Marine sediments are the major reservoir of methane on earth, where it is produced by organic matter mineralization and water-rock interactions. Despite a gross production of ~400 Tg per year of this potent greenhouse gas, sediments only contribute to a very minor part of the atmospheric methane pool. Microorganisms mediating the Anaerobic Oxidation of Methane (or AOM) coupled to sulphate reduction indeed retains over 80% of this methane flux and are thus major players in sediment carbon and sulphur cycles, and climate control.

During this doctoral research, I studied the links between the activity and community structure of marine sediments microorganisms and their environment in order to better understand the functioning of these microbial ecosystems.
A JULIE, LOUISE, ET AVA.

UN CLIN D’OEIL DE DARWIN
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MICROBIAL ECOLOGY OF CARBON AND SULPHUR CYCLES IN DEEP-SEA CARBONATE MOUNDS AND MUD VOLCANOES

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences.
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Reading this, one may think that this thesis is the result of a collective work. Well, it truly is!

Loïs, September 2011.
“The value of having for a time rigorously pursued a rigorous science does not rest especially in its results: for in relation to the sea of worthy knowledge, these will be but a negligible little drop. But it brings forth an increase of energy, of deductive ability, of persistence; one has learned to gain one's purpose purposefully. To this extent, in respect to all one does later, it is very valuable to have once been a scientific man.”

F. NIETSZCHE

*Human, all too human.*

A book for free Spitsits
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CURRICULUM VITAE
List of abbreviations

ANME   Annaerobic Methanotroph
AOM(R)  Anaerobic Oxidation of Methane (Rates)
AVS    Acid Volatile Sulphide
BSR    Bottom Simulating Reflectors
CARD-FISH Catalyzed Reporter Deposition – Fluorescence In Situ Hybridization
cm bsf centimeter below the seafloor
Gt Giga Ton
GoC Gulf of Cadiz
GoM Gulf of Mexico
IODP Integrated Ocean Drilling Program
m bsf meter below the sea floor
m bsl meter below sea level
MG Methanogenesis
    H-MG Hydrogenotrophic Methanogenesis
    Ac-MG Acetotrophic Methanogenesis
    Meth-MG Methylotrophic Methanogenesis
MV Mud Volcano
    CAMV Captain Arutyunov Mud Volcano
    CRMV Carlos Ribeiro Mud Volcano
    HMMV Håkon Mosby Mud Volcano
R/V Research Vessel
ROV Remotely Operated Vehicle
SIMS Secondary Ion Mass Spectrometry
SMTZ Sulphate Methane Transition Zone
SRB Sulphate reducing Bacteria
SR(R) Sulphate Reduction (Rates)
TRS Total Reducible Sulphur

Cruises

MD04  R/V Marion Dufresne  2004 Gulf of Cadiz
MSM1/3 R/V Maria S. Merian  2006 Gulf of Cadiz
JC10  R/V James Cook  2007 Gulf of Cadiz
MD08  R/V Marion Dufresne  2008 Gulf of Cadiz
TTR   R/V Prof. Logachev Training Through Research Cruises
A large fraction of oceanic and continental organic matter is buried in marine sediments, where it is degraded by the action of microorganisms or by heating along the geothermal gradient. Methane is the major end product of this process, and \( \sim 10^7 \) Tg of this hydrocarbon are stored in marine sediments, either in fossil reservoirs or enganged in ice-like solid gas hydrates. Potentially, marine sediments thus constitute a vast source of this greenhouse gas, which as a more than 20-fold higher radiative forcing effect than the one of CO\(_2\) on a 100 years basis. However, methane is retained in the sediment during its migration toward the sediment surface by the microbially mediated anaerobic oxidation of methane (AOM). This reaction uses the seawater sulphate diffusing in the sediment to oxidise methane into carbonate before it reaches the ocean water. Hence AOM is a key reaction for climate control as it considerably mitigates methane content in the atmosphere. Marine sediments, despite being the main methane reservoir on earth, only accounts for 2\% of the global methane emissions, i.e. less than termite gut, ruminant enteric fermentation or rice paddies emissions.

Further, in areas of intense upward flux, methane promotes the development of thriving ecosystems on the seafloor that only rely on this chemical energy rather than on direct sunlight and photosynthetic primary production. These ecosystems sustain a high diversity of micro-, meio- and macrofauna that sharply contrasts with typical deep-sea environments. By controlling the methane flux and releasing sulphide as a by-product of the associated sulphate reduction, AOM is at the base of these ecosystems and exert a strong ecological control on the rest of these chemosynthetic communities.
SUMMARY

Hence, microorganisms mediating AOM have a central place at the interface between different biological, geological and geochemical processes, all influencing their activity and distribution. The study of their ecology and biogeography is thus of importance to understand global geochemical cycles and anticipate the effect of physical and chemical changes in the oceans.

Surprisingly, despite the wide distribution of AOM and the complexity of these processes, only three closely related Archaea have been identified as anaerobic methanotrophs (ANME), acting in concert with a few groups of sulphate reducing bacteria (SRB). Despite the seemingly simple biochemical reaction carried out by this microorganisms, and the considerable scientific efforts dedicated to this process, the AOM biochemical mechanism remains unknown and the microorganisms involved have resisted repeated isolation attempts that would have allowed the elucidation of their functioning. In this context, the study of these microorganisms in their natural environments remains a very efficient study approach.

The aim of the present work is thus to gain a better knowledge of the ecology, activity and biogeochemical role of these microorganisms. In this work, an integrated microbial ecosystem functioning approach was used to elucidate the different feedback mechanisms between environmental parameters, microbial activity and community structure. Natural AOM hot spot ecosystems on the seafloor such as mud volcanoes (MV) displaying a wide variety of habitat characteristics were used as natural laboratories. This work has been undertaken during three European scientific expeditions in the Gulf of Cadiz, between the southern Portuguese and Spanish margins, and the western Moroccan margin. This zone of broad methane migration toward the seafloor encompasses a wide diversity of geological, oceanographic and geochemical conditions, setting up a large array of habitats suitable for comparative studies.

Marine carbonate mounds represent the geological fingerprint of a widespread strategy of life through earth history. Modern carbonate mounds built by cold water coral represent conspicuous examples of such strategy. In the first part of this work, we describe a new type of cold-water coral carbonate mounds, where methane migration strongly overprints the geochemistry and diagenetic processes of these sediments. AOM activity in the Alpha mound has a significant impact on the sulphur cycle, on mound’s dissolved inorganic carbon (DIC) budget and isotopic signature. Further, this reaction has the potential to modify the carbonate chemistry and alter the coral preservation state in the subsurface. This study highlights the
importance of diagenetic processes that followed the mound’s formation, but that were not related to the biology of corals, the main mound builders.

The second part of this thesis shows that carbonate production during AOM can lead to the formation of massive carbonate hardgrounds in the crater of the Darwin MV. In turn, the self-sealing of these sediments by carbonate induced the relocation of the geofluid flux at the rim of the carbonate crust. Methane was apparently channelled through discrete pathways, as a very heterogeneous AOM activity distribution along the rim has been observed. As a result, both zones of very high and no activity were found. The former exhibited the highest methane turnover measured thus far in the Gulf of Cadiz. There, AOM was mediated by consortia of ANME-2 and DSS sulphate reducers that were consistently forming shell type aggregates of ANME cells surrounded by DSS cells. Based on our observations and on the current knowledge on methane-derived carbonate, a development model for this original MV is proposed.

The third part of this thesis focuses on the AOM microbial ecology under hypersaline conditions. Density driven faulting of the sediments by salt masses (i.e. salt diapirs) often occurs and provides an important escape pathway for over-pressurised fluid. Very little is known about the methane turnover in these conditions and AOM inhibition due to hypersalinity can potentially release large amounts of methane in the ocean. This study of the hypersaline Mercator MV shows that, despite the very low energetic yield of AOM, methane consumption occurred at salinity up to halite saturation (5.8 M NaCl), i.e. about ten times seawater salinity. Further, hypersaline conditions exerted a strong pressure toward ANME-1 cells, and no other ANME cells where present. The comigration of evaporite-derived sulphate together with methane was a distinct feature in this habitat and promoted AOM activity through extended depth. This was contrasting with the standard salinity Captain Arutyunov MV where AOM occurred at the discrete interface between methane migrating from below and sulphate diffusing from seawater. Overall, these results are challenging our current view of hypersaline conditions as a strong selection factor toward high-energy yield metabolisms.

In a companion paper, geochemical modelling permitted to constrain the methane flux at different sites of the Carlos Ribeiro MV, in the deep part of the Gulf of Cadiz. AOM and SR turnover rates were measured after recovery of the sediments at the same locations. These yielded turnover estimates that where at least an order lower than the previously estimated methane and sulphate fluxes. This underestimation of in situ activity of microorganisms may be
attributed to the difference of methane solubility between surface and in situ hydrostatic pressure. Rates calculated with realistic in situ methane concentrations were indeed similar to the geochemical model outputs. In these sediments, AOM was mostly mediated by ANME-2 and -3 cells, but the dominance of cells from the GoM Arc I group in the AOM zone suggested that these may also play a role in AOM.

This work shows that AOM mediating microorganisms can thrive in a wide range of habitats where they efficiently retain methane carbon in the sediments. Despite the low energy that can be conserved from AOM for maintenance metabolism and biomass production, ANME cells have the ability to face highly energy-demanding conditions such as hypersalinity, nitrogen fixation, formation of dense aggregates and a probable syntrophic metabolism with SRB. Hence, these organisms are remarkably adapted to environments characterised by energy stress. In low redox gradients that are close to the thermodynamic equilibrium, the presence of available energy strongly depends on the relative concentration of substrates and products. The ability to revert metabolic pathways according to environmental conditions, as proposed for AOM, may thus constitute a distinct evolutionary feature of subsurface microorganisms.
CHAPTER I. General

Introduction
1. Structure and properties of methane

Methane is the simplest hydrocarbon molecule, composed of one carbon covalently bound to four hydrogen atoms. Its molecular weight is of 16 g mol$^{-1}$. The melting point of methane is -183 °C and its boiling point is at -164 °C under standard conditions (273K, 1 bar). The solubility of methane in seawater at 1 bar is only ~1.4 mM, and increases with pressure and thus with water depth (Figure I-1). Methane solubility also strongly decreases with salinity (Figure I-1).

Methane has the capacity to form gas hydrate, i.e. methane molecules encaged in a water lattice (Figure I-2). Pressure/Temperature conditions of hydrate stability match an important part of ocean subseafoor, and hydrates are thought to constitute an enormous reservoir of organic carbon in marine sediments. The presence of hydrates in marine sediments can be detected by seismic survey, as the interface between the lower hydrate layer and free gas in sediment typically yield a bottom simulating reflectors or BSR in seismographs.

![Figure I-1 Maximum solubility of methane in seawater as a function of depth at temperatures (4 and 13°C) relevant for the studies presented in this thesis (left), or as a function of salinity (right). Note the difference of scale between methane solubility at surface and at 350 m water depth (the latter is the depth of the hypersaline MV studied in Chapter IV). Data from thermodynamic models of (Duan and Mao, 2006)](image)

Methane is one of the most reduced organic carbon compounds, due to the four C-H bonds with the C atom being in the –IV redox state, and the redox potential of the CO$_2$ / CH$_4$ couple being of $E_0 = -240$ mV (Thauer et al., 1977). In addition, methane has a high energy of
activation (434 kJ mol$^{-1}$ for the first C-H bound) and its oxidation thus requires the action of oxygen radical chemistry (Strous and Jetten, 2004, and references therein) or specific enzyme cofactors. Finally, methane is a potent greenhouse gas in the atmosphere and is over 20 times more effective than CO$_2$ in trapping radiative energy (IPCC, 2007) on a 100 year basis.
2. Methane reservoirs and fluxes

2.1 Marine sediments and global methane budget

Sediment methane and hydrates constitute the major methane pool on earth, with an average estimated mass of between 500 and 10,000 Gt of methane carbon (Figure I-3). Most recent estimates indicate a distribution of ~3000 Gt as hydrates and ~2000 Gt as gas bubbles. (Reeburgh, 2007, and references therein). In comparison, the methane hydrate carbon pool present in permafrost soils or shallow sediments -the second major hydrate reservoir- is much lower, with estimates of ~ 400 Gt (Macdonald, 1990b). Such reservoirs are not yet commercially exploited but there is a strong ongoing research on the extraction methods that could harvest methane from hydrates fields (Milkov and Sassen, 2002).

The greenhouse effect of methane, together with the hydrate reservoir sensitivity to ocean temperature, are of concern for climate change studies as a small bottom water temperature increase could cause the destabilisation of hydrates, liberate free methane that could potentially further contribute to global warming. Hence, the release of a small fraction of the methane from the hydrate pool could have a considerable effect on the atmospheric methane, which is only of 4 Gt C (Dlugokencky et al., 1998). Marine and permafrost are likely to respond differently to global temperature increase: marine hydrates can be close to sediment surface and thus immediately affected by slight temperature increase. In addition, Dickens (2001) estimated that an increase of 5 ºC of ocean water would divide by a factor 2 the extent of the deep-sea hydrate stability zone. Due to the depth of hydrate stability zones in permafrosts, these are less likely to respond quickly to surface temperature changes, although permafrost thawing and warmer water incursion could accelerate the hydrate dissociation process. Hence, the understanding of possible source and sinks of methane are of considerable importance to produce relevant climate change scenarios.

2.2 Methane sources in marine sediments

In marine sediments, methane originates from three different sources: abiotic, abiogenic and biogenic. Abiotic methane is the result of seawater interaction with ultramafic rocks in hydrothermal systems at mid oceanic ridges. There, olivine ((Fe,Mg)2SiO4) in recently erupted
rocks is transformed to serpentine ($\text{Mg}_3\text{Si}_2\text{O}_5(\text{OH})_4$) and $\text{H}_2$ is released during the Fe(II) oxidation to Fe(III) (Charlou et al., 1998). Methane is apparently formed, from dissolved $\text{CO}_2$ and $\text{H}_2$, by the Fisher–Tropsch reaction at $T>300$ ºC and $P>500$ bar in the presence of metal catalysts. Such mechanisms are thought to produce the methane found in hydrothermal fluid such as in Lost City vent fields in the mid Atlantic ridge (Boetius, 2005).

Abiogenic methane is produced by the abiotic alteration of biologically produced organic matter. In sediments, abiogenic methane mainly results from the heating of buried organic matter along the geothermal gradient. This thermogenic methane is typically formed in subductive convergent margins where thick sediment layers are progressively heated in contact with the earth mantle.

Biogenic methane is exclusively produced by the archaeal group of methanogens. The low potential of the $\text{CH}_4/\text{CO}_2$ redox couple indicates that the biological formation of methane occur in reducing environment, and consistently, methanogens are generally strict anaerobes. Methanogens can use $\text{H}_2 + \text{CO}_2$, acetate or methyl groups of compounds such as methylamines, methylsulphides, and methanol as substrates. The biological formation of methane is thus the terminal step in the degradation of organic matter after the cleavage of macromolecules to monomers and the monomer fermentation resulting in $\text{CO}_2$, $\text{H}_2$, and volatile fatty acids. In marine sediments, the depth distribution of terminal electron acceptors involved in organic matter oxidation mirrors the decreasing energetic yield of the redox reactions involved. Hence, most favourable acceptors such as $\text{O}_2$, $\text{NO}_3^-$, Mn(IV), Fe(III) and $\text{SO}_4^{2-}$ are consumed in this order in the upper sediment layers, and methanogenesis using the least favourable electron acceptor $\text{CO}_2$, is mostly restricted to the sulphate free environments (Figure I-4).

Unlike in the atmosphere where it is readily oxidised by hydroxyl radicals deriving from the ozone photodissociation (Logan et al., 1981), biological processes are the main sinks of methane in soil, sediments and oceans. In presence of oxygen, methane is oxidized by methanotrophic bacteria possessing the methane monooxygenase. This enzyme involves oxygen radical chemistry for the activation of methane and the cleavage of the first C-H bond. The oxidation of methane with nitrite has been recently discovered and involves similar mechanisms, since some bacteria are able to generate oxygen from nitrite disproportionation. In anaerobic conditions, and in the absence of nitrite, the activation mechanism of methane is not yet understood but probably involves metal catalytic properties of the Ni(I)-containing F430 cofactors present in methanogens and anaerobic methanotrophs (Kruger et al., 2003; Mayr et al., 2008; Scheller et al., 2011).

Methane produced in the deep sediment strata migrates toward the surface and penetrates in the gradient of electron acceptors that are diffusing downward from seawater (Figure I-4). The anaerobic oxidation of methane (AOM) coupled to sulphate reduction (SR) is the major sink of methane in marine sediments. This microbially mediated reaction consumes ca. 382 Tg of methane per year, which correspond to ca. 80-90% of the global methane gross production from this environment (Reeburgh, 2007, and references therein), thus reducing the net emission by a factor 5 to 10.

Methane can also be oxidized aerobically in the sediments. However, the rapid consumption of oxygen by heterotrophic microorganisms in the first centimetres of sediment restricts the niche of aerobic methanotrophic bacteria to surface sediments and oxygenated water column. Recently, two additional mechanisms have been proposed for the anaerobic oxidation of methane. (Beal et al., 2009) have shown that Mn(IV) and Fe(III) could be used as terminal electron acceptor. In addition, it has been demonstrated that nitrite can oxidize methane through a novel oxygenic pathway (Raghoebarsing et al., 2006; Ettwig et al., 2008; Ettwig et al., 2009; Ettwig et al., 2010). However, the significance of these pathways is not yet fully understood. Concentration of nitrate, the precursor of nitrite, is very low in seawater (3 orders lower than sulphate), and the presence of oxidized metal ions in anoxic sediments is possible (D'hondt et al., 2004; Parkes et al., 2005) but has only been observed in specific geological settings so far. Hence, due to the relatively small pool of oxidized N, Fe and Mn species, these pathways could...
only play a minor role in the ocean methane budget. These different mechanisms are apparently efficient methane sinks, as it is estimated that the net contribution of ocean to the atmospheric methane only represents <2% (Reeburgh, 2007).
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Figure I-2 Left: molecular representation of methane hydrate, i.e. a methane molecule (black/green) encaged in a water lattice (red/white) (reproduced from the ecosystems course curriculum Texas A&M university). Right: The domain of hydrate stability is illustrated in function of temperature and water depth instead of pressure (reproduced from U.S. Nat. Energy Tech. Lab.). Here, a water depth of 1200 m is assumed, but theoretically methane hydrate can start to form below 300 m water depth.

Figure I-3: Summary of the global methane reservoirs, fluxes and turnover (adapted from Prof. Reeburgh personal page at the University of California, Irvine www.ess.uci.edu/~reeburgh/fig9.html and (Reeburgh, 2007). Despite that marine sediments are the principal methane reservoir, they only contribute to ~1% to the annual methane flux toward the atmosphere.
Figure I-4 In marine sediments, the vertical stratification of redox reactions involved in organic matter oxidation follows the decreasing energy yield of these reactions. CO$_2$ is the less favourable electron acceptor and is used below the sulphate reduction zone. (reproduced from Fenchel and Jørgensen, 1977)
4. **Anaerobic oxidation of methane: biogeochemistry and ecology**

4.1 Stoichiometry and energetic yield

The AOM reaction couples the reaction of methane oxidation to sulphate reduction according to the following stoichiometry:

$$\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HS}^- + \text{HCO}_3^- + \text{H}_2\text{O} \quad \text{Equation (1)}$$

The free energy that can be harvested by microorganisms from this redox reaction is $\Delta G^o = -16.9 \text{ kJ mol}^{-1}$ in standard conditions, but it is estimated that this yield can reach ~40 kJ mol$^{-1}$ *in situ*. In the particular case of syntrophy between a sulphate reducing Bacteria (SRB) and an anaerobic methanotrophs (ANME) Archaea, the available energy has to be shared between both partners. These values are very close to the minimum energy yield requirement to ensure ATP production and maintenance metabolism (4 to 10 kJ mol$^{-1}$) (Alperin and Hoehler, 2009). Such low-energy way of life has major implications for the ecology of AOM mediating communities. These microorganisms have a very slow growth rate of 3 to 7 month (Girguis et al., 2005; Nauhaus et al., 2005). This, together with the apparent syntrophic nature of AOM hindered successful isolation attempts, making physiological studies difficult. Secondly, such a low yield implies that the pathways involved can be extremely dependent on the substrate and product *in situ* concentrations, i.e. the electron flow through dissimilatory energy conservation pathway can be reverted in function of the available electron sources and sinks. (See “methanogenic activity of AOM organisms section” below)

4.2 Geochemistry:

In most sedimentary settings, AOM mediating microorganisms are active in a narrow depth interval, where both gradients of downward diffusing sulphate from seawater, and upward migrating methane overlap. Hence, the presence of a sulphate to methane transition zone (SMTZ) is typical for AOM activity. The depth and extend of this zone is primarily governed by the flux of methane (Borowski, 1996). In diffusion-controlled environments, the SMTZ can be located at hundreds of meter below the seafloor, and can spread over ten’s of meters (see for instance Webster et al., 2009). In high fluid advection environments and high methane flux, the gradients are very steep and the SMTZ is restricted to a few cm immediately
below the sediment surface (see for instance Niemann et al., 2006a; Niemann et al., 2006b). Eventually, when advective fluid flux is too high to allow downward sulphate diffusion, methane freely escapes the sediment and is partly oxidized by aerobic methanotrophs at the surface (Niemann et al., 2006a).

### 4.3 Microorganisms mediating AOM

Three groups of Archaea have been shown to mediate the anaerobic oxidation of methane (ANME-1, -2 and -3). These ANME cells all belong to the phylum Euryarchaeota and are closely related to methanogens. Based on the observation of tight consortia of ANME and SRB using in situ fluorescence labelling (FISH) (Boetius et al., 2000), SRB are thought to function in syntrophy with ANME, by carrying the sulphate reduction half-reaction in AOM. SRB associated with ANME cells are related to *Desulfosarcina* Deltaproteobacteria (ANME-1 and -2 Knittel et al., 2003; Knittel et al., 2005; Schreiber et al., 2010) or *Desulfobulbus* (ANME-3 Losekann et al., 2007). However, (Pernthaler et al., 2008) have shown that other bacterial clades could be occasionally involved in consortia with ANME-2 cells.

### 4.4 Mechanisms.

The biochemical mechanisms involved in AOM is not yet elucidated, highlighting the continuous need of environmental studies that can bring additional knowledge on the functioning of these communities. Metagenomic studies have shown that ANME cells posses all the genes (but *mcrA*) involved in the sequential reduction of CO$_2$ to CH$_4$ (Hallam et al., 2004; Meyerdierks et al., 2010) and are thus thought to mediate AOM through reverse methanogenesis (Hallam et al., 2004; Thauer, 2011). Besides, inhibition experiments have demonstrated the obligate syntrophic nature of AOM with sulphate reduction, as none of the two reactions proceeds further when the other one is inhibited (Alperin and Reeburgh, 1985; Hoehler et al., 1994; Nauhaus et al., 2002). However, neither the metabolic link between AOM and SR reactions, nor the physical link between ANME and SRB living in aggregates, has been elucidated. Hypothetically, the diffusion of a chemical carrier would transfer electrons resulting from methane oxidation in ANME cells to SRB cells where they would be involved in sulphate reduction. In this hypothesis, which is similar to the well-known interspecies hydrogen transfer (Stams and Plugge, 2009, and references therein), an increase of ANME-SRB cell distance has
a dramatic effect on the thermodynamic yield of the reaction. Ultimately the physical separation of cells with distance in the $\mu$m order (depending on the in situ conditions, Sorensen et al., 2001) would stop the AOM reaction. To date, the addition of a wide range of possible electron carrier (Nauhaus et al., 2005) didn’t succeed in uncoupling AOM from SR. An exception is the interesting proposition of (Moran et al., 2008) of methyl sulphide as a possible intermediate. This scenario uses a reverse methyltrophic methanogenesis pathway (coupling CO$_2$ reduction and CH$_4$ oxidation) to produce CH$_3$-SH, the latter being used as electron donor for SR. Accordingly, Moran et al. found that the addition of CH$_3$-SH inhibited 68% of the AOM activity. However, this result could apparently not be reproduced in other laboratories (Knittel and Boetius, 2009). Hence, to date, and in spite of considerable efforts from many different groups, the fundamental AOM mechanisms remain elusive.

4.5 Methanogenic activity of AOM communities

An increasing number of studies have reported a dual methanotrophic-methanogenic activity in environmental samples (Orcutt et al., 2005) or in vitro (Treude et al., 2007; Orcutt et al., 2008b; House et al., 2009). Using parallel $^{14}$C-CO$_2$ and $^{14}$C-CH$_4$ radiotracer experiments, these authors found that methanogenesis (MG) was active in AOM zones with MG rates corresponding to 5-20% of the AOM rates. This finding further support the hypothesis of a reverse methanogenesis pathway involved in AOM. However, the role of this two-way reaction is not known. House et al. (2009) found large heterogeneities of carbon isotopic values within ANME-1 populations. The authors proposed that some cells could have a methanotrophic metabolism (more negative $\delta^{13}$C values, see section 5 below), whereas other could have a methanogenic metabolism (less negative $\delta^{13}$C values). Such interpretation is supported by thermodynamic models (Alperin and Hoehler, 2009) predicting that the presence of fermentation end products such as acetate or H$_2$ could act as a thermodynamic switch between AOM and MG: when fermentation processes release H$_2$ and increase its concentration above a certain threshold, then AOM becomes thermodynamically unfavourable whereas MG becomes exergonic. Hence, if ANME cell can use the methanogenic pathway in both directions, AOM should probably be very sensitive to H$_2$ levels. Such energetic constrains have been invoked to explain the lack of AOM activity in an H$_2$-rich brine pool in the Gulf of Mexico (Joye et al., 2009)
4.6 AOM community structure

Thermodynamic constraints on AOM predict that, assuming a chemical electron carrier hypothesis, there is a direct link between AOM mediating community structure (ANME-SRB distance) and their activity. ANME-2 cells are mostly found in tight association with SRB cells in shell type (ANME-2b) or mixed-type (ANME-2a) consortia (Knittel and Boetius, 2009; Figure I-5, and occasionally as monospecific clusters (Treude et al., 2005c). ANME-1 cells, on the other hand, seem to be often found as single cells, chains of ANME-1 cells, monospecific aggregates or in loose association with SRB cells (Figure I-5). It is thus unclear whether ANME-1 cells require a tight association with SRB. Strikingly, only the mixed-type of ANME-2a is a thermodynamically sound arrangement, maximizing exchange surface between ANME and SRB (Alperin and Hoehler, 2009). Using microscale reaction / transport models, these authors have shown that the formation of the conspicuous shell-type cluster has an energy cost for both partners. Hence, the reason for the formation of such aggregates could be related to yet unknown environmental parameters. The finding of ANME cells with a methanogenic activity (previous section) has the potential to resolve the apparent ANME-SRB cell distance conundrum, as isolated ANME cells or monospecific aggregates could have a methanogenic activity, and would thus not need to be in the vicinity of electron sinks such as SRB.

4.7 Microbial ecology

In general, a given methane seep environment, or a depth horizon is dominated by a single type of ANME cells, thus suggesting the existence of ANME ecotypes (Knittel and Boetius, 2009). The environmental parameters controlling the AOM community structure are not always clear. However, several patterns emerge from the numerous environmental studies of methane seeps.

(i) The structure of microbial community and the type of ANME involved seem to be governed by methane flux. This was evident from studies in Hydrate Ridge for instance, where sediments bearing high methane flux below *Beggiatoa* mats and lower flux below *Calyptogena* fields are dominated by ANME-2a and ANME-2b respectively (Knittel et al., 2005). Similarly, the AOM microbial community structure was dependant of the methane flux zonation at the Haakon Mosby MV in the Barent Sea (de Beer et al., 2006; Niemann et al., 2006a). (ii) ANME depth distribution (Knittel et al., 2005; Yanagawa et al., 2011) indicates a niche separation between ANME-1 and ANME-2, as the former are consistently found in the deeper, methane-rich and sulphate-poor or -depleted sediments, whereas ANME-2 are located at the transition zone. (iii)
Hypersaline environments seem to select for ANME-1 and exclude other ANME types (Lloyd et al., 2006; Yakimov et al., 2007; Niederberger et al., 2010).

4.8 AOM and authigenic carbonate precipitation.

The precipitation of carbonate in methane seeps has been widely observed (Greinert et al., 2002; Luff and Wallmann, 2003; Mazzini et al., 2004; Luff et al., 2005; Stadnitskaia et al., 2008) and such carbonate constitute an indication for paleoseeps occurrences (Peckmann and Thiel, 2004; Birgel and Peckmann, 2008). Striking examples of authigenic carbonate include the vast chemohemers found off South Carolina on the Hydrate ridge or the massive carbonate chimneys cropping out the Black Sea sediments (Michaelis et al., 2002). Such phenomenon is thought to occur due to rapid alkalinity production at high AOM turnover (equation 1). In addition, based on the analysis of alkalinity production and weak acids/bases exchanges, (Soetaert et al., 2007) have shown that AOM reactions tend to force a pH evolution toward a stable value around 7.9, which is above the critical pH for CaCO₃ precipitation.

4.9 Open questions and current challenges in the comprehension of AOM mechanism, ecology and methane budget: a summary.

Although research on methane turnover in marine sediment has occupied a very large part of marine sciences during the last decade, the comprehension AOM at the ecological, biochemical and geochemical level remains one of the largest challenges in (marine) microbiology.

• The elucidation of the metabolic pathway involved in the methane oxidation coupled to sulphate reduction is probably the major question remaining unanswered to date. A growing body of (meta-)genomic evidence suggest that AOM is mediated by the same enzymes involved in methanogenesis, but acting in reverse (*i.e.* the reverse methanogenesis hypothesis). However, 8 electrons are generated during AOM, and the transport mechanism as well as the final destination (electron acceptor), are not yet clear. A chemical shuttle such as H₂, acetate, formate or methanol could not be identified. Moreover alternative electron acceptors other than SO₄²⁻ (NO₂⁻, FeIII and MnIV) have been recently discovered but their contribution to AOM activity in sediments is not known.

• Another metabolic conundrum is the recent discovery of a dual AOM / methanogenic activity of sediments microorganisms under methanotrophic conditions. Such activity could be due to different cells having different activities, or a simple back reaction of the ANME
metabolic pathway involved in methanotrophy. Even more surprising was the conclusion of microscale modeling studies suggesting that most Archaea at cold seep could in fact be normal methanogens with few being methanotrophs. Methane production and consumption by methane seep microorganisms will thus deserve more attention.

- The identification of several Archeal phylotypes that can apparently mediate AOM raises the question of the existence of ANME ecotypes. The set of environmental conditions, or niches, selecting for particular ecotypes are poorly constrained and it remains not clear why certain group dominate particular AOM ecosystems. More generally, environmental factors besides methane and sulphate concentrations, affecting AOM microbial communities composition and activity are not well constrained.

- Conversely, the activity of AOM microorganisms can have an enormous impact on their environments, mostly through the production of sulphide and solid carbonate. The exploration of deep-sea methane seeps has only started to unveil the diversity and the extent of these ecosystems, their structure and dynamics, but our current catalogue is probably far from complete.

- Most of our knowledge on AOM ecophysiology derives from measures carried out at the sea surface, on board of research vessels upon sediment recovery (ex situ) or in on-shore laboratories (in vitro). However, very few data exist on the comparison between such data and the in situ processes. In particular, the metabolic rate at which AOM occurs is proportional to the methane concentration in situ. Since such data is usually not directly measureable, a large incertitude remains concerning the specific energy yield of the AOM reaction and the true methane turnover in situ.

- Finally, if AOM microbial communities constitute an efficient benthic filter against methane release in the water column, very little is known about their capacity to cope with increasing methane flux in a context of ocean warming, acidification and methane hydrate dissociation.
5. Tracking the origin and fate of methane with carbon stable isotopes.

The natural carbon pool contains both stable carbon isotopes $^{12}$C and $^{13}$C. Biological assimilation or transformations during the carbon cycle tend to preferentially use the lighter $^{13}$C. Hence, organic matter derived from photosynthesis has a typical depletion in $^{13}$C ($\delta^{13}$C) of about -20‰ compared to a reference value (0‰ is set according to the standard Vienna Pee Dee Belemnite or VPDB value). Such value differs according to the carbon fixation pathways used by plants and cyanobacteria: the more common C3 metabolism has an isotopic signature comprised between -24 and -33‰ whereas the C4 metabolism has a signature between -16 and -10‰. The crassulacean acid metabolism (CAM) used by plants in arid environments produces a stable isotope pool with values between -20 and -10‰. In turn, methane originating from organic matter mineralization will further fractionate this pool differently according to the mechanism of methane formation (Whiticar, 1999): biogenic methane will have a typical $\delta^{13}$C of between -50 and -100‰ VPDB, whereas methane thermogenesis has a lower fractionation effect (-25 to -50‰). Hence, both the carbon assimilatory (biosynthesis of membrane lipids and other macromolecules) and dissimilatory (mineralization, methanogenesis, AOM) processes can be tracked based on $\delta^{13}$C carbon values. In addition to geochemical evidences, the presence of highly fractionated carbon in archaeal and bacterial membrane lipids provided the first diagnostic of the microbial nature of AOM (Hinrichs et al., 1999) and is still a high throughput method for AOM community analysis (Niemann and Elvert, 2008; Wegener et al., 2008; Rossel et al., 2011). Stable isotope fractionation also potentially permits to discriminate between methane derived inorganic carbon, dissolved (i.e. DIC) or as solid carbonates, which have a lighter signature, and other DIC sources. In practice, a natural sample often contains a DIC/carbonate pool of mixed origin of which the relative contribution can be determined if the end members values are known.
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Figure I-5 Epifluorescence micrographs of different ANME single cells and aggregates visualized by FISH or CARD-FISH. (a) Single ANME-1 cells living in a microbial mat from the Black Sea. (b) Mat-type consortia formed by ANME-1 (red) and DSS cells (green). (c–e) Mixed-type consortia of ANME-2a (red) and DSS (green) cells observed in different seep sediments. (f–g) Shell-type consortia of ANME-2c (red) and DSS (green) cells and (h) single ANME-2c cells observed in different seep sediments. (i) ANME-3/Desulfobulbus consortia. (picture and caption reproduced from (Knittel and Boetius, 2009))

Figure I-6 16S rRNA genes based phylogenetic trees showing the different deltaproteobacterial (left) and archaeal (right) partners known to be involved in AOM (shown in red). Phylotypes that are suspected to be involved in AOM are shown in blue. Several groups closely related to sulphate reducing bacteria (Seep SRB-1 to -4) are exclusively found in methane seeps, but only SeepSRB1-c (Schreiber et al., 2010) and a group related to Desulfobulbus (Losekann et al., 2007) have been shown to be involved in consortia with ANME cells. The trees where constructed with the maximum likelihood method (see Chapter VI: experimental procedures)
6. Mud Volcanoes

6.1 Subseafloor origin and relation to environmental settings

Mud volcanoes (MVs) are positive relief formed by the extrusion of low-density subsurface sediment on the seafloor (Figure I-7). Most MVs are found in compressional settings, such as convergent margins (Kopf, 2002). The pressure build up in the deep sedimentary layers causes clay mineral dewatering and tend to fluidize surrounding sediments, allowing their extrusion through upper layers. In additions, in convergent settings, the progressive heating of the buried sedimentary organic matter leads to the formation of thermogenic gases and further contribute to the pressure build-up and to the fluidization and buoyancy of the sediment masses through formation of free gas inclusions. Hence, mud expelled on the seafloor often contains high amounts of gas, mainly methane, in the form of solute, free gas, or hydrates, and clasts that where mobilized during the ascent of the fluid through lithified sediment horizons.

6.2 Fluid conduit

In general, upward fluid migration is allowed or facilitated by the presence of fault systems (Kopf, 2002). These can either result from tectonic activity, or from diapiric motion of sediment masses of lower density that are migrating upward and fracturing the overlaying strata. Such low density masses includes evaporitic (gypsum, halite) or low grain density clay
minerals (kaolinites, smectites, vermiculites) that have the potential to include high amount of water.

6.3 Methane emission

Mud volcanoes are major escape conduits for deep methane reservoir. It has been estimated that 30-70 Tg yr\(^{-1}\) of methane are transferred toward the MV surfaces (Kopf, 2002, 2005) However, the fate of this methane is not clear, as most of it can be oxidized by AOM, and the fraction that escapes the sediment is probably oxidized aerobically in the water column. In case of gas bubble formation however, considerable amount of methane can reach the atmosphere (Solomon et al., 2009). Overall, in spite of numerous studies on the gross methane emission, the contribution of deep-sea mud volcanoes to the atmospheric methane pool is not yet fully constrained.
7. **The Gulf of Cadiz.**

7.1 **Origin and geology of the Gulf of Cadiz.**

The Gulf of Cadiz is located at the diffuse boundaries of oceanic and continental margins on the one hand, and of the Iberian and African plate boundaries, in the prolongation of the E.-W. Gloria fault, on the other hand. It is delimited by the South Portuguese and Iberian margins, and the Moroccan margin in the North and East, by the Horseshoe abyssal plain and the Gorringe Bank in the West, and by the Seine abyssal plain in the South (Figure I-8).

Since the inception of the Irish margin in the Triassic (~230 My), the Gulf of Cadiz underwent many transformations due to interacting tectonic and depositional processes. The margin evolved from a passive margin in the Mezozoic to an active margin in the Cenozoic (Maldonado and Nelson, 1999, and references therein) due to the African and European plates motion. Tectonic activity is at the origin of major features of the Gulf, such as the thick flysch on the eastern part, developed during orogenesis of the Gibraltar Arc. Secondly, important tectonically induced sediment displacement that occurred in the Tortonian led to the formation of the large allochtonous unit of the Gulf of Cadiz or AUGC (Medialdea et al., 2004; Medialdea et al., 2009) and constitutes the thickest unit of the -up to 14 km- sedimentary cover. The AUGC, often referred as olistostrome and of which the origin is still debated (Gutscher et al., 2002), is composed of huge volume of sediments from the Triassic to Neogene, including mud and Triassic salt. Moreover, numerous faults and seabed morphologies resembling accretionary wedges could also be related to compressive tectonic activities, including possible currently active subduction (Gutscher et al., 2002). The region is undergoing current seismic activity as witnessed by the >8.5 magnitude Lisbon earthquake in 1755, which together with the resulting tsunami, almost razed the city.
Figure I-8 Situation and geological map of the Gulf of Cadiz (reproduced from (Medialdea et al., 2009))
In term of deposition, seven different sedimentary regimes were identified, from Early Mezozoic carbonate platform formation to contemporary human cultural regimes (<5000 y) that influenced sediment deposition in major rivers basins located in the Gulf (Maldonado and Nelson, 1999).

### 7.2 Hydrologic regime in the Gulf of Cadiz

The Gulf of Cadiz waters are dominated by two different water masses. The surficial North-Atlantic waters generally flow eastward with a net inflow into the Mediterranean Sea (Nelson et al., 1999), possibly as a compensation of a net loss of water by evaporation in the later. This water mass, of lower temperature and salinity than the Mediterranean seawater has a relatively lower density and is located in the first 300 m of the water column. Conversely, the warmer (~13ºC) but more saline Mediterranean outflow waters (MOW) that are flowing westward through the strait of Gibraltar have a higher density and occupy the zone below 300 m (Nelson et al., 1993). After the strait, the circulation of the MOW is strongly influenced by the seabed topography that features numerous ridges and valleys, and by the continental slope that induces a westward density driven current acceleration. Toward the deep abyssal plains in the West, bottom water temperature decrease toward more typical deep-sea temperatures (~4 ºC).

### 7.3 Origin and seafloor expression of hydrocarbon migration in the Gulf of Cadiz.

The hydrocarbon source rocks in the Gulf of Cadiz are complex and not fully identified, in part due to the composite sedimentary structure described above. A mixed thermogenic and biogenic origin of gases in the migrating fluid, as indicated by the composite methane $\delta^{13}C$ signature (~ -50‰ VPDB) and the relative content of higher hydrocarbons indicate that multiple sources are involved (Mazurenko et al., 2003; Stadnitskaia et al., 2006; Hensen et al., 2007) Hence, it is likely that methane and higher volatile hydrocarbons are produced by thermal cracking of buried organic matter at great depth (between 5 and 14 km below the sea floor or bsf), at temperature >150ºC, as well as by microbial methanogenesis (Stadnitskaia et al., 2006; Hensen et al., 2007) possibly originating from Miocene black shales (Medialdea et al., 2009). The upward migration of fluid and fluidized mud, with velocities up to cm yr$^{-1}$ (Hensen et al., 2007) is provoked by a combination of compressive tectonic stress, presence of
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deep-rooted faults deriving from tectonic or diapiric activity, and clay mineral dewatering during the transformation of e.g. illite to smectite. During the ascending migration, fluid can also be affected by secondary processes such as evaporite leaching leading to enrichment with regard to $\text{Na}^+$, $\text{Cl}^-$, $\text{Ca}^{2+}$, $\text{SO}_4^{2-}$, and admixing with seawater in shallow subsurface (Hensen et al., 2007).

As a result, numerous seafloor expressions of this fluid migration have been identified in the Gulf of Cadiz. These have been classified in three main categories (Leon, 2006):

(i) Pockmarks: these circular negative reliefs on the seafloor that can reach up to 800 m diameter and up to 18 m depth in the Gulf of Cadiz and are formed by the expulsion of gas from surface sediments (Baraza and Ercilla, 1996; Somoza, 2003)

(ii) Over 50 mud volcanoes have been identified in the Gulf of Cadiz (Figure I-8). They are characterized by the presence of reduced mud breccia containing gas, occasionally in the form of hydrates, and hydrogen sulphide as a result of AOM. The accumulation of erupted material form typical cone shaped positive relief on the seafloor. These can reach 3500 m in diameter and reach 300 m height (Medialdea et al., 2009). Seismic studies have shown that the formation of these mud volcanoes is tightly linked to salt or marl diapiric activity underneath, and to compressive tectonic-derived fault systems, both providing feeding conduits between the deep-sited reservoirs and the seafloor (Medialdea et al., 2009).

(iii) Methane derived authigenic carbonate structures, in the form of slabs, crusts, chimneys, or even massive carbonate mounds (Leon, 2006).
8. Carbonate mounds

8.1 A life strategy throughout earth history

The term carbonate mound broadly defines structures built by living organisms on the seafloor by different processes, at different geological times, but that have in common to create massive (m to tens of km wide) topographic height composed for a large part of carbonate. This brief literature review is based on thorough review work of M. Hovland (Deep-Water Coral Reefs, Unique Diversity Hot-Spots, 2008) and J.-P. Henriet and A. Foubert (Nature and Significance of the Recent Carbonate Mound Record The Mound Challenger Code, 2009), and the reader is redirected to this publications for “in-depth” review of the occurrence, distribution, geology and biology of these structures. The formation of carbonate structures by living organisms, or bioherms, was first recognised in Archaean rocks (~ -3.5 Gy), where microorganisms formed carbonate stromatolites. Their number and diversity expended during the Proterozoic, and some modern examples can still be observed today, e.g. in the Shark Bay (Australia). In these structures, carbonate formation is related to the mineralisation of organic matter deriving from cyanobacteria phototrophic activity (Taylor et al., 1993). With the onset of metazoan organisms in the late Precambrian (~ -650 My), new types of carbonate mounds appeared, involving sponges, corals or bryozoans. In these mounds, carbonate was derived from the accumulation of skeletal carbonate, early diagenetic processes associated with biomass mineralization by microorganisms, and photosynthesis. Hence, behind the relatively simple definition of carbonate mounds provided a the beginning of this section, resides in fact a complex interplay of biological activities, spanning from microorganisms photosynthesis and organic matter mineralisation to ecosystem engineering metazoans, of oceanographic constraints such as primary productivity, seawater level, temperature and mixing regimes, and of geological properties of the underlying seabed. However, the unifying trait of these different mound forms resides in the local accumulation of carbonate resulting from enhanced local biological activity.

8.2 Modern cold-water coral carbonate mounds

In contrary to their tropical counterparts, these corals are azooxanthellate, i.e. they do not possess algal symbionts, but both reef types are comparable in term of productivity and biodiversity (Roberts et al., 2006). The development of the cold-water coral reef like structures
along continental margins may lead to the formation of massive carbonate mounds. As for the Devonian mounds shown in Figure I-9 and Figure I-10, these mounds seem to result from various processes including coral growth controlled by hydraulic conditions, locally enhanced organic matter supply, glacial interglacial alternations, and microbial biomineralisation. An important step forward in understanding the distribution of these mounds along the continental margins was the discovery by Dullo et al. (2008) that most thriving cold water corals were lying within a seawater density envelope of sigma-theta ($\sigma_{\theta}$) = 27.35 to 27.65 kg m$^{-3}$. This provided a strong support for a hydraulic forcing on the development of these mounds. However, based on overlaps between mound distribution and possible subseafloor hydrocarbon deposits and seepage routes, several authors also proposed an internal forcing hypothesis connecting the two observations (Henriet et al., 1998; Naeth et al., 2005; Hovland, 2008). Possible links between hydrocarbon seepage and mound distribution and persistence across long geological times include methane and other hydrocarbons derived carbonate (See section 4.8), which could consolidate the mound sediment matrix, or provide a hard ground substratum on the sea floor for the settlement of sessile cold-water corals. The recent drilling of the challenger mound (Off Ireland) during the integrated ocean drilling program (IODP) leg 307 (Ferdelman et al., 2006) has shown that such mechanisms was unlikely, as a sulphate to methane transition zone was only present below the mound’s base, and that on mound carbonate isotopic composition did not support an hydrocarbon origin (Takashima et al., 2009).
Figure I-9 Beauchateau carbonate mound (A), and Fort Condé mud mound (B) in the south of Belgium near Couvin. These Devonian mounds were built by various processes involving sponges, corals, brachiopods, crinoids, iron oxidizing bacteria and probably organic matter mineralisation. Different geological sequences are apparently related to sea water level variations, and changes of bottom water conditions. Reproduced from (Boulvain, 2001).

Figure I-10 Kess-Kess Devonian mounds in the Moroccan Anti Atlas. The reef or hydrothermal nature of the mounds is still debated (Cavalazzi et al., 2007). Picture from the Cocarde project. www.cocarde.eu.

Figure I-11 Major constituents of cold-water coral carbonate mounds. Left: Large Lophelia pertusa colony (Photo M. Roberts), and Madrepora oculata (Photo U.S. National Oceanographic and Atmospheric Administration / Coral Reef Information System).
8.3 Carbonate mounds in the Gulf of Cadiz

The El Arraiche mud volcano field is located in the shallow area (700-450 mbsf) of the Moroccan margin. It features several active mud volcanoes such as Mercator MV, Al Idrissi MV and Gemini MV, witnessing repeated event of fluid flow and mud extrusions over the last 2.4 Ma, as revealed by seismic data acquired during R/V Belgica and R/V Prof. Logachev TTR 12 cruises (Van Rensbergen, 2005). Mud volcanoes in the El Arraiche field are mainly clustered along two ridges: Renard and Vernadsky ridges, rising about 300 m above the seafloor and characterized by steep escarpments on their flanks (Figure I-12, Figure I-13). These ridges were interpreted as related to fault systems, and thus constitute privileged pathway for deep fluid up flow. In this context, the discovery of fossil cold-water coral carbonate mounds on top of the Renard Ridge was of utmost interest, and offered a new opportunity to address the question of an internal forcing of carbonate mounds. These carbonate mounds are located close to the inferred hydrocarbon seeps resulting from over pressurized sediment zones in contact with MV conduits (Figure I-14). In addition, patches of living or dead corals were also reported near Captain Arutyunov MV (this thesis), Darwin MV (this thesis), Meknes MV (Hovland, 2008), Rabat MV and Tasyo MV (Akhmanov, 2001), Pipoca MV and Almazan MV (Somoza, 2003) and Al Idrissi MV (Henriet et al., 2009), suggesting that both cold water corals carbonate mounds and fluid extrusion though the seafloor are often associated in this region.
Figure I-12 The El Arraiche mud volcano field, located on the Moroccan Margin. The Pen Duick escarpment and the Renard ridge, where several cold-water coral carbonate mounds were discovered, are located within a zone of hydrocarbon seepage, as witnessed by numerous mud active mud volcanoes in the vicinity (reproduced from Van Rensbergen, 2005).

Figure I-13: Topography of the Pen Duick escarpment and the Renard Ridge. In the S.-W. of the Gemini MV, several carbonate mounds built by cold-water corals crop up from the escarpment (white dots) and apparently root on the base of the what has been interpreted as a fault bounded cliff (Foubert et al., 2008). Methane biogeochemistry was investigated in the Alpha mound (nbr. 292). Numbers refer to the cores recovered during the MSM1/3 cruise aboard the R/V Maria S. Merian (2006). Depth range: -300 mbsl (purple) –700 mbsl (yellow).

Figure I-14: Seismic profiles (data from T. van Weering, NIOOZ, The Nederlands) across the Pen Duick escarpment (PD) and the Gemini MV (GMV), indicating zones of overpressurized sediments (OP) in contact with MV conduits. Inferred seeps (IS) based on the seismic data apparently occur near carbonate mounds. Reproduced from (Hovland, 2008).
9. **General goal and outline of the thesis:**

Microbial communities mediating AOM are key players in the control of greenhouse gas emissions, benthic habitats ecology and ecosystem engineering. However, their study is hampered by their slow growth, the difficulties associated with their isolation, the remoteness of their deep-sea habitats, and the extreme conditions associated with these habitats. Hence, in parallel to geochemical, metagenomic or in vitro approaches, integrated studies of natural methane seep environments have proven to be a successful approach for the comprehension of the ecology of AOM microbial communities.

This work aims at a better comprehension of the ecology of these communities by studying **AOM microbial ecosystem functioning** in different natural environments, *e.g.* understanding the feedbacks between **environmental parameters, microbial community structure** and **relevant microbial activities**.

This approach is summarised in the opposite scheme: with an extensive characterisation of the different summits (environment, activity, community) in a range of different deep sea methane seeps habitats, we aim at understanding the interactions between them (arrows).

**Chapter II** addresses the question of a possible internal forcing in the **Alpha mound**, a cold-water coral carbonate mounds located in the Pen Duick Escarpment, in the Gulf of Cadiz. The aim of this chapter is thus to evaluate the presence of methane migration through such structure, and it’s possible impact on the carbonate budget, the DIC stable isotope signature, and microbial abundance and activity.

**Chapter III** focuses on the recently discovered **Darwin MV**, which is covered for a large part by a thick carbonate platform. We examine the impact of this unusual structure on the methane flux and microbial activity distribution. In conclusion we provide a possible model that integrate the results of this study and all previous observations of this MV. I show that the concept of “the self-sealing nature of methane seeps” developed first by (Hovland, 2002) may...
explain the current distribution of AOM microbial activity at this MV.

Chapter IV compares the microbial community structure and methane cycling in the hypersaline sulphate rich sediments of Mercator MV with the standard salinity sediment of Captain Arutyunov MV. Based on current knowledge on metabolism selection in hypersaline environment, it was unlikely to find AOM activity at Mercator MV. I show however that AOM and SR were active and in spite of an apparent inhibition, consumed a significant part of the rising methane flux. The presence of sulphate from evaporite leaching stimulated AOM and compensated the salinity inhibition. Further, the AOM community was remarkable in that AOM was exclusively mediated by ANME-1 cells, which only proceeded in a methanotrophic mode, and showed only distant interactions with SRB cells.

Chapter V focuses on the Carlos Ribeiro MV. Methane and sulphate fluxes estimated in a companion paper by Vanneste et al. (in press) were compared to AOM and SR rates obtained with radiotracer measurements. I show that reaction transport modelling exceeded microbial activity by one order of magnitude. However, these conflicting data may result from an underestimation of the in situ methane pool, as reassessment of the microbial activity based on in situ methane concentration tend to converge toward modelled methane flux. In addition I show that archaeal community in the crater centre was dominated by GoM Arc I cells, which has been only found in mud volcanoes so far and thus constitute a candidate phylotype that could be involved in AOM.

Chapter VI is a general discussion covering the recent studies on the Alpha mound and other carbonate mounds in the vicinity in the perspective of the question raised in Chapter II. The functioning of the different AOM ecosystems in the four MV studied in this thesis are also reviewed, in term of microbial diversity, community structure and possible AOM mechanism.

Similar experimental procedures were used for the studies described in Chapter III to V. These are extensively described in Chapter VII.
CHAPTER II. Anaerobic Oxidation of Methane in a Cold-Water Coral Carbonate Mound from the Gulf of Cadiz

CHAPTER II
ABSTRACT

The Gulf of Cadiz is an area of mud volcanism and gas venting through the seafloor. In addition, several cold-water coral carbonate mounds have been discovered at the Pen Duick escarpment amidst the El Arraiche mud volcano field on the Moroccan margin. One of these mounds -named Alpha mound- has been studied to examine the impact of the presence of methane on pore-water geochemistry, potential sulphate reduction (SR) rate and dissolved inorganic carbon (DIC) budget of the mound in comparison with off-mound and off-escarpment locations. Pore water profiles of sulphate, sulphide, methane and DIC from the on-mound location showed the presence of a sulphate to methane transition zone at 350 cm below the sea floor. This was well correlated with an increase in SR activity. $^{13}$C-depleted DIC at the transition zone (-21.9 ‰ vs. Vienna Pee Dee Belemnite) indicated that microbial methane oxidation significantly contribute to the DIC budget of the mound. The Alpha mound thus represents a new carbonate mound type where the presence and anaerobic oxidation of methane has an important imprint on both geochemistry and DIC isotopic signature and budget of this carbonate mound.
1. **Introduction**

Cold-water corals are commonly found on the continental margins in a wide range of depth and latitude (Roberts et al., 2006). Unlike their tropical counterparts, cold-water coral are azooxanthellate and thus do not rely on the activity of a photosynthetic symbiont to develop, but rather on local supply of organic matter. Under favorable environmental conditions (Dullo et al., 2008), these organisms can form important carbonate structures or carbonate mounds remaining in the geological records (De Mol, 2002; Kenyon, 2003; Wheeler et al., 2007; Frank et al., 2009). They are mainly composed of the stony corals species *Lophelia pertusa* and *Madrepora oculata* (De Mol, 2002), which constitute thriving habitats for numerous benthic species through the development of reef-like frameworks (Costello, 2005). Cold-water carbonate mounds have thus been recognized as hotspot ecosystems on the continental margins (Weaver, 2004). Several mound provinces have been described on the southwest Irish margin where they often host prosperous ecosystems with living corals on their surface (Porcupine bank and Rockall through) (Henriet et al., 1998; Huvenne, 2002; Kenyon, 2003). In this area, carbonate mounds are situated between 500 and 1000 meters water depth, rising above the seafloor from a few meters to 250 meters and some of them are 2 kilometers in diameter. In the Belgica mound province (Porcupine bank), the first scientific drilling of a carbonate mound (Challenger mound) during the Integrated Ocean Drilling Program (IODP), leg 307 (Ferdelman et al., 2006) showed that such mounds may constitute an important carbonate sink (Titschack et al., 2009), and constitute a unique microbial habitat compared to other sub seafloor environments (Webster et al., 2009). First results from this expedition indicated that the geochemistry and diagenetic processes within the Challenger mound are not influenced by methane and other hydrocarbons (Ferdelman et al., 2006).
The Gulf of Cadiz is an important zone of hydrocarbon-rich fluids migration. Tectonic compression and Olistrostone development led to the formation of numerous mud volcanoes, diapiric cones and ridges, and pockmarks with several occurrences of gas hydrates (Kenyon, 2002; Pinheiro, 2003; Somoza, 2003; Van Rensbergen, 2003, 2005; Kenyon, 2006; Leon, 2006). Methane migration and its subsurface removal due to microbial anaerobic oxidation of methane (Niemann et al., 2006b) led to the formation of authigenic carbonates as attested by the presence of $^{13}$C depleted clasts, slabs or chimneys (Leon, 2006). Recently, several fossil cold-water coral carbonate mounds -almost devoid of living corals- have been described in this area suggesting that these margins once provided suitable conditions for the development of cold-water coral carbonate mounds (Van Rensbergen, 2003, 2005; Foubert et al., 2008). The discovery of such mounds on the top of the Pen Duick escarpment (Foubert et al., 2008) in the Al Arraich mud volcano field is of particular interest to study mound formation processes and sedimentary diagenesis in a context of hydrocarbon migration.

In marine sediments, microorganisms mediating Anaerobic Oxidation of Methane (AOM) indeed consume methane at the expense of sulphate according to the equation (Hoehler et al., 1994; Nauhaus et al., 2005):

$$\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HCO}_3^- + \text{HS}^- + \text{H}_2\text{O} \quad \text{(equation 1)}$$

When present, AOM has a strong impact on the overall geochemistry and diagenetic processes in the sediment. In particular, the production of carbonate alkalinity can lead locally to carbonate precipitation (Ritger et al., 1987; Luff and Wallmann, 2003; Luff et al., 2004; Stadnitskaia et al., 2008).

In this study, we describe methane related geochemical processes in the Alpha mound, located at the Pen Duick escarpment in the Gulf of Cadiz, as well as two reference locations off-mound and off-escarpment. We show that unlike other carbonate mounds described so far, methane is
present in the sediments of the Alpha mound, and a front of AOM at 350 cmbsf has a profound influence on both the geochemistry and the DIC budget of this mound.

Figure II-1 The Gulf of Cadiz (A) is a zone of mud volcanisms, spanning from Portuguese and Spanish margin in the north and Moroccan margin in the east, to Atlantic abyssal plains in the west. The zone of study (B, 5m contour line spacing) is located in the Al Arraich mud volcano field, on the Moroccan margin. Several cold-water coral carbonate mounds are located along the Pen Duick escarpment (delimited by dashed lines in B and C), a 4km long and 80 m high fault-bound cliff. The Alpha mound is a 30m high carbonate mound in the eastern part of the escarpment. Note the presence of Gemini and Lazarillo de Tormes mud volcanoes in the vicinity of the escarpment. C: maps of the escarpment showing that the cold-water carbonate mounds are rooting on the plateau in the N. E. side and on the foot of the escarpment in the S.W. side.
2. Results

2.1 Site description

The Pen Duick escarpment, a 4 km long fault-bounded cliff on the western part of the El Arraiche mud volcano field (Moroccan margin, Figure II-1A and B), was mapped by multibeam bathymetry during the CADIPOR Cruise 2002 on board of the R/V Belgica (Van Rensbergen, 2005). The escarpment’s base is at about 700 mbsl (meters below the sea level), and rises to a plateau at 520 mbsl. Fifteen carbonate mounds of height ranging from 20 to 60 meters crop out of the top of this cliff (Figure II-1C). The Alpha mound is located at the southeastern end of the escarpment, near the Gemini mud volcano. It is 50 m high with a slope inclination between 15° and 25°. The on-mound station was located near the mound summit, and cold-water coral debris constituted a large part of the recovered sediments (Foubert 2008). The off-mound station was located on the escarpment next to the base of the mound, and the off-escarpment station, 2 km away from the escarpment (Figure II-1B).

<table>
<thead>
<tr>
<th>Core name</th>
<th>Core type</th>
<th>Length [m]</th>
<th>Location</th>
<th>Depth [m]</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD04-2804</td>
<td>CASQ Kasten core</td>
<td>6</td>
<td>6°47.0072215'W 35°17.451'N</td>
<td>500</td>
<td>On-mound</td>
</tr>
<tr>
<td>MD04-2809</td>
<td>Calypso Piston core</td>
<td>15</td>
<td>6°47.14183'W 35°17.56383'N</td>
<td>540</td>
<td>Off-mound</td>
</tr>
<tr>
<td>MD04-2806</td>
<td>Calypso Piston core</td>
<td>24</td>
<td>6°49.6793'W 35°18.5367'N</td>
<td>640</td>
<td>Off-escarpment</td>
</tr>
<tr>
<td>MSM1/3-294</td>
<td>Gravity core</td>
<td>4.2</td>
<td>06°47.012'W 35°17.458'N</td>
<td>534</td>
<td>On-mound</td>
</tr>
</tbody>
</table>

Table II-1 Location and feature of the cores used in this study
CHAPTER II

2.2 Pore water geochemical profiles.

On-mound, sulphate concentration decreased with depth, forming a gradient from seawater concentration values (28 mM) at the sediment-water interface to complete depletion at 400 cm below the sea floor (cmbsf) (Figure II-2A). This concentration gradient corresponded to a downward sulphate flux of 54.0 mmol m\(^{-2}\) yr\(^{-1}\) (Table II-2). Conversely, methane concentration increased with depth, forming a gradient from complete depletion at 300 cmbsf to 2.0 mM at the bottom of the core (580 cmbsf). This concentration gradient corresponded to an estimated upward methane flux of 17.0 mmol m\(^{-2}\) yr\(^{-1}\). These two gradients defined a sulphate to methane transition zone (SMTZ) between 300 and 400 cmbsf. Coinciding with this SMTZ, a sulphide concentration peak was observed with maximum sulphide concentration of 3.6 mM at 350 cmbsf.

On-mound, Dissolved Inorganic Carbon (DIC) increased with depth from 1 mM at the sediment surface up to 32.0 mM at the SMTZ whereas $\delta^{13}$C-DIC decreased with depth, with the lowest value ($-21.9\%_e$ vs. Vienna Pee Dee Belemnite or VPDB) coinciding with the SMTZ. Below, $\delta^{13}$C-DIC remained stable around -16%\(_e\).

In order to gain further knowledge on the origin of methane present on-mound, the carbon stable isotopic signature of methane were measured at the bottom of the on-mound core: methane $^{13}$C depletion was $-51.1\%_e$ at 580 cmbsf, $-51.8\%_e$ at 530 cmbsf and $-52.2\%_e$ at 500 cmbsf.
Figure II-2 On-mound (A) pore-water geochemical profiles of sulphate, methane, sulphide, DIC and $\delta^{13}$C-DIC as well as SR rates (SRr) and cell counts. Off-mound (B) and off-escarpment (C) pore-water sulphate, methane, sulphide profiles. ●: data from MD04 cores. ○: data from MSM1/3 core (Table II-1). The two datasets are similar and indicate that the cores used for geochemical measurement and activity test correspond to the same setting. Dashed lines indicating estimated gradients were fitted manually.

A SMTZ was also present at off-mound and off-escarpment locations (Figure II-2B and C), but deeper in the sediment, in the 500-900 cmbsf, and 1000-1500 cmbsf respectively. Sulphate concentration gradients corresponded to downward sulphate fluxes of 22.6 and 12.5 mmol m$^{-2}$ yr$^{-1}$ respectively (Table II-2). Methane was present in the deeper part of the core at both locations, with maximum concentration of 0.9 mM and 0.6 mM respectively. At both sites, sulphide concentration showed a maximum within the SMTZ.
2.3 On-mound location: in vitro SR rates and cell counts

The SR rate measurements revealed the presence of relatively high but scattered SR between 50 and 300 cmbsf. Relatively low or no SR activity was detected between 300 and 450 cmbsf with a discrete peak (0.09 nmol/cm³/day) at 350 cmbsf. The latter coincided with the sulphide increase at the SMTZ (Figure II-2). In the upper interval, depth integrated rates corresponded to a sulphate conversion of 126.0 mmol m⁻² yr⁻¹, and 4.6 mmol m⁻² yr⁻¹ in the lower zone.

In order to assess the impact of the presence of an AOM activity zone on the microbial community within the on-mound sediments, we performed cell counts by direct microscopic observation of fluorescently stained cells (AODC). Direct cell counts showed that cell number decreased between sediment surface and 50 cmbsf and then remained constant around 2x10⁸ cells cm⁻³ of sediment, down to 350 cmbsf. Below, the cell number decreased to 6x10⁷ cells cm⁻³ at 420 cmbsf.

<table>
<thead>
<tr>
<th>Core name</th>
<th>Porosity at SMTZ</th>
<th>Gradients [μmol cm⁻³]</th>
<th>Fluxes [mmol m⁻² years⁻¹]</th>
<th>Methane flux/sulphate flux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sulphate</td>
<td>Methane</td>
<td>Σ Sulphide</td>
</tr>
<tr>
<td>On-mound</td>
<td>0.59</td>
<td>0.0889</td>
<td>0.0182</td>
<td>0.1247</td>
</tr>
<tr>
<td>Off-mound</td>
<td>0.56</td>
<td>0.0389</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Off-escarpment</td>
<td>0.58</td>
<td>0.0208</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table II-2 Estimated concentration gradients, diffusive fluxes and methane / sulphate flux ratio for the on-mound, off-mound and off-escarpment cores. Concentrations are given in mM in pore-water.
3. Discussion

3.1 Anaerobic oxidation of methane in Alpha mound.

Our results show that methane was present in shallow subsurface sediment of the Alpha mound. On-mound, migrating methane was anaerobically consumed forming a SMTZ associated with a peak of sulphide at the interface between 300 and 400 cmbsf. Such SMTZ typically results from the microbially mediated AOM reaction coupled to SR (Iversen, 1985; Niewohner, 1998). The presence of a microbial community carrying AOM was supported by the observation of a SR activity peak coinciding with the SMTZ. In addition, DIC was produced at this depth and displayed a typical light $^{13}$C signature. Therefore, the presence of methane and its consumption had a significant impact on both the geochemistry and microbial activity of the Alpha mound sediment. Beyond the parameters analyzed in this study, AOM is likely to alter other biogeochemical processes: previous report have indeed shown that i) AOM derived sulphide can reduce Fe III and form stable complexes with Fe II, thus reducing the pool of reactive iron available for diagenetic processes such as dissimilatory iron reduction (Thamdrup et al., 1994; Jorgensen et al., 2004; Poulton et al., 2004; Lovley, 2006). ii/ AOM derived DIC can precipitate and form authigenic carbonate, a well documented process at cold-seep settings (Aloisi et al., 2002; Luff and Wallmann, 2003; Luff et al., 2004). However, less is known about methane-derived carbonate in deeper SMTZ and lower methane flux. In such situation, the effect of DIC production can be balanced by diffusion in sediment and precipitation due to supersaturation is less likely to occur. Yet, authigenic carbonate (nodules) in a geochemical environment comparable to Alpha mound has been described for instance in the Gulf of Mexico (Ussler III, 2006; Chen et al., 2007) or as $^{13}$C depleted carbonate in the sediments of the Chilean margin (Treude et al., 2005b). Therefore, carbonate formation due to methane derived DIC is also probably occurring in Alpha mound.
CHAPTER II

3.2 Origin of methane and DIC budget in the Alpha mound.

Considering that thermogenic methane has a typical $\delta^{13}C$ signature between -50 and -20‰ (Schoell, 1980, 1988) and biogenic methane in marine sediment, a signature between -110 and -60‰ (Rice and Claypool, 1981; Schoell, 1983; Whiticar et al., 1986; Whiticar, 1999), we can therefore take an average of -35‰ for the former and -85‰ for the latter. According to these end-member values, on-mound methane with $\delta^{13}C = -51.8$‰ would thus originate for 66% from a thermogenic source. These results are conform with previous studies on the origin of methane in the Gulf of Cadiz methane seeps (Stadnitskaia et al., 2006), indicating a mixed but dominantly deep thermogenic origin of migrating methane. Therefore, the main source of methane responsible for the formation of a SMTZ and the AOM activity in the Alpha mound does not derive from microbial methanogenesis in the mound itself, but rather from deeper sources.

Further, the contribution of AOM to the total DIC budget in the on-mound core can be estimated based on a simple two sources isotope-mixing model involving AOM derived DIC and non-AOM derived DIC. The mixing of these two sources results in a bulk DIC with an intermediate isotopic signature. In this model:

$$F_{AOM} \times \delta^{13}C_{AOM} + F_{non-AOM} \times \delta^{13}C_{non-AOM} = \delta^{13}C_{bulk} \quad (Equation \ 2)$$

$$F_{AOM} + F_{non-AOM} = 1 \quad (Equation \ 3)$$

where $F_{AOM}$ and $F_{non-AOM}$ are the contribution of each source (in %) and $\delta^{13}C_{AOM}$ and $\delta^{13}C_{non-AOM}$, the isotopic signature of each source. As shown in Figure II-2, all methane was consumed during AOM reaction, excluding fractionation effect between the source methane and the resulting DIC. Hence, AOM derived DIC has a similar $\delta^{13}C$ than its methane source, e.g. $\delta^{13}C_{AOM} = -51.7$‰. Besides AOM, the main DIC sources derive from nanofossils calcite ooze (coccolith and forams) and coral fragments (Foubert et al., 2008). The former displays values between 0 and -2‰ as measured in two close locations (Foubert et al., 2008).
The later is mainly composed of aragonite and typically displays $\delta^{13}$C values comprised between 0 and -10‰ within single fragments (Blamart, 2005). Hence a range of 0 to -10‰ is a safe approximation for non-AOM DIC isotopic signature in this carbonate mounds. Using these values in the isotope-mixing model, AOM accounted for 28-42% of bulk DIC at the SMTZ, and 14-30% below this depth. AOM thus significantly contributed to the DIC budget in the on-mound core.

3.3 Fluxes and rates

On-mound, sulphate consumption was in good agreement with the sulphide production (54 and 47 mmol m$^{-2}$ yr$^{-1}$ respectively) based on diffusion model. Highest sulphide concentrations were observed in the SMTZ and AOM was likely to provide most of sulphide observed in this location. Based on the stoechiometry of the AOM reaction (equation 1) and on both methane and sulphate flux estimation, it is also possible to determine the contribution of methane oxidation to the overall SR in the on-mound location. Methane flux estimation should however be interpreted with caution as degassing during recovery and sampling likely occurred and because of a low-resolution sampling in the SMTZ. In these conditions, it was difficult to infer both methane flux and migration regime and thus the actual AOM contribution to the overall SR. Based on the data available, we estimated that 33% of the diffusing sulphate (e.g. 17.8 mmol m$^{-2}$ yr$^{-1}$) was consumed by the AOM reaction.

The peak of SR activity (4.6 mmol m$^{-2}$ yr$^{-1}$) at the SMTZ (-350 cmbsf) was interpreted as AOM dependant SR, whereas the activity between 50 and 300 cmbsf was likely due to organoclastic SR. The AOM dependant SR was certainly underestimated, as AOM strongly depends on methane concentration (Nauhaus et al., 2005). During in vitro incubation, the methane concentration corresponded to saturation at 1 bar and 4°C (~1.4 mM), whereas in situ this concentration can potentially reach ~60 mM (Duan and Weare, 1992) This difference between
in situ and in vitro conditions can explain the discrepancies observed between AOM dependant sulphate consumption based on potential rates measurements (4.6 mmol m$^{-2}$ yr$^{-1}$) and on methane flux calculation (17.8 mmol m$^{-2}$ yr$^{-1}$).

The sulphate flux was higher on-mound than off-mound and off-escarpment (Figure II-2). Downward sulphate fluxes are usually driven by both organoclastic and AOM dependant SR when methane is present. Off-mound and off-escarpment, organic content due to sedimentary processes were probably similar, given the proximity of these two stations. Hence, differences between sulphate fluxes in these locations were mainly controlled by methane. Our results suggest that the methane flux was thus higher on the escarpment than in the surrounding sediments. Such enhanced methane migration could be related to the fault system underlying the escarpment and/or to mud volcanic activity as mud breccia and clasts have been observed at 170 cmbsf in the flank of the mound (Core AT570G, Foubert et al., 2008). Sulphate profiles presented in this study are comparable to other locations where a SMTZ due to the presence of methane has been observed, such as in the Blake Ridge sediments (Borowski, 1996, 2000), in the Kattegat and Skagerrak sediments (Iversen, 1985), in the upwelling of Namibia (Niewohner, 1998), or on the Chilean margin (Treude et al., 2005b).

Parkes et al. (Parkes et al., 1994; Parkes et al., 2000) determined an average of the prokaryotic cell number as a function of sediment depth based on their observations in various sedimentary settings. Total cell counts in Alpha mound sediment remained close to this average and in all cases, within the interval containing 95% of the counts by Parkes et al.. Moreover, cell counts within the AOM zone did not significantly differ from counts above and below this zone. Therefore, the presence of a microbial community mediating the AOM reaction did not stimulate the overall cell number in the on-mound sediment as compared to sediment without methane. These observations are coherent with several previous studies: cell counts did not increase within a deep SMTZ (150-200 mbsf) in the Porcupine seabight (Webster et al., 2009),
nor did it in a shallower SMTZ’s (150-200 cmbsf) in the Black sea sediments (Knab et al., 2009). These observations probably reflect the fact that AOM mediating microrganisms have very low growth rate (Girguis et al., 2005; Nauhaus et al., 2007) and although they may metabolize substrates at high rates, they do not induce an overall cell number increase in sediment horizons where AOM occurs, at least in low methane flux environments.

3.4 Comparison with other mound settings

The recent drilling of the Challenger mound during the Integrated Ocean Drilling Program (IODP) Expedition 307 on the S.W. Irish margin (Ferdelman et al., 2006; Webster et al., 2009) constitutes a reference for comparison with the Gulf of Cadiz Alpha mound. Both mounds are outcropping structures built by reef forming cold water corals and lie in similar depths on the continental margin. Challenger mound is higher (130 m) and wider (1000 m at its base) than the Alpha mound in this study (30 m and 200 m respectively). In both locations, sulphate gradients from mound summit down to a SMTZ have been observed. In the Challenger mound however, the transition zone is wider (50 mbsf) and much deeper (150-200 mbsf) than in Alpha mound, and is located below the mound’s base. As a consequence, it has been suggested that AOM did not participate in the mound formation processes, and organoclastic SR may drive the carbonate diagenesis in the Challenger mound. In contrast, our results showed that AOM driven SR is an important microbial process in the on-mound location. Hence, Alpha mound represent a new mound type where reef-framework forming scleratinians and methane driven DIC production -and probably carbonate precipitation- both contribute to the mound processes.
CHAPTER II

4. Conclusion

To our knowledge, we describe here the first occurrence of a cold-water carbonate mound coinciding with current hydrocarbon migration. Methane migration and anaerobic oxidation in this mound markedly shaped the geochemistry of the sediments and participated to the DIC production in the on-mound location. Alpha mound therefore constitutes an original mound setting where methane-related diagenesis participated to the mound’s processes. Further studies on the Gulf of Cadiz carbonate mounds should provide new insights on the initiation and development of cold-water coral carbonate mounds in a context of hydrocarbon migration. In particular, the recovery of the entire mound sediment sequence and the mound base would allow to study the time scale of both coral development an hydrocarbon migration, and to examine if methane derived authigenic carbonate could facilitate the settlement of these corals. Besides, to generalize the present results to the entire mound, other on-mound coring is needed to assess the heterogeneity of such methane related processes within the mound. These questions are part of the rational for the recently submitted Integrated Ocean Drilling Program pre-proposal 673 for a scientific drilling of the Alpha mound: “Atlantic Mound Drilling 2: Morocco Margin”.

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5. Material and methods

5.1 Sampling procedure

For sulphate, methane and DIC measurements, three cores were taken in June 2004 during the oceanographic cruise PRIVILEGE by the R/V Marion Dufresne (Table II-1). On-mound, a large square-section (25x25 cm) Kasten type core (CASQ core MD04-2804) was recovered from the top of the Alpha mound. In addition, giant “calypso” piston cores were deployed at two reference locations: on the escarpment (off-mound core MD04-2809) and at the foot of the Pen Duick escarpment (off-escarpment core MD04-2806). The CASQ core was sampled within 4 hours upon recovery at deck ambient temperature (15°C). Piston cores were split in halves and sampled within 10 hours after recovery.

From each core, between 2 and 8 mL of pore-water were taken each 20 cm using Rhizon membranes (standard pore size 0.1 µm) (Seeberg-Elverfeldt, 2005) plugged into glass vacuum tubes (Eijkelkamp, Giesbeek, The Netherlands). Samples were then stored and transported at –20°C.

In addition, gravity core MSM1/3-294 was taken at the same on-mound location during the MSM1/3 cruise in May 2006, aboard the R/V Maria S. Merian. Upon recovery, the core was cut in sections and stored in a cold room immediately. Pore-water was extracted by plugging Rhizons membranes into predrilled sampling port in the plastic core liner. After pore-water collections, the core sections where stored at 4°C in trilaminate PE/Al/PE bags under anaerobic conditions for further in vitro studies in the home laboratory.
5.2 Gas analysis

For methane and light hydrocarbons measurements, a “headspace method” adapted to Rhizon sampling was used: each pore-water tube was thawed and shaken to allow dissolved gas to be transferred to the headspace. The tubes were weighed to determine the amount of pore-water collected. After equilibration of the headspace to ambient pressure with Nitrogen, 1 mL of headspace was injected in a gas chromatograph (Carlo Erba Instruments, Wigan, UK) equipped with a Varian PoraplotQ (25m x 0.53mm) column and a flame ionization detector. The column temperature was initially set at 30°C for 3 min. and was then increased to 90°C with a 30°C/min. rate, and finally to 190 °C with a 10°C/min. Headspace concentrations were determined using a standard mixture and pore-water gas concentrations were calculated according to the amount of pore-water collected in each tube. For dissolved inorganic carbon (DIC) measurements, 1mL of pore-water was acidified with 0.33 mL of phosphoric acid (Borowski, 1996) in 5 mL gas-tight argon-flushed bottles. 1 mL of the headspace was injected in a Shimazu gas chromatograph (GC-14B) equipped with a Hayesep D column (Alttech Associates, Lokeren, Belgium) and a thermal conductivity detector.

5.3 Carbon Isotopes

Delta$^{13}$C-CH$_4$ was measured from pore-water sample headspace, and $\delta^{13}$C-CO$_2$ from DIC bottle headspace. One mL of gas at the appropriate dilution was analyzed, using gas cryotraping and cryofocusing (and subsequent combustion to CO$_2$ for CH$_4$) on a trace gas preparation unit (AHCA-TGII, PDZ-Europa, UK) coupled to an isotope ratio mass spectrometer (IRMS) (20-20, Sercon, UK). The $\delta^{13}$C value of the laboratory reference was -36.9 ‰ and was measured against reference CH$_4$ (Air Liquide, Belgium) with a $\delta^{13}$C of -38.4
‰ determined gravimetrically. δ^{13}C results are expressed in the Vienna Pee Dee Belemnite (VPDB) scale.

### 5.4 Sulphate and sulphide concentration

Sulphate concentration in pore-water was measured by anion exchange chromatography (Gieskes, 1991) with a IC 761 (Metrohm, Herisau, Switzerland). The sulphide concentration was measured by the colorimetric method (: 0.5 mL of pore-water preserved in a zinc acetate solution was mixed with 0.4 mL of Cline Reagent under anoxic conditions. After 20 minutes, the optical density was measured at 670 nm (UVIKON930 Spectrophotometer).

### 5.5 Flux calculation

In order to estimate the flux of sulphate and sulphide in the three locations, the different gradients were considered linear, corresponding to steady-state situations. In this case, the estimated gradient obtained by linear regression of the curve is introduced in the Ficks’s first law:

\[
J = \phi D_s \frac{\Delta C}{\Delta x} \quad (1) \quad \text{With } D_s = \frac{D_0}{1 - \ln(\Phi^2)} \quad \text{ (equation 1)}
\]

Where \( J \) is the flux (mmol m\(^{-2}\) yr\(^{-1}\)), \( D_s \) is the sediment diffusion coefficient (cm\(^2\) yr\(^{-1}\)) corrected according to porosity and sea bottom temperature (Boudreau, 1997), \( D_0 \) is the molecular diffusion coefficient, \( \phi \) is the porosity of the sediments, and \( \Delta C/\Delta x \) is the estimated gradient (represented by dashed lines in pore-water profiles, Figure II-2). Porosity was determined from the weight loss per volume of wet sediment after drying at 60°C for 48 hours. Porosity values at the sulphate to methane transition zone were used for flux calculation.
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5.6 In vitro SR rates

Sediment slurries (12 mL, final sediment dilution 1/6) were prepared with sediment from core MSM1/3-294 from each 25 cm interval along the core, and each 10 cm within the sulphate to methane transition zone (SMTZ) according to methods described by (Nauhaus et al., 2002). Each slurry was incubated at 4°C in triplicate in presence of 5µl of $^{35}$S-SO$_4^{2-}$ (60 kBq) and dissolved methane as sole electron donor. After 118 days, sulphate turnover was determined by the cold distillation method according to (Treude et al., 2003)and (Kallmeyer et al., 2004). SR rates were calculated according to:

$$SRr = \frac{TRI^{35S}}{35SO_4^{2-} + TRI^{35S}} \times \left[ SO_4^{2-} \right] \frac{t}{t}$$

where TRI$^{35S}$ is the activity (in Bq) of total reduced inorganic sulphur intermediate, $^{35}$SO$_4^{2-}$ the activity of sulphate, $\left[ SO_4^{2-} \right]$ the sulphate concentration and t the incubation time in days. Results are given as potential rates per cm$^3$ of undiluted sediment.

5.7 Direct Cell Count

Acridine Orange Direct Counts (AODC) on sediment from core MSM1/3-294 was carried following the method described in (Fry, 1988): On board, 1 cm$^3$ sediment plug was taken with sterile 5 mL cut-off syringe and transferred in a sterile vial containing 4 mL of filter sterilized (0.2 µm) 2% formaldehyde in artificial seawater. In the laboratory, 15 µl of this slurry was filtered through a 0.2 µm black polycarbonate membrane (Millipore, Belgium) mounted on a filtration column. The sediment was then stained on the membrane with 1 mL of a 5 mg.L$^{-1}$ acridine orange solution (Sigma, Belgium) for 1 minute, and rinsed with 3 mL of sterile artificial seawater. The membrane was dried and mounted on a microscope slide with a minimum of paraffin oil. Slides were viewed under blue excitation light in an epifluorescence
microscope (Zeiss, Germany). A minimum of 100 cells was counted per membrane. At least two membranes per sample were prepared and results are given as average of duplicate counts.
ACKNOWLEDGMENTS

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AOM IN THE CARBONATE MOUND ALPHA
CHAPTER III. Heterogeneous Methane Geochemistry and Anaerobic Oxidation in the Carbonate-Sealed Darwin Mud Volcano, Gulf of Cadiz.

CHAPTER III
ABSTRACT

The Darwin mud volcano (MV) is a newly discovered cold seep in the Gulf of Cadiz (East Atlantic margin) that displayed a conspicuous structure. The MV crater was covered by a thick methane-derived carbonate platform of ca. 8000 m², colonized by chemosynthetic bivalves. Such structure prevented the methane-laden fluid to escape through the crater and induced seep relocation at the rim of the carbonate platform. There, strong spatial variations in methane flux and anaerobic oxidation (AOM) were observed, suggesting that relocation occurs through preferential channeling pathways. In particular, we identified a microbial activity hot spot bearing maximum AOM rate of 260 nmol cm⁻³ d⁻¹ at 1.7 cm below the sediment surface. This was twice as high than 2 m away, and three orders of magnitude higher than in other stations around the central crater. There, the AOM microbial community was dominated by ANME-2/DSS consortia that formed typical shell type aggregates, contrasting with other ANME cells that were present as single cells. Our seafloor and biogeochemical observations are consistent with the concept of cold seeps self-sealing by methane–derived carbonates, and a development model for this MV is proposed in the context of this hypothesis.
CHAPTER III

1. Introduction

In marine cold seeps, methane migrating toward sediment surface is oxidized by anaerobic oxidation of methane (AOM) coupled to sulphate reduction (SR):

\[ \text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HS}^- + \text{HCO}_3^- + \text{H}_2\text{O} \]  
\[ \text{equation 1} \]

The formation of a sulphate to methane transition zone (SMTZ) resulting from the local consumption of both substrates is a typical proxy for the presence and activity of AOM mediating microorganisms. Moreover, the production of bicarbonate and alkalinity during the AOM reaction supports carbonate precipitation (Berner, 1980):

\[ \text{Ca}^{2+} + 2 \text{HCO}_3^- \Leftrightarrow \text{CaCO}_3(s) + \text{CO}_2 + \text{H}_2\text{O} \]  
\[ \text{equation 2} \]

Authigenic methane-derived carbonate formation at cold seeps has been extensively studied (Greinert et al., 2002; Luff and Wallmann, 2003; Mazzini et al., 2004; Luff et al., 2005; Reitner et al., 2005; Stadnitskaia et al., 2008; Bahr et al., 2009): carbonate crusts are formed at the AOM activity front, and grow downward due to the activity of microbial mats carrying AOM on their lower surface. The accumulation of methane-laden fluid below the crust may lead to horizontal fluid migration followed by seep relocation at the rim of the crust (Naudts et al., 2010). Carbonate formations can also take the shape of slabs (Mazzini et al., 2004) or chimneys, depending on the structure of the sediment layers or fluid channeling pathways (Mazzini et al., 2008). In extended zones of high fluid flux, such mechanisms can lead to the formation of massive carbonate chemoherm (Bohrmann et al., 1998; Greinert et al., 2001).

AOM is mediated by Archaea closely related to methanogens of the orders *Methanosarcinales* (ANME-2 and -3) and *Methanomicrobiales* (ANME-1) often co-occurring in consortia with sulphate reducing bacteria (SRB) of the genera *Desulfosarcina/Desulfobulbus* (Knittel and Boetius, 2009, and reference therein). These observations led to the hypothesis of a syntrophic relationship between both cell types (Hoehler et al., 1994; Boetius et al., 2000).

In the Gulf of Cadiz, complex tectonic activity (Gutscher et al., 2002; Medialdea et al., 2009), olistostrome development (Maldonado and Nelson, 1999; Medialdea et al., 2009), and salt diapirism (Fernandez-Puga et al., 2007) are at the origin of hydrocarbon migration through the seafloor, and of the formation of numerous seep related structures (Somoza, 2003) such as mud volcanoes (MV) (Pinheiro, 2003; Van Rensbergen, 2005, Chapter IV and V; Niemann et al., 2006b), and hydrocarbon-derived carbonate (Diaz-del-Rio et al., 2003; Leon, 2006; Magalhaes
and Pinheiro, 2009). Recent exploration (Kopf et al., 2003; Masson et al., 2006; Weaver and Masson, 2007) shed light on the Darwin MV, a new seafloor structure at ~1100 m water depth that differs from other Gulf of Cadiz MV studied to date in exhibiting a massive carbonate platform in its center. Colonisation by chemosynthetic bivalves (Genio et al., 2008; Vanreusel et al., 2009) suggests that fluid migration and subseafloor microbial processes are still active.

The aim of this study is to assess the current geochemical and microbial activity in term of methane turnover at this original MV, to relate these to the microbial community structure and to the description of meiofaunal assemblages in the companion paper of (Pape et al., in press). We propose a development model for this MV that accounts for the different observations of Darwin MV and uses previous studies on carbonate development and fluid dynamics at cold seep.
2. **Results**

2.1 **Sampling site**

Darwin MV was located on the Moroccan Margin, at 1100 m water depth (Figure III-1A). This structure was mapped by high-resolution bathymetry using the remotely operated vehicle (ROV) ISIS during the JC10 cruise aboard the R/V James Cook in 2007. It was 55 m high, 900 m wide at the base and had a distinct “crater” of about 100 m in diameter. Its structure was markedly different from other MV’s described thus far in the Gulf of Cadiz in that a thick carbonate platform covered the crater (Figure III-1B). Chemosynthetic bivalves (*Bathymodiolus mauritanicus*, (Genio et al., 2008)) colonized the numerous fissures between slabs (Figure III-1C) and boulders (Figure III-1D). In addition, white filamentous mats were occasionally observed in the fissures and in the West Rim Site. Coring the MV centre was not possible due to the presence of such hard ground, and only North, South and West Rim sites were sampled (Figure III-1B and Table III-1).

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**Figure III-1** Location, map and observation of the Darwin MV. (A.) The Darwin MV is located in the Gulf of Cadiz, on the Moroccan margin at 1100 m water depth. (B.) The positive relief structure of the Darwin MV shown in the high-resolution bathymetry map. The white circle broadly indicates the location of thick carbonate platform covering the crater center. (C.) Fissures between carbonate slabs are colonized by living chemosynthetic bivalves (in black) and empty shells (in white). (D.) Carbonate boulders in the crater center.
At this latter station, a small sediment area (100 cm²) was covered with white filamentous mats, and coring by mean of a small push cores deployed with the ROV provoked a strong release of gas bubbles from the sediment (video available online \(^1\), \(^2\)). This site, called “Seep site” in a companion paper studying the meiofaunal assemblage at Darwin MV (Pape et al., *in press*), was surrounded by shell and *Lophelia*-like coral debris. A second push core was taken 2 m away of the Seep site, as in the study of Pape et al. and a reference site located at 1 km was sampled for comparison with non-seep sediments.

<table>
<thead>
<tr>
<th>Station</th>
<th>Core Name</th>
<th>Core Type</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Depth (m)</th>
<th>Length (cm)</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference site</td>
<td>JC10-26</td>
<td>Mega Core</td>
<td>35°23.965N</td>
<td>7°11.121W</td>
<td>1145</td>
<td>36</td>
<td>GC (CH₄, H₂, SO₄, HS⁻, Ac), AOM, SR, Ac-MG, Meth-MG, H-MG</td>
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<tr>
<td>JC10-25</td>
<td>Piston Core</td>
<td>35°23.965N</td>
<td>7°11.120W</td>
<td>1145</td>
<td>507</td>
<td>AOM, SR, Ac-MG, Meth-MG, H-MG</td>
<td></td>
</tr>
<tr>
<td>Black Spot (BS)</td>
<td>JC10-036-PUC5</td>
<td>ISS-Push Core</td>
<td>35°23.541N</td>
<td>7°11.508W</td>
<td>1111</td>
<td>20</td>
<td>GC⁺ (CH₄, SO₄, HS⁻), AOM, SR, Etane (H₂), CARD-FTSY</td>
</tr>
<tr>
<td>2m from BS</td>
<td>JC10-036-PUC12</td>
<td>ISS-Push Core</td>
<td>35°23.541N</td>
<td>7°11.509W</td>
<td>1111</td>
<td>11</td>
<td>GC⁺ (CH₄, SO₄, HS⁻), AOM, SR</td>
</tr>
<tr>
<td>South Rim</td>
<td>JC10-038</td>
<td>Piston Core</td>
<td>35°23.473N</td>
<td>7°11.493W</td>
<td>1105</td>
<td>78</td>
<td>GC (CH₄, H₂, SO₄, HS⁻, Ac), AOM, SR, Ac-MG, Meth-MG, H-MG</td>
</tr>
<tr>
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<td>Mega Core</td>
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<td>7°11.454W</td>
<td>1104</td>
<td>22</td>
<td>GC (CH₄, H₂, SO₄, HS⁻, Ac), Ac-MG, Meth-MG, H-MG</td>
</tr>
<tr>
<td>JC10-039</td>
<td>Gravity Core</td>
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<td>7°11.450W</td>
<td>1104</td>
<td>43</td>
<td>GC (CH₄, H₂, SO₄, HS⁻, Ac), Ac-MG, Meth-MG, H-MG</td>
<td></td>
</tr>
</tbody>
</table>

Table III-1 Coordinates and features of the cores used in this study, with the overview of the analysis performed on each core. Geochemistry (GC): porewater concentration of methane, hydrogen, sulphate, sulphide and acetate. AOM: anaerobic oxidation of methane. SR: sulphate reduction. Ac-MG: acetotrophic methanogenesis. Meth-MG: methylotrophic (methylamine) methanogenesis. H-MG: hydrogenotrophic methanogenesis. *: porewater geochemistry data reproduced from Pape et al. (*in press*).

### 2.2 In western Rim at the Seep site

At the BS site, AOM activity was present from sediment surface down to 16.5 cm below the seafloor (cm bsf) (Figure III-2). Volumetric AOM rates (AOMR) were higher near the surface (260 nmol cm⁻³ d⁻¹ at 1.7 cm bsf) and broadly decreased with depth with scattered values. SR activity followed a similar trend, with maximum SR rates (SRR) near the surface (197 nmol cm⁻³ d⁻¹ at 1.7 cm bsf). Below, SRR decreased with depth to a minimum rate of 8.2 nmol cm⁻³ d⁻¹ at 18.5 cm bsf. Depth integrated rates ∑AOMR and ∑SRR were 4197 and 2477 mmol m⁻² yr⁻¹ respectively over the core length. At the same BS site, Pape et al. (*in press*) reported a steep sulphate concentration gradient between the surface (29 mM) down to the 11 cm bsf (3 mM) indicating a downward sulphate flux, and a methane gradient toward surface between 7 cm bsf (1 mM) and 1 cm bsf (0.1 mM) indicating an upward methane flux. Below, methane values were more scattered, probably due to degassing during core recovery.

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\(^1\) http://www.youtube.com/watch?v=K-U8sQ5nAxs&feature=channel

\(^2\) http://www.youtube.com/watch?v=ZCzZeCqeehQ
Figure III-2 Geochemistry and microbial activity at the Seep site. SR and AOM activity (grey area). Note the methane, sulphate and sulphide profiles are from a second push core located ~ 20 cm away from the core used for activity measurements; geochemical data are reproduced from Pape et al. (in press).

Bacterial and Archaeal clone libraries were constructed from sediment in the 1-2 cm bsf interval, where AOMR and SRR were highest. In general, bacterial diversity was high, with clones belonging to 9 different phyla, and with most OTU only containing one single representative sequence. 15/67 sequences belonged to *Deltaproteobacteria* (Figure III-3), with sequences falling in the Seep SRB1-a and -e groups (Schreiber et al., 2010), and in the short chain hydrocarbon oxidizing (SCHO) SRB (Kniemeyer et al., 2007) closely related to *Desulfosarcina* (DSS). In addition, 7/61 clones were closely related to aerobic methanotrophs.
Figure III-3 Phylogenetic tree showing the affiliation of deltaproteobacterial sequences. The tree was based on a set of 1026 nearly full-length 16S rDNA sequences (>1400 bp) affiliated with Deltaproteobacteria and that had a high alignment score. The tree was reconstructed with the maximum likelihood method, in combination with filter excluding the most variable position among reported Deltaproteobacteria sequences. Calculations were performed on the PhyML server (Guindon et al. 2010) using the aLRT branch support calculation method.
Figure III-4 Phylogenetic tree showing the affiliation of bacterial sequences (except Deltaproteobacteria). The tree was obtained by inserting clone library bacterial sequences and their close relatives into the All-Species Living Tree LTP100 release (Yarza et al., 2008; 2010) using parsimony criteria and without allowing changes in the overall tree topology. A filter excluding the most variable position was used.

(Methylococacceae), and belonged to the HMMV Meth I, Meth II and MPH groups (Losekann et al., 2007) (Figure III-4). Most known anaerobic methanotrophs (ANME) were represented in the archaeal clone library (Figure III-5): ANME-2a (13/32) and -2c (2/36) (Boetius et al., 2000), ANME-1b (6/36) (Hinrichs et al., 1999) and ANME-3 (1/36) (Niemann et al., 2006a). Remaining Euryarchaeota belonged to the GoM Arc 1 group (1/36), Marine Benthic Group E (1/36), Deep Sea Euryarchaeotic Group (DSEG, 1/36). Crenarchaeal sequences (4/36) were all closely related to the Nitrosopumilus maritimus isolate (Konneke et al., 2005).
Figure III-5 Phylogenetic tree showing the affiliation of archaeal sequences. The tree was based on a set of 365 nearly full-length (>1400 bp) 16s rDNA genes with a high alignment score and was reconstructed using the maximum likelihood method, in combination with filter excluding the most variable position among reported archaeal sequences. Calculations were carried on the PhyML server (Guindon et al., 2009).

In the same sediment horizon, the presence and distribution of ANME cells and their putative bacterial syntrophic partner were investigated by CARD-FISH labeling and microscopic observations (Figure III-6). Most archaean cells were involved in shell-type clusters, typically forming the core of these aggregates (Figure III-6A). Using group-specific probes, cells forming the inner core of these consortia were identified as ANME-2 cells (Figure III-6B and C), whereas...
DSS cells were forming an outer layer (Figure III-6D). ANME-1 positive cells were present, with a coccoid shape (Figure III-6E). ANME-3 cells were rarely observed and were found as single or paired cells (Figure III-6F).

Figure III-6 Epifluorescence microscopy imaging of selected groups from the Seep site microbial community (1-2 cm bsf). DAPI stained cells appear in blue in each panel. Positive CARD-FISH cells (B to F) appear in green. (A) FISH stained cells stained with the Cy3 monolabeled Arc-915 probe (red) specific for the domain Archaea. CARD-FISH stained cells with (B) the Arc-915 probe, (C) the EelMS-932 probe specific for ANME-2 cells, (D) the DSS probe specific for *Desulfosarcina/Desulfoococcus* including most Seep SRB cells, (E) the ANME1-350 probe specific for ANME-1 cells, and (F) the ANME3-1249 probe specific for ANME-3 cells. Scale bar: 5 µm.
2.3 In western rim, 2 m away of the Seep site

At short distance from the Seep site (2m), both AOM and SR activities were lower next to the sediment surface and increased with depth, reaching maximum activity at 11.5 cm bsf (Figure III-7). At this depth, AOMR was of 88 nmol cm\(^{-3}\) d\(^{-1}\) and SRR of 148 nmol cm\(^{-3}\) d\(^{-1}\), which translated into \(\sum\)AOMR of 1169 mmol m\(^{-2}\) yr\(^{-1}\) and \(\sum\)SRR of 1075 mmol m\(^{-2}\) yr\(^{-1}\). Pape et al. (in press) reported sulphate, sulphide and methane profiles in an adjacent core. As for the BS site, these are reproduced here as an indication of the probable geochemistry in these sediments.

![Figure III-7 Geochemistry and microbial activity 2m away of the Seep site. SR and AOM activity (grey area). Note that methane, sulphate and sulphide profiles are from a second push core adjacent from the core used for activity measurements; the geochemical profiles are reproduced from Pape et al. (in press).](image)

2.4 South Rim

At the Southern Rim Site (Figure III-8), sulphate and methane concentrations formed a sulphate to methane transition zone (SMTZ) between 45 and 60 cm bsf: in this interval, sulphate decreased from 24 mM to 0.25 mM and methane increased from 0.01 mM to 0.58 mM. Sulphide concentration was maximum within the SMTZ (1.6 mM at 64 cm bsf) and steeply decreased above and below. Peaks of both AOM and SR activity were present in the SMTZ with maximum values of 2.4 and 0.7 nmol cm\(^{-3}\) d\(^{-1}\) respectively at 51 cm bsf. Within the SMTZ, \(\sum\)AOM and \(\sum\)SRR were of 17.3 and 44 mmol m\(^{-2}\) yr\(^{-1}\). Above the SMTZ (0-45 cm bsf), sulphate concentration remained close to seawater values, between 24 and 28 mM, and SR was present with scattered rates between 0 and 3.5 nmol cm\(^{-3}\) d\(^{-1}\). There, methane was below 0.01 mM and AOMR were very low or zero. In this interval, \(\sum\)SRR was of 109.8 mmol m\(^{-2}\) yr\(^{-1}\), which translates into an overall \(\sum\)SRR of 153 mmol m\(^{-2}\) yr\(^{-1}\) in this site. Methanogenesis (MG) with
AOM ACTIVITY AT DARWIN MV

(H-MG) or acetate (Ac-MG) was absent in this site, in spite of the presence of these substrates: an H2 concentration peak of 230 nM was present at 58 cm bsf, and acetate concentration was between 22 and 51 µM throughout the core. Methylamine methanogenesis (Met-MG) was only present above the SMTZ with maximum rates of 5.3 pmol cm⁻³ d⁻¹.

Figure III-8 Geochemical and microbial activity profiles at the South Rim. AOM, SR and MG microbial activity rates (grey area) are plotted with their respective substrates (circles), i.e. sulphate, methane, H₂, and acetate. DIC is expressed as dissolved CO₂ (squares). Meth-MG rates were calculated based on an arbitrary methylamine concentration of 5µM, and should thus be considered as potential rates.

2.5 North Rim

At Northern Rim site (Figure III-9), no SMTZ was observed, as sulphate concentration remained constant with depth, methane concentration was very low, between 0.5 and 2.5 µM, and sulphide concentration was below detection limit at all depths. Acetate concentration linearly increased with depth from 11.5 to 27.5 µM, at the exception of a peak of 68 µM at 37.5 cm bsf. Significant methanogenesis was present at this site with H-MG rates of 6 pmol cm⁻³ d⁻¹ at 7 cm bsf and of 1 pmol cm⁻³ d⁻¹, and Met-MG rates of 4.6 pmol cm⁻³ d⁻¹ at 17.5 and 1.4 pmol cm⁻³ d⁻¹ at 27.5 cm bsf, but no Ac-MG was observed.
Figure III-9 Geochemical and microbial activity profiles at the North Rim. Empty symbols: surface sediment recovered with a multicore. Plain symbol: data from the gravity core at the same site. Sulphate, methane, sulphide and DIC (expressed as dissolved CO$_2$) concentration profiles. MG microbial activity rates (grey area) are plotted with their respective substrates (circles), i.e. H$_2$, and acetate. Meth-MG rates were calculated based on an arbitrary methylamine concentration of 5µM, and should thus be considered as potential rates.

2.6 Reference Site

At the reference site, sulphate concentration decreased linearly with depth from 27 mM at sediment surface down to 13.5 mM at 480 cm bsf. In this interval, SRR remained below 1 nmol cm$^{-3}$ d$^{-1}$, and sulphide concentration was below detection limit. Methane concentration was very low and decreased from deep sediments (0.97 µM at 480 cm bsf) to sediment surface (0.09 µM), with AOMR values close to detection limits (max. rate of 0.04 nmol cm$^{-3}$ d$^{-1}$) as $^{14}$C-CH$_4$ turnover was close to turnover blank values at this site. H$_2$ and DIC were detected throughout the core, with maximum concentration of 7280 nM at 169 cm bsf and 1.8 mM at 474 cm bsf respectively. However, no H-MG activity was observed at all depths. Similarly, in spite of acetate being present throughout the core (15-33 µM), a single peak of Ac-MG of 0.11 pmol cm$^{-3}$ d$^{-1}$ was present at 30 cm bsf. No Met-MG activity was detected in this site.
Figure III-10 Geochemical and microbial activity profiles at the Reference site. Empty symbols: surface sediment recovered with a multicorner. Plain symbol: data from the gravity core at the same site. AOM, SR and MG microbial activity rates (grey area) are plotted with their respective substrates (circles), i.e. sulphate, methane H$_2$, and acetate.
CHAPTER III

3.  Discussion

3.1  AOM activity distribution

At the seep site, AOM and SR rates were the highest measured in the Gulf of Cadiz with maximum volumetric rates one order higher than rates measured at the crater center of Capt. Aryutinov MV ((Niemann et al., 2006b) and Chapter IV) or Carlos Ribeiro MV (Chapter V). The relatively lower AOM and SR activity 2 m away from the seep site was consistent with the observation of a strong horizontal geochemical gradient at this site (Pape et al., in press), as methane and sulphide were absent 5 and 10 m away from the seep site. In addition, these results support the conclusion of Pape et al. who proposed that a higher methane flux and AOM activity could account for the differences of meiofauna composition and distribution between Seep site and 2 m from Seep site. Moreover, AOM activity at Southern Rim site was three orders lower than at the seep site and no indication of AOM activity was detected in the North rime site, indicating that methane flux and its consumption in subsurface sediments strongly varied at both the meter and decameters scales around the carbonate platform covering the MV center. These observations are in sharp contrast with the typical MV structure of a focused fluid flow at the MV crater center, with methane flux and AOM activity decreasing toward the crater rim. Such structure is illustrated by the studies of the Carlos Ribeiro MV (Vanneste et al., 2011) and Chapter V). The differences between sites that are equally distant from the MV center are interpreted as a result of focused fluid flow through discrete channeling pathways towards the rim of the MV.

3.2  Microbial community structure at the seep site.

In the high AOM activity sediment at the seep site, members of the ANME-2 phylotype dominated the archaeal community, but most other known methanotrophs were present. In addition, sequences belonging to the putative syntrophic SRB partner of ANME-2 cells (Seep SRB1, Knittel et al., 2005; Schreiber et al., 2010) were also found. These results are consistent with the ANME ecotype hypothesis of a single ANME phylotype dominating a given AOM community (Knittel and Boetius, 2009). All the ANME-2 cells were involved in dense shell type aggregates, whereas the more rare ANME-3 and ANME-1 positive cells were found as single cells. The factors triggering the formation of dense consortia are unknown, but Alperin and Hoehler (2009) predicted that the presence of organic matter fermentation end products such as H₂ and acetate could play an important role. Since elevated concentration of these end products
could make methanotrophic activity endorganic, the presence of SRB cells that can efficiently scavenge these products could promote the formation of such shell type aggregates. Although these end products were not quantified at the seep site, the high microbial and meiofaunal biomass are good proxies for the presence of organic matter and our observations are in line with the Alperin and Hoehler hypothesis. The presence, in the same sediments, of aerobic methanotrophs closely related to the ones found in the surface of Håkon Mosby Crater Center (HMMV Met I, II and MPH groups) was remarkable, as these usually do not coexist with the oxygen sensitive ANME cells (Boetius and Joye, 2009). Similarly, sequences closely related to Nitrosopumilus maritimus, an Archaeon that uses oxygen for ammonium oxidation, were also found. The presence of both cell types can be explained either by the reworking of surface sediments during sampling, or by the presence of aerobic micro-niches in the upper 1.5 cm sediments layer, possibly due to bioturbation by the dense meiofauna at the Seep site surface sediment (Pape et al., in press).

3.3 Activity coupling in Darwin MV sediments.

The AOM reaction (equation 1) predicts that methane and sulphate turnover should follow a 1:1 ratio in the AOM zones of cold seeps. In practice, SRR often exceeds AOMR due to the presence of alternative electron donors for sulphate reduction, such as non-methane hydrocarbons present in migrating fluid, or sedimentary organic matter (Bowles et al., 2011). At Seep site and 2m from the Seep site, both AOM and SR activity profiles showed similar trends, and 2m from the Seep site, the $\sum_{SRR} : \sum_{AOM}$ ratio was 1.08. These observations indicated a strong coupling between both AOM and SR activities. However, at Seep site, AOM exceeded SR with a $\sum_{SRR} : \sum_{AOM}$ ratio of 0.59, thus deviating from the 1:1 ratio predicted by AOM stoichiometry. This could potentially be due to strong horizontal variations of the methane flux, leading to different geochemistry and activities in sediments sampled for AOM and SR rates measurements. At South Rim site, SR activity in the first 50 cmbsf was not coupled to AOM and was interpreted as organotrophic sulphate reduction. Below, both AOM and SR peaks coincided with a $\sum_{SRR} : \sum_{AOM}$ ratio of 8.3 close the average ratio of 10.7 for methane seep site (Bowles et al., 2011). This indicated that AOM only partly accounted for the overall SR activity and downward sulphate flux at the South Rim site. The remaining sulphate flux is attributed to organoclastic sulphate reduction, as shown by the presence of AOM-independent SR activity above the AOM zone.
Besides, several in vitro studies have shown that AOM, in spite of a net methane consumption, may also produce methane at a rate of ~10% of the AOM rate either in situ (Orcutt et al., 2005) or in vitro (Treude et al., 2007; Orcutt et al., 2008b; House et al., 2009). The absence of methane formation in the AOM zone of the South Rim site indicated that this AOM side reaction does not necessarily occur. These results were similar to those obtained in other Gulf of Cadiz MV such as Capt. Arutyunov MV, Mercator MV (Chapter IV) and Carlos Ribeiro (Chapter V). This ability to both oxidize and produce methane does not seem to depend on ANME types present, as these MV were dominated by different ANME cells (ANME-1 at Mercator MV, ANME-2 at CAMV and ANME-2 and 3 at CRMV), and could thus be rather triggered by environmental factors. At Darwin MV, methane production was rather interpreted as conventional methanogenesis. Since methylamine is a non-competitive MG substrate (Oremland and Polcin, 1982), the presence of Met-MG activity in the sulphate zone at South and North Rim sites was coherent. However, H-Mg activity at the North Rim site, and Ac-MG at the reference site were more unexpected as sulphate reducers usually outcompete methanogens for these substrates (Kristjansson et al., 1982; Schonheit et al., 1982)

3.4 Proposition of a development model for the Darwin MV

Typical MVs, such as Carlos Ribeiro and Capt. Arutyunov MVs (next chapters), have a rather predictable physical and biogeochemical structure: deep rooted-fluid migrating through a central conduit formed a mud dome on the seafloor. Concentric rims formed by previous eruptive events surround a “crater eye” indicating the most recent mud eruption. Both geochemical and ex-situ rates measurement (Vanneste et al., 2011), Chapter IV and V- have shown that such structure was correlated with decreasing methane flux along a radial axis from the crater eye towards the crater rim. The Darwin MV obviously deviated from this model and the fluid dynamics in this MV was less predictable. Based on the current knowledge on methane-derived carbonate formation at cold seep sites, we propose a development model for the Darwin MV that can account for previous observations of this structure and for the methane flux and microbial activity distribution described in this study. We posit that intense methane migration first occurred in the crater center. Its anaerobic oxidation by AOM microorganisms led to the formation of methane derived carbonate crust and boulders as observed on the MV center, which then developed into a compact chemoherm, as for instance in the Hydrate Ridge (Greinert et al., 2001). The methane origin of the carbonate matrix, demonstrated by its low δ13-C signature
(Nekhorosheva and Blinova, 2009), is supporting this interpretation. Intense AOM activity at the base of the carbonate layer would lead to sulphide production and the development of a chemotrophic fauna at the surface, such as the dense *Bathymodiolus* fields (Genio et al., 2008; Rodrigues et al., 2010). As carbonate crust developed downward (Reitner et al., 2005; Mazzini et al., 2008), the sulphate flux through the carbonate diminished, preventing further methane oxidation at the base of the crust. The concurrent decrease of upward sulphide flux triggered the decline of the *Bathymodiolus* population. Overpressure due to fluid accumulation below the chemoherm, possibly at the origin of the observed fissure network, provoked the relocation of a major part of the fluid flux toward the rim of the chemoherm through discrete channels. Such fluid dynamics would follow the mechanism described by (Naudts et al., 2010) of cold seeps on the New Zealand margin. In this study, the authors identified different stages of methane-derived carbonate formation. The methane flux relocation was clearly visible in the form of gas bubble formation at the rim of the carbonate crusts exposed on the seafloor. Such mechanism could thus explain the high and focused methane flux and the high AOM activity at the Seep site, the relatively low AOM activity at the South site, and the apparent lack of significant methane flux at the North rim site.
CHAPTER III

4. Conclusion

This study of the Darwin MV provides elements of the functioning of singular cold seep covered by a thick carbonate layer and illustrates the “self-sealing” nature of some of these structures (Hovland, 2002). This peculiar structure may explain the strong horizontal variations of methane flux and AOM activity by focusing the migrating fluid flux to discrete hot spot at the rim of the carbonate chemoherm. Such seep relocation may lead to very high microbial activity, as in the Seep site and 2m away, with the highest AOM rates and methane turnover measured thus far in the Gulf of Cadiz. The wealth of chemosynthetic fauna shells on the MV center, with only few living specimen, has been interpreted as a decline of the fluid emission at this site (Vanreusel et al., 2009). We argue that the global activity of this MV is difficult to estimate due to its structure, and would require an intensive sampling effort around the rim in order to provide global estimate of fluid emission and methane turnover.
CHAPTER IV. Anaerobic oxidation of methane in hypersaline cold seep sediments.

Submitted to Applied and Environmental Microbiology as L. Maignien, R. J. Parkes, B. Cragg, H. Niemann, K. Knittel, S. Coulon, A. Akhmetzhanov, P. Weaver, N. Boon. Anaerobic oxidation of methane in hypersaline sediments of Mercator Mud Volcano in the Gulf of Cadiz
ABSTRACT

Life in hypersaline environments could be limited by bioenergetic constraints. Microbial activity at the thermodynamic edge, such as the anaerobic oxidation of methane (AOM) coupled to sulphate reduction (SR) is thus unlikely to thrive in these environments. In this study, carbon and sulphur cycling were investigated in the hypersaline cold seep sediments of Mercator mud volcano (MV) and compared to the adjacent non-hypersaline Captain Arutyunov MV. AOM activity - albeit partially inhibited- was still present at a salinity of 292 g L\(^{-1}\) (ca. 8 fold seawater concentration) with rates of 2.3 nmol cm\(^{-3}\) d\(^{-1}\), and even in saturating conditions with rates of 0.5 nmol cm\(^{-3}\) d\(^{-1}\). Methane and evaporite-derived sulphate co-migrated in these sediments, which in combination with the partial inhibition, resulted in an AOM activity that was spread over wide depth intervals. Up to 79% of total cells in the AOM zone were identified as ANME-1 by fluorescence in situ hybridisation (FISH). No other ANME type could be detected either by FISH or in 16s rDNA gene libraries, indicating a possible salinity selection toward ANME-1. The depth distribution of methanogenesis rates did not match AOM rates nor ANME-1 cells counts, thus arguing for a strict methanotrophic role of these cells in Mercator MV sediments. Most ANME-1 cells formed monospecific chains and were generally distant from sulphate reducing bacteria or other cells. At all sites, AOM activity co-occurred with SR activity and sometimes significantly exceeded it. Possible causes of these unexpected results are discussed. This study demonstrates that in spite of a low energy yield, microorganisms carrying AOM can thrive in salinity up to saturation.
CHAPTER IV

1. Introduction

Marine mud volcanoes (MV) are formed by the extrusion of fluidized sediments of deep origin to the sea floor (Milkov, 2000; Dimitrov, 2002; Kopf, 2002; Niemann and Boetius, 2010). Erupted material (mud breccia) often contains high amounts of methane, and occasionally higher hydrocarbons (Kopf, 2002 and reference therein). The migration of hydrocarbons towards the sediment surface typically fuels a great variety of microbes and symbiotic fauna that harvest energy from these reduced molecules, directly or indirectly, using seawater electron acceptors (e.g. O2, NO3, SO4). Hydrocarbon seepage therefore promotes the development of thriving cold-seep ecosystems, which support high standing stocks of free living and symbiotic chemosynthetic microbes. One of the most important processes at cold seeps is the anaerobic oxidation of methane (AOM) coupled to sulphate reduction (SR) (Knittel et al., 2005), and references therein:

\[
\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HS}^- + \text{HCO}_3^- + \text{H}_2\text{O} \quad \Delta G^0 = -16.9 \text{kJ mol}^{-1}
\]

On a global scale, this reaction retains more than 80% of uprising CH4 in the sea floor (Reeburgh, 2007), and references therein.

AOM is performed by three groups of Euryarchaeota related to methanogens: ANME-1, ANME-2, and ANME-3 (Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001b, 2002b; Knittel et al., 2005; Niemann et al., 2006a). These anaerobic methanotrophs often form consortia with sulphate-reducing bacteria (SRB) of the genera Desulfosarcina / Desulfococcus (ANME-1 and -2) or Desulfobulbus (ANME-3) (Knittel et al., 2003; Losekann et al., 2007). Based on these observations, a syntrophic relationship between these microorganisms has been postulated (Boetius et al., 2000; Orphan et al., 2001a) with an up to now unidentified Interspecies Transfer Agent (ITA) mediating electron transfer from methane oxidising ANME cells to their sulphate-reducing partners. The typical geochemical signature of AOM activity in marine sediments is the presence of a so-called sulphate-methane transition zone (SMTZ), i.e. opposed gradients of downward diffusing sulphate and upward migrating methane (Reeburgh, 2007). Microorganisms mediating AOM are usually present and active in a narrow depth interval at the interface between these gradients (Knittel and Boetius, 2009). In addition to this sulphate dependent mode, AOM may also be coupled to the reduction of oxidised metal species such as Fe(III) and Mn(IV) (Beal et al., 2009) and N species (Raghoebarsing et al., 2006; Hu et al., 2009; Ettwig et
al., 2010). So far, evidence for an environmental significance of these pathways, particularly in marine environments, is missing.

Based on an inventory of known metabolisms able to thrive in hypersaline environments, it has been proposed that the upper limit of salt concentration at which energy conservation can occur “primarily depends on bioenergetic constraints”, and secondly on the “mode of osmotic adaptation used” (Oren, 1999; Oren, 2011). Balancing cell osmotic pressure by active ion pumping or the production of compatible solutes is indeed energetically costly and apparently excludes dissimilatory pathways characterized by low energy yields. From an energetic standpoint, it is thus surprising that a few studies could detect AOM in hypersaline environments (Oren, 2011). Yet, the influence of salinity on AOM activity and microbial community composition is not well constrained. One study, for instance, found geochemical and molecular evidence for AOM activity mediated by ANME-1/SRB in hypersaline sediment of the Gulf of Mexico (Lloyd et al., 2006), while a recent study on a deep-sea brine pool and a MV, in contrast, suggested that presence of hydrogen prevented AOM activity in spite of the presence of both methane and sulphate (Joye et al., 2009). However, circumstantial evidence indicate a dominance of ANME-1 over the other ANME groups in hypersaline settings, thus suggesting that this clade might be more tolerant to high salinity (Daffonchio et al., 2006; Harrison et al., 2009; Niederberger et al., 2010; La Cono et al., 2011). Nevertheless, significant ANME-1 populations are also found in non-hypersaline methane seeps, e.g. in the Eel River Basin, the Black sea microbial mats, the Hydrate Ridge, or the Tommeliten seeps (Addendum II, p.114, and (Orphan et al., 2002a; Knittel et al., 2005; Niemann et al., 2005; Treude et al., 2005c).

Recently, an hypersaline mud volcano (named Mercator MV after the Belgien cartographer) was discovered by Van Rensbergen et al. (2005) in the Gulf of Cadiz, East Atlantic Large amounts of methane were reported (Nuzzo et al., 2009; Scholz et al., 2009) in this structure. This hence provides a very good opportunity to study the microbial ecology of methane and sulphur cycling in an extreme hypersaline environment. Here, we document for the first time the microbial activity and community structure in relation to geochemical parameters at Mercator MV, with the aim to understand the functioning of AOM communities under extreme hypersaline conditions. Our results are compared with those obtained from Captain Arutyunov MV (CAMV), a non-hypersaline MV in the vicinity of the Mercator MV.
CHAPTER IV

2. Results

2.1 Geochemistry

Mercator MV was sampled in the crater centre as well as at three rim sites (Figure IV-1 and Figure IV-7). At Mercator MV crater centre site, gas was venting from the seafloor and large halite (NaCl) and gypsum (CaSO₄) crystals were recovered from the sediments between 150 and 200 cm below the sea floor (cmbsf). CAMV was sampled in the crater centre site. For comparison with non MV sediments, a reference station was sampled 2.4 km N.-E. of Mercator MV. Details of the high-resolution bathymetry method, sampling sites description and coring devices are given in supplementary information.

![Figure IV-1 Study location and sampling stations. (A) Gulf of Cadiz bathymetric map showing the location of the two mud volcanoes investigated in this study. (B) High-resolution bathymetric maps of the Mercator mud volcano, located in the shallow region of the Gulf of Cadiz. This MV was cored in the Crater Centre (JC10-09 and -19), Northern Rim (JC10-11), and Southern Rim (JC10-13 and -15). Depth range: -350 m (yellow), -500 m (blue).](image)

The salinity in the sediments of Mercator MV and CAMV was approximated by the chloride concentration (Figure IV-2). At the Mercator MV crater centre (cores JC10-09 and -19), chloride concentration at 10 cmbsf was already 3.5-fold (2011 mM) higher than seawater values and further increased with depth along concave up gradients, up to halite saturation level (~5800 mM) at 220 cmbsf. At the rim stations (cores JC10-11, -13 and -15), chloride concentration formed a quasi-linear gradient with depth from seawater concentration (~580 mM) at the surface up to 3213 mM at 67.5 cmbsf. Both at the reference station (cores JC10-02 and -04) and CAMV
crater Centre (core JC10-66), chloride concentration reflected seawater concentration at the sediment surface and remained constant with depth.

<table>
<thead>
<tr>
<th>Location</th>
<th>Station</th>
<th>Core Name</th>
<th>Core Type</th>
<th>Latitude</th>
<th>Longitude</th>
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<th>Length (cm)</th>
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<td>6°40.251W</td>
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</tbody>
</table>

Table IV-1 Sampling stations. Size, stations location and core description in the Mercator MV and the Captain Arutyunov MV (CAMV)

Three methane rich habitats with decreasing salt contents (chlorinity gradients) were selected for further detailed studies on geochemistry and microbial activity: (i) hypersaline sediments (core JC10-19) hereafter named Mercator MV Crater Centre, (ii) sediments with lower but still elevated salinity (cores JC10-13 and JC10-15) hereafter named Mercator MV Rim 1 and Rim 2 respectively, and (iii) sediments with regular seawater salinity (core JC10-66) hereafter named CAMV Crater Centre.

![Figure IV-2 Sediment salinity. Comparison of chloride concentration profiles used to estimate salinity in the sediments of Mercator MV and CAMV.](image-url)
CHAPTER IV

**Methane** – At all three locations, an upward migration of methane in the sediment was indicated by elevated methane concentrations in deeper section of the cores, generally decreasing toward surface (Figure IV-3). These results are in agreement with previous publications on the geochemistry of the chosen structures (Hansen et al., 1998; Van Rooij, 2005; Niemann et al., 2006b; Nuzzo et al., 2009; Scholz et al., 2009). At Mercator MV Crater Centre, methane concentration decreased from 3 mM at 75 cmbsf to 0.6 mM at the sediment surface. At Mercator MV Rim 1, the methane decreased from 0.23 mM at 21 cmbsf to 5.10^{-3} mM at 11.5 cmbsf, and similar trend was observed at Mercator MV Rim 2. At CAMV Crater Centre, methane concentration steeply decreased from 1.2 mM at 26.5 cmbsf to near background concentrations at 21.5 cmbsf, and methane was depleted in the upper 20 cm of the core.

**Sulphate** - Sulphate concentration was close to seawater values at the sediment surface (28-30 mM) in all cores (Figure IV-3). At Mercator MV, sulphate first decreased with depth down to 13.2 mM at Crater Centre or to 24.4 mM at Rim 2 (at 162 cmbsf and 42 cmbsf respectively), but then increased again with depth, reaching maximum values of 18.5 and 28.3 mM respectively. At Mercator MV Rim 1, sulphate concentration remained constant down to 28 cmbsf. At CAMV crater Centre, sulphate concentration steeply decreased and reached 2.8 mM at 22.5 cmbsf, and was below detection limit below 55 cmbsf.

**Sulphide** - At all Mercator MV sites, we could not detect any soluble sulphide (ΣH2S, HS-, S2-) (data not shown), although the decreasing sulphate contents and sulphate reduction rates (next section), indicated sulphide production. However, at least some of the sulphide could be precipitated because acid volatile sulphide (AVS) content was elevated (0.5 mmol L^{-1} of sediment) at Mercator MV Rim 2 (32 cmbsf, data not shown). In contrast, at CAMV Crater Centre, we could detect substantial amounts of (dissolved) sulphide peaking with 3.2 mM at 22.5 cmbsf (Figure IV-3D), i.e. at the interface between the methane and sulphate gradients.

**Acetate and hydrogen** - Acetate was present in the pore water of all sites (Figure IV-3) with decreasing concentrations from the deeper layers (between ~40 µM at Mercator Rim sites to ~90 µM at Mercator Crater Centre) toward the surface (5-20 µM). At CAMV Crater Centre, a peak of acetate (107 µM) occurred at 40 cmbsf. Substantial amounts of dissolved dihydrogen, mostly as discrete concentration peaks, could be detected in all four cores (Figure IV-3). Maximum hydrogen concentration ranged from 0.7 nM at Mercator crater Rim 2 to 4.4 nM at Mercator crater Centre.
Figure IV-3 Geochemistry and microbial activity at Mercator MV and CAMV. AOM, SR and MG activities (triangle) at each station plotted with respective substrate/product concentration profiles (methane,
sulphate/sulphide, hydrogen, acetate). Methylamine methanogenesis rates are given based on a methylamine concentration of 5 µM in sediment pore water (see Material and Methods). Note the scale difference for methane at Mercator MV Crater Centre (A), and for SRR and methane at CAMV (D). Error bars correspond to ± one standard error of the mean (n=3) when replicates were taken.

2.2 Microbial activity.

(i) Anaerobic Oxidation of Methane

Significant rates of AOM (AOMR) could be detected in all habitats investigated in this study (Figure IV-3). At Mercator MV Crater Centre (Figure IV-3A), AOM activity was present throughout the entire core, with two zones of apparently higher activity: just below the surface, with a maximum AOMR of 6 nmol cm⁻³ d⁻¹ at 21 cmbsf, and at greater depth (170 cmbsf) with a maximum AOMR of 2.3 nmol cm⁻³ d⁻¹. Notably, both maxima coincided with a decrease in methane concentrations (Figure IV-3). At Mercator MV Rim 1 and Rim 2 (Figure IV-3B and C), AOM activity was present in near surface sediments with rates of 0.8 and 2 nmol cm⁻³ d⁻¹ respectively, and decreased with depth to values close or below detection limit. At 25 cmbsf, AOMR increased and two activity peaks of 3.0 and 3.6 nmol cm⁻³ d⁻¹ were detected between 30 and 40 cmbsf at Rim 1, and a single peak of 8.5 nmol cm⁻³ d⁻¹ at Rim 2. At the latter, AOM activity extended down to the bottom of the core, with rates between 1 and 1.7 nmol cm⁻³ d⁻¹. At CAMV Crater Centre (Figure IV-3D), a distinct peak of AOM activity (11.4 nmol cm⁻³ d⁻¹) was found between 28 and 31 cmbsf, which vertically coincided with the sulphide peak and the depth of the sulphate-methane transition. AOM was close to the detection limit above this active zone. As already observed by (Niemann et al., 2006b), weak AOMR (0.5 to 1.3 nmol cm⁻³ d⁻¹) were detected below the SMTZ between 47 and 297 cmbsf.

(ii) Sulphate reduction.

At Mercator Crater Centre (Figure IV-3A), SRR showed three maxima: immediately below the surface (SRR of 5.58 nmol cm⁻³ d⁻¹ at 5 cmbsf), at 56 cm (2.36 nmol cm⁻³ d⁻¹), and at 220 cmbsf (7.47 nmol cm⁻³ d⁻¹). At Mercator MV Rim 1 (Figure IV-3B), SR was scattered over depth, but showed a general increase towards 30 cmbsf, broadly corresponding to depth of the two AOM maxima. At Mercator MV Rim 2 (Figure IV-3C), SR was maximal just beneath the sediment surface (9.6 nmol cm⁻³ d⁻¹), and showed a second weaker maximum of 1.83 nmol cm⁻³ d⁻¹ at the same depth (31 cmbsf) where also the AOM maximum was found. At CAMV Crater Centre, in contrast to the rather weak coupling between AOM-SR found at Mercator MV, SR
peaked (24.13 nmol cm\(^{-3}\) d\(^{-1}\)) just in the same sediment horizon (28-31 cmbsf) where also AOM was maximal.

Depth integrated AOM rates (\(\Sigma AOMR\)) and SR rates (\(\Sigma SRR\)) were comprised between 147 and 898 mmol m\(^{-2}\) yr\(^{-1}\), and 326 and 1803 mmol m\(^{-2}\) yr\(^{-1}\) respectively (Table IV-2). Remarkably, AOMR exceeded SRR in several sediments intervals. This difference was significant (no overlap of 95% confidence intervals) at Mercator MV Rim 2 (37.5 cmbsf) and in most sediment horizons between 7 and 29 cmbsf at Mercator MV Crater Centre. As a consequence, \(\Sigma AOMR\) exceeded \(\Sigma SRR\) by a factor 2.8 at Rim 2 in the 27-77 cmbsf interval, 1.23 over the entire core (Table IV-2), and 2.2 at Crater Centre between 7 and 49 cmbsf.

<table>
<thead>
<tr>
<th>Station</th>
<th>Core</th>
<th>Depth integrated rates [mmol m(^{-2}) yr(^{-1})]</th>
<th>(\Sigma SRR) (Total)</th>
<th>(\Sigma SRR) (SMTZ)</th>
<th>(\Sigma AOMR) (Total)</th>
<th>(\Sigma AOMR) (SMTZ)</th>
<th>(\Sigma AOMR) (Total) / (\Sigma SRR) (Total)</th>
<th>(\Sigma AOMR) (Total) / (\Sigma SRR) (SMTZ)</th>
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</thead>
<tbody>
<tr>
<td>JC10</td>
<td>Crater Centre</td>
<td>19</td>
<td>1803</td>
<td>n.a.</td>
<td>898</td>
<td>n.a.</td>
<td>0.50</td>
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</tr>
<tr>
<td>JC10</td>
<td>Mercator MV Rim 1</td>
<td>13</td>
<td>326</td>
<td>n.a.</td>
<td>80</td>
<td>n.a.</td>
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<tr>
<td>JC10</td>
<td>Mercator MV Rim 2</td>
<td>15</td>
<td>458</td>
<td>n.a.</td>
<td>562</td>
<td>n.a.</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>CAMV</td>
<td>Crater Centre</td>
<td>56</td>
<td>435</td>
<td>232</td>
<td>147</td>
<td>0.63</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

Table IV-2 Areal microbial activity rates. Areal rates \(\Sigma SRR\) and \(\Sigma AOMR\) were obtained by integrating volumetric anaerobic oxidation of methane rates (AOMR) and sulphate reduction rates (SRR) over the entire core (total) or the over the sulphate to methane transition zone (SMTZ). The AOM contribution to SR over the entire core (resp. over the SMTZ) is given by the ratios \(\Sigma AOMR\) (total) / \(\Sigma SRR\) (total) (resp. \(\Sigma AOMR\) (total) / \(\Sigma SRR\) (SMTZ)). In the absence of an SMTZ in Mercator MV sediments, the latter ratio does not apply to these sites. n.d.: not determined. n.a.: not applicable.

(iii) Methanogenesis

With the exception of Mercator MV Crater Centre, methanogenesis (M) using different substrates could be detected in all cores, albeit at very low rates and at discrete depth horizons. No methanol methanogenesis was observed in any of the sites investigated. In principal, we found a vertical succession of methylotrophic (Meth MG) with methylamine as a substrate in the shallowest horizons, followed by acetotrophic (Ac) and then by hydrogenotrophic (H) MG. At Mercator MV RIM 1 (Figure IV-3B), Meth-MG peaked with 8.1 pmol cm\(^{-3}\) d\(^{-1}\) at 3 cmbsf, Ac-MG with 2.3 pmol cm\(^{-3}\) d\(^{-1}\) at 13 cmbsf and H-MG with 50.5 pmol cm\(^{-3}\) d\(^{-1}\) at 17 cmbsf. At RIM 2 (Figure IV-3C), a peak of Meth-MG was also present near sediment surface, but we could not
detect H-MG or Ac-MG. Similarly, at CAMV Crater Centre, H-MG was absent, but we found two peaks of Ac-MG at 55 and 300 cmbsf (~0.13 pmol cm⁻³ d⁻¹) and a near surface peak in Meth-MG (Figure IV-3D).

2.3 Microbial community structure

The structure of the microbial community involved in AOM in hypersaline sediments of Mercator MV Rim 1 was investigated by constructing both archaeal (85 clones) and bacterial (75 clones) 16S rRNA gene clone libraries with DNA extracted in the 32-33 cmbsf interval where AOM was maximum. Sequences belonging to the ANME-1b clade (Hinrichs et al., 1999) largely dominated the archaeal library (n=69/85, Figure IV-4), followed by sequences belonging to the GoM-Arc1 clade (n=14/85) (also called ANME-2d in Mills et al., 2003; Mills et al., 2004; Lloyd et al., 2006). Most bacterial sequences were affiliated with sulphate-reducing bacteria (SRB) belonging to the Deltaproteobacteria (n=50/75, Figure IV-5). Of these, sequences belonging to the Seep-SRB1 cluster of putative syntrophic ANME partners these, sequences belonging to the Seep-SRB1 cluster of putative syntrophic ANME partners (Knittel et al., 2003) constituted the largest fraction (n=39/75) of the bacterial diversity, falling in the Seep-SRB1-c (n=30/75), Seep-SRB1-d (n=7/75) and Seep-SRB1-a (n=2/75) subgroups (Schreiber et al., 2010).
Figure IV-4 Affiliation of Archaeal 16S rRNA sequences from Mercator MV Rim 1 (32-33 cmbsf). The phylogenetic tree was reconstructed based on a subset of 340 sequences using the maximum likelihood method (RaxML) filtering out positions according to base frequencies among Archaea (50% cutoff). Results from 1000 bootstrap analysis are indicated as percentage.
Figure IV-5 Affiliation of Bacterial 16s rDNA sequences from Mercator MV Rim 1 (32-33 cmbsf). The phylogenetic tree was reconstructed based on a subset of 195 sequences using the maximum likelihood method (RaxML) filtering out positions according to base frequencies among *Deltaproteobacteria* (50% cutoff). Results from 1000 bootstrap analysis are indicated as percentage.
The in-situ abundance and distribution of known archaeal anaerobic methanotrophs (ANME-1, -2 and -3) was further investigated in the three Mercator MV cores applying CARD-FISH. Confirming the results of the clone library, ANME-1 was the only group of known methanotrophs detected at Mercator MV. Members of this clade were found at several depth intervals. At Mercator MV Rim 1, ANME-1 cells were dominant at 29 cmbsf, accounting for 79% (±0.8 % standard error) of the DAPI stained (n=848) cells (Figure IV-3C). At the same depth, 75% of the cells belonged to the domain Archaea, indicating that most of these were ANME-1 cells (results not shown). Bacteria accounted for 21% of DAPI stained cells, of which 53% (e.g. 11% of the total cell number) belonged to the genus Desulfosarcina / Desulfococcus, which includes the Seep-SRB cluster of putative ANME partners (results not shown). In general, ANME-1 cells were rod-shaped, formed chains of 2 to 16 cells (Figure IV-6A and B) and were not associated with other type of cells. Less than 10% of ANME-1 cells formed large clusters (Figure IV-6C and D) comprising few non-ANME-1 coccoid cells. At Mercator MV Rim 2, ANME-1 cells were only observed at 42.5 cmbsf and constituted 58% (±10%) of the DAPI stained cells (n=180) (Figure IV-3C). There, cells were smaller (~0.7 µm), with coccoid or short rod shapes and found as single cells or chains of 2 to 4 cells (Figure IV-6E and F). Very few DAPI-stained cells and no ANME-1 cells were detected in the Mercator MV Crater Centre at the investigated depths. In contrast, in the AOM zone (30 cmbsf) of the CAMV Crater Centre, the presence of ANME-2 cells (previously found by Niemann et al., 2006b) was confirmed by FISH assays. There, ANME-2 cells occurred as shell type aggregates, surrounded by other non-ANME cells (Figure IV-6G and H).
Figure IV-6 Direct microscopic observations of AOM communities of CARD-FISH and DAPI stained cells at Mercator MV. Monospecific chains of rod-shaped cells positive for the ANME-1 probe (A and B), or aggregates comprising ANME-1 positive cells (C and D) observed at Mercator MV crater rim 1 (32 cmbsf). E and F: small cocci or short rod-shaped ANME-1 positive cells observed at Mercator MV crater rim 2 (41 cmbsf). Overlay of DAPI stained (blue) and ANME-2/Cy3 (red) stained cells images (double stained cells appear in purple). White scale bars: 5 µm.
3. Discussion

3.1 AOM activity in hypersaline and non-hypersaline sediments

For a long time, hypersaline environments were considered as hostile or even biogeochemical dead ends. Indeed, the immense ionic strength and osmotic pressure appear to exclude most life on Earth, yet specialised microbes and a few eukaryotes have accomplished to minimise the negative effects of hypersalinity, apparently allowing them to thrive in these challenging environments (Boetius and Joye, 2009). Because of the many biochemical constraints associated to life in hypersalinity, these environments are believed to select for metabolisms associated with elevated energy yield (Oren, 2011). AOM is characterised by one of the lowest known energy yields among microbial energy conserving reactions. In the particular case of the putative syntrophic nature of AOM, the energy is shared, which further reduces the energy yield for each syntrophic partner. Despite these constraints, previous studies could already show that AOM can occur in hypersaline environments such as Mono- (Joye et al., 1999) and Big Soda lake (Iversen et al., 1987) at salinity of 88-90 g L\(^{-1}\) (~1.6 M Cl\(^{-}\)). In marine cold seep sediments, Lloyd et al. (2006) provided geochemical and molecular evidence for AOM activity at 146 g L\(^{-1}\) (~2.5 M Cl\(^{-}\)). Our results significantly extend the salinity range for AOM, showing that substantial rates (2.3 nmol cm\(^{-3}\) d\(^{-1}\) at Mercator MV Crater Centre) can be attained under hypersaline conditions at 263 g L\(^{-1}\) (4.5 M Cl\(^{-}\)), and even at halite saturating conditions (340 g L\(^{-1}\) or 5.8 M Cl\(^{-}\)) with rates of 0.5 nmol cm\(^{-3}\) d\(^{-}\). Hence, ANME-1 cells detected in extreme hypersaline environments, such as the Bannock, l’Atalante, and Thetis deep sea brine pools, with respective salinities of 148 g L\(^{-1}\) (Daffonchio et al., 2006), 230 g L\(^{-1}\) (Yakimov et al., 2007) and 348 g L\(^{-1}\) (La Cono et al., 2011), could carry AOM. However, these results are in contrast to the current concept that hypersaline conditions select for high-energy yield metabolisms. It is not clear how microbial communities mediating AOM can implement energy demanding osmoregulatory mechanisms (e.g. ion pumping, production of compatible solutes) unless the low AOM energy yield is balanced by a very high methane turnover per cell.

In spite of high methane and sulphate concentrations at Mercator MV sites, AOMR was below 8.5 nmol cm\(^{-3}\) d\(^{-1}\), i.e. up to 3 orders of magnitude lower than the maximum AOMR observed elsewhere (Knittel and Boetius, 2009). Moreover, we found a rather wide vertical distribution of AOM activity at all Mercator MV sites. In contrast, at CAMV, characterised by background
CHAPTER IV

salinity, AOM activity was confined to a discrete sediment horizon as already found previously at this structure (Niemann et al., 2006b) and at other non-hypersaline seep systems (Knittel and Boetius, 2009). This strongly suggests that AOM was inhibited at the level of cell activity or population growth, probably as a result of the extreme hypersaline conditions at Mercator MV. This interpretation is supported by the results of (Nauhaus et al., 2005), Figure III-2 showing that AOM rates tend to decrease with increasing salt concentration above seawater salinity during in vitro incubations with seep sediments.

Maximum AOMR at CAMV were higher compared to any of the Mercator MV stations, but depth integrated areal rates ($\sum\text{AOMR}$) were 6.1- and 3.8-fold lower than in Mercator MV Crater Centre and Rim 2, respectively (Table IV-2). In contrast to regular ocean sediments, Mercator MV hypersaline sediments were characterized by elevated sulphate levels due to the leaching of sulphate-rich evaporite strata by the ascending fluid (Scholz et al., 2009). In this system, sulphate was therefore not only transported from seawater downwards into the sediment, but also upward together with other ions, hydrocarbons and fluids. Furthermore, the inhibition of AOM in these hypersaline sediments prevented a rapid consumption of sulphate from both sources and upward migrating methane, and therefore, the formation of a typical SMTZ. Instead, the hampering of methane and sulphate turnover led to a very broad AOM zone where methane and sulphate overlap and in which AOM-communities are spread-out vertically. Consequently, such vertical extension of microbial activity resulted in higher turnover per unit area and thus compensated the reduction of volumetric rates to some extend.

Our results provide new insights on the origin of the gas venting observed during video surveys (Van Rooij, 2005). Shallow sub-seafloor internal seismic reflectors resembling bottom-simulating reflectors (BSR) were identified in Mercator MV Crater Centre sediments, suggesting the presence of gas hydrates near the sediment surface (Depreiter et al., 2005). Based on these observations, it was proposed that such gas venting could be caused by methane hydrate dissociation (Van Rooij, 2005). Our results show that the main trigger for gas venting was rather the inhibition of the AOM microbial activity in this hypersaline environment.

3.2 AOM community structure in hypersaline sediments of Mercator MV.

The Mercator MV environment closely resembles the Gulf of Mexico gassy sediment described by (Lloyd et al., 2006), in term of hypersalinity and by the fact that ANME-1 cells
dominated both environments. Therefore, extremely saline environments may well exert a selective pressure toward ANME-1, as already proposed by (Lloyd et al., 2006) and (Yakimov et al., 2007). This would also explain the findings of ANME-1 in brines in the eastern Mediterranean (Daffonchio et al., 2006; Yakimov et al., 2007; La Cono et al., 2011). Since ANME-1 cells have also been reported in the CAMV Crater Centre site (Niemann et al., 2006b) and other non-hypersaline environments (see Addendum II of this chapter, p.114), a negative selection towards other ANME groups thus appears to be the most probable ANME selection mechanism in hypersaline cold seeps. Such selection pattern could be related to the comparatively low effect of ionic strength on Archaea in general and ANME-1 in particular. The permeability of archaeal membranes comprising isoprenoidal glycerol ethers is generally lower than that of bacterial membranes comprising fatty acid glycerol esters (Valentine, 2007). Low membrane permeability reduces the energy loss associated to random ion exchange between the cyto- and ectoplasm. Specifically, ANME-1 comprises high contents of membrane spanning lipids, so called glycerol dialcyl glycerol tetraethers - GDGTs (Niemann and Elvert, 2008). Membranes composed of GDGTs are at the lower end of permeability when comparing typical membrane lipids (Yamauchi et al., 1993; Valentine, 2007). This could also explain the apparently negative selection of hypersalinity towards ANME-2 and -3 as these organisms feature membranes composed of diethers characterised by higher membrane permeability. In addition, genes coding for manosylglycerate and di-myoinositol-phosphate synthesis pathways were identified in the ANME-1 genome (Meyerdierks et al., 2010). These two compatible solutes are widely employed by halophile microorganisms to increase their turgor pressure (Roberts, 2004; da Costa and Empadinhas, 2008). These compounds may thus also confer hypersalinity resistance to the ANME-1 cells.

In Mercator MV Rim 1 sediments, ANME-1 cells were mainly present as monospecific chains, with no apparent contact with any other cell type. Such organization has already been reported in most previous ANME-1 microscopic observations (excepted in the conspicuous Black Sea mats and occasionally in Eel River basin sediments, see supplementary information). This thus seems to be the dominant spatial organisation of ANME-1 cells, irrespective of the hypersaline conditions found at Mercator MV. However, increasing ANME-to-SRB cell distance has a detrimental effect on AOM energy yield (Sorensen et al., 2001; Alperin and Hoehler, 2009). In our microscopic observations, distances between ANME-1 cells and the closest non-ANME-1 cell typically exceed 150 µm, the size of the observation grid. Such distance could be an artefact from CARD-FISH sample preparation, but (Orphan et al., 2002b) found similar
bacteria-free ANME-1 in the absence of disruptive treatments. Such existence of bacteria free ANME-1 population is also supported by the observation of large patches of DSS free ANME-1 aggregates in subsurface microbial mats in the Black sea sediments (Treude et al., 2005c). In addition, at 29 cmbsf in Mercator MV Rim 1 sediments, the microbial community was composed of 79% of ANME-1 and 11% DSS cells, resulting in an ANME:SRB ratio of 7:1. Such ratio strongly differs from the typical 1:3 ratio observed in shell type consortia (Orcutt and Meile, 2008a) and necessarily imposes distances between both partners. Consequently, our observations suggest that monospecific chains of ANME-1 cells are able to carry out AOM even if their distance to SRB cells exceeds the previously published critical distance (typically 5 µm under the Hydrate Ridge conditions. Alperin and Hoehler, 2009). Hence, in this system, AOM could involve an electron transport mechanism different that the commonly proposed AOM/SR coupling involving a chemical ITA diffusion between both partners. Alternatively, ANME-1 cells could overcome this thermodynamic constraint by carrying out both parts of the redox reaction, i.e. the oxidation of methane and the reduction of sulphate (Hansen et al., 1998; Orphan et al., 2002b). However, (Meyerdierks et al., 2010) could not find known genes involved in dissimilatory sulphate reduction in the ANME-1 genome (~80% coverage). Another possibility explaining the apparent separation of both syntrophic partners could be a direct electron shuttling between distant ANME and SRB cells through a conductive matrix. In mesocosms experiments with marine surface sediments, diffusion-independent electron shuttling over 1.2 cm distance has been shown (Nielsen et al., 2010). The expression of genes coding for membrane c-type cytochromes by ANME-1b cells (Meyerdierks et al., 2010) would be in line with this hypothesis of a direct electrons shuttling (Mehta et al., 2005). However, evidence for electroactivity of ANME-1 or Seep-SRB cells are lacking.

### 3.3 Reaction coupling between AOM, SR and MG

At CAMV Crater Centre, we found that depth of SRR and AOMR maxima coincided and areal SRR ($\Sigma$SRR) exceeded areal AOMR ($\Sigma$AOMR) within the SMTZ. Based on these areal rates, 63% of the sulphate reduction in the AOM zone was due methane oxidation (Table IV-2). This was thus compatible with a coupling between these reactions with an equimolar consumption of both substrates (equation 1). Several factors can account for the remaining 37% sulphate reduction activity in the AOM zone: organoclastic sulphate reduction through mineralization of higher hydrocarbons co-migrating with methane (Niemann et al., 2006b;
Kniemeyer et al., 2007; Bowles et al., 2011), or sedimentary organic matter from microbial or seep fauna necromass (Hilario and Cunha, 2008; Niemann et al., 2009; Sommer et al., 2009). Relatively low concentrations of higher hydrocarbons were found at CAMV (Niemann et al., 2006b). It is thus likely that the substantially higher $\Sigma$ SRR are fuelled by a combination of AOM, hydrocarbons and necromass oxidation.

We also found the reverse situation where $\Sigma$ AOMR consistently exceeded $\Sigma$ SRR over large depth intervals at Mercator MV Crater Centre or Rim 2 and at CAMV Crater Centre between 43 and 300 cmbsf. These remarkable results suggested the presence of alternative electron acceptors, other than sulphate, in both MV sediments. Among potential electron acceptors, Fe(III) and Mn(IV) or nitrate/nitrite were shown to be utilised in alternative modes of AOM. Although speculative at present, it therefore seems possible that AOM at Mercator MV might, partially, be independent from SR. Although the presence of oxidized Mn, Fe or N species in subseafloor sediments and brines is possible (D‘hondt et al., 2004; Parkes et al., 2005), there is, to our knowledge, no such data available for Mercator MV that would substantiate this aspect further. At CAMV, the intrusion of seawater within the ascending fluid (Hensen et al., 2007) could be the source of such electron acceptors.

Interestingly, similar decoupling of AOM and SR were already observed in conditions of very low or no sulphate, either in vitro (Hansen et al., 1998; Beal et al., 2011), or in environmental samples, at the base of or below SMTZs (Hansen et al., 1998; Joye et al., 2004; Niemann et al., 2006b; Parkes et al., 2007), which apparently correspond to the preferential habitat for ANME-1 cells in non-hypersaline methane bearing marine sediments (Knittel et al., 2005; Yanagawa et al., 2011). These observations suggest that ANME-1 dominated AOM communities could potentially decouple AOM and SR, as observed in the present study.

Recent work (House et al., 2009) showing important isotopic carbon heterogeneities among ANME-1 cells from Eel River Basin sediments, together with the presence of significant methanogenesis rates, indicates that ANME-1 cells may switch from a methanotrophic to a methanogenic metabolism. Similar conclusions arose from micrometer scale reaction/transport modeling (Alperin and Hoehler, 2009) showing that the concentration of reduced fermentation product -such as H$_2$- could act as a thermodynamic switch between the two metabolisms. This is in agreement with the increasing number of studies reporting a dual methanotrophic-methanogenic activity in environmental samples (Orcutt et al., 2005) or in vitro (Treude et al., 2007; Orcutt et al., 2008b). In contrast, since the vertical distribution of AOM and MG rates did
not match in any of the sites investigated, it seems that ANME cells did not operated in such dual mode within AOM zones of Mercator and CAMV, and have rather a strict methanotrophic metabolism.

3.4 Methanogenesis rates and zonation.

MG rates observed in this study were thus attributed to “true” methanogenesis. These were however too low and within too narrow sediment intervals to significantly contribute to the methane pool at all investigated sites. In general, the presence of sulphate is thought to exclude methanogenesis from marine sediments (Oremland and Taylor, 1978), as SRB generally outcompete methanogens for the utilisation of H₂ or acetate (Schonheit et al., 1982). In line with this general rule was the presence of Ac-MG rates only below the SMTZ at CAMV Crater Centre. However, departing from such zonation, peaks of Ac-MG and H-MG also occurred in the sulphate zone in Mercator MV Rim 1 sediments. Similar observations were made in the Napoli MV hypersaline sediments of the East Mediterranean sea (Lazar et al., 2011). It has been shown that, in the presence of sulphate, methylotrophic methanogens can still metabolize methyl moieties but mostly redirect them through the reverse methanogenesis oxidative pathway, with CO₂ as final product rather than CH₄ (Finke et al., 2007). In this case, sulphate reduction act as a sink for the electrons released during methyl oxidation via interspecies H₂ transfer. Similar process could occur with acetate as electron donor (Phelps et al., 1985; Achtnich et al., 1995) and could explain the residual Ac-MG rate observed at Mercator MV Rim 1. This interpretation of residual Ac-MG was supported by the very high peak of acetate oxidation to CO₂ at 13 cmbsf (7847 pmol cm⁻³ d⁻¹, data not shown) compared to the CH₄ formation (2.4 pmol cm⁻³ d⁻¹) at the same depth. The interpretation of the H-MG activity peak was more problematic, as SRB usually maintain H₂ levels that are inhibitory for methanogens (Kristjansson et al., 1982). However, it is possible that energetic constraints imposed by hypersaline conditions prevented SRB to consume hydrogen down to such low level, thus opening a narrow window for hydrogenotrophic methanogenesis activity along salinity gradients. However, to our knowledge, H₂ kinetics of consumption by SR and MG in varying salinity that could support this interpretation is lacking.

The Met-MG activity in sulphate rich near-surface sediments at all sites excepted Mercator MV Crater Centre was coherent with the fact that methylated amines are non-competitive substrate that cannot be used by SRB (Oremland and Polcin, 1982). The lack of Met-MG activity below was unlikely due to an increasing salt inhibition as methylotroph are among the most halotolerant methanogens (McGenity, 2010). This rather suggests that the source of substrate for
Met-MG was near-surface diagenetic processes, such as for instance glycin and betain degradation (Oren, 1990).
4. Conclusion

In brine sediments of Mercator MV, the presence of both sulphate and methane in the ascending fluid fuels substantial rates of AOM and SR. Unlike at regular ocean sediments, no clear sulphate-methane transition zone is present, but AOM and SR are distributed over a wide depth interval. However, most probably as a result of the hypersaline conditions, AOM is partially inhibited, so that maximum volumetric rates are orders of magnitude lower in comparison to other cold seeps. At Mercator MV, AOM is mediated by ANME-1, which mostly occurs as monospecific cell-chains and constitutes up to 79% of the microbial community. This, together with previous findings of ANME-1 in hypersaline settings, suggests that this ANME cluster is well adapted to elevated salinity, despite the low energy conserved from the AOM reaction. We also found that AOM and SR can be uncoupled with AOM significantly exceeding SR over wide depth intervals. Hypothetically, this could be related to the utilisation of electron acceptors other than sulphate for AOM. Finally, unlike in several other seep sites, methanogenesis is apparently not coupled to AOM as such activity was not detected where AOM was maximum.
ACKNOWLEDGMENTS

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5. ADDENDUM I: BATHYMETRY, SAMPLING AND SEDIMENT DESCRIPTION

5.1 Sampling site, seafloor observations and sedimentology

Mercator MV was located in the shallow region of the Gulf of Cadiz, amidst the El Arraich mud volcano province (Van Rensbergen, 2005) at 350 m water depth. CAMV was located deeper (1300 m water depth), about 100 km N.-E. of Mercator MV (Figure IV-1A). In the Gulf of Cadiz, thermogenic methane is formed at about 4000 m water depth and sediment fluidization occurs due to clay mineral dewatering (transformation of smectite to illite) with typically low ion contents of pore waters (Nuzzo et al., 2009; Scholz et al., 2009). However, the upward migrating fluids at Mercator MV are enriched in sodium, chloride and sulphate, probably by mixing with a subsurface evaporite strata. Evaporite leaching is much more pronounced in Mercator MV compared to CAMV (Scholz et al., 2009). Mercator MV (Crater Centre and Rim sites) and CAMV (Crater Center) as well as a reference site were sampled.

At Mercator Crater Centre (core JC10-009 and -019), sediments recovered by gravity and piston coring mainly consisted of mud breccia and were characterized by a very low porosity, decreasing from 50% near the seafloor down to 15% at 32 cmbsf (JC10-009) or 115 cmbsf (JC10-019). In addition, large crystals of Gypsum (Figure IV-7A) and Halite (Figure IV-7B), identified based on their morphology, were recovered from the Mercator MV crater centre. At Rim 1 (core JC10-013) and Rim 2 (core JC10-015) sites, sediments mainly consisted of an
alternation of compact and “foamy” mud breccia, with porosity comprised between 40% and 60%, overlain by a 10 cm layer of brown hemipelagic sediments. Observations carried out with the ROV Isis at Mercator MV revealed an active methane seep in the centre of the crater: we found gas venting from small holes (ca. 5 cm in diameter), confirming previous observations of (Van Rooij, 2005). On the contrary, no gas seepage could be found at CAMV. The CAMV Crater Centre core was recovered from the location previously sampled by (Niemann et al., 2006b) (core GeoB 9041-12). Facies and fauna associated with these mud volcanoes are further described in (Niemann et al., 2006b) and (Vanreusel et al., 2009).
### 6. ADDENDUM II: Abundance and consortia structure involving ANME-1 in methane seeps

<table>
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<th>Location</th>
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<th>ANME (%)</th>
<th>DSS (%)</th>
<th>ANME-2:ANME-3</th>
<th>Cell Nbr</th>
<th>Structure of ANME-1 cells and ANME-1/DSS associations</th>
<th>Salinity</th>
<th>AOMR:SR (nmol cm(^{-3}) d(^{-1}))</th>
<th>Reference</th>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>292</td>
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<tr>
<td></td>
<td>220</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>326</td>
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</tr>
<tr>
<td>Mercator MV Rim1</td>
<td>31</td>
<td>79</td>
<td>10</td>
<td>No</td>
<td>-</td>
<td>Chains of 2 to 16 ANME-1 cells &lt;10% ANME-1 cells formed large aggregates with non ANME-1 cells</td>
<td>104</td>
<td>3.5:2</td>
<td>This study</td>
</tr>
<tr>
<td>Mercator MV Rim2</td>
<td>32</td>
<td>59</td>
<td>-</td>
<td>No</td>
<td>-</td>
<td>Cocc or short rod pair of cells, positive for ANME-1 probe</td>
<td>104</td>
<td>8.4:1.8</td>
<td></td>
</tr>
<tr>
<td>East Mediterranean sea</td>
<td>0-2</td>
<td>5</td>
<td>11</td>
<td>No</td>
<td>20.45</td>
<td></td>
<td>116(^{(4)})</td>
<td>2:1050</td>
<td>Omoregie et al., 2009</td>
</tr>
<tr>
<td>Napoli MV</td>
<td>2-4</td>
<td>9</td>
<td>7</td>
<td>No</td>
<td>1.80</td>
<td></td>
<td>3:1050</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-12</td>
<td>22</td>
<td>0</td>
<td>No</td>
<td>1.99</td>
<td>Single ANME-1 cells or cell filaments, no consortia</td>
<td>2:1000</td>
<td>2:1000</td>
<td>(Omoregie et al., 2009)</td>
</tr>
<tr>
<td>East Mediterranean sea</td>
<td>0-2</td>
<td>&gt;0</td>
<td>7</td>
<td>13;&lt;1</td>
<td>3.52</td>
<td></td>
<td>35(^{(3)})</td>
<td>-</td>
<td>60:450</td>
</tr>
<tr>
<td>North Alex MV</td>
<td>2-4</td>
<td>&gt;0</td>
<td>8</td>
<td>No</td>
<td>3.62</td>
<td></td>
<td>30:250</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-12</td>
<td>1</td>
<td>3</td>
<td>7;&lt;1</td>
<td>2.70</td>
<td></td>
<td>60:450</td>
<td></td>
<td>(Omoregie et al., 2009)</td>
</tr>
</tbody>
</table>
### AOM IN AN HYPERSALINE ENVIRONMENT

<table>
<thead>
<tr>
<th>Location</th>
<th>Temperature</th>
<th>pH</th>
<th>Salinity</th>
<th>Dominant Feature</th>
<th>Community Description</th>
<th>Environment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Plußsee</td>
<td>8-15</td>
<td>0.1-1</td>
<td>&lt;1 / 0.5</td>
<td>0.005</td>
<td>Single cells or short chains of up to 5 cells, no consortia</td>
<td>Freshwater</td>
<td>(Eller et al., 2005)</td>
</tr>
<tr>
<td>Hydrate Ridge Beggia()toa mat</td>
<td>3</td>
<td>1-15</td>
<td>Yes</td>
<td>Yes</td>
<td>Mostly as single cells without any directly associated bacterial or archaeal partner. Chains of 2 to 10 cells. Spherically aggregated ANME-1 cells rarely detected.</td>
<td>33</td>
<td>(Knittel et al., 2005)</td>
</tr>
<tr>
<td>Calyptogena field</td>
<td>3</td>
<td>8</td>
<td>Yes</td>
<td>Yes</td>
<td>Single cell / filaments</td>
<td>33</td>
<td>(Orphan et al., 2002b)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21</td>
<td></td>
<td></td>
<td>Filaments near DSS aggregates</td>
<td>33</td>
<td>(Orphan et al., 2004)</td>
</tr>
<tr>
<td>Eel River basin</td>
<td>6-12</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>Interspersed ANME-1 filaments and DSS</td>
<td>33</td>
<td>(House et al., 2009)</td>
</tr>
<tr>
<td>Eel River basin</td>
<td>2-15</td>
<td>Yes</td>
<td>-</td>
<td>Yes</td>
<td>ANME-1 rods and filaments</td>
<td>33</td>
<td>1-55: -</td>
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<tr>
<td>Haakon Mosby MV</td>
<td>9-10</td>
<td>&lt;0.5</td>
<td>7</td>
<td>No</td>
<td>Cell filaments</td>
<td>33</td>
<td>(Losekann et al., 2007)</td>
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<tr>
<td>Gulf of Mexico Brine Pool</td>
<td>1</td>
<td>2.7</td>
<td>10</td>
<td>4.6</td>
<td>5.4</td>
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<td>0.3:100</td>
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<td>Gulf of Mexico Hydrate</td>
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<td>19</td>
<td>25.9</td>
<td>3.0</td>
<td>2.2</td>
<td>31</td>
<td>1.2:700</td>
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<tr>
<td>Black sea</td>
<td>250-2000(^{(1)})</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>0.3</td>
<td>~20</td>
<td>~1.5: -</td>
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\(^{(1)}\) Indicates references for specific locations and data sources.
<table>
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<tr>
<th>Water column</th>
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<tr>
<td><strong>Lost Hammer terrestrial Arctic hypersaline seep</strong></td>
<td>-</td>
<td>3.4</td>
<td>No</td>
<td>No</td>
<td>0.00045</td>
<td>No DSS</td>
<td>240</td>
<td>-</td>
</tr>
<tr>
<td>(Niederberger et al., 2010)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Baltic sea Eckernförde bay</strong></td>
<td>0-40</td>
<td>low</td>
<td>Yes</td>
<td>Yes</td>
<td>0.14</td>
<td>Short filament of 4-6 rectangular cells</td>
<td>SW</td>
<td>5-120: -</td>
</tr>
<tr>
<td>(Treude et al., 2005a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>North sea Tommeliten seep</strong></td>
<td>170</td>
<td>13</td>
<td>-</td>
<td>No</td>
<td>0.12</td>
<td>Single ANME-1 cells or short chains of up to 3 cells, no consortia</td>
<td>Seawater</td>
<td>2:2</td>
</tr>
<tr>
<td>(Niemann et al., 2005)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Black sea Carbonate Chimney mat</strong></td>
<td>0</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
<td>-</td>
<td>. Interspersed ANME-1 and DSS cells or large aggregates . Much lower DSS number compared to ANME-1 and to ANME-2/DSS aggregates . Large area (up to 400 µm in diameter) of DSS free ANME-1 aggregates.</td>
<td>Seawater</td>
<td>-</td>
</tr>
<tr>
<td>(Michaelis et al., 2002)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td>(Blumenberg et al., 2004)</td>
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</tr>
<tr>
<td>(Knittel et al., 2005)</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(Treude et al., 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Black sea subsurface mat</strong></td>
<td>11-13</td>
<td>40(2)</td>
<td>10</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>~20</td>
<td>1500:1800</td>
</tr>
<tr>
<td>(Treude et al., 2005c)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Black sea Sedimentary</strong></td>
<td>Yes</td>
<td>No</td>
<td>ANME-1 in Large aggregates of short chains</td>
<td>~20</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Reitner et al., 2005)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Table IV-3 Studies using (CARD)-FISH to describe the abundance, distribution and community structures involving ANME-1.

(1): cm water depth. (2): evaluated by slot blot hybridization of rRNA. (3): data from (Feseker et al., 2010) (4): Chloride profile was not given in (Omoregie et al., 2009), and was thus estimated from the Cl⁻ profile published in (Lazar et al., 2011).
Publications on AOM communities involving ANME-1 cells, their quantification and structure relative to DSS cells or other ANMEs are briefly reviewed in Table IV-3. In all studies, ANME and DSS cells were quantified by (CARD-)FISH cell counts, excepted in (Treude et al., 2005c) where dot slot was also used. In these studies, 3 types of ANME-1 organisation were reported: (A) single cells or chains of ANME-1 cells with no apparent association (i.e. contact or small distance) with other non-ANME-1 cells, (B) large ANME-1 aggregates interspersed with fewer non-ANME-1 single cells or aggregates (including DSS cells) and (C) large aggregates of interspersed ANME-1 and DSS single cells. (A) was by far the dominant type and was observed in almost all methane seep sites where ANME-1 were reported. It is possible however, that individual cells or filaments are detached from large aggregates during FISH procedures. In (B) type of aggregates, a syntrophic association between ANME-1 and DSS involving electron carrier diffusion was not evident as ANME-1/DSS distances were often very large. (Treude et al., 2007) reported for instance DSS free aggregates as large as 400µm in diameter. (C), which would resemble the ANME-2/DSS type of association was only occasionally observed in Eel River sediments and in Black sea chimneys mats. Hence, ANME-1 dominated communities, tend to have higher ANME:DSS cells ratio, and higher ANME/DSS cell-cell distances. Besides, these observations support two other characteristic patterns of ANME-1 distribution. As already proposed by (Knittel et al., 2005), the proportion of ANME-1 cells, relatively to ANME-2 cells, generally increases with depth and could possibly relate ANME-1 ecological niche to low sulphate environments. Such pattern was observed in Napoli MV, Gulf of Mexico brine pool and hydrate bearing sediments, Hydrate ridge Beggiatoa mat and Calyptogena (Table 1) and was reported by recent studies using phospholipid (Rossel et al., 2011) and molecular (Yanagawa et al., 2011) markers. Moreover, ANME-1 dominated the hypersaline environments reviewed (Napoli MV, Lost Hammer terrestrial seep, Mercator MV) and other ANME types were not present. A similar pattern was supported by (Lloyd et al., 2006) and (Yakimov et al., 2007) using archaeal clone libraries. However, such pattern was also observed in non hypersaline conditions, in a subsurface Black sea mat (Treude et al., 2005c).
CHAPTER V. Methane oxidation at the Carlos Ribeiro Mud Volcano: Ex-situ Microbial Activity vs. Geochemical Modeling.

ABSTRACT

The determination of the methane flux and consumption rates by microorganisms is of importance to constrain the methane budget and the carbon cycling in cold seep sediments. In this study, the rates of methane oxidation (MO) and sulphate reduction (SR) measured in the deep sea Carlos Ribeiro mud volcano (CRMV) sediments by ex situ radiotracers turnover were one order lower than the modelled methane and sulphate flux resulting from reaction transport geochemical models described in a companion paper (Vanneste et al., 2011). This apparent inconsistency could be explained by estimating in situ microbial activity rates based on modelled methane profiles. This suggested that the difference of methane solubility between in situ vs. ex situ conditions can lead to a large underestimation of the in situ rates. A small fraction of the methane flux was oxidised in the bioirrigated shallow sediment subsurface, probably by an aerobic process, but the largest part was consumed by anaerobic oxidation of methane (AOM) coupled to sulphate reduction (SR), involving all known types of anaerobic methanotrophs (ANME-1, -2 and -3). However, the AOM community in the MV crater centre was different than other known seep sites as it was dominated by GoM-Arc1 Archaea and butane/propane oxidising sulphate reducing Bacteria.
1. Introduction

Anaerobic Oxidation of Methane (AOM) coupled to sulphate reduction (SR) is the most significant microbial process at methane cold seeps such as mud volcanoes (Knittel and Boetius, 2009).

\[
\text{CH}_4 + \text{SO}_4^{2-} \rightleftharpoons \text{HS}^- + \text{HCO}_3^- + \text{H}_2\text{O} \quad \Delta G^0'=-16.9 \text{ kJ mol}^{-1} \quad \text{Equation 1}
\]

This reaction is performed by various Archaea closely related to methanogens forming distinct phylogenetic groups: ANME-1, ANME-2, and ANME-3 (Hinrichs et al., 1999; Boetius et al., 2000; Orphan et al., 2001a; Orphan et al., 2001b; Knittel et al., 2005; Lloyd et al., 2006; Losekann et al., 2007). These anaerobic methanotrophs often form tight associations with sulphate-reducing bacteria (SRB) of the genera *Desulfosarcina / Desulfococcus* (ANME-1 and -2) or *Desulfobulbus* (ANME-3) (Knittel et al., 2003; Losekann et al., 2007), and a syntrophic relationship between these microorganisms has been proposed (Boetius et al., 2000; Orphan et al., 2001b). However, the presence of monospecific aggregates or single ANME cells in active AOM zones (Losekann et al., 2007), CHAPTER III and IV, and the results from reaction/transport models of ANME/SRB aggregates indicate that cell contact is not required for AOM activity (Alperin and Hoehler, 2009). The typical geochemical signature of AOM activity in sediments is the presence of a sulphate-methane transition zone (SMTZ), *i.e.* opposed gradients of downward diffusing sulphate and upward migrating methane. Microorganisms mediating AOM are usually present and active in a narrow depth interval at the interface between these gradients.

Subsurface methane flux and turnover can be quantified by two complementary approaches. On the one hand, reaction transport models (RTM) integrate sulphate diffusive fluxes and fluid advection rates in order to constrain the methane concentration profile and flux (Boudreau, 1997). On the other hand, the ex-situ approach, consisting in injecting radiolabeled substrates in sediment samples upon core recovery (Iversen et al., 1987; Fossing and Jørgensen, 1989), permits to measure methane and sulphate turnover and identify active AOM sediments horizons. However, large discrepancies have been observed between AOM turnovers estimated by these two approaches. This is illustrated by study of the Captain Arutyunov MV (CAMV) in the Gulf of Cadiz: RTM resulted in a methane flux of 6268 mmol m$^{-2}$ yr$^{-1}$ (Hensen et al., 2007; Vanneste et al., 2011), whereas ex-situ AOM and SR areal rates were of 383 and 577 mmol m$^{-2}$ yr$^{-1}$ (Niemann et al., 2006b). We hypothesise that the
differences in methane solubility between in situ and ex situ conditions may explain these discrepancies.

In this chapter, we describe the community structure involved in AOM at three different sites of the CRMV and determined the major microbial activities in these sediments (AOM, SR and Methanogenesis or MG). Further, we use realistic methane concentrations from a geochemical model combined with the AOM rate dependency on substrate concentration (Jin and Bethke, 2002, 2003, 2005; Dale et al., 2008; Knab et al., 2008) to estimate the in situ methane turnover.
2. Description of the model used to estimate in situ AOM rates

The AOM rate \( V_{\text{AOM}} \) dependency on substrate concentration (Jin and Bethke, 2002, 2003, 2005; Dale et al., 2008; Knab et al., 2008) can be expressed as a function of maximum rate \( V_{\text{max}} \):

\[
v_{\text{AOM}} = V_{\text{max}} \cdot F_K \cdot F_T \tag{Equation 2}
\]

According to this model, the maximum specific rate is modulated by a Michaelis–Menten kinetic factor \( F_K \),

\[
F_K = \frac{[CH_4]}{K_{CH_4} + [CH_4]} \cdot \frac{[SO_4^{2-}]}{K_{SO_4^{2-}} + [SO_4^{2-}]}
\tag{Equation 3}
\]

and a thermodynamic factor \( F_T \) accounting for activity inhibition when the reaction yield \( \Delta G \) approaches a threshold value \( \Delta G_{\text{th}} \) corresponding to the smallest amount of energy that must be conserved for ATP formation (Jin and Bethke, 2005):

\[
F_T = 1 - e^{\left( \frac{\Delta G + \Delta G_{\text{th}}}{RT} \right)} \tag{Equation 4}
\]

Based on Equations 2 to 4, in situ AOM rates \( V_{\text{AOM,IS}} \) can thus be estimated from measured ex situ rates \( V_{\text{AOM,ES}} \) by:

\[
V_{\text{AOM,IS}} = \frac{V_{\text{AOM,ES}} \cdot V_{\text{MAX,IS}} \cdot F_{K,IS} \cdot F_{T,IS}}{V_{\text{MAX,ES}} \cdot F_{K,ES} \cdot F_{T,ES}} \tag{Equation 5}
\]

Following the same assumptions than in (Dale et al., 2006; Knab et al., 2008), i.e \( K_{\text{CH}_4} \gg [\text{CH}_4] \), \( F_K \) becomes:

\[
F_K = \frac{K_{CH_4}^{-1}}{K_{SO_4^{2-}} \cdot [SO_4^{2-}]} \tag{Equation 6}
\]

Replacing this expression of \( F_K \) in Equation 5, and since \( V_{\text{MAX}}, K_{\text{CH}_4}, K_{SO_4^{2-}} \) and \([SO_4^{2-}]\) do not vary between in situ and ex situ conditions, Equation 5 becomes:

\[
V_{\text{AOM,IS}} = \frac{V_{\text{AOM,IS}} \cdot [CH_4]_{\text{IS}} \cdot \frac{\Delta G_{\text{th}} + \Delta G_{\text{th}}}{RT \cdot \chi}}{[CH_4]_{\text{ES}} \cdot \frac{\Delta G_{\text{th}} + \Delta G_{\text{th}}}{RT \cdot \chi} - e^{\left( \frac{\Delta G_{\text{th}} + \Delta G_{\text{th}}}{RT \cdot \chi} \right)}} \tag{Equation 7}
\]
ΔG is calculated according to:

$$\Delta G = \Delta G^0 + R \cdot T \cdot \ln \frac{\gamma_{\text{HS}^-} [\text{HS}^-] \cdot \gamma_{\text{HCO}_3^-} [\text{HCO}_3^-]}{\gamma_{\text{CH}_4} [\text{CH}_4] \cdot \gamma_{\text{SO}_4^{2-}} [\text{SO}_4^{2-}]}$$

Equation 8

where $V_{\text{AOM,ES}}$ is the ex situ AOM rate measured by radiotracer turnover, $[\text{CH}_4]_{\text{IS}}$ is the in situ methane concentration modelled in (Vanneste et al., 2011), $[\text{CH}_4]_{\text{ES}}$ is the ex situ methane concentration measured during the radiotracer turnover determination ((Treude et al., 2003). For calculations, we used $R=8.3144 \text{ J.mol}^{-1}\text{.K}^{-1}$ for the gas constant, $T=277.1 \text{ K}$ as the absolute in situ temperature, $\chi=2$ as a stoichiometric factor (Jin and Bethke, 2005; Knab et al., 2008), $\Delta G_{\text{BO}}=10 \text{ KJ.mol}^{-1}$ as the critical yield value for energy conservation (Jin and Bethke, 2005; Knab et al., 2008). For $\Delta G$ calculation, we used the activity coefficient values provided in (Alperin and Hoehler, 2009), i.e.

$$\gamma_{\text{HS}^-} = 1.03 \quad \gamma_{\text{HCO}_3^-} = 0.532 \quad \gamma_{\text{CH}_4} = 1.24 \quad \gamma_{\text{SO}_4^{2-}} = 0.104$$

$[\text{HCO}_3^-]$ and $[\text{HS}^-]$ values were taken from (Vanneste et al., 2011). At three locations studied, our depth scale was corrected by fitting sulphate and chloride profile to the profiles from (Vanneste et al., 2011). Note that unlike the Crater Eye and the Off-center sites, the Rim site of our study had no counterpart in the study of Vanneste et al. However, their “Margin” site presented similar sulphate and chloride profiles, and data from this site was used as a comparison for the Rim site of our study.

Estimated in situ areal AOM rates were calculated by integrating (by the rectangle method) the in situ volumetric rates resulting from this model within the boundaries of the SMTZs.
3. Results

3.1 Sampling site

The CRMV is located on the south Portuguese margin, in the deep part of the Gulf of Cadiz at water depth of 2200 m. The crater diameter was ca. 1500 m and mud accumulations formed dome-like structure of ca. 80 m height. Successive mud eruptions formed concentric rims that are visible on the high-resolution bathymetric map. The CRMV was sampled at the Crater Eye and Off Centre sites that were also used for reaction transport modelling in (Vanneste et al., 2011). In addition, we sampled a radial mudflow at the Crater Margin (Figure V-1 and Table V-1).

![Figure V-1](image_url) The Carlos Ribeiro mud volcano (CRMV) was located in the deep basin of the Gulf of Cadiz (N.-E. Atlantic). The CRMV base was at 2300 m water depth. The crater was about 140 m high and 360 m wide. Circles indicate the location of the sampling sites. The mud volcano was sampled in the Crater Eye and Off Centre sites that were used for geochemical modelling in the study of (Vanneste et al., 2011). In addition, the Crater Rim site was also sampled. Circle colours correspond to sampling devices used at each site. (red: megacore, green: gravity core and blue: piston core). Depth range: -2170 m (brown), -2200 m (green).
Table V-1 Core depth and position at the three CRMV sites of this study. Sediments were also taken 2.9 km S.-W. of CRMV for reference.

3.2 Geochemistry

Methane was present in the deeper sediment strata at the three locations investigated, with maximum values around 2 mM (Figure V-2). Methane concentration decreased toward sediment surface, indicating an upward methane flux. A large part of this methane flux was consumed in the sediment subsurface, as shown by the steep concentration gradients between 26 and 6 cm below the sea floor (cmbsf) at Crater Eye, 44 and 14 cmbsf at Off Centre, and 41 and 21 cmbsf at Crater Margin sites. However, residual methane concentrations were still present above these intervals (between 0.002 mM at Crater Rim and 0.022 mM at Crater Eye station).

Sulphate concentration remained close to seawater values (~28 mM) along depth intervals varying from 7 cmbsf (at Crater Eye and Rim sites) to 14.5 cmbsf (at Off Centre site) (Figure V-2). Below, sulphate concentration steeply decreased down to values <1mM at depth of 27, 32 and 42 cmbsf at Crater Eye, Off Centre and Rim sites respectively. Below these depths, sulphate concentration gradually reached values below detection limits.

A peak of sulphide concentration was present at the interface of methane and sulphate gradients, with maximum values of 0.3, 2.6 and 1.8 mM at Crater Eye, Off Centre and Crater Rim respectively, and reached minima between 10 and 20 µM near sediment surface.

<table>
<thead>
<tr>
<th>Station</th>
<th>Core Name</th>
<th>Core Type</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Depth (m)</th>
</tr>
</thead>
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<tr>
<td>Reference</td>
<td>JC10-044</td>
<td>Mega Core</td>
<td>35°46.042N</td>
<td>8°26.554W</td>
<td>2344</td>
</tr>
<tr>
<td></td>
<td>JC10-045</td>
<td>Piston Core</td>
<td>35°46.044N</td>
<td>8°26.556W</td>
<td>2345</td>
</tr>
<tr>
<td>Crater Eye</td>
<td>JC10-053</td>
<td>Piston Core</td>
<td>35°47.259N</td>
<td>8°25.320W</td>
<td>2174</td>
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<tr>
<td>Off Center</td>
<td>JC10-048</td>
<td>Gravity Core</td>
<td>35°47.225N</td>
<td>8°25.292W</td>
<td>2177</td>
</tr>
<tr>
<td></td>
<td>JC10-050</td>
<td>Mega Core</td>
<td>35°47.221N</td>
<td>8°25.292W</td>
<td>2176</td>
</tr>
<tr>
<td>Crater Rim</td>
<td>JC10-054</td>
<td>Mega Core</td>
<td>35°47.300N</td>
<td>8°25.219W</td>
<td>2179</td>
</tr>
</tbody>
</table>
3.3 Ex situ microbial activity

(i) Sulphate Reduction

Peaks of sulphate reduction activity (SR) were present in sediment subsurface within the SMTZ at all locations (Figure V-2). Maximum SR rates (SRR) were of 23, 8.9 and 9.5 nmol cm\(^{-3}\) d\(^{-1}\) at Crater Eye (11 cmbsf), Off Center (23 cmbsf) and Crater Rim (37 cmbsf) sites respectively. At the later, SRR peaks of 5.4 and 7.8 nmol cm\(^{-3}\) d\(^{-1}\) were also present at 19 and 25 cmbsf. SRR were generally very low or null below the SMTZ and near sediment surface. Areal SRR at Crater Eye, Off Centre and Crater Rim sites were of 584, 688 and 475 mmol m\(^{-2}\) yr\(^{-1}\) respectively when the entire core depth was considered for integration of the volumetric rates (Table V-2).

(ii) Methane Oxidation

In general, three zones of methane oxidation (MO) could be distinguished. In the shallow subsurface at Off Centre and Rim sites, MO rates were of 12.6 and 6.3 nmol cm\(^{-3}\) d\(^{-1}\) at 3 and 1 cmbsf respectively (Figure V-2). Below, MO rates decreased steeply down to values of 0.5 nmol cm\(^{-3}\) d\(^{-1}\) at 13 cmbsf and 0.1 nmol cm\(^{-3}\) d\(^{-1}\) at 11 bsf in the Off Centre and Rim stations respectively. Such shallow subsurface activity was not present at Crater Eye station, possibly due to loss of surface sediments during piston coring.

Within the SMTZ of the three locations, a peak of MO was present with maximum MO rates of 11.2 nmol cm\(^{-3}\) d\(^{-1}\) in Crater Eye site at 13 cmbsf, 15.5 nmol cm\(^{-3}\) d\(^{-1}\) in Off Centre site at 35.5 cmbsf and 5.1 nmol cm\(^{-3}\) d\(^{-1}\) in Crater Rim site at 19 cmbsf. In the Crater Eye site, the depth of the MO maximum coincided with the depth of the SR peak. In the Off Centre site, there was an apparent depth offset between MO and SR maxima. However, SR measurement was lacking at the depth of the MO peak (35.5 cmbsf). In the SMTZ of the Rim site, the depth of MO peak corresponded to the upper SR maximum (19 cmbsf).

Below the SMTZs, MO was present but at very low rates (around 1 nmol cm\(^{-3}\) d\(^{-1}\)) at Crater Eye and Off Centre sites. At Crater Rim sites, the bottom of the core corresponded to the lower boundary of the SMTZ and MO data was not available below the SMTZ.

Areal MO rates were of 257, 787 and 206 mmol m\(^{-2}\) yr\(^{-1}\) over the entire core lengths and of 208, 455 and 138 mmol m\(^{-2}\) yr\(^{-1}\) within the SMTZ at Crater Eye, Off Centre and Crater Rim sites respectively (Table V-2).
Figure V-2 Microbial activity rates and geochemical profiles. First column: Sulphate reduction rates plotted with sulphate and sulphide concentration profiles. Second column: methane oxidation rates plotted with methane concentration profile. Third column: estimated in situ methane oxidation rates based on ex-situ methane turnover and methane profile from reaction / transport models. Model results are presented in (Vanneste et al., 2011), and are reproduced here (methane and sulphate profiles). Fourth column: proportion of the three types of ANME cells observed at different sediment depth.
Figure V-3 Microbial activity rates and geochemical profiles II. Methanogenic substrate concentration (H₂ and acetate) and hydrogenotrophic (H-MG), Acetotrophic (Ac-MG) and methylamine methyltrophic (Met-MG) methanogenesis rates.
(iii) Methanogenesis

In general, methanogenesis (MG) rates were low and restricted to narrow depth intervals (Figure V-3). Hydrogenotrophic methanogenesis (H-MG) and acetotrophic methanogenesis (Ac-MG) were only present in the shallow subsurface sediments at Off Centre site (Figure V-3), with a maximum H-MG rate of 7.8 pmol cm\(^{-3}\) d\(^{-1}\) at 1.5 cmbsf, and Ac-MG rate of 10.1 pmol cm\(^{-3}\) d\(^{-1}\) at 11 cmbsf. At Crater Eye site, a peak of methylamine methanogenesis (Meth-MG) was observed in shallow subsurface sediments, with Meth-MG rate of 0.23 pmol cm\(^{-3}\) d\(^{-1}\) at 8.5 cmbsf. Below, Meth-MG was present at very low rates (<0.01 pmol cm\(^{-3}\) d\(^{-1}\)). At the Off Centre site, a Meth-MG peak of 0.18 pmol cm\(^{-3}\) d\(^{-1}\) was present at 25.5 cmbsf. No methanol methanogenesis was observed in any of the sites investigated.

3.4 Estimation of in situ AOM rates

In situ MO rates within the SMTZ of each site were evaluated based on Equation 5. Estimated rates presented a peak within the SMTZ of each of the three sites, with maximum values of 157, 61 and 72 nmol cm\(^{-3}\) d\(^{-1}\) at Carter Eye, Off Centre and Crater Rim sites respectively (Figure V-2). Below, significant rates (between 3 and 32 nmol cm\(^{-3}\) d\(^{-1}\)) were observed down to the lower boundary of the SMTZ at the three locations. Areal MO rates based on these estimated in situ volumetric rates were of 2505 mmol m\(^{-2}\) yr\(^{-1}\) at Crater Eye site, 2235 mmol m\(^{-2}\) yr\(^{-1}\) at Off Centre site, and 2263 mmol m\(^{-2}\) yr\(^{-1}\) at Crater Rim site (Table V-2).

3.5 AOM microbial community structure

The structure of the microbial community involved in methane oxidation activity in the SMTZ of the Crater Eye site was investigated by constructing both archaeal (65 clones) and bacterial (71 clones) 16S rRNA gene libraries from the 15-17 cmbsf sediment interval. Amplified Ribosomal DNA Restriction Analysis (ARDRA) resulted in 34 archaeal and 48 bacterial groups displaying different restriction patterns for which at least 2 representative clones were sequenced. Among Archaea (Figure V-4), sequences belonging to the GoM Arc I clade defined by by (Lloyd et al., 2006) constituted the largest group (n=27). In addition, most known anaerobic methanotrophs groups were represented with sequences belonging to ANME-1b (n=2), ANME-2a (n=7), ANME-2c (n=10) and ANME-3 (n=11). The remaining sequences belonged to the class of Thermoplasmata (n=8). Most bacterial sequences were affiliated with sulphate-reducing bacteria (SRB) belonging to the Deltaproteobacteria (n=39, Figure V-5). Off these, sequences belonging to the short-chain hydrocarbon oxidiser group
(Butane12-GMe cells and Bus5 strain) defined by (Kniemeyer et al., 2007) constituted the largest group (n=31). Sequences belonging to the Seep-SRB1 clade of putative SRB syntrophs involved in AOM (Knittel et al., 2003; Schreiber et al., 2010) were also present in the library: Seep-SRB1-b (n=1), Seep-SRB1-e (n=3) and Seep-SRB1-f (n=2)

Figure V-4 Affiliation of archaeal sequences from the Crater Eye AOM zone (15-17 cmbsf). The tree was reconstructed by the maximum likelihood method (RaxML), excluding most variable position from the alignment (base frequency below 50% among archaeal sequences).
Figure V-5 Affiliation of deltaproteobacteria sequences from the Crater Eye AOM zone (15-17 cmbsf). The tree was reconstructed by the maximum likelihood method (RaxML), excluding most variable position from the alignment (base frequency below 50% among deltaproteobacteria sequences).
The presence of ANME-1, -2 and -3 cells in the CRMV sediments was confirmed by direct observations of CARD-FISH labelled cells using group specific probes. At Crater Eye site, ANME-2 and ANME-3 cells were present between 8.5 and 23.5 cmbsf (2.3 to 32.3 % of the DAPI stained cells) and at 18.5 and 23.5 cmbsf (6.5 and 1.5% of the DAPI stained cells) respectively (Figure V-2A). At Off Centre site, ANME-1 and ANME-2 cells were only present at 40.5 cmbsf, coinciding with the MO peak (respectively 16 and 6.5% of DAPI stained cells) (Figure V-2B). At Rim site (Figure V-2C), ANME-2 cells (1.4 to 37.5% of DAPI stained cells) were found between 22.5 and 32.5 cmbsf, whereas ANME-3 cells were only observed at 22.5 and 29.5 cmbsf (5.9 and 14.5% of the DAPI stained cells respectively). ANME-2 and -3 cells were both involved in very different structures: they occasionally formed dense shell-type consortia surrounded by non-ANME cells (Figure III-6A and B), but were mostly present as large size (Figure III-6C and D) or small size (Figure III-6E and F) loose associations with non-ANME cells, or as isolated single cells (Figure III-6G and H). In addition, ANME-2 were also found associated as dense cell aggregates (Figure III-6I), as paired cells (Figure III-6J), or as dense monospecific aggregates (Figure III-6K and L).

<table>
<thead>
<tr>
<th>Station</th>
<th>Core</th>
<th>modeled flux (a) [mmol.m-2.yr-1]</th>
<th>Depth integrated rates (b) [mmol.m-2.yr-1]</th>
<th>Total ex situ MO rates [mmol.m-2.yr-1]</th>
<th>Estimated in situ MO rates [mmol.m-2.yr-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crater Eye</td>
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<td>3164</td>
<td>584</td>
<td>208</td>
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<tr>
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<td>2368</td>
<td>2735</td>
<td>688</td>
<td>455</td>
</tr>
<tr>
<td>Crater Rim</td>
<td>54</td>
<td>n.d.</td>
<td>n.d.</td>
<td>475</td>
<td>138</td>
</tr>
</tbody>
</table>

Table V-2 Reaction rates and geochemical fluxes. (a): modeled flux calculated by reaction / transport models. Data from (Vanneste et al., 2011). (b): Methane and sulphate turnover based on ex situ methane and sulphate rates at 1 bar and after recalculation using in situ methane concentrations. $\Sigma$SRR and $\Sigma$AOMR correspond to depth integrated volumetric rates of SR (integrated over the entire core) and AOM (integrated over the SMTZ).
Figure V-6 Microscopic observations of fluorescently labeled ANME cells. Overlay of CARD-FISH and DAPI stained cells (green) and DAPI only stained cells (blue). (A) ANME-2 cells in Off Center at 40.5 cmbsf; (B) ANME-3 cells Crater Rim, 28 cmbsf; (C) ANME-2 cells in Off Centre at 40.5 cmbsf; (D) ANME-3 cells in Crater Rim at 33 cmbsf; (E) ANME-2 cells in Crater Rim at 13.5 cmbsf; (F) ANME-3 cells in Crater Eye at 18.5 cmbsf; (G) in Crater Rim at 28 cmbsf; (H) ANME-3 cells in Crater Eye at 13.5 cmbsf. ANME-2 cells in Crater Rim at 33 cmbsf (I and J), and in Off Centre at 40.5 cmbsf (K and L).
CHAPTER V

4. Discussion

4.1 MO, SR and MG activity coupling in CRMV sediments.

*Ex situ* MO rates indicated that methane was mostly consumed in the SMTZ of the three sites at CRMV. In addition MO and SR activity peaks coincided, thus showing that methane oxidation coupled to sulphate reduction (*i.e.* AOM) was the main sink for methane in the three zones of the MV. At Crater Eye and Margin sites, methane oxidation accounted for 29-36% of the sulphate reduction (Table 2). However, these ratios between methane and sulphate turnover rates may be overestimated, as *ex situ* rates were measured at atmospheric pressure (see next section). The higher ratio (66%) in the Off Centre site probably resulted from a sampling artefact: SR rate was not measured at 35.5 cmbsf where MO rates were highest. This likely led to an underestimation of the areal *ex situ* SRR. As discussed elsewhere (Bowles et al., 2011), non-AOM SR at cold seeps sites has been commonly observed and may exceeds methane turnover, on average, by a factor 10. Such activity could result from organic matter mineralization or oxidation of hydrocarbons containing 2 or more carbons (C2+) that are co-migrating with methane. The latter was supported by the presence of C2+ hydrocarbons in CRMV sediments (Vanneste et al., 2011) and by the presence of butane / propane oxidizing sulphate-reducing bacteria in the SMTZ of the Crater Eye site (see below).

Besides AOM, low but significant MO rates were present in the shallow subsurface (0-14 cmbsf) at Off Centre and Crater Margin sites, with no corresponding SR peaks. These rates mostly resulted from high methane turnover, since methane concentration was very low. The presence of a methane tail between the SMTZ and the surface was probably due to a thermodynamic inhibition of AOM at low methane concentration (Dale et al., 2008). In these zones, oxygen rather than sulphate was possibly the terminal electron acceptor for methane oxidation. Such situation of deep (10 cm) penetration of oxygen in the sediment has already been described at the CAMV (Sommer et al., 2009) where chemosyntetic tubeworms irrigate shallow subsurface sediments with oxic seawater. Using $^{13}$C-methane profiles, Sommer et al. (2009) have shown that such bioirrigation was responsible for aerobic methane oxidation in the shallow subsurface. Large populations of chemosynthetic tubeworms have been observed in the CRMV sediments (Hilario and Cunha, 2008; Vanreusel et al., 2009). Moreover, the constant sulphate concentration in the 0-10 cmbsf interval, above the sharp gradients of the SMTZ, indicated that bioirrigation occurred in the CRMV sediments at these two CRMV sites.
Weak MO rates (0-3 nmol cm\(^{-3}\) d\(^{-1}\)) were present in the deep part of the cores, whereas sulphate was entirely depleted. Similar rates were also observed at CAMV Crater Centre site (Niemann et al. 2006a; Chapter IV). This activity cannot be attributed to trace methane oxidation during methanogenesis, as described in (Zehnder and Brock, 1979), as MG rates were below detection limit at the corresponding depths. Possible pathways for such MO activity thus remain elusive.

Similarly to Mercator MV and CAMV in the Gulf of Cadiz (Chapter III and IV), MG rates were very low or null in the sediment of CRMV. Several studies have shown that seep sediments also produced methane during AOM, at rates of up to 10% of the AOM rates (Treude et al., 2007; Orcutt et al., 2008b). The lack of significant MG rates in the AOM zones of CRMV indicated that such coupling is absent in CRMV sediments and thus does not always occur.

### 4.2 Estimated in situ methane consumption in CRMV sediments.

We used modelled in situ methane profiles and measured ex situ methane turnover in order to provide an estimation of the in situ AOM rates. With this method, the estimated in situ areal AOM rates were one order higher than the measured ex-situ areal AOM rates, but were in the same order than the downward sulphate flux estimated by RTM (Table V-2; (Vanneste et al., 2011)). Besides AOM, heterotrophic sulphate reduction is the other probable sulphate-consuming pathway in these sediments. However, several observations suggested that this pathway was a very minor sulphate sink in CRMV sediments: the low organic content of the Gulf of Cadiz mud volcanoes sediments (Stadnitskaia et al., 2006), the quasi absence in CRMV sediments of hydrogen that typically results from organic matter fermentation, the low SR rates outside AOM zones, and the peak of sulphide coinciding with AOM activity peaks. Therefore, the major part of the sulphate flux should be attributed to AOM dependant sulphate reduction. However, the ex-situ areal AOM rates at the three CRMV stations were one order lower than the RTM estimated sulphate fluxes, suggesting that these rates were most probably underestimating the in situ rates. On the other hand, estimated in situ areal AOM rates were on the same order than the sulphate flux estimated by (Stadnitskaia et al., 2006) As a consequences, AOM-dependant sulphate reduction rates were also probably underestimated by ex situ measurements and our method would thus predict a ~10 fold increase of estimated in situ areal SRR. Whether this method provided accurate estimates of the true in situ AOM rates is still debatable. The method used to estimate in situ turnover rely on modeled methane
profiles that can vary according to several partially constrained parameters, such as boundary conditions, rate constant or microbial activities distribution. Better geochemical profiles will probably result from the development of in situ methods, e.g. submersible mass spectrometer devices (Wankel et al., 2010)

Most of the differences between ex situ and estimated in situ rates were due to the kinetic inhibition of the lower methane concentration at ambient pressure. The thermodynamic correction factor \( (F_{kIS}/F_{kES}) \) indeed only contributed for 0.6 to 7.4% of the estimated in situ turnover at CRMV Off Canter and Crater Eye respectively. This was in agreement with the observations from (Dale et al., 2008; Knab et al., 2008; Wankel et al., 2010) that a kinetic inhibition modulated the AOM rates amplitude, whereas a thermodynamic inhibition rather controlled the microbial activity depth distribution in Skagerrak sediments (Norwegian trench).

4.3 AOM community structure at CRMV.

In the three locations investigated, ANME cells represented a large part of the microbial community, with ANME-2 cells dominating in term of both cell number and depth distribution. In addition, the ANME distribution correlated well with the AOM activity peaks. Sequences belonging to the Seep-SRB1 group of putative syntrophic SRB (Knittel et al., 2003; Schreiber et al., 2010) were also detected in the AOM zone of the Crater Eye. These observations are thus in agreement with previous studies of cold seep ecosystems showing that AOM is mediated by ANME cells (Knittel and Boetius, 2009). ANME-2 and -3 cells were mostly found as single cells, monospecific ANME aggregates or mixed-type consortia, and very few shell-type consortia were present in the three locations. Based on bioenergetics considerations, (Alperin and Hoehler, 2009) proposed that high hydrogen concentration resulting from fermentative processes in organic sediments might promote the formation of shell-type consortia, with ANME cells having a H-MG activity rather than AOM. Following this hypothesis, the generally low organic content of the Gulf of Cadiz MV sediments (0.2-0.5 wt%) (Stadnitskaia et al., 2006), may thus explain this low abundance of shell-type consortia.

Sequences belonging to the GoM Arc I Archaea clade belonging to the methanogen branch of Archaea formed the most abundant archaeal group in the AOM zone of CRMV Crater Eye. However, a methanogenic role, at least using the conventional substrates tested in this study, can be excluded since MG rates were very low or zero at this site. GoM Arc I sequences distribution showed a strong bias toward AOM zones off mud volcanoes from the Gulf of Cadiz (Chapter IV), East Mediterranean Sea (Heijs et al., 2005; Omorogie et al., 2008;
Pachiadaki et al., 2010) and Gulf of Mexico (Lloyd et al., 2006). Further, the GoM Arc I clade is a sister group of the AOM Associated Archaea (AAA) clade (Fig. 6; (Knittel and Boetius, 2009). A methanotrophic role of AAA has been recently suggested by Schubert et al. (2011), describing large aggregates of $^{13}$C-depleted AAA cells in the putative AOM zone of an alpine lake. These observations, together with our finding of GoM Arc I sequences in the AOM zone of CRMV, thus provide some motives to further explore the potential of GoM Arc I cells as possible methane oxidizers in cold seeps environments.

A large part of the bacterial sequences (31/71) found in the AOM zone of the CRMV Crater Eye site formed a monophyletic group that included the Butane12-GMe cells and the Bus5 strain: two butane and propane oxidising sulphate reducers (Kniemeyer et al., 2007). To our knowledge, environmental sequences belonging to this group have not yet been reported (in the Silva 104 database). Our results thus provide additional evidence that AOM zones of methane cold seeps are suitable environments for the Butane12-GMe cells. Their presence in the Crater Eye sediments was probably due to the co-migration of butane and propane together with methane, as shown by the measurements of C2+ hydrocarbons concentration at Crater Eye and Off Centre sites (Vanneste et al., 2011).
5. Conclusion

The sediments of CRMV displayed some typical features of cold seep sites, such as shallow SMTZ with AOM coupled to SR consuming the major part of the ascending methane. However part of the methane flux was apparently oxidised aerobically in the bioirrigated shallow subsurface zone of the sediment. Microbial communities involved in AOM included all known ANME types and most of the putative SRB syntrophic partners, but the dominance of GoM-ArcI Archaea and of butane / propane oxidising SRB at the Crater Eye site were remarkable attributes compared to other AOM communities described thus far. In these sediments, ANME-2 and -3 cells were mostly found as loosely organized consortia or single cells, rather than typical shell-type clusters. This could be due the low content of organic matter and fermentation by-products. Finally, the combination of realistic methane profiles from geochemical models with \textit{ex situ} microbial activity turnover, provided AOM rates compatible with the estimated sulphate and methane fluxes at the CRMV. The systematic use of such approach should thus help to better constrain the methane flux and turnover in deep-sea sediments.
CHAPTER VI. General Discussion
Recent development of Gulf of Cadiz carbonate mound studies based on the results in Chapter II contributed to a more complete picture of the mound processes in this location. Besides, the comprehensive description of methane related microbial processes in Chapters III to V provides the basis for a comparative microbial ecology overview in these cold seeps environments. These aspects will be further developed in this section and evaluated along the larger ecological concept of life in low-energy environments.

1. **What is the contribution of AOM to cold-water carbonate mound processes?**

   In the Chapter II of this thesis, it has been demonstrated that the geochemistry and the microbial activity in the Alpha carbonate mound is strongly influenced by the presence of a methane source in the underlying sediments. These results raised new questions on the role of AOM in carbonate mound environments. What is the influence of AOM on the sediment lithology in a carbonate mound? How does it change the stable isotope record and influence carbonate precipitation/dissolution reactions? Does it influence other microbial processes and diagenetic processes? Are methane and AOM common features of all carbonate mounds on the Pen Duick escarpment? To address these questions, a new multidisciplinary research cruise was organized on board of the R/V Marion Dufresne in July 2008 (MD 169 MICROSYSTEMS cruise). In addition to Alpha Mound, two other mounds were sampled along the escarpment and were named Beta and Gamma mounds (number 252 and 254 in the map Figure I-13). A brief review of these new results (Larmagnat and Neuweiler, 2011; Templer et al., 2011; Van Rooij et al., 2011; Wehrmann et al., 2011) and their significance is discussed in this section.

   i) Alpha mound was sampled in its summit, with an ~70 m offset comparing to the core used in chapter II. Unexpectedly, the sediment geochemistry of the two cores was very different: the sulphate profile gradient was smaller and did not reach complete depletion in the new core. Hence, assuming that the sulphate profile is controlled by methane and AOM, this observation implies that methane distribution is very heterogeneous in the Alpha mound and suggest the existence of preferred channelling pathways rather than uniform upward diffusion.

   ii) Methane and AOM rates where detected (AOMR of <1 nmol cm$^{-3}$ d$^{-1}$) in the Beta mound but not in the Gamma mound, showing that methane flux is not a common feature of all these mounds. This observation questions the links between methane and carbonate mound development in this region. If cold-water coral would have benefited from the development of
methane derived carbonate on the sea-floor, then all mounds should witness of at least paleoseepage proxies. However, more compelling evidence for a genetic link between mounds and methane should be sought in the deeper strata underlying these structures. Hence the scientific drilling proposal currently in review at the Integrated Ocean Drilling Program (IODP 673, 'Atlantic Mound Drilling 2: Morocco Margin') for a scientific drilling of the Alpha mound will should document this aspect further.

iii) Pen Duick carbonate mounds have been found to be extremely rich with regard to oxidised iron. In Gamma mound with no AOM, organic matter mineralisation is thus probably coupled to dissimilatory iron reduction by heterotrophic iron reducing bacteria (Lovley, 1991) In Alpha and Beta mound however, where AOM produced sulphide, iron precipitates as iron sulphide, and mineralization mostly occurred through the sulphate reduction pathway.

iv) In term of subseafloor microbial metabolism, AOM appears to be the most active compared to other processes, as ATP measurements showed significant values only in the AOM zones. This further confirms that the in vitro sulphate reduction rates observed in Chapter II, that were lower in the AOM zone than in the organoclastic sulphate reduction zone, are probably largely underestimated due to the lower methane solubility in vitro. As expected, microbial community structure profiles evaluated by fingerprinting techniques show significant shifts in the AOM zones toward the presence of AOM mediating microorganisms.

v) These studies provided a compelling explanation for the apparent coral dissolution in methane bearing carbonate mounds. Such observations were counterintuitive at first sigh since AOM tend to induce carbonate precipitation. However AOM derived sulphide reoxidation, a process that lowers the pH, has been shown to induce carbonate dissolution. Hence, periods of sediment oxidation by seawater following periods of methane flux decline may provoke such cold water coral fossils dissolution in methane bearing sediments.

Based the Chapter II and these new results, the Alpha and Beta mounds were clearly identified as new cold water coral carbonate mound types distinct from the numerous mounds found along the European margins, in term of biogeochemistry and diagenetic processes. Hence, these new mound types can serve as comparison for the comprehension of the processes involved in the numerous paleomound systems that have thrived during most of the earth history. In particular, this shows that the inorganic carbon pool isotopic signature is not necessarily coupled to the trophic regime of the main mound builders. The low $\delta^{13}C$ values observed in alpha mound could have indeed suggested that corals had relied on methane or other hydrocarbon as a food source. Rather, our results are highlighting the importance of post-
CHAPTER VI

formation microbial diagenetic processes such as AOM in the geochemistry of the mound sediments.
2. **Comparison of the microbial community diversity in the three MV and possible environmental controls.**

The factors influencing the microbial diversity in natural ecosystems is a central question in current microbial ecology, and the principles underlying patterns of biodiversity of Bacteria and Archaea are only starting to be unveiled (Horner-Devine et al., 2004; Martiny et al., 2006; Prosser et al., 2007; Ramette and Tiedje, 2007; Fierer, 2008; Green et al., 2008). Moreover, the significance of functional redundancies within microbial communities is still poorly understood: if indeed several phylogenotypes are able to carry out the same reaction -as for ANME-1 -2 and -3 cells- then what factor determines the presence and dominance of each of these phylogenotypes? Here, we compare both archaeal and bacterial diversity (Shannon Index), observed richness and estimated richness (Chao1 index) in all mud volcanoes investigated in Chapter III, IV and V. The three diversity parameters were clearly highest at Darwin MV West Rim site than in the other MV’s (Figure VI-1). Compared to other sites, the Darwin West Rim habitat was more complex or heterogeneous, as shown by the presence of both aerobic methanotrophs bacteria and ammonium oxidizing Archaea, along with oxygen sensitive SRB and AOM microorganisms within the same sediment sample (1 cm depth interval), thus indicating a wide diversity of ecological niches at this site. This habitat also sustains a higher energy flux / productivity, as shown by the higher AOM activity at this site and the presence of gas at saturation concentration in near surface sediment indicating a high methane flux. Conversely, both archaeal and bacterial observed diversity (Sobs, Figure VI-1) and estimated richness (Chao I) were lowest at Mercator Rim site, where ANME-1b strongly dominated. At this site, habitat conditions exerted a strong selective pressure due the extreme hypersaline conditions. Therefore, our results are in agreement with the propositions that habitat heterogeneity and higher productivity can promote higher bacterial and archaeal diversity, whereas diversity are lower in more extreme and challenging habitats (Horner-Devine et al., 2004; Fierer, 2008).
Assessing the microbial diversity with clone libraries is a powerful tool when studying low diversity environments such as the Mercator MV. Most OTU where represented by a large number of sequences and estimated richness was close to observed richness. These were indications of good coverage of both archaeal and bacterial diversity. However, in heterogeneous environments bearing large number of different microbial cells, good diversity coverage necessitates larger sequencing efforts that may become costly and time consuming. Hence if this approach remains valid to determine the major taxa present, high throughput sequencing techniques should be used when the goal is to compare diversity patterns based on statistical clustering analysis between high diversity habitats. In the last phase of this PhD work, multiplex pyrosequencing of 16S phylotags was used for the microbial community fingerprinting of all AOM zones investigated in this thesis. Based on this large number of sequences (~250 000), each representing a single cell of the community, it is likely that this approach will yield a better picture of each community and some yet undetected patterns in AOM community structure.
At the phylotype level, our results at Mercator MV, together with previous studies of hypersaline methane seeps mentioned in Chapter IV, indicate that hypersalinity has a selective effect toward ANME-1 cells. The fact that only ANME-1 cells were found in clone libraries and microscopic observations (Chapter IV) also indicated that hypersalinity excluded other ANME phylotypes.

In this work, the presence of other selective factors was less clear. For instance, methane flux toward the sea floor has often been invoked as a determinant parameter in selecting different AOM microbial communities and ANME cell types (Knittel et al., 2005; Niemann et al., 2006a). In this line, (Niemann et al., 2006a) demonstrated that the amplitude of methane flux clearly caused an ecological zonation at Hakon Mosby Mud Volcano (HMMV) in the Barent Sea, with different ANME’s and aerobic methanotrophs mediating methane oxidation. In our study, such influence could not be clearly observed as ANME-2 cells constituted a large part of the microbial communities in both high flux (Darwin MV) and lower flux (CRMV). In addition, Chapter V and (Vanneste et al., 2011) have shown that methane flux was decreasing from the crater centre toward the rim of the mud volcanoes. However, no clear ANME distribution pattern could be observed within such natural gradient (Chapter V). Hence, it is possible that methane flux do exert a role in shaping cold seep microbial communities, but probably at larger flux amplitude than the one observed in the investigated mud volcanoes.

Finally, the ability of AOM communities to form massive carbonate structure on the seafloor has an indirect influence of the community structure. As shown by the study of Darwin MV (Chapter III), the formation of such carbonate hard grounds induces not only methane flux relocation at the rim of these hard grounds, but also the focusing of this flux. Hence, this process promotes the formation of very active and very diverse microbial communities, but in discrete hotspots.
3. Are Gom-Arc I cells novel AOM mediating Archaea?

At CRMV, the archaeal community involved in AOM was dominated by sequences of the GoM-Arc1 phylotype. Members of this group were also abundant in Darwin MV west Rim. Such dominance of GoM-Arc1 has no analogues among reported AOM communities. In this section we discuss the possibility for these cells to form a novel ANME type.

The closest relative of the GoM Arc I group is the AOM associated Archaea group, or AAA (Knittel and Boetius, 2009) (Figure I-6). Members of AAA were initially found together with NC10 bacteria in an enrichment mediating the limnic anaerobic oxidation of methane coupled to denitrification N-AOM (Raghoebarsing et al., 2006). Despite the report (Ettwig et al., 2008; Ettwig et al., 2010) of NC10 bacteria mediating N-AOM alone, a methanotrophic role of AAA is supported by the reports of (Hu et al., 2009) showing the enrichment of AAA cells in a reactor performing N-AOM at 35°C, and of (Schubert et al., 2011) describing large aggregates of 13C-depleted AAA cells in the putative AOM zone of an alpine lake. Hence, despite the fact that GoM Arc I are not monophyletic with other ANME groups, their strong affiliation with AAA argues for a methanotrophic role of GoM Arc I.

Secondly, the GoM Arc I group falls within the large branch of methanogens. This specialized branch of Euryarchaeota is characterized by the presence in its members of the unique methanogenesis pathway as the sole energy conservation metabolism. The only exception is the presence of anaerobic methanotrophs (ANME), which also possess such pathway (Hallam et al., 2004; Meyerdierks et al., 2010), but apparently use it in the reverse order (reverse methanogenesis hypothesis). Hence, It is very likely that GoM Arc I are either methanotrophs or methanogen. However, in CRMV sediments and Mercator MV sediments where GoM arc I constituted a large part of the microbial community, methanogenesis activity based on a wide range of substrate was absent, but AOM activity was high. Hence these observations also strongly argue for a methanotrophic role of GoM Arc I cells.

Thirdly, environmental molecular surveys indicate that GoM Arc I distribution show a strong bias toward AOM zones off mud volcanoes from the Gulf of Cadiz (Chapter III, IV and V), East Mediterranean sea (Heijs et al., 2005; Heijs et al., 2007; Omoregie et al., 2009; Pachiadaki et al., 2010) and Gulf of Mexico (Lloyd et al., 2006, Mills et al., 2003, 2004). This pattern of distribution thus also argues for a participation in AOM.

The formation of typical consortia of GoM Arc I with DSS cells would have provided further evidence for their role in AOM. In spite of repeated attempts to observe GoM Arc I...
cells by fluorescence microscopy using group specific probes, these cells could not be detected. In parallel however, a clear fluorescence signal of recombinant *E. coli* expressing the GoM Arc I 16s rDNA gene was observed, demonstrating that the probe hybridisation was not affected by steric hindrance or 16s rRNA secondary structure. Since ANME cells resisted all cultivation attempts thus far, the demonstration of methane assimilation based on $^{13}$C stable isotope profiles of the ANME cell content by FISH and NanoSIMS (secondary ion mass spectrometry) is currently the golden standard to identify a methanotrophic metabolism (Orphan et al., 2002b; Treude et al., 2007). Hence, FISH labelling of GoM Arc I cells clearly deserves further efforts, as their successful identification by fluorescence microscopy would open the way for NanoSIMS experiments and the elucidation of the metabolism of this conspicuous archaeal group.
4. How to better evaluate methane profile, flux and turnover from deep-sea marine sediments?

The Chapters I to V of the thesis clearly illustrated the difficulty to quantify deep-sea benthic processes when gas solutes are involved. The very large difference of methane solubility between *in situ* and *ex situ* conditions indeed hampers the reconstruction of methane concentration profile and the evaluation of methane flux and turnover rates. Two methods are currently in use to estimate these parameters: reaction-transport models and microbial activity measurement using labelled substrates. Each method bears some uncertainties. On the one hand, the former do not involve true microbial activity but only infer possible activity based on solute profiles. The limit of such method can be well illustrated by the situation at Mercator MV (Chapter IV): in the quasi-absence of sulphate gradient and of sulphide due to its precipitation as AVS, a reaction transport modelling approach would have concluded that AOM was completely inhibited hypersaline conditions. On the contrary, our results have demonstrated that AOM was active with significant areal rates. The major drawback of the *ex situ* method, on the other hand, is the very low methane solubility at ambient pressure compared to the deep sea (see Figure I-1), which may lead to a large underestimation of the *in situ* methane pool size. Since AOM rates are calculated by multiplying the measured turnover by the size this methane pool, *ex situ* rates underestimate *in situ* rates when the *in situ* methane pool exceeds the maximum methane solubility at ambient pressure. In the Chapter V, we have proposed a recalculation method for AOM rates measured *ex situ* that permitted to reduce the gap between rates with the two methods.

Recent development in deep-sea instrumentation should permit gaining precision in these estimates. Firstly, using a submersible *in situ* membrane inlet mass spectrometer, (Wankel et al., 2010) could accurately measure methane in a deep-sea brine pool from the Gulf of Mexico. Similar to our conclusions in Chapter V, they found that AOM rates corrected for *in situ* methane concentration were 35-40 fold higher than *ex situ* rates. Hence, if coupled to an *in situ* sediment pore water extraction system, such apparatus has the potential to provide more accurate methane profiles. Secondly, (Parkes et al., 2009) described a set of tools for deep-sea sediments sampling, recovery and in vitro incubation without depressurisation during the entire process. With some modifications, such tools could be used for the injection of radiolabeled...
substrates in sediment maintained at in situ conditions of pressure and temperature as well as for the recovery of the \textit{in situ} methane pool.

The determination of accurate activities is important not only for the estimation of methane turnover and emission from methane seep sites, but is also determinant for the understanding of this metabolism that has a critical energy yield under methane concentration at ambient pressure. The determination of AOM and SR cell specific rates is thus a key parameter to assess how much energy is truly gained by these microorganisms.
5. **Solving a bioenergetic gap: other mechanisms for AOM?**

AOM coupled to SR has a weak energy yield under standard conditions (-16.9 kJ mol\(^{-1}\)), and a slightly higher yields under *in situ* conditions (up to ~40 kJ mol\(^{-1}\)). Such yields are higher than the minimum energy quantum that can support the formation of ATP, and has thus the potential to support at least cellular maintenance metabolism (3-10 kJ mol\(^{-1}\)) (Hoehler et al., 1994; Alperin and Hoehler, 2009). However, in the case of AOM, and assuming a syntrophic mechanism between ANME and SRB, the conserved energy has to be shared by both partners. Moreover, the dominant hypothesis for AOM functioning invokes the electron transfer via a chemical shuttle such as H\(_2\), acetate, formate, or methylsulphide (Nauhaus et al., 2002; Nauhaus et al., 2005; Moran et al., 2008). Hence, increasing distance between ANME and SRB further decreases the overall energy yield of the AOM reaction (Figure VI-2). In other words, AOM reaction stops if SRB do not maintain the level of the reduced shuttle produced by ANME at a very low level. This constrain is common with known cases of interspecies electron transfer (Stams and Plugge, 2009): for instance, butyrate can be fermented in acetate + H\(_2\) by a bacteria only if H\(_2\) is maintained very low (1 Pa) by the activity of a methanogen: \(4H_2 + CO_2 \rightarrow CH_4 + 2H_2O\) within tight aggregates of both syntrophs.

(Figure VI-2 Free energy yield of methane oxidation (MO, plain line) and sulphate reduction (SR, dashed line) as a function of H\(_2\) as a possible interspecifics transfer agent. Assuming a minimum free energy yield of -10 KJ mol\(^{-1}\) for cell maintenance, this graph defines a hydrogen concentration range (\(\Delta ITA\)) for which the coupling of MO to SR is energetically favorable (reproduced from (Alperin and Hoehler, 2009)). Since hydrogen concentration gradient between the hydrogen producer and the hydrogen scavenger is directly dependant on the cell-cell distance, AOM energetic yield is strongly constrained by such distance. (Alperin and Hoehler, 2009) calculated that, in the favorable conditions of the Hydrate Ridge methane seep sediments, an increase of 5 \(\mu\)m between an ANME and a SRB cell correspond to a free energy loss 30%).
Without formally disproving it, previous observations and results from this thesis do not argue for interspecies hydrogen transfer in AOM. Firstly, the morphology of ANME and SRB do not conform to such model. In shell type of aggregates (Figure I-5), the ANME-SRB exchange surface is minimal and (Alperin and Hoehler, 2009) calculated that such aggregates has a high energy cost for both partner. Secondly, most ANME-1 cells (Chapter IV and addenda of this chapter), and many ANME-2 and -3 cells found at CRMV (Chapter V) have been observed to live without direct bacterial partner. Thirdly, none of the electron shuttle that has been tested can uncouple AOM and SR, nor enrich for a single partner.

We thus propose that, with such a lack of coherence between these observations and the proposed mechanism, one should continue to explore alternative mechanisms for AOM. For instance, a single cell could mediate both reactions. None of the known sulphate reduction genes has been found in metagenomic studies of ANME cells (Hallam et al., 2004; Meyerdierks et al., 2010), but it is possible that such genes are parts of the genomes that were not sequenced, or that sulphate could be reduced by a novel pathway. Alternatively, there are growing evidences that microbial cells can exchange electrons directly through conductive appendages (nanowires) (Reguera et al., 2006; Reguera, 2011), or through membrane bound cytochromes (Mehta et al., 2005; Reguera, 2011; Summers et al., 2011). In mesocosms experiments with marine surface sediments, diffusion-independent electron shuttling over 1.2 cm distance has been shown (Nielsen et al., 2010). The expression of genes coding for membrane c-type cytochromes by ANME-1b cells (Meyerdierks et al., 2010) would be in line with this hypothesis of a direct electrons shuttling, i.e. electrical current between different microbial cells (Mehta et al., 2005). The nature of such conductive matrix is not clear, but the high amounts of AVS at Mercator mud volcano (Chapter IV) indicated the presence of the conductive iron sulphide. However, evidence for electroactivity of ANME-1 or Seep-SRB cells are lacking. To address this question, we built a microbial fuel cell able to host AOM communities from the investigated mud volcanoes. In absence of sulphate or other electron acceptors besides the anode, the anodic potential of the fuel cell remained very low around -200 mV during three months. Considering that the electrical circuit was closed with a 10 kΩ resistance and that methane was the sole electron donor, these preliminary results indicated that methane was probably oxidised and electrons directly flowing through the fuel cell circuit. Hence, this line of research should certainly be continued.

Finally, different ANME phylotypes may widely differ in term of phylogenetic affiliation (Figure I-6), morphology (Chapter II, IV and V), genomics (Hallam et al., 2004; Meyerdierks et al., 2010), ecological niches (see discussion of Chapter IV), and capacity to form organized
aggregates with SRB (Chapter III, IV and V). There is thus a good possibility that different ANME groups could find different strategies to carry out the AOM reaction. This was the rational of the study of Nauhaus and colleagues (Nauhaus et al., 2005) studying the impact of different environmental parameters on ANME-1 or ANME-2 dominated communities. However, the Black Sea ANME-1 community of this study also bears a large amount of ANME-2 cells, which may have interfered with the results of this comparative study. In Chapter IV, we have found that hypersalinity is probably a very good selective parameter for ANME-1 cells. We thus argue that salt concentration should be used to cultivate and maintain stable ANME-1 communities in vitro, and perform physiological analysis to elucidate the specific metabolism of this group.
6. **What can trigger the formation of shell type aggregates of ANME and SRB?**

Since its discovery (Boetius et al., 2000), conspicuous aggregates of Archaea and Bacteria have raised large interests and fuelled numerous studies (Knittel and Boetius, 2009). The reason is that such high level of organisation has to be strongly supported by an ecological or thermodynamic determinism, making these aggregates an interesting model for microbial ecology. Initially, the formation of such aggregates has been attributed to the syntrophic nature of AOM consortia between a methanogen functioning in reverse and a sulphate reducer. However, microscale reaction transport models (Alperin and Hoehler, 2009) have demonstrated that not only is contact between partners unnecessary for AOM functioning, but also that shell type organisation is detrimental to the overall AOM energy yield in a syntrophic perspective. This finding thus calls for alternative models.

Alperin and Hoehler (2009) have proposed that organic matter fermentation could be responsible for the formation of these aggregates. H$_2$ in sediment impedes AOM functioning, as suggested by *in situ* studies (Joye et al., 2009). Hence, by consuming fermentation-derived hydrogen in the outer layer, sulphate reducers would maintain low H$_2$ levels within the aggregates and allow AOM. Further, this model implies that most ANME cells would function as hydrogenotrophic methanogens, and only a minor part of the inner core would mediate AOM, relegating this reaction to a side reaction of organic matter mineralization. In this view, the high yield of organic matter mineralisation would fuel the low-yield AOM reaction.

Alternatively, based on a comparison of AOM communities in different environments, and previous work on ANAMMOX (anaerobic ammonium oxidation), one could speculate that oxygen could be considered as a selection factor for AOM microorganisms. Under controlled aerobic conditions, OLAND (Oxygen-Limited Autotrophic Nitrification/Denitrification) community is organized in clusters where layers of ammonium- and nitrite- oxidising bacteria, both consuming oxygen, surrounds a core of oxygen-sensitive ANAMMOX cells (Vlaeminck et al., 2010) Figure VI-3. In this case, the shielding of ANAMMOX cells from oxygen has been invoked to explain such organisation. This hypothesis also considers that SRB are acting as a shield for the ANME cells of the inner core, as suggested by the aggregates geometry. By producing large amounts of sulphide, which spontaneously reacts with oxygen, SRB would
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prevent any oxygen incursion inside the aggregates and protect the very oxygen sensitive ANME cells.

We argue that similar processes could lead to the formation of highly organized ANME2/SRB clusters in near surface, bioturbated gassy sediments. The consistent position of SRB cells outside of the shell-type clusters, whereas ANME-2 cells are forming the cluster core (Chapter III) would be in agreement with such protective mechanisms. At CRMV, where the AOM zones are deeper and well below the possible oxygen incursion zone by bioturbation or bioirrigation, shell type consortia were only rarely observed (Chapter V).

Moreover, (Dekas et al., 2009) have shown that ANME-2 cells are capable of nitrogen fixation. This highly oxygen-sensitive enzymatic pathway is only performed in the very inner core of the shell type aggregates (Figure VI-4). These observations are thus in line with the oxygen shielding hypothesis.
Figure VI-3 Microbial consortia performing the oxygen limited autotrophic denitrification (OLAND) provide a good model for the study of microbial aggregate formation. Such consortia are formed when oxygen is present. Oxygen is consumed by ammonium (green) and nitrite (blue) oxidizing bacteria. Such geometry prevents oxygen to penetrate in the inner core of the aggregate where oxygen-sensitive cells perform the anoxic ammonium oxidation (ANAMMOX). Reproduced from (Vlaeminck et al., 2010)

Figure VI-4 Patterns of nitrogen fixation in shell type AOM consortia involving ANME-2 cells (red) and DSS cells (green). Nitrogen fixation revealed by NanoSIMS and fluorescence microscopy occurs mostly in the inner core of the aggregates and is mediated by ANME-2 cells. Reproduced from (Dekas et al., 2009)
7. Perspectives

Despite low AOM energy yield, ANME cells live in extremely hypersaline, or in negative temperature habitats. They are able to fix nitrogen for biomass synthesis, one of the most energy-demanding metabolisms. They are also able to invest energy in the formation of highly organized consortia. Hence, all of the currently available data fits with the concept developed by (Valentine, 2007) of adaptation of Archaea to energy stress (illustrated in Figure VI-5). In this review of archaeal energy conservation metabolisms, cellular biochemistry and environmental distribution highlights that Archaea are well adapted to energy stress. In the particular case of AOM, energy stress is intrinsic of the reaction yield itself and of its syntrophic functioning. Such considerations constitute an incentive to reconsider our views of microbial ecology in low-energy environments. This domain of research has indeed built upon observations of the surface world where energy is abundant due to the presence of photosynthesis: this reaction maintains a very large redox gradient by reducing carbon to organic molecules and oxidizing water into oxygen. This considerable amount of energy dictates the ecology of surface microorganisms, with generally fast metabolism turnover and relatively short generation times. In this “hyperenergetic” environment, energy-conserving reactions are mostly irreversible because of the very high energetic steps involved. In a “hypoenergetic” environment such as the deep subsurface biosphere, microorganisms have to conserve energy from electrons flowing along very small redox gradients. Maintenance energy should be kept minimal, with generation times orders higher than those in surface life. Biological energy quantum, the minimum energy that can be conserved to sustain life, has to be lower. Yet, deep subsurface constitute the major reservoir of microorganisms on earth, estimated to amounts up to two third of the earth prokaryotic biomass (Whitman et al., 1998). ANME cells have been found in 111 Myrs old sediments at 1600 mbsf (Roussel et al., 2008). Hence, in conditions close to thermodynamic equilibrium, the capacity to revert metabolic pathways to harvest energy from small environmental changes is probably a distinct feature of the deep biosphere microorganisms.
Figure VI-5 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. Only the type strain for each species is shown, and an average temperature is given for species that have a range for the optimal growth temperature. The overlaid blue ‘zone’ comprises environmental conditions to which bacteria are best adapted, the overlaid pink zone comprises conditions to which archaea are best adapted, and the grey zone represents those conditions to which both archaeal and bacterial species are well adapted. Data were compiled from Bergey’s Manual of Systematic Bacteriology and from the primary literature. Reproduced from (Valentine, 2007)

Figure VI-6 Schematic view of the latent redox mechanisms that might impact methane biogeochemistry and microbial activity in subsurface sediments. At top: radiolysis of water can potentially produce oxidants for methane. Note that a balanced material cycle is possible, with the potential to regenerate all starting material, but also note that the relative proportions of chemicals such as methane and acetate (Ac) could vary. At bottom, a potential scheme by which halogenated organic matter might be liberated from sediment and draw reducing equivalents away from methanogenesis. Reproduced from (Valentine, 2011)
Radiolysis of water as source of hydroxyl radicals, or halorespiration using halogenated compounds released during refractory organic matter slow degradation reviewed in (Valentine, 2011); Figure VI-6), have the potential to sustain such metabolisms. There is no doubt that new ecological principles, novel metabolic pathways and energy conserving reactions, as intriguing as in AOM microorganisms, will be revealed by studying this environment.
CHAPTER VII. Experimental Procedures
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Since the same approach has been employed to study the four different mud volcanoes, the Material and Methods of Chapter III to V display large overlaps. Hence, the methods that were common to these chapters are reviewed in this single separated section.

Bathymetric data and sediment samples were acquired during the JC-10 cruise aboard the RV James Cook (2007) using the Remote Operated Vehicle (ROV) ISIS, sediment surface corer (mega corer), as well as gravity and piston corer.

1. Microbathymetry.

The microbathymetry data processing utilized the following field data produced by the ROV ISIS: the sonar head data, ultra-short baseline acoustic positioning, Doppler navigation data and altitude data from the vehicle’s sensors. The processing was done with the Caraibes package (V 3.3) developed by the IFREMER and custom-tailored for the ROV ISIS specifications. The processing workflow included, integration of ISIS sonar head data with attitude sensors data and conversion into the CARAIBES native format, calculation of the true water depth using vehicles altitude and pressure sensors data, cleaning of the bathymetry data (removal of extreme values), filtering and smoothing of navigation data, merging of sonar head and navigation data and gridding and output of XYZ data. The resulted grids were then imported into Gilden Software’s Surfer (TM) and ESRI’s ArcGIS (TM) for further processing, mapping and visualization. Surfer was used for corrections of obvious depth and lateral misfits of adjacent swaths, and merging of the individual swaths into a complete coverage. The coverage was then imported into ArcGIS and used for the production of maps, data visualization and sampling plans.

2. Coring and Sampling

Sediments were recovered by gravity-, piston-, or mega corer coring (GC, PC and MC, respectively). Subsequently to recovery, GCs and PCs were cut into 1 m sections and stored at 4°C until further processing (less than 10 h after recovery). MCs as well as GC and PC core sections were sub-sampled by extruding sediments from the core using a plunger.
EXPERIMENTAL PROCEDURE

3. Geochemistry

Pore water was extracted from 25 ml sediment using a pore water press (Reeburgh, 1967) equipped with 0.45 µm pore size nitrocellulose filters. Porewater was then filtered through 0.1 µm pore size filters prior to sample storage. For sulphide measurement, 1 ml of pore water was preserved in 500 µL zinc acetate solution (10% w/v). Acid volatile sulphide (AVS) was extracted from sediment sealed in a glass jar by adding 6N HCl under continuous N₂ flow collected in a 7 mL Zn acetate trap, using the experimental setup described in (Kallmeyer et al., 2004). Concentration of both dissolved and AV sulphide was measured calorimetrically using the methylene blue method (Cline, 1969). Sulphate, chloride and acetate were measured by ion chromatography as described in (Parkes et al., 2007).

Methane dissolved in sediments/porewater was analysed according to (Parkes et al., 2007). In short, 2 cm³ of sediment (sampled with a 5 ml cut-off syringe) were transferred into a glass vial containing 6 mL NaOH (2.5%, w/v), which was then immediately sealed with a butyl rubber septum and stored upside down. Methane collected in the headspace was analysed by gas chromatography and flame ionisation detection (Perkin Elmer/Arnel Natural Gas Analyser).

4. Ex situ rate measurements.

Radiolabeled tracer turnover during short-term sediments incubations is a very efficient method to determine how much substrate is metabolized per unit of time. The turnover is then given by the radioactivity of the product divided by the initial radioactivity contained in the substrate, assuming that radioactivity of the sediment prior to the experiment is null or neglectible compared to the one introduced by the radiolabeled tracer. Alternatively, substrate turnover is sometimes determined by stable isotope labelled substrate, thus avoiding inconvenience and safety measures associated with the use of radioactive products. Such approach is often used to follow substrate turnover in long term sediment incubation in the lab (see for instance (Beal et al., 2009). In these homogenized and slurried sediment incubations, the substrate turnover is determined by the evolution of the stable isotope profile with time. However, in the case of short-term incubations with undisturbed sediments, of which the aim is to study the microbial metabolism as close as possible to in situ conditions, such approach would introduce incertainties linked with the stable isotopic profile of inorganic carbon and sulphur pools already present in sediments prior to incubation with labelled substrates. Radiolabeled substrates have thus been widely used for the determination of AOM SR and MG
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turnover rates in marine sediment in *ex situ* (on board, immediately after sediment recovery) conditions.

For AOM and SR rates measurements, core-sections were sub-sampled with mini cores (polycarbonate tube, diameter 2.5 cm, length 20 cm) with silicon-sealed side injection ports spaced every 2 cm, or with glass barrels (diameter 1 cm, length 6 cm) with a syringe plunger at one end, and a butyl rubber stopper at the other end as injection port (for details see (Treude et al., 2005b). Within 2 hours after sampling, methane radiotracer (25 µl of saturated aqueous $^{14}$CH$_4$ solution, 5 KBq) or sulphate radiotracer (5 µl of aqueous $^{35}$SO$_4^{2-}$ solution, 60 KBq) was injected through the injection ports, and samples were incubated for 24 hours in the dark at *in situ* temperature (e.g. 13° for Mercator MV stations and 4°C for CAMV, T was measured during ROV dives). After incubation, AOM and SR reactions were stopped by transferring the samples into glass flasks containing 25 ml NaOH (2.5% w/v), or 50 mL plastic tubes containing 20 mL zinc acetate solution (20% w/v) respectively. Methane and sulphate turnover were determined as described elsewhere (Jørgensen, 1978; Treude et al., 2003; Kallmeyer et al., 2004). Briefly, the methane pool present in each incubation ([CH$_4$]) was determined by gas chromatography using a thermal conductivity detector. The radioactive methane pool $^{14}$CH$_4$ in each sample was then determined by stripping the incubation headspace out with natural air flow, oxidising it through 850 °C in line furnace, and trapping the resulting carbon dioxide in phenylethylamine. The radioactive inorganic carbon resulting from microbial methane oxidation was then extracted from the samples by acidification with 6N HCl and trapping the evolving $^{14}$CO$_2$ gas in a phenylethylamine trap. For sulphate reduction turnover measurement, the total reduced inorganic sulphur (TRIS) fraction in sediment incubation was reduced to sulphide by addition of Cr(II). The TRIS fraction was extracted by addition of 6N HCl under a flow of N$_2$, and collected in a Zn-Acetate trap. The radioactivity in the different fractions was measured by adding scintillation cocktail (UltimaGold, PerkinElmer). AOM and SR rates were determined as a product of the turnover and methane or sulphate concentration respectively:

$$AOM = \frac{^{14}CO_2}{^{14}CO_2 + ^{14}CH_4} \times \frac{[CH_4]}{t}$$

$$SRR = \frac{TRIS}{TRIS + 35SO_4^{2-}} \times \frac{[SO_4^{2-}]}{t}$$

where $t$ is inbation time. For AOM rates, methane concentration was corrected for maximum solubility at ambient pressure and in situ salinity (Duan and Mao, 2006).

Rates of methanogenesis were measured as described in (Parkes et al., 2007). Briefly, $^{14}$C labeled carbonate (10 KBq/µl), acetate (6 KBq/µl), methanol (2.05 KBq/µl), or
methylamine (7.25 KBq/µl) were injected into either mini cores (diameter 1.5 cm, length 10 cm) through silicone-filled side injection holes (4µl substrate per 1 cm depth interval), or 10 ml cut off syringes closed with rubber stoppers (7.5 µl substrate per syringe), and incubated at in situ temperature for 7 to 21 hours in nitrogen flushed gas tight bags. Microbial reactions were then stopped by transferring incubations into glass jars containing 7 mL of 1M NaOH. In the laboratory, ^14C methane was determined as for AOM. Substrate turnover was determined according to the radioactivity of the trapped CO₂ and the amount of added label in the product pool. Rates were calculated as for AOM and SR rates by multiplying the radiolabeled substrate turnover per time unit by the substrate concentration (see above). For methylamine methanogenesis, rates were obtained by multiplying the substrate turnover value by an arbitrary methylamine concentration of 5µM (as methylamine concentration in pore water was consistently below detection limit). Me-MG rates should thus be considered as maximum potential rates rather than actual ex situ rates.

5. Nucleic acid extraction and amplification.

Environmental DNA was extracted from five replicates, each of 0.3 g sediment, using the PowerSoil DNA extraction Kit (MoBio Labconsult, Brussels, Belgium) according to manufacturer recommendations. 16S rRNA genes were amplified using Arch20f/Uni1392r primer pair for Archaea (Kane et al., 1993; Massana et al., 1997) and GM3f/GM4r primer pair for Bacteria (Muyzer et al., 1995). PCR conditions were as in (Losekann et al., 2007). PCR were carried out in triplicate, amplicons were pooled and purified (PCR purification kit, Qiagen).


Purified PCR products were ligated in the PCR-2.1 vector and cloned into Top10 chemocompetent cells using TOPO-TA cloning kit (Invitrogen, Merelbeke, Belgium). The size of the insert was verified with a colony PCR on overnight grown positive transformants using M13 vector specific primer pair (Invitrogen). M13 PCR amplicons were used for amplified ribosomal DNA restriction analysis (ARDRA) of the libraries with AluI and RsaI (Fermentas, St. Leon-Rot., Germany). Digested DNA was run on a 3% agarose gel and images were analyzed with the Bionumerics software (Bionumerics, Kortrijk, Belgium). Clones were clustered according to restriction pattern using the Jaccard similarity index, and when a cluster contained multiple clones, at least 2 representative clones were sequenced. Bidirectional
sequencing was carried on ABI-3730xl sequencer (Applied Biosystem, Lennik, Belgium) at the Genetic Service Unit of the Ghent University Hospital using M13 vector primers.

7. Sequence accession numbers.

Sequences from this study are published in GenBank under the following accession numbers:


8. **Phylogenetic tree reconstruction and phylotype inference.**

All sequences were aligned with the online SINA webaligner of SILVA (Pruesse et al., 2007); http://www.arb-silva.de), and imported together with closely related sequences in the ARB SILVA database release 102. Sequences were further clustered by operational taxonomic unit (OTU, 98% similarity cutoff) with the MOTHUR software (Schloss et al., 2009). Phylogenetic trees were reconstructed with the ARB software package (Ludwig et al., 2004) using the maximum likelihood method RaxML implemented in ARB (Chapter IV) or the PhyML server (http://www.atgc-montpellier.fr/phyml) (Guindon et al., 2009) in combination with filters removing the most variable position of the alignments (maximum frequency filter implemented in the ARB software package) filtering out most variable positions of the alignment (50% base frequency filter).

9. **(Catalyzed reporter deposition)-fluorescence in situ hybridization (FISH and CARD-FISH) cell staining and microscopic observations.**

Upon core retrieval, 2 cm$^3$ of sediment were fixed in filter-sterilized (0.2 µm) formaldehyde in seawater solution (3% v/v final concentration) for 4 hours at 4°C. FISH and CARD-FISH procedures were carried out according to (Pernthaler et al., 2001; Pernthaler et al., 2002). For CARD-FISH, the following modifications were applied: for permeabilisation of rigid archaeal cell walls, 15 µg/mL proteinase K was used (3 min. at room temperature); for bacterial cell wall permeabilisation, 10 mg/mL lysozyme (15 min. at 37 °C) was used. Horseradish-peroxidase- (CARD-FISH) or Cy3-labeled (FISH) probes targeting the following groups were used: probes Eub338 I-III (Daims et al., 1999) specific for most Bacteria (35% formamide in hybridisation buffer or FA), probe Arch915 (Stahl and Amann, 1991) specific for most Archaea (35% FA), probe ANME1-350 (Boetius et al., 2000) specific for ANME-1 (40% FA), probe EelMS-932 (Boetius et al., 2000) specific for ANME-2 (40% FA), probe ANME-3-1249 with unlabeled helper oligonucleotides ANME3-1243h and 1249h (Losekann et al., 2007) specific for ANME-3 (40% FA) and probe DSS658 (Manz et al., 1998) specific for the Desulfosarcina/Desulfococcus branch of Deltaproteobacteria (60% FA), including Seep-SRB subgroups. Cells were counterstained with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Hybridized cells were examined under an epifluorescence microscope (Carl Zeiss Axioplan, Jena, Germany). For each sample and probe, 50 independent microscopic fields of view were counted, corresponding to approximately 400-800 DAPI-stained cells.
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Lycée Lakanal, Sceaux, France  

Training & experiences

- STAMPS: Strategies and Approaches for the Analysis of Microbial Population Stucture MBL, Woods Hole, USA.  
Analysing microbial community structure with next generation DNA sequencing methods.  
August 2011, 10 days,.  
- Advanced Bioinformatics course, Ghent University:  
Building of a BioPerl/MySQL based script to handle high throughput (454) 16s phylotags datasets, access it via a web interface (apache/php), and analyze it with the “R”/Vegan packages. 15 days, Feb 2010.
- ARB software and molecular phylogeny course, Max Planck Institute – Bremen, Germany
- Microbial activity measurement using radiotracers Max Planck Institute – Bremen.
- Microbial ecology and ecosystem functioning workshop, Lunz, Austria.
  European Science Foundation exploratory workshop on the developments in the field of theoretical microbial ecology and ecosystem functioning. Sept. 2007.
- Microbial Diversity Course, Marine Biology Laboratory, Woods Hole, MA, USA.
  The aim of this 6 weeks course was to study microbial processes in their environmental context, including a very broad range of microbial way of life. Emphasis was put on cultivation and in vitro technique, as well as molecular methods and bioinformatics tools. (06/2005 to 08/2005)
- Lab of Microbial ecology and technology, Gent University, Belgium. (PhD position)
  Microbial ecology of carbonate mounds, cold seeps and mud volcanoes (gulf of Cadiz): linking environmental parameters with microbial activity and community structure. Keywords: Ecosystem functioning, Anaerobic oxidation of methane, radiotracer, (CARD-) FISH, DGGE, archaeal and bacterial 16s clone libraries, phylogenetic tree reconstruction, 16s phylotags pyrosequencing. (Current project). Participation to other projects in marine geochemistry and microbial ecology (GI tract, aquaculture, Microbial Fuel Cells, high pressure reactors).
- Lab of fungal genomics, Wageningen University, The Netherlands. (predoc. Position)
  Fundamental studies on the A. niger secretion pathway using transcriptome analysis with microarrays and protein fluorescence tagging approaches. (02/2004 to 12/2004) Keywords: secretion pathway / microarray / ER protein / FRET.
- Lab of molecular immunology and biotoxins, CICESE, Ensenada. Mexico. (Msc. thesis)
  Creation and selection of shark NAR antibodies and human scFv antibody variable domain against a bee toxin using phage display technique. (01/2002 to 01/2003). Keywords: Antibody Library / Recombinant protein expression / Library panning / Novel Antigen Receptor. Report available

Language

- French: mother tongue
- English: read, spoken and written (TOEFL result: 560pts)
- Spanish: read, spoken and written
- German and Dutch: good notions

Scientific expeditions

- R/V Marion Dufresne, 10 days, July 2008, Gulf of Cadiz. Coord. of Microbiology team
- R/V Darwin and ROV Isis: 20 days, May 2007, Gulf of Cadiz. Microbiology team
- R/V Maria S. Merian: 50 days, May 2006, Gulf of Cadiz. Microbiology team
IT knowledge

- **Molecular ecology and bioinformatics:** proficiency in ARB, unifrac, xploreseq, EstimateS, EbioX, ImageJ, Pintail, Mothur, Qiime… Good notions of Unix, Perl, and MySQL scripting and applications.
- **Geo Information Systems:** ArcGIS, GrassGIS.

Publications


Marzorati, M., **Maignien**, L., Verhelst, A., Luta, G· Sinnott, R., Boon, N., Van de Wiele, T., and Possemiers, S. Use of the barcoded pyrosequencing in an ecological survey of a simulator of the human gastrointestinal tract to follow up a prebiotic treatment. *Submitted*

Y. Zhang, **L. Maignien**, N. Stadnitskaia, P. Boeckx, N. Boon. Stratification in Ginsburg Mud Volcano determines microbial community structure and activity in response to methane and sulfate supply. *Submitted*


**Conference Abstracts of Oral Presentations**


Other interests and activities.

- Alto Saxophone (“Jour de Fête” Big Band, Brussels).
- Sailing Race on 470 and cruiser/racer (Tour de France Voile, Spi Dauphine, World Student Championship, etc…).
- Diving. (PADI Open Water Diver)