A Geomicrobiological Investigation of Sub-surface Mud Volcano Sediments from the Gulf of Cadiz

Julia Claire Sas

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Cardiff University
School of Earth and Ocean Sciences

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Summary

Submarine mud volcanoes (MVs) are a type of cold seep environment where sediment, hydrocarbons and other reduced compounds are channelled upwards to the seafloor from significant depth. These sites can be ecological hotspots because of the potential microbial substrates present in the MV ejecta, and are a potential habitat for deep-sourced prokaryotes (*Bacteria* and *Archaea*). The microbial communities in sub-surface sediments from four separate MVs in the Gulf of Cadiz (Capt. Arutyunov, Bonjardim, Meknes and Porto) were investigated using a multidisciplinary approach. This involved cultivation and culture–independent molecular genetic-based methods, supplemented by basic pore water geochemistry, activity measurements and direct cell counts. Cultivation-independent 16S rRNA and functional (*mcrA* and *dsrA*) gene analyses revealed that the prokaryotes present in the MV sediments were often most closely related to uncultivated organisms. Phylogenetic groups representing major components of the sediment community in these sites included ANME-2a, ANME-1a, Miscellaneous Crenarchaeota Group, *Deltaproteobacteria* and the JS1 candidate division, though community composition varied significantly between MV samples and with depth. Variation in community composition with depth through MV craters paralleled changes in pore water geochemistry indicating this is an important parameter influencing prokaryotic distribution in MV sediments. While containing certain phylogenetic groups 'characteristic' of the deep biosphere, the MV sediments also contained groups commonly associated with near-surface seep environments, suggesting the mud breccia had become colonised by organisms adapted to the present *in situ* conditions over time. Cultivation analysis showed novel organisms and important functional groups (methanogens and sulphate-reducers) could be cultivated from MV sediment. Pure cultures obtained from Capt. Arutyunov included a putative new species of *Arcobacter* named "*Candidatus* Arcobacter subtericola" and species belonging to the genera *Pseudomonas*, *Marinobacter*, and *Halomonas*. Enrichments from Meknes contained *Bacteria* from the groups *Bacteroidetes, Fusobacteria*, *Firmicutes, Spirochaetes, Desulfobulbaceae* and *Desulfovibrio*, and *Archaea* belonging to the genera *Methanogenium* and *Methanococcoides*.

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* The results of this work are presented, described and discussed in Appendix II

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Chapter 1 – General Introduction

Section 1.1 provides a general introduction to the marine sub-surface biosphere and the main types of metabolism that drive biogeochemical cycling in anoxic marine sediments. In **section 1.2** submarine mud volcanism is introduced and a case study of the well-studied Haakon Mosby mud volcano is presented. This case study serves to introduce the various physical, chemical and biological aspects of submarine mud volcanism and allow comparisons to be made with the present work. Literature on prokaryotic population diversity in other mud volcano environments is also summarized in this section. In **section 1.3** the Gulf of Cadiz study area is introduced. **Section 1.4** provides an overview of the methodological approach used, and the project aims are outlined in **section 1.5**.

1.1 Introduction to the marine sub-surface biosphere

Marine sediments cover more than 2/3 of the Earth's surface and the sub-marine biosphere extends down to at least 1,626 meters below seafloor (mbsf), ~111 Ma and 60-100°C (Rousell *et al.*, 2008). The prokaryotes (*Bacteria* and *Archaea*) living within sub-marine sediments are estimated to constitute approximately 70% of all prokaryotic biomass on Earth (Whitman *et al.*, 1998) and are responsible for driving major biogeochemical processes, such as organic matter oxidation and associated re-mineralization, methanogenesis, methanotrophy, sulphate reduction and metal reduction (Jørgensen, 2000; D'Hondt *et al.*, 2004). Prokaryotic cell numbers decrease exponentially with depth from an average around 10^9 cells per cm^3 in the near-surface to as low as 10^4 cells per cm^3 at depth (Fig 1.1, Parkes *et al.*, 1994). The decrease in cell numbers reflects the fact that labile organic matter is preferentially consumed in the near-surface, which leaves only low quantities of increasingly recalcitrant material remaining during burial for further metabolism (Westrich & Berner, 1984; Parkes *et al.*, 2000). Commensurate with this, microbial activities in the sub-surface are low with metabolic rates 10^3 – 10^5 times lower than in organic-rich near-surface sediments (Lovley & Chapelle, 1995).

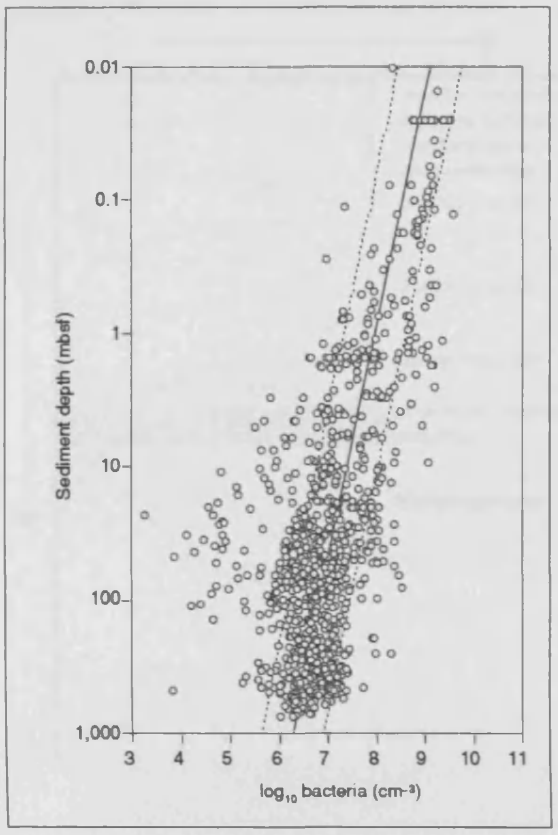


Fig 1.1 Depth distribution of prokaryotic cells in marine sediments. Data points represent AODC counts from a wide variety of sediment types (carbonates, turbidites, gas hydrate bearing, sapropel containing, hydrothermal); sediment depths (to ~800 m); sediment ages (to ~ 15 My); sediment temperatures (to ~150°C) and water depths (to ~5800 m). Solid line represents the general regression line model. Dashed lines are the upper and lower 95% prediction limits. Reproduced from Telling *et al.*, (2004), after Parkes *et al.*, (1994).

Biogeochemical processes in the marine sub-surface are zoned. Typically, electron acceptors (e.g. O_2 and SO_4^{2-}) diffuse into the sediment from the overlying seawater, or accumulate during sedimentation (e.g. metal oxides), and are respired by prokaryotes with depth in a predictable cascade according to their energetic efficiencies. Oxygen is reduced first, and then nitrate, manganese (Mn^{4+}) oxides, iron (Fe^{3+}) oxides, sulphate and finally carbon dioxide. This sequence of reactions is reflected in the pore water chemistry (Jørgensen, 2000; Telling *et al.*, 2004), hence, the measurement of pore water chemical profiles is a useful guide to which biogeochemical reactions are dominating at a given depth within the sediment. An idealized marine sediment pore water profile is shown in Fig 1.2.

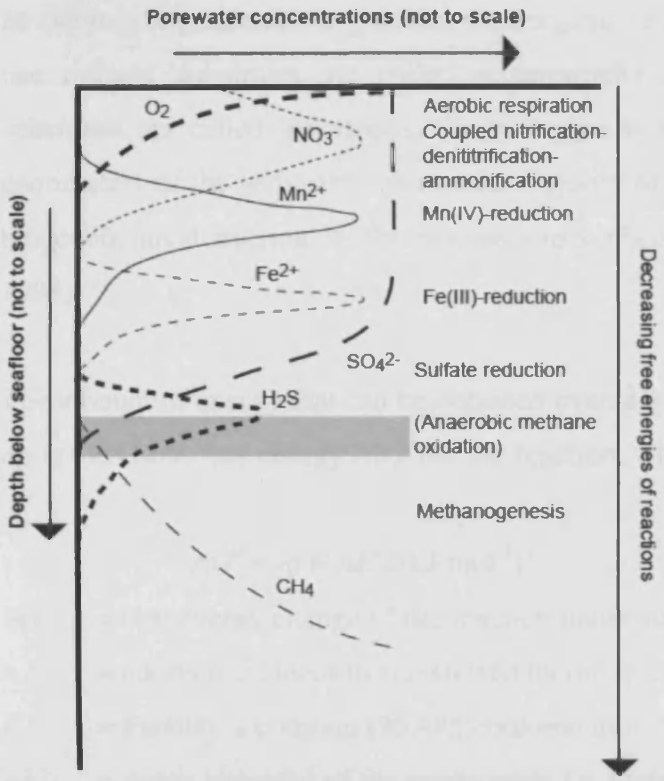


Fig 1.2 Pore water profile in idealized marine sediment. The geochemical sequence is determined by the free-energy yield of the various redox reactions shown (see text). Oxygen is consumed first, followed by nitrate, manganese (Mn^{4+}) oxides, iron (Fe^{3+}) oxides, sulphate and finally CO_2. Aerobic ammonia oxidation (nitrification) and anaerobic methane oxidation are also shown. Note depth is not to scale. Reproduced from Telling *et al.*, 2004.

There are several factors determining the exact nature of the biogeochemical zonation at a specific location: the quantity of available organic matter, the sediment porosity and permeability, presence of near-surface bioturbation, the presence of sub-surface geochemical and/or lithological interfaces, and upward advecting fluids and gases, can all result in perturbations from the norm (e.g. Coolen *et al.*, 2002; Inagaki *et al.*, 2003; D'Hondt *et al.*, 2004; Parkes *et al.*, 2005). Furthermore, the distributions of certain physiological groups of microbes often deviate from those predicted solely on the basis of standard thermodynamic considerations. For example, methanogenesis and methanogens are frequently detected in zones of abundant sulphate (e.g. Parkes *et al.*, 1990; Mitterer *et al.*, 2001; D'Hondt *et al.*, 2004; Parkes *et al.*, 2005; Kendall *et al.*, 2007), which contradicts standard models predicting sulphate reducers will consistently out-compete methanogens (Schönheit *et al.*, 1982; Kristjansson *et al.*, 1982; Lovley *et al.*, 1982; Lovley & Klug, 1983; Conrad *et al.*, 1986).

The prokaryotes responsible for biogeochemical cycling in the marine sub-surface are phylogenetically and physiologically diverse, and complex inter-dependent metabolic reactions often take place. Sub-surface organisms may grow fermentatively, by disproportionation, or by respiring electron acceptors. Substrates (electron donors) may

be organic (e.g. acetate or glucose) or inorganic (e.g. H_2, Fe^{2+} or H_2S). Organisms that use organic substrates are called organotrophs and organisms that use inorganic substrates are called lithotrophs. Since oxygen is typically removed in the upper few centimeters of the sediment column the majority of metabolic processes responsible for biogeochemical cycling in the marine sub-surface are anaerobic (e.g. Parkes *et al.*, 1993).

The amount of energy that can be obtained from a given metabolic reaction is dependent upon the Gibbs free energy (ΔG^0) of the reaction, which is defined as follows:

$$\Delta G^0 = -n\ F\ \Delta E'_0\ (\text{kJ mol}^{-1})$$

ΔG^0 = free energy change of the reaction under standard conditions

n = number of electrons transferred by the reaction

F = Faraday's constant (96,485 coulomb mol^{-1})

$\Delta E'_0$ = redox potential of the compounds i.e. their inherent potential to gain or donate electrons relative to a hydrogen standard (0.0 V)

Net reactions are energy yielding (exergonic) when electron donors that have a more negative E'_0 are coupled to electron acceptors that possess a more positive E'_0 (Fig 1.3).

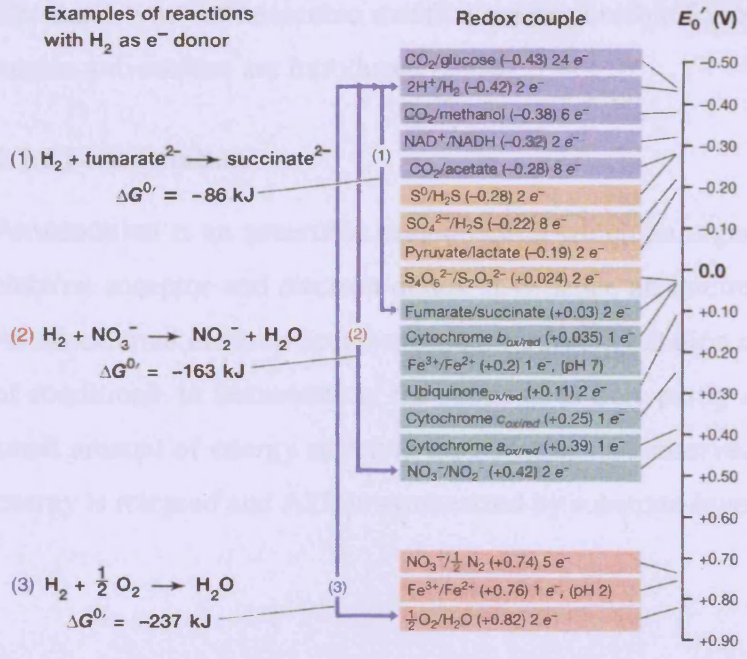


Fig 1.3 The redox tower. Redox couples are ordered from strongest reductants at the top to strongest oxidants at the bottom. The greater the difference in reduction potential between electron donor and electron acceptor the greater the energy released when the two react. This is demonstrated in the examples where H_2 reacts with three different electron acceptors: fumarate, nitrate and oxygen. Taken from Madigan & Martinko (2006).

The energy released during the redox reaction must be conserved (i.e. not lost as heat) in order to drive cellular functions. In cells high-energy phosphate bonds are used to store energy that can subsequently be used to drive energy demanding reactions. The most important high-energy phosphate compound is adenosine triphosphate (ATP) but other high-energy compounds are also important, for example acetyl-CoA, as synthesized by fermentative organisms. It should be noted that theoretical predictions of redox reactions that may sustain life are not always correct because in nature conditions may deviate from the standard conditions (1 M concentration of reactants and products, 1 atm, pH 7) used in calculations. For example, through the formation of syntrophic relationships organisms can prevent the detrimental build up of metabolic products in order to maintain a thermodynamically favorable state for the energy-yielding reaction to proceed. Hydrogen, which is a common end product of anaerobic metabolism, can exert a greater thermodynamic control on metabolic reactions than any other pore water constituent and hence the removal of produced hydrogen (a valuable electron donor for a syntrophic partner) is a common strategy in anaerobic syntrophic relationships. For this reason, syntrophy has also been called interspecies H_2 transfer (Conrad *et al.*, 1986). Such metabolic cooperation can allow extremely efficient catabolic systems and the organisms in question to function at close to thermodynamic equilibrium (Jackson & McInerney, 2002).

The main types of anaerobic metabolism responsible for biogeochemical cycling in the marine sub-surface are introduced below.

1.1.2 Fermentation

Fermentation is an anaerobic catabolism in which an organic compound serves as both electron acceptor and electron donor; it is hence an internally balanced redox reaction. As no external electron acceptor is required, fermentation can occur under a wide range of conditions. In fermentation, the substrate is only partly oxidized and therefore only a small amount of energy stored in the substrate is conserved. During most fermentations energy is released and ATP is synthesized by substrate-level phosphorylation (Fig 1.4 a).

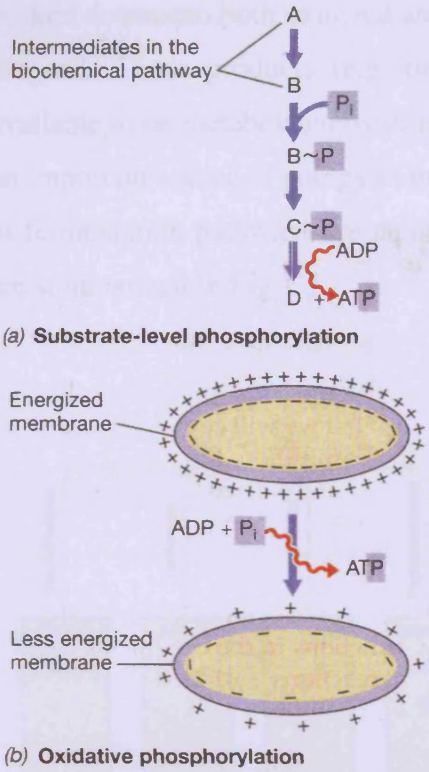


Fig 1.4 Modes of bacterial energy conversion
(a) In fermentation ATP is produced via enzymatic reactions directly connected to the metabolism of the substrate. A phosphate group gets added to some intermediate in the biochemical pathway and eventually gets transferred to ADP to form ATP. This process is known as substrate-level phosphorylation.
(b) In respiration ATP is produced via membrane-mediated processes not directly connected to the substrate. In this case a proton gradient is established across the cell membrane, and is used to drive ATP synthesis (by way of a membrane protein enzyme complex called ATP synthase), or for some other energy-demanding activity such as substrate uptake or locomotion. This process is called oxidative phosphorylation. Taken from Madigan & Martinko (2006).

However, the fermentation of certain substrates (e.g. the dicarboxylic acids oxalate and succinate) yields insufficient energy to synthesize ATP by substrate-level phosphorylation, yet fermentation of these compounds can still support growth. In these unusual cases, fermentation of the substrate is coupled to membrane-bound, energy-linked ion pumps, and H^+ or Na^+-translocating ATPases in the membrane are used to drive ATP synthesis (Madigan, 2006). This finding is important as it shows that any chemical reaction that yields less than the 31.8 kJ required to make one ATP cannot be excluded as a potential growth-supporting reaction for a bacterium. ATP production remains a possibility if the reaction can be coupled to an ion gradient and can yield at least the energy required to pump a single H^+ or Na^+ across the membrane (Madigan, 2006).

Regardless of the mechanism of ATP formation during fermentation, the amount of ATP produced is always low by comparison with aerobic catabolism. The ATP yield of a fermentation reaction is dependent on the pathway used and can range from 0.3 – 4 mol ATP per mol substrate (Müller, 2001). Fermenters can degrade a wide range of organic molecules (e.g. polysaccharides, proteins, amino acids, long chain organic acids, long chain alcohols, sugars and aromatic compounds) but in doing so they only release a small amount of the potential energy in the compound. The fermented substrate is

broken down into both oxidized and reduced end products, which are then excreted from the cell. These products (e.g. organic acids, alcohols, NH_3 CO_2 and H_2) are then available to be metabolized by other physiological groups and in this way fermenters are an important source of energy in marine sediments (Telling *et al.*, 2004). The main types of fermentation pathways are named after the main fermentation products produced and are summarized in Fig 1.5.

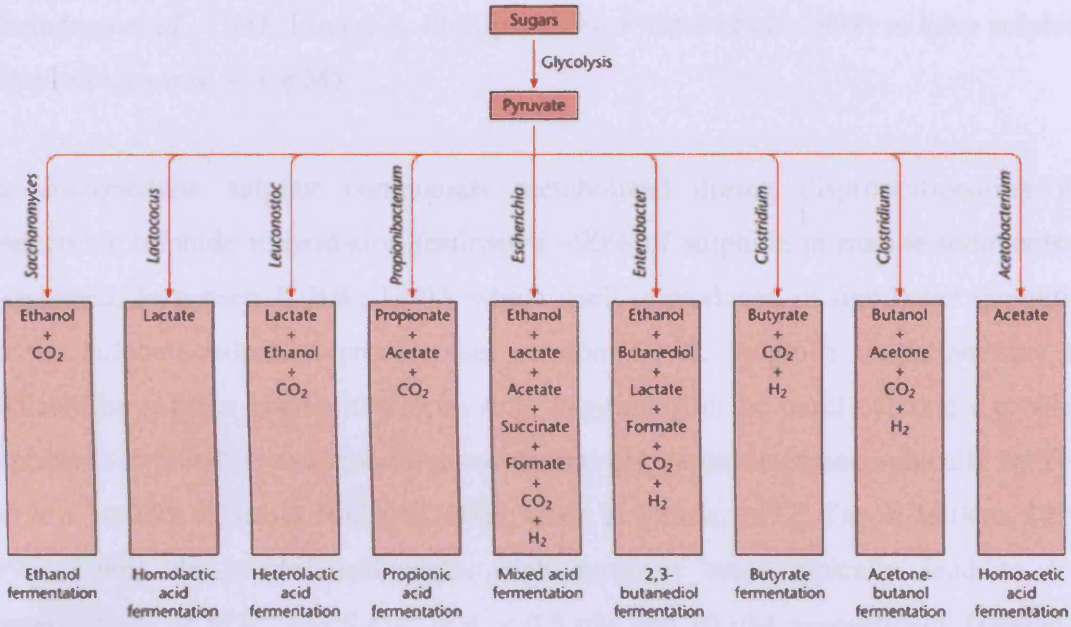


Figure 1.5. Major pathways for fermentation of sugars showing the end products formed and the organisms involved. Taken from Müller, (2001)

The ability to ferment spans many phylogenetic groups but some fermentations, e.g. of unusual organic compounds, are carried out by only a specialist group of organisms or a single bacterium. Organisms of the genus *Clostridium* are important fermenters in marine sediments.

1.1.3 Disproportionation

Disproportionation is an inorganic fermentation in which a compound of intermediate oxidation state (e.g. elemental sulphur, S^0; sulphite, SO_3^{2-} and thiosulphate, $S_2O_3^{2-}$) is split into two new compounds, one more reduced and one more oxidized than the original compound, for example, according to the equation below:

$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- \qquad \Delta G^{0'} = -21.9 \text{ kJ}$$

The oxidation of the intermediate compound drives a proton motive force that can be used to make ATP but the exact biochemical pathways are not well understood (Cypionka *et al.*, 1998). Under standard conditions, disproportionation of thiosulphate and sulphite are exergonic, whereas the disproportionation of elemental sulphur is endergonic (Bak & Cypionka, 1987). However, the free energy yields can become more favorable where sulphide scavenging agents are present, e.g. Fe^{3+} and Mn^{4+} oxides, (Thamdrup *et al.*, 1993; Lovley & Phillips, 1994; Finster *et al.*, 1998) to keep sulphide concentrations low (< 1 mM).

The intermediate sulphur compounds metabolized during disproportionation are products of sulphide re-oxidation (estimated ~90% of sulphide in marine sediments is re-oxidized, Jørgensen & Bak, 1991), which itself is produced in significant quantities via the sulphate-reduction process (see section 1.1.7). Sulphide oxidation may be mediated by sulphur chemolithotrophs (e.g. *Beggiatoa*) or be purely abiotic e.g. when sulphide is exposed to oxic conditions at neutral pH, or oxidized anaerobically by Fe^{3+} and Mn^{4+} oxides (Cline & Richards, 1969; Chen & Morris, 1972; Yao & Millero, 1993; 1995; 1996). In natural sediments, high turnover rates typically lead to low concentrations of SO_3^{2-} and $S_2O_3^{2-}$ (e.g. < 0.5 µM and 10 µM, respectively, Habicht *et al.*, 1998) but S^0 is rather more common e.g. in oxidized near-surface sediments (Troelsen & Jørgensen, 1982; Thode-Andersen & Jørgensen, 1989; Thamdrup *et al.*, 1994; Canfield & Thamdrup, 1996; Böttcher & Thamdrup, 2001).

It has been shown that disproportionation reactions represent important metabolic pathways in marine sediments (Jørgensen, 1990; 1991) and that disproportionating bacteria can be widespread and abundant in these environments (Thamdrup *et al.*, 1993; Finster *et al.*, 1998), particularly where sulphide scavengers are available. However, relatively little is known about the quantitative significance of sulphur disproportionation in the environment, but it is hoped that isotope studies can be used to resolve this (Habicht *et al.*, 1998), since sulphur transformations result in isotope fractionations of $^{34}S/^{32}S$ and $^{18}O/^{16}O$. The use of stable isotopes is complex, e.g. because of the superimposition of different reductive and oxidative process (Böttcher *et al.*, 2005), but it has recently been demonstrated that the different types of sulphur-based metabolism impart different multiple isotope signatures on the compounds involved, and

these can indeed be used to discriminate between the different pathways (e.g. Johnston *et al.*, 2005a; 2005b).

Since its discovery as a form of energy conservation (Bak & Cypionka, 1987), disproportionation has been recognized in numerous genera of SRB, although not all can use the process to support growth (Krämer & Cypionka, 1989). Well-studied organisms capable of growth by disproportionation include the freshwater bacterium *Desulfovibrio sulfodismutans* (Bak & Cypionka, 1987; Bak & Pfennig, 1987), *Desulfobulbus propionicus* (Lovley & Phillips, 1994) and the apparently obligately disproportionating organism *Desulfocapsa sulfoexigens* (Finster *et al.*, 1998; Frederiksen & Finster, 2004). Until recently the process was believed to be restricted to SRB of the *Deltaproteobacteria* (Canfield *et al.*, 1998), but it has also been reported in the facultative anaerobic organism *Pantoea agglomerans* strain SP1 (Obraztsova *et al.*, 2002), which is a member of the *Gammaproteobacteria* (Francis *et al.*, 2000). This organism was reported to grow via sulphur disproportionation coupled to the reduction of Fe^{3+}, Mn^{4+} and soluble Cr^{6+} (Obraztsova *et al.*, 2002) and therefore offers a further suggestion that this mode of metabolism may be more important than previously thought.

1.1.4 Nitrate reduction/denitrification

Nitrate is an energetically favorable electron acceptor for anaerobic metabolism (Fig 1.3) but its availability is limited in the marine environment. Nitrate reduction involves the formation of gaseous nitrogen compounds (N_2O, NO and N_2) in a process called denitrification (see Zumft, 1997 for a review). Nitrate reduction proceeds in three stages: first nitrate (NO_3^-) is reduced to nitrite (NO_2^-), then nitrite is reduced to nitrous oxide (N_2O) via (NO) and finally the N_2O may be reduced to N_2 (Fig 1.6).

Some organisms e.g. *Escherichia coli* can only carry out the first step. They are hence incomplete nitrate reducers. Also, some prokaryotes may reduce nitrate to ammonium (NH_4^+) in a dissimilative process termed ammonification (Jørgensen & Sørensen, 1985). Many denitrifying prokaryotes are members of the *Proteobacteria* and are facultative anaerobes, preferring to use oxygen, if it is present, rather than nitrate. All enzymes used in nitrate reduction are repressed by oxygen.

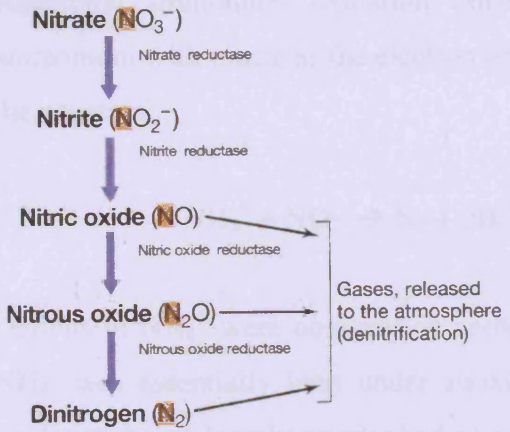


Fig 1.6 Dissimilatory reduction of nitrate. Taken from Madigan & Martinko (2006).

Nitrate reducers tend to be metabolically diverse with the ability to use a range of other electron acceptors (e.g. Fe^{3+}). They can degrade a range of organic substrates (Sørensen, 1987; Coates *et al.*, 2002) and some may also grow by fermentation. The organism *Pseudomonas stutzeri* has been a model organism in the study of the dissimilatory nitrate reduction process (Berks *et al.*, 1995).

Nitrate concentrations in seawater are low, in the µM range, and hence nitrate reduction in marine sediments is relatively unimportant in terms of total organic matter degradation. Studies of continental margin sediments typically report a contribution of denitrification of < 10% (Jørgensen & Sørensen, 1985; Jørgensen, 1983; Middleburgh *et al.*, 1996; Laursen & Seitzinger, 2002). Filamentous sulfur bacteria (*Thioploca, Thiomargarita* and *Beggiatoa*), which are commonly found in sulphide-rich sediments, may accumulate NO_3^- at intracellular concentrations of >100 mM (Fossing *et al.*, 1995; McHatton *et al.*, 1996; Schulz *et al.*, 1999). However, studies indicate that this NO_3^- is not directly available for sediment denitrification, but rather is used in dissimilatory reduction to NH_4^+ (Sayama, 2001; Zopfi *et al.*, 2001).

1.1.5 Anaerobic ammonium oxidation

Anaerobic ammonium oxidation (anammox) involves the anaerobic oxidation of ammonium with nitrite as the electron acceptor and yields gaseous nitrogen according to the equation:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \Delta G^{0'} = -357 \text{ kJ}$$

Deficits in NH_4^+ were observed in anoxic basins over 40 years ago but the belief that NH_4^+ was essentially inert under anoxic conditions led to microbial anaerobic NH_4^+ oxidation being largely overlooked as a part of the biological nitrogen cycle (Strous *et al.*, 2004). The process of microbial NH_4^+ oxidation with NO_2^- as electron acceptor under anaerobic conditions was first confirmed in a denitrifying pilot plant (Mulder *et al.*, 1995) and has since been demonstrated to occur in the natural environment (e.g. Thamdrup & Dalsgaard, 2002; Kuypers *et al.*, 2003; 2005; Trimmer *et al.*, 2006). Anammox organisms are now recognized as important organisms in the global biogeochemical nitrogen cycle and the process may be responsible for ~50% of the global removal of fixed nitrogen in the oceans (Arrigo *et al.*, 2005). The relative importance of anammox to N_2 production in marine sediments is highly variable accounting for between 20 – 80% of total N_2 production (Dalsgaard *et al.*, 2005; Engstrom *et al.*, 2005; Hulth *et al.*, 2005; Schmid *et al.*, 2007). The factors regulating its importance (e.g. organic matter content, nitrate availability, environmental conditions) are not well understood (see Dalsgaard *et al.*, 2005 and Brandes *et al.*, 2007 for discussion), as studies are still in their infancy. The available evidence suggests that the relative significance of anammox in N_2 production is highest at greater water depths, and therefore that sediment mineralization rates may be the most important factor (Dalsgaard *et al.*, 2005). In the environment, anammox organisms are active over a temperature range of at least –1 – 24°C and the process can also occur at high salinity (Meyer *et al.*, 2005; Rysgaard & Glud 2004).

All known anammox bacteria have a specialized cell compartment, called the anammoxosome, in which the anammox reaction is believed to take place (Jetten *et al.*, 2001; van Niftrik *et al.*, 2004). It is hypothesized that NH_4^+ is oxidized inside the organelle via hydrazine (N_2H_4) and hydroxylamine (NH_2OH) intermediates and a proton

motive force is created across the membrane, which is used to produce ATP (Dalsgaard *et al.*, 2005). The unique lipids that surround the anammoxosome form an unusually dense membrane, which probably acts to protect the rest of the cell from the toxic intermediates, produced during the anammox reaction.

Anammox organisms were previously considered to be obligate chemolithoautotrophs (Strous *et al.*, 1999), however, genome and experimental studies have indicated they have a more versatile metabolism and can also use organic compounds as a carbon source (Güven *et al.*, 2005; Strous *et al.*, 2006). Anammox bacteria were not believed to reduce nitrate but recent experiments have shown anammox bacteria can use organic acids as electron donors to reduce nitrate to N_2 via nitrite (Kartal *et al.*, 2007a). Organic acid oxidation was observed simultaneously with the anammox process. The pathway of N_2 production from NO_3^- in this situation is unclear but two possibilities are recognized (Fig 1.7).

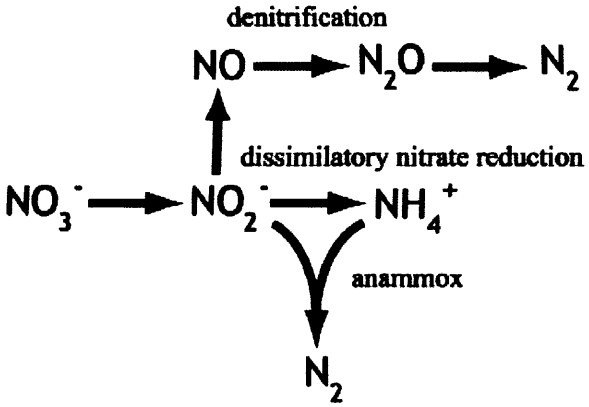


Fig 1.7 Two possible routes of nitrate reduction by anammox organisms. N_2 is either produced by 'classical' denitrification, or NO_3^- is reduced to NO_2^- then to NH_4^+, which is then combined with nitrite to form N_2 via the anammox pathway. Taken from Kartal *et al.*, (2007a).

The ability to reduce nitrate suggests anammox organisms are less dependant on external sources of ammonium that first thought, and may still be underestimated in their contribution to the removal of fixed nitrogen from the environment (Kartal *et al.*, 2007).

Known anammox organisms fall within the domain *Bacteria.* They have not yet been isolated in pure culture but have been enriched to sufficient densities in laboratory and commercial bioreactors to allow their study. Currently 4 genera have been proposed: *Candidatus* "Brocadia", *Candidatus* "Kuenenia", *Candidatus* "Scalindua" and *Candidatus* "Anammoxoglobus" (Kartal *et al.*, 2007b and references therein). All marine anammox organisms are members of the genus *Scalindua.* These organisms are

though to form a deeply branching lineage of the order *Planctomycetales*, and show a high level of genus level diversity.

1.1.6 Metal reduction (Mn^{4+} and Fe^{3+})

Some microorganisms have the capacity to conserve energy from the anaerobic respiration of metal oxides in dissimilatory metabolism. This form of respiration was discovered relatively recently (Balashova & Zavarzin, 1980; Lovley *et al.*, 1987; Lovley & Phillips, 1988) and thus compared to other respiratory processes the study of metal reduction is still in its infancy. As metal oxides can also be reduced abiotically, e.g. by humic compounds, other reduced metal species and H_2S, it has been difficult to distinguish between biotic and abiotic processes in nature. The ferric iron Fe^{3+} and manganic Mn^{4+} ions are the most important metal electron acceptors due to their chemical stability and the abundance of iron and manganese in the natural environment. Most organisms that reduce Fe^{3+} can also reduce Mn^{4+} and *vise versa* (Lovley *et al.*, 2004). Other metal ions (e.g. U^{6+}, Cr^{6+}, SeO_4^{2-} and AsO_4^{3-}) can also act as electron acceptors but are of much lower importance due to their scarcity in the environment. The most prevalent forms of Fe^{3+} and Mn^{4+} are Fe^{3+} and Mn^{4+} oxides, which are insoluble except at very low pH. Metal-reducing organisms thus have to overcome the problem of transferring intercellular-derived electrons onto an insoluble, extracellular electron acceptor. Some organisms e.g. some *Geobacter* species require physical contact with the metal oxides (i.e. via microbial nanowires) in order to reduce them (see Lovley *et al.*, 2004; Reguera *et al.*, 2005), whereas others e.g. *Schewanella* and *Geothrix* do not require direct contact (see Weber *et al.*, 2006). The latter produce chelators that solubilize the metal oxide so it can be taken into the cell (Nevin & Lovley, 2002b) and/or release electron shuttling compounds such as quinones that transfer electrons to the unattached metal oxides (Rosso *et al.*, 2003; Mehta *et al.*, 2005). The relative importance of natural chelators and extracellular quinones in bacterially-mediated Fe^{3+} reduction has yet to be determined but the available evidence suggests that in all but the most organic rich sediments, and where populations of Fe^{3+} reducers are high, direct reduction of insoluble Fe^{3+} oxides (e.g. ferrihydrite) is likely to be the most important mechanism of Fe^{3+} oxide reduction (Nevin & Lovley, 2002b).

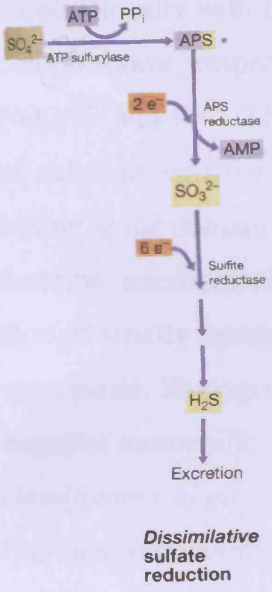
The reduction potentials of the Fe^{3+}/Fe^{2+} and Mn^{4+}/Mn^{2+} couple are quite high ($E^{0'}$ = +0.20 V and +0.80 V, respectively, at pH 7) and therefore a range of organic and inorganic electron donors can theoretically be coupled to Fe^{3+} and Mn^{4+} reduction. In most sedimentary environments the source of electron donors for metal reduction is the complex organic matter deposited with the sediments (Lovley, 1991). In hydrothermal environments the inorganic electron donors hydrogen and sulphur are believed to be important, but detailed studies quantifying the importance of microbial chemolithotrophic processes have yet to be conducted *in situ* (Lovley *et al.*, 2004). Regarding organic electron donors, the current evidence suggests that although complex organic compounds (e.g. sugars and amino acids) can be coupled to metal reduction in pure cultures, fermentative organisms will out-compete metal reducers for these compounds in sediments (Lovley & Phillips, 1989). It is therefore believed that it is the simpler fermentation products (e.g. acetate) derived from the complex organic matter that serve as the primary electron donors for metal reduction in sediments (Lovley, 2000a). Often the organic compounds coupled to metal reduction will be completely oxidized to CO_2.

Metal-reducing organisms are phylogenetically dispersed throughout the *Bacteria* and *Archaea* (see Weber *et al.*, 2006) and include obligate and facultative anaerobes, gram-positive and gram-negative organisms, and organisms growing in psychrophilic to hyperthermophilic temperature ranges (Lovley, 2000b; Lovley *et al.*, 2004). The fact that deeply branching hyperthermophilic *Archaea* have the ability to respire Fe^{3+} compounds (commonly with hydrogen as the sole electron donor) suggests iron reduction may represent the earliest form of microbial respiration (Vargas *et al.*, 1998; Kashefi & Lovley 2003). It is noteworthy, however, that other phylogenetically distinct organisms have different mechanisms for metal reduction, thus suggesting the capacity for this mode of metabolism may have evolved independently several times through the course of evolution (Lovley *et al.*, 2004). Two well-studied groups of Fe^{3+} reducers are the facultatively anaerobic genus *Shewanella* in the *Gammaproteobacteria* and the family *Geobacteraceae* in the *Deltaproteobacteria*. The importance of *Shewanella* in the natural environment has been questioned (see Lovley *et al.*, (2004) and references therein) but the *Geobacteraceae* have indeed been shown to dominate in sediments where Fe^{3+} reduction is important (Stein et al., 2001; Anderson *et al.*, 2003b).

The difficulty in separating biotic from abiotic processes in the natural environment has meant the importance of microbial Fe^{3+} and Mn^{4+} reduction in anaerobic organic matter degradation of has only recently been appreciated (Thamdrup, 2000). In near-surface marine sediments where metal concentrations are high and bioturbation acts to accelerate the recycling of reduced metal compounds back to the oxic/anoxic interface where they are re-oxidized, metal reduction can account for between 50-100% of the overall organic carbon oxidation (Aller, 1990; Canfield *et al.*, 1993a,b). However, in deep water pelagic sites where metal concentrations are much lower and bioturbation is less prevalent metal reduction is correspondingly of lower importance (Thamdrup, 2000). In the deeper marine sub-surface only the recalcitrant, i.e. crystalline, forms of metal oxides remain (e.g. goethite, hematite and magnetite) and there is considerable debate as to whether these compounds can act as electron acceptors in sub-surface systems (see Lovley *et al.*, 2004; Weber *et al.*, 2006 and references therein). However, clear evidence for both Mn^{4+} and Fe^{3+} reduction in deep marine sediments has been presented (D'Hondt *et al.*, 2004; Parkes *et al.*, 2005). Fe^{3+} reduction has been demonstrated at temperatures between 4 – 121°C with pure cultures (Finneran *et al.*, 2003; Holmes, 2004d and Kashefi & Lovley, 2003), indicating that biologically relevant temperatures are not prohibitory to the Fe^{3+} reduction process. The wide range of pH conditions under which biologically mediated Fe^{3+} reduction has been demonstrated (Kusel *et al.*, 199; Ye *et al.*, 2004) further testifies to the ubiquity of the process.

1.1.7 Sulphate reduction

Several inorganic sulphur compounds (S^0, $S_2O_3^{2-}$, SO_2, SO_3^{2-} and SO_4^{2-}) can act as electron acceptors in anaerobic dissimilatory metabolism. The latter - sulphate - is one of the major seawater anions (average 28 mM concentration) and hence is the most important sulphur compound reduced in the seawater-derived pore water of anoxic marine sediments. The other sulphur compounds are typically present in μM concentrations (see section 1.1.3).



The reduction of SO_4^{2-} to H_2S is an eight electron reduction and proceeds through a number of intermediate stages (Fig 1.8). The SO_4^{2-} ion is chemically stable and hence must first be activated before it can be reduced. Activation requires ATP and leads to the formation of APS (adenosine phosphosulfate). The APS is then reduced to sulphite (SO_3^{2-}), which is then further reduced to sulphide by the enzyme dissimilatory sulphite reductase (*dsr*). The electron transport reactions that occur during this process lead to the formation of a proton motive force, which drives the synthesis of ATP.

Fig 1.8 Pathway of dissimilative sulphate reduction. Taken from Madigan & Martinko (2006).

Growth yields suggest SRB produce one mole ATP per mole SO_4^{2-} reduced to HS^-, but additional ATP can also be synthesized when lactate or pyruvate act as electron donors. In these circumstances the high-energy intermediates acetyl-CoA and acetyl-phosphate are produced and these can also be used to produce ATP by substrate-level phosphorylation. With H_2 as the electron donor the general equation for sulphate reduction is:

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \qquad \Delta G^{0'} = -152 \text{ kJ}$$

The main electron donors used in sulphate reduction in marine sediments are H_2, volatile fatty acids and amino acids (Parkes *et al.*, 1989) although more complex organic matter can also be used, e.g. by the spore-forming, gram positive SRB (Daumas *et al.*, 1988;

Tasaki *et al.*, 1991; 1993). A few specialized SRB can even use crude oil as an electron donor for sulphate reduction under anoxic conditions (Wilkes *et al.*, 2000; Cravo-Laureau *et al.*, 2004a; 2004b and references therein). Physiologically, SRB may be divided into 2 groups – those capable of the complete oxidation of low molecular weight fatty acids, particularly acetate, to CO_2 (e.g. *Desulfobacter, Desulfococcus,* and *Desulfosarcina*), and those that only incompletely oxidize organic substrates to acetate (e.g. *Desulfovibrio, Desulfotomaculum Desulfobulbus* and the archaeon *Archaeoglobus*). Certain SRB (*Desulfosarcina, Desulfonema, Desulfobacterium, Desulfotomaculum* and some *Desulfovibrio* spp. have the ability to grow chemolithotrophically and autotrophically with H_2, SO_4^{2-} and CO_2 as the sole electron donor, electron acceptor and carbon source, respectively. Many SRB can also use additional electron acceptors e.g. NO_3^-, S^0 and Fe^{3+}. The majority of SRB are obligate anaerobes. In total over 40 genera of sulphate-reducing prokaryotes are known. As all but one genus (*Archaeoglobus*) belong to the domain *Bacteria* the collective abbreviation SRB is conventionally used to describe microorganisms carrying out the dissimilatory sulphate reduction process, though strictly speaking the term SRP (sulphate-reducing prokaryotes) may be more appropriate. Phylogenetically, 4 groups can be distinguished (Castro *et al.*, 2000): gram-negative mesophilic SRB (e.g. *Desulfovibrio*); gram-positive spore forming SRB (e.g. *Desulfotomaculum* and *Desulfosporosinus*); thermophilic bacterial SRB (e.g. *Thermodesulfovibrio*) and thermophilic *Archaea* (*Archaeoglobus*).

Rates of sulphate reduction in marine sediments vary over six orders of magnitude, from a few picomole cm^{-3} d^{-1} in very deep sediments to tens of micromole cm^{-3} d^{-1} in near-surface coastal sediments (e.g. Canfield & Teske, 1996; Ferdelman, *et al.*, 1997; Rudnicki *et al.*, 2001). The rate and vertical extent of sulphate reduction in the sediment column varies as a function of organic matter availability (Westrich & Berner, 1984). Where labile organic matter is abundant, sulphate may be depleted within the upper few tens of cm (e.g. Skan Bay, Alaska, Reeburgh, 1980; Shaw *et al.*, 1984; Kendall *et al.*, 2007), whereas in open-ocean sites where concentrations of labile organic matter are low, sulphate may remain for >100 mbsf (e.g. Woodlark Basin, Off-shore New Guinea; Wellsbury *et al.*, 2002). In organic-rich, shallow water sediments, sulphate reduction can account for up to 75% of all organic matter degradation (Wellsbury *et al.*, 1996). In deep water ocean basin sediments the contribution of sulphate reduction to total organic matter degradation may be zero (Jørgensen, 1983; Canfield, 1991).

Within the open-ocean deep sub-surface, in low-organic carbon sediments, sulphate reduction may occur at rates just above detection limit but this still can be quantitatively important. For example, in the Woodlark Basin (ODP Leg 180, site 1115, 1,150 m water depth), an area representative of large areas of marine sediments, sub-surface sulphate reduction reached maxima of only ~4 nmol cm^{-3} d^{-1} yet when the whole sediment column was considered it was shown that the majority (72%) of sulphate reduction actually occurred below 20 mbsf (Wellsbury *et al.*, 2002).

A significant proportion of marine sub-surface sulphate reduction is fuelled by the anaerobic oxidation of methane (AOM, see section 1.1.8), which is mediated by a consortium of prokaryotes including SRB (Boetius *et al.*, 2000). Beneath 1.5 m most of the sulphate consumed in marine sediments is attributed to this process (Parkes & Sass, 2007). In continental margin sediments, anaerobic oxidation of methane has been estimated to account for 5-20% of the total sulphate reduction (Kasten & Jørgensen, 2000). Sulphate reduction is thus stimulated in zones where AOM rates are elevated, e.g. gas hydrate-bearing sediments and cold seeps (Boetius *et al.*, 2000; Knittel *et al.*, 2005). This may also include mud volcano sediments since mud volcanoes are a significant source of methane (see section 1.2). Sulphate reduction may also be stimulated in sediments where there are additional sulphate sources. Such sources include deep-sourced, sulphate-rich brines (Mather & Parkes, 2000; Parkes *et al.*, 2005) and that from the anaerobic oxidation of sulphide minerals (Bottrell *et al.*, 2000).

Although microbially-mediated sulphate reduction is one of the most important processes in marine sediments, knowledge of the organisms responsible for it, particularly in the deep sub-surface, is rather limited. The available evidence (phylogenetic and cultivation based) suggests different SRB communities may inhabit near-surface and deeper sub-surface layers (Bidle *et al.*, 1999; Li *et al.*, 1999; Köpke *et al.*, 2005), perhaps as a function of the different physical conditions and substrate concentrations that exist in these environments. Certainly, sub-surface SRB seem to be adapted to their *in situ* conditions (e.g. temperature and pressure, Bale *et al.*, 1997) and are more successfully cultivated with low substrate concentration media (Köpke *et al.*, 2005). Interestingly, SRB are not always detected in the marine sub-surface by molecular genetic methods (Kormas *et al.*, 2003; Reed *et al.*, 2002) even when sulphate

reduction has been demonstrated to occur and specific SRB-targeted (*dsrA* gene) primers are used (Parkes *et al.*, 2005). A possible explanation for this is that sulphate reducers, while active, are present in low abundance in the deep marine sub-surface (Parkes & Sass, 2007). This explanation is supported by the observation that even in near-surface coastal sediments where sulphate reduction is intense, SRB account for <20% of the total microbial population (e.g. Nedwell, 2005). An alternative, but not mutually exclusive, reason is that sub-surface sulphate reduction is carried out by unidentified, as-yet-uncultivated SRB, which may not be identified with the current oligonucleotide primers and probes used to detect SRB.

1.1.8 Anaerobic methane oxidation

Anaerobic oxidation of methane (AOM) describes the process whereby methane is oxidized anaerobically to CO_2 (in the form of HCO_3^- in near-neutral pH aqueous solutions). It is an important, but not well understood, biogeochemical process that regulates the amount of methane, a potent greenhouse gas, escaping into the atmosphere. It is estimated that ~80% of methane produced in marine sediment is consumed by AOM, and where conditions are suitable, the microbial filter may be efficient enough that 100% of the sub-surface methane is consumed anaerobically within the sediment column (see Hinrichs & Boetius, 2002 and references therein).

Pore water profiles indicate that AOM often occurs within the overlap of the sulphate and methanogenic zones, where methane rising from depth encounters sulphate diffusing from above (Fig 1.2 and Fig 1.9).

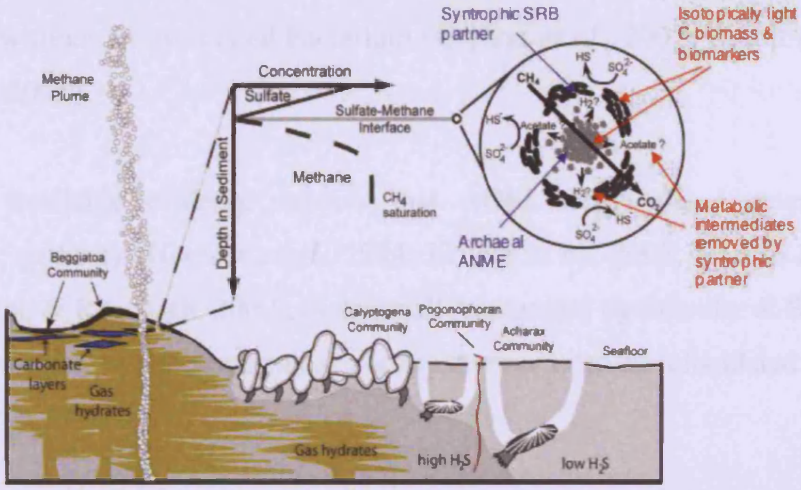


Fig 1.9 Schematic diagram showing the processes and phenomena associated with methane flux in marine sediments. The position of the AOM zone (situated at the sulphate-methane transition) and the relationship of the associated sediment sulphide boundary to benthic fauna are shown. The insert provides an enlarged view of ANME aggregates. Supplied by John Parkes.

This observation led to early predictions that anaerobic methane oxidation might be coupled to sulphate reduction (e.g. Barnes & Goldberg, 1976; Reeburgh, 1976; Martens & Berner, 1977; Iversen & Jørgensen, 1985), with AOM-mediating organisms acting in syntrophic association with sulphate reducers. Various lines of evidence now support this proposed partnership (see Valentine, 2002 for a review). Hence the net syntrophic reaction is:

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad \Delta G^{0'} = -25 \text{ kJ}$$

The organisms mediating AOM remain as-yet-uncultured, however combinations of molecular and biomarker analyses have implicated three distinct archaeal phylogenetic groups – the so-called ANME groups (Boetius *et al.*, 2000). The ANME-1 and ANME-2 (closely related to the *Methanosarcinales*) groups are commonly found in consortium with SRB of the *Desulfosarcina/Desulfococcus* cluster (termed Seep-SRB1) (e.g. Hinrichs *et al.*, 1999; Boetius *et al.*, 2000; Orphan *et al.*, 2001; Knittel *et al.*, 2003), whereas the more recently discovered ANME-3 group appear to act in association with SRB of the *Desulfobulbus* cluster (Niemann *et al.*, 2006). The role of the SRB is believed to be the removal of an up until now undetermined intermediate, in order to maintain thermodynamically favorable conditions for AOM to proceed (see Fig 1.9). However, recently the strict requirement of a tightly coupled syntrophic partner has been

questioned, as evidence exists that in some situations the *Archaea* may be able to oxidize CH_4 without an associated bacterium (Orphan *et al.*, 2002; Orcutt *et al.*, 2005; Treude *et al.*, 2005).

The current available evidence favours that AOM occurs via a reversal of the methanogenic pathway (Hoehler *et al.*, 1994; Krüger *et al.*, 2003; Hallam *et al.*, 2003; 2004; Valentine & Reeburgh, 2000), as originally suggested by Zehnder & Brock (1979; 1980), however the exact biochemical pathway remains to be elucidated (Valentine, 2002; Reeburgh, 2007).

Incubation studies using cold seep sediment from Hydrate Ridge, Cascadia Margin demonstrated that AOM rates were optimal at 4-16°C (Nauhaus *et al.*, 2002), and elevated methane pressures [1.4 MPa, corresponding to 21 mM dissolved CH_4 (12°C)] had a stimulating effect on methane-dependant sulphide production (Nauhaus *et al.*, 2002; 2005). Long-term incubations indicate AOM consortia have a doubling time of approximately 7 months (growth rate of 0.003 d^{-1}) (Nauhaus *et al.*, 2007). This is significantly lower than measured for conventional SRB, which have doubling times in the range of hours – to days (Aeckersberg *et al.*, 1998; Galushko *et al.*, 1999).

The position and vertical extent of the AOM zone in the sediment column (see Reeburgh, 2007 review for ranges) is dependent on several factors: organic matter availability (electron acceptors are depleted more quickly where microbial activity is elevated); the rate of methane supply from depth; the methane partial pressure; and the occurrence of gas hydrate deposits. In advective systems (e.g. marine methane seeps, gas hydrate bearing sediments and mud volcanoes) AOM rates are higher by several orders of magnitude than in diffuse systems (Krüger *et al.*, 2005) since advection is far more efficient in supplying methane from depth. However, if advection rates are too high the electron acceptors (primarily sulphate) necessary to sustain the process may be impeded from penetrating the sediment by diffusion, thus allowing the methane to escape un-oxidized to the water column (e.g. <40% of the CH_4 flux from the Haakon Mosby MV was anaerobically oxidized, Niemann *et al.*, 2006a). The global rate of AOM has been estimated between 70 – 300 Tg CH_4 per year (Reeburgh *et al.*, 1991; Hinrichs & Boetius, 2002). However, this value is not well constrained due to the fact the majority of data used in the estimate came from sites with elevated methane supply (not

representative of the majority of marine sediments), and because of the inherent limitations of *ex situ* rate measurements (laboratory conditions deviated from *in situ* conditions, particularly with regards to pressure; samples can be disturbed by sampling etc, Valentine, 2002; Krüger *et al.*, 2005). In some sediment sites AOM rates are closely coupled to sulphate-reduction rates (Hinrichs & Boetius, 2002; Nauhaus *et al.*, 2005), while in others sulphate reduction rates exceed those of AOM indicating additional sulphate-reducing substrates such as hydrocarbons are consumed (Joye *et al.*, 2004). There is significant potential for AOM and methanogenesis to co-occur in marine sediments (Krüger *et al.*, 2005). For example, a recent study by Parkes *et al.*, (2006) conducted with non-seep sediments from Skagerrak, showed that these two processes were finely balanced (AOM and methanogenesis both 1.70 mmol m^2 d^{-1}) and intimately related, with products of AOM being recycled back into methane.

AOM is a geologically significant process since it leads to an increase in pore water alkalinity, which favors the precipitation of carbonate (e.g. Bohrmann *et al.*, 1998; Suess *et al.*, 1999; Peckmann *et al.*, 2001; Michaelis *et al.*, 2002; Zhang *et al.*, 2003 Treude *et al.*, 2005), for example according to the reaction:

$$CH_4 + SO_4^{2-} + Ca^{2+} \rightarrow CaCO_{3(s)} + H_2S + H_2O$$

These carbonates will carry the isotopically light signature of the methane from which they were derived, and can therefore be used as an indication of AOM in the recent or distant geological past. Isotopically light biomarkers have also been recovered from carbonates in the Black Sea, further supporting their connection to AOM (Peckmann *et al.*, 2001). Conversely, the aerobic oxidation of methane by methanotrophic organisms results in an increase in acidity, favoring carbonate dissolution (Reeburgh, 2007).

As the AOM process produces hydrogen sulphide (see equations above), sulphide concentrations frequently exceed 15 mM in seep sites where AOM rates are high (Valentine, 2002). This sulphide, while toxic for many organisms, can provide a substrate to support significant populations of specially adapted chemosynthetic (thiotrophic) communities. The presence of thiotrophic microbial mats (e.g. *Beggiatoa*) and megafauna communities with thiotrophic endosymbionts (tube worms and

mollusks) are hence good indicators of high AOM rates in the sediment below (see Fig 1.9).

It is noteworthy that recently microbes capable of oxidizing methane anaerobically with nitrate have been reported (Thauer & Shima, 2006; Raghoebarsing *et al.*, 2006). These organisms were recovered from a drainage ditch where nitrate concentrations were high due to agricultural runoff. The importance of this process in ocean sediments is not believed to be significant (Reeburgh, 2007) due to nitrate concentrations being far lower, but this remains to be investigated.

1.1.9 Methanogenesis

Methanogenesis describes the biological production of methane and occurs under strictly anaerobic, low redox, conditions by *Archaea* (Smith & Hungate, 1958; Zehnder, 1978). Three nutritional classes of methanogens are recognized on the basis of the carbon precursor used: CO_2-reducing methanogens, methylotrophic methanogens and acetoclastic methanogens. CO_2-reducing methanogens generally use hydrogen as an electron donor and carry out the reaction:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O \qquad \Delta G^{0'} = -131\ kJ$$

However formate ($HCOO^-$), carbon monoxide and some organic compounds (e.g. alcohols) can also act as electron donors. Methylotrophic methanogens reduce methylated compounds (e.g. methanol, methylamines, methylmercaptan and dimethylsulphide). This can occur in either of two ways. If an external electron donor, e.g. H_2, is available then the below reaction (using methanol as the model methyl substrate) can occur:

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \qquad \Delta G^{0'} = -113\ kJ$$

In the absence of an external electron donor, some CH_3OH can be oxidized to CO_2, which generates electrons used to reduce other molecules of CH_3OH to CH_4:

$$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O \qquad \Delta G^{0'} = -319\ kJ$$

Acetoclastic methanogenesis involves the cleavage of acetate to CO_2 plus CH_4:

$$CH_3OO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G^{0'} = -31 \text{ kJ}$$

ATP is synthesized during methanogenesis by a proton motive force, with the necessary energy being conserved from the terminal step in the metabolism. Substrate-level phosphorylation, reportedly does not occur in methanogens (Madigan & Martinko, 2006). The specific biochemical pathways of methanogenesis are complex (see Fig 1.10).

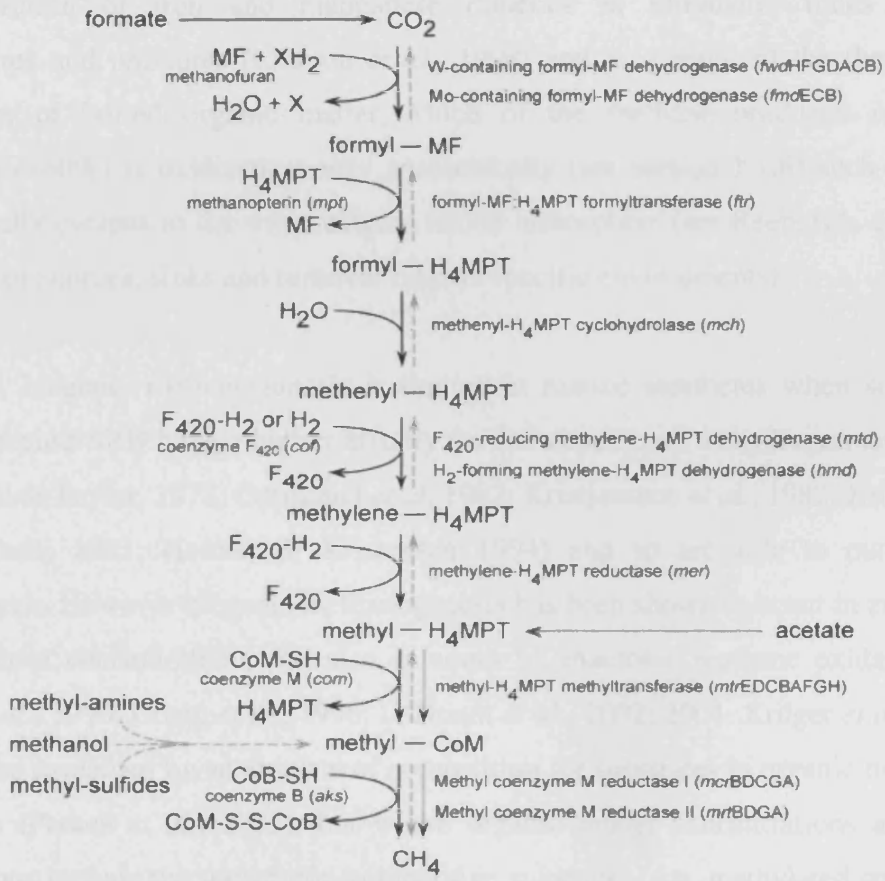


Fig 1.10 Pathways of methanogenesis: hydrogenotrophic (solid black arrows), acetoclastic (solid grey arrows) and methylotrophic (broken gray arrows). The hydrogenotrophic pathway can operate from formate or H_2/CO_2, with formate or H_2 as electron donors respectively. The aceticlastic pathway obtains electrons from the oxidation of CO produced by the splitting of acetate. The methylotrophic pathway can operate in two ways: (1) the methylated C-1 compound is a source for a methyl group as well as electrons (one molecule oxidized to CO_2 provides electrons for three molecules to be reduced to methane; and (2) the methylated C-1 compound is reduced to methane using electrons from H_2.Taken from Bapteste *et al.* (2005).

Methanogenesis is important in the marine sub-surface as it represents the terminal step of organic matter degradation (Kiene, 1991). Methanogenesis amounts to ~0.1% of ocean primary productivity and is most prevalent in areas with high sedimentation rates (Reeburgh, 2007), since the burial efficiency of organic carbon is highest in these areas (Henrichs & Reeburgh, 1987).

Globally, methane production in marine sediments is very significant – between 75 and 320 Tg (1 Tg = 10^{12}g) y^{-1} (Valentine, 2002) and isotope studies[1] indicate that most of the methane produced in marine sediments, including that locked up in gas hydrates, is biogenic in origin (Claypool & Kvenvolden, 1983; Cragg *et al.*, 1996; Whiticar, 1999; Kvenvolden, 2002). Abiotic methane production also occurs, e.g. via seawater-induced serpentinization of iron and manganese minerals in ultramafic rocks at high temperatures and pressures (Charlou *et al.*, 1998) and as a result of the thermogenic breakdown of buried organic matter. Much of the methane produced in marine sediments (~80%) is oxidized, mostly anaerobically (see section 1.1.8) such that very little actually escapes to the water column and/or atmosphere (see Reeburgh, 2007 for a summary of sources, sinks and turnover rates in specific environments).

Generally, biogenic methanogenesis is limited in marine sediments when sulphate is present because SRB have a higher affinity for substrates such as hydrogen and acetate (Oremland & Taylor, 1978; Oremland *et al*, 1982; Kristjansson *et al.*, 1982; Kristjansson & Schonheit, 1983; Holmer & Kristensen 1994) and so are able to out-compete methanogens. However biogenic methanogenesis has been shown to occur in zones with high sulphate concentrations and also in zones of anaerobic methane oxidation (e.g. Parkes *et al.*, 1990; Cragg *et al.*, 1996; D'Hondt *et al.*, 2002; 2004; Krüger *et al.*, 2005). This can be explained by an absence of competition for substrates in organic matter-rich sediments (Parkes *et al.*, 2005), but where organic matter concentrations are lower, explanations include the use of non-competitive substrates, e.g. methylated compounds (Oremland *et al.*, 1982; Whiticar 1999); the possibility that methanogens are better

[1] Stable isotope analysis can be used as a method of determining methane source, since prokaryotes will discriminate against the heavier ^{13}C isotope, such that biogenic methane is depleted in ^{13}C. Stable isotope results are expressed in "del" notation as deviation in parts per thousand (‰) from a standard (Pee dee belemnite, PDB). Bacterial methane is generally considered to have a $\delta^{13}C$ of less than –50‰, thermogenic methane –20‰ - –50‰ (see Whiticar, 1999).

adapted to the *in situ* conditions (Telling *et al.*, 2004); that methanogens may require lower energy yields than SRB (Hoehler *et al.*, 2001); or because the free energy yields deviate from those predicted under standard conditions (Stumm & Morgan, 1996; D'Hondt *et al.*, 2004).

H_2/CO_2 methanogenesis is thought to be the dominant mode of methanogenesis in marine sediments, since acetate is preferentially depleted by SRB in the near-surface (Whiticar, 1999). However, at depth (c.a. >400 mbsf), where sulphate has been depleted, acetoclastic methanogenesis may be important since it has been demonstrated that acetate can be produced by the temperature activation of recalcitrant forms of organic matter (Wellsbury *et al.*, 1997; Parkes *et al.*, 2007). The relative importance of methylotrophic methanogenesis in marine sediments remains largely untested. Methylated substrates are not competed for by sulphate-reducers, i.e. they are noncompetitive substrates, and hence may fuel methanogenic pathways in the presence of sulphate and even within oxygenated waters (Sieburth, 1993a, b, c). Such compounds can be supplied by the bacterial degradation of compounds such as lignins, pectin, choline, creatine and betaine (Oremland *et al.*, 1982), but ambient concentrations and pool sizes largely remain un-quantified in the marine environment (Reeburgh, 2007). In *Thioploca*-dominated sediments off the coast of Chile, radiotracer studies showed methylamine was the dominant methanogenic substrate, but methane production was low (<0.5 nmol cm^{-3} d^{-1}) (Ferdelman *et al.*, 1997). Interestingly, it has been suggested that methanogens may also be responsible for AOM in some environments, with methane production or consumption being dependant upon environmental conditions (Orcutt *et al.*, 2005).

Taxonomically there are several orders of methanogens, all of which fall within the *Euryarchaeota*: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales* and *Methanopyrales*. Most of the methanogenic orders are comprised of H_2/CO_2 methanogens. The exceptions are within the order *Methanosarcinales,* which contains genera with the ability to use H_2/CO_2, methyl compounds and acetate (*Methanosarcina*); just methyl compounds (*e.g. Methanococcoides*) or just acetate (*Methanosaeta*) as substrates for methanogenesis (Madigan & Martinko, 2006). We know relatively little about marine sediment methanogens since <10% of all cultured methanogens come from ocean sediments.

Furthermore, most cultured methanogens do not closely match environmental DNA sequences (Newberry *et al.*, 2004; Inagaki *et al.*, 2006) and do not adequately reflect the range of methanogenic sequences found in DNA surveys using 16S rRNA gene and/or methanogen-specific *mcrA* gene (encoding methyl coenzyme M reductase I; Fig 1.10) primers.

1.1.10 Acetogenesis

Acetogenesis describes the biological production of acetate involving the reduction of CO_2 via the acetyl-CoA pathway. Some organisms produce acetate via other pathways (e.g. the reductive citric acid cycle) as part of their metabolism, but strictly speaking these organisms are not acetogens (Drake, 1994). Some acetogens (e.g. *Acetobacterium woodii* and *Clostridium aceticum*) can grow either chemoorganotrophically, e.g. by the fermentation of sugars (Fig 1.11):

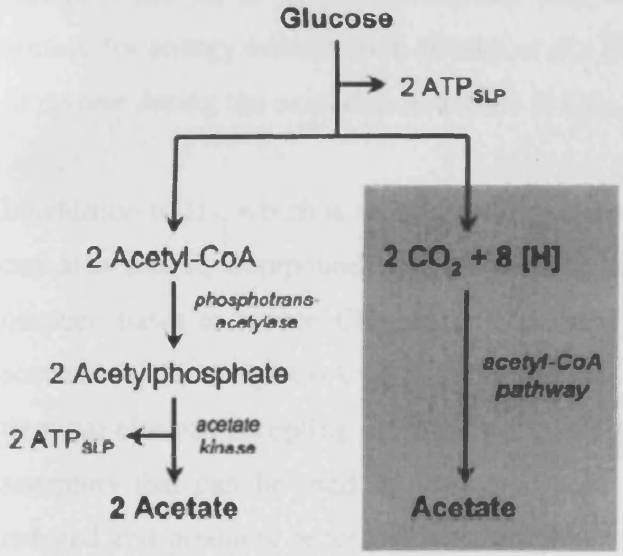


Fig 1.11 Homoacetogenic conversion of glucose to acetate.
4 ATP are produced during this process by substrate level phosphorylation (SLP). Taken from Drake, (2002).

Or chemolithotrophically by reducing CO_2 with hydrogen:

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \Delta G^{0'} = -100 \text{ kJ}$$

Energy is conserved during acetogenesis via H^+ or Na^+ gradients and substrate-level phosphorylation (Madigan & Martinko, 2006). The acetyl-CoA pathway involves the reduction of CO_2 in two linear branches (Fig 1.12).

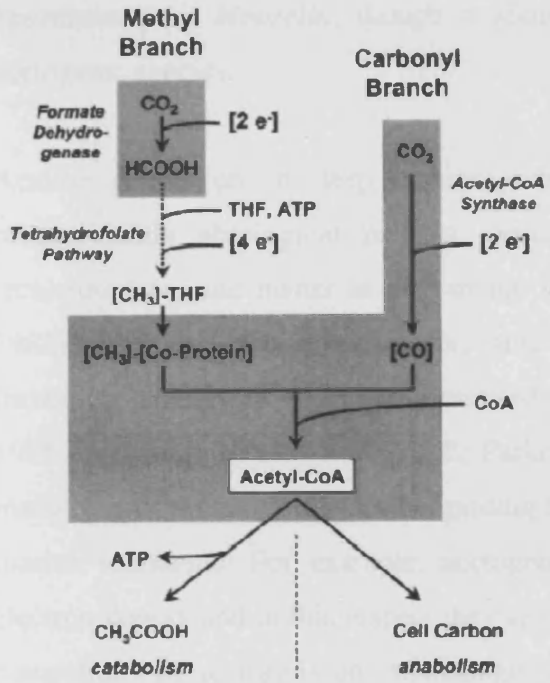


Fig 1.12 The acetyl-CoA pathway.
One branch sees one molecule of CO_2 reduced to the methyl group of acetate via a series of reactions involving the coenzyme tetrahydrofolate, and the other sees one molecule of CO_2 reduced to the carbonyl group of acetate via the enzyme *carbon monoxide (CO) dehydrogenase*. The two carbon atoms are combined to form acetyl-CoA, which is then converted to acetate. The pathway does not just serve as an energy conserving process for acetogens but can also be used to fix carbon for biomass production during autotrophic growth. Taken from Drake, (2002).

Other organisms (methanogens and SRB) also use this pathway during autotrophy, though it has yet to be proven whether they can use it for the reductive synthesis of acetate for energy conservation (Drake *et al.*, 2002). The pathway may also be utilized in reverse during the oxidation of acetate to CO_2 by acetotrophic methanogens and SRB.

In addition to H_2, which is the main electron donor used by acetogens, some organisms can also use C_1 compounds, sugars, organic acids, amino acids, alcohols and some nitrogen bases to reduce CO_2. It is noteworthy that although the reduction of CO_2 to acetate via the acetyl-CoA pathway is the hallmark of acetogens, this is not the only terminal electron accepting process utilized. In fact there are a variety of other electron acceptors that can be used by acetogens (see Drake *et al.*, 2002), which means the reduced end products generated by acetogens is actually quite diverse. Such metabolic versatility may also go some way to explaining the success of acetogens in the environment despite their thermodynamic disadvantages and necessary competition with other organisms (see below).

Acetogens are found in at least 20 different genera and the total number of validly described species is around 100 (Drake *et al.*, 2002). Most of these genera (17) are affiliated with gram-positive bacteria with low G+C content (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999) but other only distantly related phylogenetic groups are also known (Drake, 1994). Important genera include *Clostridium*, *Acetobacterium*,

Sporomusa and *Moorella*, though it should be noted the former also include non-acetogenic species.

Acetate generation in deep marine sediments was previously believed to be a predominantly abiological process, occurring from the thermogenic alteration of recalcitrant organic matter as part of the fossil fuel generation process (Cooles *et al.*, 1987). However, the potential for, and possible significance of, bacterial acetate formation has recently been demonstrated in several studies (e.g. Chapelle & Bradley, 1996; Wellsbury *et al.*, 1997; 2002; Parkes *et al.*, 2007). Nevertheless, there are still many questions to be addressed regarding the ecological importance of acetogenesis in marine sediments. For example, acetogens must compete with other organisms for electron donors and in this respect they are theoretically disadvantaged. The most direct competitors of acetogens are methanogens. Autotrophic methanogenesis has a greater standard free energy yield than autotrophic acetogenesis (-135.6 kJ per mole methane versus –104.6 kJ per mole acetate, respectively) and hence methanogens can maintain lower H_2 thresholds (Cord-Ruwisch *et al.*, 1988; Kotsyurbenko *et al.*, 2001) giving them the competitive advantage. However, investigation of other habitats has shown that for a variety of possible reasons (e.g. temperature, pH and physical proximity to substrate source) *in situ* conditions may actually favor acetogens (Phelps & Zeikus, 1984; Jones & Simon, 1985; Conrad *et al.*, 1989; Krumholz *et al.*, 1999; Kotsyurbenko *et al.*, 2001). How such findings translate in marine sub-surface ecosystems remains to be elucidated.

The matter of unraveling the significance of bacterially mediated acetate generation is further complicated by the fact acetate itself is an important bacterial substrate, and hence becomes rapidly oxidized or consumed by other organisms. This makes the activities of acetogens difficult to assess under field conditions. One study of marine sediments from the Woodlark Basin showed that despite active rates of acetoclastic methanogenesis, acetate concentrations remained relatively constant (<10 μM) with depth suggesting that active acetate generation, presumably by the acetogens that were present, was also occurring (Wellsbury *et al.*, 2002). Furthermore, discrete increases in acetate concentrations have been detected in pore water from some sedimentary environments. For example, acetate was found to increase significantly (up to 15 mM)

beneath the hydrate stability zone in hydrate-bearing sediments of Blake Ridge despite the temperature of the sediments not exceeding 30°C (Wellsbury *et al.*, 2000).

The broad phylogenetic diversity of acetogens has precluded the use of 16S rRNA targeted oligonucleotide probes and primers in the quantitative assessment of acetogens in the environment, but advances are being made with functional gene-targeted approaches (e.g. Lovell & Hui, 1991; Leaphart & Lovell, 2001). Formyltetrahydrofolate synthase (FTHFS), which is involved in the methyl branch of the acetyl-CoA pathway, is the enzyme targeted, and such methods may shed further light on this enigmatic but potentially very important group of organisms.

1.2 Introduction to Sub-marine Mud Volcanism

Mud volcanoes occur in both the terrestrial and sub-marine setting and are distributed globally (see Dimitrov, 2003). Specifically, sub-marine mud volcanoes are considered in the present study, though the term mud volcano (MV) is used throughout the thesis for brevity. Sub-marine MVs have only been known to exist for a few decades, following the development of technologies such as swath bathymetry, side-scan sonar, seismic profiling and sub-marine coring. MVs are constructional features on the seafloor that result from the extrusion of sediment, fluid and gas (mostly methane) from depth. The temperature of the extruded material as it reaches the seafloor is low (e.g. < 25°C; Kaul *et al.*, 2006) and hence they are considered to be a type of cold seep. Mud volcanism is a geological phenomenon that is brought about by a variety of often interplaying processes, e.g. structural or tectonic compression, fluid volume increases (due to thermal expansion, diagenetic mineral dehydration reactions and hydrocarbon generation), and as a consequence of high sedimentation rates (see Kopf, 2002; Dimitrov, 2003 and references therein). Ultimately, these processes lead to the development of overpressured pore fluids at depth. If trapped, e.g. beneath impermeable salt or shale layers, eventually the fluid pressures may exceed the horizontal stress plus the tensile strength of the overlying rock burden, causing extension hydro-fractures to open up (e.g. Osborne & Swarbrick, 1997). Once a hydro-fracture has formed, the velocity of upward moving pore fluids can exceed the grain fall velocity, causing particles to become entrained as fluidized sediment (Davies & Stewart, 2005). The buoyant mudflow rises upwards under pressure but on breaching the seafloor the energy is dispersed, causing the entrained sediment to be deposited and the mud volcano crater to develop (Fig 1.13).

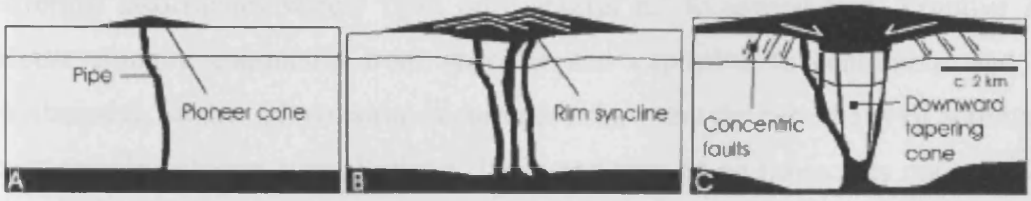


Fig 1.13 Simple schematic showing 3-stage development of a mud volcano. (a) upward rising mud breaches the seafloor forming a pioneer cone (b) mud extrusion continues, possibly with the development of additional feeder channels (c) burden of extruded mud combined with loss of volume at depth results in crater collapse (Taken from Davies & Stewart, 2005).

More than one feeder channel may supply a given mud volcano, and collapse features, due to the discharge of significant volumes of underlying sediment are common (Fig 1.13). The upward-moving fluidized sediment may also erode the surrounding country rock as it discharges, which is why at outcrop mudflows are often found to be rich in wall-rock breccia. The location of the fluid escape pathways may have a deep structural control, for example at fault intersections (Pinheiro *et al.*, 2003), and typically numerous individual MVs are found together in what are termed mud volcano fields. MVs are dispersed widely on the seafloor and are found in water depths ranging from a few hundred to a few thousand metres. The source of the mobilized mud may be several kilometers below the seafloor (Kopf, 2002).

MVs form in several types of geological setting, most commonly along active compressive margins (e.g. Nankai Trough region), but also within large deltaic or fan complexes (e.g. Niger and Amazon regions respectively) and in areas of salt diapirism (e.g. Gulf of Mexico). At present, the number of documented occurrences is around 1,700 (Kopf, 2002) though some authors believe the total number is far greater and may be up to 100,000 (Milkov *et al.*, 2003). Certainly more are continually being discovered with the improvement of bathymetric imaging techniques and the progression of ocean surveying.

The term 'mud volcano' is used very broadly and encompasses structures displaying a very wide range of physical characteristics (Dimitrov, 2002). For example, submarine MV craters range from having a negative relief with respect to the surrounding sea floor (due to crater collapse) to mud cones a few hundred metres in elevation. Their areal coverage also ranges widely from only several m^2 to several km^2. Eruptive activity occurs along a continuum from sporadic and explosive to continuous and placid. Furthermore, studies of terrestrial occurrences show that the periodicity of activity varies considerably between sites (Kalinko, 1964) and may be on timescales not conducive to scientific study (e.g. periods of quiescence >65 years, Dimitrov, 2002). There are no statistical data yet available on the periodicity of sub-marine MV activity as long-term observations are yet to be made. Such factors prohibit broad generalizations to be made between, or even within, a given MV region and question, for example, the accuracy of calculations that seek to quantify the collective gas flux from MVs. These estimates vary

considerably e.g. ~1 – 33 Tg yr^{-1} (Kopf, 2002; Milkov, 2003 and references therein) and reflect the limited data currently available.

In addition to the long established interest in MVs from geologists, these features have recently started to receive attention from a microbiological perspective (Pancost *et al.*, 2000; Pimenov *et al.*, 2000c; Aloisi *et al.*, 2002; Werne *et al.*, 2004; Stadnitskaia *et al.*, 2005; Heijs *et al.*, 2005; 2006; Bouloubassi *et al.*, 2006; de Beer *et al.*, 2006; Martinez *et al.*, 2006; Niemann *et al.*, 2006a, 2006b). This interest stemmed from reports that microbial mats and megafauna (e.g. clams, bivalves and tubeworms) could be seen colonizing the craters of MVs, particularly where active seeping was observed. MVs were thus identified as potential hotspot ecosystems on the seafloor, analogues perhaps to their high-temperature counterparts hydrothermal vents. Work on MVs from a perspective of biosphere-geosphere coupling is in its infancy, and much of the available literature was published only after the start of the present study (see references above).

Fig 1.14 shows the different phases of activity that occur during the lifetime of a 'typical' sub-marine mud volcano.

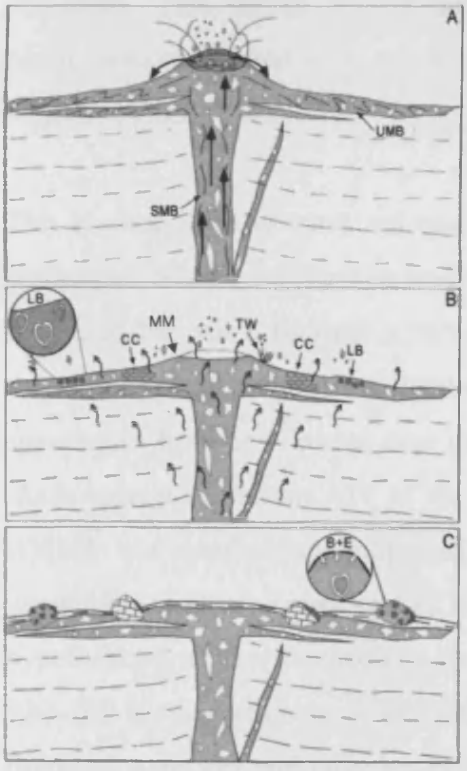


Fig 1.14. Schematic diagram showing the different phases of activity of a submarine MV. A) Active eruption of the MV gives rise to a cone-shaped edifice consisting of un-sheared mud supported breccias (UMB) interbedded with hemipelagic sediments. Sheared mud breccias (SMB) are formed in the conduits along which the plastic sediments are squeezed. B) Quiescent period characterized by slow and diffuse degassing. At this time colonies of chemosynthetic and chemosymbiotic taxa - e.g. microbial mats (MM), lucinid bivalves (LB) and tube worms (TW) can become established around the seep. Carbonate crusts (CC) may also develop at this time due to anaerobic methane oxidation. C). Extinction of the MV at which time degassing stops and the cone begins to be eroded by marine currents. Carbonate crusts may be exposed, bored (B) and encrusted (E) by sessile fauna. Mud breccia becomes draped by hemipelagic sediment. Adapted from Clari *et al.*, 2004.

MVs are sites of fluid flow – in the present or the past. They apparently sustain hot-spot ecosystems because MV ejecta contain compounds that can act as energy sources for chemosynthetic prokaryotic life. This increased energy supply creates the potential for increased microbial activities and an intensification of biogeochemical processes. An important prokaryotic substrate discharged by MVs is methane, as this is the main component (~99%) of gas expelled from MV vents (Dimitrov, 2003; Kopf, 2002). Other higher hydrocarbons, volatile fatty acids and minerals containing reduced metals may also be important substrates for bacterial growth in MV sediments. Mega fauna may also be supported by deep sourced energy if they harbour chemosynthetic endosymbionts (Sibuet & Olu, 1998; Levin, 2005). In addition to the increased energy supply at MVs, these sites are also interesting microbiological targets since it can be hypothesized that the deep-sourced sediments and fluids rising through MVs may bring deep biosphere prokaryotes up to the more accessible shallow sub-surface.

1.2.1 Case study: The Haakon Mosby mud volcano

Below a case study of the well-studied Haakon Mosby mud volcano (HMMV) is presented. This serves to introduce the various physical, chemical and biological phenomena associated with sub-marine volcanism, and allows comparisons to be made with the present work.

The Haakon Mosby mud volcano is situated at a water depth of 1250 m in the Norwegian Sea on the Norwegian-Barents-Spitsbergen margin. It was first discovered in 1990 and has since become a natural laboratory for the study of mud volcanism and associated phenomena. Investigation of the site by a variety of complimentary approaches has been intense over the last few years, which probably makes it the best characterized submarine MV in the world (see Milkov *et al.*, 2004 for a review). The HMMV is a circular feature approximately 1.5 km wide with a relief of about 10 m and the whole structure covers an area of 0.74 km^2 (Jerosch *et al.*, unpublished results). It is hypothesized to have formed as a result of a submarine landslide in the Late Pleistocene 330–200 Ky ago (Laberg *et al.*, 1993; Vogt *et al.*, 1999), and is still actively venting fluidized sediment and large quantities of methane gas to the seafloor today (Sauter *et al.*, 2006). The source depth of the extruded mud is believed to be 2-3 km (Vogt *et al.*, 1999).

1.2.1.1 Authigenic carbonates

The precipitation of authigenic carbonate crusts at MVs and other cold seeps is well documented (e.g. Aloisi *et al.*, 2000; Peckmann *et al.*, 2001; Greinert *et al.*, 2002), and these deposits are also found at HMMV (Lein *et al.*, 2000a). Carbon isotope analysis of HMMV carbonates showed depletion in ^{13}C indicating the major source of carbon was microbially oxidized methane (Lein *et al.*, 2000a); see section 1.1.8. Lein *et al.*, (2000a) found the carbonate crusts associated with sites of intense AOM, although, interestingly, a later study by de Beer *et al.* (2006) failed to find carbonate crusts (or even increase in pH) even in zones where AOM rates were high.

1.2.1.2 Gas hydrates

Gas hydrate occurrence is another phenomena commonly associated with mud volcanoes (e.g. Ben-Avraham *et al.*, 2002; Aloisi *et al.*, 2000; Hein *et al.*, 2006; Schmidt *et al.*, 2005; Werne *et al.*, 2004). Gas hydrates are crystalline, ice-like, non-stoichiometric compounds in which methane (concentrated 164 x by volume at standard conditions) is caged by frozen water, and are indicative of high gas flux environments (Sassen *et al.*, 2001). The reader is referred to Milkov (2005) and references therein for further information. Gas hydrates have repeatedly been recovered from HMMV and are observed in sediments mainly as vein-fillings, which can be up to several cm in thickness (Ginsburg *et al.*, 1999; Milkov *et al.*, 2004), though finely disseminated hydrate is also observed. Gas hydrate at HMMV is present near the seafloor (0-3 mbsf) in the crater, but the largest accumulations are found at greater depth (1-3 mbsf) in the peripheral zone (Milkov *et al.*, 2004). The depth extent of the hydrate remains untested due to an absence of deep coring at the site. Estimates based on pore water chlorinity analysis suggests gas hydrate can comprise up to 25% of the sediment by volume at HMMV, although average values are around 1% (Ginsburg *et al.*, 1999). The dominant component of the gas is methane (>99%) and based on isotopic analysis ($\delta^{13}C$-isotope value of -60‰) has a largely microbial origin (Milkov *et al.*, 2004). Gas hydrate has been reported to occur up to 750 m from the HMMV crater (Ginsburg *et al.*, 1999).

1.2.1.3 Geochemistry

Pore water measurements made from the crater, outer crater (hummocky periphery) zone, and background site at the HMMV (Lein *et al.*, 1999; Ginsburg *et al.*, 1999) have revealed distinct geochemical profiles (Fig 1.15).

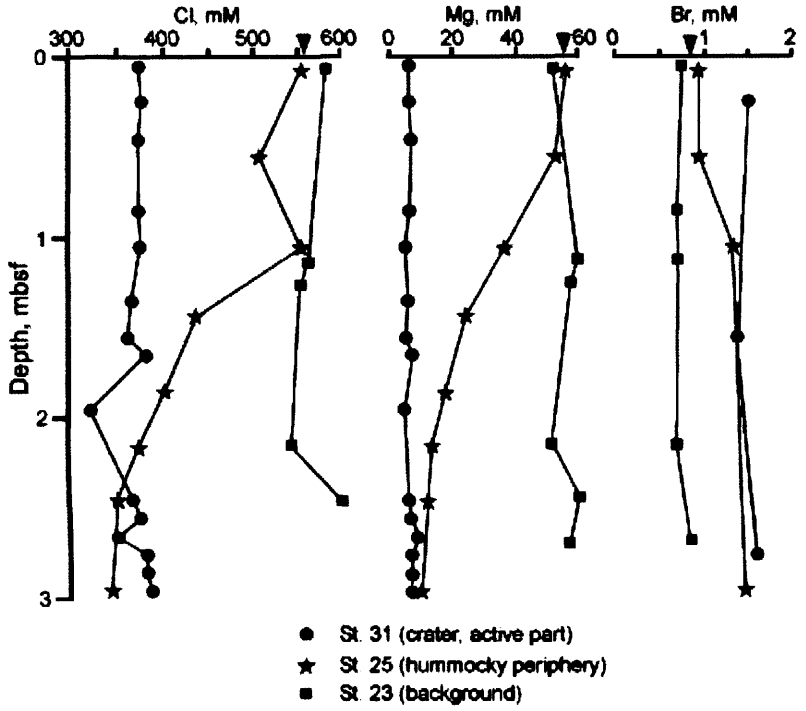


Fig 1.15. Concentration of Cl^-, Mg^{2+}, and Br^- in sediment pore water from the crater, outer crater (hummocky periphery) zone, and a background site at the HMMV. Taken from Milkov *et al.*, 2004.

Pore water in the active crater had lower salinity (~22‰), increased Br^- and I^-, and decreased major cation (Mg^{2+}, Ca^{2+}, Na^+) concentrations relative to seawater (Milkov *et al.*, 2004). The relatively low concentrations of major ions were attributed to clay mineral dehydration reactions at depth (Milkov *et al.*, 2004). The increases in Br^- and I^- were attributed to the dehydration of organic matter (e.g. Martin *et al.*, 1996). Pore water in the background site had ionic and isotopic compositions consistent with seawater. In the outer crater (hummocky periphery) zone the pore water profile was indicative of mixing between deep-sourced MV water (as measured at the crater) and seawater. Low salinity and low major ion concentrations were observed in the deeper sections, whereas seawater compositions were measured in the upper parts. The occurrence of gas hydrate in this zone was proposed as a further contributor to pore water freshening, i.e. due to gas hydrate dissociation (see Ginsburg *et al.*, 1999).

1.2.1.4 MV Ecology

Three broad zones with distinct fluid flow regimes are recognized at the HMMV, and these can be correlated with distinct habitat types (Fig 1.16).

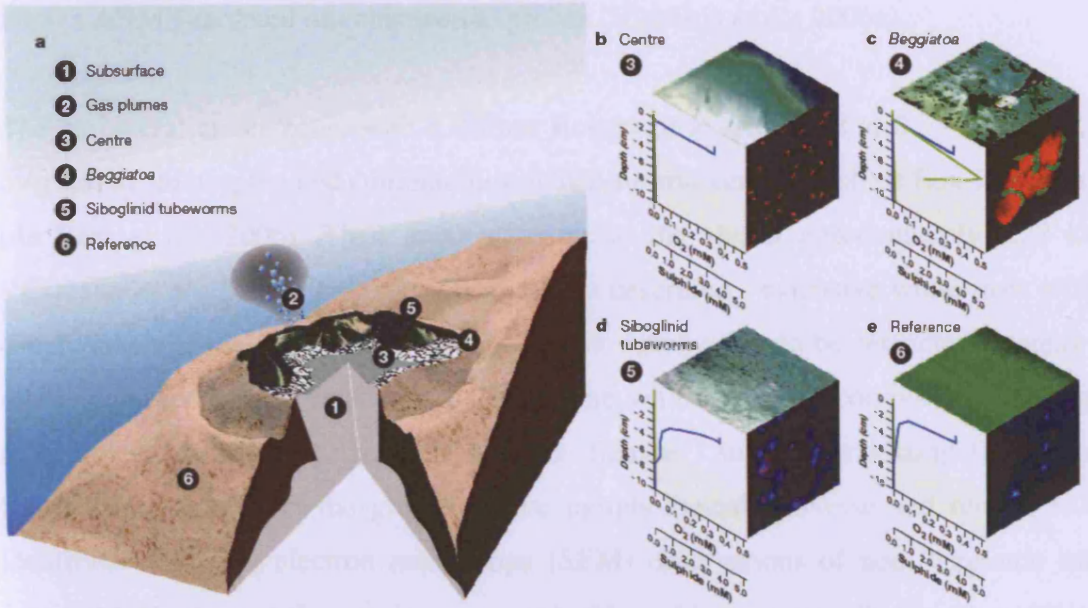


Fig 1.16. Schematic diagram showing the zones of distinct fluid flow and associated habitat types recognizable at HMMV. Images of the sea floor (top panel), idealized concentration gradients (oxygen, blue and sulphide, green) and micrographs of dominant microbes are shown as 3D cubes. The central crater (b) is colonized by aerobic methanotrophs (*Methylococcales*); the peripheral zone (c) is colonized by *Beggiatoa* mats on the surface and ANME-3 (red)/DBB (green) aggregates in the shallow sub-surface (<3 cmbsf); and the outer zone (d) is colonized by siboglinid tubeworms, and ANME-2/DSS aggregates at depth (>60 cmbsf). A reference site (e) is also shown. Vertical height exaggerated 50-fold. DBB – *Desulfobulbus*-related SRB; DSS – *Desulfosarcina/Desulfococcus*-related SRB. Taken from Niemann *et al.*, (2006a).

In the central crater (0.11 km^2) where flow velocities are estimated at 3-6 m yr^{-1} (de Beer *et al.*, 2006) and where near-surface temperature gradients reach 3°C m^{-1} (Kaul *et al.*, 2006) there is an absence of thiotrophic microbial mats and no accumulations of sulphide. This is believed to be due to an inhibition of AOM, caused by a lack of suitable electron acceptors in the rising methane-rich fluids (de Beer *et al.*, 2006). High prokaryotic cell numbers (approximately 3.6 x 10^9 cells cm^{-3}) were detected in the upper most surface of recent crater mudflows. Approximately half of these cells were identified by fluorescent in situ hybridization (FISH) to be aerobic methanotrophic bacteria of the orders *Methylococcales* and *Methylophaga* (Niemann *et al.*, 2006a). Cell numbers quickly decreased to ~10^7 cells cm^{-3} below 5 cm reflecting the very limited oxygen penetration in the crater, and hence narrow habitat range of the aerobic methanotrophs (Niemann *et al.*, 2006; de Beer *et al.*, 2006). At their maximal abundance

and activity, methanotrophic communities showed relatively low potential rates of activity (~0.1 fmol CH_4 per cell d^{-1}), possibly reflecting the low ambient sediment surface temperature (-1°C) (Niemann *et al.*, 2006a). AOM was not detected in the central crater and ANME cells were not microscopically detectable by FISH using all known ANME-targeted oligonucleotide probes (Niemann *et al.*, 2006a).

The peripheral crater zone, with a diffuse flow regime calculated at 0.3 – 1 m yr^{-1}, is overlain by thiotrophic mat communities, which survive on the sulphide flux from below (de Beer *et al.*, 2006). Their areal coverage at the site is approximately 0.22 km^2 (Niemann *et al.*, 2006). Two types of mats are described - extensive white mats which overly much of the diffuse zone and grey mats which seem to be restricted to areas of recent gas seepage (de Beer *et al.*, 2006). The white mats are composed of a single morphotype identified as the large (10 µm diameter) sulphide oxidizing filamentous bacteria *Beggiatoa*, but the grey mats are morphologically diverse and remain to be identified. Scanning electron microscope (SEM) observations of non-*Beggiatoa* mats from HMMV showed them to be composed of long filamentous cells (length >100 µm, width 2-8 µm) short rods and cocci (Pimenov *et al.*, 2000c). These thinner filamentous cells were suggested to be related to the sulphide-oxidizing genera *Leucothrix* and *Thiothrix* as found at deep-sea hydrothermal vents (Pimenov *et al.*, 2000b). Large spherical cells with sulphur globules have also been reported in non-*Beggiatoa* mats from HMMV and their morphological similarity to *Thiomargarita* cells has been noted (de Beer *et al.*, 2006). *Beggiatoa* sp. can oxidize sulphide anaerobically with nitrate, producing sulphate or sulphur and ammonium as products (Vargas & Strohl 1985; McHatton *et al.*, 1996), and this pathway is thought to occur at the HMMV site based on geochemical and fluid flow data (de Beer *et al.*, 2006). Below the *Beggiatoa* mats in the upper few centimeters of the sediment, a steep sulphide peak is observed, which is likely to result from AOM. Increased biomass levels were detected at the sulphide peak, due to dense cell aggregates (20 x 10^6 cm^{-3}) of a new type of archaeal – sulphate-reducing consortia, which comprised 80% of the total cells (Niemann *et al.*, 2006). The archaea in this new consortium are named ANME-3, and the sulphate-reducer partner was identified as a close relative of *Desulfobulbus spp.* rather than the *Desulfosarcina/Desulfococcus*-related SRB commonly associated with the ANME-1 and ANME-2 groups (e.g. Hinrichs *et al.*, 1999; Knittle *et al.*, 2003).

The outer zone (approximately 300 – 400 m from the geographic centre) of HMMV is devoid of bacterial mats but is instead populated by symbiotic tubeworms (*Pogonophora*), which cover an area of approximately 0.41 km^2 (Niemann *et al.*, 2006a). The presence of these organisms in an area of very low net upward flux (0.4 m yr^{-1}) can partly be explained by their ability to 'bioirrigate' the sediment, i.e. penetrate down into the sediment where reduced compounds occur (de Beer *et al.*, 2006). *In situ* microprobe measurements indicated oxygen penetration is between 3 - 10 cmbsf in tubeworm habitats, and temperature profiles suggested advective exchange of seawater and pore water in the upper (ca 5 cm) sediment where tubeworms are present (de Beer *et al.*, 2006). The tubeworms are hypothesized to transport reduced compounds from depth into the aerobic zone where they may be oxidized by their bacterial endosymbionts. *Pogonophora* are thus suspected of harboring methane and possibly sulphide oxidizing bacteria (Pimenov *et al.*, 2000; de Beer *et al.*, 2006). Sulphate decreases much more slowly in sediments occupied by tube worms compared to those overlain by *Beggiatoa* mats and may not be depleted until several decimeters below seafloor (de Beer *et al.*, 2006). Maximum AOM rates occur at 60-90 cmbsf in this zone, between the base of the tubeworms and the hydrate layer (Niemann *et al.*, 2006). The organisms responsible are ANME-2/DSS aggregates, which are found at cell densities of 5.5 x 10^6 aggregates cm^{-3} (Niemann *et al.*, 2006a).

The methane emitted from the HMMV accounts for >99% of the hydrocarbons released and is of a mixed microbial/thermogenic origin, with a $\delta^{13}C$-isotope signature of -60‰ (Damm & Budéus 2003; Milkov *et al.*, 2004). Methane-oxidation is the main energy source for microbial biomass in all habitats of the HMMV (Niemann *et al.*, 2006a) but not all the expelled methane is consumed. The sum total of methane consumption at HMMV was calculated as 5.0 x 10^6 mol yr^{-1}, which reflected relative rates in the center, *Beggiatoa*, and tubeworm habitats of up to 0.2, 1.8 and 3.0 x 10^6 mol yr^{-1}, respectively (Niemann *et al.*, 2006a). This equates to <40% removal of the total methane flux (13-40 x 10^6 mol yr^{-1}) according to data collected in 2003 (Sauter *et al.*, 2006; Niemann *et al.*, 2006). Anaerobic methane oxidation is the dominant process of methane consumption (37%) at HMMV due to the very limited habitat range of aerobic methanotrophs. The success of the ANME organisms at the site can partly be attributed to their ability to profit from association with other organisms (*Beggiatoa* and tubeworms), which act to

replenish their electron acceptor supply through the re-oxidization of sulphide (Niemann *et al.*, 2006a).

1.2.2 Prokaryotic diversity in sub-marine MV environments

A major aim of the present work was to characterize the diversity of prokaryotic populations in Gulf of Cadiz MVs through the use of culture-independent, PCR-based methods. The following section specifically reviews the available literature on other sub-marine MV environments that have been investigated by this approach. It is note worthy at this point that apart from organisms involved in AOM, there have been no previous systematic attempts to cultivate or isolate MV sediment organisms.

1.2.2.1 Gulf of Mexico MVs

The Gulf of Mexico is characterized by a variety of hydrocarbon seep phenomena including mud volcanoes (MacDonald *et al.*, 2000). Active gas seeps and areas where gas hydrate outcrop on the seafloor have been studied quite intensively from a geochemical and microbiological perspective (Lanoil *et al.*, 2001; Joye *et al.*, 2004; Mills *et al.*, 2003; 2004; 2005; Zhang *et al.*, 2003; Knittel *et al.*, 2005; Lloyd *et al.*, 2006) but the MV systems are less well characterized. One hypersaline MV 'GB425' from the northern continental slope of the Gulf of Mexico (600 m water depth) was recently investigated by a culture-independent molecular-based approach (Martinez *et al.*, 2006). The authors extracted genomic DNA and RNA from three sediment depths in the MV (0-2, 6-8 and 10-12 cm) in order to investigate vertical changes in both the total prokaryotic community composition (DNA libraries) and the metabolically active component (RNA libraries).

Distinct differences in the composition and patterns of the total prokaryotic population (rDNA libraries), and the metabolically active component (crDNA libraries) were found relative to sediment depth (Fig 1.17). At all sediment depths *Bacteria* showed greater phylogenetic diversity than *Archaea* (Martinez *et al.*, 2006).

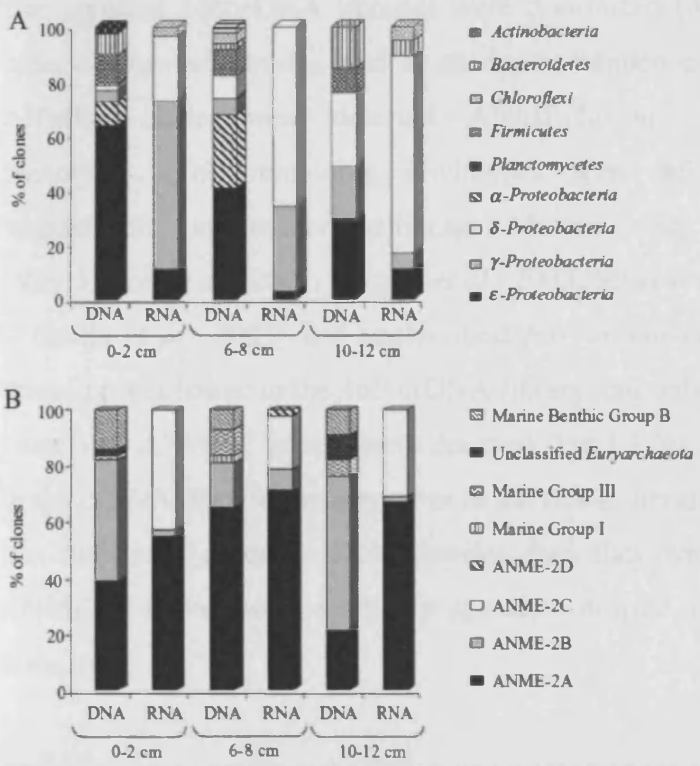


Fig 1.17 (A) Summary of bacterial phylogenetic lineages detected in 16S rDNA (DNA) and 16S crDNA (RNA) clone libraries from the GB425 MV in the Gulf of Mexico. (B) Summary of archaeal phylogenetic lineages detected in 16S rDNA (DNA) and 16S crDNA (RNA) clone libraries from the GB425 MV in the Gulf of Mexico. Calculations were based on the total number of clones associated with phylotypes from which a representative clone had been sequenced. Taken from Martinez *et al.* (2006).

The majority of bacterial clones in the 16S rDNA libraries (79%) belonged to the *Proteobacteria*, of which 55% were related to the *Epsilonproteobacteria* sub-group. The abundance of *Epsilonproteobacteria*-related clones decreased with increasing sediment depth, while the number of *Delta-* and *Gammaproteobacteria* increased. The latter two sub-groups showed the greatest phylogenetic diversity of the proteobacterial lineages detected. Non-*Proteobacteria*-related clones (see Fig 1.17) individually made up less than 10% of the total rDNA clones.

Proteobacteria also dominated the crDNA library (90%). The most abundant phylotypes were *Deltaproteobacteria,* not *Epsilonproteobacteria* as in the rDNA library. In contrast to the rDNA library *Gammaproteobacteria* dominated the near-surface crDNA library suggesting these were among the most metabolically active *in situ* organisms at this depth. In the two deeper samples *Deltaproteobacteria* came to dominate, perhaps reflecting their adaptation to anoxic conditions and association with anaerobic sulphate-reduction (Martinez *et al.*, 2006). Non-*Proteobacteria*-related clones (*Bacteroidetes* and *Chloroflexi*) were present in some of the crDNA libraries but represented only minor components. Other non-*Proteobacteria*-related groups detected in the rDNA library were absent from the crDNA libraries.

The archaeal 16S rDNA libraries were dominated (80%) by clones belonging to the order *Methanosarcinales,* and at all depths sequences related to the AOM-associated ANME-2 clades were detected. ANME-2a and ANME-2b were the dominant phylotypes. The remaining phylotypes were related to the uncultivated and metabolically uncharacterized lineages Marine Group I and III (van der Maarel *et al.*, 1998; Lanoil *et al.*, 2001; Inagaki *et al.*, 2003; Mills *et al.*, 2003), Marine Benthic Group B (Mills *et al.*, 2003) and unclassified *Euryarchaeota* (Teske *et al.*, 2002). Archaeal diversity was lower in the 16S crDNA library and only phylotypes related to the AOM-associated ANME-2 groups were detected (Fig 1.17b). ANME-2a clones were dominant in the crDNA libraries as they were in the rDNA libraries. ANME-2b clones were much less numerous in the crDNA libraries than they were in the rDNA libraries, while ANME-2c clones were relatively abundant despite a complete absence in the rDNA libraries.

The differences between the rDNA and crDNA libraries in this study were interpreted as a reflection of which organisms were the most metabolically active *in situ.* Cell numbers were reported as comparable at each depth ($6.2 - 9.6 \times 10^9$ g^{-1}) and therefore environmental parameters were implicated as the controlling factors in determining different levels and types of metabolic activity. Suggestions made as to the possible environmental function/ecological niche of certain phylogenetic groups remained tentative due to a lack of geochemical data and low sequence similarity with characterized organisms. The authors noted the inherent technical biases in DNA and RNA-based molecular approaches (e.g. see Baker *et al.*, 2003) as a possible additional factor contributing to the discrepancies. It should also be noted that such RNA-based approaches, which seek to use rRNA as a measure of bacterial activity according to the observed correlation between rRNA content and growth rate in laboratory cultures (Rosset *et al.*, 1966; Nomura *et al.*, 1984; Kemp *et al.*, 1993), may not be applicable in oligotrophic marine sediment environments where prokaryotes exist under non-steady state growth conditions (see Kerkhof & Kemp, 1999). Marine sediment prokaryotes may be metabolically active but not actively dividing, or dividing only very slowly.

The vast majority of phylotypes in all of the twelve Martinez *et al.* (2006) rDNA and crDNA libraries represented as-yet-uncultivated lineages, which is commonly the case with molecular-based studies of methane seep environments. Furthermore, many

phylotypes were closely related to sequences detected in other environmentally similar but geographically distant sites, as also found by other groups (e.g. Hinrichs *et al.*, 1999; Boetius *et al.*, 2000; Orphan *et al.*, 2001a; Nauhaus *et al.*, 2002; Mills *et al.*, 2003; 2004; 2005).

1.2.2.2 Eastern Mediterranean MVs

Due to the tectonic convergence of the African and Eurasian plates an accretionary prism, the Mediterranean Ridge, occurs beneath the Mediterranean Sea. Along some areas of the ridge mud diapers have formed, which breach the sediment surface in several localities to produce fields of mud volcanoes. One prominent MV field is the Olimpi field situated south of Crete. Here mud volcanism is commonly associated with brine seepage due to the deformation of underlying Messinian evaporates. Another important area of mud volcanism in the Eastern Mediterranean is the tectonically complex Anaximander Mountain area, situated offshore Turkey. Messinian evaporates are absent in the Anaximander Mountain basins and hence this field lacks the brine seepage associated with the Olimpi field (Charlou *et al.*, 2003; Zitter *et al.*, 2005). MVs in the Olimpi field (e.g. Napoli and Milano MVs) and the Anaximander Mts (e.g. Kazan and Amsterdam MVs) have been extensively studied by a variety of geochemical and geophysical approaches since the 1980s, and in this respect they are well characterized (e.g. Camerlenghi *et al.*, 1992; Charlou *et al.*, 2003; Werne *et al.*, 2004; 2005; Zitter *et al.*, 2005; Haese *et al.*, 2003; 2006). However, knowledge of the microbiology of the MVs is much more limited. Carbonate crusts from some of the MVs have been investigated by lipid biomarker and 16S rRNA PCR-based methods (Aloisi *et al.*, 2002; Heijs *et al.*, 2006; Bouloubassi *et al.*, 2006), and one study used 16S rRNA PCR-based methods to determine the community structure of a MV microbial mat (Heijs *et al.*, 2005). The MV sediments themselves remain uncharacterized.

In the study by Heijs *et al.* (2006), DNA was extracted from powdered carbonate crust material from three Eastern Mediterranean MVs (Napoli, 1945 m water depth; Kazan, 1706 m water depth and Amsterdam, 2034 m water depth) and subject to PCR amplification with domain-specific 16S rRNA bacterial and archaeal primers. The study aimed to characterize the carbonate crust population and assess the possible role of prokaryotes in carbonate crust formation, since isotopic evidence suggested

anaerobically oxidized methane was a source of the crust carbon (Aloisi *et al.*, 2002). Phylogenetic analysis of bacterial and archaeal 16S rRNA gene libraries revealed highly diverse microbial communities and these communities were different in the different crusts (Fig 1.18).

The majority of bacterial phylotypes belonged to the *Proteobacteria*, of which the *Alpha-* and *Gamma-* sub-groups were the most dominant in all three crusts. Additional bacterial lineages were present in lower abundance but were not detectable in all crusts (Fig 1.18). *Archaea* were only detected in two of the three crusts analyzed and overall diversity was lower than for the *Bacteria*. Some archaeal phylotypes were related to ANME groups but the majority of *Archaea* were not related to organisms associated with AOM. Interestingly, different ANME groups dominated the two different crusts analyzed. Non-ANME archaeal phylotypes were related to the ubiquitous Marine Group I group, *Thermoplasmales* and a hitherto unclassified novel group of *Crenarchaeota*, previously identified in deep-sea sediments.

The majority of the microbial sequences obtained from the crusts were novel with <97% similarity to other known sequences. Nevertheless, based on the physiology of related isolates it was tentatively suggested that aerobic heterotrophy, sulphide oxidation, methylotrophy, sulphate reduction and AOM were all metabolic processes possibly conducted by members of the carbonate crust community.

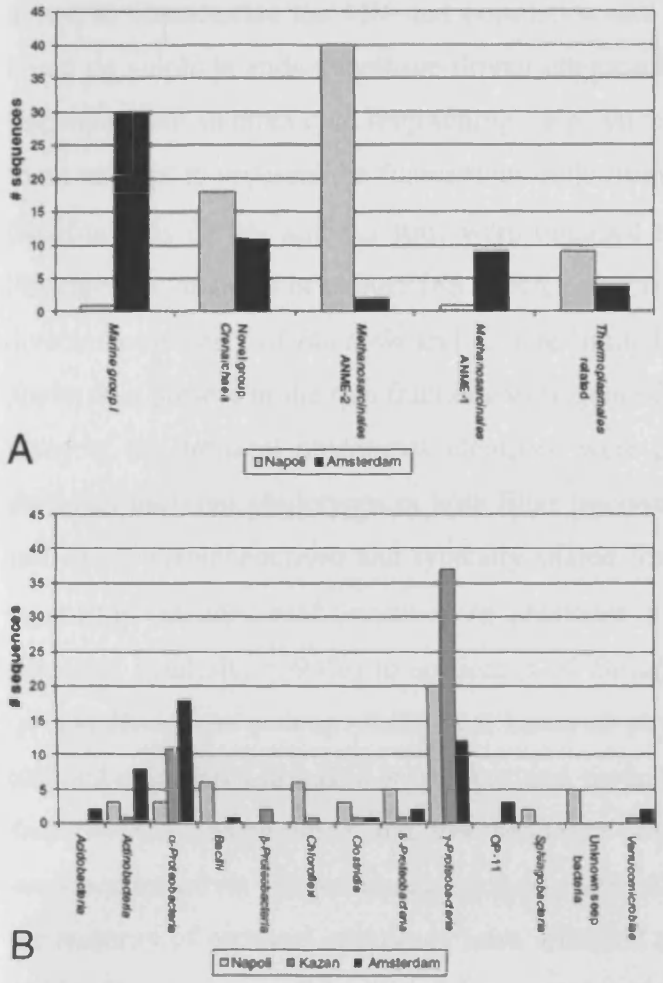


Fig 1.18 Phylogenetic group distributions of the archaeal (A) and bacterial (B) 16S rRNA gene sequences detected in Eastern Mediterranean deep-sea carbonate crusts. N.B. archaeal 16S rRNA genes could not be amplified from the Kazan MV. Taken from Heijs *et al.* (2006).

Sequences related to the genera *Halomonas*, *Halovibrio* and *Marinobacter* were present at relatively high abundance in two and three of the carbonate crusts studied, respectively. It was suggested that these organisms might play a role in carbonate crust formation, which is supported by laboratory studies of similar organisms (Rivadeneyra *et al.*, 1998; 1999). These authors did not comment on possible controls on community structure in the different MVs, but suggested that different populations of microorganisms may carry out similar processes that facilitate the carbonate crust formation. The abundance of non-ANME-related archaeal sequences in association with the strongly negative ^{13}C values of diagnostic archaeal lipids (Bouloubassi *et al.*, 2006) was proposed as evidence for a wider diversity of AOM mediating organisms than previously thought (Heijs *et al.*, 2006; Bouloubassi *et al.*, 2006).

A white microbial mat containing large filaments as well as various coccoid- and rod-shaped organisms was recovered from the Milano MV (2000 m water depth) and investigated by phylogenetic and lipid biomarker methods (Heijs *et al.*, 2005). The study

aimed to characterize the MV mat population and assess whether the community was based on sulphide and/or methane-driven chemoautotrophy, as has been implicated for microbial mats in other cold seep settings (e.g. Michaelis *et al.*, 2002; Mills *et al.*, 2004). In an attempt to separate the filamentous cells from the smaller associated prokaryotes, two fractions (5 μm and 0.2 μm) were obtained from the mat material by filtration. Phylogenetic analysis of cloned 16S rRNA genes from the two mat fractions revealed a diverse community of *Bacteria* and a more limited diversity of *Archaea*. The bacterial phylotypes present in the two fractions were generally different from one another, while many of the archaeal phylotypes identified were common to both mat fractions. The abundant bacterial phylotypes in both filter fractions included *Alpha-, Gamma-, Delta-* and *Epsilonproteobacteria* and typically shared low sequence similarity with cultured organisms. *Deltaproteobacteria* were abundant in the 0.2 μm fraction and shared moderate similarity (>94%) to sequences of *Desulfocapsa sulfoexigens*, *Desulfovibrio* sp. and *Desulfosarcina* sp. Additional bacterial phylotypes were moderately related to cultured organisms involved in sulphur and methane cycling; e.g. *Thiomicrospira* sp., *Sulfurimonas autotrophica*, but low sequence similarity meant functional inferences remained tentative. The archaeal phylotypes were all members of the *Euryarchaeota* and the majority of archaeal sequences were affiliated to the ANME-2 groups (Heijs *et al.*, 2005). The presence of these organisms suggested a potential for AOM within the mat community but the importance of this process was questioned by their lipid data, as ANME signature lipids were not detected.

Based on their combined phylogenetic and lipid data Heijs *et al.* (2005) concluded that the mat community may be sustained by a combination of methane- and sulphide-driven chemotrophy, both aerobic and anaerobic, with *Bacteria* as the dominant community members. The filamentous organisms in the mat were not conclusively identified. It was noted that freeze-thawing of the mat material prior to analysis may have introduced a bias in the relative abundances of the sequences obtained, such that their results may not necessarily have reflected the *in situ* structure of the mat community.

1.3 The Gulf of Cadiz Study Area and its Associated Mud Volcanism

This section is a general introduction to the Gulf of Cadiz study area and its associated mud volcanism. More detailed descriptions of the sites investigated during the present study are given in chapters 3 and 4.

The Gulf of Cadiz is situated in the NE Atlantic. The area is bounded in the north by the southern coasts of Portugal and Spain, and in the south by the western coast of Morocco (Fig 1.19).

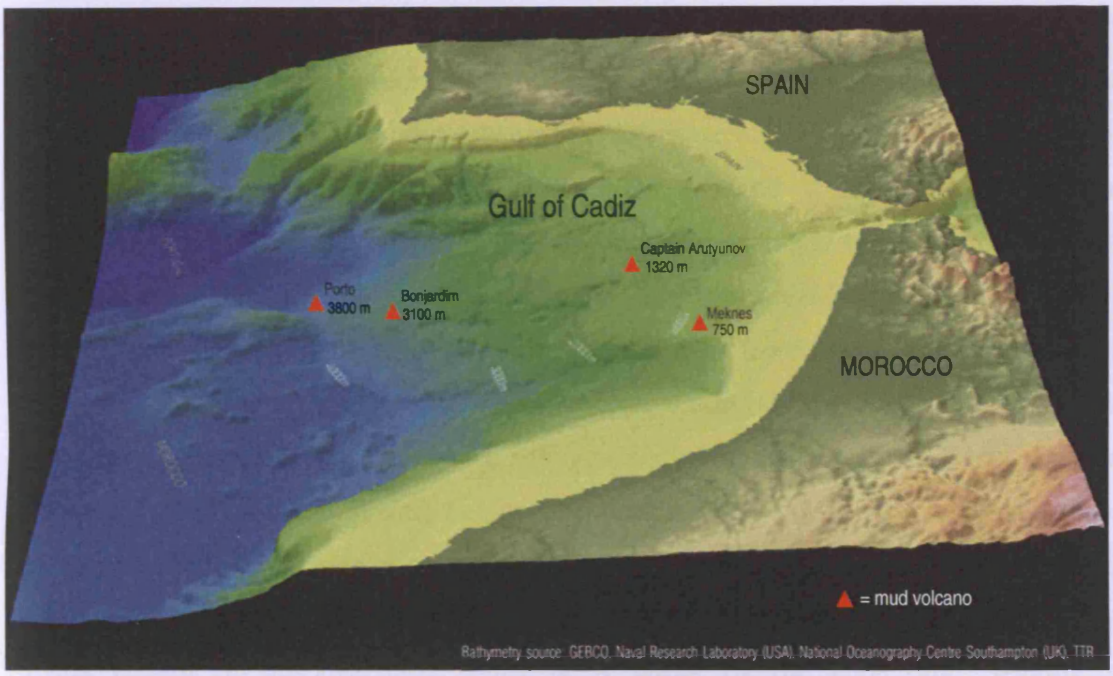


Fig 1.19. Overview map of the Gulf of Cadiz study area, showing the approximate locations and water depths of the four mud volcanoes investigated during the present study.

It is a structurally complex area that has undergone a multifarious tectonic evolution (e.g. Maldonado *et al.*, 1999; Medialdea *et al.*, 2004). Compressive tectonics act in the area due to the NW convergence of the African and Eurasian plates (Gardner, 2001; Pinheiro *et al.*, 2003), and have resulted in the formation of several types of fluid escape features (e.g. pock marks, carbonate chimneys and MVs) at the seafloor. Features hypothesized to be MVs based on their size, structure and backscatter intensity were first identified in the Gulf of Cadiz during an American naval survey cruise in 1992 (Gardener, 2001). A follow up cruise in 1999 (Leg 2 of TTR-9) confirmed the MV interpretation after gravity cores recovered mud breccia and gas hydrates from the sites of high backscattering (Gardener, 2001). Since that first exploratory cruise the presence

of more than 30 MVes have been confirmed in the area, spanning water depths of 200 m to 4 km. More discoveries are expected in the area as surveys continue.

Both recently active and inactive MVes have been identified on the basis of geochemical data and/or the relative thickness of hemipelagic sediment draping over the extruded mud breccia. Numerous seismic profiles of MVes in the region show stacked outflow deposits at the crater flanks (e.g. Van Rensbergen *et al.*, 2005; Niemann *et al.*, 2006). These are interpreted as mud breccia lenses inter-fingered between hemipelagic sediments, and are believed to testify episodic but long-lived mud volcanism in the area (Van Rensbergen *et al.*, 2005). The Gulf of Cadiz MVes are believed to be a consequence of the area's compressive tectonic regime acting upon sediments that were deposited at a time when basement subsidence rates were high (Maldonado *et al.*, 1999). These rapidly deposited sequences, collectively referred to in the area as the 'olistostrome unit', represent an allochthonous body of sediment that was eroded from the Betic Cordillera in Spain and the Rif Massif in Morocco (Maldonado *et al.*, 1999; Somoza *et al.*, 2003). Such rapid deposition rates might have lead to the formation of overpressure compartments by disequilibrium compaction (Osborne & Swarbrick 1997), as has been hypothesized in models of MV genesis in the South Caspian Basin (Davies & Stewart, 2005). Disequilibrium compaction occurs when, due to rapid sedimentation, subsidence and low sediment permeability, pore water cannot escape from the deposited sediments at a sufficient rate to let them compact normally. In this situation a large share of the weight of the overlying sediment is borne by the pore fluid, leaving it overpressured (Xie *et al.*, 2001). Hydrocarbon generation may have further contributed to the overpressure of sediments in the Gulf of Cadiz, though the importance of this is not well constrained (Van Rensbergen *et al.*, 2005). The fact several Gulf of Cadiz MVes align along major NW-SE and NE-SW faults suggests the escape pathways of the overpressure fluids may have a deep structural control (Gardener, 2001; Pinheiro *et al.*, 2003).

Since the start of the present study, Niemann *et al.* (2006b) have described, and measured microbial methane turnover at, several Gulf of Cadiz MVs including two MVs (Captain Arutyunov and Bonjardim) that were investigated during this work. The main findings of this recently published work are summarized below and are discussed in more detail in relevant sections later in the thesis.

Pore water analyses and methane oxidation measurements revealed the methane rising from the Gulf of Cadiz MV craters was completely consumed within the seabed. This was largely confirmed by limited visual observations, which detected only scare traces of methane seepage and relatively low densities of seep related biota on the seafloor (Niemann *et al.*, 2006b).

Distinct sulphate-methane transition zones (SMTZs) were observed within the sub-surface of Gulf of Cadiz MV craters (between 20 – 200 cmbsf), which overlapped with high sulphide concentrations, suggesting that the methane was consumed by AOM with sulphate as the electron acceptor. This was supported by maxima in *ex situ* AOM and sulphate reduction (SR) rates at the SMTZs. There was deviation from the expected 1:1 stoichiometry between AOM and SR rates, as well as between the sulphate and methane fluxes at the MVs, indicating electron donors other than methane were also being used for SR (Niemann *et al.*, 2006b). Geochemical data suggested higher hydrocarbons were a significant source of the additional SR observed, as reported in cold seep sites in the Gulf of Mexico (Joye *et al.*, 2004).

In comparison to other methane seeps (e.g. Hydrate Ridge, the Gulf of Mexico and Haakon Mosby MV) AOM activity (<383 mmol m^{-2} yr^{-1}) and diffusive methane fluxes (<321 mmol m^{-2} yr^{-1}) in the Gulf of Cadiz were low to mid range. Based on methane flux and microbial turnover rates, Capt. Arutyunov was the most active of the seven sites investigated by Niemann *et al.* (2006), followed by Bonjardim.

Total community DNA was extracted from the crater SMTZ (30 – 40 cmbsf) of Capt. Arutyunov only. The DNA was amplified with universal bacterial and archaeal 16S rRNA gene primers and clone libraries constructed. Nine phylogenetic groups were identified in the archaeal library (39 clones sequenced). The majority of sequences were related to AOM-mediating ANME groups. The ANME-2a, ANME-2c and ANME-1 groups made up 59, 3 and 18% of all archaeal sequences, respectively). The other archaeal sequences were affiliated with uncultured groups: Marine benthic group D (8%), Marine benthic group B (3%) and unclassified archaea (10%). The bacterial 16S rRNA gene library (47 clones sequenced) consisted of 10 uncultivated bacterial lineages. The majority of sequences (81%) belonged to the Seep-SRB1 group in the

Deltaproteobacteria, which have been identified as the bacterial partners of the ANME-1 and ANME-2 organisms (Knittel *et al.*, 2003). The other bacterial phylotypes were represented by single sequences and were affiliated with *Clostridia* (2%), *Spirochaetes* (4%), *Gammaproteobacteria* (2%), other *Deltaproteobacteria* (4%) and unclassified bacteria (6%), including JS1. The clone library results are discussed in comparison with findings of the present study in chapters 3 and 5.

1.4 Overview of Methodological Approach

The present study used a multidisciplinary approach to investigate the microbial ecology of Gulf of Cadiz MVs. Both cultivation dependent and cultivation independent approaches were used to assess prokaryotic diversity. Some sediment samples (Meknes and Porto MVs) were also investigated by other methods – acridine orange direct counts (AODC), pore water geochemical analysis and potential activity measurements. Figure 1.20 below summaries the methodological approach used. Full experimental details are given in chapter two.

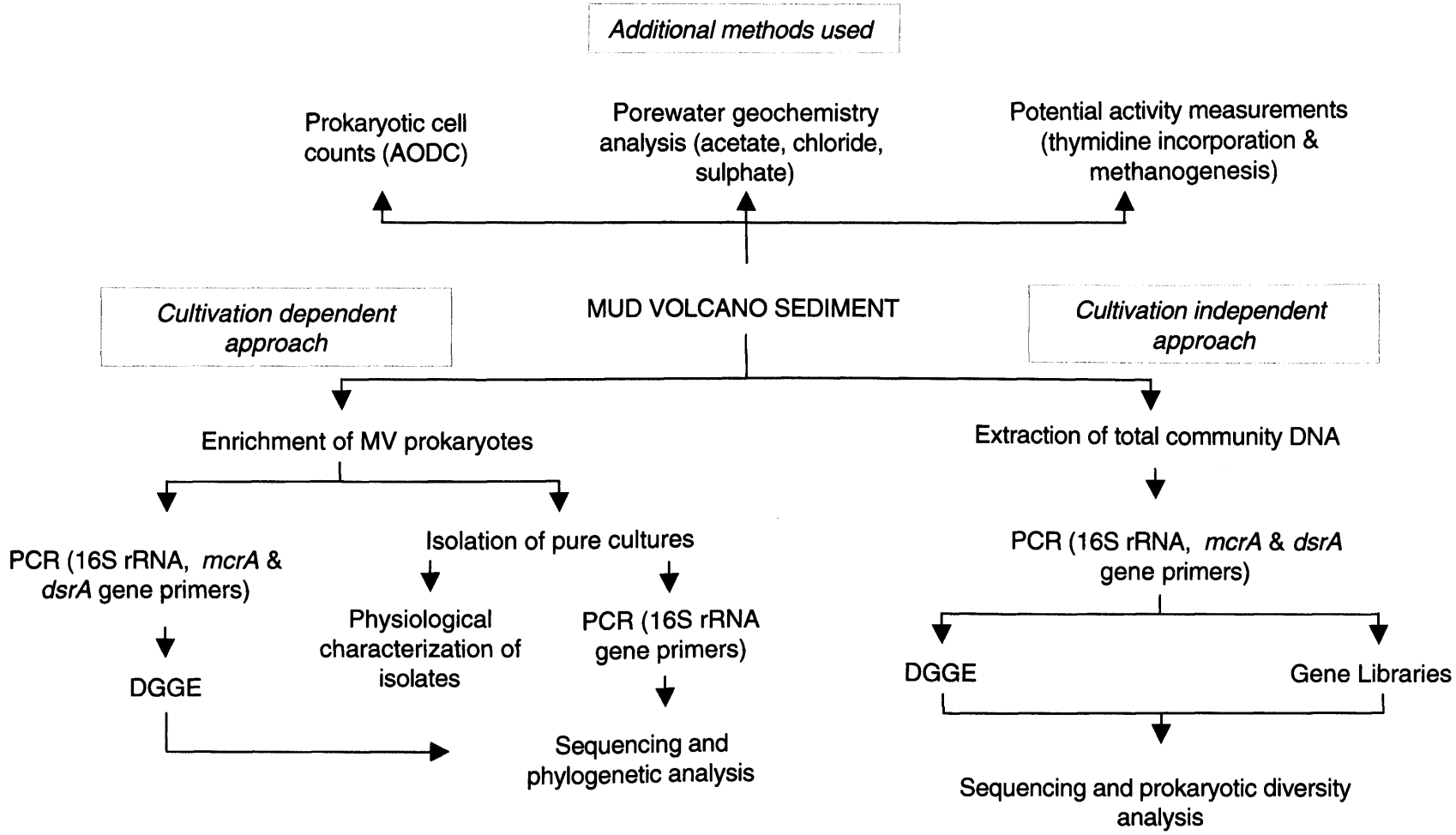


Fig 1.20 Summary of methodological approach used in this study

1.5 Project aims

In this project sediments from several Gulf of Cadiz MVs were studied to gain an understanding of microbial diversity, microbial community composition and biogeochemical processes in these hitherto poorly studied environments. The main objectives were:

- To provide a taxonomic census of prokaryotes in the MV environment.
- To contrast community structure, microbial activities and biogeochemistry between different Gulf of Cadiz MV craters.
- To examine spatial variation in microbial populations and biogeochemical processes across one MV - crater, slope and reference site.
- To contrast Gulf of Cadiz MV populations with those from other MV and non-MV environments.
- To establish whether deep biosphere signature microorganisms were detectable in near-surface mud breccia, i.e. were mud volcanoes 'a window to the deep biosphere?'
- To formulate hypotheses about MV community function and control.
- To enrich, isolate and characterize prokaryotes from the MV environment.

Chapter 2 – Materials and Methods

2.1 Sample collection and storage

The samples used in this study were collected from two separate cruises to MV fields in the Gulf of Cadiz between 2003 and 2005. All the methods described in this chapter were conducted by the author, unless otherwise stated.

2.1.1 Captain Arutyunov and Bonjardim MVs

Samples were collected by a colleague during Leg 2 of cruise SO175, a.k.a. Gibraltar Arc Processes (GAP), onboard the R/V *Sonne* at the end of 2003. Sediment cores targeted in the crater of two MVs, the 1321 m water depth Captain Arutyunov MV and the 3087 m water depth Bonjardim MV were recovered with a 1.6 tonne, 6 m long gravity corer. Once on deck the cores were cut into 1 m sections and spilt lengthways. The samples used for this study were taken from the lower part of the cores as composite samples and were stored in the dark at 4°C in sealed bags flushed with oxygen-free nitrogen until arrival at Cardiff University. Sample details are given in Table 2.1.

Table 2.1 Captain Arutyunov and Bonjardim MV sample details.

Sample code	Target	Lat.	Long.	Water depth (m)	Horizon sampled (cmbsf)	Description
GeoB 9072-1	Crater of Captain Arutyunov mud volcano	35:39,71	7:19,95	1320	126 – 270	Mud breccia (gas hydrate bearing)
GeoB 9051-2	Crater of Bonjardim mud volcano	35:27,61	9:00,03	3100	205 – 240	Mud breccia (no gas hydrate reported)

The recovered sediment, still in their original sample bags, were transferred to the Cardiff laboratory 6 months later and sealed for a week inside anaerobic jars at 4°C until sub-sampling. Sub-sampling was conducted inside an anaerobic cabinet (Forma Scientific), which had first been thoroughly cleaned and wiped down with ethanol. A sterile scalpel blade was used to remove the outer portions of mud that had been in contact with the bag, and sub-cores of mud from the 'pristine' interior of the sample were extracted using sterile (foil wrapped and autoclaved for 20 min at 121°C) 5 ml

syringes with their luer end removed. The sediment was diluted 1:10 with sterile mineral salts solution inside the anaerobic cabinet to produce an inoculum for cultivation (see section 2.4). Mineral salts solution contained the following (g l^{-1}): NaCl (23), $MgCl_2.6H_2O$ (3), KCl (0.5), NH_4Cl (0.25), KH_2PO_4 (0.2) and $CaCl_2.2H_2O$ (0.15). The salts solution was autoclaved at 121°C for 45 min, removed from the autoclave whilst hot then cooled and dispensed under filtered N_2:CO_2 (80:20).

Additional samples were taken for molecular analysis. These samples were taken with an ethanol-wiped spatula and immediately stored frozen at –80°C in sterile universal tubes until analysis (see section 2.3). It should be noted that although the precise depth origin of the samples taken was not known (Table 2.1) they were sufficient to meet the stated objectives of the study (section 1.5).

2.1.2 Meknes and Porto MVs

Samples were collected onboard the R/V *Professor Logachev* during Leg 4 of the TTR-15 (Training Through Research) cruise in July 2005. Sampling was from two active MVs: the 750 m water depth Meknes MV on the Moroccan Margin and the 3850 m water depth Porto MV on the Portuguese Margin. A transect of sites were sampled across the Meknes MV (see Fig 2.1) whereas only the crater of the Porto MV was sampled. Full details of the samples collected are shown in Table 2.2.

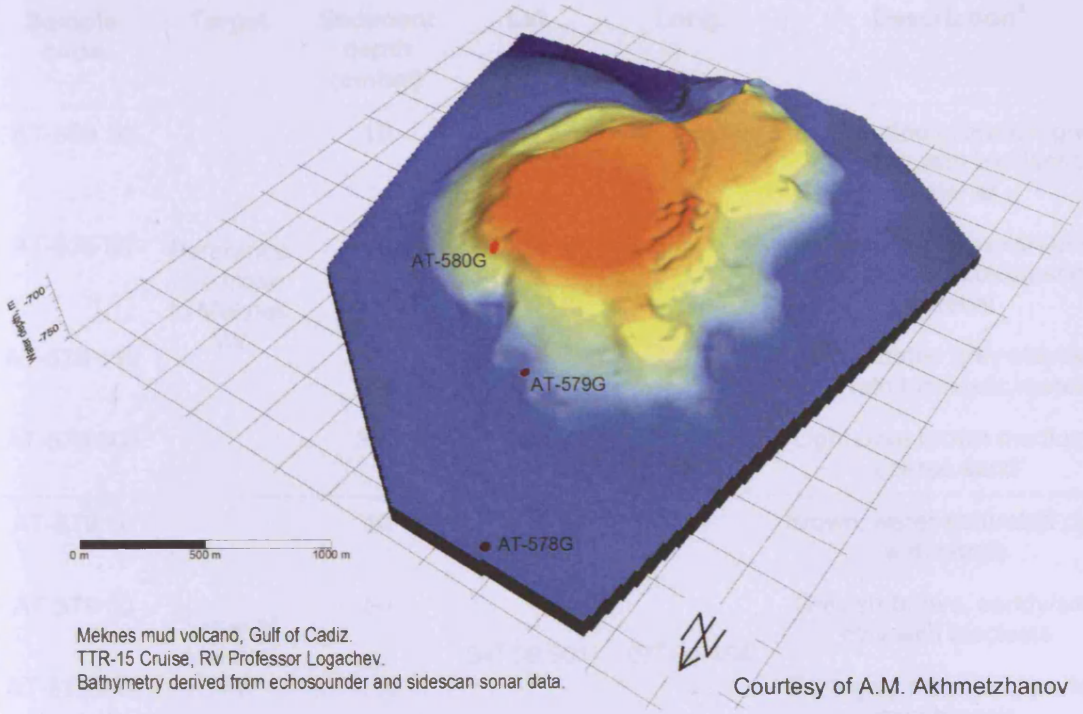


Figure 2.1. Locations of the transect sites sampled across the Meknes mud volcano, Latitude and Longitude positions are given in Table 2.2

Table 2.2 Meknes and Porto MV sample details

Sample code	Target	Sediment depth (cmbsf)	Lat.	Long.	Description[a]
AT-578 10	Reference site close to Meknes MV	10	34° 59,492'	07° 04,546'	Bioturbated brownish-grey silty clay with bioclastic material
AT-578 50		50			Bioturbated brownish-grey silty clay with bioclastic material
AT-578 110		110			Bioturbated grey clayey sand with bioclastic material
AT-578 300		300			Light olive brown medium-coarse sand
AT-579 10	Slope of Meknes MV	10	34° 59,301'	07° 04,464'	Brown, water-saturated clay with corals
AT-579 50		50			Greyish-brown, sandy/silty clay with bioclasts
AT-579 110		110			Dark grey matrix-supported mud breccia
AT-579 125		125			Dark grey matrix-supported mud breccia
AT-580 10	Crater of Meknes MV	10	34° 59,203'	07° 04,352'	Dark grey mud breccia
AT-580 50		50			Dark grey matrix-supported mud breccia
AT-580 65		65			Dark grey matrix-supported mud breccia
AT-596 10	Crater of Porto MV	10	35° 33,811'	09° 30,532'	Brown water-saturated clay with foraminifera
AT-596 50		50			Grey matrix-supported mud breccia
AT-596 110		110			Grey matrix-supported mud breccia
AT-596 200		200			Very dark grey matrix-supported mud breccia. Porous due to de-gassing

[a] TTR-15 Shipboard party

All samples were obtained with a 1500 kg 6 m long gravity corer (14.7 cm internal diameter). On deck, the sediment cores were extruded from the core liner, cut into 60 cm sections and sliced lengthways with a wire. Sub-samples were taken aseptically from core halves immediately after splitting, using sterile (foil wrapped and autoclaved) syringes with their luer-end removed, unless otherwise stated. All samples were taken from the central 'pristine' parts of the sediment cores i.e. no sediment that had been in direct or close contact with the core liner was collected.

Syringe samples taken for molecular analysis were wrapped in cling film and immediately frozen (–20°C onboard ship, then –80°C in the laboratory).

Samples for enrichment and cultivation were placed inside wine bags with an activated Anaerocult® catalyst (Merck, Darmstadt, Germany) to remove oxygen and create an anaerobic atmosphere, heat-sealed and stored at 4°C. This temperature represented close to *in situ* conditions and hence would help prevent perturbation of the original community structure prior to selective enrichment in the laboratory.

Samples for total counts were collected in sterile 5 ml syringes. A 1 ml sediment plug from the central part of the syringe was extruded into a glass vial containing 9 ml of sterile 2% formaldehyde in mineral salts. The vials were crimp sealed and shaken vigorously to ensure dispersal of the sediment and fixing of the cells.

Samples for potential activity measurements were taken with 20 cm by 26 mm ID acrylic tubes with injection ports drilled into them every centimetre. The injection ports were sealed for sampling with a silicon-based aquarium sealant. The minicores, sharpened at one end, were inserted lengthways into halved sediment cores with the aid of a hand-held vacuum pump to keep disturbance of the sediment to a minimum. After sampling the minicores were sealed at each end with red rubber bungs and placed inside wine bags (Gruber-Folien, Straubing, Germany) with an activated Anaerocult® catalyst (Merck, Darmstadt, Germany). The bags were heat-sealed and stored at 4°C. These samples were processed within a week of return to the Cardiff laboratory (see section 2.3).

2.2 Sediment characterisation

2.2.1 Geochemistry

Only pore water from the Meknes and Porto MVs were analysed. Samples originally intended for pore water analysis were taken using 20 ml syringes and stored at 4^0C inside anaerobic bags, but due to technical difficulties (contamination of pore water from acetate-containing filters) alternative samples were later used. Experience with the original samples showed little pore water could be extracted from the very compacted mud breccia and therefore pore water recovery was optimised with the later samples. Frozen samples, originally taken as spare material for molecular genetic analysis, were used for pore water extraction by centrifugation. Samples were allowed to defrost overnight at 4°C before approximately 1.5 ml portions were transferred to microfuge tubes (Eppendorf) using a clean spatula. Centrifugation was conducted for 5 min at 16,100 g then the supernatants from each horizon were combined inside fresh microfuge tubes and subjected to further centrifugation to remove any remaining sediment particles.

The extracted pore water was analysed for sulphate, chloride and volatile fatty acids by ion chromatography using a Dionex® ICS-2000 system equipped with a Dionex® AS50 autosampler. Chromatographic separation was conducted on two Ionpac® AS15 columns in series with a Dionex® Anion Self-Regenerating Suppressor (ASRS®-ULTRA II 4-mm) unit, in combination with a Dionex® DS6 heated conductivity cell. An eluent flow rate of 1.0 ml/min was used with the following gradient programme: 6.0 mM KOH (38 min), 16.0 mM KOH/min to 70 mM (17 min), 64.0 mM KOH/min to 6.0 mM (12 min). The concentrations in samples were calculated within the linear range using standard calibration curves. Standard deviations (1 σ) were calculated for all data from the replicate measurement of appropriate known standards. The lower detection limit of the system for acetate and sulphate was 1 μM and 10 μM, respectively.

2.2.2 Total cell counts

The total number of prokaryotic cells in the sediment and the proportion that were active/growing, as indicated by dividing or recently divided cells, was determined by the acridine orange direct counting (AODC) method based on the general recommendations of Fry (1988). Acridine orange differentially stains cells green against a background orange stain of inorganic particles when viewed under blue epifluorescent light. The total population was enumerated by extrapolation of microscopic cell counts.

Samples were taken as described in section 2.1.2 (Meknes and Porto) or by adding 9.5 ml of 1:10 slurry (see section 2.1.1) to 0.5 ml of 40% filtered formaldehyde (Captain Arutyunov and Bonjardim). Sub-samples of the fixed slurries were dispensed into another 10 ml of 0.1-µm filter-sterilized 2% formaldehyde in artificial seawater. The quantities were selected to achieve 50 – 75% particle coverage on the membrane to aid efficient and reliable counting (Fry, 1988). The sub-samples were stained with 50 µl (1 gl^{-1}) acridine orange (BDH Chemicals Ltd, Poole, England) for 3 min before vacuum filtering onto a black polycarbonate (0.2 µm pore size) membrane. Membranes were rinsed with a further 10 ml of 2% filtered-sterilized formaldehyde and mounted on a microscope slide in a medium of paraffin oil under a cover slip. Membranes were viewed under incident illumination with an Axioskop microscope (Zeiss, Germany) fitted with a 50 W mercury vapour lamp, a wide-band interference filter set for blue excitation, a 100x (numerical aperture = 1.32) Leitz Fluotar objective lens and 8x eyepieces. A total of 200 fields of view (FOV) or 200 cells were counted for each membrane and at least 2 membranes examined per sample. Standard deviations and 95% confidence limits were calculated, with further membranes counted as necessary. Care was taken to discriminate between bacterial-shaped objects and other green-stained organic material (e.g. Fig 2.2, panel d). Cells seen on the face of sediment particles were doubled in the calculations to account for others that may have been hidden from view, cells on the side of a particle were not doubled (e.g. Fig 2.2, panel b). Dividing and divided cells were counted separately from those 'on' and 'off' particle; these were cells with a clear invagination (e.g. Fig 2.2, panel c) or pairs of cells with identical morphology (e.g. Fig 2.2, panel a), respectively. Dividing and divided cells were both counted once, and numbers of divided cells were subsequently doubled in the calculation (see below).

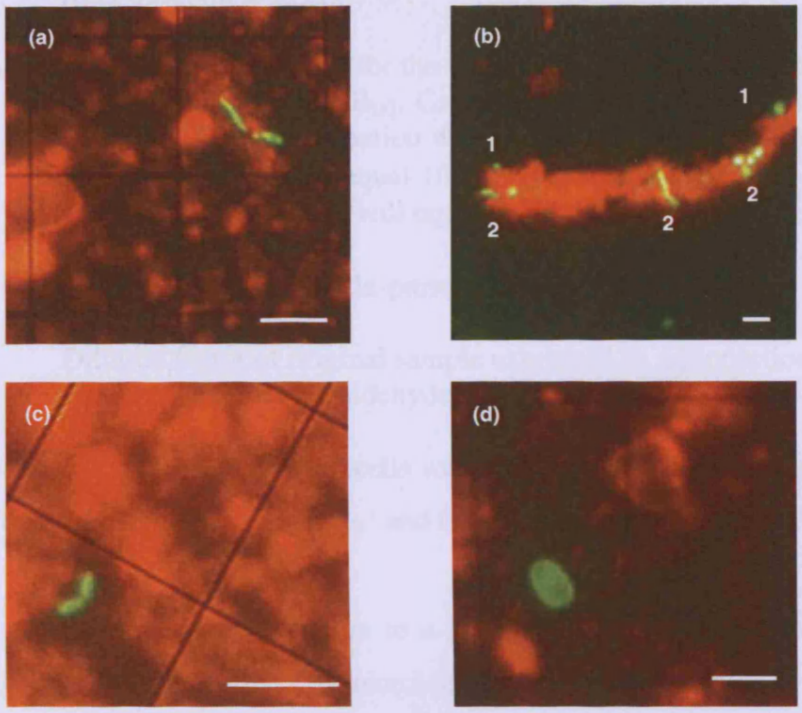


Fig 2.2. Illustrative photos showing cell distinctions made during AODC procedure, (a) divided cell; (b) cells counted off particle, marked '1', or on particle, marked '2'; (c) dividing cell; (d) non-bacterial stained particle. Scale bars – 5 μm.

Total cell counts were calculated from the equation:

$$\left(\left(\left(\frac{2C_{ON} + C_{OFF} + C_{DG} + 2C_{DD} + \left[\frac{C_{ON}}{2C_{ON} + C_{OFF}} \times (C_{DG} + 2C_{DD}) \right]}{VIEW} \right) \times A \right) - B_T \right) \times \frac{1000}{V_{CT}}$$

$$D$$

C_{ON} and C_{OFF}: Number of cells counted ON and OFF particles. Transparent particles e.g. diatom frustules, are not included.

C_{DG} and C_{DD}: Number of cells observed DIVIDING (cells with clear invagination or two touching cells with identical morphology) and DIVIDED (cells with identical morphology but clear space between them). N.B. cells counted in these two categories are not tallied under ON and OFF particle categories.

VIEW: Total number of FOV observed per membrane.

A: Filter area ratio; total countable area of the filter divided by the area of filter observable in one FOV.

B_T and B_D: Blank correction terms for the total cell number (B_T) and the dividing and divided cell numbers (B_D). Calculated from counts of blank membranes and using the same equation with the omission of the correction term. In this instance V_{CT} will equal 10050 (10 ml formaldehyde + 50 µl acridine orange solution) and D will equal 1.

V_{CT}: Volume of formaldehyde-preserved sample stained (µl).

D: Dilution factor of original sample expressed as a proportion, e.g. 1 cm^3 of sediment in 9 ml formaldehyde will give D = 0.1.

The numbers of dividing and divided cells were calculated from the same equation with the omission of the terms '$2C_{ON} + C_{OFF}$' and the substitution of B_D for B_T.

Total cell counts were plotted relative to a regression line of average total cell count data, as complied by the Cardiff Geomicrobiology Research Group since the late 1980s, (e.g. Parkes *et al.*, 1994).

2.2.3 Potential activity measurements

Sediments from the Meknes and Porto MVs only were analyzed for potential activity. The samples were collected and briefly stored as described in section 2.1.2. All incubation times used in potential activity measurements were based on previous experience of the Cardiff Geomicrobiology Research Group (e.g. Parkes *et al.*, 2005).

2.2.3.1 Thymidine incorporation

Three weeks after sampling, the 4°C-stored minicores were removed from anaerobic bags and injected at 2 cm intervals with 10 µl of tritiated [^{3}H]-thymidine solution (specific activity 37 Mbq ml^{-1}, Amersham, UK). The syringe was withdrawn slowly whilst injecting to leave a thin line of isotope through the centre of the core. At least 2 cm was left un-injected at either end of the core as these layers may have been disturbed. Cores from the Meknes and Porto MVs were incubated at close to *in situ* temperature (4°C) for 4 or 8 h respectively. Porto sediment was incubated for longer as deeper water sites typically have lower activities. Incubations were terminated by thoroughly mixing

each injected 2 cm sediment slice with 5 ml ice-cold 10% (w/v) trichloroacetic acid inside a 50 ml centrifuge tube. A 2 cm collar was used to gauge accurate recovery of each injected slice as the sediment was progressively extruded from the minicore with a plunger. The fixed samples were stored at 4°C until processing.

Several fractions were processed in order to account for the fate of all injected isotope (Fig 2.3). Centrifugation values are given in g, the appropriate RPM can be calculated from the equation below:

$$RPM = \sqrt{\frac{g \times 1{,}000{,}000}{11.18 \times R}}$$

Where RPM = revolutions per minute; g = g-force; R = average sample radius in rotor (cm).

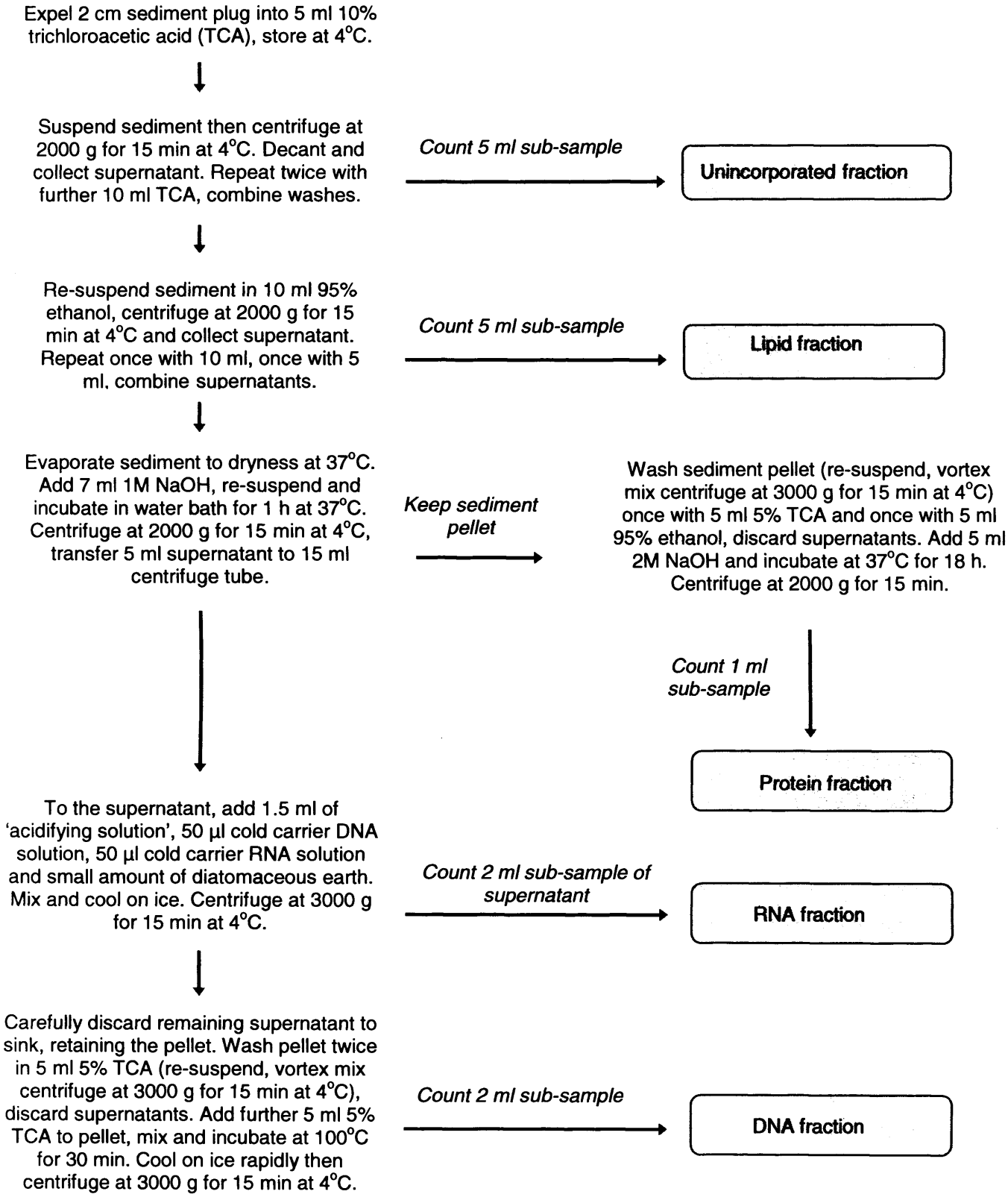


Fig 2.3 Flow diagram showing processing steps of the thymidine incorporation method. 'Acidifying solution was 20% w/v TCA in 3.6 M HCl; DNA solution was 1 mg ml^{-1} in Milli-Q water (Sigma D-6898); RNA solution was 1 mg ml^{-1} in Milli-Q water (Sigma R-7125); diatomaceous earth 'Kieselguhr' (Sigma). References: Karl (1982); Craven & Karl (1984) and Wellsbury *et al.*, (1996).

Radioactivity was counted in OptiPhase HiSafe-3 scintillant (Perkin Elmer, UK) in a TRI-CARB 2900TR liquid scintillation counter (Packard). The potential rate of thymidine turnover d^{-1} (incorporation into bacterial DNA) was calculated from the following equation:

$$\text{Turnover } d^{-1} = \frac{\left[A \;/\; DPM_{IN} \right]}{\left[\text{Incubation time (hr)} \;/\; 24 \right]}$$

Where:

A = Volume corrected DPM_{OUT} [measured DPM x (Total DNA sample vol (ml) / vol counted (ml)) x dilution factor]. Dilution factor is necessary due to the addition of DNA and RNA carriers plus 'acidifying solution' (see Fig 2.3).

DPM_{IN} = Activity injected per sample

2.2.3.2 CO_2/H_2 methanogenesis

Samples from the Meknes and Porto MVs were injected, as described above, with 2 µl sodium [^{14}C]bicarbonate (specific activity 18.5 MBq ml^{-1}, Amersham, UK) and incubated at 4°C for 2 and 3 days, respectively. Incubations were terminated by extruding individual sediment slices into glass bottles containing 7 ml of 1 M NaOH and a magnetic stirring bar. A 2 cm collar was used to accurately recover each injected slice as the sediment was progressively extruded from the minicore with a plunger. Bottles were sealed with red rubber bungs and mixed vigorously to dispense the sediment then stored upside down to avoid gas loss. Samples were processed through a methane-furnace rig using a method adapted from Cragg *et al.* (1992). Inlet and outlet needles were inserted through the rubber bungs and the samples mixed on a magnetic stirrer whilst the headspace gas was sparged with a carrier gas of nitrogen:oxygen (99:1) for 20 min at 70 ml min^{-1}. The headspace gas was blown through the rig through indicating silica-gel desiccant, a CO_2 trap (Supelco, UK) and a second desiccant trap to ensure removal of any $^{14}CO_2$ carried over. The gas was then passed over copper oxide in a furnace (Carbolite, UK) at 800°C where the $^{14}CH_4$ was oxidised to $^{14}CO_2$. The oxidized gas was then collected by bubbling through two scintillation vials placed in series

containing OptiPhase HiSafe-3 scintillant (Perkin Elmer, UK) and 7% (v/v) β-phenethylamine.

The radioactivity was counted with a liquid scintillation analyzer (TRI-CARB 2900TR, Packard) and relative rates of bicarbonate turnover were calculated. Due to a lack of total CO_2 pool data, potential rates were expressed as substrate turnover per day (24 h) according to the following equation:

$$\text{Turnover } d^{-1} = \frac{\left[DPM_{OUT} \times C \right] / DPM_{IN}}{\left[\text{Incubation time (hr)} / 24 \right]}$$

Where:

DPM_{OUT} is the average measured disintegrations per minute

C is an isotope fractionation correction factor (1.06 for acetate, 1.12 for bicarbonate)

DPM_{IN} is the activity per µl multiplied by the volume injected into sediment slice

2.2.3.3 Acetoclastic methanogenesis

Samples from the Meknes and Porto MVs were injected as described, with 2 µl sodium [^{14}C]acetate (specific activity 7.4 MBq ml^{-1}, Amersham, UK) and incubated at 4°C for 8 and 24 h respectively. The incubations were terminated and processed as described above. Potential relative rates of acetate turnover per day were calculated according to the equation above.

2.3 Culture-independent PCR-based methods

2.3.1 DNA extraction

Total community genomic DNA was extracted from 'pristine' MV sediment that had been stored frozen since collection (Meknes and Porto MV), or frozen since sampling of stored samples (Capt Arutyunov and Bonjardim), see section 2.1. In order to prevent contamination, sampling was conducted inside a sterile flow cabinet using an ethanol-soaked, flamed metal spatula. The MV sediment was sampled in 5-6 ~1 g portions to optimise the yield of good quality DNA. The sediment sub-samples were placed inside Lysing Matrix E Tubes and a bead-beating DNA extraction performed using the FastDNA® SPIN® Kit for Soil (Q-biogene, CA, USA) according to the manufacturer's protocol with the modifications of Webster *et al.*, (2003). The modifications were as follows: 20 µl (200 µg) of polyadenylic acid (poly A) was added to the lysis mixture to reduce DNA loss through binding to sediment clays, the volumes of other lysis mixture reagents were slightly reduced as necessary to accommodate the increased sediment volume, and spin times were extended throughout the procedure. Additionally, the matrix binding time was increased to 45 min, DNA was eluted in 100 µl nuclease-free H_2O (Severn Biotech Ltd, Kidderminster, UK), and Hi-Yield Nucleic Acid Recovery Tubes (Robbins Scientific, CA, USA) were used.

DNA from the replicate samples were combined, concentrated and purified by dialysis in Microcon YM-100 centrifugal filter devices (Millipore, MA, USA) before finally eluting in 50 µl nuclease-free H_2O. The relative DNA yield was assessed by visualising 10 µl aliquots of each extract by gel electrophoresis (1.2% w/v agarose gel stained with 0.5 µg ml^{-1} ethidium bromide run at 80 V for 45 min in 1 x TAE buffer). TAE buffer was 40 mM Tris acetate, 20 mM acetic acid and 0.5 M EDTA [pH 8.0]. The DNA was compared to the HyperLadder I DNA quantification marker (Bioline, London, UK) using the Gene Genius Bio Imaging System (Syngene, Cambridge, UK). DNA samples were aliquoted into several batches and stored at –80°C to minimise degradation of the DNA template over time.

Positive (estuarine or soil sediment) and negative (reagent only) controls were included with each new set of samples and/or new extraction kit to both monitor the effectiveness of the extraction procedure and as contamination controls, respectively.

2.3.2 Amplification of 16S rRNA gene sequences

Universal bacterial and archaeal 16S rRNA gene primers were used for the amplification of both short (DGGE screening) and long (gene cloning) PCR products. The 16S rRNA primer sets and PCR conditions used during this study are detailed in Tables 2.3 and 2.4, respectively.

Table 2.3. Primers used in this study for the amplification of 16S rRNA gene sequences.

Amplification target	Primer pair	Sequence 5'-3'[a]	Binding position[b]	Approximate product size	Reference
General 16S rRNA for *Bacteria* (Gene cloning & sequencing)	27F 1492R	AGA GTT TGA TCM TGG CTC AG GGT TAC CTT GTT ACG ACT T	8-27 1492-1510	1502 bp	Lane (1991)
General 16S rRNA for *Bacteria* (Gene cloning & sequencing)	27F 907R	AGA GTT TGA TCM TGG CTC AG CCG TCA ATT CMT TTR RAG TTT	8-27 907-1017	1009 bp	Lane (1991)
General 16S rRNA for *Bacteria* (DGGE bands & sequencing)[c]	357F-GC[d] 518R	CTT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	341-357 518-534	193 bp	Muyzer *et al.* (1993)
General 16S rRNA for *Archaea* (Gene cloning & sequencing)	109F 958R	ACK GCT CAG TAA CAC GT YCC GGC GTT GAM TCC AAT T	109-125 958-976	867 bp	Grosskopf *et al.* (1998) DeLong (1992)
General 16S rRNA for *Archaea* (DGGE bands & sequencing)[c]	SAf[e] PARCH	CCT AYG GGG CGC AGM RGG TTA CCG CGG CKG CTG	341-357 519-533	192 bp	Nicol *et al.* (2003) Øvreas *et al.* (1997)

[a] K = G or T, M = A or C, R = A or G, W = A or T, Y = C or T.
[b] Based on *Escherichia coli* numbering (Brosius *et al.*, 1978).
[c] Sequencing of DGGE bands was conducted according to the method of O'Sullivan *et al.* (2008), see section 3.4
[d] For DGGE, this primer has the following GC-clamp at the 5' end, CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G
[e] Primer SAf is a mixture of primers SA1f (CCT AYG GGG CGC AGC AGG) and SA2f (CCT ACG GGG CGC AGA GGG) at a molar ratio of 2:1

Table 2.4. Summary of PCR conditions used for 16S rRNA gene amplification

Primer pair	Initial denaturation step	Denaturing, annealing and extension cycle		Final elongation step	Reference
27F – 1492R 27F – 907R	95°C for 2 min	94°C for 30 s 52°C for 30 s 72°C for 90 s	x 35 increasing by 1 s every cycle	72°C for 5 min	Lane (1991)
357F-GC – 518R	95°C for 5 min	94°C for 30 s 55°C for 30 s 72°C for 60 s	x 10	72°C for 10 min	Muyzer *et al.* (1993)
		92°C for 30 s 52°C for 30 s 72°C for 60 s	x 25		
109F – 958R	98°C for 30 s	92°C for 45 s 45°C for 45 s 72°C for 42 s	x 35	72°C for 5 min	Newberry *et al.* (2004)
SAf – PARCH	95°C for 5 min	94°C for 30 s 53.5°C for 30 s 72°C for 60 s	x 5	72°C for 10 min	Nicol *et al.* (2003)
		92°C for 30 s 53.5°C for 30 s 72°C for 60 s	x 30		

PCRs were conducted using reagents from commercial PCR kits (Bioline). Reaction mixtures (49 µl) were prepared as follows: 5 µl, (50x) *Taq* buffer; 1.5 µl, $MgCl_2$ (50 mM); 0.5 µl Combined dNTPs (25 mM); 0.5 µl, of each primer (20 pmol μl^{-1}); 1 µl, BSA (bovine serum albumin 10 mg ml^{-1}, added only to first-round PCR reactions with environmental template to prevent non DNA molecules from inhibiting the PCR reaction); 40.75 µl, sdH_2O and 0.25 µl *Taq* Polymerase (Bioline). One microlitre of DNA was added to each reaction mixture as template. The sdH_2O used in the PCR reaction was either 0.1 µm filtered, twice-autoclaved ultra-pure water or nuclease free H_2O (Severn Biotech, Kidderminster, UK). PCR reactions were carried out in either a Dyad Thermal Cycler gradient block DNA Engine (MJ Research, Boson, USA) or a PTC-200 Gradient Cycler DNA Engine (MJ Research, Boson, USA).

Positive control DNA extracted from pure cultures was included in each batch of PCR amplifications to check that reactions were successful. Negative PCR controls were also included as a contamination control by substituting 1 µl DNA template for 1 µl sdH_2O. In the case of nested PCR reactions, the negative control amplification product from the first round reaction was used in addition to a fresh sdH_2O only negative control. *Escherichia coli* sequences amplified during nested reactions were considered to be experimental artefacts derived from the PCR reagents (Rochelle *et al.*, 1992b; Webster *et al.*, 2003) and discounted from results.

Template from negative DNA extraction controls were also examined by nested PCR amplification using universal 16S rRNA primers for both *Bacteria* and *Archaea*. Amplified products from DNA negative controls were screened by DGGE and bands sequenced to identify contaminants. Contaminating sequences were discounted from results.

A series of template dilutions were used to optimize PCR reactions and to reduce inhibitory compounds e.g. humic acids, pigments and heavy metals (Rochelle *et al.*, 1992a; Webster *et al.*, 2003; Newberry *et al.*, 2004). The amplified products from undiluted, 1:10 and 1:100 diluted DNA templates were screened by DGGE (see section 2.3.4) to both assess the reproducibility of banding patterns and the relative diversity detected from different dilutions.

2.3.3 Amplification of the dissimilatory sulphite reductase (*dsrA*) gene

The *dsrA* gene, coding for the enzyme catalysing the terminal step in sulphate-reduction, was targeted as a proxy for the presence of SRB in the MV environment. Two different primer sets were used to amplify the *dsrA* gene from sediment DNA from the Meknes and Porto MVs: DSR-1F and DSR-4R (Wagner *et al.*, 1998), and DSR-1F+ and DSR-R (Kondo *et al.*, 2004). These primers yielded products of approximately 1900 bp and 220 bp, respectively. Primer details and PCR conditions used are given in Tables 2.5 and 2.6. Reaction mixtures (total volume, 49 µl) were prepared as described (section 2.3.2) except the volume of 50 mM $MgCl_2$ was doubled to 3 µl. Direct amplifications with both primer sets were conducted in addition to a nested approach in which 1 µl of the DSR-1F – DSR-4R primer products were re-amplified with the DSR-1F+ - DSR-R primers. Direct and nested products obtained with the DSR-1F+ - DSR-R primer set were screened by DGGE to assess *dsrA* gene diversity at each horizon (see section 2.3.5). Selected samples were also analysed by *dsrA* gene library construction, as described in sectin 2.3.7. Function gene clones were kindly plated out and amplified by a colleague.

2.3.4 Amplification of the methyl coenzyme M reductase (*mcrA*) gene

The *mcrA* gene, coding for the enzyme catalysing the terminal step in CH_4 production in the methanogenesis pathway was targeted as a proxy for the presence of methanogens and ANME organisms in the MV environment. Two different primer sets targeting the *mcrA* gene were used: the ME1 and ME2 primers (Hales *et al.*, 1996), and the LutonF and LutonR primers (Luton *et al.*, 2002), which yielded products of approximately 760 bp and 425 bp, respectively. Reaction mixtures (49 µl) were prepared as described (section 2.3.2). Primer details and PCR conditions are given in Table 2.5 and 2.6. Direct and nested products obtained with the LutonF – LutonR primer set were screened by DGGE to assess *mcrA* gene diversity (see section 2.3.5). Selected samples were also analysed by *mcrA* gene library construction, as described in sectin 2.3.7. Function gene clones were kindly plated out and amplified by a colleague.

Table 2.5 Primers used to amplify *dsrA* and *mcrA* gene sequences

Amplification target	Primer pair	Sequence 5'-3'[a]	Binding position[b]	Approximate product size	Reference
General α-subunit of methyl coenzyme M reductase (*mcrA*)	ME1 ME2	GCM ATG CAR ATH GGW ATG TC TCA TKG CRT AGT TDG GRT AGT	684-703 1424-1444	760 bp	Hales *et al.* (1996)
General α-subunit of methyl coenzyme M reductase (*mcrA*)	LutonF LutonR	GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC TTC ATT GCR TAG TTW GGR TAG TT	1015-1046 1480-1502	425 bp	Luton *et al.* (2002)
General α and β-subunits of dissimilatory sulphite reductase gene	DSR 1F DSR 4R	ACS CAC TGG AAG CAC G GTG TAG CAG TTA CCG CA	421-436 2347-2363	1,900 bp	Wagner *et al.* (1998)
General α-subunit of dissimilatory sulphite reductase gene	DSR 1F+ DSR R	ACG CAC TGG AAG CAC GGC GG GTG GMR CCG TGC AKR TTG G	421-440 623-641	220 bp	Kondo *et al.* (2004)

[a] D = G or A or T, H = A or T or C, K = G or T, M = A or C, R = A or G, W = A or T, Y = C or T.
[b] *McrA* genes correspond to *Methanosarcina barkeri* (Accession no. Y00158) numbering, and *Methanothermobacter marburgensis* numbering (Lueders *et al.*, 2001) respectively. *Dsr* genes correspond to *Desulfovibrio vulgaris* (DSM644^T) numbering

Table 2.6. PCR conditions used with *dsrA* and *mcrA* gene primers

Primer pair	Initial denaturation step	Denaturing, annealing and extension cycle		Final elongation step	Reference
ME1 – ME2	94°C for 5 min	92°C for 45 s 50°C for 45 s 72°C for 1 min 45 s	x 35	72°C for 5 min	Newberry *et al.* (2004)
LutonF – LutonR[a]	94°C for 5 min	94°C for 60 s 52°C for 60 s 72°C for 1 min 30 s (Ramp to 72°C at 0.1°C s^{-1})	x 10	72°C for 6 min	Luton *et al.* (2002)
		94°C for 60 s 52°C for 60 s 72°C for 1 min 30 s	x 25		
DSR 1F – DSR 4R	95°C for 2 min	94°C for 30 s 54°C for 45 s 72°C for 90 s	x 35	72°C for 7 min	Wagner *et al.* (1998)
DSR 1F+ – DRS R	94°C for 1 min	94°C for 30 s 60°C for 30 s 72°C for 60 s	x 30	72°C for 7 min	Kondo *et al.* (2004)

[a] Primers are degenerate, ramp in temperature between annealing temperature and extension temperature slowed to 0.1°C s^{-1} for first 10 cycles to allow extension of mismatched primers

2.3.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE screening was performed using the D-Code™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) with 1 mm-thick (16 x 16 cm glass plates). PCR products (ca. 200 ng) were separated on 8% (w/v) polyacrylamide gels (Acrylogel 2.6 solution, acrylamide: N,N'-methylenebisacrylamide; 37:1; BDH Laboratory Supplies, Poole, UK) with a denaturant gradient between 30% and 60% (16S rRNA gene fragments), 35% and 50% (*mcrA* gene fragments) or 35% and 65% (*dsrA* gene fragments). One hundred per cent denaturing conditions are defined as 7 mol l^{-1} urea and 40% (v/v) formamide. Gels were poured with the aid of a 50 ml volume Gradient Mixer (Fisher Scientific, Loughborough, UK) and run in 1x TAE buffer (40 mM Tris acetate, 20 mM acetic acid and 0.5 M EDTA [pH 8.0]). Electrophoresis was conducted at 60°C and 80 V for the first 10 min then 200 V thereafter, for a total of for 5 h. The gels were stained with 1 µl ml^{-1} SYBR Gold nucleic acid stain (Molecular Probes, Leiden, The Netherlands) for 30 min and viewed under UV. Gel images were captured with a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

Individual bands were excised from DGGE gels with a sterile scalpel blade under UV light. Bands were placed in sterile PCR tubes and twice washed for 10 min in 100 µl molecular-grade water. After washing, the bands were allowed to air-dry in a laminar flow cabinet, crushed and re-suspended in 10-15 µl of sterile distilled water, depending on the band's intensity. DNA was left to elute at 4°C for at least 1 h before freezing at –20°C. The thawed supernatant was used as template for re-amplification by PCR using the original DGGE PCR primers, unless otherwise stated. If the recovery of a band was problematical, for example due to potential contamination from adjacent bright bands in the same lane, the PCR product was re-analysed by DGGE to confirm recovery of the target band and to check purity of the product. Where re-analysis showed products not to be pure a second excision and re-amplification of the target band was conducted before sequencing.

2.3.6 Sequencing of DGGE fragments

Re-amplified band products were de-salted by dialysis with molecular grade water using Microcon YM-50 centrifugal filter devices (Millipore Corporation, Billerica, MA) then sequenced. In the case of the bacterial and archaeal 16S rRNA genes, sequencing was conducted with either the 518R (*Bacteria*) or PARCH (*Archaea*) primers (Table 2.3), though later, following the development of an improved DGGE band sequencing method, the M13F primer was used (see below). The improved method involved first re-amplifying bands with a modified version of the original DGGE primers. These modified primers consisted of the appropriate DGGE primer linked to either the M13F or M13R primer via either a G-C or A-T region [357F or SAf primer with 5' CAG GAA ACA GCT ATG ACG GGC GGG GCG GGG GCA CGG GGG G 3' at the 5' end; and 518R or PARCH with 5' GTA AAA CGA CGG CCA GTA AAT AAA ATA AAA ATG TAA AAA AA 3' at the 5' end]. Sequencing these products with the unmodified M13F primer [5' GTA AAA CGA CGG CCA GT 3'] allowed the ends of the relatively short DGGE band sequences to be fully sequenced. (O'Sullivan *et al.*, 2008) Functional gene products, *dsrA* and *mcrA*, were sequenced with the DSRA 1F+ or LutonF primers, respectively (Table 2.5).

Sequences were obtained courtesy of the Cardiff School of Biosciences Molecular Biology Support Unit using an ABI 3100 Prism Genetic Analyzer automated capillary sequencer (Applied Biosystems, Forster City, CA) using BigDye terminator chemistry (Version 3.1). The machine was later updated (Oct '06) to an ABI 3130xl model.

2.3.7 Cloning, sequencing and phylogenetic analysis

Gene libraries were constructed from near full-length bacterial and archaeal 16S rRNA gene sequences amplified from MV sediments. These products from 5 replicate PCR reactions were re-amplified with partial-length primers and screened by DGGE to assess reproducibility and diversity, as recommended by Webster *et. al.* (2003). The full-length amplicons were pooled and ligated with pGEM-T Easy vector (Promega Corporation, Madison, WI) at 4°C overnight using optimised insert:vector ratios, according to the manufacturer's instructions. Transformation reactions were carried out with heat shock of alpha-select competent cells of *Escherichia coli* (Golf Efficiency, DH5alpha related

strain, Bioline) and plated on Luria Bertani (LB) agarose media containing ampicillin (80 μg ml^{-1}). Recombinant transformants were selected by blue-white screening. Clones were amplified by PCR with M13 primers (section 2.3.6) and the product size checked against the HyperLadder I (Bioline) DNA marker to confirm the presence of inserts of the expected size.

Randomly selected clones were screened by DGGE initially to assess species richness and to guide the level of sequencing required to adequately capture the diversity. Screened clones were initially sequenced with the 27F or 109F primer (Table 2.3). The partial sequences were grouped into operational taxonomic units (OTU) with the BLASTClust programme (http://www.ncbi.nlm.nih.gov/BLAST) using a 97% similarity threshold. Thresholds of 95% and 99% were also trialled in the analysis but made little difference to the taxonomic groupings, (see section 3.2.2). Sequences were analysed by the phylotype-richness estimators recommended by Kemp & Aller (2004) to ensure the gene libraries sufficiently represented the diversity in the source environment. Representative clones from each of the identified OTUs were additionally sequenced with the 907R and 1492R (bacteria), or 958R (archaea) primer. Near full-length (~1500 bp) sequences were assembled from the partial sequences using the Phrap programme (P. Green, unpublished data) after base calling by Phred (Ewing & Green, 1998a; 1998b). The assembled sequences were manually scrutinized in Consed (Gordon *et al.*, 1998) before use to ensure accuracy of the assemblies. The bacterial 16S rRNA sequence library was checked for chimeras using the Mallard software package (Ashelford *et al.*, 2006, available at: www.cardiff.ac.uk/biosi/research/biosoft/) but no chimeras or otherwise anomalous sequences were detected. This was indicated by the absence of outliers in the generated deviation from expectation (DE) plot (Fig 2.4).

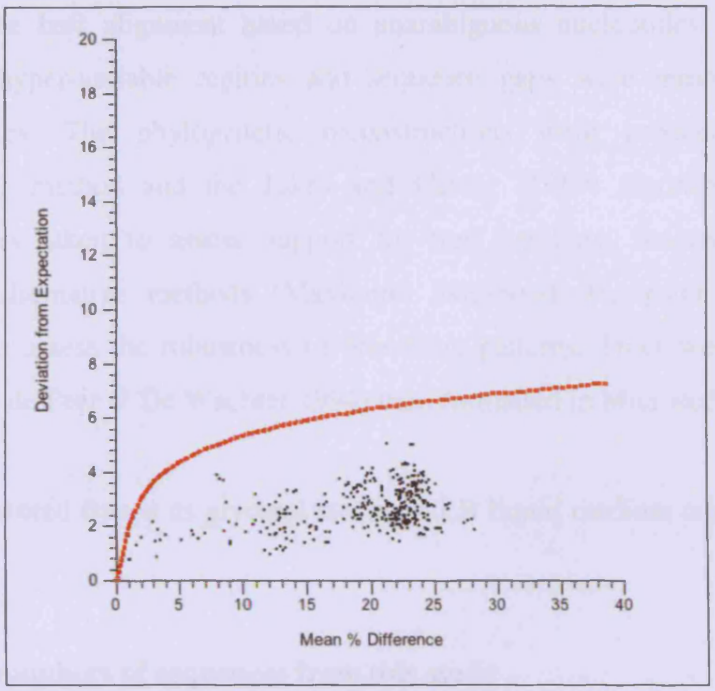


Fig 2.4 Deviation from expectation (DE) plot generated from an alignment of the Capt A bacterial 16S rRNA gene library sequences and selected sequences from related type species. Each plotted DE value represents a separate sequence comparison using the Pintail algorithm (Ashelford *et al.*, 2005). The dotted line represents the 100% cutoff line used for identifying anomalous sequences with unusually high DE values. *Escherichia coli*^{T} X80725 was used as the reference sequence.

Representative archaeal sequences (n = 4) were checked for anomalies with the Pintail programme (Ashelford *et al.*, 2005, available at: http://www.cardiff.ac.uk/biosi/research/biosoft/), as it is more appropriate for smaller datasets. The type strain sequences of *Methanosarcina barkeri* (AJ012094) and *Desulfurococcus fermentans* (AY264344) were used for querying *Euryarchaeota* and *Crenarchaeota* clones, respectively. Low Deviation from Expectation (DE) values were reported for each of the comparisons indicating that percentage differences between query and subject sequences were roughly equivalent to evolutionary difference and not due to sequence anomalies, data not shown.

Closest relatives of each OTU were identified by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and representative related sequences downloaded for construction of phylogenetic reconstructions. Related type strain sequences were retrieved where possible to provide a general evolutionary context to clones and their near relatives. Sequence alignments were constructed in ClustalX

(Thompson *et al.*, 1997), manually edited in BioEdit (Hall, 1999) then re-aligned in ClustalX until the best alignment based on unambiguous nucleotides was achieved. During editing hyper-variable regions and sequence gaps were removed from the aligned sequences. The phylogenetic reconstructions were produced using the neighbour-joining method and the Jukes and Cantor (1969) algorithm with 1000 bootstrap samples taken to assess support for tree topology. Selected trees were reproduced by alternative methods (Maximum likelihood, Dempster *et al.*, 1977; Kimura, 1980) to assess the robustness of branching patterns. Trees were rendered in TREECON (Van de Peer & De Wachter 1994) then formatted in Microsoft PowerPoint.

All clones were stored frozen as glycerol stocks in LB liquid medium containing 80 μg ml^{-1} ampicillin.

2.3.8 Accession numbers of sequences from this study

FN397806 – FN397820	Bacterial 16S rRNA clones from Capt. A MV sediment gene library
FN397889 – FN397892	Archaeal 16S rRNA clones from Capt. A MV sediment gene library
FN397893 – FN397902	Bacterial 16S rRNA gene sequences from Capt. A and Bonjardim MV pure culture isolates
FN547389 – FN547396	Archaeal 16S rRNA sediment DGGE band sequenes from Meknes and Porto MVs
FN547397 – FN547399	Bacterial 16S rRNA sediment DGGE band sequences from Meknes and Porto MVs
FN547400 – FN547405	Archaeal 16S rRNA enrichment DGGE band sequences from Meknes MV sample 580A enrichments
FN548082 – FN548096	Bacterial 16S rRNA enrichment DGGE band sequences from Meknes MV sample 580A enrichments
FN553435 – FN553439	*McrA* gene sequences from Meknes and Porto sediment gene libraries
FN554544 – FN554551	*McrA* sediment DGGE band gene sequences from Meknes and Porto MVs
FN554552 – FN554555	*DsrA* sediment DGGE band gene sequences from Meknes and Porto MVs
FN554556 – FN554564	*DsrA* gene sequences from Meknes and Porto sediment gene libraries
FN554565 – FN554567	*DsrA* enrichment DGGE band sequences from Meknes MV sample 580A enrichments
FN554568 – FN554572	*McrA* enrichment DGGE band sequences from Meknes MV sample 580A enrichments

2.4 Cultivation methods

2.4.1 Preparation of MV enrichments

Several types of anaerobic media were prepared to specifically target different functional groups of interest. The following groups were targeted: hydrocarbon-degrading heterotrophic SRB (Captain Arutyunov and Bonjardim samples only), general heterotrophic SRB, autotrophic SRB, heterotrophic methanogens, autotrophic methanogens, heterotrophic acetogens and autotrophic acetogens. An additional general enrichment medium containing 0.5 g l^{-1} yeast extract as the substrate was included for the Meknes and Porto MV samples to promote growth. All media were made in 2 litre batches inside anaerobic media vessels. To prepare the media, basal mineral salts and all heat-stable additions were dissolved in reverse osmosis water and autoclaved for 45 min at 121°C. Media were then removed whilst hot from the autoclave and cooled under N_2:CO_2, unless otherwise stated. Once cool, sterile heat-sensitive additions (e.g. vitamins, acetate and carbonate buffer) were added and the pH adjusted to approximately pH 7.2 (unless otherwise stated) with sterile 1 M HCl or sodium bicarbonate. All media were reduced with sterile 1 M sodium sulphide (1.5 mM final concentration). The redox-sensitive colour indicator resazurin was added to all media (1 mg l^{-1}) as a guide to Eh; this indicator undergoes a reversible change from the pink coloured compound resorufin, to the colourless compound dihydroresorufin at Eh below -110 mV and hence can be used to verify oxygen-free conditions (Tratnyek *et al.*, 2001).

Enrichments with Captain Arutyunov and Bonjardim MV sediment were prepared in sterile 7 ml glass vials. These media were dispensed aseptically inside a gassing hood under filtered N_2:CO_2 (Fig 2.5). A gassing jet was used to briefly flush the headspace of the vials before they were closed with sterile butyl rubbers stoppers and crimp-sealed prior to inoculation.

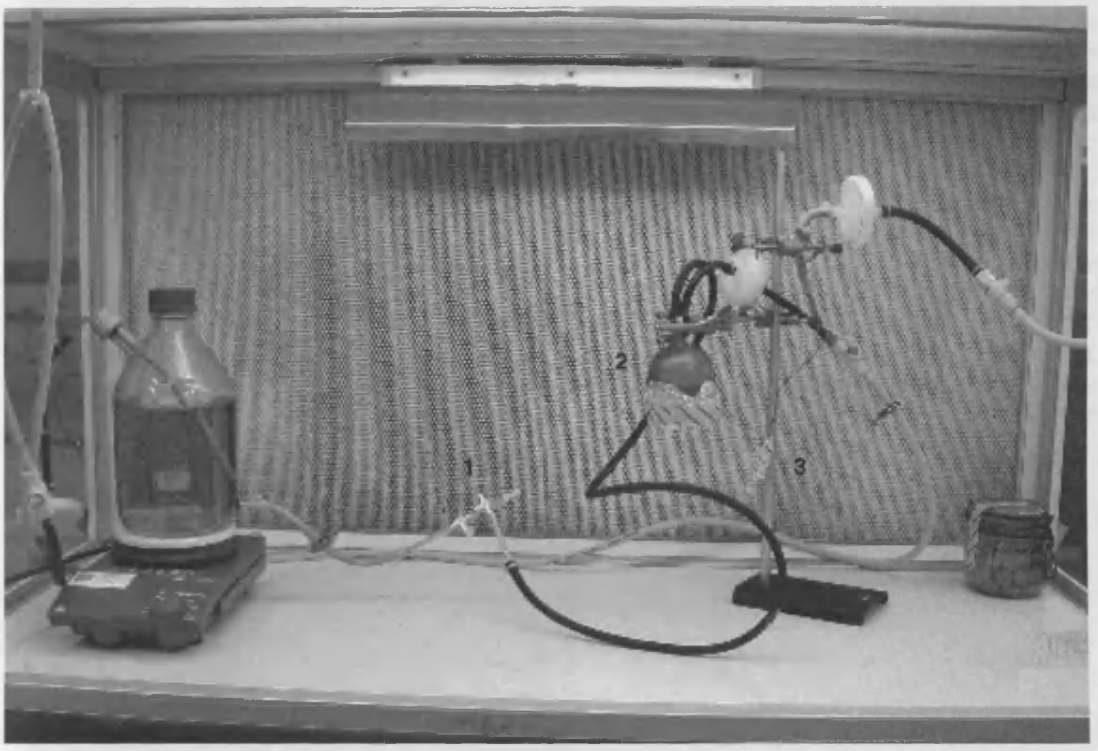


Fig 2.5 Apparatus used for dispensing Captain Arutyunov and Bonjardim MV enrichment media. 1, 3-way taps used to control flow of media into the gassing hood; 2, gassing hood used to maintain a clean oxygen free atmosphere for media dispensing; 3, gassing jet used to flush headspace of enrichment vials before sealing.

Enrichments with Meknes and Porto MV sediment samples were prepared under N_2/CO_2 in sterile 100 ml screw cap glass bottles (all heterotrophic media) or under H_2/CO_2 in sterile 15 ml Hungate tubes (autotrophic media). The bottled enrichments were completely filled to prevent oxidation by the headspace overtime, and tightly sealed with Teflon-sprayed screw caps. The Hungate tubes were filled two-thirds with media and sealed with thick butyl rubber stoppers (Bellco, Vineland, New Jersey, USA) under screw caps to preserve the anaerobic headspace. Bottle media were dispensed aseptically using a modified apparatus (Fig 2.6). Autotrophic media were dispensed using 10 ml pipettes with filters inside the anaerobic cabinet.

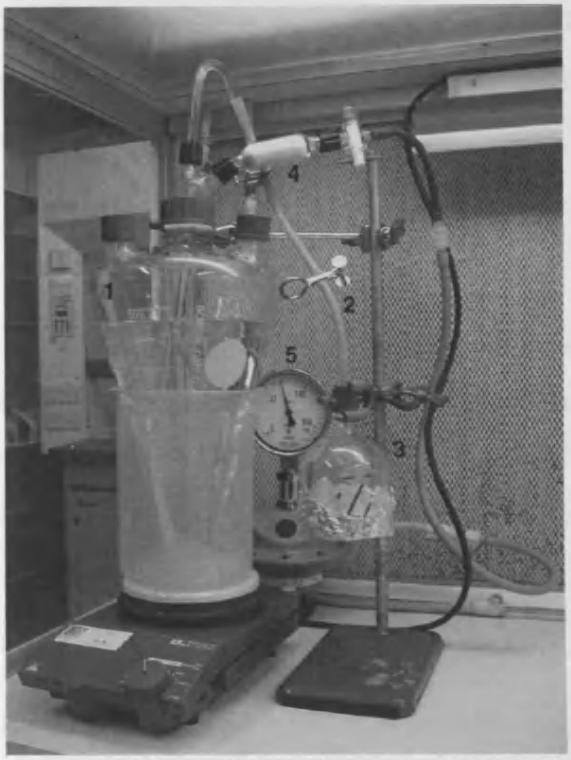


Fig 2.6 Apparatus used for dispensing the bottled Meknes and Porto enrichment media. 1, Modified glass vessel featuring a tapered bottom to preserve surface area of headspace gas to volume ratio, a gas inlet and a media outlet (through a rubber stopper in the centre), and two screw cap openings to allow gas venting and sterile additions to be made; 2, finger-controlled clamp used for fine control of media flow; 3, glass hood used to provide a clean environment in which to dispense media; 4, cotton wool plug to filter incoming gas; 5, pressure regulator (TESCOM) for controlling gas flow.

2.4.2 Enrichment media recipes

All media recipes were based on the bicarbonate buffered saltwater medium of Widdel & Bak (1992) and contained the following per litre: NaCl, 20 g (23 g added to non SRB media); Na_2SO_4, 4 g (omitted from non SRB media); $MgCl_2.6H_2O$, 3 g; KCl, 0.5 g; NH_4Cl, 0.25 g; KH_2PO_4, 0.2g; $CaCl_2.2H_2O$, 0.15 g; yeast extract, 0.1 g (added to provide cofactors and stimulate growth); 2.5 ml chelated trace element solution (see appendix I); 2.5 ml vitamin solution (see appendix I); 30 ml 1 M bicarbonate buffer. All media were injected with a 2.5% inoculum of 1:10 sediment slurry prepared in anoxic mineral salts. The various individual recipes were prepared as follows:

2.4.2.1 Hydrocarbon-degrading SRB medium

A 2% toluene stock solution was prepared in pharmaceutical-grade non-fluorescent mineral oil (Lamotte, Bremen, Germany) that had been autoclaved and cooled under N_2 before use. The toluene was sterilized by filtration using a glass vacuum pump filter unit and a sterile 0.2 µm cellulose acetate filter (Whatman). Sterile toluene was added to the mineral oil inside the anaerobic cabinet and stored in a sterile glass solvent bottle. Fifteen microliters of the stock solution was added to 5 ml of enrichment medium via a

glass syringe soaked overnight in ethanol. These vials were incubated upside down to prevent long-term contact of the hydrophobic, buoyant hydrocarbon with the butyl stoppers (Aeckersberg *et al.*, 1991).

2.4.2.2 Heterotrophic SRB medium

Sterile stock solutions of acetate and lactate (2 M) were prepared and stored as individual aliquots. Lactate was added (2.5 ml l^{-1}) to the medium before autoclaving to give a final concentration of 5 mM. The volatile acetate solution was added (7.5 ml l^{-1}) aseptically to cool sterile medium to give a final concentration of 15 mM.

2.4.2.3 Autotrophic SRB medium

The medium was prepared as described (section 2.4.1 and 2.4.2) except H_2:CO_2 (80:20) gas was used to cool and dispense the media. A 100 kPa (1 bar) over pressure of filtered H_2:CO_2 was injected into the vials following inoculation using a gas regulator (TESCOM).

2.4.2.4 Heterotrophic methanogen medium

The medium was prepared as described with the omission of sulphate from the mineral salts. Sterile 2 M acetate solution was added to the cooled medium as the sole substrate at a final concentration of 15 mM.

2.4.2.5 Autotrophic methanogen medium

The medium was prepared as described for autotrophic SRB (section 2.4.2.3) except sulphate was omitted from the mineral salts.

2.4.2.6 Heterotrophic acetogen medium

The medium was prepared as described (section 2.4.1 and 2.4.2) but with the inclusion of 6.6 g of 2-bromoethanesulfonic acid (30 mM final concentration) in the basal medium to inhibit growth of methanogens (Oremland & Capone 1988). Ethanol was used as the

sole substrate and was added from a sterile 1 M stock solution to give a final concentration of 15 mM. The pH of the media was lowered to ~6.3 with sterile 1 M HCl as this was found to be more selective for acetogens (Schink, 1994).

2.4.2.7 Autotrophic acetogen medium

The medium was prepared as described (section 2.4.2.6) without sulphate in the basal salts The media was cooled and dispensed under H_2:CO_2 (80:20) and a 100 kPa (1 bar) overpressure of filtered H_2:CO_2 was injected to vials after inoculation, using a gas regulator (TESCOM).

2.4.2.8 Yeast extract-containing general enrichment medium

This media was prepared as described (section 2.4.1 and 2.4.2) and contained 0.5 g l^{-1} yeast extract, added from a twice-filtered sterile (0.2 µm) 10% stock solution. This media was only used for Meknes and Porto MV enrichments.

2.4.3 Incubation of enrichments

The Capt. Arutyunov and Bonjardim enrichments were inoculated with 0.5 ml of 10% sediment slurry and incubated at incremental steps of approx 5°C intervals inside purpose-built temperature gradient systems from 0°C to ~120°C (Fig 2.7).

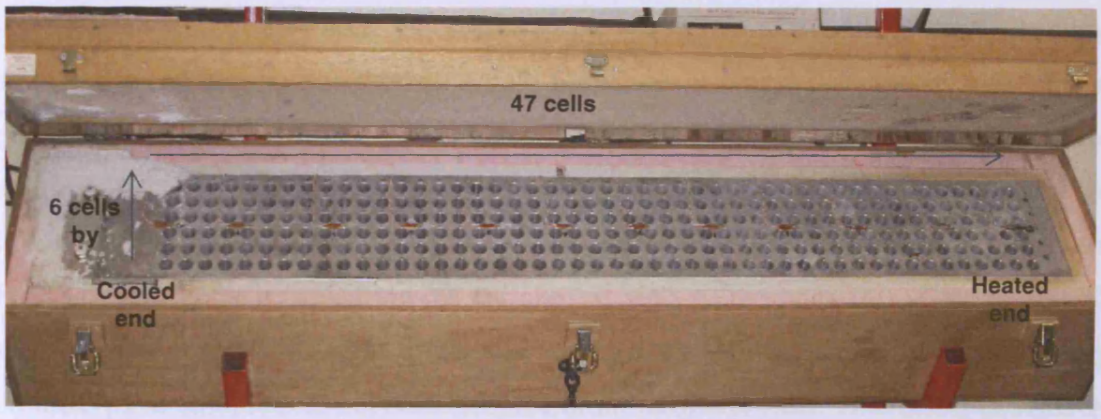


Fig 2.7 Example of a temperature gradient system used for incubating enrichments from the Capt. Arutyunov and Bonjardim MVs. Based on the design of Jørgensen *et al.* (1992).

Temperature gradient systems were aluminium blocks that had hollow cells drilled into them sufficient for incubating 2 x 7 ml glass vials. They were heated at one end with heating rods, and cooled at the opposite end with cooling channels regulated by a circulating chiller unit (Huber, Offenburg, Germany). The desired temperature gradient for the system was achieved by adjusting the degree of heating. A safety cut out system was fitted to prevent over-heating and loss of samples. The incubation blocks were cased in wood and insulated with foam. The temperature was recorded at twelve points along the gradient with fixed temperature sensors and the temperature of individual incubation cells calculated. The systems were manufactured in the Department of Earth Sciences workshops, Bristol University.

The Meknes and Porto MV heterotrophic enrichments were incubated in the dark at 25°C and 7°C. The autotrophic enrichments were incubated at 25°C. The number of incubation temperatures used was fewer than for Capt. Arutyunov and Bonjardim MVs (section 2.4.4) because more samples (8 instead of 2) were studied. The low incubation temperature (7°C) was chosen to represent close to *in situ* conditions. The higher incubation temperature was chosen to promote faster growth and also as an alternative approach to enriching MV (potentially sub-surface adapted) microorganisms.

2.4.4 Monitoring enrichments

Capt. Arutyunov and Bonjardim enrichments were incubated for 4 months and routinely visually assessed for growth. However, visual assessment alone was often found to be inconclusive and so epifluorescent microscopic assessments were also done. Because assessing every vial by this method would have been too time consuming, only one in every three incubation temperatures were examined by microscopy. The approximate temperatures of the enrichments assessed by epifluorescence microscopy were as follows: 5, 30, 58 and 82°C. Duplicates from the selected temperatures were assessed. Prior to microscopic examination the enrichment vials were vortex mixed for at least 30 sec to disperse any cell aggregations and dislodge sediment-attached cells. Depending on the type of enrichment, i.e. heterotrophic or autotrophic, either filtered N_2 or H_2:CO_2 (80:20) was used for sampling. The sterile syringe and needle were flushed with the appropriate gas three times before injection into the enrichment. The needle was injected at a shallow angle through the thicker outer part of the butyl stopper in order to minimise

permanent puncturing of the seal. It was found this was necessary in order to prevent oxygen leaking into the anaerobic enrichment over time. A 50 µl sub-sample was removed from each enrichment vial, fixed in 10 ml of 2% formaldehyde (marine salinity), and stained with 50 µl acridine orange (1 g l^{-1}). It was not necessary to count the cells present in the sub-samples at this stage but a semi-quantitative assessment of cell concentrations was made that could be used to decide whether isolation attempts were justified. Isolation attempts were made from enrichment vials that contained 5 or more cells in the majority of fields of view. Each slide was observed for 5 min.

The progress of the Meknes and Porto MV enrichments was made by visual assessment every few weeks during which time any signs of positive growth (e.g. turbidity, blackening of sediment) were noted. Enrichments with a visual indication of growth were further analysed by phase contrast microscopy. Microscopy was conducted under a light microscope (Axioskop, Zeiss, Germany) equipped with a x100 magnification phase-contrast oil immersion objective and x10 eyepiece. Microscope slides were prepared by placing a small amount of liquid enrichment under a cover slip using a flamed inoculating loop. Enrichments that showed a significant number of cells (typically > 5 per FOV) were selected for sub-culturing.

2.4.5 Isolation of MV microorganisms

Isolation attempts were made from Capt. Arutyunov and Bonjardim MV enrichments selected on the basis of microscopy results (section 2.4.4). A richer medium containing commonly used bacterial substrates (yeast, peptone, glucose and lactate) was used for the isolation attempts because growth even in the positive enrichments was still relatively weak (see section 3.3.1). The marine YPG medium described by Süß *et al.*, (2004) was selected as it had previously been used in the successful cultivation of a broad range of sub-surface microorganisms (Süß *et al.*, 2004; Köpke *et al.*, 2005; Batzke *et al.*, 2007). The isolation attempts were made under both aerobic and anaerobic conditions as a further way of maximising the diversity of the isolates, and because it has previously been shown that many culturable sub-surface bacteria can in fact grow under aerobic conditions (Toffin *et al.*, 2004; Süß *et al.*, 2004; Köpke *et al.*, 2005; Batzke *et al.*, 2007). The YPG medium was made up in 1 litre Duran bottles and contained the following per litre: 24.32 g NaCl; 10 g $MgCl_2.6H_2O$; 1.5 g $CaCl_2.2H_2O$;

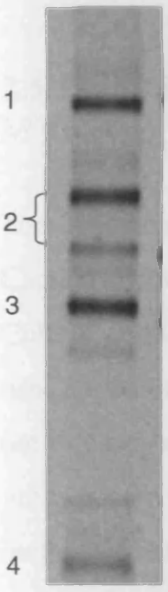
0.66 g KCl; 4 g Na_2SO_4; 1 ml SL 10 trace element solution (Widdel *et al.*, 1983), 0.2 ml selenite tungstate solution (Widdel & Bak, 1992), 0.03 g yeast extract; 0.06 g peptone; 5 ml 1 M sodium lactate and 2.38 g HEPES buffer. One ml of the following stock solutions were also added: 0.84 M KBr; 0.4 M H_3BO_3; 0.15 M $SrCl_2$; 0.4 M NH_4Cl; 0.04 M KH_2PO_4 and 0.07 M NaF. The pH was adjusted to 7.2 – 7.4 using 4 M NaOH before sterilization at 121°C for 45 min inside an autoclave. The following sterile additions were made to the medium once cool: sodium carbonate solution (final concentration 2.4 mM), 2 ml 5x concentrated vitamin solution (Balch *et al.*, 1979); 1.2 ml 1 M (0.2 µm filter-sterilized) glucose solution and 1 ml 1 M sodium thiosulphate. It was not necessary to adjust the pH of the completed medium. Aerobic plates were prepared by re-heating the medium to ~50°C in a water bath and adding (4%) sterile molten agar (Oxoid, Basingstoke). Unspecified substrates and any inhibitory compounds were removed from the agar by thoroughly washing it with reverse osmosis water prior to autoclaving. Aerobic plates were poured inside a clean laminar flow cabinet and allowed to cool overnight. Anaerobic plates were prepared from media reduced with Na_2S (1 mM final concentration) that had been cooled under filtered nitrogen. The sterile additions and molten agar were added to re-heated medium inside a clean anaerobic cabinet and then the plates poured. All sub-cultures were incubated at close to their original enrichment temperature. Anaerobic plates were streaked inside the anaerobic cabinet using sterile single-use plastic inoculation loops, then wrapped with Nescofilm (Alfresa Pharma Corporation, Osaka, Japan) and placed inside anaerobic jars or bags with an activated Anaerocult® (Merck, Darmstadt, Germany) catalyst according to the manufacturer's instructions. Anaerotest® indicators strips (Merck, Darmstadt, Germany) were used to monitor the anaerobic atmosphere of each jar/bag, and all were shown to remain anaerobic throughout their use. Contamination controls were routinely conducted when working inside the anaerobic cabinet by leaving a plate open for several minutes whilst sub-culturing. These plates were incubated for several months, but no contaminant was ever detected.

Colonies growing on solid aerobic media were purified by repeated sub-culturing. After a minimum of three successful re-streaks of a single colony type, purity was checked by phase contrast microscopy. Once purity was supported by microscopy, isolates were grown up in liquid YPG medium and samples taken for DNA extraction. Colonies

growing on solid anaerobic YPG media were also purified by single colony isolation over at least three successive stages. The 16S rRNA gene sequences of the anaerobic isolates were amplified (section 2.3.2) directly from colony material, which was transferred to sterile PCR tubes using autoclaved wooden cocktail sticks, inside the anaerobic cabinet.

Sub-cultures were made from positive Meknes and Porto MV enrichments by transferring between 5 and 1% inoculum (depending on cell density and growth rate), into fresh bottles of the same liquid media. After several successful transfers, some isolation attempts were made on solid versions of the same media. However, due to time constraints pure culture isolates were not pursued from the Meknes and Porto MV enrichments.

2.4.6 DGGE screening of Capt. Arutyunov and Bonjardim MV isolates and preparation of a standard DGGE marker



DGGE screening (section 2.3.5) was used to verify the purity of Capt. Arutyunov and Bonjardim isolates and assign them to different operational taxonomic units (OTUs). Partial length bacterial 16S rRNA genes were amplified from pure cultures with distinct colony morphologies and combined to produce a 'standard' DGGE marker (Fig 2.8). This marker was used to compare against all the subsequently screened isolates. The DGGE marker was also used in subsequent molecular genetic work.

Fig 2.8. Standard DGGE marker produced from isolated MV organisms. The DGGE marker contained combined partial 16S rRNA gene fragments from the following isolated organisms: *Pseudomonas stutzeri*, band 1; *Marinobacter*, band 2 (double operon); *Arcobacter*, band 3 and *Halomonas*, band 4.

2.4.7 16S rRNA gene sequence identification of enriched and isolated MV organisms

DNA was extracted from approximately 1 ml of liquid enrichment or liquid pure cultures and spun down for 5 min at 16,100 g in microfuge tubes. The supernatant was discarded and DNA extracted from the cell pellet by the freeze-thaw method. The cell pellet was re-suspended by pipette action in 50 µl of sterile 5% Chelex solution (Bio-

Rad). The tubes were boiled in a water bath for 5 min then immediately quenched on ice for a further 5 min. The process was repeated then the tubes were centrifuged for 1 min at 16,100 g to remove the cell debris from the DNA-containing supernatant.

One microliter of DNA was amplified by PCR with general bacterial 16S rRNA gene primers as described (section 2.3.2). Primers 357F and 518R (Muyzer *et al.*, 1993) were used for DGGE analysis, and primers 27F and 1492R (Lane, 1991) were used for sequencing. DNA from Meknes and Porto enrichment cultures was also amplified with general archaeal primers to screen for the presence of *Archaea*. Primers SAf (Nicol *et al.*, 2003) – PARCH (Øvreas *et al.*, 1997) were used for DGGE analysis, and primers 109F (Grosskopf *et al.*, 1998) – 958R (DeLong, 1992) were used for sequencing where appropriate. All primer details are given in Tables 2.3 and 2.4.

The 16S rRNA gene products from enrichment and colony PCR reactions were screened by DGGE and bands sequenced as described (section 2.3.5).

2.5 Preliminary phenotypic characterisation of organisms isolated from MVs

Ten representative strains of the microorganisms isolated in pure culture from the Captain Arutyunov and Bonjardim MVs were selected for further characterisation. Culture collections were not fully developed from the Meknes and Porto MVs due to time constraints. Eight of the ten selected strains grew well under aerobic conditions and are referred to as 'aerotolerant strains'. The two other strains were more fastidious and grew poorly or not at all in the presence of oxygen, because of this characterisation methods used sometimes varied.

2.5.1 Electron donor tests

The utilization of 54 potential organic substrates was tested with all ten strains under aerobic conditions in 96-well microtitre plates (N.B. two of the substrates, glutamate and proline, became contaminated and were disregarded from the data). Individual substrates were selected to cover a broad range of substrate types that may be present in the marine environment (complex organics, polysaccharides, disaccharides, monosaccharides and

their derivates, amino acids, organic acids, alcohols, aromatic and heterocyclic compounds; see Table 2.7).

Table 2.7. Organic substrates tested for growth with selected MV isolates

Substrate class	Compound [formula]	Conc. (mM)
Alcohols	Methanol [CH_4O]	5
	Ethanol [C_2H_6O]	10
	Propanol [C_3H_8O]	5
	Butanol [$C_4H_{10}O$]	5
Unsaturated diol	Ethylene glycol [$C_2H_6O_2$]	5
Saturated triol	Glycerol [$C_3H_8O_3$]	5
Sugar alcohol (polyol)	Mannitol [$C_6H_8(OH)_6$)	5
Aromatic aldehyde (+ OH group)	Vanillin [$C_8H_8O_3$]	1
Aromatic aldehyde	pOH-Benzaldehyde [C_7H_6O]	1
Aromatic organic acid	Benzoate [$C_7H_5O_2$-]	1
	3,4,5-Trimethoxybenzoate [$C_{10}H_{12}O_5$]	1
Organic (fatty) acid	Acetate [$C_2H_3O_2^-$]	10
	Lactate [$C_3H_6O_3$]	10
	Formate [CH_2O_2]	5
	Propionate [$C_3H_6O_2$]	5
	Butyrate [$C_4H_8O_2$]	2.5
	Valerate [$C_5H_{10}O_2$]	2.5
	Hexanoate [$C_6H_{12}O_2$]	2.5
	Octanoate [$C_8H_{16}O_2$]	2.5
	Gluconate [$C_6H_{12}O_7$]	5
	Salicylate [$C_7H_6O_3$]	1
Dicarboxylic acid	Malonate [$C_3H_4O_4$]	5
	Succinate [$C_4H_6O_4$]	5
	Fumarate [$C_4H_4O_4$]	5
	Malate [$C_4H_6O_5$]	5
	Tartrate [$C_4H_6O_6$]	2.5
Tricarboxylic acid	Citrate [$C_6H_8O_7$]	2.5
Carboxylic amino acid	Glutamate [$C_5H_9NO_4$]	5
Amino acids	Alanine [$C_3H_7NO_2$]	10
	Arginine [$C_6H_{14}N_4O_2$]	5

Table 2.7 cont. Organic substrates tested for growth with selected MV isolates

Substrate class	Compound [formula]	Conc. (mM)
Amino acids	Cysteine [$C_3H_7NO_2S$]	5
	Isoleucine [$C_6H_{13}NO_2$]	5
	Proline [$C_5H_9NO_2$]	2
	Serine [$C_3H_7NO_3$]	10
	Phenylalanine [$C_9H_{11}NO_2$]	2
Amino acid mixture	Caseine hydrolysate	0.05%
Amino sugar	Glucosamine [$C_6H_{14}NO_5$]	5
Monosaccharide	Glucose [$C_6H_{12}O_6$]	5
	Fructose [$C_6H_{12}O_6$]	5
	Arabinose [$C_5H_{10}O_5$]	5
	Rhamnose [$C_6H_{12}O_5$]	5
	Mannose [$C_6H_{12}O_6$]	5
	Xylose [$C_5H_{10}O_5$]	5
Disaccharide	Trehalose [$C_{12}H_{22}O_{11}$]	2.5
	Sucrose [$C_{12}H_{22}O_{11}$]	2.5
	Maltose [$C_{13}H_{24}O_{11}$]	2.5
	Cellobiose [$C_{12}H_{22}O_{11}$]	2.5
Polysaccharide	Cellulose [$(C_6H_{10}O_5)_n$]	0.05%
	Starch [$(C_6H_{10}O_5)_n$]	0.1%
	Chitin [$(C_8H_{13}NO_5)_n$]	0.05%
	Xylan	0.05%
	Laminarin [$C_{12}H_{22}O_{11}(C_6H_{10}O_5)_n$]	0.05%
Amines	Methylamine [CH_3NH_2]	10
	Choline [$(CH_3)_3N^+CH_2CH_2OHX^-$]	10
Alcohol amine	Ethanolamine [C_2H_7NO]	10
Peptides	Peptone	0.05%

Stock solutions of each substrate were prepared at appropriate concentration from high-grade chemicals and sterilized by autoclaving or filtration (0.2 μm pore size). Twenty microliters were added aseptically to 160 μl of HEPES-buffered mineral salts (section 2.1.1) in 96-well microtitre plates prior to inoculation. Plates were inoculated with 20 μl of twice washed cells re-suspended in substrate-free mineral salts. Two substrate-free

controls were included for each of the strains to quantify 'background growth' (i.e. growth on internal storage compounds within the inoculum cells) and an inoculum-free, substrate only well included for contamination control. All strains were tested on the various substrates in replicate. Plates were incubated at 10°C for 6 weeks. Growth was assessed by fluorescent nucleic acid staining and measured on a fluorescence microtitre plate reader (Martens-Habbena & Sass, 2006). Twenty five microliters of SYBR Green I (Molecular Probes, Leiden, The Netherlands) working solution (1000-fold dilution of stock solution in filter-sterilized TE buffer; 10 mM Tris-HCl, 1 mM EDTA, pH 8) was added to 100 µl of each substrate test in black 96-well microtitre plates and incubated overnight in the dark. The fluorescence was then measured on a plate reader (FluroCount™ with Xenon Fiber Optic light source, Packard) using a 485/530 nm excitation/emission wavelength. Plates were shaken for two seconds before reading. The read length was 0.5 s. When analysing the fluorescence data only substrates that produced fluorescent signals greater than four times the average background growth of the strain were considered positive. Wells measuring between two and four times the average background growth were scored as weak growth. Additionally, when the substrate itself was found to produce a fluorescent signal, this was first subtracted from the data before assigning growth scores. Growth scored qualitatively from the processed fluorescence data was verified against visual results to check for errors (Fig 2.9).

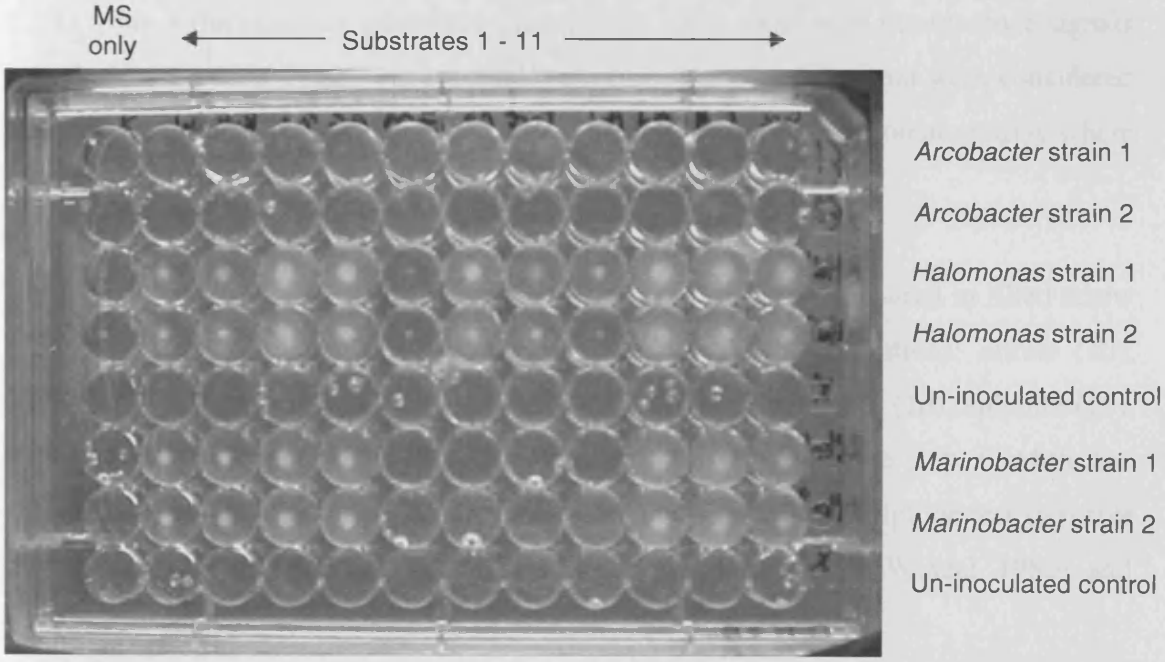


Fig 2.9 Example of an organic substrate assay prepared in a 96 well plate. Growth was assessed semi-quantitatively by fluorescent nucleic acid staining as described in text and compared to visual results for quality control. MS = Mineral salts.

Strains affiliated with *Arcobacter* sp. that did not grow well under aerobic conditions were also tested for growth on the various substrates (Table 2.7) under anaerobic conditions in filled screw-cap tubes containing anoxic mineral salts. In these tests nitrate (10 mM) was used as the electron acceptor.

2.5.2 Electron acceptor tests

Electron acceptor tests with the aerotolerant strains were set up in deep 96 well plates. The electron acceptors tested and their concentrations (mM) were as follows: nitrate (10), nitrite (3), Fe(III) hydroxide (~20), Mn(IV) dioxide (~20), sulphate (10), thiosulphate (10), dimethyl sulfoxide (DMSO) (10), trimethylamine N-oxide (TMAO) (10), the analogue for humics anthraquinone-2,6-disulphonate (AQDS) (5), selenate (5), fumarate (10). Acetate and lactate were included as electron donors at 5 mM concentration each. A control lane containing only the electron donors was also prepared. Wells were inoculated with 20 µl of 1:50 diluted culture, sealed with sterile lids (CAPMAT, Beckman, Fullerton, CA) and incubated at 25°C in gas-tight bags containing an oxygen-consuming catalyst (Anaerocult® A mini, Merck, Darmstadt, Germany) and an oxygen indicator strip (Anaerotest®, Merck, Darmstadt, Germany). Growth was assessed after 8 weeks visually and by fluorimetry as described (section 2.5.1) using a fluorescence microtitre plate reader. Only wells with fluorescence signals greater than 4x the average 'background' (electron donors only) signal were considered positive. Plate reader results were confirmed visually and by ion chromatography where necessary.

Electron acceptor tests with the *Arcobacter*-related strains were prepared in filled screw cap tubes. The following compounds were tested (mM concentration): nitrate (10), nitrite (2), Fe(III) hydroxide (~20), Mn(IV) dioxide (~20), sulphate (10), sulphite (15), thiosulphate (10), sulphur (10), DMSO (10), TMAO (10), fumarate (10). Growth was assessed after 3 months by visual inspection, by microscopy, the sulphide test (positive if a brown precipitate forms after the addition of acidified $CuSO_4$, Widdel, 1980), and ion chromatography, where necessary.

2.5.3 Fermentation tests

The fermentation of amino acids (0.05% caseine hydrolysate) and glucose (10 mM) was tested for each strain in filled tubes of mineral salts. The tubes were inoculated with 200 µl of culture and left to incubate for 6 weeks. Growth was assessed either visually or by microscopy when ambiguous.

2.5.4 Temperature range for growth

Growth at 4, 7, 10, 15, 25, 37, 42 and 45°C was tested with the aerotolerant strains on solid YPG marine medium, wrapped with Parafilm (Alcan packaging, WI, USA). All incubations lasted at least 6 weeks. The upper temperature range for growth was confirmed where necessary inside the temperature gradient system (see section 2.4.3). Liquid YPG medium was inoculated with dilute cultures and the development of turbidity noted. Negative results were confirmed by microscopy. Growth at 0°C was tested with the aerotolerant strains in aerobic liquid YPG medium (7.5% salinity) incubated at 0°C inside a temperature gradient system. Growth was determined visually and negative results confirmed by microscopy after 8 weeks.

The temperature range for growth of one of the two oxygen-sensitive (*Arcobacter*-related) strains was determined in liquid anoxic basal medium with 10 mM acetate and nitrate. These incubations were conducted in parallel inside a temperature gradient system from 5 – 54°C. As above, growth was determined with the plate reader method by comparing the fluorescence of the incubated cultures against that of frozen samples taken at the start (time zero) following inoculation (section 2.5.1). The temperature range for growth of the second oxygen-sensitive strain was not tested due to its later isolation and time constraints.

2.5.5 pH range for growth

The pH range for growth was tested in liquid marine YPG medium adjusted across a range of pH (4.5 – 9.5) using appropriate buffers (Table 2.8).

Table 2.8 Biological buffers used for pH tests with selected MV isolates

Buffer	pKa (25°C)	Effective pH range
Formate	3.75	3.0 – 4.5
MES	6.1	5.5 – 6.7
PIPES	6.76	6.1 – 7.5
HEPES	7.48	6.8 – 8.2
TRIS	8.06	7.5 – 9.0
Taurine	9.06	8.4 – 9.6

Two-thirds filled screw cap tubes were used with the aerotolerant strains, whereas completely filled screw cap tubes, plus 10 mM nitrate (electron acceptor), were used with the oxygen-sensitive strains. Each test contained a 5% inoculum and was incubated in replicate at 25°C for 4 weeks. Growth was assessed visually by noting the presence or absence of turbidity/cell aggregates and results were confirmed by microscopy where necessary.

2.5.6 Salinity range for growth

The salinity range for growth of the aerotolerant organisms was tested in 96-well plates using 1:100 diluted cultures as inoculum. YPG medium was prepared at three salinities: fresh, 1 g l^{-1} (salinities 0.1 - 3%); marine, 35.18 g l^{-1} (salinities 3.5 – 9.7%); and hypersaline, 100.06 g l^{-1} (salinities 10 – 25%) then amended with an appropriate amount of salt concentrate solution, 308.75 g l^{-1}, to achieve the desired salinities. Salinity tests were prepared in replicate, incubated at 25°C and checked for growth after 4 weeks. Un-inoculated contamination control wells were included on the plates. Growth at 0% salinity was tested in sterile MilliQ water with 2 mM glucose or 10 mM alanine as substrate. The salt transferred with the inoculum after two sub-cultures totalled 0.00175%. The salinity range of the oxygen-sensitive strains was tested by the same method but plates were incubated inside anaerobic bags. Growth of oxygen-sensitive strains was checked by microscopy and using the plate reader (section 2.5.1) after 4 weeks.

2.5.7 Microscopy

Cell morphology of the isolated strains was observed in exponential phase cultures by phase contrast microscopy with an Axioskop microscope (Zeiss, Germany) fitted with a 100x (numerical aperture = 1.32) Leitz Fluotar objective lens and 8x eyepieces. Images of selected FOV were taken using a Nikon Coolpix 4.0 megapixel digital camera pointed down the eyepiece. Each image was taken in duplicate (one including the 10 x 10 μm counting grid) so an accurate scale could be shown.

2.6 Further phenotypic tests with a selected novel isolate

One of the isolated organisms proposed to represent a new species in the genus *Arcobacter* (see section 5.5.5) was subjected to a more detailed characterisation. The additional methodologies conducted with this organism are given below. Two strains were characterised and tests were conducted with at least one of the two strains.

2.6.1 Disproportionation reactions

The ability of the two *Arcobacter*-related stains to grow by disproportionation was tested in filled screw cap tubes. Thiosulphate (10 mM) and sulphite (10 mM) disproportionation was tested and growth assessed after 3 months by the sulphide test using acidified $CuSO_4$ (5 mM $CuSO_4$ in 50 mM HCl) (Widdel, 1980).

2.6.2 Lipid analysis

Fatty acid composition was determined from crushed freeze-dried cell material by the Microbial Identification System protocol (MIDI; http://www.midi-inc.com). Lipids from the whole cell hydrolysate were saponified, methylated and extracted into the organic phase before analysis by gas chromatography (Model 6890N, Agilent Technologies).

An additional ultrasonic extraction was made using a modified Bligh-Dyer protocol (Rutters *et al.*, 2002) with a solvent mixture of methanol/dichloromethane/phosphate buffer (pH 7.4) in the proportions 2:1:0.8 (v/v). The supernatants of the first and following 5 re-extractions were collected in an opaque separation funnel. Dichloromethane and phosphate buffer were added to the extraction mixture to achieve a

ratio of 1:1:0.9 (v/v). After phase separation, the organic phase was drained and the aqueous phase re-extracted 4 times with dichloromethane. The combined extracts were evaporated to dryness in a heated aluminium block under N_2 then stored at –20°C. Total lipid extracts were fractionated into neutral lipids, free fatty acids (FFA) and polar lipids (including phospholipids) using an aminopropyl bond elut solid-phase extraction (SPE) cartridge with 100 mg sorbent mass and 1 ml capacity. After conditioning/pre-extracting of the cartridge with 12 ml of n-hexane, the lipid extract was dissolved in 0.5 ml of 2:1 (v/v) dichloromethane:isopropanol (0.5 ml) and added to the top of the sorbent. Fractions were eluted sequentially with 7 ml of 2:1 (v/v) dichloromethane:isopropanol (neutral lipids), 7 ml of 2% glacial acetic acid in diethyl ether (FFA) and 7 ml of methanol (polar lipids). The fractions were dried under a stream of N_2 and stored at -20°C. The polar lipid fraction containing PLFA was transesterified with 1 ml of 14% BF3 in methanol solution at 50°C for 1 h. The methyl esters obtained were analysed with a gas chromatograph equipped with a flame ionisation detector (GC-FID; Varian 3500) on a Supelco Omegawax 320 column (polyethylene glycol phase, 30 m x 0.32 mm i.d., 0.25 μm film). Hydrogen was used as carrier gas, and the temperature of the oven was programmed as follows: 50–100°C at 10°C min^{-1}, 100–240°C at 4°C min^{-1} (20 min isothermal). Quantification of each lipid was calculated using the peak areas, and by comparison with the known amount of a co-injected standard (C12 FAME or C19 alkane). Tentative identification based on retention time was confirmed on a GC-MS (Carlo Erba 5160) equipped with a Chromapack CPWax-52 CB column (50 m x 0.32 mm i.d., 0.12 μm film). Helium was used as carrier gas (10 psi head pressure), and the GC was coupled via a heated transfer line (320°C) to a Finnigan MAT 4500 quadropole mass spectrometer scanning in the range of 50–650 m/z with a cycle time of 1 s. The same temperature program as for the GC-FID experiments was used.

No significant difference was found between whole cell hydrolysate fatty acid and phospholipid-derived fatty acid (PLFA) compositions, indicating that most of the recovered fatty acids were derived from PLFAs.

2.6.3 G +C DNA base composition

The DNA base composition of strain CpA_a5 was determined at the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) with the

following procedure: DNA was isolated using a French pressure cell and purified on hydroxyapatite according to the procedure of Cashion *et al.* (1977). DNA was hydrolyzed with P1 nuclease and the nucleotides dephosporylized with bovine alkaline phosphatase (Mesbah *et al.*, 1989); the resulting deoxyribonucleosides were then analyzed by HPLC. The HPLC system (Shimadzu Corp., Japan) consisted of the following modules: LC-20AD solvent delivery module, DGU-3A online degasser, CTO-10AC column oven, SIL-9A automatic sample injector, SPD-6A UV spectrophotometric detector and a C-R4AX Chromatopac integrator. The analytical column was a VYDAC 201SP54, C_{18}, 5 µm (250 x 4.6 mm) equipped with a guard column 201GD54H (Vydac, Hesperia, CA, USA). Chromatography conditions were adapted from Tamaoka & Komagata (1984): temperature 45°C, 10 µl sample, solvent 0.3 M $(NH_4)H_2PO_4$/acetonitrile, 40:1 (v/v). pH 4.4, 1.3 ml/min. Reference DNA from non-methylated Lambda-DNA (Sigma), GC-content 49.858 mol%, and 3 strains for which complete genome sequences were published (http://ergo.integratedgenomics.com/GOLD/): *Bacillus subtilis* DSM 402, 43.518 mol% G+C; *Xanthomonas campestris* pv. *Campestris* DSM 3586^T, 65.069 mol% G+C); *Streptomyces violaceoruber* DSM 40783, 72.119 mol% G+C, were used for calibration. G+C was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) according to the method of Mesbah *et al.*, (1989).

2.6.4 Determination of dry weight

Two litres of basal medium containing 10 mM acetate and nitrate was prepared as described previously (section 2.4). The medium was inoculated with a 1% inoculum and incubated for 6 days at 25°C until significant turbidity had developed. The culture was harvested by repeated centrifugation (3 x 10 min at 5,100 g, followed by 2 x 10 min at 6,500 g) at 4°C. Ammonium acetate buffer (150 mM) was used to wash the cells and remove salts. The cell pellet was re-suspended in 4 ml ammonium acetate solution and transferred to a small pre-weighed furnaced glass beaker. The pellet was left to dry in an oven at 90°C until a constant weight had been reached.

2.6.5 Determination of cell size

Photographs of exponential-stage cultures stained with acridine orange viewed on a 10 by 10 μm counting grid were taken under epifluorescent light using an Axioskop microscope (Zeiss, Germany). The images were imported into Microsoft PowerPoint, magnified and the size of single cells (n = 120) determined relative to a scale bar. The mean was calculated from the normal distribution of data.

2.6.6 Scanning electron microscopy (SEM)

For SEM analysis growing cell cultures were harvested by centrifugation and fixed in a 1:10 solution of 25% glutaraldehyde in 0.1 M PBS (pH 7.4) buffer for 30 min. The sample was washed in PBS buffer then subjected to post fixation in a mixture of 2 parts 1% osmium tetroxide and 1 part 2.5% glutaraldehyde for 1 h. The sample was progressively dehydrated in an increasing concentration (50% - 100%) of ethanol solution before drying under pressure (80 bar) in a critical point dryer CPD 030 (Balzers) over a temperature increase of 4–45°C for 2 h. The sample was then spattered with gold and examined with a scanning electron microscope (XL 20 SEM, Phillips).

2.6.7 Transmission electron microscopy (TEM)

For TEM imaging, concentrated culture was negatively stained with methylamine tungstate, phoshotungstic acid and uranyl acetate solutions (4% final concentration) to optimise the results. The stained culture suspension was allowed to air dry on a polyvinyl butyral-coated copper mesh then excess stain was removed by washing in double distilled water. Meshes were imaged using a transmission electron microscope (EM 208, Phillips).

Chapter 3 – Molecular and Cultivation-Based Analysis of the Captain Arutyunov and Bonjardim MVs

3.1 Introduction

The work described in this chapter was conducted as an initial investigation of Gulf of Cadiz MV sediments, which were sampled more extensively later in the project. The samples described were collected by a colleague, Marianne Nuzzo, during a research cruise at the end of 2003 (see section 2.1.1). Samples were from two relatively active MVs situated at different water depths in the Gulf of Cadiz – Capt. Arutyunov (1320 m) and Bonjardim (3090 m). As one aim of the project was to explore the potential 'window' that MVs may provide to the deeper sub-seafloor biosphere, analyses were restricted to lower anoxic parts of sediment cores comprising extruded mud breccia, rather than upper parts which may have contained sediment and prokaryotes of hemipelagic origin. This work describes the first study to investigate microbial diversity in sub-surface MV mud breccia below the sulphate-methane transition zone.

3.1.1 Capt. Arutyunov MV site description

The Capt. Arutyunov MV was discovered in 2002 during the TTR-12 cruise on board the R/V *Professor Logachev* (Kenyon *et al.*, 2003), and was named after the late Captain of the ship. It is a conical structure ~2 km wide at its base, ~100 m high with a single 300 m wide concentric crater at its top (Kenyon *et al.*, 2003). It is located at 07° 20.00 N, 35° 39.67 W in the central part of the Gulf of Cadiz in a water depth of 1320 m (Fig 1.19). Seismic profiles over the feature (Fig 3.1) have revealed the presence of stacked outflow lenses at the MV flanks. These are interpreted as mud flows inter-bedded between hemipelagic sediments, and are believed to be indicative of long, episodic activity at this site (Kenyon *et al.*, 2003). Cores recovered from the site predominantly contain mud breccia and only have a relatively thin layer of oxidized hemipelagic sediment at their upper surface. The mud breccia is noted to smell strongly of H_2S and often contains gas hydrate crystals up to several centimetres in size (e.g. Niemann *et al.*, 2006b). The mud breccia is low in organic carbon, with a reported total organic carbon (TOC) value of 0.35% (Ed Hornibrook, personal communication).

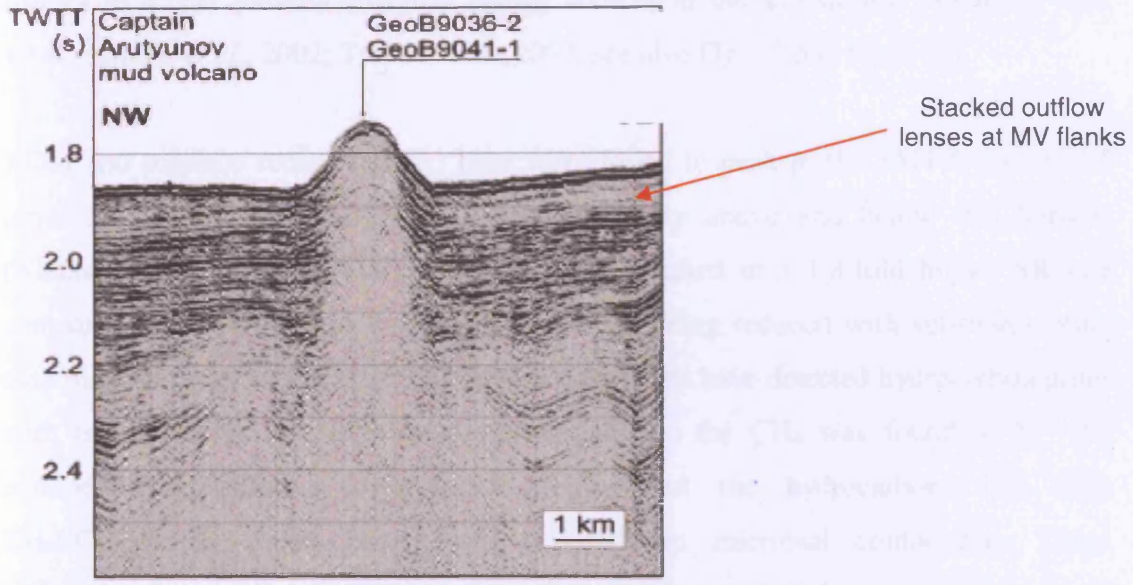


Fig 3.1 Seismic section through the Capt. Arutyunov MV showing stacked outflow lenses at the flanks. Reproduced from Niemann *et al.* (2006b), modified after Kenyon *et al.* (2003). TWTT - two-way travel time (sec).

Geochemical and lipid biomarker data obtained during sample collection for the present study, showed that methane was almost completely consumed by AOM within the sub-surface of the Capt. Arutyunov crater (Niemann *et al.*, 2006b). The methane concentrations were very low (<0.001 mM) in the uppermost sediment (0-20 cmbsf) but were above saturation at atmospheric pressure below the sulphate reduction zone (Niemann *et al.*, 2006b). The sulphate reduction zone was measured at a depth of 25 – 40 cmbsf (Niemann *et al.*, 2006b). This is shallow compared to other MVs in the Gulf of Cadiz and is taken as evidence of this MV's relatively high activity. However, it should be noted that in the context of other global MVs, the activity of this site can only be described as moderate (Niemann *et al.*, 2006b). For example, the Haakon Mosby MV (see section 1.2.1) on the Barents Sea margin, and many Mediterranean MVs have considerably more intense fluid flows, which push the SMTZs to at, or very near the sediment surface. As a result, these more active MVs typically support extensive chemosynthetic microbial mat communities on the sediment surface (e.g. Sassen *et al.*, 1993; Barry *et al.*, 1996; Milkov, 2000 and Joye *et al.*, 2004). Microbial mats have not been reported at Capt. Arutyunov but the site instead hosts communities of small pogonophoran tubeworms (Kenyon *et al.*, 2003; Niemann *et al.*, 2006b) and bivalves e.g. *Acharax* sp. (M. Cuhna, unpublished results), which reflects the ability of these

species to access reduced chemical energy sources in the sub-surface (Sibuet & Olu, 1998; Sahling *et al.*, 2002; Treude *et al.*, 2003; see also Fig 1.9 and Fig 1.16).

AOM and sulphate reduction (SR) rates were found to peak in the SMTZ (11 and 25 nmol cm^{-3} d^{-1}, respectively) and decreased sharply above and below this horizon (Niemann *et al.*, 2006b). The areal integration resulted in a 1.8-fold higher SR rate compared to AOM suggested some sulphate was being reduced with substrates other than methane. Analysis of Capt. Arutyunov pore fluids have detected hydrocarbon gases with unusual isotopic and molecular compositions; the CH_4 was found to be ^{13}C-enriched (suggesting a thermogenic origin) but the hydrocarbons had high $CH_4/(C_2H_6+C_3H_8)$ ratios (suggesting a significant microbial component). These compositions could not be explained by simple mixing between conventional thermogenic and microbial sources and instead a geothermal origin has been proposed (Hensen *et al.*, 2007). Fluids from the Capt. Arutyunov crater are slightly enriched in chloride, which suggests there is dissolution of salt deposits at depth beneath this site. Chloride concentrations above 600 mM were frequently measured during a recent cruise to the site (C. Hensen, unpublished results).

Niemann *et al.* (2006b) constructed a bacterial and an archaeal 16S rRNA gene library from the SMTZ of the Capt. Arutyunov crater (30 – 40 cmbsf). Nine phylogenetic groups were identified in the archaeal library (39 clones sequenced). The majority of sequences were related to AOM-mediating ANME groups. The ANME-2a, ANME-2c and ANME-1 groups made up 59, 3 and 18% of all archaeal sequences, respectively. The other archaeal sequences were affiliated with uncultured groups: Marine benthic group D (8%), Marine benthic group B (3%) and unclassified *Archaea* (10%). The bacterial 16S rRNA gene library (47 clones sequenced) consisted of 10 uncultivated bacterial lineages. The majority of sequences (81%) belonged to the Seep-SRB1 group in the *Deltaproteobacteria*, which have been identified as the bacterial partners of the ANME-1 and ANME-2 organisms (Knittel *et al.*, 2003). The other bacterial phylotypes were represented by single sequences and were affiliated with *Clostridia* (2%), *Spirochaetes* (4%), *Gammaproteobacteria* (2%), other *Deltaproteobacteria* (4%) and unclassified *Bacteria* (6%).

3.1.2 Bonjardim MV site description

The Bonjardim MV was discovered during TTR-10 in July/August 2000 (Kenyon *et al.*, 2001). It occurs in the Deep Portuguese Margin Field at 09° 00.00 N, 35° 27.65 W in a water depth of 3090 m (Fig 1.9). Side-scan data acquired by the US Naval Research laboratory showed it as an approximately circular feature with a diameter of ~1 km and a height of ~100 m (Pinheiro *et al.*, 2003). It is considered active on account of its thin covering of hemipelagic sediment, high concentration of methane in the sub-surface (Niemann *et al.*, 2006b) and the presence of chemosynthetic mega fauna (Pinheiro *et al.*, 2003). Gas hydrates have been frequently detected at Bonjardim with aggregates up to 6 cm found during a recent cruise (TTR-16, unpublished results). Measurements taken during sample collection for the present study, measured the SMTZ between 45 and 70 cmbsf in the Bonjardim crater (Niemann *et al.*, 2006b). Similarly to Capt. Arutyunov, there was a complete consumption of methane within the SMTZ and a corresponding peak in sulphide indicating AOM coupled to the reduction of sulphate occurs at the site (Niemann *et al.*, 2006b). Below the SMTZ *ex situ* methane concentrations were above saturation at atmospheric pressure (Niemann *et al.*, 2006b). AOM and SR rates peaked in the SMTZ (2.6 and 15.4 nmol cm^{-3} d^{-1}, respectively) but showed a high standard error over replicate measurements, possibly as a result of high small-scale variability on a metre scale (Niemann *et al.*, 2006b). Areal SR rates were 19-fold higher than AOM rates suggesting these two processes are not closely coupled at Bonjardim (Niemann *et al.*, 2006b). Lipid biomarker analysis revealed a moderate increase in diagnostic archaeal and bacterial lipid concentrations within the SMTZ but these generally had less depleted ^{13}C values than were measured at Capt. Arutyunov, and isotopic analysis of dominant phospholipid fatty acids indicated AOM is not the main energy and carbon source at this site (Niemann *et al.*, 2006b). Analysis of the Bonjardim hydrocarbons revealed a high concentration of C_2+ compounds, suggesting that the gases at this site are predominantly of thermogenic origin (Mazurenko *et al.*, 2002; Stadnitskaia *et al.*, 2006; Niemann *et al.*, 2006b). In contrast to Capt. Arutyunov, fluids from the site have low chloride concentrations (C. Hensen, unpublished results), which may be due to mineral dehydration processes occurring at depth (Ginsburg *et al.*, 1999).

3.1.3 Scientific objectives

The scientific aims of this part of the project were as follows:

- To characterise and compare the sub-surface microbiology of two different Gulf of Cadiz MV craters using molecular genetic techniques and culturing
- To enrich and isolate MV-sourced prokaryotes into pure culture for the first time
- To assess to what extent MVs are "windows to the deep biosphere"

3.2 PCR-based analysis of the Capt. Arutyunov and Bonjardim MV sediment

Sediment for PCR-based analyses was sampled from the inner 'pristine' parts of mud breccia recovered from below the SMTZ in the crater sub-surface of the Capt. Arutyunov and Bonjardim MVs (section 2.1.1). The Capt. Arutyunov mud breccia sample was taken from sediment in the depth range 126 – 270 cmbsf and the Bonjardim mud breccia sample was taken from sediment in the depth range 205 – 240 cmbsf. Although the precise depth origin of these samples was not known they were sufficient to meet the stated objectives of the study (section 1.5). Full details of sample handling and relevant methodologies used are given in chapter 2.

3.2.1 DNA extraction, PCR amplification of 16S rRNA gene sequences and DGGE analysis

Following DNA extraction and cleaning (section 2.3), the two MV samples were analysed by gel electrophoresis to assess the relative quantities of DNA obtained (Fig 3.2).

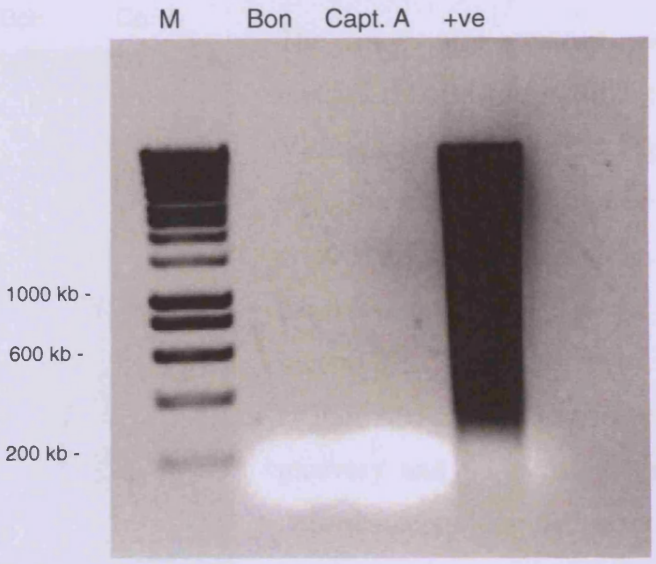


Fig 3.2. Visualization of DNA extracted from the Capt. Arutyunov and Bonjardim MVs by gel electrophoresis. Lanes were as follows: M, HyperLadder I DNA quantification marker; Bon, 25% of DNA extraction product from Bonjardim mud volcano; Capt. A, 25% of DNA extraction product from Capt. Arutyunov mud volcano; +ve, 25% of DNA extraction product from 1 g tidal flat sediment, Portishead (positive control). Values shown to the left of the DNA marker refer to fragment size in kilobases (kb).

DNA was not visible in the Bonjardim sample and was only barely visible in the Capt. Arutyunov sample despite both extractions representing the pooled product from 5 x 1 g of sediment. In contrast the positive control shown in Fig 3.2 represented the product from just 1 g of tidal flat sediment. The very low amount of extractable DNA from the MV samples was consistent with low total prokaryotic cell counts (log AODC values of 6.44 and 6.93 total cells/cm^3 from the Bonjardim and Capt. Arutyunov MVs, respectively).

To test if the extracted MV DNA was amplifiable by PCR the samples were initially amplified with the 357F – 518R general bacterial 16S rRNA gene primers (Muyzer *et al.*, 1993; see section 2.3.2). Products were successfully obtained from both samples during the first round of PCR, and as an initial assessment of diversity, these partial length bacterial 16S rRNA PCR products were screened by DGGE (section 2.3.5).

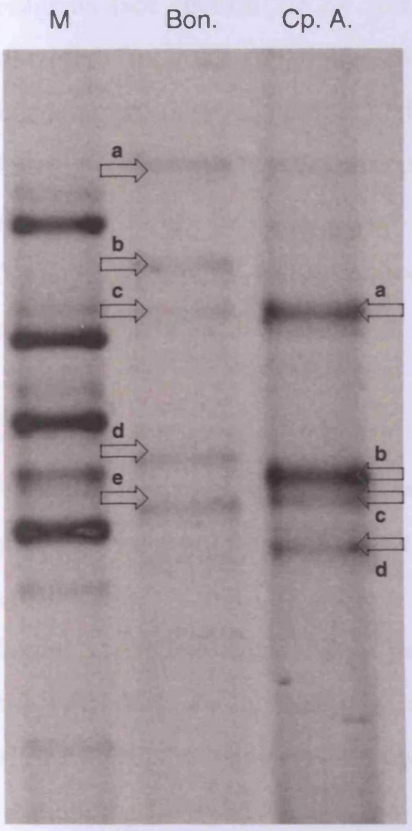


Fig 3.3. DGGE analysis of bacterial 16S rRNA genes amplified from the subsurface of Capt Arutyunov and Bonjardim MVs. Lane marked M was a DGGE marker. Arrows indicate bands extracted for re-amplification, see text.

The community structure, as revealed by this method (Fig 3.3), was different at the two sites. Visible bands (labelled in Fig 3.3) were excised from the gel, cleaned and re-amplified with the original DGGE primers in an attempt to identify the bands by sequencing (see section 2.3.6). The re-amplified products were run on a second DGGE gel prior to sequencing to confirm recovery and purity of the targeted bands, but unfortunately, despite several attempts, good quality sequences could not be obtained from any of the DGGE bands from the Capt. Arutyunov sample, and only from three of the five DGGE bands from the Bonjardim sample (bands Bon a, b and e), see Table 3.1. The Capt. Arutyunov bands labelled 'b' and 'c' could not be separated from each other (two bands were always present when the re-amplified product from just one of the bands was re-run on DGGE) and the chromatographs from Capt. Arutyunov bands 'a' and 'd', repeatedly showed a mixed signal suggesting that, despite the appearance of single bands, more than one bacterial 16S rRNA gene had denatured at that position on the DGGE gel.

Comparative sequence analysis (BLAST) revealed four of the five Bonjardim DGGE band sequences were members of the *Gammaproteobacteria*, though the sequences were all different from one another. The remaining DGGE band sequence was affiliated with the *Deltaproteobacteria* (Table 3.1). Two of the successfully sequenced *Gammaproteobacteria* Bonjardim DGGE bands (Bon. a and Bon. b), were affiliated to the ubiquitous *Pseudomonas* genus (see Spiers *et al.*, 2000 and references therein). Multiple 100% sequence matches were obtained for both of these bands in the BLAST search, and in both cases the matching sequences were recovered from a very wide variety of habitats. A third Bonjardim *Gammaproteobacteria* DGGE band sequence matched sequences of *Escherichia coli*, which suggested it was a contaminant from PCR

reagents (see section 2.3.2), and this result was therefore discounted. Chromatographs obtained from the remaining two Bonjardim DGGE bands were of low quality and hence no meaningful phylogenetic information could be determined beyond the division level. It is noteworthy that the pure culture isolates obtained from the Bonjardim MV sediment during this study were also members of the *Pseudomonas* genus (see section 5.4). These strains (98% related to each other) both shared 94 and 98% sequence similarity with the Bonjardim 'a' and 'b' DGGE bands, respectively.

As an initial analysis of archaeal diversity at the two sites, sediment DNA was amplified with the 109F – 958R general archaeal 16S rRNA gene primers (section 2.3.2). PCR products were not obtained in the first round reaction but nested PCR reactions using the smaller SAf – PARCH primer pair did yield faintly visible products from both MV samples. Analysis by DGGE again showed a difference in community structure between the two sites, as indicated by a difference in the DGGE banding pattern (data not shown), but the bands visible on the gel were very faint and could not be identified by sequencing.

Table 3.1 Results of Capt. Arutyunov and Bonjardim bacterial 16S rRNA gene DGGE band sequencing

Band identifier (Fig 3.3)	Sequence length (bp)	Closest match in BLASTN database (Accession number)[a]	Identities (%)	Phylogenetic affiliation	Origin of closest matching sequence(s)
Cp. A a		Mixed sequence signal			
Cp. A b		Unable to separate bands			
Cp. A c		Unable to separate bands			
Cp. A d		Mixed sequence signal			
Bon. a	194	Clone SML_216_47 *Pseudomonas sp.* (DQ446028)	194/194 (100)	*Gamma-proteobacteria*	Multiple sequence matches. Various environments.
Bon. b	194	Strain SMW6 *Pseudomonas sp.* (DQ887516)	194/194 (100)	*Gamma-proteobacteria*	Multiple sequence matches. Various environments.
Bon. c		Mixed sequence		*Gamma-proteobacteria*	
Bon. d		Mixed sequence		*Delta-proteobacteria*	
Bon. e	194	Strain K12 *Escherichia coli.* (U00096)	194/194 (100)	*Gamma-proteobacteria*	Multiple sequence matches. Various environments.

[a] **Where multiple sequence matches were identified, the top match listed in the BLAST results pages was arbitrarily selected for inclusion in the table.**

3.2.2 Construction of representative bacterial 16S rRNA gene libraries

Near full-length bacterial 16S rRNA genes could not be amplified by PCR from the Bonjardim MV despite several attempts using different methods (re-extracting fresh DNA, addition of BSA to PCR reaction mixture, and trial of different DNA template dilutions). Partial length 16S rRNA gene products were obtained from Bonjardim with the 27F – 907R primer pair but the success of these reactions was variable. It was decided that any resulting clone library data may be unreliable and therefore no bacterial 16S rRNA gene library was constructed from this site.

Near full-length (~1450 bp) bacterial 16S rRNA genes were reproducibly amplified from the Capt. Arutyunov MV in five replicate PCR reactions. These products were then analysed by DGGE (Fig 3.4).

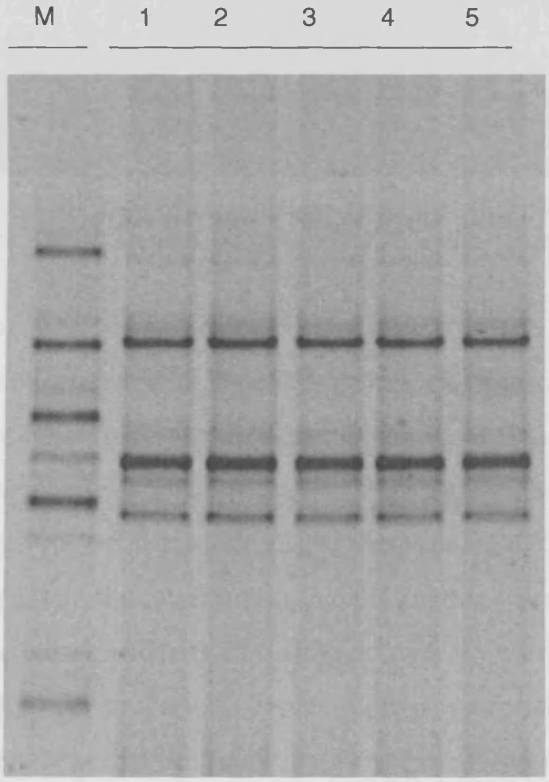


Fig 3.4. DGGE analysis of Capt. Arutyunov bacterial 16S rRNA amplicons obtained from five parallel PCR reactions. Lane M, standard DGGE marker (see section 2.4.6); lanes 1 – 5, products from five replicate reactions re-amplified with primers 357F – 518R (see Table 2.3).

DGGE analysis showed that the amplicons obtained were consistent across the five PCR reactions and that the banding pattern matched that obtained by direct amplification of

the sediment DNA with the 357F-GC – 518R DGGE primers (see Fig 3.3). The products from these five reactions were pooled and cloned.

DGGE analysis of 44 randomly selected clones suggested a moderate level of diversity in the Capt. Arutyunov MV bacterial 16S rRNA gene library (Fig 3.5).

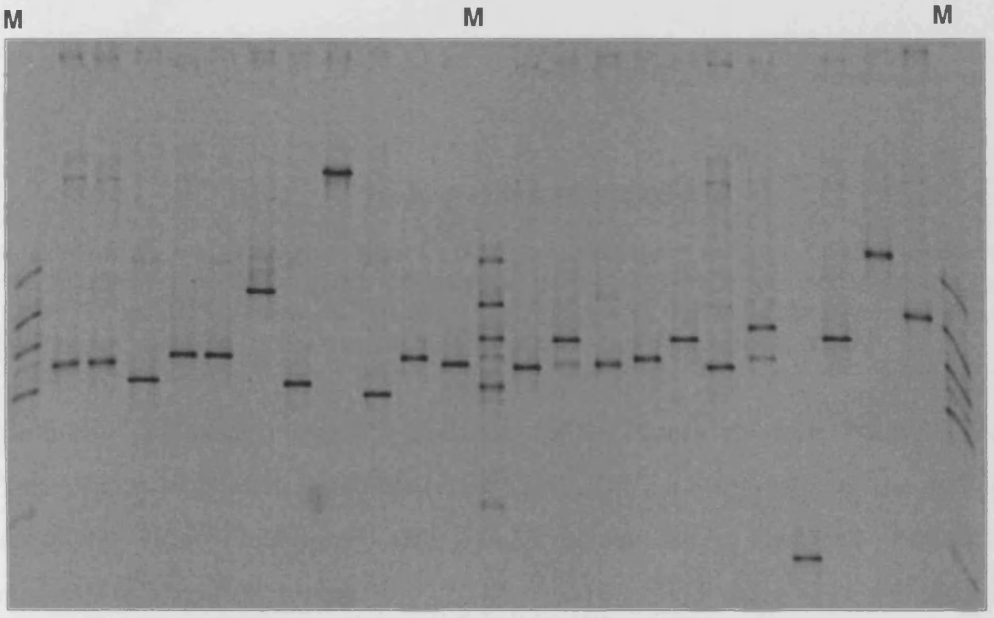


Fig 3.5 Example of a DGGE gel used to screen cloned bacterial 16S rRNA genes amplified from Capt. Arutyunov sediment. Lanes marked 'M' were a DGGE marker (see section 2.4.6).

To capture the level of diversity indicated by the initial DGGE analysis, a total of 58 clones were initially sequenced with the 27F primer. A threshold of 97% sequence similarity was used to distinguish the different Operational Taxonomic Units (OTUs) present in the partial-sequence (~680 bp) library. Phylotype-richness estimators (Kemp & Aller 2004) applied to the bacterial Capt. Arutyunov 16S rRNA gene library sequences produced an asymptotic maximum, indicating that after the initial sequencing effort the library could be considered of sufficient size to yield a stable estimate of phylotype richness. An accumulation curve plotted from the randomized clone phylotype data (Fig 3.6) supported the findings of the phylotype-richness estimators and showed that few additional phylotypes were likely to be recovered by further sequencing efforts.

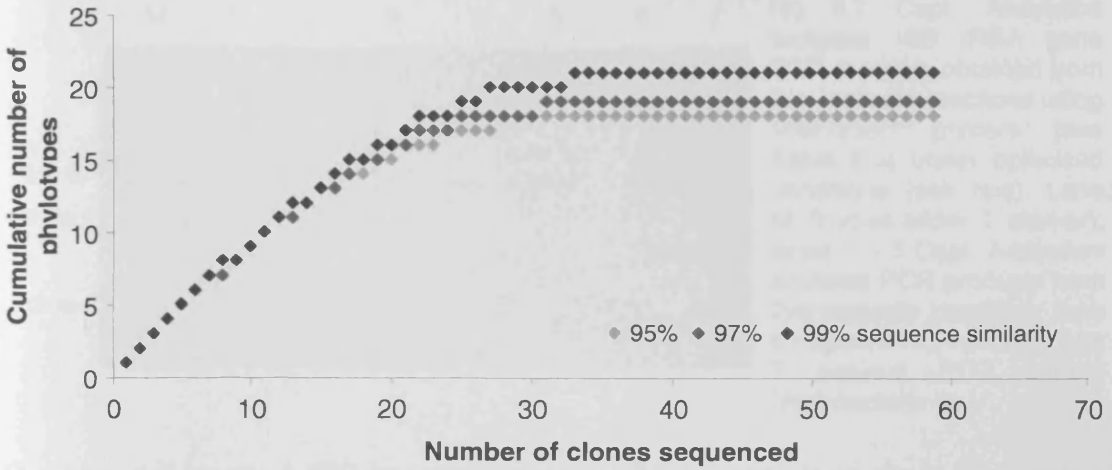


Fig 3.6. Accumulation curve plotted from Capt. Arutyunov partial length (~680 bp) bacterial 16S rRNA gene library data. The different series show cumulative phylotype data calculated on the basis of 95, 97 and 99% sequence similarity. The library reached a stable asymptote indicating additional phylotypes were unlikely to be recovered by further sequencing efforts.

Representative clones were selected from each of the identified phylotypes (OTUs) and near full-length sequences assembled (section 2.3.7). Re-analysis of the full-length representative clone sequences with a 97% sequence similarity threshold to differentiated different phylotypes showed there to be 15 individual OTUs in the dataset, which was slightly less than had been identified on the basis of comparing the partial length sequences (n = 18).

3.2.3 Construction of representative archaeal 16S rRNA gene libraries

Near full-length archaeal 16S rRNA genes could not be amplified by PCR from the Bonjardim MV despite several attempts using different methods (re-extracting fresh DNA, addition of BSA to PCR reaction mixtures and trial of different DNA template dilutions); hence no archaeal gene library was constructed for this site.

Following initial difficulty in obtaining near full-length archaeal 16S rRNA gene products from the Capt. Arutyunov MV optimized PCR reactions were used. The PCR reactions were optimized through the addition of bovine serum album to the reaction mixture (Kreader, 1996), and by increasing the number of reaction cycles to 40. Five replicate reactions under these optimized conditions were conducted using the 109F – 958R primers and were all successful in yielding clear archaeal 16S rRNA gene PCR products in the first round (Fig 3.7).

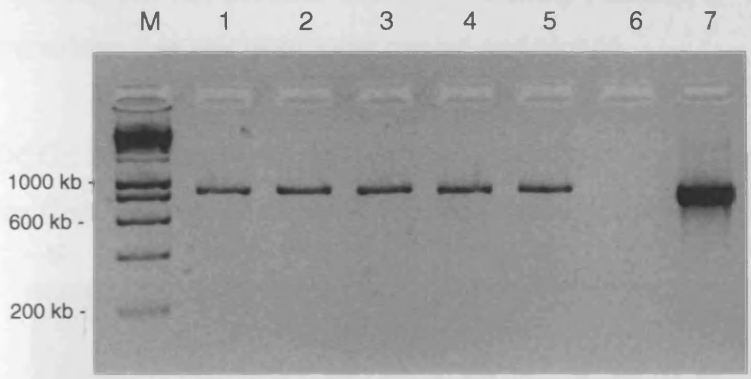


Fig 3.7 Capt. Arutyunov archaeal 16S rRNA gene PCR products obtained from five replicate reactions using 109F/958R primers (see Table 2.3) under optimized conditions (see text). Lane M (HyperLadder I marker); lanes 1 – 5 Capt. Arutyunov archaeal PCR products from five replicate reactions; lane 6 negative PCR control; lane 7 positive PCR control (*Halobacteria* sp.).

The near full-length (~950 bp) archaeal 16S rRNA genes from these five replicate reactions were re-amplified with the SAf – PARCH primers and analysed by DGGE (Fig 3.8).

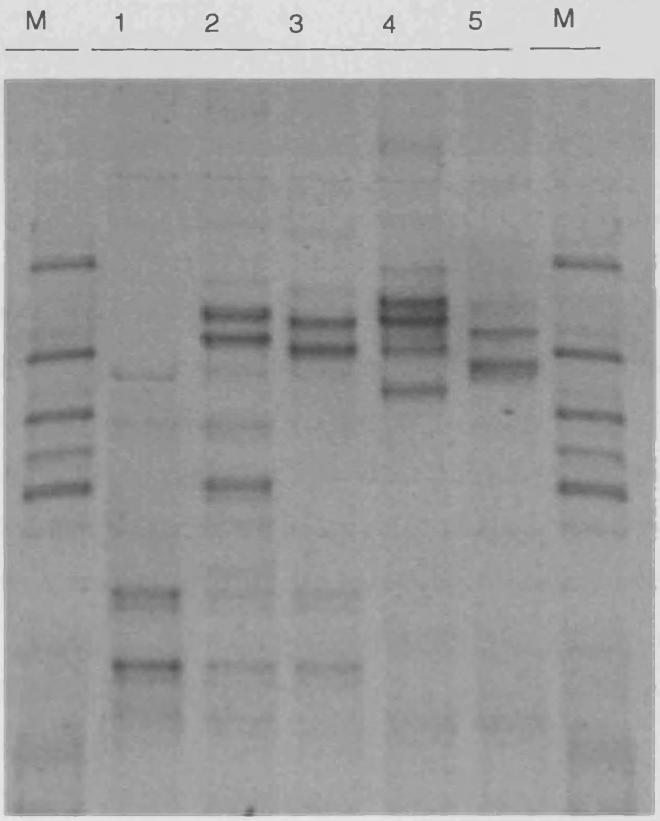


Fig 3.8 DGGE analysis of Capt. Arutyunov archaeal 16S rRNA gene products obtained from five parallel PCR reactions. Lane M, standard DGGE marker (see section 2.4.6); lanes 1 – 5 Capt. Arutyunov archaeal PCR products from five replicate reactions re-amplified with SAf – PARCH primers (see Table 2.3).

DGGE analysis suggested archaeal diversity in the Capt. Arutyunov sediment was relatively low and in contrast to the bacteria, the gene products from the replicate PCR

reactions did not produce the same banding patterns on the DGGE gel. The products from these five reactions were pooled and cloned.

DGGE screening of 40 randomly selected clones supported there being low diversity in the Capt. Arutyunov archaeal 16S rRNA gene library (Fig 3.9).

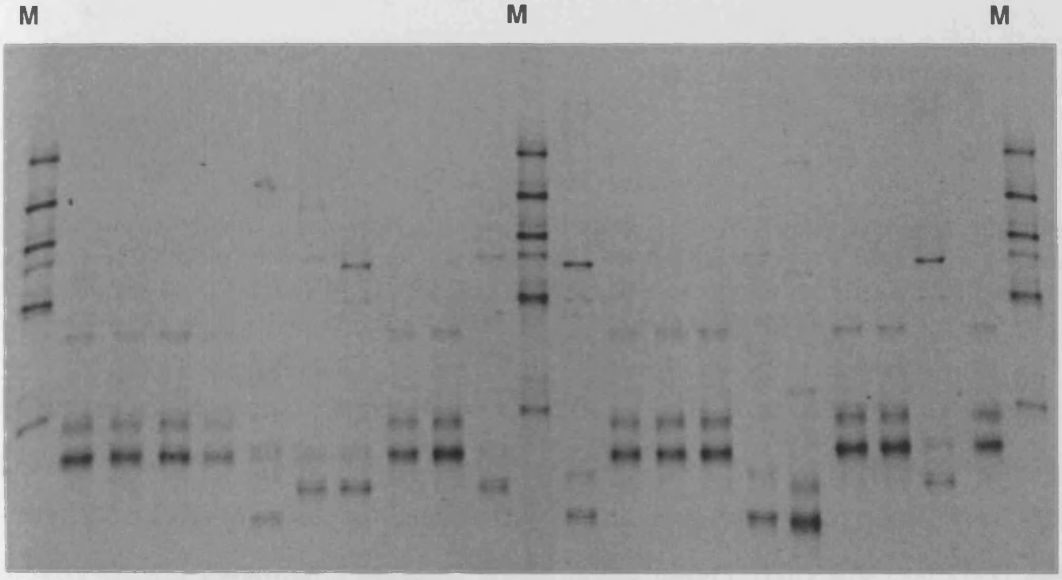


Fig 3.9 Example of a DGGE gel used to screen cloned archaeal 16S rRNA genes amplified from the Capt. Arutyunov MV. The lanes labelled 'M' contained a DGGE marker (see section 2.4.6).

Forty archaeal clones were initially sequenced with the 109F primer and individual OTUs (phylotypes) were discriminated on the basis of having <97% sequence similarity to other clones in the library. Subjecting the partial length (660 bp) archaeal clone sequences to analysis with the phylotype richness estimators recommended by Kemp & Aller (2004) revealed that no additional sequencing was necessary as the libraries were already saturated with respect to diversity. An accumulation curve plotted from the randomized clone phylotype data supported the findings of the phylotype-richness estimators and indicated additional phylotypes were unlikely to be recovered by further sequencing efforts (Fig 3.10).

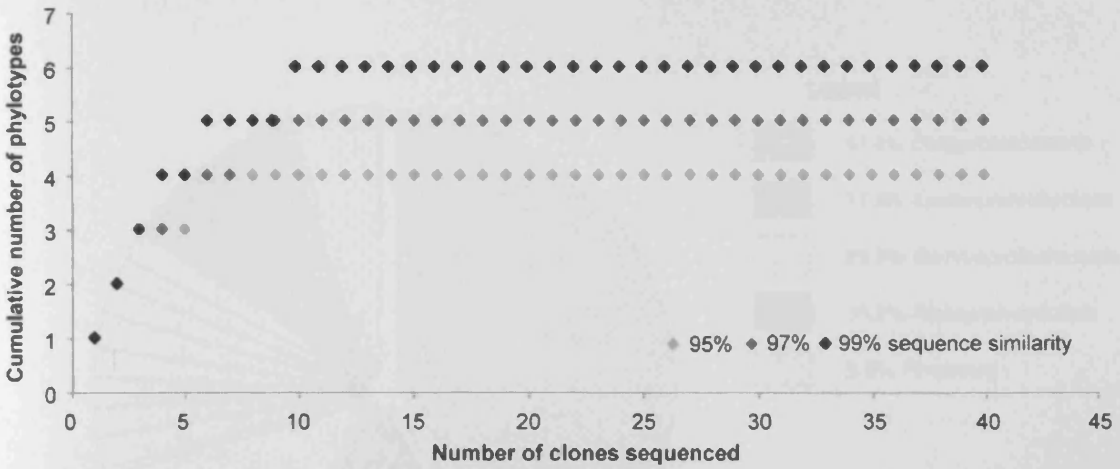


Fig 3.10. Accumulation curve plotted from phylotype data of partial archaeal 16S rRNA sequences in the Capt. Arutyunov gene library. The different series show cumulative phylotype data calculated on the basis of 95, 97 and 99% sequence similarity. The asymptote shows additional phylotypes were unlikely to be obtained with further sequencing efforts.

Representative members of each of the identified OTUs were additionally sequenced with the 958R primer and near full-length archaeal 16S rRNA sequences assembled (section 2.3.7). Re-analysis of the near full-length sequences using a 97% sequence similarity cut off identified 4 separate OTUs in the library. This was one OTU fewer than had been identified in the partial length sequence dataset.

3.2.4 Bacterial diversity in the Capt. Arutyunov MV

The 15 different phylotypes identified in the Capt. Arutyunov near full-length bacterial 16S rRNA gene library (n = 58) belonged to 5 separate taxonomic groups (Fig 3.11). The vast majority of sequences in the library (95%) were members of the *Proteobacteria*. The only other division detected was the *Firmicutes* (5%). *Deltaproteobacteria* constituted 41% of the gene library and represented one dominant phylotype (the most abundant phylotype in the library) and one further phylotype that was detected only once. *Gammaproteobacteria* constituted 26% of the library and represented nine separate phylotypes, eight of which were only detected once. Sequences affiliated with the *Epsilonproteobacteria* made up 17% of the library and represented two separate phylotypes. *Alphaproteobacteria* sequences made up 10% of the library and represented a single phylotype. A single phylotype belonging to the *Firmicutes* made up the remaining 5% of the library.

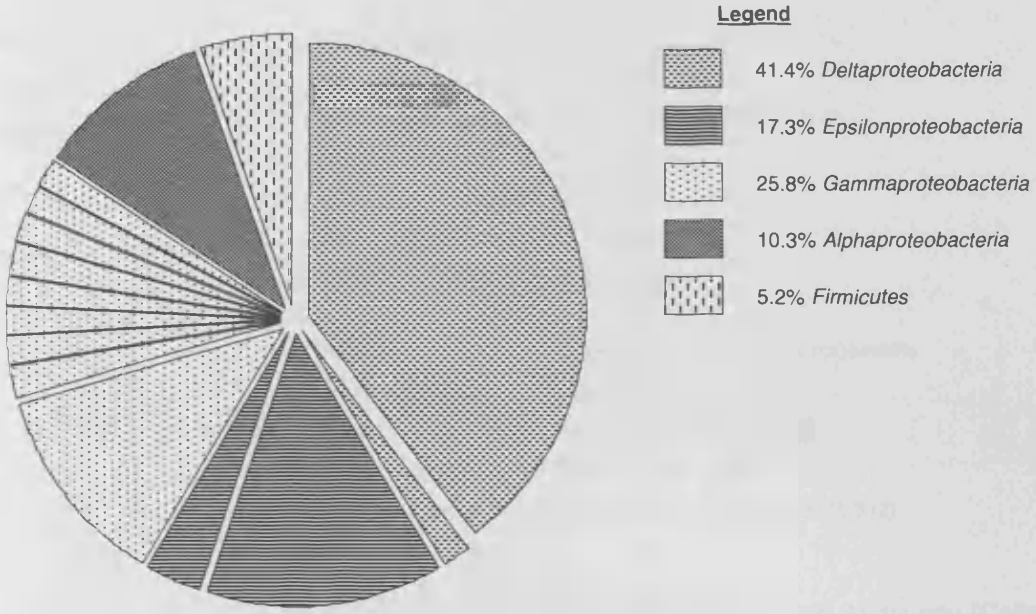


Fig 3.11 Pie chart showing the taxonomic groupings and relative abundance of OTUs (>97% sequence similarity) identified within the Capt. Arutyunov bacterial 16S rRNA gene library (n = 58). Shading indicates different taxonomic groups, seqments indicate the number of individual OTUs within each group.

Phylogenetic trees were produced separately for each taxonomic group identified in the bacterial 16S rRNA gene library and show the nearest cultured and uncultured relatives of the Capt. Arutyunov clone sequences (Figs 3.12 – 3.16). Sequences of representative isolates cultivated from the Capt. Arutyunov and Bonjardim MVs as part of the present study (see section 3.3) were included, where appropriate, for comparison. A habitat key was also included with each tree in an attempt to identify patterns in the environmental distribution of uncultivated taxa and offer insight into their ecological niche and/or potential function.

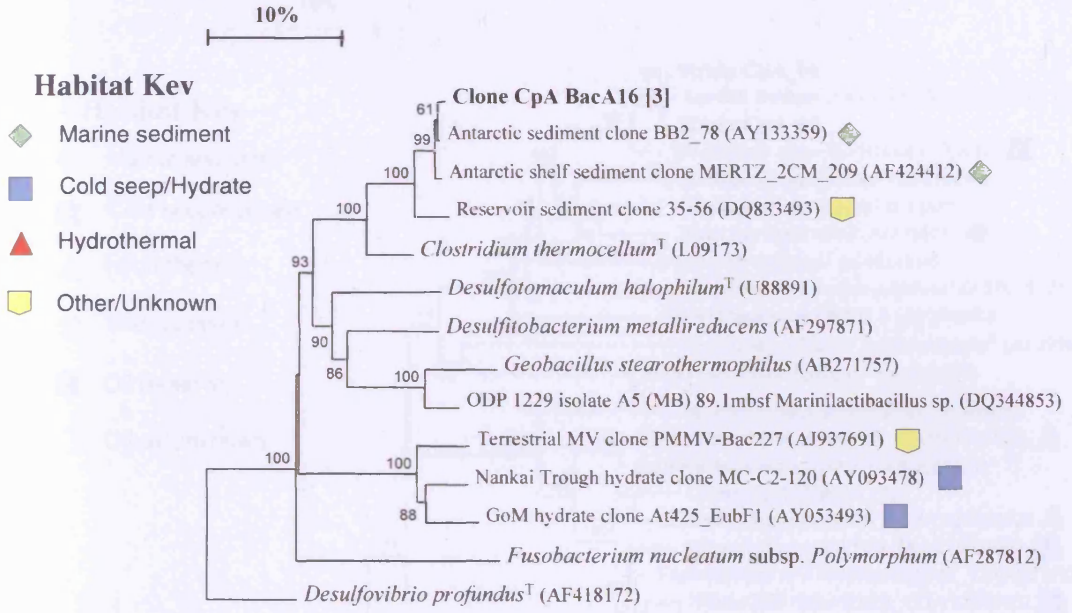


Fig 3.12 Neighbour-joining tree showing the phylogenetic position of the Capt. Arutyunov (CpA) *Firmicute* clones relative to other environmental clones and representative type strains within the Division. The reconstruction was based on an alignment of 1119 nucleotide positions using the Jukes and Cantor (1969) algorithm. *Desulfovibrio profundus*^{T} was used as outgroup. Numbers at nodes represent percentage bootstrap values after 1000 samples. Values in square brackets indicate the number of clones represented by the individual OTU shown. Accession numbers are shown in parentheses.

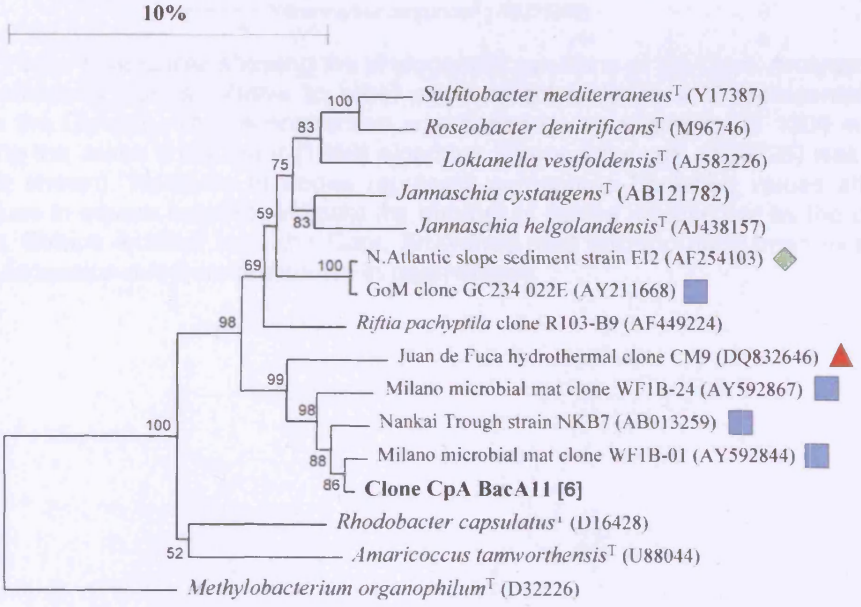


Fig 3.13. Neighbour-joining tree showing the phylogenetic position of the Capt. Arutyunov (CpA) *Alphaproteobacteria* clones relative to other environmental clones and representative type strains within the Division. The reconstruction was based on an alignment of 1172 nucleotide positions using the Jukes and Cantor (1969) algorithm. *Methylobacterium organophilum*^{T} was used as outgroup. Numbers at nodes represent percentage bootstrap values after 1000 samples. Values in square brackets indicate the number of clones represented by the individual OTU shown. Accession numbers are shown in parentheses.

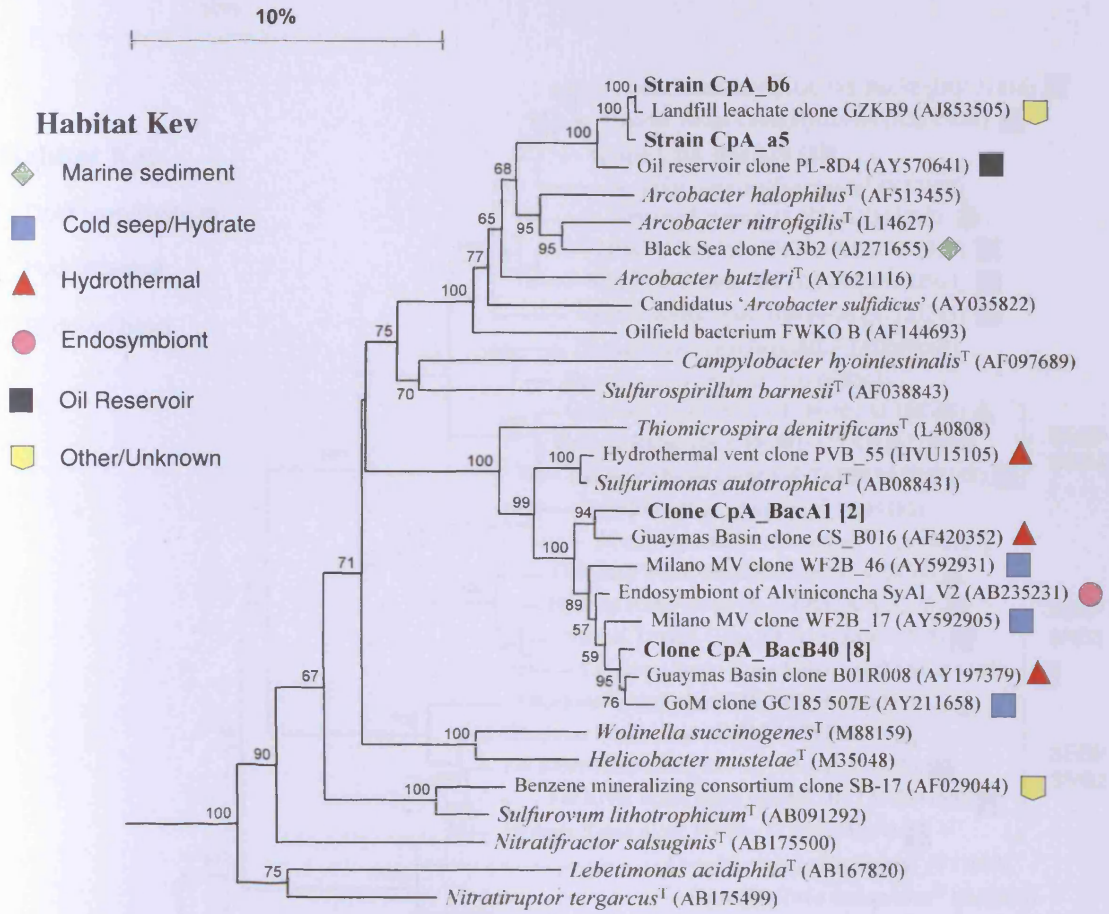


Fig 3.14 Neighbour-joining tree showing the phylogenetic positions of the Capt. Arutyunov (CpA) *Epsilonproteobacteria* clones relative to other environmental clones and representative type strains within the Division. The reconstruction was based on an alignment of 1300 nucleotide positions using the Jukes and Cantor (1969) algorithm. *Escherichia coli*^{T} (X80725) was used as outgroup (not shown). Numbers at nodes represent percentage bootstrap values after 1000 samples. Values in square brackets indicate the number of clones represented by the individual OTUs shown. Strains isolated from the Capt. Arutyunov mud volcano have been included for comparison. Accession numbers are shown in parentheses.

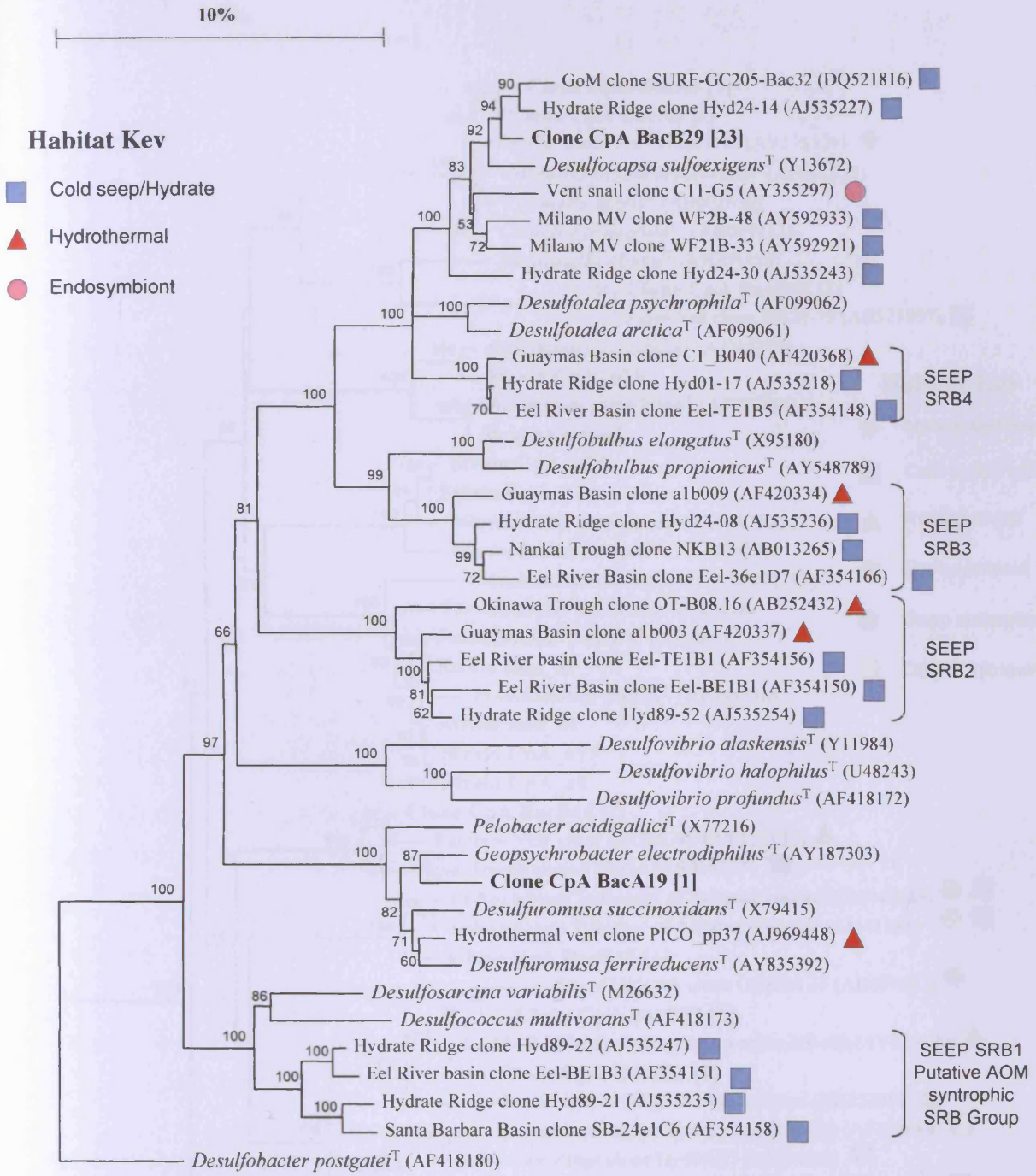


Fig 3.15 Neighbour-joining tree showing the phylogenetic positions of the Capt. Arutyunov (CpA) *Deltaproteobacteria* clones relative to other environmental clones and representative type strains within the Division. The reconstruction was based on an alignment of 1313 nucleotide using the Jukes and Cantor (1969) algorithm. *Desulfobacter postgatei*^{T} was used as outgroup. Numbers at nodes represent percentage bootstrap values after 1000 samples. Scale bar represents 10% sequence divergence. Values in square brackets indicate the number of clones represented by the individual OTUs shown. The various SEEP groups of *Deltaproteobacteria*, as defined by Knittel *et al.*, (2003) have been highlighted for reference. Accession numbers are shown in parentheses.

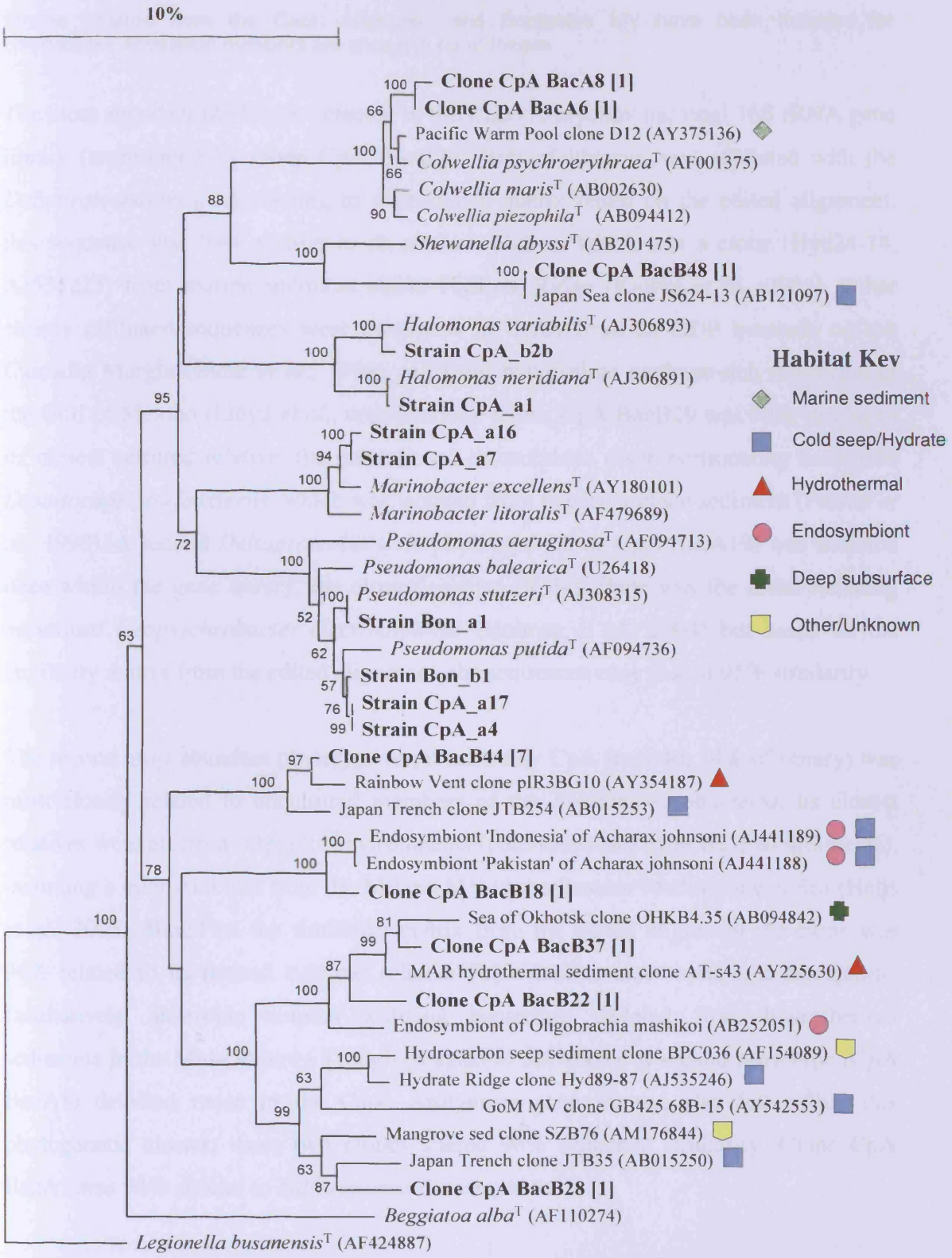


Fig 3.16 Neighbour-joining tree showing the phylogenetic positions of the Capt. Arutyunov (CpA) *Gammaproteobacteria* clones relative to other environmental clones and representative type strains within the Division. The reconstruction was based on an alignment of 1287 nucleotide positions using the Jukes and Cantor (1969) algorithm. *Legionella busanensis*^{T} was used as outgroup. Numbers at nodes represent percentage bootstrap values after 1000 samples. Values in square brackets indicate the number of clones represented by the individual OTUs shown.

Strains isolated from the Capt. Arutyunov and Bonjardim MV have been included for comparison. Accession numbers are shown in parentheses.

The most abundant phylotype detected in the Capt. Arutyunov bacterial 16S rRNA gene library (represented by clone CpA BacB29, 40% of library), was affiliated with the *Deltaproteobacteria*. According to a similarity matrix based on the edited alignment, this sequence was 99% similar to its nearest relative, which was a clone (Hyd24-14, AJ535227) from marine sediment above Hydrate Ridge (Knittel *et al.*, 2003). Other closely affiliated sequences were recovered in fluids from an ODP borehole on the Cascadia Margin (Bidle *et al.*, 1999) and from hypersaline methane-rich sediments in the Gulf of Mexico (Lloyd *et al.*, unpublished). Clone CpA BacB29 was 98% similar to its closest cultured relative, the sulphur and thiosulphate disproportionating bacterium *Desulfocapsa sulfoexigens*, which was isolated from marine surface sediment (Finster *et al.*, 1998). A second *Deltaproteobacteria* phylotype (clone CpA BacA19) was detected once within the gene library, the closest relative of this clone was the metal reducing bacterium *Geopsychrobacter electrodiphilus* (Holmes *et al.*, 2004) but based on the similarity matrix from the edited alignment, the sequences only shared 95% similarity.

The second most abundant phylotype (represented by CpA BacB40, 14% of library) was most closely related to uncultured members of the *Epsilonproteobacteria*. Its closest relatives were all from sulphidic environments (cold seeps and hydrothermal sediments), including a microbial mat from the Milano MV in the Eastern Mediterranean Sea (Heijs *et al.*, 2005). Based on the similarity matrix from the edited alignment the clone was 96% related to its nearest cultured relative *Sulfurimonas autotrophica*, a mesophilic, facultatively anaerobic sulphur-oxidizing bacterium isolated from hydrothermal sediments in the Mid-Okinawa Trough (Inagaki *et al.*, 2003). A second phylotype (CpA BacA1) detected twice in the Capt. Arutyunov gene library, also fell within this phylogenetic cluster; these two clones shared 96% sequence similarity. Clone CpA BacA1 was 94% similar to *Sulfurimonas autotrophica*.

Twenty six percent of the library was composed of *Gammaproteobacteria*. Nine separate OTUs were identified within this sub-phylum making it the most genetically diverse phylogenetic group of the library. Eight of the OTUs occurred only once in the library but the ninth was represented by 7 sequences. This more abundant OTU,

represented by clone CpA BacB44, shared 99% sequence similarity with a clone from hydrothermal sediments of the Rainbow Vent on the Mid-Atlantic Ridge (Nercessian *et al.*, 2005) and 97% sequence similarity with a clone from a deep cold seep in the Japan Trench (Li *et al.*, 1999). It represents a deeply branching uncultivated lineage within the *Gammaproteobacteria* and as such, is of unknown function. Four other *Gammaproteobacteria* clones, CpA BacB28, CpA BacB48, CpA BacA22[a] and CpA Bac B18 were most closely related to sequences from cold seep environments and also were only very distantly related to any cultured organisms. Two other clones, CpA BacA8 and CpA BacA6 were >98% related to cultured *Colwellia* species. *Colwellia*-like sequences have been recovered from mesophilic (DeLong *et al.*, 1993; Zeng *et al.*, 2005) and even hydrothermal systems (Moyer *et al.*, 1995) but all the cultured representatives are from cold marine environments and are typically psychrophilic and are characterized as facultatively anaerobic chemoorganotrophs (Junge *et al.*, 2006). The remaining two *Gammaproteobacteria* clones, CpA BacB37 and CpA BacB22, fell within another uncultivated lineage and shared their greatest sequence similarity (98 and 95%) with sequences from hydrothermal sediment and a *Siboglinidae* polychaete endosymbiont, respectively.

The remaining clones represented members of the *Alphaproteobacteria* and *Firmicutes*. The *Firmicute* clone (CpA BacA16), representative of three sequences in the library was >99% similar to sequences recovered from marine sediment and only 91% similar to the nearest cultured organism, *Clostridium thermocellum*. The *Alphaproteobacteria* clone (CpA BacA11), representative of six sequences in the library was 99% similar to a clone (WF1B-01, AY592844) recovered from a microbial mat of the Milano submarine MV. It was 95% similar to its nearest cultured relative *Nereida ignava* (Pujalte *et al.*, 2005).

[a] N.B. clone CpA BacA22 was mistakenly omitted from the phylogenetic reconstruction in Fig 3.16 but is most closely related to the clone Hyd89-87 (AJ535246).

3.2.5 Archaeal diversity in the Capt. Arutyunov MV

The 4 phylotypes identified in the Capt. Arutyunov 16S rRNA gene library belonged to 3 taxonomic groups (Fig 3.17). The uncultivated ANME-2 group, affiliated to the *Euryarchaeota,* was most abundant taxa in the archaeal library (53% of sequences) and was represented by a single phylotype. The second most abundant taxa was the uncultivated *Miscellaneous Crenarchaeotic Group* (44% of sequences), and contained two different phylotypes, one of which was detected just once in the library. The one remaining phylotype, detected on just a single occasion, was affiliated with the *Euryarchaeota* and belonged to the genus *Methanococcoides.*

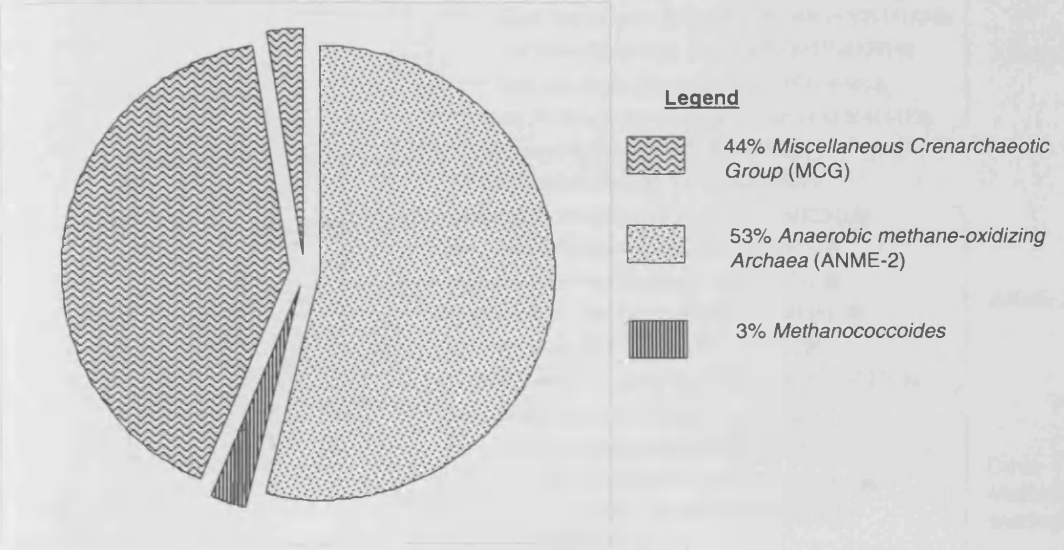


Fig 3.17 Pie chart showing the taxonomic groupings and relative abundance of OTUs identified within the Capt. Arutyunov archaeal 16S rRNA gene library (n = 40). Shading indicates different taxonomic groups, segments indicate individual OTUs.

Neighbour-joining trees constructed separately for the *Euryarchaeota* and *Crenarchaeota*, show the phylogenetic positions and nearest neighbours of Capt. Arutyunov 16S rRNA archaeal clones (Fig 3.18 and Fig 3.19). A habitat key showing the environmental distribution of related uncultivated taxa was included as for the *Bacteria.*

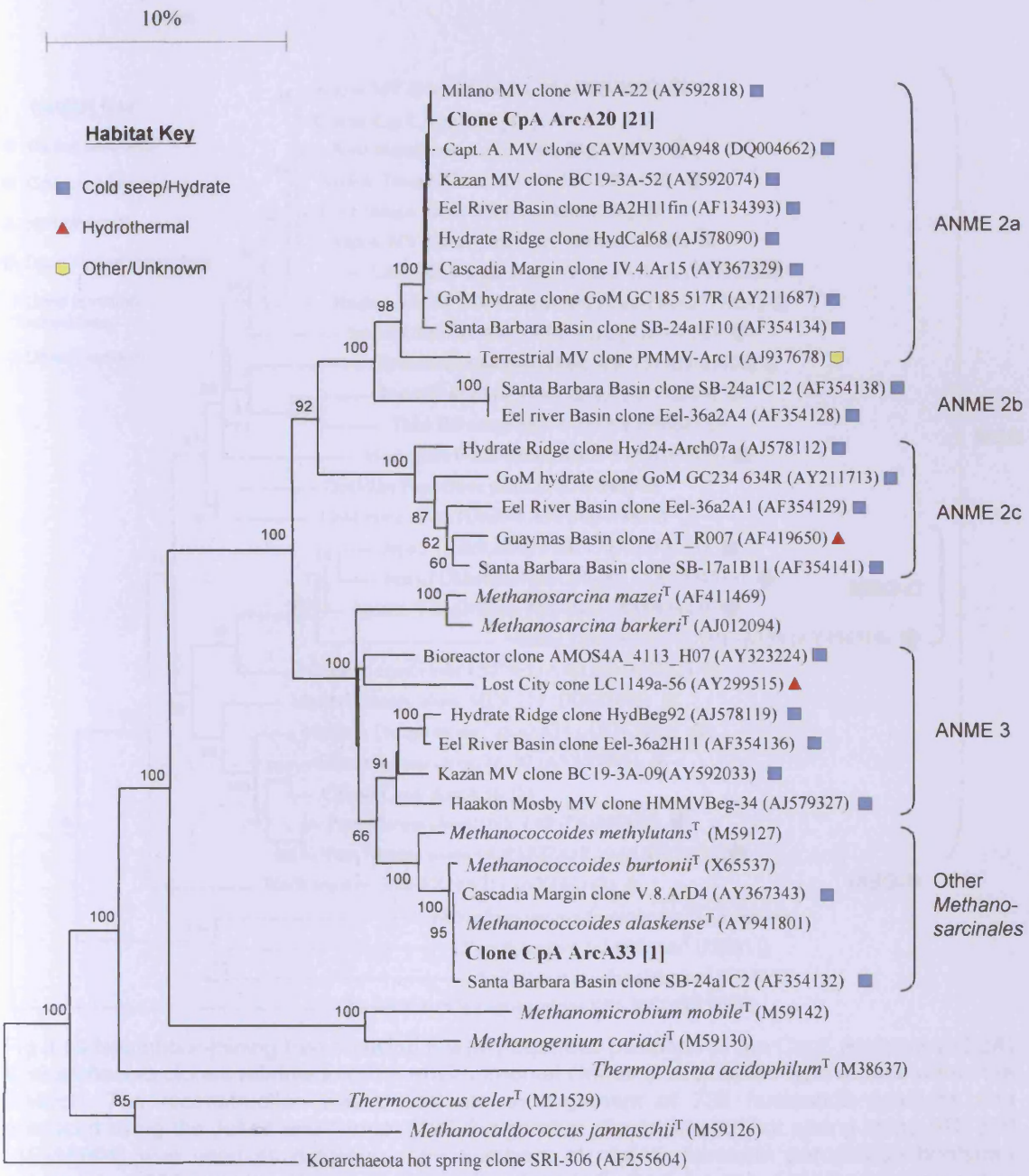


Fig 3.18 Neighbour-joining tree showing the phylogenetic positions of the Capt. Arutyunov (CpA) *Euryarchaeota* clones relative to other environmental clones and selected type strains within the Division. The reconstruction was based on an alignment of 709 nucleotide positions and produced using the Jukes and Cantor (1969) algorithm. Korarchaeota hot spring clone SRI-306 (AF255604) was used as outgroup. The numbers at nodes represent percentage bootstrap values after 1000 samples. Values in square brackets indicate the number of clones represented by the individual OTUs shown. Accession numbers are shown in parentheses.

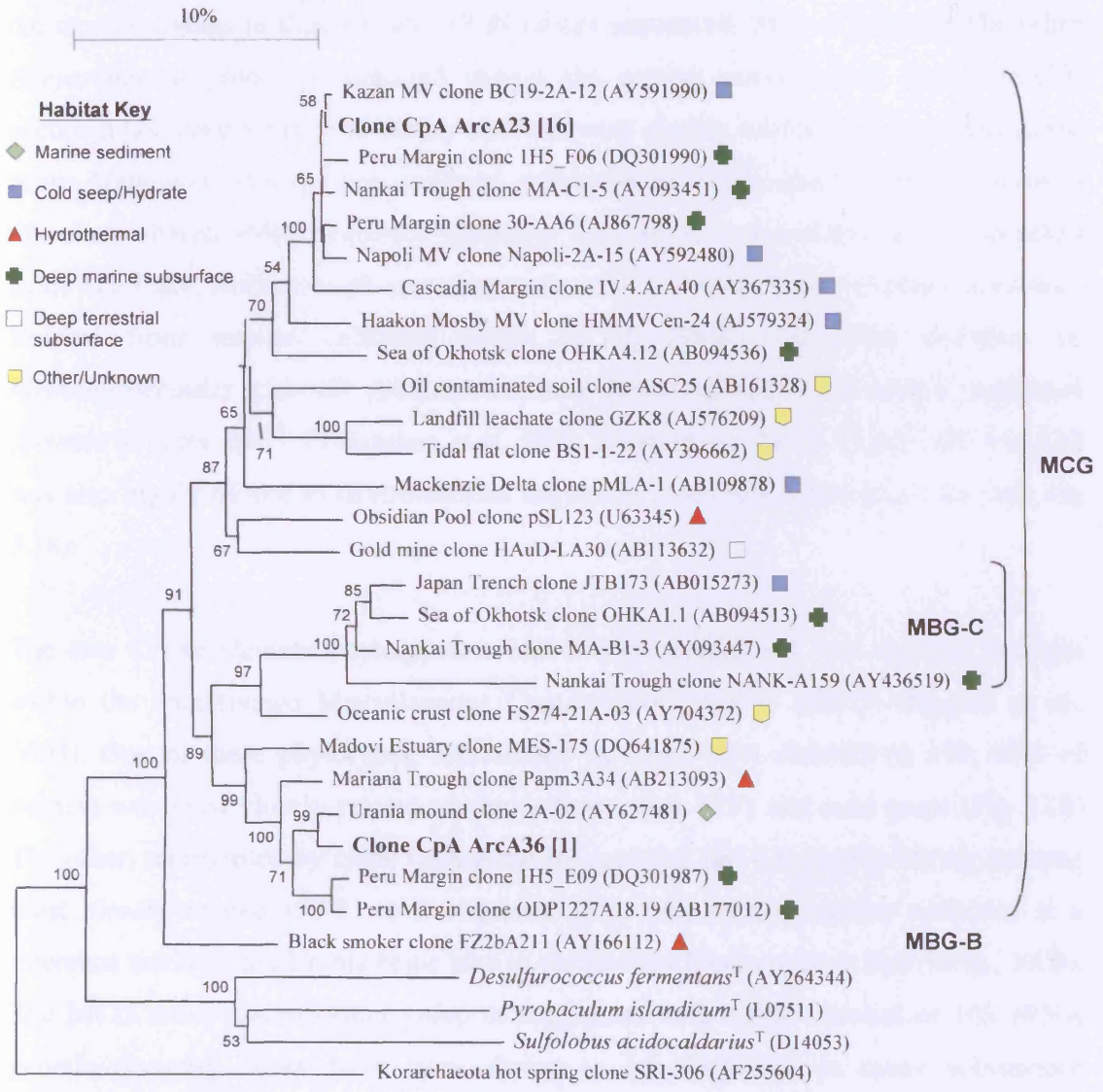


Fig 3.19 Neighbour-joining tree showing the phylogenetic positions of the Capt. Arutyunov (CpA) *Crenarchaeota* clones relative to other environmental clones and selected type strains within the Division. The reconstruction was based on an alignment of 735 nucleotide positions and produced using the Jukes and Cantor (1969) algorithm. Korarchaeota hot spring clone SRI-306 (AF255604) was used as outgroup. The numbers at nodes represent percentage bootstrap values after 1000 samples. Values in square brackets indicate the number of clones represented by the individual OTUs shown. Accession numbers are shown in parentheses.

The most numerous *Euryarchaeota* phylotype, represented by clone CpA ArcA20 (n = 21, 53% of library), was a member of the uncultivated ANME-2a group, which are known to mediate the AOM process (Orphan *et al.*, 2001; see also section 1.1.8). This group of highly related sequences have all been recovered from methane-rich habitats, including other MVs (see Fig 3.18). It is notable that one of the close relatives of clone CpA ArcA20 (CAMV300A948, DQ004662) was from the sulphate-methane transition zone (SMTZ) of the Capt. Arutyunov crater during the microbial diversity survey of

Niemann *et al.*, (2006b), see section 1.3. As in the present study, this sequence was also the most abundant in their library (23/39 clones sequenced, 59% of library). The other *Euryarchaeota* phylotype detected during the present study, clone CpA ArcA33, occurred just once within the library and was very closely related to the methanogenic genus *Methanococcoides*. The similarity matrix based on the edited alignment showed this clone shared >99% sequence similarity with *Methanococcoides burtonii*, isolated from Ace Lake, Antarctica (Franzmann *et al.*, 1992), and *Methanococcoides alaskense*, isolated from marine sediment (Singh *et al.*, 2005). Described members of *Methanococcoides* can all produce methane from methylamines and/or methanol (Sowers & Ferry, 1983; Franzmann *et al*, 1992; Singh *et al.*, 2005). Clone CpA Arc A33 was also highly related to environmental sequences from other cold seep sites (see Fig 3.18).

The two *Crenarchaeota* phylotypes in the library represented two separate lineages within the uncultivated Miscellaneous Crenarchaeotic Group (MCG) (Inagaki *et al.*, 2003). One of these phylotypes, represented by clone CpA ArcA23 (n =16, 41% of library) was most closely related to clones from other MVs and cold seeps (Fig 3.18) The other, represented by clone CpA ArcA36, occurred just once in the library and was most closely related (98%) to a sequence from near-surface marine sediment at a reference site near the Urania brine lake in the Eastern Mediterranean Sea (Heijs, 2005). The MCG are a cosmopolitan group of organisms with a high amount of 16S rRNA genetic diversity. They have been shown to be ubiquitous in many sub-surface environments (Barns *et al.*, 1994; Coolen *et al.*, 2002; Inagaki *et al.*, 2003, 2006; Parkes *et al.*, 2005; Webster *et al.*, 2006; Sorensen & Teske 2006), and the fact they have been detected, often as the dominant archaeal phylotype, in such a wide range of environments suggests they are physiologically flexible or that significant physiological diversity exists within this group (Sorensen & Teske 2006).

3.2.6 Discussion of cultivation independent molecular genetic analysis of Capt. Arutyunov and Bonjardim MVs

3.2.6.1 Extraction and amplification of DNA from MV sediment, and the construction of representative 16S rRNA gene libraries

DNA extracted from the Capt. Arutyunov and Bonjardim sediment was not readily visible by standard staining after agarose gel electrophoresis, indicating that the DNA yield from the sub-surface mud breccias was very low (e.g. ~1 ng/sample). Low DNA yield is reported as a common problem in the investigation of marine sub-surface sediments by PCR-based methods (e.g. Rochelle *et al.*, 1992; Reed *et al.*, 2002; Kormas *et al.*, 2003; Webster *et al.*, 2003; 2006) and presents a significant challenge to working with such samples. As prokaryotic cell counts normally decrease rapidly with depth in marine sediments (Parkes *et al.*, 1994, see also section 1.1), DNA yields typically also decreases as the sample depth increases. The fact these relatively shallow MV sediments had such a low DNA yield could be a reflection of their deeper sub-surface origin.

16S rRNA gene PCR amplification was particularly difficult from Bonjardim, which agreed with the lack of any visible DNA in the extracts obtained from this site. The only PCR products amplifiable from this sample were short (~200 bp) bacterial 16S rRNA gene fragments; longer bacterial 16S rRNA gene fragments and archaeal 16S rRNA gene fragments were not obtained, despite several attempts using optimized protocols. It has been found previously that PCR success is inversely related to the size of the gene fragment targeted for amplification (e.g. Webster *et al.*, 2006), and this relationship can be attributed to the fact PCR reaction kinetics favour the amplification of smaller gene fragments (Kleter *et al.*, 1998), and because shorter gene fragments are more likely to be preserved as molecular fossils in sub-surface sediment (Coolen & Overmann, 1998). One of the DGGE bands from the Bonjardim sample matched sequences of *E. coli* suggesting it was derived from the PCR reagents rather than the sediment (see section 2.3.2) and therefore a cautionary view of the authenticity of the other DGGE bands is required. As a result of the difficulty in obtaining suitable PCR products, it was not possible to further analyse the prokaryotic diversity at Bonjardim by 16S rRNA gene cloning, and the results suggest the horizon was largely biologically inert at the time of sampling. This may be a reflection of its situation >135 cm below the SMTZ, i.e. below a supply of electron acceptors, the low TOC content (< 0.5%) of Bonjardim mud breccia (Stadnitskaia *et al.*, 2008c) and the deep sub-surface source of the sediment.

Furthermore, published observational, lipid biomarker and metabolic rate measurements all indicated the current relative inactivity of this particular site (Niemann *et al.*, 2006b; Hensen *et al.*, 2007; Stadnitskaia *et al.*, 2008c). Low biomass issues should be considered when planning future diversity studies of sub-surface mud breccia samples, for example sample collection should reflect the fact that it might be necessary to extract and pool DNA from an even greater volume of sediment.

Near full-length bacterial 16S rRNA genes (~1500 bp) were readily obtained from the Capt. Arutyunov sample, consistent with the presence of some visible DNA (> 1 ng) in the extract (Fig 3.2). DGGE analysis of the near full-length products obtained from five parallel PCR reactions produced a consistent banding pattern (Fig 3.4), which matched that obtained by direct amplification of the MV DNA with the bacterial DGGE primers (Fig 3.3). These results indicated that the bacterial DNA from Capt. Arutyunov was of sufficient quality to produce a stable representation of diversity at the site. Near full-length archaeal 16S rRNA gene products (~850 bp) were only obtained from the Capt. Arutyunov sample after BSA was added to the PCR reaction mixture and the number of PCR reaction cycles was increased. Hence, the amplification of archaeal DNA required optimized protocols, whereas the amplification of bacterial DNA did not. Reed *et al.* (2002) reported similar differences in amplification success of bacterial and archaeal 16S rRNA genes from the same sample, and suggested this may have reflected the relative proportions of bacterial and archaeal cells in the original sediment, i.e. that *Bacteria* may have outnumbered *Archaea* in the sediment. This may also explain the present findings. When the archaeal 16S rRNA gene products from five parallel reactions were re-amplified and screened by DGGE they did not produce a consistent banding pattern. This may have reflected a bias in the first round PCR reactions due to a very low initial concentration of archaeal DNA in the Capt. Arutyunov sample (Chandler *et al.*, 1997; Webster *et al.*, 2003). In accordance with the findings of Webster *et al.* (2003) that low biomass marine sediment samples may be susceptible to PCR bias due to low DNA yields, the present study demonstrates the benefits of screening and pooling 16S rRNA gene products from parallel PCR reactions in order to produce representative clone libraries.

3.2.6.2 Prokaryotic diversity at and below the Capt. Arutyunov SMTZ

Comparing the results of the present study to those of Niemann *et al.* (2006b), who constructed bacterial and archaeal 16S rRNA gene libraries from the Capt. Arutyunov SMTZ, suggest the prokaryotic community structure is significantly different at and below the SMTZ. Archaeal diversity was higher at the SMTZ than below it; only four archaeal phylotypes were detected in the present study but nine archaeal phylotypes were detected at the SMTZ (Niemann *et al.*, 2006b). The two archaeal 16S rRNA gene libraries shared one common taxon – the ANME-2a group, which was dominant in both libraries (59% of sequences at the SMTZ and 53% of sequences in the present study). The other archaeal phylotypes detected at the SMTZ (ANME-2c, ANME-1, Marine benthic group D, Marine benthic group B and unclassified archaea; Niemann *et al.*, 2006b) were not detected in the present study. Bacterial diversity was slightly higher in the 16S rRNA gene library obtained in the present study than in the Niemann *et al.* (2006b) library constructed from the SMTZ (15 phylotypes and 10 phylotypes, respectively). There were no common bacterial phylotypes between the two libraries, but both libraries contained bacterial 16S rRNA gene sequences most closely related to uncultivated lineages, many of which had previously been detected in other marine seep sites. At the SMTZ the bacterial phylotypes were affiliated to Seep-SRB1; an uncultivated cluster in the *Desulfosarcina/Desulfococcus* group (*Deltaproteobacteria*), *Stenotrophomonas (Gammaproteobacteria), Clostridia, Spirochaetes* and unclassified bacteria (Niemann *et al.*, 2006b). Sequences belonging to the Seep-SRB1 group were the dominant bacteria (81% of clones) in the SMTZ library, with all other phylotypes being detected on just a single occasion (Niemann *et al.*, 2006b). The Seep-SRB1 are the sulphate-reducing *Bacteria* that act as syntrophic partners with ANME organisms in AOM-mediating consortia (Knittel *et al.*, 2003; 2005), hence their presence was consistent with the occurrence of sulphate-dependent AOM activity at the SMTZ (Niemann *et al.*, 2006b). Their absence in the bacterial library obtained in the present study is consistent with the depletion of sulphate at the depth sampled and raises the question as to whether the ANME sequences detected in the archaeal 16S rRNA gene library actually represented actively metabolizing organisms. There have been reports that under certain circumstances ANME-2 organisms may have an ability to assimilate methane without a bacterial partner (Orphan *et al.*, 2001; 2002), and it is notable that low rates of AOM activity were measured below the SMTZ at Capt. Arutyunov (Fig 4,

Niemann *et al.*, 2006b). Low rates of AOM activity were also recently measured below the SMTZ at another marine sediment site (Parkes *et al.*, 2007) and ANME sequences have been detected in other sulphate-depleted sub-surface settings (e.g. Skan Bay, Alaska; Kendall *et al.*, 2007) showing their occurrence in these environments clearly warrants further investigation. The present study in conjunction with results published by Niemann *et al.* (2006b) indicate that different prokaryotic populations exist at different depths within MV sediment, and that, more generally, compared with more active seep sites prokaryotic diversity in the Capt. Arutyunov MV is low.

3.2.6.3 Physiological considerations

Phylogenetic analysis (Figs 3.12 – 3.19) showed that many of the OTUs present in the Capt. Arutyunov 16S rRNA gene libraries were most closely related to uncultured environmental clones and only distantly related to cultured isolates. This observation was consistent with findings of other PCR-based surveys undertaken in similar environments e.g. cold seeps (Reed *et al.*, 2002; Mills *et al.*, 2004) and deep hydrate bearing sediments (Inagaki *et al.*, 2006). A lack of close characterised relatives makes speculation about the ecophysiology of organisms represented by cloned sequences problematical. It has been shown that 16S rRNA gene sequence similarity is not always a good indicator of physiological or genomic similarity (e.g. Achenbach & Coates, 2000) and the lower the 16S rRNA sequence match between two organisms the less likely it is that they will shared a common metabolic function. However 16S rRNA gene sequence similarities of >97% are typically indicative of relatedness at the species level where physiological capabilities should be more conserved (Stackebrandt & Goebel, 1994). Therefore where the near-full length Capt. Arutyunov clone sequences shared >97% 16S rRNA similarity with characterised organisms some inferences about potential physiologies could be made.

Sequences with significant similarity to characterised organisms included the most dominant sequence in the Capt. Arutyunov bacterial 16S rRNA gene library (clone CpA BacB29, 40% of library), which was 98% similar to *Desulfocapsa sulfoexigens* a mesophilic anaerobic bacterium that grows chemolithoautotrophically exclusively by the disproportionation of inorganic sulphur compounds (Finster *et al.*, 1998). Also detected was the *Epsilonproteobacteria* clone CpA BacB40 (14% of the library), which shared

just under 97% (96%) sequence similarity with the organism *Sulfurimonas autotrophica* isolated from hydrothermal sediments. The genus *Sulfurimonas* was recently amended by Takai *et al.*, (2006) and currently contains three species: *S. autotrophica*, *S. denitrificans (formerly Thiomicrospira denitrificans)* and *S. paralvinellae*, all of which are mesophilic, facultatively anaerobic chemolithoautotrophs and associated with sulphidic environments. The two *Firmicute* clones in the library (CpA BacA8 and CpA BacA6) shared >98% sequence similarity to members of the genus *Colwellia*, which contains species of generally psychrophilic marine bacteria (Jung *et al.*, 2006), all of which require salts for growth and can be characterised as facultatively anaerobic chemoorganotrophs.

The archaeal 16S rRNA gene library comprised the ANME-2a group, which have been directly implicated through combined fluorescent *in situ* hybridization and secondary ion mass spectrometry analyses (Orphan *et al.*, 2001) to be one of the anaerobic methanotrophic organisms responsible for the anaerobic oxidation of methane (AOM) (see section 1.1.8). It has been suggested these organisms can assimilate methane with or without a bacterial partner depending of environmental conditions (Orphan *et al.*, 2001, 2002), and under certain circumstances they may also have the ability to generate some methane, albeit during, rather than instead, of performing AOM (Orcutt *et al.*, 2008). A sequence closely related (>99%) to *Methanococcoides* sp. was present as a single clone in the archaeal 16S rRNA library. Described members of the *Methanococcoides* are united physiologically by their ability to catabolize methylamines, which may be present in the marine environment as plant and animal degradation products, e.g. from the breakdown of osmoregulatory compounds. Two of the three described species, *M. methylutans* and *M. burtonii*, can also grow on methanol (Sowers & Ferry, 1983; Franzmann *et al.*, 1992). The available clone library data suggested *Methanococcoides* was not an abundant component of the Capt. Arutyunov MV community but its presence is interesting since *Methanococcoides* sp. were confirmed during cultivation studies with sediment from the Meknes MV (see section 4.4.2), and methanol and methylamines have consistently been shown to stimulate methanogenesis in a range of Gulf of Cadiz MVs, including Capt. Arutyunov (B. Cragg unpublished results). The MCG, which comprised the remaining 41% of the archaeal library, are a cosmopolitan group of organisms that have never successfully been cultivated. Therefore evidence for their physiological and ecological role(s) remains purely circumstantial. However, given the

fact they have been detected, often as the dominant archaeal phylotype, in a wide range of terrestrial and marine sub-surface environments (Barns *et al.*, 1994; Coolen *et al.*, 2002; Inagaki *et al.*, 2003, 2006; Parkes *et al.*, 2005; Webster *et al.*, 2006; Sorensen & Teske 2006) would suggest members of the MCG are physiologically flexible or that significant physiological diversity exists within this group (Sorensen & Teske 2006).

Notwithstanding the limitations of using phylogenetic data alone to make physiological inferences, the sequences related to described organisms detected in the Capt. Arutyunov MV were consistent with an anaerobic community mineralising organic matter and cycling simple carbon (including methane) and sulphur compounds.

3.2.6.4 Mud volcanoes as windows to the deep biosphere?

One of the original aims of this work was to investigate whether relatively near-surface MV mud breccia harboured prokaryotes that came from greater depth. Some phylotypes present in the Capt. Arutyunov bacterial and archaeal 16S rRNA gene libraries (e.g. the MCG and some *Gammaproteobacteria* sequences) were most closely related to clones from the deep marine sub-surface (see Figs 3.12 – 3.19), perhaps supporting a deeper sediment origin of these organisms. However, the majority of phylotypes were most closely related to clones detected in near-surface marine sediment habitats, including other cold seeps (Figs 3.12 – 3.19). This suggests that over time the Capt. Arutyunov mud breccia has been colonized by organisms adapted to the present *in situ* conditions. Hence, overall it seems near-surface mud breccia may contain mixed communities with both deep and near-surface origins.

3.3 Enrichment and isolation of prokaryotes from Capt. Arutyunov and Bonjardim MV sediment

3.3.1 Capt. Arutyunov and Bonjardim sediment slurry enrichments

This section reports the first attempt to investigate prokaryotic diversity in sub-surface mud breccia sediment using as systematic cultivation-based approach. Enrichments were performed in 7 different types of liquid media, designed to target a broad range of physiological groups: autotrophic SRB, heterotrophic SRB, hydrocarbon-degrading SRB, autotrophic methanogens, heterotrophic methanogens, autotrophic acetogens and heterotrophic acetogens. The enrichments were incubated between 4 and 120°C inside a temperature gradient system in order to allow the growth of psychrophilic through to hyperthermophilic organisms potentially present within the mud breccia sediment. In total 356 enrichments were prepared. All enrichment media were anoxic and based on the saltwater medium of Widdel & Bak (1992). The main selective features of each media type and the physiological groups targeted are summarised in Table 3.2 (see section 2.4 for full details).

Table 3.2 Summary of the main selective features of the Capt. Arutyunov and Bonjardim enrichment media

Target group	Selective features of media			
	Sulphate	pH	Inhibitor	Substrate (conc)
Autotrophic SRB	+	7.2	-	H_2:CO_2
Heterotrophic SRB	+	7.2	-	Acetate (15 mM) + Lactate (5 mM)
Hydrocarbon-degrading SRB	+	7.2	-	Toluene (0.25 mM in aqueous phase)
Autotrophic Methanogens	-	7.2	-	H_2:CO_2
Heterotrophic Methanogens	-	7.2	-	Acetate (15 mM)
Autotrophic Acetogens	-	6.3	BESA[a]	H_2:CO_2
Heterotrophic Acetogens	-	6.3	BESA	Ethanol (15 mM)

[a] BESA - 2-bromethanesulfonic acid (6.6 g l^{-1}) added during medium preparation as an inhibitor of methanogens (e.g. Schink, 1994; Haveman & Pedersen, 2002 and Scholten *et al.*, 2000).

The enrichment vials were visually inspected periodically, but after 4 months of incubation none of the vials showed conclusive signs of growth. The observations that were made over this time period are summarised in Table 3.3.

Table 3.3 Summary of observations made during visual assessment of Capt. Arutyunov and Bonjardim MV temperature gradient sediment slurry enrichments

Observation	Interpretation
Change in indicator colour from colourless to pink	Indication of media redox becoming less negative. This was most likely a result of oxygen penetration of the media due to random failure of butyl stoppers over time
Slight turbidity of the enrichment	Possible indication of biomass production or alternatively due to suspension of the fine sediment fraction of the inoculum
Aggregations of particles at the headspace/medium interface	Possible indication of biomass production but alternatively due to a collection of low density inorganic particles
Development of rigid and/or shiny films at the headspace/medium interface and on the inside walls of the glass vials particularly in higher temperature enrichments (> 40°C)	Almost certainly the result of inorganic precipitations e.g. carbonate compounds and not an indication of growth. Such films were also observed in the high temperature un-inoculated controls
Enrichment dried up (all vials incubated over 90°C)	High temperature caused steam formation inside the vials; the increased pressure resulted in failure of the butyl stoppers at the point of injection and therefore liquid dried up

Despite the absence of conclusive visual signs of growth (e.g. strong turbidity, biofilm development, blackening due to sulphide production), selected enrichment vials were further assessed by epifluorescence microscopy as it is known that environmental bacteria can grow very slowly and only to low cell densities that are not always detectable with the naked eye. Due to the amount of time required to screen the enrichments by this method only the vials incubated at 5, 30, 58 and 82°C (both replicates) were examined. Quantitative cell counts were not made but an enrichment was considered positive if cells were identifiable in the majority of fields of view over a 5 minute screening period (see section 2.4.4).

Many of the vials screened by epifluorescence microscopy contained identifiable cells but these were often in low abundance (i.e. less than ten cells found during a 5 min screening period). However, others did contain quite high cell numbers, despite their lack of visual signs of growth (e.g. Fig 3.20).

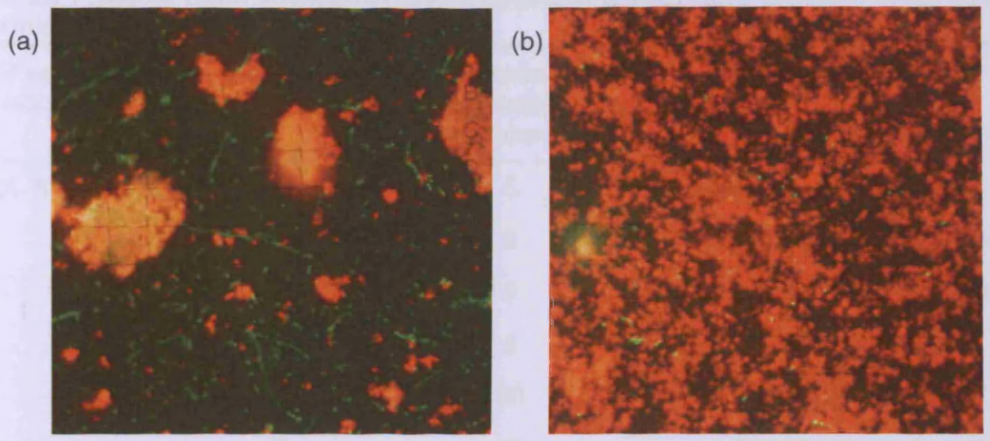


Fig 3.20 Selected microscope images of positive Capt. Arutyunov enrichment cultures stained with acridine orange and viewed under UV (green = cells, orange = inorganic particles). (a) heterotrophic SRB medium incubated at 33°C; (b) heterotrophic SRB medium incubated at 7°C. One grid square length is 10 µm.

On a few occasions cells were detectable in enrichments that had been incubated at temperatures at or above 58°C but these were never in numbers high enough to justify isolation attempts. The high temperature enrichment vials were more typified by an occurrence of large flat particles with a green colouration (Fig 3.21). These possibly corresponded to the film-like precipitates that were often observed during visual assessment of such vials (Table 3.3).

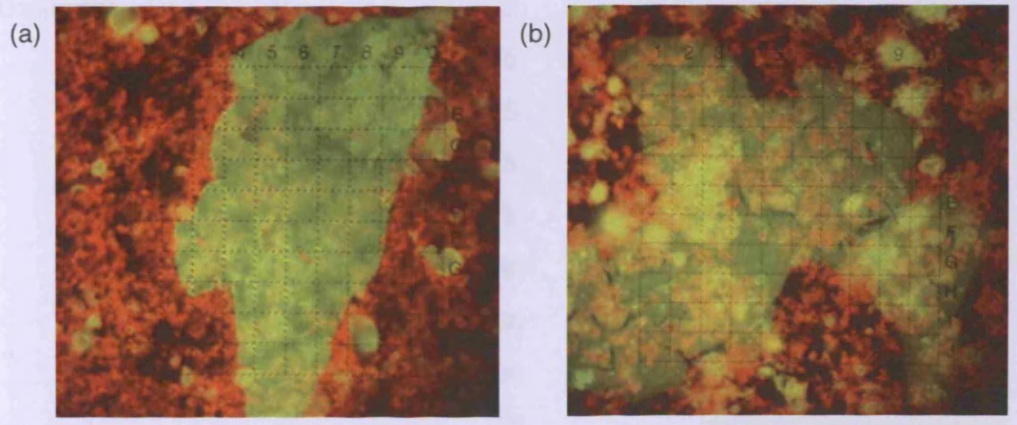


Fig 3.21 Selected microscope images of negative high temperature Capt. Arutyunov enrichment cultures stained with acridine orange and viewed under UV (green = suspected inorganic precipitates, orange = inorganic particles). (a) autotrophic SRB medium incubated at 56°C; (b) autotrophic SRB medium incubated at 82°C. Note: these observations were not restricted to just this media type.

Twenty-four of the 112 enrichments examined were determined to be positive following assessment by epifluorescent microscopy (Table 3.4).

Table 3.4 Positive Capt. Arutyunov and Bonjardim temperature gradient enrichments, as determined by microscopy.

MV sediment Inoculum	Replicate	Approximate incubation temperature (°C)	Medium
Capt. Arutyunov	B	5	Autotrophic acetogen
	A	5	Heterotrophic methanogen
	B	5	Autotrophic methanogen
	B	5	Heterotrophic SRB
	A	30	Autotrophic acetogen
	B	30	Autotrophic acetogen
	B	30	Heterotrophic methanogen
	A	30	Autotrophic methanogen
	B	30	Autotrophic methanogen
	A	30	Hydrocarbon-degrading SRB
	B	30	Hydrocarbon-degrading SRB
	A	30	Heterotrophic SRB
	B	30	Heterotrophic SRB
	A	30	Autotrophic SRB
	B	30	Autotrophic SRB
	A	30	Heterotrophic acetogen
Bonjardim	B	5	Autotrophic acetogen
	B	5	Heterotrophic methanogen
	A	5	Hydrocarbon-degrading SRB
	A	5	Heterotrophic SRB
	B	5	Heterotrophic SRB
	A	30	Heterotrophic SRB
	A	30	Autotrophic SRB
	A	30	Heterotrophic acetogen
	B	30	Heterotrophic methanogen
	A	30	Hydrocarbon-degrading SRB

As Table 3.4 shows which enrichments became positive was quite random. Sometimes both replicates of the same medium at the same temperature were determined to be positive, whereas on other occasions just one of the replicates was positive. Also the medium type seemed to have little affect on enrichment success.

Sub-samples from the positive enrichments were streaked out on aerobic and anaerobic agar plates of YPG medium in an attempt to isolate colonies (see section 2.4.5).

Unlike any of the temperature gradient incubated enrichments, two of the room temperature (RT) enrichments did go on to develop visual signs of growth. These were enrichments of Capt. Arutyunov sediment in heterotrophic SRB medium (designated replicates X and Y) and both were turbid and had a very thin biofilm at their headspace/medium interface after 4 months. These enrichments were also selected for isolation attempts and were sub-cultured into the same liquid medium. A three stage 1:25 dilution series (4% starting inoculum) was made as an alternative approach to isolation from these enrichments. RT enrichments that did not show visible signs of growth were not analysed further.

3.3.2 Capt. Arutyunov and Bonjardim sub-cultures

After four weeks of incubation several sub-cultures showed signs of growth. This included the sub-cultures made on solid aerobic and anaerobic YPG medium inoculated from the positive temperature gradient enrichments, and also those inoculated as part of the anaerobic heterotrophic SRB liquid media dilution series. Colonies developed on many of the YPG plates, while positive growth was indicated in the RT liquid enrichment sub-cultures by the development of visible turbidity. The first sub-culture of replicate X and the first two sub-cultures of replicate Y became positive. Sub-samples were taken from the original enrichments and the positive liquid sub-cultures, amplified with general bacterial 16S rRNA gene primers and analysed by DGGE. Three distinct DGGE bands were present in the replicate X samples (Fig 3.22) and two bands, equivalent to A and B in Fig 3.22, in the replicate Y samples (data not shown). The enrichments were not analyzed for the presence of *Archaea*.

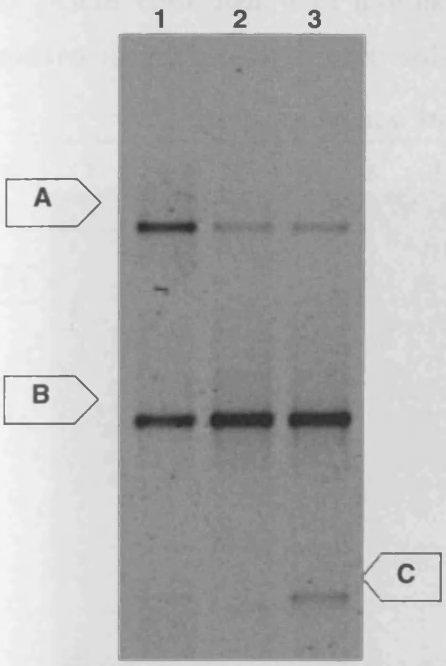


Fig 3.22 DGGE screen of a positive heterotrophic SRB medium room temperature enrichment and subsequent sub-cultures (replicate X). Lane 1, original enrichment; lane 2, sub-culture 1; lane 3, sub-culture 2. Band position A, *Pseudomonas*-related organism; band position B, *Arcobacter*-related organism; band position C, un-resolvable by sequencing.

Sequencing the DGGE bands showed band 'A' represented an organism affiliated to *Pseudomonas* and band 'B' represented an organism related to *Arcobacter*. The band labelled 'C' could not be resolved by sequencing as a mixed sequence signal was always obtained, which was probably due to interference from DNA from the strong *Arcobacter* band at position 'B' above. Since DGGE analysis showed the highest positive dilution still contained a mixed culture additional isolation attempts were made by streaking out sub-samples of the positive enrichments onto aerobic and anaerobic YPG plates as for the positive temperature gradient enrichments.

3.3.3 Isolation of pure cultures

After four weeks of incubation at the same temperature as the source enrichment, visible colonies developed on many of the YPG plates incubated under both aerobic and anaerobic incubations. Individual colonies were selected from these plates for purification by repeated re-streaking (minimum of three times) if they displayed a distinct morphology, but also if they had been inoculated from a different enrichment vial. Using this conservative method a total 56 strains were isolated (16 from Bonjardim enrichments and 40 from Capt. Arutyunov enrichments). DGGE was used to assess the purity of the isolated strains and to sort them into different phylotypes according to their banding pattern (section 2.4.6). DGGE analysis of both the aerobic and anaerobic isolates revealed there were only four phylotypes in the Capt Arutyunov culture collection (e.g. Fig 3.23) and only a single phylotype in the Bonjardim culture collection (data not shown). During the screening period it was consistently shown that the results

of DGGE correlated with morphological observations of colonies, and this allowed putative identification of some isolates to be made on a visual basis alone.

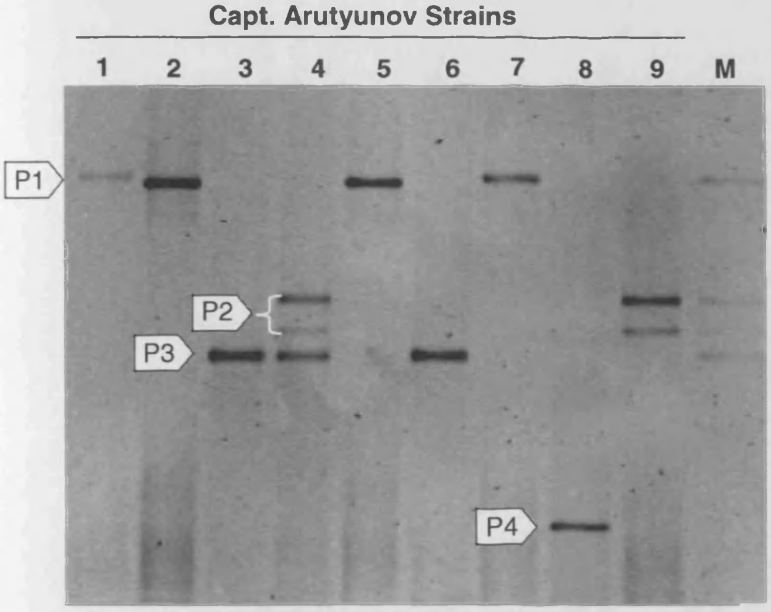


Fig 3.23 Example of a DGGE gel used to screen isolates from the Capt. Arutyunov culture collection. Lanes 1 – 9 contained the bacterial 16S rRNA genes amplified from individual strains following purification over at least 3 successive re-streaks. Lane M was an early version of the DGGE marker used to identify and assign different phylotypes (see section 2.4.6). Banding patterns labelled P1 – P4 show the different phylotypes present in this culture collection

Sequencing the 16S rRNA gene of bacterial strains representative of the four phylotypes present in the Capt. Arutyunov culture collection identified them as belonging to four different genera: *Pseudomonas*, *Marinobacter* [double band due to double 16S rRNA gene operon (Klappenbach *et al.*, 2001)] *Arcobacter* and *Halomonas,* while sequencing a strain representative of the single phylotype present in the Bonjardim culture collection showed it belonged to the genus *Pseudomonas* (see Table 3.5).

Additional sequencing of randomly selected isolates showed that banding pattern was a reliable indicator of identity and did not detect any further phylotypes. Full details of the isolates present in the Capt. Arutyunov and Bonjardim culture collections are summarized in Table 3.6

Table 3.5 Results of bacterial 16S rRNA gene sequencing of phylotypes within the Capt. Arutyunov and Bonjardim culture collections

MV	Phylotype (see Fig 3.23)	Sequence length (bp)[c]	Closest match in BLASTN database (Accession number)[a]	Identities (%)	Phylogenetic affiliation	Origin of closest matching sequence(s)
Capt. Arutyunov.	P1	605	Pseudomonas sp. JPL-1 (AY030314)	603/603 (100)	*Pseudomonas*	Strain JPL-1
	P2	135	Marinobacter sp. ws22 (AJ704789)	135/135 (100)	*Marinobacter*	Deep-sea station of the Pacific Nodule Province
	P3	620	Uncultured bacterium clone "GZKB9" (AJ853504)	614/6220 (99)	*Arcobacter*	Landfill leachate, Guangdong Province, China
	P4	146	Halomonas isolate HY-B2 (AJ294346)	144/146 (98)	*Halomonas*	Hydrothermal fluids of the North Fiji Basin
Bonjardim	P5[b]	144	Pseudomonas stutzeri strain 24a50 (AJ312171)	144/144 (100)	*Pseudomonas*	Soil

[a] Where multiple sequence matches were identified, the top match listed in the BLASTN results pages was arbitrarily selected for inclusion in the table.
[b] Not shown in fig 3.23, taken from a representative of the one phylotype in Bonjardim culture collection.
[c] Some isolates were sequenced with the 27F primer while others were sequenced with the 518R primer resulting in different sequence lengths.

Table 3.6 Summary of strains in the Capt. Arutyunov and Bonjardim MV culture collections

The strain names indicate isolation from either Capt. Arutyunov (CpA) or Bonjardim (Bon) MV sediment. Original enrichment media is abbreviated as follows: Auto SRB – Autotrophic SRB medium, Het SRB – Heterotrophic SRB medium, HC SRB – Hydrocarbon utilizing SRB medium, Auto Meth – Autotrophic methanogen medium, Het Meth – Heterotrophic methanogen media, Auto Aceto – Autotrophic acetogen medium, Het Aceto – Heterotrophic acetogen medium.

Strain name	Original enrichment media	Isolation conditions	Enrichment/isolation temperature	Putative phylotype according to DGGE band position
CpA_a1*	Het. Meth.	Aerobic	6°C	*Halomonas*
CpA_a2	Het. Meth.	Aerobic	6°C	*Pseudomonas*
CpA_a3	Auto Aceto	Aerobic	33°C	*Pseudomonas*
CpA_a4*	Het Aceto	Aerobic	33°C	*Pseudomonas*
CpA_a5*	Auto Meth	Aerobic	33°C	*Arcobacter*
CpA_a6	Duplicate[a] of strain CpA_a5			
CpA_a7*	Auto SRB	Aerobic	33°C	*Marinobacter*
CpA_a8	Duplicate of strain CpA_a7			
CpA_a9	Het SRB	Aerobic	Room temperature	*Pseudomonas*
CpA_a10	Het SRB	Aerobic	Room temperature	*Pseudomonas*
CpA_a11	Duplicate of strain CpA_a9			
CpA_a12	Het SRB (X)	Aerobic	Room temperature	*Pseudomonas*
CpA_a13*	Het SRB (X1)	Aerobic	Room temperature	*Marinobacter*
CpA_a14	Het SRB (X1)	Aerobic	Room temperature	*Pseudomonas*
CpA_a15	Het SRB (Y2)	Aerobic	Room temperature	*Arcobacter*
CpA_a16	Auto SRB	Anaerobic	33°C	*Mixed – Marinobacter and Arcobacter*
CpA_a17*	Auto Aceto	Anaerobic	33°C	*Pseudomonas*
CpA_a18	Auto. Meth.	Anaerobic	33°C	*Arcobacter*
CpA_a19	Duplicate of strain CpA_a19			
CpA_a20	Het Aceto	Anaerobic	33°C	*Pseudomonas*
CpA_a21	Duplicate of strain CpA_20			
CpA_a22	Auto Aceto	Anaerobic	33°C	*Pseudomonas*
CpA_a23	Het SRB (X1)	Anaerobic	33°C	*Marinobacter*
CpA_a24	Het SRB (X1)	Anaerobic	33°C	*Halomonas*
CpA_a25	Het. SRB (Y2)	Anaerobic	33°C	*Pseudomonas*
CpA_b1	Auto. Meth.	Aerobic	6°C	*Pseudomonas*
CpA_b2*	Auto. Meth.	Aerobic	6°C	*Halomonas*

Table 3.6 cont. Summary of strains in the Capt. Arutyunov and Bonjardim MV culture collections

Strain name	Original enrichment media	Isolation conditions	Approximate enrichment/isolation temperature	Putative phylotype according to DGGE band position
CpA_b3	Het. SRB	Aerobic	6°C	*Pseudomonas*
CpA_b4	Het. SRB	Aerobic	6°C	*Halomonas*
CpA_b5	Auto. Meth.	Aerobic	33°C	*Pseudomonas*
CpA_b6*	HC SRB	Aerobic	33°C	*Arcobacter*
CpA_b7	Auto. Aceto.	Aerobic	33°C	*Pseudomonas*
CpA_b8	Auto. SRB	Aerobic	33°C	*Pseudomonas*
CpA_b9	Het. Meth.	Anaerobic	33°C	*Arcobacter*
CpA_b10	Auto. SRB	Anaerobic	33°C	*Pseudomonas*
CpA_b11	Auto. Meth.	Anaerobic	33°C	*Pseudomonas*
CpA_b12	Duplicate of strain CpA_b11			
CpA_b13	Duplicate of strain CpA_b9			
CpA_b14	Auto. Aceto.	Anaerobic	33°C	*Pseudomonas*
CpA_b15	Duplicate of strain CpA_b14			
Bon_a1*	Auto. SRB	Aerobic	33°C	*Pseudomonas*
Bon_a2	Het. SRB	Aerobic	33°C	*Pseudomonas*
Bon_a3	Duplicate of strain Bon_a2			
Bon_a4	Het. Aceto.	Aerobic	33°C	*Pseudomonas*
Bon_a5	Duplicate of strain Bon_a4			
Bon_a6	Auto. SRB	Anaerobic	33°C	*Pseudomonas*
Bon_a7	Duplicate of strain Bon_a6			
Bon_a8	HC SRB	Anaerobic	33°C	*Pseudomonas*
Bon_a9	Duplicate of strain Bon_a8			
Bon_a10	Het. Aceto.	Anaerobic	33°C	*Pseudomonas*
Bon_a11	Duplicate of strain Bon_a10			
Bon_b1*	Het. SRB	Aerobic	6°C	*Pseudomonas*
Bon_b2	Auto. Aceto.	Aerobic	6°C	*Pseudomonas*
Bon_b3	Het. Meth.	Aerobic	33°C	*Pseudomonas*
Bon_b4	Duplicate of strain Bon_b3			
Bon_b5	Het. Meth.	Anaerobic	33°C	*Pseudomonas*

[a] Strains were considered duplicates if isolated twice under the same conditions and from the same original enrichment source.
*Identifies the strains selected for full 16S rRNA gene sequencing and physiological characterisation, see chapter 5.

N.B. The assignment as either an 'a' or 'b' isolate was a reflection of which of the two replicate temperature gradient enrichments the strains were isolated from.

In total 31 strains from Capt. Arutyunov enrichments, and 10 strains from Bonjardim enrichments were isolated, not including duplicates (see footnote to Table 3.6). Organisms affiliated to the genus *Pseudomonas* were the most frequently isolated in both the MV culture libraries (19 isolates from the Capt. Arutyunov enrichments and 10 isolates from the Bonjardim enrichments). *Halomonas, Marinobacter* and *Arcobacter* sp. were isolated on 4, 3 and 5 separate occasions from the Capt. Arutyunov sediment, respectively. Similarly to the *Pseudomonas* isolates these organisms all came from more than one enrichment source. What was striking from the culture collection results was the consistent isolation of the same phylotypes from different enrichments, i.e. there was no correlation between enrichment media type, incubation temperature or aerobic/anaerobic incubation and the ultimate isolation of a given phylotype (see section 3.3.4).

Two representative strains of each isolate/OTU in the Capt. Arutyunov and Bonjardim culture collection were selected for physiological characterisation and more detailed phylogenetic analysis (see Table 3.6). The results of this work are presented in Chapter 5.

3.3.4 Discussion of Capt. Arutyunov and Bonjardim enrichments and isolates

Enrichments incubated inside temperature gradient systems for four months did not develop biomass in quantities sufficient to be detected visually. However significant cell numbers were detected in some enrichments following screening by epifluorescent microscopy. This highlights the limitations of visual assessment alone as a guide to growth of environmental bacteria in defined, low-substrate liquid enrichments. Growth may be relatively slow over the timescales typically employed in laboratories and more sensitive screening techniques are needed to avoid potentially interesting cultures being discarded as false negatives. Due to the large number of enrichments prepared with the Capt. Arutyunov and Bonjardim sediment not all could be assessed by microscopy and as a result some positive enrichments may have been lost.

Two enrichments incubated outside the temperature gradient systems at room temperature (RT) did develop visibly detectable growth but, for reasons that are not clear, the enrichments incubated inside the temperature gradient systems did not. The same media type from the same approximate temperature inside temperature gradient (33°C) showed significant cell numbers when examined microscopically (see Fig 3.20), and DGGE analysis showed the same organisms were present in the RT enrichments as were subsequently isolated from the temperature gradient enrichment vials.

As mentioned previously, the low diversity of the culture collection from the MV enrichments was striking. Repeated isolation of strains of the same organism occurred regardless of the enrichment media or isolation conditions used, i.e. aerobic/anaerobic incubation or incubation temperature. This would suggest the isolated organisms were able to grow (or at least survive) under a wide range of enrichment conditions but did not out-compete each other under these conditions. The isolates could only be grown to significant numbers once a richer medium (YPG) was used, and then subsequently purified when the physical isolation technique of repeated re-streaking on agar plates was used. The 16S rRNA gene analysis results from the liquid dilution series (Fig 3.22) and single colonies from agar plates (e.g. Fig 3.23) showed some of the strains obtained could indeed co-exist in culture. Laboratory contamination could be reasonably ruled out as a source of the organisms as their isolation was consistently random from across the range of original enrichments (e.g. it was not restricted to a single batch of media or day of preparation). Furthermore, not all enrichments produced positive sub-cultures and stringent contamination controls were used throughout the development of the culture collection. Checks of the effectiveness of such controls (e.g. open agar plates left out during a batch of streaking) were consistently negative. Isolates affiliated with the group *Pseudomonas* were cultured most often and were found in both libraries making these the most likely candidates as contaminants. However, the full 16S rRNA gene sequencing and physiological work conducted as part of the detailed characterisation work showed the two MV samples yielded different strains, again making contamination an improbable explanation (see section 5.4).

One necessary consideration when interpreting the cultivation results from the Bonjardim and Capt. Arutyunov MVs is that the community could have shifted from that *in situ* during sample storage, which was sub-optimal for these samples (see section

2.1.1). Previously it has been shown that sub-surface (terrestrial) sediments yield a greater number of aerobic heterotrophic bacteria following sample storage, though interestingly, with little or no increase in total cell numbers (Haldeman *et al.*, 1994; 1995; Fredrickson *et al.*, 1995; Chandler *et al.*, 1997b; Brockman *et al.*, 1998). However, that the community had dramatically shifted from that reasonably expected to be present *in situ* in the MV sub-surface environment (e.g. organisms with anaerobic metabolisms suited to subseafloor conditions/organisms related to other seep-related taxa), was not borne out by the results of the molecular genetic study (see section 3.2.4), although the samples for molecular analysis were taken from the stored sediment at the same time as those used for culturing. This, therefore, suggests perturbation of the original community structure was not a significant problem for these samples, but that the taxa obtained by cultivation methods only represented a very small and relatively unrepresentative subset of those revealed by molecular methodologies, as was also found with other sub-seafloor sediments (e.g. D'Hondt *et al.*, 2004; Toffin *et al.*, 2004). That these samples appeared not to have been significantly affected by their sub-optimal storage might be a reflection of the low substrate content of the mud breccia, the compacted nature of the sediment impeding pore water movement over time, and that the core material remained intact.

Systematic records of cell morphologies observed during the epifluorescent microscopic screening of enrichment vials were not made, but anecdotal observations suggested cell types additional to those obtained in the culture collections (see chapter 5) were present in the original enrichments. It is possible that other organisms present in the original enrichments were not suited to colony formation on solid media and hence were excluded from the culture collection. The majority of strains isolated were most closely related to other cultured organisms reflecting the suitability of these phylotypes to enrichment and isolation under laboratory conditions. Both the Capt. Arutyunov and Bonjardim culture collections were dominated by strains of the *Pseudomonas* genus suggesting an enrichment bias towards this particular group. *Pseudomonas* organisms are known to be particularly suited to growth under standard laboratory conditions, and this is reflected by the large number of characterised isolates within the group, i.e. 169 species and 8 sub-species (Euzéby, 1997, accessed 04/2007).

The culture collection also contained a large number of duplicate strains, which resulted from a conservative approach to distinguishing different organisms on the basis of morphology alone. Colonies with minor morphological differences were isolated separately but were often found to represent the same phylotype (OTU) after subsequent DGGE screening. The *Pseudomonas* sp. strains were particularly susceptible to duplicate isolation as these colonies displayed moderate morphological variation, for example in their average colony size and degree of pigmentation.

3.4 Chapter 3 synthesis and summary

3.4.1 Comparison of diversity revealed by cultivation and PCR-based approaches in the Bonjardim and Capt. Arutyunov MVs

Bacterial diversity in the sub-surface of Bonjardim was very low according to both cultivation and PCR-based approaches. A lack of reproducibly amplifiable near full-length 16S rRNA PCR products from the Bonjardim sediment DNA precluded a detailed molecular-based analysis of diversity at the site. The assessment of diversity was therefore limited to the results of DGGE screening short length sediment-derived PCR products, and by cultivation. *Pseudomonas* was the only genus detectable in the Bonjardim sub-surface by both these approaches (sections 3.2.1 and 3.3.3). The two Bonjardim strains isolated and selected for further characterisation work (98% related to each other, see Chapter 5) both shared 94% and 98% sequence similarity with the bacterial 16S rRNA gene DGGE bands 'Bon a' and 'Bon b', respectively (see Table 3.1). In this case the results of molecular and cultivation work concurred in identifying only very limited prokaryotic diversity restricted exclusively to members of the ubiquitous *Pseudomonas* group. However, it has been frequently observed that cultivation and molecular approaches yield fundamentally different results (e.g. Amann *et al.*, 1995; Pace 1997; Dunbar *et al.*, 1997; Zengler *et al.*, 2002), and this was indeed the case in the study of prokaryotic diversity in the Capt. Arutyunov sample. Here the specific findings of the PCR-based and cultivation approaches differed significantly. The bacterial and archaeal 16S rRNA gene libraries detected sequences that tended to group with other uncultivated molecular-derived sequences (sections 3.2.4 and 3.2.5), while the majority of the strains isolated from the sediment were related to other cultured organisms (Table 3.5).

Generally, the cultivation based results seemed to reflect the effect of enrichment bias towards a minority culturable fraction, within an already low diversity community.

3.4.2 Prokaryotic diversity in the Bonjardim and Capt. Arutyunov sub-surface

Prokaryotic diversity at the Bonjardim site was extremely limited suggesting the horizon sampled for the present study was relatively microbiologically inactive. This is probably a reflection of the sample horizon being ~205 mbsf (205 – 240 cmbsf, pooled sample), within deep-sourced mud breccia, and at least 135 cm below the reaction front of the sulphate-methane transition zone (45 – 70 cmbsf) where prokaryotic activity is stimulated. Furthermore, fluid flow was low at the site and the available observational and geochemical data all suggested the site to be relatively inactive at the time of sampling (section 3.1.2, Niemann *et al.*, 2006b).

The prokaryotic diversity detected at Captain Arutyunov (126 – 270 cmbsf, pooled sample), even when the results of the PCR and cultivation-based approaches were combined, was low when compared to other global cold seep sites. However, microbial diversity studies of other cold seep sediments have until now mainly been restricted to active near-surface sediments and/or those within the SMTZ (e.g. Li *et al.*, 1999a, 1999b; Mills *et al.*, 2003; Lee *et al.*, 2004; LaMontagne *et al.*, 2004; Joye *et al.*, 2004; Arakawa *et al.*, 2006; Fang *et al.*, 2006; Niemann *et al.*, 2006a, 2006b; Wegener *et al.*, 2008a) with very little attention given to the associated deeper methanogenic sub-surface sediments. This makes the results of the present study in some ways more akin to those of sub-surface deep biosphere methanogenic sediment diversity studies, albeit within a different environmental context [e.g. Newberry *et al.*, 2004 (4,800 m water depth, low TOC marine sediments from the Nankai Trough, clone library at 4 mbsf); Parkes *et al.*, 2005 (150 m water depth, high TOC marine sediments from the Peru Margin, clone library at 35 mbsf); Inagaki *et al.*, 2006 (5,086 m water depth, methane hydrate-bearing marine sediments from the Peru Trench, clone library at ~20 mbsf); Reed *et al.*, 2002 (945 m water depth, low TOC marine sediments from the Nankai Forearc Basin, clone library at 165.5 mbsf)]. The 16S rRNA clone library data obtained in these studies often used different methodological approaches to the present study (e.g. different DNA extraction protocols and/or domain-specific 16S rRNA gene primers), which prevents direct quantitative comparison, however qualitative assessments of prokaryotic diversity can be made. Overall the most common bacterial groups were *Gammaproteobacteria* (abundant in 3 out of 4 libraries), the candidate division JS1 (abundant in 3 out of 4 libraries) and the diverse *Chloroflexi* group (abundant in 2 out of

4 libraries). Other groups detected in these libraries included *Betaproteobacteria*, *Planctomycetes*, *Deltaproteobacteria*, Gram-Positive *Bacteria*, *Bacteroidetes*, *Cyanobacteria* and the deep-branching group NT-B2 though they typically represented minority components of the detected communities and were often unique to their libraries (see Fry *et al* 2008, Fig 1). The dominant archaeal phylotypes detected in the methanogenic marine sub-surface to date have been the crenarchaeotal groups Marine Benthic Group B (dominant in 3 out of 4 libraries) and the Miscellaneous Crenarchaeotic Group (present in 3 out of 4 libraries). Unfortunately, these are two poorly defined phylogenetic groups unrelated to any cultured species, and thus their physiological function remains speculative. Based on the evidence to date it has been suggested that the Marine Benthic Group B are active members of sub-surface SMTZ environments (Biddle *et al.*, 2006; Sørensen & Teske 2006) and are the dominant *Archaea* in methane hydrate-bearing sediments (Inagaki *et al.*, 2006), while the Miscellaneous Crenarchaeotic Group are a metabolically and phylogenetically diverse group (~20% sequence variation) that utilize complex carbon compounds (Biddle *et al.*, 2006; Webster *et al.*, 2006a; Teske & Sørensen 2008). Other archaeal taxa detected as minor components in the methanogenic marine sub-surface include the crenarchaeotal groups Marine Group 1 and Marine Benthic Group A, and the euryarchaeotal groups South African Gold Mine Group, Marine Benthic Group D and methanogens (see Fry *et al.*, 2008, Fig 2). Figures 3.11 and 3.17 show that the community structure in Capt. Arutyunov differed significantly to what may perhaps be considered to be a prokaryotic assemblage indicative of the methanogenic sub-surface marine biosphere, i.e. according to the results of the studies referred to above. Although whether it is the case that the methanogenic submarine biosphere really has a 'signature community' is uncertain given our very limited sample coverage of subseafloor sediments at this time. What is noticeable is that the prokaryotic community of Capt. Arutyunov shares common taxa with previously studied seep environments (e.g. Li *et al.*, 1999; Lanoil *et al.*, 2001; Knittel *et al.*, 2003; Heijs *et al.*, 2005), for example *Deltaproteobacteria* and *Epsilonproteobacteria* implicated to have metabolisms based on sulphur compounds, and ANME organisms, perhaps suggesting the mud breccia of Capt. Arutyunov has become re-colonized by seep-associated organisms over geological time.

A comparison between the Niemann *et al.* (2006b) Capt. Arutyunov bacterial 16S rRNA gene library and that constructed in the present study, suggests the microbial community structure changes with depth through the crater, presumably reflecting the contrasting

geochemical conditions that prevail within and below the SMTZ. The two libraries were consistent, however, in the presence of a bacterial community of limited diversity; just ten OTUs were recovered in the Niemann *et al.* (2006b) library, and fourteen in the present study. Furthermore, the findings of the present study agree with the assessment of Niemann *et al.* (2006b) in that the Bonjardim site is, generally, less active than the Captain Arutyunov site.

Chapter 4 – Geomicrobiological Investigation of the Porto and Meknes MVs

4.1 Introduction

This chapter reports the study of samples collected during a cruise to the Gulf of Cadiz in July 2005 (section 2.1.2). Two sites were investigated – the deep water Porto MV and the moderate water depth Meknes MV. Analysis of the Porto site was restricted to a single core taken from the MV crater, while three cores taken along a transect (crater, slope and an off MV pelagic control) were studied from the Meknes site (Fig 2.1). Several depth horizons were investigated through the cores: 10, 50 and 110 centimetres below seafloor (cmbsf) and 10 cm from core bottom. However, the core from the Meknes crater was short, which only allowed sampling to a depth of 65 cmbsf. The main emphasis of the chapter is on the investigation of the Meknes transect samples, with a comparison to the Porto crater given where appropriate.

4.1.1 Porto site description

The Porto MV was discovered during the TTR-15 cruise and was selected for sampling for the present study as it represented a new interesting and apparently active site. The sub-circular, relatively flat feature is centred at approximately 35° 33.78 N, 09° 30.45 W and occurs in ~3850 m water depth on the Portuguese Deep Water margin (see Fig 1.19). The core recovered from the site mostly contained matrix-supported mud breccia overlain by approximately 40 cm of brown hemipelagic clay. Parts of the core were very dark in colour and a strong smell of H_2S was detected. Lower parts of the sediment section were porous, possibly as a result of degassing (TTR-15 shipboard party). Cores taken from the site during subsequent cruises recovered gas hydrates and living specimens of chemosynthetic *Siboglinidae* pogonophorans and *Acharax* bivalves (MSM cruise 1 leg 3 results), further indicating Porto as a presently active MV/seep environment.

4.1.2 Meknes site description

The Meknes MV was discovered in 2004 during the TTR-14 cruise. It is situated in the SE part of the Gulf of Cadiz centred at approximately 34° 59.10'N, 07° 04.38'W

in a water depth of ~750 m (see Figs 1.19 and Fig 2.1). Video observation made over the structure revealed heavily disturbed greenish mud breccia exposed at the surface of the crater (Cunha *et al.*, 2005). Coring the crater recovered sulphidic compacted mud breccia with evidence of gas hydrates. The MV is considered active on account of the chemosynthetic fauna assemblages (*Acharax* bivalves and *Siboglinid* pogonophoran worms) found at the crater (Cunha *et al.*, 2005), the apparent recent extrusion of mud breccia, and high concentrations of methane in the sub-surface (up to 48 ml/l) (Blinova *et al.*, 2005). Methane $\delta^{13}C$ isotopic values reached –82.53‰ PDB at the site (Blinova *et al.*, 2005), suggesting the gas contains a significant microbial component. Fluids from the Meknes crater have low chloride concentrations (see section 4.2.2) with respect to seawater, which are presumably the result of clay mineral dehydration reactions occurring at depth (Ginsburg *et al.*, 1999).

4.1.3 Scientific objectives

The scientific aims of this particular work were as follows:

- Investigate the spatial variability of the following across an active MV (Meknes):
    - Pore water geochemistry (sulphate, chloride and volatile fatty acids (VFAs).
    - Total prokaryotic cell count.
    - Bacterial and archaeal 16S rRNA gene diversity.
    - Bacterial and archaeal functional gene diversity (*dsrA* and *mcrA*).
    - Potential microbial metabolic activity (H_2:CO_2 methanogenesis, acetoclastic methanogenesis and thymidine incorporation).
    - Culturability.
- Characterise the microbial population of a further Gulf of Cadiz MV crater (Porto) and compare to findings from other sites.
- Enrich and, if possible, isolate further MV prokaryotes.

4.2 Characterisation of the Meknes and Porto MV samples

Information on sample lithology is given briefly in Table 2.2 courtesy of the TTR-15 shipboard party. The following sections describe sediment characterisation work conducted as part of the present study to provide a context for the main PCR- and cultivation- based results.

4.2.1 Prokaryotic cell counts

Total prokaryotic cell numbers were determined by the AODC method (section 2.2.2) at each of the horizons studied (see Fig 4.1). Percentages of all cells involved in division (i.e. combined divided and dividing cell counts), and the percentage of divided cells only were also determined (Fig 4.2).

Total prokaryotic cell counts at the pelagic control site adjacent to the Meknes MV (AT-578) closely followed the trend of the global average in all 4 horizons. The 10 cmbsf sample at the Meknes slope site (AT-579) was situated within a hemipelagic interval and contained 8.49 log mean total cells/cm^3, which also agreed with global average total cell count data for that depth. The lower 50 cmbsf sample, situated very close to the lithological boundary between hemipelagic sediment and mud breccia, contained total cell counts (7.47 log mean total cells/cm^3) equivalent to the lower 95% prediction limit for that depth. Counts within the dark-grey matrix supported mud breccia at 110 and 125 cmbsf were also lower than was predicted from the global regression line (7.42 and 7.34 log mean cells/cm^3 respectively). The Meknes crater site (AT-580) was interesting as cell counts were elevated above the global average in the 10 cmbsf horizon (9.25 log mean cells/cm^3) but lower than the global average in the two lower samples (7.51 and 7.76 log mean cells/cm^3) despite the fact all three horizons were within extruded mud breccia. In the Porto crater the upper 10 cmbsf sample was from an interval of hemipelagic sediment and contained 8.06 log mean total counts/cm^3, which was lower than the global average for this sediment depth.

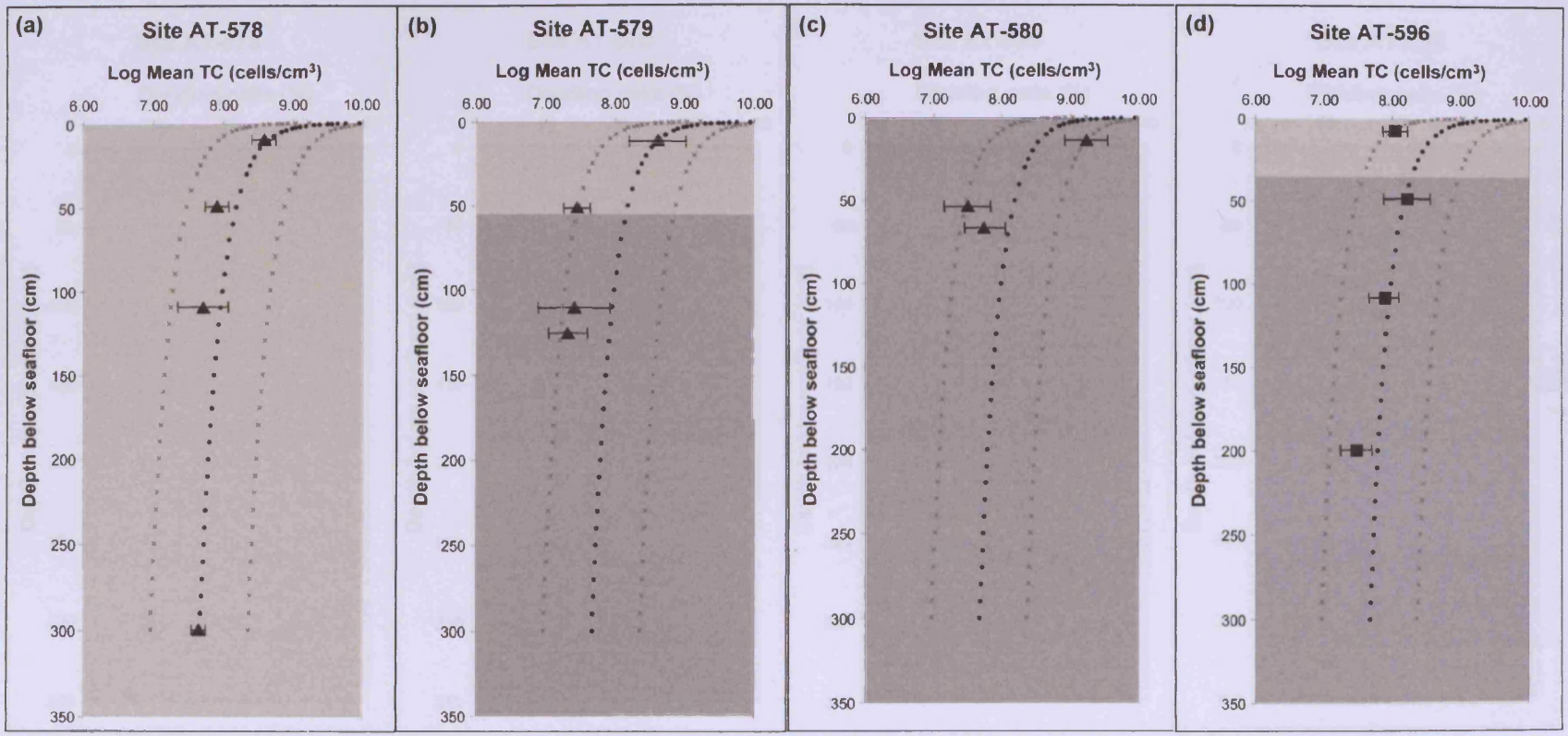


Fig 4.1 Total prokaryotic cell counts as determined by AODC (section 2.2.2) from Meknes (filled triangles) and Porto (filled squares) samples. Panel (a) site AT-578, pelagic reference core taken off vent near Meknes; panel (b) site AT-579, flank of Meknes MV; panel (c) site AT-580, crater of Meknes MV; panel (d) site AT-596, crater of the Porto MV. Light and dark grey shading indicates hemipelagic sediment and mud breccia respectively. Error bars show 95% confidence limits of count values, which were all <0.5. Small filled circles represent the global average total cell count regression line (Parkes *et al.*, 1994), crosses adjacent to this trend represent upper and lower 95% prediction limits.

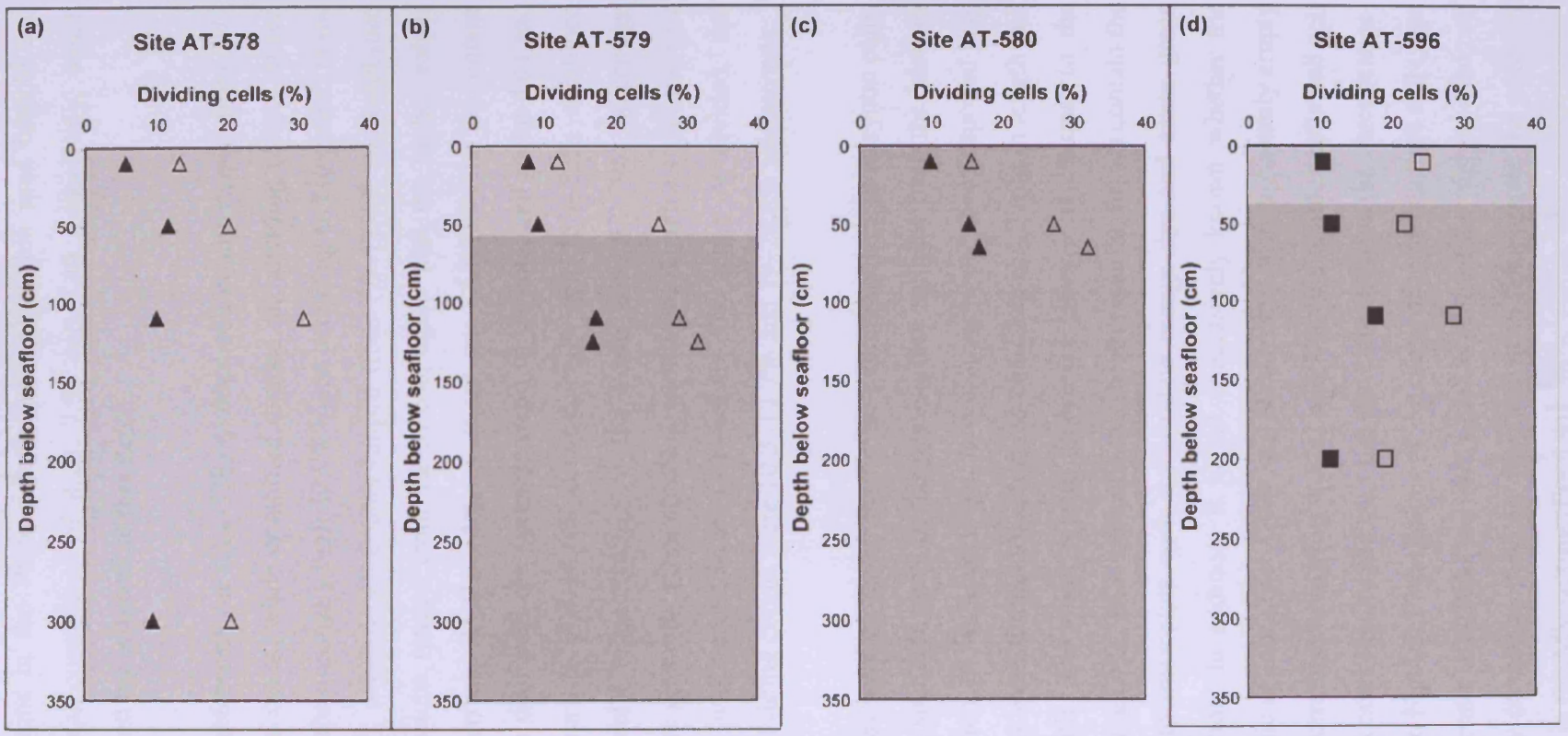


Fig 4.2 Percentages of divided cells (filled symbols) and combined divided and dividing cells (open symbols) from Meknes (triangles) and Porto (squares) samples. Panel (a) site AT-578, pelagic reference core taken off vent near Meknes; panel (b) site AT-579, flank of Meknes MV; panel (c) site AT-580, crater of Meknes MV; panel (d) site AT-596, crater of the Porto MV. Light and dark grey shading indicates hemipelagic sediment and mud breccia respectively.

The deeper three horizons in the Porto sample were all within mud breccia and contained log mean total counts/cm^3 of 8.23, 7.91 and 7.49 respectively, which mimicked the global trend for sediment at this depth.

In the Meknes pelagic reference site (AT-578) divided cell counts ranged from 5.7–11.7% of the mean total count, while combined divided and dividing counts ranged from 13.3–30.9%, with the unusually high 30.9% value occurring at 110 cmbsf. In the Meknes slope (AT-579), divided cell counts ranged from 7.9–17.4% and combined divided and dividing counts ranged from 12–31.6%, again with the higher values occurring in the sub-surface. At the Meknes crater site (AT-580) the percentage dividing cell count data displayed the opposite trend to the total cell count data and increased in the sub-surface (10–16.6% divided cells and 15.7–32% combined divided and dividing cells respectively). At the Porto site (AT-596) percentage dividing cell counts were generally more constant with depth though a slight increase in both measurements was recorded at 110 cmbsf. The range of divided, and combined divided and dividing counts were 10.3–17.7% and 19–28.6% respectively.

Varied cell morphologies were observed during the enumeration of prokaryotic cells. This included some unusual cell types in the near-surface samples from the Meknes transect (10 cmbsf), which, to my knowledge, have not previously been reported (Fig 4.3). These unusual cells were filamentous, formed chains up to ~2 mm in length and featured an outer sheath. The outer sheath fluoresced orange in contrast to the enclosed green fluorescing cells. This indicated the sheath material did not contain the nucleic acids required to intercalate with the acridine orange dye and cause green fluorescence on excitation. In addition, it is not conclusively known whether the sheath material is organic of inorganic. Outer cell sheaths that were apparently empty of internal cell chains were also seen (Fig 4.3 [c] and [f]). Filamentous cells and cell chains were observed in other deeper samples but these did not have the outer orange-stained sheaths shown in Fig 4.3. Photographs of the range of representative cell types present in the Meknes crater 10 cmbsf sample, which contained the highest total cell counts measured in any of the samples, are also shown to demonstrate the range of cell types that can exist in the MV setting (Fig 4.4).

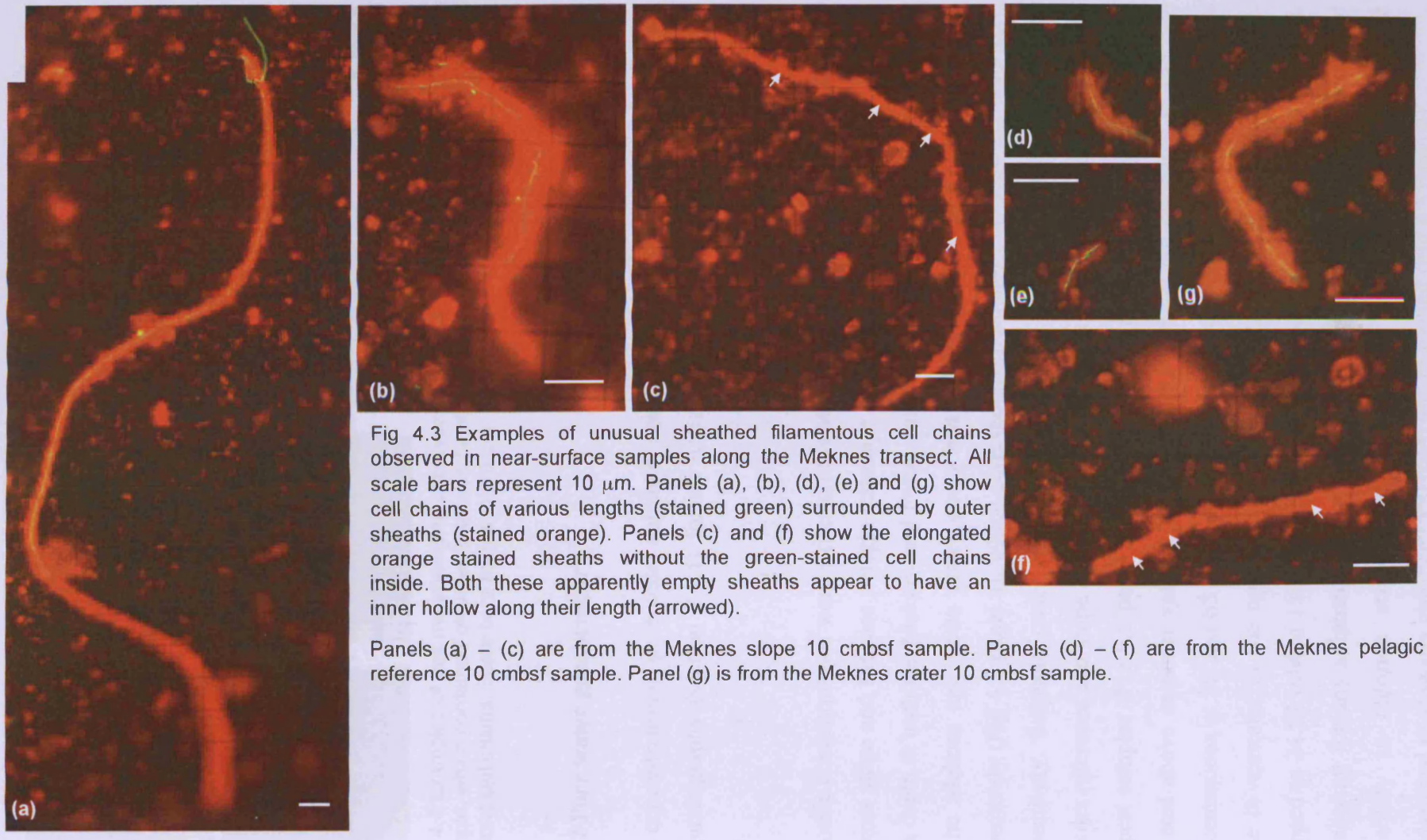


Fig 4.3 Examples of unusual sheathed filamentous cell chains observed in near-surface samples along the Meknes transect. All scale bars represent 10 μm. Panels (a), (b), (d), (e) and (g) show cell chains of various lengths (stained green) surrounded by outer sheaths (stained orange). Panels (c) and (f) show the elongated orange stained sheaths without the green-stained cell chains inside. Both these apparently empty sheaths appear to have an inner hollow along their length (arrowed).

Panels (a) – (c) are from the Meknes slope 10 cmbsf sample. Panels (d) – (f) are from the Meknes pelagic reference 10 cmbsf sample. Panel (g) is from the Meknes crater 10 cmbsf sample.

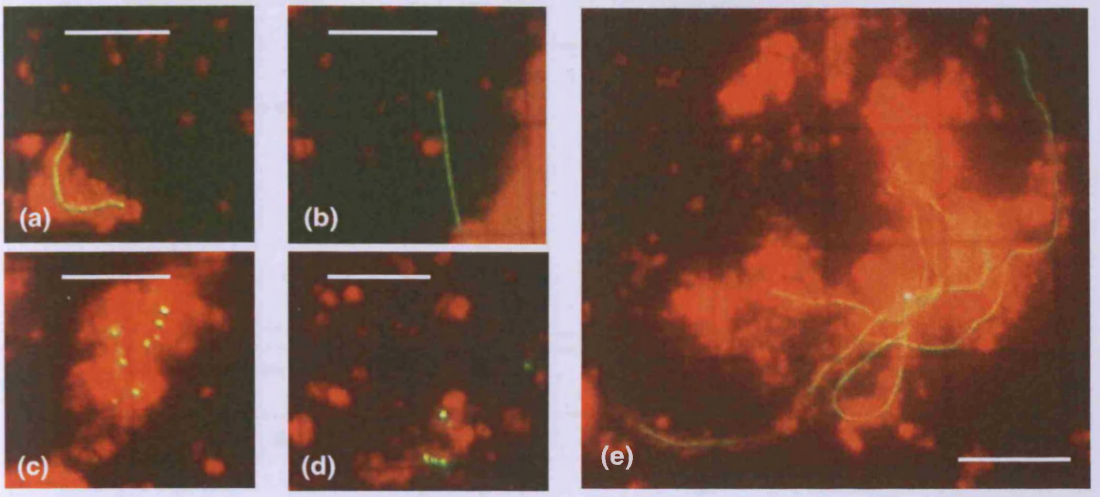


Fig 4.4 Examples of cell morphologies observed in the Meknes crater 10 cmbsf mud breccia sample, which contained elevated total cell numbers. (a) and (b) long rods; (c) and (d) cocci; (e) long cell chains. Scale bars represent 10 µm.

4.2.2 Pore water geochemistry

The concentration of pore water sulphate, chloride and acetate was determined by ion chromatography (section 2.2.1) for all horizons studied within the four cores (Fig 4.5).

Chloride concentrations were close to seawater values at all horizons analyzed in the Meknes slope and Meknes pelagic reference cores, but showed a consistent decrease with depth at both the Meknes and Porto crater sites. The deepest horizons measured in the Meknes and Porto crater cores had chloride values of 332 and 389 mM respectively (Fig 4.5 panel a), which corresponds to 60 and 71% seawater chlorinity, respectively. Sulphate concentrations followed the trend of chloride in both the Meknes reference and slope cores, and remained at over 25 mM concentrations in the deepest samples (Fig 4.5 panel b). In the Meknes crater core, sulphate was slightly elevated above seawater values (30.55 mM) at 10 cmbsf but decreased to sub mM concentrations at 50 and 65 cm depth. In the Porto crater core, sulphate values were close to seawater in the upper two horizons (29.22 and 28.96 mM, respectively), dropped to 24.57 mM at 110 cm depth, and decreased to 1.18 mM at 210 cm depth. To account for any sulphate removal due to pore water dilution and to correct for variability in chloride concentration values, all sulphate concentrations were standardized to seawater chloride (550 mM) and re-plotted (Fig 4.5 panel d).

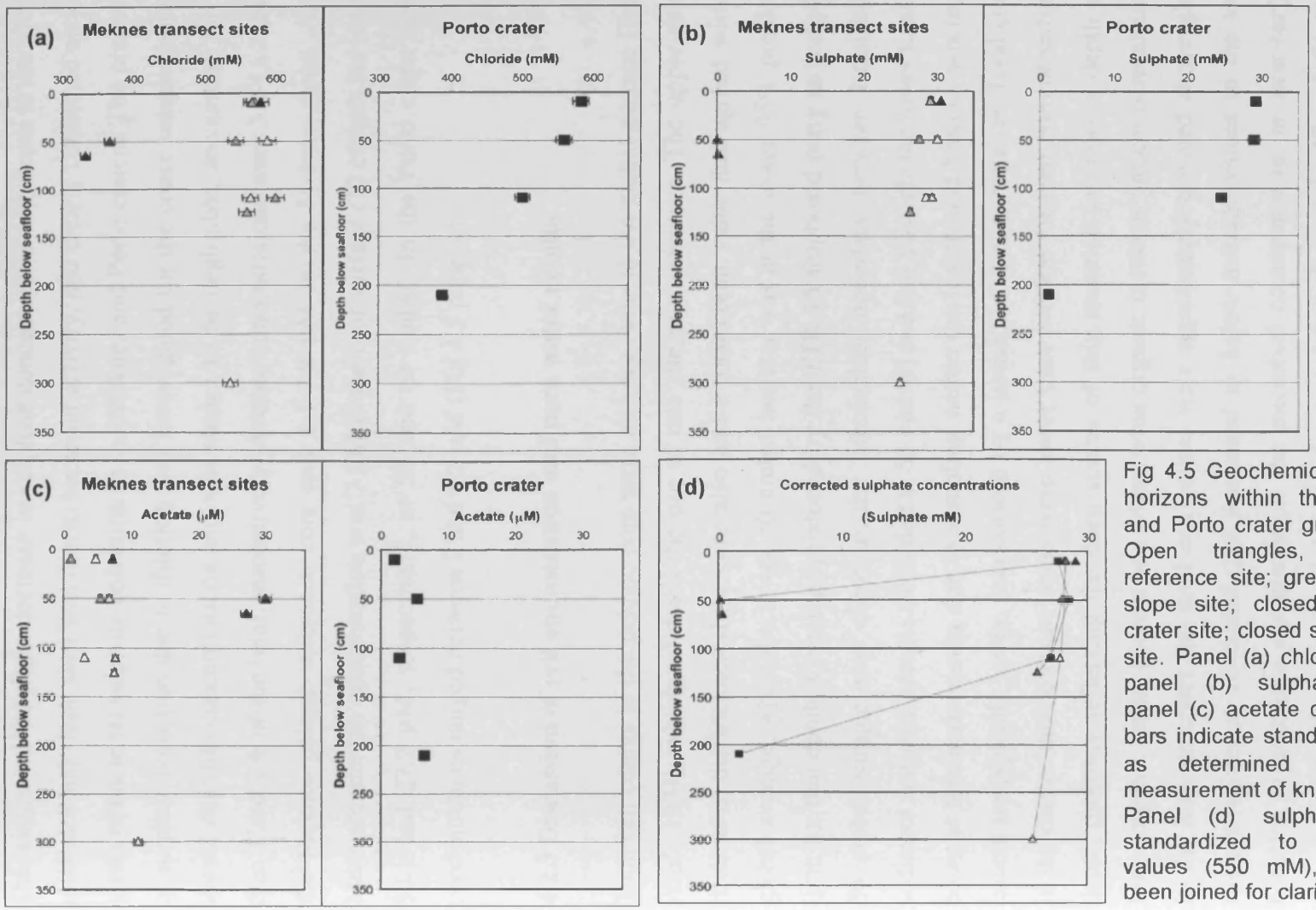


Fig 4.5 Geochemical data for selected horizons within the Meknes transect and Porto crater gravity core samples. Open triangles, Meknes pelagic reference site; grey triangles, Meknes slope site; closed triangles, Meknes crater site; closed squares, Porto crater site. Panel (a) chloride concentrations; panel (b) sulphate concentrations; panel (c) acetate concentrations; Error bars indicate standard deviations (1 σ) as determined by the replicate measurement of known standards.
Panel (d) sulphate concentrations standardized to seawater chloride values (550 mM), data points have been joined for clarity.

This suggested the slight decrease in sulphate concentration with depth at the Meknes reference and slope sites was due to bacterial activity, and clearly confirmed elevated levels of bacterial sulphate reduction in the Meknes and Porto craters. The proportion of sulphate reduction due to dilution was investigated for the crater samples with the lowest chloride concentrations and was shown to be negligible, accounting for less than 1 and 2% of the total, respectively. Acetate concentrations were 3.2-11.2 μM in the Meknes pelagic reference core and 1.1-7.8 μM in the Meknes slope. At the Meknes crater the concentration was 7 μM in the near-surface (10 cmbsf) but reached 30.1 and 27.2 μM, respectively, at 50 and 65 cmbsf. In the Porto crater acetate concentrations ranged between 2 and 6.5 μM (Fig 4.5 panel c).

4.2.3 Discussion of cell enumeration and pore water results

Total cell counts in the pelagic core were all very close to the global average (Parkes *et al.*, 1994), which validated the use of this site as a reference. The upper pelagic horizon in the Meknes slope core also had a total count value that agreed with the global average, while the lower 50 cmbsf horizon was at the lower 95% prediction limit for that depth. Core logs produced by the TTR-15 shipboard party indicated the 50 cmbsf sample was close to the lithological boundary between hemipelagic sediment and mud breccia (recorded at 55 cmbsf) and it is possible the lower total cell count at this horizon was due to sampling within the lower mud breccia, where cell counts are typically lower, necessitated by a limitation of core material. Total counts in the deeper mud breccia layers were lower than average for their current sediment depth, probably reflecting the deep source of this material, i.e. from a depth with lower organic matter content and where what organic matter is present is recalcitrant. In the Meknes crater the total cell counts were significantly elevated above global average values at 10 cmbsf, but decreased to below average values in the lower horizons suggesting a stimulation of the microbial community in the near-surface. Pore water data from the Meknes crater (section 4.2.2) showed the 10 cmbsf interval contained 29 mM sulphate, while the lower horizons had <1 mM sulphate as a result of sulphate reduction. The situation of the 10 cmbsf horizon above the SMTZ, may explain the apparent stimulation of the microbial community, i.e. with the community exploiting the increased availability of more oxidized electron acceptors (e.g. sulphate) and an increased flux of reduced deep geosphere energy sources in the

crater mud breccia, where fluid flow and energy supply is presumably greater. For example, in the crater elevated acetate concentrations were found at 50 and 65 cmbsf but these were reduced to μM concentrations in the near-surface sulphate reduction zone (see Fig 4.5).

In contrast, total cell counts at the Porto crater (3,850 m) were lower than the global average in the overlying pelagic horizon, probably reflecting the very low energy supply (particulate organic carbon) that reaches pelagic sediment at this water depth (Jahnke, 1996). Counts in the sub-surface mud breccia were closer to the global average for their depth, which is perhaps surprising given the inferred deep source of this sediment. It is possible the prokaryotic population in sub-surface mud breccia of the Porto crater have been stimulated by a supply of geosphere energy coinciding with a relatively high concentration of electron acceptors such as sulphate at these depths (Fig 4.5). The measurement of lower cell counts in the pelagic layer relative to the mud breccia horizon below was independently supported by the relative yields of DNA obtained from these two samples (see section 4.3.1).

Calculating the percentage of total cell counts that were comprised of combined dividing and divided cells, produced values that were higher than expected, i.e. above literature values for these sediment depths. To further assess the reliability of the data, and to account for the possibility that short cell chains were miscounted as actively dividing cells, the percentages were recalculated from the number of divided cells only. Divided cells (i.e. identical cells with a clear separation between them [e.g. Fig 2.2, panel a]) are much less prone to being wrongly categorized and can still provide a conservative measure of relative microbial activity in a sample. This showed that for certain horizons (e.g. the Meknes reference 110 cmbsf sample) the combined data did seem artificially high and for this reason only the divided cell count data are discussed further. However, divided cell count data must also be viewed with some caution as an indicator of microbial growth, since sediment bacteria may remain attached for long and variable periods of time following division (Newell & Fallon, 1982; Fallon *et al.*, 1983; Parkes *et al.*, 1990). This can be a function of both the particular organisms present and the physical character of the sediment matrix (Wellsbury *et al.*,

1996). These data are therefore best interpreted within the context of other relevant data – geochemistry, enrichment data and DNA yields, etc.

The percentage of divided cells remained fairly constant in the reference site, which was consistent with a lack of chemical interfaces in the pore water chemistry. The percentage of divided cells in the Meknes slope site increased slightly from just below 10% in the two upper hemipelagic horizons to just above 15% in the lower mud breccia horizons. The reason for this increase is unclear as there was no corresponding evidence for microbial stimulation in the pore water data but it is notable that this increase corresponded with the lithological change from hemipelagic to mud breccia sediment. In the Meknes crater divided cell counts also increased slightly with depth, which was contrary to the trend of the total cell counts. These increases coincided with a 3-fold increase in acetate concentration but it is not clear whether the acetate concentration increase was a result of bacterial stimulation or a response to the shift from a sulphate reducing to a methanogen community. In the Porto crater divided cell counts were fairly constant at around 10% of the total. The one higher value (18%) at 110 cmbsf coincided with the start of rapid sulphate reduction (Fig 4.5).

In the Meknes and Porto craters the pore water chloride concentrations consistently decreased with depth. The most likely explanation for this drop in chlorinity is pore water dilution caused by an upward advection of fresher fluids from depth (Ginsburg *et al.*, 1999; Niemann *et al.*, 2006; Haese *et al.*, 2006; Hensen *et al.*, 2007). At temperatures 80–100°C the hydrous clay mineral smectite is diagenetically altered to illite, and inter-crystalline water is released (Colten-Bradley, 1987). Upward advection of fluid from the depths where this reaction takes place can reasonably be assumed to occur in the two relatively active MV craters where the freshening is observed. The alternative possibility that the freshening was a result of gas hydrate dissociation (e.g. Martin *et al.*, 1996) seemed less likely since there were few instances of macroscopic hydrate in the cores studied and the chloride concentrations appeared to decrease linearly rather than erratically.

Correcting the pore water sulphate concentrations to a standard (seawater) chloride value showed the sulphate removal in the Meknes and Porto craters was significantly

above (>98%) that which could be explained by the dilution alone. Under the prevailing conditions (i.e. in situ temperature and pressure) this extra sulphate removal could only be attributed to bacterial sulphate reduction and not thermochemical sulphate reduction. The use of chloride-corrected sulphate concentrations as an indicator of bacterial sulphate reduction was possible since chloride can be assumed to be a conservative tracer under the in situ conditions, and there was no evidence to suggest the relative abundances of chloride and sulphate in the crater pore water would naturally be very different to that of modern seawater (e.g. because of brine incursions or because the mud breccia pore water was derived from something other than seawater).

The acetate concentrations in the Porto crater, Meknes slope and the pelagic reference site were low (< 10 μM), and in the range measured at similar sediment depths in low organic carbon marine sediments e.g. the Southern Ocean (Wellsbury *et al.*, 2001) and Woodlark Basin (Wellsbury *et al.*, 2002). The acetate concentration in the Meknes crater site was also low in the 10 cmbsf sediment horizon but increased substantially (x3) in the two lower horizons, despite pore water freshening at these depths. Sulphate concentrations in these lower horizons were <1 μM, showing the acetate increases occurred below the sulphate-reduction zone, presumably within the zone of methanogenesis. The fact methanogens have a higher minimum threshold concentration for acetate utilization than sulphate reducers (Schönheit *et al.*, 1982) could explain this finding as this would theoretically lead to a drop in the degree of acetate removal below the sulphate-reduction zone.

4.3 PCR-based analysis of the Meknes and Porto MV sediment

4.3.1 DNA extraction results

Assessment of the DNA extracts (pooled product from 6 x 0.8 g of sediment) by standard gel electrophoresis (see section 2.3.1) showed visible DNA (≥1 ng 10 μl^{-1}) was obtained from 9 of the 15 horizons studied, and that the DNA extracted was of high molecular weight. The intensity of the fluorescent signal was used semi-quantitatively to assess the DNA concentration in each sample (Table 4.1). DNA yield generally corresponded to total prokaryotic cell counts as determined by AODC (see section 4.2.1) and was typically highest in near-surface horizons. Fig 4.6 shows the two highest DNA yields obtained, which were from the Meknes crater 10 cmbsf and Porto crater 50 cmbsf samples, respectively. Visible DNA was not obtained from reagent-only extractions.

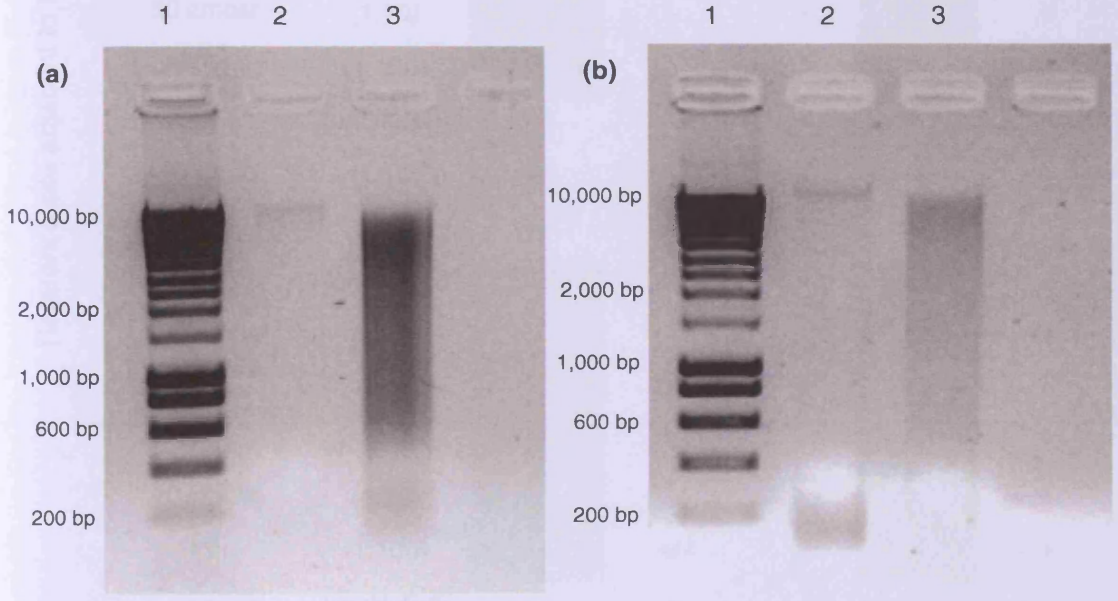


Fig 4.6 Relative quantities of DNA extracted from selected Meknes and Porto MV horizons. Panel (a) Lane 1, HyperLadder I marker; lane 2, Meknes slope 10 cmbsf sample; lane 3, Meknes crater 10 cmbsf sample. Panel (b) Lane 1, HyperLadder I marker; lane 2, Porto 10 cmbsf sample; lane 3, Porto 50 cmbsf sample. Values shown to the left of the DNA marker show relative DNA fragment size in base pairs (bp). For quantitative purposes, the 10,000, 2,000, 1,000, 600 and 200 bp bands represent 100, 20, 100, 60 and 20 ng DNA, respectively.

4.3.2 Amplification of bacterial 16S rRNA gene sequences

The amplification of bacterial 16S rRNA genes from each of the 15 samples was conducted with 3 different dilutions of DNA template (undiluted, 1:10 and 1:100) to

provide different template/inhibitor concentrations to maximise PCR success. Both direct and nested reactions were conducted using general bacterial primers (section 2.3.2) and the results of the various reactions are summarized in Table 4.1.

Table 4.1 DNA extraction and 16S rRNA gene amplification results from Meknes and Porto MV samples.
The relative yield of DNA from each of the horizons and the relative success of the various PCR reactions were scored semi-quantitatively according to the intensity of the florescent signal observed after standard gel electrophoresis. Grey shading indicates reactions that were not conducted.

Core location	Horizon [Visible DNA]	Template	27F – 907R Nested	27F – 1492R Direct	357F – 518R		
					Direct	Nested 907R	Nested 1492R
Reference site adjacent to Meknes MV	578 A	(undiluted)		-	-		-
	10 cmbsf	(1:10)		-	-		-
	[+]	(1:100)		+/-	-		+
	578 B	(undiluted)		-	-		-
	50 cmbsf	(1:10)		-	-		+
	[+/-]	(1:100)		-	-		+
	578 C	(undiluted)	+/-	-	-	+	-
	110 cmbsf	(1:10)	+/-	-	-	+	-
	[-]	(1:100)	+	-	-	+	-
	578 D	(undiluted)		-	-		+
	300 cmbsf	(1:10)		-	-		+
	[-]	(1:100)		-	-		+
Slope of Meknes MV	579 A	(undiluted)		-	-		-
	10 cmbsf	(1:10)		+	-		+
	[+]	(1:100)		+/-	-		+
	579 B	(undiluted)	-	-	-	-	-
	50 cmbsf	(1:10)	-	-	-	+	-
	[+/-]	(1:100)	-	-	-	+	-
	579 C	(undiluted)	-	-	-	+/-	-
	110 cmbsf	(1:10)	-	-	-	+	-
	[-]	(1:100)	+/-	-	-	+	+
	579 D	(undiluted)		+/-	-		+
	125 cmbsf	(1:10)		-	-		+
	[-]	(1:100)		-	-		+

Table 4.1 cont. DNA extraction and 16S rRNA gene amplification results from Meknes and Porto MV samples.
The relative yield of DNA from each of the horizons and the relative success of the various PCR reactions were scored semi-quantitatively according to the intensity of the florescent signal observed after standard gel electrophoresis. Grey shading indicates reactions that were not conducted.

Core location	Horizon [Visible DNA]	Template	27F – 907R Nested	27F – 1492R Direct	357F – 518R		
					Direct	Nested 907R	Nested 1492R
Crater of Meknes MV	580 A	(undiluted)		-	-		++
	10 cmbsf	(1:10)		++	++		++
	[+]	(1:100)		+	++		++
	580 B	(undiluted)	+/-	-	+/-	++	+
	50 cmbsf	(1:10)	+	-	+/-	-	++
	[+/-]	(1:100)	+	-	+/-	++	++
	580 C	(undiluted)	-	-	+	+/-	+/-
	65 cmbsf	(1:10)	+	-	-	+	-
	[-]	(1:100)	+	-	-	+	+
Crater of Porto MV	596 A	(undiluted)	-	-	-	-	-
	10 cmbsf	(1:10)	+/-	-	-	+	+/-
	[+/-]	(1:100)	+	-	-	+	+
	596 B	(undiluted)	-	-	-	-	-
	50 cmbsf	(1:10)	+	-	-	+	+
	[+]	(1:100)	+	-	+	+	+
	596 C	(undiluted)		-	-		-
	110 cmbsf	(1:10)		-	-		+
	[+/-]	(1:100)		-	-		+/-
	596 D	(undiluted)		-	-		+
	200 cmbsf	(1:10)		-	-		+
	[-]	(1:100)		-	-		-

Successful first round PCR reactions with the 27F – 1492R primers were, in all but one case, restricted to near-surface sediment samples where DNA concentrations were highest. Typically dilution of the template was required to achieve PCR success, suggesting PCR-inhibitory compounds were co-extracted with the sediment DNA (Rochelle *et al.*, 1992; Webster *et al.*, 2003). Direct amplifications with the 357F – 518R primer pair were more successful overall than the 27F – 1492R primer pair but

surprisingly some samples that had been successfully amplified with the larger product 27F – 1492R primer pair were not amplified with the short product 357 – 518R primers, suggesting that not all reactions were optimal and/or there was some effect from primer bias. One round of nested PCR was successful in obtaining PCR products from 12 out of the 15 horizons, however for the remaining 3 horizons two rounds of nested PCR were required (Table 4.1). In all nested reactions the negative PCR controls from the previous rounds were also re-amplified. In several cases these produced a visible product, which were included in the DGGE analysis (section 4.3.3).

4.3.3 DGGE screening of bacterial 16S rRNA gene amplicons

Products from successful PCR reactions were screened by DGGE to assess the bacterial 16S rRNA gene diversity in each of the horizons, and to compare diversity obtained from different template dilutions. Where PCR products were obtained from more than one template dilution, DGGE screening consistently showed the same dominant bands were present. This indicated the diversity detectable by PCR was stable at different DNA concentrations (e.g. Fig 4.7). Nested reactions, as expected, produced higher yields of the targeted 16S rRNA genes than direct amplification of the sediment DNA with short DGGE primers alone.

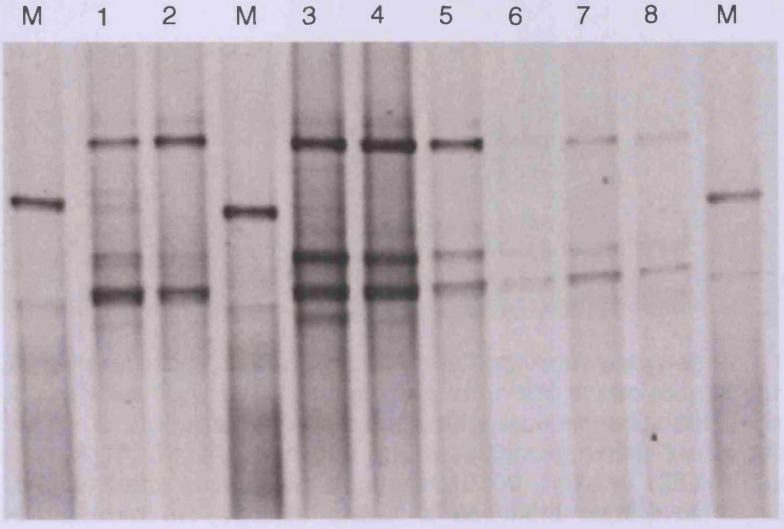


Fig 4.7 Example of a DGGE screen of bacterial 16S rRNA gene products obtained by direct and nested PCR from one MV sediment sample using variously diluted DNA templates. The example shown is from the Meknes crater 50 cmbsf sample. Lane 1, product from 1:100 diluted DNA after two rounds of nested PCR; lane 2, product from undiluted DNA after two rounds of nested PCR; lane, 3 product from 1:100 diluted DNA after one round of nested

PCR; lane 4, product from 1:10 diluted DNA after one round of nested PCR; lane 5, product from undiluted DNA after one round of PCR; lane 6, product from 1:100 diluted DNA after direct PCR; lane 7, 1:10 diluted DNA after direct PCR and lane 8, undiluted DNA after direct PCR. Lanes marked M were a DGGE marker. Note there was no product after two rounds of PCR from the 1:10 diluted DNA due to failure of the final PCR reaction.

To compare bacterial diversity in the three Meknes MV sites, the amplified products from all 11 of the horizons studied were profiled on a single DGGE gel (Fig 4.8). The screened products represented the 16S rRNA gene amplicons obtained from 1:100 diluted templates after one or two rounds of nested PCR, as necessary. This indicated that out of all the samples analyzed the bacterial 16S rRNA gene diversity was highest (greatest number of bands) at 10 cmbsf in the Meknes crater. This was also the horizon with the highest DNA yield and the highest total cell counts. Substantial numbers of bands were also present in the two lower depths of the Meknes crater.

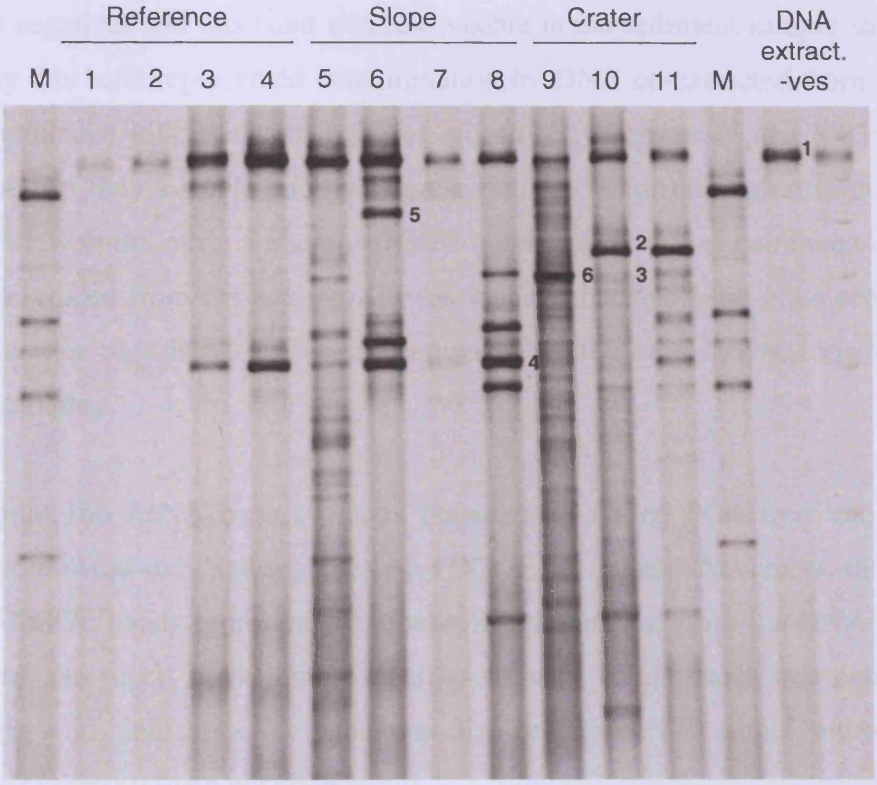


Fig 4.8. DGGE gel profiling of the 16S rRNA gene amplicons obtained from the Meknes MV samples. Products were from 1:100 diluted DNA after one or two rounds of nested PCR, as required. Lanes 1 – 4, products from the pelagic reference site adjacent to the Meknes site at sediment depths of 10, 50, 110 and 300 cm, respectively; lanes 5 – 8, products from the slope of the Meknes crater at sediment depths of 10, 50, 110 and 125 cm, respectively; lanes 9 – 11, products from the Meknes crater at sediment depths of 10, 50 and 65 cm, respectively. Lanes marked M were a DGGE marker. Lanes labelled DNA extraction negatives contained the product from the reagent-only extractions amplified after one round of nested PCR. Numbered bands were excised and sequenced, see Table 4.2.

High bacterial 16S rRNA gene diversity, as estimated by the number of DGGE fragments, was also detected at 10 cmbsf in the Meknes slope, which also coincided with a relatively high DNA yield (Fig 4.6) and relatively high total cell counts (section 4.2.1). Diversity was much lower in the other Meknes samples, which corresponded to the very low or non-detectable concentrations of DNA in these extracts. Surprisingly however, this also included the 10 cmbsf sample from the pelagic reference site, which had a relatively high DNA concentration (Table 4.1). The failure to obtain 16S rRNA gene products from this sample was not pursued due to focus on crater samples, but may have been due to sub-optimal PCR conditions in the initial round of PCR or a persistent presence of PCR inhibitory compounds. Fig 4.8 also shows the products obtained following nested PCR of the DNA extraction negatives (reagent-only). The same single band was visible in two separate DNA extraction negatives and this band was also visible in the sediment sample lanes. It is very likely this band represented contamination by DNA co-extracted from reagents in the commercial kits used (Rochelle *et al.*, 1992a; Webster *et al.*, 2003; section 2.3.2). The fact only a single band was present and it occurred at a position that did not interfere with the other authentic DGGE bands meant this contaminant could be easily be excluded from the analysis. It was notable that this band often represented the dominant or only band visible in products from the very low/non-visible DNA sediment samples.

The bacterial 16S rRNA gene products obtained by nested PCR from the 4 Porto crater samples were screened separately by DGGE. At 10 and 200 cmbsf, the only or dominant DGGE bands represented probable contamination from the DNA kit (data not shown). However, at 50 cmbsf a different very bright band was consistently present (Fig 4.9), and this same band was also present at 110 cmbsf, although at a much lower intensity (data not shown).

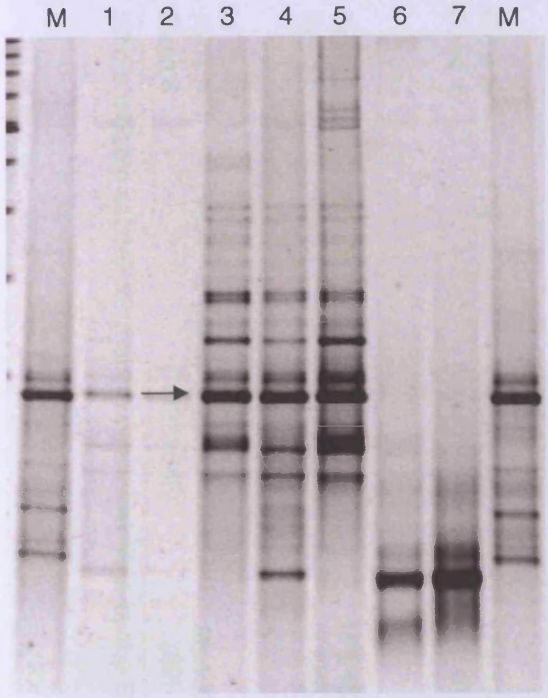


Fig 4.9 DGGE gel screening the 16S rRNA gene amplicons obtained from the Porto 50 cmbsf sample. Lane 1, product from direct PCR of 1:100 diluted DNA; lane 2 product from 1:10 diluted DNA after one round of nested PCR; lane 3, product from 1:100 diluted DNA after one round of nested PCR; lane 4 product from 1:10 diluted DNA after two rounds of nested PCR; lane 5 product from 1:100 diluted DNA after two rounds of nested PCR; lane 6 product obtained by re-amplifying the negative from the first round of PCR; lane 7 product obtained by re-amplifying the negative control from the second round of PCR. Lanes marked M were a DGGE marker. The band referred to in the text is marked with an arrow.

4.3.4 Sequencing bacterial 16S rRNA gene DGGE bands

Individual bands were excised from the DGGE gels and sequenced to identify the corresponding organisms where possible (see section 2.3.6). When numerous bands were present in a sample lane, the re-amplified band products were re-analysed by DGGE to confirm purity and recovery of the targeted band. Inspection of the second gels often showed that fainter bands could not be recovered on the first attempt because DNA from adjacent bands, particularly when bright, were also present as a significant component of the re-amplified product. A further attempt to recover the fainter bands from the second DGGE gel was sometimes successful. Bands that were successfully sequenced are numbered in Fig 4.8 and their closest sequence matches are given in Table 4.2.

Table 4.2 Identity of bacterial 16S rRNA gene fragments recovered from DGGE bands from Meknes MV samples.
Note that only representative bands are labelled in the corresponding figure for clarity, but further sequencing showed that where bands had migrated to the same position on the DGGE gel they consistently denoted the same 16S rRNA gene sequence.

Band identifier	Sequence length (bp)	Closest match[a] in BLASTN database (Accession number)	Identities (%)	Phylogenetic affiliation	Origin of closest matching sequence(s)
Fig 4.8 Band 1	165	Clone 61-01-24c007 (DQ316806)	165/165 (100)	*Betaproteobacteria*	Contaminated sediment, human and veterinary samples, gas hydrate, deep terrestrial sub-surface
Fig 4.8 Band 2	177	Clone NANK-B7 (AY436531)	177/177 (100)	JS1 candidate division	Anaerobic marine sediment, gas hydrate associated sediment, tidal flat sediment
Fig 4.8 Band 3	140	Clone SURF-GC205-Bac33 (DQ521818)	140/140 (100)	*Epsilonproteobacteria*	Hydrothermal vent environment, methane-rich (cold seep) sediment, tidal flat sediment, mud volcano mat, harbour sediment
Fig 4.8 Band 4	174	Clone G2r0 (AM421676)	174/174 (100)	*Gammaproteobacteria*	Lake water, *Escherichia coli* strain ATCC 25922, volcanic soil, anaerobic baffled reactor, estuarine sediment enrichments, *Shigella flexneri* 5 strain 8401, *Escherichia coli* 536, animal guts
Fig 4.8 Band 5	165	Staphylococcus sp. TD12 (DQ978269)	165/165 (100)	*Firmicutes*	Oyster digestive tissue, manufacturing environments and the workplace, various *Staphylococcus* species, rhizosphere, agricultural sources, clinical samples, seawater, soil, activated sludge
Fig 4.8 Band 6	110	Clone BJS8R-080 (AB239068)	98/109 (89)	*Epsilonproteobacteria*	Cold seep sediment

[a] Where multiple sequences matches were identified by the BLAST search, the entry at the top of the subject list was included in the table.

Three of the six DGGE bands sequenced from the Meknes samples were identified as probable contaminants of either the DNA extraction or PCR kits used. Several DGGE bands represented by band '1' in Fig 4.8, which included the bands observed in the DNA extraction negatives, were sequenced and all were identified as *Betaproteobacteria*. According to NCBI database sequence matches these sequences were related to uncultured members of *Burkholderia* and *Herbaspirillum*. Multiple 100% matches were identified by the BLASTN searches and the reported sources for the matching sequences included both clinical and environmental sources (Table 4.2). The findings of the present study however, does cast doubt as to whether the environmental sequences are really representative of indigenous sequences to those environments, as opposed to unrecognised contaminants. The second and third suspected contaminants were members of the *Gammaproteobacteria* and *Firmicutes,* respectively. DGGE band 4, which was present in nested PCR negatives (data not shown) and several of the low DNA samples, was 100% similar to sequences that included several *Escherichia coli* strains. *Escherichia coli* is a known contaminant of commercial PCR kits (see section 2.3.2) and given that this band was present in nested PCR negatives makes it a very strong candidate as a contaminant. Once again it is notable that other matching sequences were reported as indigenous environmental sequences in the NCBI database (Table 4.2). DGGE band 5 was closely related to various *Staphylococcus* species and the matching sequences in the NCBI database were reported from a wide variety of sources including workplace environments. The ubiquity of *Staphylococcus* species and its detection after two rounds of nested PCR with a low biomass sample makes this sequence also a likely laboratory contaminant.

The remaining bands identified by sequencing were those that appeared brightest in the Meknes crater samples. DGGE band 2 was dominant in both the 50 and 65 cmbsf samples (Fig 4.8) and according to the BLASTN search was a member of the JS1 candidate division. The 100% sequence matches included clones obtained from sub-surface sediment in the Nankai Trough e.g. NANK-B7 (Newberry *et al.*, 2004), methane-hydrate bearing sediment on the Cascadia Margin e.g. ODP1251B1.5 (Inagaki *et al.*, 2006), sub-seafloor sediments of the Sea of Okhotsk e.g. clone OHKB16.55 (Inagaki *et al.*, 2003) and DGGE band sequences from tidal flat enrichment cultures e.g. 13CJS1-44 (Webster *et al.*, 2006a). The sequence represented by band 3 (shown more clearly in Fig 4.7) was identified as a member of the

Epsilonproteobacteria and was 100% similar to sequences from various marine environments including hydrothermal vents (e.g. Kormas *et al.*, 2006; Nakagawa *et al.*, 2005), near-surface brine pool sediment from the Gulf of Mexico (Lloyd *et al.*, 2006), cold seep sediment from the base of Florida Escarpment (Reed *et al.*, 2006), a mud volcano microbial mat (Heijs *et al.*, 2005) and tidal flat sediment (Kim *et al.*, 2004). Band 6 was also identified as a member of the *Epsilonproteobacteria* and was 89% similar to clone BJS8R-080 isolated from cold seep sediment in the Japan Sea (Arakawa *et al.*, 2006). However, due to the sub-optimal quality of this sequence affiliations above the division level should be viewed with caution.

The numerous fainter DGGE bands present in the Meknes crater and 10 cmbsf slope samples could not be identified by sequencing as re-analysis of the amplified products by DGGE consistently showed multiple sequences to be present in the amplified product.

The dominant bacterial 16S rRNA gene DGGE band sequence (see Fig 4.9) from the Porto 50 cm depth sample was identified as a member of the JS1 candidate division (Webster *et al.*, 2004). The sequence shared 100% sequence similarity across 195 bp with clone FE2BotBac14 (AY769033) from cold seep sediment from the Florida Escarpment (Reed *et al.*, 2006) and other JS1 clones from the Sea of Okhotsk (Inagaki *et al.*, 2003) and the Japan Trench (Li *et al.*, 1999). The sequence similarity between the Meknes JS1 sequence (see Table 4.2) and the Porto JS1 sequence was 96%.

4.3.5 Amplification of archaeal 16S rRNA gene sequences

Archaeal phylotypes were detected in 7 of the 15 sediment horizons investigated (Fig 4.10). The brightest archaeal 16S rRNA gene PCR products were obtained from the Meknes crater, near-surface Meknes slope and Porto crater samples, which agreed well with relative DNA yields and levels of bacterial diversity. Archaeal 16S rRNA genes were not detected in samples from the Meknes reference site, which were also negative for the bacteria (section 4.3.2). It is notable that one sample (Meknes slope 50 cmbsf) was weakly positive after the first round reaction (109F – 958R primer set) but failed to amplify in the second round (SAf – PARCH primer set), suggesting a

mismatch between the second round primers and the amplified DNA. It has been shown recently that the SAf – PARCH primers fail to detect the ubiquitous MCG group (Webster *et al.*, 2006), which may explain this finding.

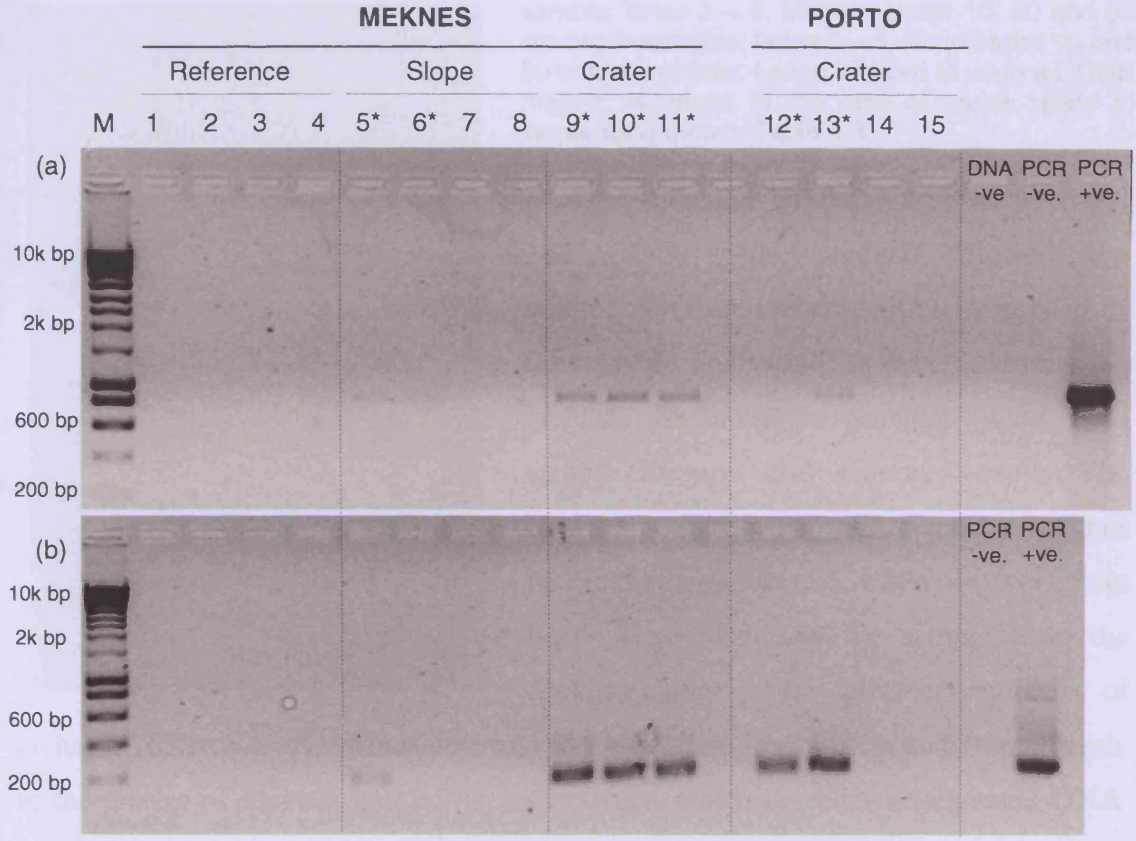


Fig 4.10 Results of direct and nested PCR reactions conducted with Meknes and Porto MV samples using general archaeal 16S rRNA gene primers. Panel (a) first round reaction results, panel (b) second round (nested) reaction results. Lanes 1 – 4 samples from 10, 50, 110 and 300 cm depths in the Meknes reference site; lanes 5 – 8 samples from 10, 50, 110 and 125 cm depths in the Meknes slope site; lanes 9 – 11 samples from 10, 50 and 65 cm depths in the Meknes crater site; lanes 12 – 15 samples from 10, 50, 110 and 200 cm depths in the Porto crater site. The * symbol denotes samples that were positive in the first and/or second round of PCR. Lane M was HyperLadder I Marker. DNA –ve was an amplification of a reagent only DNA extraction, PCR – ve was an amplification without any DNA template added.

4.3.6 DGGE screening of archaeal 16S rRNA gene amplicons

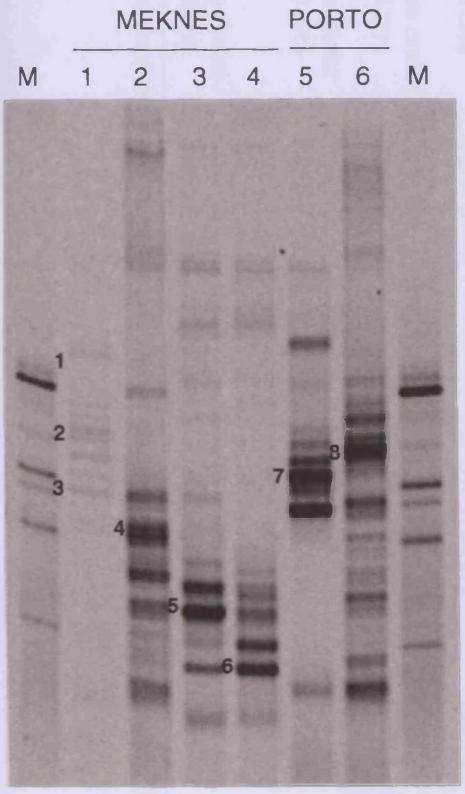


Fig 4.11 DGGE screen of archaeal 16S rRNA genes detected by PCR in Meknes and Porto MV samples. Lane 1, Meknes slope 10 cm depth sample; lanes 2 – 4, Meknes crater 10, 50 and 65 cm depth samples; lanes 5 – 6, Porto crater 10 and 50 cmbsf samples; Lanes labelled M were a DGGE marker. Numbers to the right of bands relate to sequencing data in Table 4.3

DGGE screening of the amplified archaeal 16S rRNA gene products showed the community structure was different in each of the samples analysed (Fig 4.11) and thus the archaeal community changed with both sample location and sediment depth. The brightest bands were visible in the Meknes and Porto crater samples, while only very faint bands were visible in the sample from the Meknes slope. The greatest number of archaeal 16S rRNA gene bands occurred in the samples from 10 cm and 50 cm depth in the craters of Meknes and Porto, respectively, which agreed with elevated DNA yields and total prokaryotic cell numbers in these horizons.

4.3.7 Sequencing archaeal 16S rRNA gene DGGE bands

Sequence analysis of archaeal 16S rRNA gene DGGE bands revealed the identity of dominant community members, which are shown in Table 4.3.

Table 4.3 Identity of archaeal 16S rRNA gene fragments recovered from DGGE bands from Meknes and Porto MV samples.

Sample site (sediment depth)	Band identifier	Sequence length (bp)	Closest match[a] in BLASTN database (Accession number)	Identities (%)	Phylogenetic affiliation	Origin of closest matching sequence(s)
Meknes slope (10 cm)	Fig 4.11 Band 1	129	Clone Urania-1A-28 (AY627452)	119/122 (97%)	*Crenarchaeota* – Marine Group I	Marine sediment (mound near Urania brine Lake & Kazan mud volcano)
	Fig 4.11 Band 2	131	Clone Napoli-1A-47 (AY592465)	124/126 (98%)	*Crenarchaeota* – Marine Group I	Marine sediment, carbonate crusts, mud volcanoes, lake sediment, rice paddy soil, tropical seawater tank substratum
	Fig 4.11 Band 3	120	Clone CCA47 (AY179969)	83/92 (90%)	*Euryarchaeota*	Anoxic marine sediment, mud volcano sediment
Meknes crater (10 cm)	Fig 4.11 Band 4	128	Clone 1H5_H04 (DQ301995)	111/119 (93%)	*Euryarchaeota* – Marine Benthic Group D	Peru Margin ODP Site 1227
Meknes crater (50 cm)	Fig 4.11 Band 5	111	Clone PL-10A3 (AY570687)	85/86 (98%)	*Euryarchaeota* – ANME-1a	Low-temperature biodegraded oil reservoir, methane-rich marine sediments
Meknes crater (65 cm)	Fig 4.11. Band 6	111	Clone PL-10D2 (AY570642)	101/105 (96%)	*Euryarchaeota* – ANME-1a	Low-temperature biodegraded oil reservoir, methane-rich marine sediments
Porto crater (10 cm)	Fig 4.11 Band 7	125	Clone MN13BT4-157 (AY593263)	124/126 (98%)	*Crenarchaeota* – Marine Group I	Carbonate crust on Amsterdam mud volcano, Haakon Mosby MV sediment, marine sediments, marine organisms, tidal flat sediment
Porto crater (50 cm)	Fig 4.11 Band 8	131	Clone Napoli-1A-11 (AY592444)	124/127 (97%)	*Crenarchaeota* – Marine Group I	Surface sediment of Napoli mud volcano

[a] Where multiple sequences matches were identified by the BLAST search, the entry at the top of the subject list was included in the table

In the Meknes slope 10 cmbsf sample bands 1 and 2 were members of the *Crenarchaeota* and were most closely related to sequences affiliated to Marine Group 1 (MG1; Table 4.3). The matching sequences were reported from a variety of environments including a MV in the Eastern Mediterranean (e.g. Heijs, 2005). The third Meknes slope sequence was a member of the *Euryarchaeota* but its low similarity to database entries precluded a higher phylogenetic assignment. In the Meknes crater 10 cmbsf sample, only the most dominant band was successfully sequenced. This band was identified as a member of Marine Benthic Group D (MBG-D) of the *Euryarchaeota* and was most closely related to a clone from ODP site 1227 on the Peru Margin (Sorensen & Teske 2006). The MBG-D is an uncultivated group found ubiquitously in marine environments (Hinrichs *et al.*, 1999; Knittel *et al.*, 2005; Orphan *et al.*, 2001; Takai & Horikoshi 2000; Vetriani *et al.*, 1999 and Kendall *et al.*, 2007). In the deeper depths of the Meknes crater, *Euryarchaeota* sequences related to the ANME-1 group were found. Band 5 from 50 cmbsf in the Meknes crater was most closely related to sequences from a low temperature biodegraded Canadian oil reservoir (Grabowski *et al.*, 2005) and band 6 was most closely related to sequences from the biodegraded oil reservoir, cold seep sediments on the Cascadia Margin (Lanoil *et al.*, 2005) and anaerobic methane rich sediments from the Gulf of Mexico (Hinrichs *et al.*, 1999). In the Porto crater two bands were successfully sequenced and both were members of the *Crenarchaeota* related to MG1; band 7 was most closely related to sequences from a variety of marine sources including cold seeps, and the closest match to band 8 was a clone recovered from a near-surface sediment layer of the Napoli MV in the Eastern Mediterranean (Heijs, 2005).

Archaeal 16S rRNA gene bands were sometimes visible in pairs on the DGGE gels (Fig 4.11). This is a known phenomenon with the degenerate primers used (G. Webster, personal communication) and sequencing confirmed that these bands were in fact representative of the same organism (data not shown).

4.3.8 Amplification of function gene, *mcrA* and *dsr*A, sequences

Direct and nested PCR reactions were carried out with undiluted, 1:10 and 1:100 diluted DNA template using functional gene primers to detect functional groups of

interest (methanogens/methanotrophs and sulphate reducers) in the Meknes and Porto MV samples. The results of the various reactions are summarized in Table 4.4.

Table 4.4 Detection of *mcrA* and *dsrA* functional genes in Meknes and Porto MV samples by direct and nested PCR
Results were scored as -, negative; +/-, faint band present; +, clear band present

Site (water depth)	Depth (cmbsf)	Template dilution	*mcrA* gene targeted primers			*dsrA* gene targeted primers		
			ME1 – ME2 (direct)	LutonF – LutonR (direct)	LutonF – LutonR (nested)	DSR 1F – DSR 4R (direct)	DSR 1F+ – DSR R (direct)	DSR 1F+ – DSR R (nested)
Meknes reference site 762 m	10	undiluted	-	-	-	-	-	+/-
		1:10	-	-	-	+/-	+	+
		1:100	-	-	-	-	+/-	+
	50	undiluted	-	-	-	-	-	-
		1:10	-	-	-	-	-	-
		1:100	-	-	-	-	-	-
	110	undiluted	-	-	-	-	-	-
		1:10	-	-	-	-	-	-
		1:100	-	-	-	-	-	-
	300	undiluted	-	-	-	-	-	-
		1:10	-	-	-	-	-	-
		1:100	-	-	-	-	-	-
Meknes slope 747 m	10	undiluted	-	-	-	-	-	-
		1:10	-	-	-	-	-	+
		1:100	-	-	-	-	-	+
	50	undiluted	-	-	-	-	-	-
		1:10	-	-	-	-	-	+
		1:100	-	-	-	-	-	-
	110	undiluted	-	-	+	-	-	+/-
		1:10	-	-	+	-	-	+
		1:100	-	-	-	-	-	-
	125	undiluted	-	-	-	-	-	+
		1:10	-	-	-	-	-	-

Table 4.4 cont. Detection of *mcrA* and *dsrA* functional genes in Meknes and Porto MV samples by direct and nested PCR
Results were scored as -, negative; +/-, faint band present; +, clear band present

Site (water depth)	Depth (cmbsf)	Template dilution	*mcrA* targeted primers			*dsrA* targeted primers		
			ME1 – ME2 (direct)	LutonF – LutonR (direct)	LutonF – LutonR (nested)	DSR 1F – DSR 4R (direct)	DSR 1F+ – DSR R (direct)	DSR 1F+ – DSR R (nested)
Meknes crater 705 m	10	undiluted	-	-	-	-	-	+
		1:10	-	+	+	+	+	+
		1:100	-	+/-	+	+/-	+	+
	50	undiluted	+	+	+	+	+	+
		1:10	-	+	+	-	+/-	+
		1:100	-	+/-	+	-	-	+
	65	undiluted	+	+	+	+	+	+
		1:10	-	+	+	-	+/-	+
		1:100	-	+/-	+	-	-	+
Porto crater 3878 m	10	undiluted	-	-	-	-	-	-
		1:10	-	-	-	-	-	+
		1:100	-	-	-	-	-	+
	50	undiluted	-	-	-	-	-	+/-
		1:10	-	-	+	-	+	+
		1:100	-	-	+	-	+/-	+
	110	undiluted	-	-	-	-	-	+/-
		1:10	-	-	-	-	+	+
		1:100	-	-	-	-	-	+
	200	undiluted	-	-	-	-	-	-
		1:10	-	-	-	-	-	+/-
		1:100	-	-	-	-	-	-

Direct PCR with the ME1 – ME2 (Hales et al., 1996) and the LutonF – LutonR (Luton et al., 2002) primer sets detected mcrA genes at all depths in the Meknes crater but were negative for all samples from the Meknes slope and reference site. Nested PCR (ME1 – ME2 products re-amplified with LutonF – LutonR primers) detected mcrA genes in one additional sample from 110 cmbsf in the Meknes slope (Table 4.4). Direct PCR with the DSR1F – DSR4R (Wagner et al., 1998) and the DSR1F+ - DSRR (Kondo et al., 2004) primer sets detected dsrA genes at all depths in the Meknes crater and the 10 cmbsf sample from the Meknes pelagic reference site. Nested PCR also detected dsrA genes at all depths in the Meknes slope.

At the Porto site direct PCR with mcrA-targeted primers did not detect mcrA genes in any of the depths analysed but nested PCR detected mcrA genes in the 50 cmbsf horizon. Direct PCR with the DSR1F+ - DSRR primer set detected dsrA genes at 50 and 110 cmbsf in the Porto MV, while nested PCR additionally detected dsrA genes at 10 cmbsf.

All PCR negatives in these reactions were clear. Template dilution had a clear affect on the success of first-round functional gene-targeted PCR reactions (Fig 4.12 and Table 4.4). In the Meknes crater the 10 cmbsf sample (highest DNA concentration) required 1:10 or 1:100 dilution before PCR reactions were successful, suggesting the presence of PCR inhibitory compound in the templates, while the two deeper samples (lower DNA template concentration) were most successful with undiluted templates and became less successful as dilution increased. In the Porto crater 1:10 or 1:100 dilution was required before reactions were successful, also suggesting PCR inhibitors were an issue in these samples.

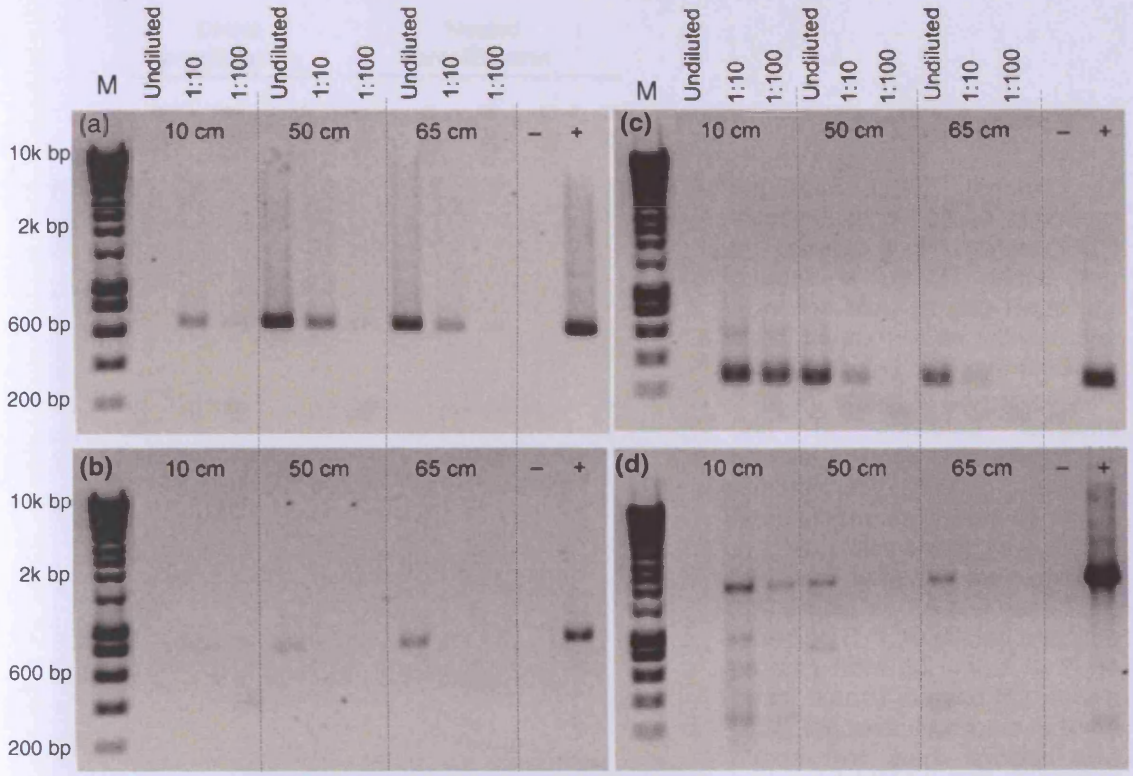


Fig 4.12 Example of the affect template dilution had on PCR success using functional primers. Gels show the result of direct functional gene targeted PCR reactions with Meknes MV crater samples. Panel (a) LutonF – LutonR *mcrA* gene primer set; panel (b) ME1 – ME2 *mcrA* gene primer set; panel (c) DSR1F+ - DSRR *dsrA* gene primer set; panel (d) DSR1F – DSR4R *dsrA* gene primer set. Lanes marked M are HyperLadder I.

4.3.9 DGGE and clone library analysis of functional (*mcrA* and *dsrA*) genes from the Meknes and Porto MV sites

4.3.9.1 *McrA* genes

To compare the diversity of functional genes in the Meknes and Porto sites, and to separate individual genes for sequencing selected samples were analysed by DGGE (see 2.3.5). These results were then compared to those obtained by cloning functional gene products. Results of DGGE screening *mcrA* gene products are shown in Fig 4.13 and results of cloning *mcrA* gene products are shown in Fig 4.14.

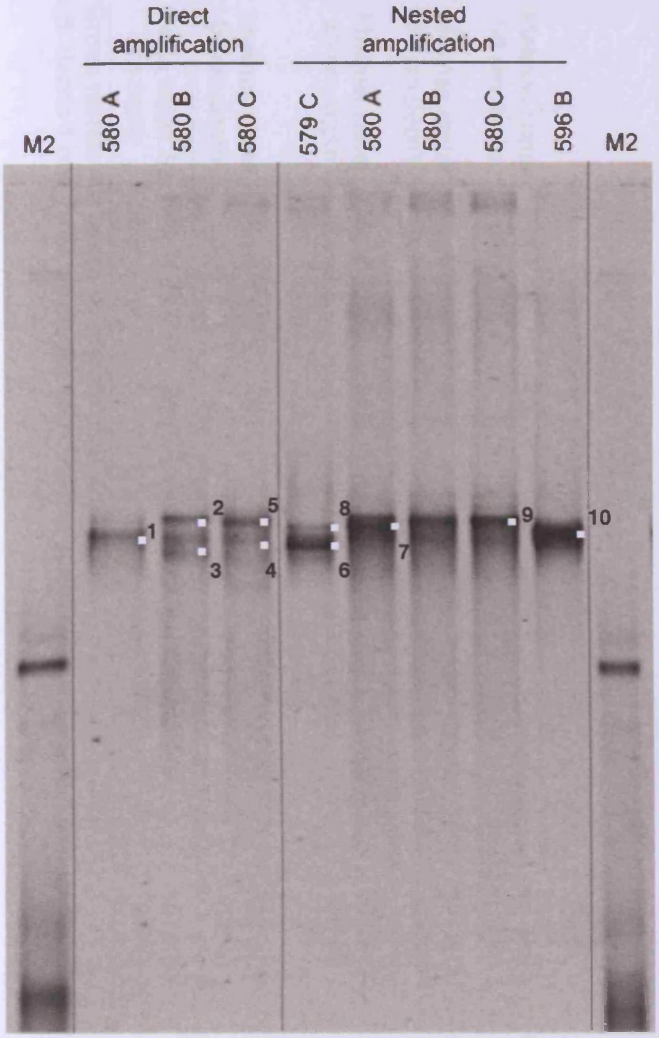


Fig 4.13 DGGE analysis of selected *mcrA* genes amplified by direct and nested PCR (LutonF – LutonR primer set) from the Meknes and Porto MV sites. Lanes are as follows: 580 A, 1:10 diluted template products from 10 cmbsf in Meknes crater; 580 B, undiluted template products from 50 cmbsf in Meknes crater; 580 C, undiluted template products from 65 cmbsf in the Meknes crater; lane 579 C, undiluted template product from 110 cmbsf in the Meknes slope; lane 596 B, 1:10 diluted template product from 50 cmbsf in Porto crater. Lanes marked M2 were a DGGE marker. Numbers indicate bands that were excised and sequenced (Table 4.5).

DGGE analysis suggested that the diversity of *mcrA* genes in each sample was low but that different *mcrA* genes were present at the different sites. The bands labelled in Fig 4.13 were excised and sequenced with the LutonF primer (Table 4.5).

Table 4.5 Closest matches to *mcrA* DGGE band sequences obtained from the Meknes and Porto MV sites

Sample	Band identifier	Sequence length (bp)	Closest match in BLASTN database (Accession number)	Identities (%)	*mcrA* gene group[a] (phylogenetic affiliation)	Origin of closest sequence match
Meknes slope 110 cm depth	Fig 4.13. Band 6	403	No matches (V. good sequence quality)	None	Novel *mcrA* group	
Meknes slope 110 cm depth	Fig 4.13. Band 8	397	No matches (V. good sequence quality)	None	Novel *mcrA* group	
Meknes crater 50 cm depth	Fig 4.13. Band 2	416	Clone 0418F12 (BX649197)	374/416 (89%)	*mcrA* group a (ANME-1)	Fosmid library from a methane-rich Black Sea microbial mat, various methane rich marine sediments (Monterey Canyon, Gulf of Mexico, Blake Ridge, Eel River)
Meknes crater 50 cm depth	Fig 4.13. Band 3	407	Clone F17.1_30A02 (AY324363)	371/407 (91%)	*mcrA* group a (ANME-1)	Methane rich marine sediment (Monterey Canyon)
Meknes crater 65 cm depth	Fig 4.13. Band 4	415	Clone T201_30C04 (AY324373)	391/414 (94%)	*mcrA* group a (ANME-1)	Methane rich marine sediment (Eel River)
Meknes crater 65 cm depth	Fig 4.13.. Band 5	416	Clone F17.1_30A02 (AY324363)	383/417 (91%)	*mcrA* group a (ANME-1)	Various methane rich marine sediments (Monterey Canyon, Gulf of Mexico, Blake Ridge, Eel River)
Meknes crater 65 cm depth	Fig 4.13. Band 9	405	Clone T201_30C04 (AY324373)	387/403 (96%)	*mcrA* group a (ANME-1)	Eel River marine sediment
Porto crater 50 cm depth	Fig 4.13. Band 10	408	Clone KM-m-2.28 (AB233462)	367/380 (96%)	*mcrA* group e (Methanosarcinales)	Nankai Trough marine sediments

[a] Group names are based on Hallam *et al.*, 2003.

N.B. Bands 1 and 7 (10 cm depth) from Meknes crater could not be identified by sequencing (see text)

DGGE bands from 10 cmbsf in the Meknes crater (bands 1 and 7 in Fig 4.13) could not be identified by sequencing as the chromatograph indicated that more than one *mcrA* gene occupied the bands. In the deeper samples of the Meknes crater (50 and 65 cmbsf) '*mcrA* group a' sequences were detected. The DGGE band sequences were highly related to each other and were 89–96% related to sequences from a Black Sea microbial mat (Kruger *et al.*, 2003) and methane rich sediment from the Gulf of Mexico (unpublished), Monterey Canyon, Blake Ridge and Eel River (Hallam *et al* 2003). Hallam *et al.* (2003) showed that '*mcrA* group a' is equivalent to the ANME-1 16S rRNA gene phylogenetic group.

In the Porto crater (50 cmbsf) the *mcrA* DGGE band was 96% related to a '*mcrA* group e' sequence from Nankai Trough sediments (Nunoura *et al.*, 2006). The '*mcrA* group e' is affiliated to the *Methanosarcinales* (Hallam *et al.*, 2003) but the corresponding 16S rRNA phylotype has yet to be conclusively constrained, though it has been suggested '*mcrA group e*' is probably related to ANME archaea (Nunoura *et al.*, 2006; 2008).

Clone libraries constructed with *mcrA* gene products (LutonF – LutonR primer set) from three samples: 110 cmbsf in the Meknes slope (undiluted), 50 cmbsf in the Meknes crater (undiluted) and 50 cmbsf in the Porto crater (1:10 dilution) showed the DGGE approach had provided a good summary of *mcrA* gene diversity. The dominant *mcrA* groups identified by DGGE band sequencing were also the dominant groups in the *mcrA* gene libraries (Fig 4.14).

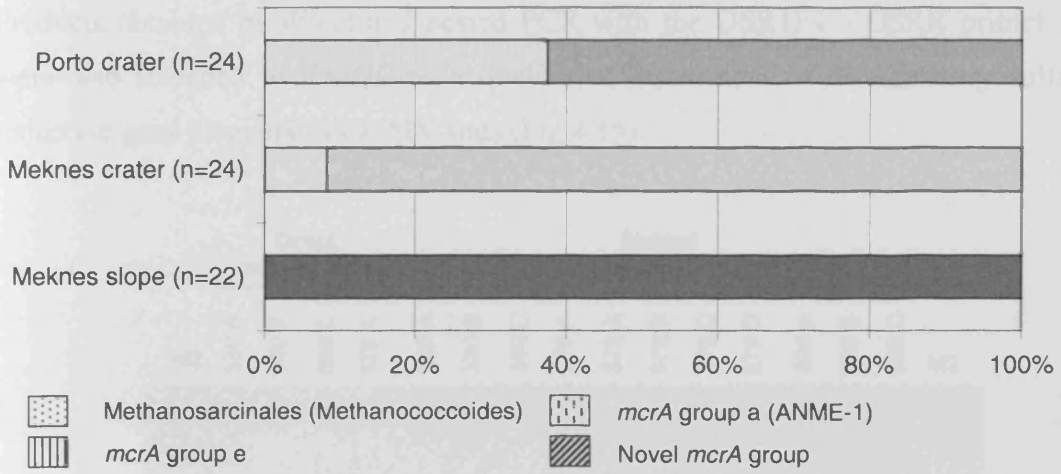


Fig 4.14 Meknes and Porto MV *mcrA* gene library results. Porto Crater library was from 50 cmbsf, Meknes crater library from 50 cmbsf and Meknes slope library from 110 cmbsf. Shading indicates different mcrA gene groups based on the names of Hallam *et al.* (2003). The corresponding 16S rRNA phylogenetic groups are given in brackets where these have been determined (Springer *et al.*, 1995; Hallam *et al.*, 2003).

In the Meknes slope *mcrA* gene library (n=22) 100% of the sequences were affiliated to a single novel phylogenetic group. The cloned sequences were the same as those obtained from the DGGE bands (Table 4.5) and shared only low similarity (88%) to a *mcrA* gene sediment sequence (clone TopMcrA17) from the Pearl Estuary, China (Jiang *et al.*, 2008, unpublished GenBank description). In the Meknes crater *mcrA* gene library (n=24) the majority of sequences (92%) were the same as those identified from the DGGE gel and were most closely related to '*mcrA* group a' (ANME-1) (Hallam *et al.*, 2003). The remaining 8% of the library represented 2 sequences that were 95% similar to the *mcrA* gene of *Methanococcoides burtonii* DSM 6242 (Springer *et al.*, 1995).

In the Porto crater *mcrA* gene library (n=24) 63% of the sequences matched those from the DGGE gel and were 94–96% related to '*mcrA* group e'. The remaining sequences, not detected by DGGE band sequencing, were 98% similar to a clone (B09) from hydrothermal sediment in the Guaymas Basin (Dhillon *et al.*, 2005) and 96% similar to the *mcrA* gene of *Methanococcoides methylutans* (Springer *et al.*, 1995).

4.3.9.2 *DsrA* genes

Products obtained by direct and nested PCR with the DSR1F+ - DSRR primer set were also screened by DGGE as a qualitative assessment of dissimilatory sulfate reductase gene diversity in the MV sites (Fig 4.15).

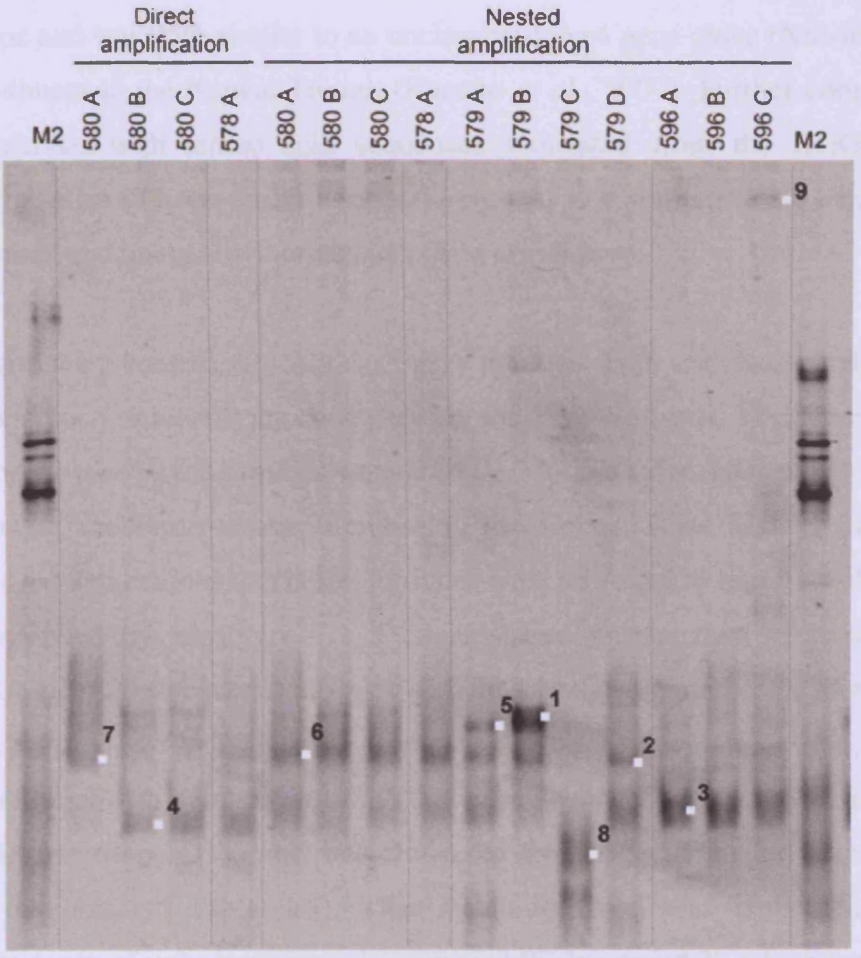


Fig 4.15 DGGE analysis of *dsrA* genes amplified by direct and nested PCR (DSR1F+ - DSRR primer set) from Meknes and Porto MV sites. Lanes were as follows (template dilution): 580 A, (1:10) product from 10 cmbsf in Meknes crater; 580 B, (undiluted) product from 50 cmbsf in Meknes crater; lanes 580 C, (undiluted) product from 65 cmbsf in the Meknes crater; lane 578A, (1:10) product from 10 cmbsf in Meknes reference; lane 579 A, (1:10) product from 10 cmbsf in Meknes slope; lane 579 B, (1:10) product from 50 cmbsf in Meknes slope; lane 579 C (undiluted) product from 110 cmbsf in the Meknes slope; lane 579 D, (1:10) product from 125 cmbsf in Meknes slope; lane 596 A (1:10) product from 10 cmbsf in Porto crater; lane 596 B (1:10) product from 50 cmbsf in Porto crater; lane 596 C (1:10) product from 110 cmbsf in Porto crater. Lanes marked M2 were a DGGE marker. Numbers indicate bands that were excised and sequenced.

The DGGE banding pattern suggested there was low *dsrA* gene diversity in all the samples but that *dsrA* gene diversity changed with sample location and sometimes sediment depth. The *dsrA* gene DGGE bands were not as well defined as the *mcrA* or

16S rRNA gene DGGE bands (Fig 4.11 and 4.13) and sequencing the excised bands was not always successful. Four of the nine bands excised from the gel (bands 1, 2, 5 and 8) yielded moderate to very good quality sequences (~160 bp long) but the others produced mixed or poor quality sequences. However, only 1 of the 4 successfully resolved sequences (band 1 in Fig 4.15) matched any *dsrA* gene nucleotide sequences in the NCBI database. The identified band (Band 1) was from 50 cm depth in the Meknes slope and was 95% similar to an unclassified *dsrA* gene clone (Ntd-I09) from deep-sea sediment in the Nankai Trough (Kaneko *et al.*, 2007). Further comparative sequence analysis with amino acid sequences translated from the DGGE band sequences (i.e. BLASTX searches) also only returned low similarities to *dsrA* genes from uncultured and unclassified organisms (data not shown).

Clone libraries were constructed with *dsrA* gene products from selected samples as an additional approach to identifying *dsrA* genes in the MV sediments. The same sample depths as for the *mcrA* gene libraries were used i.e. Meknes slope 110 cmbsf; Meknes crater 50 cmbsf and Porto crater 50 cmbsf. In the Meknes slope library, 14 clones were sequenced and examined. These sequences were all found to represent the same OTU[2], represented by phylotype D1_53, and shared greater than 98% sequence similarity. Comparative sequence analysis with the translated amino acids identified a *dsrA* clone (Colne dsrA OTU-089) from the Colne Estuary to be the nearest phylogenetic neighbour (Kondo *et al.*, 2007). The sequence shared 95% similarity with the Meknes Slope OTU and was recovered from a predominantly freshwater region of the estuary (Table 4.6). This matching clone was part of a wider phylogenetic group of unknown physiology named 'Colne group 2', which was found throughout all 4 sites of the Colne Estuary investigated by Kondo *et al.* (2007). Further comparison of the Meknes Slope OTU with the sub-directory of the BLASTX database containing only *dsrA* genes from cultured organisms, revealed the closest described relative was the organism *Carboxydothermus hydrogenoformans* strain z-2901, from the *Thermoanaerobacterales* family in the *Firmicutes* (Wu *et al.*, 2005), though the two sequences shared only 75% similarity (Table 4.6).

[2] There is no consensus on how to distinguish species or genera using *dsrA* sequence similarity data but a relatively conservative value of 90% was chosen for this study. The taxa (OTU) defined for the purpose of this analysis are likely to be at least distinct at the species level (Kondo *et al.*, 2007).

Table 4.6 Sequence identities of bacterial *dsrA* genes from Meknes and Porto gene libraries

Clone Library	OTU[a]	No. of clones	Closest phylotype in BLASTx database (Protein ID)	Identities (%)	Origin of closest sequence match	Reference	Closest described relative in BLASTx database[b]	Identities (%)
Meknes slope 110 cm depth	D1_53	14	Colne dsrA OTU-089 (BAF56375)	71/74 (95)	Estuarine sediments from a predominantly freshwater region of the Colne Estuary	Kondo *et al* (2007)	*Carboxydothermus hydrogenoformans* strain z-2901	54/72 (75)
Meknes crater 50 cm depth	D2_16	1	Clone BSII-2 (CAJ84864)	69/71 (97)	SMTZ (188 cm depth) in Black Sea Sediment	Leloup *et al* (2007)	*Desulfatibacillum alkenivorans* strain Ak-01	60/71 (84)
	D2_65	1	AB-DGGE-11 (CAL64105)	47/50 (94)	Marine sediment from Aarhus Bay	Leloup (2006) *unpublished*	*Desulfatibacillum alkenivorans* strain Ak-01	37/50 (74)
Porto crater 50 cm depth	D3_1	2	Clone dsr-121 (ACB05453.1)	72/73 (98)	30-35cm depth in gas hydrate-bearing sediment Gulf of Mexico	Ye *et al* (2008) *in press*	*Desulfatibacillum alkenivorans* strain Ak-01	59/72 (81)
	D3_16	5	Clone L. Suigetsu dsrA OTU-86 (BAF80572)	67/74 (90)	0-4cm depth in sulphate reducing sediment of Lake Suigetsu	Kondo & Butani (2007)	*Carboxydothermus hydrogenoformans* strain z-2901	55/72 (76)
	D3_54	2	Clone dsr-190 (ACB05459)	66/66 (100)	30-35cm depth in gas hydrate-bearing sediment Gulf of Mexico	Ye *et al* (2008) *in press*	*Desulfovibrio desulfuricans subsp. desulfuricans* strain G20	52/66 (78)
	D3_8	2	Clone Ntd-V05 (BAE96515)	72/73 (98)	Nankai Trough deep sea sediment	Kaneko *et al* (2007)	*Carboxydothermus hydrogenoformans* strain z-2901	55/72 (76)
	D3_3	1	Colne dsrA OTU-033 (BAF56319)	70/70 (100)	Estuarine sediments from a predominantly marine region of the Colne Estuary	Kondo *et al* (2007)	*Syntrophobacter fumaroxidans* strain MPOB	58/69 (84)
	D3_14	1	Clone L. Suigetsu dsrA OTU-71 (BAF80557)	68/74 (91)	0-4cm depth in sulphate reducing sediment of Lake Suigetsu	Kondo & Butani (2007)	*Carboxydothermus hydrogenoformans* strain z-2901	54/72 (75)

[a] OTUs were defined as sequences with greater than 90% similarity to each other.
[b] Closest described relatives were determined by comparing the *dsrA* sequences from this study with those in the NCBI reference sequence protein (refseq_protein) sub directory of the BLASTx database.

Clones from the Meknes MV 50 cmbsf crater library were sequenced and examined but were found often not to be the correct length for the region of the *dsrA* gene targeted, and were hence shown to represent erroneously cloned PCR artefacts. Indeed, inspection of the original PCR reaction gels (Fig 4.12) showed some products other than the target size expected, and it seemed these additional products had often been preferentially cloned. Out of the clones sequenced from this library only 2 were found to be *dsrA* gene sequences. These sequences were analysed and shown to represent two distinct OTUs (Table 4.6). The first was 97% similar to a clone (BSII-02) recovered from the SMTZ in sediment from the Black Sea (Leloup *et al.*, 2007), and was 84% similar to its closest described relative *Desulfatibacillum alkenivorans* strain Ak-01, which is a member of the *Desulfobacterales* family in the *Deltaproteobacteria* (Copeland *et al.*, unpublished). The closest phylotype, clone BSII-02, represented the most common OTU in the library derived from the SMTZ of the Black Sea sediment, and its phylogenetic affiliation with *Desulfosarcina*, *Desulfococcus* and other species of the *Desulfobacteraceae* led the authors to assert that it could represent the *dsrA* gene of an AOM-catalysing SRB (Leloup *et al.*, 2007). The second OTU in the Meknes MV crater library was 94% similar to a *dsrA* DGGE sequence (AB-DGGE-11) obtained from sediment from Aarhus Bay (Leloup, 2006 unpublished). The closest described relative was the same as for the first OTU in this library although the sequence similarity was much lower at just 74%. The two OTUs in the Meknes MV crater library shared only 85% sequence similarity and therefore may represent distinct phylotypes at the genus level.

A total of 13 clones were sequenced from the library constructed from the Porto crater 50 cmbsf sample. Using the 90% similarity level to discriminate different taxa, 6 different OTUs were found (Table 4.6). The most abundant OTU represented 5 sequences and was 90% similar to a clone (dsrA OTU-86) from the near-surface of (saline) sulphate reducing sediment in Lake Suigetsu, Japan (Kondo & Butani, 2007). This clone formed part of a monophyletic group without any close cultured relatives, and was hence of unknown function. Comparing the Porto OTU with sequences from described organisms revealed only a low similarity (76%) with the Firmicute *Carboxydothermus hydrogenoformans* strain z-2901 (Wu *et al.*, 2005). Three further OTUs from the Porto library were represented by two sequences each, the first two were most closely related to clones from a library constructed with gas hydrate

bearing sediment from 30-35 cm depth in the Gulf of Mexico, clones dsr-121 and dsr-190, respectively (Ye *et al.*, 2008 in press). The first was 81% similar to the *dsrA* gene from *Desulfatibacillum alkenivorans* strain Ak-01, in the *Desulfobacterales* family of the *Deltaproteobacteria* (Copeland *et al.*, unpublished) and the second was 78% similar to the *Deltaproteobacteria Desulfovibrio desulfuricans subsp. desulfuricans* strain G20 (Copeland *et al.*, unpublished). The final of the 3 OTUs represented by two sequences in the Porto library was 98% similar to a clone (Ntd-V05) recovered from sediment from the Nankai Trough (Kaneko *et al.*, 2007). The matching clone was part of a deeply branching lineage (Dsr Group V) highly affiliated with clones from the Guaymas Basin (Dhillon *et al.*, 2003), the group was unrelated to any cultured organisms but was related to *dsrAB* clones widely present in marine environments (Kaneko *et al.*, 2007). This MV clone shared 76% sequence similarity with the Firmicute *Carboxydothermus hydrogenoformans* strain z-2901 (Wu *et al.*, 2005). The two remaining OTUs were each represented by a single sequence in the library (Table 4.6). The first was 100% similar to a clone (dsrA OTU-33) from a predominantly marine region of the Colne Estuary (Kondo *et al.*, 2007). This matching clone fell into a group of several OTUs phylogenetically related to the *Syntrophobacteraceae* group (Kondo *et al.*, 2007) and indeed the closest cultured relative of the clone from this study was the Deltaproteobacterium *Syntrophobacter fumaroxidans* strain MPOB (Copeland *et al.*, unpublished). The final OTU in the library shared 91% similarity with a clone (dsrA OTU-71) from near-surface sulphate reducing sediment in Lake Suigetsu, Japan (Kondo & Butani, 2007) and its closest cultured relative (75% sequence similarity) was the Firmicute *Carboxydothermus hydrogenoformans* strain z-2901 (Wu *et al.*, 2005). The three individual MV libraries were collectively analysed but there was no sequence similarity above 90% between their respective OTUs.

4.3.10 Discussion of molecular genetic results for Meknes and Porto MVs

4.3.10.1 Methodological considerations: PCR inhibitors, gene template concentrations, exogenous DNA and DGGE band sequencing

During the extraction procedure it was noticed that many of the sample supernatants had a brown colouration and that the degree of colouration was greatest in samples that yielded the highest concentrations of DNA. The discolouration of the supernatant

may have resulted from the co-extraction of inhibitory compounds such as humic acids and would explain why some samples, particularly those with relatively high initial DNA concentrations, required dilution before PCR reactions were successful. By trialling several template dilutions with bacterial 16S rRNA and functional gene-targeted primers then scrutinizing DGGE band profiles or the relative PCR product brightness, the diversity detectable by PCR was maximised. Only a single template dilution (1:100) was trialled with the archaea 16S rRNA gene PCR reactions as this was the most successful for the bacteria, but the later functional gene PCR results suggested this might have resulted in a loss of detectable archaeal diversity. The results of taxonomic and functional gene PCR reactions with the Meknes and Porto MV samples showed that consideration of the relative levels of gene template and PCR inhibitors in a sample is important when seeking to maximise the detectable diversity. It was shown with the Meknes MV crater samples that PCR inhibitors were a greater problem with the 10 cmbsf sample with a high initial DNA concentration, and thus dilution was beneficial, but that in the two deeper samples where DNA concentration was lower and PCR inhibitors were less of a problem, dilution actually became detrimental to PCR success. It can be assumed that different genes (e.g. bacterial and archaeal 16S rRNA and *mcrA* and *dsrA*) will be present in different amounts in a given DNA extract (e.g. Aller & Kemp 2008; Lipp *et al.*, 2008 and references therein) and thus the level of dilution required to reveal the maximal diversity may depend on the genes being targeted. It is recommended, therefore, that to fully optimize the detectable diversity several DNA template dilutions should be trialled with each set of different gene-targeted primers in an attempt to reach the most favourable ratio of targeted genes to PCR inhibitors in the sample.

It was clear from DGGE analysis and sequencing results that some exogenous DNA had been co-extracted with sediment DNA during the extraction procedure. The fact all plasticware was routinely sterilised by autoclaving and/or UV exposure before use makes the kit reagents the most likely source of this contaminating DNA. Unpublished results suggest the poly-adenosine added to optimise extraction was a probable source of the contamination (G. Webster personal communication). Fortunately, the contamination was limited to a single 16S rRNA gene sequence and once identified could easily be excluded from the analysis. Close relatives of the contaminating sequence, a member of the *Betaproteobacteria* related to the

Burkholderia/Herbaspirillum group, have previously been identified as possible contaminants of molecular reagents in other studies using low DNA templates (e.g. Tanner *et al.*, 1998). These findings emphasize the importance of identifying all possible sources of contamination so they are not erroneously included in environmental diversity data.

4.3.10.2 Analysis of functional gene diversity using a DGGE-sequencing approach

The present study used a combined DGGE-sequencing approach to qualitatively screen functional gene diversity and identify dominant functional gene groups present in the MV samples. The results were then verified against functional gene library data collected from selected samples. For the *mcrA* gene, results of the DGGE-sequencing approach agreed very well with clone library data in respect of the relative level of diversity and identity of dominant gene groups. Additional phylotypes were sometimes identifiable by gene cloning compared to the DGGE approach alone but their relative abundance in the gene libraries indicated these additional sequences were not representative of the dominant organisms. Initial results of DGGE screening partial *dsrA* gene fragments (~160 bp) showed that with some further optimization to enable greater sequencing success (e.g. different denaturant gradients and/or adding a GC clamp to one of the primers, Wilms *et al.*, 2007), and with further expansion of public *dsrA* gene sequence databases, this method could also provide a useful approach to assessing *dsrA* gene diversity and determining the dominant groups present in a sample. However, the clone libraries constructed from amplified *dsrA* gene products exposed a greater level of diversity, particularly in the Porto crater sample, than was suggested by DGGE results alone, e.g. compare Fig 4.15, lane 596B and Porto clone library results. It can be concluded therefore that a DGGE-sequencing approach as described here offers a quick and reliable method of screening functional gene diversity and identifying dominant functional gene groups in environmental samples, which may be of particular use when a high throughput method is required, but clone libraries are necessary to expose the fuller details of community composition.

4.3.10.3 16S rRNA and functional gene diversity in the Meknes and Porto MV sites

Bacteria belonging to the JS1 candidate division (Webster *et al.*, 2004) were identified as dominant community members in both the Meknes and Porto MV craters. This group has previously been detected as an abundant or dominant component of the bacterial community in studies of several marine environments: deep sub-seafloor sediments, e.g. Nankai Trough (Newberry *et al.*, 2004), Sea of Okhotsk (Inagaki *et al.*, 2003), Peru Margin (Webster *et al.*, 2006a), Skagerrak (Parkes *et al.*, 2007); methane hydrate and cold seep sediments (Li *et al.*, 1999; Reed *et al.*, 2002; 2006; Inagaki *et al.*, 2006); shallow hydrothermal marine sediments (Teske *et al.*, 2002), and also in tidal flat sediments (Webster *et al.*, 2004; 2006b; 2007). The group has also been found in benzene-degrading enrichments (Phelps *et al.*, 1998) and in acetate-utilizing sulphate-reducing enrichments (Webster *et al.*, 2006b), yet its biogeochemical role still remains to be elucidated (Webster *et al.*, 2007). In stratified sediments JS1 has previously been observed to have an apparent preference for organic-rich sediment horizons, though the associated organic matter may be recalcitrant in nature (Inagaki *et al.*, 2006; Webster *et al.*, 2007). Furthermore, it has also been suggested that JS1 has a preference for low-sulphate concentration (e.g. ≤ 10 mM) conditions (Webster *et al.*, 2007), however, the findings of the present study do not always support these proposed associations. In this study JS1 16S rRNA genes were found to be abundant in the 50 cmbsf horizon of the Porto MV crater and in the 50 and 65 cmbsf horizons of the Meknes MV crater, according to PCR-DGGE analysis. Interestingly, these horizons in the two MVs had very different geochemical conditions. At the depth in Porto MV where JS1 dominated, sulphate concentrations were 29 mM and acetate concentrations were ~5 μM, whereas at the depths where JS1 appeared dominant in Meknes MV the sulphate concentration was ~0.1 mM and acetate was ~30 μM. As the PCR-DGGE analysis was conducted with general 16S rRNA primers rather than JS1 targeted primers these data can be considered reliable. This would suggest that JS1 is not restricted to specific biochemical niches, which may help explain its wide distribution. However, this does not offer insight as to it biogeochemical function. The JS1 sequences identified in the two sites shared just 96% sequence similarity across ~180 bp suggesting significant phylogenetic and, by inference, significant physiological differences may exist with the group.

The *Archaea* detected in the MV craters by molecular genetic analysis mostly comprised phylotypes associated with AOM, though the phylogenetic groups differed according to site. In the Meknes MV crater (50 cmbsf) ANME-1 sequences dominated according to both the 16S rRNA and *mcrA* gene data. This group is commonly associated with seep/methane-containing sediments and have been found as a dominant community member in a Black Sea microbial mat (Knittel *et al.*, 2005) and a North Sea gas seep (Niemann *et al.*, 2005). Notably, no putative SRB syntrophic partners were detected at this depth during the *dsrA* gene analysis (section 4.3.9). Additionally to ANME-1, sequences belonging to the methylotrophic methanogen *Methanococcoides* were found, and it is noteworthy this organism was successfully enriched from the upper 10 cmbsf horizon (see section 4.4.2). Over half (63%) of the sequences in the *mcrA* gene library from the Porto MV crater (50 cm depth) were affiliated with '*mcrA* group e', which is believed to represent ANME organisms (Nunoura *et al.*, 2006; 2008). The corresponding 16S rRNA gene library did not contain ANME sequences, but rather was dominated by phylotypes affiliated with the *Crenarchaeota* Marine Group 1. The remaining sequences in the Porto MV crater *mcrA* library belonged to *Methanococcoides* suggesting this may be a common organism in the MV environment.

4.4 Culture-based analysis of the Meknes and Porto MV sediment

4.4.1 Culturability of prokaryotes in the Meknes and Porto MV sites

Culturable microbial diversity was investigated in 8 samples from the two MV sites: 110 cmbsf, Meknes reference; 110 cmbsf, Meknes slope; 10 and 50 cmbsf, Meknes MV crater, and 10, 50 110 and 200 cmbsf, Porto MV crater. Seven different types of enrichment media were used. Six were targeted to specific functional groups of interest (heterotrophic SRB, heterotrophic methanogens, heterotrophic acetogens, autotrophic SRB, autotrophic methanogens and autotrophic acetogens) and one was a general enrichment medium containing yeast extract as the substrate (see section 2.4.2). Autotrophic enrichments were incubated at 25°C and heterotrophic enrichments were incubated at both 7 and 25°C (see section 2.4.3).

Overall, the enrichment success was quite low, with growth detected in only 12 of the 88 enrichments after 6 months of incubation. The 12 positive enrichments were from near-surface depths in the Meknes and Porto craters (Table 4.7).

Table 4.7 Details of positive enrichments from Meknes and Porto MV samples and whether successful sub-cultures were achieved
Sample 580 A was 10 cmbsf in Meknes MV crater; sample 580 B was 50 cmbsf in Meknes MV crater; sample 596 A was 10 cmbsf in Porto MV crater.

Sample	Enrichment medium	Substrate	Incubation temperature	Successfully sub-cultured
580 A	General heterotroph	Yeast extract	25°C	Yes
580 A	Heterotrophic SRB	Acetate and lactate	25°C	Yes
580 A	Heterotrophic methanogen	Acetate	25°C	No
580 A	Heterotrophic acetogen	Ethanol	25°C	No
580 A	Autotrophic SRB	H_2:CO_2	25°C	No
580 A	Autotrophic methanogen	H_2:CO_2	25°C	No
580 A	Autotrophic acetogen	H_2:CO_2	25°C	No
580 A	General heterotroph	Yeast extract	7°C	Yes
580 A	Heterotrophic SRB	Acetate and lactate	7°C	Yes
580 B	Yeast extract	Yeast extract	7°C	No
596 A	Yeast extract	Yeast extract	25°C	No
596 A	Heterotrophic SRB	Acetate and lactate	25°C	No

Cultivation success was highest with sediment from 10 cmbsf in the Meknes MV crater. Nine of the 11 enrichments inoculated with this sediment developed growth after 1 month. Additionally, one enrichment type from 50 cmbsf in the Meknes MV crater also became positive, which was the media containing yeast extract aimed at general heterotrophs, incubated at 7°C. Two enrichments inoculated with sediment from 10 cmbsf in the Porto MV crater became positive though cell numbers were lower (data not shown). These were the yeast extract containing general heterotrophic medium incubated at 25°C and the heterotrophic SRB medium also incubated at 25°C.

4.4.2 Analysis of enrichments series from the Meknes MV crater

Four successful subcultures were achieved from the 12 positive enrichments shown in Table 4.7, and these were all with sediment from 10 cm depth in the Meknes crater. The new subcultures grew well and typically developed visible turbidity in around 1 week. Over a period of 6 months 4 successive subcultures were made from each. DNA was extracted from each of the cultures (original enrichments plus 4 subcultures) and amplified with universal bacterial and archaeal 16S rRNA gene primers to investigate the composition of the enrichment via DGGE. PCR amplification showed *Archaea* were present in 16/20 samples (Fig 4.16) and *Bacteria* were present in all samples (data not shown).

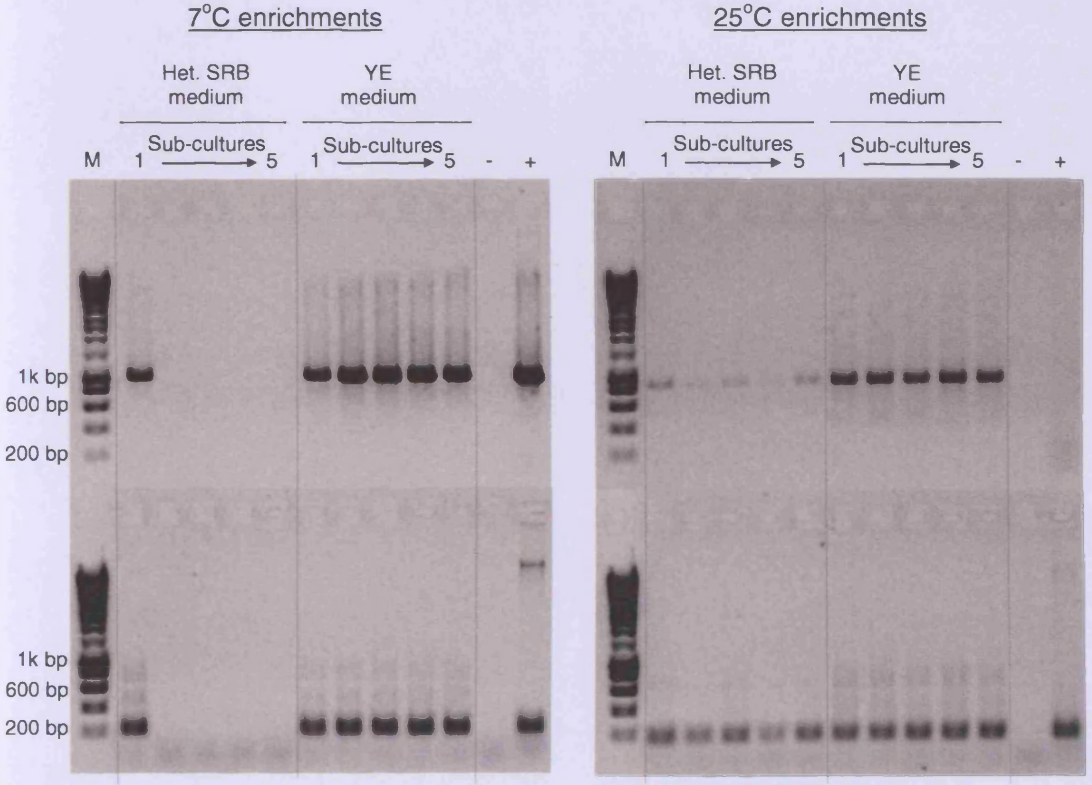
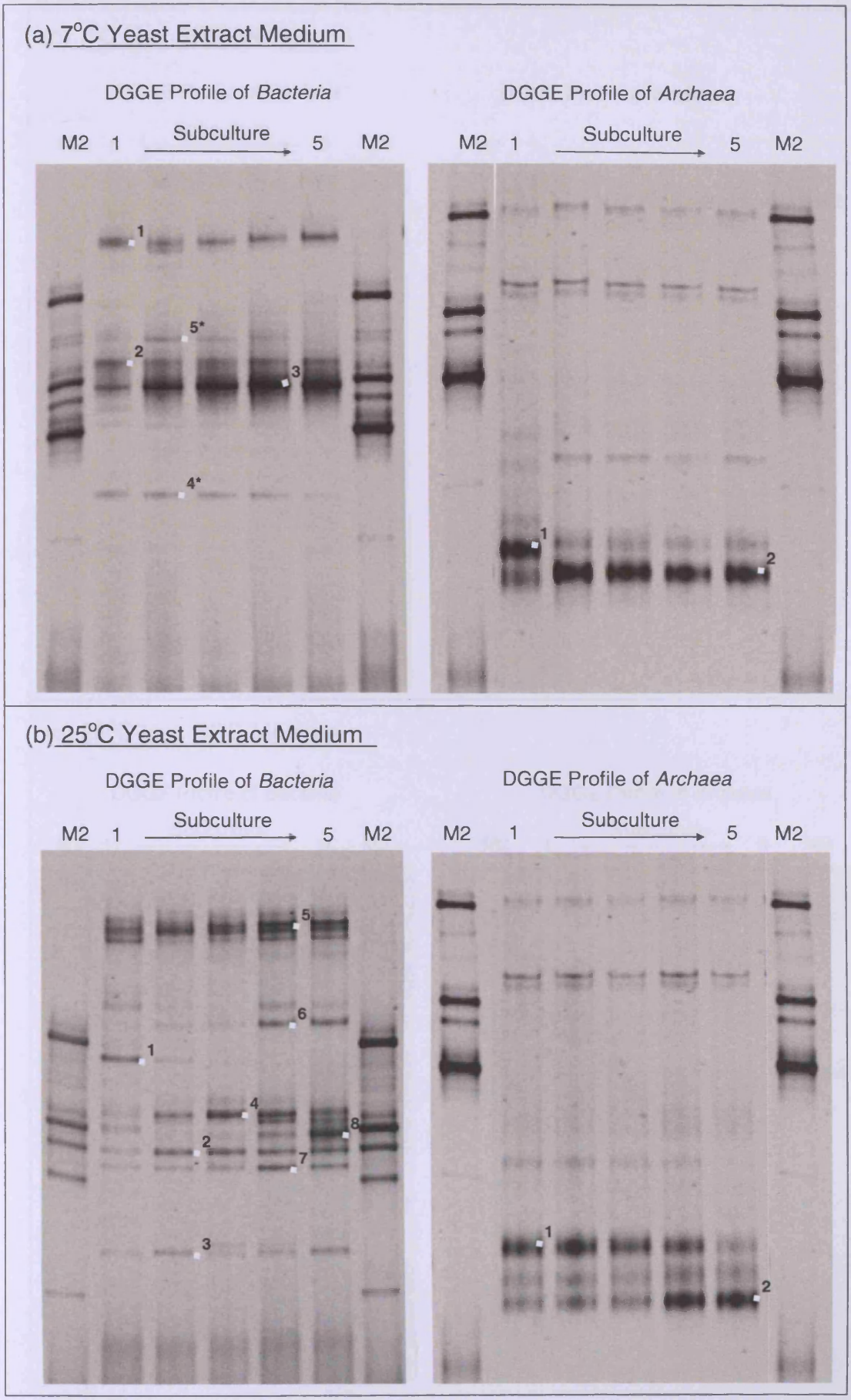


Fig 4.16 Results of direct (top panels) and nested (bottom panels) PCR with general archaeal 16S rRNA gene primers and DNA from positive Meknes crater 10 cm depth subcultures. Sub-cultures labelled 1 were the original enrichments. Lane M is HyperLadder I, lanes marked – and + were the PCR negatives and positives respectively.

Archaea were readily detectable in all yeast extract enrichments incubated at both 7 and 25°C. *Archaea* were also readily detectable in the initial 7°C heterotrophic SRB enrichment but were absent in the successive sub-cultures. The relative intensity of archaeal PCR products obtained from the 25°C heterotrophic SRB enrichments suggested *Archaea* were less abundant under these particular growth conditions but

that they represented an actively growing population, as they were detectable in all subcultures.

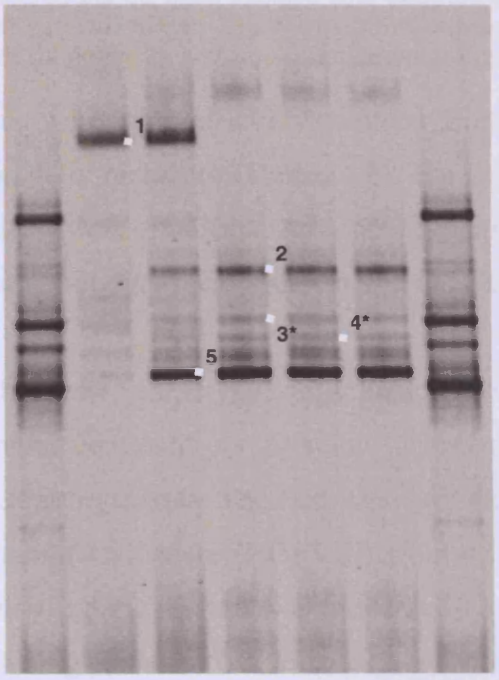
DGGE screening showed that each of the 4 enrichment types had a distinct community structure (Fig 4.17).


(a) 7°C Yeast Extract Medium
DGGE Profile of *Bacteria*
M2 1 Subculture 5 M2
1
2
5*
3
4*
DGGE Profile of *Archaea*
M2 1 Subculture 5 M2
1
2
(b) 25°C Yeast Extract Medium
DGGE Profile of *Bacteria*
M2 1 Subculture 5 M2
5
6
1
4
8
2
7
3
DGGE Profile of *Archaea*
M2 1 Subculture 5 M2
1
2

(c) 7°C Het. SRB Medium

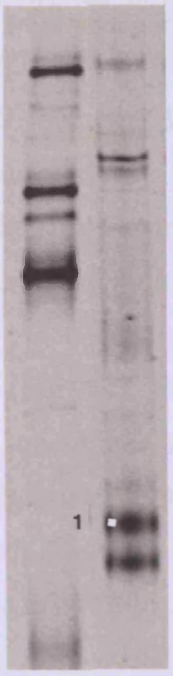
DGGE Profile of *Bacteria*

M2 1 Subculture → 5 M2



DGGE Profile of *Archaea*

M2 1

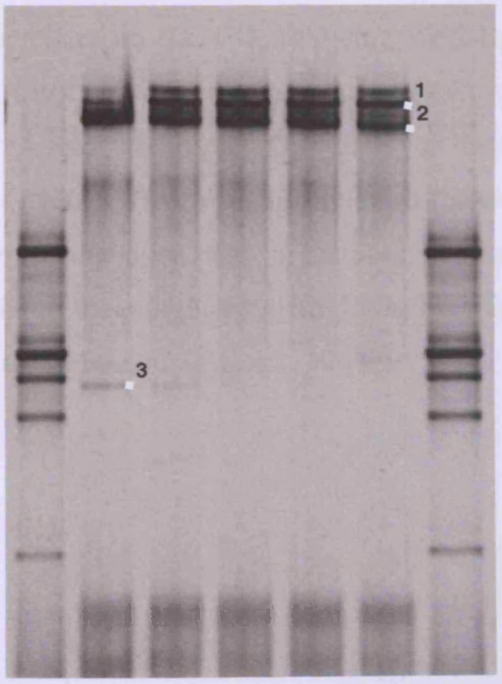


N.B Archaea were absent from subcultures 2 - 5

(d) 25°C Het. SRB Medium

DGGE Profile of *Bacteria*

M2 1 Subculture → 5 M2



DGGE Profile of *Archaea*

M2 1 Subculture → 5 M2

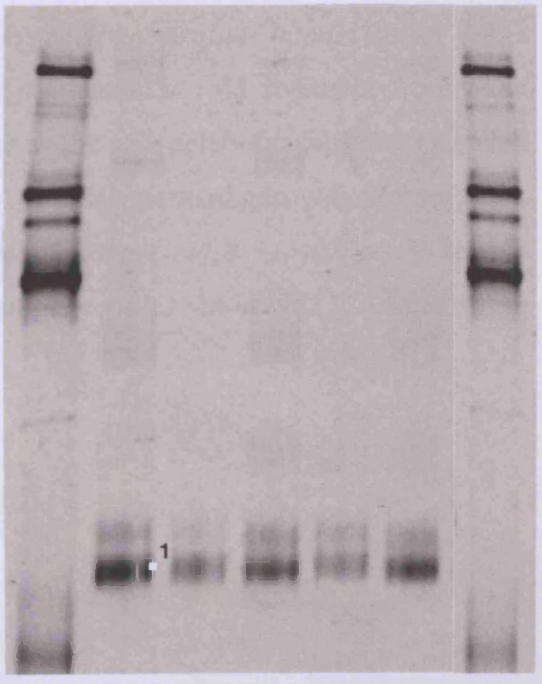


Fig 4.17 DGGE profiles of bacterial and archaeal 16S rRNA gene products amplified from the Meknes MV crater 10 cmbsf enrichment cultures. Panel (a) yeast extract medium enrichment series incubated at 7°C, panel (b) yeast extract medium enrichment series incubated at 25°C, panel (c) heterotrophic SRB medium enrichment series incubated at 7°C. Note only the initial enrichment contained *Archaea*, panel (d) heterotrophic SRB medium enrichment series incubated at 25°C. In all cases the lanes marked 1 were the original enrichments. Heterotrophic SRB medium contained acetate (5 mM) and lactate (15 mM) as substrates. Numbered bands were excised and sequenced (*Bacteria*), or corresponded to samples from which near full-length 16S rRNA gene products were sequenced (*Archaea*), see Table 4.8. Bands marked with an * could not be sequenced and may have represented PCR artefacts.

Bands were excised and sequenced from the bacterial DGGE gels to identify the community members (Tables 4.8). For the Archaea near full-length 16S rRNA gene products were amplified and sequenced, as often only a single archaeal phylotype was present, however the corresponding DGGE bands are indicated in Fig 4.17

In the yeast extract 7°C enrichment series (Fig 4.17 panel a and Table 4.8), the bacterial community was stable across all subcultures and was composed of two different organisms. The first was very closely related (99% sequence similarity) to a *Bacteroidetes* isolate (NO5VIII) from a North Sea tidal flat (B. Köpke unpublished results) and the second was moderately related (94%) to an obligatory anaerobic H_2-producing fermentative *Fusobacteria* isolate from cyclic nitramine polluted marine sediment (Zhao *et al.*, 2004). Two other DGGE bands present on the bacterial gel could not be sequenced and may have represented PCR artefacts. The archaeal community was comprised of two methanogens belonging to the orders *Methanomicrobiales* and *Methanosarcinales*. In the initial enrichment an organism 98% related to the CO_2-reducing methanogen *Methanogenium boonei* strain AK-7 was detected. This strain was isolated from permanently cold sediment from Skan Bay, Alaska, and used CO_2 plus H_2 or formate as the sole catabolic substrate (Kendall *et al.*, 2007). However, in the subcultures a different methanogen was present. This other methanogen was a member of the *Methanosarcinales* most closely related (99%) to the obligately methylotrophic *Methanococcoides* strain NaT1, isolated from marine sediments (Tanaka, 1994).

Table 4.8 Closest matches to bacterial and archaeal 16S rRNA gene DGGE bands from Meknes crater 10 cm depth enrichments.
Bac. – Bacteria Arc. – Archaea

Enrichment	Band identifier	Sequence length (bp)	Closest match in BLASTN database (Accession number)	Identities (%)	Phylogenetic affiliation	Origin of closest matching sequence(s)
Yeast extract medium incubated at 7°C	Fig 4.17 (a) Bac. Band 1	179	Isolate NO5VIII (AJ786089)	168/169 (99%)	*Bacteroidetes*	Tidal flat sediment
	Fig 4.17 (a) Bac. Band 2	163	Isolate HAW-EB21 (AY579753)	145/153 (94%)	*Fusobacteria*	Marine sediments containing cyclic nitramine explosives, sea water, Basalt from extinct smoker pipe, *Paralvinella palmiformis* (palm worm) mucus secretions, tidal flat sediment
	Fig 4.17 (a) Bac. Band 3	160	Same as band 2	151/158 (95%)	*Fusobacteria*	Same as band 2
	Fig 4.17 (a) Arc. Band 1	794	*Methanogenium boonei* strain AK-7 (DQ177343)	779/792 (98%)	*Methanogenium*	Cold marine anoxic sediments
	Fig 4.17 (a) Arc. Band 2	803	Isolate NaT1 (Y16946)	791/798 (99%)	*Methanococcoides*	Marine sediments
Yeast extract medium incubated at 25°C	Fig 4.17 (b) Bac. Band 1	193	Clone LV60-CY-19 (AY642583)	182/195 (93%)	*Firmicutes*	Alkaline lake microbialites
	Fig 4.17 (b) Bac. Band 2	168	Sapropel isolate J151 (AJ630153)	167/167 (100%)	*Firmicutes*	Deep-sea sediment, tidal flat sediment
	Fig 4.17 (b) Bac. Band 3	171	Clone DR546BH1103001SAD18 (DQ234642)	164/171 (95%)	*Firmicutes*	Terrestrial subsurface water, gold mine borehole water, anoxic hypersaline sediment, hydrothermal vent
	Fig 4.17 (b) Bac. Band 4	170	Clone T37 (Z94008)	163/170 (95%)	*Firmicutes*	Activated sludge

Table 4.8 cont. Closest matches to bacterial and archaeal 16S rRNA gene DGGE bands from Meknes crater 10 cm depth enrichments.
Bac. – Bacteria Arc. – Archaea

Enrichment	Band identifier	Sequence length (bp)	Closest match in BLASTN database (Accession number)	Identities (%)	Phylogenetic affiliation	Origin of closest matching sequence(s)
Yeast extract medium incubated at 25°C cont.	Fig 4.17 (b) Bac. Band 5	193	*Spirochaeta litoralis* (M88723)	187/195 (95%)	*Spirochaetes*	Marine mud
	Fig 4.17 (b) Bac. Band 6	188	Clone CH5_3f_BAC (AY672529)	179/191 (93%)	*Bacteroidetes*	Hydrothermal vent, permanently cold marine sediments, biofilm from sulfidic cave stream
	Fig 4.17 (b) Bac. Band 7	188	Isolate DG890 (AY258122)	179/188 (95%)	*Bacteroidetes*	Marine dinoflagellate, sea water
	Fig 4.17 (b) Bac. Band 8	161	Isolate HAW-EB21 (AY579753)	148/152 (97%)	*Fusobacteria*	Marine sediments containing cyclic nitramine explosives, sea water, basalt from extinct smoker pipe, Paralvinella palmiformis mucus secretions, tidal flat
	Fig 4.17 (b) Arc. Band 1	535	SBAK-CO_2-reducing enrichment-2 clone (DQ280483)	532/537 (99%)	*Methanogenium*	Cold marine anoxic sediments
	Fig 4.17 (b) Arc. Band 2	454	Isolate NaT1 (Y16946)	443/455 (97%)	*Methanococcoides*	Marine sediments
Het. SRB medium incubated at 7°C	Fig 4.17 (c) Bac. Band 1	180	Isolate NO5VIII (AJ786089)	163/165 (99%)	*Bacteroidetes*	Tidal flat sediment
	Fig 4.17 (c) Bac. Band 2	194	Isolate *Desulfotalea psychrophila* LSv54 (CR522870)	190/196 (96%)	*Desulfobulbaceae*	Permanently cold Arctic marine sediments

Table 4.8 cont. Closest matches to bacterial and archaeal 16S rRNA gene DGGE bands from Meknes crater 10 cm depth enrichments.
Bac. – Bacteria Arc. – Archaea

Enrichment	Band identifier	Sequence length (bp)	Closest match in BLASTN database (Accession number)	Identities (%)	Phylogenetic affiliation	Origin of closest matching sequence(s)
Het. SRB medium incubated at 7°C cont.	Fig 4.17 (c) Bac. Band 5	196	Isolate *Desulfotalea arctica* LSv514 (AF099061)	187/195 (95%)	*Desulfobulbaceae*	Permanently cold Arctic marine sediments
	Fig 4.17 (c) Arc. Band 1	768	Isolate NaT1 (Y16946)	759/767 (98%)	*Methanococcoides*	Marine sediments
Het. SRB medium incubated at 25°C	Fig 4.17 (d) Bac. Band 1	174	Isolate Desulfovibrio sp. strain HAW-EB18 (AY579755)	174/174 (100%)	*Desulfovibrio*	Estuarine microbial mat, marine sediments, marine sediments containing cyclic nitramine explosives, tidal flat sediment
	Fig 4.17 (d) Bac. Band 2	174	Isolate Desulfovibrio sp. NA302 (AJ866944)	174/174 (100%)	*Desulfovibrio*	Tidal flat sediment
	Fig 4.17 (d) Bac. Band 3	176	Clone MN16BT2-16 (AF361654)	157/175 (89%)	*Deltaproteobacteria*	Carbonate crust from Napoli mud volcano, marine sediment above Hydrate Ridge
	Fig 4.17 (d) Arc. Band 1	571	Isolate NaT1 (Y16946)	566/574 (98%)	*Methanococcoides*	Marine sediments

In the 25°C yeast extract enrichment series (Fig 4.17, panel b) a more diverse bacterial community was present, which consisted of various *Firmicutes, Bacteroidetes* and *Fusobacteria*-related organisms (Table 4.8). The sequence similarity to closest matches in the BLASTN database was typically ≤95% despite excellent sequence quality, suggesting many of these enriched *Bacteria* might represent novel species or even genera. One organism however was 100% similar to sapropel isolate J151, isolated from sapropel 1 at site 575 (cruise M51/3 of the R/V Meteor) in the Eastern Mediterranean Sea. This isolate was obtained under anoxic conditions using a mixture of amino acids as substrate, while an identical isolate was obtained from the sediment surface of the same core under anoxic conditions using monomer medium (Süß *et al.*, 2004; H Sass, pers. comm.). The archaeal community was composed of methanogens most closely related to the same species as found in the 7°C yeast extract enrichments (Table 4.8). DGGE profiling suggested the *Methanogenium* organism and *Methanococcoides* organisms co-existed in some of the subcultures but that Methanogenium dominated the initial enrichment, while *Methanococcoides* dominated the final subculture (Fig 4.17, panel b). The *Methanogenium* and *Methanococcoides* methanogen sequences from the two yeast extract enrichment series incubated at the two different temperatures shared 99 and 97% similarity, respectively, indicating they were strains of the same species.

In the 7°C incubated heterotrophic SRB enrichments the community structure differed between the initial enrichment and the subsequent sub-cultures (Fig 4.17, panel c). In the initial enrichment a single dominant bacterial band representing a member of the *Bacteroidetes* was detected but disappeared after the second subculture. This band sequence was closely related to isolate NO5VIII and was phylogenetically identical to the 'Band 1' sequence from the 7°C yeast extract enrichment (Fig 4.17, panel a and Table 4.8). A methanogen most closely related to *Methanococcoides* strain NaT1 was detected in the initial enrichment but was absent from the subcultures (Fig 4.17, panel c). In the four 7°C heterotrophic SRB subcultures two additional bacterial DGGE bands were successfully sequenced (bands '2' and '5'). These band sequences were 93% similar to each other, and shared moderate sequence similarity (95–96%) to the SRB *Desulfotalea psychrophila* and *Desulfotalea arctica,* respectively, which were both isolated from cold marine sediments (Knoblauch *et al.*, 1999b). Analyzing re-amplified band products on a second DGGE gel showed that two fainter bands present

(bands '3' and '4' in Fig 4.17, panel c) could not be recovered due to interference from DNA of band 5 (data not shown).

The enriched organisms in the 25°C heterotrophic SRB enrichments were represented by two bright DGGE bands (bands '1' and '2' in Fig 4.17, panel d), which were present throughout the series, and also one faint DGGE band (band '3') that was only present in the initial enrichment. The two bright DGGE bands were 98% similar to each other and shared 100% sequence similarity with *Desulfovibrio* strain HAW-EB18, a strictly anaerobic SRB capable of degrading cyclic nitramines (Zhao *et al.*, 2004), and as yet uncharacterised *Desulfovibrio* strains isolated from tidal flat sediment from the North Sea Coast (Sass, unpublished results), respectively (Table 4.8). The most closely related characterised organism to the sequences (97 - 98% sequence similarity) was *Desulfovibrio acrylicus* a mesophilic SRB that can grow on lactate (van der Maarel *et al.*, 1996). The minor bacterial band in the initial enrichment was affiliated with the *Deltaproteobacteria* and was distantly related (~86%) to environmental clones from a MV carbonate crust (Heijs *et al.*, 2006) and sediment from Hydrate Ridge (Knittel *et al.*, 2003). Throughout the 25°C heterotrophic SRB enrichment series a single methanogen closely related (98%) to *Methanococcoides* strain NaT1 was present (Fig 4.17, panel d and Table 4.8).

4.4.3 Functional gene diversity in the Meknes MV crater enrichments

Functional (*dsrA* and *mcrA*) genes were amplified by direct PCR with two different primer sets from selected enrichment samples (see chapter 2.3). Appropriately sized products were analysed by DGGE as described for Meknes sediment samples, and bands were sequenced where possible to identify the closest matches of the functional genes present (Table 4.9).

Table 4.9 Success of amplifying functional genes (*mcrA* and *dsrA*) from selected enrichment samples, and the identity of functional genes present according to comparative sequence analysis of DGGE band sequences.

Sample	PCR success with *mcrA* primers		Closest match of DGGE band sequence in BLASTX database (Accession number)	Sample	PCR success with *dsrA* primers		Closest match of DGGE band sequence in BLASTX database (Accession number)
	ME1 – ME2	LutonF – LutonR			DSR1F – DSR4R	DSR1F+ – DSRR	
Yeast extract 7°C initial enrichment	+	+	98% similar to Enrichment clone McrA2 (ACD65220)	Yeast extract 7°C final enrichment	+	+	94% similar to Enrichment clone NKeSL1 (ACH73209)
Yeast extract 7°C final enrichment	+	+	99% similar to *Methanococcoides burtonii* DSM 6242 (ABE53268)	Yeast extract 25°C final enrichment	+	+	ND
Yeast extract 25°C initial enrichment	+	+	98% similar to Enrichment clone McrA2 (ACD65220)	Het. SRB 7°C initial enrichment	-	+/-	ND
Yeast extract 25°C final enrichment	+	+	98% similar to *Methanococcoides burtonii* DSM 6242 (ABE53268)	Het. SRB 7°C final enrichment	-	-	ND
Het. SRB 7°C initial enrichment	-	+	97% similar to *Methanococcoides burtonii* DSM 6242 (ABE53268)	Het. SRB 25°C initial enrichment	-	+	96% similar to Enrichment clone NKeSL1 (ACH73209)
Het. SRB 25°C initial enrichment	-	+	98% similar to *Methanococcoides burtonii* DSM 6242 (ABE53268)	Het. SRB 25°C final enrichment	+	+	94% similar to Desulfovibrio sp. TBP-1 (AAK48723))

Primers used for *mcrA* and *dsrA* gene DGGE analysis were LutonF - LutonR and DSR1F+ – DSRR, respectively.
ND – no data due to unsuccessful band sequencing.

A range of *mcrA* gene sequences were successfully detected with the LutonF – LutonR primer set (see Tables 2.5 and 2.6) in all methanogen-containing enrichments tested. Two of the methanogen enrichment samples failed to amplify with the ME1 – ME2 primer set (see Tables 2.5 and 2.6) and product yields were general lower than obtained with LutonF – LutonR primer set (data not shown), indicating the ME1 – ME2 primers were less sensitive under the conditions used. PCR amplification with *dsrA* gene-targeted primers indicated that reaction success was dependant on what organisms were present in the enrichments and also on which primer set was used. Results of comparative sequence analysis of *mcrA* and *dsrA* DGGE bands (Table 4.9) corresponded well with the phylogenetic affiliations determined on the basis of 16S rRNA gene data (Table 4.8). In the initial 7 and 25°C yeast extract enrichments, the *mcrA* genes were most closely related to a methanogen clone of unknown phylogenetic affiliation from an enrichment culture inoculated with sediment from an estuary (Jiang *et al.*, unpublished), but were 97% similar to the *mcrA* gene of *Methanogenium organophilum* DSM 3596 (Nunoura *et al.*, 2008). 16S rRNA gene analysis revealed the closest matches to the methanogens in these enrichments was *Methanogenium boonei* strain AK-7, and an enrichment clone also affiliated with *Methanogenium*, respectively. In the final sub-cultures of the 7 and 25°C yeast extract enrichment series, *mcrA* genes most closely related to *Methanococcoides burtonii* DSM 6242 (Copeland *et al.*, unpublished) were detected, while 16S rRNA gene analysis had shown *Methanococcoides* sp. isolate NaT1 to be the closest match to the enrichment organisms. The final heterotrophic SRB 25°C enrichment samples contained organisms with 100% 16S rRNA gene sequence similarity to uncharacterised *Desulfovibrio* species (Table 4.8), and contained *dsrA* genes most similar (<96%) to *dsrA* gene sequences affiliated with *Desulfovibrio* species (Table 4.9). The relatively low *dsrA* sequence similarity is likely to be a reflection of the limited *dsrA* gene sequences currently available in public databases rather than an indication of phylogenetic difference. *DsrA* gene products could not generally be amplified from the heterotrophic SRB 7°C enrichments confirming reports that the primers used do not target *Desulfotalea* species (Zverlov *et al.*, 2005). The faint product obtained from the initial heterotrophic SRB 7°C enrichment was most likely derived from another sulphate-reducing organism that was lost during sub-culturing. Surprisingly, *dsrA* genes were also amplified from the final 7 and 25°C yeast extract enrichments (other subcultures were not tested). These enrichments did not contain

sulphate-reducers according to the 16S rRNA DGGE data (Fig 4.17 and Table 4.8), suggesting that the sulphate-reducers from which these products were derived were present in low abundance. The *dsrA* DGGE band from the final 7°C yeast extract enrichment was sequenced and was found to share low similarity to the same *Desulfovibrio* isolate *dsrA* gene database sequence as matched the heterotrophic SRB 25°C enrichment genes. The *dsrA* genes sequenced from the enrichment samples shared 94–100% similarity with each other.

Comparison of the *dsrA* genes obtained from the Meknes MV crater enrichments and those obtained from the Meknes MV sediment *dsrA* gene libraries revealed no significant similarity (≤67%), which is consistent with the enrichment SRB being affiliated with *Desulfovibrio*, and the sediment library SRB being affiliated with *Desulfobacterales*. However, comparison of the *mcrA* genes obtained from the Meknes MV crater sediment enrichments, and those detected in the Meknes MV crater sediment *mcrA* gene libraries, strongly suggested that the same organism was present in both. Eight percent (n=2) of the Meknes MV crater sediment *mcrA* gene library was comprised of sequences belonging to *Methanococcoides*, and these sequences shared ≥99% sequence similarity with the *mcrA* genes amplified from the enrichments, indicating these culture attempts were successful in enriching an indigenous organism of functional significance from the crater.

4.4.4 Discussion of enrichment work

Overall, culturability was low in the MV sediments. However, at 10 cmbsf in the Meknes MV crater where high DNA yields and high prokaryotic total cells counts were obtained, growth was stimulated in the majority of enrichments prepared, and survived into subculture under 4 of the growth conditions tested. In these positive Meknes MV crater enrichments several physiological and phylogenetic groups of *Bacteria* were successfully cultivated along with two methanogenic *Archaea*. Growth was also detected in the 10 cmbsf hemipelagic sediment from the Porto MV crater but the prokaryotes did not survive into subculture and remained unidentified. No growth was detected in the deeper mud breccia sediment from the Porto MV crater or in 110 cmbsf samples from the Meknes MV slope or reference site.

Initially it seemed surprising that methanogens were cultivated from the near-surface sediment of Meknes MV crater, as sulphate was present in seawater concentrations at this depth and sulphate-reducers are known to out-compete methanogens for certain substrates e.g. acetate and hydrogen (Oremland & Taylor, 1978; Kristjansson *et al.*, 1982; Lovley *et al.*, 1982). However, the two particular methanogens present in the MV enrichments (identified on the basis of both 16S rRNA and *mcrA* gene sequence data) were members of the genera *Methanogenium* and *Methanococcoides*, which have been cultivated from sulphate-rich sediments before (Singh *et al.*, 2005; Kendall *et al.*, 2007), and therefore appear adapted to growth in the presence of sulphate. For example, all known species of the genus *Methanococcoides* do not grow on H_2:CO_2 formate or acetate, which are competitive with SRB, but instead grow solely on methanol and methylamines (Boone *et al.*, 2001; Singh *et al.*, 2005), which are not known to be utilized by SRB (e.g. Sowers & Ferry, 1983). *Methanogenium* species do grow on the competitive substrates hydrogen and formate but some can also use alcohols (1-propanol, 2-propanol and 2-butanol) as electron donors for methanogenesis (Sowers, 1995). The fact the *Methanogenium* organism was less successful in the MV co-cultures than the *Methanococcoides* organism supports the assumption that the latter should have a better competitive edge due to its exclusive use of non-competitive substrates. It is interesting to note that while all known *Methanococcoides* species grow only on methanol and methylated compounds (Singh *et al.*, 2005) these compounds were not added as substrates to the enrichments in which the *Methanococcoides* organism was found. It remains undetermined therefore what the *Methanococcoides* species in these particular enrichments were metabolizing, as the fact they were clearly present throughout 4 subcultures indicated an actively growing population. It is possible that the *Methanococcoides* organisms were growing on methylated compounds transferred with the sediment inoculum and/or produced as degradation products by the other organisms living in co-culture. For example many osmolytes, which contribute a significant fraction of organic matter in marine sediments (King, 1984), are methylated. Furthermore, Fiebig & Gottschalk (1983) have demonstrated that *Desulfovibrio* species, which were living in co-culture with *Methanococcoides* in the 25°C heterotrophic SRB enrichments, can provide a source of trimethylamine to methanogens in co-culture through the degradation of choline, though this was not specifically added to the MV enrichments. Further work is needed to investigate these interesting findings, however it is likely

that breakdown products from the added yeast extract or lactate were providing a source of substrates for the methanogens growing in these enrichments.

It remains to be determined how important a process methanogenesis is in the sulphate zone of the Meknes MV crater. Methanogen 16S rRNA gene sequences were not detected by PCR-DGGE analysis of 10 cmbsf Meknes MV crater sediment, but *mcrA* genes belonging to *Methanococcoides* were detected at this depth by gene cloning and these were virtually identical to those amplified from the enrichments. The potential for hydrogenotrophic and acetoclastic methanogenesis was investigated using stored cores, but did not detect methanogenesis at 10 cmbsf in the Meknes MV crater (see Appendix II). However, members of *Methanococcoides* are methylotrophic methanogens, and the potential for methylotrophic methanogenesis was not tested. The potential activity of this organism therefore remains undetermined in the MV environment. The importance of methylotrophic methanogenesis is poorly constrained in the marine sediment environment generally, but the presence of viable *Methanococcoides* in the Meknes MV sediment, and the findings of other studies which have detected methylotrophic methanogens in sulphate zones (e.g. Hydrate Ridge, Kendall & Boone 2006 and Skan Bay, Alaska; Kendall *et al.*, 2007), shows this process warrants further investigation in the future.

Cultivation results for the *Bacteria* highlighted the benefits of using different growth conditions (substrate and incubation temperature) with the same inoculum. Using this approach a variety of different *Bacteria* from important functional groups (fermenters and sulphate-reducers) were detected in the MV sediment. The heterotrophic SRB enrichment medium successfully selected for sulphate-reducers at both temperatures tested, though temperature determined which particular SRB were present. At 7°C the enriched SRB were most closely related (95–96%) to psychrophilic sulphate-reducers from permanently cold Arctic marine sediments (Knoblauch *et al.*, 1999b), while at 25°C the SRB were closely related to the mesophilic organism *Desulfovibrio acrylicus* (van der Maarel *et al.*, 1996). In yeast extract containing enrichments fermentative bacteria dominated, but functional gene analysis of these enrichments suggested sulphate reducers were also present in low abundance. The fermentative organisms were generally most closely related to organisms from other marine environments although sequence similarity with described species was often low,

suggesting the presence of several novel species. The lack of closely related described species meant specific physiologies could not be inferred for the majority of the *Bacteria* cultivated in the yeast extract-containing enrichments, and this would have to be determined through isolation and physiological testing. Isolations attempts were begun from these enrichments with some success but results are not available to be presented here. How abundant such fermentative organisms were as a percentage of the total population *in situ* remains to be quantitatively determined. Based on the thymidine incorporation measurements conducted and the PCR-based 16S rRNA gene data, there is no evidence to suggest that they were the numerically dominant community members, which would agree with the restricted energy supply available for such organisms in mud breccia with a low total organic carbon content. It is possible therefore that the fermenters were present *in situ* as a relatively minor component of the mud breccia community.

4.5 Chapter 4 synthesis and summary

4.5.1 Considerations of depth and spatial variability in pore water geochemistry

The pore water geochemical profiles varied between the two crater sites and those taken adjacent to and on the slope of the Meknes MV. In the Porto and Meknes MV crater sites pore water in the deepest samples contained 30 and 40% less Cl^- than standard (i.e. seawater derived) pore water, respectively. Similar pore water freshening with depth has been reported for other MV/cold seep sites globally, e.g. The Haakon Mosby MV (Ginsburg *et al.*, 1999; Niemann *et al* 2006a); The Olimpi MV field and the Anaximander Mts in the Eastern Mediterranean Sea (Haese *et al* 2006; Dählmann & de Lange 2003); MVs within the Barbados Accretionary Complex (Martin *et al.*, 1996); MVs along the convergent continental margin off Costa Rica (Hensen *et al.*, 2004); and Bonjardim, Ginsburg, Yuma and Gemini MVs in the Gulf of Cadiz (Mazurenko *et al.*, 2003; Hensen *et al.*, 2007). The likely explanation for this is upward advection of freshened fluid from depth where diagenetic clay mineral dehydration is taking place, typically at temperatures 60–150°C (Kastner *et al.*, 1991). The pore water measurements taken from outside the craters confirm that the influence of upward migrating fluids is spatially limited to the MV vents only. Notably, not all MVs in the Gulf of Cadiz show such a decrease in Cl^- concentration

with depth through their craters; pore water measurements from Capt. Arutyunov, Hesperides and Mercator MV have revealed an enrichment of Cl^- with depth with respect to seawater (Hensen *et al.*, 2007 and M Haeckel, unpublished data), which is thought to be attributable to the leaching of NaCl from localised sedimentary halite deposits, penetrated by the upward migrating freshened diagenetically influenced pore water (Hensen *et al.*, 2007).

Sulphate removal with depth in the Meknes and Porto MV craters was well beyond that which could be explained by pore water dilution alone and could only be reasonably attributed to bacterial sulphate reduction. Levels of bacterial sulphate reduction were highest in the Meknes MV crater site where sulphate decreased to concentrations <1 mM by 50 cmbsf. There was some appreciable bacterial sulphate removal in the Meknes MV slope and pelagic reference site (Fig 4.5, panel b) but pore water data indicated that the majority of bacterial activity was focussed within the crater. The Porto MV crater was less active in terms of sulphate reduction than the Meknes MV crater, with 1.18 mM sulphate still remaining in the deepest Porto MV sample measured (210 cmbsf). Many of the other MVs/advective cold seep sites studied to date have had SMTZs at or near the sediment surface (e.g. Haakon Mosby MV and seep sites studied in the Gulf of Mexico, Black Sea, Japan Sea and Eastern Mediterranean), rather than buried within the sediment column as at Meknes and Porto MV, and are hence characterised by the presence of significant communities of microbial mats at the sediment/water interface, nourished to a large extent by the sulphide produced during the AOM reaction (section 1.1.8). A further consequence of the narrow, surface/near-surface SMTZs in these previously studied sites has been a release of methane to the overlying water column, and sometimes atmosphere, as the resident microbial organisms (and their multicellular syntrophic partners) have been unable to consume all the reduced energy sources (principally methane) rising from depth, due partly to a lack of suitable electron acceptors with which to oxidise them (Niemann *et al.*, 2006a). In the Meknes and Porto MVs where fluid flow is relatively low and sulphate penetrates over several decimetres into the sediment column reduced compounds rising from depth can be consumed within the sediment, meaning these sites, as others previously investigated in the Gulf of Cadiz (Niemann *et al.*, 2006b), are unlikely to be significant sources of methane to the hydrosphere.

4.5.2 Cultivation success with Meknes and Porto MV sediments

The cultivation work presented in this chapter, in addition to that presented in chapter 3, represented the first systematic attempt to cultivate indigenous prokaryotes belonging to important functional groups from submarine MV sediments. Enrichment success was highest in the Meknes MV crater site at 10 cmbsf, where all other data indicated a stimulation of microbial activity. Here, 7 of the medium types incubated at 25°C became positive, in addition to a further 2 media types (general heterotroph and heterotrophic SRB) incubated at 7°C. One sample from 50 cmbsf in the Meknes MV crater incubated at 7°C in general heterotrophic medium also became positive. Enrichment success was also achieved with sediment from the Porto MV at 10 cm depth using heterotrophic SRB and general heterotrophic medium incubated at 25°C. Unfortunately, not all positive enrichments could be successfully brought into sub-culture (Table 4.7) and in these cases the enriched organisms remained unidentified, but where enrichments were successfully sub-cultured targeted functional groups of interest were obtained (fermenters, SRB and methanogens, see Table 4.8), which can be considered a success as such organisms are notoriously difficult to grow in the laboratory despite many concerted efforts (e.g. Toffin *et al.*, 2004; D'Hondt *et al.*, 2004; Biddle *et al.*, 2005a). Furthermore, the organisms identified in the Meknes MV enrichments were not always closely related to known isolates and sometimes shared their greatest phylogenetic similarity with environmental clones rather than cultured organisms. The further investigation of these enrichments and efforts to isolate pure cultures from them is suggested as future work (see chapter 6).

4.5.3 Prokaryotic diversity in the Meknes and Porto MVs

Prokaryotic diversity was not resolved to such a high level of detail in the Meknes and Porto MV samples as it was in the single Capt. Arutyunov MV sample (see chapter 3), since clone libraries were only constructed for functional genes rather than 16S rRNA genes. Instead bacterial and archaeal diversity were investigated using PCR-DGGE analysis, band sequencing and comparative sequence analysis; a method that was more appropriate for the larger number of samples and which could still identify trends in community structure and the dominant community members.

Archaeal diversity according to PCR-DGGE analysis of 16S rRNA genes and band sequencing was lower than bacterial diversity in both the Meknes and Porto MV sediments successfully analysed, which is a pattern common to other seep sites (Li *et al.*, 1999; Heijs *et al.*, 2005; Knittel *et al.*, 2005; Fang *et al.*, 2006; Kormas *et al.*, 2008; Wegener *et al.*, 2008a; chapter 3). *Archaea* were not detectable in all samples amplified with universal archaeal primers (Fig 4.10). This suggested either an absence of *Archaea* in these sediments, that archaeal cell numbers were extremely low and hence below detection by standard PCR-based analysis, that the *Archaea* present were missed by the primers employed, or that DNA extraction and/or PCR amplification were sub-optimal. The sediments where *Archaea* were not detected were those where other analyses (sea-floor observation, pore water analysis and total AODC cell counts) suggested activity was lowest, e.g. through the Meknes MV reference site, perhaps making the latter explanation less likely. According to inspection of amplified products by gel electrophoresis, *Archaea* were most abundant in the Meknes and Porto MV crater samples (Fig 4.10), however the *Archaea* that were detected in the two craters were different. *Archaea* were also present in the upper parts of the Meknes MV slope sample, albeit in apparently lower numbers (Fig 4.10). In Meknes MV crater at 10 cmbsf where sulphate concentrations were still at seawater concentration the euryarchaeotal Marine Benthic Group D was the only group detected, but in the 50 and 65 cmbsf samples where sulphate had dropped to μM concentrations euryarchaeotal ANME-1a sequences were the only ones detectable by both 16S rRNA and *mcrA* gene analysis, indicating the MV crater's AOM zone had been reached (see Table 4.3 and Table 4.5). Marine Benthic Group D (synonymous with the Deep-Sea Archaeal Group) have been identified as a dominant member of the archaeal community in a wide range of sediment types but these are typically anoxic and often associated with methane (see Teske & Sørensen 2008). It has been suggested that Marine Benthic Group D organisms may benefit directly or indirectly from AOM in marine sediments (Sørensen & Teske 2006) and their close proximity to the AOM zone in the Meknes MV crater may support this assertion. The ANME-1a sub-group, identified here as the only detectable archaeon in the lower parts of the Meknes MV crater core, have previously been found to represent 10% of all archaeal clones in microbial mats sampled from areas of active gas seepage and carbonate reef formation in anoxic waters of the Black Sea (Knittel *et al.*, 2005). This group has also been detected in several other seep sites including those in the Eel River and Santa

Barbara Basin (Hinrichs *et al.*, 1999; Orphan *et al.*, 2001), Gulf of Mexico (Lanoil *et al.*, 2001), Eastern Mediterranean (Heijs *et al.*, 2006), Aarhus Bay (Thomsen *et al.*, 2001), and also in hydrothermal sediments of the Guaymas Basin (Teske *et al.*, 2002), though its relative abundance was not always determined. What determines the relative abundance and distribution of ANME-1a *Archaea* and their relatives ANME-1b (<96% 16S rRNA similarity) and ANME-2 (<85% 16S rRNA similarity) in nature is unknown, given that they perform the same function (AOM) and can co-exist (Knittel *et al.*, 2005). It has been suggested that oxygen sensitivity may be a controlling factor in the relative distribution of ANME-1 and ANME-2 groups (Knittel *et al.*, 2005) though more data is needed to determine how important this factor is globally. A phylogenetic study of higher resolution than the one presented here is recommended for Gulf of Cadiz MV crater sediments such as Meknes, as if they do exclusively contain a particular ANME sub-group, this data coupled with detailed measurements of key environmental parameters (e.g. temperature, salinity, pressure, oxygen concentration, fluid flow rate and electron donor/acceptor concentrations) may shed light on this important outstanding question.

Crenarchaeotal Marine Group I sequences were present in the upper 10 cmbsf sample of the Meknes MV slope sample, along with a euryarchaeotal sequence which could not be assigned to a higher phylogenetic group (Table 4.3). Interestingly, two novel *mcrA* sequences were also detected in this horizon (Table 4.5), which despite excellent sequence quality did not match any existing *mcrA* sequences held in the GenBank databases. It remains unknown however whether these novel *mcrA* sequences were from the loosely assigned euryarchaeotal organism detected in this horizon by 16S rRNA analysis, or from another organism not detected by 16S rRNA gene analysis.

In the Porto MV crater *Archaea* were only detectable in the upper 10 and 50 cmbsf samples by combined 16S rRNA and *mcrA* PCR-DGGE analysis. At both these depths sulphate remained at seawater concentrations and only crenarchaeotal Marine Group I were detected by 16S rRNA analysis, in addition to a single *mcrA* group e sequence (*Methanosarcinales*-associated) detected at 50 cmbsf (Tables 4.3 and Table 4.5). The construction of a *mcrA* gene library from the Porto MV crater 50 cmbsf sample confirmed the presence of *mcrA* group e and additionally detected an *mcrA*

gene affiliated to *Methanococcoides* (Fig 4.14). The Marine Group I *Archaea* from both the Meknes MV slope and Porto MV crater site, were most closely related to clones from marine sediments, including those from other MVs (Table 4.3). Marine sediment-associated sub-groups have previously been identified within the Marine Group I (e.g. Sørensen *et al.*, 2004), which is a phylogenetic group that was first discovered in, and is commonly associated with seawater samples (DeLong, 1992; Teske & Sørensen 2008). However, a more detailed phylogenetic analysis (e.g. cloning and phylogenetic tree construction) would be needed to determine to what extent the sequences identified here fall within a marine sediment or MV-associated sub-group of the wider Marine Group I. ANME organisms were not detected in any of the Porto MV crater samples by 16S rRNA gene analysis, but it has been suggested that the *mcrA* group e, as detected at 50 cmbsf in Porto MV by *mcrA* gene PCR-DGGE analysis and *mcrA* clone library construction is affiliated with an ANME organism (Nunoura *et al.*, 2006; 2008), though this is to be confirmed. The 50 cmbsf Porto MV sample would be right on the upper edge of the AOM zone according to sedimentary pore water results (Fig 4.5).

Phylogenetic analysis of the bacteria present in the various Meknes and Porto MV samples was hampered to a degree by a methodological pitfall of the PCR-DGGE method. Despite a large number of bacterial 16S rRNA sequence bands being identified in several samples, including those from the Meknes MV crater (all depths), the Meknes MV slope at 10 cmbsf and the Porto MV crater at 50 cmbsf (see Fig 4.8), only a few of them could be identified by DGGE band sequencing. Often when fainter bands were excised, re-amplified and sequenced a mixed sequence was obtained indicating DNA from two or more different 16S rRNA gene sequences were present at the target DGGE band position. Indeed this could be confirmed by re-running the amplified target band on a second DGGE gel, where the target band itself plus additional bands were visible (data not shown). This is a known problem and occurs because small amounts of DNA from overlying and/or adjacent DGGE bands can co-migrate with other sequences and then obscure the sequence of the target band following re-amplification by PCR, which amplifies the 'contaminating' sequence(s) to detectable levels. This is a particular problem when certain 16S rRNA gene sequences are present in high concentrations relative to other 16S rRNA gene sequences, as was often the case in the samples analysed here. Sequences belonging

to the JS1 candidate division of bacteria were the dominant component of the bacterial community in the Meknes MV crater 50 and 65 cmbsf samples, and the Porto MV crater 50 cmbsf sample according to PCR-DGGE analysis (see Fig 4.8 and Fig 4.9). These largely prevented other sequences in the samples from being resolved by DGGE band sequencing, with the exception of two *Epsilonproteobacteria* sequences identified in the Meknes MV crater 10 cmbsf and 50 cmbsf samples. The predominance of JS1 in the two separate MV craters was interesting since the JS1 candidate division currently has no cultured representatives, has been found in a wide variety of both coastal and deep marine sub-surface environments, including those from the deep biosphere (e.g. Webster *et al.*, 2004), has an unknown physiology (Webster *et al.*, 2007), and has often previously only been detectable through the use of JS1-specific primers. The detection of JS1 in relatively high abundance in both the Meknes and Porto MV craters may offer them as environments in which to focus future research into this group of poorly understood organisms.

Cloning and phylogenetic analysis of *dsrA* genes from 3 sites (Meknes MV slope 110 cmbsf, Meknes MV crater 50 cmbsf and Porto MV crater 50 cmbsf) revealed different SRB were present in each location. A single phylotype was detected in the Meknes slope sample and was most closely related to an estuarine-derived sequence (Table 4.6). In the Meknes MV crater sample, cloning was hindered by the amplification of PCR artefacts but two phylotypes were detected, one of which was most closely affiliated to a sequence proposed to be derived from a putative AOM-catalysing SRB, which would tie in with the presence of ANME-1a organisms at this horizon. SRB diversity was highest in the Porto MV sample, with 6 different OTUs identified. The *dsrA* genes detected were most closely affiliated to clones retrieved from lake sediment, gas hydrate-bearing sediment and marine sediment, including sediment from Nankai Trough. However, low sequence similarity to their nearest relatives and a lack of equivalence to a corresponding organism with 16S rRNA gene identity, prohibits further comment on the organisms represented by these genes. The presence of these diverse *dsrA* genes does however suggest sulphate-reduction is an important metabolic process at this horizon.

Chapter 5 – Characterisation of isolates

5.1 Introduction

Ten representative pure culture isolates from the Capt. Arutyunov and Bonjardim MV culture collections were selected for further characterisation: *Halomonas* strains CpA_a1 and CpA_b2b; *Marinobacter* strains CpA_a7 and CpA_a13; *Pseudomonas* strains Cpa_a4, CpA_a17, Bon_a1 and Bon_b1 (most closely affiliated to the *Pseudomonas stutzeri* genomovar group); and *Arcobacter* strains CpA_a5 and CpA_b6. Phylogenetic analyses were conducted using near-full length 16S rRNA gene sequences, and the nearest described species to each of the MV isolates were used as a basis for identification. Phenotypic tests were conducted to allow physiological comparisons with near phylogenetic neighbours and to investigate the potential environmental roles of the isolates. Metabolic capabilities of the isolates were investigated using comprehensive substrate tests, which included a broad range of environmentally relevant organic compounds and electron acceptors. The methodologies used are given in chapter 2, with relevant sections indicated where appropriate within the text.

5.2 Description of *Halomonas* MV isolates

5.2.1 Introduction to the *Halomonas* genus

The *Halomonas* genus belongs to the order *Oceanospirillales* in the *Gammaproteobacteria,* and was originally described by Vreeland *et al.* (1980) with a single species *H. elongata* isolated from a saltern. The species of the genus *Halomonas* were described as straight or curved Gram-negative rods that may be motile or non-motile, are catalase-positive and aerobic, with the ability to grow over a wide range of salt concentrations (Vreeland *et al.*, 1980; James *et al.*, 1990; Dobson & Franzmann, 1996). Some species of the genus can also grow anaerobically by nitrate reduction (Mata *et al.*, 2002). At the time of writing, there are 38 validly described species in the *Halomonas* genus (J. P. Euzéby [http://www.bacterio.cict.fr/] accessed 03/07). The described organisms have been isolated from a variety of habitats including seawater, low temperature hydrothermal fluids, saline soil, hypersaline lake water, seafood, and even a wall mural (e.g. Yoon *et al.*, 2001; 2002;

Heyrman *et al.*, 2002; Kaye *et al.*, 2004; Quillaguaman *et al.*, 2004; Romano *et al.*, 2005). These source environments represent a wide range of salinities, temperatures, hydrostatic pressures and pH. It has been shown that the considerable physiological diversity in the genus is discordant with the phylogenetic diversity, and this incongruence between phenotypic and 16S rRNA gene based phylogeny has presented a significant challenge to achieving a coherent taxonomic classification for the group. Consequently, considerable taxonomic revisions have been made during the classification of the currently recognised species. In an attempt to assist the classification of this heterogeneous group two large comparative studies were recently conducted. The first, by Arahal *et al.* (2002a), was a phylogenetic analysis based on both 16S and 23S rRNA gene sequences from described members of the family *Halomonadaceae*. It resolved two phylogenetic sub-groups within the *Halomonas* genus (sub-group 1, containing 5 species and sub-group 2, containing 7 species), but six other *Halomonas* species did not fall into either two sub-groups or group with each other. However, despite identifying considerable phylogenetic differences within the genus Arahal *et al.* (2002a) were unable to support splitting it based on the available physiological data. A later study by Mata *et al.* (2002) was a detailed polyphasic phenotypic analysis of 21 *Halomonas* type strains based on 234 tests. Cluster analysis of phenotypic data with a similarity level of 62% defined three separate 'phena' within the genus: phenon A, which contained 5 species unified by their ability to produce acids from a range of sugars; phenon B, which contained 12 species without the general physiological traits of phenon A or C, and phenon C, which contained 3 strains grouped together on the basis of their more limited substrate range. Unfortunately, however, there was little agreement between the sub-classifications of the two studies. For example, members of all three of the phena recognized by Mata *et al.* (2002) were present in the 16S rRNA subgroup 2 of Arahal *et al.* (2002a). Since the time of these comparative studies several new species have been assigned to the genus, and have often further exposed inconsistencies between 16S rRNA based data and phenotypic results (e.g. Kaye *et al.*, 2004; Romano *et al.*, 2005).

Halomonas species represent one of the most abundant groups of culturable organisms in the marine environment (e.g. Kaye & Baross 2000; Sass *et al.*, 2001), their broad range of metabolic capabilities and tolerance of physical extremes are probable factors contributing to their repeated laboratory cultivation.

5.2.2 Enrichment and isolation

Strain CpA_a1 was isolated on solid YPG medium (section 2.4.5) under aerobic conditions at 6°C. The inoculum from which it was isolated came from an enrichment targeting heterotrophic methanogens (see section 2.4.2.4) and contained 15 mM acetate as the main substrate.

Strain CpA_b2b was isolated on solid YPG medium (section 2.4.5) under aerobic conditions at 6°C. The inoculum from which it was isolated came from an enrichment targeting autotrophic methanogens (see section 2.4.2.5) and contained H_2:CO_2 (80:20) as the sole substrate.

5.2.3 Phylogenetic analysis of MV *Halomonas* isolates

Phylogenetic analysis conducted with all the available validly described *Halomonas* 16S rRNA gene sequences revealed that both of the strains isolated from the Capt. Arutyunov mud breccia were most closely affiliated to *Halomonas* group 2 species (Arahal *et al.*, 2002b). A phylogenetic reconstruction produced from the 16S rRNA genes of these species revealed the strains' closest relatives within this group (Fig 5.1).

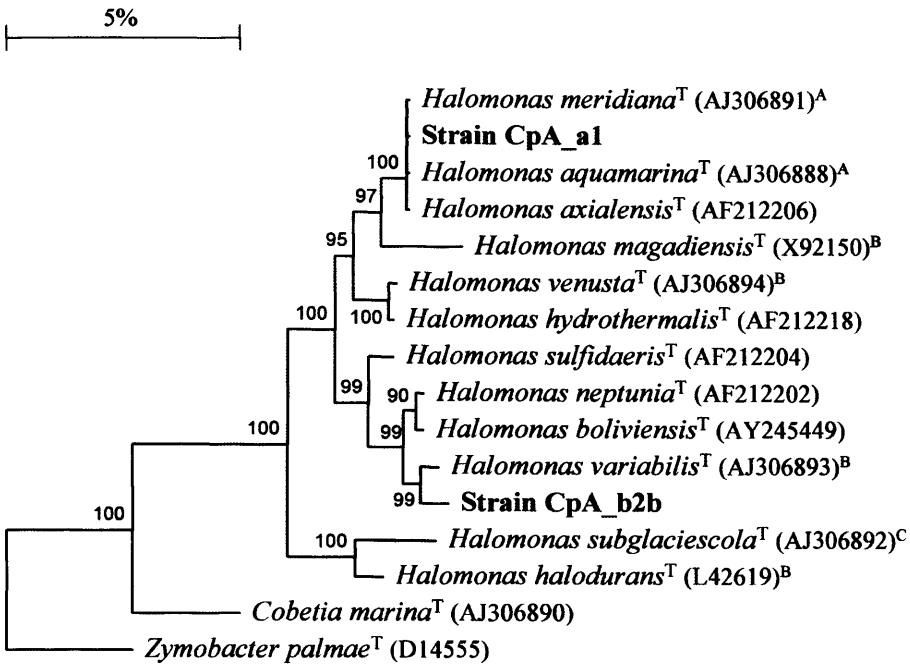


Fig 5.1. Neighbour-joining tree showing the phylogenetic position of the two Capt. Arutyunov MV *Halomonas* isolates and their relationship to type strains of the group 2 sub-group (Arahal *et al.*, 2002b). The reconstruction was based on an alignment of 1352 nucleotide positions using the Jukes and Cantor (1969) algorithm. Numbers at nodes represent percentage bootstrap values after 1000 samples. Accession numbers are shown in parentheses. Scale bar indicates 5% sequence divergence. Capital letters in superscript after accession numbers indicate phenotypic groupings according to Mata *et al.*, (2002). *Cobetia marina* and *Zymobacter palmae* were used as outgroups.

Strain CpA_a1 shared its greatest 16S rRNA gene sequence similarity (99.7%) equally with the species *H. meridiana*, *H. aquamarina* and *H. axialensis*, while strain CpA_b2b was most similar (99.1%) to *H. variabilis*. The two strains shared 96.2% 16S rRNA gene sequence similarity with each other (Table 5.1).

Table 5.1 Similarity matrix of CpA MV isolates and *Halomonas* group 2 16S rRNA gene sequences, calculated from an edited alignment of 1352 unambiguous nucleotide positions.

	H. halodurans	*H. subglaciescola*	**Strain CpA_a1**	*H. meridiana*	*H. aquamarina*	*H. axialensis*	*H. magadiensis*	*H.hydrothermalis*	*H.venusta*	**Strain CpA_b2b**	*H.variabilis*	*H.boliviensis*	*H.neptunia*	*H.sulfidaeris*
H. halodurans	ID	0.969	0.948	0.949	0.949	0.948	0.941	0.953	0.952	0.946	0.946	0.946	0.944	0.951
H. subglaciescola	0.969	ID	0.947	0.948	0.947	0.947	0.941	0.952	0.950	0.944	0.946	0.945	0.946	0.952
Strain CpA_a1	0.948	0.947	ID	0.997	0.997	0.997	0.977	0.979	0.979	0.962	0.964	0.967	0.966	0.974
H. meridiana	0.949	0.948	0.997	ID	0.998	0.998	0.978	0.980	0.979	0.963	0.965	0.968	0.966	0.976
H. aquamarina	0.949	0.947	0.997	0.998	ID	0.998	0.979	0.980	0.980	0.963	0.965	0.968	0.966	0.975
H. axialensis	0.948	0.947	0.997	0.998	0.998	ID	0.979	0.981	0.980	0.963	0.965	0.968	0.966	0.975
H. magadiensis	0.941	0.941	0.977	0.978	0.979	0.979	ID	0.971	0.974	0.954	0.954	0.957	0.953	0.960
H.hydrothermalis	0.953	0.952	0.979	0.980	0.980	0.981	0.971	ID	0.997	0.967	0.966	0.971	0.971	0.977
H.venusta	0.952	0.950	0.979	0.979	0.980	0.980	0.974	0.997	ID	0.968	0.967	0.972	0.968	0.974
Strain CpA_b2b	0.946	0.944	0.962	0.963	0.963	0.963	0.954	0.967	0.968	ID	0.991	0.985	0.985	0.977
H.variabilis	0.946	0.946	0.964	0.965	0.965	0.965	0.954	0.966	0.967	0.991	ID	0.988	0.988	0.980
H.boliviensis	0.946	0.945	0.967	0.968	0.968	0.968	0.957	0.971	0.972	0.985	0.988	ID	0.994	0.982
H.neptunia	0.944	0.946	0.966	0.966	0.966	0.966	0.953	0.971	0.968	0.985	0.988	0.994	ID	0.983
H.sulfidaeris	0.951	0.952	0.974	0.976	0.975	0.975	0.960	0.977	0.974	0.977	0.980	0.982	0.983	ID

Table 5.1 shows that 16S rRNA gene sequence similarity was very high, not only between the CpA MV isolates and certain described species but also between several of the validly described species themselves. This situation prohibits any further interpretation of the MV isolate phylogenetic data to be made in isolation of additional phenotypic data.

5.2.4 Phenotypic analysis of MV *Halomonas* isolates

Strains CpA_a1 and CpA_b2b both formed convex, cream-coloured, opaque colonies with a smooth shiny surface when grown on solid YPG media. Cells of both strains were motile rods and did not produce endospores under any of the conditions tested. The cells of both strains were not uniformly opaque under phase contrast light but had some dark patches, which might indicate the presence and uneven distribution of carbon stores within the cell (Fig 5.2).

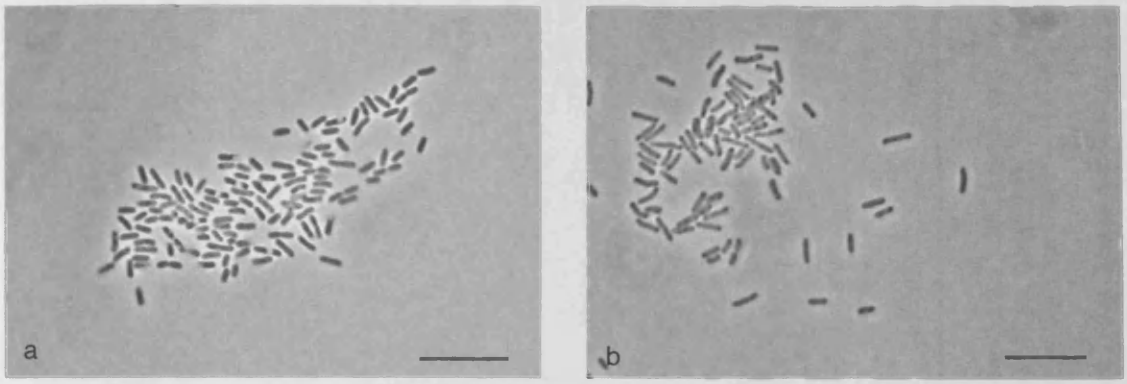


Fig 5.2. Phase contrast images of isolated *Halomonas* MV strains. Panel a, strain CpA_a1; panel b, strain CpA_b2b. Images show cell morphology during exponential growth phase. Images taken after 3 days incubation at 25°C in YPG medium. Scale bar = 10 μm.

Tests were conducted to investigate the physiological characteristics of the two isolates (section 2.5) and to place them in context of the wider *Halomonas* genus. The results were compared to the literature data available for near phylogenetic (16S rRNA gene based) relatives and are summarized in Table 5.2.

Table 5.2. Differences among new isolates and related type strains of the *Halomonas* 16S rRNA group 2 species (Arahal *et al.*, 2002a)

Strains: CpA_a1; *H. meridiana* DSM 5425^T data from James *et al.*, (1990), Mata *et al.*, (2002); *H. aquamarina* DSM 30161^T data from, Dobson & Franzmann (1996), Mata *et al.*, (2002); *H. axialensis* DSM 15723^T data from Kaye *et al.*, (2004); CpA_b2b; *H. neptunia* DSM 15720^T data from Kaye *et al.*, (2004); *H. boliviensis* DSM 15516^T data from Quillaguaman *et al.*, (2004); 8, *H. variabilis* DSM 3051^T data from Fendrich (1988), Dobson & Franzmann (1996), Mata *et al.*, (2002).

Characteristic	**CpA_a1**	*H. meridiana*[a]	*H. aquamarina*[a]	*H. axialensis*	**CpA_b2b**	*H. neptunia*	*H. boliviensis*	*H. variabilis*[b]
Isolated from:	Mud volcano sediment	Cold hypersaline lake	Temperate ocean	Low temp. hydrothermal fluid	Mud volcano sediment	Hydrothermal plume	Soil around hypersaline lake	Hypersaline lake
Growth properties:								
Salinity range (%)	0.1 - 25	0.01 – 25	0 – 20	0.5 – 24	0.1 - 25	0.5 – 27	0 – 25	7 – 29
pH range	5.7 – 9.3	5 – 10	5 – 10	5 – 12	5.7 – 9.3	5 – 12	6 – 11	6 – 9
Temperature range (°C)	0 – 43	-5 – 45	5 – 40	-1 – 35	0 – 40	-1 – 35	0 – 45	15 – 37
Growth on:								
Fructose	+	-	-	+	(+)	+	+	+
L-Arabinose	-	-	+	+	(+)	+	+	-
Rhamnose	-	-	-	-	-	+	-	-
Mannose	-	-	-	-	-	+	+	+
Xylose	+	-	-	-	(+)	+	+	-
Trehalose	+	+	+	-	+	+	+	+
Maltose	+	+	-	+	+	+	+	+
Cellobiose	-	-	-	-	+	+	-	+

Table 5.2. cont. Differences among new isolates and related type strains of the *Halomonas* 16S rRNA group 2 species (Arahal *et al.*, 2002a)

Characteristic	**CpA_a1**	*H. meridiana*[a]	*H. aquamarina*[a]	*H. axialensis*	**CpA_b2b**	*H. neptunia*	*H. boliviensis*	*H. variabilis*[b]
Ethanol	-	+	+	+	-	+	ND	+
Propanol	-	ND	ND	-	-	-	ND	ND
Glycerol	+	+	+	-	+	+	-	+
Mannitol	+	+	-	+	+	+	-	+
Acetate	+	-	+	-	(+)	+	ND	+
Lactate*	+	+	+	-	+	-	ND	+
Formate	-	+	-	ND	-	ND	ND	-
Propionate	+	-	+	-	+	-	ND	+
Butyrate	+	ND	ND	+	-	+	ND	ND
Gluconate	+	-	+	ND	+	ND	-	+
Malonate	ND	-	+	-	ND	+	ND	-
Succinate	+	+	+	-	+	+	ND	+
Fumarate	+	-	+	+	+	+	ND	+
Malate	+	ND	ND	+	+	+	ND	ND
Tartrate	(+)	ND	ND	+	-	+	ND	ND
Citrate	+	-	+	-	(+)	+	-	+
L- Alanine	+	+	+	-	(+)	+	ND	+
L- Arginine	+	ND	ND	-	+	+	ND	ND

Table 5.2 cont. Differences among new isolates and related type strains of the *Halomonas* 16S rRNA group 2 species (Arahal *et al.*, 2002a)

Characteristic	**CpA_a1**	*H. meridiana*[a]	*H. aquamarina*[a]	*H. axialensis*	**CpA_b2b**	*H. neptunia*	*H. boliviensis*	*H. variabilis*[b]
Isoleucine	+	+	+	ND	+	ND	ND	+
Serine	(+)	+	-	-	-	-	ND	+
Phenylalanine	-	+	-	ND	-	ND	ND	+
Glucosamine*	-	+	+	-	(+)	-	ND	+
Starch	-	-	+	ND	-	ND	ND	-
Other phenotypic properties:								
Facultative anaerobe	+	-	-	+	+	+	-	-
Anaerobic NO_3^- reduction	+	ND	ND	+	+	+		ND

Traits are scored as: +, positive; (+), weak positive; -, negative; ND, not determined
Traits that were all positive: Cell morphology (rods), motility, growth on glucose, and growth on sucrose.
* Determined with Biolog plates for *H. meridiana, H. aquamarina, and H. variabilis* (Mata *et al.*, 2002)
[a] Grouped in phenon A according to Mata *et al.*, (2002), based on clustering of type species of *Halomonas* on the basis of 57 diagnostic phenotypic characteristics.
[b] Grouped in phenon B according to Mata *et al.*, (2002), see above.

The two strains CpA_a1 and CpA_b2b were able to grow across a relatively wide range of temperature, salinity and pH, in agreement with the characteristics of their phylogenetic relatives. Salt was required for growth; cells did not divide when inoculated into Milli-Q water containing 2 mM glucose as substrate (final salinity after inoculum added was 0.00175% NaCl). Both CpA_a1 and CpA_b2b were able to utilize a broad range of substrates for growth (62% and 54% of those tested, respectively), although the substrates used differed slightly between the two strains. For example, strain CpA_a1 was able to grow on some organic acids (butyrate, valerate) and aromatic compounds (p-OH benzaldehyde, benzoate) that did not support growth of CpA_b2b. Conversely, strain CpA_b2b grew on cellobiose but this was not used by strain CpA_a1. Another slight difference between the two strains was their temperature range for growth. Strain CpA_a1 grew from 0°C to 45°C on solid media whereas strain CpA_b2b grew from 0°C to 40°C. The upper range for growth was also tested in liquid media inside a thermal gradient system. The upper range for strain CpA_b2b was confirmed at 40°C (no growth at 43°C). Strain CpA_a1 did grow at 43°C in side the thermal gradient system but not at 45°C. Microscope images of strain CpA_a1 growing at 45°C on solid media were taken and showed all the cells were deformed and not dividing properly (Fig 5.3).

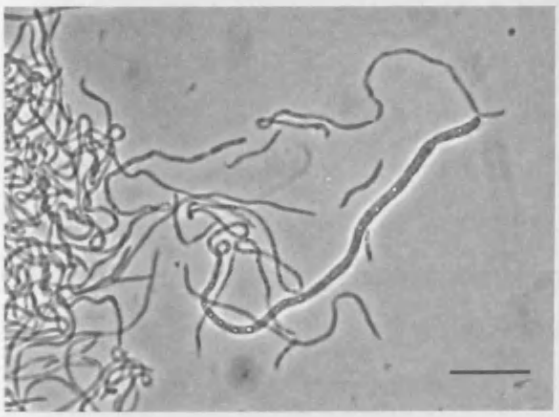


Fig 5.3. Microscope image of strain CpA_a1 cells growing at 45°C on solid media. Scale – 10 μm.

Microscopic assessment of growth at 43°C in liquid media showed some normal looking cells but others that were highly elongated (data not shown). It seems therefore that the very upper limit for *normal* growth is around 43°C for strain CpA_a1 (Table 5.2). It should be noted that both strains CpA_a1 and CpA_b2b were enriched and isolated at 6°C.

Both strains grew across the full pH range tested (5.5–9.5), however re-measuring the pH after growth revealed that both strains had altered the pH of the media to between pH 7–8. It is not known whether the pH change occurred as a prerequisite to growth (cell division), through metabolic activity of non-dividing cells or simply as a result of metabolic activity during growth. Additional tests with different media buffers

were conducted to firmly ascertain at what pH range these cells could divide (see Table 5.2).

Growth of strain CpA_a1 was supported by glucose fermentation but strain CpA_b2b was not. The fermentation of amino acids was negative as tested by microscopy and the sulphide test (Widdel 1980); apparent growth by amino acid fermentation in the first test was shown to be background growth following a second sub-culture, i.e. the growth in the first test was as a result of compounds transferred with the inoculum and/or because of cell storage compounds.

A range of alternative electron acceptors were tested with acetate and lactate as the electron donors. Visual and processed fluorescence data (see section 2.5.2) indicated both CpA_a1 and CpA_b2b only used nitrate as an electron acceptor. Nitrite was not used according to fluorescence data, and this was further confirmed by ion chromatography. Gas bubbles were not observed in the positive nitrate wells and analysis of the supernatant by ion chromatography (both replicates) showed a production of nitrite (data not shown). The two strains were therefore shown to be capable of incomplete dissimilatory nitrate reduction to nitrite.

5.2.5 Discussion

Comparing the phenotypic traits of the MV isolates with those of their near phylogenetic neighbours reported in the literature revealed some interesting differences. What is noticeable is that the two MV isolates are more similar to each other than their closest relatives according to purely 16S rRNA-based phylogeny. Strain CpA_a1 and CpA_b2b produced a common result for 86% of the electron donors tested, both were facultatively anaerobic and able to reduce nitrate anaerobically. The strains also had the same salinity and pH range for growth. There was less similarity between the two MV *Halomonas* isolates and their closest 16S rRNA gene sequence matches according to the available literature data (see Table 5.2). Obviously some differences in phenotype data may be due to variations in experimental procedures (e.g. Mata *et al.*, 2002) but it may also simply reflect the adaptation of the two isolates to the same environment. A recent study by Kaye *et al.* (2004) investigating the physiologies of six *Halomonas* isolates from hydrothermal

vent environments also reported greater phenotypic similarity between their isolates than the described organisms with which they shared greater 16S rRNA gene sequence similarity.

Despite their broad physiological similarities, some differences between the isolates were identified. The strains had moderately different 16S rRNA gene sequences (96% similarity) and some different substrate capabilities. Additionally, CpA_a1 had a temperature range for growth at least 3°C higher than strain CpA_b2b and could grow by glucose fermentation. These differences suggest the two *Halomonas* strains may be members of different species; further analysis, e.g. DNA-DNA hybridization experiments, would be needed to confirm this. The ability to utilize different substrates may be what allows these two organisms to co-exist in the same environmental setting.

5.2.6 Description of strain CpA_a1

Cells were short fat round end rods capable of motility and were primarily found as single cells. Endospores were not observed. Colonies grown on solid YPG media were cream-coloured, opaque with a smooth shiny surface and a convex form.

Growth occurred between 0–43°C, pH 5.5–9.5 and salinities between 0.1–25%.

The organism used ethylene glycol, glycerol, mannitol, pOH-benzaldehyde, benzoate, acetate, lactate, propionate, butyrate, valerate, hexanoate, octanoate, gluconate, salicylate, succinate, fumarate, malate, tartrate, citrate, alanine, arginine, isoleucine, serine, glucose, fructose, xylose, trehalose, sucrose, maltose, yeast extract, casamino acid mixture and peptone for growth.

Methanol, ethanol, propanol, butanol, vanillin, 3,4,5-trimethoxybenzoate, formate, malonate, cysteine, phenylalanine, glucosamine, arabinose, rhamnose, mannose, cellobiose, cellulose, starch, chitin, xylan and laminarin did not support growth. Glucose was fermented but amino acids were not.

Nitrate was the only alternative electron acceptor used. Ion chromatography confirmed this was reduced to nitrite.

5.2.7 Description of strain CpA_b2b

Cells were short fat round end rods capable of motility and were primarily found as single cells. Endospores were not observed. Colonies grown on solid YPG media were cream-coloured, opaque with a smooth shiny surface and a convex form.

Growth occurred between 0–40°C, pH 5.5–9.5 and salinities between 0.1–25%.

The organism used ethylene glycol, glycerol, mannitol, acetate, lactate, propionate, hexanoate, octanoate, gluconate, malonate, succinate, fumarate, malate, citrate, alanine, arginine, cysteine, isoleucine, glucosamine, glucose, fructose, arabinose, xylose, trehalose, sucrose, maltose, cellobiose, cellulose, casamino acid mixture, yeast extract and peptone for growth.

Methanol, ethanol, propanol, butanol, vanillin, pOH-benzaldehyde, benzoate, 3,4,5-trimethoxybenzoate, formate, butyrate, valerate, tartrate, salicylate, serine, phenylalanine, rhamnose, mannose, starch, chitin, xylan and laminarin did not support growth. Glucose and amino acids were not fermented.

Nitrate was the only alternative electron acceptor used. Ion chromatography confirmed this was reduced to nitrite.

5.3 Description of *Marinobacter* MV isolates

5.3.1 Introduction to the *Marinobacter* genus

The genus *Marinobacter* was proposed by Gauthier *et al.* (1992) to accommodate a new species of gram-negative, moderately halophilic *Gammaproteobacteria; Marinobacter hydrocarbonoclasticus*, that used a variety of hydrocarbons as the sole source of carbon and energy. A second species, *Marinobacter aquaeolei,* originally described by Stan-Lotter *et al.* (1999), was recently reclassified as a later heterotypic synonym of *M. hydrocarbonoclasticus* (Márquez & Ventosa, 2005). In the last 3 years a further 14 species assigned to the *Marinobacter* genus have been described (Gorshkova *et al.*, 2003; Martin *et al.*, 2003; Shieh *et al.*, 2003; Yoon *et al.*, 2003; 2004; Romanenko *et al.*, 2005; Takai *et al.*, 2005; Shivaji *et al.*, 2005; Green *et al.*, 2006; Kim *et al.*, 2006; Liebgott *et al.*, 2006).

Cultivation-based studies have revealed the *Marinobacter* genus is widely distributed in the marine environment. Described species have a variety of marine fluid (Gauthier *et al.*, 1992; Yoon *et al.*, 2003; Yoon *et al.*, 2004; Shivaji *et al.,* 2005; Rontani *et al.*, 2003), marine sediment (Gorshkova *et al.*, 2003; Romanenko *et al.*, 2005; Kim *et al.*, 2006; Takai *et al.*, 2005) or biological sources (Romanenko *et al.*, 2005; Green *et al.*, 2006). Species belonging to this genus have also been cultured from terrestrial saline environments (Shieh *et al.*, 2003; Martin *et al.*, 2003) and wine barrel de-calcification wastewater (Liebgott *et al.*, 2006). Both strict aerobes and facultative aerobes are present within the group (some species use nitrate as an alternative electron acceptor), and despite their association with saline environments not all the species require salt for growth. *Marinobacter* are gram-negative, heterotrophic, motile, non-spore forming, rod-shaped bacteria.

5.3.2 Enrichment and isolation

Strain CpA_a7 was isolated from a positive enrichment culture originally designed to grow autotrophic sulphate-reducing bacteria (SRB). The enrichment was incubated at 30°C inside a thermal gradient system. The strain was purified by repeated single colony isolation under aerobic conditions on solid YPG medium at 30°C. Strain CpA_a13 was isolated from a positive enrichment culture designed to target heterotrophic SRB. The enrichment was incubated at room temperature. The strain was also purified by repeated single colony isolation under aerobic conditions on solid YPG medium at 30°C.

5.3.3 Phylogenetic analysis of MV *Marinobacter* isolates

Analysis of the 16S rRNA gene sequences of the MV isolates, described *Marinobacter* species and selected environmental strains showed that strains CpA_a7 and CpA_a13 were highly related to each other (99.7%) and their closest relative was the recently described type strain of *Marinobacter vinifirmus* (Liebgott *et al.*, 2006) (Fig 5.4).

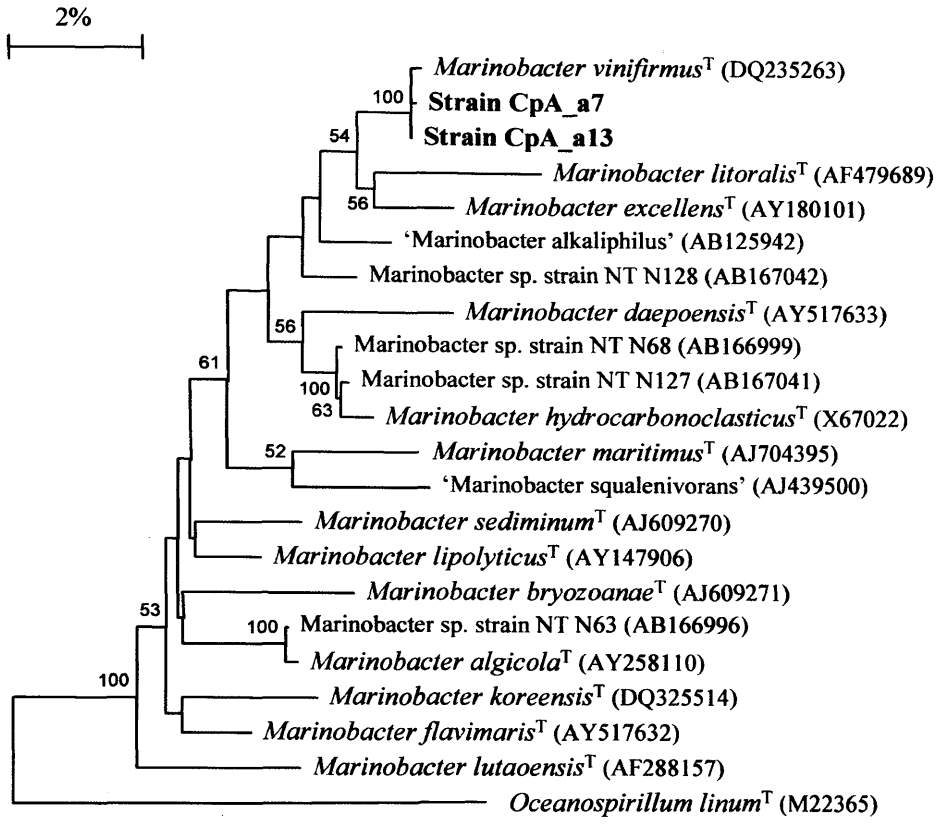


Fig 5.4 Neighbour-joining tree showing the phylogenetic relationship of the two MV *Marinobacter* strains to described species and selected environmental strains. The reconstruction was based on an alignment of 1397 nucleotide positions using the Jukes and Cantor (1969) algorithm. Numbers at nodes represent percentage bootstrap values after 1000 samples. Accession numbers are shown in parentheses. Scale bar indicates % sequence divergence. *Oceanospirillum linum* was used as outgroup.

The MV strains were also closely related (>97% 16S rRNA gene sequence similarity) to the described organisms *M. excellens, M. alkaliphilus, M. hydrocarbonoclasticus, M. daepoensis* and *M. litoralis*, and Marinobacter strains NT N127, NT N128 and NT N68 isolated from deep-sea cold seep sediment in Nankai Trough (Okamoto, T & Naganuma, T, unpublished results). The very close affiliation to *Marinobacter vinifirmus* is a little surprising given this organism's source (wine-barrel-decalcification wastewater) and some of its reported phenotypic properties (see below). However, as shown for the *Halomonas* and *Pseudomonas* genera (see sections 5.2 & 5.4), 16S rRNA gene sequence similarity alone does not always correlate with physiological similarity.

5.3.4 Phenotypic analysis of MV *Marinobacter* isolates

Strains CpA_a7 and CpA_a13 both formed small, opaque, shiny, cream-coloured colonies with irregular edges when grown aerobically on solid YPG media. When picking colonies it was noted that they had penetrated down into the agar, possibly indicating the organism's preference for microaerophilic/anaerobic conditions. Cells form homogenous suspensions in liquid media. Both strains were rods capable of motility and did not produce endospores under any of the conditions tested. Phase contrast images are shown in Fig 5.5.

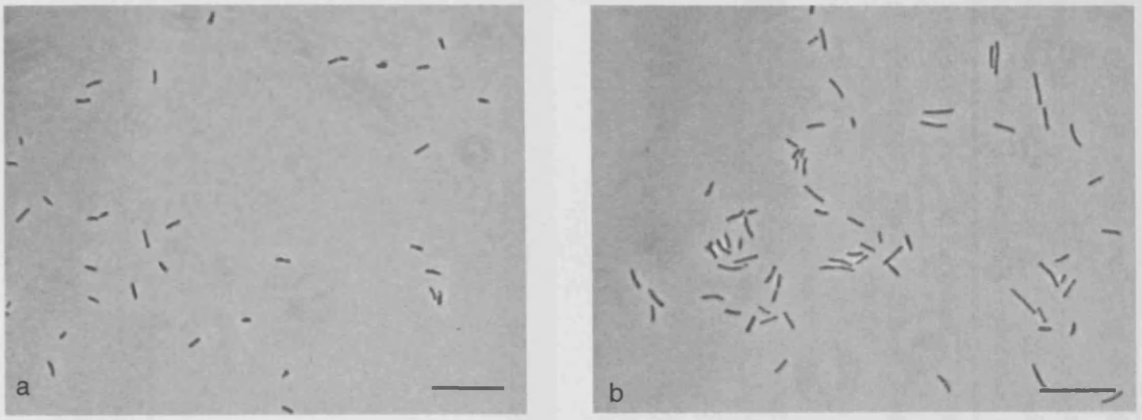


Fig 5.5. Phase contrast images of isolated *Marinobacter* MV strains. Panel a, strain CpA_a7; panel b, strain CpA_a13. Images show cell morphology during exponential growth phase. Images taken after 3 days incubation at 25°C in YPG medium. Scale bar = 10 μm.

A variety of growth tests were carried out on the two MV Marinobacter isolates to provide a physiological context for the organisms. These data were compared with literature data for the organisms' closest phylogenetic relatives (>97% sequence similarity) and are summarized in Table 5.3.

Table 5.3 Phenotypic properties of MV isolates and related type strains of *Marinobacter*

Strains: CpA_a7, present study; CpA_a13, present study; M. vinifirmus DSM 17747^T, data from Liebgott *et al.*, (2006); *M. excellens* KMM 3809^T, data from Gorshkova *et al.*, (2003); *M. litoralis* JCM 11547^T, data from Yoon *et al.*, (2003), Takai *et al.*, (2005); *M. alkaliphilus* JCM12291^T, data from Takai *et al.*, (2005); *M. daepoensis* DSM 16072^T, data from Yoon *et al.*, (2004); *M. hydrocarbonoclasticus*, ATCC 49840^T, data from Gauthier *et al.*, (1992), Takai *et al.*, (2005).

Characteristic	**CpA_a7**	**CpA_a13**	*M. vinifirmus*	*M. excellens*	*M. litoralis*	*M. alkaliphilus*	*M. daepoensis*	*M. hydrocarbonoclasticus*
Isolated from:	Sub-surface MV mud breccia	Sub-surface MV mud breccia	Wine barrel decalcification waste water	Radionuclide-contaminated marine sediment	Seawater	Sub-surface alkaline mud from a serpentine MV	Seawater	Seawater near petroleum refinery
Growth properties:								
Salinity range (%)	0.1 - 25	0.1 - 25	0 – 20	1 – 15	0.5 – 18	0 – 21	ND – 18	0.5 – 20.5
pH range	5.5 – 9.5	5.5 – 9.5	6.5 – 8.4[a]	6.0 – 10.0	4.5 - ND	6.5 – 11.4	5.5 -	6.0 – 9.5
Temperature range (^oC)	0 - 42	0 - 42	15 – 50	10 – 41	4 – 46	10 – 45	ND – 45	10 – 45
Growth on:								
Glucose	-	-	-	-	-	+	-	-
Fructose	-	-	(+)	+	-	+	-	-
Rhamnose	-	-	-	+	-	ND	ND	ND
Sucrose	-	-	-	-	-	-	ND	+
Maltose	-	-	-	+	-	+	ND	-
Cellobiose	-	-	-	ND	-	ND	ND	+
Glycerol	-	-	ND	-	-	+	-	-

Table 5.3 cont. Phenotypic properties of MV isolates and related type strains of *Marinobacter*

Characteristic	CpA_a7	CpA_a13	*M. vinifirmus*	*M. excellens*	*M. litoralis*	*M. alkaliphilus*	*M. daepoensis*	*M. hydrocarbonoclasticus*
Mannitol	-	-	-	+	-	ND	-	-
Acetate	+	+	+	ND	ND	+	ND	+
Lactate	+	+	+	-	ND	+	ND	+
Succinate	+	+	+	+	+	-	+	+
Citrate	-	-	-	-	ND	-	ND	+
L- Alanine	(+)	+	ND	-	-	+	-	-
Serine	-	-	ND	+	-	ND	-	-
Other phenotypic properties:								
Facultative anaerobe	+	+	-	+	-	+	+	+
Anaerobic NO_3^- reduction	+	+	-	+	-	+	+	+
Fermentation of glucose	ND	ND	-	+	-	ND	ND	-
Fumarate as e-acceptor	+	(+)	ND	ND	ND	+	ND	ND

[a] Indicates optimal pH, the full pH range of growth is not stated, although the authors note no growth at pH 4.5

The two strains had very similar physiology, with both strains using the same 15 substrates for growth (29% of those tested). Many organic acids were utilized as sole carbon and energy sources, as was the amino acid alanine and the polysaccharide cellulose. Other sugars and amino acids did not support growth. Neither strain grew on alcohols or aromatic compounds. Full details of the substrates used are stated in the species description (section 5.3.6). Both strains grew over a wide range of temperatures, pH and salinities in accordance with the known characteristics of the *Marinobacter* genus. The MV isolates were facultatively anaerobic with the ability to use nitrate, nitrite (both reduced to N_2) and fumarate as alternative electron acceptors.

Microscopic and visual assessment revealed some increase in biomass for both strains in both the initial glucose and amino acid fermentation tests. However, experience of these strains showed they could produce an appreciable amount of biomass when transferred into substrate free medium, presumably by growing on internal storage compounds and/or substrates transferred with the inoculum. A sulphide test (Widdel 1980) conducted with a second sub-culture of the amino acid fermentation cultures produced a very slight reaction for strain CpA_a7, but strain CpA_a13 was negative. Growth by glucose fermentation remained to be conclusively proven for strains CpA_a7 and CpA_a13.

5.3.5 Discussion

Phylogenetic and physiological tests revealed virtually no differences between the two MV *Marinobacter* isolates strongly suggesting they represented two strains of the same bacterium. Despite a very high 16S rRNA gene sequence similarity to the species *Marinobacter vinifirmus*, the MV isolates differed physiologically from this organism in a number of ways. The MV isolates were facultatively anaerobic, whereas Liebgott *et al.* (2006) reported that the *M. vinifirmus* type strain was a strict aerobe incapable of grow under anaerobic conditions on acetate, citrate, succinate or glucose as substrate, with or without nitrate as an electron acceptor. Four of the five other closely related strains to the MV isolates were however reported to be facultatively anaerobic (see Table 5.3). Furthermore, there were significant differences in the growth properties of *M. vinifirmus* and the MV isolates. The temperature minimum for growth of CpA_a7 and CpA_a13 was more than 10°C

lower than the minimum reported for *M. vinifirmus*, while *M. vinifirmus* was capable of growth at higher temperatures (45-50°C) than the MV isolates. The shift in temperature range between the MV isolates and *M. vinifirmus* presumably reflects their adaptation to two quite different habitats – deep-sea sub-surface mud breccia and wine-barrel decalcification wastewater, respectively. It was difficult to directly compare the pH range between the MV isolates and *M. vinifirmus*, as only the *optimal* pH range (plus no growth at pH 4.5) was reported by Liebgott *et al.* (2006). Furthermore, Liebgott *et al.* (2006) did not state whether appropriate buffers were used during their pH tests, an absence of which can result in misleading data (Sorokin, 2005). Work conducted during the present study showed that the MV *Marinobacter* isolates were capable of shifting the pH of liquid medium by up to 2 pH units when it was not adequately buffered. Salt tolerance was higher in the MV isolates than reported for *M. vinifirmus* (250 versus 200 g l^{-1} respectively) and higher than that reported for any of the MV isolates' other near phylogenetic neighbours. Liebgott *et al.* (2006) state that *M. vinifirmus* did not require NaCl for growth, an ability within the *Marinobacter* shared only by *M. aquaeolei*[3] (Nguyen *et al.*, 1999). However, it was not stated in the publications whether a series of sub-cultures were made in order to dilute out all salt carried over in the inoculum, or whether all minor sources of Na were omitted from the basal medium used. The specific requirement for NaCl was not tested during the present study but a requirement for salts was, and this showed the MV isolates did require some salt (0.1%) for growth. As the *M. vinifirmus* type strain is the only representative of this species and the experimental approaches of Liebgott *et al.* (2006) differed to those used in the present study, it is difficult to assess to what extent the differences in phenotypic results reflect different methodologies rather than real differences between the bacteria. However, although the MV isolates shared 99.7% similarity with *M. vinifirmus* it has been shown many times that comparison of the 16S rRNA gene does not always produce sequence clusters of physiologically similar organisms. This is easily explicable when it is considered that the 16S rRNA gene is not likely to be involved in determining ecological differences between bacterial populations, since mutations of this gene are not involved in population-specific adaptations in the same way protein coding genes are (see Palys *et al.*, 1997).

[3] Now reclassified as a later synonym of *M. hydrocarbonoclasticus* (Marquez & Ventosa, 2005).

Based on the available data, CpA_a7 and CpA_a13 seemed to be strains of the same bacterium, and this organism may warrant classification as a new species within the *Marinobacter* genus. However, further genetic (i.e. DNA G+C ratio and/or DNA-DNA similarity between the closest phylogenetic relative) and phenotypic information would be required before this could be validated. It would also be desirable to test the phenotypic properties of *M. vinifirmus* and other relatives of strains CpA_a7 and CpA_a13 under comparable experimental conditions.

5.3.6 Description of strains CpA_a7 and CpA_a13

Cells are rods capable of motility and did not produce endospores under any of the conditions tested. Homogenous cell suspensions are produced when grown in liquid media. Colonies are small, shiny, opaque, cream-coloured colonies with irregular edges that penetrate down into the agar.

Growth occurred between 0–42°C, pH 5.5–9.5 and salinities between 0.1–25%.

The organism used acetate, lactate, propionate, butyrate, valerate, hexanoate, octanoate, succinate, fumarate, malate, alanine, casein hydrolysate, fructose, trehalose, cellulose, and peptone for growth.

Methanol, ethanol, propanol, butanol, ethylene glycol, glycerol, mannitol, vanillin, pOH-benzaldehyde, benzoate, 3,4,5-trimethoxybenzoate, formate, gluconate, salicylate, malonate, tartrate, citrate, arginine, cysteine, isoleucine, serine, phenylalanine, glucosamine, glucose, arabinose, rhamnose, mannose, xylose, sucrose, maltose, cellobiose, starch, chitin, xylan and laminarin did not support growth. Fermentation of glucose and amino acids has yet to be demonstrated conclusively.

Oxygen, nitrate, nitrite (both reduced to N_2) and fumarate were utilized as electron acceptors.

The strains were isolated from sub-surface mud breccia from the crater of the Capt. Arutyunov MV in the central part of the Gulf of Cadiz.

5.4 Description of *Pseudomonas* MV isolates

5.4.1 Introduction to the species *Pseudomonas stutzeri* and associated genomovars

The genus *Pseudomonas* was introduced in the 19th Century by Migula (Palleroni, 1984) and at the time of writing contained 169 species and 8 subspecies with validly approved names (J. P. Euzéby [http://www.bacterio.cict.fr/] accessed 03/07). The genus has undergone extensive revision through molecular systematic analysis. Consideration of the genus as a whole is not appropriate here (see Anzai *et al.*, 2000; Kersters *et al.*, 1996; Moore *et al.*, 1996 for reviews); rather it is necessary only to consider those members closely related to the strains isolated during the present study. All four of the MV *Pseudomonas* strains characterised were most closely related to the species *Pseudomonas stutzeri* according to their 16S rRNA gene sequences. *P. stutzeri* is a non-fluorescent pseudomonad that is frequently isolated from a wide variety of environmental habitats (see Bennasar *et al.*, 1998a; Sikorski *et al.*, 2002a; Lalucat *et al.*, 2006). The bacterium grows on a wide range of organic substrates aerobically, or anaerobically coupled to denitrification. It is considered an exceptional species due to a remarkable physiological and biochemical versatility, and an unusually high genetic diversity (Cladera *et al.*, 2006). In fact the genetic diversity of *P. stutzeri* strains is the highest yet described for a single species (Rius *et al.*, 2001; Sikorski *et al.*, 2002b; Cladera *et al.*, 2004; 2006). Recent work on the various pure culture strains available has established a basis for grouping the known isolates into several genomic variants termed 'genomovars' (Rossello *et al.*, 1991). Genomovars (gv) are genomic groups, which lack a clear taxonomic status. A Genomovar group may be re-classified under a new species name once consistent discriminative phenotypic traits are identified that allow it to be distinguished from all related groups. However, this has seldom been possible for the genomovars of *P. stutzeri* despite several large-scale comparative phenotypic studies (Gavini *et al.*, 1989; Rossello *et al.*, 1991; Rossello-Mora *et al.*, 1994). An exception is *P. balearica*, which was formerly classified as gv 6 (Bennasar *et al.*, 1996). Currently at least 18 genomovars are recognized within *P. stutzeri* based on 16S rRNA gene sequences and DNA-DNA hybridization analysis (Sepulveda-Torres *et al.*, 2001; Garcia-Valdes *et al.*, 2003; Sikorski *et al.*, 2005).

5.4.2 Enrichment and isolation

Strain CpA_a4 was isolated from an anoxic 29°C temperature gradient enrichment originally designed to grow heterotrophic acetogens. It was isolated by repeated re-streaking of single colonies grown from the enrichment on solid YPG medium at 30°C under aerobic conditions. Strain CpA_a17 came from an anoxic 31°C temperature gradient enrichment designed to grow autotrophic acetogens. It was also isolated on solid YPG medium (incubated at 30°C) but under anaerobic conditions. Strain Bon_a1 was isolated from an anoxic 33°C temperature gradient enrichment designed to grow autotrophic sulphate-reducing bacteria (SRB). Single colonies of Bon_a1 were grown from the enrichment on solid YPG medium (aerobic conditions at 30°C) and purified by repeated re-streaking. Strain Bon_b1 came from an anoxic 7°C temperature gradient enrichment designed to grow heterotrophic SRB. It was isolated at the same temperature on aerobic YPG medium by repeatedly re-streaking single colonies.

5.4.3 Phylogenetic analysis of MV *Pseudomonas stutzeri*-associated isolates

In order to constrain the phylogeny of the 4 MV isolates the 16S rRNA genes were sequenced over >1400 bp and compared to reference sequences for each of the 18 recognized genomovars (Lalucat *et al.*, 2006), and some other selected sequences from the *Pseudomonas* genus (Fig 5.6).

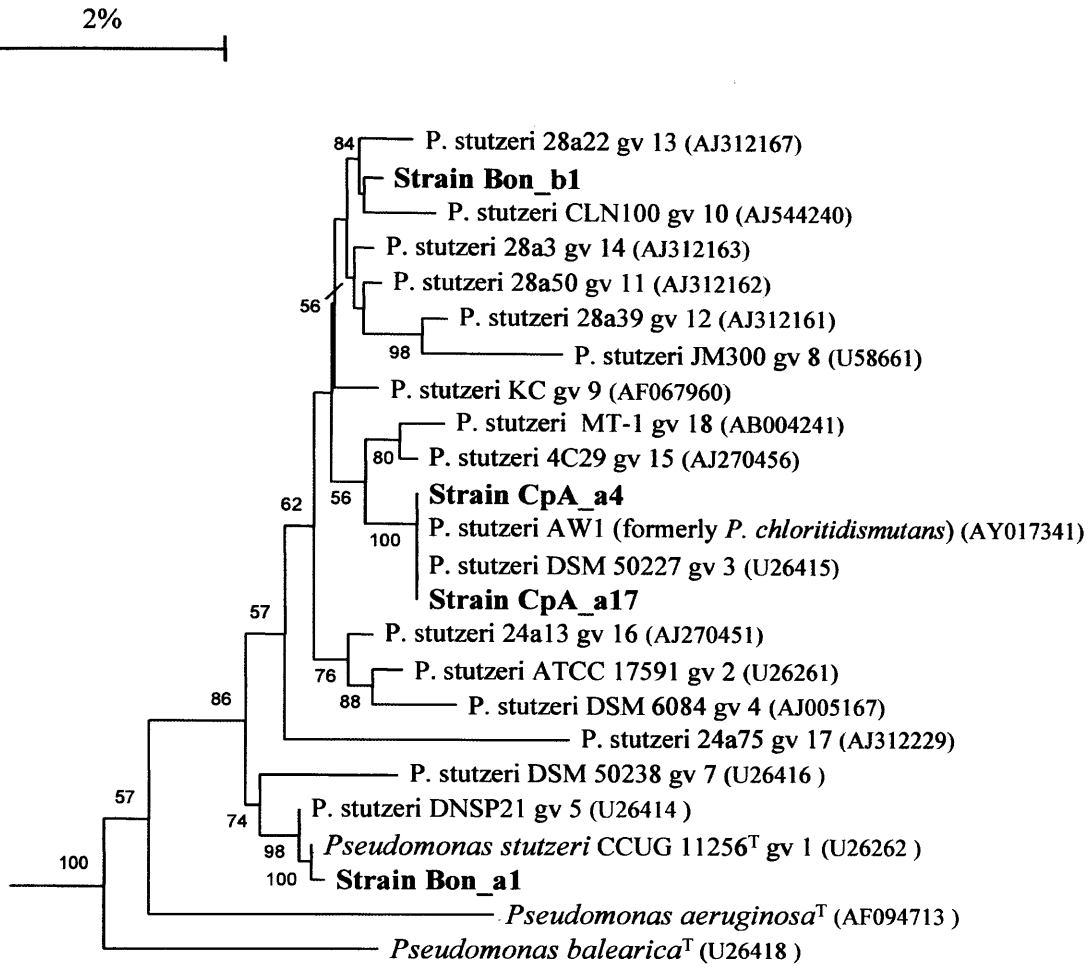


Fig 5.6 Neighbour-joining tree showing the phylogenetic relationship of the 4 MV *P. stutzeri* strains to the 18 genomovars (gv) identified within the *P. stutzeri* species (Lalucat *et al.*, 2006), and other related type strains of the genus. The reconstruction was based on an alignment of 1059 unambiguous nucleotide positions using the Jukes and Cantor (1969) algorithm. Numbers at nodes represent percentage bootstrap values after 1000 samples. Accession numbers are shown in parentheses. Scale bar indicates 2% sequence divergence. *E. coli* (ATCC 11775^T) used as outgroup (not shown).

Fig 5.6 shows that strains isolated from the two MV sites are phylogenetically distinct. The Capt. Arutyunov isolates represent two near identical strains (99.9% sequence similarity), which are 100% similar to *P. stutzeri* strain DSM 50227, the reference strain for genomovar 3, and 99.9% similar to Pseudomonas stutzeri strain AW1. Strain AW1 was originally described as a novel species – *P. chloritidismutans* - on account of a low DNA-DNA hybridization value with the *P. stutzeri* type species and its ability to couple chlorate reduction but not nitrate reduction to growth (Wolterink *et al.*, 2002). However, its species status was recently questioned by Cladera *et al.* (2006) after variants of the strain capable of anaerobic nitrate reduction were obtained following sub-cultivation under selective conditions. It seems likely therefore that this species will be re-classified as a strain of genomovar 3 and the two

Capt. Arutyunov MV strains are further members of this group. Strains Bon_a1 and Bon_b1 were found to share 98.3% sequence similarity with each other and were not so clearly affiliated with one particular genomovar. Strain Bon_a1 was 99.9% similar to the *P. stutzeri* type species but also 99.8% similar to the reference sequence for genomovar 5, while strain Bon_b1 shared 99.3% sequence similarity with the reference sequences for both genomovars 13 and 10. Clearly DNA-DNA hybridization studies would be required to fully resolve the taxonomic status of these two strains.

5.4.4 Phenotypic analysis of MV Pseudomonas stutzeri-associated isolates

Strain CpA_a4 formed relatively large (up to ~ 7 mm) irregular translucent wet-looking colonies with a wrinkled surface. They grew proud from the agar surface and were typically as high as they were wide. The colonies were hard and rigid and sometimes caused the agar to sink down around their edges. Cells were vibrioid – curved rods with round ends, capable of motility and sometimes observed growing in capsules of exopolysaccharide (EPS) (Fig 5.7, panel a).

Strain CpA_a17 had colony and cell morphology identical to strain CpA_a4.

Strain Bon_a1 colonies were small (sub millimetre) in size, with a sticky texture and a yellow pigmentation. They had a convex form and a smooth shiny surface. Cells were vibrioid – curved rods with round ends, capable of motility.

Strain Bon_b1 also had colony and cell morphology as described for strain CpA_a4.

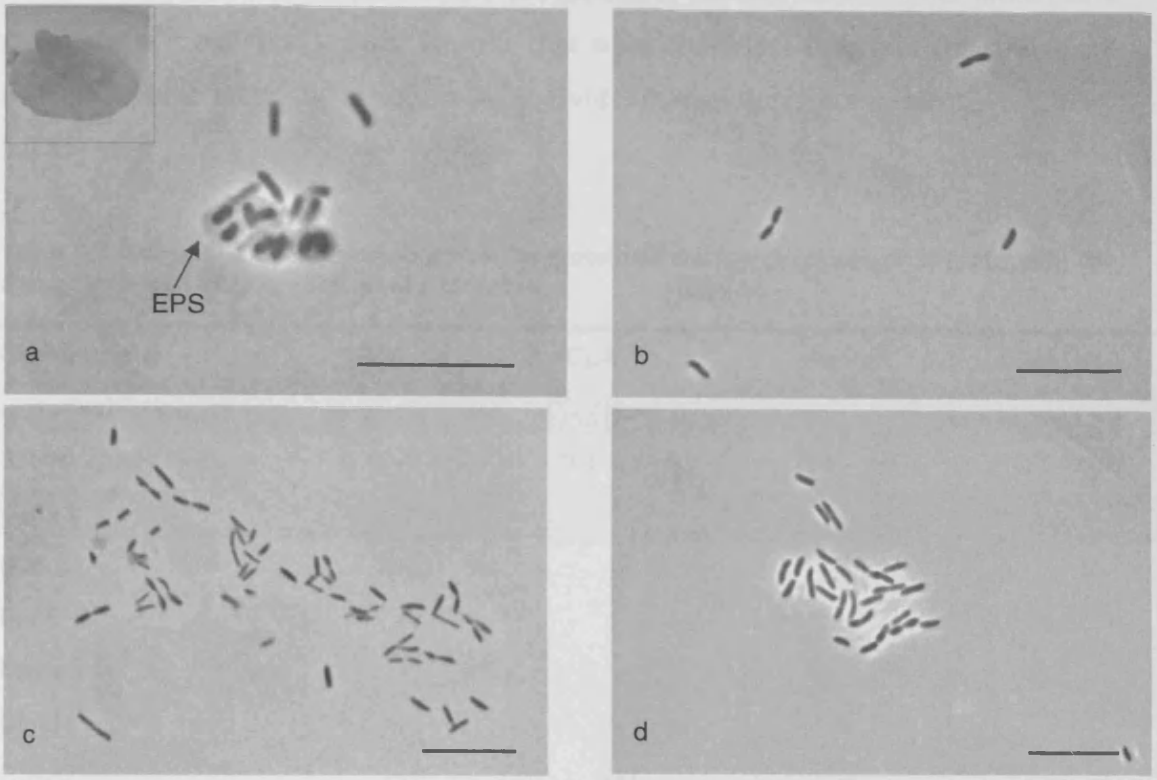


Fig 5.7 Phase contrast images of isolated *Pseudomonas stutzeri*-related MV strains. Panel a, strain CpA_a4 (note the capsules of EPS surrounding some of the cells) insert shows the characteristic colony morphology displayed by this organism; panel b, strain CpA_a17; panel c, strain Bon_a1; panel d, strain Bon_b1. Pictures show cell morphology during exponential growth phase, images taken after 3 days incubation at 25°C in YPG medium. Scale bar = 10 µm. N.B. colony shown in insert = approx 7 mm.

Several very detailed characterisation studies have been conducted with *P. stutzeri* strains and related species in an attempt to assist taxonomic classification (e.g. Gavini *et al.*, 1989; Rossello *et al.*, 1991; Rossello-Mora *et al.*, 1994) but all have failed in grouping the individual *P. stutzeri* genomic groups on a physiological basis. Owing to the comparatively limited phenotypic characteristics analysed during the present study and the considerable physiological heterogeneity present within the *P. stutzeri* species, it was considered unlikely that any meaningful information would be obtained by comparing the phenotypic traits of the MV isolates with the other described strains of *P. stutzeri*. Additionally, many of the substrates tested during the present study were not reported on in the published physiological studies with other stains. Instead therefore, the four MV strains were compared with each other to assess the phenotypic diversity present within a group of *P. stutzeri* strains isolated from the same type of habitat.

Table 5.4 summarises the phenotypic properties of the MV isolates that produced a variable result between strains. Results that were consistent for all of the strains are listed below the table and stated in the individual strain descriptions (sections 5.4.5 – 5.4.8).

Table 5.4 Summary of the variable results obtained during physiological tests with the *Pseudomonas stutzeri*-related MV isolates.

Characteristic	CpA_a4	CpA_a17	Bon_a1	Bon_b1
Growth properties:				
Salinity range (%)	0.1 – 12.5	0.1 – 12.5	0.1 – 10	0.1 - 10
Growth on:				
Starch	(+)	(+)	-	-
Sucrose	+	+	-	-
Maltose	+	+	-	-
Citrate	+	+	+	-
Glycol	(+)	(+)	+	+
Benzoate	-	-	+	+
pOH-Benzaldehyde	-	-	+	+
Fermentation of glucose	+	+	-	-

Growth was scored as +, positive; (+), weak positive; -, negative (see section 2.5.1).
Substrates that were positive for all strains: growth on ethylene glycol, glycerol, mannitol, acetate, lactate, propionate, butyrate, valerate, hexanoate, octanoate, gluconate, malonate, succinate, fumarate, malate, alanine, isoleucine, glucose, fructose, casamino acid mixture, yeast extract and peptone.
Substrates that were negative for all strains: growth on methanol, ethanol, propanol, butanol, vanillin, 3,4,5-trimethoxybenzoate, formate, salicylate, tartrate, cysteine, serine, arginine, phenylalanine, glucosamine, arabinose, rhamnose, mannose, trehalose, xylose, cellulose, cellobiose, chitin, xylan and laminarin.

Strains CpA_a4 and CpA_a17 were identical in their metabolic capabilities, with both strains using 50% of the substrates tested. Strains Bon_a1 and Bon_b1 were very similar in their metabolic capabilities, with Bon_a1 using 48% and Bon_b1 using 46% of the substrates tested.

All strains grew between 0 – 42°C on solid YPG media (section 2.5.4). No growth occurred at 45°C. All strains grew between pH 5.5 – 9.5 (section 2.5.5). Strains CpA_a4 and CpA_a17 grew between 0.1 and 12.5% salinity, with no growth was observed at 15 or 0.00175% salinity. Strains Bon_a1 and Bon_b1 grew between 0.1

and 10% salinity, with no growth was observed at 12.5 or 0.00175% salinity (section 2.5.6).

A range of alternative electron acceptors were tested with the MV *P. stutzeri* isolates (section 2.5.2). Both Nitrate and nitrite supported growth with all four strains when 5 mM acetate and lactate were used as the electron donors. Fluorescence measurements in nitrate and nitrite-containing wells were significantly above that observed in control wells containing the electron donors only. Gas bubbles were also observed in the test wells of both replicate tests with all four strains, suggesting the nitrate and nitrite were reduced through to N_2.

5.4.5 Discussion

Some notable differences were found between the *P. stutzeri* strains isolated during the present study and interestingly these seemed to reflect the strains' isolation source. Of the 6 substrate tests that differed, 5 varied according to whether the strains were isolated from the Capt. Arutyunov or the Bonjardim MV craters. The similarity between the two selected Capt. Arutyunov strains is perhaps unsurprising given their highly similar 16S rRNA gene sequences but it is perhaps more surprising for the two Bonjardim strains which seemed to belong to distinct genomovars. The Capt. Arutyunov strains differed from the Bonjardim strains in their ability to grow on the disaccharides sucrose and maltose, and also on starch (weak growth). It is notable that growth on starch and maltose are considered to be two of the characteristics that distinguish *P. stutzeri* strains from other pseudomonads (Lalucat *et al.*, 2006), and so the apparent inability of the Bonjardim strains to grow on these substrates may question their taxonomic affiliation with this group, however more data would be required to resolve this. Both Bonjardim strains were able to grow on the aromatic compounds p-hydroxybenzaldehyde and benzoate, while the Capt. Arutyunov strains could not. Growth on aromatics has previously been shown to be a variable trait within the *P. stutzeri* species (Rossello-Mora *et al.*, 1994). The Capt. Arutyunov strains and the Bonjardim strains also differed in their salinity range for growth, with the Capt. Arutyunov strains showing the wider range, up to 12.5%.

Consistent with their affiliation to *Pseudomonas stutzeri* all of the MV strains were facultatively anaerobic with the ability to denitrify. This ability suggests these organisms may represent active members of the prokaryotic community in the MV sub-surface environment, where conditions are anaerobic.

5.4.6 Description of strains CpA_a4 and CpA_a17

Colonies may grow relatively large (up to ~ 7 mm), are irregular, translucent and wet looking with a wrinkled surface. They grow proud from the agar surface and are typically as high as they are wide. The colonies are hard and rigid and sometimes cause the agar to sink down around their edges. Cells are rod shaped, capable of motility and are sometimes surrounded by capsules of EPS, sporulation was not observed. Growth occurs at 0–42°C (no growth at 45°C), at pH 5.5–9.5 (no growth at pH 4.5), and at sea salt concentrations of 1–125 g l^{-1} (no growth at 0.0175 or 150 g l^{-1}). Facultatively anaerobic. Able to use nitrate and nitrite as alternative electron acceptors, by coupling growth to denitrification. Able to grow on starch, ethylene glycol, glycerol, mannitol, acetate, lactate, propionate, butyrate, valerate, hexanoate, octanoate, gluconate, malonate, succinate, fumarate, malate, citrate, alanine, isoleucine, glucose, fructose, sucrose, maltose, casamino acid mixture, yeast extract and peptone. No growth on methanol, ethanol, propanol, butanol, vanillin, 3,4,5-trimethoxybenzoate, formate, salicylate, tartrate, cysteine, serine, arginine, phenylalanine, glucosamine, arabinose, rhamnose, mannose, trehalose, xylose, cellulose, cellobiose, chitin, xylan or laminarin. The 16S rRNA gene sequence was 100% similar to *P. stutzeri* strain DSM 50227, the reference strain for genomovar 3.

5.4.7 Description of strain Bon_a1

Colonies are small (sub millimetre) in size, with a sticky texture and a yellow pigmentation. They have a convex form and a smooth shiny surface. Cells are vibrioid – curved rods with round ends, capable of motility. Sporulation was not observed. Growth occurs at 0–42oC (no growth at 45oC), at pH 5.5–9.5 (no growth at pH 4.5), and at sea salt concentrations of 1–100 g l-1 (no growth at 0.0175 or 125 g l-1). Facultatively anaerobic. Ability to use nitrate and nitrite as alternative electron acceptors, by coupling growth to denitrification. Able to grow on ethylene glycol,

glycerol, mannitol, acetate, lactate, propionate, butyrate, valerate, hexanoate, octanoate, gluconate, malonate, succinate, fumarate, malate, citrate, alanine, isoleucine, glucose, fructose, benzoate, p-OH-benzaldehyde, casamino acid mixture, yeast extract and peptone. No growth on methanol, ethanol, propanol, butanol, vanillin, 3,4,5-trimethoxybenzoate, formate, salicylate, tartrate, cysteine, serine, arginine, phenylalanine, glucosamine, sucrose, maltose, arabinose, rhamnose, mannose, trehalose, xylose, cellulose, cellobiose, starch, chitin, xylan or laminarin. The 16S rRNA gene sequence was 99.9% similar to the Pseudomonas stutzeri type species CCUG 11256T.

5.4.8 Description of strain Bon_b1

Colonies can grow relatively large (up to ~ 7 mm), are irregular, translucent and wet looking with a wrinkled surface. They grow proud from the agar surface and are typically as high as they are wide. The colonies are hard and rigid and sometimes cause the agar to sink down around their edges. Sporulation was not observed. Growth occurs at 0–42°C (no growth at 45°C), at pH 5.5–9.5 (no growth at pH 4.5), and at sea salt concentrations of 1–100 g l^{-1} (no growth at 0.0175 or 125 g l^{-1}). Facultatively anaerobic. Ability to use nitrate and nitrite as alternative electron acceptors, by coupling growth to denitrification. Able to grow on ethylene glycol, glycerol, mannitol, acetate, lactate, propionate, butyrate, valerate, hexanoate, octanoate, gluconate, malonate, succinate, fumarate, malate, alanine, isoleucine, glucose, fructose, benzoate, p-OH-benzaldehyde, casamino acid mixture, yeast extract and peptone. No growth on methanol, ethanol, propanol, butanol, vanillin, 3,4,5-trimethoxybenzoate, formate, salicylate, tartrate, citrate, cysteine, serine, arginine, phenylalanine, glucosamine, sucrose, maltose, arabinose, rhamnose, mannose, trehalose, xylose, cellulose, cellobiose, starch, chitin, xylan or laminarin. The 16S rRNA gene sequence was 99.3% sequence similarity with the reference sequences for both genomovars 13 (DSM 17087) and 10 (strain CLN100).

5.5 Description of *Arcobacter* MV isolates

5.5.1 Introduction to the *Arcobacter* genus

The *Epsilonproteobacteria* comprises organisms of the phylogenetically distinct rRNA superfamily VI and is a taxonomically diverse group, including both commensals and pathogens (*Campylobacter*, *Helicobacter*, *Wolinella*) and free-living organisms with important biogeochemical roles in marine and terrestrial ecosystems (e.g. *Sulfurospirillum*, *Nautilia*, *Caminibacter*, *Thiovulum*). The *Arcobacter* genus was introduced to the group to accommodate phylogenetically and phenotypically distinct organisms that had previously been classified as campylobacters (Vandamme *et al.*, 1991). *Arcobacter nitrofigilis* is the type species of the genus and was isolated from the roots of the salt marsh plant *Spartina alterniflora* (McClung *et al.*, 1983). The genus is unusual within the *Epsilonproteobacteria* as it contains both clinical/veterinary-associated species (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius*) and environmental species with no known pathogenicity (*A. nitrofigilis* and *A. halophilus*). The traditional interest in *Arcobacter* has been in its role as a potential human and animal pathogen (Mansfield & Forsythe 2000; On 2001; Lehner *et al.*, 2005). However, this, and the few described free-living representatives, does not adequately reflect the potential importance of this genus in the environment. Culture and molecular based studies have revealed a wide habitat range for organisms affiliated to *Arcobacter*, including brackish lakes (Maugeri *et al.*, 2000); hypersaline lakes (Teske *et al.*, 1996); estuaries (Covert *et al.*, 2001); sea water (Eilers *et al.*, 2000a, Wirsen *et al.*, 2002, Fera *et al.*, 2004); marine sediments (Llobet-Brossa *et al.*, 1998, Thamdrup *et al.*, 2000, Wirsen *et al.*, 2002, Bowman & McCuaig 2003); cold seep sediments (Arakawa & Kato unpublished); oil reservoirs and storage cavities (Voordouw *et al.*, 1996, Watanabe *et al.*, 2000, Telang *et al.*, 1999, Grabowski *et al.*, 2005); landfill sites (Huang *et al.*, 2005); marine fauna (Romero *et al* 2002, Frias-Lopez *et al.*, 2002); activated sludge (Snaidr *et al.*, 1997) and hydrothermal vent environments (Moussard *et al.*, 2006; Sievert *et al.*, 2007). The environmental organisms that have been obtained in pure culture are implicated in nitrogen and sulphur cycling (Teske *et al.*, 1996, Wirsen *et al.*, 2002, Donachie *et al.*, 2005), however the scarcity of pure cultures and a lack of comprehensive metabolic

characterisations in the published literature makes the environmental relevance of this widespread organism difficult to fully assess.

5.5.2 Enrichment and isolation

Strain CpA_a5 was enriched at 31°C inside a temperature gradient system in anoxic liquid medium that was originally designed to target autotrophic methanogens (no sulphate, H_2:CO_2 as substrate). Sub-samples of the positive enrichment were transferred onto solid YPG medium and incubated at 30°C under both anaerobic and aerobic conditions. CpA_a5 was isolated from the aerobic plate by repeatedly re-streaking single colonies. CpA_b6 was also isolated under aerobic conditions on solid YPG media at 30°C. It came from a positive anoxic temperature gradient enrichment (30°C) designed to target SRB capable of growing on aromatic hydrocarbons (sulphate present, toluene as substrate).

5.5.3 Phylogenetic analysis of MV *Arcobacter* isolates

Phylogenetic analysis of near full-length 16S rRNA gene sequences from the two isolated MV strains CpA_a5 and CpA_b6 revealed they were most closely related to organisms affiliated to the genus *Arcobacter*, though the sequence similarity to all described members of this group was low (<93%). A phylogenetic reconstruction was produced using selected pure culture and environmental sequences of the genus *Arcobacter* (Fig 5.8).

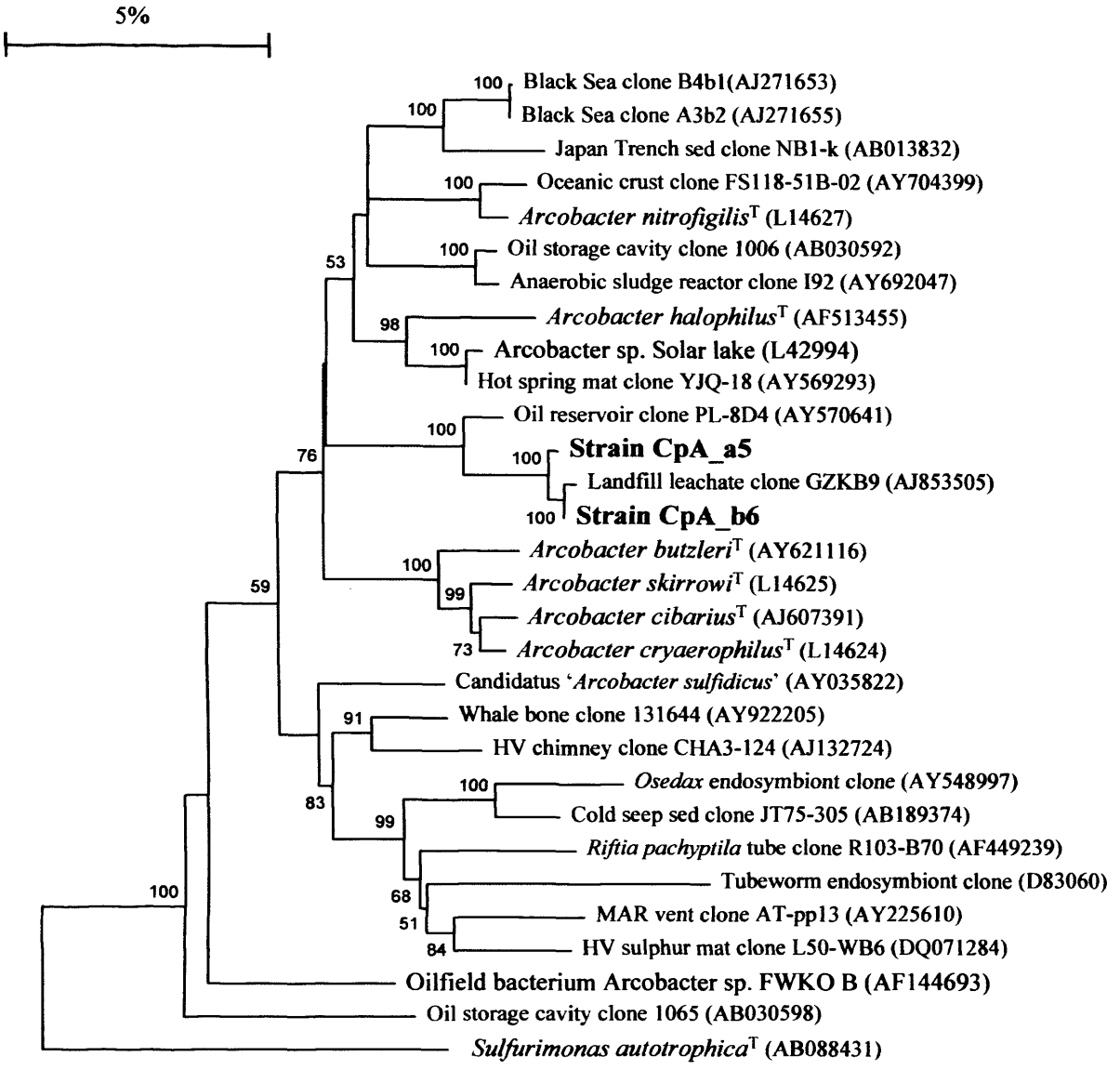


Fig 5.8 Neighbour-joining tree showing the phylogenetic position of the two Cp. A. MV *Arcobacter*-related isolates to selected pure culture and environmental clone sequences within the *Arcobacter* genus. The reconstruction was based on an alignment of 1275 nucleotide positions using the Jukes and Cantor (1969) algorithm. Numbers at nodes represent percentage bootstrap values after 1000 samples. Accession numbers are shown in parentheses. Scale bar indicates 5% sequence divergence. *Sulfurimonas autotrophica*^{T} was used as outgroup.

The reconstruction shows that the two MV isolates (99.5% sequence similarity) occupy a distinct lineage within the *Arcobacter*, known previously only from molecular-based studies. The closest phylogenetic neighbour of the MV isolates (>99% sequence similarity) was a clone recovered as a minor component (2.2% of library) from the leachate of a municipal solid waste landfill (clone GZKB9, accession no. AJ853504; Huang *et al.*, 2005). The 16S rRNA gene sequence similarity between the MV strains and validly described species of *Arcobacter* is given in Table 5.5.

Table 5.5 Similarity matrix of MV strains and related *Arcobacter* isolates for which near-full length 16S rRNA gene sequences are available, calculated from an edited alignment of 1275 unambiguous nucleotide positions.

	Strain CpA_a5	Strain CpA_b6	Arcobacter sp. Solar lake	*Arcobacter halophilus*	*Arcobacter nitrofigilis*	*Arcobacter cryaerophilus*	*Arcobacter cibarius*	*Arcobacter skirrowii*	*Arcobacter butzleri*
Strain CpA_a5	ID	99.5	92.9	92.8	92.7	92.6	92.4	93.0	92.7
Strain CpA_b6	99.5	ID	93.2	93.0	92.7	92.7	92.6	93.2	92.7
Arcobacter sp. Solar lake	92.9	93.2	ID	96.0	94.1	91.9	92.4	92.3	92.7
Arcobacter halophilus	92.8	93.0	96.0	ID	94.1	92.0	92.5	92.3	92.7
Arcobacter nitrofigilis	92.7	92.7	94.1	94.1	ID	93.5	93.8	93.7	93.9
Arcobacter cryaerophilus	92.6	92.7	92.0	92.0	93.5	ID	98.7	98.3	97.3
Arcobacter cibarius	92.4	92.6	92.5	92.5	93.8	98.7	ID	98.1	97.4
Arcobacter skirrowii	93.0	93.2	92.3	92.3	93.7	98.3	98.1	ID	96.9
Arcobacter butzleri	92.7	92.7	92.7	92.7	93.9	97.3	97.4	96.9	ID

The 16S rRNA gene similarity between the MV strains and the validly described species of *Arcobacter* is within the range (90-96%) recognized for genus-level differentiation (Gillis *et al.*, 2001), suggesting that on a purely phylogenetic basis the MV strains could represent a new genus within the *Epsilonproteobacteria*. However, the phylogenetic position of the MV strains falls well within the broad '*Arcobacter* cluster' of 16S rRNA gene sequences defined by Campbell *et al.* (2006) and a genus-level distinction is not supported by the available phenotypic data (see section 5.5.4). The phylogenetic evidence clearly supports that strains CpA_a5 and CpA_b6 represent at least novel species within the genus *Arcobacter*.

5.5.4 Phenotypic analysis of MV *Arcobacter* isolates

Strains CpA_a5 and CpA_b6 both formed small (≤ 1 mm), opaque, cream-yellow pigmented, irregular shaped colonies on solid YPG media. The colonies grew down into the agar, which was probably as a response to the organism's preference for

microaerophilic/anaerobic conditions. Cells were curved – helical shaped and highly motile. Fig 5.9 shows phase contrast images of both CpA_a5 and CpA_b6.

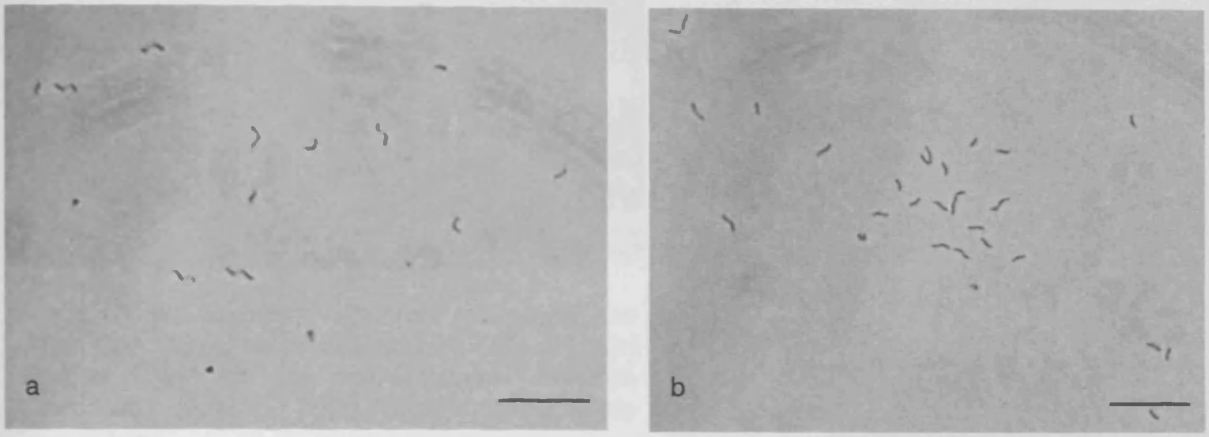


Fig 5.9. Phase contrast images of isolated *Arcobacter*-related MV strains. Panel a, strain CpA_a5; panel b, strain CpA_b6. Pictures show cell morphology during exponential growth phase. Images taken after 3 days incubation at 25°C in YPG medium. Scale bar = 10 µm.

The average cell size of strain CpA_a5 was determined to be 2.7 µm long by 0.5 µm wide, according to measurements of 120 individual cells (section 2.6.5).

Strain CpA_a5 was additionally examined under higher magnification by TEM and SEM microscopy (sections 2.6.6 and 2.6.7), which allowed visualisation of the flagella (Fig 5.10). Using these techniques a single polar flagellum was frequently observed.

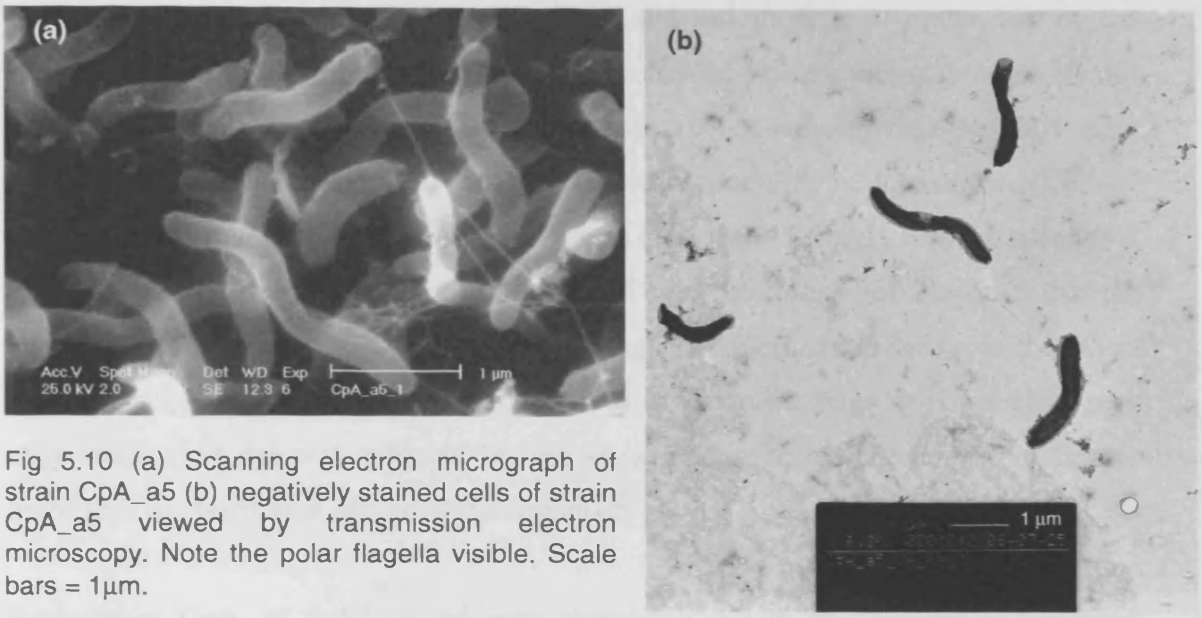


Fig 5.10 (a) Scanning electron micrograph of strain CpA_a5 (b) negatively stained cells of strain CpA_a5 viewed by transmission electron microscopy. Note the polar flagella visible. Scale bars = 1µm.

The temperature range for growth of strain CpA_a5 was determined inside a temperature gradient system in liquid basal medium containing 5 mM acetate and nitrate as substrates (section 2.5.4). Positive growth temperatures were determined visually by noting the development of turbidity and validated at the extremes by ion chromatography i.e. by confirming depletion of the substrates relative to a time zero un-inoculated control sample. Strain CpA_a5 was shown by this method to grow between 9–38oC, with no growth occurring at 4.5 and 41oC after three weeks of incubation. The temperature range of strain CpA_b6 was not determined.

The pH range of both MV Arcobacter strains was tested using appropriate buffers in completely filled tubes of YPG medium containing nitrate as the electron acceptor (section 2.5.5). Both strains grew between pH 6.1–7.4, with no growth occurring at 5.7 or 8.9. The strains did not alter the pH of the media during growth.

The salinity range for growth of strains CpA_a5 and Cp_b6 was tested under anaerobic conditions on 96-well plates using YPG media and 5 mM nitrate as the electron acceptor (section 2.5.6). Growth of strain CpA_a5 occurred at 2 and 5% according to measurement of the fluorescent signal of DNA stained cells (SYBR Green I) with a plate reader (Martens-Habbena, & Sass, 2006), and this was further confirmed by phase contrast microscopy. The greatest growth of strain CpA_a5 occurred at 5% salinity and indeed a small pellet developed at this salinity. No growth occurred at 1 or 7%. All un-inoculated controls were negative according to plate

reader data and microscopy. Strain CpA_b6 did not grow well under any of the salinities tested according to data from the plate reader. Phase contrast microscopy did detect strain CpA_b6 cells at 5% and 2% salinity but showed that the cells were typically semi-transparent rather than opaque suggesting they may have lost viability. Phase contrast microscopy did not detect strain CpA_b6 cells at ≤1 or ≥7% salinity. A possible explanation for the lack of growth of CpA_b6 during these tests is that the cells received a damaging oxygen shock during the experimental set up as the plates were prepared on the bench before incubation in anaerobic bags. It is not clear why only strain CpA_b6 was affected by the same experimental procedure but further highlights the fastidious nature of the organism.

Both strains CpA_a5 and CpA_b6 were tested for growth on a variety of substrates selected to represent a wide variety of organic and inorganic compounds likely to occur in the marine environment (section 2.5.1). The substrates that supported growth were quite limited for the two strains, being restricted to methanol (only with 1mM of acetate added as carbon source), acetate, lactate, propionate, succinate, fumarate, malate, glutamate, peptone and yeast extract. Weak growth occurred with formate (when 1mM acetate was added), xylan, and with cysteine and casein hydrolysate according to the sulphide test.

No growth occurred with methanol (without carbon source), ethanol, propanol, butanol, 1,2-propandiol, glycerol, mannitol, polysorbate 80 (Tween 80), vanillin, benzaldehyde, benzoate, 3,4,5-trimethoxybenzoate, formate (without carbon source), butyrate, valerate, hexanoate, octanoate, crotonate, pyruvate, gluconate, salicylate, glycolate, ascorbate, isoleucine, proline, serine, glucosamine, glucose, fructose, arabinose, rhamnose, mannose, galactose, trehalose, sucrose, maltose, cellobiose, cellulose, starch, chitin, laminarin and methylamine.

Results were inconclusive for the following substrates: malonate, tartrate, citrate, niacin, alanine, arginine, glutamine, phenylalanine, xylose, choline and ethanolamine. These tests were negative by visual assessment and did not produce gas (N2) on opening of the screw-cap test tubes, but the results were not confirmed by microscopy, as the other tests were, due to time constraints.

Amino acids were weakly fermented by both strains (confirmed by the sulphide test and microscopy) but glucose was not.

The strains were also tested with a range of electron acceptors (section 2.5.2). Growth occurred with nitrate, fumarate and thiosulphate. Weak growth occurred with sulphur, which was confirmed by the development of a brown colouration during the sulphide test (Widdel, 1980). Weak growth also occurred with iron(III) hydroxide. Growth with oxygen was only supported under microaerophilic conditions. No growth occurred with TMAO, DMSO, sulphite, nitrite, selenate and manganese dioxide.

The major fatty acids were determined for strain CpA_a5 using freeze-dried cells grown at 25oC in mineral salts containing 5mM acetate and nitrate as substrates (section 2.6.2). The dominant fatty acids were found to be C16:0, C16:19(Z) and C18:111(Z), the full profile is shown in table 5.6. Fatty acid compositions of the other validly described Arcobacter species are also shown in the table for comparison although these species were studied using different growth conditions (on Blood Agar at 37oC). It is well documented that fatty acid profiles are influenced to some extent by cultivation conditions (e.g. Männistö & Puhakka, 2001), however, the fatty acid profile of strain CpA_a5 had a degree of variation comparable with other described species of Arcobacter.

Strain CpA_a5 was catalase positive as indicated by the production of O2 following the addition of hydrogen peroxide in glycerol (1:1).

Table 5.6 Fatty acid composition of strain CpA_a5 and validly described members of the *Arcobacter* genus

Strains: 1, CpA_a5 present study; 2, *A. nitrofigilis* CCUG 15893^T data from Vandamme *et al.*, 1992; 3, *A. halophilus* ATCC BAA-1022^T data from Donachie *et al.*, 2005; 4, *A. cryaerophilus* (subgroup 1) data from Vandamme *et al.*, 1992; 5, *A. butzleri* data from Vandamme *et al.*, 1992; 6, *A. skirrowii* data from Vandamme *et al.*, 1992. Fatty acid data is not available for *A. cibarius*.

Blank cells represent data not determined or results < 1%. Literature fatty acid data is given for cells grown at 37°C on blood agar.

	Fatty acid content (% of total)					
Strain:	**1**	2	3	4	5	6
Saturated fatty acids						
$C_{12:0}$	1.7	7.2	8.2	3.7	6.8	9.1
$C_{14:0}$	1.4	4.8	5.0	1.8	4.8	2.4
$C_{16:0}$	16.6	32.0	19.3	18.7	19.6	22.2
$C_{18:0}$	1.4	-	-	-	-	-
Unsaturated fatty acids						
$C_{12:1}$	1.1	-	-	-	-	-
$C_{14:1}^{7(Z)}$	-	-	-	9.2	4.9	1.4
$C_{16:1}^{9(Z)}$	42.7	30.9	37.9	45.2	19.2	22.8
$C_{16:1}^{9(E)}$	-	-	-	-	13.8	7.8
$C_{16:1}$ iso 1	1.6	-	-	-	-	-
$C_{16:1}$ iso 2	2.4	-	-	-	-	-
$C_{18:1}^{11(Z)}$	22.9	12.8	21.7	10.6	11.6	19.7
Hydroxy fatty acids						
$C_{12:0}$ 3OH	2.2	5.5	2.7	-	-	-
$C_{14:0}$ 2OH	2.2	-	-	-	-	-
$C_{14:0}$ 3OH	1.9	5.3	-	9.4	17.4	9.2

N.B. Data given in Vandamme *et al.* (1992) were average fatty acid compositions for multiple strains of the named species.

The DNA G + C content of strain CpA_a5 was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (see section 2.6.3) and compared to literature data for other validly described Arcobacter sp. (Table 5.7). The G + C content of strain CpA_b6 was not determined.

Table 5.7. G + C content (mol%) for strain CpA_a5 and validly described members of the *Arcobacter* genus
Strains: 1, CpA_a5 present study; 2, *A. nitrofigilis* CCUG 15893^T data from Vandamme *et al.*, 1991; 3, *A. halophilus* ATCC BAA-1022^T data from Donachie *et al.*, 2005; 4, *A. cryaerophilus* CCUG 17801^T data from Vandamme *et al.*, 1991; 5, *A. butzleri* ATCC 49616^T data from Vandamme *et al.*, 1992; 6, *A. skirrowii* CCUG 10374^T data from Vandamme *et al.*, 1992; 7, *A. cibarius* CCUG 48482^T data from Houf *et al.*, 2005.

	DNA base composition of *Arcobacter* sp.						
Strain:	1	2	3	4	5	6	7[a]
G + C content (mol%)	33.7	29.0	35.0	28.6	28.0	29.0	27.7

[a] Average value, Houf *et al.* (2005) gave the range of G + C mol% for the four reference strains of *A. cibarius* as 27.2 – 28.2. The exact value of the type strain was not given.

5.5.5 Discussion

The available phylogenetic and phenotypic data strongly suggest CpA_a5 and CpA_b6 are strains of the same organism. The species is distantly related to previously described members of the genus *Arcobacter* according to 16S rRNA gene sequence data (≤93% similarity), and forms a distinct phylogenetic branch within the group (Fig 5.8). The organism does however fall firmly within the *Arcobacter* clade according to the 16S rRNA gene sequence data of (Campbell *et al.*, 2006).

A meaningful phenotypic comparison between the MV isolates and described members of the *Arcobacter* genus could not be made due to the fact all validly described members of the genus have been investigated using techniques appropriate to the association *Arcobacters* can have with clinical and veterinary samples (e.g. On, 1996; Atabay *et al.*, 1998; 2006), including even those isolated as free-living organisms from the environment (Donachie *et al.*, 2005). The *Arcobacter* genus is unique within the *Epsilonproteobacteria* in its inclusion of both pathogenic and free-living environmental species, and it is likely some taxonomic revision will have to be made in the future given the different ecological ranges and roles of the organisms therein. It is recommended that a polyphasic investigation of representative cultured *Arcobacters*, including the phenotypic tests used in this study, be conducted as future work to address this. Moreover, the lack of environmentally relevant phenotypic tests conducted with the free-living *Arcobacters* may mean important ecological functions are being overlooked. In addition to obtaining comparable phenotypic data, DNA-DNA hybridization tests between the species represented by strain CpA_a5 and

described members of the *Arcobacter* genus is necessary to assist in defining its exact taxonomic status (Stackebrandt *et al.*, 2002). It is noteworthy that DNA-DNA hybridization tests conducted between the obligate halophile *A. halophilus* isolated as a free-living organism from a hypersaline lagoon in Hawaii, and other described *Arcobacters* showed only between 12 – 4% re-association (Donachie *et al.*, 2005).

Some putative *Arcobacter* species without standing in nomenclature e.g. "*Candidatus* Arcobacter sulfidicus", isolated from coastal seawater (Wirsen *et al.*, 2002); Arcobacter sp. Solar Lake, isolated from a sample of cyanobacterial mat originally from Solar Lake, Egypt (Teske *et al.*, 1996); and Arcobacter sp. FWKO B, isolated from oil-field brine (Gevertz *et al.*, 2000) have been tested for growth with some environmentally relevant substrates. For example, all of these strains are able to oxidize H_2S to S_0; a function not demonstrated experimentally with the MV strains, but one that would be relevant to the MV environment where reduced sulphide is present in the extruded sediment, e.g. as a result of AOM (Niemann *et al.*, 2006). Similar to CpA_a5 and CpA_b6, none of these additional putative *Arcobacter* strains could grow in air, but could use O_2 as an electron acceptor at lower concentrations (Arcobacter sp. Solar Lake up to 20%, Teske *et al.*, 1996; Strain FWKO B in 1% O_2, Gevertz *et al.*, 2000 and *Candidatus* Arcobacter sulfidicus in 1% O_2, Wirsen *et al.*, 2002). Only one of these additional strains – Arcobacter sp. Solar Lake – is chemoorganotrophic like strains CpA_a5, CpA_b6 and all validly described members of the genus. Both strains *Candidatus* Arcobacter sulfidicus and strain FWKO B were shown to be autotrophs unable to grow on all organic substrates tested (Gevertz *et al.*, 2000; Wirsen *et al.*, 2002). These strains were also both unable to completely reduce nitrate. This data was recognized as a significant divergence from the phenotype of the *Arcobacter* genus but the available phylogenetic, morphological and other physiological data supported their association with this group (Wirsen *et al.*, 2002; Campbell *et al.*, 2006). Such physiological variation within a discrete phylogenetic clade presents further taxonomic complications for the genus *Arcobacter* and further highlights the need for additional phenotypic and genotypic data from these organisms.

5.5.5.1 Strain CpA_a5 as the type strain ('*Candidatus* Arcobacter subtericola') of a putative new species within the genus *Arcobacter*

Two strains of curved – helical, highly motile bacteria were isolated from sub-surface mud volcano sediment taken from the Captain Arutyunov mud volcano located in the Gulf of Cadiz. The taxonomic position of the novel strains was preliminarily investigated using a polyphasic approach, in which strain CpA_a5 was investigated in detail. Sequencing of near-full length fragments of the 16S rRNA gene revealed the two strains were 99.5% similar to each other and 93% similar to their nearest neighbour *Arcobacter skirrowii.* The organism had a narrow substrate range; restricted to some organic and amino acids, plus methanol when acetate is added as a carbon source (see description below) and is microaerophilic. Nitrate was reduced to N_2 and cells were catalase positive. The G+C content was 33.7 mol%. These data indicate that the organism is a member of the genus *Arcobacter* but its phylogenetic distinctiveness and differential phenotypic properties signify it should be considered to represent a novel species. CpA_a5 is the type strain of the putative species and the name '*Candidatus* Arcobacter subtericola' is proposed (*subter* L. prep. below, underneath; *cola* L. suff. from L. masc. n. *incola*, inhabitant; N.L. masc. n. *subtericola*, the one who lives underneath).

5.5.6 Description of strains CpA_a5 and CpA_b6

Cells are curved - helical, 2.7 μm long and 0.5 μm wide, and non-spore-forming. Cells are highly motile by means of a single polar flagellum. Colonies are small (≤ 1 mm), opaque, cream-yellow pigmented, and irregular shaped on solid YPG media, which grow downwards below the agar surface. Cells are catalase positive and microaerophilic. Growth occurs between 9–38°C, with no growth at 4.5 or 41°C. The salinity range is 2–5% and the pH range is 6.1–7.4. Growth occurs with methanol and formate when 1mM acetate is added as a carbon source, and with the carboxylic acids acetate, lactate, propionate, succinate, fumarate, malate and glutamate. The amino acid cysteine and casein hydrolysate are used weakly, and peptone, xylan and yeast extract also support growth. Aromatic compounds higher alcohols and carbohydrates are not used as energy sources. Nitrate, fumarate and thiosulphate act as electron acceptors and weak reactions are also observed with sulphur and iron (III) hydroxide.

Amino acids are weakly fermented but glucose is not. The predominant fatty acids are $C_{16:0}$, $C_{16:1}{}^{9(Z)}$ and $C_{18:1}{}^{11(Z)}$. The G+C content of CpA_a5 is 33.7 mol%.

The strains were isolated from sub-surface mud breccia from the crater of the Capt. Arutyunov MV in the central part of the Gulf of Cadiz.

Chapter 6 – General Discussion

6.1 Introduction

Sub-surface sediment samples from MVs in the Gulf of Cadiz collected between 2003 and 2005 were the basis of a multidisciplinary study designed to investigate the geomicrobiology of these hitherto poorly understood habitats. Principle objectives of the study were as follows:

- To compile a microbial census for the samples studied using complimentary PCR-based molecular genetic techniques.
- To place molecular genetic results in context by measuring key environmental data such as total prokaryotic (AODC) cell count, *ex situ* metabolic activity (via radioisotopes), porosity, sediment type and basic pore water geochemistry.
- To assess to what extent MV craters offer a readily accessible 'window to the deep biosphere'.
- To enrich MV sediment organisms and obtain pure culture isolates from these environments for the first time.
- To characterise MV sediment isolates using environmentally relevant tests.
- To compare and contrast the findings from different Gulf of Cadiz MV samples both with each other and with results from other relevant sediment diversity studies.

Firstly in this chapter, the main finds of the thesis are summarized, then a general discussion addresses the principle themes and develops discussions from previous chapters where sample results were often considered in isolation to other findings in the thesis. Finally, future work is recommended.

6.2 Summary of main findings

Bacteria showed greater phylogenetic diversity than *Archaea* in all the MV sediments studied according to analysis of 16S rRNA and functional genes by cloning and PCR-DGGE.

The vast majority of sequences detected by molecular genetic methods were most closely related to uncultivated organisms, and often fell into groups for which there are currently no cultured representatives (e.g. ANME, JS1, MCG).

Proteobacteria dominated the bacterial community in the sub-surface of Capt. Arutyunov below the SMTZ, according to 16S rRNA gene cloning. Within the *Proteobacteria* the following sub-groups were detected: *Deltaproteobacteria* (41%), *Gammaproteobacteria* (26%), *Epsilonproteobacteria* (17%) and *Alphaproteobacteria* (10%). The only other bacterial group detected was the *Firmicutes*.

ANME-2 16S rRNA gene sequences were present below the SMTZ of the Capt. Arutyunov MV though sequences related to previously identified putative bacterial partners were not present in the corresponding bacterial 16S rRNA gene library.

ANME-1a were the only known AOM-mediating sequences detected in the crater of Meknes MV according to PCR-DGGE analysis and *mcrA* gene cloning. This is the first report of a natural sedimentary ANME population apparently consisting only of ANME-1a, and the first report of a MV sediment environment dominated by ANME-1a. No putative bacterial syntrophic partner was detected by PCR-DGGE 16S rRNA gene analysis but one *dsrA* gene clone was affiliated to a putative AOM SRB, see Table 4.6.

JS1 were an abundant component of the bacterial community in both the Meknes and Porto MV according to PCR-DGGE analysis. This is the first time this as-yet-uncultured group has been reported in such abundance in MV sediment. JS1 were absent from the Capt. Arutyunov sample analysed in this study.

Functional genes (*mcrA* and *dsrA*) associated with methanogenesis/AOM and sulphate-reduction were readily detectable by PCR in Meknes and Porto MV crater sediments, suggesting these processes are important in the MV environment. Further, in some cases the genes' host organism could be identified through sequence comparison, indicating such analyses offer a useful approach to learning more about the organisms responsible, particularly as public sequence repositories grow.

McrA genes obtained from sediment DNA, analysis of 16S rRNA and *mcrA* genes from enrichment cultures, combined with the results of potential methanogenic activity measurements, suggest methylotrophic methanogenesis may be the dominant methane-producing pathway in the MV environment.

Community composition at the phylotype level varied between samples from different MVs, as well as with depth through a given MV, based on the findings of this and other complimentary studies. The vertical changes in community composition often paralleled changes in pore water geochemistry, suggesting pore water geochemistry is an important parameter influencing prokaryotic distribution in MV sediments and/or is a parameter being influenced by the relative distribution of different prokaryotes in these sediments.

At a higher taxonomic level (Class) there are certain groups, i.e. *Deltaproteobacteria*, *Chloroflexi* and ANME-2, which have commonly been reported in MV sediment diversity studies to date. The present study also detected *Deltaproteobacteria* and ANME-2 organisms in several MV sediment samples, though *Chloroflexi* were not detected in any of the samples analysed. However, results suggest that community composition can vary significantly from site to site, and even within a single site. This is likely a reflection of the variable nature of MV environments, which, for example, can differ significantly in terms of eruptive activity, pore water geochemistry (e.g. chloride and sulphate concentrations), fluid flow regime, extent of bioturbation, water depth and methane/high hydrocarbon ratios.

While harbouring some phylogenetic groups 'characteristic' of the deep biosphere, e.g. JS1 and MCG, comparisons to other microbial diversity studies suggest the Gulf of Cadiz mud breccia communities contain organisms adapted to the present

conditions (i.e. that of a near-surface cold seep), e.g. *Epsilonproteobacteria*, *Deltaproteobacteria* and ANME. This suggests the mud breccia sediment has become re-colonized over time, and hence any original deep-biosphere derived community has become partly over-printed by one adapted to the near-surface seep sediment conditions. Therefore, the question of whether mud volcanoes offer a more accessible 'window to the deep biosphere' would best be investigated using, as far as possible, freshly extruded sediment.

Pore water chloride concentration consistently decreased with depth through the Meknes and Porto MVs. This was presumably a result of dilution by upward advecting fresher fluids sourced from depths where diagenetic mineral dehydration reactions were taking place.

Microscope observations made during AODC analysis showed varied cell morphologies are present in MV environments, these included long and short rods, cocci and rods forming long cell chains. Sometimes the latter long cell chains were encased in an outer sheath of unknown composition, an observation not known to have been previously reported in other marine sediments (Figs 4.3 and 4.4).

Sub-surface MV crater sediments are a source of novel, culturable organisms. Enrichments of sediment from the Meknes MV contained *Bacteria* belonging to *Bacteroidetes, Fusobacteria, Firmicutes, Spirochaetes, Desulfobulbaceae* and *Desulfovibrio*, and *Archaea* belonging to *Methanogenium* and *Methanococcoides*. Pure culture isolates obtained from sediment from Capt. Arutyunov MV included a putative new species of *Arcobacter* named "*Candidatus* Arcobacter subtericola" and species belonging to the genera *Pseudomonas*, *Marinobacter*, and *Halomonas*.

6.3 Microbial diversity in sub-surface Gulf of Cadiz MV sediments and comparisons to other work

A common finding of microbial diversity studies of MV sediments is that *Bacteria* show greater phylogenetic diversity than *Archaea* (Martinez *et al.*, 2006; Niemann *et al.*, 2006b; Heijs *et al.*, 2007; 2008; Kormas *et al.*, 2008) and this was supported by

the findings of the present study. A further similarity to these other studies was that the majority of sequences identified were not closely related (≤ 97% sequence similarity) to cultured, physiologically characterised, organisms, with significant numbers of the sequences detected belonging to phylogenetic groups for which no physiological data is available. This is a common finding in many environmental diversity studies and frustrates attempts to use phylogeny as an indicator of prokaryotic function.

For comparative purposes, results from published 16S rRNA gene library prokaryotic diversity studies of marine MV environments were compiled along with the results from Capt. Arutyunov from this study (Table 6.1, *Bacteria* and Table 6.2, *Archaea*). The available literature data were all from near-surface MV sediments (<40 cmbsf), which highlights the current under-sampling of deeper sub-surface marine MV sediments, as targeted in the present study. While it is necessary to acknowledge that the studies referenced used different methodological approaches (e.g. different sample handling methods, different nucleic acid extraction protocols and/or different domain-specific 16S rRNA gene PCR primers), qualitative comparisons can be made. The results show that while prokaryotic communities in marine MV sediments can vary greatly in their composition at the phylotype level on a fine (i.e. decimetre) scale both between and within individual sites (i.e. along a depth profile of the same sediment core), a limited number of groups at the taxonomic level of Class appear to dominate these environments, particularly *Deltaproteobacteria, Chloroflexi* and ANME-2 (see Tables 6.1 and 6.2).

The bacterial community detected in Capt. Arutyunov MV by 16S rRNA gene cloning in the present study was dominated by proteobacterial sequences from within the Delta- (41%), Gamma- (26%), Epsilon- (17%) and Alpha- (10%) sub-groups. The only other phylogenetic group detected was the *Firmicutes*. This differed from the community composition in the bacterial 16S rRNA library from the SMTZ of Capt. Arutyunov reported by Niemann *et al.*, (2006b), which was dominated by sequences affiliated with the AOM process, shown through *ex situ* radio-tracer studies to be occurring at this horizon. In the SMTZ library the vast majority (81%) of sequences were related to the Seep-SRB1 group of *Deltaproteobacteria*, identified as the putative syntrophic partners of ANME-1/ANME-2 organisms in AOM-mediating

consortia (Knittel *et al.*, 2003). *Gammaproteobacteria* and other *Deltaproteobacteria* were present as minor components of the library but *Epsilonproteobacteria* were apparently absent and sequences affiliated to *Clostridia* and *Spirochaetes* were detected, though these groups were absent in the library from the present study (Table 6.1).

Such community composition differences can be seen on even smaller scales in MV sediments from the Eastern Mediterranean (Tables 6.1 and 6.2). In the study by Heijs *et al.* (2008) each sample harboured a different community at the phylotype level irrespective of which MV site or sediment depth it was taken from, though the authors reported that at higher taxonomic levels (taxonomic rank of Class) similar phylogenetic groups were found in the different sediments and that similar depth layers tended to contain similar communities. For example, the *Chloroflexi* group was present in all samples and was a dominant component of the library in 6 out of 11 samples, and *Actinobacteria* dominated the surface sample libraries in 3 of the 4 sites.

In another recently published study, which investigated microbial community composition in two very closely spaced samples (15 and 20 cmbsf) from the Kazan MV in the Eastern Mediterranean, the majority of phylotypes detected were found in both horizons (Kormas *et al.*, 2008; Tables 6.1 and 6.2). Similarly, the study by Martinez *et al.* (2006) of a hypersaline MV in the Gulf of Mexico, which looked at vertical changes in bacterial and archaeal community composition using three very closely spaced near-surface samples (0-2, 6-8 and 10-12 cmbsf), found that on this scale the same phylogenetic groups (taxonomic rank of Class) were present (though the relative abundances changed), and that the same phylotypes were often present in the different samples.

Table 6.1 Comparison of bacterial 16S rRNA gene library results from this and other studies of MV sediments

Reference	This study	Niemann *et al.*, 2006b	Kormas *et al.*, 2008		Heijs *et al.*, 2008											Martinez *et al.*, 2006		
Site name	**Capt. A**	**Capt. A**	**Kazan**		**Kazan**			**Napoli**				**Amsterdam**		**Urania**		**GB425**		
Location	GoC	GoC	EM, AM		EM, AM			EM, OF				EM, AM		EM		GoM		
Water depth	1,320 m	1,320 m	1,673 m		1,673 m			1,910 m				1,995 m		3,342 m		600 m		
Sample depth (cmbsf)	126-270	30-40	15	20	0-6	6-22	22-34	0-8	8-18	18-27	27-28	0-14	14-31	0-10	10-20	0-2	6-8	10-12
Sample characteristics	MB, Below SMTZ	MB, SMTZ	GH, SMTZ	GH, SMTZ	nd	nd	nd	nd	nd	nd	nd	nd	nd	Control site, high carbon		nd	nd	nd
Sulphate (mM)	nd	<15	≤1	≤1	31.4	32.7	3.3	33	30	25.8	19.5	33.3	34.6	31.2	34.4	nd	nd	nd
Chloride (mM)	nd	nd	nd	nd	627	612	377	858	1150	1742	2197	632	633	622	624	95‰	104‰	110‰
Clones sequenced	58	47	69	79	47	43	44	67	51	63	57	54	71	48	66		143[a]	
No. clones in bacterial phylogenetic groups																		
Acidobacteria	-	-	-	-	5 (4)	1 (1)	-	1 (1)	-	-	-	4 (4)	1 (1)	4 (4)	-	-	-	-
Actinobacteria	-	-	3 (1)	4 (1)	4 (2)	9 (3)	-	3 (1)	2 (2)	-	1 (1)	13 (8)	6 (4)	3 (2)	-	12% (3)	10% (1)	9% (2)
Alphaproteobacteria	6 (1)	-	-	-	-	2 (2)	-	3 (3)	1 (1)	-	11 (2)	-	1 (1)	11 (8)	28 (2)	9% (1)	27% (1)	-
Bacilli	-	-	-	-	1 (1)	-	-	-	-	2 (2)	10 (6)	-	-	2 (2)	10 (5)	-	-	-
Betaproteobacteria	-	-	-	-	-	-		-	-	2 (1)	11 (2)	-	3 (2)	1 (1)	13 (1)	-	-	-
CFB-group	-	-	-	-	-	-		-	1 (1)	2 (1)	3 (1)	1 (1)	-	1 (1)	-	7% (1)	1.5% (1)	14% (1)
Chloroflexi (GNS)	-	-	-	-	5 (5)	7 (1)	17 (3)	13 (10)	5 (3)	5 (3)	14 (8)	13 (9)	12 (8)	13 (8)	6 (6)	-	4% (1)	-
Clostridia	-	1 (1)	-	-	1 (1)	1 (1)	-	-	-	-	-	-	-	-	-	-	-	-
Deltaproteobacteria	24 (2)	40 (3)	40 (2)	42 (2)	-	3 (3)	8 (3)	5 (5)	4 (4)	7 (5)	2 (1)	9 (8)	16 (10)	5 (3)	1 (1)	1.5% (1)	8% (2)	28% (4)
Epsilonproteobacteria	10 (2)	-	-	-	-	-	-	1 (1)	-	-	-	1 (1)	-	2 (2)	-	65% (4)	2% (1)	30% (2)
Firmicutes	3 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.5% (1)	-
Fusobacteria	-	-	-	-	-	-	-	-	-	-	-	-	1 (1)	-	-	-	-	-
Gammaproteobacteria	15 (9)	1 (1)	-	-	4 (3)	3 (3)	-	2 (2)	3 (2)	-	-	8 (6)	4 (4)	2 (1)	-	4% (3)	6% (2)	19% (3)
Nitrospira	-	-	-	-	2 (2)	2 (1)	-	-	-	-	-	-	-	2 (2)	-	-	-	-
Planctomycetes	-	-	-	-	4 (3)	1 (1)	2 (2)	16 (7)	8 (8)	11 (10)	2 (2)	4 (3)	7 (7)	1 (1)	3 (3)	1.5% (1)	-	-
Spirochetes	-	2 (2)	5 (1)	2 (1)	-	-	-	-	-	-	-	-	1 (1)	-	-	-	-	-
JS1 candidate division[b]	-	-	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-	-	-
WS3 candidate division	-	-	16 (1)	25 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OP11 candidate division	-	-	5 (1)	6 (1)	2 (2)	-	3 (3)	1 (1)	1 (1)	1 (1)	1 (1)	-	2 (1)	-	3 (1)	-	-	-
Other/unclassified bacteria	-	3	-	-	9	4	4	4	6	13	2	1	17	1	2	-	-	-

Dominant and second most dominant phylogenetic group in library.

MB – Mud breccia; Below SMTZ – below sulphate-methane transition zone; SMTZ – sulphate-methane transition zone; GH – Gas hydrate present.

GoC – Gulf of Cadiz; EM - Eastern Mediterranean; OF – Olimpi Field; AM – Anaximander Mountains; GoM – Gulf of Mexico.

Numbers in brackets show number of individual phylotypes within the different phylogenetic groups.

[a]Total number of clones sequenced across the 3 16S rRNA libraries, numbers of phylotypes were determined from Fig 2 and percentage values were estimates extracted from Fig 4 in the Martinez *et al.*, 2006 publication. N.B. The data shown from Martinez *et al.*, is for their DNA (not RNA) results only.

[b]N.B. Niemann *et al.*, 2006b and Heijs *et al.*, 2008 included JS1 candidate division sequences with 'unclassified bacteria' in their classification of clone sequences.

Table 6.2 Comparison of archaeal 16S rRNA gene library results from this and other studies of MV sediments

Reference		This study	Niemann *et al.*, 2006b	Kormas *et al.*, 2008		Heijs *et al.*, 2008										Martinez *et al.*, 2006		
Site name		**Capt. A**	**Capt. A**	**Kazan**		**Kazan**			**Napoli**				**Amsterdam**		**Urania**		**GB425**		
Location		GoC	GoC	EM, AM		EM, AM			EM, OF				EM, AM		EM		GoM		
Water depth		1,320 m	1,320 m	1,673 m		1,673 m			1,910 m				1,995 m		3,342 m		600 m		
Sample depth (cmbsf)		126-270	30-40	15	20	0-6	6-22	22-34	0-8	8-18	18-27	27-28	0-14	14-31	0-10	10-20	0-2	6-8	10-12
Sample characteristics		MB, Below SMTZ	MB, SMTZ	GH, SMTZ	GH, SMTZ	nd	nd	nd	nd	nd	nd	nd	nd	nd	Control site, high carbon		nd	nd	nd
Sulphate (mM)		nd	<15	≤1	≤1	31.4	32.7	3.3	33	30	25.8	19.5	33.3	34.6	31.2	34.4	nd	nd	nd
Chloride (mM)		nd	nd	nd	nd	627	612	377	858	1150	1742	2197	632	633	622	624	95‰	104‰	110‰
Clones sequenced		39	39	43	49	47	46	51	32	46	37	10	49	29	54	33		134[a]	
No. clones in archaeal phylogenetic groups																			
Euryarchaeota	AMNE-1	-	7 (1)	-	-	-	-	-	-	-	-	-	-	7 (2)	-	-	-	-	-
	ANME-2	1 (1)	24 (2)	2 (1)	49 (4)	-	16 (2)	5 (1)	-	-	-	-	-	1 (1)	-	-	2% (1)	1% (1)	6% (1)
	Marine Benthic Group D	-	3 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	1% (1)	9% (2)	6% (2)
	Methanogens	1 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Halobacteriales-related	-	-	-	-	1 (1)	3 (1)	2 (2)	-	2 (1)	3 (2)	3 (3)	-	-	-	5 (3)	-	-	-
	Thermoplasmatales-related	-	-	-	-	-	1 (1)	2 (1)	7 (6)	14 (7)	3 (1)	3	3 (2)	2 (1)	-	-	-	-	-
	Unclassified *Euryarchaeota*	-	4 (2)	-	-	-	4 (2)	-	6 (4)	8 (3)	8 (5)	-	21 (5)	3 (3)	-	3 (1)	3% (1)	-	10% (1)
Crenarchaeota	Marine Group I	-	-	-	-	6 (1)	3 (2)	-	19 (8)	6 (4)	3 (2)	-	5 (3)	-	54 (15)	-	-	3% (1)	-
	Marine Benthic Group B	-	1 (1)	-	-	-	19 (12)	2 (2)	-	16 (9)	-	-	-	-	-	5 (3)	14% (1)	7% (1)	8% (1)
	Miscellaneous Crenarchaeota Group	17 (3)	-	-	-												-	-	-

Dominant and second most dominant phylogenetic group in library.

MB – Mud breccia; Below SMTZ – below sulphate-methane transition zone; SMTZ – sulphate-methane transition zone; GH – Gas hydrate present.

GoC – Gulf of Cadiz; EM - Eastern Mediterranean; OF – Olimpi Field; AM – Anaximander Mountains; GoM – Gulf of Mexico.

Numbers in brackets show number of individual phylotypes within the different phylogenetic groups.

[a]Total number of clones sequenced across the 3 16S rRNA libraries, numbers of phylotypes were determined from Fig 3 and percentage values were estimates extracted from Fig 4 in the Martinez *et al.*, 2006 publication. N.B. The data shown from Martinez *et al.*, is for their DNA (not RNA) results only.

N.B. Heijs *et al.*, 2008 did not distinguish between Marine Benthic Group B and the Miscellaneous Crenarchaeota Group.

Examining the results for the *Archaea* (Table 6.2) shows that the anaerobic methane-oxidizing ANME-2 group has been the dominant archaeal group found in MV sediments to date, including the Capt. Arutyunov sample investigated in the present study. It should be noted however that significant phylogenetic variation (minimum of ~87% sequence similarity) exists within this group (Orphan *et al.*, 2001), and therefore several distinct phylotypes, and potentially ecotypes, are sometimes present in a library. Interestingly, PCR-DGGE and *mcrA* gene library results from the Meknes MV contradicted this trend, with only sequences affiliated with ANME-1 being detected (see chapter 4). ANME-1 sequences have not been reported to dominate the archaeal community of MV sediments before, though they have sometimes been present as minor components (Niemann *et al.*, 2006b; Heijs *et al.*, 2008; Table 6.2). Other archaeal groups detected in 16S rRNA gene libraries from MV environments were most often groups without any cultured relatives and for which ecophysiology has yet to be conclusively determined, e.g. Marine Group 1 and Marine Benthic Group B (Table 6.2).

It is not clear what determines variation in community composition at these scales in MV environments, but as sampling increases, we learn more about the distribution and function(s) of different phylogenetic groups, and complimentary data on geochemistry and lithology are made available, it should be possible to determine to what extent community compositions are consistent with prevailing local conditions (e.g. geographical location, pore water chemistry, sediment type, electron donor type/concentration), or are simply the result of stochastic events. For example, previously it has been suggested that the distribution of certain phylogenetic groups in a given sediment core follows changes in sediment type, when geochemical conditions are broadly consistent (e.g. Inagaki *et al.*, 2003; Webster *et al.*, 2007). The PCR-DGGE based survey of shallow, sub-surface tidal flat sediment cores by Webster *et al.* (2007) found *Chloroflexi* dominated deeper sandy layers of one core, whereas JS1 was dominant in the deeper muddy layers of another, when analysed with non-specific JS1 targeted 16S rRNA gene primers. Broadly similar geochemical conditions led the authors to suggest JS1 may have been better adapted to the small grain size and associated small pore size in the mud, and therefore able to out-compete *Chloroflexi* in these layers. A similar attempt to rationalize the difference in relative abundance of the ANME-1 and ANME-2 groups in seep environments has

been made by Knittel *et al.* (2005). ANME-2 were reported to dominate in surface sediments of Hydrate Ridge (>90% total biomass), with ANME-1 increasing with depth in the underlying sediment layers, whereas in the Black Sea ANME-1 accounted for ~50% of cells in surface microbial mats, and ANME-2 were restricted to microenvironments within the mats and accounted for <1% of total cells (Knittel *et al.*, 2005). The authors suggested oxygen concentrations might have influenced which group came to dominate, with the ANME-1 seemingly the more sensitive to oxygen (not present in the anoxic bottom waters of the Black Sea). However, based on 16S rRNA clone library results, ANME-2 has been found to dominate over ANME-1 in environments where oxygen penetration is unlikely, for example the sub-surface of Capt. Arutyunov MV as investigated in this study and by Niemann *et al.* (2006b), so to what extent this parameter alone can explain their relative distribution and abundance is uncertain. Community compositions may be influenced to some extent by stochastic events, which, for example, may restrict the population from which the environment ultimately selects. This may explain why in some instances different organisms with similar ecophysiologies perform the same key environmental functions in different samples, e.g. the different anaerobic methane oxidising ANME groups in the sub-surface sediments of different MVs. Results of this study show the distribution of dominant prokaryotic groups in MV sediments parallels changes in pore water geochemistry, and hence pore water geochemistry is a key parameter influencing and/or being influenced by the distribution of phylotypes in the marine MV environment.

It was not possible to analyse sediment from Bonjardim MV by 16S rRNA gene cloning due to a lack of good-quality amplifiable DNA. Furthermore, the sediments of Meknes and Porto MVs were investigated using the alternative techniques of PCR-DGGE profiling to provide a more high throughput method and allow the analysis of a larger number of samples. Analysis of prokaryotic diversity by PCR-DGGE along a depth profile in Meknes and Porto MV allowed for an initial assessment of how community structure varies with depth through a MV's crater. In Meknes, the greatest number of bands, i.e. putative bacterial phylotypes, were detected in the near-surface sample (10 cmbsf) and decreased with depth (Fig 4.8). Interestingly, each of the three depths studied had a dominant band in their respective DGGE profile, a proxy by which abundance in the source environment can be inferred. Re-amplification and

sequencing showed this belonged to a member of the *Epsilonproteobacteria* in the upper-most sample (10 cmbsf) and to a member of the candidate division JS1 in the lower two horizons (50 and 65 cmbsf, see Table 4.2). These differences in dominant bacterial 16S rRNA genes paralleled differences in pore water chemistry; in the upper horizon where *Epsilonproteobacteria* appeared to dominate, sulphate was still present at sea water concentrations and acetate values were low, but sulphate had dropped to micro-molar concentrations and acetate concentration increased where JS1 appeared to dominate (Fig 4.5). Archaeal 16S rRNA genes also varied within the upper and two lowermost horizons of Meknes MV. In the near-surface Marine Benthic Group D appeared to dominate, whereas in the two lowermost horizons ANME-1 organisms were dominant (Fig 4.11 and Table 4.3). In Porto MV an assessment of community structure through a depth profile could not be made as 16S rRNA genes were only amplifiable from the 50 cmbsf sample (*Bacteria*), and 10 and 50 cmbsf sample (*Archaea*), respectively. The dominant bacterial species at 50 cmbsf in Porto was a member of JS1, though it was phylogenetically distinct (96% sequence similarity) from the sequence detected in Meknes. The dominant archaeal sequences belonged to Marine Group I in both the horizons studies, though the DGGE profile (Fig 4.11) suggested other archaeal species were present in the lower of the two horizons, where total cell counts (Fig 4.1) and DNA concentrations (Fig 4.6) were also slightly higher. This was confirmed by later analysis of functional genes (see below). The apparent dominance of a single bacterial and archaeal phylotype in the Meknes and Porto MV samples meant identifying other subsidiary species was not possible by re-amplification of the additional DGGE bands, due to interference from co-migration of DNA from the dominant species.

McrA and *dsrA* functional genes were detected by PCR at all depths through the Meknes crater, and at 50 cmbsf (*mcrA*) and 10, 50 and 110 cmbsf (*dsrA*) in the Porto crater (Table 4.4), suggesting these MVs are sites of active methanogenesis and/or AOM and sulphate reduction. Some additional horizons outside the Meknes MV crater also contained functional genes (*mcrA* detected at 110 cmbsf of Meknes slope and *dsrA* detected at all depths in the Meknes slope, and in the 10 cmbsf sample from the Meknes reference site) suggesting these processes are also ongoing in these sediments. Sequencing of *mcrA* DGGE bands identified them as belonging to a novel *mcrA* group in the Meknes slope, to ANME-1a at 50 and 65 cm depth in Meknes

crater, and to *mcrA* group e of the *Methanosarcinales*, most likely representing ANME-2a (Nunoura *et al.*, 2006; 2008; Lösekann *et al.*, 2007), at 50 cm depth in Porto MV (Table 4.5). The Meknes crater 10 cmbsf *mcrA* sequences remained unidentified due to a mixed sequence signal. Cloning *mcrA* genes from selected horizons (110 cmbsf of Meknes slope, 50 cmbsf of Meknes crater and 50 cmbsf of Porto crater) confirmed the findings of the DGGE sequence analysis, and further detected some *Methanosarcinales* (*Methanococcoides*) sequences in the two MV craters. These represented 8% and 37% of the Meknes and Porto *mcrA* gene libraries, respectively (Fig 4.14). The study by Kormas *et al.* (2008) investigated *mcrA* genes in sediments of Kazan MV but less than 4% of clones across the two libraries belonged to ANME-1, all others being affiliated to sub-groups of ANME-2. Another analysis of *mcrA* genes in MV sediments from the Haakon Mosby MV by Lösekann *et al.* (2007) detected a small number of sequences (3%) belonging to *mcrA* group e (putatively ANME-2a) with the majority belonging to a novel *mcrA* clade (designated *mcrA* group f), believed to represent ANME-3 *Archaea*. Further, the study of sediments from the terrestrial Paclele Mici MV in the Carpathian Mts, Romania by Alain *et al.* (2006) only detected *mcrA* group e sequences. These publications, and the other results of this study, show that Meknes MV is so far unique as a MV crater harbouring ANME-1a as the only AOM group, as confirmed by both 16S and *mcrA* gene analysis.

A study of hypersaline sediments overlying a brine pool methane seep in a MV region of the Gulf of Mexico (Green Canyon 205) only detected sequences affiliated to ANME-1b when analysed with universal archaeal 16S rRNA gene primers, ANME-2 targeted 16S rRNA gene primers, and *mcrA* gene primers (Lloyd *et al.*, 2006). No ANME-1a sequences were detected. This was reported as the first study to describe a natural sedimentary ANME population consisting of only ANME-1b *Archaea* (Lloyd *et al.*, 2006), and similarly the study reported here is the first study to describe a natural sedimentary ANME population consisting of only ANME-1a *Archaea*. In the Gulf of Mexico sediments the authors suggested the site's unusually high salinity (2,200 mM) was the cause of the limited ANME population, i.e. the sediments contained a high-salt-adapted subpopulation of ANME-1b, which could exist where other ANME groups could not. Interestingly, the two horizons where ANME-1a were detected in Meknes MV had relatively low chloride concentrations (366 and 332 mM,

respectively) compared to normal seawater (550 mM). Whether the presence of ANME-1a in Meknes MV was because of an adaptation of the sub-group to relatively low salt concentrations remains to be confirmed, but suggests relative salinity is a parameter that should be considered when investigating the biogeography of ANME organisms.

Identifying *dsrA* genes from Meknes and Porto MV by DGGE band sequencing was successful for 1 out of the 9 bands re-amplified; 5 of the bands produced poor or mixed sequence signals, and 3 band sequences did not share significant similarity to *dsrA* gene sequences currently held in public databases despite excellent sequence quality. The successfully sequenced band was from 50 cm depth in the Meknes slope and was related (95%) to an unclassified *dsrA* gene clone (Ntd-I09) from deep-sea sediment in the Nankai Trough (Kaneko *et al.*, 2007). *DsrA* gene libraries were constructed at the same depths as used for *mcrA* gene libraries (110 cmbsf of Meknes slope, 50 cmbsf of Meknes crater and 50 cmbsf of Porto crater) as an alternative technique to identify the *dsrA* phylotypes present. At 110 cmbsf of Meknes slope only one phylotype was present in the library and it shared 95% sequence similarity to a clone from the Colne Estuary (Table 4.6). At 50 cmbsf in Meknes crater 2 phylotypes were present: one most closely related to a clone from the SMTZ in Black Sea sediment, and one most closely related to a clone from marine sediment in Aarhus Bay (see Table 4.6). As this was the horizon where ANME-1a sequences were detected, it is noteworthy that neither of these were affiliated with SRB currently recognised as syntrophs with ANME consortia. However, only 1 out of 58 *dsrA* clones detected in hypersaline Gulf of Mexico sediments harbouring ANME-1b sequences belonged to putative AOM-consortia associated SRB (Lloyd *et al.*, 2006), and it has been suggested that ANME-1 may be capable of mediating AOM without a bacterial partner in some situations (Sørensen *et al.*, 2001; Orphan *et al.*, 2002; Orcutt *et al.*, 2005; Treude *et al.*, 2005; 2007).

The greatest diversity in *dsrA* genes was found in the 50 cm depth sample of Porto. Here 6 different phylotypes (OTUs) were detected and comparative sequence analysis suggested these represented several different groups at the taxonomic rank of Class, though sequence similarity to described organisms was low (Table 4.6). This is the first study to report on the distribution and identity of *dsrA* genes in sediment from

MV craters. The only other similar study published to date is the investigation of *dsrA* genes in hypersaline sediments overlying a brine pool methane seep in an area associated with mud volcanism by Lloyd *et al.* (2006). These authors found *dsrA* genes to be more diverse than *mcrA* genes in the three sediment depths analysed, and that the majority of *dsrA* clones were affiliated with hydrocarbon degraders, which was congruous with there being petroleum in the source sediments, though novel groups with no close cultured relatives were also present in significant abundance (Lloyd *et al.*, 2006).

The abundance of JS1 sequences in sub-surface sediments from the Meknes and Porto MV craters (detected using universal bacterial 16S rRNA primers) was not expected, as this group has not been reported as a dominant component of MV sediments before (Table 6.1). However, it has been present as a minor component in some libraries, albeit categorised in these studies as 'unclassified *Bacteria*' (Niemann *et al.*, 2006b; Heijs *et al.*, 2008). The problem of mis- or non-identification of candidate groups such as JS1 continues to hinder efforts to learn more about a groups' range of habitat types and biogeography etc, through the comparison of published studies. The horizons in the two MVs where JS1 apparently dominated were geochemically distinct with respect to relative sulphate and acetate concentrations, which was consistent with the sequences being phylogenetically different. Such phylogenetic, and, by inference, potential physiological differences between members of the JS1 candidate division may help explain the cosmopolitan distribution of this group within different environmental sediment types (see Fry *et al.*, 2008; Webster *et al.*, 2004).

6.4 MV sediments as 'windows to the deep biosphere'

The fact that MVs are sites of sediment, fluid and gas explosion make them a logical potential target for attempting to sample deep-sourced subseafloor biosphere organisms in a more accessible environment. However, it is necessary to consider two points: firstly, the timing of emplacement of a given sediment package within a MV is usually poorly constrained, and therefore how long it may have had to 'equilibrate' to a near-surface adapted community is unknown; and secondly, there are no clear

criteria by which you could distinguish any detected organisms as being deep biosphere-derived.

A recent review of prokaryotic diversity studies from ODP and other deep sediment cores by Fry *et al.* (2008) shows that deep subseafloor biosphere sediments harbour a broadly diverse prokaryotic population with great variability in composition between, and often within, sites. While some phylogenetic groups have been described as 'characteristic' of the deep marine biosphere (e.g. JS1, *Chloroflexi*, *Gammaproteobacteria*, Miscellaneous Crenarchaeotic Group and Marine Benthic Group B), these same groups are also found in other habitats [shallow sedimentary, aquatic and/or terrestrial, e.g. see Teske & Sørensen (2008) for a review of the distribution of the archaeal groups], and therefore their presence in a sample cannot be used as a conclusive indication of a deep marine biosphere source. Further, given that the deep sediment 16S rRNA based diversity studies conducted to date are yet to detect any taxa that can be said to be truly unique to the deep biosphere, it is difficult to assess the source of a prokaryotic population based on 16S rRNA phylogeny alone. Thus, this particular question is difficult to answer with the data currently available from this and other complementary studies. The sediments analysed during the present study contained phylotypes associated with seep environments (e.g. those belonging to *Epsilonproteobacteria*, *Deltaproteobacteria* and ANME) as well as those that have been found to be abundant in deep sediment cores (e.g. those belonging to JS1 and MCG), suggesting the community detectable in the deep-sourced mud breccia today has adapted to the present *in situ* conditions over time. 16S rRNA gene sequences related to obligate, anaerobic thermophiles (and/or piezophiles) may suggest provenance from the deeper sub-surface biosphere, and *Thermoplasmatales*-related sequences, indicative of thermophiles (including acidophiles) were major components of 16S rRNA libraries from certain MV samples from the Eastern Mediterranean (Heijs *et* al., 2008; Table 6.2). This perhaps provides preliminary circumstantial evidence that MVs can indeed transport detectable microorganisms from the deep warm sub-surface to the cooler near-surface.

A phenotypic assessment of isolates from MV sediments, particularly in terms of their adaptation to elevated temperatures and pressures, would be instructive as to whether viable microorganisms sourced from deeper depths were present in a MV

environment. Such a study would be best conducted with (as far as possible) freshly extruded sediment, though practically it would be severely impeded by the current very limited ability (generally <0.1%) to successfully cultivate deep biosphere microorganisms (e.g. Cragg *et al.*, 1992; Wellsbury *et al.*, 2002; Toffin *et al.*, 2004; D'Hondt *et al.*, 2004), and further by the logistical difficulties with determining physiological ranges and optima at high temperatures and pressures. However, it is hoped with technological advances that allow manipulation of sediments and enrichments without depressurization, e.g. HYACINTH coupled to the Deep IsoBUG system (Schultheiss *et al.*, 2006; Parkes *et al.*, 2009 *submitted*) these barriers will be lowered in the future. The MV isolates obtained during the present study were not isolated at elevated temperatures, though these were attempted for Capt. Arutyunov and Bonjardim sediments (see chapter 3), but generally had a broad temperature tolerance, i.e. growth up to ~40°C, which is equivalent to ~1.3 km depth based on the 30°C/km geothermal gradient for this area of the Gulf of Cadiz (Casas *et al.*, 2003). However, in the majority of cases this temperature tolerance was also shared by the isolates' phylogenetic neighbours, despite their source from temperate environments where an adaptation to elevated temperatures would most likely not be tested (see chapter 5). A tolerance to elevated pressures was not tested with the isolates.

Building on the PCR based analysis and cultivation attempts reported here, additional techniques may help address this question in the future. For example, recent metagenomic evidence from Peru Margin ODP sediments suggests subseafloor sediment derived communities are distinct from those in surface pelagic environments, e.g. the authors reported a decrease in genes for locomotion and cell communication with depth in metagenomes from successive depths in ODP Site 1229 (Biddle *et al.*, 2008), and therefore metagenomic analysis may provide a tool with which to discriminate a deep sediment derived community in a MV environment in the future.

A further consideration when assessing to what extent MV sediments can provide a 'window to the deep biosphere', is what is actually meant by the term 'deep biosphere' (a.k.a. deep marine biosphere and deep marine sub-surface). This term has been examined recently by Teske & Sørensen (2008), and rationalised as "sediment layers with distinct microbial communities that lack a microbial imprint of water

column communities", which would necessarily be locally variable. The authors suggested that sediment layers between this boundary and the sediment-water interface should be considered as the 'shallow sub-surface' and would be characterized by a mix of sediment and water column communities. Other authors have defined the deep subseafloor biosphere as marine sediments of >1 mbsf (e.g. Fry *et al.*, 2008) on an operational basis, i.e. that at this depth the habitat is likely to have become isolated from the surface environment, with sediment ages ~1,500 years [e.g. based on an average sedimentation rate of 0.07 cm/yr (Van Green *et al.*, 1997)], though locally variable. Near-surface MV environments, like other types of cold seep, are sites of elevated microbial activity where microbially driven geochemical change happens on a much narrower depth scale than at non-seep sediment locations. As a consequence of this, community composition changes on a fine scale and soon becomes distinct from a typical water column/surface sediment community, e.g. one that would reflect the availability of oxygen, nitrate and other efficient electron acceptors. Using the latter definition, some MV sediments would therefore *be* part of the deep marine biosphere in their own right, though they may not necessarily harbour any deeper sub-surface organisms that may have lived in the sediment at its source depth. However, the *potential* of MVs to harbour deep-sediment derived microorganisms, emplaced to the near-surface through the mechanics of mud expulsion, makes them distinct from environments such as tidal flats, which have also been studied as a more accessible proxy to the deeper subseafloor biosphere (e.g. Wilms *et al.*, 2006a; Webster *et al.*, 2007).

In summary, MVs are interesting microbial habitats where novel culturable organisms are present (see chapters 4 and 5), enigmatic phylogenetic groups (e.g. JS1 MCG and Marine Group 1) can sometimes dominate (see chapters 3 and 4) and important ecophysiological groups (e.g. anaerobic methane oxidisers) exert environmentally relevant geochemical change (Niemann *et al.*, 2006a; Nuzzo *et al.*, 2008). For these reasons alone, MVs are microbial habitats that warrant further investigation. Whether or not MVs can offer 'windows' into deeper sub-surface habitats through the extrusion of sediments hosting deep biosphere-adapted organisms sustained by deep energy sources is probably best examined using samples of freshly extruded sediment that have not had time to equilibrate to their new near-surface position. The sediments analysed during the present study contained phylotypes associated with seep

environments (e.g. those belonging to *Epsilonproteobacteria*, *Deltaproteobacteria* and ANME) as well as those that have been found to be abundant in deep sediment cores (e.g. those belonging to JS1 and MCG), suggesting original mud breccia communities can become overprinted by organisms that are adapted to the current environmental conditions over time.

6.5 Enrichments and isolates

A culture-based survey of Meknes and Porto MV sediments taken at intervals along a depth profile of 10 – 50 cmbsf and 10 – 200 cmbsf, respectively, showed that culturable diversity was highest in the near-surface. This related to a horizon of stimulated microbial activity and increased microbial diversity within mud breccia at the Meknes site, and the hemipelagic sediment layer that overlay extruded mud breccia at the Porto site, (although in the latter case the cell numbers were relatively low and the enriched organisms did not survive into sub-culture). The near-surface layer of the Meknes crater yielded higher cell numbers in the enrichments, and organisms were successfully transferred into sub-culture from 4 separate enrichment types. The four different sets of growth conditions used in the cultivation of Meknes crater organisms showed that for the *Bacteria*, enrichment conditions significantly influenced organisms became dominant. For example, the SRB enriched at 7°C were most closely related to psychrophilic SRB of the *Desulfobulbaceae* isolated from permanently cold arctic marine sediments, whereas the SRB enriched at 25°C in the same medium were most closely related to mesophilic *Desulfovibrio* species (Table 4.8). Furthermore, *Bacteroidetes* and *Fusobacteria* were present in the 7°C yeast extract medium enrichments, whereas when incubated at 25°C these groups were present in addition to members of the *Firmicutes* and *Spirochaetes* (Table 4.8). Enrichment conditions had less of an effect on the archaeal populations present, possibly due to the lower overall diversity and culturability of *Archaea* within these sediments.

Results from Capt. Arutyunov and Bonjardim MV showed that culturability was very low at greater depths (≥125 cmbsf) within MV sediments, away from geochemical interfaces and efficient electron acceptors, although novel organisms were present

(see chapter 5). The majority of the organisms obtained in pure culture (those belonging to *Pseudomonas, Halomonas* and *Marinobacter*) could be considered as generalists with a broad tolerance to changing environmental conditions (e.g. pH, temperature and salinity, see descriptions of strains in chapter 5), which is possibly why they were suited to laboratory culture. However, the other genus cultured – *Arcobacter* – represented an organism with few previously cultured relatives, particularly from environmental samples, and which was only distantly related to its closest cultured relatives (< 93%). Molecular studies have indicated the habitat range of this genus is much wider than the sources of cultured representatives suggest (see section 5.5.1), and the members are implicated in nitrogen and sulphur cycling (Teske *et al.*, 1996; Wirsen *et al.*, 2002; Donachie *et al.*, 2005). The *Arcobacter* isolates were fastidious in nature and more sensitive to changes in environmental parameters compared to the other isolates, as demonstrated by a narrower growth range of pH, temperature and salinity, and a more restricted substrate range (see section 5.5.6).

The isolates obtained from the MV sediments were not phylogenetically related to 16S rRNA gene sequences obtained directly by PCR, which highlights once again how molecular and culture-based analyses of environmental samples can produce fundamentally different results (Amann *et al.*, 1995; Hugenholtz & Pace, 1996; Pace, 1997; Rappe & Giovannoni 2003; Hugenholtz *et al.*, 1998; Stackebrandt & Tindall, 2000 and Breznak, 2002). However, this should not deter efforts in cultivating organisms from environmental samples, as cultures are necessary for establishing new taxa with standing in nomenclature. Further, they are required for metabolic characterization and in establishing optimal, upper and lower physiological limits for growth. This in turn will make clone library data more powerful, i.e. through assigning potential metabolic functions and environmental limits to taxonomic sequences, and thus move us away from the unsatisfactory situation where no physiological data is available for whole phylogenetic groups. Recently, metagenomic studies (e.g. Tyson *et al.*, 2004) have sought to circumvent the issues of non-culturability by amplifying whole genomes directly from environmental DNA in order to, for example, explore which taxonomic and metabolic groups dominate at particular depths. Such studies are an important indicator of the overall metabolic potential of an environment (Tringe *et al.*, 2005), but ideally will link the detected genes to specific groups of microorganisms. However, such analyses rely on

comparison of the environmental metagenome with identified genomic sequences held in databases, and to this end are still constrained to some extent by the limitations in culturing environmental organisms, since pure cultures are still the most useful source of definitively identified genes. For example, in the study by Biddle *et al.* (2008), the authors made a metagenomic analysis of subseafloor sediments of the Peru Margin using whole-genome amplification and pyrosequencing in an attempt to address questions of microbial and metabolic diversity, but were unable to identify up to 85% of the genes from the environment due to a lack of homologous genes in sequence databases.

6.6 Future work

6.6.1 Investigate the relative distribution of ANME groups in Gulf of Cadiz MV sediments

Analysis of sediment from the sub-surface of Capt. Arutyunov by PCR amplification and cloning of 16S rRNA gene sequences only detected AOM mediating *Archaea* of the ANME-2 sub-group (see chapter 3). These organisms were also the dominant archaeal phylotype at the SMTZ of this MV according to Niemann *et al.* (2006b), though ANME-1 organisms were also detected at lower abundance in this horizon (18% of library). In contrast, 16S rRNA gene PCR-DGGE analysis and cloning of *mcrA* gene sequences from Meknes MV sediment only detected AOM mediating *Archaea* of the ANME-1 sub-group (see chapter 4). ANME-1 and ANME-2 are often, but not always, found to co-habit, with one of the groups tending to dominate, e.g. ANME-1 in Black Sea microbial mats and ANME-2 in surface sediments of Hydrate Ridge (Knittel *et al.*, 2005; Nauhaus *et al.*, 2005; Lloyd *et al.*, 2006 and references therein). This is an ecological puzzle since both organisms perform the same environmental function, and the factors that determine the environmental distribution and relative abundance of these groups remain poorly understood (Knittel *et al.*, 2005; Orcutt *et al.*, 2005; Nauhaus *et al.*, 2005; Treude *et al.*, 2007). To what extent the distribution of these organisms is stochastic is not yet clear. If it is confirmed that different MV craters in the Gulf of Cadiz harbour different relative abundances of AOM-mediating phylotypes, as suggested by the results of the present study, closer

inspection of the prevailing environmental conditions (e.g. sediment type, porosity, temperature, pressure, salinity, oxygen concentration, electron acceptor abundance) may offer insights into these important outstanding questions in microbial biodiversity.

6.6.2 Target the JS1 candidate division in Gulf of Cadiz MV sediments, e.g. for metagenomic analysis, enrichment and potential isolation

Organisms belonging to the JS1 candidate division (Webster *et al.*, 2004) were identified as the dominant bacterial phylotype in both the Meknes and Porto MV crater sub-surface sediment by 16S rRNA PCR-DGGE analysis, with the sequence similarity between the phylotypes from the two sites measured at 96% across ~180 bp (see chapter 4). Organisms belonging to the candidate division JS1 were dominant in a number of 16S rRNA gene libraries from the deep marine sub-surface (Rochelle *et al.*, 1994; Inagaki *et al.*, 2003; Kormas *et al.*, 2003; Newberry *et al.*, 2004; Webster *et al.*, 2006a; Parkes *et al.*, 2007), methane hydrate and cold seep sediments (Li *et al.*, 1999; Reed *et al.*, 2002; 2006; Inagaki *et al.*, 2006), shallow hydrothermal marine sediments (Teske *et al.*, 2002) and tidal flat sediments (Webster *et al.*, 2004; 2006b; 2007), and they were previously detected in other MV sediments (Kazan MV, Heijs *et al.*, 2007; Capt. Arutyunov, Niemann *et al.*, 2006b), though not at the apparent abundance detected in the Meknes and Porto craters. The horizons where JS1 dominated in the Meknes and Porto MVs were subject to significantly different prevailing geochemical conditions in terms of relative sulphate concentration, and to a lesser extent acetate concentration. This suggests the group can thrive in different biogeochemical niches and may help explain its cosmopolitan distribution. The biogeochemical role(s) of the group is yet to be determined, however a recent stable-isotope probing study of enrichments including JS1 organisms suggested the group could incorporate acetate and glucose (or glucose metabolites) under anaerobic low SO_4^{2-} sulphate-reducing conditions (Webster *et al.*, 2006b). It is suggested that relatively easily accessible sediments where JS1 appear to dominate the bacterial community, such as in the 50 cmbsf horizon of Porto MV and the 50–65 cmbsf horizon of Meknes MV, would make ideal samples with which to further study the interesting JS1 candidate division, which represents a deeply branching bacterial lineage typical of, but not restricted to, the marine sub-surface (Teske, 2006), for

which there are currently no cultured representatives. Specific techniques employed to this end could include direct cell capture of JS1 via JS1 targeted probes and magneto-FISH; a method of selectively capturing microorganisms from the environment using combined fluorescence *in situ* hybridization and immunomagnetic cell capture (Pernthaler *et al.*, 2008), followed by genome-directed isolation of JS1 (Tyson *et al.*, 2005b) from the enriched sample. Even without successful isolation, a metagenomic study of a JS1 enriched sample, would likely reveal new insights into the metabolic potential of this organism.

6.6.3 Investigate the importance of methylotrophic methanogenesis in the MV environment, and pursue isolation of the organisms responsible

Methanococcoides sp. were successfully enriched from Meknes MV sediment sampled from 10 cmbsf (section 4.4.2), *mcrA* genes believed to belong to *Methanococcoides* sp. were detected in the 50 cmbsf samples from Meknes and Porto MV (section 4.3.9), and a *Methanococcoides* sp. was the only known methanogen identified by 16S rRNA gene cloning analysis of the Capt. Arutyunov MV sediment (section 3.2.5). Furthermore, unpublished work showed that methanol and methylamine additions to sediments from a range of Gulf of Cadiz MVs consistently resulted in stimulation of CH_4 production (Cragg and Parkes, unpublished results). Analysis of 16S rRNA genes showed that *Methanococcoides* sp. was the dominant methanogen in these enrichments. This archaeon has been successfully enriched and is in the process of being purified. In comparison, stimulation of CH_4 production by the supposedly more environmentally important methanogenic substrates H_2/CO_2 and acetate was more limited and fewer enrichments were obtained. This poses the interesting question as to if methyl-substrates are more important for CH_4 production in MV environments. The relative importance of methylotrophic methanogenesis in the marine environment remains largely untested and may be currently underestimated owing to the assumption that methyl compounds are in low abundance and are of relative insignificance compared to other methanogenic substrates (Reeburgh, 2007). However, the results presented here suggest it may be of significance, at least in certain environments such as MV craters, and therefore warrants further investigation. The fact that compounds used in methylotrophic methanogenesis are non-competitive makes the methylotrophic methanogenic

pathway and methylotrophic methanogens of potential importance in environments where sulphate is present such as within and above the SMTZ of MV craters. Targeted enrichment and attempted cultivation of methanogens from MV environments (e.g. following on from the work presented in chapter 4), in addition to conducting radio-tracer studies with methylotrophic methanogenic substrates, would help explore the significance this process, and would increase our knowledge of ocean-sediment derived methanogens, which currently represent a very small proportion (<10%) of the cultured methanogenic community.

6.6.4 Pursue isolation of other MV organisms from the successful Meknes enrichments

In addition to methanogens, organisms identified as belonging to other functional groups of interest (fermenters and SRB) were also successfully enriched from the crater of Meknes MV, and were maintained over 4 subsequent sub-cultures but were not pursued into pure culture due to time constraints (see chapter 4). Both the fermenters and SRB present in the enrichments included organisms with low (≤ 95%) 16S rRNA gene sequence similarity to previously characterised organisms and therefore may represent novel species.

6.6.5 Complete necessary data to enable the submission of "*Candidatus* Arcobacter subtericola" as a novel bacterial species

Among the pure cultures isolated from the Capt Arutyunov MV sediment (see chapter 5) was a candidate for a novel species of *Epsilonproteobacteria* named "*Candidatus* Arcobacter subtericola". The species description presented in section 5.5 is almost of sufficient detail to be the basis of a novel species description paper. However, crucially DNA/DNA hybridization need to be conducted for "*Candidatus* Arcobacter subtericola" and its nearest neighbours according to 16S rRNA gene analysis, and it is therefore suggested that this, as a minimum, should be completed so that such a submission can be made. It would also be instructive to conduct a polyphasic taxonomic investigation of other cultured free-living environmental *Arcobacters*, as previously these have often been examined using phenotypic tests more suited to their clinical and veterinary counterparts.

Appendix I

Trace element recipe

Chelated trace element solution contained the following per litre (final conc $\mu M\ l^{-1}$ in media): Nitrilotriacetic acid, 6 g (78); $FeSO_4.7H_2O$, 555 mg (5); Na_2SeO_3, 2 mg (0.4); $CoCl_2.6H_2O$, 380 mg (4); $MnSO_4.4H_2O$, 2.7 g (30); $Na_2MoO_4.2H_2O$, 38 mg (0.1); $Na_2WO_4.2H_2O$, 3 mg (0.02); $ZnSO_4.7H_2O$, 575 mg (5); $AlCl_3.6H_2O$, 48 mg (0.5); $NiCl_2.6H_2O$, 10 mg (0.02); H_3BO_3, 50 mg (2); $CuCl_2.2H_2O$, 5 mg (0.1). The nitrilotriacetic acid was dissolved in 800ml of MilliQ water and the pH adjusted to 6.5 with KOH. The reagents were added in the order shown, then the volume made up to 1 litre and the final pH adjusted to 7. Individual aliquots were dispensed before autoclaving at 121°C for 20 min.

Vitamin solution recipe

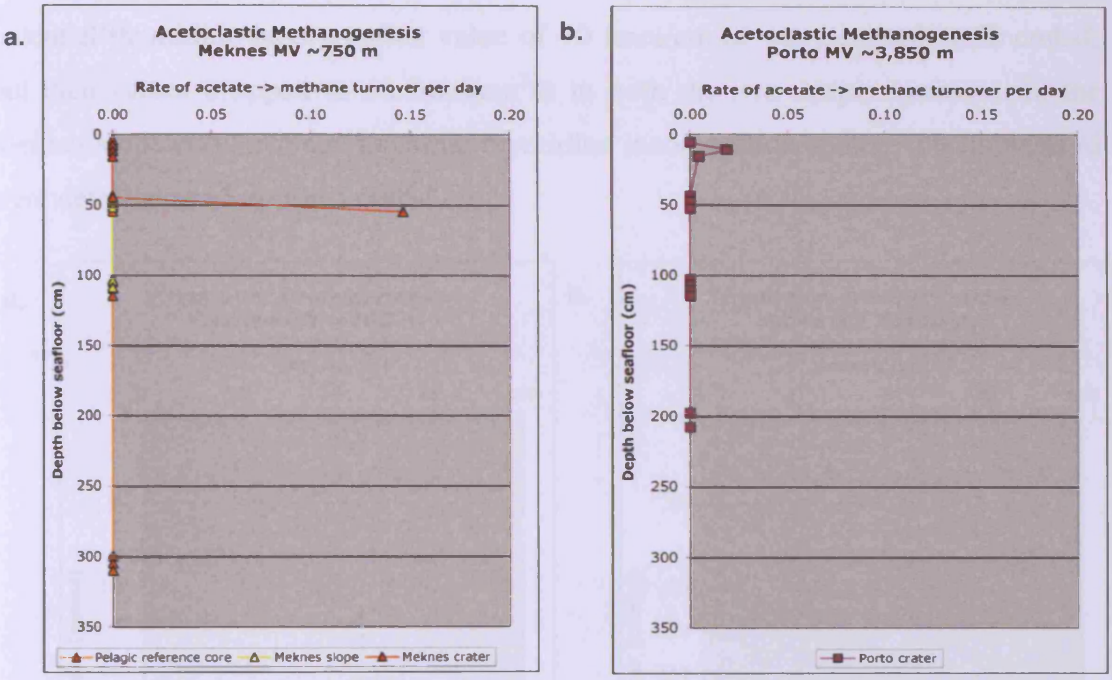
Vitamin solution contained the following per litre (final conc. $\mu M\ l^{-1}$ in media): Biotin, 20 mg (0.2); Folic acid, 9 mg (0.05); Pyridoxine HCl, 40 mg (0.5); Thiamine HCl, 70 mg (0.5); Riboflavin, 45 mg (0.1); Nicotinic acid, 40 mg (0.75); Calcium pantothenate, 55 mg (0.25); Vitamin B_{12}, 28 mg (0.05); Aminobenzoic acid, 30 mg (0.5) and Lipoic acid, 20 mg (0.25). Compounds were dissolved in MilliQ water and sterilized by filtration (0.2 μm). Individual stock aliquots were dispensed and stored in the dark at 4°C.

Appendix II

Potential activity measurements

The results of the radiotracer activity measurements are presented, described and discussed below. Relevant methods are described in section 2.2.3.

The potential for acetoclastic methanogenesis in the Meknes and Porto MV samples was very limited (Fig AII.1a and AII.1b).

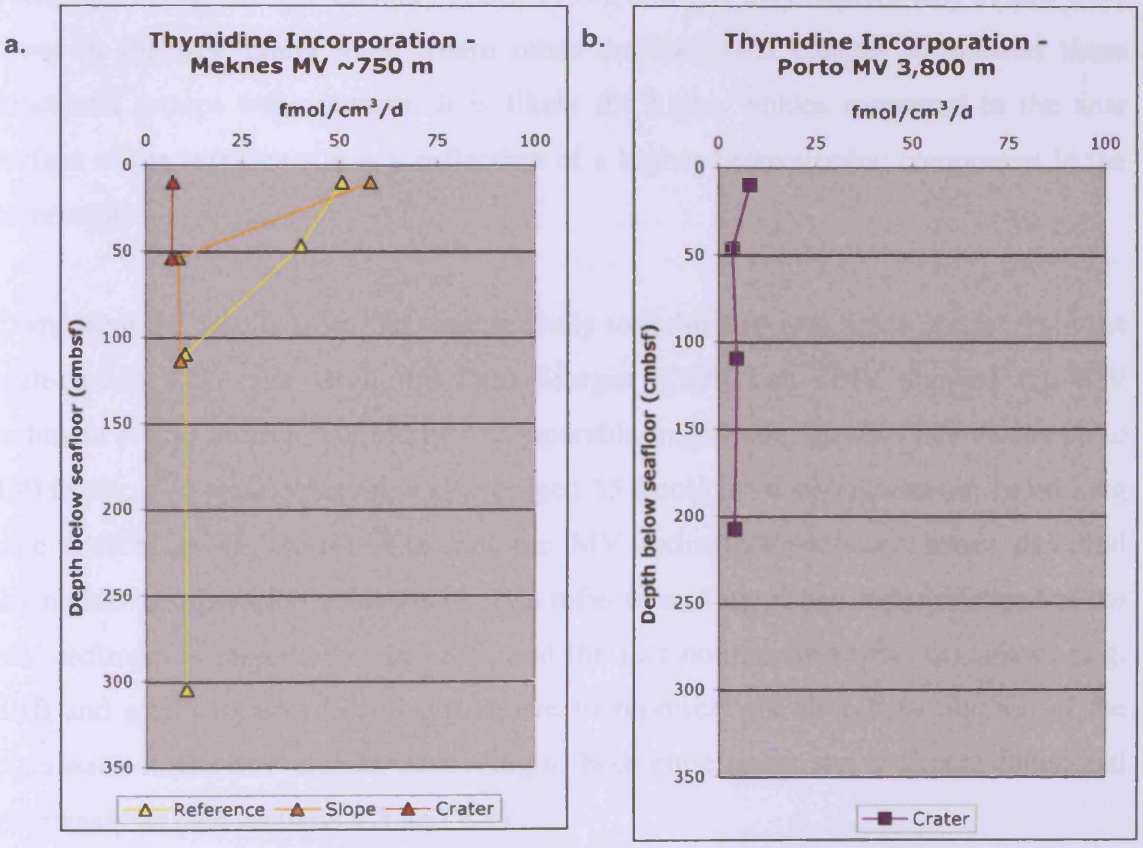


Figs AII.1a and AII.1b Graphs showing potential acetoclastic methanogenesis turnover day^{-1} values in *ex situ* cores from the Meknes MV reference, slope and crater, and Porto MV crater cores, respectively.

Injections of radio-labelled acetate were made through the Meknes MV crater core at 4, 8, 12, 16, 45 and 55 cmbsf. At 55 cmbsf a turnover day^{-1} value of 0.146 was detected, but all other horizons were below detection. All horizons in the Meknes slope and Meknes reference core were below detection limit. Injections were made through the Porto MV crater core at 6, 11, 16, 44,48, 53, 103, 107, 111, 115, 198 and 208 cmbsf. At 11 cmbsf a turnover value of 0.019 was detected, at 16 cmbsf a turnover day^{-1} value of 0.04 was detected, at all other horizons potential activity was below detection.

There was no detectable potential for methanogenesis via CO_2-reduction in any of the horizons tested throughout the four cores investigated, data not shown.

The potential for thymidine incorporation in the 10 cmbsf Meknes crater sample was low compared to the same depth horizon in both the slope and reference core; 7 versus 58 and 59 fmol/cm^3/d, respectively. The potential for thymidine incorporation in the 10 cmbsf crater of Porto was also low, with a mean value of 8 fmol/cm^3/d detected. Potential thymidine incorporation values were below 10 fmol/cm^3/d throughout both the Meknes and Porto MV cores. In the Meknes reference core a potential thymidine incorporation value of 40 fmol/cm^3/d was detected at 50 cmbsf, but then values dropped to 10 fmol/cm^3/d in both the two deeper horizons. In the Meknes slope core, average potential thymidine incorporation values of 9 fmol/cm^3/d were detected at 55 and 115 cmbsf.



Figs AII.2a and AII. 2b Graphs showing potential thymidine incorporation values (calculated as the mean of three 5 cm spaced injections where core material allowed) as measured in *ex situ* sediment cores from the Meknes MV reference, slope and crater, and Porto MV crater sites, respectively.

The very limited potential hydrogenotrophic and acetoclastic methanogenesis activity in the *ex situ* sediment cores fits with the molecular genetic and enrichment data, which shows methylotrophic methanogenesis is likely to be the dominant methanogenic pathway in the MV crater environment. It is therefore suggested potential methylotrophic methanogenesis activity is measured in MV crater cores as future work.

As thymidine is one of the four bases of DNA, rates of thymidine incorporation into prokaryotic DNA was measured as a quantitative proxy of bacterial growth in the MV environment, however there are a number of provisos to the method (see Dixon & Turley, 2001 for discussion). For example, several groups of sub-surface prokaryotes (acetogens, methanogenic *Archaea* and many sulphate-reducing *Bacteria*) do not take up thymidine from the environment but instead synthesize it within their cells (e.g. Wellsbury *et al.*, 1993; Wellsbury *et al.*, 1994), and this may explain why values were lower in the MV crater sites where other methods (see chapter 4) showed these functional groups were present. It is likely the higher values measured in the near surface of the referece site is a reflection of a higher heterotrophic component in the community.

Comparing the results from the present study to published thymidine results from the high-carbon 1229 site from the Peru Margin (ODP Leg 201), showed the MV sediment results were lower but of a comparable magnitude. In site 1229 values up to 130 fmol/cm^3/d were detected, and averaged 55 fmol/cm^3/d throughout the 185m long core (Parkes *et al.*, 2005). The fact the MV sediments produced lower potential thymidine incorporation values is likely a reflection of the lower carbon content of the MV sediment compared to site 1229, and the fact non-heterotrophic organisms (e.g. SRB and methanogens) have been shown to represent the dominant fraction of the population in the MV craters, according to both enrichment and sediment functional gene analysis (see sections 4.3 and 4.4).

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