP 5890 gas chromatograph equipped with FID and TCD detectors. The 2-sigma uncertainty is better than 4 % (Donval *et al.*, tted). Results are expressed as microlitre per litre of sediment ($\mu$L/L).

Pore-waters were extracted on board from MD06-3019 and MD06-3022 cores from end of each core segment by centrifuging sediment samples (10,000 rpm over 30 min). The dissolved anions ($SO_4^{2-}$, $Cl^-$) and cations ($Na^+$, $Ca^{2+}$, $Mg^{2+}$) were determined from diluted pore-waters using a Dionex DX100 ion chromatograph. The IAPSO standard seawater was used for calibration and quality control. Results are expressed as millimole per litre pore-water (mM).

### DNA extractions and PCR amplification

To avoid contaminations, all manipulations were carried out in a PCR cabinet exclusively dedicated to the present study (Biocap™ RNA/DNA, erlab®), using Biopur® 1.5 mL Safe-Lock micro test tubes (Eppendorf™), Rnase/Dnase Free Water (MP Biomedicals™) and UV-treated (> 60 min) plasticware and pipettes.

DNA was extracted, pooled and purified from 5×1 g of uncontaminated frozen sample following a modified FastDNA® Spin Kit for Soil (Bio101 Systems, MP Biomedicals™) protocol (Webster *et al.*, 2003; Roussel *et al.*, 2009).

All amplifications were performed using a "GeneAmp PCR system" 9700® (Applied Biosystems™). All PCR mixtures (50 $\mu$L) contained 5 $\mu$L of DNA template, 1X Taq DNA polymerase

buffer (MP Biomedicals™), 1 $\mu$L of dNTP (10 mM of each dATP, dCTP, dGTP and dTTP), 10 $\mu$M of each primer and 0.5 $\mu$L of Taq DNA polymerase (MP Biomedicals™). Negative controls were also carried out with DNA extractions performed with no sample. For all controls, no PCR products were detected.

Archaeal 16S rRNA gene amplification was conducted by nested PCR with combination of primers A8f-A1492r in the first round (Casamayor *et al.*, 2000), with A344f-A915r in the second round (DeLong, 1992; Eden *et al.*, 1991). PCR cycles for the first round (A8f/A1492r), and for the second round (A344f/A915r) were as previously described (Roussel et al., unpublished). To minimize PCR bias, five independent PCR products from the first round were pooled and purified (QIAquick PCR purification Kit; Qiagen™) and used as template for the second round. This nested PCR was necessary to obtain visible PCR products on a 0.8 % (w/v) agarose gel stained with ethidium bromide.

A portion of the *amoA* gene (635 bp) was amplified with primers Arch-amoAF and Arch-amoAR (Francis *et al.*, 2005), and the following reaction conditions were performed: 1 cycle of 5 min at 95°C, 35 cycles of 45 s at 94°C, 60 s at 53°C and 60 s at 72°C, and 1 cycle of 15 min at 72°C.

### RNA extractions and RT-PCR amplification

Total RNA was extracted from each uncontaminated frozen sample (5×1 g) using the FastRNA® Pro soil direct Kit (Bio101 Systems, MP Biomedicals™) as previously described (Roussel et al., unpublished), with the following modifications: the addition of 170 $\mu$g poly-adenylic acid, tubes kept on ice and extended spin. After bead-beating on a FastPrep FP120 homogenizer (Bio101 Systems, MP Biomedicals™), the $\frac{3}{4}$ of the aqueous phases were transferred to a new tube before the remaining aqueous phases were homogenized a second time. After the addition of the 660 $\mu$L of isopropanol (100 %), the tubes were incubated 60 min at –20°C followed by centrifugation at 20,000×g for 15 min at 1°C. In order to increase the RNA yield, the extraction procedure was ended after the first ethanol wash and diluted in 100 $\mu$L of DEPC water. To digest trace amounts of DNA, the extraction products were immediately pooled and 150$\mu$L were incubated 1 hour at 37°C with 1X of TURBO DNase® buffer and 18U of TURBO DNase® (Ambion™). The digestion was stopped by adding EDTA to a final concentration of 15 mM and heating 10 min at 65°C. The product was finally concentrated and purified with the RNeasy minikit (Qiagen™), following manufacturer's instructions, to give a final volume of 100 $\mu$L.

The purified RNA product was immediately serially diluted (1 to 50 times) and reverse transcribed using the OneStep RT-PCR kit (Qiagen™), according to the manufacturer's instructions, with combination of 16S rRNA primers for *Archaea* with A8f-A1492r and the following touchdown PCR protocol as previously described (Roussel *et al.*, 2009). To obtain visible products, a nested PCR was performed as described for the 16S rRNA gene amplification. Nested PCR assays, using the 16S rRNA primers for *Archaea*, without the reverse transcribed step, showed

no DNA contamination.

## DGGE analysis

In order to obtain the general archaeal 16S rRNA gene depth diversity, a PCR-DGGE analysis was performed. To avoid background interference, visualization of unspecific fragments, and to increase sensitivity and resolution, nested PCR was performed as described for the archaeal 16S rRNA gene amplification using a Cy3 labelled reverse primer Saf-PARCH 519r*Cy3 or A344fGC-A915r*Cy3. All manipulations were performed in the dark. The touchdown PCR protocol was as previously described (Nicol *et al.*, 2003; Casamayor *et al.*, 2000). The PCR products were analyzed by DGGE using a DCode Universal Mutation Detection System® (BioRad™) on a 1 mm thick (16×16 cm) polyacrylamide gel (acrylamide/bisacrylamide, 40 %, 37,5:1, BioRad™) prepared with 1xTAE buffer (pH 8, 40 mM de Tris Base, 20 mM acetic acid, 1 mM d'EDTA, (MP Biomedicals™)) and poured with a "Gradient maker" (Hoefer SG30®). For Saf-PARCH 519r*Cy3 PCR products, the 8 % (w/v) polyacrylamide gel had a denaturant gradient between 30 and 60 %. For A344fGC-A915r*Cy3 PCR products, the 6 % (w/v) polyacrylamide gel had a denaturant gradient between 40 and 70 %. Electrophoresis was carried in 1xTAE buffer at 60°C for 330 min at 200 V (initially at 80 V for 10 min). The gel was scanned using a Phospho fluorimager Typhoon 9400® (Amersham Biosciences™). Prior to band excision as described previously (Wilms *et al.*, 2006), the gel was stained with SYBRGold® nucleic acid gel stain for 20 min, and washed for 10 min with 1xTAE buffer and visualized with a Dark Reader transilluminator (Clare Chemicals, Dolores, CO). The DGGE profiles were analysed by cluster analysis using the software package GelCompar II version 5.10 (Applied Maths, St-Martens-Latem, Belgium) as described elsewhere (Wilms *et al.*, 2006).

## Co-Migration-DGGE analysis (CM-DGGE)

Co-Migration-DGGE analysis (CM-DGGE), a new approach based on DGGE was developed in order to obtain the general archaeal depth diversity and associated active fraction. After amplification of the PCR products, using two different fluorescent reverse labelled primers from either total DNA or cDNA of a same sample, these were pooled and loaded into the same lane. Archaeal 16S rRNA gene amplification was performed with primers A344fGC-A915r or Saf-PARCH 519r, labelled with either Cy3 or Cy5, following touchdown PCR protocol as previously described (Nicol *et al.*, 2003; Casamayor *et al.*, 2000). The DGGE analysis and gel conditions were the same as described for the DGGE analysis, except that loading and migration were performed in the dark. The gel was scanned using a Phospho fluorimager Typhoon 9400® (Amersham Biosciences™).

### Cloning and sequencing

According to archaeal DGGE profiles, 21 DNA-derived 16SrRNA gene, two RNA-derived 16S rRNA gene and two DNA-derived *amoA* gene clone libraries were constructed. To minimize PCR bias (Polz and Cavanaugh, 1998), five independent PCR products were pooled, purified (QIAquick PCR purification Kit; Qiagen™), and cloned into *Escherichia coli* (XL10-Gold; Stratagene™) using the pGEM-T Easy vector system I (Promega™) following the manufacturer's instructions. Positive transformants were screened by PCR amplification of the insert using the vector-specific M13 primers. Plasmid extraction, purification and sequencing of the insert, were carried out by the sequencing Ouest-Genepole platform® of Roscoff Marine laboratory (France).

### Phylogenetic analysis

Chimeras (Cole *et al.*, 2003) were excluded from the clone libraries and a total of 771 sequences (including those from the 16S rRNA gene and *amoA* gene) were used for further phylogenetical analysis. The phylogenetic placement was carried out using NCBI BLAST search program within GenBank (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.*, 1990). The 16S rRNA sequences (∼553 bases) were then edited in the BioEdit 7.0.5.3 program (Hall, 1999) and aligned using CLUSTALW (Thompson *et al.*, 1994). The phylogenetic trees were constructed by the PHYLO-WIN program (http://pbil.univ-lyon1.fr/) (Galtier *et al.*, 1996) with neighbour-joining method (Saitou and Nei, 1987) and Jukes and Cantor correction. The *Thermococcales* dataset was further analysed by Bayesian method. Bayesian trees were inferred using MrBayes 3.1.2. (Huelsenbeck and Ronquist, 2001). The Markov Chain Monte Carlo search was run with 4 chains for 2,000,000 generations, with trees being sampled every 100 generations. Stabilization of the chain parameters (tree likelihood, $\alpha$ shape parameter, and proportion of invariant sites) was verified with the program ModelTest version 3.7.2 (Posada and Crandall, 1998). The first 5,000 trees were discarded (burnin), keeping only trees generated after those parameters stabilized. Phylogenetic trees were viewed using the program TreeDyn (Chevenet *et al.*, 2006). The nonchimeric *amoA* sequences (∼635 bases) were translated into amino acids using BioEdit and then aligned using CLUSTALW, and the PHYLO-WIN program with neighbor-joining algorithm and PAM distance (Dayhoff *et al.*, 1978) was then used for phylogenetic tree construction. For the 16S rRNA and amoA phylogenetic reconstruction, the robustness of inferred topology was tested by bootstrap resampling (1,000), values over 50 % are shown on the trees. The richness from the clone libraries was estimated, with the rarefaction curves at 99 %, 97 % and 95 % sequence identity levels, using the DOTUR program (Schloss and Handelsman, 2005). Operational taxonomic units (OTUs), using a 97 % sequence similarity, were generated with the SON program (Schloss and Handelsman, 2006), and the percentage of coverage (Cx) of the clone libraries was calculated by Good's method (Good, 1953) as described by Singleton and colleagues (Singleton *et al.*, 2001). Statistical estimators, the

significance of population differentiation among clone libraries ($F_{ST}$) (Martin, 2002), and the exact tests of population genetic differentiation (Raymond and Rousset, 1995), were calculated using Arlequin 3.11 (Excoffier *et al.*, 2005).

### Nucleotide sequence accession numbers

The sequences are available from GenBank database under the following accession numbers and names: 16S rRNA gene and rRNA (AM989356 to AM989452) and *amoA* gene (AM988840 to AM988859).

## Acknowledgements

The authors are deeply indebted to Yvon Balut, chief of the operation on board the R.V. Marion Dufresne and to the I.P.E.V. (Institut Paul Emile Victor). We also thank Jean-Paul Foucher, chief scientist of ZoNéCo 12. All of the crew and the Scientific Party were crucial in this effort, especially Laetitia Hervé for the sampling efforts, and Frauke Klingelhoefer and Emmanuelle Cosquer for kindly providing the bathymetric map and seismic profiles. This work was supported by the Université de Bretagne Occidentale, the Centre National de la Recherche Scientifique and the Région Bretagne. We are also grateful to Ouest-Génopole for the use of their facilities. Erwan Roussel was supported by a grant from the Ministère de la Recherche.

## Supplementary material

**Supplementary material**

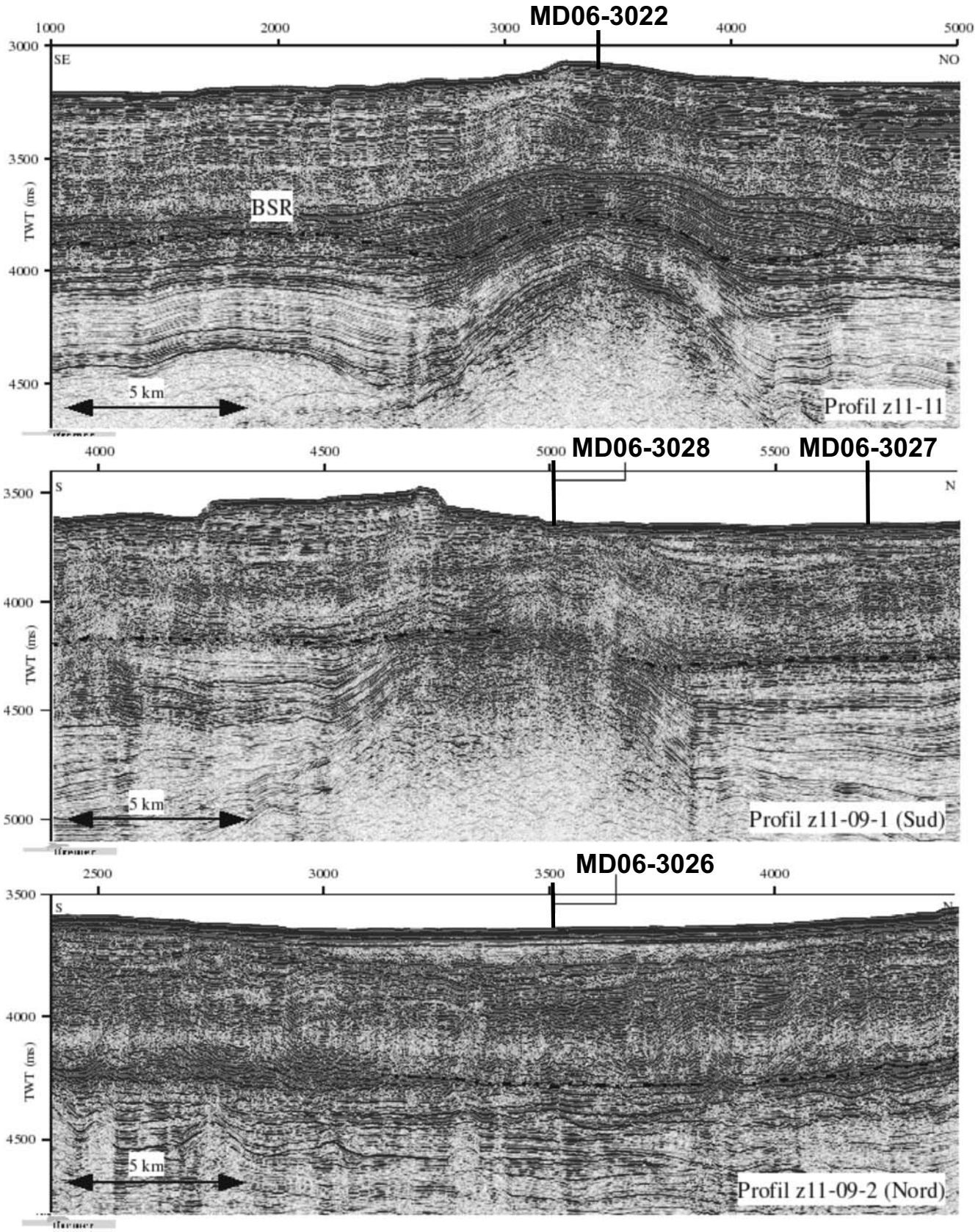



**Fig. S1.** Seismic profiles of the Fairway Basin on which are located the position of the sites MD06-3022, MD06-3026, MD06-3027 and MD06-3028. Figure modified from Foucher et al. (2006).

| Site | MD06-3018 | | MD06-3019 | | | | | |
|---|---|---|---|---|---|---|---|---|
| Depth (mbsf) | 12 | 24 | 0 | 0.2 | 0.4 | 0.8 | 1.5 | 6 |
| Quartz | 2.5 | 2.4 | 11.9 | 12.4 | 9.8 | 6.9 | 10.0 | 6.7 |
| Calcite | 93.9 | 93.5 | 59.6 | 57.1 | 63.3 | 81.4 | 77.6 | 81.1 |
| Aragonite | 0.0 | 0.0 | 0.0 | 7.0 | 5.5 | 0.0 | 0.0 | 0.0 |
| Chlorite | 1.5 | 1.4 | 4.8 | 4.2 | 3.3 | 1.5 | 1.6 | 1.9 |
| Kaolinite | 0.0 | 0.0 | 6.6 | 2.7 | 2.1 | 1.0 | 1.0 | 1.2 |
| Montmorillonnite | 2.1 | 2.1 | 5.0 | 4.3 | 3.4 | 2.9 | 3.1 | 3.5 |
| Halite | 0.0 | 0.7 | 8.1 | 7.0 | 5.5 | 3.1 | 3.3 | 3.8 |
| Albite | 0.0 | 0.0 | 4.1 | 5.3 | 4.2 | 3.2 | 3.4 | 1.8 |
| Clinoenstatite | 0.0 | 0.0 | 0.0 | 0.0 | 3.0 | 0.0 | 0.0 | 0.0 |

**Table. S1.** Mineral contents (wt.% normalized to 100%) of bulk samples from the cores MD06-3018 and MD06-3019.

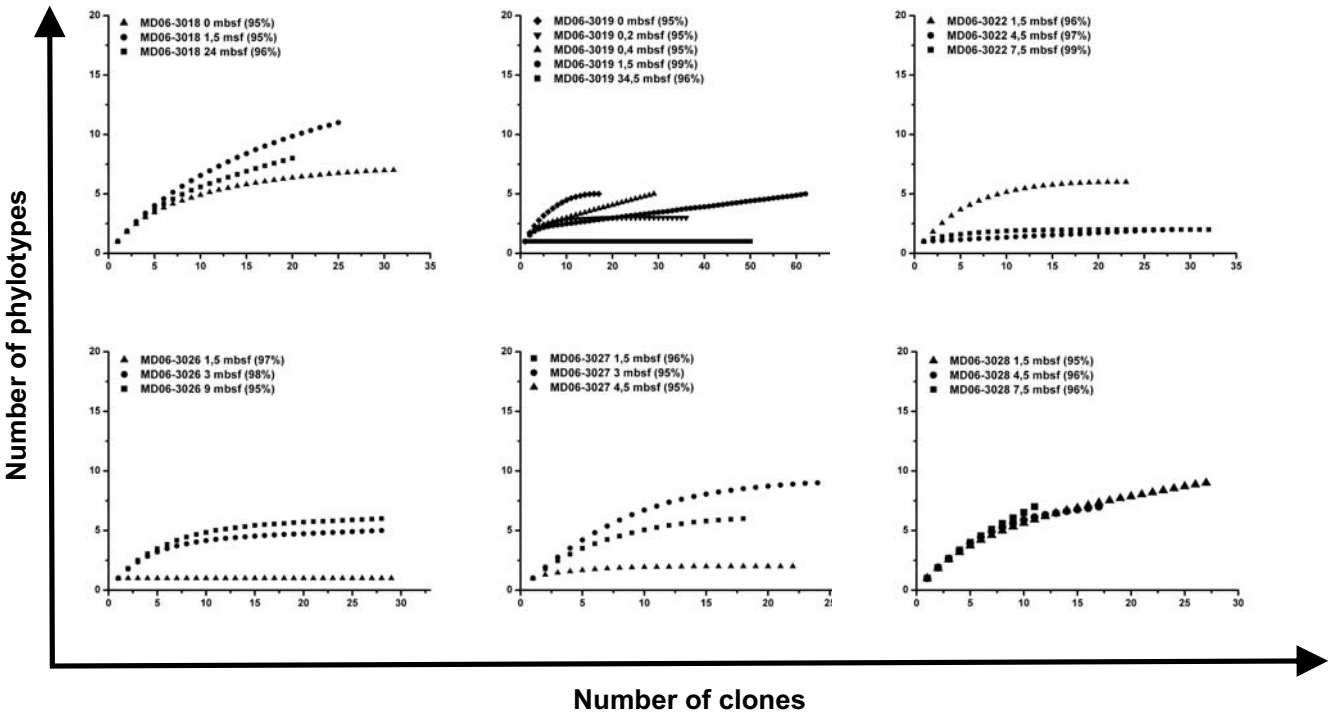



**Fig. S2.** Rarefaction curves for the 16S rRNA gene clone libraries from the Fairway and New Caledonia Basin sites (Schloss and Handelsman, 2005). The sequence identity levels are represented in brackets.

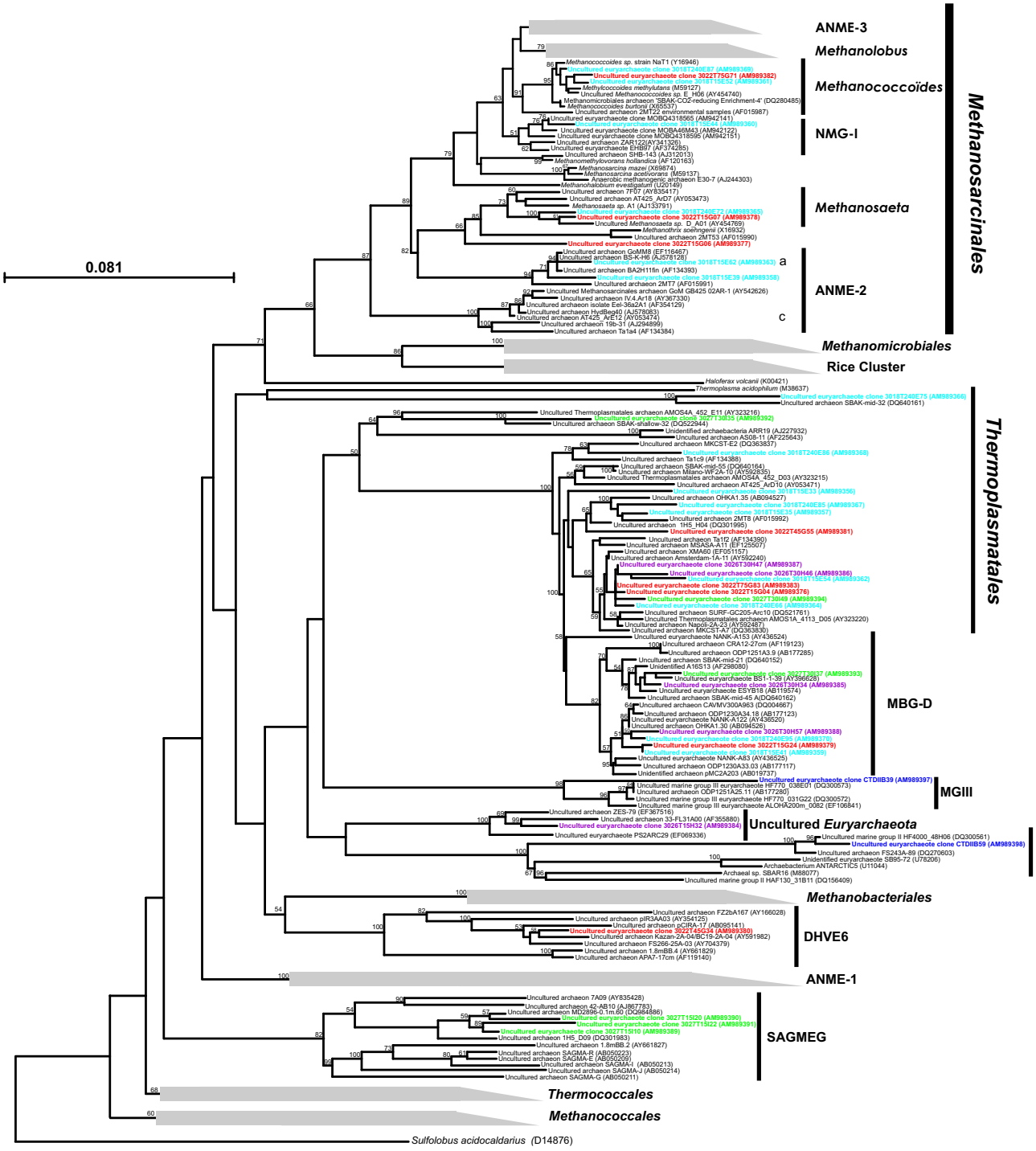



**Fig. S3.** Phylogenetic tree representing the Euryarchaeota 16S rRNA gene, except the Thermococcales lineage, sequences DNA- and RNA derived. RNA-derived sequences are underlined. Each phylotype is represented by one sequence with ≥97% similarity grouping. The tree was constructed using the neighbour-joining method with Jukes and Cantor correction. Bootstrap values <50% are not shown. ANME: anaerobic methane oxidizers, NMG-1: Novel Methanosarcinales group 1, SAGMEG: South African Gold Mine Euryarchaeotic Group, DHVE6: Deep-sea Hydrothermal Vent 6, MBG-D: Marine Benthic Group D, MG: marine groups.

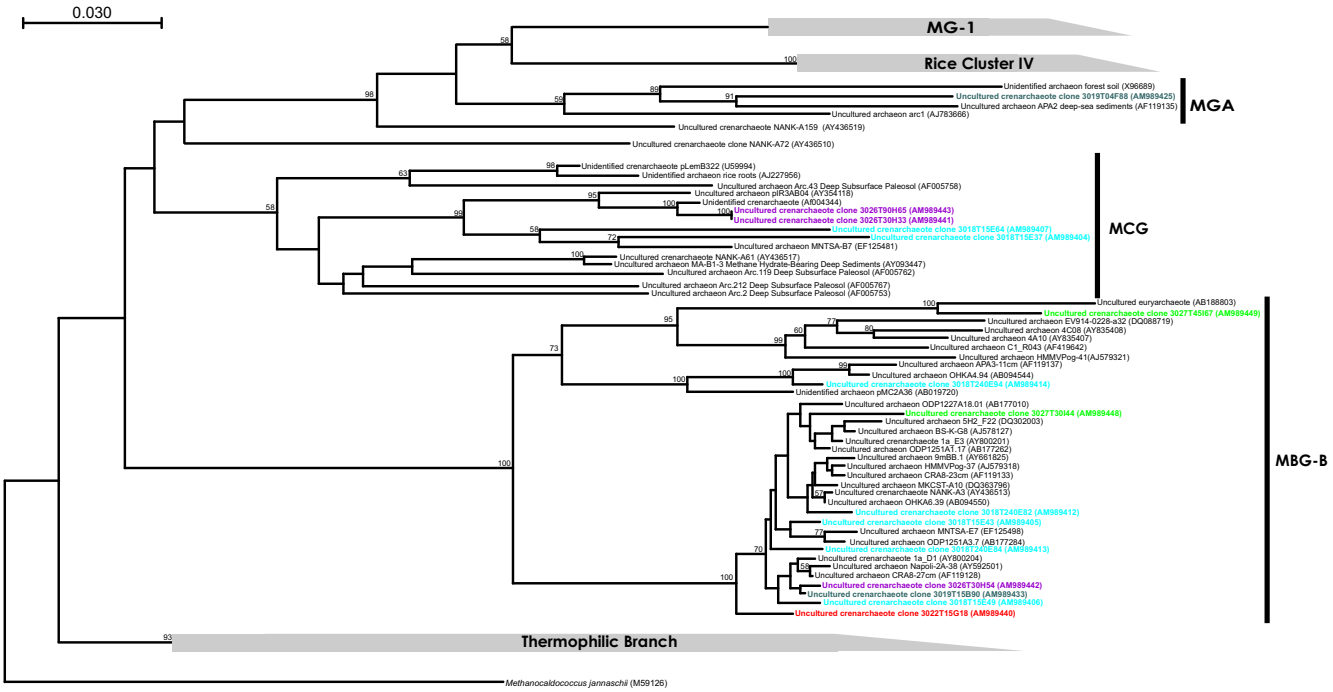



**Fig. S4.** Phylogenetic tree representing the Crenarchaeota 16S rRNA gene sequences, except the MG-1 lineage, DNA- and RNA derived. RNA-derived sequences are underlined. Each phylotype is represented by one sequence with ≥97% similarity grouping. The tree was constructed using the neighbour-joining method with Jukes and Cantor correction. Bootstrap values <50% are not shown. MCG: Miscellaneous Crenarchaeotal Group, MBG-B: marine benthic group B, MBG-A: Marine Benthic Group A.

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## 2.4 Extending the Sub–Sea-Floor Biosphere

# Science, vol. 320, 1046 (2008)

Erwan G. Roussel, Marie-Anne Cambon Bonavita, Joël Querellou, Barry A. Cragg, Gordon Webster, Daniel Prieur and R. John Parkes

Sub-seafloor sediments may contain two-thirds of Earth's total prokaryotic biomass. However, this has its basis in data extrapolation from 500-meter to 4-kilometer depths, whereas the deepest documented prokaryotes are from only 842 meters. Here, we provide evidence for low concentrations of living prokaryotic cells in the deepest (1,626 meters below the sea floor), oldest (111 million years old), and potentially hottest ( 100°C) marine sediments investigated. These Newfoundland margin sediments also have DNA sequences related to thermophilic and/or hyperthermophilic *Archaea*. These form two unique clusters within *Pyrococcus* and *Thermococcus* genera, suggesting unknown, uncultured groups are present in deep, hot, marine sediments ( 54° to 100°C). Sequences of anaerobic methane-oxidizing *Archaea* were also present, suggesting a deep biosphere partly supported by methane. These findings demonstrate that the sub-seafloor biosphere extends to at least 1,600 meters below the sea floor and probably deeper, given an upper temperature limit for prokaryotic life of at least 113°C and increasing thermogenic energy supply with depth.

*Les sédiments marins profonds pourraient contenir les deux-tiers de la biomasse microbienne. Bien que cette hypothèse soit basée sur l'extrapolation de calculs appliqués à des sédiments d'une profondeur allant de 500 m à 4 km sous la surface du sédiment, la présence de procaryotes n'a été à ce jour démontrée que jusqu'à 842 m de profondeur. Nous présentons ici des preuves de vie procaryote présente à de faibles concentrations cellulaires, jusqu'à une profondeur de 1626 m sous la surface du sédiment. Ces échantillons représentent les sédiments les plus profonds, les plus anciens (111 millions d'années) et les plus chauds (100°C) jamais analysés. Ces sédiments provenant de la marge de Terre-Neuve présentent aussi des séquences d'ADN affiliées à des Archaea potentiellement (hyper)thermophiles, suggérant l'existence de nouveaux groupes de microorganismes non-cultivés issus de sédiments marins et chauds (54 à 100°C). La présence de séquences affiliées à des Archaea ayant la capacité d'oxyder le méthane en anaérobie suggère aussi l'existence d'une biosphère profonde alimentée par du méthane. Cette étude démontre que la biosphère de sub-surface dans les sédiments marins s'étend jusqu'au moins 1600 m sous la surface du plancher océanique. Sachant que la température maximale connue à laquelle la vie persiste est d'au moins 113°C, il est probable que la profondeur maximum n'a pas encore été atteinte.*

# Extending the Sub–Sea-Floor Biosphere

Erwan G. Roussel,[1] Marie-Anne Cambon Bonavita,[1] Joël Querellou,[1] Barry A. Cragg,[2] Gordon Webster,[2] Daniel Prieur,[1] R. John Parkes[2]*

An extensive, global, sub–sea-floor biosphere has recently been documented (*1*), with the deepest sedimentary prokaryotes so far confirmed at 842 m depth, ~55°C, and 3.5 million years (My) old (*2*). It has been suggested that the sub–sea-floor biosphere may contain two-thirds of Earth's total prokaryotic biomass (*3*), but this extrapolation requires analysis of prokaryotic populations at greater depths. Here, we provide evidence for living prokaryotic cells in 1626 mbsf (meters below the sea floor) sediments that are 111 My old and at 60° to 100°C.

Nine deep sediment samples (*4*) (860 to 1626 mbsf) from the Newfoundland Margin [Ocean Drilling Program (ODP) leg 210, site 1276] were analyzed (*5*). These age from 46 to 111 My and are mainly hemipelagic mudrocks with interbedded gravity-flow deposits, formed during the opening of the northern Atlantic Ocean (*4*). An igneous sill at ~1613 mbsf (~10-m thick) is a trap for gases (methane, Fig. 1) and fluids. On the basis of the local thermal gradient and the measured thermal conductivity (*4*), the temperature of the deepest sample is calculated to be between 60° and 100°C. The upper temperature is close to the highest temperature for prokaryotic growth (~113°C) and where thermogenic alteration of organic matter can occur. The occurrence of thermogenic reactions and high temperatures (~80° to >100°C) in the deeper part of the core is supported by the presence of high methane concentrations and higher molecular weight hydrocarbons (*4*).

Intact prokaryotic cells were detected by microscopy in all samples, and many were dividing cells. Depth profiles of cell numbers fluctuate around $1.5 \times 10^6$ cells $ml^{-1}$ (Fig. 1), consistent with the general depth distribution of prokaryotic cells from other sub–sea-floor sediments (*1*). Overall, cell numbers were significantly correlated ($P < 0.01$) with the organic matter hydrogen index (HI), which reflects organic matter reactivity for microorganisms (*6*) and implies that some of the cells were metabolically active. This is consistent with the presence of dividing cells (Fig. 1) and the detection of live cells using Live/Dead (Molecular Probes Incorporated, Eugene, OR) staining (~60% viable cells). Percentages of dividing cells of the total count are in the range of previous results (0 to 4.8%) (*1*), except for higher values in the deepest sample (1626 mbsf, 11.8%). This increase in the proportion of dividing cells is probably related to the dramatic increase in concentrations of methane and higher hydrocarbons together with the generally elevated organic carbon concentrations, both providing potential energy sources (Fig. 1) (*4*).

Successful archaeal 16*S* ribosomal RNA (rRNA) gene amplification also strongly suggests that the cells are from living prokaryotes because preservation of extracellular DNA in 46 to 111 My old, hot (60° to 100°C) sediments is unlikely. In addition, some detected sequences are related to thermophiles and/or hyperthermophiles, which matches in situ temperatures. The resulting clone libraries show a low diversity of Archaea (Fig. 1), with thermophilic *Pyrococcus* dominating the 958-m depth, and then, as soon as methane increases above background concentrations, potential anaerobic methane-oxidizing (ANME) sequences became dominant (fig. S1). Surprisingly, there were no ANME sequences in the deepest sample (1626 mbsf) despite the highest methane concentrations; however, at 60° to 100°C this may be above the upper temperature limit for ANME prokaryotes (*7*). In addition, the *Pyrococcus* and *Thermococcus* sequences (fig. S1) in the deepest sample may belong to high-temperature Archaea that are able to use the thermogenic higher hydrocarbons that accumulate below the sill.

These data provide direct evidence that significant prokaryotic populations are present in marine sediments at depths greater than a kilometer and as old as 111 My. This study also suggest that Archaea capable of anaerobic oxidation of methane and novel members of the high-temperature Thermococcales (*Pyrococcus* and *Thermococcus*) can dominate deep and hot sediments where there are thermogenic energy sources.

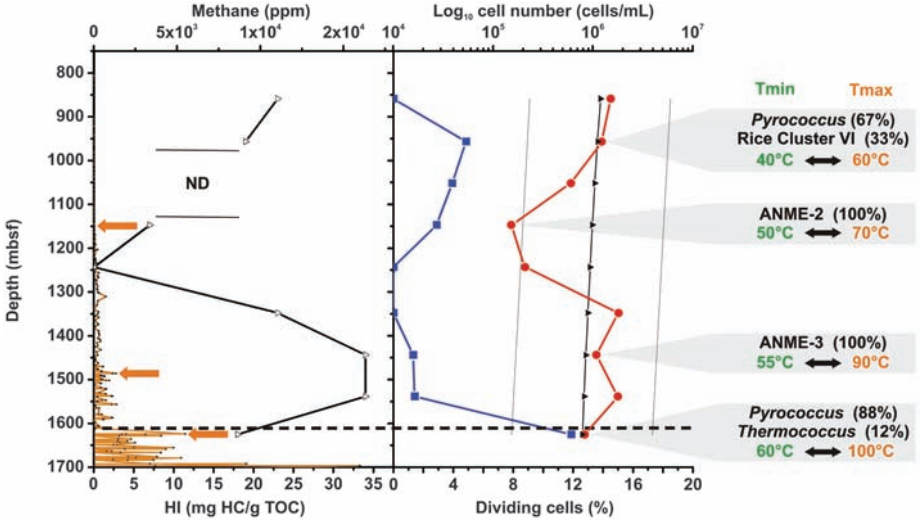


**Fig. 1.** Depth profiles of methane (black dots with orange line), prokaryotic cells (red circles), and % dividing cells (blue squares) determined by acridine orange staining. Regression equation (*1*) for prokaryotic cells in marine sediments (solid triangles), prediction limits (...). Orange arrows show local increases in methane. HI (open triangles) measured as mg of hydrocarbon (HC) per g of total organic carbon (TOC). ND, not determined. Dominant archaeal 16*S* rRNA gene sequences and in situ temperature range are on the right at the depths obtained. The diabase sill is shown as a bold horizontal dashed line.

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7. J. Kallmeyer, A. Boetius, *Appl. Environ. Microbiol.* **70**, 1231 (2004).
8. We thank members of the Leg 210 cruise, especially J. C. Sibuet, for obtaining samples and K. E. Louden for helpful discussions and providing the thermal gradient data. This study used data provided by the ODP (www-odp.tamu.edu) and was partly funded from Natural Environment Research Council, UK. GenBank accession numbers are AM418589 to AM418604.

## Supporting Online Material

www.sciencemag.org/cgi/content/full/320/5879/1046/DC1
Materials and Methods
Fig. S1

21 December 2007; accepted 10 March 2008
10.1126/science.1154545

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# Supporting Online Material for
## Extending the sub-seafloor biosphere.

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**This PDF file includes:**



Materials and methods:

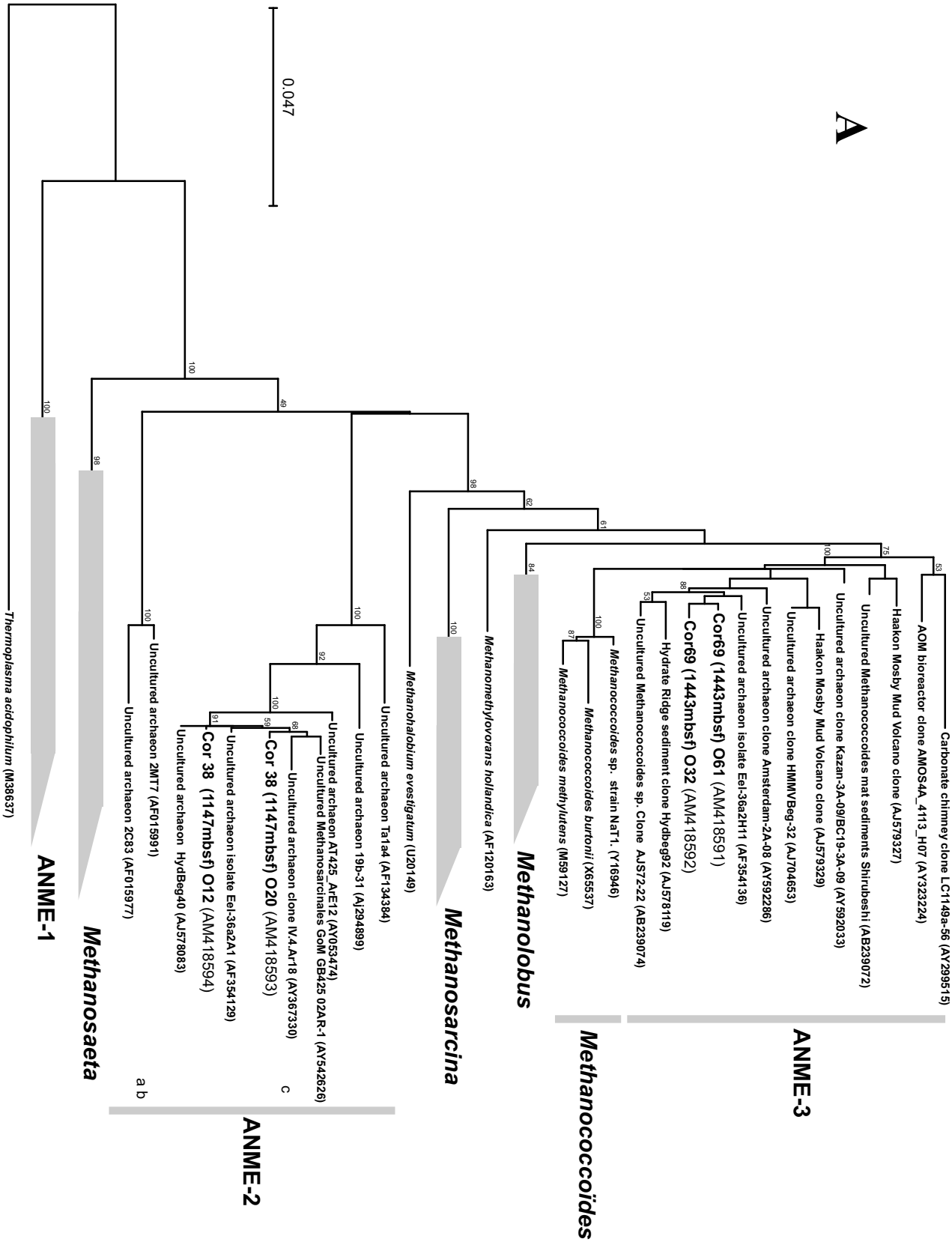
**Sampling, total prokaryotic count and live/dead staining.** Nine samples were collected below 800 m from Site 1276 (4560 m water depth) on the Ocean Drilling Program (ODP) Leg 210 in the Newfoundland Margin (*1*). Samples were stored on board ship for enrichment cultures (4°C) or molecular analysis (-20°C), prokaryotic counts were conducted on both sample sets. Sediment cores were aseptically and anaerobically subsampled (*2*). Particulate tracers were deployed during drilling to check for any potential contamination from seawater during drilling. No contamination was detected in the subsampled cores. Total prokaryotic counts and dividing/divided cells were determined by AODC (*3*). Two types of dividing cells were counted, dividing cells, which are co-joined cells of identical morphology with an invagination between the two cells, and divided cells, which are two adjacent cells of identical morphology with a visible space between cells. No significant differences in the cell counts occurred between the 4°C and -20°C stored samples.

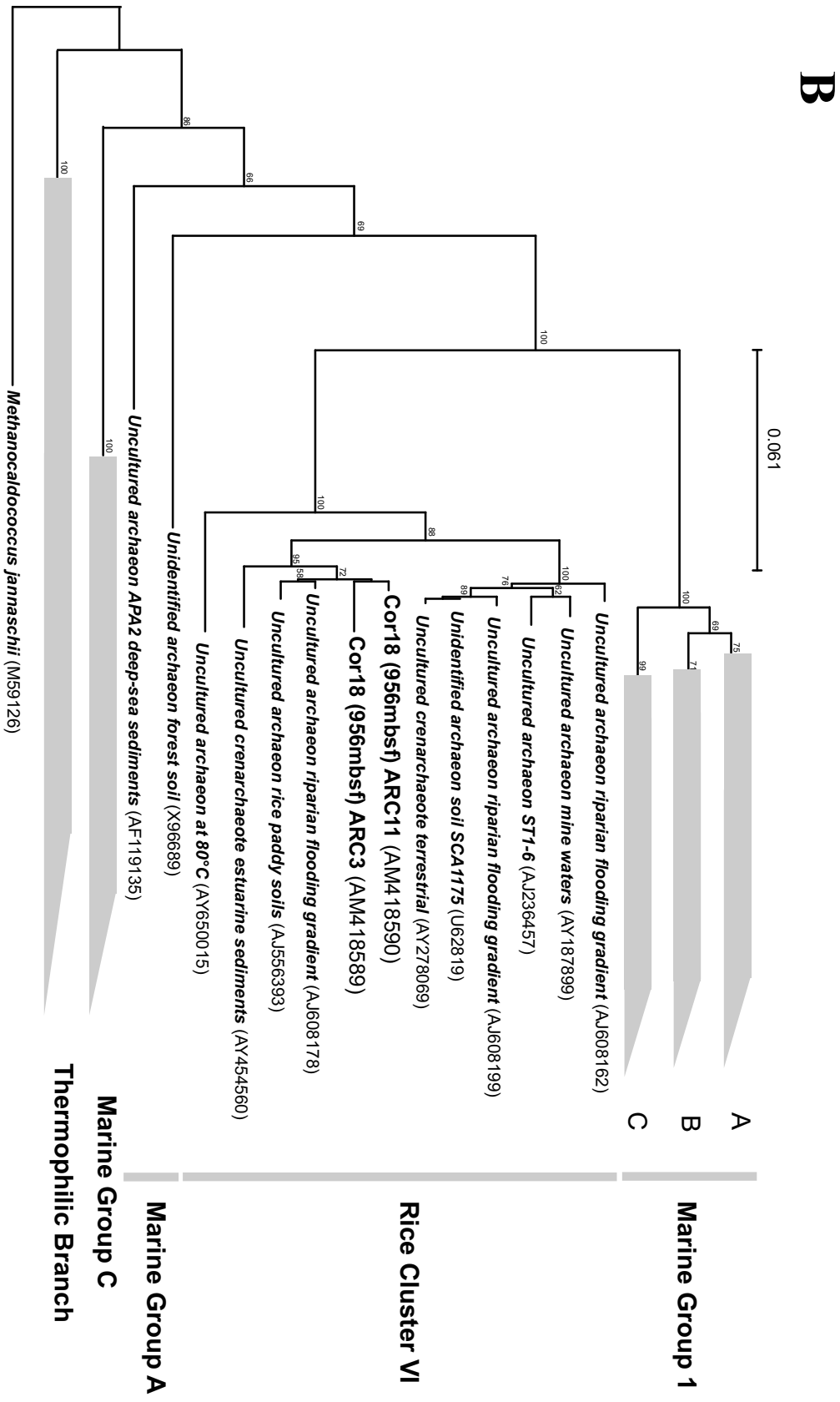
For BacLight LIVE/DEAD® analysis, live prokaryotic cells with intact membranes fluoresce green, whilst dead cells with damaged membranes fluoresce red (*4*). A sediment subsample (1g) was diluted $10^4$ times in filtered (0.1 µm pore-size) sterilized artificial seawater, the LIVE/DEAD BacLight bacterial viability stain was then added

following manufacturer's instructions (Molecular Probes). The solution was filtered through a polycarbonate (0.2 µm pore-size) membrane and then mounted and viewed under an epifluorescence microscope.

**DNA extraction and PCR amplification of Archaeal 16S rRNA gene.** All manipulations were carried out in a dedicated PCR cabinet. DNA was extracted from 5g of uncontaminated frozen sample (-20°C) following a modified FastDNA Spin Kit for Soil (Bio101) protocol (*5*). Extractions with no sample were carried out as negative controls. Nested PCR were conducted with combination of 16S rRNA gene primers for *Archaea* with 8f (5'-CGG TTG ATC CTG CCG GA-3') and 1492r (5'-GGC TAC CTT GTT ACG ACT T-3') in the first round, with 344f (5'-AYG GGG YGC ASC AGG SG-3') and 915r (5'-GTG CTC CCC CGC CAA TTC CT-3') in the second round. Five independent PCR products from the first round were pooled and purified (QIAquick PCR purification Kit; Qiagen) and used as a template for the second round.

**Cloning, sequencing and phylogenetic analysis.** Five independent PCR products from each depth (957.72, 1148.34, 1444.10, 1626.40 metres below sea floor [mbsf]) were pooled and purified (QIAquick PCR purification Kit; Qiagen) and cloned into *Escherichia coli* using the pGEM-T Easy vector system (Promega). Inserts of clones were confirmed by using the vector-specific M13 primers. Plasmid extraction, purification and sequencing of the insert, at least twice for both strands, were carried out by the sequencing Ouest-Genepole Facility of Roscoff Marine laboratory (France). Each ~553 base pair sequence was checked for chimeras and BLAST analysed against GenBank (http://www.ncbi.nlm.nih.gov/blast). Sequences were aligned with SEAVIEW program (http:// pbil.univ-lyon1.fr/) and phylogenetic trees were constructed by the PHYLOWIN program (http:// pbil.univ-lyon1.fr/) with neighbour-joining and Jukes and Cantor correction.


A
0.047
Carbonate chimney clone LC1149a-56 (AY299515)
AOM bioreactor clone AMOS4A_4113_H07 (AY323224)
Haakon Mosby Mud Volcano clone (AJ579327)
Uncultured Methanococcoides mat sediments Shirubeshi (AB239072)
Uncultured archaeon clone Kazan-3A-09/BC19-3A-09 (AY592033)
Haakon Mosby Mud Volcano clone (AJ579329)
Uncultured archaeon clone HMMVBeg-32 (AJ704653)
Uncultured archaeon clone Amsterdam-2A-08 (AY592286)
Uncultured archaeon isolate Eel-36a2H11 (AF354136)
Cor69 (1443mbsf) O61 (AM418591)
Cor69 (1443mbsf) O32 (AM418592)
Hydrate Ridge sediment clone Hydbeg92 (AJ578119)
Uncultured Methanococcoides sp. Clone AJS72-22 (AB239074)
Methanococcoides sp. strain NaT1. (Y16946)
Methanococcoides burtonii (X65537)
Methanococcoides methylutens (M59127)
ANME-3
Methanococcoïdes
Methanolobus
Methanomethylovorans hollandica (AF120163)
Methanosarcina
Methanohalobium evestigatum (U20149)
Uncultured archaeon Ta1a4 (AF134384)
Uncultured archaeon 19b-31 (AJ294899)
Uncultured archaeon AT425_ArE12 (AY053474)
Uncultured Methanosarcinales GoM GB425 02AR-1 (AY542626)
Uncultured archaeon clone IV.4.Ar18 (AY367330)
Cor 38 (1147mbsf) O20 (AM418593)
Uncultured archaeon isolate Eel-36a2A1 (AF354129)
Cor 38 (1147mbsf) O12 (AM418594)
Uncultured archaeon HydBeg40 (AJ578083)
Uncultured archaeon 2MT7 (AF015991)
Uncultured archaeon 2C83 (AF015977)
c
a b
ANME-2
Methanosaeta
ANME-1
Thermoplasma acidophilum (M38637)


B
0.061
Uncultured archaeon riparian flooding gradient (AJ608162)
Uncultured archaeon mine waters (AY187899)
Uncultured archaeon ST1-6 (AJ236457)
Uncultured archaeon riparian flooding gradient (AJ608199)
Unidentified archaeon soil SCA1175 (U62819)
Uncultured crenarchaeote terrestrial (AY278069)
Cor18 (956mbsf) ARC11 (AM418590)
Cor18 (956mbsf) ARC3 (AM418589)
Uncultured archaeon riparian flooding gradient (AJ608178)
Uncultured archaeon rice paddy soils (AJ556393)
Uncultured crenarchaeote estuarine sediments (AY454560)
Uncultured archaeon at 80°C (AY650015)
Unidentified archaeon forest soil (X96689)
Uncultured archaeon APA2 deep-sea sediments (AF119135)
Methanocaldococcus jannaschii (M59126)
A
B
C
Marine Group 1
Rice Cluster VI
Marine Group A
Marine Group C
Thermophilic Branch

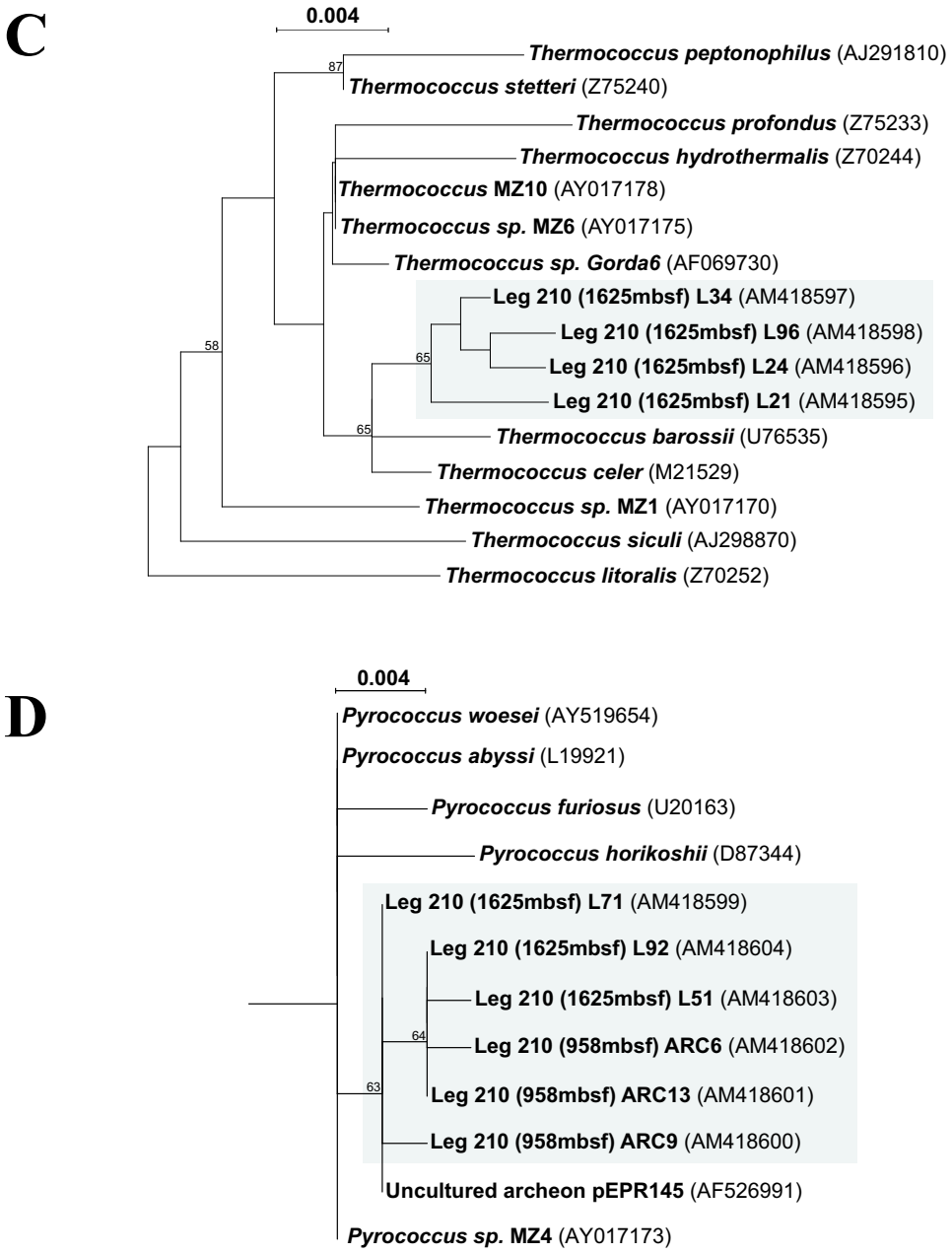



**Figure S1** Phylogenetic trees of archaeal 16S rRNA gene sequences from deep sediments of the Newfoundland margin (depth, 956 mbsf; 1147 mbsf; 1443 mbsf; 1625 mbsf). Tree topologies was inferred by neighbour-joining analysis on ~550 bases with Jukes and Cantor correction. Bootstrap support values over 50% (1,000 replicates) are shown. **A** *Euryarchaeota*-related sequences, **B** *Crenarchaeota*-related sequences, **C** *Thermococcus*-related sequences, **D** *Pyrococcus*-related sequences.

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S2 P. Wellsbury, I. Mather, & R. J. Parkes, in *Proc. Ocean Drilling Program, Scientific Results*, Volume 180, 1 (2001).
S3 B.A. Cragg, M. Summit & R.J. Parkes, in *Proc. Ocean Drilling Program, Scientific Results*, Volume. 169, 1 (2000).
S4 N.V. Queric, T. Soltwedel, W.E. Arntz, *J. Microbiol Methods* **57**, 351 (2004).
S5 G. Webster, C. J. Newberry, J. C. Fry, & A. J. Weightman, *J. Microbiol. Methods* **55**, 155 (2003).

## 2.5 Archaeal communities associated with Atlantic ultramafic hydrothermal systems

# In preparation

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Although some studies are controversial, several terrestrial (hyper)thermophilic chemoautolitrophic communities were shown recently to probably sustain by using $H_2$ as unique energy source (Anderson *et al.*, 1998; Chapelle *et al.*, 2002; Spear *et al.*, 2005; Stevens and Mc Kinley, 1995; Stevens and Mckinley, 2000). As already shown in the "Geological and geochemical context" section, sulphur cycling could be, together with methanogenesis, the dominant metabolic process in deep sea hydrothermal systems. However, some hydrothermal environments can release high concentrations of $H_2$ and $CO_2$ and low sulphide and sulphate concentrations. It was suggested that the high hydrogen content released by ultramafic-hosted hydrothermal systems could fuel the ecosystems at these sites (Charlou *et al.*, 2002; Takai *et al.*, 2004).
Hence, in order to investigate the diversity and characterize the possible metabolic pathways of hydrothermal subsurface communities associated to ultramafic-hosted hydrothermal systems, we characterized, using 16S rRNA gene and functional genes, the molecular genetic diversity of methanogens, methanotrophs and sulfate-reducers associated with fluid, chimney and sediments from three ultramafic-hosted hydrothermal sites: Rainbow, Lost City and Ashadze. As large data sets were obtained, statistical approaches were performed in order to correlate their phylogeny with variable environmental factors.

*Bien qu'elles soient controversées, certaines études montrent l'existence de communautés (hyper)thermophiles chimio-lithoautotrophes alimentées par de l'hydrogène comme seule source d'énergie (Anderson* et al.*, 1998; Chapelle* et al.*, 2002; Spear* et al.*, 2005; Stevens and Mc Kinley, 1995; Stevens and Mckinley, 2000). Comme cela a été décrit dans la section "Geological and geochemical context" , les cycles du soufre et du méthane pourraient représenter les processus dominants dans les systèmes hydrothermaux. Par ailleurs, certains environnements hydrothermaux libèrent d'importantes quantités d'hydrogène et de dioxyde de carbone, et de faibles quantités de soufre. C'est pourquoi, il a été suggéré que l'hydrogène libéré au niveau de systèmes ultramafiques pourrait alimenter les écosystèmes de ces sites (Charlou* et al.*, 2002; Takai* et al.*, 2004).*

*Afin de caractériser la diversité de certaines voies métaboliques des procaryotes associés à la sub-surface des trois sites ultramafiques (Rainbow, Lost City et Ashadze), le gène codant pour l'ARNr 16S et des gènes de fonction ont été analysés. Ces résultats ont été interprétés grâce à des outils statistiques afin de mettre en évidence de potentielles corrélations entre les facteurs biotiques et abiotiques.*

# ARCHAEAL COMMUNITIES ASSOCIATED WITH ATLANTIC ULTRAMAFIC HYDROTHERMAL SYSTEMS

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## Abstract

The distribution of archaeal communities, methanogens, methanotrophs and sulphate-reducers in three Atlantic ultramafic-hosted hydrothermal systems (Rainbow, Ashadze, Lost City) was compared using 16S rRNA clone libraries and functional genes (*mcrA, pmoA* and *dsrA*). Here we show that the microbial communities associated with the hydrothermal habitats (fluids and chimneys) are significantly different from the sea water communities. The Lost City hydrothermal field, characterized by high alkaline warm fluids (pH $>$ 11; T $<$ 95°C) contains a singular archaeal diversity mostly composed of unaffiliated *Methanosarcinales*. Interestingly, the ar-

chaeal communities associated with the recently discovered Ashadze site, the deepest known hydrothermal field, is characterized by putative extreme halophiles. Moreover, sequences related to the rarely detected *Nanoarchaeota* phylum were retrieved from the Rainbow hydrothermal field. The methanogenic *Methanococcales* is the most widespread distributed archaeon among the hot ($> 350°C$) ultramafic-hosted hydrothermal system environments. Most of the lineages detected are linked to methane and hydrogen cycling, suggesting that in ultramafic-hosted hydrothermal systems, large methanogenic and methanotrophic communities could be fuelled by hydrothermal fluids highly enriched in methane and hydrogen.

## Introduction

Deep-sea hydrothermal environments are characterized by intense physico-chemical gradients providing a large range of habitats for chemolithoautotrophic microorganisms (Kelley *et al.*, 2002). Until recently, most of the studies of the microbial diversity associated to deep-sea hydrothermal environments have mainly investigated basaltic-hosted hydrothermal systems (Kelley *et al.*, 2002). However, recent studies showed that ultramafic-hosted hydrothermal systems contained specific microbial communities (Brazelton *et al.*, 2006; Perner *et al.*, 2007). To date, only three ultramafic sites were fully described (Rainbow, Lost City and Logatchev) and were characterized by high concentrations of methane and hydrogen, in contrast with basaltic-hosted hydrothermal systems (Charlou *et al.*, 2002; Kelley *et al.*, 2001; Schmidt *et al.*, 2007). However Ashadze, a novel undescribed hydrothermal site, potentially hosting ultramafic rocks, was recently reported on the Mid-Atlantic Ridge (MAR) (Bel'tenev *et al.*, 2005; Fouquet *et al.*, 2007; Charlou *et al.*, 2007). Ultramafic hydrothermal fluids are highly enriched in methane and hydrogen as a result of serpentinization reactions between the ultramafic rocks and seawater (Allen and Seyfried, 2004). Moreover, ultramafic-hosted hydrothermal systems could supply twice as much chemical energy as basaltic-hosted hydrothermal systems (McCollom, 2007). Hence, most of the prokaryotes found at these sites seemed to be related to methane and hydrogen cycling (Perner *et al.*, 2007; Boetius, 2005).

Although microbial communities occupy both aerobic and anaerobic habitats, anaerobic hyperthermophilic *Archaea* were reported to be associated with the hottest parts of these environments, and were therefore likely to be entrained by hydrothermal fluids from subsurface ecosystems (Deming and Baross, 1993*a*; Kelley *et al.*, 2002; Takai *et al.*, 2004). Moreover, *Archaea* encompass up to 33-50 % of the total microbial community in deep-sea hydrothermal environments (Harmsen *et al.*, 1997; Nercessian *et al.*, 2003) and could be fuelled by inorganic compounds (Amend and Shock, 2001).

Although, an increasing number of thermophilic prokaryotes are cultivated from hydrothermal environments (Huber *et al.*, 2002*a*; Reysenbach *et al.*, 2006; Miroshnichenko and Bonch-Osmolovskaya, 2006; Wagner and Wiegel, 2008), molecular phylogenetic approaches have revealed several new lineages (Kormas *et al.*, 2006; Takai and Horikoshi, 1999; Nercessian, 2003;

Moussard *et al.*, 2006*a*). Metagenomic approaches and functional gene analyses have successfully characterized the metabolic and physical proprieties of these communities (Moussard *et al.*, 2006*b*; Moussard *et al.*, 2006*c*; Nercessian *et al.*, 2005*a*). Moreover, to our knowledge, 16S rRNA molecular approaches have rarely compared the microbial diversity from multiple different hydrothermal sites (Lopez-Garcia *et al.*, 2003*a*; Lopez-Garcia *et al.*, 2003*b*).
In the present study, we characterized the molecular genetic diversity, using 16S rRNA gene and functional genes of methanogens, methanotrophs and sulfate-reducers associated with three ultramafic-hosted hydrothermal sites: Rainbow, Lost City and Ashadze. As these hydrothermal fluids are highly enriched in methane and hydrogen, it is crucial to identify specific prokaryotic communities possibly associated with potential subsurface chemolithoautrophic ecosystems. Hence, the aim of this study was to compare the microbial communities of these ultramafic-hosted hydrothermal sites using statistical approaches, in order to correlate their phylogeny with variable environmental factors.

## Results and discussion

### Site and sample description

A total of 15 samples, encompassing fluids, chimney fragments and sediments, were retrieved from three MAR hydrothermal sites: Rainbow, Lost City and Achadze (Figure 2.17A). All the hydrothermal fluid samples at Rainbow were retrieved from the "*termitière*" chimney group (Figure 2.17E), except the "PP27 swarm" sample which was obtained in close proximity to the shrimp swarms on the side of the PP27 chimney. The "*termitière*" chimney group was composed of both diffuse and black smoker venting. The Rainbow sediment samples were retrieved nearby the hydrothermal chimneys and were dominantly made of pelagic sediment (98 % calcite) with small amount of hematite indicating a small hydrothermal contribution (Figure 2.17D). The Lost City fluid sample was obtained from one of hottest (93°C) venting areas of this site, which was located above a flange (Figure 2.17C). The two Ashadze and Logatchev vent fields are all located at13°N and 14°45 N region of the Mid-Atlantic Ridge (Bel'tenev *et al.*, 2005; Bogdanov *et al.*, 1997). This part of the MAR is characterized by basalt compositions indicating that anomalously enriched mantle domains are involved in the melting region (Dosso *et al.*, 1993), and by numerous outcrops of serpentinized mantle-derived rocks (Cannat *et al.*, 1997; Bougault *et al.*, 1993). Ashadze is characterized by an ultramafic rock environment (Fouquet *et al.*, 2007; Charlou *et al.*, 2007; Charlou, tted). Several groups of active 1 m to 2 m high chimneys were observed at Ashadze 1. The fluid and chimney fragments were obtained from 2 different active chimneys in a unique group near the SE-2 marker (Figure 2.17F).

In the light of their geological settings and mineral and fluids compositions, Rainbow and Lost City are also are also typical of ultramafic environments vents (Kelley *et al.*, 2005; Charlou *et al.*, 2002; Fouquet *et al.*, tted). Hence these three sites have much higher hydrogen ($< 16$ mM)

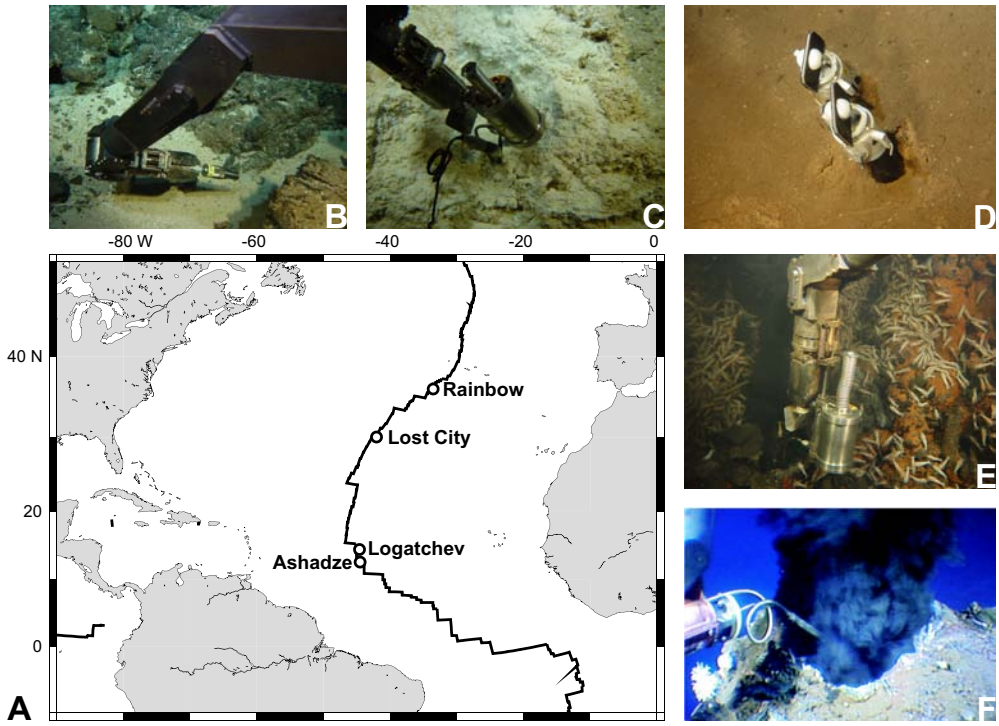


FIG. 2.17: A. Map of the location of Atlantic ultramafic sites. B. Photograph of the sediment sampling at Lost City hydrothermal field. C. Photograph of the fluid sampling using titanium syringe at Lost City hydrothermal field. D. Photograph of the sediment sampling using push-core devices at Rainbow hydrothermal field. E. Photograph of the fluid sampling using titanium syringe at Rainbow hydrothermal field. F. Photograph of temperature measurements at Ashadze 1 hydrothermal field.

and abiogenic methane ($< 2.5$ mM) concentrations than the MAR basaltic-hosted hydrothermal sites (Kelley *et al.*, 2005; Charlou *et al.*, 2002; Proskurowski *et al.*, 2008; Charlou, tted), as a result of the serpentinization processes, caused by the ultramafic-water interaction.
To date, Lost City is a unique off-axis hydrothermal site expulsing fluids with a high pH ($\sim$11), as opposed to the other known ultramafic environments that are acidic (Rainbow, Logatchev, pH = $\sim$3). Therefore, the dilution of the hydrothermal fluid sample was estimated according to pH measurements. For this study, the maximum temperature measured at Ashadze was 353°C and was in the same range at Rainbow (324°C), whereas at Lost City a lower maximum temperature (93°C) was recorded. Still, these moderate to high fluid temperatures are probably linked to magmatic heating processes (Allen and Seyfried, 2004).

### Archaeal 16S rRNA gene diversity based on clone libraries

Fourteen different 16S rRNA gene clone libraries were constructed, representing a total of 610 sequences. All the DNA-derived 16S rRNA PCR products from all the samples were screened

by DGGE prior cloning, in order to estimate the archaeal phylogenetic diversity of each sample (Webster *et al.*, 2003). After technical optimisation and removal of soluble PCR inhibitors and in order to obtain sufficient PCR product for cloning, archaeal amplifiable DNA from all samples was retrieved by nested PCR. The molecular techniques (PCR and cloning), used to build clone libraries, are known to be inherently biased (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998; von Wintzingerode *et al.*, 1997; Nocker *et al.*, 2007). However, we assume that the biases can be equal for all the samples as they were analysed under the strictly the same conditions (storage, DNA extraction, PCR amplification, cloning) (von Wintzingerode *et al.*, 1997).
The coverage values for the 16S rRNA clone libraries ranged from 68 to 97 % (Figure 2.18). On the whole, rarefaction curves were curvilinear for all the libraries confirming sufficient sampling effort (Figure S1). The whole 16S rRNA gene sequences, were assigned to 95 OTUs based on a 95 % genus level of phylotype differentiation (Schloss and Handelsman, 2004). The number of OTUs per clone library ranged from 5 to 19 and the Shannon-Weaver index of diversity ranged between 0.63 and 3.04 (Figure 2.18). The archaeal diversity indexes were all in the same range, except the fluid associated to Lost City which, as previously described, displayed the lowest diversity (Figure 2.18) (Schrenk *et al.*, 2004). Interestingly, the highest diversity indexes were observed in the sediment samples, as a consequence of the very high intra-phylum observed within the Marine Group I. Moreover according to pH measurements, the archaeal diversity in the hydrothermal fluids was always the most reduced in the less diluted fluids, suggesting that the archaeal diversity was linked to environmental factors.

On the whole, 16S rRNA sequences, were related to *Euryarchaeota* (51 %), *Crenarchaeota* (48 %) and *Nanoarchaea* (1 %). The 16S rRNA clone libraries obtained from hydrothermal environments were dominated by sequences related to Euryarchaeota (69 %), whereas sequences related to *Crenarchaeota* were a majority in the sediment (92 %) and sea water samples (70 %). The archaeal phylogenetic diversity was quite high, forming a total of 21 different lineages (Figure 2.18 and 2.19 and 2.20). Excepted for Lost City, the overall hydrothermal samples contained 6 different lineages, which was in agreement with most of the published studies on hydrothermal environments (Nercessian *et al.*, 2003; Kormas *et al.*, 2006; Pagé *et al.*, 2008; Schrenk *et al.*, 2003; Takai *et al.*, 2001*a*; Takai *et al.*, 2004, for e.g.).
The *Methanococcales* were detected in 7 of the 9 clone libraries obtained from hydrothermal samples (fluid or chimney) (Figure 2.18). *Methanococcales Archaea* are strictly anaerobic autotrophic methanogens, using hydrogen and carbone dioxide or formate as energy sources (Whitman *et al.*, 2001). Moreover, strains affiliated to *Methanocaldococcus infernus* and to *Methanopyrus* were successfully cultured from hydrothermal chimneys samples from Rainbow and Achadze (L'Haridon and Jeanthon, personal communication) The methanogenic potentials of the *Methanococcales* at Rainbow and Ashadze were confirmed by the detection of *mcrA* genes related to *Methanothermococcus thermolithotrophicus* ($>$ 97 % similarity) and *Methanocaldo-*

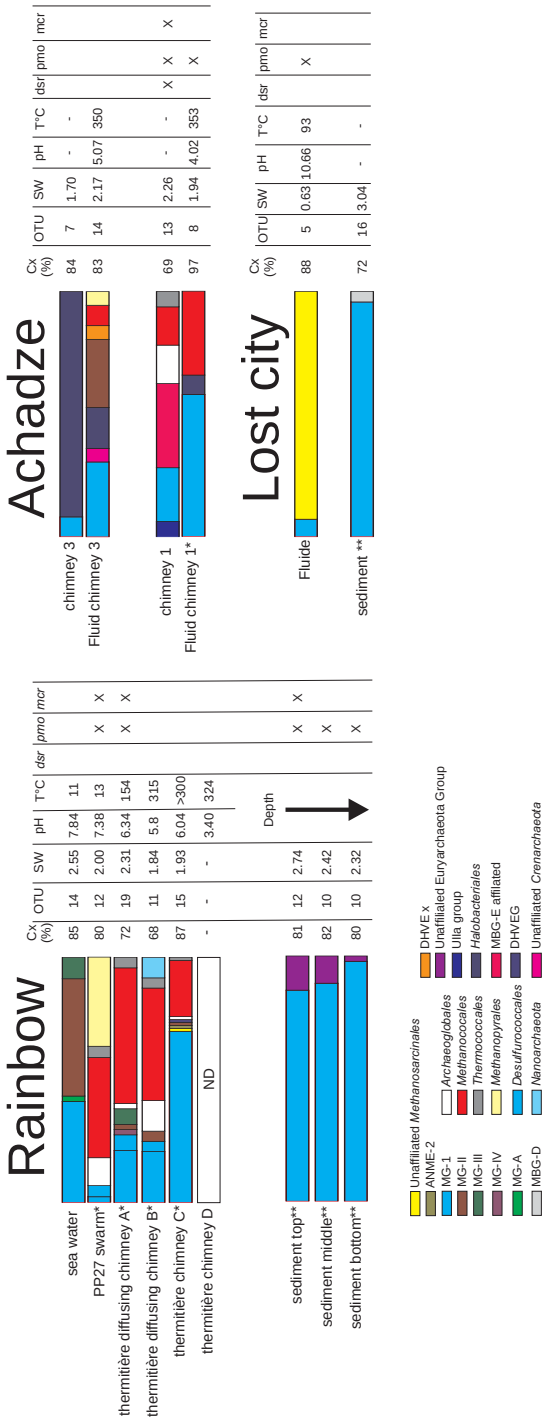


FIG. 2.18: Distribution of the archaeal phylogenetic communities based on 16S rRNA gene from 3 ultramafic-hosted hydrothermal sites. The phylogenetic affiliation of each clone sequence was determined by similarity analysis. The relative abundance of each phylotype was calculated and represented in a column diagram. Cx indicates the percentage of coverage of each clone library. OTU indicates the number of operational taxonomic units (95 %) for each clone library. SW indicates the Shannon-Weaver index of diversity. *dsr, pmo* and *mcr*, respectively indicate positive amplification of the functional genes. The asterisks indicate groups of clone libraries with insignificant ($P < 0.001$) differences between all the diversity indices ($F_{ST}$ and the exact test method). ANME-2: anaerobic methane oxidizers, DHVEG: Deep-sea Hydrothermal Vent Group, MBG-D: Marine Benthic Group D, MBG-A: Marine Benthic Group A, MG-1 (II, III, IV): Marine Group 1 (II, III, IV), MBG-E: Marine Benthic Group E, ND: Not Detected.

*coccus infernus* (> 97 %) (Figure 2.22). Hyperthermophilic or thermophilic members of the *Methanococcales* are commonly cultured, and detected with molecular tools, from marine hydrothermal vent systems (Kelley *et al.*, 2002; Nercessian *et al.*, 2003; Pagé *et al.*, 2008; Perner *et al.*, 2007; Schrenk *et al.*, 2003; Takai *et al.*, 2004, for e.g.). However, Schrenk and colleagues reported that *Methanococcales* encompassed a low proportion (< 5 %) of the hydrothermal prokaryotic communities, whereas on different hydrothermal sites Takai and colleagues reported proportions up to 76.5 % (Schrenk *et al.*, 2003; Takai *et al.*, 2004). These differences could be linked to environmental factors such as the hydrogen production, which could fuel these communities.

Sequences related to the *Thermococcales* and *Archaeoglobales* lineages were also detected in 5 of the 9 clone libraries obtained from hydrothermal samples (fluid or chimney) (Figure 2.18). Members of the *Thermococcales* order are mainly characterized as thermophilic to hyperthermophilic anaerobic heterotrophs that ferment peptides and sugars, and their growth can also be stimulated by sulphur reduction (Miroshnichenko and Bonch-Osmolovskaya, 2006). *Archaeoglobales Archaea* are also involved in the sulphur cycle as hyperthermophilic sulphate reducers (Miroshnichenko and Bonch-Osmolovskaya, 2006). However, some members of the *Thermococcales* were also able of growing on acetate utilising Fe(III) (Summit and Baross, 2001*b*) or capable of lithotrophic growth on carbon monoxide coupled with hydrogen production (Sokolova *et al.*, 2004). A new specie of the *Archaeoglobaceae* was recently reported as the first dissimilatory Fe(III)-reducing prokaryote obligately growing autotrophically on hydrogen (Kashefi *et al.*, 2002). It has been suggested that *Thermococcales* like the (hyper)thermophilic members of the *Methanococcales* order could inhabit sub-seafloor ecosystems (Summit and Baross, 1998; Summit and Baross, 2001*a*; Takai *et al.*, 2004; Kelley *et al.*, 2002), and could be part of a hydrogen-driven subsurface lithoautotrophic microbial ecosystems (Nealson *et al.*, 2005).

To date, *Methanopyrales* were never detected by molecular methods on the MAR, probably a consequence of the restricted number of microbial studies of the MAR, though the first isolated relative originates from a hydrothermal system north of Iceland (Kurr *et al.*, 1991). Sequences related *Methanopyrales* were also rarely detected elsewhere (Takai *et al.*, 2004; Ehrhardt *et al.*, 2007; Pagé *et al.*, 2008; Nercessian, 2003). However in this study, eighteen sequences related to *Methanopyrus kandleri* (> 96 % similarity) were detected from Rainbow and Ashadze fluids. Takai and colleagues recently reported an isolate related to *Methanopyrus kandleri* capable of methanogenesis with $H_2/CO_2$ under hydrostatic conditions and at 122°C (Takai *et al.*, 2008). *mcrA* gene sequences related to *Methanopyrus kandleri* (88 % similarity) were also detected at Rainbow (Figure 2.22), suggesting that the *Methanopyrales* detected were capable of methanogenesis. Moreover, within the methane cycling communities associated to Rainbow, putative methanotrophic ANME-2 sequences were detected, suggesting occurrence of anaerobic methane

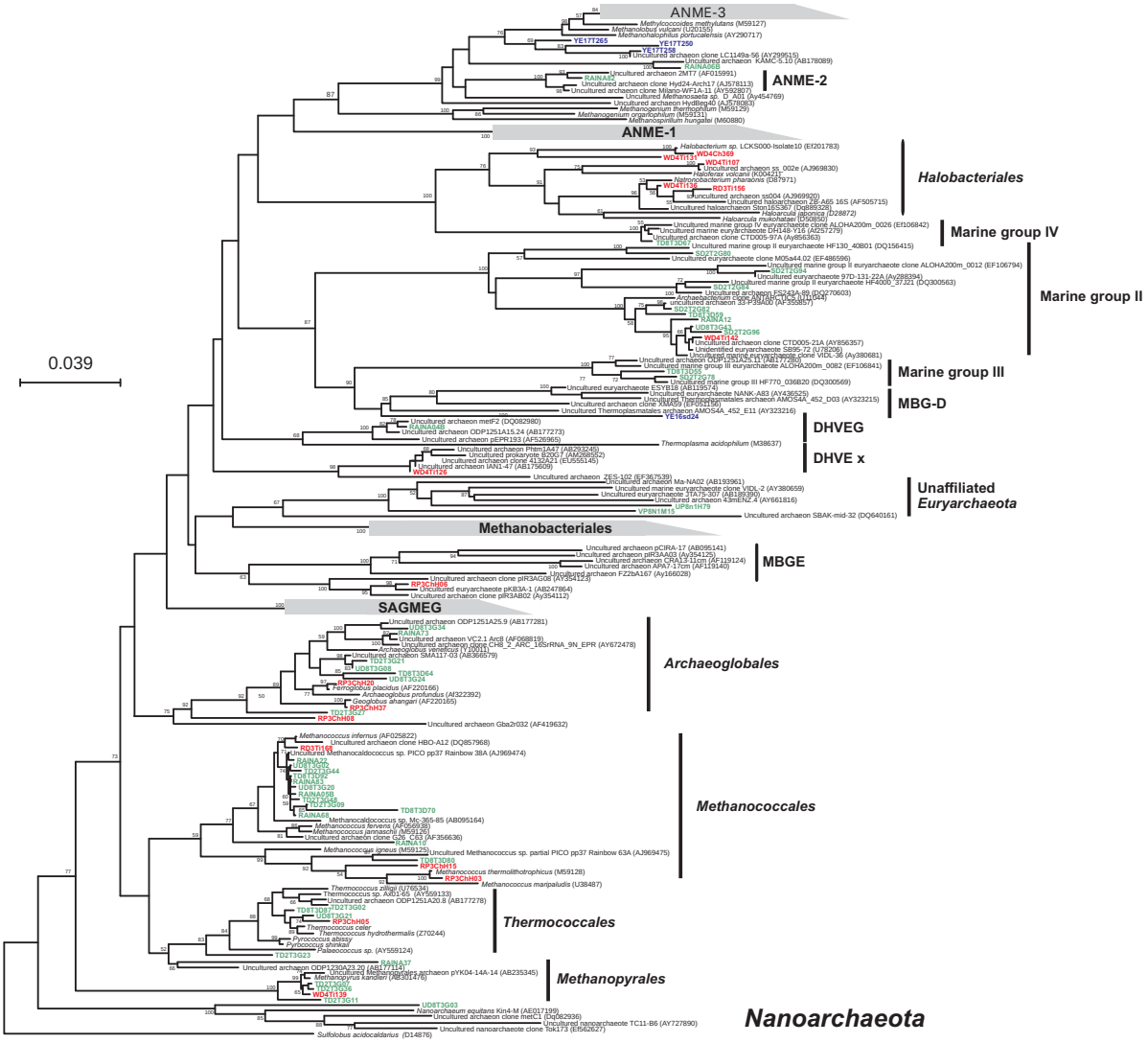


FIG. 2.19: Phylogenetic tree representing the *Euryarchaeota* 16S rRNA gene. Each phylotype is represented by one sequence with $> 97\,\%$ similarity grouping. The tree was constructed using the neighbor-joining method with Jukes and Cantor correction. Bootstrap values $< 50\,\%$ are not shown. Red sequences: Ashadze clone libraries. Green sequences: Rainbow clone libraries. Blue sequences: Lost City clone libraries. ANME: anaerobic methane oxidizers, DHVE: Deep-sea Hydrothermal Vent, MBG-D: Marine Benthic Group D, MBG-E: Marine Benthic Group E, SAGMEG: South African Gold Mine Euryarchaeotic Group.

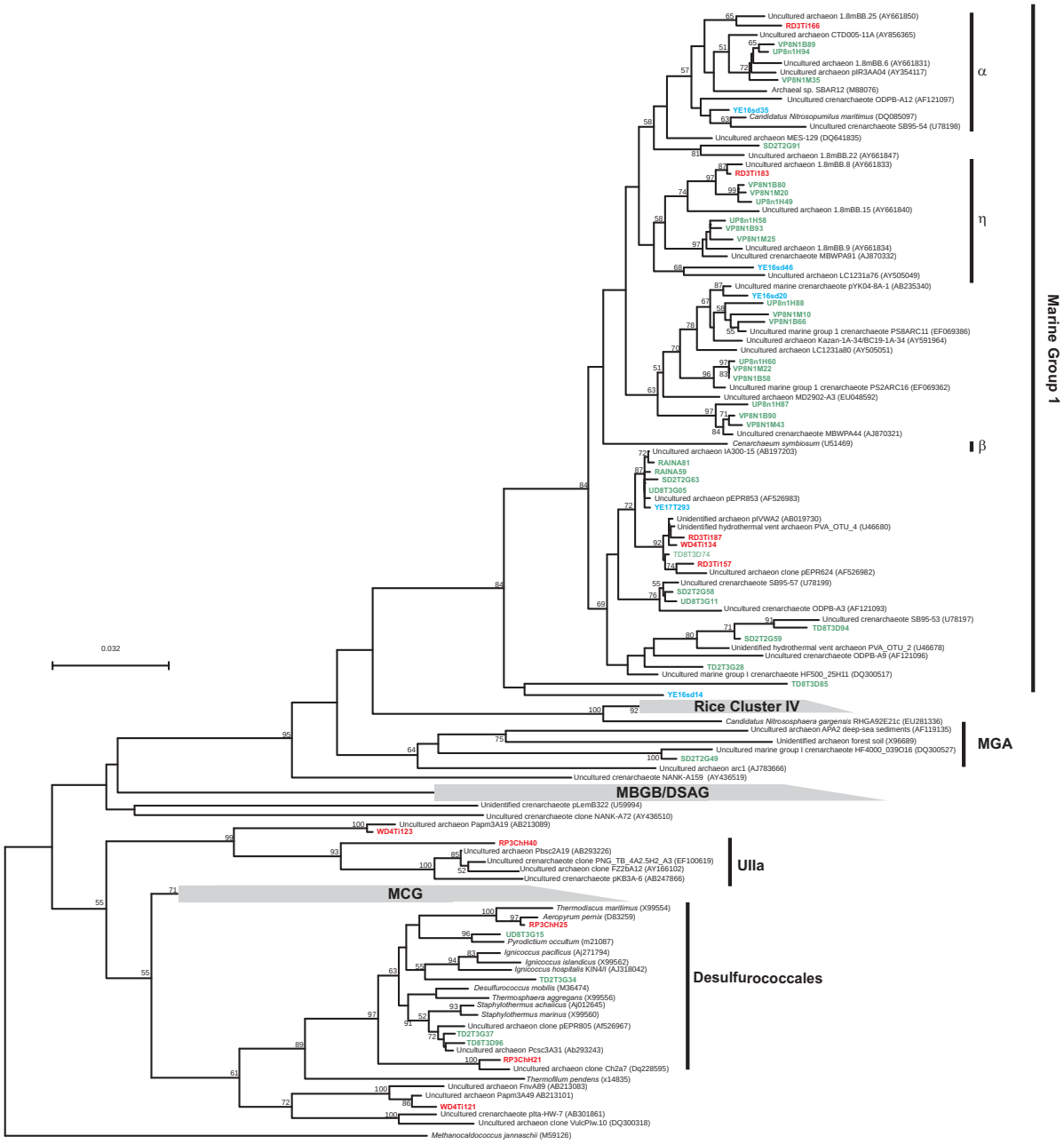


FIG. 2.20: Phylogenetic tree representing the *Crenarchaeota* 16S rRNA gene sequences. Each phylotype is represented by one sequence with $> 97$ % similarity grouping. The tree was constructed using the neighbor-joining method with Jukes and Cantor correction. Bootstrap values $< 50$ % are not shown. Red sequences: Ashadze clone libraries. Green sequences: Rainbow clone libraries. Blue sequences: Lost City clone libraries. MCG: Miscellaneous Crenarchaeotal Group, MBG-B: marine benthic group B, MBG-A: Marine Benthic Group A.

oxidation communities associated to anoxic habitats below 90°C (Kallmeyer and Boetius, 2004). Interestingly, 16S gene sequences related to *Halobacteriales* were only detected from Ashadze, which is to date the deepest known hydrothermal site (Figure 2.18 and 2.19). The highest similarity with a cultured relative was *Natronomonas pharaonis* (98 %), an extremely haloalkaliphilic archaeon. The occurrence of halotolerant prokaryotes in hydrothermal environments, growing at higher NaCl concentrations than most of marine *Bacteria*, was previously reported. Due to phase separation it is admitted that venting of a condensed vapor phase with low salinity will generate a high salinity phase a depth. This phase may be venting later or be trapped in the subsurface environments. In addition some authors have suggested a double diffusive hydrothermal system where brines are trapped in the deepest part of the system and exchange only heat with the upper convective system (Fouquet *et al.*, 1993*a*; Bischoff and Rosenbauer, 1989). If partially cooled, this deep high salinity reservoir may constitue and extensive location for halotolerant prokaryotes. Hence it was also suggested that these communities could be associated with a sub-vent ecosystem (Kaye and Baross, 2000; Takai *et al.*, 2001*a*).

The recently discovered novel Nanoarchaeota phylum has shown a wide distribution in high temperature ecosystems (Hohn *et al.*, 2002; Huber *et al.*, 2002*a*), and may represent pioneering communities in deep-sea hydrothermal vents (McCliment *et al.*, 2006). *Nanoarchaeota* could represent a fast-evolving euryarchaeal lineage related to *Thermococcales* (Brochier *et al.*, 2005). Hence, in this study, the detection of sequences related to *Nanoarchaeota* in the Rainbow hydrothermal system (Figure 2.19) shows that nanoarchaeal habitat extends to the deep hot marine hydrothermal systems of the MAR. Interestingly, the same site Crenarchaeota were retrieved with 94 % similarity to the hyperthermophilic chemolithoautotrophic suphur and hydrogen utilizing *Ignicoccus hospitalis*, a member of the *Desulfurococcales* order (Figure 2.20). The nano-sized *Nanoarchaeota* were previously described to have a symbiotic relationship with *Ignicoccus hospitalis*, a strain isolated from the Kolbeinsey Ridge, north of Iceland (Paper *et al.*, 2007).

As previously described, the off-axis Lost City hydrothermal system is remarkable by its geological, geochemical and biological settings (Kelley *et al.*, 2005). The archaeal diversity associated with hot and very alkaline ($< 95$°C, pH $> 10$) Lost City hydrothermal fluid was limited to unaffiliated *Methanosarcinales* and to Marine Group I sequences (Figure 2.18, 2.19 and 2.20). The detected unaffiliated *Methanosarcinales* sequences matched with the Lost City *Methanosarcinales* cluster (99 % similarity) described by Schrenk and colleagues (Schrenk *et al.*, 2004), suggesting that these *Archaea* were involved in methane cycling processes (Boetius, 2005; Schrenk *et al.*, 2004). However, no *mcrA* gene sequences were detected at Lost City, as a probable consequence of low cell densities in the hot ($< 95$°C) Lost City fluids (Schrenk *et al.*, 2004). The occurrence of molecular genetic evidences in hot and very alkaline fluids also suggests that the Lost City *Methanosarcinales* have physiological potentials beyond the capacities of any known

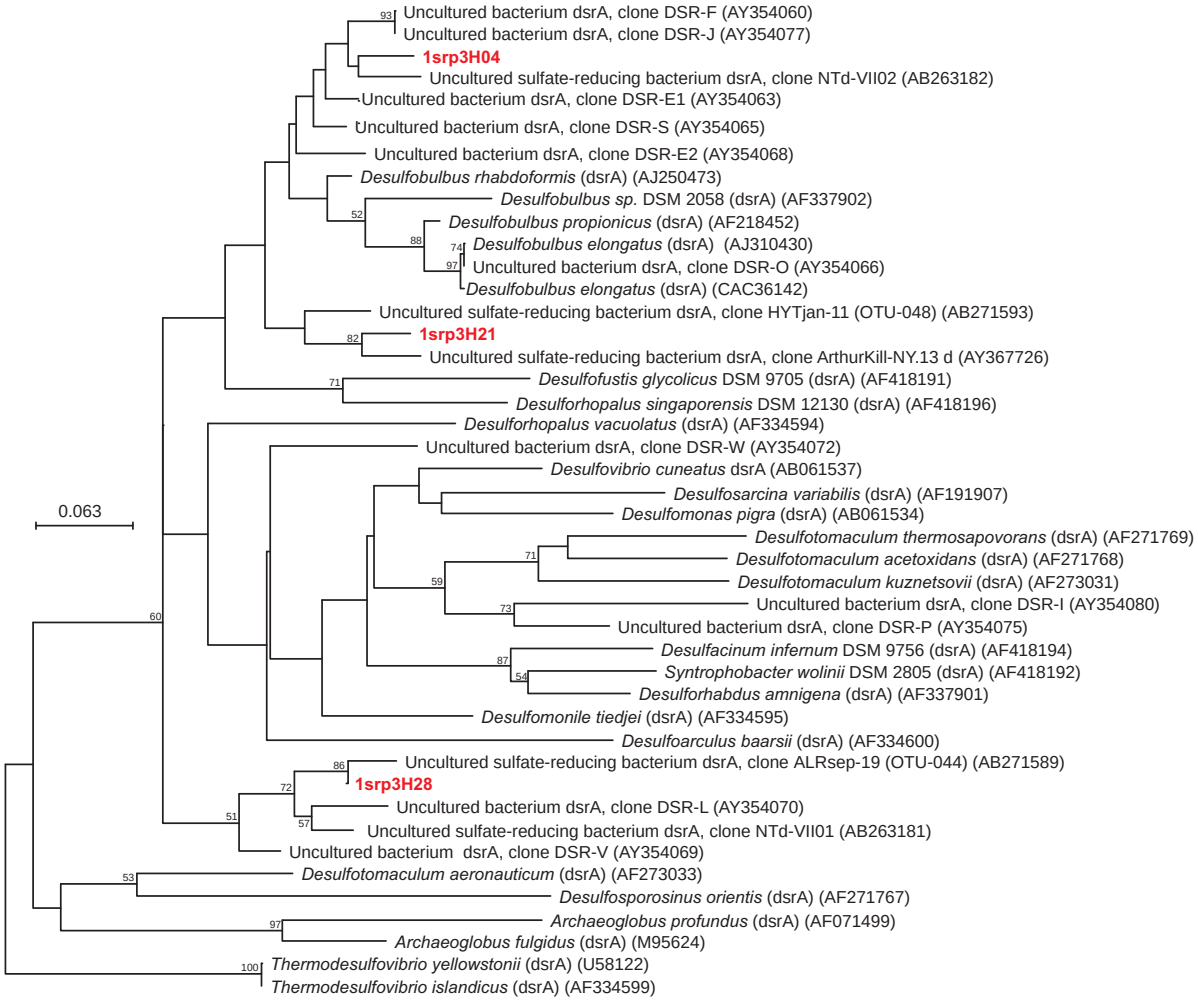


FIG. 2.21: Phylogenetic tree based on translated, partial amino acid sequences of *dsr* gene. The tree was constructed using the neighbor-joining method using PAM distance (Dayhoff *et al.*, 1978). The robustness of inferred topology was tested by the bootstrap. Bootstrap values $< 50$ % are not shown.

cultured isolates (Mesbah and Wiegel, 2008).

### Marine Group 1 distribution

Sequences related to the Marine Group I (MG-1) were detected in 13 of the 14 clone libraries (Figure 2.18 and 2.20), representing the most ubiquitous lineage found in the MAR ultramafic-hosted hydrothermal environments. The archaeal diversity of the seawater clone library was significantly different from all the other clone libraries ($P < 0.001$), and was dominated by sequences related to the MG-1 (41 %) and Marine Group II (48 %). However, Takai and colleagues showed that the highest proportion of MG-1 members in hydrothermal environments was found in the sea water adjacent to the hydrothermal emissions (Takai *et al.*, 2004). MG-1 are widespread in our hydrothermal fluid clone libraries, suggesting MG-1 could be the result of the mixing with the ambient seawater.
MG-1 *Archaea*, identified as aerobic autotrophic ammonia oxidizers (Konneke *et al.*, 2005; Francis *et al.*, 2005; Hallam *et al.*, 2006), are commonly found in seawater and marine sediments, forming several phylogenetic clusters with currently cultured relatives (Konneke *et al.*, 2005; Preston *et al.*, 1996). Moreover, based on the analysis of the first sequenced genome of a cultured relative (*Crenarchaeum symbiosum*), the MG-1 were recently proposed as a novel archaeal phylum named *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008). The MG-1 sequences also dominated the sediment 16S rRNA gene clone libraries ($> 86$ %) as commonly found in suboxic marine surface sediments (Inagaki *et al.*, 2001; Teske and Sorensen, 2008; Roussel *et al.*, 2009). Furthermore, a moderately thermophilic ammonia-oxidizing crenarchaeote was recently isolated from the Siberian Garga hot spring (Hatzenpichler *et al.*, 2008) and a thermophilic origin for anaerobic ammonium oxidation was suggested (Canfield *et al.*, 2006). Hence, according to their widespread dissemination in hydrothermal systems and to physico-chemical gradients, MG-1 could therefore be responsible for a large proportion of the ammonium oxidation in hydrothermal systems.

### Diversity based on functional gene clone libraries

The diversity of four *mcrA* libraries was limited to sequences related to the $H_2/CO_2$ methanogens *Methanococcales* and *Methanopyrales* orders (Figure 2.22), congruently with the 16S rRNA clone libraries (Figure 2.19). The operon coding for the MCR-I, which includes McrA subunit, is found in all known methanogens (Reeve *et al.*, 1997). The *mcrA* sequences from Rainbow and Ashadze matched with the two groups of uncultured methanogenic *Archaea* previously retrieved from Rainbow (Nercessian *et al.*, 2005*a*), suggesting that these could be long-term stabilized population in these chemically slow evolving environments (Charlou *et al.*, 2002).
Sequences related to *dsrA* gene were only detected from one fluid sample located at the Ashadze chimney 1 (Figure 2.18 and 2.21). Interestingly, *dsrA* sequences clustered with sequences previously detected in methane-rich hydrothermal systems and related to the *Desulfobulbaceae*

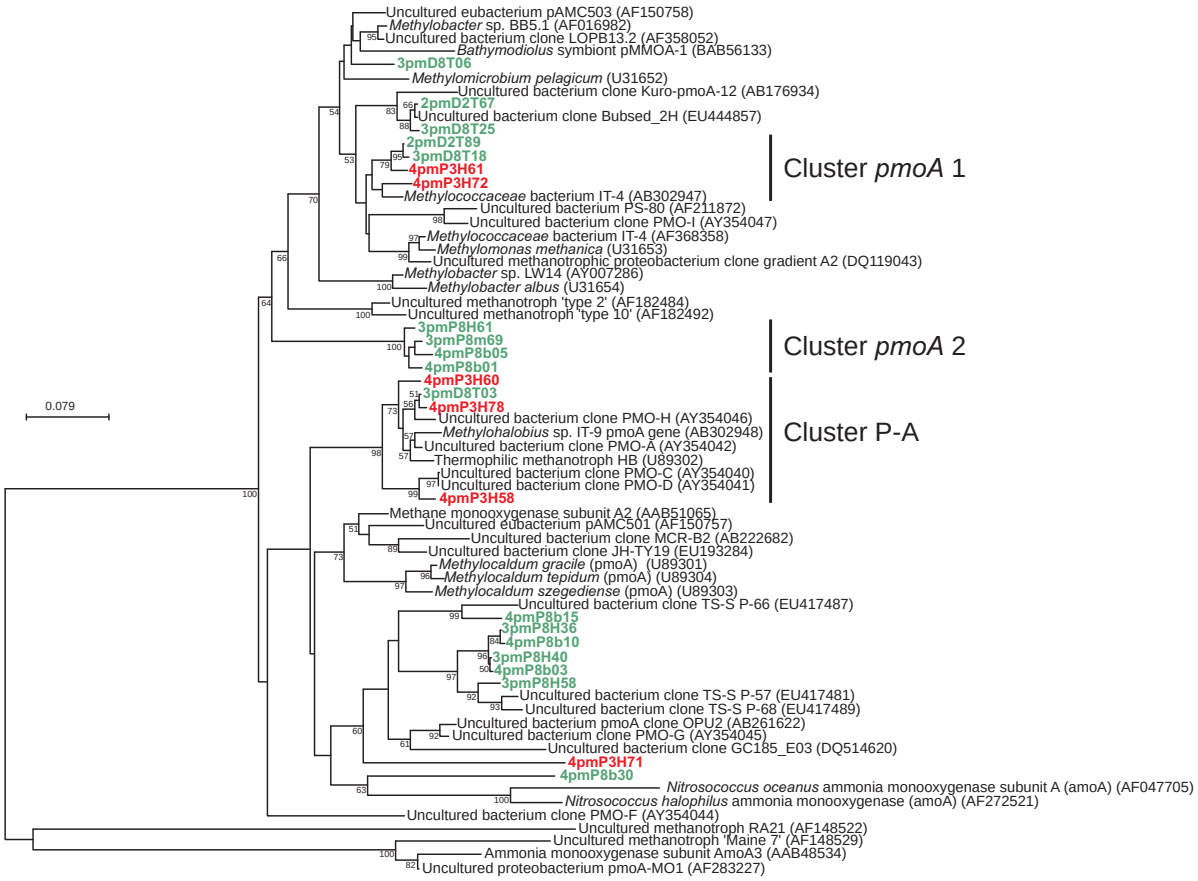


FIG. 2.22: Phylogenetic tree based on translated, partial amino acid sequences of *pmo* gene. The tree was constructed using the neighbor-joining method using PAM distance (Dayhoff *et al.*, 1978). The robustness of inferred topology was tested by the bootstrap. Bootstrap values $< 50$ % are not shown.

family (Nercessian *et al.*, 2005*a*; Teske *et al.*, 2002), indicating that these putative sulphate-reducing *Bacteria* could be linked to these specific environmental conditions. Besides, some members of *Desulfobulbaceae* can live syntrophically with ANME-3 members (Niemann *et al.*, 2006).

The *pmoA* gene was the most widespread functional gene detected, as a PCR amplification was obtained on 8 of the 15 samples tested (Figure 2.22 and 2.23). However, the phylogeny of the *pmoA* gene was poorly resolved, the bacterial *pmoA* gene being distantly related to the ammonia monooxygenase subunit A (*amoA*) (Holmes *et al.*, 1995; Nicol and Schleper, 2006), as revealed by incongruence between tree topologies performed with different phylogenetic methods (Figure 2.22). Two groups of *pmoA* sequences from Rainbow and Ashadze clustered (cluster *pmoA* 1 and cluster P-A) with sequences related to thermophilic methylotrophes (Hirayama *et al.*, 2007; Inagaki *et al.*, 2003*b*). Moreover, a group of sequences (cluster *pmoA* 2)

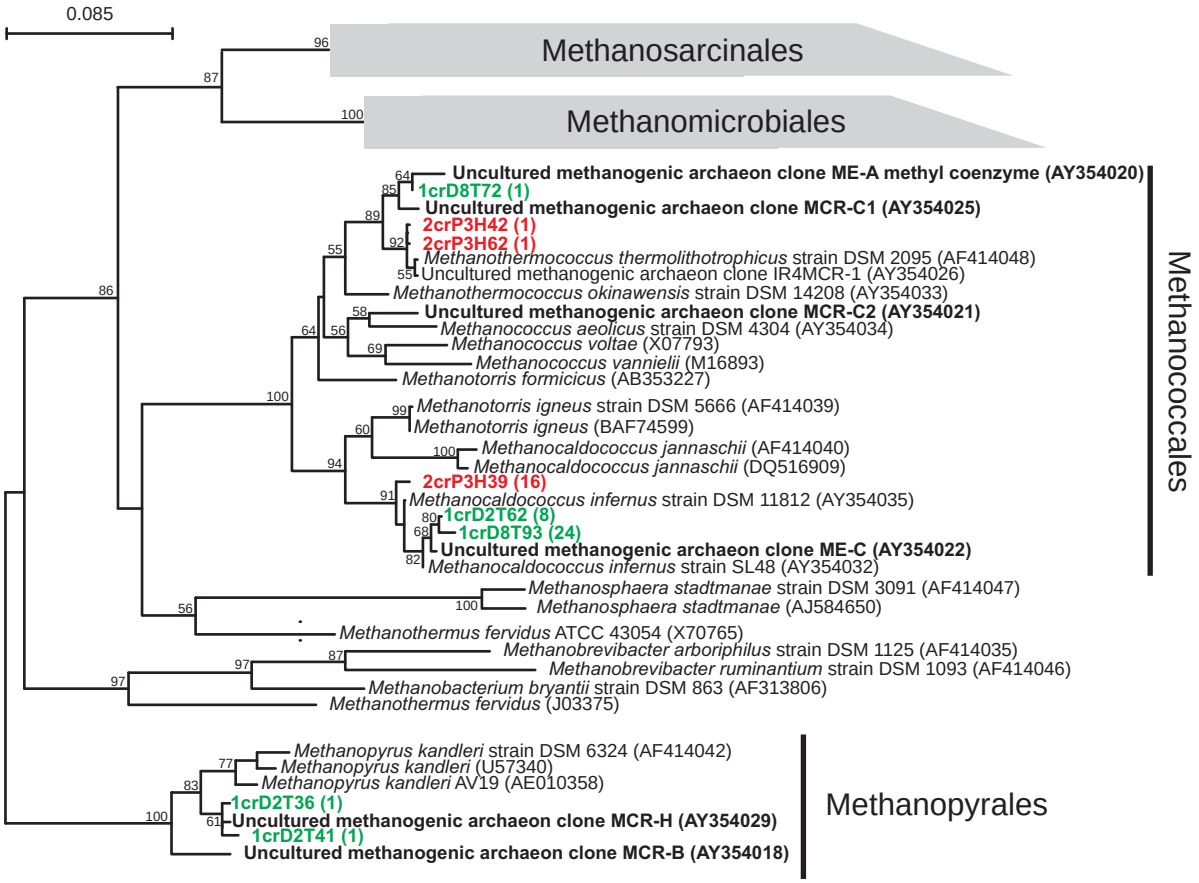


FIG. 2.23: Phylogenetic tree based on translated, partial amino acid sequences of *mcr* gene. The tree was constructed using the neighbor-joining method using PAM distance (Dayhoff *et al.*, 1978). The robustness of inferred topology was tested by the bootstrap. Bootstrap values $< 50$ % are not shown.

from Rainbow with no close related sequence was also detected (Figure 2.23). Methanotrophic *Bacteria* were shown to be the dominant symbionts in *Bathymodiolus* species at the Rainbow hydrothermal field, suggesting that the symbionts could be also present in the adjacent seawater. However, Nercessain and colleagues did not detect any free-living symbionts (Nercessian *et al.*, 2005*b*). Therefore, the prevalence of methanotrophic *Bacteria* in ultramafic-hosted hydrothermal environments could be linked to the high methane concentrations released.

## Archaeal community distributions

Insignificant $F_{ST}$ and P tests ($P < 0.001$) suggested that sequences from the Rainbow hydrothermal fluids and Ashadze chimney 1 clone libraries (Figure 2.18) were from similar lineage distributions and were indistinguishable from the combined communities (Martin, 2002). Moreover, all sediment clone libraries were also indistinguishable from the combined communities (Fig-

ure 2.18). Therefore, in order to analyse and identify potential relation between environmental factors and the archaeal communities in the ultramafic-hosted hydrothermal systems, a redundancy analyses (RDA) were calculated as a triplot displaying environmental measurements, sites and archaeal lineages or putative metabolic/physiological traits (Figure 2.24). The RDA based on environmental measurements (pH, temperature, depth), sites and archaeal lineages ($R^2 = 0.97$, $Ra^2 = 0.84$, $P = 0.001$) showed that 54 % of the overall variability was explained by the first two axes ($P < 0.003$) (Figure 2.24A). Similar results were also obtained for RDA of OTUs defined at 97 % and 95 %, and using presence/absence matrices (data not shown). The RDA plot revealed a correlation between *Methanococcales* ($P < 0.001$) and *Thermococcales* ($P < 0.05$) lineages and the Rainbow fluids. Correlations were also observed between MG-1 lineage and the hydrothermal sediments ($P < 0.01$), and between the unaffiliated *Methanosarcinales* cluster and the Lost City fluids ($P < 0.0001$). However, no correlation was found between environmental measurements and sites or lineages.

As traits are possibly the fundamental units of biodiversity and biogeography (Weiher and Keddy, 1995; Green *et al.*, 2008), we performed a RDA based on environmental measurements (pH, temperature, depth), sites and putative metabolic/physiological traits ($R^2 = 0.99$, $Ra^2 = 0.92$, $P = 0.001$). Although 16S rRNA genes do not provide a direct link to physiology or metabolic capacities, the analysis was performed assuming that each lineage could be characterized by a metabolic or physiological trait as presented in table S1. The RDA showed that 62 % of the overall variability was explained by the first two axes ($P < 0.05$) (Figure 2.24B). The RDA plot revealed a correlation between $H_2/CO_2$ ($P < 0.0005$), chemoorganotrophic elemental sulphur utilizing ($P < 0.01$) *Archaea* and the Rainbow fluids. Ammnonia oxidizing *Archaea* were also correlated with the Rainbow and Lost City sediments ($P < 0.01$).
In this study, 6 of the 21 archaeal lineages detected were probably involved in hydrogen or methane cycling processes. McCollom showed that ultramafic-hosted hydrothermal systems could provide twice as much chemical energy as comparable basaltic-hosted systems, suggesting that these ecosystems could be mainly fuelled by the hydrothermal fluids highly enriched in hydrogen and methane (McCollom, 2007). Hence, mesophilic to thermophilic methane-oxidizing *Bacteria* could dominate the moderate oxic habitats in the mixing environment as revealed by the *pmoA* gene analyses. The detection of ANME-2 members suggests that moderate thermophilic ($< 90°C$) methanotrophes could occur in probably restricted anoxic habitats, as a consequence of the very high oxygen and temperature gradiants. Moreover, the Lost City uncultured *Methanosarcinales* cluster possibly encompassing methane cycling members could be specifically associated to a cooler ($< 95°C$) and very alkaline habitat. Conversely, the (hyper)thermophilic methanogen *Methanococcales* order and the *Thermoccocales* order could be typical members of the hot anoxic microbial ecosystem that probably extends below the Rainbow hydrothermal system seafloor.

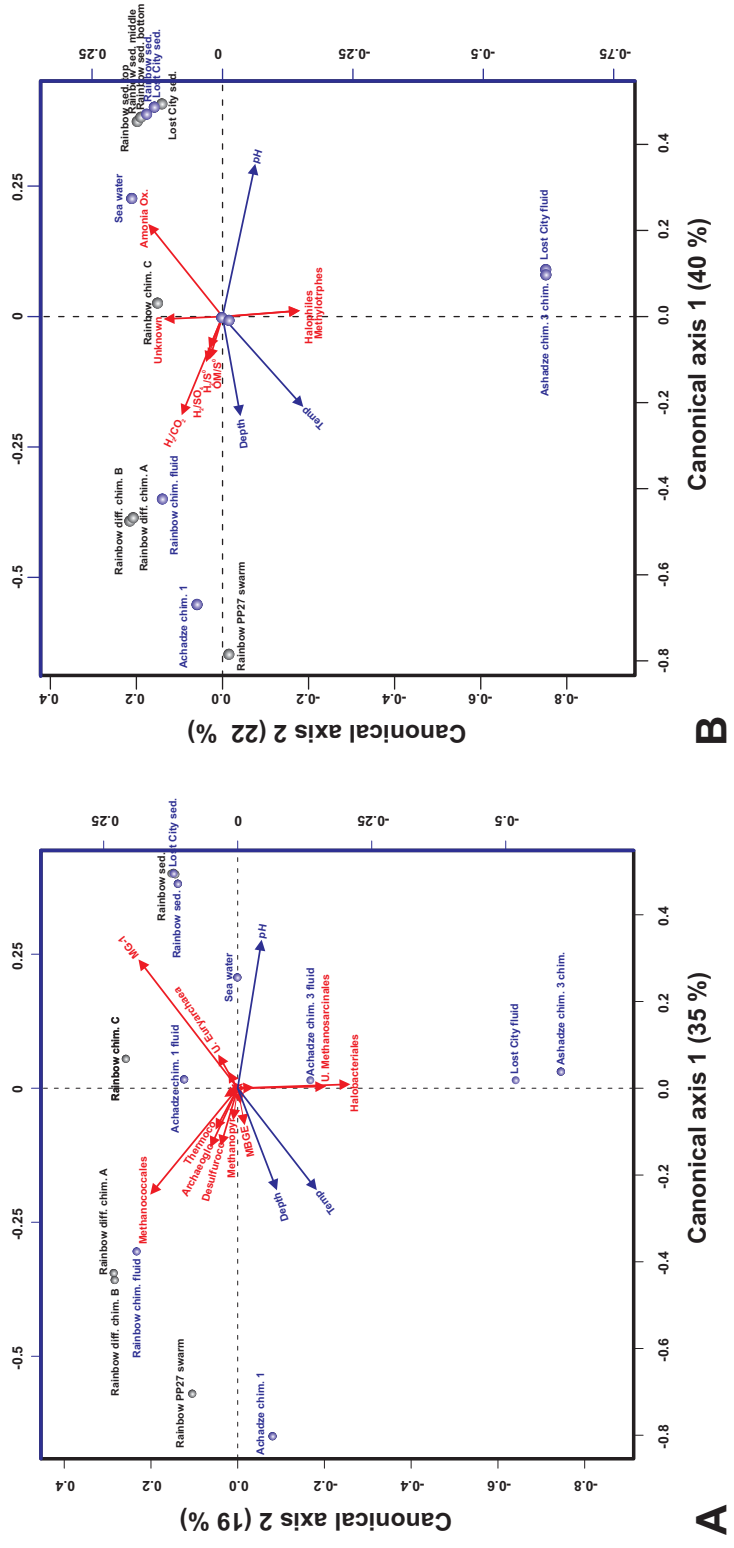


FIG. 2.24: Triplot of redundancy analysis (RDA) integrating archaeal lineages (A) or metabolic/physiologic traits (B), environmental variables and sites. The assumed metabolic or physiologic traits for each lineage are described in Table S1. The measured environmental variables and archaeal lineages or metabolic/physiologic traits are shown as arrows. The vector orientations represent the direction of strongest change; vector lengths correspond to relative importance.

## Experimental procedures

### Site description and sampling

Fluid, chimney and sediment samples were collected during the scientific cruises EXOMAR (2005), SERPENTINE (2007) and MoMARDREAM-Naut (2007) conducted with the R. V. "*L'Atalante*" and "*Pourquoi pas ?*" and using the ROV "*Victor 6000*" and DSV "*Nautile*". The three hydrothermal fields explored, Rainbow (36°13'N; 33°54'W; ∼2,300 m depth), Lost City (30°07'N; 42°07'W; ∼750 m depth) and Ashadze 1 (12°58'N; 44°51'W; ∼4,090 m depth) were all located in the area of the MAR (Figure 2.17).
The fluid samples retrieved from Rainbow site were collected at the "*termitière*" chimney (36°13'76"N; 33°54'16"W; 2,294 m depth) and the sediment close to the hydrothermal area (36°13'76"N; 33°54'04"W; 2,287 m depth).The fluid samples retrieved from the Lost City site were collected from a flange near the EXOMAR 11 Marker (30°07'43"N; 42°07'16"W; 748 m depth) and the surface sediment in the immediate periphery of the site (30°07'57"N; 42°07'05"W; 752 m depth). The fluid and associated chimney samples retrieved from Ashadze 1 site were collected from two chimneys in the SE2 area (12°58'33"N; 44°51'78"W; 4,097 m depth).
All fluid samples were collected using titanium syringes and analysed as described elsewhere (Charlou *et al.*, 2002). On board, the fluid samples were immediately removed aseptically from the titanium syringes and stored at -80°C for molecular genetic analyses. On board the sediment cores (∼20 cm in length, 5 cm diameter) collected from Rainbow, using a push-core device operated by the arm of the DSV "*Nautile*", were sectioned in three equal samples and were designated as top, middle and bottom. The sediment surface sample from Lost City was collected using PSDE system (Kato, unpublished). The chimney fragment and sediment samples were stored aseptically at -80°C for molecular genetic analyses.
As a control, seawater from the water column was also collected from the Rainbow site (36°13'76"N; 33°54'06"W; 2,291 m depth).

### DNA extractions and PCR amplification

To avoid contaminations, all manipulations were carried out in a PCR cabinet (Biocap™ RNA/DNA, erlab®, using Biopur® 1.5 mL Safe-Lock micro test tubes (Eppendorf™), Rnase/ Dnase Free Water (MP Biomedicals™) and UV-treated (> 60 min) plasticware and pipettes.
DNA extraction from fluids was performed from 50 mL of fluid left to thaw on ice prior centrifugation (15,000 g for 60 min). Supernatant was carefully discarded and DNA was extracted from the pellet, following a modified FastDNA® Spin Kit for Soil (Bio101 Systems, MP Biomedicals™) protocol (Webster *et al.*, 2003; Roussel *et al.*, 2009). The DNA extractions from sediments and chimney fragments were also performed using the modified FastDNA® Spin Kit for Soil as described elsewhere (Roussel *et al.*, 2009).
All amplifications were performed using a "GeneAmp PCR system" 9700® (Applied Biosys-

tems™). All PCR mixtures (50 $\mu$L) contained 5 $\mu$l of DNA template, 1X Taq DNA polymerase buffer (MP Biomedicals™), 1 $\mu$L of dNTP (10 mM of each dATP, dCTP, dGTP and dTTP), 10 $\mu$M of each primer and 0.5 $\mu$L of Taq DNA polymerase (MP Biomedicals™). Negative controls were also carried out with DNA extractions performed with no sample. For all controls, no PCR products were detected. Inhibition of PCR amplification by soluble contaminants in the DNA extracts was also tested as described elsewhere (Juniper *et al.*, 2001). For all DNA extracts, no PCR inhibition was observed.

Archaeal 16S rRNA gene amplification was conducted by nested PCR with combination of primers A8f-A1492r in the first round (Casamayor *et al.*, 2000), with A344f-A915r in the second round (DeLong, 1992; Eden *et al.*, 1991). PCR cycles for the first round (A8f/A1492r), and for the second round (A344f/A915r) were as previously described (Roussel *et al.*, 2009). To minimize PCR bias, five independent PCR products from the first round were pooled and purified (QIAquick PCR purification Kit; Qiagen™) and used as template for the second round. This nested PCR was necessary to obtain visible PCR products on a 0.8 % (w/v) agarose gel stained with ethidium bromide.

A portion of the *mcrA* gene was amplified using the ME primers (Hales *et al.*, 1996) with the following reaction conditions as described elsewhere (Roussel *et al.*, 2009). A fragment of the *pmoA* gene was amplified using the pmoA189-mb661 primer couple (Holmes *et al.*, 1995; Costello and Lidstrom, 1999) with the following reaction conditions: 1 cycle of 4 min at 92°C, 35 cycles of 1 min at 92°C, 1.5 min at 55°C and 1 min at 72°C, and 1 cycle of 9 min at 72°C. A portion of the *dsrA* gene was amplified using the DSR1f-DSR-R primers (Kondo *et al.*, 2004) with the following reaction conditions: 1 cycle of 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 54°C and 2.5 min at 72°C, and 1 cycle of 8 min at 72°C. For all functional genes, two rounds with the previous reaction conditions were required to obtain visible amplification products. An aliquot (5 $\mu$L) of 3 pooled PCR products of the primary amplification was used as template for the second amplification round.

## DGGE analysis

In order to obtain the general archaeal 16S rRNA gene diversity associated to the hydrothermal environment and to compare it with the seawater diversity, a preliminary CM-DGGE analysis was performed as described elsewhere (Roussel *et al.*, sion).

After amplification of the nested PCR products, using two different fluorescent reverse labelled primers from total DNA from either a hydrothermal sample or seawater, these were pooled and loaded into the same lane. Archaeal 16S rRNA gene amplification was performed with primers Saf-PARCH 519r, labelled with either Cy3 (hydrothermal samples) or Cy5 (seawater), following touchdown PCR protocol as previously described (Nicol *et al.*, 2003). All manipulations were performed in the dark. The PCR products were analyzed by DGGE using a DCode Universal Mutation Detection System® (BioRad™) on a 1 mm thick (16×16 cm) 8 % (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 40 %, 37,5:1, BioRad™) with a denaturant gra-

dient between 30 and 70 % prepared with 1X TAE buffer (pH 8, 40 mM de Tris Base, 20 mM acetic acid, 1 mM d'EDTA, MP Biomedicals™) and poured with a "Gradient maker" (Hoefer SG30®). Electrophoresis was carried in 1X TAE buffer at 60°C for 330 min at 200 V (initially at 80 V for 10 min). The gel was scanned using a Phospho fluorimager Typhoon 9400® (Amersham Biosciences™). The DGGE profiles were analysed by cluster analysis using the software package GelCompar II version 5.10 (Applied Maths, St-Martens-Latem, Belgium) as described elsewhere (Wilms *et al.*, 2006).

### Cloning and sequencing

According to archaeal DGGE profiles, fourteen 16S rRNA gene, one *dsrA* gene, four *mcrA* gene, and eight *pmoA* gene clone libraries were constructed. To minimize PCR bias (Polz and Cavanaugh, 1998), five independent PCR products were pooled, purified (QIAquick PCR purification Kit; Qiagen™), and cloned into *Escherichia coli* (XL10-Gold; Stratagene™) using the pGEM-T Easy vector system I (Promega™) following the manufacturer's instructions. Positive transformants were screened by PCR amplification of the insert using the vector-specific M13 primers. Plasmid extraction, purification and sequencing of the insert, were carried out by the sequencing Ouest-Genepole platform® of Roscoff Marine laboratory (France).

### Phylogenetic analysis

Chimeras (Cole *et al.*, 2003) were excluded from the clone libraries and a total of 759 sequences (including those from the 16S rRNA gene and functional genes) were used for further phylogenetical analysis. The phylogenetic placement was carried out using NCBI BLAST search program within GenBank (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.*, 1990). The 16S rRNA sequences (∼553 bases) were then edited in the BioEdit 7.0.5.3 program (Hall, 1999) and aligned using CLUSTALW (Thompson *et al.*, 1994). The phylogenetic trees were constructed by the PHYLO-WIN program (http://pbil.univ-lyon1.fr/) (Galtier *et al.*, 1996) with neighbor-joining method (Saitou and Nei, 1987) and Jukes and Cantor correction. The nonchimeric *mcrA* (∼0.76 kp), *pmoA* (∼0.51 kb) and *dsrA* (∼0.22 kp) sequences were translated into amino acids using BioEdit and then aligned using CLUSTALW, and the PHYLO-WIN program with neighbor-joining algorithm and PAM distance (Dayhoff *et al.*, 1978) was then used for phylogenetic tree construction. For the all phylogenetic reconstruction, the robustness of inferred topology was tested by bootstrap resampling (1,000), values over 50 % are shown on the trees. The richness from the clone libraries was estimated, with the rarefaction curves at 99 % , 97 % and 95 % sequence identity levels, using the DOTUR program (Schloss and Handelsman, 2005). Operational taxonomic units (OTUs), using a 97 % sequence similarity, were generated with the SON program (Schloss and Handelsman, 2006), and the percentage of coverage (Cx) of the clone libraries was calculated by Good's method (Good, 1953) as described by Singleton and colleagues (Singleton *et al.*, 2001). Statistical estimators, the significance of population differ-

entiation among clone libraries ($F_{ST}$) (Martin, 2002), and the exact tests of population genetic differentiation (Raymond and Rousset, 1995), were calculated using Arlequin 3.11 (Excoffier *et al.*, 2005).

### Statistical analyses

In order to analyse and interpret the relation between environmental factors and the microbial communities, canonical redundancy analyses were performed (Rao, 1964). RDA was described as a series of multiple regressions followed by a principal component analysis based on linear (Euclidean distance) relationships between variables (Legendre and Legendre, 1998; Vanwijngaarden *et al.*, 1995; Braak, 1995). Each variable of a Y table, which contains community data, was regressed on a X table, which contains the explanatory variables (i.e. environmental factors). Variables corresponding to community data were transformed with Hellinger transformation. Legendre and Gallagher showed that this transformation makes species abundance data amenable to analyses like RDA (Legendre and Gallagher, 2001). Analyses were performed with the statistical software package R Ver. 2.7.0 (RDevelopmentCoreTeam, 2008) and the function "rdaTest" following the algorithm described in elsewhere (Legendre and Legendre, 1998). A plot of the results of the RDA was drawn, with the function "graph.rdaTest". The adjusted coefficient of determination ($Ra^2$) corrects for the number of explanatory variables in the model and for the number of observations. It provides an unbiased estimate, in RDA, of the real contributions of the explanatory variables to the explanation of a community data table (Peres-Neto *et al.*, 2006).

### Nucleotide sequence accession numbers

The sequences are available from GenBank database under the following accession numbers and names: 16S rRNA gene (XXXXXXXX to XXXXXXXX), *mcrA* gene (XXXXXXXX to XXXXXXXX), *dsrA* (XXXXXXXX to XXXXXXXX) and *pmoA*(XXXXXXXX to XXXXXXXX).

## Acknowledgements

We thank Anne Godfroy, Françoise Gaill chief scientists of Exomar and MoMARDREAM-Naut cruises. All crew members and the Scientific Party were crucial in this effort, especially the ROV "*Victor 6000*" and DSV "*Nautile*" crews for the sampling efforts. This work was supported by the Université de Bretagne Occidentale, the Centre National de la Recherche Scientifique and the Région Bretagne. We are also grateful to Ouest-Génopole for the use of their facilities. Erwan Roussel was supported by a grant from the Ministère de la Recherche.

## Supplementary material

Figure S1: Rarefaction curves for the 16S rRNA gene clone libraries from Rainbow, Lost City and Ashadze hydrothermal sites (Schloss and Handelsman, 2005). The sequence identity levels are represented in brackets.

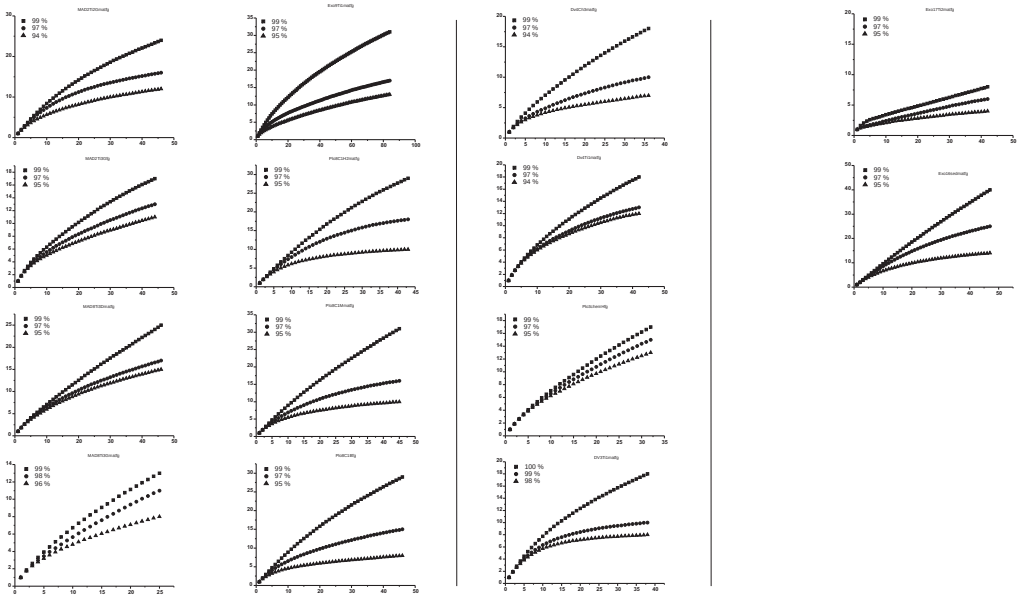

Table S1: Inferred metabolic or physiological traits assumed from each archaeal lineage as described in the Bergey's Manual of Systematic Bacteriology.

| Phylogenetic lineage | Putative traits |
|---|---|
| Marine Group 1 | Ammonia oxidation |
| Marine Group A | Unknown |
| UIIa | Unknown |
| Desulfurococcales | $H_2/S^0$ |
| Unaffiliated Crenarchaeota | Unknown |
| Unaffiliated Methanosarcinales | Methylotrophes |
| ANME-2 | Anaerobic Methane Oxidation |
| Halobacteriales | Halophiles |
| Marine Group IV | Unknown |
| Marine Group II | Unknown |
| Marine Group III | Unknown |
| MBGD | Unknown |
| DHVEG | Organic matter/S0 |
| DHVEx | Unknown |
| Unaffiliated Euryarchaeota | Unknown |
| Marine Benthic Group E | Unknown |
| Archaeoglobales | Organic matter/$SO_4$ |
| Methanococcales | $H_2/CO_2$ |
| Thermococcales | Organic matter/$S^0$ |
| Methanopyrales | $H_2/CO_2$ |
| Nanoarchaeota | Unknown |

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# Chapter III – Synthesis, discussion, conclusion and perspectives

# CHAPTER 3

# SYNTHESIS, DISCUSSION, CONCLUSION AND PERSPECTIVES

**Contents**



In order to determine the main environmental factors driving the archaeal diversity in the sub-seafloor, the present study was mainly focused on the 16S rRNA and functional gene diversity associated with four different sub-seafloor ecosystems. As previously described, the current cultural approaches are not yet adapted to the analysis of the overall prokaryotic communities in sub-surface environments, and molecular approaches are to date the most relevant.
The limits of the deep biosphere remain unclear and their definition can probably vary according to environmental conditions. However, the deep biosphere prokaryotic communities are characterised by typical members, with for most of them, unknown physiologies. Hence, according to the results obtained in previous studies, the limits and composition of the archaeal deep biosphere will be discussed first. The widespread microbial life at depth in the deep sub-seafloor sediments and crust could be sustained by energy supply coming from chemical sources, due to fluids that migrate upward from deeper levels in the Earth. These chemical energy sources could originate from thermogenic or radiolytic reactions, suggesting the possible existence of a deep hot biosphere and this will be discussed in a second section. In the last

section the possible dissemination and evolution mechanisms of sub-surface communities will be considered, and the possible existence of a deeply buried virosphere will be discussed. Several conclusions will be drawn from these studies and be presented in the last section.

## 3.1 Environmental factors driving the prokaryotic diversity

### 3.1.1 Defining the limits of the deep biosphere

According to the most recent definition, the limits of the deep biosphere are locally variable (Teske and Sorensen, 2008). For instance, several typical sediment sub-surface *Bacteria* and *Archaea* are readily detected in 3–4 m deep coastal sediments, and this would not be considered as "deep sub-surface". Conversely, in some deep marine sediments, the deep sub-surface could occur within 1–2 m of the sediment surface. Hence, Teske and colleagues defined the deep sub-surface as "the sediment layers with distinct microbial communities that lack a microbial imprint of water column communities".

The study of the coastal sediment of the Marenne-Oleron bay showed no strong variation between the composition of the surface archaeal phylotypes and the deeper communities (study 2). Besides, these sediments are coastal, and in a range of 3 mbsf, the typical deep biosphere lineages (MCG and MBG-B) detected along the sediment column suggest that according to the previous definition, these sediments could be considered as part of the deep biosphere since no typical archaeal phylotypes from the water column were detected.

Hence, the boundary between surface and deep sub-surface in these sediments is difficult to define as a consequence of the high OM imput all along the sediment column that evens the prokaryotic diversity.

The study of sediments with much lower OM input showed a different picture (study 3). All the sediment archaeal phylotypes were clearly different from the water column phylotypes, which were also part of the MG-1. A clear transition was observed below 1.5 mbsf between the sediment surface archaeal communities and the deeper *Archaea*, mainly composed of typical deep biosphere lineages. A similar transition between sediment surface was previously observed by Inagaki and colleagues at ∼4 mbsf in sediments of the West Philippine Basin (Inagaki *et al.*, 2001). Hence, the lower OM input could increase the depth of the oxic and sub-oxic zone subsequently modifying the archaeal diversity.

The definition of the limits of the deep biosphere could therefore be more precise, and be as follows "the sediment layers with distinct microbial communities that lack a microbial imprint of sediment section directly in contact with the column". However, in study 4, we show that the deep biosphere extends to at least 1,600 mbsf and probably deeper given an upper temperature limit for prokaryotic life of at least 122°C and increasing thermogenic energy supply at depth. Hence, it is likely that microbial processes in the deep biosphere are stretched in both time and space unlike spatially compressed ecosystems (e.g. biofilms, surface sediments, microbial

mats), as the energy sources in the deep biosphere are expanded in space, and can be observed on meter and even kilometre scales. This perspective offers an interesting opportunity to study fine scale processes.
In contrast to deep sub-surface sediments, hydrothermal environments are defined by strong physical and chemical gradients over small spatial scales. However, similar archaeal communities were usually associated to hydrothermal fluids and chimneys (study 5), encompassing lineages such as *Thermococcales* and *Methanococcales*. Still, in study 5, no *Archaea* were detected in the less diluted hydrothermal fluids suggesting that these sub-seafloor lineages could originate from a shallow sub-surface as the deeper zones are well above the given upper limit for prokaryotic life. These observations were also supported by Takai and colleagues, which also showed that high temperature fluids had a lower cell density (Takai *et al.*, 2004). However, as previously suggested the members of these lineages could represent indicators of sub-seafloor ecosystems as they are regularly found in fluids migrating from the deep sub-seafloor.

### 3.1.2 The deep biosphere archaeal communities

#### 3.1.2.1 The Marine Group 1 (MG-1): a surface lineage?

One of the most widespread lineages of all the studies (studies 3 and 5) was the MG-1. This lineage was detected in the marine water column, the marine surface sediments and in the hydrothermal environments. Although not representing a typical deep biosphere lineage, the MG-1 *Archaea* are interesting because as they could be aerobic autotrophic ammonia oxidizers (Konneke *et al.*, 2005; Francis *et al.*, 2005; Hallam *et al.*, 2006), and are commonly found in seawater and marine sediments (Inagaki *et al.*, 2001; Teske and Sorensen, 2008), forming several phylogenetic clusters with currently two cultured relatives (Konneke *et al.*, 2005; Preston *et al.*, 1996). Moreover, based on the analysis of the first sequenced genome of a cultured relative (*Crenarchaeum symbiosum*), the MG-1 were recently proposed as a novel archaeal phylum named *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008).
The active MG-1 phylotypes retrieved in the sub-oxic to anoxic sediments in study 3 were different from those found in the oxic seawater, suggesting that specific MG-1 subclades could be adapted to suboxic conditions (Teske and Sorensen, 2008). Morover, *amoA* genes related to MG-1 were only detected at depths containing MG-1, suggesting that these communities could be fuelled by ammonium and dioxygen originating from seawater. However, *Crenarchaeota* may be the dominant contributors to nitrification in both surface sediments and pelagic environments, and although archaeal genes for ammonia oxidation have been found in other environments, none have been detected in the sub-surface metagenome (Biddle *et al.*, 2008). Even so, as the majority of the metagenome did not match database sequences, little information about possible ammonium oxidation in the deep sub-surface can be deduced for this study. Interestingly, the MG-1 lineage was widespread in the hydrothermal environment and usually

harbored the same phylotypes as the seawater, suggesting that the MG-1 from the seawater were also entrained in the hydrothermal circulation. Takai and colleagues also showed that a higher proportion of MG-1 was detected in deep-sea hydrothermal environments than in normal deep and surface seawaters.
Furthermore, a moderately thermophilic aerobic ammonia-oxidizing crenarchaeote was recently isolated from the Siberian Garga hot spring (Hatzenpichler *et al.*, 2008). A thermophilic origin for anaerobic ammonium oxidation was also suggested (Canfield *et al.*, 2006). The hydrothermal environments were shown to have a very high primary production, releasing considerable amounts of dissolved organic compounds into the ambient seawater. Hence, the extensive seawater circulation in hydrothermal systems could fuel these ammonia oxidizers, suggesting that MG-1 could therefore be responsible for a large proportion of the ammonium oxidation in hydrothermal systems.

#### 3.1.2.2 Typical deep biosphere inhabitants

The study of coastal and open ocean sediment (study 2 and 3) showed that some lineages were systematically detected. The MCG, MBG-B and MBG-D were the most commonly detected and represent lineages previously described as ubiquitous in the deep biosphere (Teske and Sorensen, 2008, for review). These lineages occur in a wide range of habitats encompassing coastal, methane bearing and open ocean sediments, suggesting a broad adaptation to a wide range of organic matter contents and to various environmental conditions. As described in the first chapter the MBG-B could be related to methane cycling processes, whereas the MCG are suspected to be heterotrophic anaerobes that utilize and assimilate complex organic substrates. However, the archaeal SAGMEG and DHVE6 lineages were only retrieved in the open ocean sediments. Although these lineages are also regularly detected in deep marine sediments, their distribution seems more limited, possibly indicating a more specific environmental and possibly oligotrophic conditions. However, these lineages are adapted to a low temperature deep sediment environment as none were detected in higher temperature sediments or in hydrothermal environments.
Although very rarely detected with molecular genetic tools (Biddle *et al.*, 2008; Parkes *et al.*, 2005), methane cycling prokaryotes seem to play a key role in the geochemical cycles in the deep sub-seafloor sediments (D'Hondt *et al.*, 2002, for e.g.). However, methyl-coenzyme M reductase (*mcr*) genes (study 2), putative methanotrophs (ANME-1, ANME-2, ANME-3), acetoclastic methanogens (*Methanosaeta*), hydrogenoclastic (*Methanomicrobiales*) and methylotrophic methanogens (*Methanococcoides*), were detected in several sediment environments (study 2, 3, 4). However, as contamination by exogenous DNA was of particular concern (see methodological section), the detection of rare sub-seafloor sediment lineages, such as putative methane cycling *Archaea*, was probably related to methodological implications. PCR-based surveys are biased, as between 5 to 71 % of the archaeal diversity can be detected using the

commonly described primers (Teske and Sorensen, 2008). Thus, the several DNA extractions followed by pooling and concentration of several PCR and nested PCR products, combined to the use of a primer (A344f) containing degeneracies (Teske and Sorensen, 2008), may reduce PCR biases and facilitate the detection of these rare deep sub-seafloor lineages. These results are in agreement with the recent findings of Biddle and colleagues (Biddle *et al.*, 2008), suggesting that the putative methane cycling lineages are not the dominant fraction of the overall archaeal diversity in the deep biosphere. It is interesting to note that the ANME lineages seem to dominate specific niches, such methane bearing sediments (study 2 and 4) or deeply buried SMTZ (Biddle and Teske, 2008), suggesting that these putatives methanotrophes could require relatively high methane concentrations and, also restricted to an upper temperature of ∼90°C, could possibly utilize thermogenic methane as a deep energy source (study 4). However, as previously suggested, the MBG-B lineage could also be involved in methane cycling although their environmental requirements are still undetermined.

#### 3.1.2.3 (Hyper)thermophilic inhabitants: a deep hot biosphere community?

According to the analysis of several deep biosphere environments (study 3, 4 and 5) putative lineages such as the *Methanococcales* and *Thermococcales* were suggested to be archaeal indicators of deep hot sub-seafloor ecosystems. Although, hyperthermophilic or thermophilic members of the *Methanococcales* are commonly cultured, and detected with molecular tools, from marine hydrothermal vent systems, their distribution varies according to the environmental condition prevailing at each hydrothermal site. The strictly anaerobic autotrophic methanogenic *Methanococcales* are the most widespread distributed archaeon among the hot ($>$ 350°C) ultramafic-hosted hydrothermal system environments. Most of the lineages detected are linked to methane and hydrogen cycling, suggesting that in ultramafic-hosted hydrothermal systems, large methanogenic and methanotrophic communities could be fuelled by hydrothermal fluids highly enriched in methane and hydrogen. However, *Methanococcales* were only detected in hydrothermal environments, in contrast to the *Thermococcales* that were also detected in deep marine sediments.

The low archaeal diversity in several deep sub-seafloor sediments (study 3 and 4) is dominated by *Thermococcales*, a putative thermophilic *Euryarchaeota* order commonly found at hydrothermal vent sites and representing an excellent indicator of sub-seafloor ecosystems. Sequences affiliated to putative *Thermococcales* have been previously detected in other cold (1-12°C) deep marine sediments and their occurrence was usually interpreted as a deposition consequent to fluid migration, or as buried microbial relicts, representing "fossil DNA". Even though the minimum temperature required for the growth of a *Thermococcus* is 40°C, *Thermococcales* can survive over long periods in cold (4°C) oxygenated samples, and were shown to be living (study 3), possibly allowing a wide dissemination in marine environments. However, a distinct *Thermococcus* cluster (DSSTG) was detected at the two deep sub-surface sediment sites (study 3

and 4), suggesting that this phylotype may represent a unique "deep hot biosphere community". Although Thomas Gold in 1992 suggested that a deep hot biosphere could be fuelled by thermogenic compounds (Gold, 1992), little evidence of such existence has been since discovered. Moreover, it was recently suggested that radiolysis of water in the deep sub-surface could also fuel prokaryotic communities (Blair *et al.*, 2007). Although, *Thermococcales* are known to grow heterotrophically and autotrophically, yet none were shown to utilise hydrogene. However, as the *in situ* temperatures of the Newfoundland margin sediments (study 4) are within the growth range of the *Thermococcales* (40 to 105°C) and potential substrates for both metabolismes are present (reactive organic matter which can also thermogenically produce $H_2$ and inorganic carbon), it seems reasonable to conclude that the organisms from which these sequences are derived were metabolically active in these sediments. This suggests that these microorganisms have slow growth rates or/and specific growth requirements that have yet to be identified, which is the case for the majority of prokaryotes in the environment.

#### 3.1.2.4 Why *Archaea* could rule the deep biosphere?

As seen in the previous section *Archaea* were suggested to be the dominant domain in the hydrothermal or sub-seafloor biosphere. It was suggested that the prokaryotes in the deep subsurface sediments could have doubling times in the range of 300 to 3000 years, suggesting that they have very slow growth if not maintenance or survival modes (Jorgensen, 2008). Hence, these energy modes require low energy requirements (Figure 3.1a), such as those found in deep sub-surface sediments.

It is usually assumed that *Archaea* and the bacterial evolution results must derive from genetic and biochemical adaptations to their environment. Valentine recently suggested that *Archaea* should be better adapted than *Bacteria* to extreme low-energy conditions, whereas *Bacteria* are more successful in dynamic environments (Valentine, 2007, for review). The adaptation of *Archaea* to extreme low-energy conditions is a result of distinctive characteristics such as the lipid-membrane composition and several secondary adaptations such as specific catabolic pathways and mechanisms of energy conservation. The lower membrane permeability resulting from the lipid structure could lower the demand for maintenance energy of an archaeal cell relative to a bacterial cell. Moreover, distinctive mechanisms of energy conservation such as methanogenesis and anaerobic methane oxidation, are specific catabolic pathways providing adaptation to chronic energy stress. These adaptations of *Archaea* to energy stress provide a competitive advantage under a range of environmental conditions such as extreme, temperature, pH and probably energy limited environments (e.g. Figure 3.1b). Hence, these adaptations could explain why *Archaea* seem to dominate the hydrothermal and sub-seafloor biosphere.

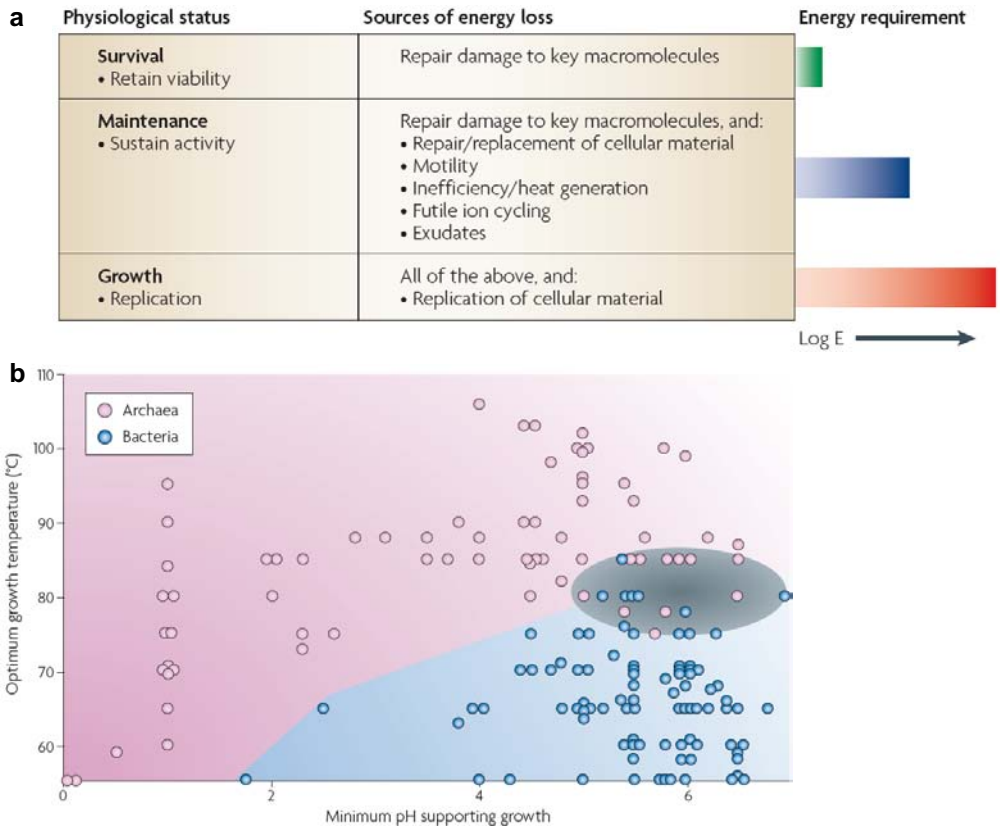


FIG. 3.1: a The energy requirements for conditions of survival, maintenance and growth are shown. The ratio of energy requirements (survival/maintenance/growth) was recently estimated at $1/10^3/10^6$. b Temperature and pH requirements for growth distinguish thermophilic *Bacteria* and *Archaea*. 72 archaeal species that represent 32 genera are included (pink dots), as are 107 bacterial species that represent 61 genera (blue dots). Methanogenic *Archaea* are excluded; from (Valentine, 2007).

## 3.2 The unexplored deep sub-surface

### 3.2.1 Evolution and the slow pace of life in the deep biosphere

The mechanisms driving the evolution in the deep biosphere remain unknown. In order to understand the evolution of these sub-seafloor prokaryotes, it is imperative to analyse how they segregate in the face of ecological pressures. However, the physical, chemical and biological factors controlling evolution in the deep biosphere drastically vary from deep sub-surface sediments to a deep sub-seafloor hydrothermal environment.

Claiming, "everything is everywhere" implies that microorganisms have such enormous dispersal capabilities that they rapidly erase the effects of past evolutionary and ecological events (Martiny *et al.*, 2006, for review). As seen in the previous sections, it is interesting to note that the prokaryotic generation times in the deep sub-surface sediments are so slow that the evolution

and dispersal process may be very different in these habitats, possibly approaching the extreme limits of life. Valentine suggested that chronic energy stress unifies the archaeal ecology, but it is also a powerful and divergent selective pressure for prokaryotic evolution (Valentine, 2007). Although *Archaea* and *Bacteria* coexist throughout much of the biosphere, and as they are known to readily exchange genetic information which partially sustains evolution (Allers and Mevarech, 2005), the environmental conditions of deep sub-surface sediments may limit these exchanges. The sediments in most of the sub-seafloor zones are relatively impermeable to prokaryotic diffusion, and very few motility genes were retrieved from the deep sub-seafloor sediments (Biddle *et al.*, 2008), suggesting that the prokaryotic dispersal is limited and that the contact between individual prokaryotes may be a very rare event. Moreover, Jørgensen recently estimated the occurrence of this event to every 3 millions years (Jorgensen, 2008). Hence, the direct exchanges of genetic information would be an insignificant factor in promoting evolution. However, local variation in these mechanisms could occur according to possible vertical and horizontal fluid fluxes located in specific zones, such as those close to the basaltic crust or to strongly faulted seafloor (e.g. study 3). Interestingly, in the present study the comparison of the 16S rRNA gene sequences from the DSSTG (study 3 and 4) to all known *Thermococcales*, which have a highly conserved 16S rRNA gene sequence, showed that half of the nucleotide substitutions were located at conserved nucleotide sites, mostly within functional structures of ribosomal RNA. This suggests that the members of this new cluster within *Thermococcales* could have evolved independently from the probably faster evolving *Thermococcales* from hydrothermal vents. However, up to 90 % of the total DNA in shallow marine sediments is extra cellular and can be partially preserved to some extent during burial (Dell'Anno and Danovaro, 2005; Danovaro *et al.*, 1999). As direct genetic exchanges are probably limited between deep marine sediments prokaryotes, the horizontal transfer of genetic information through transformation by extracellular DNA could represent an alternative pathway. Transduction by virus could also be a pathway for genetic horizontal transfers although depending on the dissemination of viral particles; this will be discussed in the next section.

Unlike deep sub-surface sediments, hydrothermal sub-surface environments are characterised by strong physical and chemical gradients, high-energy fluxes and dynamic fluid mixing. Hence, evolution mechanisms could be enhanced compared to deep sub-surface sediments. For example, the high fluid fluxes in the oceanic crust might be responsible for the dispersal of prokaryotic communities over kilometre scales. Moreover, among environmental extremes accommodated by *Archaea*, high temperature has particular significance for genomic integrity, as it directly destabilizes the structure of DNA possibly resulting in rearrangements, mutation, and gene loss or acquisition, suggesting strong consequences on the evolutionary mechanisms (Grogan, 2004; Grogan, 2000). Several extremophilic *Archaea* were shown to have an extensive G2 phase leading to polyploidy (Breuert *et al.*, 2006), possibly reducing the chance that deleterious recessive mutations become homozygous, and possibly increasing the gene diversification resulting in the acquisition of new functions. Moreover, archaeal polyploidy could have unsuspected

consequences on the quantification of archaeal communities when using quantitative PCR. Interestingly, two species of the *Thermococcales* order are capable of cell fusion, suggesting important consequence on their evolution processes (Kuwabara *et al.*, 2005; Kuwabara *et al.*, 2007). Moreover, some evidences show that prokaryotes colonizing thermophilic environments undergo genetic differentiation through genetic mobile elements (plasmids, viruses or transposition of insertion sequences) brought on by high frequency genetic drift resulting from physical isolation (Escobar-Paramo *et al.*, 2005, for e.g.).

### 3.2.2 A sub-surface virosphere?

As seen in the previous section, viral populations could play an important role in cell death and in the horizontal transfer of genes between microorganisms. However, the relative abundances and role of virus in the sub-seafloor remain largely unknown. Although viruses were shown to be abundant in the sediment surface (Danovaro *et al.*, 2008*b*; Drake *et al.*, 1998; Hewson and Fuhrman, 2003; Mei and Danovaro, 2004; Middelboe *et al.*, 2003), the number of studies of the viral communities in the deep sub-surface remain very limited (Kyle *et al.*, 2008; Bird *et al.*, 2001).

Estimations of the relative abundances of viruses in surface and sub-surface sediments suggest that they could exceed those in the water column ($10^5$ and $10^8$ particles $mL^{-1}$) by 10–1,000 times (Danovaro *et al.*, 2008*a*, for review). However, since the energy availability and the dissemination of microorganisms are limited in the deep sub-surface sediments, several question can be addressed regarding how dynamic viral communities could be sustained. For instance, the slow growth of the hosts (prokaryotes), consequently to the limited resources, should also restrict the dissemination of lytic viruses. Temperate viruses could also be limited since they would represent handicap for the host.

Hence, it would be revealing to compare the virus communities from depths where prokaryotic abundances are greater than the general trend, such as at the SMTZ, in order to identify a relation between the viruses and their hosts. Alternately, the majority of the viruses detected in deep marine sediments could simply be a consequence of the burial of surface viruses. However, virus communities in the deep marine sediments could represent large genetic pools, deeply impacting the evolution of the prokaryote communities.

In contrast to deep sub-surface sediments, sub-seafloor crustal environments are more favourable for the development of dynamic viral communities. Surface hydrothermal environments have already been shown to harbour a large diversity of viruses (Prangishvili *et al.*, 2006, for review). Moreover, an initial metagenomic analysis indicates that temperate viruses in hydrothermal fluids appear to have a high density of temperate viruses and lysogenic hosts, and that these viral populations contain an extraordinarily high frequency of novel genes (Figure 3.2). Hence, it appears likely that temperate viruses play a key role in the ecology of prokaryotes within the deep sea hydrothermal systems. Moreover, as these results were obtained from diffusive

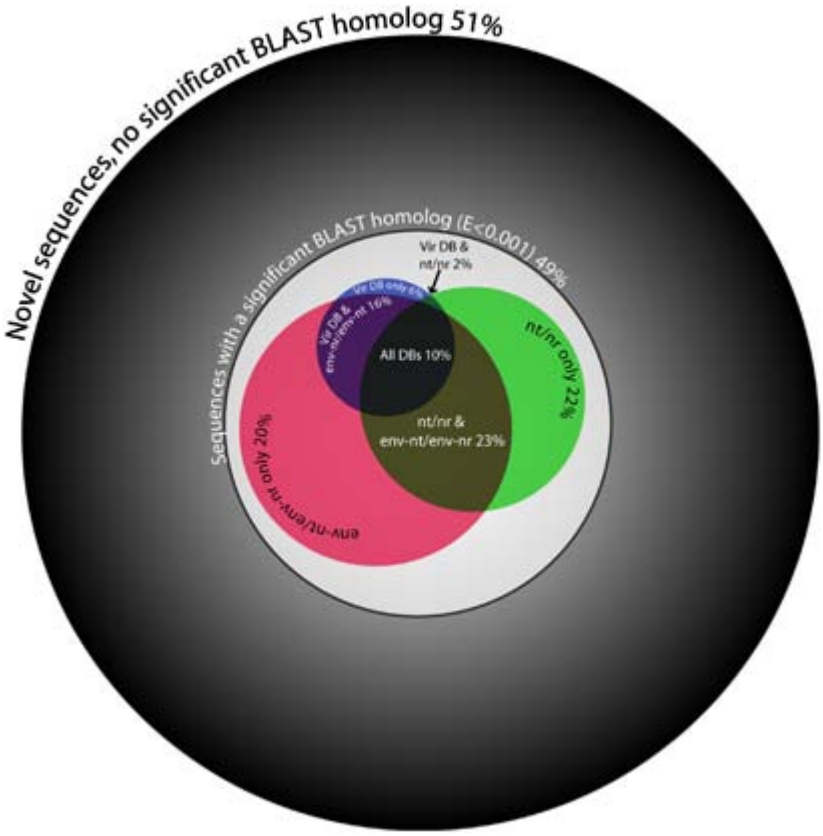


FIG. 3.2: Metagenomic analysis of the hydrothermal vent viral communities. Venn diagram of BLAST search results by database and relationship of sequences with a significant BLAST homolog to all sequences. Circle size represents the number of sequences within each category and are drawn to scale. Areas of circle intersections approximate the frequency of BLAST homologs across multiple databases. Outermost black circle represents all sequences, whereas the inner white circle represents all sequences with a significant BLAST homolog, from (Williamson *et al.*, 2008).

hydrothermal fluids, it could be possible to some extent to extrapolate them to the deep crustal sub-seafloor.

## 3.3 Conclusion and outlook

The present study described the prokaryotic ecosystems associated with deep marine sub-surface. As, to date, most of the phylotypes in the deep sub-surface remain uncultured, molecular approaches were used to analyse the prokaryote diversity. This study mainly focused on the comparison and description of archaeal communities associated to several sub-surface environments, as these are considered to probably be the dominant domain in the sub-surface.

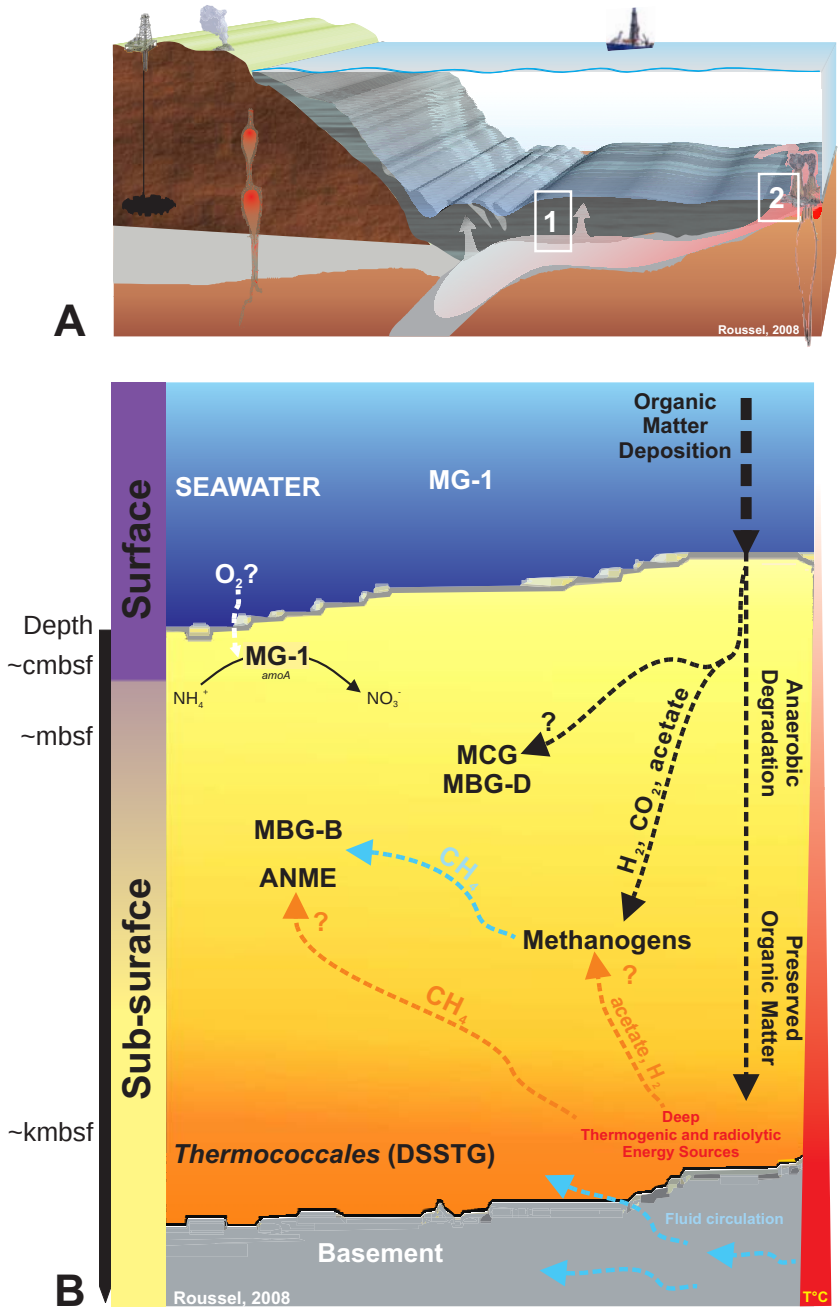


FIG. 3.3: Possible distribution and processes of the archaeal communities associated with the deep biosphere. A. Cross-section of the seafloor and seabed structures. The white squares point out the deep sub-surface sediment ecosystem (1) and the hydrothermal sub-surface (2). The red arrows show the possible localization of the deep hot biosphere communities. B. Cross-section of the seafloor and seabed structures associated with the deep sub-surface sediment ecosystem (1).

### 3.3.1 Deep sub-seafloor marine sediments

The definition and limits of the deep biosphere was specified and the possible environmental factors controlling the archaeal distribution were investigated. The wide distribution of the MBG-B, MCG and MBG-D in the deep sub-surface sediments suggests that these lineages are indigenous and adapted to the environmental factors. It was suggested that the MCG could be heterotrophic anaerobes that utilize and assimilate complex organic substrates (Teske and Sorensen, 2008). The present studies also detected rare lineages that are seldom detected in deep sub-surface sediments although they play a key role in the biogeochemical processes of the deep biosphere, such as methanogenic and methanotrophic *Archaea*. Although the frequently detected MBG-B could enter the methane cycle, it was suggested that putative methanotrophic communities such as ANME *Archaea* could utilize methane from various origins such as from thermogenic sources (Figure 3.3). Interestingly, the present study also extends also the marine sub-seafloor biosphere to at least 1,626 mbsf, and probably deeper, considering the higher limit of prokaryotic growth. The occurrence of DSSTG *Archaea*, a cluster of putative sub-surface thermophilic *Archaea* from the *Thermococcales* order, as deep as 1,626 mbsf points out the possible existence of a deep hot biosphere.
However, the analysis of classical 16S rRNA genes gives very limited information on the ecological role of prokaryotes. Hence metagenomic approaches such the one recently published by Biddle and colleagues give a broader picture of the metabolic and physiological capabilities of these deep prokaryote communities (Biddle *et al.*, 2008). The hypotheses inferred from these *in silico* predictions should however be confirmed by autecological methods. The increasing knowledge on the deep biosphere communities tends to indicate that the indigenous or "true" sub-surface microorganisms are probably very slow growing, and have very specific growth requirements. Hence new innovative cultural approaches, such high-throughput methods could allow to empirically test a larger number of growth requirements such as oligotrophic conditions.

### 3.3.2 The sub-surface of hydrothermal environments

Although the archaeal communities retrieved at the Atlantic hydrothermal ultramafic sites are similar to those detected in other hydrothermal systems, hydrogen utilizing microorganisms seem to dominate the diversity. Hence, *Methanococcales* were suggested to be members, together with the *Thermococcales*, of a deep hydrothermal sub-surface community. Although, the distribution of these lineages are limited by temperature, they could extend from the oxic-anoxic mixing zones, where the fluids are enriched in hydrogen and carbon dioxide to the higher temperature limit for life (Figure 3.4). The communities could also circulate and disseminate through the large crustal fluid circulation. Moreover, the oxic recharging zone could be dominated by MG-1. Although the phylogenic diversity of this new phylum is very large, they could represent putative ammonium oxidizing *Archaea*.

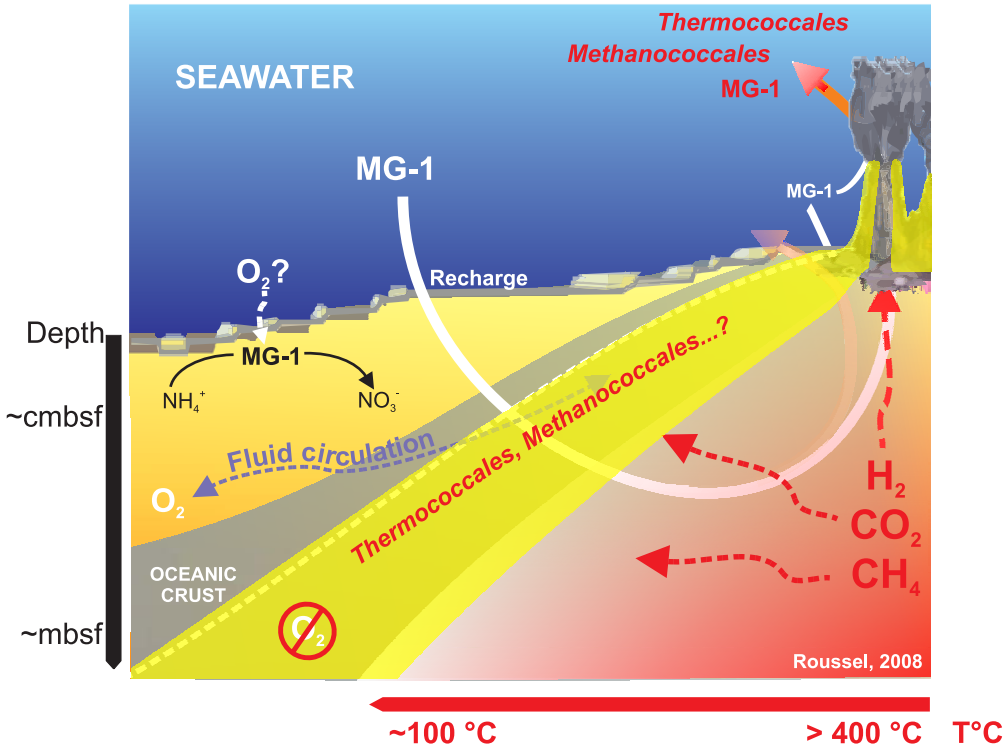


FIG. 3.4: Cross-section of the seafloor and seabed structures associated with the deep subsurface hydrothermal ecosystem (see 2 of figure 3A). The white dotted line indicates the position of the oxic-anoxic limit.

### 3.3.3 Deeper prospective...

The physiology, metabolic capabilities, dissemination and evolution processes of the deep biosphere prokaryotic communities remain still unclear. Moreover, the occurrence and possible interactions with the prokaryotes, of viral communities should be defined. As the analysis of the deep biosphere prokaryotic communities is mainly limited by technical issues, new approaches should be investigated. Hence, *in situ* experimentations, such as CORK microbiological observatories associated to sensors (pressure, temperature, strain and tilt sensors) and samplers (OSMO), are ultimately the best mechanisms for determining the Geosphere-Biosphere interactions. The spacial and time scales at wich these processes occur should although be determined. Moreover, the limits of the deep biosphere remain unknown and their definition would help to understand how possible life forms would sustain on other planets.

*Ce travail de thèse décrit les écosystèmes archéens associés à la sub-surface du plancher océanique. Ainsi, comme la plupart de ces microorganismes restent non-cultivés, des approches moléculaires ont été utilisées afin de décrire la diversité archéenne, ce domaine étant considéré comme celui dominant la sub-surface des environnements marins.*

### *Les sédiments marins profonds*

*La définition et les limites de la biosphère profonde ont été spécifiées ainsi que les facteurs environnementaux contrôlant la distribution des communautés archéennes. L'ubiquité de la distribution des groupes MBG-B, MCG et MBG-D dans les sédiments de sub-surface suggère que ces groupes sont parfaitement adaptés aux conditions environnementales de ces écosystèmes. Il a été précédemment suggéré que les MCG pourraient être des hétérotrophes anaérobies capables d'utiliser des substrats organiques complexes (Teske and Sorensen, 2008). Cette étude a également permis de mettre en évidence la présence de phylotypes rarement détectés dans les sédiments de sub-surface, comme les Archaea méthanogènes et méthanotrophes, bien qu'elles jouent un rôle essentiel dans les processus biogéochimiques. Les MBG-B sont fréquemment détectés et probablement impliqués dans le cycle du méthane. Nous suggérons également que les ANME pourraient utiliser le méthane d'origine thermogénique et/ou biogénique (Figure 3.3). Cette étude étend aussi la profondeur de la biosphère à au moins 1626 m sous la surface du sédiment et probablement plus si l'on tient compte de la température maximale à laquelle la vie peut se développer. La présence des DSSTG, un groupe de thermophiles putatifs de l'ordre des Thermococcales, démontre l'existence possible d'une biosphère profonde et chaude.*
*Néanmoins, l'analyse du gène codant pour l'ARNr 16S ne donne qu'une information limitée sur la position écologique des procaryotes étudiés. Ainsi, des approches basées sur la métagénomique comme celle récemment publiée par Biddle et al., présentent un tableau plus complet des capacités physiologiques et métaboliques de ces procaryotes (Biddle* et al.*, 2008). Les hypothèses proposées par ces études in silico nécessitent néanmoins d'être confirmées par des approches « autécologiques ». Le nombre croissant d'études des communautés procaryotes associées à la biosphère profonde semble indiquer que les microorganismes indigènes ont probablement des temps de génération très élevés ainsi que des conditions de croissance très spécifiques. C'est pourquoi des méthodes de cultures innovantes, comme les techniques à hauts débits, permettraient par une approche empirique de tester de nombreuses conditions de cultures différentes.*

### *La sub-surface des environnements hydrothermaux*

*Bien que les communautés archéennes détectées au niveau des sites ultramafiques hydrothermaux de l'océan Atlantique soient similaires à celles détectées dans d'autres systèmes hydrothermaux, les procaryotes utilisant l'hydrogène semblent être dominants. Ainsi, cette étude suggère que les Méthanococcales et les Thermococcales détectées dans les fluides hydrothermaux sont*

*issues de communautés de sub-surface. La distribution de ces phylotypes pourrait être limitée par la température d'une part, et par des conditions oxiques d'autre part (Figure 3.4). Ces communautés pourraient aussi circuler et se disséminer grâce à la circulation de fluides dans la croûte basaltique. Le groupe des MG-1 semble dominer les zones oxiques. Bien que la diversité intra phylum de ce groupe soit très large, il pourrait s'agir principalement d'Archaea capable d'oxyder l'ammonium.*

### *Autres perspectives*

*Les capacités physiologiques et métaboliques ainsi que les processus de dissémination et d'évolution des procaryotes de la biosphère profonde restent mal connus. De plus, la présence de communautés virales et leurs interactions possibles avec des communautés procaryotes restent à définir. Comme l'étude de ces communautés procaryotes est principalement limitée par des considérations techniques, de nouvelles approches doivent être appliquées. Ainsi, des approches in situ comme l'utilisation d'observatoires équipés de différents capteurs (pression, température, souches procaryotes) pourraient constituer les meilleurs outils pour comprendre les interactions entre la biosphère et la géosphère. Les échelles de temps et d'espace, auxquelles ces processus s'établissent, devront aussi être déterminées. Enfin, les limites de notre biosphère étant encore indéterminées, leurs définitions permettraient de comprendre comment la vie aurait pu s'établir sur d'autres objets célestes.*

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# Appendix

APPENDIX A

# DIVERSITY AND DISTRIBUTION OF METHANE OXIDIZING MICROBIAL COMMUNITIES ASSOCIATED WITH DIFFERENT FAUNAL ASSEMBLAGES IN A GIANT POCKMARK OF THE GABON CONTINENTAL MARGIN

## In revision

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# DIVERSITY AND DISTRIBUTION OF METHANE OXIDIZING MICROBIAL COMMUNITIES ASSOCIATED WITH DIFFERENT FAUNAL ASSEMBLAGES IN A GIANT POCKMARK OF THE GABON CONTINENTAL MARGIN

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## Abstract

A giant 800 m diameter pockmark named REGAB was discovered on the Gabon continental margin actively emitting methane at a water depth of 3200 m. The microbial diversity in sediments from four different assemblages of chemosynthetic organisms, Mytilidae, Vesicomyidae, Siboglinidae and a bacterial mat, was investigated using comparative 16S rRNA gene sequence analysis. Aggregates of anaerobic methanotrophic archaea (ANME-2) and bacteria of the *Desulfosarcina/Desulfococcus* cluster were found in all four chemosynthetic habitats. Fluorescence *in situ* hybridization targeting the ANME-2/*Desulfosarcina/Desulfococcus* aggregates showed their presence few centimeters (3-5 cm) below the surface of sediment. 16S rRNA gene sequences from all known marine ANME groups were detected in the pockmark sediments, as well as from both known bacterial partners. The archaeal diversity was limited to the ANME cluster for all investigated samples. The bacterial diversity included members of the *Proteobacteria*, *Bacilliales*, *Cytophaga/Flavobacteria*, *Verrucomicrobia*, JS1 and *Actinobacteria* clusters. Bacterial 16S rRNA gene sequences related to those of known sulfide oxidizing symbionts were recovered from tissues of several invertebrates including vesicomyid clams and siboglinid tubeworms of REGAB.

## Keywords

REGAB, AOM, ANME, chemosynthetic ecosystem, pockmark, cold seep, faunal assemblage, microbial phylogeny, FISH

## Introduction

Deep-sea cold-seep ecosystems are characterized by high fluxes of hydrocarbons and sulphide originating from the subsurface seabed by thermogenic or microbial production. Chemotrophic microorganisms, either free-living or in symbiosis with invertebrates, are abundant at these sites where they derive energy from the oxidation of sulphide or methane (Paull , 1984; Sibuet and Olu, 1998; Pancost *et al.*, 2000; Valentine *et al.*, 2000; Orphan *et al.*, 2001; Tunnicliffe *et al.*, 2003). Deep-water cold seeps often show substantial accumulations of methane in the seafloor in gaseous, liquid and solid form, the latter also known as gas hydrates (Kvenvolden, 1998; Milkov and Sassen, 2002). A major fraction of the methane rising from deeper sediments to the seafloor is consumed by microorganisms within the seabed, mainly through the anaerobic oxidation of methane (Reeburgh, 1976). The anaerobic oxidation of methane (AOM) by sulphate reduction leads to the formation of sulphide and carbonate (Barnes and Goldberg, 1976 ; Reeburgh, 1976). This process is mediated by yet uncultivated methanotrophic archaea related to the methanogens, and sulphate reducers of the Deltaproteobacteria clade (Hinrichs and Boetius 2002). The sulphate-reducing bacteria (SRB) and anaerobic methanotrophic archaea (ANME)

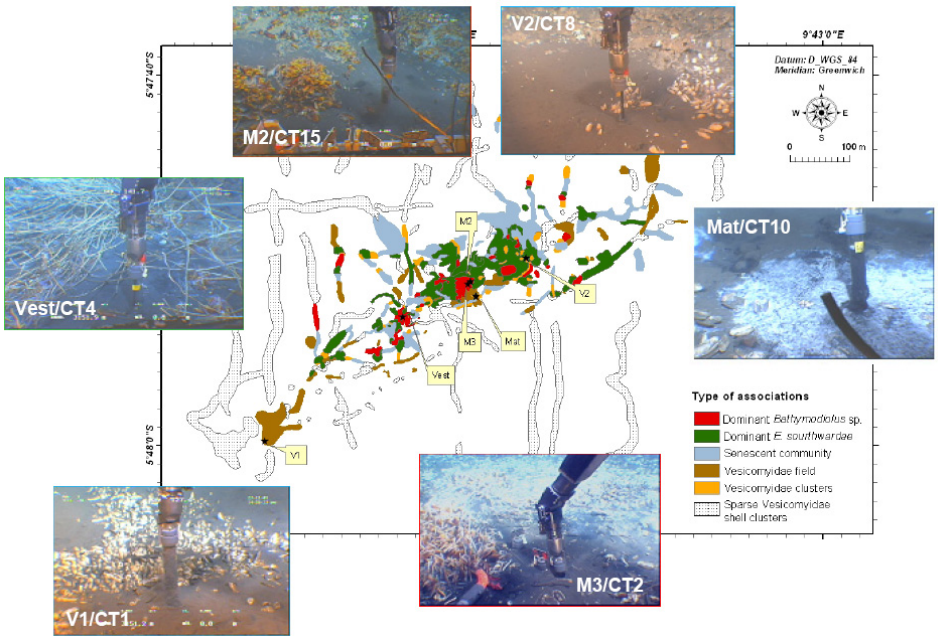


FIG. A.1: Map of the REGAB site (adapted from the Olu-Le Roy *et al.* 2007a) and sediment sampling sites.

often form aggregates and occur at very high abundances in cold-seep sediments (Boetius *et al.*, 2000; Orphan *et al.* 2001). Several distinct phylogenetic clusters of *Archaea* are involved in AOM as shown in various observations including proteomic and metagenomic data (Knittel *et al.* 2005, Krüger *et al.* 2003; Hallam *et al.* 2004; Meyerdierks *et al.*, 2005). The ANME-1 group is related to the *Methanosarcinales* and *Methanomicrobiales* orders (Hinrichs *et al.* 1999), the ANME-2 and ANME-3 groups are related to the *Methanosarcinales* (Boetius *et al.*, 2000; Niemann *et al.*, 2006). The ANME occur in sediments in consortium with sulphate-reducing bacteria (Michaelis *et al.*, 2005; Knittel *et al.*, 2005) or as single cells or small assemblages (Orphan *et al.*, 2001; Knittel *et al.*, 2005). ANME-1 and ANME-2 are generally associated with sulphate-reducing bacteria of the *Desulfosarcina/Desulfococcus* cluster, and ANME-3 with the *Desulfobulbus* genus (Losekann *et al.*, 2007). The AOM process provides high fluxes of sulfide, which nourishes several types of microbe-invertebrate symbioses such as bivalves from the Lucinidae, Thyasiridae, Solemyidae and Vesicomyidae families, as well as annelid polychaetes of the family Siboglinidae (Goffredi *et al.*, 2004 ; Duperron *et al.*, 2007a ; Bright and Giere, 2005). Mussels from cold seeps (family Mytilidae) host methanotrophic bacteria (Cavanaugh *et al.*, 1987; Barry *et al.*, 2002) or live in dual symbiosis with both methanotrophic and thiotrophic bacteria (Won *et al.*, 2003; Duperron *et al.*, 2005; Duperron *et al.*, 2007b). In most cold-seep environments, such chemosynthetic organisms dominate the community biomass and form specific habitats generally associated with high AOM rates.

Pockmarks are a special type of cold-seep characterized by negative seafloor features caused

by the seepage of fluids through the seabed (Hovland and Judd, 1988). They generally occur in unconsolidated fine-grained sediments as cone-shaped circular or elliptical depressions ranging from a few metres to 300 or more in diameter and from 1 m to 80 m in depth. The giant 800 m diameter pockmark REGAB was discovered along the Gabon continental margin at a water depth of 3200 m during the ZAIANGO cruise by geophysical surveys with the research vessel Suroit (April 2000, Ifremer). REGAB is characterized by high methane emissions and by the presence of methane hydrates in subsurface and surface sediments (Charlou *et al.*, 2004). First dives on REGAB using the remotely operated vehicle (ROV) VICTOR 6000 (cruises ZAINGO and BIOZAIRE I - 2001) showed the occurrence of dense communities of typical seep-associated invertebrates, some of which represent chemosynthetic symbioses (Ondréas *et al.*, 2005; Olu-Le Roy *et al.*, 2007a). Among these were Mytilidae (Bathymodiolus aff. boomerang, Olu-Le Roy *et al.* 2007b), Vesicomyidae bivalves (see Cosel and Olu-Le Roy, this volume), and Siboglinidae polychaetes (Vestimentifera, *Escarpia southwardae* n. sp., Andersen *et al.*, 2005). A few mats of giant sulfide oxidizing bacteria have also been observed in the pockmark. Three main faunal assemblages were observed (Figure 1): Mytilidae and Siboglinidae occur in the centre area of the pockmark associated with carbonate crusts, and Vesicomyidae mostly in the periphery on soft sediments. The bacterial mats patches were observed close to Vesicomyidae. Olu *et al.* (2007a) found that methane concentrations in the bottom water were the main factor explaining the distribution and high spatial variability of the faunal assemblages. Methane measurements in these habitats suggested that the *Bathymodiolus* species are exposed to highest methane concentrations while Vesicomyidae and Siboglinidae occur at the periphery of methane-emitting patches, experiencing lower methane concentrations. The occurrence of *Bathymodiolus* mussels in very large and dense aggregates in the most methane-rich areas is probably linked to their dual symbiosis with methane and sulphide-oxidizing Gammaproteobacteria (Duperron *et al.* 2005). Vesicomyidae and Siboglinidae were assumed to harbour only sulphide-oxidizers (Olu-Le Roy *et al.*, 2007a). In this study, we present a molecular survey of microbial communities associated with different types of habitats in the REGAB giant pockmark. The two main goals were to assess microbial diversity associated with the different faunal assemblages including their bacterial symbionts, and to assess the distribution of microorganisms involved in AOM in the seafloor.

## Materiel and methods

### Sample collections

During the BIOZAIRE II cruise in November 2001, aboard the research vessel L'Atalante, sediment samples were collected with push cores operated by the ROV VICTOR 6000 (Figure 1). The target sites in the REGAB pockmark at 3152 m depth included sites M2 and M3 close to Mytilidae (dives 146 and 147, cores CT15 and CT2 respectively), sites V1 and V3 close to Vesicomyidae (dives 145 and 147, cores CT1 and CT8 respectively), site Vest close to

Siboglinidae (dive 146, core CT4) and site "Mat"on bacterial mat (dive 146, core CT10) (Table 1). Lengths of sediment cores were 15 cm except for M2, V3 and Vest sites because of carbonate concretions below 8, 10 and 10 cm sediment depth, respectively. The site nomenclature follows the one that had been used before (Olu-Le Roy *et al.*, 2007a; Duperron *et al.*, 2007). Description of the faunal communities associated with the sites can be found in Olu-Le Roy *et al.*, 2007a. Siboglinidae, Mytilidae and Vesicomyidae were collected by the ROV grab and rapidly dissected on board. Bivalve gills and siboglinid trophosomes were frozen for phylogenetic analysis of bacterial symbionts.

### Methane concentration measurement

Fluids were sampled using the water sampling pump and collected in 200 ml titanium bottles at the different sites (Table 1). Methane concentrations were measured as described previously (Olu *et al.*, 2007a).

### Fluorescence *in situ* hybridization (FISH)

Subsamples of sediment cores were sliced into 1 cm intervals and fixed for 2h with 2 % formaldehyde solution. After washing with PBS (10 mM sodium phosphate, 130 mM NaCl), samples were stored in PBS/ethanol (1/1) at -20°C until used in the laboratory. Fixed sediment samples were diluted ten times in PBS/ethanol and sonicated 20s. Twenty $\mu$L were filtered on 0.2 $\mu$m GTTP polycarbonate filters. Separated hybridizations with two monolabelled probes were done on half cut filters. Microbial aggregates were counted after hybridization with Cy3 monolabelled probes, probe EelMS932 (5'-AGCTCCACCCGTTGTAGT-3') for archaea ANME-2 and probe DSS658 (5'-TCCACTTCCCTCTCCCAT-3') for sulfate-reducing bacteria (*Desulfosarcina /Desulfococcus*). After staining with 4,6-diamidino-2-phenylindole (DAPI), aggregates were counted with the appropriate fluorescent filters on a Olympus BH-2 microscope. For each sample slide, 500 microscope fields were counted and reported as number of aggregates per ml.

### DNA extraction

DNA from the different samples (0.8 g per sample) was extracted with FastDNA SPIN kit for soil (Bio 101 System, Qbiogen) with the modifications previously described by Webster *et al.* (2003). The DNA extraction and amplification procedures gave good results on all samples except the bacterial mat sample. Therefore, the DNA extraction of the bacterial mat sample (dive 146 core CT10) was carried out on 5×0.8 g replicates. The replicates were pooled, concentrated and purified with YM-100 centrifugal filters (Millipore Corporation) to give a final volume of 70 $\mu$L.

## PCR and cloning

For cores CT1 and CT2, amplifications were performed using the 16S rRNA gene universal primers for Bacteria or Archaea: U1492r (5'-GTT ACC TTG TTA CGA CTT-3') as reverse universal primer, E8f (5'-AGA GTT TGA TCA TGG CTC AG-3') for Bacteria and A8f (5'-CGG TGG ATC CTG CCG GA-3') for Archaea. The amplifications for cores CT4, CT8, CT15 and Bacteria in core CT10 were performed with: U1407r (5'-GAC GGG CGG TGW GTR CAA-3') as reverse universal primer, E338f (5'-ACT CCT ACG GGA GGC AGC-3') for Bacteria and E344f (5'-AYG GGG YGC ASC AGG SG-3') for *Archaea*.
For amplification of *Archaea* in core CT10, nested PCR was conducted with combination of 16S rRNA gene primers for *Archaea* with 8f (5'-CGG TTG ATC CTG CCG GA-3') and 1492r (5'-GGC TAC CTT GTT ACG ACT T-3') in the first round, with 344f (5'-AYG GGG YGC ASC AGG SG-3') and 915r (5'-GTG CTC CCC CGC CAA TTC CT-3') in the second round. Five independent PCR products from the first round were pooled and purified (QIAquick PCR purification Kit, Qiagen) and used as a template for the second PCR round.
The bulk DNA was amplified in a 50 $\mu$L reaction mix containing (final concentration): 1X Taq DNA polymerase buffer (Q biogen, Strasbourg, France), 20 $\mu$M of each dNTP, 20 $\mu$M of each primer and 0.5 $\mu$L of Taq DNA polymerase (Q Biogen, Strasbourg, France). Reaction mixtures were held at 94°C for 3 min followed by 30 cycles of 94°C for 60 s, 49°C (except for primer couples U1407r-E338f, A1492r-A344f and A915r-A344f: 54°C, 55°C and 57°C respectively) for 90 s and 72°C for 120 s, with a final extension step of 6 min at 72°C. PCR products were then visualized on a ethidium bromide containing agarose gel before cloning. Before cloning, two to four PCR products were pooled, purified and concentrated using the QIAquick PCR purification kit (Quiagen SA, Grenoble, France) following the manufacturer's instructions. Clone libraries were constructed by transforming *E. coli* TOP10F'using the TOPO TA Cloning kit (Invitrogen Corp., San Diego CA USA) according to the manufacturer's protocol. Clones harbouring the expected insert were then cultured and treated for sequencing on the "Ouest Genopole Plateform"(Roscoff, France, `http//www.sb-roscoff.fr/SG/`) on a Abi prism 3100 GA (Applied Biosystem), using the big-Dye Terminator V3.1 (Applied Biosystem) following the manufacturer's instructions.

## Phylogenetic analyses

To determine preliminary phylogenetic affiliations, sequences were compared to those available in databases using the BLAST network service (Altschul *et al.*, 1990). Alignments of 16S rRNA gene sequences were performed using CLUSTALW (Thompson *et al.*, 1994), further refined manually using SEAVIEW (Galtier *et al.*, 1996). The trees were constructed by PHYLO-WIN (Galtier *et al.*, 1996). Only homologous positions were included in the phylogenetic comparisons. For the 16S rRNA phylogenetic reconstruction, the robustness of inferred topology was tested by bootstrap resampling (1,000) (Felsentein, 1985) of the tree calculated

on the basis of evolutionary distance (neighbour-joining algorithm with Kimura 2 correction). For the Gamma and and Deltaproteobacteria phylogenetic reconstruction, the Maximum Likelihood was used with 100 bootstraps resampling. Sequences displaying more than 97 % similarity were considered to be related, and grouped in the same phylotype.

### Nucleotide sequence accession numbers.

Sequences have been deposited at EMBL for partial 16S rRNA gene sequences. The clones were named according to the sample core number (CTi), and A for *Archaea* and B for *Bacteria*. For the symbionts, the entire name of the host was used. The accession numbers are AM888202 to AM888272.

## Results and discussion

### Distribution of AOM consortia

Fluorescence *in situ* hybridization of pockmark sediments showed abundant microbial aggregates targeted with ANME-2 and DSS probes. All the counted aggregates were also visualized with DAPI staining. The aggregates consisted of ANME-2 archaea surrounded by sulphate-reducing bacteria of the *Desulfosarcina/Desulfococcus* cluster (Figure 2). No monospecies aggregates of ANME-2 were found as previously detected in cold seep sediments from Eel River Basin (Orphan *et al.*, 2002). Depth profiles of the number of AOM consortia differed between the habitats (Figure 3). Sediment from the Mytilidae site M3 contained the highest number of aggregates ($1.8 \times 10^7$ cm$^{-2}$) integrated over 15 cm depth. This was twice as much as at the Vesicomyidae site V1 ($0.99 \times 10^7$ cm$^{-2}$) and ten times more than at the microbial mat site ($0.16 \times 10^7$ cm$^{-2}$). At all sites except the Vest site the surface sediments did not contain aggregates. The reason could be the depletion of methane in the surface layers and/or the oxygenation of the surface sediments. The maximum of aggregate counts in sediments from the microbial mat site occurred between 5 and 6 cm ($7 \times 10^5$ cm$^{-3}$) and between 10 and 11 cm for the Vesicomyidae site ($30 \times 10^5$ cm$^{-3}$). The density of aggregates for the Mytilidae sites (M2 and M3) showed a maximum between 13 and 14 cm ($28 \times 10^5$ cm$^{-3}$). At the microbial mat site, aggregates were restricted to the uppermost centimetres (between 3 and 8 cm) whereas at all other sites the aggregate numbers did not decrease with depth. We assume that the bioturbation by the bivalves and hence oxygenation in surface and greater availability of sulfate in subsurface sediments, can explain the deeper distribution of aggregates in M3 and V1 sites compared to the bacterial mat site. Distribution of aggregates in sediments below siboglinid tubeworms (Vest site) showed no trend with sediment depth. Aggregates were detected in the first ten centimetres of sediment. Tubeworms use their roots to take up hydrogen sulphide, which fuels the thiotrophic symbiotic bacteria in their trophosome (Julian *et al.*, 1999; Freytag *et al.*, 2001). Then the tubeworms release sulfate across their roots into the surrounding

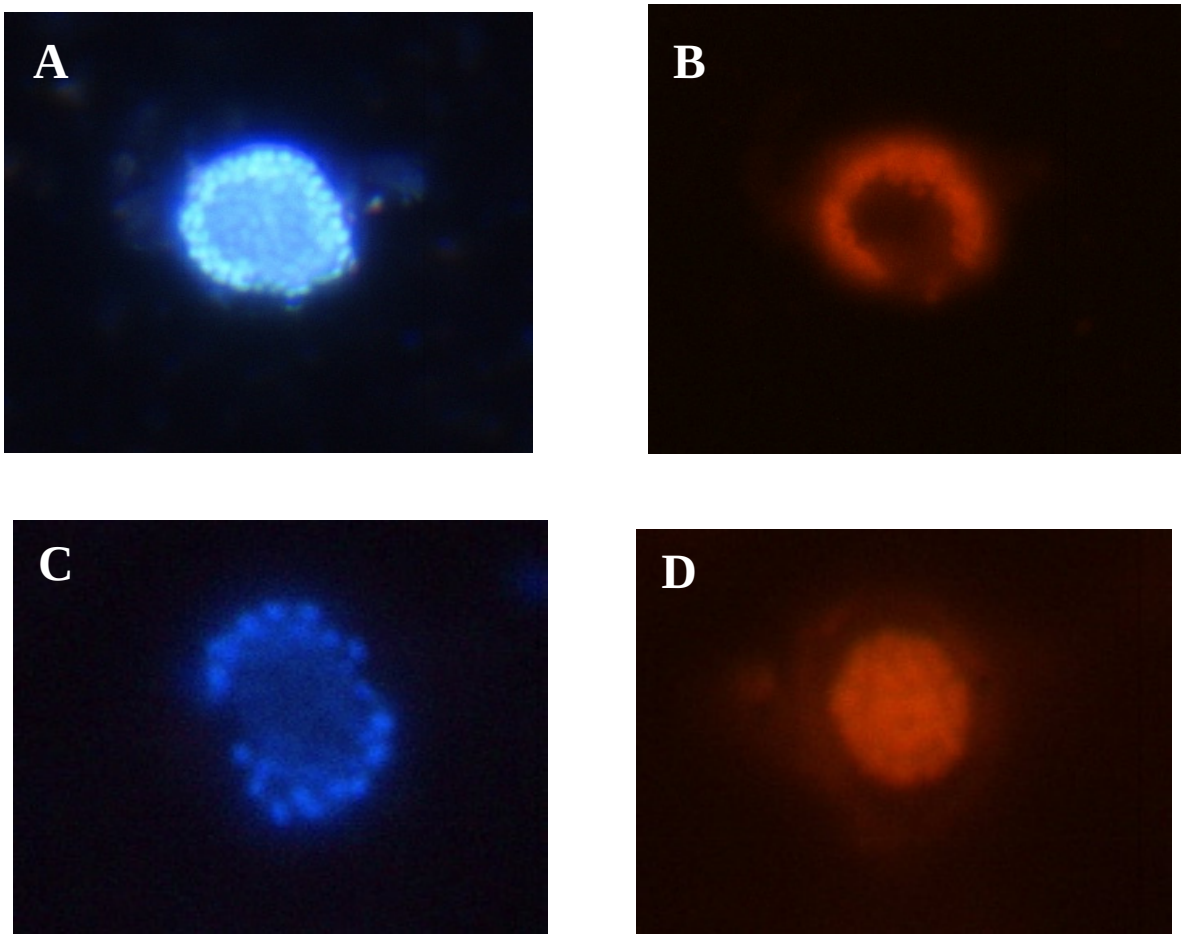


FIG. A.2: Consortia of ANME-2 *archaea* and sulfate-reducing *bacteria* DSS in sediments of REGAB visualized by fluorescence microscopy and FISH. (A) DAPI staining of aggregate. (B) Corresponding FISH image of same aggregate with probe DSS658 specific for sulfate-reducing bacteria (*Desulfosarcina/Desulfococcus*). (C) DAPI staining of aggregate. (D) Corresponding FISH image of same aggregate with probe EelMS932 specific for ANME-2

sediments (Dattagupta *et al.*, 2008), probably creating variable microhabitats for the AOM consortia. Moreover, Vestimentiferan tubeworm assemblages are usually associated with reduced sediments where oxygen is rapidly consumed in the first centimetres (Ritt, Sarrazin *et al.* in prep).

The densities of microbial aggregates in the pockmark sediments follow the same trend as the methane concentrations in the bottom waters, which were much higher at M3 (23.5 $\mu$M) than at V1 (0.9 $\mu$M) (Table 1). Probably bottom water concentrations are linked to the gradient in total methane fluxes from the seafloor to the hydrosphere, decreasing from the mussel sites to the bacterial mats.

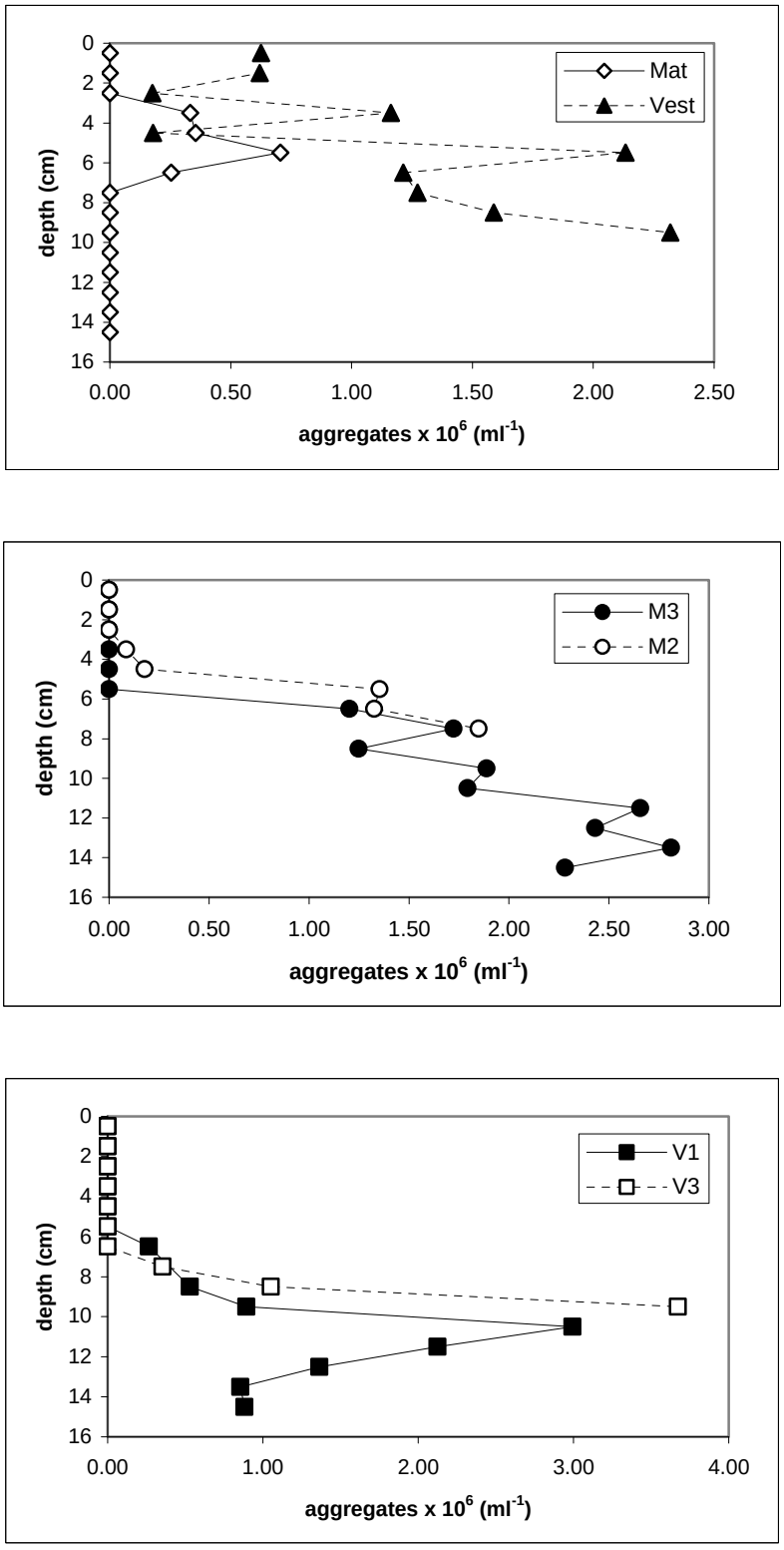


FIG. A.3: Detection and quantification of AOM consortia in REGAB sediments by FISH (archaeal probe EelMS932, and bacterial probe DSS658)

| Sampling site | Sample name | Fauna | Methane concentration (µM) | DNA extraction cm |
|---|---|---|---|---|
| M2 | CT15 | *Bathymodiolus* sp. | 33.7 | 5-8 |
| M3 | CT2 | *Bathymodiolus* sp. | 11.8 | 10-15 |
| V1 | CT1 | Vesicomyidae | 0.9 | 5-10 |
| V3 | CT8 | Vesicomyidae | 4.4 | 5-10 |
| Vest | CT4 | *Escarpia* sp. | 0.7 | 5-10 |
| Mat | CT10 | Bacterial mat | n. d. | 5-10 |

n.d.: not determined

Table A.1: Sampling sites and methane concentrations measured above sediment-water interface. DNA was extracted on sub-sample of each core and the extracted sample is indicated in centimeter below the surface.

## Symbiont sequences

Invertebrate communities at REGAB are dominated by taxa previously reported to harbour chemosynthetic bacterial symbionts, including Mytilidae, Vesicomyidae and Siboglinidae (Fisher, 1990). Specimen of the mytilid *Bathymodiolus* sp. from the Gabon margin were investigated previously and shown to harbour two phylotypes related to sulphide- and methane-oxidizing symbionts (Duperron *et al.* , 2005). No evidence for methane-oxidizing symbionts was found in the bacterial clone libraries from the two invertebrate species investigated here (Figure 6). A single phylotype, related to the sulphide-oxidizing endosymbiont of *Calyptogena pacifica* was recovered from the vesicomyid clam. Vesicomyid clams are known to harbour chemoautotrophic symbionts and to colonize sulphide-rich habitats at vents and seeps (Peek *et al.*, 1998). Symbiosis in the tubeworm was also investigated, and preliminary results based on electron micrographs suggested the presence of sulphide-oxidizing symbionts (Andersen *et al.*, 2005) confirmed by the sequence obtained here. Although FISH was not performed on animal samples, the close relationship between sequences recovered and known symbionts suggests that these sequences represent the dominant symbionts, although the presence of additional symbionts remains to be addressed in more specific studies. Symbiosis is known to be of prime importance in the productivity of cold seep communities (Sibuet and Olu, 1998), and recent results confirm that availability of energy in the form of methane and sulfide to the symbiosis is a key parameter for distribution and structure of communities at REGAB (Duperron *et al.*, 2005, Olu *et al.*, 2007a).

## Evidence of AOM in REGAB pockmark through molecular survey

DNA was extracted, according to FISH results, from each sample where the AOM consortia were most abundant (Table 1). As the present study was mainly focused on the identification of the microorganisms involved in AOM in the seafloor, only a limited number of clones were sequenced. For the CT10 sample from the thiotrophic mat, the use of nested PCR was necessary to obtain a correct amplification of the archaeal 16S rRNA genes, confirming the low abundance of *Archaea* in this sample. From the Mat sample, we sequenced 78 clones from the bacterial library and 39 from the archaeal one. For each other sediment sample, 25 to 30 clones were sequenced both for *Archaea* and *Bacteria*. 18 to 20 sequences were suitable to be used for phylogenetic analyses, the others 10 being too short for proper phylogenetic analysis.

**Archaeal diversity.** In all samples, the archaeal diversity comprise only members of the ANME-1b, ANME-2 (a, b and c) and ANME-3 clusters (Table 2 and Figure 4). For the CT10 sample from the thiotrophic mat, nearly half of the sequences obtained were related to ANME-2a and formed two clusters closely associated to uncultured *Methanosarcinales* ($>$ 98 % similarity) and to clones retrieved at Kazan mud volcano (ANME-2ab). The other sequences are related to the ANME-3 group, forming a cluster closely related to environmental sequences from methane-rich sites such as Eel River Basin. The seawater overlying the mytilid sites M2 and M3 displayed the highest methane concentration (Table 1), corresponding to higher densities of aggregates in the seafloor. As encountered in other studies, few archaeal sequences were retrieved (Inagaki *et al.* , 2004) despite the high abundance of aggregates according to FISH results (this study, Lösekann *et al.* , 2007). All archaeal sequences were affiliated to the ANME-2c and ANME-3 groups. For the Vesicomydae and Siboglinidae fields, few archaeal sequences were retrieved and these were also all affiliated to ANME clusters.

| Sample origin | Sequence type | Phylogenetic affiliation | Closest match by BLASTN search (accession number) | % of similarity | Number of related clone (similarity above 97%) |
|---|---|---|---|---|---|
| Microbial Mat (CT10) Partial sequences 1000 bp for bacteria 500 bp for archaea | CT10B1049 | Epsilonproteobacteria | Uncultured clone NKB11 (AB011363) | 98 | 1 |
| | CT10B1068 | | Uncultured clone C1_B005 (AF420358) | 98 | 1 |
| | CT10B1056 | | Uncultured clone R76-B76 (AF449250) | 98 | 10 |
| | CT10B1047 | Alphaproteobacteria | Clone LC1-35 (DQ289905) | 99 | 5 |
| | CT10B1069 | Gammaproteobacteria | Clone Hyd89-87 (AJ535246) | 98 | 2 |
| | CT10B1042 | | Clone Milano Mud Volcano (AY592847) | 98 | 1 |
| | CT10B1054 | Deltaproteobacteria | Clone Hyd89-22 (AJ535247) | 96 | 20 |
| | CT10B1030 | | Clone GoMGC2324463 (AM745212) | 98 | 1 |
| | CT10B1015 | CFB | Clone HMMVPog-1 (AJ704701) | 98 | 11 |
| | CT10B1072 | Bacilliales | *Clostridium* sp. Kas303 (AB114244) | 98 | 6 |
| | CT10B1025 | Verrucomicrobiales | Chlamydial symbiont (EF177461) | 92 | 3 |
| | CT10B1031 | GNS | Clone GoM161-bac49 (AM745150) | 99 | 9 |
| | CT10B1057 | Actinobacteria | Clone Kazan 2B 21 (AY592145) | 98 | 5 |
| | CT10B1020 | | Clone GoM Gb425 (AY542578) | 99 | 7 |
| | CT10B1059 | | Clone Kazan 2B 10 (AY592135) | 99 | 1 |
| | CT10B1048 | | Clone Kazan 2B 12 (AY592136) | 99 | 1 |
| | CT10A1044 | ANME-2 | Clone HydBeg46 (AJ578097) | 99 | 12 |
| | CT10A1032 | | Clone Kazan 3A 05 (Ay592029) | 97 | 3 |
| | CT10A109 | | Clone HydBeg22 (AJ578118) | 98 | 1 |
| | CT10A1024 | ANME-3 | Clone fos0625e3 (CR937011) | 98 | 5 |
| | CT10A1045 | | Clone fos0625e3 (CR937011) | 99 | 10 |
| Mytilid fields (CT15 partial sequences 1000 bp, CT2 full length sequences) | CT2B294 | Alphaproteobacteria | Clone Kazan 2B 34 (AY592158) | 96 | 3 |
| | CT15B04 | Gammaproteobacteria | Guaymas clone C1-B038 (AF420367) | 94 | 4 |
| | CT15B17 | | Clone GoM Gb425 (AY542558) | 95 | 1 |
| | CT15B19 | Deltaproteobacteria | Clone GoM HDB-20 (AY542210) | 95 | 2 |
| | CT2B216 | | Clone Eel-36e1G12(AF354163) | 99 | 6 |
| | CT2B287 | | Clone Hyd01-30 ( AJ535242) | 98 | 2 |
| | CT15B02 | CFB | Clone GoM161-Bac19 (AM745134) | 97 | 2 |
| | CT2B280 | Bacilliales | Clone RL199-aaj41a08 (DQ793227) | 95 | 1 |
| | CT2B214 | Verrucomicrobiales | Clone LD1-PA20 (AY114314) | 89 | 1 |
| | CT2B224 | GNS | Clone C5LK53 (AM086136) | 97 | 2 |
| | CT15B21 | | Clone C1B046 (AF419699) | 96 | 1 |
| | CT2B276 | JS1 | Clone Hyd01-10 (AJ535219) | 92 | 5 |

Table A.2: Closest 16S rRNA gene sequence matches to our clone type sequences using the NCBI BLASTN search tool.

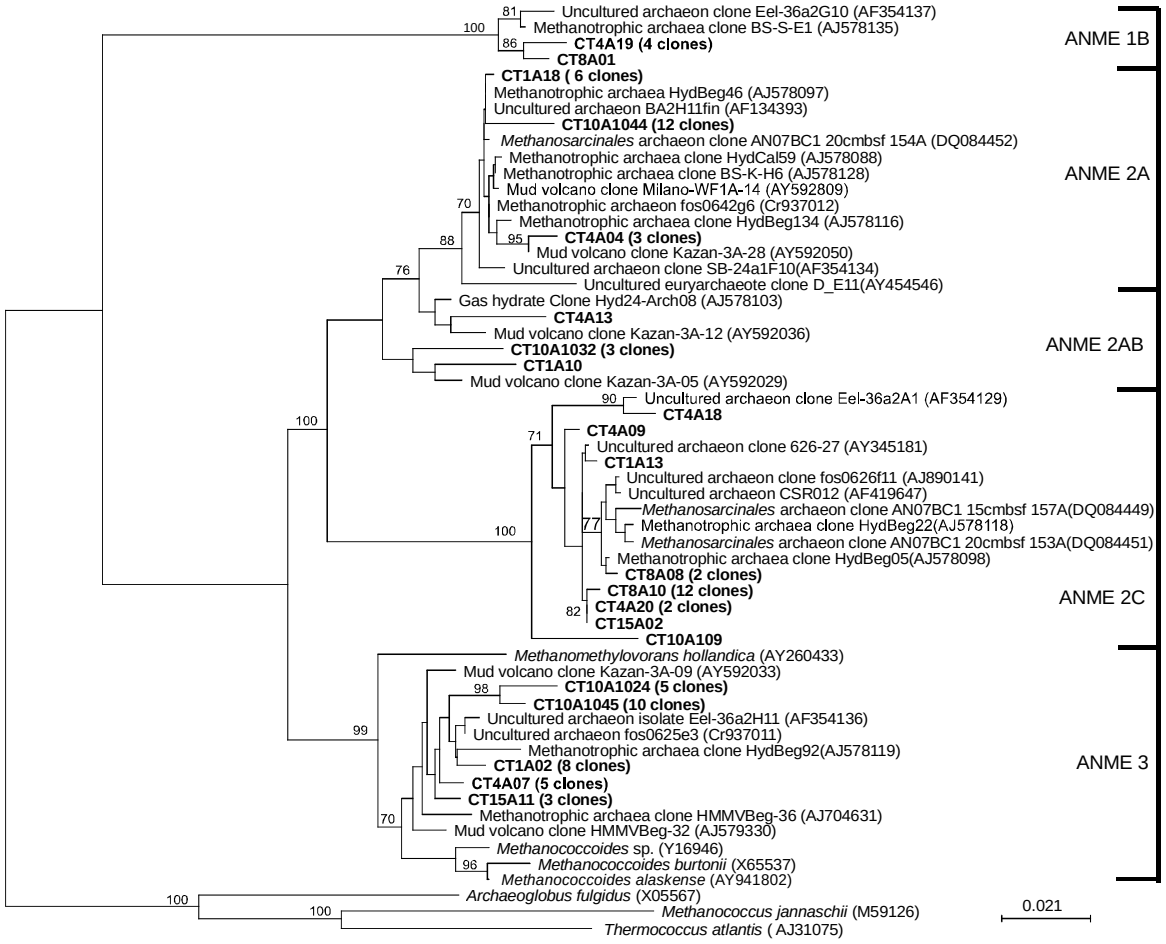


FIG. A.4: Phylogenetic tree of Archaea obtained using Neighbour-joining analysis with bootstrap resampling (1,000 replicates), based on 558 base pairs. Topologies were confirmed with Maximum Parsimony and Maximum Likelihood. Bootstraps values are indicated on nodes above 70 %. The outgroup sequence used was *Thermococcus atlantis*.

This relative low archaeal diversity is to be compared to other methane rich environments such as Kazan mud volcano (Heijs *et al.* , 2007), Haakon Mosby mud volcano (Niemann *et al.* , 2006), Black-Sea (Treude *et al.* , 2005, Knittel *et al.* , 2005), Hydrate Ridge (Knittel *et al.* , 2005), Eel River basin (Orphan *et al.* , 2001), Ryukyu Arc seeps (Inagaki *et al.* , 2004) and Gulf of Mexico (Lloyd *et al.* , 2006). In all these methane-rich environments, archaeal diversity is clearly dominated by ANME members but in most of these sites, sequences related to other *Euryarcheota* (e.g. *Thermoplasmales*) or *Crenarcheota* (marine benthic group B, C) were also found, which is not the case here (Knittel *et al.* 2005). However, this apparently low diversity may also be related to the low number of sequences analysed for this preliminary study.

**Bacterial diversity.** The bacterial diversity is apparently higher than the archaeal one (Table 2 and Figure 5 and 6), as described for the other environments cited above. Nevertheless, the sequences obtained for each sample are all affiliated to a small number of phylogenetic groups.

Twenty six sequences were affiliated to the Epsilonproteobacteria cluster (Figure 5), most of them being closely related to sequences retrieved from deep-sea sediments (NKB sequences) and to methane-rich environments. Some sequences are related to environmental clones associated to chemosynthetic deep-sea marine invertebrates and could thus represents sulfur oxydizers.

Few sequences (CT10 and CT2) were related to the Alphaproteobacteria, *Agrobacterium-Halophaga* division ($>$ 98 % similarity), showing similar results to those reported from Kazan Mud volcano (Heijs *et al.* , 2007) and Eel River basin (Orphan *et al.* , 2001). This group, closely related to *Hyphomicrobium* sequences, could represent aerobic methylotroph species.

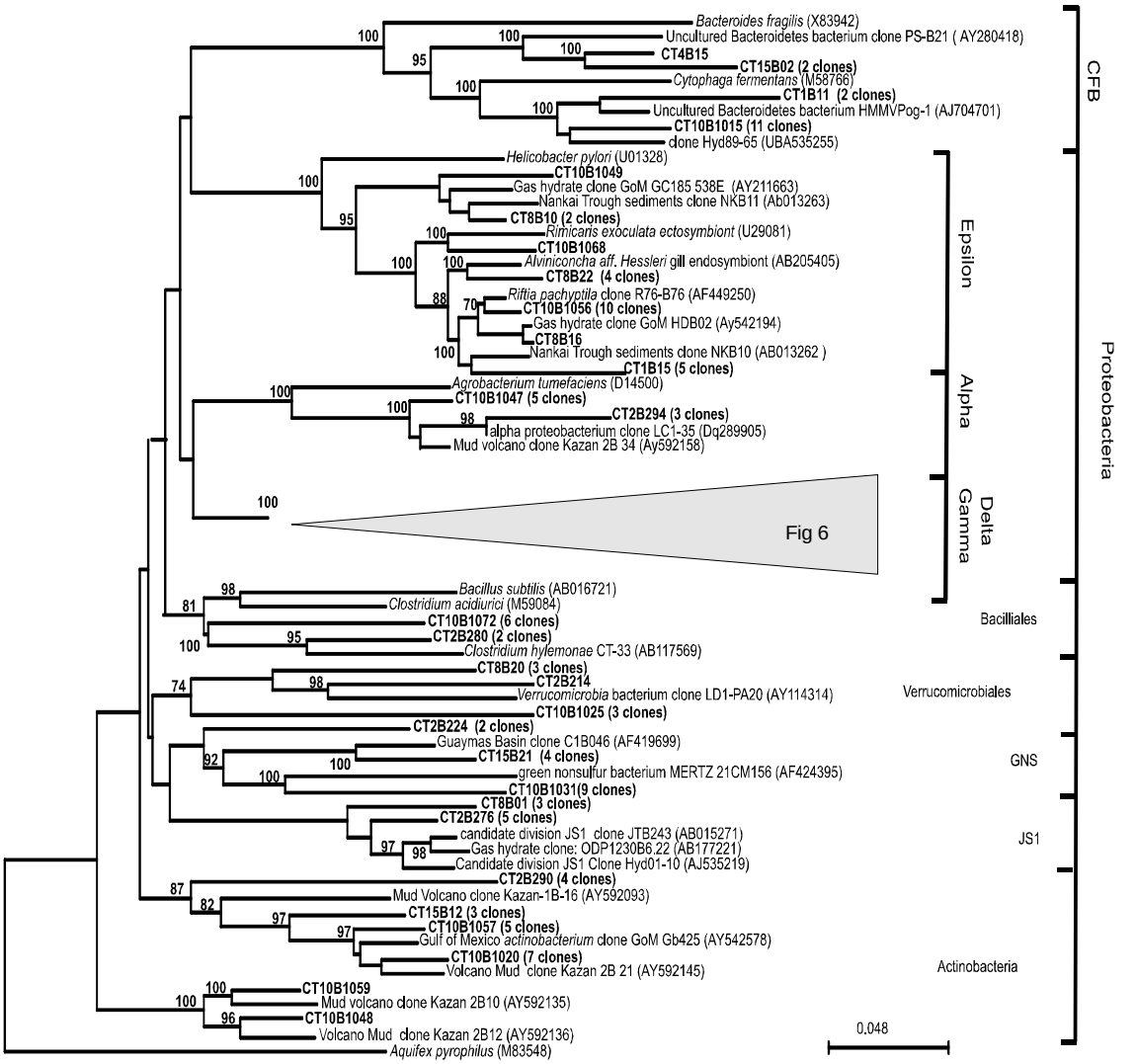


FIG. A.5: Phylogenetic tree of Bacteria obtained using Neighbor-joining analysis with bootstrap resampling (1,000 replicates), based on 812 base pairs. Topologies were confirmed with Maximum Parsimony and Maximum Likelihood. Bootstraps values are indicated on nodes above 70 %. The outgroup sequence used was *Aquifex pyrophilus*.

Fourteen sequences were related to the Gammaproteobacteria and three of them were related to mytilid-associated methanotrophic endosymbiont sequences ($>$ 98 %). This result could suggest the occurrence of free-living, close relatives of mussel symbionts in the environment. A more general survey of methanotrophs is needed, including a complete phylogenetic analysis of the first centimetres where lipid signatures indicate the presence of aerobic methanotrophs (Bouloubassi pers. com.).

Relatives of the Deltaproteobacteria dominated the bacterial clone libraries with 42 sequences (Figure 6). Most of the clones were related to the DSS subgroup (*Desulfosarcina/Desulfococcus* relatives) to which the bacterial partners in AOM belong. Some sequences were closely related to the DBB subdivision (*Desulfobulbus* relatives), which also includes bacteria involved in AOM associated with members of the ANME3 archaeal group (Niemann *et al.* , 2006).

Other clones were related to clusters usually encountered in deep-sea sediments such as Bacilliales (8 clones), *Verrucomicrobiales* (7 clones), Green non sulphur (16 clones), *Actinobacteria* (21 clones), and the *Cytophaga – Flexibacter – Bacteroidetes* (CFB) (19 clones). These clusters are generally supposed to be heterotrophic and present quite versatile groups found in many environments (Alain *et al.* , 2002). In the present study, our clone sequences are mostly related to environmental sequences originated from methane and hydrocarbon-rich areas (Kazan mud volcano, Golf of Mexico or Guaymas Basin).

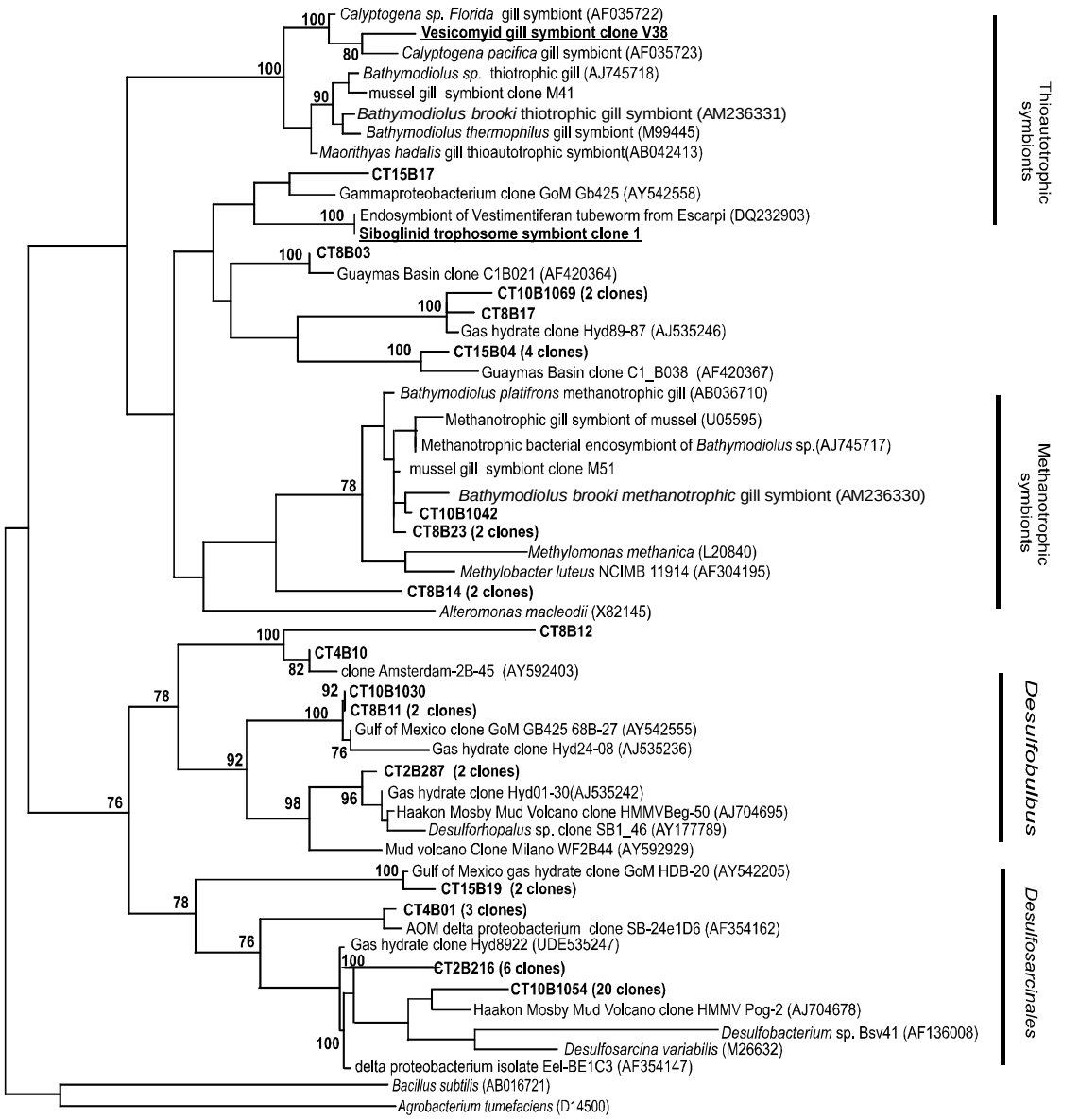


FIG. A.6: Phylogenetic tree obtained using Maximum Likelihood analysis with bootstrap resampling (100 replicates), based on 561 base pairs. Bootstraps values are indicated on nodes above 70 %. The outgroup sequences used were *Agrobacterium tumerfaciens* and *Bacillus subtilis*.

Members of the JS1 group (8 clones) were also retrieved under the mat sample and from the mytilid field. Representative sequences of the JS1 group have previously been found in methane-rich subsurface sediments where gas hydrates are present (Webster *et al.* , 2004). This suggests that some members of the JS1 division may be associated with methanogenic consortia. JS1 inhabit strictly anoxic organic-rich environments (Webster *et al.* , 2006).
Results from this preliminary study clearly indicate that key microorganisms mediating the anaerobic oxidation of methane occur below each type of faunal assemblage (thiotrophic mat, Mytilidae, Vesicomyidae) on the REGAB pockmark. Too few sequences were recovered from the Siboglinidae fields to draw any conclusion, but these preliminary results seem to confirm also the presence of AOM consortia under the Siboglinidae fields. Interestingly, all known marine AOM-mediating groups were present in clone libraries of the pockmark sediments. Below the *Bathymodiolus* field, aggregates were most abundant but the archaeal PCR amplification yielded poor results. The low number of ANME-related sequences in sediments characterized by high methane concentration such as observed here was previously reported (Inagaki *et al.* , 2004, Losekann *et al.* , 2007) and could be due to PCR inhibition or biases (Lösekann *et al.* , 2007). In the absence of quantitative data regarding abundances, the relative importance of each group cannot be assessed. Nevertheless, the occurrence of strongly 13C-depleted archaeal lipids (such as archaeol, sn-2 hydroxyarchaeol, crocetane and PMI), clearly points out to substantial biomass of anaerobic methanotrophic *archaea* (Bouloubassi, this volume). Their composition and relative abundance indicate a methanotrophic archaeal community dominated by ANME-2 members. In addition, the co-occurrence of 13C-depleted monoalkyl glycerol ethers (MAGE) indicates the presence of sulphate reducing bacteria among the *Desulfosarcinales* group, also implicated in AOM with ANME-2. The downcore distribution of archaeal and bacterial (SRB) lipids provides evidence for dominant anaerobic oxidation of methane (AOM) and concomitant sulphate reduction below the upper 2 cm layer of the sediments (Bouloubassi pers. comm.). Within the surface sediment layer, AOM-related biomarkers show low concentrations, whereas, in contrast, amounts of $^{13-}$C depleted diploptene are highest, most likely reflecting aerobic methanotrophy in the zone where oxygen penetrates from the overlying bottom water (Bouloubassi this volume).

## Conclusion

The sediments collected from four main types of chemosynthetic habitats of the REGAB pockmark, characterized by aggregations of Mytilidae, Vesicomyidae, Siboglinidae and thiotrophic bacterial mats, have been found to contain abundant AOM consortia. The ANME-2/DSS shell-type aggregates were concentrated a few centimetres below the surface (3-5 cm). Highest densities of AOM consortia were discovered at the Mytilidae sites, which also showed the highest methane concentrations in the bottom waters.
Due to the necessary use of 30 PCR cycles, and the investigation of limited numbers of clones,

we were not able to give an exhaustive description of the diversity of microbial communities present on the REGAB site. Nevertheless, representatives of all ANME groups were found at REGAB, comprising all recovered archaeal sequences. Bacterial diversity in clone libraries from the REGAB sediment was relatively high for the number of clones examined and included sequences related to aerobic methanotrophs, sulphide oxidizing and sulphate reducing bacteria, as well as diverse heterotrophs. The bacterial 16S rRNA genes associated with the 3 main types of chemosynthetic invertebrate symbioses of mytilids, vesicomyids and siboglinids were closely related to known thiotrophic symbionts of the respective groups. Only the mytilids contained a second symbiont related to aerobic methanotrophs. This indicates that the rich chemosynthetic communities of the REGAB pockmark are mostly dependent on AOM as the sulphide generating process in this ecosystem.

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APPENDIX B

# POSTER 1 – VINGT MILLE LIEUES SOUS LA TERRE: UNE BIOSPHÈRE PROFONDE ET CHAUDE?

## Rencontre des jeunes chercheurs du Grand Ouest (CIES); France, Rennes, 2005

E.G. Roussel, M.A. Cambon-Bonavita, J. Querellou and D. Prieur.

# Vingt Mille Lieues Sous La Terre:

## Une biosphère profonde et chaude?

**Erwan Roussel, Marie-Anne Cambon-Bonavita, Joël Querellou et Daniel Prieur**

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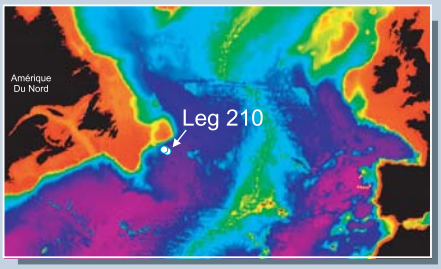
## LES SÉDIMENTS MARINS PROFONDS: UNE 2ÈME BIOSPHÈRE ?

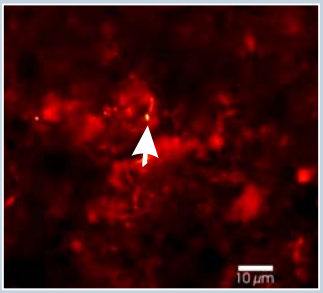
- Les sédiments marins recouvrent les 2/3 de la planète terre.
- En 1955, la vie semblait impossible en dessous de 7m de sédiment marin...
- En 1997 la présence de microbes a été démontrée sous 800m de sédiments marins, datés de 15 millions d'années.
- 10% du carbone organique de la planète terre serait contenu dans les sédiments marins...




Carte bathymétrique du Bassin de Terre Neuve

## LA VIE SOUS 1600 MÈTRES DE SÉDIMENT!

- En 2003, la campagne océanographique ODP leg 210 a permis de recueillir des sédiments à 1625m sous la surface du sédiment, à 4560m sous la mer, datés de 106 millions d'années!
- Des micro-organismes vivants ont pu être observés et cultivés, de l'ADN détecté et caractérisé.




Photographie en microscopie de micro-organismes (flèche blanche) sur le sédiment (rouge)

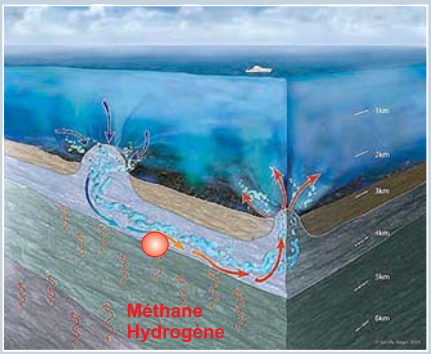
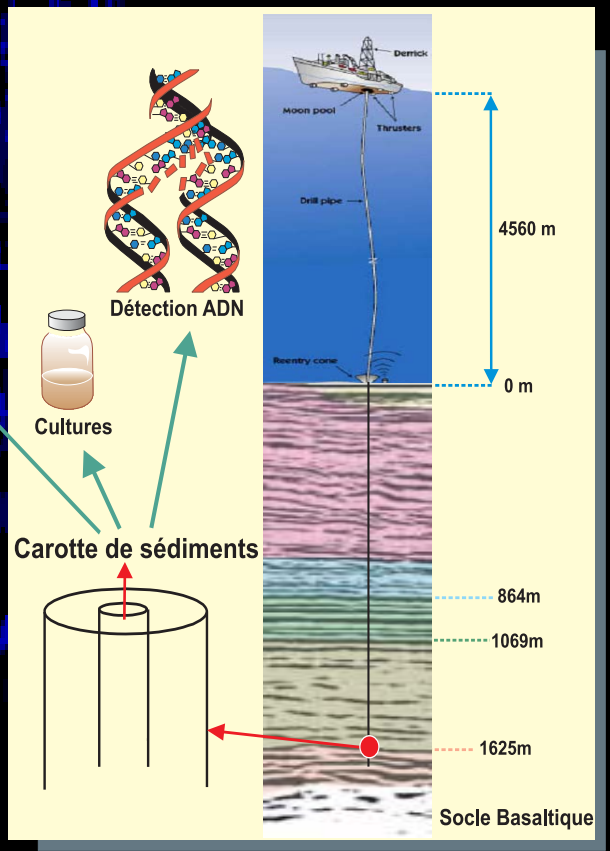



Schéma hypothétique d'une biosphère profonde et chaude. Les micro-organismes (●) sont alimentés par des composés comme le méthane et l'hydrogène.

- Certains de ces micro-organismes utiliseraient le méthane et l'hydrogène.
- D'autres sont capables de résister à des températures de plus de 100°C.
- Ces micro-organismes pourraient être issus d'une biosphère profonde et chaude.




## Une Biosphère Profonde: Les Enjeux

- Impact sur les stocks de méthane (gaz à effet de serre) et le cycle du carbone.
- L'existence d'une biosphère profonde appuierait l'hypothèse d'une vie microbienne souterraine dans le sédiment de la planète Mars et des satellites de Jupiter.

APPENDIX C

# POSTER 2 – DIVERSITY OF *BACTERIA* IN LOW-BIOMASS DEEP SUB-SURFACE SEDIMENTS COMPARED TO A 16S RDNA CONTAMINANT LIBRARY

## Extremophiles; France, Brest, 2006

E.G. Roussel, E. Delage, F. Duval, R.J. Parkes, B. Cragg, J. Querellou, D. Prieur, and M.A. Cambon-Bonavita.

# Diversity of *Bacteria* in low-biomass deep sub-surface sediments compared to a 16S rDNA contaminant library

**E. Roussel, E. Delage, F. Duval, R.J. Parkes, B. Cragg, J. Querellou, D. Prieur, M.A. Cambon Bonavita**

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## INTRODUCTION

- Recent studies of the sub-seafloor have demonstrated the existence of large and active microbial populations buried as deep as 800 m below the seafloor (mbsf). Meanwhile, the microbial diversity below 800 mbsf remains unknown. This deep biosphere could have an impact on the global biochemical cycling (1).
- Bacterial diversity from samples containing very low biomass from the sedimentary column of the Newfoundland basin (Fig.1) were retrieved during the ODP leg 210 from 800 to 1739 mbsf (46 to 106 million years old) under 4560 m of overlaying water.

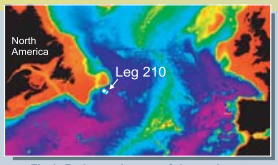


**Fig.1- Bathymetric map of the conjugate Newfoundland and Iberia margins (2).**

- The crucial issue for optimization of molecular ecological methods to investigate the Bacterial diversity of low biomass sediment samples is the Dna extraction. This approach is more likely to introduce contaminating rDNA during experimental procedures preceding the PCR amplification.
- The aim of this study was to optimize the DNA extraction procedure from low biomass sediments and to identify potential contaminants from Bacterial molecular diversity from the the deepest and oldest (106 million years old) samples ever studied .

## RESULTS

| Decontamination* | Yes | | No | | No | |
|---|---|---|---|---|---|---|
| Lysis° | Bead beating | | | | SDS/ Sarkozyl/ proteinase K | |
| DNA purification and DNA elution° | FastDNA® Spin kit for Soil | Phenol-chloroform /isopropanol /ethanol/ Dnase-free water | FastDNA® Spin kit for Soil | Phenol-chloroform /isopropanol /ethanol/ Dnase-free water | FastDNA® Spin kit for Soil | Phenol-chloroform /isopropanol /ethanol/ Dnase-free water |
| Cell density of the sample (cells/mL) 5 x $10^8$ | + | – | + | – | + | – |
| 7 x $10^7$ | + | – | + | – | – | – |
| $10^5$ | – | – | – | – | – | – |
| Negative control | + | – | + | ± | ± | ± |

**Fig.2- Comparison of the combination of different DNA extraction methods assessed by PCR-amplification on sediments with different cell densities from the West African Equatorial margin (BioZaire1) site CRAVO and from the Newfoundland margin (ODP leg 210) site 1276 at 1147 mbsf. +/-: poor PCR reproductibility**

- Six DNA extractions methods were tested, using a combination previously published methods and the FastDNA spin kit for soil () for different cell densities from the West African Equatorial margin (BioZaire1) site CRAVO and from the Newfoundland margin (ODP leg 210) site 1276 at 1147 mbs. DNA amplification were carried out, using PCR (primers 8F, 1407R).
- A 16S rDNA gene library derived from control extractions and PCR amplification (primers 8F, 1407R) that did not contain an environmental sample was also analyzed. The 16S rDNA sequences for Bacteria were analyzed from clone libraries.
- As previously published (3) the most reliable methode, of those tested, to extarct DNA from low biomass sediment is the FastDNA® Spin kit for Soil (Fig 3).
- A decontamination procedure of the DNA extration reagents and plasticware does not reduce the amount of contaminants (Fig 3).

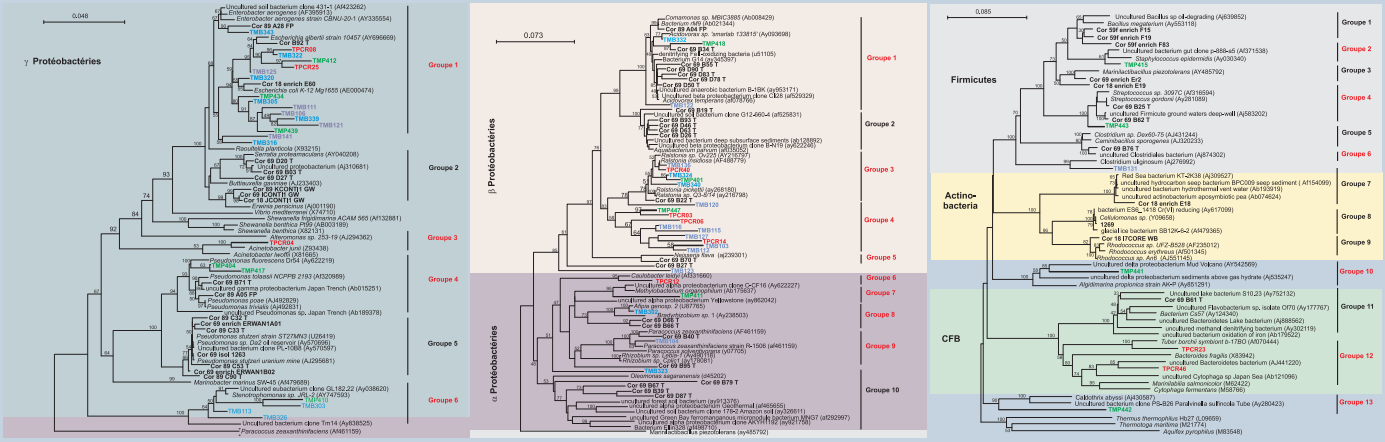


**Fig. 3 -Phylogenetic trees of all the bacterial 16S rDNA sequences obtained from the contaminant clone library (in blue, green and red ) and the ODP leg 210 sediments clone library (in bold black) from four depths. The sequences were aligned using CLUSTAL W. The tree was constructed using neighbor-joining with PHYLOWIN. Only the Bootstrap values over 50 are shown on the nodes. For each sample a single clone is represented.**

- Analysis of the gene library of 16S rDNA from the negative extraction control revealed a diverse collection of contaminant related to the following genera: _*Ralstonia*, *Herbaspirilum*, *Caulobacter*, *Escherichia*, *Acinobacter*, *Tuber borchii symbiont* and *uncultured Bacteroides* (PCR reagents), _*Escherichia* (Taq polymerase), _*Bradyrhizobium*, *Parracoccus*, *Clostridium*, *Acidovorax* and *Stenotrophomonas* (FastDNA® Spin kit for Soil), _*Methylobacterium*, *Algidimarina* and *Caldothrix* (phenol-chloroform), *Pseudomonas tolaasii*, *Staphylococcus* and *Streptococcus* (laboratory).(Fig 4).

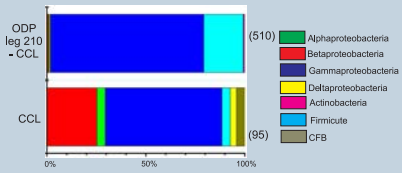


**Fig.4- Bacterial biodiversity based on the relative abundances of clones of each phyla within the contaminant clone library (CCL) and the ODP leg 210 sediments clone library from four depths (OPD leg 210). The phylogenetic affiliation of each clone sequence was determined by similarity analysis of 911-922 bp of 16S rRNA gene sequences and classified with the. Numbers of clones examined at each depth are indicated in parentheses.**

- Majority of sequences of the gene library of 16S rDNA from ODP samples are affiliated to *Proteobacteria* and *Firmicutes* (Fig 4).
- A majority of the 16S rDNA sequences are affiliated to sequences of microorganisms found on plants or in soil.

## CONCLUSIONS

- The the most reliable methode to extarct DNA from low biomass sediment is the FastDNA® Spin kit for Soil
- Majority of contaminants are Beta and Gamma Proteobacteria from the extraction and PCR reagents
- The phylogenetic analysis of clone libraries retrieved from in the deepest (1625 mbsf) and oldest (106 Ma) marine sediments ever studied. Was dominated by sequences of uncultured *Proteobacteria* and *Firmicutes* retrieved from sediment environment. Some sequences were also assigned to new genera.

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***ACKNOWLEDGEMENTS***
*The authors would like to thank Adam Klaus, Texas A&M University, Jean-Claude Sibuet, IFREMER, Plouzané for access to the ODP leg 210 samples and Gordon Webster, University of Cardiff for his advice in molecular biology.*

APPENDIX D

# POSTER 3 – ARCHAEAL METHANE CYCLING COMMUNITIES ASSOCIATED WITH GASSY SUBSURFACE SEDIMENTS OF MARENNES-OLÉRON BAY (FRANCE)

## ISME12; Australia, Cairns, 2008

E.G. Roussel, A.L. Sauvadet, J. Allard, C. Chatudeau, P. Richard, M.A. Cambon-Bonavita and E. Chaumillon.

# Archaeal methane cycling communities associated with gassy subsurface sediments of Marennes-Oléron bay (France)

**Erwan G. Roussel**[1], AL Sauvadet[1], J. Allard[2], C. Chaduteau[3], P. Richard[2], M.A. Cambon Bonavita[1]& E. Chaumillon[2]

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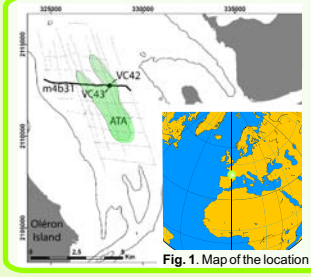
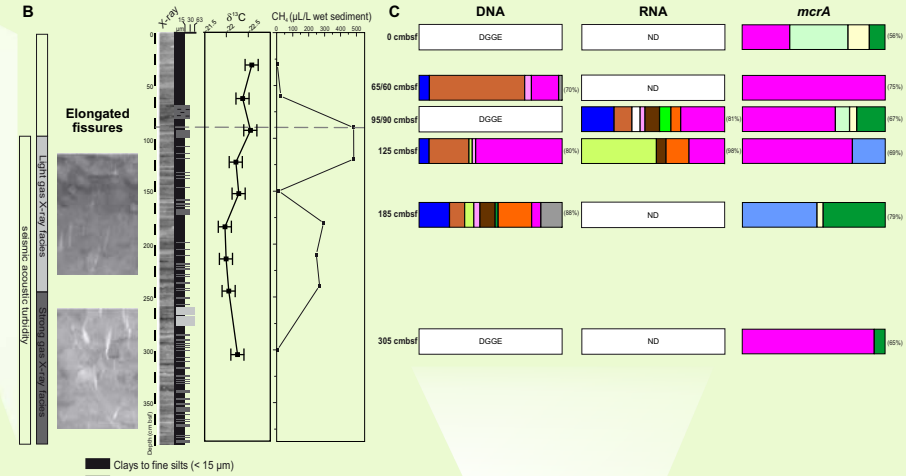



**Fig. 1**. Map of the location of Marennes-Oléron bay in France. Green area: acoustic turbidity.

- Over 85% of $CH_4$ emission, one of the most important greenhouse gases, are biogenically produced by methanogenic *Archaea*, mainly in marine sediments[1].
- Over 30% of the world's shallow-water areas produce methane bubbles[2]. Deltas and estuaries commonly constitute appropriate settings for the generation of shallow biogenic gases, due to the rapid accumulation of sediments with high organic matter concentrations.
- Over 80% of the biogenic $CH_4$ which rises from anoxic marine sediment is consumed by anaerobic methane oxidization microbial communities (ANME).
- Only a few studies have found microbial methane cycling communities in non seep or gas hydrate coastal sediments. Recent studies on Marennes-Oléron bay, a macro-tidal bay located on the French Atlantic coast, have revealed kilometer-scale acoustic turbidity, interpreted as an accumulation of free gas in the sediment.
- The study of coastal gassy sediments submitted to high environmental and anthropic variables, which modify the microbial niches, would help to define the biogeochemical habitats of the deep biosphere communities.
- The present study aims at determining, by an integrated seismic, sedimentologic, biogeochemical and molecular genetic approach, whether active methane cycling microbial communities are involved in the accumulation of gas bubbles in the Marennes-Oléron bay sediments.


**Fig.2. (A)** Interpretation of VHR seismic profile showing the geometry of the sediment infilling of the northern Marennes-Oléron Bay. **(B)** Detailed description of the studied vibra core. From left to right: X-ray image, sedimentary facies, variations in $\delta^{13}C$ and $CH_4$ concentrations. **(C)** Representation of the relative abundance of each archaeal phylotype per clone library based on 16S rRNA gene, crRNA and mcrA gene. 11 different clone libraries (376 sequences) were constructed according to the DGGE patterns in order to select the depths with the most representative archaeal phylogenetic distribution of each core. Percentage of coverage of each clone library is indicated in brackets. DNA designs the DNA-derived libraries; RNA, the RNA-libraries; *mcrA*, the *mcrA* gene libraries.




## High methane concentration in the gassy sediments

- The acoustic turbidity layer was correlated with the highest sedimentation rates of the bay.
- Elongated fissures demonstrated the existence of methane bubbles in the sediment.
- $CH_4$ concentration increased with depth, reaching a maximum peak at the acoustic turbidity upper boundary.
- Relatively large amounts of organic matter (0.83 % C) combined with the highest sedimentation rates of the bay create ideal settings for active methanogenic microbial communities producing high amounts of biogenic methane.

*cmbsf: centimetres below seafloor*

## Archaeal diversity depth distribution

- The archaeal phylogenetic diversity was high, forming a total of 11 different lineages.
- 3 ANME lineages dominated the 16S rRNA (34 %) and the mcrA libraries (74 %).
- ANME-1 depth-dependant distribution tends to support the hypothesis that they are more sensitive to oxygen, to lower temperatures[3] and to high methane flow rates than AMNE-2[4].
- RNA-derived libraries (95 and 125 cmbsf) were also dominated by sequences related to these ANME lineages (36 %), suggesting that these *Archaea* were also metabolically active in depths where the methane concentration was the highest. AOM could represent one of the major archaeal microbial activities in these sediments.
- Sequences affiliated to archaeal methanogenic lineages (*Methanococcoïdes*, *Methanosarcina*, *Methanosaeta* and *Methanomicrobiales*) represented an important component of the total libraries (26 %) .
- The *Crenarchaeota* sequences were related to the Marine Benthic Group B (MBG-B) and Miscellaneous Crenarchaeotic Group (MCG) lineages, representing 35 % of the 16S rRNA clone libraries. The MBG-B *Archaea* were found to be most active where the methane concentration was the highest (95 cmbsf).

## Three potential new lineages with no closely related culture isolates were detected in high methane concentration zones.

- The 1st novel lineage formed an independent cluster named Novel *Methanosarcinales* group 1 (NMG-1) within the methane cycling *Methanosarcinaceae* family, and sequences were closely related to environmental clones from low salinity methane-harboring environment (e.g. estuary sediments).
- The 2nd novel lineage, designed as the Coastal Sediment group-1 (CSG-1), deeply branching within *Euryarchaeota* phylum, was distantly related to the *Methanosarcinales* family (highest similarity to pure culture, 86% with *Methermicoccus shengliensis*).
- The 3rd cluster groups with the VAL III sequences, a novel lineage distantly related to *Methanobacteriales* and *Methanococcales*.
- As all 3 lineages were related to methanogens or environmental clones found in methane-bearing environments, and as 2 were only found active at the highest methane concentrations, we suggest these lineages could be methane cycling *Archaea*.

## Conclusion

- In Marennes-Oléron Bay, the acoustic turbidity upper boundary matched with the highest concentrations of free methane, and was the only section where active *Archaea* were detected, using reverse-transcribed rRNA.
- 16S rRNA and *mcrA* genes clone libraries were dominated by sequences affiliated to methane cycling communities (ANME, *Methanosarcinales*, *Methanomicrobiales* and MBG-B).
- 3 metabolically active novel uncultivated lineages, related to putative methane cycling *Archaea*, seem specifically associated to these methane bearing sediments. The distribution of the specific methane cycling communities (e.g. ANME) in shallow gassy sediments helps to define their biogeochemical habitats.

The authors thank the officers and crew of RV Côtes de la Manche (CNRS/INSU). We are also grateful to Ouest-Génopole for the use of their facilities. E. Roussel was supported by a grant from the Ministère de la Recherche.. Support from the Life, Earth and Environmental Sciences (LESC) of the European Science Foundation made this poster presentation possible (www.esf.org/lesc).

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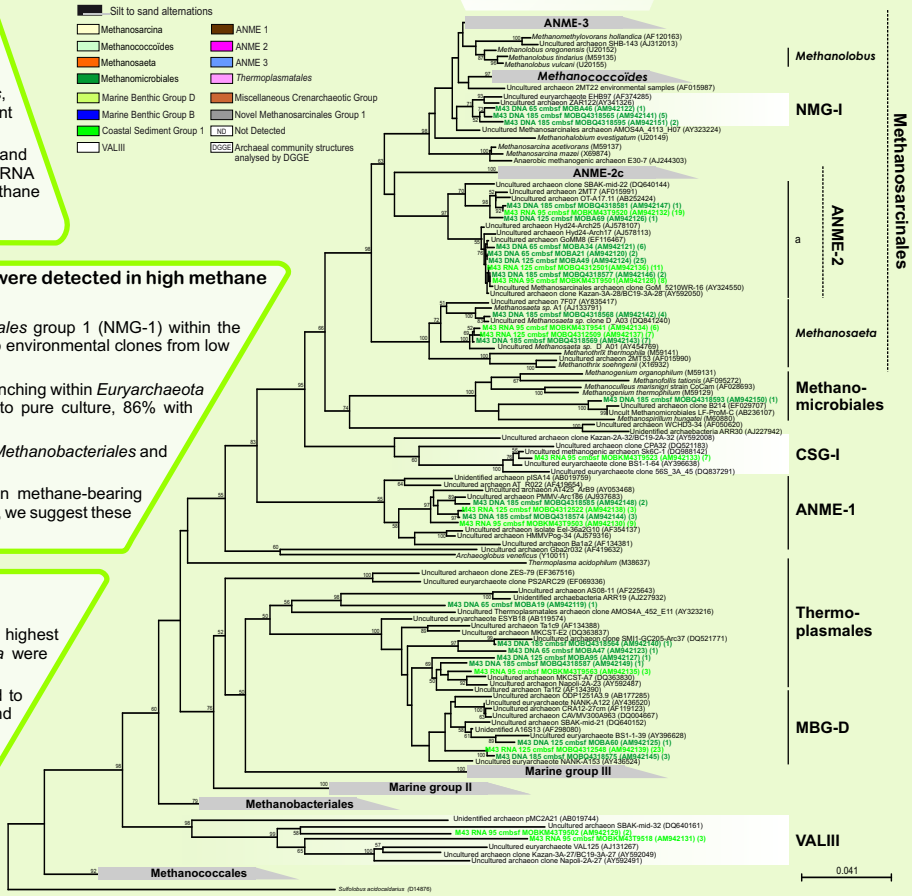


**Fig. 3**. Phylogenetic tree representing the *Euryarchaeota* 16S rRNA gene sequences DNA- and RNA derived. CSG-1: Coastal Sediment Group 1, MBG-D: Marine Benthic Group D, NMG-1: Novel *Methanosarcinales* group 1, VALIII: VAL III cluster

*ISME12, Cairns, Australia*
*08/17/2008 - 08/22/2008*

APPENDIX E

# POSTER 4 – ARCHAEAL COMMUNITIES ASSOCIATED WITH DEEP MARINE SEDIMENTS: FROM THE SURFACE TO A DEEP HOT BIOSPHERE?

## ISME12; Australia, Cairns, 2008

E.G. Roussel, A.L. Sauvadet, R.J. Parkes, C. Chatudeau, B.A. Cragg, G. Webster, Y. Fouquet, J. Querellou, D. Prieur and M.A. Cambon-Bonavita.

# Archaeal communities associated with deep marine sediments: From the surface to a deep hot biosphere ?

**Erwan G. Roussel**[1], A.L. Sauvadet[1], R.J. Parkes[2], C. Chaduteau[3], B.A. Cragg[2], G. Webster[2], Y. Fouquet[2], J. Querellou[1], D. Prieur[1] & M.A. Cambon Bonavita[1]

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- The sub-seafloor biosphere may contain 2/3 of Earth's total prokaryotic biomass[1], and extends to at least 1600 mbsf[2].The large and active microbial populations buried in these sediments play a key role in global biogeochemical[3] cycles. However, little is known about these prokaryotic communities.
- The prokaryotic cell density decreases with depth. However, local increases can occur in response to the adaptation of microbial communities to specific geochemical environments[3,4].
- The boundary between surface and subsurface could be defined as a change of the microbial community composition, shifting from surface (e.g. the water column) to deep subsurface communities.
- The aim of the present study was to compare the archaeal diversity distribution using DGGE, 16S rRNA gene clone libraries and functionl genes from surface to subsurface sediments with different biogeochemical habitats. Analyses were performed on piston core sediments in a range of 0 to 34.5 mbsf (~2500 m water depth) from the New-Caledonia Basin (ZoNéCo 12 cruise) **(Fig. 1)**. Archaeal indicators of deep hot subseafloor ecosystems were also investigated, as some sites were characterized by a strongly faulted cold seafloor (3028), associated with deep vertical fluid migration.
- The active fraction of the archaeal community in deep-sea sediments was assessed by a new molecular approach: Co-migration denaturant gradient gel electrophoresis (CM-DGGE).

mbsf: meters below the seafloor, DGGE: Denaturant Gel Gradient Electrophoresis

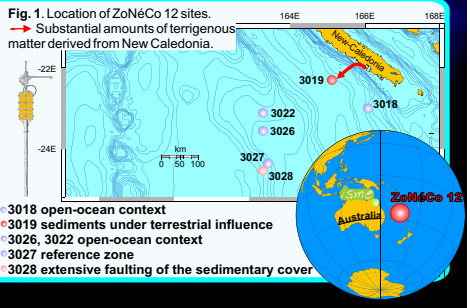


**Fig. 1**. Location of ZoNéCo 12 sites. → Substantial amounts of terrigenous matter derived from New Caledonia.

## Community structure and diversity depth distributions

- Prokaryotic cell counts (AODC) were within the limits of the Parke's regression equation. Prokaryotic abundances at the open-ocean sites (3022, 3026 and 3028) were ~3.5 times lower than at the site under terrestrial influence (3019).
- The dendrograms of DGGE patterns displayed 2 major clusters **(Fig. 2)**. Cluster A: from 0 to 1.5 mbsf, exclusively affiliated to MG-1. **Cluster B**: below 1.5 mbsf, 6 phylotypes (Rice cluster V, *Thermoplasmatales*, MBG-D, SAGMEG, unclassified *Euryarchaeota*) were detected.
- The sediment-derived archaeal clone library diversity clearly differs from the seawater. Euryarchaeal lineages represented less than 1% in the clone libraries from surface sediments (0 to 1.5 mbsf), whereas below they represent 59% **(Fig. 3)**.

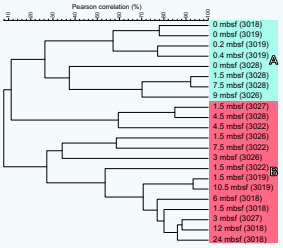


**Fig. 2.** Cluster analysis of PCR-DGGE band patterns of archaeal 16S rRNA genes of cores from different sites, using primers: Saf-PARCH 519r.

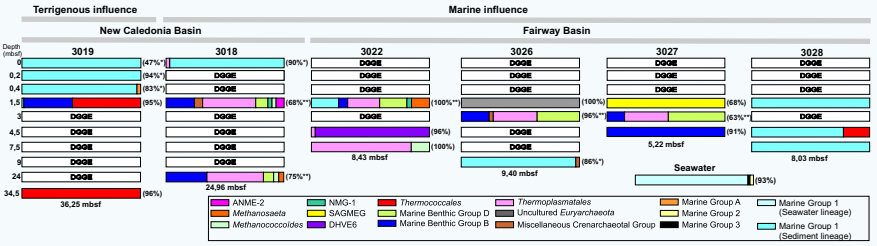


**Fig. 3.** Representation of the relative abundance of each archaeal phylotype per clone library based on 16S rRNA gene. 25 different clone libraries (771 sequences) were constructed according to the DGGE paterns in order to select the depths with the most representative archaeal phylogenetic distribution of each core.Percentage of coverage of each clone library is indicated in brackets. (*) indicate groups of clone libraries with insignificant ($P < 0.01$) differences between all the diversity indices ($F_{ST}$ and the exact test method).

- *Euryarchaeota* phylogenetic diversity was high. Sequences related to the MBG-B and ubiquitous *Thermoplasmatales* and MBG-D lineages were found in 44% of the libraries below 1.5 mbsf **(Fig.3)**. MBG-B sequences, *Crenarchaeota* possibly linked to anaerobic methane oxidization (AOM)[5].
- Interestingly, sequences related to methane cycling ANME-2 and a Novel *Methanosarcinales* group 1 (NMG-1)[6], a new phylotype in the *Methanosarcinaceae* family, were also detected. These sequences grouped with environmental clones from a sulfide rich spring and from estuary sediments **(Fig. 3, and ISME poster 188)**.
- Archaeal amplifiable DNA was obtained from all depths, whereas amplifiable RNA was only obtained above 1.5 mbsf and was not detected at any other depth, suggesting that *Archaea* communities were most active between 0 to 1.5 mbsf **(Fig. 4 and 6)**.

## The metabolically active surface archaeal community

- *Marine Group 1* (MG-1), ubiquitous putative ammonia-oxidizing *Archaea* in marine environments, dominated all the clone libraries and DGGE community structures above 1.5 mbsf **(Fig. 2 and 3)**.
- The active fraction of the archaeal community (RNA-derived) was compared to the DNA-derived archaeal community by Co-Migration DGGE, a new molecular approach based on DGGE. We showed that the archaeal communities were most active between 0 to 1.5 mbsf, as no complementary ribosomal DNA (crDNA) was detected below 1.5 mbsf **(Fig. 4 and 6)** and was dominated by the MG-1.
- *amoA* encodes the enzyme responsible for catalyzing ammonia oxidation. Archaeal *amoA* gene sequences related to MG-1 were detected, demonstrating that active ammonia-oxidizing *Archaea* dominated the suboxic surface archaeal communities **(Fig. 5)**.

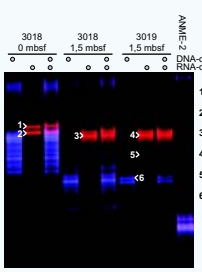


**Fig. 4.** CM-DGGE analysis of archaeal 16S rRNA genes DNA-derived and RNA-derived from sites 3018 and 3019. PCR products were amplified with the Saf-PARCH 519r*Cy5 or Saf-PARCH 519r*Cy3 primer set.

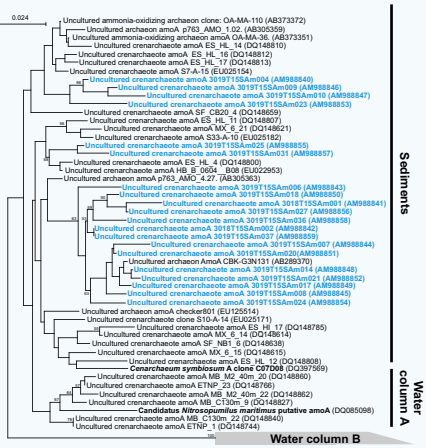


**Fig. 5.** Phylogenetic tree based on translated, partial amino acid sequences of *amoA* gene.

## To the deep subsurface...

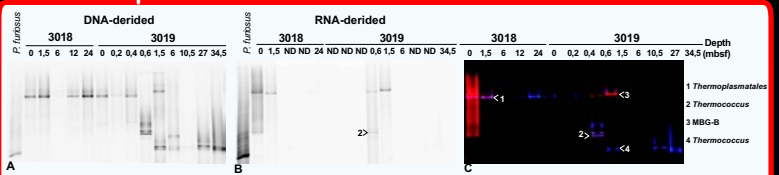


**Fig. 6.** CM-DGGE analysis of archaeal 16S rRNA genes DNA-derived and RNA-derived of the cores from sites 3018 and 3019. **(A)** DNA-derived (A344fGC-A915r*Cy5). **(B)** RNA-derived (A344fGC-A915r*Cy3). **(C)** both DNA- and RNA-derived.

- At site 3019 below 1.5 mbsf, *Thermococcales*, a putative (hyper)thermophylic *Euryarchaeota*, was the unique lineage detected by DGGE and clone library analysis **(Fig. 3 and 6)**. These sequences formed a unique cluster within the genera *Thermococcus* (TG-1), suggesting a same origin **(Fig. 7)**.
- A crDNA fragment related to the *Thermococcales* order was detected only at site 3019 at 0.6 mbsf, indicating the presence of living *Thermococcales* **(Fig. 6)**. Their distribution was correlated with the occurrence of authigenic minerals, such as aragonite.
- The 3019 site shows no signs of fluid migration and is located in a canyon deriving terrigenous matter from New Caledonia. An active alkaline hydrothermal field was revealed in the south-west lagoon of New Caledonia **(Fig. 1)**.
- A second cluster within the *Thermococcales* comprised sequences detected at the strongly faulted site (3028). These sequences grouped in a new cluster with sequences from deep hot subseafloor sediments (1626 mbsf)[2] **(Fig. 7)**.

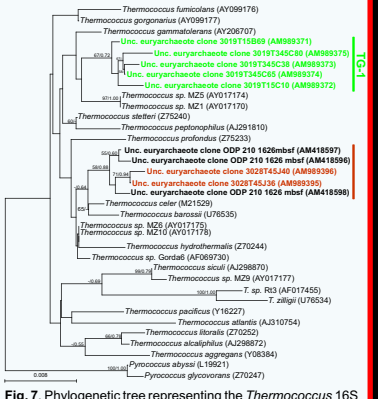


**Fig. 7.** Phylogenetic tree representing the *Thermococcus* 16S rRNA gene sequences. Tree topology was inferred by neighbor-joining analysis. Bootstrap support values over 50% (1,000 replicates) and bayesian posterior probabilities are indicated at nodes.

## Conclusion

- This study shows the transition between active surface archaeal communities, a component of which is ammonium oxidizers, and probably of less active deep typical sub-seafloor lineages.
- The occurrence of living putative thermophiles in cold marine sediments suggests that the dispersion of the hydrothermal archaeal communities could either occur by rafting through the surface seawater from New-Caledonia on biotic or terrigenous substrates or by fluid migration through the sub-seafloor.

The authors are deeply indebted to Yvon Balut, chief of the operation on board the R.V. Marion Dufresne and to the I.P.E.V. (Institut Paul Emile Victor). We also thank Jean-Paul Foucher, chief scientist of ZoNéCo 12. All of the crew and the Scientific Party were crucial in this effort, especially Laetitia Hervé for the sampling efforts. We are also grateful to Ouest-Génopole for the use of their facilities. Support from the Life, Earth and Environmental Sciences (LESC) of the European Science Foundation made this poster presentation possible (www.esf.org/lesc).

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# INDEX

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# Résumé

La biosphère de sub-surface océanique représente les deux tiers de la biomasse de la Terre. Ces importantes populations microbiennes enfouies sous la surface du plancher océanique jouent un rôle clé dans les cycles biogéochimiques. Bien que la profondeur maximale de cette biosphère de sub-surface ne soit toujours pas déterminée actuellement, celle-ci serait limitée par des facteurs physico-chimiques tels que la température ou l'absence de source d'énergie. Certains microorganismes non-cultivés du domaine des *Archaea* jouent probablement un rôle majeur dans l'écosystème sédimentaire marin profond et hydrothermal.

Afin de contourner les limitations des approches culturales, les outils moléculaires sont principalement utilisés pour l'étude des communautés microbiennes de la biosphère profonde. Ainsi, dans le but de déterminer les facteurs environnementaux contrôlant la distribution des *Archaea* dans la biosphère profonde océanique, ce travail de recherche a comparé la diversité moléculaire (ARNr 16S et les gènes codant pour des enzymes spécifiques de différentes voies métaboliques) associée à quatre écosystèmes différents de sub-surface : les sédiments marins profonds côtiers, océaniques, ceux des marges continentales, et l'environnement hydrothermal.

Ce travail de recherche a permis (i) de caractériser la distribution des communautés d'*Archaea* dans différents contextes biogéochimiques, (ii) de mettre en évidence de nouveaux phylotypes, (iii) de proposer une nouvelle définition de la sub-surface profonde, (iv) d'étendre la profondeur de la biosphère marine profonde jusqu'à au moins 1626 m sous la surface du sédiment. Ainsi, cette étude montre que la distribution des différentes communautés d'*Archaea* de la surface jusqu'à la sub-surface est liée aux différentes conditions physico-chimiques. De plus, dans des environnements présentant une température plus élevée, des thermophiles putatifs pourraient être des représentants d'une biosphère profonde et chaude.

**Mots Clés:** Biosphère profonde; *Archaea*; *Bacteria*; 16SrRNA; *amoA*; *mcrA*; *dsrA*; *pmoA*; DGGE; CM-DGGE; ultramafique; écosystème hydrothermal; sédiment.

# Abstract

The sub-seafloor biosphere may contain two thirds of Earth's total prokaryotic biomass. The large and active microbial populations buried in the sub-seafloor play a key role in global biogeochemical cycles. However, little is known about these prokaryotic communities. The depth limit of this sub-seafloor biosphere is still unreached, and elevated temperatures as well as insufficient energy sources are the likely factors limiting life at depth. Interestingly, archaeal communities with unknown physiologies and no cultured relatives seem to have a key role in deep marine sediments and hydrothermal ecosystems.

As most deep biosphere microorganisms detected so far have been extremely resistant to cultivation, molecular approaches remain to date the most appropriate tools for the analysis of the overall prokaryotic communities in sub-surface environments. In order to determine the main environmental factor driving the archaeal diversity in the sub-seafloor, the present study is mainly focused on the comparison of the 16S rRNA (RNA- or DNA- derived) and functional gene diversity associated with four different sub-seafloor ecosystems: deep coastal, margin and open-ocean sediments, and hydrothermal environment.

The present study (i) shows the distribution of the archaeal communities within different biogeochemical habitats, (ii) reveals new phylogenetic lineages, (iii) proposes a different definition of the deep sub-surface, (iii) expands the known sub-seafloor biosphere to at least 1,626 meters below seafloor. The archaeal lineages seem to be specifically distributed from the surface to the deep sub-subsurface according to the physical and chemical conditions. In higher temperature environments, such as very deep sub-surface sediments, or in hydrothermal environments, putative (hyper)thermophilic *Archaea* may represent indicators of a deep hot biosphere.

**Keywords:** Deep biosphere; *Archaea*; *Bacteria*; 16SrRNA; *amoA*; *mcrA*; *dsrA*; *pmoA*; DGGE; CM-DGGE; ultramafic; hydrothermal system; sediment.