A Survey of Culturable Methanogens in Contrasting Marine Sediments, their Substrates and Physiological Characteristics

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Summary

Few culture-based surveys have been undertaken on methanogens in contrasting marine sediments. This project conducted a comprehensive study of methanogens in a range of marine sediments including tidal-flats, a shallow bay, deep-water mud volcanoes and Guaymas Basin hydrothermal sediments. Nine of the sixteen recognised methanogen genera were cultured ranging from only one methanogen genus at mud volcano sites to eight in tidal-flats. At a number of these sites, culture-independent techniques (16S rRNA and *mcrA* gene sequences) had either failed to detect the presence of methanogens or had under-estimated the methanogen diversity.

Acetate and hydrogen utilizing methanogens (including two strains of the genus *Methanococcus*) were cultured from marine sediments with high sulphate content. But by far the most abundant genus was *Methanococcoides* being isolated from seven of the sites investigated, including the deepest (in terms of water depth) non-thermophilic methanogens so far isolated. Study of the isolated *Methanococcoides* strains has extended the substrate range of the genus by identifying of two new directly utilized methylated substrates (betaine and choline). The substrate range of this genus has been further extended by demonstrating dimethylsulphide utilization, a compound only previously known as a growth substrate in closely related genera.

In addition to substrate tests, representative strains from each of the sites investigated were physiology characterized including temperature, pH and Na^+ concentration ranges. Members of the genus *Methanococcoides* are mesophilic/ psychrotolerant, neutrophilic and halotolerant. Their substrates are limited to methylated compounds. For the first time strains of mesophilic methanogens (*Methanococcoides*) were tested for their ability to grow under elevated hydrostatic pressure, up to 70 MPa.

In conclusion, methylotrophic methanogens play an important role in marine sediment methanogenesis with the methylotrophic genus *Methanococcoides* being widespread. The range of methylotrophic substrates is wider than previously described and further work is required to determine the full extant of methylated compounds as substrates for methanogenesis. Molecular based studies often fail to identify the presence of methanogens that are subsequently detected in culture-based studies.

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Chapter 1
Marine Methanogens

1.1 Introduction

Methane is produced biogenically by microorganisms and by the thermogenic hydrolysis of organic matter, and abiogenically. Methanogens are specialized microorganisms that produce methane as the major end product of metabolism; they are strict anaerobes and are members of the prokaryote domain *Archaea* (Section 1.2). Methanogens are found in many habitats including anaerobic digestors, landfill sites, intestines of ruminants and other animals, rice paddies, soils, freshwater and marine sediments (Lange & Ahring, 2001). Methanogenesis is the final step in the anaerobic breakdown of organic matter (Lange & Ahring, 2001).

Whilst methane is the main metabolic product of methanogens; methane may also be produced from 'minimethane' anaerobic microorganisms that produce small quantities of methane as a side product of metabolism (Whitman *et al.*, 2006) For example, the acetogen *Clostridium thermoautotropicum* when cultivated under CO-dependent chemolithotropic conditions will produce trace amounts of methane (Savage *et al.*, 1987). Methane can also be produced aerobically in seawater; methylphosphonate decomposes to produce methane in a process enhanced by nitrogen-fixing microorganisms (Karl *et al.*, 2008). Methyl phosphonic acid can also be degraded by different *Escherichia coli* strains, which utilized phosphonic acid as the sole phosphorus source with resulting methane formation (Matys *et al.*, 1996). A general feature of the archaeal genus *Archaeoglobus* is the emission of trace amounts of methane (Mori *et al.*, 2008).

Methane can be produced abiogeneically by the metamorphism of graphite, the thermal decomposition of siderite and the reduction of carbon oxides by Fischer-Tropsch-type reactions (Equations 1.1 and 1.2) (Fiebig *et al.*, 2007).

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (Equ.1.1)

$CO + 3H_2 \rightarrow CH_4 + H_2O$ (Equ.1.2)

Methane could also be produced from HCO_3^- under reducing hydrothermal conditions in the presence of a hydrothermally formed Ni-Fe alloy catalyst (Equation 1.3)(Horita & Berndt, 1999). This form of methane production maybe widespread; the oceanic crust is largely composed of ultramafic rocks that contain a large proportion of serpentinite with Ni-Fe alloy (Horita & Berndt, 1999).

$HCO_3^- + 4H_2 \rightarrow CH_4 + OH^- + 2H_2O$ (Equ.1.3)

Methane (CH_4), the simplest alkane, is a colourless and odourless gas in which the valence of carbon is -4, is the most reduced carbon molecule. Like water vapour and carbon dioxide, methane is a greenhouse gas. Although the atmosphere consists of only about 1.7 ppm of methane it is a potent greenhouse gas 24 times more effective in trapping infrared radiation than carbon dioxide (IPCC, 2007). The atmospheric methane concentration has increased substantially over the past two hundred years (IPCC, 2007). The major sources of atmospheric methane include wetlands, landfills, termites, rice cultivation and enteric fermentation in animals (IPCC, 2007), Table 1.1.

In marine sediments methane can be present as free gas, gas dissolved in porewater and as gas hydrate (Lösekann *et al.*, 2007). Under certain conditions of pressure, temperature and salinity, methane can combine with water to form gas hydrates (Dickens, 2003). Gas hydrates are composed of "cages" composed of water molecules, which trap gas molecules and gas hydrates are found in the form of nodules, veins, pore fillings and surface crusts (Kvenvolden, 2000). The gas they contain is almost pure methane (>99%) with the methane almost entirely derived from microbial activity (Dickens, 2003). The atmospheric emission of methane from gas hydrates (the largest global reservoir of methane) has been implicated in sudden climatic change in the geological past, for example, the event known as the Paleocene-Eocene Thermal Maximum may have been the result of a catastrophic release of methane (Bice & Marotzke, 2002).

Table 1.1 Sources of methane emission and estimates of annual emissions compiled from a number of studies (IPCC, 2007). Geological sources (mainly biogenic) include emissions through geological faults, mud volcanoes and submarine gas seeps.

Source	Methane emission ($Tg\ yr^{-1}$)
Natural Sources	
Wetlands	100-231
Termites	20-29
Geological sources	40-60
Hydrates	4-5
Oceans	4-15
Wildfires	2-5
Agricultural Sources	
Ruminants	76-92
Rice cultivation	31-112
Non-agricultural anthropogenic sources	
Waste disposal/ landfill	35-69
Biomass burning	14-88
Coal mining	30-46
Oil/ gas industry	36-68

Methanotrophs are microorganisms that can consume methane under oxic or anoxic conditions. In anoxic conditions methane in marine sediments is oxidized in the sulphate to methane transition zone (SMTZ) where upwardly diffusing methane coincides with downwardly diffusing sulphate from seawater (Hinrichs & Boetius, 2002). The anaerobic oxidation of methane (AOM) is a major sink for methane in marine sediments oxidizing up to 90% of the methane produced (Hinrichs *et al*., 2000). In aerobic surface sediments, oxygen-dependent methane oxidation also contributes to the reduction of methane emissions (King, 1992). As a result of AOM and aerobic methane oxidation, marine sediments contribute only about 2% of the annual methane flux to the atmosphere (Cicerone and Oremland, 1988). In diffuse seabed systems, the methane flux is consumed by methanotrophic *Archaea.* At cold seeps and sediments such as intertidal mudflats methane may find its way to the atmosphere due to the rapid advection of methane from the subsurface (Knittel & Boetius, 2009).

Kinetic isotope effects associated with the production of methane lead to changes in the isotopic composition of methane (Reeburgh, 2007). The ratio of carbon isotopes in methane can be used to distinguish between methane of microbial and thermogenic origin. Values of $\delta^{13}C$ for thermogenic methane are in the range –

50‰ to –20‰ compared with values of –110‰ to –50‰ for methane of microbial origin (Whiticar, 1999). The methane in gas hydrates generally has $\delta^{13}C$ values –56‰ to –73‰ indicating a biogenic origin (Reeburgh, 2007). Also, $\delta^{13}C$ values reflect methanogenic substrates with the hydrogen pathway producing methane ranging from –110‰ to –60‰ and the acetate pathway producing methane with $\delta^{13}C$ values ranging from –65‰ to –50‰ (Whiticar *et al.*, 1986).

1.2 Energetics and Energy Conservation

Prokaryotes are responsible for driving major biogeochemical processes often involving reduction-oxidation (redox) reactions (Jørgensen, 2000). Redox reactions, the transfer of electrons from one substance to another, are the basis of energy generation in microorganisms (Madigan & Martinko, 2006). Electrons obtained from an electron donor are transferred via a number of intermediate enzymes and cofactors, usually involving an electron transport chain, to a terminal electron acceptor (TEA). The net free energy change is expressed as the Gibbs free energy ($\Delta G^{o'}$) (Equation 1.4). If $\Delta G^{o'}$ is negative then the reaction is exergonic and can be biologically catalysed, if it is positive then the reaction is endergonic and an input of energy is required to drive the reaction (Jørgensen, 2000). For a detailed discussion of the calculation of $\Delta G^{o'}$ for microbial reactions see Thauer *et al*. (1977). It is thought that prokaryotes require a minimum energy of approximately -20 kJ mol^{-1} to exploit a reaction's free energy change (Schink, 1997). However, syntrophs and their partners (Section 1.3.1) can operate close to thermodynamic equilibrium (Jackson & McInerney, 2002).

$$\Delta G^{o'} = -nF\Delta E^{o'} \text{ [kJ mol}^{-1}\text{]} \qquad \text{(Equ. 1.4)}$$

Where $\Delta G^{o'}$ is the free energy at standard conditions (pH 7, 25 ^{o}C and 1 molar concentrations of products and reactants), *n* is the number of electrons transferred by the reaction, F is Faraday's constant (96.48 kJ/V) and $\Delta E^{o'}$ is the difference in the redox potential of the oxidation and reduction reactions.

Reactions are exergonic if the electron donors with a more negative $E^{o\prime}$ are coupled to electrons acceptors that have a more positive $E^{o\prime}$. If the external electron acceptor is oxygen then this process is known as aerobic respiration and this type of microbial respiration yields the most energy (Madigan & Martinko, 2006). If any other external electron acceptor is used, for example, nitrate (NO^{3-}), ferric iron (Fe^{3+}) or sulphate (SO_4^{2-}) then the process is known as anaerobic respiration and this is less energetically favourable. The higher energy gain of aerobic respiration is due to the positive reduction potential of O2. Electron acceptors are utilized in the order $O_2 > NO^{3-} > Mn(IV) > Fe(III) > SO_4^{2-} > CO_2$ which reflects the decreasing free energies of the redox reactions (Figure 1.1), so prokaryotes with the most energy yielding (exergonic) reaction dominate until the electron acceptor is depleted (Telling *et al.*, 2004).

The energy released during redox reactions must be conserved by microorganisms in order for them to carry out energy-requiring reactions. Oxidative phosphorylation involves the synthesis of adenosine triphosphate (ATP) through the generation of a proton motive force (PMF) across the cell membrane. This is achieved by electron transport systems, which are a series of membrane-bound electron carriers. As electrons are passed from electron carrier to electron carrier, protons are transported to the outside of the cell to produce an electrochemical gradient, the PMF, across the cell membrane. This drives ATP synthesis via the activity of the enzyme ATPase as protons move back into the cell driven by the PMF.

Anaerobic catabolism can occur without an external electron acceptor. In fermentation an organic compound serves as both the electron donor and electron acceptor, it is an internally balanced rather than an externally balanced redox reaction (Madigan & Martinko, 2006). Fermentation is an inefficient means of energy conservation in comparison to oxidative phosphorylation. For fermentors, substrate level phosphorylation is often the only mechanism available for energy conservation; this is the synthesis of high-energy phosphate bonds by the reaction of inorganic phosphate with an organic substrate. Where there is insufficient energy to directly couple energy conservation to ATP they may use ion pumps to

generate a proton or Na^+ gradient across the cell membrane. The products of fermentation *e.g*. acetate, ethanol, hydrogen and lactate are substrates for other microorganisms often in syntrophic relationships with fermentors, including for some substrates, methanogens.

1.3 Marine Sediments

1.3.1 Introduction

Marine sediments cover over two-thirds of the Earth's surface and are estimated to contain 70% of the prokaryote biomass on Earth (Whitman *et al.*, 1998). The order in which electron acceptors are used by marine microorganisms is reflected in marine sediment (Telling *et al.*, 2004), an idealized profile through marine sediment is illustrated in Figure 1.1. Electron acceptors that yield higher free energy than sulphate are usually consumed within the first few cm of the sediment (D'Hondt *et al.*, 2002). The concentration of sulphate at the surface is around 50 times higher than the sum of all other higher electrode potential electron acceptors (D'Hondt *et al.*, 2002).

Sulphate is the primary factor controlling the distribution of microorganisms in marine sediments (Capone & Kiene, 1988). For example, a study of a river mudbank (Asleworth Quay, River Severn, UK) and an intertidal mudflat (Aust Warth, Severn Estuary, UK) found that anaerobic processes dominated the mineralisation of organic carbon at both sites, however, the freshwater site was dominated by methanogenesis whilst the marine site, with a high sulphate concentration, was dominated by sulphate-reduction (Wellsbury *et al.*, 1996). The depths to which sulphate-reduction dominates varies, in organic-rich coastal sediments, microbial activity depletes sulphate and other electron acceptors (except CO_2) within a few metres of the sediment surface, in organic-poor sediment, sulphate can penetrate tens to hundreds of metres into the sediment (D'Hondt *et al.*, 2002).

Organic matter in marine sediments is broken down by the action of a number of groups of microorganisms. Polymers, such as polysaccharides, proteins, nucleic acids, and lipids, are converted to oligomers and monomers (sugars, amino acids, fatty acids, and glycerol) in a process that usually involves the action of extracellular hydrolytic enzymes (Figure 1.2) (Schink, 1997). The oligomers and monomers are degraded further by the action of primary fermentors and secondary fermentors (syntrophic bacteria) (Figure 1.2). Major fermentation pathways are illustrated in Figure 1.3.

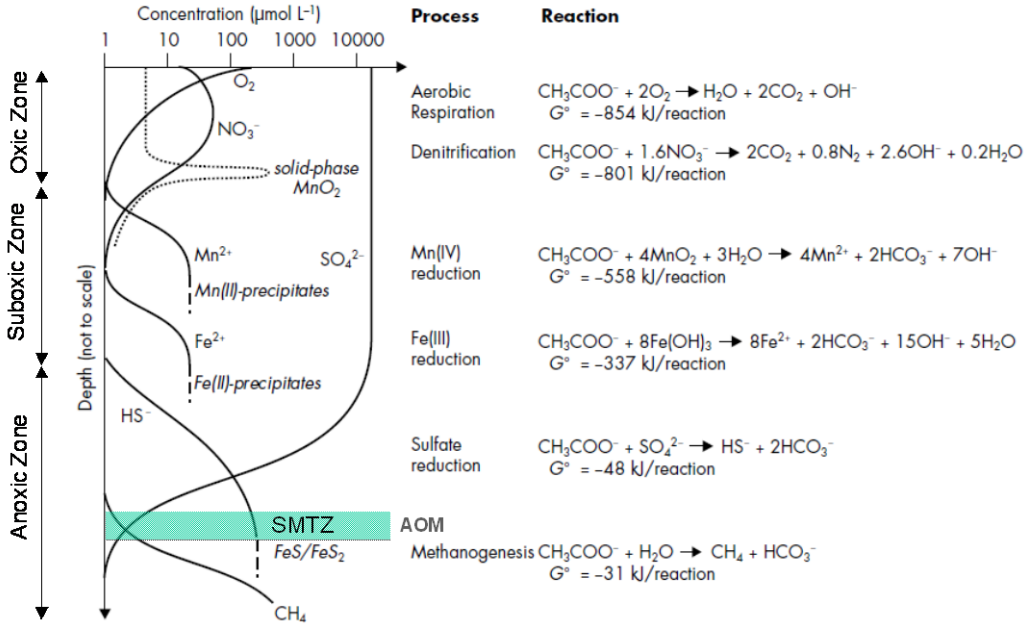


Figure 1.1 Idealized profile through marine sediment (modified from Konhauser, 2007). Electron acceptors are utilized in the order O_2 > NO_3^- > Mn(IV) > Fe(III) > SO_4^{2-} >CO_2. Methanogenesis is not significant until sulphate has become exhausted and occurs below the sulphate to methane transition zone (SMTZ). Methane is anaerobically oxidized (AOM) in the SMTZ resulting in the 'concave up' distribution of methane (Reeburgh, 2007). The suboxic zone is where oxygen is depleted yet the redox potential is kept positive due to the availability of NO_3^-, Mn(IV) and Fe(III). Also shown are the free energy yields per reaction for each of the terminal electron acceptors for the same substrate.

Sulphate-reducing bacteria use sulphate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulfide (Muyzer & Stams, 2008). In the presence of an excess of sulphate, sulphate-reducers outcompete methanogens and syntrophic methanogenic communities for the common substrates hydrogen and acetate (Muyzer & Stams, 2008). Sulphate-reducers are able to grow on a wide range of substrates including amino acids,

sugars, methanol, methanethiol, aromatic hydrocarbons such as ethylbenzene, and long-chain alkanes (Muyzer & Stams, 2008). As sulphate-reducers are metabolically versatile, collectively they can use all products of primary fermentors (Widdel *et al.*, 1988), Figure 1.2. In such circumstances, sulphate-reducers do not depend on the action of syntrophic fermentations.

Methanogens use a limited range of growth substrates (for example, acetate, hydrogen, methylated compounds and some alcohols), they are unable to use some of the products of primary fermentors, such as fatty acids longer than two carbon atoms, and, branched-chain and aromatic fatty acids, the action of an additional group of fermenting bacteria (secondary fermentor or syntrophic bacteria) is required (Schink, 1997). The action of these bacteria converts the primary fermentation products to acetate and hydrogen. (Figure 1.2).

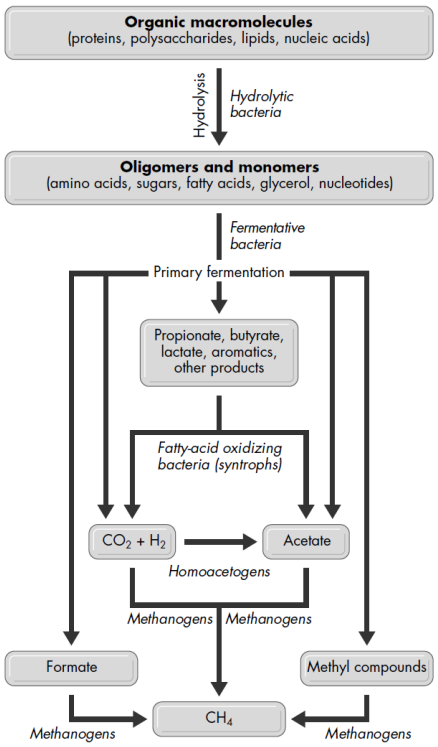


Figure 1.2 The sequential microbial degradation of organic macromolecules in anoxic environments. Organic macromolecules are hydrolysed by hydrolytic bacteria, monomers are fermented by bacteria to give fermentation products that can be utilized by methanogens as growth substrates (Konhauser, 2007). However, in sediments with high sulphate concentrations, sulphate-reducing bacteria will out-compete methanogens for the substrates acetate and hydrogen. Many methyl compounds are used by methanogens but are not used by sulphate-reducers and are known as non-competitive substrates.

Figure 1.3 illustrates the fermentation products of sugars by different groups of bacteria.

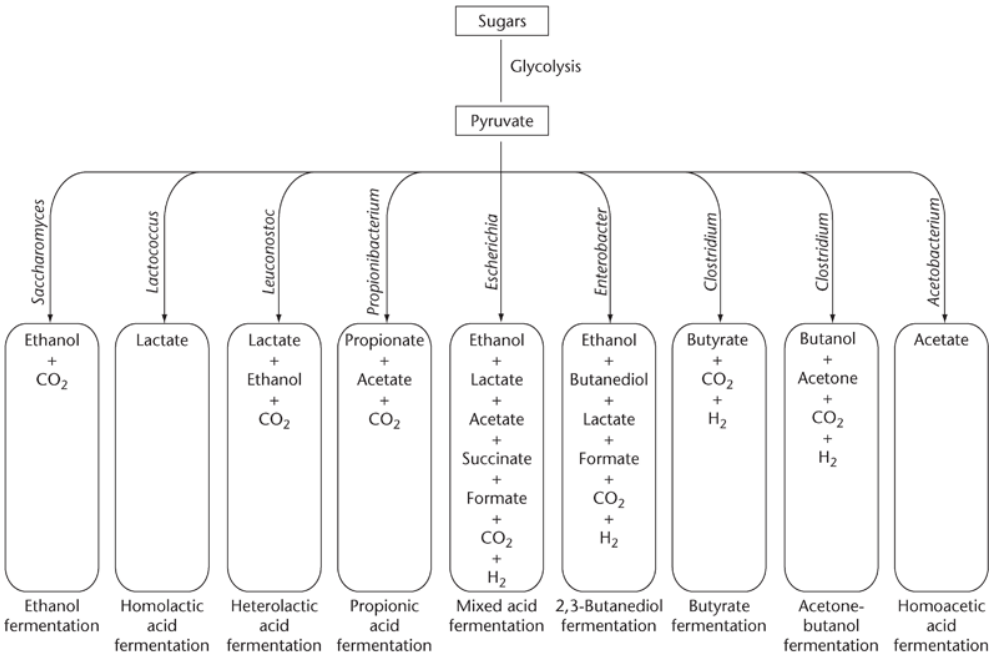


Figure 1.3 Major pathways in the fermentation of sugars indicating the products formed and the microorganisms involved. Diagram from Müller (2008).

1.3.2 Syntrophy

Syntrophy is a form of symbiotic cooperation (Schink, 1997). Organisms in a syntrophic relationship are able to degrade substances that neither could degrade on its own. For example, the hydrogen produced by fermenting bacteria can be used by methanogens and in doing so they maintain a low hydrogen concentration; a high hydrogen concentration would make the reaction thermodynamically unfavourable and inhibit fermentation (Schink, 1997). This utilization of hydrogen is known as interspecies hydrogen transfer.

A classic example of syntrophy is "*Methanobacillus omenlianskii*" which is a coculture of two syntrophic microorganisms, strain S and strain M.o.H. with ethanol as the substrate. The reaction given in Equation 1.5 cannot be carried out by strain S (bacteria) under standard conditions, as it is endergonic ($\Delta G^{o'}$ is positive). The syntrophic partner, strain M.o.H. (a methanogen), consumes

hydrogen (Equation 1.6) and this keeps its concentration low allowing the first reaction to proceed (Schink, 1997). The overall reaction is given by Equation 1.7.

$$2\ CH_3CH_2OH + 2\ H_2O \rightarrow 2\ CH_3OO^- + 2\ H^+ + 4\ H_2 \quad \text{(Equ. 1.5)}$$
$$\Delta G^{o'} = +\ 19\ kJ/\ 2\ mol\ ethanol$$

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \quad \text{(Equ. 1.6)}$$
$$\Delta G^{o'} = -\ 131\ kJ/\ mol\ methane$$

$$2\ CH_3CH_2OH + CO_2 \rightarrow 2\ CH_3OO^- + 2\ H^+ + CH_4 \quad \text{(Equ. 1.7)}$$
$$\Delta G^{o'} = -\ 112\ kJ/\ mol\ methane$$

Numerous compounds that can be degraded syntrophically. Aromatic compounds such benzoate (Jackson & McInerney, 2002) and long-chain alkanes such as hexadecane (Gray *et al.*, 2011) can be broken down to acetate and hydrogen if conditions are made exergonic by the removal of hydrogen by methanogens. The overall reactions for the syntrophic breakdown of benzoate and hexadecane to acetate and hydrogen are given by Equations 1.8 and 1.9 respectively.

$$Benzoate^- + 7\ H_2O \rightarrow 3\ CH_3COO^- + HCO_3^- + 3\ H^+ + 3H_2 \quad \text{(Equ. 1.8)}$$

$$4\ Hexadecane + 64\ H_2O \rightarrow 32\ CH_3COO^- + 32\ H^+ + 68\ H_2 \quad \text{(Equ. 1.9)}$$

1.3.3 Competition Between Microorganisms

In marine sediments there are three groups of microorganisms in competition for hydrogen as a growth substrate. Sulphate-reducers can out-compete homoacetogenic bacteria and methanogens for hydrogen. Methanogens are able to outcompete homoacetogenic bacteria under conditions of low hydrogen concentration; however, homoacetogens appear to be better adapted to low temperatures than methanogens (Kotsyurbenko *et al.*, 2001).

Sulphate concentrations > 3 mM are regarded as non-limiting for sulfate-reducers (Boudreau & Westrich, 1984), in marine systems sulphate reduction is the most important anaerobic process due to the high concentration of sulphate in seawater,

approximately 29 mM (Reeburgh, 2007). Sulphate-reducers will outcompete methanogens for common substrates such as acetate and hydrogen; sulphate reducers have both a higher affinity than methanogens for these substrates and a lower threshold value for them. For example, *Methanosarcina barkeri* has been shown to have a much lower affinity for acetate than *Desulfobacter postgatei*, they have K_s values of 3 and 0.2 mM respectively (Schönheit *et al.*, 1982). *Methanobrevibacter arboriphilus* has been demonstrated to have a K_s value for hydrogen of 6 μM, which was six times higher than determined for *Desulfovibrio vulgaris* (Kristjansson *et al.*, 1982). The substrate thresholds are a result of the thermodynamic effect of acetate and hydrogen concentrations on the yield of free energy. ΔG´ can be calculated using Equation 1.10.

$$\Delta G' = \Delta G^{o'} + RT \ln[C]^c[D]^d/[A]^a[B]^b \qquad \text{(Equ. 1.10)}$$

Where [A] and [B] are the concentrations of the reactants, and [C] and [D] are the concentrations of the products. *R* is the ideal gas constant and *T* the absolute temperature in Kelvin. Calculations show that the utilization of acetate and hydrogen become energetically unfavourable for methanogens at higher concentrations than for sulphate-reducing bacteria (Zinder, 1993) and this is backed up by experimental results. Examples of acetate and hydrogen thresholds for sulphate-reducing bacteria, methanogens and acetogens are given in Table 1.2. The acetate threshold concentration for the methanogen genus *Methanosarcina* is lower than that for the methanogen genus *Methanosaeta* (Table 1.2) this is thought to be a consequence of the different enzymes used to activate acetate (Section 1.4).

Owing to their lower threshold values, acetate and hydrogen utilizing methanogens are rapidly out-competed by sulphate-reducers for these substrates. For example, sulphate reduction was stimulated in sediment collected from intertidal mudflats in San Francisco Bay by the addition of acetate or hydrogen and sulphate inhibited methanogenesis from these substrates (Oremland & Polcin, 1982). A study of intertidal sediments along the Brittany coast found that sulphate reduction was responsible for 99% of the acetate consumed (Winfrey & Ward, 1983).

Table 1.2 Example acetate and hydrogen minimum threshold concentrations for acetogens, methanogens and sulphate-reducing bacteria.

	Threshold Concentration		Reference
	Acetate (μM)	Hydrogen (nM)	
Methanogens			
Methanosarcina barkeri 227	1180		Westermann *et al*. (1989)
Methanosarcina barkeri MS^T	850		Grosßkopf *et al*. (1998)
Methanosarcina thermophila CAL-1	190		Min & Zinder (1989)
Methanosaeta concilii Opfikon	7		Jetten *et al*. (1990)
Methanosaeta concilii VeAc9	<10		Grosßkopf *et al*. (1998)
Methanosaeta thermoacetophila CALS-1	12		Min & Zinder (1989)
Methanospirillum hungatei		23	Cord-Ruwisch *et al*. (1988)
Methanobacterium formicicum		21	Cord-Ruwisch *et al*. (1988)
Sulphate-Reducing Bacteria			
Desulfobacca acetoxidans DSM 11109	<15		Oude Elferink *et al*. (1998)
Desulforhabdus amnigenus DSM 10338	<15		Oude Elferink *et al*. (1998)
Desulfovibrio desulfuricans strain		6.8	Cord-Ruwisch *et al*. (1988)
Acetogens			
Acetobacterium carbinolicum		713	Cord-Ruwisch *et al*. (1988
Acetobacterium woodii		390	Cord-Ruwisch *et al*. (1988)

1.3.4 Interactions Between Sulphate Reducers and Methanogens

Sulphate-reducers are not only found in high sulphate conditions. *Desulfobulbus*, for example, in the absence of sulphate can ferment lactate and ethanol to acetate and propionate but only when in a syntrophic partnership with hydrogen-consuming methanogens (Bryant *et al.*, 1977a).

Finke *et al.* (2007) has shown that some methylotrophic methanogens growing in the presence of sulphate-reducers will leak hydrogen. The sulphate-reducers consume the hydrogen and maintain low hydrogen concentrations. The hydrogen leakage results in a potentially favourable free energy yield for the methanogen. Thus methanogens utilising non-competitive substrates such as methylated amines could be supplying the substrate requirements of sulphate-reducing bacteria.

1.3.5 Non-Competitive Substrates

Substrates that are utilize by methanogens but not by sulphate-reducing bacteria are known as non-competitive substrates. These substrates include, methylamines (methylamine, dimethylamine and trimethylamine) and possibly methylated

sulfides (DMS and methanethiol) and methanol. Studies have shown that at high concentrations (mM range), DMS and methanethiol are mainly converted to methane and at low concentrations (μM range) they are mainly consumed by sulphate-reducing bacteria (Kiene *et al.*, 1986; Kiene & Visscher, 1987). *Desulfosarcina* strain SD1 isolated from a mangrove sediment (Mtoni, Tanzania) was the first marine sulphate-reducing bacterium reported to use DMS and methanethiol (Lyimo *et al.*, 2009a). The situation is similar with methanol, at low concentrations (μM range) sulphate-reducers appear to outcompete methanogens whilst the reverse is true at higher concentrations (>1 mM) (King, 1984).

A study of a San Francisco Bay saltmarsh (Palo Alto, California, USA) found that methane produced from methanol was not affected by the presence of sulphate; the methane production rate and amounts of methane produced where not changed by the addition of 20 mM sulphate (Oremland *et al.*, 1982). Methanol was found to accumulate in sediments when methanogenesis was inhibited and the immediate and linear production of $^{14}CH_4$ from [^{14}C] methanol indicated that the methanogens were adapted to methanol utilization. A study of a San Francisco Bay intertidal mudflat found that the addition of methanol, trimethylamine, or methionine stimulated methanogenesis but did not stimulate sulphate-reduction (Oremland & Polcin, 1982). A radiotracer study at the Bay of Concepción (Chile) found that methane production was almost exclusively from methylamines despite high sulphate reduction rates (170–4670 nmol cm^{-3} d^{-1}) (Ferdelman *et al.*, 1997). The addition of methylamine to beach, estuarine mudflat and salt-marsh sediment from locations along the Brittany coast, stimulated methanogenesis in the presence of sulphate, and the radiotracer [^{14}C]methylamine was rapidly converted to $^{14}CH_4$ and $^{14}CO_2$ (Winfrey & Ward, 1983).

1.3.6 Sources of Non-Competitive Substrates

1.3.6.1 Trimethylamine

Trimethylamine can be produced from betaine (*N*,*N*,*N*-trimethylglycine), choline (*N*,*N*,*N*-trimethylethanolamine) and trimethylamine *N*-oxide (TMAO). Osmoregulation in prokaryotes is assisted by the accumulation of compatible solutes (Yancey *et al.*, 1982). These low molecular weight compounds that

include glycine betaine and trimethylamine *N*-oxide are responsible for osmotic balance and at the same time are compatible with the cell's metabolism. Compatible solutes maintain a higher intracellular osmotic potential than that of the extracellular environment thereby keeping the cellular turgor pressure constant by preventing water loss by osmosis under marine conditions (Yancey *et al.*, 1982). Choline is widely distributed in membrane lipids (King, 1984).

A. *TMAO*

TMAO ($(CH_3)_3NO$) can be reduced to trimethylamine by bacteria, e.g. *Salmonella typhimurium* and *Rhodopseudomonas capsulatus* (Strom *et al.*, 1979). Production of TMAO has been shown to occur in 20 species of marine macro-algae where concentrations can exceed 700 mmol g^{-1} dry weight of tissue (Fujiwara-Arasaki & Mino, 1972). It is thought to function mainly in osmoregulation (Fujiwara-Arasaki & Mino, 1972). It is also found in fish, 2-4% of the dry weight of a teleost fish is TMAO (Strom *et al.*, 1979).

B. *Betaine*

Betaine can be anaerobically degraded to trimethylamine by a number of processes. *Eubacterium limosum* is able to ferment betaine to *N*,*N*-dimethylglycine with the methyl group being converted to acetate or butyrate (Muller *et al.*, 1981), Equation 1.11.

7 betaine + $2CO_2$ → 7 *N*,*N*-dimethylglycine + 1.5 acetate + 1.5 butyrate (Equ. 1.11)

Sporomusa ovata strain $H1^T$ and *Sporomusa sphaeroides* strain E^T can also ferment betaine; in this case the products are acetate, trimethylamine, *N*,*N*-dimethylglycine, NH_3, and CO_2 (Möller *et al.*, 1984). *Clostridium sporogenes* (DSM 79) is able to produce trimethylamine and acetate from betaine by utilizing L-alanine, L-isoleucine, L-leucine or L-valine as an electron donor in a Stickland reaction (a coupled oxidation-reduction reaction) where the betaine acts as the electron acceptor (Naumann *et al.*, 1983), equation 1.12.

R-CH(NH_2)-COOH + 2 betaine + 2 H_2O → R-COOH + CO_2 + NH_3 + 2 trimethylamine + 2 acetate (Equ. 1.12)

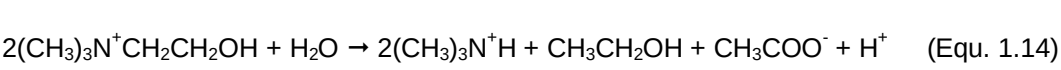
Clostridium sporogenes is unable to utilise betaine as a sole growth substrate (Naumann *et al.*, 1983). The sulphate–reducer *Desulfuromonas acetoxidans* from intertidal mud has been shown to ferment betaine (Heijthuijsen & Hansen, 1989), Equation 1.13.

$$4 \text{ betaine} + 2\ H_2O \rightarrow 4 \text{ trimethylamine} + 3 \text{ acetate} + 2\ CO_2 \qquad \text{(Equ. 1.13)}$$

With sulfur as an electron acceptor the reminder of the acetate formed by the cleavage of betaine is oxidized to CO_2 with the formation of hydrogen sulfide (Heijthuijsen & Hansen, 1989).

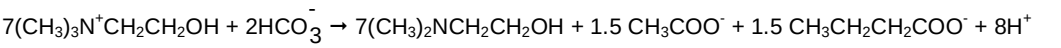
C. *Choline*

Whilst, choline has not been shown to be a direct catabolic substrate for methanogenesis (*e.g.* Hippe *et al.* (1979) it can be degraded to compounds that can be utilized by methanogens. *Desulfovibrio desulfuricans* has been determined to degrade choline to trimethylamine, acetate and ethanol (Hayward & Stadtman, 1959), Equation 1.14.



$$2(CH_3)_3N^+CH_2CH_2OH + H_2O \rightarrow 2(CH_3)_3N^+H + CH_3CH_2OH + CH_3COO^- + H^+ \qquad \text{(Equ. 1.14)}$$

The same products have been noted for the degradation of choline by *Clostridium* sp. (Bradbeer, 1965). *Eubacterium limosum* has also been shown to grow on choline but rather than produce trimethylamine it produces *N*, *N*-dimethylethanolamine along with acetate and butyrate (Muller *et al.*, 1981), Equation 1.15.



$$7(CH_3)_3N^+CH_2CH_2OH + 2HCO_3^- \rightarrow 7(CH_3)_2NCH_2CH_2OH + 1.5\ CH_3COO^- + 1.5\ CH_3CH_2CH_2COO^- + 8H^+ \qquad \text{(Equ. 1.15)}$$

Desulfovibrio desulfuricans isolated from mud in a stagnant stream can degrade choline to trimethylamine, acetate and ethanol (Hayward & Stadtman, 1959), Equation 1.16.

$$2(CH_3)_3N^+CH_2CH_2OH + H_2O \rightarrow 2(CH_3)_3N^+H + CH_3COOH + CH_3CH_2OH \quad \text{(Equ. 1.16)}$$

In a coculture experiment with *Desulfovibrio* strain G1 and *Methanosarcina barkeri* strain Fusaro (DSM 804) choline was degraded to methane, ammonia, hydrogen sulfide, and carbon dioxide in the presence of sulphate (Fiebig & Gottschalk, 1983). This was not an example of a syntrophic relationship, as the presence of *Methanosarcina barkeri* did not affect choline degradation. Equation 1.17 gives the overall reaction.

$$4(CH_3)_3N^+CH_2CH_2OH + SO_4^{2-} \rightarrow 4(CH_3)_3N^+H + 4CH_3COO^- + 3H^+ + HS^- \quad \text{(Equ. 1.17)}$$

Eubacterium limosum is also able to grow anaerobically on choline producing *N, N*-dimethylethanolamine, acetate and butyrate (Muller *et al.*, 1981).

1.3.6.2 Methanol

Methanol can be produced by the anaerobic decomposition of pectin in anoxic sediments (Schink & Zeikus, 1982). Pectin is a common constituent of plant and algal cells and is a polymer of α-(l,4)-galacturonic acid that is partially methoxylated at the carboxy groups. The methoxy group is released as methanol during anaerobic decomposition. Various pectinolytic strains of *Clostridium, Erwinia,* and *Pseudomonas* species produced methanol as a major end product during growth on pectin (Schink & Zeikus, 1982).

1.3.6.3 Methylated Sulphur Compounds

Dimethyl sulfide (DMS) is produced by the degradation of sulphur-containing amino acids or by the methylation of methanethiol. Dimethylsulfoniopropionate (DMSP) is a tertiary sulfonium compound and osmoregulatory solute of many marine plants and algae (Vairavamurthy *et al.*, 1985) and is the major source of DMS in the marine environment (van der Maarel *et al.*, 1995). DMS is produced from DMSP by way of an elimination reaction that can either be catalyzed by OH^- (very slow reaction) or biologically (Kiene, 1990), Equation 1.18.

$$\underset{\text{DMSP}}{(CH_3)_2S^+CH_2CH_2COO^-} \rightarrow \underset{\text{DMS}}{(CH_3)_2S} + \underset{\text{Acrylate}}{CH_2{=}CHCOO^-} + H^+ \quad \text{(Equ. 1.18)}$$

Sulphate-reducing bacteria from anoxic intertidal sediment have been shown to carry out this reaction (Van der Maarel *et al.*, 1996). DMSP can also be demethylated to 3-methiolpropionate (MMPA). 3-methiolpropionate can be demethylated to 3-mercaptopropionate (MPA) or demethiolated producing methanethiol (Kiene & Taylor, 1988; Taylor & Gilchrist, 1991). Demethylation of DMS has been observed in *Desulfobacterium vacuolatum, Desulfobacterium niacini* and *Eubacterium limosum* (Van der Maarel *et al.*, 1996). In anaerobic conditions, DMS may be oxidized to DMSO by phototrophic bacteria (Taylor & Kiene, 1989). Dimethylsulfoxide (DMSO) can be reduced to DMS in marine environments by sulphate-reducing bacteria such as *Desulfovibrio desulfuricans* (Jonkers *et al.*, 1996).

1.4 Methanogens

1.4.1 Archaea

Life on Earth is grouped into three Domains, *Archaea*, *Bacteria* and *Eukarya* (Woese & Fox, 1977; Woese *et al.*, 1990) and the methanogens belong to the domain *Archaea*. Due to the morphological similarity of microorganisms that belong to the prokaryote domains *Archaea* and *Bacteria*, the existence of two different types of prokaryotes (single celled organisms without membrane-bound internal compartments) was not recognized until the introduction of small subunit ribosomal ribonucleic acid (rRNA) gene sequencing. The representatives of this domain were first distinguished from bacteria based on differences in transfer ribonucleic acid (tRNA) and rRNA, the cytoplasmic membrane and cell-wall composition, together with their restriction to unusual habitats (Woese & Fox, 1977; Woese *et al.*, 1990) *Archaea* were originally identified in conditions that were characterized by high salinity, high temperature and high acidity which led to *Archaea* being regarded as extremophiles.

Valentine (2007) argues that the adaptation to chronic energy stress is the crucial factor that distinguishes *Archaea* from Bacteria. *Archaea* possess biochemical mechanisms that allow them to cope with chronic energy stress such as low-

permeability membranes, which results in less energy being lost during the maintenance of a chemiosmotic potential. This is claimed to reduce the maintenance energy (the minimum energy required to maintain cellular activity) for *Archaea* compared with *Bacteria*.

Some of the differences between *Archaea*, Bacteria and Eukarya are presented in Table 1.3. *Archaea* are more related to Eukarya in terms of molecular and gene structure (Whitman *et al.*, 2006).

Table 1.3 Summary of major differences between *Archaea*, *Bacteria* and *Eukarya* (adapted from Madigan and Martinko, 2006).

Characteristic	*Archaea*	*Bacteria*	*Eukarya*
Prokaryotic cell structure	Yes	Yes	No
Cell wall	Muramic acid absent	Muramic acid present	Muramic acid absent
Membrane lipids	Ether linked	Ester linked	Ester linked
Ribosomes (mass)	70S	70S	80S
Initiator tRNA	Methionine	Formylmethionine	Methionine
RNA polymerases	Many (8-12 subunits each)	One (4 subunits)	Three (12-14 subunits each)
Transcription factors required	No	Yes	No
Spore formation	Absent	Present in some	Absent

All *Archaea* lack a cell wall that contains muramic acid (Albers & Meyer, 2011). The most common archaeal cell envelope consists of a single (glyco)protein surface layer (S-layer) that is directly associated with the plasma membrane being attached to it by its carboxyl-terminal transmembrane domain. The only methanogens to lack a S-layer are members of the genera *Methanobrevibacter* and *Methanosphaera* (Figure 1.4). In some *Archaea* the cell envelope also consists of polymers such as pseudomurein (containing L-talosaminuronic acid) and methanochondroitin, pseudomurein is similar to bacterial peptidoglycan (murein). *Archaea* with pseudomurein have a rigid cell wall, cell walls with only the S-layer provide limited support and cells are osmotically fragile (Whitman *et*

al., 2006). The S-layer may also be found on the outside (Figure 1.4) (Albers & Meyer, 2011). The most complex cell envelopes are found in the genera *Methanosaeta* and *Methanospirillum*. Both genera form filamentous chains enclosed by a proteinaceous sheath. Individual cells of each genus are surrounded by an inner cell wall (Figure 1.4). Cells of *Methanosarcina* can form aggregates and the cell aggregate produces a cell wall polymer called methanochondroitin due to its similarity to chondroitin, a component of vertebral connective tissue matrix (Albers & Meyer, 2011).

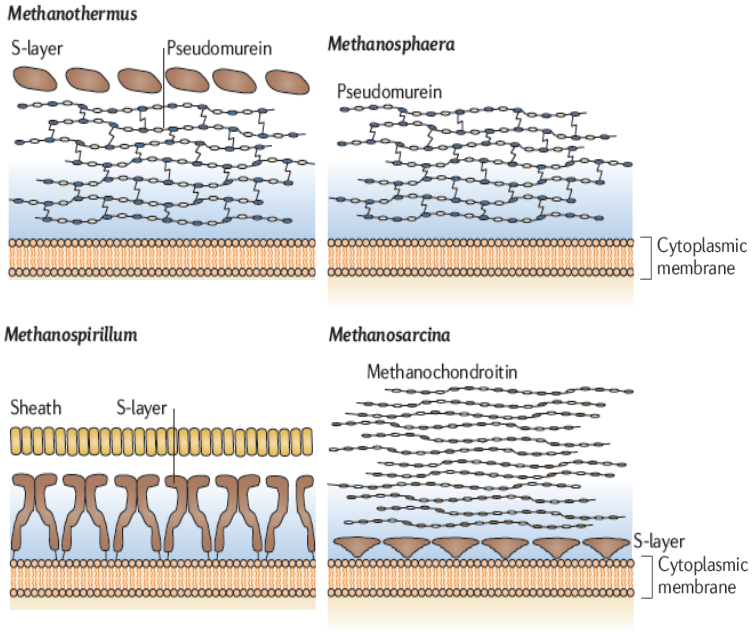


Figure 1.4 The cell membranes of selected methanogens illustrating the protein S-layer and pseudomurein layers. Modified from Albers & Meyer (2011).

As the cell wall structure of *Archaea* differs from that of bacteria they are insensitive to antibiotics that target cell wall synthesis such as ampicillin, penicillin and vancomycin. The differences in protein synthesis between *Archaea* and *Bacteria* also make them insensitive to antibiotics such as kanamycin, which interferes with bacterial protein synthesis (Whitman *et al.*, 2006).

The cell membrane lipids of *Archaea* also differ from those the Bacteria. Archaeal polar lipids consist of a core lipid (the hydrophobic part) that is attached to the polar head group. The core lipids of *Archaea* are generally fully saturated

isopranoid chains that are attached via ether bonds to the 2,3-*sn* carbon positions of the glycerol backbone (Sprott, 2011). These differenced are illustrated in Figure 1.5. The core lipids of *Eukaryotes* and *Bacteria* are generally unbranched fatty acyl chains (often unsaturated) that are attached to the 1,2-*sn* glycerol carbons via ester bonds (Figure 1.5). Whilst ether bonds can be found in *Bacteria* and *Eukarya*, ester bonds are not found in *Archaea* (Sprott, 2011).

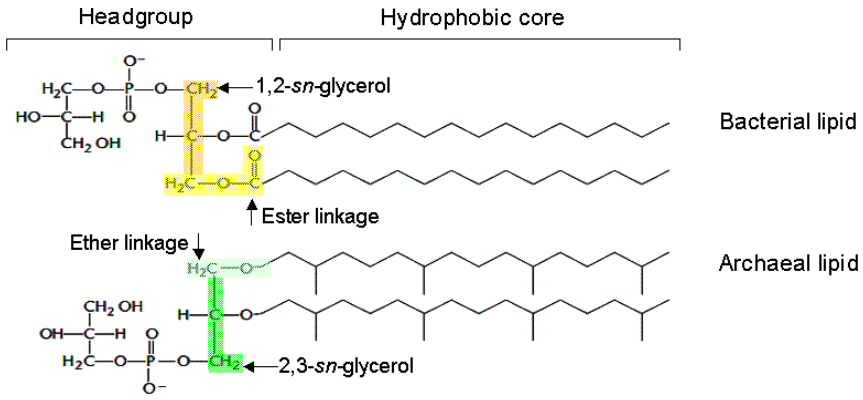


Figure 1.5 The differences between Archaeal and Bacterial lipids. Modified from Sprott (2011).

1.4.2 Archaeal Phyla

Two phyla of *Archaea* are recognized (Woese *et al.*, 1990), the *Crenarchaeota* and the *Euryarchaeota*, and others are proposed: *Korarchaeota* (Barns *et al.*, 1996), *Nanoarchaeota* (Huber *et al.*, 2002) and *Thaumarchaeota* (Brochier *et al.*, 2005). There are four orders of *Crenarchaeota*, *Acidilobales*, *Desulfurococcales*, *Sulfolobales* and *Thermoproteales* and eleven orders of *Euryarchaeota* of which five are non-methanogenic: *Archaeoglobales*, *Halobacteria*, *Picrophilales*, *Thermococcales* and *Thermoplasmatales* (Euzéby, 2011).

1.4.3 Methanogen Phylogeny

The methanogens are phylogenetically diverse, they are classified into six orders: *Methanobacteriales*, *Methanocellales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanopyrales* (Table 1.4 and Figure 1.6). Prokaryote phylogenetic is based on the results of comparative analysis of the evolutionary conservative 16S rRNA genes. The methanogen orders are based on 16S rRNA gene sequence similarities of less than 82% (Liu & Whitman, 2008). The orders

are divided into 13 families (< 88-93% 16S rRNA gene sequence identity) and 31 genera (< 93-95% 16S rRNA gene sequence identity). A sequence identity of 98% or less is evidence for a separate methanogen species (Whitman *et al.*, 2006).

Table 1.4 Orders, Families and Genera of the methanogenic *Archaea* based on 16S rRNA gene sequences (Euzéby, 2011).

Order	Family	Genus
Methanobacteriales	*Methanobacteriaceae*	*Methanobacterium*
		Methanobrevibacter
		Methanosphaera
		Methanothermobacter
	Methanothermaceae	*Methanothermus*
Methanococcales	*Methanococcaceae*	*Methanococcus*
		Methanothermococcus
	Methanocaldococcaceae	*Methanocaldococcus*
		Methanotorris
Methanocellales	*Methanocellaceae*	*Methanocella*
Methanomicrobiales	*Methanomicrobiaceae*	*Methanomicrobium*
		Methanoculleus
		Methanofollis
		Methanogenium
		Methanolacinia
		Methanoplanus
		Methanosphaerula
	Methanospirillaceae	*Methanospirillum*
	Methanocorpusculaceae	*Methanocorpusculum*
	Not assigned to a Family	*Methanocalculus*
		Methanoregula
Methanosarcinales	*Methanosarcinaceae*	*Halomethanococcus**
		Methanosarcina
		Methanococcoides
		Methanohalobium
		Methanohalophilus
		Methanolobus
		Methanomethylovorans
		Methanimicrococcus
		Methanosalsum
	Methanosaetaceae	*Methanosaeta***
	Methermicoccaceae	*Methanococcus*
Methanopyrales	*Methanopyraceae*	*Methanopyrus*

* *Halomethanococcus* may be considered as a later heterotypic synonym of *Methanohalophilus* (Paterek & Smith, 1988).

** The genus name *Methanosaeta* has recently been declared illegitimate and should be replaced with the genus name *Methanothrix* (Garrity *et al.*, 2011). The Family name *Methanosaetaceae* is also illegitimate.

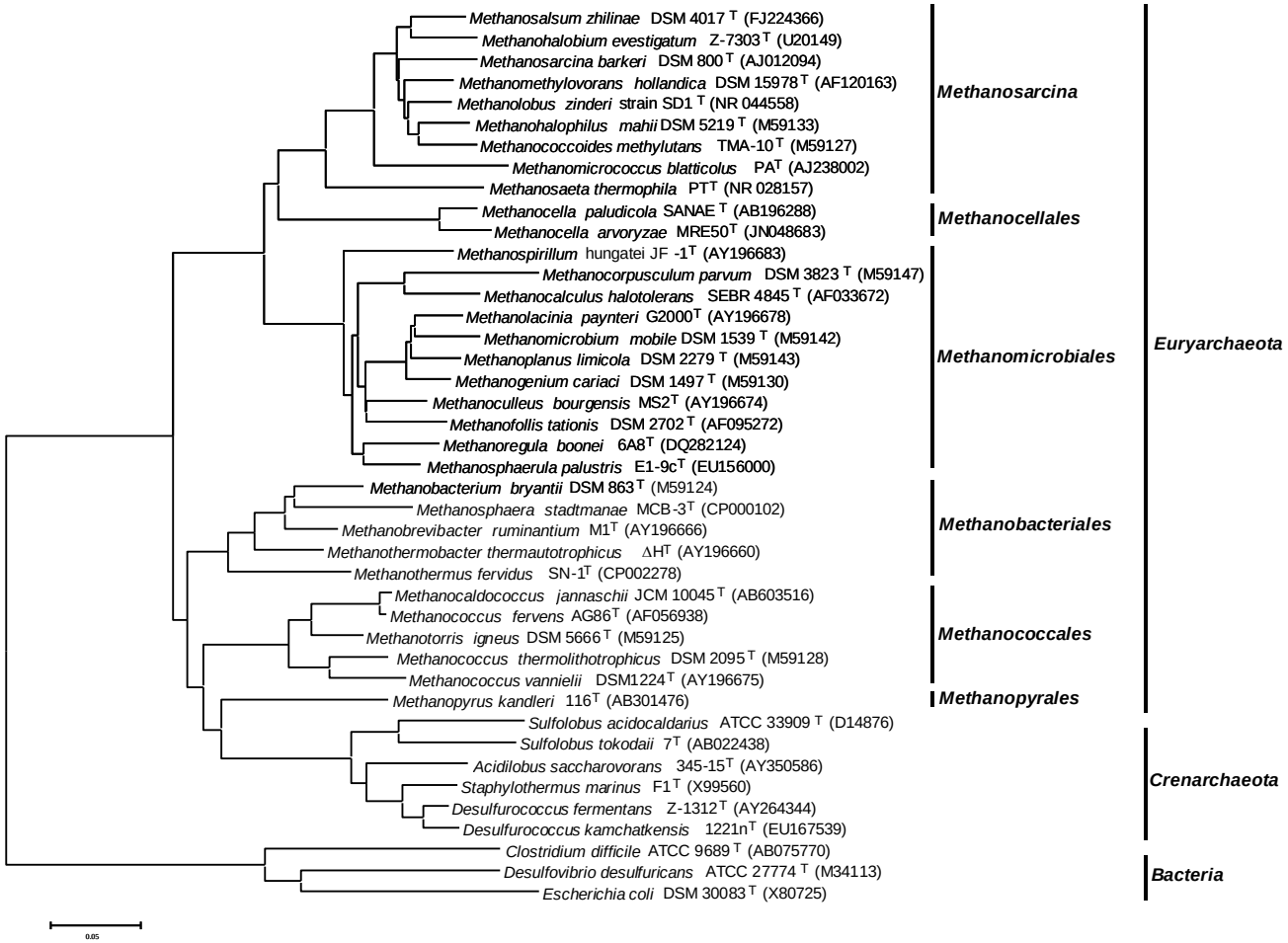


Figure 1.6 Phylogenetic tree (neighbour-joining) based on 16S rRNA gene sequences showing the relationship of the methanogen orders, *Crenarchaeota* and *Bacteria*. Accession numbers in brackets. Bar, 0.05 substitutions per site.

Methyl coenzyme M reductase (Section 1.3) is an alternative phylogenetic marker for methanogens. Methyl coenzyme M reductase consists of two alpha (*mcrA*), two beta (*mcrB*), and two gamma (*mcrG*) subunits. The subunits are phylogenetically conserved (Hallam *et al.*, 2003a) and on the basis of the comparison of available 16S rRNA and mcrA gene sequences of methanogens, the mcrA gene was demonstrated to be an alternative phylogenetic marker showing similar relationships as those seen based on the 16S rRNA gene (Luton *et al.*, 2002).

1.4.3.1 *Methanobacteriales*

The order *Methanobacteriales* contains two families and five genera (Table 1.4). Members of the order *Methanobacteriales* generally utilise H_2/CO_2 for

methanogenesis although some can also use formate or secondary alcohols (Whitman *et al.*, 2001). The *Methanobacteriales* have a cell wall composed of pseudomurein with the family *Methanothermaceae* also having a protein surface layer (Whitman *et al.*, 2001). Morphologically members of this order can be rod or coccoid in shape. Members of the genus *Methanosphaera* can only produce methane by the reduction of methanol with hydrogen. This is the only genus outside of the order *Methanosarcinales* that can utilize methanol for methanogenesis (Whitman *et al.*, 1992).

1.4.3.2 *Methanococcales*

This order contains two families and four genera (Table 1.4). Both genera of the family *Methanocaldococcaceae* contain hyperthermophiles, as does the genus *Methanothermococcus* of the family *Methanococcaceae*. Members of this order use H_2/CO_2 for methanogenesis and have cell walls with a protein S-layer (Whitman *et al.*, 2001).

1.4.3.3 *Methanomicrobiales*

The order *Methanomicrobiales* contains three families and eleven genera (Table 1.4). Members of the order *Methanomicrobiales* utilise H_2/CO_2 for methanogenesis. Some species can also use formate or secondary alcohols as electron donors. Acetate is not a substrate for methanogenesis; however, many species require acetate as a carbon source (Whitman *et al.*, 2001). This is the most morphologically varied of the methanogenic orders; they can be coccoid, rod, plate or spiral in shape (Whitman *et al.*, 2001). Cell walls have a glycoprotein S-layer and may also have an exterior sheath (Whitman *et al.*, 2001).

1.4.3.4 *Methanocellales*

The order *Methanocellales* contains one family and one genus (Euzéby, 2011). This order was previously known as Rice Cluster I (RC-1) and, was known only from 16S rRNA gene sequences from rich paddy soil until the first representative was isolated in 2008, *Methanocella paludicola* (Sakai *et al.*, 2008). Both species can utilize H_2/CO_2 and formate for methanogenesis (Sakai *et al.*, 2008; Sakai *et al.*, 2010).

1.4.3.5 *Methanosarcinales*

The order *Methanosarcinales* consists of three families (Table 1.4). Members of the order *Methanosarcinales* are the most metabolically versatile of all the methanogens. They can produce methane by the disproportionation of methyl-group containing compounds, the fermentation of acetate and the reduction of CO_2 with H_2 (Whitman *et al.*, 2001). However, they do not utilise formate. All methanogen that can utilise methylated compounds without hydrogen are found in this order. They also have diverse morphologies including cocci, pseudosarcinae, and sheathed rods. Members of the genus *Methanosaeta* (*Methanothrix*) are the only methanogens that utilise only acetate for methanogenesis. Cell walls of the *Methanosarcinales* have a S-layer (Whitman *et al.*, 2001).

1.4.3.6 *Methanopyrales*

The order *Methanopyrales* is represented by only one species, the hyperthermophilic *Methanopyrus kandleri* (Euzéby, 2011). H_2/CO_2 is used for methanogenesis and cell walls contain pseudomurein (Whitman *et al.*, 2001). *M. kandleri* has a temperature range of 84–110 ºC, it is found in marine hydrothermal systems (Kurr *et al.*, 1991).

1.4.4 Uncultured *Archaea*

1.4.4.1 Methanogens

As well as the recognized archaeal taxa there are novel phylogenetic groups of detected by culture-independent studies. For example, Zoige cluster I (ZC-I, Zoige wetlands, Tibetan Plateau) is an uncultured methanogen cluster known from 16S rRNA and *mcrA* gene sequencing (Grosskopf *et al.*, 1998). Other *Archaea*l lineages are suspected of containing methanogens, for example, the Mediterranean Sea Brine Lake candidate division 1 (MSBL1) which has been identified in Mediterranean hypersaline basins (Bannock, Discovery, L'Atalante and Urania) by 16 rRNA gene based analysis (van der Wielen *et al.*, 2005). Methanogenesis occurs in all of these Mediterranean basins but few 16S rRNA gene sequences of known methanogens have been identified, the majority of archaeal 16S rRNA gene sequences discovered belong to MSBL1 which makes

MSBL1 the prime candidate for methanogenesis in these basins (van der Wielen *et al.*, 2005).

1.4.4.2 Anaerobic Oxidation of Methane

The anaerobic oxidation of methane (AOM) is carried out by *Euryarchaeota* known as *an*aerobic *me*thanotrophs (ANME) that form three clusters based on 16S rRNA gene sequences (Knittel & Boetius, 2009). The clusters ANME-1 and ANME-2 are related to the methanogen order *Methanosarcinales.* The third cluster, ANME-3, is related to the methanogen order *Methanomicrobiales*. Hoehler *et al*. (1994) proposed that in sulphate containing sediments methane oxidation is brought about by a syntrophic partnership of ANME and sulphate-reducing bacteria whereby the by-products of AOM were used by sulphate-reducing bacteria, Equation 1.19.

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad \text{(Equ. 1.19)}$$
$$\Delta G^{o'} \quad -16.6 \text{ kJ mol}^{-1}$$

It is possible that methanogens are responsible for both methane production and consumption in a syntrophic relationship with sulphate-reducing bacteria via the process of 'reverse methanogenesis' (Knittel & Boetius, 2009). On the basis of the presence of ANME-1 within methane-producing sediments and a re-assessment of the published literature it has recently been suggested that ANME-1 are methanogens (Lloyd *et al.*, 2011).

1.4.4.3 Other Archaeal Groups

There are numerous groups of *Archaea* that are uncultured and whose physiology is unknown. For example, Marine Benthic Group D (MBG-D) is a cluster of closely related gene sequences affiliated with the *Euryarchaeota*, sharing a common ancestry with the aerobic moderate thermophile, *Thermoplasma acidophilum* (Vetriani *et al.*, 1999). Other examples include the Miscellaneous Crenarchaeotic Group (MCG) which are common in marine sediment 16S rRNA clone libraries (Teske & Sørensen, 2008) and Marine Group I (MG-I) which were originally identified in seawater (DeLong, 1992) but are also found in marine sediments (Teske, 2006).

1.5 Methanogenesis

Methanogens make use of a limited range of substrates for energy metabolism. Substrate usage can be divided into three groups all of which result in the production of methyl coenzyme M (methyl-CoM) (Lessner, 2009)(Figure 1.7). The last step, common to all three groups, reduces methyl-CoM to methane by methyl coenzyme M reductase (Mcr). The direct electron donor is Coenzyme B (CoB) and oxidized CoB forms a heterodisulfide with CoM (CoM-S--S-CoB). CoM-S--S-CoM is reduced with electrons from coenzyme F_{420} to regenerate CoM-SH and CoB-SH (Figure 1.7). Both the methyl transfer to CoM and the reduction of the heterodisulfide are involved in energy conservation (Liu & Whitman, 2008). The pathways of methanogenesis can be broadly categorized into three groups each of which results in the formation of a methyl group:

1. The reduction of carbon dioxide. Carbon dioxide is reduced to a methyl group using electrons derived from the electron donor, which is usually hydrogen (Lessner, 2009). Many hydrogenotrophic methanogens can also use formate as an electron donor in a process whereby four formate molecules are oxidized to carbon dioxide before one carbon dioxide molecule is reduced to methane. Secondary alcohols *e.g.* 2-propanol, 2-butanol, and cyclopentanol can also be used by some methanogens to provide electrons for the reduction of carbon dioxide. These are first oxidized to ketones by coenzyme F_{420}-dependent secondary alcohol dehydrogenases (Adf). Ethanol may also be used and this is oxidized to acetate via a nicotinamide adenine dinucleotide phosphate (NADP)–dependent alcohol dehydrogenase.

2. The fermentation of acetate. The carbon–carbon bond of acetate is cleaved to produce a methyl and a carbonyl group, the methyl group is reduced to methane with electrons originating from oxidation of the carbonyl group to carbon dioxide (Lessner, 2009). In contrast, bacteria oxidize both the methyl and carboxyl groups to carbon dioxide. Prior to cleavage, acetate needs to be activated. *Methanosarcina* sp. activate acetate to acetyl-CoA in a reaction catalyzed by acetate kinase and phosphotransacetylase. These enzymes are not present in

Methanosaeta spp., instead CoA synthetase catalyzes the activation of acetate to acetyl-CoA (Ferry, 1992).

3. The disproportionation of methylated compounds. The methylated compounds include methanol, methylated amines (methylamine, dimethylamine, trimethylamine, and tetramethylammonium), and methylated sulphides (methanethiol and dimethylsulphide). The methyl groups are transferred to a cognate corrinoid protein and then to CoM to form methyl-CoM (Lessner, 2009). The activation and transfer of the methyl group requires substrate specific methyltransferases. The electrons required for the reduction of methyl groups to methane are obtained from the oxidation of additional methyl groups to carbon dioxide (the reverse of hydrogenotrophic methanogenesis). Three methyl groups are reduced to methane for every molecule of carbon dioxide formed (see Table 1.5). This is a disproportionation (dismutation) reaction as the oxidation of one substrate molecule is used to reduce another substrate molecule.

The reduction of carbon dioxide by hydrogen is the most energetically favourable reaction and the least favourable is the fermentation of acetate (Table 1.5). The free energy values in Table 1.5 were calculated for standard conditions (neutral pH, 25 ºC and 1 molar concentrations of products and reactants) under *in situ* concentrations of the free energy available to the methanogen would be lower.

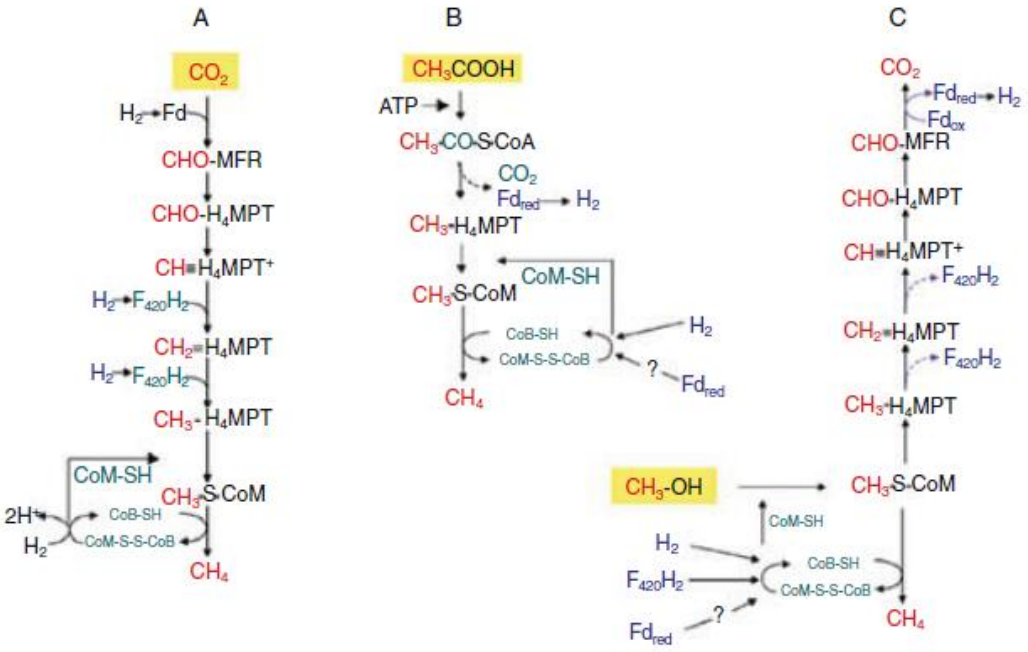


Figure 1.7 Methanogenesis from H_2/CO_2 (A), acetate (B) and methanol (C). Although initially different, the three pathways have a common intermediate; Methyl-coenzyme M (CH_3-S-CoM. Methyl-coenzyme M is converted to methane and the heterodisulfide of coenzyme M and coenzyme B (CoM-S-S-CoB). CoM-S-S-CoB functions as the terminal electron acceptor. Diagram from Hedderich & Whitman (2006).

Table 1.5 Comparison of the Gibbs free energy ($\Delta G^{o'}$) yield of various methanogenic reactions, data from Thauer (1977).

Reaction	$\Delta G^{o'}$ kJ/ reaction*
$4CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2$	-136
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	-136
$4\ Formate \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	-130
$2\ Ethanol + CO_2 \rightarrow CH_4 + 2\ Acetate$	-116
$Methanol + H_2 \rightarrow CH_4 + H_2O$	-113
$4\ Methanol \rightarrow 3\ CH_4 + HCO_3^- + H^+ + H_2O$	-315 (-105)
$4\ Trimethylamine + 9\ H_2O \rightarrow 9\ CH_4 + 3HCO_3^- + 3H^+ + 4\ NH_4^+$	-669 (-74)
$2\ Dimethylamine + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + H^+ + 2\ NH_4^+$	-220 (-73)
$4\ Methylamine + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + H^+ + 4\ NH_4^+$	-225 (-75)
$2\ Dimethylsulfide + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + H_sS$	-222 (-74)
$4\ 2\text{-}Propanol + CO_2 \rightarrow CH_4 + 4\ Acetone + 2\ H_2O$	-37
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31

* Values in brackets are for kJ per mole methane; the free energy from methanogenic reactions is commonly quoted as per mole methane.

Due to their limited range of substrates, methanogens depend on the metabolism of other prokaryotes to break down more complex organic compounds into suitable substrates. Hydrolytic, fermenting and syntrophic bacteria are involved in degrading organic macromolecules to methanogenic substrates such as acetate and hydrogen (Figure 1.2).

1.6 Culture and Isolation of Methanogens

The presence of methanogens in many environments has been predicted from PCR-based surveys. However, methanogens are difficult to isolate as many are not amenable to laboratory cultivation and some require long incubation times for growth to occur (Garcia *et al.*, 2000). As obligate anaerobes, methanogens are sensitive to low levels of oxygen (< 10 ppm)(Garcia *et al.*, 2000). Methanogens need to be cultured in pre-reduced media and require a redox potential of about –330 mV for growth (Lange & Ahring, 2001). The insensitivity of methanogens to certain antibiotics has been used to advantage in the culture of methanogens by eliminating contaminating bacteria (Garcia *et al.*, 2000).

The isolation of *Methanococcus vannielii* from San Francisco Bay by Stadtman and Barker in 1951 was the first reported isolation of a methanogen from a marine environment (Stadtman & Barker, 1951). To date (December 2011), 111 species of methanogens have been described (Euzéby, 2011) but not all have been isolated from the marine environment. In comparison to the 111 described species of methanogen there are a total of 435 species of *Archaea* and 10,680 species of *Bacteria* (Euzéby, 2011).

1.7 Objectives

The main objectives of this project were to further explore the culturable diversity of marine methanogens by:

- enriching methanogens from a variety of marine environments.
- obtaining pure methanogen cultures from these environment.
- characterizing and identifying isolated methanogens and comparing them to known isolates.
- testing for possible new methanogenic substrates.

The environments to be studied were:

Tidal-flat sediments from the Severn and Tamar Estuaries.
Mud volcano sediments from the Gulf of Cadiz, Eastern Mediterranean and Black Sea.
Sediment from the SMTZ of Aarhus Bay.
Sediment from the cool upper sedimentary layer of Guaymas Basin, which is underlain by a hydrothermal system.

Chapter 2
Materials and Methods

2.1 Site Descriptions

2.1.1 Aarhus Bay

2.1.1.1 Site

Aarhus Bay is a shallow (average water depth 15 m) semi-enclosed basin located in eastern Denmark between the Kattegat and the Great Belt (Figure 2.1). The pre-Quaternary surface of Aarhus Bay consists of Tertiary clays covered by glacial tills from the Main Weichselian advance (circa 20,000 years ago) and ice-marginal deposits from the succeeding Young Baltic advance (Jensen & Bennike, 2009). Post glaciation isostatic rebound reduced the sea level resulting in Aarhus Bay becoming dry land with bogs and lakes around 11,000 –12,000 years ago (Jensen *et al.*, 2002). The area was flooded by the Littorina Sea transgression (9,500 years ago) resulting in the deposition of marine sand and mud (Jensen & Bennike, 2009). Over the past 6,000 years a marine regression and changes in hydrographic conditions within Aarhus Bay have given rise to areas of erosion and sedimentation. The central basin is dominated by mud and at present sandy-mud is being deposited in the eastern central part of Aarhus Bay. The N-W and S-E parts of the Bay are characterised by mixed sediments and incised channels.

Free methane gas occurs (indicated by acoustic blanking on seismic profiles) in the central area of the bay where more than 4-5 m thickness of mud is present but gas also occurs at shallower depths (even at the seabed) in areas of high sedimentation rate, such as the eastern part of the bay (Jensen & Bennike, 2009).

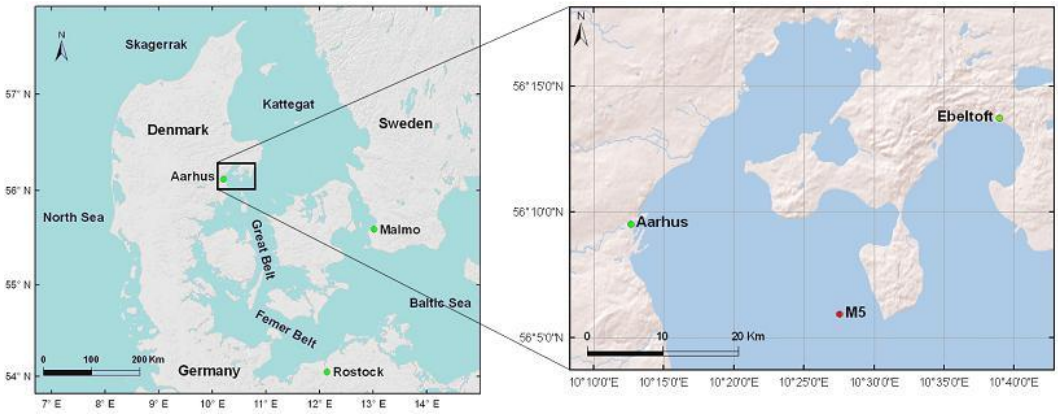


Figure 2.1 Location of Aarhus Bay (Denmark) and the sample site M5.

2.1.1.2 Sediment Samples

Two 12 cm diameter cores were taken from station M5 (Figure 2.1) in Aarhus Bay with a gravity corer during the METROL (Methane Flux Control in Ocean Margin Sediments) cruise HN04F in December 2004. The cores (165GC, 56º 6.201´N 10º27.467´E and 167GC, 56º 6.202´N 10º 27.467´E) were cut into 1 m sections and immediately 3 cm^3 samples were taken at various depths for methane analysis in order to identify the sulfate methane transition zone (SMTZ) (Webster *et al.*, 2011). The top 1 m of the cores contained the SMTZ and were sealed in gas-tight aluminium bags with a nitrogen atmosphere and stored at 4 ^oC (Webster *et al.*, 2011). In the laboratory the 0.4-0.8 mbsf sections (including the SMTZ) of the two cores were mixed and used to make a 25% (v/v) sediment slurry with anaerobic artificial marine medium (Webster *et al.*, 2011).

An additional core was taken in May 2010 (Britta Gribscolt, University of Aarhus) and was stored at 4 ^oC at Cardiff University until required.

2.1.2 Dvurechenskii MV (Black Sea)

2.1.2.1 Site

The Black Sea is the World's largest anoxic water body. It is bordered by Bulgaria, Georgia, Turkey, Romania, Russia and the Ukraine (Figure 2.2). It is 2.0 km to 2.2 km deep and anoxic below approximately 100 m. The Black Sea is composed of two basins, eastern and western, separated by the Andrusov Ridge.

The Black Sea formed is a result of back-arc extension associated with the subduction of the African and Arabian tectonic plates during the late Cretaceous (Nikishin *et al.*, 2003). During the Eocene, the Black Sea became subject to a compressional regime and subsequent subsidence resulted in the formation of the two basins.

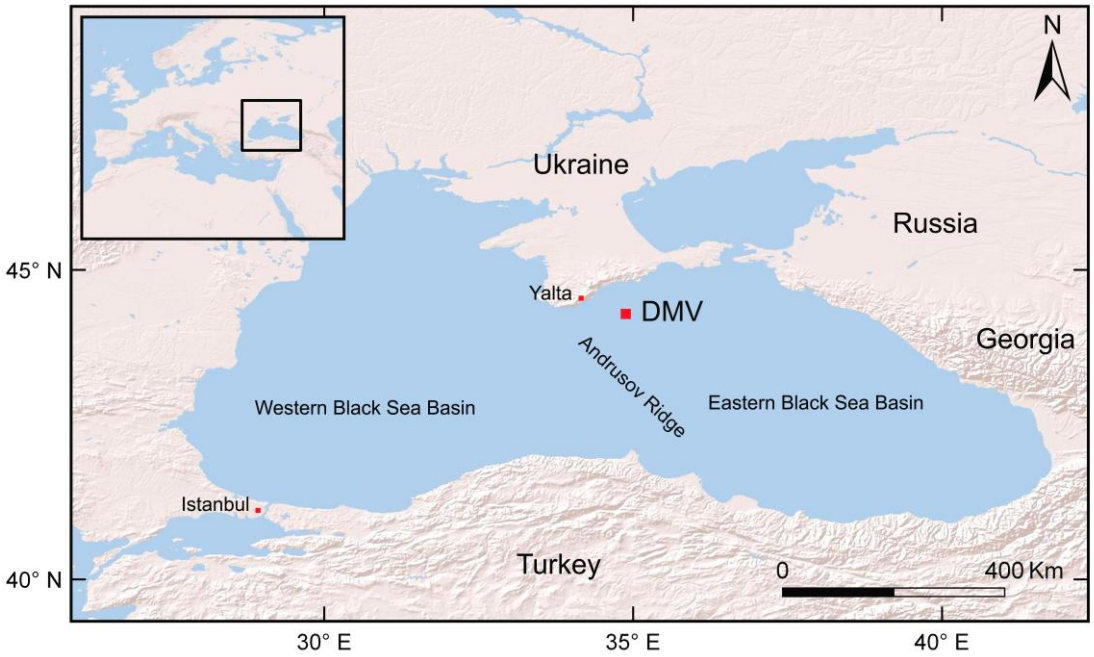


Figure 2.2 Dvurechenskii mud volcano is located in the Sorokin Trough of the Eastern Black Sea Basin.

The Sorokin Trough is a 150 km long, 45-50 km wide NE-SW oriented depression, which runs along the southeastern margin of the Crimean Peninsula and has water depths ranging from 600 to 2100 m. The Trough is considered to be a foredeep of the Crimean mountains (Krastel *et al.*, 2003). The Sorokin Trough contains numerous mud volcanoes. These are the result of compressional tectonics between the Shatsky Ridge in the east, the Tetyaev Rise in the south and the Crimean Peninsula in the north. The resultant overpressure causes the rise of the clay of the Oligocene to Lower Miocene Maikopian Formation through the overlying Pliocene to Quaternary sediments giving rise to many NE-SW trending diapiric ridges in the Sorokin Trough (Bohrmann *et al.*, 2003). The Dvurechenskii mud volcano is located in the Sorokin Trough, it is 800 m in diameter, 80 m in height, flat topped and at a water depth of 2055 m (Wallmann *et al.*, 2006).

An acoustic survey of mud volcanoes in the Sorokin Trough has shown the mud volcanoes, with the exception of Kazakov mud volcano, are located above mud diapers (Krastel *et al.*, 2003). The same survey imaged mudflows extending down the flanks of Dvurechenskii mud volcano. This extrusion of warm mud from depth has resulted in relatively high temperatures in the uppermost sediments; temperatures of 16.5 ^{o}C have been recorded in the axial and southern parts of the mud volcano summit (Bohrmann *et al.*, 2003) and 20.5 ^{o}C at 2 mbsf at the summit (Feseker *et al.*, 2009). In other areas the sediment temperature equals the bottom water temperature of approximately 9 ºC. At the mud volcano crater organic carbon content of the mud is between 0.9-1.3 % and is covered with a thin layer (average thickness 2 cm) of pelagic sediment (Wallmann *et al.*, 2006)

Gas samples taken from pressurized sediment cores were found to be dominated by methane and the molecular ratio, C_1/C_{2+} = 1760 (Feseker *et al.*, 2009), suggesting that the low molecular weight hydrocarbons located in shallow sediments of Dvurechenskii mud volcano were of microbial origin (Bernard *et al.*, 1976).

2.1.2.2 Sediment Samples

Sediment cores for this study were collected during cruise M72/2 of the *RV Meteor* in March 2007. Cores were taken from the geographic centre of Dvurechenskii mud volcano and from a reference site to the north of the mud volcano (Table 2.1, Figure 2.3). Onboard ship, samples were taken from various depths of the core using sterile 50 ml syringes with their luer ends removed (Dr Barry Cragg). After sub-sampling, the syringes were sealed with a rubber bung or Parafilm and stored at 4°C in a gas-tight bag under nitrogen for later laboratory use.

Table 2.1 Coordinates of the two Dvurechenskii MV sites from which the cores used in this study were taken during cruise M72/2 of the*RV Meteor* in March 2007.

Site	Coordinates		Water depth (M)
	Latitude	Longitude	
Geographic Centre - site 269	44^{o} 16.951´N	34^{o} 58.933´E	2051
Reference site - site 313	44^{o} 17.263´N	34^{o} 59.005´E	2075

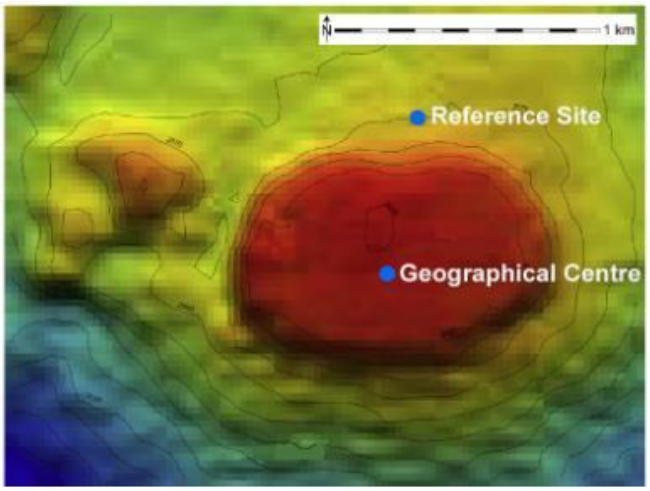


Figure 2.3 Locations of core sites at the Dvurechenskii mud volcano (contours at 5 m intervals). Modified after Krastel *et al*. (2003).

2.1.3 Eastern Mediterranean Mud Volcanoes

Mud volcanoes have been discovered at a number of sites in the Eastern Mediterranean including the Mediterranean Ridge and the Anaximander Mountains (Figure 2.4). The eastern Mediterranean is generally an area of deep oxygen and sulfate penetration into the seafloor resulting from low rates of organic matter mineralization and low microbial cell numbers (Boetius & Lochte, 1996).

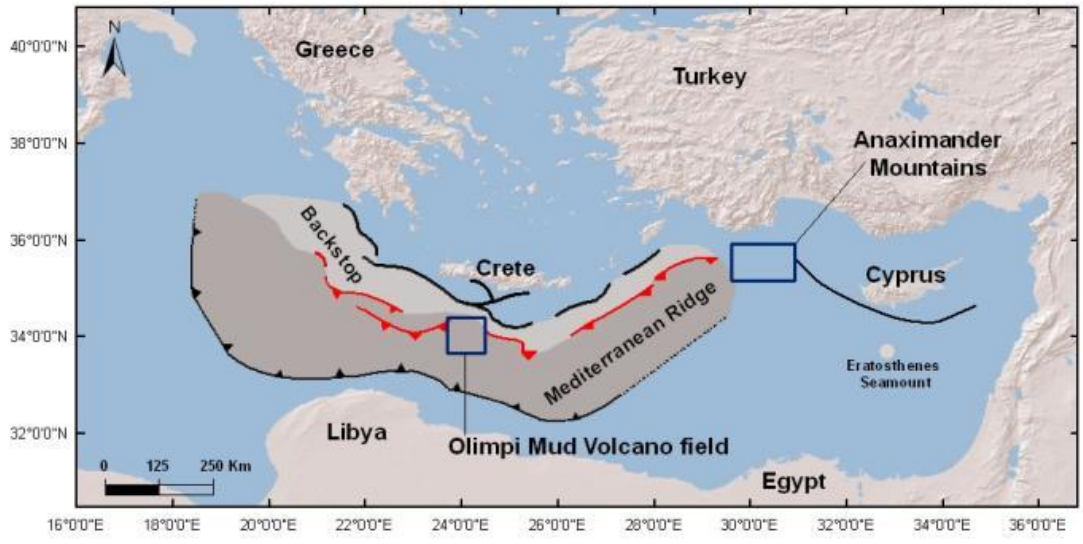


Figure 2.4 Location of the Olimpi mud volcano field and the Anaximander Mountains in the Eastern Mediterranean sea. Based on Zitter *et al*. (2005).

2.1.3.1 Amsterdam Mud Volcano

The Anaximander Mountains (Figure 2.4) are a complex of three seamounts (Anaximander, Anaximenes and Anaxogoras) located between the Cyprus and Hellenic arcs (Zitter *et al.*, 2005). They are considered to be southward rifted blocks of south-western Turkey (Woodside *et al.*, 1998). The mud volcanoes of the Anaximander Mountains where discovered by a multibeam bathymetric survey carried out by the *RV L'Atalante* operating as part of the Dutch ANAXIPROBE project. Amsterdam mud volcano, the most active mud volcano in the Anaximander Mountains, is located on the southern flanks of the Anaximenes seamount (Lykousis *et al.*, 2009). It is has a flat top, it is circular shaped with dimensions of 6 x 5 km and the summit is at 2025 m water depth (Lykousis *et al.*, 2009). There are extensive mud breccia flows with the clasts derived from Late Cretaceous limestones, Paleocene siliciclastic rocks, Eocene biogenic limestones and Miocene mudstones (Lykousis *et al.*, 2009). Methane hydrates were found at the Amsterdam mud volcano during the MEDINETH expedition in 1999 but brines have not been found in the Anaximander area (Charlou *et al.*, 2003). The transformation of smectite to illite in the deep subsurface would release intercrystalline water resulting in the decreased salinity of pore water in surface sediments throughout the Anaximander Mountain area (Haese *et al.*, 2006).

2.1.3.2 Napoli Mud Volcano

The Mediterranean Ridge is an arcuate swelling in the Eastern Mediterranean (Figure 2.4). It extends more than 1500 km from the Ionian deep basin in the west to the Levantine basin to the east with a width varying from 150 km to 300 km. Like other accretionary complexes the Mediterranean Ridge has many fluid seeps and mud volcanoes. Most of the mud volcanoes on the seafloor of the central Mediterranean Ridge are due to the convergence of the African and Aegean tectonic plates which resulted in the mobilization of overpressured sediments (Huguen *et al.*, 2004). Five mud volcano fields have been identified; the Cobblestone area, Pan-di-Zucchero, Prometheus II, Olimpi and the United Nations Rise (Huguen *et al.*, 2004).

The Olimpi field is located south of Crete and covers an area of more than 6000 km^2 (Huguen *et al.*, 2005). Napoli mud volcano, located along a backthrust fault, is one of Olimpi field's most prominent features. This mud volcano is lens shaped, 100 m high and flat topped (Huguen *et al.*, 2005). The flat top is covered with brine lakes, diagenetic carbonate patches, active fluid vents and bacterial mats (Huguen *et al.*, 2005). The brine, compared to normal Mediterranean seawater, is enriched in Cl^- and Na^+ but depleted in Br^-, Mg^{2+}, K^+, Ca^{2+}, Sr^{2+}, and S_4^{2-} (Charlou *et al.*, 2003). The Mediterranean Ridge is underlain by thick Messinian (Late Miocene) evaporites (Huguen *et al.*, 2004) and the significant enrichment of $\delta^{18}O$ in the brines compared to ambient seawater is consistent with an evaporated seawater origin (Charlou *et al.*, 2003). Cl^- concentrations up to nine times higher than sea water have been found previously (Haese *et al.*, 2006).

Methane is emitted by Napoli mud volcano generating plumes 100-200 m high, a methane concentration of 463 nmol kg^{-1} has been recorded close to the seafloor (1947 m depth) (Charlou *et al.*, 2003). In comparison the background concentration in the Olimpi field area is approximately 0.8-0.9 nmol kg^{-1}. The *in situ* near bottom conditions of depth, salinity and temperature (13 ^oC, 19–20 MPa) are close to the destabilization boundary for gas hydrates, however, gas hydrate destabilization was not identified (Charlou *et al.*, 2003).

Napoli mud volcano methane to ethane ratios (>1000) and $\delta^{13}C$ (methane) values −65.6‰PDB) indicate that the methane has a biogenic origin (Charlou *et al.*, 2003). The large variation in the CH_4/He ratio of brines in the Olimpi area indicates that the sources of CH_4 and He are not related and this is consistent with a biogenic origin for the methane (Charlou *et al.*, 2003). Biomarkers diagnostic of archaea have been detected in sediments from a seep on Napoli mud volcano (Pancost *et al.*, 2000). The most abundant compounds were diphytanylglycerol diether, a lipid diagnostic of archaea, and *sn*-3-hydroxyarchaeol; hydroxyarcheaol is predominantly found in methanogens (Koga *et al.*, 1998).

2.1.3.3 Amsterdam and Napoli Mud Volcano Cores

Sediment cores for this study were collected in October 2007 during Leg 1 of the MEDECO (Mediterranean Deep-Sea Ecosystems) cruise of the RV *Pourquoi*

Pas? Gravity cores MED-KUL3 (0.16 m depth) and MED-KUL6 (0.62 m depth) were taken from Napoli and Amsterdam mud volcanoes respectively. Core sampling was carried out at Cardiff University; samples were taken for culturing, and sulphate, chloride and methane measurements.

2.1.4 Gulf of Cadiz Mud Volcanoes

The Gulf of Cadiz is located to the west of the strait of Gibraltar between the Iberian Peninsula and Morocco at the boundary of the African and Eurasian tectonic plates (Figure 2.5). The tectonic history of this area is complex; the area has undergone periods of extension, compression and strike-slip motion since the Triassic (Maldonado *et al.*, 1999). The area is covered with Mio-Pliocene sedimentary deposits up to a thickness of 14 km (Thiebot & Gutscher, 2006).

This area has undergone several episodes of rifting since the Mesozoic (Maldonado *et al.*, 1999). During the Tortonian (late Miocene), a large olistrostome complex composed of eroded material from Spain and Morocco was emplaced in the Gulf of Cadiz in an accretionary wedge-type environment (Maldonado *et al.*, 1999; Medialdea *et al.*, 2004).

Numerous mud volcanoes were discovered in the Gulf of Cadiz during the UNESCO Training Through Research (TTR) cruises of *RV Prof. Logachev* during 1999-2000 (Kenyon *et al.*, 2000); (Gardner, 2001). The mud volcanoes are a consequence of the current compression regime (plate convergence 4 mm yr^{-1} (Argus *et al.*, 1989) leading to the dewatering of the sediments (Díaz-del-Río *et al.*, 2003). The mud volcanoes are generally located along major E-W trending strike-slip faults or at the intersection of these faults with arcuate faults resulting from the formation of the Gibraltar Arc (Pinheiro *et al.*, 2003). The Gulf of Cadiz also contains cold seeps, carbonate mounds and hydrocarbon-rich gas vents (Baraza & Ercilla, 1996; Gardner, 2001; Kenyon *et al.*, 2000).

Six mud volcanoes were investigated in this study; Bonjardim, Carlos Ribeiro Captain Arutyunov, Darwin, Meknes, and Mercator (Figure 2.8).

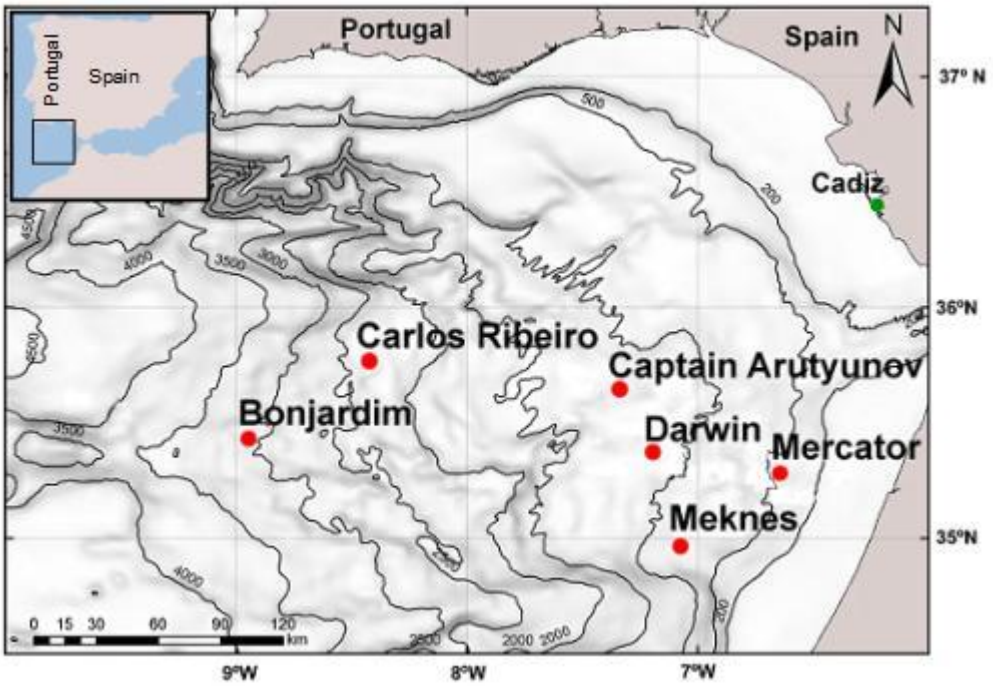


Figure 2.5 Mud volcanoes of the Gulf of Cadiz. Bonjardim and Carlos Ribeiro mud volcanoes (located in the Deep South Portuguese Field), and Captain Arutyunov, Darwin, Meknes, and Mercator mud volcanoes located in the Western Moroccan Field. Modified from Rodrigues *et al*. (2011).

2.1.4.1 Deep Portuguese Field Mud Volcanoes

Bonjardim (35°26.6'N, 09°00.0'W) was discovered during the TTR-10 cruise in 2000 (Kenyon *et al*., 2001). Bonjardim MV is located 3090 m below sea level and it as an approximately circular structure with a diameter of ~1 km and a height of ~100 m (Pinheiro *et al*., 2003). Gas hydrates have been recovered from the Bonjardim mud volcano (Pinheiro *et al*., 2003). Surface sediment (0-52 cm) methane concentrations were < 0.001 mM with a SMTZ at 45 to 70 cm below the sediment surface (Niemann *et al.*, 2006).

Carlos Ribeiro MV (35°47.217'N, 08°25.313'W) was also discovered during the TTR-10 cruise in 2000 (Kenyon *et al*., 2001). It is conical in shape with a height of ~ 80 m and a diameter of ~1.5 km and is at a water depth of 2200 m (Pinheiro et al, 2003). Sediments consisted of poorly sorted highly gasified mud breccia with a strong H_2S smell. The lack of a pelagic veneer indicated recent mud volcano activity (Pinheiro et al, 2003).

2.1.4.2 Western Moroccan Field Mud Volcanoes

Darwin MV (35°23.53'N, 07°11.48'W) was discovered in 2006 during the cruise of the *RS Charles Darwin* (Masson & Berndt, 2006). It is 40 m high at a water depth of 1100 m. Captain Arutyunov MV (35°39.67'N, 07°20.00'W) was discovered during the TTR-12 cruise of *RV Professor Logachev* in 2002 (Kenyon *et al*., 2003). It is at a water depth of 1320 m, has a conical shape ~2 km wide at its base, ~100 m high with a 300 m wide concentric crater on top (Kenyon *et al*., 2003). Surface sediment (0-20 cm) methane concentrations were < 0.001 mM, gas hydrates were found in the cores and a SMTZ at 25 to 40 cm below the sediment surface (Niemann *et al.*, 2006).

Mercator MV is one of eight mud identified volcanoes during a survey undertaken by the *RV Belgica* in 2002 and further studied during Leg 2 of TTR-12 (Van Rensbergen *et al.*, 2003). It is an asymmetrical mud volcano that lies at a water depth of 350 m, has a maximum height of 141 m and a maximum diameter of 2.5 km (Van Rensbergen *et al.*, 2005)

Meknes MV (34°59.10'N, 07°0.38'W) was discovered in 2004 during the TTR-14 cruise. Meknes MV is at a water depth of ~750 m.

2.1.4.3 Sediment Samples

During the JC10 cruise of RRS *James Cook* in May 2007 push cores were collected from Mercator, Darwin and Carlos Ribeiro mud volcanoes using ROV *Isis*. Other cores collected during the Cadiz 1 cruise of *RV Maria S. Merian*.

2.1.5 Severn and Tamar Estuaries

2.1.5.1 Portishead

Portishead is located on the Severn Estuary to the west of Bristol (Figure 2.6). The Severn Estuary is a funnel shaped estuary with extensive intertidal mudflats, sandflats and a tidal range (~15m) that is second only to that of the Bay of Fundy, Canada (Archer & Hubbard, 2003).

The area of interest is an intertidal mudflat at Woodhill Bay, Portishead (51º29´31´´N, 2º46´29´´W). The mudflat is backed by rock outcrops and saltmarshes and is sheltered from the strong currents of the main estuary channel (Whitehouse & Mitchener, 1998); at spring tides the speed of the current at nearby Avonmouth exceeds 1.5 m s^{-1} (Dyer, 1984). The mudflat bed level varies on a tide by tide basis, the bed level being approximately 10mm higher after a spring tide (Whitehouse & Mitchener, 1998). The annual change in bed level is of the order of 100 mm with seasonal storms, the subaerial environment and biostabilization all playing a significant role (Whitehouse & Mitchener, 1998).

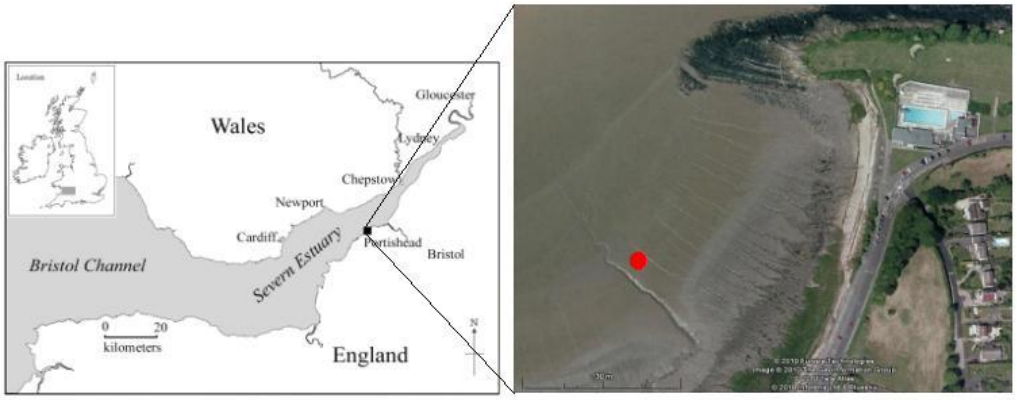


Figure 2.6 Severn Estuary intertidal flat at Woodhill Bay, Portishead. Photograph (Google Earth) looking north towards Battery Point. Sampling site indicated by the red dot.

2.1.5.2 Tamar

The River Tamar (Figure 2.7) is situated in southwest England (Long. 4°11′ W Lat. 50°23′ N) to the west of Plymouth. It begins in north Cornwall and flows southward to Plymouth Sound and is joined by two tributaries, the rivers Tavy and Lynher. The estuary is tidal from Gunnislake weir to Plymouth Sound with a spring tidal range at Devonport of 6·5 m (Miller, 1999). The estuary is a ria (drowned river valley) formed during the Flandrian (Holocene) marine transgression (Uncles *et al.*, 2003). The estuary is generally shallow with water depths of about 5 m, however, a deep-water channel (up to 40 m deep) extends downstream from Torpoint (Miller, 1999). The rising sea levels of the marine transgression led to the deposition of large quantities of muddy sediments (Uncles

et al., 2003). Extensive intertidal mudflats are located in the lower reaches of the estuary around the mouth of the Lynher and St John's Lake (Miller, 1999).

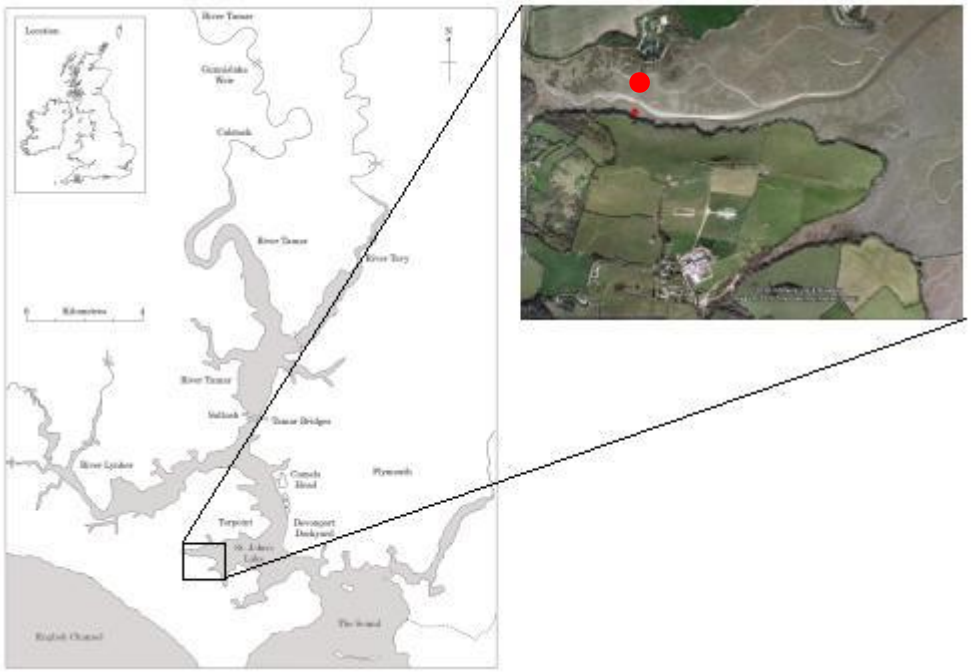


Figure 2.7 Location of the Tamar Estuary, S-W England and the sampling site (St. John's Lake, Cornwall). Red circle on aerial photograph indicates core collection site. Map modified from Miller (1999), aerial photograph Google Earth. Sampling indicated by a red dot.

2.1.5.3 Sediment Samples

Sediment cores (diameter 10 cm, depth 40 cm) were collected at low tide from the tidal flat at Portishead in June 2009 (Dr Erwan Roussel) and in December 2010 (Dr Erwan Roussel and Andrew Watkins) to provide sediment for enrichment under pressure. Cores were collected from Tamar in June 2009 (Prof. John Parkes and Dr Falko Mathes).

2.1.6 Guaymas Basin

2.1.6.1 Site

Guaymas Basin, which has a deep-sea hydrothermal system, is located in the central portion the Gulf of California (Figure 2.8). The Gulf of California is a

narrow sea formed by continental rifting located between Baja California and mainland Mexico at the boundary of the North American and Pacific tectonic plates. The rift system consists of long transform faults and short spreading centres (Lonsdale, 1989).

The spreading axis at Guaymas Basin is offset by a transform fault (Von Damm *et al.*, 1985) and is covered by sediments 1-2 km thick which are intruded 300-400 m below the sediment surface by magmatic sills (Lizarralde *et al.*, 2011). Guaymas Basin is one of the world's most biologically productive areas due to coastal ocean upwelling (Von Damm *et al.*, 1985). The sediments are enriched in organic carbon ranging from 3% to 12% (Lanza-Espino & Soto, 1999); the organic carbon content of ocean basin sediments is generally <0.6% (Barber, 1968).

Hydrothermal fluid vents at the sea floor by way of 'black smokers' at temperatures up to 350 °C or by way of slow seepages where temperatures at the sediment surface are at the ambient water temperature (2 °C) and increase to 50 °C at 40 cm depth (Jannasch *et al.*, 1989). Pyrolysis of sedimentary organic matter produces petroleum hydrocarbons (Simoneit & Lonsdale, 1982; Simoneit *et al.*, 1992) and methane (Lizarralde *et al.*, 2011).

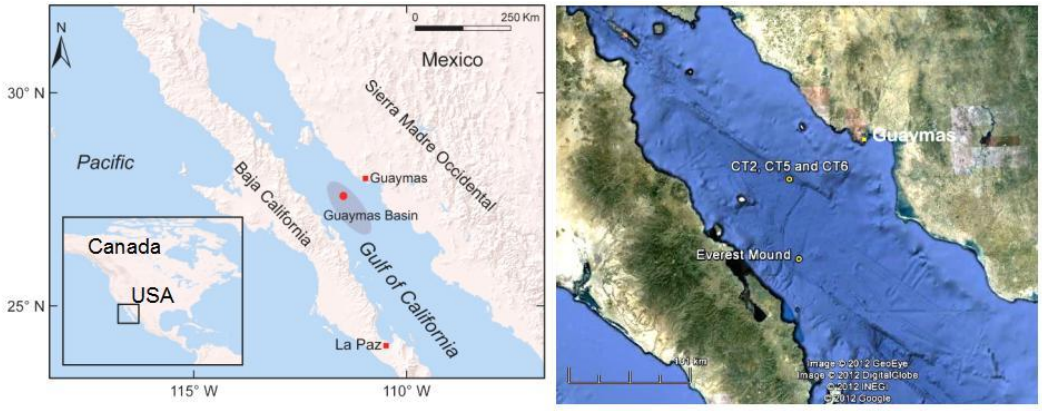


Figure 2.8 Location of sample sites CT2, CT5 and CT6, the northern trough of Guaymas Basin, Gulf of California, Mexico.

2.1.6.2 Sediment Samples

Cores were taken from sites CT2, CT5 and CT6 in the northern trough of Guaymas Basin (Table 2.2) and were supplied by Dr Laurant Toffin. Culture work was undertaken on samples taken from the upper 7 cm of the cores.

Table 2.2 Coordinates and water depths of the Guaymas Basin sample sites.

Site	Coordinates		Water depth (M)
	Latitude	Longitude	
CT2	27° 35′ 34.50″ N	111° 28′ 59.04″ W	1570
CT5	27° 35′ 35.24″N	111° 28 ′57.71″ W	1573
CT6	27° 35′ 34.94″ N	111° 28′ 58.95″ W	1570

2.2 Cultivation and Isolation

2.2.1 Preparation of Growth Media

Several types of anaerobic media were prepared in order to target methanogens or bacteria. Media was prepared in a specially designed glass vessel (Figure 2.9, after Widdel [1980]). This vessel has a gas inlet with a filter to allow flushing of the headspace with sterile gas and has an upside down conical shape to reduce differences in surface area to volume ratio, and hence in gas exchange, during dispensing. The screw cap inlets allow for the addition of temperature sensitive solutions after the vessel has been autoclaved and cooled and for release of gas during sparging.

The medium was expelled from the vessel by the gas-pressure in the headspace and was dispensed into vials/bottles under the glass bell to aid sterility.

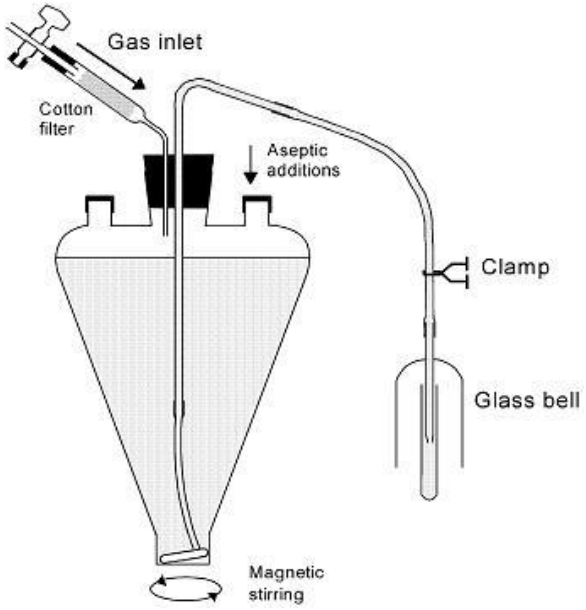


Figure 2.9 Set up for media preparation and dispensing, after Widdel (1980)

2.2.1.1 Methanogen Growth Media

Methanogens are extremely sensitive to oxygen and require strict anoxic conditions and pre-reduced media are essential for their growth and isolation. The composition of the sulphate free, bicarbonate buffered, FeS reduced, methanogen media used for enrichment and cultivation is given in Table 2.3. The composition of the trace element solution SL10 is given in Table 2.4 and the composition of the selenite-tungstate solution is given in Table 2.5. Once the medium had been made up the vessel was autoclaved at 121 ^{o}C for 60 minutes and removed from the autoclave at 75 ^{o}C. The vessel was then connected to an oxygen-free N_2/CO_2 (80/20, v/v, at 5 kPa) gas line and the headspace flushed for approximately 5 minutes to remove oxygen from the headspace. The vessel was then sealed and left to cool to room temperature under N_2/CO_2 (80/20, v/v, at 5 kPa). After cooling, the sterile solutions listed in Table 2.6 were added.

Table 2.3 Composition of basal marine medium, reduced salinity medium, freshwater medium and hypersaline media used for the cultivation of methanogens. Additions were made in the order given.

	Marine basal medium (36‰)	Reduced salinity medium (9‰)	Low salinity medium (4‰)	High salinity medium A* (126‰)	High salinity medium B* (252‰)
Distilled H_2O	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml
NaCl	24.3 g	6.0 g	3.0 g	90.6g	181.2 g
$MgCl_2 \cdot 6H_2O$	10 g	2.0 g	1.0 g	30.5 g	61 g
$CaCl_2 \cdot 2H_2O$	1.5 g	0.3 g	0.15 g	1.1 g	2.2 g
KCl	0.66 g	0.2 g	0.1 g	3.8 g	7.5 g
NH_4Cl (0.4 M)	1 ml	1 ml	1 ml	1 ml	1 ml
KH_2PO_4 (0.04 M)	1 ml	1 ml	1 ml	1 ml	1 ml
Trace elements SL10 (Table 2.4)	1 ml	1 ml	1 ml	1 ml	1 ml
SeWo (Table 2.5)	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
KBr (0.84 M)	1 ml	1 ml	1 ml	1 ml	1 ml
H_3BO_3 (0.4 M)	1 ml	1 ml	1 ml	1 ml	1 ml
$SrCl_2$ (0.15 M)	1 ml	1 ml	1 ml	1 ml	1 ml
NaF (0.07 M)	1 ml	1 ml	1 ml	1 ml	1 ml

* Sass et al. (2008)

Table 2.4 Composition of unchelated trace element solution SL10 (Widdel *et al.*, 1983). 1.5 g $FeCl_2 \cdot 4H_2O$ added to 10 ml HCl (25% solution), mixed, and distilled H_2O added. The following added and made up to a volume of 1000 ml. Autoclaved at 121°C for 30 mins.

Component	Amount (mg)
$CoCl_2 \cdot 6H_2O$	190
$MnCl_2 \cdot 4H_2O$	100
$ZnCl_2$	70
$Na_2MoO_4 \cdot 2H_2O$	36
$NiCl_2 \cdot 6H_2O$	24
H_2BO_3	6
$CuCl_2 \cdot 2H_2O$	2

Table 2.5 Composition of selenite-tungstate solution SeWo (Widdel & Bak, 1992). Autoclaved at 121°C for 30 mins.

Component	Amount (mg)
Distilled H_2O	1000 ml
NaOH	400
$Na_2SeO_3 \cdot 5H_2O$	6
$Na_2WO_3 \cdot 2H_2O$	8

Table 2.6 Solutions to be added to medium after autoclaving and cooling to room temperature.

Component	Amount (ml)
$NaHCO_3$ solution (1 M)	30
10 vitamin solution (Table 2.7)	2
Reducing agents: $FeCl_2$ (1 M in 0.1 M HCl)	0.5
Na_2S (1 M)	1.2

The $NaHCO_3$ solution was prepared in a screw top bottle and autoclaved within a plastic container in case the bottle breaks due to overpressure in the bottle. The $FeCl_2$ solution was autoclaved at 121 ºC, whilst the sulfide solution was not heated to temperatures above 109 ºC during sterilisation.

Table 2.7 Composition of the 10 vitamins solution (Balch *et al.*, 1979), filter-sterilised into a foil wrapped bottle and stored at 4°C.

Component	Amount (mg)
Distilled H_2O	1000 ml
4-Aminobenzoic acid	25
D(+) Biotin	10
Nicotinic Acid	25
Ca-D(+) Pantothenate	25
Pyridoxine-dihydrochloride (vitamin B6)	50
Folic acid	10
Lipoic acid	25
Riboflavine	25
Thiamine-hydrochloride	25
Cyanocobalamine (vitamin B12)	5

15 minutes was allowed for the iron and sulphide to react before checking the pH. If necessary, the pH was adjusted to 7.2-7.4 with sterile Na_2CO_3 (1 M) or sterile HCl (1 M) if necessary. The medium was then dispensed into sterile screw top bottles or tubes, which were filled to the top. The black FeS precipitates serve also as a redox indicator. Media without the black precipitate were re-oxidised and were discarded.

The medium was dispensed into serum bottles or anaerobic tubes (Bellco Glass Inc, New Jersey, USA), which have serum vial style necks. Sterile glass pipettes were used for transfer. Additions to the media and media dispensing was

conducted in a laminar flow cabinet. The anaerobic tube was gassed with N_2/CO_2 (80/20 v/v), via a sterile gassing cannula (Figure 2.10), both before and during medium transfer. Once the required volume has been dispensed the cannula is brought to the neck of the tube and a sterile butyl rubber stopper placed so that the cannula was against the tube wall. The cannula was removed whilst pressure was applied to the top of the stopper; the stopper was secured by twisting it into position and the tube sealed with an aluminium crimp.

The gassing cannula consisted of an autoclavable glass syringe with a rubber stopper and gas line attached to one end and a metal cannula (19G, bent through 90 ^{o}) attached to the other end (Figure 2.10). The syringe was filled with cotton wool to filter sterilize the gas and was autoclaved before use and the cannula was flame-sterilised during use.

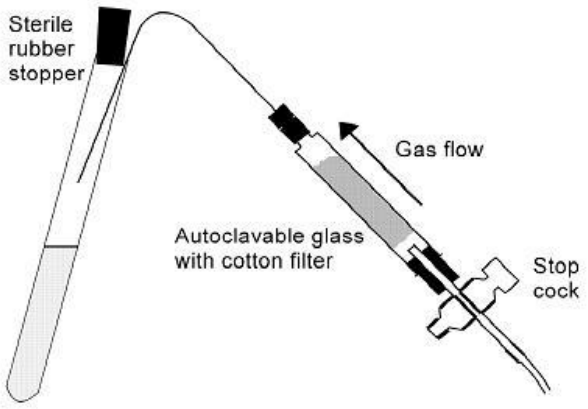


Figure 2.10 Cannula used to gas the tube headspace whilst dispensing medium.

2.2.1.2 Bacterial Growth Medium

Bacterial growth medium (for testing the purity of methanogen cultures) was prepared by adding peptone (0.06 g l^{-1}) and yeast extract (0.03 g l^{-1}) to the basal marine medium (Table 2.3) before autoclaving. The preparation of the medium was otherwise the same as that for methanogen basal marine medium.

2.2.2 Preparation of Growth Substrates

Anoxic substrate solutions were made up as sterile solutions and kept in sealed sterile serum bottles. Water (MilliQ) for making up substrates was bubbled with nitrogen for 10 minutes to remove oxygen and the headspace of the bottle gassed with nitrogen. The bottles were sealed with butyl rubber stoppers and crimped before autoclaving at 121 ºC for 30 minutes.

Hexadecane was made up as a 5% (v/v) solution in 2,2,4,4,6,8,8-heptamethyiononane.

Substrates was added to the anaerobic tubes/ serum bottles using a needle and 1 ml syringe. Before an anoxic solution was drawn up the syringe was purged with nitrogen, gas was drawn into the syringe and expelled a number of times. For growth with hydrogen the N_2/CO_2 headspace of culture tubes and bottles was replaced with H_2/CO_2 by flushing.

2.2.3 Enrichment Cultures

Aarhus Bay Enrichment cultures with acetate (10 mM), methylamine (10 mM) or H_2/CO_2 (80:20, 0.1 MPa) as substrates were prepared from unamended slurry stored at 10 °C (prepared from core 165GC by Dr Gordon Webster). All enrichment cultures were incubated at 10 °C, the *in situ* temperature. A second set of enrichment cultures (25% v/v sediment slurry) prepared using sediment from a core collected in May 2010 were prepared with a wider range of potential substrates (10mM acetate, 10mM betaine, 10 mM choline, 10 mM formate, H_2/CO_2 (80:20, 0.1 MPa), 10 mM methanol, 1 mM methanethiol, 10 mM methylamine and 10 mM 2-propanol). All of the second set of enrichment cultures was incubated at 25 ºC.

Four sets of Amsterdam MV enrichment cultures were prepared: two sets using sediment from core sections 3 (492-493 cm depth) and 10 (142-143 cm depth) with marine medium and acetate (12 mM), benzoate (1.5 mM), hexadecane (3.4 mM), H_2/CO_2 (80:20, 0.1 MPa), methanol (6 mM), methylamine (5 mM) as

substrates, and a substrateless control. Additional sets of enrichment cultures were also made up using sediment from the same sections with the same range of substrates but using a reduced salinity medium (9‰, Table 2.3). All were incubated at 14 ºC, the *in situ* temperature. All enrichment cultures were set up using a 10% v/v sediment slurry.

The Dvurechenski and Gulf of Cadiz enrichment cultures had been prepared in advance of this study by Dr Barry Cragg. For Dvurechenski and Gulf of Cadiz mud volcanoes, enrichment cultures were made up with sediment slurry (25% v/v) and a range of potential substrates added. The substrates used were acetate (12 mM), benzoate (1.5 mM), hexadecane (3.4 mM), H_2/CO_2 (80:20, 0.1 MPa), methanol (6 mM), methylamine (5 mM). All were incubated at 10 ºC. All enrichment cultures were checked for methane production before the start of this study by Dr Barry Cragg.

Guaymas Basin enrichment cultures for sediment from cores CT2, CT5 and CT6 were prepared with marine medium (Table 2.3) with acetate (10 mM), H_2/CO_2 (80:20, 0.1 MPa), methylamine (10 mM) and choline (10 mM). In addition, freshwater enrichment cultures with methylamine (10 mM) were prepared using sediment from cores CT2, CT5 and CT6. All were incubated at 25 ºC. All enrichment cultures were set up using a 25% v/v sediment slurry.

Six sets of enrichment cultures were prepared for Napoli MV; two sets using sediment from core sections 5 (60-65 cm depth) and 8 (0-5 cm depth) with marine medium and acetate (12 mM), benzoate (1.5 mM), hexadecane (3.4 mM), H_2/CO_2 (80:20, 0.1 MPa), methanol (6 mM), methylamine (5 mM) as substrates and a substrateless control. Additional sets were made up using sediment from the same two sections with the same range of substrates with hypersaline media A (126‰ salinity, Table 2.3) and B (252‰ salinity, Table 2.3). All were incubated at 14 ºC, the *in situ* temperature. All enrichment cultures were set up using a 10% v/v sediment slurry.

Portishead marine medium enrichment cultures with acetate (10 mM), H_2/CO_2 (80:20, 0.1 MPa) or methylamine (10 mM) as substrates were set up for sediment taken from 0-2 cm, 10-15 cm and 30-35 cm depth of a sediment core collected in June 2010. In addition to the marine medium cultures, freshwater medium (Table 2.3) enrichment cultures with H_2/CO_2 were prepared for each of the three depths. All enrichment cultures were incubated at 25 ºC. All Portishead enrichment cultures were set up using a 10% v/v sediment slurry except for the elevated hydrostatic pressure enrichment cultures(Section 2.4).

Tamar enrichment cultures were set up with sediment from depths of 2-4 cm and 19-21 cm with marine medium (Table 2.3) with acetate (10 mM), H_2/CO_2 (80:20, 0.1 MPa) and methylamine (10 mM) as substrates. All enrichment cultures were incubated at 25 ºC. All Tamar enrichment cultures were set up using a 10% v/v sediment slurry.

2.2.4 Isolation of Methanogens

2.2.4.1 Isolation of Methanogens using Anaerobic Agar Plates

As the FeS-containing medium cannot be autoclaved, the agar needs to be prepared separately. The agar was prepared in a wide-mouth Duran bottle (Schott, Mainz, Germany). For 100 ml of FeS reduced medium, 48 ml of 4 % agar was prepared in a 250 ml Duran bottle. The additional volume of the bottle was required for the medium, which was added later. The headspace was flushed with N_2 before autoclaving, sealed with a screw cap and autoclaved at 121 ºC for 30 minutes. The Duran bottle was removed from the autoclave at 80 ºC and immediately transferred to an anaerobic cabinet along with the medium, which had been heated to approximately 60 ºC in a water bath. The medium and substrate were added to the Duran bottle, mixed and plates poured.

A few drops of the enrichment culture were dropped onto the surface of the agar plate and streaked. Plates were incubated or stored in anaerobic jars or gas impermeable incubation bags with an Anaerocult sachet (Merck, Darmstadt,

Germany, Figure 2.11). The Anaerocult sachet absorbs oxygen and releases carbon dioxide together with some hydrogen (Dr Henrik Sass, unpublished data).

Figure 2.11 Left: anaerobic jar with an Anaerocult sachet. Right: anaerobic bag with an Anaerocult sachet (AnaeroCult A mini, Merck).

2.2.4.2 Shake Tubes – Deep-agar Dilution Series

Colonies were also isolated in agar shake tubes, which unlike Petri dishes do not require the use of an anaerobic cabinet or anaerobic bags. Using the gassing technique described for dispensing of medium into vials, 9 ml of medium was transferred to an anaerobic tube. The tube was inoculated with 1 ml of the enrichment culture using a syringe and needle and serial dilutions made from 10^{-1} to at least 10^{-5}. Substrate was then added using a 1 ml syringe and needle.

1.0 g purified agar and 25 ml medium was added to a 50 ml serum bottle sealed with a butyl rubber stopper, the headspace was gassed with nitrogen and the tubes were autoclaved at 105 ^{o}C for 30 minutes. After autoclaving the bottle was kept in a water bath at 60 ^{o}C until required. 4 ml of the agar was injected into the inoculated anaerobic tube using a 19G needle. Whilst injecting, the tube was repeatedly inverted to mix. The tube was then placed in ice water until the agar had solidified and then incubated.

A stereomicroscope was used to remove colonies from shake tubes (Figure 2.12). The tube was opened and a flame-sterilized gassing cannula (N_2/CO_2) was immediately inserted to maintain anoxic conditions. A needle and syringe was

used to remove the colony. The removed colony was injected into an anaerobic tube with liquid medium. The tube from which the colony has been removed was resealed with a sterile stopper for further incubation.

Figure 2.12 Observing colonies in an agar shake tube prior to removing colonies with a needle for inoculation. The cannula supplies N_2/CO_2 to maintain anoxic conditions.

2.2.4.3 Dilution Series

Liquid dilution series was used for the isolation of some methanogens. Balch tubes were inoculated "to extinction" with 1 ml of the enrichment culture using a syringe and needle and serial dilutions made from 10^{-1} to at least 10^{-8}. This was repeated at least three times. This method was used to isolate *Methanococcoides burtonii* DSM6424^T (Franzmann *et al.*, 1992)

2.2.4.4 Antibiotics

For the isolation of methanogens in bacteria free cultures a cocktail of four antibiotics, Ampicillin (0.5 mg/ml), Kanamycin (0.5 mg/ml), Penicillin G (0.5 mg/ml) and Vancomycin (0.1 mg/ml), were used in conjunction with both dilution series and deep agar dilution series. Antibiotics were prepared with MilliQ water and filter sterilized (0.20 µm syringe filter, Sartorius Stedim Biotech, France) and injected into a sterile serum bottle containing nitrogen.

After purification, isolates were kept in medium without antibiotics. These antibiotics have been used in the isolation of a number of methanogens (Whitman *et al.*, 2006).

2.2.4.5 Purity Checks

Methanogen cultures were checked for purity using phase contrast microscopy, epifluorescence microscopy, PCR using bacterial primers and inoculation into bacterial growth medium containing yeast extract, peptone and glucose (Section 2.2.1.2).

2.3 Characterisation of Isolated Methanogens

2.3.1 Substrates

Once isolated, methanogens were tested for growth on a range of substrates. *Methanococcoides* strains were tested with acetate (10 mM), betaine (10 mM), choline (10 mM), cyclopentanol (10 mM), dimethylglycine (10 mM), DMS (10 mM), ethanol (10 mM), formate (10 mM), H_2/CO_2 (80:20, 0.1 MPa), isopropanol (10 mM), methanethiol (1 mM), methanol (10 mM), methyliodide (1 mM), methylamine (10 mM), *N*-monomethylethanolamine (10 mM), *N*,*N*-dimethylethanolamine (10 mM), propionate (10 mM), pyruvate (10 mM), sarcosine (10 mM) and tetramethylammonium (10 mM). *Methanococcus* strains were tested with acetate (10 mM), betaine (10 mM), choline (10 mM), cyclopentanol (10 mM), ethanol (10 mM), formate (10 mM), H_2/CO_2 (80:20, 0.1 MPa), 1-propanol (10 mM), isopropanol (10 mM), methanol (10 mM), methylamine (10 mM), propionate (10 mM) and pyruvate (10 mM).

2.3.2 Temperature Range

The growth response of the pure cultures to temperature was determined in a temperature gradient block (Figure 2.13). The temperature gradient systems consist of aluminium blocks with holes drilled into them to produce incubation cells that can accommodate two 10 ml glass vials or a single 20 mm glass vial. They were heated at one end by means of heating rods, and cooled at the opposite end by a circulating chiller unit. The temperature gradient across the aluminium block was changed by adjusting the heating. The aluminium blocks were encased in wood and insulated with foam. The temperature was measured at twelve points along the gradient by means of fixed temperature sensors and the temperature of

individual incubation cells could be calculated from the linear gradient. The temperature gradient systems were manufactured in the workshops of the Department of Earth Sciences, University of Bristol.

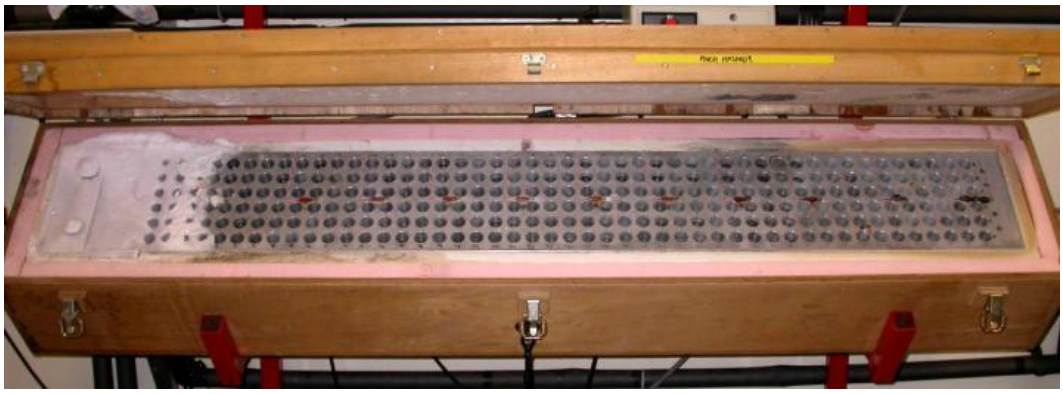


Figure 2.13 One of three temperature gradient system used for incubating pure methanogen cultures. Manufactured at the Earth Science department workshops, University of Bristol. Based on the design of Isaksen *et al*. (1994).

10 ml vials had 4 ml of marine medium with 10 mM methylamine added to them in the anaerobic cabinet to ensure oxygen free headspaces and a 5% inoculum was added to each vial. Duplicate cultures were incubated at a temperature range from 0 to 40 ^{o}C at 3–5 degree intervals. Headspace samples for gas chromatography were taken every 48 h.

2.3.3 Calculation of Q_{10} and E_a

An Arrhenius plot (plot of the log of the specific growth rate against the reciprocal of temperature) have a straight line portion whose slope is $-E_a/ 2.303R$ where R is the gas constant ($8.31 Jk^{-1} mol^{-1}$). E_a can be used to calculate the Q_{10} value, Equation 2.1

$$Q_{10} = \exp\left[\frac{E_a \cdot 10}{R \cdot T(T+10)}\right] \qquad \text{(Equ. 2.1)}$$

Where R is the gas constant and T is the temperature in Kelvin.

2.3.4 Na+ Concentration Range

The Na^+ range of methanogen isolates was tested at Na^+ concentrations of 0.05, 0.1, 0.3, 0.5, 0.7, 0.9 and 1.1 mM. Media with various concentrations of Na^+ were prepared by adding sterile anoxic stock solution (NaCl 290 g l^{-1}) to medium without NaCl or to normal salinity medium.

2.3.5 pH Range

The pH range was tested in intervals from pH 5.5 to 9.5 (5.5, 6.0, 6.4, 6.9, 7.4, 7.9, 8.5, 8.9 and 9.5). Basal medium was used for pH 7.4. Values below pH 7.4 were produced by adding sterile Na_2CO_3 to medium of pH 5.5 until the required value was reached. The pH 5.5 medium was prepared by omitting $NaHCO_3$ from basal medium and cooling it under a CO_2 headspace. Values above pH 7.4 were prepared by adding sterile Na_2CO_3 to basal medium until the required value was reached.

2.3.6 Cell Lysis

Susceptibility to sodium dodecyl sulfate (SDS) lysis was determined by dispensing two 2 ml samples of a turbid methanogen culture into centrifuge tubes (Boone & Whitman, 1988). One of the tubes had a concentrated solution of SDS added to give a final concentration of 0.01 g of SDS per litre. Susceptibility to lysis by a hypotonic solution (distilled water) was determined after centrifugation (Boone & Whitman, 1988). A sample of a turbid methanogen culture was taken and dispensed into two 2 ml centrifuge tubes and centrifuged (10000 x g for 2 minutes). The supernatants were removed, one of the samples had the cell pellet resuspended in distilled water and the other cell pellet was resuspended in uninnocculated medium. In both cases, the susceptibility to lysis was indicated by a reduction of the turbidity of the cell suspension in distilled water or SDS within 10 minutes in comparison with the control sample. Samples were also checked by microscopy.

2.3.7 Gram Staining

Gram staining was undertaken using the method of Murray *et al*. (1994) with commercially available solutions (BDH Laboratories, Poole, Dorset, UK). Methanogen cells were heat fixed on glass slides, stained with crystal violet stain

for one minute and washed with an indirect stream of distilled water. The sample was then stained with Gram's iodine solution for one minute before being washed with ethanol. Finally, the safranin counter-stain was applied for 10 seconds, washed off with distilled water and dried. The slide was examined using bright-field microscopy and comparison was made with samples of Gram negative and Gram positive bacteria.

2.3.8 Protein Concentration

Cell protein was extracted using NaOH (Hippe *et al.*, 1979), 1 ml of cell suspension was centrifuged, washed twice with 1 ml of 0.9% NaCl then resuspended in 0.5 ml of 1 M NaOH for 10 minutes. The sample was then heated for 15 s at 100°C and then cooled on ice. The sample was again centrifuged and diluted 1 in 10 with reverse osmosis water. Standards were made using bovine serum albumin (Sigma-Aldrich) in 1 M NaOH; they were diluted with reverse osmosis water to give final protein concentrations of 1-10 μg ml^{-1}. Protein concentrations were determined using the Bradford method. 0.5 ml of the Bradford reagent (Sigma-Aldrich) was added to 0.5 ml of the sample in a cuvette. The absorbance at 595 nm was measured using a spectrophotometer (Varian, Cary 50 Probe Spectrophotometer) and protein concentrations calculated from a calibration curve.

2.4 High Pressure Studies

Incubation of pure methanogen cultures (Chapter 7) and sediment slurries (Chapter 4) at elevated pressures was conducted in a stainless steel pressure vessel consisting of two end caps, with O-ring seals and a main cylinder filled with water (Figure 2.14). Liquid cultures were sealed in completely filled serum vials with butyl rubber stoppers. Serum vials were placed in the vessel, which was then completely filled with water and sealed with a lid secured by 10 steel bolts. The vessel was pressurized using a gas-powered pump (Heypac, Slough, UK); the pressure was transferred to the samples via the flexible butyl rubber stopper.

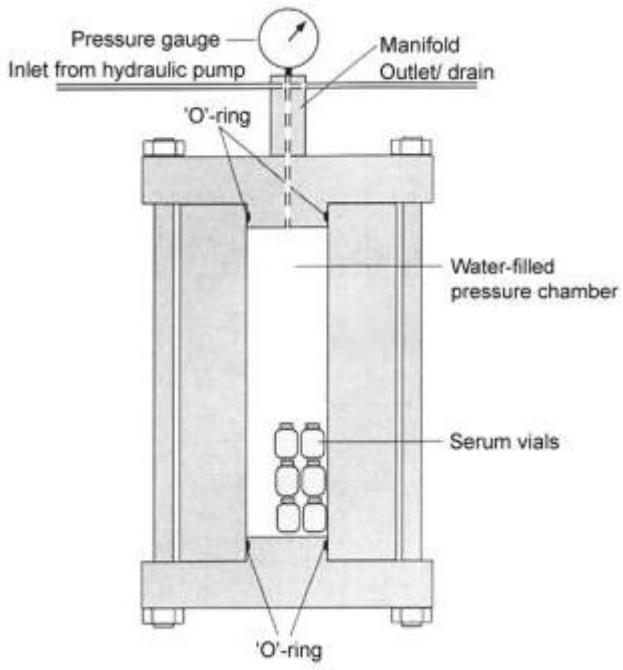


Figure 2.14 Cross-section of vessel used for cultivation at high pressures (Parkes *et al.*, 1995). Pressure vessel designed and constructed by Fred Wheeler, department workshop, Earth Sciences, University of Bristol.

For high-pressure cultivation of pure methanogen cultures, pressures of 10, 20, 40, 50, 60 and 70 MPa were used. A 5% inoculum was added to 250 ml serum bottle containing FeS reduced medium to which methylamine had been added (5 mM final concentration). The inoculated medium was magnetically stirred and medium dispensed into 25 ml serum bottles. For each pressure interval tested, four serum bottles were incubated under pressure and four incubated at atmospheric pressure. For all pressures, the incubation time was four weeks at 25 °C. Samples were taken at the start and end of the incubation for anion and cation determination.

A methanogen enrichment culture study was undertaken using elevated hydrostatic pressure and sediment from Portishead (see Figure 2.6 for the location). 25% (v/v) sediment slurries were made using sediment (15-35 cm depth and mixed before the slurry was made) that had been taken from a core obtained the previous day and stored at 4 °C overnight. The sediment slurry was dispensed into three 200 ml serum bottles and each bottle was amended with acetate, formate or methylamine as a substrate. The contents of the bottles were dispensed into 30 ml serum bottles (leaving no headspace) to give six bottles with

each substrate. Three replicates of each substrate were incubated at 0.1 and 40 MPa at 25 ºC for 90 days in a custom-made pressure vessel (Figure 2.14). At the end of the incubation period, samples were taken to determine acetate, ammonium, formate, methane, methylamine and sulphate concentrations (Section 2.5) and for archaeal 16S rRNA gene PCR-DGGE (Section 2.8.3).

2.5 Chromatography

Samples for anion and cation chromatography were prepared by centrifuging 200 ul culture samples at 5000 x g for 5 minutes.

2.5.1 Anion Chromatography

Anion chromatography was used to detect the presence of volatile fatty acids, chloride and sulfate. A Dionex® Ionchromatographic System ICS-2000 equipped with a Dionex® AS50 autosampler (Dionex, Camberley, UK) was used. Chromatographic separation was achieved by two Ionpac® AS15 columns in series with a Dionex® Anion Self-Regenerating Suppressor unit (ASRS®-ULTRA II 4-mm) in combination with a Dionex® DS6 heated conductivity cell. The instrument was calibrated using five standards of increasing concentrations of the compounds of interest. All calibrations with a calibration coefficient of less than 0.99 were rejected, the calibrations were linear.

2.5.2 Cation Chromatography

Methylated amines (methylamine, dimethylamine, trimethylamine, ethanolamine, *N*-methylethanolamine, and *N*, *N*, *N*-trimethylethanolamine) as well as ammonium were analyzed using a Dionex® DX-120 ion chromatograph (Dionex, Camberley, UK) fitted with an Ionpac® CS16 column coupled with a CSRS 300 4 mm suppressor and a conductivity detector. The chromatograph was connected to an AS40 automated sampler. Separation of sample constituents was achieved using methanesulphonic acid eluent (32 mM) at a flow rate of 0.75 ml min^{-1}. *N*, *N*-dimethylethanolamine was analysed using methanesulphonic acid eluent at 25 mM, which necessitated the running of duplicate samples. All cation chromatograph carried out by Dr Erwan Roussel. The instrument was calibrated using five standards of increasing concentrations of the compounds of interest. All

calibrations with a calibration coefficient of less than 0.99 were rejected, the calibrations were linear.

2.5.3 Gas Chromatography

Headspace gases were analyzed using a modified Perkin Elmer/Arnel Clarus 500 Natural Gas Analyser (Perkin Elmer, Waltham MA, USA). The system consists of a flame ionization detector (FID) and two combined thermal conductivity detectors (TCD). The oven temperature was 110 °C with the FID at 250 °C and the TCD at 150°C. Helium was used as the carrier gas for the FID and argon for the TCD. Three standard mixed gases (Scott Speciality Gases, Plumsteadville, Pennsylvania, USA) were used to calibrate the system. The FID was used to measure methane and the TCD to measure hydrogen and carbon dioxide. Gas samples were injected into the system via a 100 µl sampling loop. The detection limit for methane was 0.5 ppm, 70 ppm for hydrogen and 50 ppm for carbon dioxide.

The total amount of methane was calculated by measuring the headspace methane concentration and using Henry's law constant to determine the amount of methane in solution; K_{Hcc} = 0.03 (dimensionless), Dean (1992).

2.6 Microscopy

Microscopy was undertaken with a light microscope (Axioskop, Zeiss, Germany) equipped with a x 100 magnification phase-contrast oil immersion objective x10 eyepiece. Methanogen specific F_{420} autofluorescence was visualized on the same microscope using a UV light source, exciter filter BP 390-440 and, beam splitter FT 460. Images were taken using a Nikon Coolpix 4.0 megapixel digital camera attached to the eyepiece of the microscope.

2.7 Methanogen Growth Rate

The specific growth rate (µ) during the exponential growth of methanogens was calculated using linear regression from plots in which the logarithm of total methane accumulated was plotted against time and taking into account the methane produced by the inoculum (Powell, 1983).

2.8 Molecular Methods

2.8.1 DNA Extraction

DNA was extracted using the nexttec™ DNA Isolation System for Bacteria (Nexttec Biotechnologie, Leverkusen, Germany). 350 µl Prep Buffer was added to a nexttec™ cleanColumn, which was incubated for 5 minutes at room temperature before centrifuging at 350x g for 1 minute to remove excess buffer. 1.0 ml of bacterial /methanogen culture was transferred to a 1.5 ml reaction tube and centrifuged at 6000x g for 1 minute and the supernatant discarded. 90 µl Buffer B1, 10 µl lysozyme solution and 20 µl RNase A solution were added to the pellet to which was resuspend by vortexing and incubated at 60 °C for 10 minutes. 2.5 µl Buffer B2, 87.5 µl purified water and 10 µl Buffer B3, 2.5 µl of DTT (dithiothreitol**)** and 2 µl EDTA (ethylenediaminetetraacetic acid) were then added and incubated at 60 °C for 30 minutes. The lysate was transferred to the nexttec™ cleanColumn and incubated for 3 minutes at room temperature before centrifuging at 700x g for 1 minute. The cleanColumn was then discarded. The DNA yield was assessed using gel electrophoresis (1.2% w/v agarose gel stained with Sybersafe and run at 100 V for 30 min in 1 x TAE buffer) with 10 µl aliquots of each DNA extract. The TAE buffer was composed of 40 mM Tris base, 20 mM acetic acid and 0.5 M EDTA (pH 8.0). The gel was run with HyperLadder I DNA quantification marker (Bioline, London, UK). The remaining DNA extraction was frozen at -20 °C until required.

2.8.2 Polymerase Chain Reaction (PCR)

PCR was undertaken using a commercial PCR kit (Bioline). PCR was carried out under aseptic conditions to minimize contamination. Composition of the reagent mixtures is given in Table 2.8; primers are specified in Table 2.9. 1 µl of DNA was added to each reaction mixture as a template. PCR reactions were carried out in a PTC-200 Gradient Cycler DNA Engine (MJ Research, Boston, USA) with the appropriate PCR program (Table 2.10). Each PCR run included a positive control using DNA extracted from pure cultures and a negative PCR control where molecular grade water (Severn Biotech Ltd, Kidderminster, UK) was substituted for the DNA template. The DNA product was analyzed using gel electrophoresis

(1.2% w/v agarose gel stained with Sybersafe and run at 100 V for 30 min in 1 x TAE buffer) with 5 μl aliquots of each DNA product. The TAE buffer was composed of 40 mM Tris base, 20 mM acetic acid and 0.5 M EDTA (pH 8.0). The gel was run with 5 μl HyperLadder I DNA quantification marker (Bioline, London, UK). Gels were imaged using a Gene Genius Bio Imaging System (Syngene, Cambridge, UK) and photographs were taken using GeneSnap software (Syngene, Cambridge, UK). GeneTool software (Syngene, Cambridge, UK) was used to estimate DNA concentrations by comparing the intensity of DNA bands on the image with the intensity of HyperLadder I bands of known DNA concentration.

Table 2.8 Quantities of reagents used for a single PCR reaction.

PCR reagent	Volume (μl) for a single 50 μl reaction
10x PCR reaction buffer (Bioline)	10
dNTP mix (25 mM)	1
Forward primer (20 pmol μl^{-1})[a]	1
Reverse primer (20 pmol μl^{-1})[a]	1
$MgCl_2$ (50 mM)[b]	1.5
Bovine serum albumin (10 mg ml^{-1})	1
Taq DNA Polymerase (Bioline)	0.25
Molecular grade water	33.25

[a] Table 2.11.
[b] when ME1 and ME2 primers were used the amount of $MgCl_2$ was doubled and a corresponding reduction in the amount of molecular grade water was made

Table 2.9 PCR Primers used in this study.

Target gene	Primer Name	Primer sequence (5´ - 3´)	Procedure	Reference
Bacteria 16S rRNA	27F	AGAGTTTGATCMTGGCTGAG	PCR	Lane (1991)
	907R	CCGTCAATTCMTTTRRAGTTT		Lane (1991)
Archaea 16S rRNA	1AF	TCYGKTTGATCCYGSCRGAG	PCR	Embley *et al*. (1992)
	U1492R	GGTTACCTTGTTACGACTT		Lane (1991)
Archaea 16S rRNA	109F	ACKGCTCAGTAACACGT	PCR	Großkopf *et al.* (1998)
	958R	YCCGGCGTTGAMTCCAATT		Delong (1992)
Archaea mcrA	ME1f	GCM ATGCARATHGGWATGTC	PCR	Hales *et al*. (1996)
	ME2r	TCATKGCRTAGTTDGGRTAGT		Hales *et al*. (1996)
Bacteria 16S rRNA	357F-GC	CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGGCCTACG GGAGGCAGCAG	PCR-DGGE	Muyzer *et al*. (1993)
	518R	ATTACCGCGGCTGCTGG		
Archaea 16S rRNA	SAf [1]	(i) CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGG CCTAYGGGGCGCAGCAGG (ii) CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGG CCTACGGGGCGCAGAGGG	PCR-DGGE	Nicol *et al*. (2003)
	PARCH519R	TTACCGCGGCKGCTG		Øvreas *et al*. (1997)
Bacteria 16S rRNA	357F-GC-M13R	CAGGAAACAGCTATGAC GGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG	DGGE band reamplification	O'Sullivan *et al.* (2008)
	518R-AT-M13F	GTAAAACGACGGCCAG TAAATAAAATAAAAATGTAAAAAAA TTACCGCGGCTGCTGG		O'Sullivan *et al.* (2008)

Table 2.9 (continued) PCR Primers used in this study

Target gene	Primer Name	Primer sequence (5´ - 3´)	Procedure	Reference
Archaea 16S rRNA	SAF-GC-M13R[2]:		DGGE band reamplification	O'Sullivan *et al.* (2008)
	SAaf-GC-M13R	CAGGAAACAGCTATGAC GGGCGGGGCGGGGGCACGGGGGG CCTACGGGGCGCAGCAGG		
	SAbf-GC-M13R	CAGGAAACAGCTATGAC GGGCGGGGCGGGGGCACGGGGGG CCTATGGGGCGCAGCAGG		
	SAcf-GC-M13R	CAGGAAACAGCTATGAC GGGCGGGGCGGGGGCACGGGGGG CCTACGGGGCGCAGAGGG		
	PARCH519r-AT-M13R	GTAAAACGACGGCCAG TAAATAAAATAAAAATGTAAAAAA TTACCGCGGCKGCTG		O'Sullivan *et al.* (2008)
	M13F	GTAAAACGACGGCCAG	Sequencing	

[1] SAf is a mixture of (i) and (ii) in a 2:1 molar ratio (Nicol *et al.*, 2003).
[2] SAf-GC-M13R is a mixture of SAaf-GC-M13R, Sabf-GC-M13R and SAcf-GCM13R in a 1:1:1 molar ratio.
An underlined sequence correspond to a GC-clamp or AT linker.
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

Table 2.10 PCR thermocycler conditions.

Primer pair	Initial denaturation step	Denaturing, annealing and extension cycle	Final elongation step	Reference
27F & 907R	95 °C for 2 min	94 °C for 30 s 52 °C for 30 s 72 °C for 90 + 1 s per cycle } x 35	72 °C for 5 min	Lane (1991)
ME1 & ME2	94 °C for 5 min	94 °C for 45 s 50 °C for 45 s 72 °C for 1.45 min } x 35	72 °C for 10 min	Newberry *et al.* (2004)
27F & U1492R 109F & 958R	95 °C for 2 min	94 °C for 30 s 52 °C for 30 s 72 °C for 90 + 1 s per cycle } x 35	72 °C for 5 min	Lane (1991)
SAf & Parch519r	95 °C for 5 min	94 °C for 30 s 53.5 °C for 30 s 72 °C for 1 min } x 5 92 °C for 30 s 53.5 °C for 30 s 72 °C for 1 min } x 35	72 °C for 5 min	Nicol *et al*. (2003)
357F-GC & 518R	95 °C for 5 min	94 °C for 30 s 55 °C for 30 s 72 °C for 1 min } x 10 92 °C for 30 s 52 °C for 30 s 72 °C for 1 min } x 25	72 °C for 10 min	Muyzer *et al*, (1993)

2.8.3 DGGE

A nested PCR approach was used to amplify 16S rRNA genes from methanogens prior to DGGE. This involved the primer combination 109F and 958R followed by SAF-GC (GC-clamp at the 5´ end) and Parch519r (Table 2.9). SAf was used in order to obtain greater coverage of potential sequences. SAf consists of SAf1 and SAf2; SAf1 has a single degeneracy (see Table 2.9). A 2:1 mixture of the two gives rise to a 1:1:1 ratio of the different sequences (Nicol *et al*., 1993). A nested PCR approach was also used for bacterial 16S rRNA genes; a combination of 27F and 1492R followed by 357F-GC and 518R (Table 2.9). PCR was undertaken as described above with Thermocycler conditions as given in Table 2.11.

The Dcode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used to separate PCR products. 16 x 16 cm glass plates contained a 1 mm thick 8% (w/v) polyacrylamide gel (Acrylogel 2.6 solution, acrylamide:*N,N'*-methylenebisacrylamide; 37:1) with a denaturant gradient of 30- 60% (16S rRNA gene fragments) or 25% -50% (*mcrA* gene fragments). 7 M urea and 40% (v/v) formamide defined a 100% denaturing condition. The gradient was made using a 50 ml Gradient Mixer (Fisher Scientific UK Limited, Loughborough, UK) and run in 1x TAE buffer (40mM Tris base, 20 mM acetic acid and 0.5 M EDTA (pH 8.0)). Electrophoresis was initially carried out at 80 V for 10 min then increased to 200 V and allowed to run for 5 hours. The gels were stained for 20 minutes in 200 ml 1x TAE buffer with 20 µl SYBRGold nucleic acid gel stain (Invitrogen Corporation, Carlsbad, CA, USA) then viewed under UV light. Gels were imaged using a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

Individual DGGE bands were excised with a sterile scalpel blade from the gels under UV illumination. Excised bands were placed in sterile PCR tubes and washed twice for 10 min in 100 µl molecular grade water. After washing, the bands were allowed to air-dry prior to crushing and re-suspension in 10-15 µl of molecular grade water, depending on the band's intensity. Excised bands were left at 4 ^{o}C for at least 1 h to allow the DNA to elute before the bands were frozen at –20 ^{o}C.

Upon thawing the supernatant was used as a template for re-amplification using an improved DGGE band sequencing method (O'Sullivan *et al.*, 2008). This method involved reamplifying bands with a modified version of the original DGGE primers (modified bacterial primers 357F-GC-M13R and 518R-AT-M13F, modified archaeal primers SAf-GC-M13R and PARCH519r-AT-M13F). Sequencing of these PCR products with the unmodified M13F primer (see Table 2.9) allowed the ends of the relatively short DGGE band sequences to be fully sequenced.

2.8.4 Purification of DNA

Amplified DNA was purified using Microcon YM-50 centrifugal filter devices (Millipore Corporation, Bedford, MA, USA). The Microcon filter was placed in a Microcon tube; the DNA was added to the filter and washed with 500 µl molecular grade water (Severn Biotech Ltd, Kidderminster, UK) before centrifuging at 4,500 x *g* for 10 minutes. The filter was then inverted and placed in a fresh Microcon tube, 40 µl molecular grade water added and centrifuged at 800 x *g* for 4 minutes. The DNA concentration was then quantified spectrophotometrically at an absorbance of 260 nm using a NanoDrop^{TM} spectrophotometer (NanoDrop Products, Wilmington, DE, USA) at the Cardiff School of Biosciences Molecular Biology Support Unit.

2.8.5 DNA Sequencing

The Cardiff School of Biosciences Molecular Biology Support Unit carried out the sequencing using an ABI 3130xl Prism Genetic Analyzer automated capillary sequencer (Applied Biosystems, Forster City, CA) with BigDye terminator chemistry (Version 3.1).

2.8.6 Phylogenetic Analysis

Sequence chromatographs were analyzed using computer program MEGA version 5.05 (Tamura *et al.*, 2011). Closest matches to sequences were identified by searching the National Centre for Biological Information (NCBI) database using BLASTN (http://www.ncbi.nlm.nih.gov/blast). Nucleotide sequence alignments were carried out using the ClustalX routine within MEGA version 5.05. Phylogenetic trees were constructed using neighbour-joining with the Jukes and Cantor algorithm in MEGA version 5.05. Bootstrap analysis was also undertaken using MEGA version 5.05 using 1000 replicates.

2.9 Methanogen Type Species

All three *Methanococcoides* type species (*M. methylutens* DSM 11745^T, *M.burtonii* DSM 6242^T and *M.alaskense* DSM 17273^T) used in this study were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

Chapter 3
Enrichment Cultures

3.1 Introduction

This chapter describes methanogen enrichment cultures using sediment taken from Aarhus Bay (Denmark), Dvurechenski mud volcano (Sorokin Trough, Black Sea), two eastern Mediterranean mud volcanoes (Amsterdam and Napoli), six Gulf of Cadiz mud volcanoes (Bonjardim, Captain Arutynov, Carlos Ribeiro, Darwin, Meknes and Mercator), two estuaries (Portishead and Tamar) and Guaymas Basin (Baja California). The Gulf of Cadiz and Dvurechenski MV enrichment cultures were prepared in advance of this project by Dr Barry Cragg.

The enrichment cultures were set up using media described in Section 2.2. As acetate was not added as a carbon source to the medium used in this study (it was only added to some enrichment cultures as a substrate) then any requirement the methanogens would have had for acetate could have been met by acetogenic or other bacteria in the enrichment cultures.

All enrichment cultures were monitored at regular intervals for methane production and those producing methane were subcultured. The fifth subculture of each enrichment culture was examined using F_{420} autofluorescence microscopy and if the culture contained only one morphology then DNA was extracted for 16S rRNA gene sequencing (Section 2.8). The resultant sequencing chromatographs were examined for the presence of more than one sequence; in the majority of cases the sequencing chromatographs suggested that the enrichment cultures contained a single type of methanogen. If more than one morphology of methanogen was seen or the chromatographs contained more than one sequences, they were separated by PCR-DGGE and DGGE bands were excised for sequencing (Section 2.8).

In addition to these enrichment cultures, thermal gradient enrichment cultures (prepared by Dr Barry Cragg) contained a combination of potential substrates (acetate, methylamine and H_2/CO_2) and were incubated at temperatures ranging from 3 to 77 ºC at 3 to 7 ºC intervals. 10, 25, 38, 45 and 55 ºC thermal enrichment cultures was made available for subculturing by this study and subcultures were made with medium containing either acetate, H_2/CO_2 or methylamine.

Section 3.2 contains the enrichment culture results and porewater data, Section 3.3 describes the enrichment culture methanogens (methanogens of the same genus from different sites are compared with each other and the type strains of the species), and Section 3.4 contains a discussion of the sites

3.2 Enrichment Culture Results

A summary of the successful enrichment cultures and the 16S rRNA gene sequencing results are given in Table 3.1. Details of any unsuccessful enrichment cultures are given in the sections for each of the locations investigated in this study.

Table 3.1 Details of successful methanogen enrichment cultures with BLASTN search results (16S rRNA gene sequences). The identity of most methanogens was determined by direct 16S rRNA gene sequencing. Cultures marked with an asterisk had the identity of the methanogens determined by 16 rRNA gene PCR-DGGE as these cultures have been seen to contain more than one morphology using phase-contrast and autofluorescence microscopy.

Location	Enrichment culture	Depth (cm)	Temp (°C)	Substrate	Salinity	BLASTN nearest match (Accession No.)	% identity	BLASTN nearest cultured match (Accession No.)	% identity
Aarhus Bay	AM-H	40-80	25[1]	H_2/CO_2	Marine	*Methanogenium cariaci* JR1^T (M59130)	99	*Methanogenium cariaci* JR1^T (M59130)	99
	AM-M[2]	40-80	10	Methylamine	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	AM-MeOH[2]	40-80	10	Methanol	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	AB-Ace	40-80	25	Acetate	Marine	*Methanosarcina semesiae* MD1^T (NR_028182)	99	*Methanosarcina semesiae* MD1^T (NR_028182)	99
	AB-Bet	40-80	25	Betaine	Marine	*Methanococcoides alaskense* AK-5^T (NR_029122)	99	*Methanococcoides alaskense* AK-5^T (NR_029122)	99
	AB-Cho	40-80	25	Choline	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	100	*Methanococcoides methylutens* TMA-10^T (FR733669)	100
	AB-H	40-80	25	H_2/CO_2	Marine	Uncultured *Methanogenium* sp. PSW29 (EF043530)	99	*Methanogenium cariaci* JR1^T (M59130)	98
	AB-M	40-80	25	Methylamine	Marine	*Methanococcoides alaskense* AK-5^T (NR_029122)	99	*Methanococcoides alaskense* AK-5^T (NR_029122)	99
	AB-MeOH	40-80	25	Methanol	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
Guaymas Basin	CT2-M	0-7	25	Methylamine	Marine	*Methanococcoides methylutens* NaT1 (Y16946)	99	*Methanococcoides methylutens* NaT1 (Y16946)	99
	CT5-M	0-7	25	Methylamine	Marine	*Methanococcoides methylutens* NaT1 (Y16946)	99	*Methanococcoides methylutens* NaT1 (Y16946)	99
	CT6-M	0-7	25	Methylamine	Marine	*Methanococcoides alaskense* AK-5^T (NR_029122)	99	*Methanococcoides alaskense* AK-5^T (NR_029122)	99
	CT2-Cho*	0-7	25	Choline	Marine	Uncultured clone Zeebrugge_A57 (HM598511)	95	*Methanosarcina baltica* AK-5^T (AY663809)	93
						Uncultured clone Zeebrugge_A57 (HM598511)	97	*Methanosarcina baltica* AK-5^T (AY663809)	94
						Uncultured clone BCMS-19 (AJ579734)	97	*Methanococcoides methylutens* TMA-10^T (FR733669)	96
						Uncultured clone Zeebrugge_A57 (HM598511)	99	*Methanosarcina baltica* AK-5^T (AY663809)	97
	CT5-Cho	0-7	25	Choline	Marine	*Methanococcoides methylutens* MM1 (FJ477324)	100	*Methanococcoides methylutens* MM1 (FJ477324)	100
	CT2-A	0-7	25	Acetate	Marine	*Methanosarcina semesiae* MD1^T (NR_028182)	99	*Methanosarcina semesiae* MD1^T (NR_028182)	99
	CT5-H	0-7	25	H_2/CO_2	Marine	*Methanogenium cariaci* JR1^T (M59130)	99	*Methanogenium cariaci* JR1^T (M59130)	99
	CT5-MFr	0-7	25	Methylamine	Fresh	Uncultured clone LGH02-C3-9-A-5 (JQ407246)	99	*Methanosarcina semesiae* MD1^T (NR_028182)	98
Gulf of Cadiz									
Darwin MV	DMV-H	11	25*	H_2/CO_2	Marine	*Methanogenium cariaci* JR1^T (M59130)	99	*Methanogenium cariaci* JR1^T (M59130)	99
	DMV-M[2]	11	10	Methylamine	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	DMV-MeOH	11	10	Methanol	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
Darwin south rim	DMVSR-M	18	10	Methylamine	Marine	*Methanococcoides alaskense* AK-5^T (NR_029122)	99	*Methanococcoides alaskense* AK-5^T (NR_029122)	99
Cpt Arutynov MV	CAMV-M	52	10	Methylamine	Marine	Uncultured archaeon clone AMSMV-5-A48 (HQ588654)	100	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
Carlos Ribeiro MV	CRMV-M	55	10	Methylamine	Marine	*Methanococcoides burtonii* DSM 6242^T (X65537)	99	*Methanococcoides burtonii* DSM 6242^T (X65537)	99
Carlos Ribeiro MV	CRMV-MeOH	55	10	Methanol	Marine	*Methanococcoides burtonii* DSM 6242^T (X65537)	99	*Methanococcoides burtonii* DSM 6242^T (X65537)	99
Meknes MV	MKMV-M[2]	77	10	Methylamine	Marine	Uncultured archaeon clone AMSMV-5-A48 (HQ588654)	100	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	MKMV-MeOH[2]	77	10	Methanol	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
Mercator MV	MMVREF-M	20	10	Methylamine	Marine	*Methanococcoides burtonii* DSM 6242^T (X65537)	99	*Methanococcoides burtonii* DSM 6242^T (X65537)	99
Napoli MV	NMV-M[2]	0-5	14	Methylamine	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	NMV-MeOH[2]	0-5	14	Methanol	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99

Table 3.1 Continued: Details of successful methanogen enrichment cultures with BLASTN search results (16S rRNA gene sequences). The identity of most methanogens was determined by direct 16S rRNA gene sequencing. Cultures marked with an asterisk had the identity of the methanogens determined by 16 rRNA gene PCR-DGGE as these cultures have been seen to contain more than one morphology using phase-contrast and autofluorescence microscopy.

Location	Enrichment culture	Depth (cm)	Temp (ºC)	Substrate	Salinity	BLASTN nearest match (Accession No.)	% identity	BLASTN nearest cultured match (Accession No.)	% identity
Portishead	P2A	0-2	25	Acetate	Marine	Uncultured clone FeLiveControl_A_79 (GQ356882)	99	*Methanosarcina semesiae* MD1^T (NR_028182)	98
	P2H[2]	0-2	25	H_2/CO_2	Marine	*Methanococcus maripaludis* KA1 (AB264796)	100	*Methanococcus maripaludis* KA1 (AB264796)	100
	P2HFr	0-2	25	H_2/CO_2	Fresh	*Methanosaeta harundinacea* 8Ac^T (NR_043203)	98	*Methanosaeta harundinacea* 8Ac^T (NR_043203)	98
	P2M[2]	0-2	25	Methylamine	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	P10A	10-15	25	Acetate	Marine	Uncultured clone FeLiveControl_A_79 (GQ356882)	99	*Methanosarcina semesiae* MD1^T (NR_028182)	98
	P10H[2]	10-15	25	H_2/CO_2	Marine	*Methanococcus maripaludis* KA1 (AB264796)	100	*Methanococcus maripaludis* KA1 (AB264796)	100
	P10HFr	10-15	25	H_2/CO_2	Fresh	Uncultured clone: HDBW-WA01	99	*Methanoculleus submarinus* Nankai-1^T (AF531178)	98
	P10M[2]	10-15	25	Methylamine	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	P30A	30-35	25	Acetate	Marine	Uncultured clone FeLiveControl_A_79 (GQ356882)	99	*Methanosarcina semesiae* MD1^T (NR_028182)	98
	P30H	30-35	25	H_2/CO_2	Marine	*Methanococcus maripaludis* KA1 (AB264796)	100	*Methanococcus maripaludis* KA1 (AB264796)	100
	P30M[2]	30-35	25	Methylamine	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	P30HFr	30-35	25	H_2/CO_2	Fresh	*Methanoculleus submarinus* Nankai-1^T (AF531178)	98	*Methanoculleus submarinus* Nankai-1^T (AF531178)	98
Dvurechenski MV (Black Sea)	BSREF-M[2]	35	10	Methylamine	Marine	*Methanococcoides alaskense* AK-5^T (NR_029122)	99	*Methanococcoides alaskense* AK-5^T (NR_029122)	99
	BSREF-MeOH[2]	35	10	Methylamine	Marine	*Methanococcoides alaskense* AK-5^T (NR_029122)	99	*Methanococcoides alaskense* AK-5^T (NR_029122)	99
Tamar	T3A	2-4	25	Acetate	Marine	*Methanosaeta harundinacea* 6Ac^T (CP003117)	97	*Methanosaeta harundinacea* 6Ac^T (CP003117)	97
	T3E*	2-4	25	Ethanol	Marine	*Methanoculleus marisnigris* DSM 1498^T (NR_028228)	87	*Methanoculleus marisnigris* DSM 1498^T (NR_028228)	87
						Methanobacterium alcaliphilum WeN4^T (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^T (NR_028228)	100
	T3F*	2-4	25	Formate	Marine	*Methanobacterium formicium* DM 1535^T (AF028689)	100	*Methanobacterium formicium* DM 1535^T (AF028689)	100
						Methanoculleus submarinus Grau-1 (JN004139)	100	*Methanoculleus submarinus* Grau-1 (JN004139)	100
						Methanobacterium alcaliphilum WeN4^T (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^T (NR_028228)	100
	T3H*	2-4	25	H_2/CO_2	Marine	*Methanobacterium formicium* DM 1535^T (AF028689)	100	*Methanobacterium formicium* DM 1535^T (AF028689)	100
						Methanosarcina mazei Gö1 (AE008384)	100	*Methanosarcina mazei* Gö1 (AE008384)	100
						Methanospirillum hungatii JF-1^T (NR_042789)	97	*Methanospirillum hungatii* JF-1^T (NR_042789)	97
						Methanobacterium alcaliphilum WeN4^T (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^T (NR_028228)	100
	T3M[2]	2-4	25	Methylamine	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	T3AFr	2-4	25	Acetate	Fresh	*Methanoplanus limicola* M3^T (M59143)	99	*Methanoplanus limicola* M3^T (M59143)	99
	T3HFr	2-4	25	H_2/CO_2	Fresh	*Methanoculleus submarinus* Nankai-1^T (AF531178)	97	*Methanoculleus submarinus* Nankai-1^T (AF531178)	97
	T3E(38)*	2-4	38	Ethanol	Marine	*Methanosarcina mazei* Gö1 (AB065296)	100	*Methanosarcina mazei* Gö1 (AB065296)	100
						Uncultured clone SWA-0301-08 (JN398028)	99	*Methanospirillum hungatii* JF-1^T (NR_042789)	97
						Methanobacterium alcaliphilum WeN4^T (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^T (NR_028228)	100
	T3F(38)	2-4	38	Formate	Marine	*Methanobacterium alcaliphilum* WeN4^T (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^T (NR_028228)	100
	T20A	19-21	25	Acetate	Marine	*Methanosarcina semesiae* MD1^T (NR_028182)	97	*Methanosarcina semesiae* MD1^T (NR_028182)	97
	T20H*	19-21	25	H_2/CO_2	Marine	*Methanobacterium alcaliphilum* WeN4^T (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^T (NR_028228)	100
						Uncultured clone SWA-0301-08 (JN398028)	99	*Methanospirillum hungatii* JF-1^T (NR_042789)	97
	T20M[2]	19-21	25	Methylamine	Marine	*Methanococcoides methylutens* TMA-10^T (FR733669)	99	*Methanococcoides methylutens* TMA-10^T (FR733669)	99
	T20AFr*	19-21	25	Acetate	Fresh	*Methanosarcina acetivorans* C2A ^{T} (AE1010299)	100	*Methanosarcina acetivorans* C2A ^{T} (AE1010299)	100
						Uncultured clone SW0301-08 (JN398028)	99	*Methanospirillum hungatii* JF-1^T (NR_042789)	97
	T20HFr	19-21	25	H_2/CO_2	Fresh	*Methanospirillum hungatei* JF1^T (AY196683)	96	*Methanospirillum hungatei* JF1^T (AY196683)	96

3.2.1 Aarhus Bay

The successful and unsuccessful methanogen enrichment cultures, using sediment from the original and the later acquired core (core 165GC and the May 2010 core, respectively) are summarized in Table 3.2. For core 165GC, the sediment used was from a depth of 0.4-0.8 m and the sulphate concentration was below 5 mM (Figure 3.1). As a 25% v/v sediment slurry was prepared (Section 2.1.1.2), the starting sulphate concentration was below 1.25 mM; no further measurements were made. The porewater concentrations of sulphate for the May 2010 core were not measured.

Table 3.2 Summary of successful and unsuccessful Aarhus Bay enrichment cultures. Core 165GC enrichment cultures incubated at 10 °C, core May 2010 enrichment cultures incubated at 25 °C.

Core	Depth (m)	Enrichment culture	Substrate	Methanogenesis
165GC	0.4-0.8	AM-A	Acetate (10 mM)	-
		AM-H	H_2/CO_2 (80:20, 0.1 MPa)	+[1]
		AM-MeOH	Methanol (10 mM)	+
		AM-M	Methylamine (10 mM)	+
May 2010	0.4-0.8	AB-A	Acetate (10 mM)	+
		AB-Bet	Betaine (10 mM)	+
		AB-Cho	Choline (10 mM)	+
		AB-F	Formate (10 mM)	-[2]
		AB-H	H_2/CO_2 (80:20, 0.1 MPa)	+
		AB-MeOH	Methanol (10 mM)	+
		AB-Mthiol	Methanethiol (10 mM)	+[3]
		AB-M	Methylamine (10 mM)	+
		AB-2Prop	2-propanol (10 mM)	+[3]

[1] Acetogenesis when incubated at 10 °C, methanogenesis when incubated at 25 °C.
[2] Acetogenesis rather than methanogenesis.
[3] Subcultures produced methane very slowly.

Of the original set of enrichment cultures (sediment from core 165GC, Section 2.1.1.2), the acetate enrichment culture (AM-A) did not produce methane and the H_2/CO_2 enrichment culture (AM-H) produced acetate rather than methane (Table 3.2). When this enrichment culture was incubated at 25 °C methanogenesis did occur; however, growth was slow. The addition of yeast extract and acetate did not stimulate growth nor did increasing the incubation temperature to 35 °C. No attempt at isolation was made but the dominant methanogen was identified as a member of the genus *Methanogenium* (Table 3.1). Methane was produced by the methylamine and methanol enrichment culture (AM-M and AM-MeOH, Table 3.1) and members of the genus *Methanococcoides* were subsequently isolated using shake tubes (strains AM1, AM2 and AM3, see Chapters 6 and 7 for a full description of these strains).

A second set of enrichment cultures (prefixed with AB in Table 3.1), from the core taken in May 2010 (Section 2.1.1.2) were prepared with a wider range of potential substrates: acetate (10 mM), betaine (10 mM), choline (10 mM), formate (10 mM), H_2/CO_2 (80:20, 0.1 MPa), methanol (10 mM), methanethiol (1 mM), methylamine (10 mM) and 2-propanol (10 mM). All enrichment cultures produced methane although formate performed poorly in comparison with the others, formate was consumed but the end product was acetate rather than methane. Subcultures were made of all enrichment cultures except for the formate. The second methanethiol and 2-propanol subcultures produced methane very slowly and no further subcultures were made. 16S rRNA gene sequencing indicated that the betaine, choline, methanol and methylamine enrichment cultures contained members of the genus *Methanococcoides*, the acetate enrichment culture contained a member of the genus *Methanosarcina* and, the H_2/CO_2 enrichment culture contained a member of the genus *Methanogenium* (Table 3.1). The choline enrichment culture is described in Chapter 4.

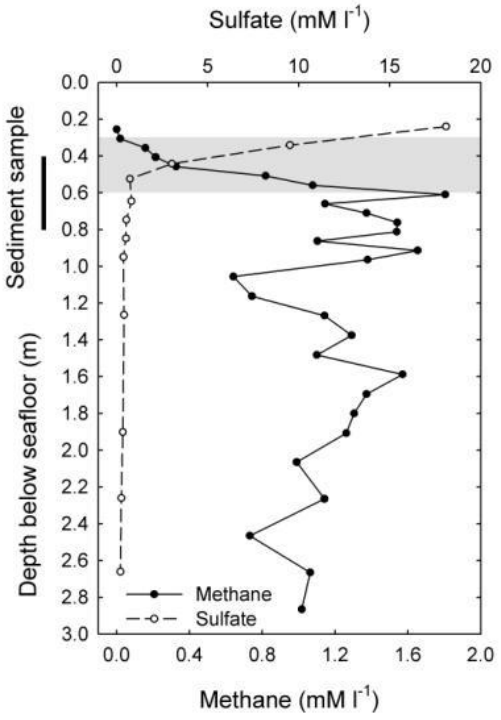


Figure 3.1 Porewater methane and sulfate profiles of core 165GC from station M5 Aarhus Bay (data from Fossing [2005]). The SMTZ is indicated by the shaded area, the sediment sample for enrichment slurries was taken from 0.4-0.8 m below seafloor.

3.2.2 Dvurechenski MV (Sorokin Trough, Black Sea)

After 250 days incubation at 10 °C (the *in situ* sediment temperature), the headspace gases were tested for the presence of methane by Dr Barry Cragg. Reference site enrichment cultures from 0.35 m depth (core 313-GC) produced methane from methanol and methylamine and were subcultured at the start of this study. Enrichment cultures for depths 1.05, 1.25, 144 and 1.94 m did not produce methane with any substrate. Sediment from 0.25 m depth at the geographic centre (the only depth studied for core 269-MUC) produced methane from methanol and methylamine and were also subcultured. For the geographic centre core 297-GC, only the 2.86 m depth enrichment culture with methylamine produced methane and was this was subcultured. Successful and unsuccessful methanogen enrichment cultures are summarized in Table 3.3.

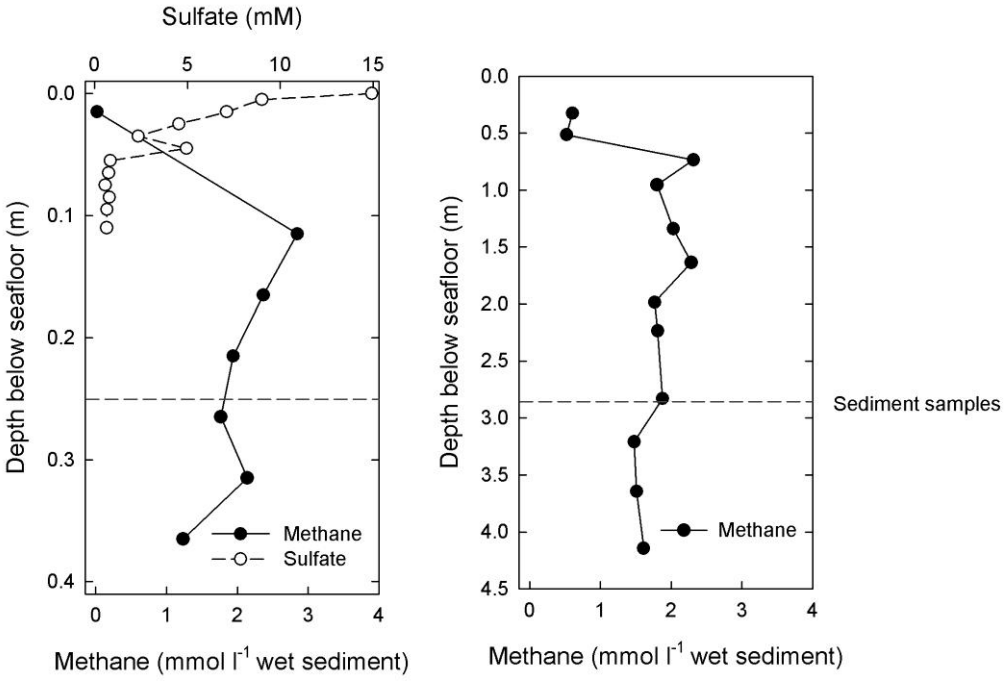
Table 3.3 Summary of Dvurechenski MV enrichment cultures (from cores 269-MUC, 297-GC and 313-GC set up by Dr Barry Cragg before the start of this study) that produced methane and where subsequently subcultured by this study. Also included are the enrichment cultures that failed to produce methane.

Incubation time (days)	Location	Core	Depth (m)	Enrichment culture	Substrate	Methanogenesis
250	Geographic centre	269-MUC	0.25	BSGC269-A	Acetate (12 mM)	-
				BSGC269-Ben	Benzoate (1.8 mM)	-
				BSGC269-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				BSGC269-Hex	Hexadecane (3.4 mM)	-
				BSGC269-MeOH	Methanol (6 mM)	+*
				BSGC269-M	Methylamine (6 mM)	+*
		297-GC	2.86	BSGC297-A	Acetate (12 mM)	-
				BSGC297-Ben	Benzoate (1.8 mM)	-
				BSGC297-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				BSGC297-Hex	Hexadecane (3.4 mM)	-
				BSGC297-MeOH	Methanol (6 mM)	-
				BSGC297-M	Methylamine (6 mM)	+*
250	Reference site	313-GC	0.35	BSREF-A	Acetate (12 mM)	-
				BSREF-Ben	Benzoate (1.8 mM)	-
				BSREF-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				BSREF-Hex	Hexadecane (3.4 mM)	-
				BSREF-MeOH	Methanol (6 mM)	+
				BSREF-M	Methylamine (6 mM)	+

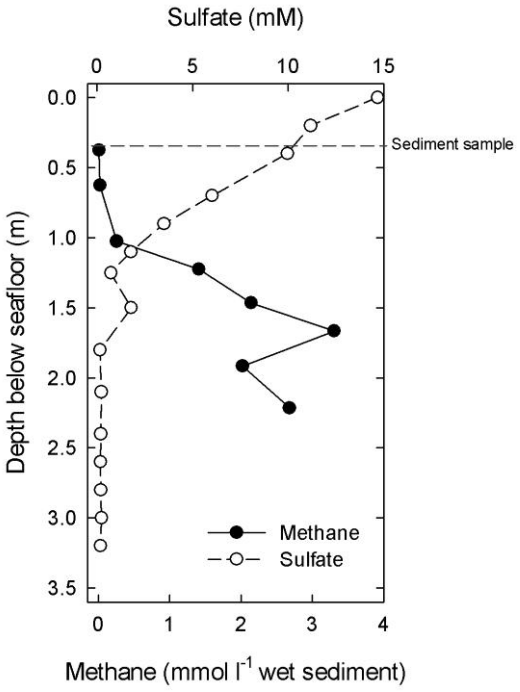
* Subcultures of these enrichment cultures did not produce methane (18 months incubation).

After 18 months incubation at 10 ºC, none of the subcultures of the original enrichment cultures from the geographic centre of Dvurechenski MV had produced methane. The subcultures of the reference site (located to the north of Dvurechenski MV) enrichment cultures were successful; methane was produced with methanol and methylamine as substrates. Members of the genus *Methanococcoides* were isolated from the enrichment cultures BSREF-M and BSREF-MeOH; two isolates were obtained from the enrichment culture BSREF-M (strains BSM1 and BSM2, both isolated by a combination of shake tubes and dilution to extinction) and one was obtained from enrichment culture BSREF-MeOH (strain BSM3, also isolated by shake tubes and dilution to extinction), Table 3.1. Strains BSM1, BSM2 and BSM3 are described in Chapters 6 and 7.

No porewater sulphate data was available for geographic centre core 297-GC. No sulphate data was available for geographic centre core 269-MUC for the depth from which sediment was taken for use in this study (2.9 m): the sulphate concentration at the lowest measured depth of this core (0.1 m) was 0.7 mM (Figure 3.2). The methane concentrations for both cores at the depth the sediment samples were taken were < 2 mmol l^{-1} wet sediment (Figure 3.2). Both sulphate and methane concentrations were measured for reference site core 313-GC, 10 mM sulphate and 0.01 mmol l^{-1} wet sediment methane (Figure 3.2). As a 25% v/v sediment slurry was prepared, the starting sulphate concentration of the enrichment cultures was one quarter of this values, 2.5 mM. No other sulphate measurements were made.



Geographic Centre Core 269-MUC **Geographic Centre Core 297-GC**



Reference Site Core 313-GC

Figure 3.2 Methane and sulphate concentrations for cores from the geographic centre of Dvurechenski MV (cores 269-MUC and 297-GC) and from the reference site (core 313-GC) located to the north of Dvurechenski MV (Figure 2.4). Dr Barry Cragg, unpublished data.

3.2.3 Eastern Mediterranean Mud Volcanoes

3.2.3.1 Amsterdam MV

Porewater data for core MEDKUL-6 showed that it was hyposaline: chloride concentrations were 600 mM at the surface, declined to 247 mM at 0.1 m depth and then ranged between 190-306 mM down to the bottom of the core (Figure 3.3). The sulphate concentrations for the depths from which sediment samples were taken were 6.7 mM (0.14 m depth, section 10) and 6.1 mM (0.49 m depth section 3). As a 10% v/v sediment slurry was prepared, the starting sulphate concentrations were 0.7 mM (0.14 m depth, section 10) and 0.6 mM (0.49 m depth section 3). No other sulphate measurements were made.

After 18 months incubation methane was produced by the section 3 (0.49 m depth) enrichment culture with marine medium and H_2/CO_2 (Table 3.4). Subcultures failed to produce methane even after 18 months incubation. Marine medium enrichment cultures (36‰ salinity) with acetate, benzoate, hexadecane, methanol and methylamine did not produce methane (two years incubation). None of the hyposaline medium (9‰ salinity) enrichment cultures (acetate, benzoate, hexadecane, H_2/CO_2, methanol and methylamine produce medium (two years incubation).

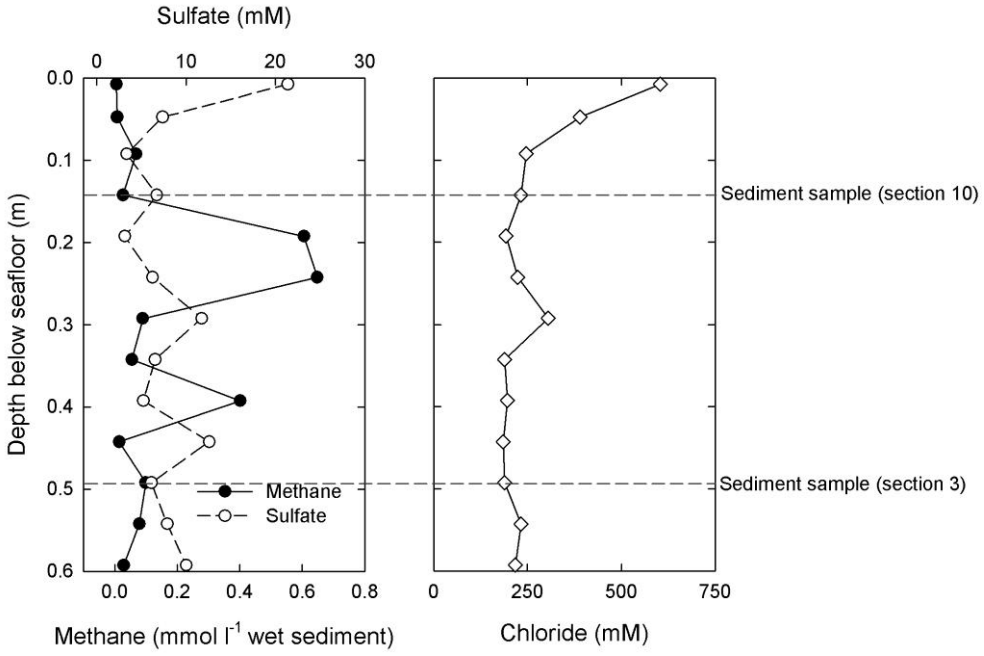


Figure 3.3 Methane and sulfate profiles of gravity core MEDKUL-6 from Amsterdam mud volcano (Dr Barry Cragg, unpublished data).

3.2.3.2 Napoli MV

The porewater of core MEDKUL-3 was hypersaline: 1578 mM Cl^- at 2.5 cm depth and 4277 mM Cl^- at 63 cm depth (Figure 3.4). Sediment from section 8 (0-5 cm depth) had the highest sulphate concentration 28 mM and section 5 (60-65 cm depth) had a sulphate concentration of 14 mM (Dr Barry Cragg, unpublished data). As a 10% v/v sediment slurry was prepared, sections 8 and 5 slurries had starting sulphate concentrations of 2.8 and 1.4 mM respectively. No other sulphate measurements were made.

Only the enrichment cultures from 0-5 cm depth (section 8) with methylamine or methanol as substrates and normal marine salinity medium produced methane (enrichment cultures NS8-M and NS8-MeOH); enrichment cultures are summarized in Table 3.4. Two strains of *Methanococcoides* were isolated, strain NM1 from enrichment cultures NS8-M and strain NM2 from enrichment culture NS8-MeOH (see Chapters 6 and 7 for details of strains NM1 and NM2), Table 3.1. Isolation was achieved using shake tubes and dilution to extinction. After 12 months incubation no other Napoli MV enrichment cultures had produced methane.

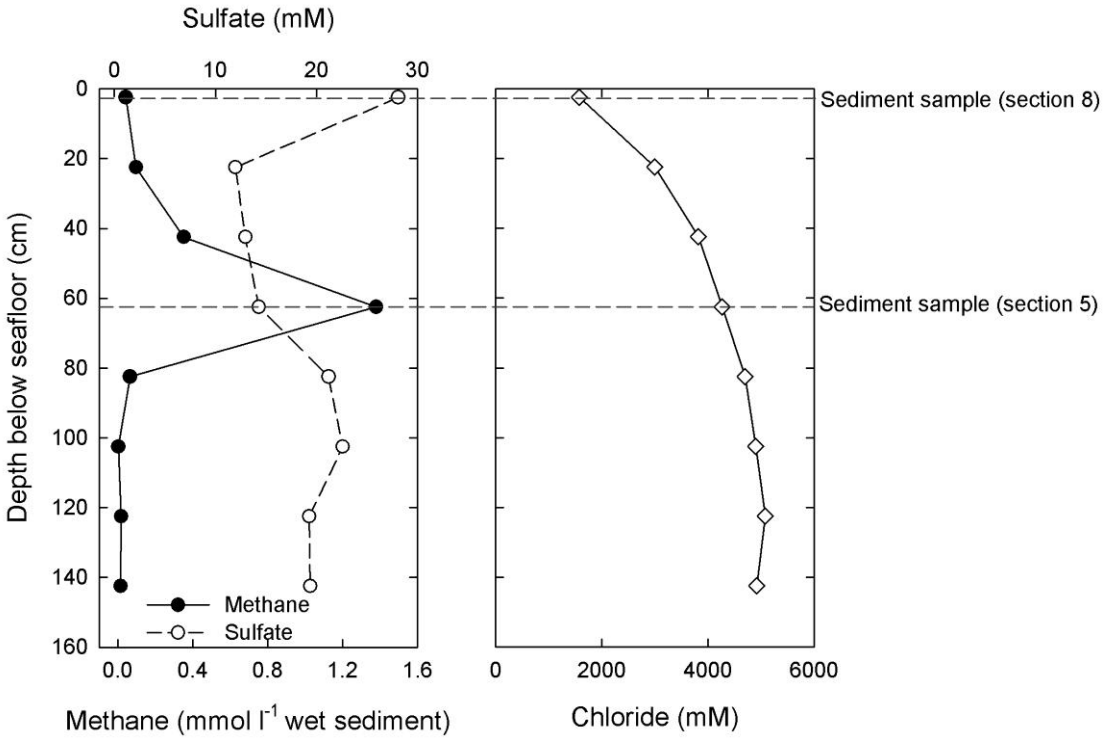


Figure 3.4 Methane and sulfate profiles of gravity core MEDKUL-3 from Napoli mud volcano. Sediment for cultivation work was taken from the indicated depths (Dr Barry Cragg, unpublished data).

Table 3.4 Summary of successful and unsuccessful Eastern Mediterranean MV methanogen enrichment cultures. No Amsterdam MV section 3 (490-495 cm depth) enrichment cultures produced methane (salinities and substrates used were the same as used for section 10). Additionally, no Napoli MV section 5 (60-65 cm depth) enrichment cultures produced methane (salinities and substrates used were the same as used for section 8).

Location	Core	Depth (cm)	Salinity	Sediment slurry	Substrate	Methanogenesis
Amsterdam	MEDKUL-6	140-145 (section 10)	Normal Marine Salinity (36‰)	AMVS10-A	Acetate (10 mM)	-
				AMVS10-Ben	Benzoate (1.5 mM0	-
				AMVS10-Hex	Hexadecane (3.4 mM)	-
				AMVS10-H	H_2/CO_2 (80:20, 0.1 MPa)	+*
				AMVS10-MeOH	Methanol (10 mM)	-
				AMVS10-M	Methylamine (10 mM)	-
			Hyposaline (9‰)	AMVS10-A-Hypo	Acetate (10 mM)	-
				AMVS10-Ben-Hypo	Benzoate (1.5 mM0	-
				AMVS10-Hex-Hypo	Hexadecane (3.4 mM)	-
				AMVS10-H-Hypo	H_2/CO_2 (80:20, 0.1 MPa)	-
				AMVS10-MeOH-Hypo	Methanol (10 mM)	-
				AMVS10-M-Hypo	Methylamine (10 mM)	-
Napoli	MEDKUL-3	0-5 (section 8)	Normal marine Salinity (36‰)	NMVS8-A	Acetate (10 mM)	-
				NMVS8-Ben	Benzoate (1.5 mM0	-
				NMVS8-Ben	Hexadecane (3.4 mM)	-
				NMVS8-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				NMVS8-MeOH	Methanol (10 mM)	+
				NMVS8-M	Methylamine (10 mM)	+
			Hypersaline A (126‰)	NMVS8-A-HA	Acetate (10 mM)	-
				NMVS8-Ben-HA	Benzoate (1.5 mM0	-
				NMVS8-Ben-HexA	Hexadecane (3.4 mM)	-
				NMVS8-H-HA	H_2/CO_2 (80:20, 0.1 MPa)	-
				NMVS8-MeOH-HA	Methanol (10 mM)	-
				NMVS8-M-HA	Methylamine (10 mM)	-
			Hypersaline B (252‰)	NMVS8-A-HB	Acetate (10 mM)	-
				NMVS8-Ben-HB	Benzoate (1.5 mM0	-
				NMVS8-Hex-HB	Hexadecane (3.4 mM)	-
				NMVS8-H-HB	H_2/CO_2 (80:20, 0.1 MPa)	-
				NMVS8-MeOH-HB	Methanol (10 mM)	-
				NMVS8-M-HB	Methylamine (10 mM)	-

*Subcultures did not produce methane.

3.2.4 Gulf of Cadiz Mud Volcanoes

The enrichment cultures listed in Table 3.5 (set up prior to this study by Dr Barry Cragg) produced methane after 168 or 290 days incubation at 10 °C (close to the *in situ* temperature of 9 °C) and were made available to this study for subculturing. 16S rRNA gene sequencing results are given in Table 3.1. Also included in Table 3.5 are unsuccessful enrichment cultures from the same cores and depths as the successful enrichment cultures. Cores and depths for which no enrichment cultures produced methane are listed in Table 3.6. As a 25 v/v sediment slurry was used for the enrichment cultures then their sulphate concentration was one-quarter of the porewater values given in Figure 3.5. No other sulphate measurements were made.

Table 3.5 Gulf of Cadiz enrichment cultures (set up by Dr Barry Cragg before the start of this study) that produced methane and were subsequently subcultured in this study. All cultures incubated at 10 ºC.

Incubation time (days)	Location	Core	Depth (m)	Enrichment culture	Substrate	Methanogenesis
168	Bonjardim MV	131	0.42	BMV-A	Acetate (12 mM)	-
				BMV-Ben	Benzoate (1.8 mM)	-
				BMV-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				BMV-HEX	Hexadecane (3.4 mM)	-
				BMV-MeOH	Methanol (6 mM)	+
				BMV-M	Methylamine (6 mM)	+
	Captain Arutynov MV	206	0.52	CAMV-A	Acetate (12 mM)	-
				CAMV-Ben	Benzoate (1.8 mM)	-
				CAMV-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				CAMV-Hex	Hexadecane (3.4 mM)	-
				CAMV-MeOH	Methanol (6 mM)	-
				CAMV-M	Methylamine (6 mM)	+
	Meknes MV	306	0.77	MKMV-A	Acetate (12 mM)	-
				MKMV-Ben	Benzoate (1.8 mM)	-
				MKMV-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				MKMV-Hex	Hexadecane (3.4 mM)	-
				MKMV-MeOH	Methanol (6 mM)	+
				MKMV-M	Methylamine (6 mM)	+
	Mercator MV	238	0.72	MMV-A	Acetate (12 mM)	-
				MMV-Ben	Benzoate (1.8 mM)	-
				MMV-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				MMV-Hex	Hexadecane (3.4 mM)	-
				MMV-MeOH	Methanol (6 mM)	-
				MMV-M	Methylamine (6 mM)	+
290	Carlos Ribeiro MV	JC10-053	0.55	CRMV-A	Acetate (12 mM)	-
				CRMV-Ben	Benzoate (1.8 mM)	-
				CRMV-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				CRMV-Hex	Hexadecane (3.4 mM)	-
				CRMV-MeOH	Methanol (6 mM)	+
				CRMV-m	Methylamine (6 mM)	+
	Darwin MV –South Rim	JC10-038	0.18	DMVSR-A	Acetate (12 mM)	-
				DMVSR-Ben	Benzoate (1.8 mM)	-
				DMVSR-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				DMVSR-Hex	Hexadecane (3.4 mM)	-
				DMVSR-MeOH	Methanol (6 mM)	-
				DMVSR-M	Methylamine (6 mM)	+
	Darwin MV (pushcore)	PUC06	0.11	DMV-A	Acetate (12 mM)	-
				DMV-Ben	Benzoate (1.8 mM)	-
				DMV-H	H_2/CO_2 (80:20, 0.1 MPa)	+
				DMV-Hex	Hexadecane (3.4 mM)	-
				DMV-MeOH	Methanol (6 mM)	+
				DMV-M	Methylamine (6 mM)	+
	Mercator MV	JC10-013	0.15	MMV013-A	Acetate (12 mM)	-
				MMV013-Ben	Benzoate (1.8 mM)	+
				MMV013-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				MMV013-Hex	Hexadecane (3.4 mM)	-
				MMV013-MeOH	Methanol (6 mM)	-
				MMV013-M	Methylamine (6 mM)	-
	Mercator MV	JC10-019	2.23	MMV019-A	Acetate (12 mM)	-
				MMV019-Ben	Benzoate (1.8 mM)	-
				MMV019-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				MMV019-Hex	Hexadecane (3.4 mM)	+
				MMV019-MeOH	Methanol (6 mM)	-
				MMV019-M	Methylamine (6 mM)	-
	Mercator MV – Ref. Site	JC10-002	0.20	MMVREF-A	Acetate (12 mM)	-
				MMVREF-Ben	Benzoate (1.8 mM)	-
				MMVREF-H	H_2/CO_2 (80:20, 0.1 MPa)	-
				MMVREF-Hex	Hexadecane (3.4 mM)	-
				MMVREF-MeOH	Methanol (6 mM)	-
				MMVREF-M	Methylamine (6 mM)	+

Table 3.6 Gulf of Cadiz enrichment cultures (set up by Dr Barry Cragg) that did not produce methane. Potential methanogen substrates for each depth were acetate (12 mM), benzoate (1.8 mM), hexadecane (2.4 mM), H_2/CO_2 (80:20, 0.1 MPa), methanol (6 mM) and methylamine (6 mM). All enrichment cultures incubated at 10 ºC.

Incubation time (days)	Location	Core	Depth (m)
168	Bonjardim MV	131	0.3, 1.67, 2.47 and 2.97
	Captain Arutynov MV	206	0.77, 1.12, 2.22 and 2.82
	Meknes MV	306	1.82
	Mercator MV	238	1.72
290	Carlos Ribeiro MV	JC10-053	0.30, 1.05, 4.25 and 5.15
	Darwin MV –South Rim	JC10-038	0.48
	Mercator MV	JC10-019	0.18, 0.38 and 1.90
	Mercator MV – Ref. Site	JC10-002	0.05

3.2.4.1 Bonjardim MV

Whilst the Bonjardim MV enrichment cultures prepared by Dr Barry Cragg produced methane with methylamine or methanol as substrates (enrichment cultures BMV-M and BMV-MeOH, Table 3.5), subcultures with the same substrates did not produce methane and were not pursued further. Sediment for enrichment cultures BMV-M and BMV-MeOH was taken from 0.42 m depth; the porewater sulphate concentration was 20 mM and the methane concentration was 0.04 mmol l^{-1} wet sediment (Figure 3.5).

3.2.4.2 Captain Arutynov MV

Captain Arutynov MV enrichment culture with methylamine (CAMV-M, Table 3.5) was successfully subcultured but methanogens were not isolated. Visible colonies did not form on agar plates or in shake tubes although methane was produced. Attempts at isolation using dilution to extinction were abandoned, as after three months incubation there was no methane production at dilutions above 10^{-3}. Methanogen sequences were, however, obtained and were found to be very similar to that of *Methanococcoides methylutens* TMA-10^T, Table 3.1. The sediment from 0.52 m depth used for enrichment culture CAMV-M had a low porewater sulphate concentration of 0.2 mM and a methane concentration of 1.7 mmol l^{-1} wet sediment (Figure 3.5)

3.2.4.3 Carlos Ribeiro MV

As with the Captain Arutynov MV enrichment cultures, the Carlos Ribeiro MV enrichment cultures were successfully subcultured but not isolated. Again they failed to form visible colonies on agar plates or in shake tubes and did not grow at dilutions higher than 10^{-3} after three months incubation. The enrichment cultures with methylamine (CRMV-M) and methanol (CRMV-MeOH) were sequenced and found to contain members of the genus *Methanococcoides* (Table 3.1). The sediment for the enrichment cultures was taken from depth 0.55 m and had a low porewater sulphate concentration (0.6 mM)(Figure 3.5).

3.2.4.4 Darwin MV

The Darwin south rim enrichment culture with methylamine as a substrate (DMVSR-M, Table 3.5) produced methane slowly and no attempt to isolate the methanogens was made. However, DNA was extracted from the culture and sequenced, it contained methanogens closely related to members of the genus *Methanococcoides* (Table 3.1). Darwin MV push-core enrichment cultures (DMV-M and DMV-MeOH, Table 3.5) grew more rapidly and an attempt to isolate methanogens was made. The methanogens did not form visible colonies on agar plates or in shake tubes; isolation from culture DMV-M was achieved using dilution to extinction. The methanogens in both cultures were identified as members of the genus *Methanococcoides* (Table 3.1). The isolated strain, strain DM1, is described in Chapters 6 and 7. A Darwin MV enrichment culture with H_2/CO_2 (DMV-H) produced methane but growth was slow and the addition of yeast extract and acetate did not increase the growth rate. Attempts at isolation using both shake tubes and dilution to extinction was unsuccessful. No porewater data was available for the push-core. The sulphate concentration of the South Rim core (JC10-038) at the depth of the sediment used for DMVSR-M was 28 mM (Figure 3.5, Dr Barry Cragg, unpublished data).

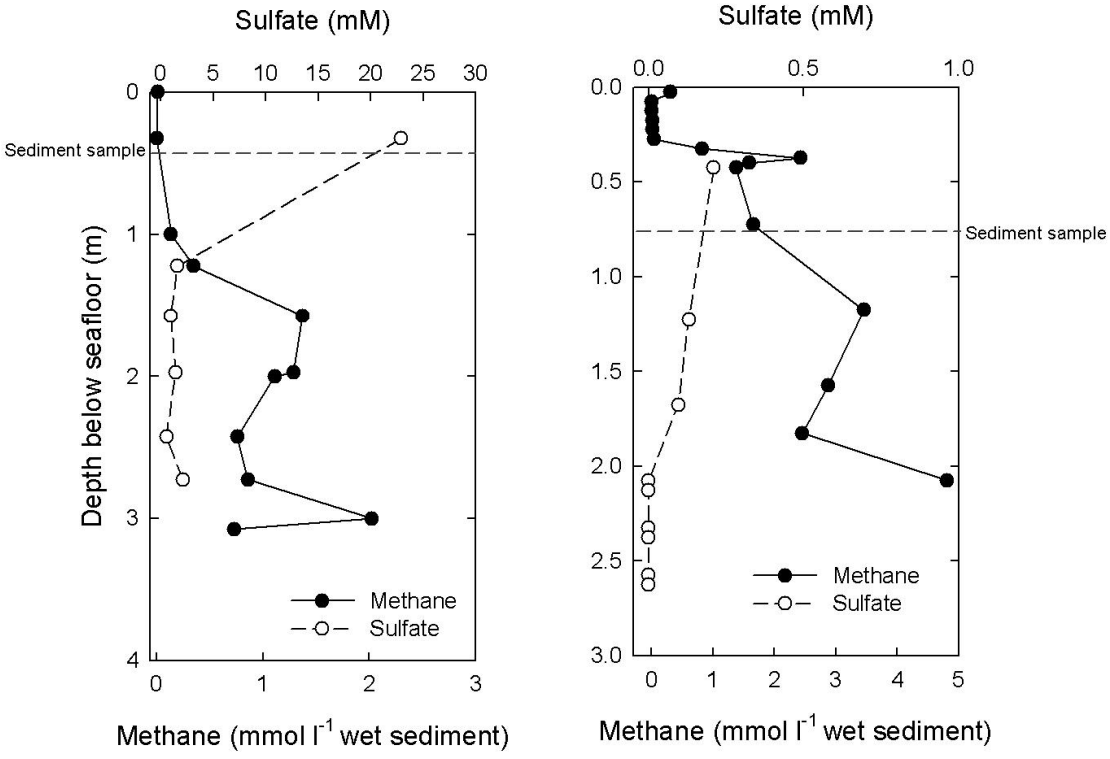
3.2.4.5 Meknes MV

Subcultures of Meknes MV sediment slurries on methanol and methylamine (MKMV-MeOH MKMV-M, Table 3.5) produced methane. An attempt was made to isolate methanogens using agar plates; growth was slow with dark coloured colonies taking at least three months to appear. Colonies were removed and transferred to a shake tube series and two strains of *Methanococcoides* were eventually isolated, strain

MKM1 from enrichment culture MKMV-M and strain MKM2 from enrichment culture MKMV-MeOH (strains described in Chapters 6 and 7). There were no porewater methane measurements made at the depth from which the sample was taken (0.77 m); at 1.2 m depth the methane concentration was 3.6 mmol l^{-1} (Figure 3.5). The sulphate concentration at 0.77 m depth was 0.1 mM (Figure 3.5).

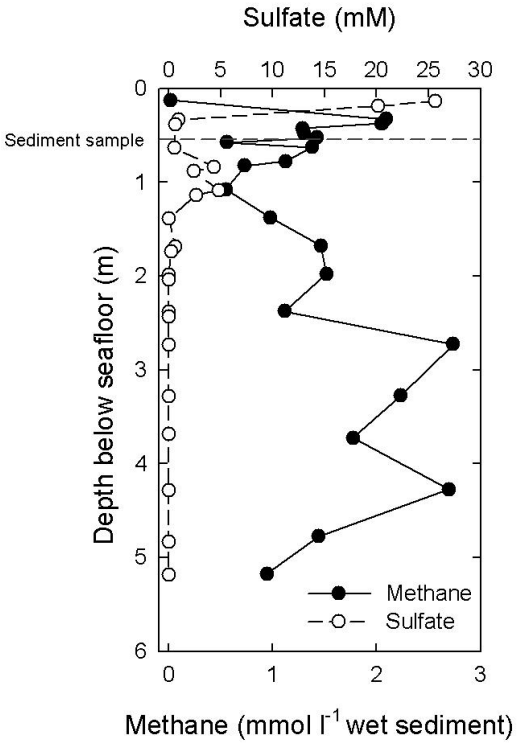
3.2.4.6 Mercator MV

The subcultures of the JC10-013 and 238 enrichment cultures (Table 3.5) were unsuccessful; methane was not detected. Subcultures of Mercator MV reference site core JC10-002 enrichment culture MMVREF-M (Table 3.5) did produce methane but growth was slow and isolation of methanogens was not attempted. The identity of the methanogen was determined; it was a member of the genus *Methanococcoides* (Table 3.1). Subcultures with benzoate or hexadecane (enrichment cultures MMV013-Ben and MMV019-Hex, Table 3.5) did not result in methane production. The porewater sulphate concentration for cores JC10-02, JC10-013 and JC10-019 at the depths the sediment samples were taken ranged from 19-34 mM (Figure 3.5). For core 238 there were no porewater sulphate measurements made at the depth from which the sample was taken (0.7 m); at 0.8 m depth the sulphate concentration was 30 mM (Figure 3.5).

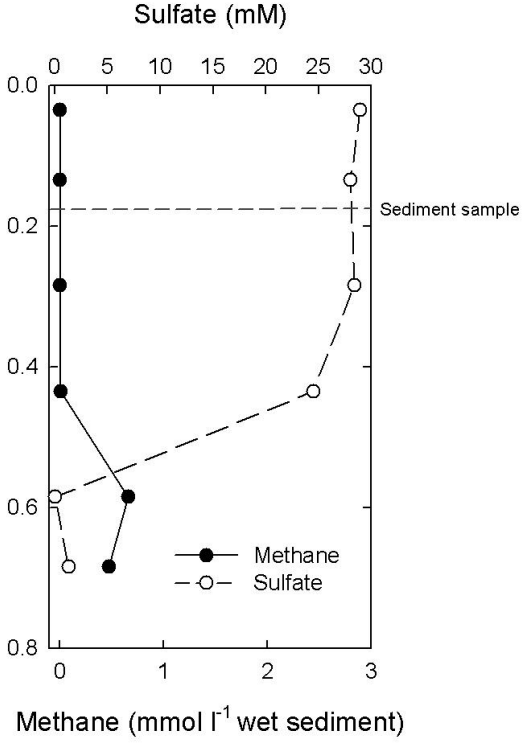


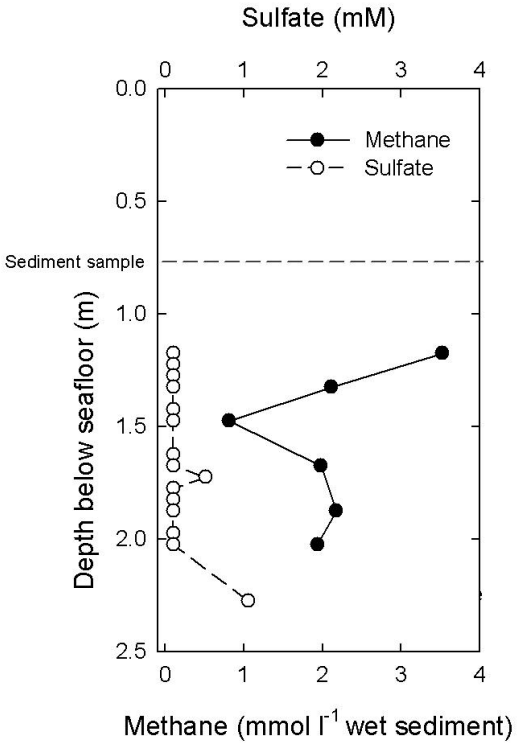
Bonjardim MV Core 131 **Captain Arutyunov MV Core 206**

Carlos Ribeiro MV Core JC10-053

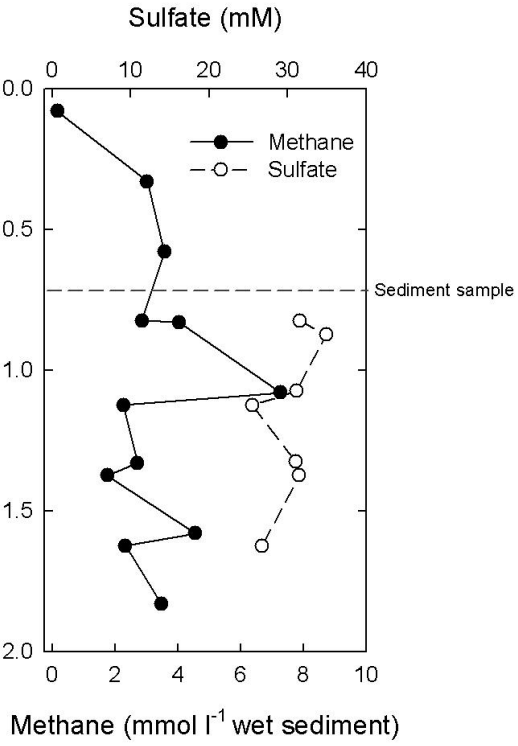


Darwin MV Core JC10-038





Meknes MV Core 306



Mercator MV Core 238

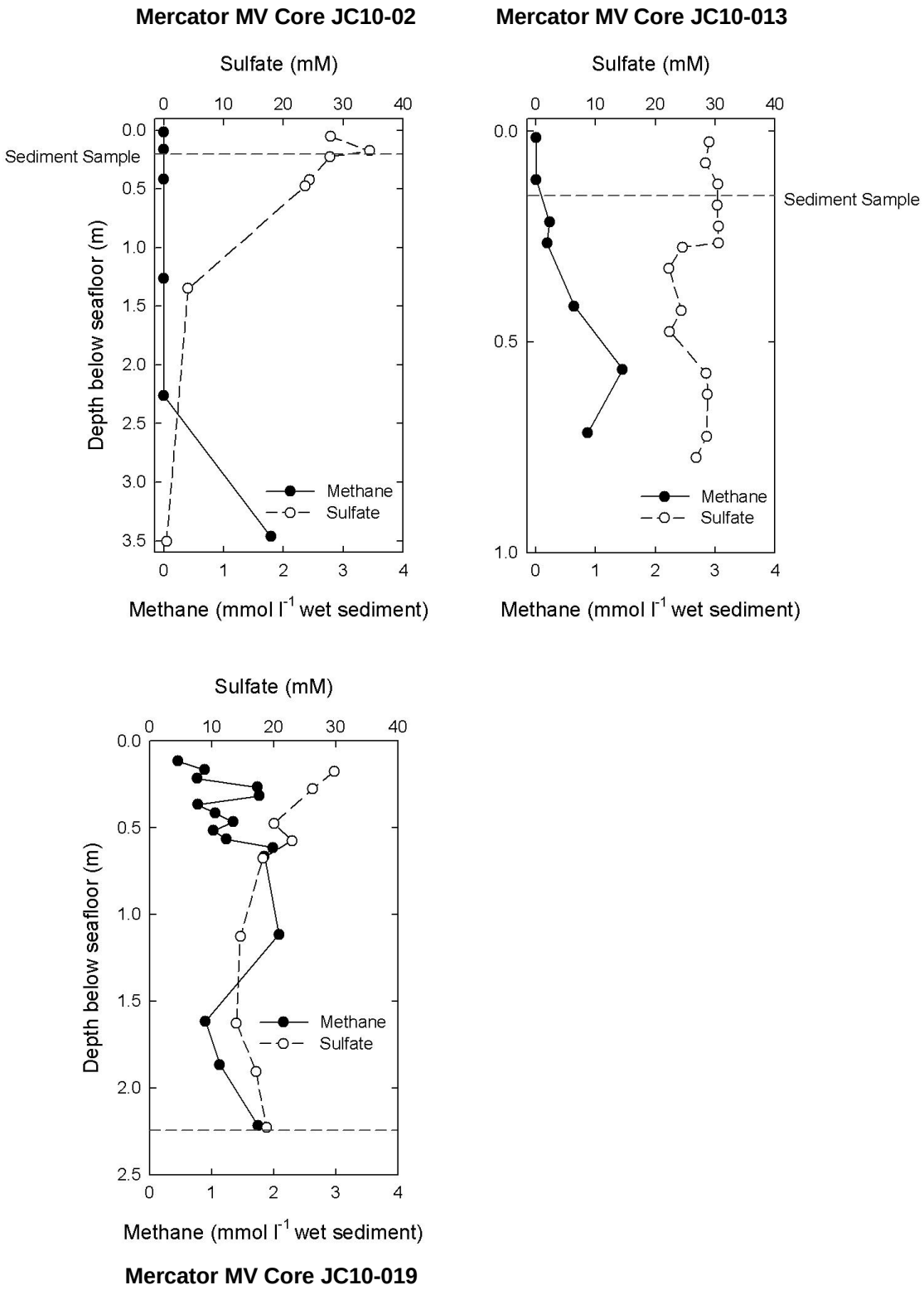


Figure 3.5 Methane and sulphate profiles of the Gulf of Cadiz sediment cores Captain Arutynov, Carlos Ribeiro, Darwin, Meknes and Mercator MVs from used in this study. The depths from which the samples were taken are indicated. Dr Barry Cragg, unpublished data.

3.2.5 Estuarine Methanogens

3.2.5.1 Portishead

The study of Portishead methanogens was in two parts: a set of enrichment cultures set up by this study and subcultures made of the Portishead temperature gradient enrichment culture experiment (Dr Barry Cragg and Dr Gordon Webster).

Sediment was taken from three depths of the June 2009 core: 0-2 cm, 10-15 cm and 30-35 cm. Marine medium enrichment cultures from all depths were set up with acetate, H_2/CO_2 and methylamine. In addition, freshwater medium enrichment cultures were prepared for all depths with H_2/CO_2. The methane and sulphate profiles are given in Figure 3.6. A 10% (v/v) sediment slurry was used so the initial sulphate concentration of the enrichment cultures were approximately 2.7 mM for depths 0-2 and 10-15 cm depth. For the 30-35 cm depth enrichment cultures the initial sulphate concentration was approximately 1.2 mM. No other sulphate measurements were made.

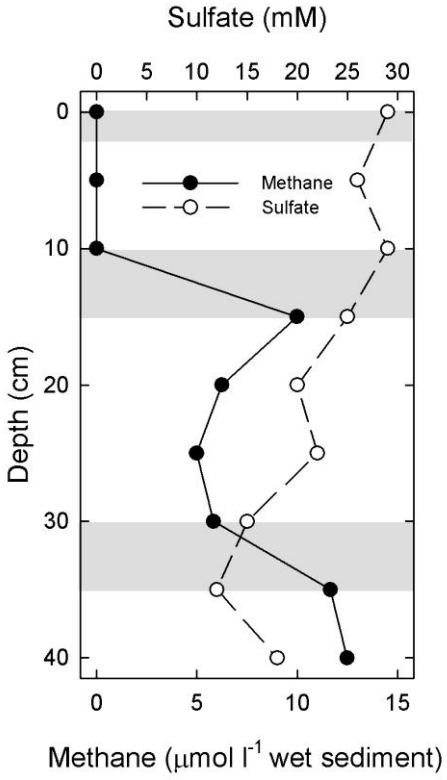


Figure 3.6 Methane and sulphate profile for Portishead sediment. The depths from which sediment was taken for use in this study are indicated by shading. Data from Webster *et al*. (2010).

All enrichment cultures produced methane. The methylamine enrichment cultures (P2A, P10A and P30A, Table 3.1) were the first to produce methane and *Methanococcoides* were isolated from all depths using dilution to extinction; strains PM1 (10-15 cm), PM2 (30-35 cm) and PM3 (0-2 cm), see Chapters 6 and 7 for details of these strains. The next enrichment cultures to produce methane were the marine medium 10-15 cm and 30-35 cm depth H_2/CO_2 enrichment subcultures (P10H and P30H, Table 3.1); *Methanococcus* were isolated from these enrichment cultures, strains PM4 and PM5 (Chapter 6). Growth was slower with the marine and freshwater 0-2 cm H_2/CO_2 enrichment cultures (P2H and P2HFr); methanogens were not isolated but were identified using 16S rRNA gene sequencing. The freshwater culture contained members of the genus *Methanosaeta* and the marine culture contained members of the genus *Methanococcus* (Table 3.1). The other freshwater enrichment cultures, depths 10-15 and 30-35 cm (P10HFr and P30HFr), contained methanogens related to the genus *Methanoculleus* (Table 3.1). The acetate enrichment cultures (P2A, P10A and P30A) produced methane at the slowest rate and no attempt to isolate methanogens was made; all enrichment cultures contained members of the genus *Methanosarcina* (Table 3.1).

The sequencing results for subcultures of the temperature gradient experiment enrichment culture are given in Table 3.7 (all Portishead temperature gradient subculture codes are prefixed with PT). The 10 ºC enrichment culture with acetate (PT10A) produced methane very slowly and no attempt at DNA sequencing was made. There was no methane production at 46 ºC and 55 ºC with substrates other than H_2/CO_2. Cultures PT46A and PT46H were observed, using F_{420} autofluorescent microscopy, to contain more than one morphotype of methanogen and the identity of the methanogens present in these cultures was determined using PCR-DGGE (Figure 3.7 and Table 3.7). Enrichment culture PT46A contained members of the genera *Methanosarcina* and *Methanoculleus*, and culture PT46H contained members of the genera *Methanobacterium* and *Methanoculleus* (Table 3.7).

Table 3.7 Phylogenetic affiliations (16S rRNA gene) of Portishead temperature gradient experiment methanogens. Subcultures PT46A and PT46H were observed to be mixed cultures using autofluorescence microscopy, their identities were determined by 16S rRNA gene PCR-DGGE (see Figure 3.7).

Culture	Temp (ºC)	Substrate	DGGE band	BLASTN nearest match (accession number)	% Identity	BLASTN nearest cultured match (accession number)	% Identity
PT10A	10	Acetate		Slow growth, not sequenced		Slow growth, not sequenced	
PT10H		H_2/CO_2		*Methanogenium cariaci* DSM1497^T (FR733663)	98	*Methanogenium cariaci* DSM1497^T (FR733663)	98
PT10M		Methylamine		*Methanococcoides methylutens* TMA-10^I (FR733669)	99	*Methanococcoides methylutens* TMA-10^I (FR733669)	99
PT25A	25	Acetate		*Methanosarcina semesiae* MD1^T (AJ012742)	98	*Methanosarcina semesiae* MD1^T (AJ012742)	98
PT25H		H_2/CO_2		*Methanogenium cariaci* DSM1497^I (FR733663)	97	*Methanogenium cariaci* DSM1497^I (FR733663)	97
PT25M		Methylamine		*Methanococcoides methylutens* NaT1 (Y16946)	99	*Methanococcoides methylutens* NaT1 (Y16946)	99
PT38A	38	Acetate		*Methanosarcina semesiae* MD1^T (AJ012742)	99	*Methanosarcina semesiae* MD1^T (AJ012742)	99
PT38H		H_2/CO_2		*Methanobacterium formicicum* MG-134 (HQ591420)	99	*Methanobacterium formicicum* MG-134 (HQ591420)	99
PT38M		Methylamine		*Methanosarcina semesiae* MD1^T (AJ012742)	99	*Methanosarcina semesiae* MD1^T (AJ012742)	99
PT46A	46	Acetate	1	*Methanobacterium alcaliphilum* WeN4^I (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^I (NR_028228)	100
			2	*Methanoculleus thermophilus* DSM 2832^I (AJ862839)	97	*Methanoculleus thermophilus* DSM 2832^I (AJ862839)	97
PT46H		H_2/CO_2	3	*Methanosarcina mazei* strain Gö1 (AE008384)	100	*Methanosarcina mazei* strain Gö1 (AE008384)	100
			4	*Methanoculleus thermophilus* CR-1 (NR_028156)	99	*Methanoculleus thermophilus* CR-1 (NR_028156)	99
PT46M		Methylamine		No methanogenesis		No methanogenesis	
PT55A	55	Acetate		No methanogenesis		No methanogenesis	
PT55H		H_2/CO_2		Uncultured archaeon clone A34 (FJ205767)	100	*Methanoculleus marisnigri* JR1 (CP000562)	98
PT55M		Methylamine		No methanogenesis		No methanogenesis	

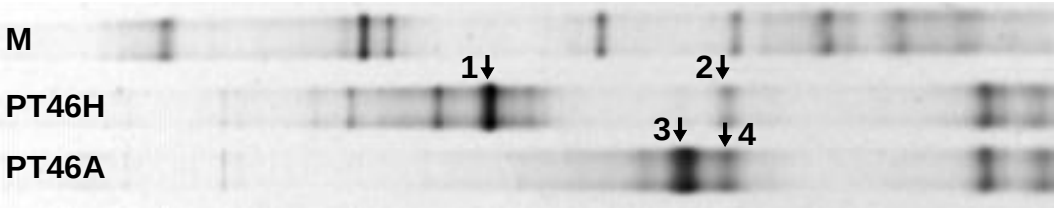


Figure 3.7 16S rRNA gene PCR-DGGE profile of Portishead temperature gradient enrichment cultures PT46A and PT46H. M, marker.

3.2.5.2 Tamar

This site was investigated by archaeal 16S rRNA gene PCR-DGGE and culturing. Samples were taken at 1, 3, 5, 10, 15 and 20 depths from the sediment core for DGGE-PCR (Figure 3.8 for the DGGE profile and Table 3.8 for the sequencing results). Based on the number of DGGE bands, the archaeal diversity increased with sediment depth from 1 to 3 cm depth (Figure 3.8); only four DGGE bands were present in the shallowest layer (1 cm depth). Bands 1.1 to 1.3 had a sequence identity of 94% to the genus *Nitrosopumilus*, within the *Crenarchaeota*, which is a marine autotrophic ammonia oxidiser (Könneke *et al.*, 2005). The band 1.4 16S rRNA gene sequence is affiliated to the Marine Benthic Group-D (MBG-D)/ *Thermoplasmatales*. Bands corresponding to bands 1.3 and 1.4 were present in every sediment depth (Figure 3.9). Bands 5.5 and 5.6 had 99 and 100% 16S rRNA gene sequence identities, respectively, to members of the Marine Crenarchaeota Group (MCG), Table 3.8. Band 20.4 had a 16S rRNA gene sequence with 98% identity to a member of Marine Group-III (MG-III), Table 3.8.

The most common sequences related to cultured *Archaea* were those related to the genus *Methanococcoides* (94%-100% sequence identity) that were present at depths of 3 and 5 cm (bands 3.4, 3.5, 3.6, 5.8 and 5.9). Members of the genus *Methanococcoides* were also isolated from 3 and 20 cm depth (see below). One sequence related to the methanogenic order *Methanomicrobiales* (band 5.7) was detected at 5 cm depth.

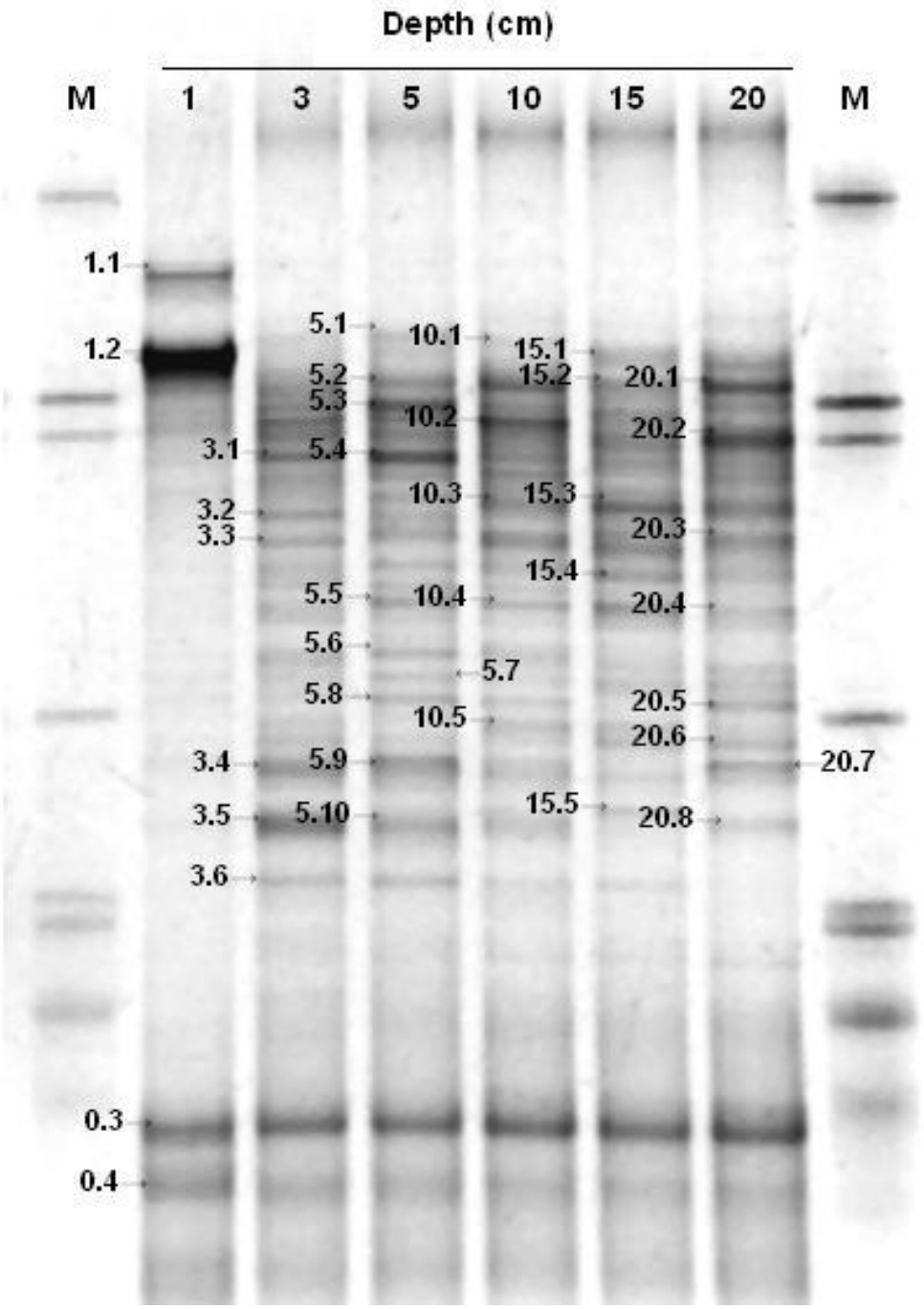


Figure 3.8 Archaeal 16S rRNA gene DGGE profile of the Tamar sediment core from which methanogens were cultured by this study. All numbered bands were excised; the BLASTN results for the excised bands are given in Table 3.8. M, marker.

Table 3.8 Phylogenetic affiliations of sequenced Tamar sediment Archaeal DGGE bands (16S rRNA gene). The numbers before the decimal point in the band numbers indicated the sediment depth in cm. Those sequences with less than 85% match to a cultivated strain are marked "No significant similarity".

DGGE Band	Nearest match by BLASTN search (accession number)	% Identity	Nearest cultivated match by BLASTN search (accession number)	% Identity	Affiliation
1.1	Uncultured archaeal clone MF30a14 (HQ230100) Fjord water, Canada	100	*Candidatus Nitropumilus* sp. NM25 (AB546961) Marine sand, Japan	95	*Nitrosopumilales*
1.2	Uncultured archaeal clone MF30a14 (HQ230100) Fjord water, Canada	100	*Candidatus Nitropumilus* sp. NM25 (AB546961) Marine sand, Japan	95	*Nitrosopumilales*
1.3	Uncultured archaeal clone SHFC733 (GU234499) Seawater, Antarctica	100	*Candidatus Nitropumilus* sp. NM25 (AB546961) Marine sand, Japan	95	*Nitrosopumilales*
1.4	Uncultured archaeal clone livecontrolA102 (FJ264792) CH_4 seep sediment, Eel River Basin, USA	92	No significant similarity		MBG-D/ *Thermoplasmatales*
3.1	Uncultured archaeal clone HSZ-Q73 (HQ267267) Yellow River sediment, China	95	No significant similarity		MBG-D/ *Thermoplasmatales*
3.4	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	100	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	100	*Methanosarcinales*
3.5	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	100	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	100	*Methanosarcinales*
3.6	*Methanococcoides methylutens* (FR733669) Marine sediment, California, USA	97	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	97	*Methanosarcinales*
5.1	Uncultured archaeal clone HSZ-Q2 (HQ267254) Yellow River sediment, China	97	No significant similarity		MBG-D/ *Thermoplasmatales*
5.2	Uncultured archaeon clone Basalt-Arch-26 (FR692167) Tamar sediment with basalt and 1% Guaymas Basin sediment	91	No significant similarity		MBG-D/ *Thermoplasmatales*
5.3	Uncultured archaeon clone BR-TN612 (EF639442) Lake Pontchartrain Basin, USA	83	No significant similarity		*Crenarchaeota*
5.4	Uncultured archaeal clone 0D_A34 (AB598177) Subseafloor sediment, Japan	86	No significant similarity		MBG-D/ *Thermoplasmatales*
5.5	Uncultured Archaeal clone Arch-SMTZ_11D (FR695318) SMTZ sediment, Aarhus Bay, Denmark	99	No significant similarity		MCG
5.6	Uncultured crenarchaeote clone ANT84-BP (GU969450) Marine sediment, King George Island, Antarctica	100	No significant similarity		MCG
5.7	uncultured archaeon clone LKS12 (AJ310861) Lake Kinnert, Israel	93	No significant similarity		*Methanomicrobiales*
5.8	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	94	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	94	*Methanosarcinales*
5.9	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	100	*Methanococcoides methylutens* TMA-10^T (FR733669) Marine sediment, California, USA	100	*Methanosarcinales*

Table 3.8 (continued) Phylogenetic affiliations of sequenced Tamar Archaeal DGGE bands (16S rRNA gene). The numbers before the decimal point in the DGGE band numbers indicated the sediment depth in cm. Those sequences with less than 85% match to a cultivated strain are marked "No significant similarity".

DGGE Band	Nearest match by BLASTN search (accession number)	% Identity	Closest cultivated BLASTN match (accession number)	% Identity	Affiliation
10.1	Uncultured archaeon clone Basalt-Arch-38 (FR692175) Tamar sediment with basalt and 1% Guaymas Basin sediment	95	No significant similarity		Euryarchaeota
10.2	Uncultured archaeal clone HSZ-Q2 (HQ267254) Yellow River sediment, China	99	No significant similarity		MBG-D/ *Thermoplasmatales*
10.3	Uncultured archaeon clone MC118_29D16 (HM601090) Marine sediment, Gulf of Mexico	98	No significant similarity		MBG-D/ *Thermoplasmatales*
10.4	Uncultured Archaeal clone Arch-SMTZ_11D (FR695318) SMTZ sediment, Aarhus Bay, Denmark	100	No significant similarity		MCG
10.5	Uncultured archaeon clone BD72AR33 (GU363084) Marine sediment, South China Sea	95	No significant similarity		Thermoproteales
15.1	Uncultured euryarchaeote clone V.8.ArD1 (AY367341) Pockmark sediment, Cacadia Margin	98	No significant similarity		Saltmarsh Group
15.2	Uncultured archaeon clone SBAK-shallow-23 (DQ522939) Marine sediment, Skan Bay, Denmark	98	No significant similarity		MBG-D/ *Thermoplasmatales*
15.3	Uncultured Archaeal clone livecontrolA102 (FJ264792) Methane seep, Eel River Basin, USA	100	No significant similarity		MBG-D/ *Thermoplasmatales*
15.4	Uncultured archaeal clone SBAK-mid-45 (DQ640162) Marine sediment, Skan Bay, Alaska, Denmark	97	No significant similarity		MBG-D/ *Thermoplasmatales*
20.1	Uncultured archaeal clone SBAK-shallow-23 (DQ522939) marine sediment, Skan Bay, Alaska	100	No significant similarity		MBG-D/ *Thermoplasmatales*
20.2	Uncultured archaeal clone MC118_29D16 (HM601090) Marine sediment, Gulf of Mexico	100	No significant similarity		MBG-D/ *Thermoplasmatales*
20.3	Uncultured archaeal clone BY5_1h3b_A025 (HQ606169) Marine sediment, South China Sea	91	No significant similarity		*Euryarchaeota*
20.4	Uncultured archaeal clone LV-Arco55 (AM943623) Sediment, hypersaline lagoon, Brazil	98	No significant similarity		MG-III

The sediment cores methane and sulphate profile is shown in Figure 3.9. Sediment was taken from a core at depths 3-4 cm and 18-21 cm and cultured with marine medium and acetate (10 mM), H_2/CO_2 (80:20) or methylamine (10 mM) as substrates. A 10% v/v sediment slurry was used so the initial sulphate concentration of the enrichment cultures were approximately 2.6 mM and 1.1 mM for depth 3-4 cm and 18-21 cm respectively. No further sulphate measurements were made. These slurries were incubated at 25 ºC.

All enrichment cultures produced methane; those with methylamine were the first to do so and methanogens were isolated from both of the methylamine cultures using dilution to extinction (strains TM1 and TM2, see Chapters 6 and 7 for further details). Subcultures of the 2 cm depth acetate amended slurries (T3A) and both H_2/CO_2 amended slurries (T3H and T20H) contained a mixed community of methanogens (determined on morphology using F_{420} autofluorescence and phase-contrast microscopy). In an attempt to separate these methanogens, a subculture of the acetate enrichment culture was made with freshwater medium (freshwater culture T3AFr) and likewise with the H_2/CO_2 enrichment cultures (freshwater cultures T3HFr and T20HFr). In addition, enrichment culture T3A was subcultured with marine medium with formate and ethanol incubated at 25 and 38 °C (cultures T3E, T3F, T3E(38) and T3F(38), Table 3.1). Ethanol can be used directly as a substrate by some methanogens, *e.g. Methanogenium organophilum* CV^T (Widdel et al., 1988) or it can be degraded by a syntrophic partnership between bacteria and methanogens. Whilst enrichment culture T20A was not mixed, a subculture of the original sediment slurry was made with acetate and freshwater medium (freshwater culture T20AFr) for comparison with the other freshwater cultures.

The use of freshwater medium in an attempt to culture only one species of methanogen from mixed cultures was successful in three cases. Culture T20H was identified by 16S rRNA gene PCR-DGGE to contain *Methanobacterium* and *Methanospirillum* (Figure 3.10 and Table 3.9), the subculture with freshwater medium (T20HFr) lead to the culture of only *Methanospirillum* (Table 3.1). The other culture that was seen to have a mixed population of methanogens (using autofluorescent and phase-contrast microscopy short rods and cocci were seen), T3A was identified by

16S rRNA gene PCR-DGGE to contain *Methanosaeta* and two other sequences with no close match to any cultivated archaeon. The freshwater subculture (T3AFr) contained methanogens with a 99% 16S rRNA gene sequence identity to *Methanoplanus limicola* $M3^T$ (Table 3.1). Culture T3H was shown by 16S rRNA gene PCR-DGGE to contain three genera, *Methanobacterium*, *Methanosarcina* and *Methanospirillum* whilst only *Methanoculleus* was identified in the freshwater subculture.

The other additional enrichment cultures (T3A, T3E, T3F, T3H, T3F(38), T3E(38), T20H and T20AFr) were not successful in enriching a single genus of methanogen as they still contained a mixed population of methanogens which were identified by 16S rRNA gene PCR-DGGE (Tables 3.1 and 3.9, and Figure 3.10).

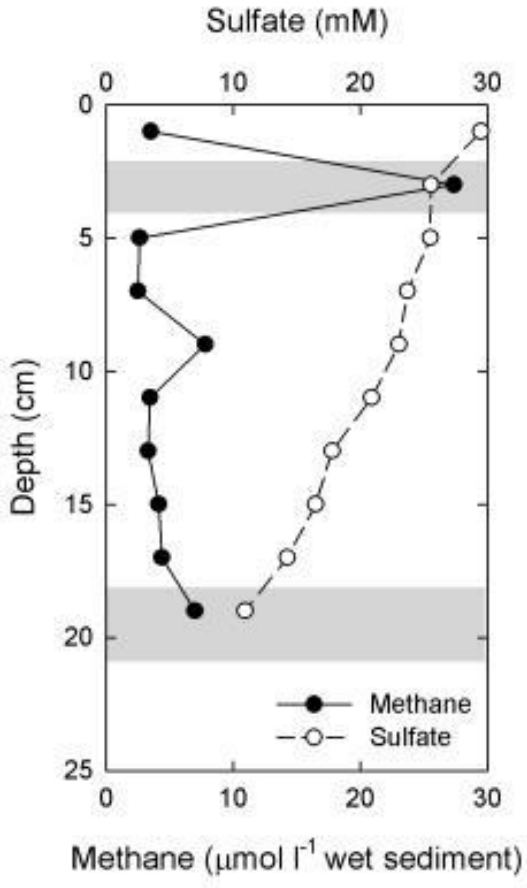


Figure 3.9 Methane and sulphate profile for the Tamar sediment core (Mathes, 2011). Shading indicates the depths from which sediment was taken for use in this study.

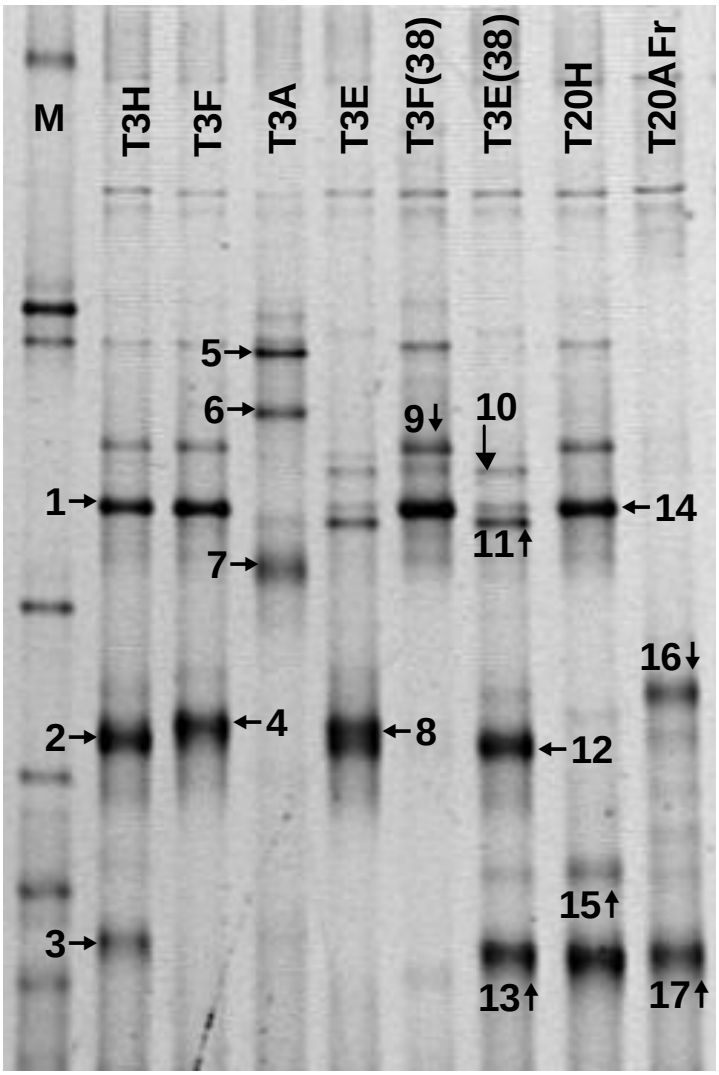


Figure 3.10 Archaeal DGGE profile of mixed Tamar enrichment cultures (16S rRNA gene). All numbered bands were excised; the BLASTN results for the excised bands are given in Table 3.9. M, marker.

Table 3.9 Phylogenetic affiliations of sequenced Archaeal DGGE bands (16S rRNA) of the mixed Tamar enrichment cultures.

Enrichment culture	DGGE Band	Nearest match by BLASTN search (accession number)	% identity	Closest cultivated by BLASTN search (accession number)	% identity	Affiliation
T3H	1	*Methanobacterium formicium* DM 1535^{T} (AF028689)	100	*Methanobacterium formicium* DM 1535^{T} (AF028689)	100	*Methanobacteriales*
T3H	2	*Methanosarcina mazei* Gö1 (AE008384)	100	*Methanosarcina mazei* Gö1 (AE008384)	100	*Methanosarcinales*
T3H	3	*Methanospirillum hungatii* JF-1^{I} (NR_042789)	97	*Methanospirillum hungatii* JF-1^{I} (NR_042789)	97	*Methanomicrobiales*
T3F	4	*Methanoculleus submarinus* Grau-1 (JN004139)	100	*Methanoculleus submarinus* Grau-1 (JN004139)	100	*Methanomicrobiales*
T3A	5	Uncultured clone MNTSA-ax6 (EF125475)	82	No significant similarity		*Euryarchaeota*
T3	6	Uncultured clone pita-HW-42 (AB301876)	88	No significant similarity		*Crenarchaeota*
T3A	7	*Methanosaeta harundinacea* 6Ac^{T} (CP003117)	97	*Methanosaeta harundinacea* 6Ac^{T} (CP003117)	97	*Methanosarcinales*
T3E	8	*Methanoculleus marisnigris* DSM 1498^{T} (NR_028228)	87	*Methanoculleus marisnigris* DSM 1498^{T} (NR_028228)	87	*Methanomicrobiales*
T3F(38)	9	*Methanobacterium alcaliphilum* WeN4^{T} (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^{T} (NR_028228)	100	*Methanobacteriales*
T3E(38)	10	Uncultured clone MC10+CO1 (HM019161)	96	No significant similarity		*Crenarchaeota*
T3E(38)	11	Uncultured clone MC10+CO1 (HM019161)	97	No significant similarity		*Crenarchaeota*
T3E(38)	12	*Methanosarcina mazei* Gö1 (AB065296)	100	*Methanosarcina mazei* Gö1 (AB065296)	100	*Methanosarcinales*
T3E(38)	13	Uncultured clone SWA-0301-08 (JN398028)	99	*Methanospirillum hungatii* JF-1^{T} (NR_042789)	97	*Methanomicrobiales*
T20H	14	*Methanobacterium alcaliphilum* WeN4^{I} (NR_028228)	100	*Methanobacterium alcaliphilum* WeN4^{I} (NR_028228)	100	*Methanobacteriales*
T20H	15	Uncultured clone SWA-0301-08 (JN398028)	99	*Methanospirillum hungatii* JF-1^{T} (NR_042789)	97	*Methanomicrobiales*
T20AFr	16	*Methanosarcina acetivorans* C2A ^{T} (AE1010299)	100	*Methanosarcina acetivorans* C2A ^{T} (AE1010299)	100	*Methanosarcinales*
T20AFr	17	Uncultured clone SW0301-08 (JN398028)	99	*Methanospirillum hungatii* JF-1^{T} (NR_042789)	97	*Methanosarcinales*

3.2.6 Guaymas Basin

Marine medium enrichment cultures with acetate (10 mM), H_2/CO_2 (80:20, 0.1 MPa overpressure) or methylamine (10 mM) were prepared for each of the three locations (CT2, CT5 and CT6, see Section 2.1.6), summarized in Table 3.10. In addition, enrichment cultures from all sites were made with freshwater medium and methylamine as a growth substrate in an attempt to culture a methanogen other than *Methanococcoides* sp. with methylamine as the catabolic substrate. Methane and sulphate profiles for all cores are illustrated in Figure 3.11. A 25% v/v sediment slurry was used so the initial enrichment culture sulphate values were one-quarter those illustrated in Figure 3.11. No subsequent sulphate measurements were made.

Table 3.10 Summary of Guaymas Basin methanogen enrichment cultures.

Core	Depth cm	Salinity	Sediment slurry	Substrate	Methanogenesis
CT2	0-7	Marine	CT2-A	Acetate (10 mM)	+
		Marine	CT2-H	H_2/CO_2 (80:20, 0.1 MPa)	-
		Marine	CT2-Cho	Choline (10 mM)	+
		Marine	CT2-M	Methylamine (10 mM)	+
		Freshwater	CT2-MFr	Methylamine (10 mM)	-
CT5	0-7	Marine	CT5-A	Acetate (10 mM)	-
		Marine	CT5-H	H_2/CO_2 (80:20, 0.1 MPa)	+
		Marine	CT5-Cho	Choline (10 mM)	+
		Marine	CT5-M	Methylamine (10 mM)	+
		Freshwater	CT5-MFr	Methylamine (10 mM)	+
CT6	0-7	Marine	CT6-A	Acetate (10 mM)	-
		Marine	CT6-H	H_2/CO_2 (80:20, 0.1 MPa)	-
		Marine	CT6-Cho	Choline (10 mM)	+
		Marine	CT6-M	Methylamine (10 mM)	+
		Freshwater	CT6-MFr	Methylamine (10 mM)	+

All marine enrichment cultures with methylamine produced methane and the methanogens were identified as members of the genus *Methanococcoides* by 16S rRNA gene sequencing; enrichment cultures CT2-M and CT5-M had 99% sequence identities to *Methanococcoides* sp. NaT1, and CT6-M had a 99% sequence identity to *Methanococcoides alaskense* AK-5^T (Table 3.1). Only one acetate enrichment culture (CT2-A) and one H_2/CO_2 enrichment culture (CT5-H) produced methane. Methanogens in enrichment culture CT2-A had a 99% 16S rRNA gene sequence identity to *Methanosarcina semesiae* MD1^T and those in CT5-H had a 99% 16S rRNA gene sequence identity to *Methanogenium cariaci* JRT^T. Freshwater enrichment cultures CT5-MFr contained methanogens with a 98% 16S rRNA gene sequence identity to *Methanosarcina semesiae* MD1^T. The enrichment cultures with choline are described in Chapter 4.

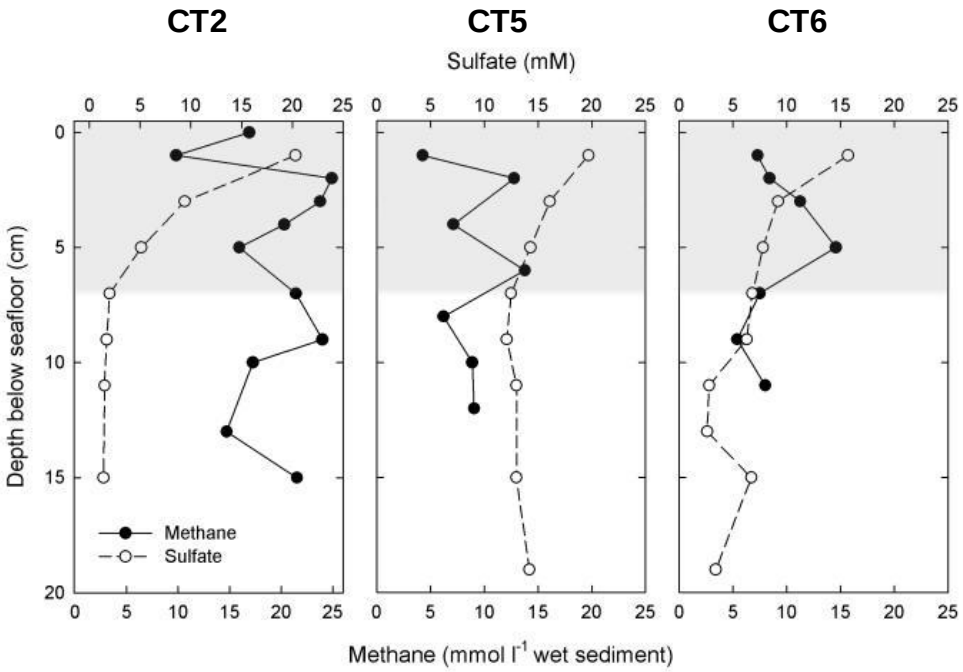


Figure 3.11 Guaymas basin methane and sulphate profiles for cores CT2, CT5 and CT6 (Dr Laurant Toffin, unpublished data).

3.3 Description of Enrichment Culture Methanogens

3.3.1 *Methanobacteriales*

The only genus of the order *Methanobacteriales* enriched in this study was *Methanobacterium* and was cultured from Portishead and Tamar sediments. Portishead enrichment culture PT38H (*M. subterraneum* A8p^T, 99% 16S rRNA sequence identity) and Tamar enrichment cultures T3H, T3F, T3E, T3E(38), T3F(38) and T20H (identified by 16S rRNA gene PCR-DGGE, Figure 3.10 and Table 3.9). The relationship of strain PT38H to representatives of the order *Methanobacteriales* is shown in Figure 3.12. Characteristics of the type strains of *M. alcaliphilum*, *M. ferruginis*, *M. formicum*, *M. palustre*, *M. petrolearium* and *M. subterraneum* are given in Table 3.11.

Enrichment culture PT38H and the six Tamer enrichment cultures contained F_{420} autofluorescent non-motile rods with rounded ends (Figure 3.13). Those cells in enrichment culture PT38H had dimensions of approximately 0.5 x 2-8 μm with dark

ends and bands visible under phase-contrast microscopy. The rods in the Tamar enrichment cultures were approximately 0.5 x 2-4 μm and were almost exclusively attached to particles of FeS or sediment. Chains of rods were seen in enrichment culture T3F(38), divisions being visible with autofluorescent microscopy (Figure 3.13). The rods in enrichment culture PT38H were seen to be attached and unattached to particles in approximately equal numbers. Morphologically, the rods in all these enrichment cultures resembled members of the genus *Methanobacterium* (Table 3.11).

Table 3.11 Characteristics of the type strains of *M. alcaliphilum, M. formicium, M. ferruginis*, *M. palustre*, *M. petrolearium* and *M. subterraneum*. Where ranges are not available the optimum values are given.

Characteristic	*M. alcaliphilum* strain WeN4^T (DSM 3387)	*M. formicum* strain MF^T (DSM 1535)	*M. ferruginis* Mic6c05^T (DSM 1974)	*M. palustre* strain F (DSM 3108^T)	*M. petrolearium* Mic5c12^T (DSM 22353)	*M. subterraneum* strain A8p^T (DSM 11074)
Habitat	Wadi el Natrum, Egypt	Sludge digester	Gas- containing brine	Peat bog	Oil storage tank sludge	Deep subterranean aquifer
Shape	Rods – singly, pairs, short chains and filaments	Rods, can form filaments & clumps	Rods	Rods and filaments	Rods	Slightly curved rods, often grows in aggregates
Size (μm)	0.5-0.6 wide 2-25 long	0.4-0.8 wide 2-15 long	0.3 wide 2.2-2.4 long	0.5 wide 2.5-5.0 long	0.3 wide 2.4-4.7 long	0-0.15 wide 0.6-1.2 long
Motility	-	-	-	-	-	-
Substrates						
H_2/CO_2	+*	+	+	+	+	+
Formate		+	-	-	-	+
Ethanol		ND	-	ND	-	-
2-propanol		+	+	+	-	-
isobutanol		+	+	+	-	-
cyclopentanol		-	+	-	-	-
Required growth factors	Trypticase Yeast extract	None	Vitamins	None	Acetate Yeast extract	None
Stimulatory factors	None	Acetate, cysteine	Acetate Yeast extract	None	None	None
Temp. range (°C)	Opt. 37	25-50	20-45	20-45	20-40	3.6-45
pH range	Opt. 8.1-9.1	Opt. 6.6-6.8	5.5-9.0	Opt. 7.0	5.5-9.0	6.5-9.2
Na^+ range (M)	ND	Opt. 0.25	0-1.2 M NaCl	0-0.3 M NaCl	0-1.2 M NaCl	0-1.4
Reference	Worakit *et al*. (1986)	Boone (1987)	Mori & Harayama (2011)	Zellner *et al*. (1989)	Mori & Harayama (2011)	Kotelnikova *et al*. (1998)

* Stated to be the sole substrate but no indication of substrates tested.

The genus *Methanobacterium* contains 18 species (Euzéby, 2011). All previously described strains of the genus *Methanobacterium* can utilise H_2/CO_2 as a substrate with *M. formicium* MF^T (Boone, 1987), *M. subterraneum* A8p^T (Kotelnikova *et al.*, 1998), *M. arcticum* M2^T(Shcherbakova *et al.*, 2011), *M. oryzae* FPi^T (Joulian *et al.*, 2000b) and *M. beijingense* 8-2^T (Ma *et al.*, 2005) also utilising formate. Only *M. formicium* MF^T (Boone, 1987), *M. ferruginis* Mic6c05^T (Mori & Harayama, 2011) and *M. palustre* F^T (Zellner *et al.*, 1998) are known to utilise 2-propanol and isobutanol. *M. ferruginis* Mic6c05^T can also utilise cyclopentanol (Mori & Harayama, 2011). *M. veterum* MK4^T is the only previously described strain to utilise methanol/H_2 and methylamine/H_2, in addition to H_2/CO_2 (Krivushin *et al.*, 2010).

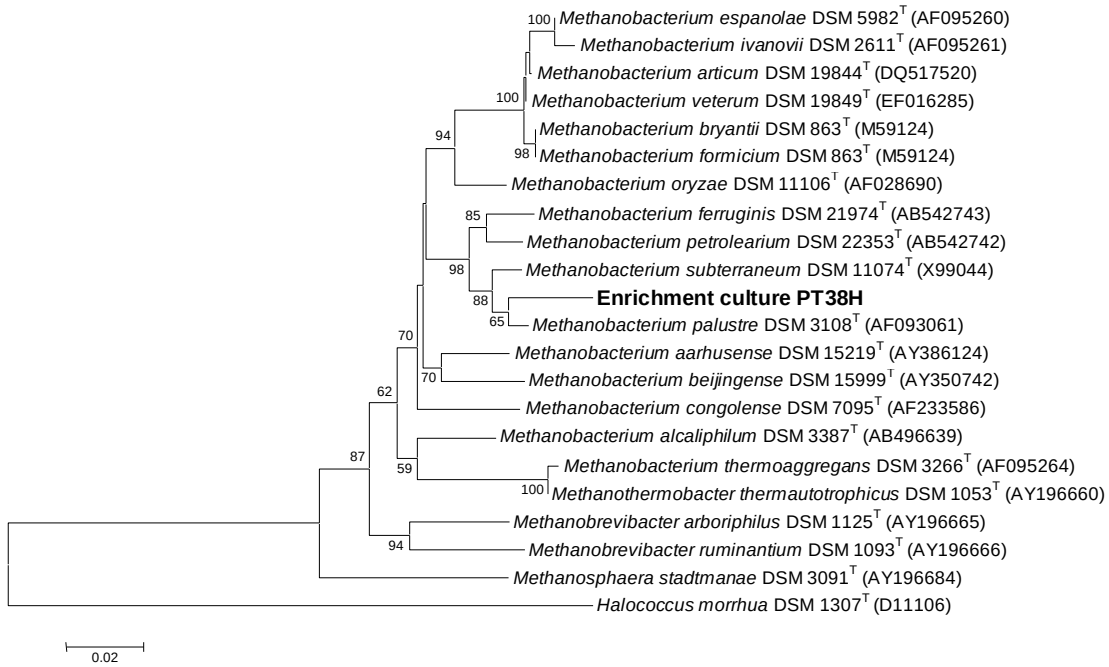


Figure 3.12 16S rRNA gene phylogenetic tree (neighbour-joining) showing the enrichment culture sequences with type strains of the genus *Methanobacterium* and representatives of other genera of *Methanobacteriales*. Numbers at nodes are percentage bootstrap values based on 1000 replicates (only bootstrap values >50 are shown). Accession numbers are given in brackets. Bar, 0.05 substitutions per site. *Halococcus morrhuae* sequence used as an outgroup.

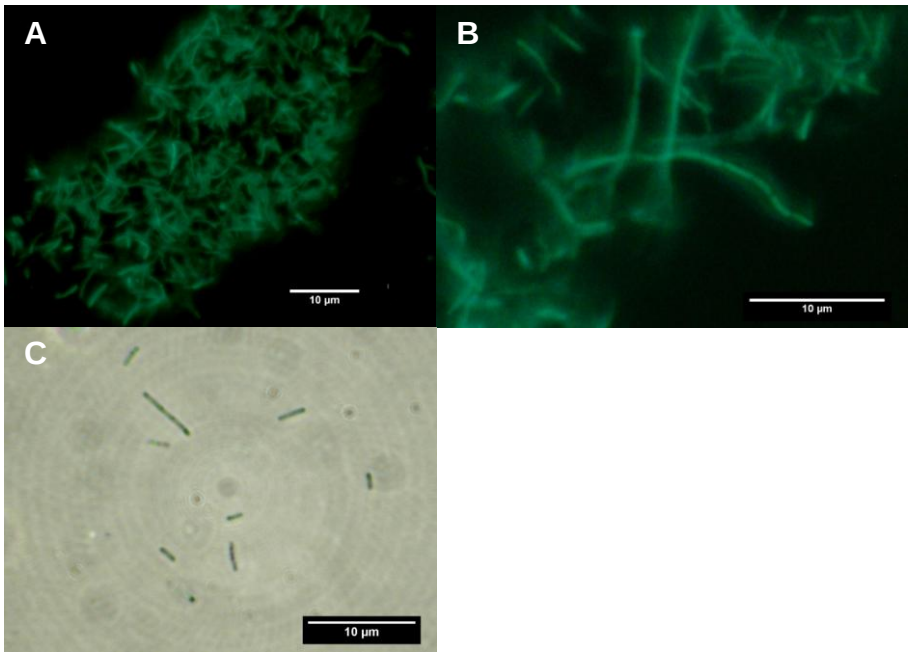


Figure 3.13 F_{420} autofluorescent photomicrographs of culture T3F(38), (A) & (B). Rods are attached to a FeS particle. Chain of rods (B). Phase-contrast photomicrograph of methanogens in culture PT38H (C). Scale bar, 10mm.

Yeast extract and peptone are required growth factors for both *M. alcaliphilum* WeN4^T (Worakit *et al.*, 1986) and *M. espanolae* GP9^T (Patel *et al.*, 1990), *M. ferruginis* Mic6c05^T requires vitamins (Mori & Harayama, 2011). Both *M. oryzae* FPi^T (Joulian *et al.*, 2000b) and *M. beijingense* 8-2^T require yeast extract (Ma *et al.*, 2005) and *M. petrolearium* Mic5c12^T requires acetate in addition to yeast extract (Mori & Harayama, 2011).

All strains are described as being non-motile. With the exception of *M. subterraneum* A8p^T (Kotelnikova *et al.*, 1998) all can occur singly, as chains (as in enrichment culture T3F(38) or filaments. *M. aarhusense* H2-LR^T is described as tending to attach to particles (Shlimon *et al.*, 2004) a feature seen in Tamar enrichment cultures.

Although the *Methanobacterium* strains enriched in this study have high 16S rRNA gene sequence identities to *Methanobacterium* from non-marine environments (Table 3.1), *Methanobacterium* is known to occur in the marine environment. *M. aarhusense* H2-LR^T is the only type strain to have been isolated from marine sediment (Shlimon *et al.*, 2004); *M. subterraneum* A8p^T (Kotelnikova *et al.*, 1998), *M. petrolearium* Mic5c12^T (Mori & Harayama, 2011) and *M. ferruginis* Mic6c05^T (Mori & Harayama, 2011) are halotolerant.

In summary, cultures with methanogens identified as *Methanobacterium* had similar morphologies to previously described strains of *Methanobacterium* and utilized H_2/CO_2 or formate as substrates, which is typical of the genus. No members of the genus have been noted to directly utilize ethanol a growth substrate, however, ethanol can be utilized in a syntrophic relationship.

3.3.2 *Methanomicrobiales*

Strains from four genera of the order *Methanomicrobiales* were enriched; *Methanoculleus* (section 3.3.2.1), *Methanogenium* (section 3.3.2.2), *Methanoplanus* (Section 3.3.2.3) and *Methanospirillum* (section 3.3.2.4). Figure 3.14 is a 16S rRNA gene phylogenetic tree including strains enriched in this study and representatives of the genera of the order *Methanomicrobiales*.

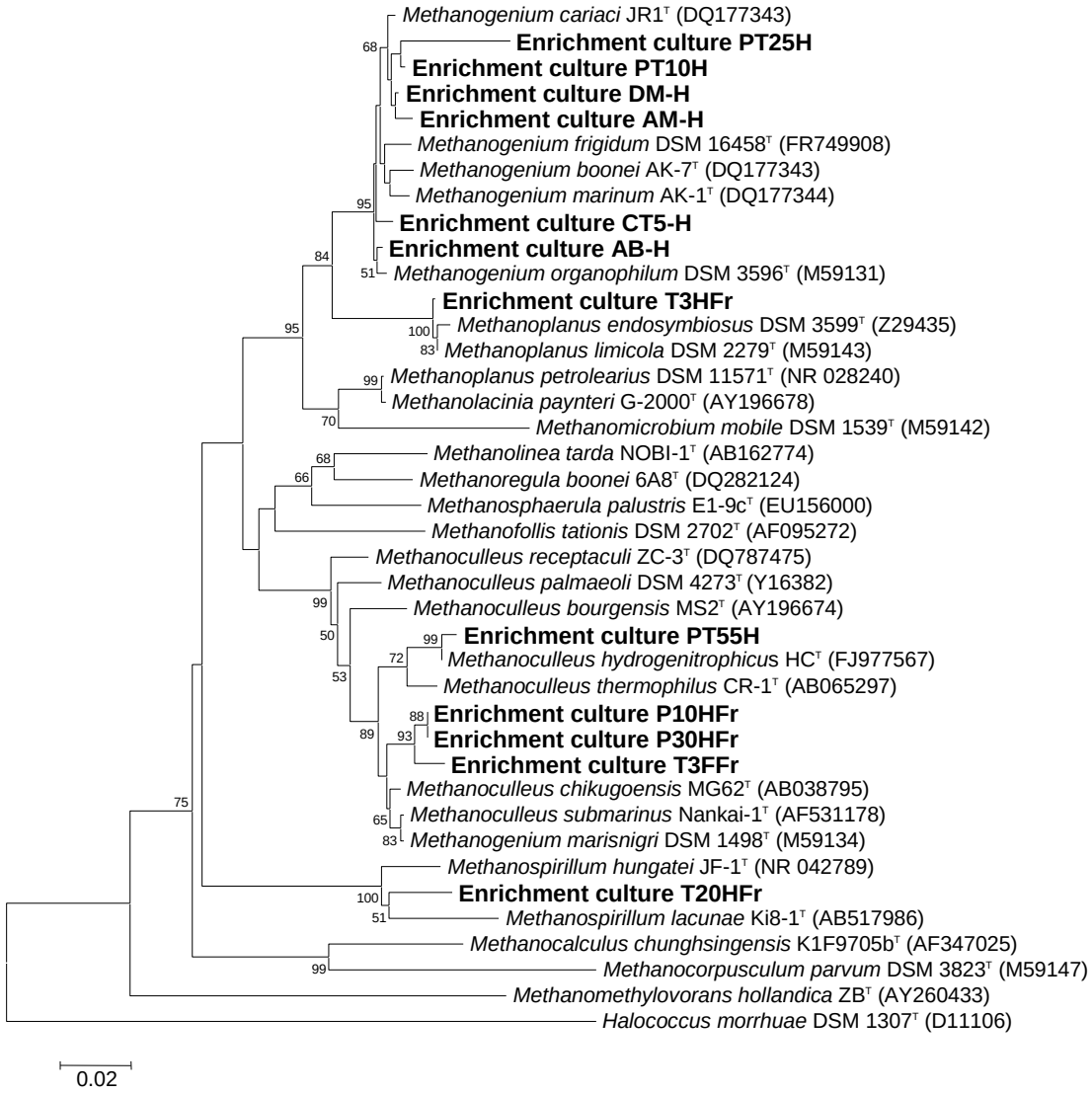


Figure 3.14 16S rRNA gene phylogenetic tree (neighbour-joining) showing the enrichment culture sequences with type strains of the genus *Methanomicrobiales*. Numbers at nodes are percentage bootstrap values based on 1000 replicates (only bootstrap values >50 are shown). Accession numbers are given in brackets. Bar, 0.05 substitutions per site. *Halococcus morrhuae* sequence used as an outgroup.

3.3.2.1 *Methanoculleus*

Seven enrichment cultures, four of which were from Portishead (P10HFr, P30HFr, PT46H and PT55H) and three from Tamar (T3F, T3HFr and T3E) contained methanogens related to the genus *Methanoculleus*, Tables 3.1 and 3.7. All cultures contained non-motile, irregular cocci approximately 1 μm in diameter that exhibited F_{420} autofluorescence. Morphologically, the autofluorescent cells could not be distinguished from members of the genera *Methanogenium* or *Methanococcus*. The

characteristics of *Methanoculleus marisnigri* JR1^T, *Methanoculleus submarinus* Nankai-1^T and *Methanoculleus thermophilus* CR-1^T are summarized in Table 3.12. All three of these type strains are able to grow in marine and low salinity conditions. The incubation temperatures of the enrichment cultures (25, 38, 46 and 55 °C) are within the known range of the genus (Table 3.12).

Members of the genus *Methanoculleus* are able to utilize H_2/CO_2 for methanogenesis and with one exception, *M. hydrogenitrophicus* (Tian *et al.*, 2010), they are also able to utilize formate (Whitman *et al.*, 2006). The type strains of *M. chikugoensis* and *M. palmolei* can also utilize 2-propanol, 2-butanol and cyclopentanol for methanogenesis (Dianou *et al.*, 2001; Zellner *et al.*, 1998). Acetate is a required growth factor for *M. submarinus* Nankai-1^T (Mikucki *et al.*, 2003) as well as *M. bourgensis* strain MS2^T (Ollivier *et al.*, 1986), *M. chikugoensis* strain MG62^T (Dianou *et al.*, 2001), *M. palmolei* strain INSLUZ^T (Zellner *et al.*, 1998) and *M. receptaculi* strain ZC-2^T (Cheng *et al.*, 2008). *M. marisnigri* strain JR1^T requires peptone or yeast extract (Maestrojuán *et al.*, 1990) and *M. thermophilus* CR-1^T has a requirement for tryptase and vitamins (Rivard & Smith, 1982).

Table 3.12 Characteristics of the type strains of *Methanoculleus marisnigri* and *Methanoculleus submarinus*.

Characteristic	*M. marisnigri* strain JR1^T (DSM1498)	*M. submarinus* strain Nankai-1^T (DSM15122)	*M. thermophilus* strain CR-1^T (DSM 2373)
Habitat	Black Sea sediment	Hydrate bearing sediment from Nankai Trough, Japanese coast	High-temp. effluent channel, nuclear power station, USA
Diameter (μm)	1-2	0.8-2.0	1.0-1.3
Motility	-	-	-
Substrates			
H_2/CO_2	+	+	+
Formate	+	+	+
2-propanol	+	ND	ND
2-butanol	+	ND	ND
3-pentanol	+	ND	ND
Required growth factors	Peptone or yeast extract	Acetate*	Trypticase & vitamins
Temperature range (°C)	10-45	11-52	37-65
pH range	5.6-7.6	4.8-8.7	6.2-7.8
Na^+ M conc. range	0-0.69	0.1-1.6	0.0-0.7
Reference	Maestrojuán *et al.* (1990)	Mikucki *et al.* (2003)	Rivard & Smith (1982)

* Peptone and yeast extract are not required but they stimulate growth

In summary, all of the *Methanoculleu*s strains enriched in this study (except for enrichment culture T3E) utilized substrates known to be substrates for members of the genus. They were morphologically similar to members of the genus and grew at temperatures and salinities that have been described for *Methanoculleus* strains. The *Methanoculleu*s strain enriched with ethanol are probably doing so in a syntrophic relationship as ethanol is not known to be a substrate for members of this genus.

3.3.2.2 *Methanogenium*

Six enrichment cultures AB-H, AM-H, DMV-H, PT10H, PT25H and CT5-H contained methanogens with high 16S rRNA gene identities to *Methanogenium cariaci* strain JR1^T, Tables 3.1 and 3.7. The methanogens exhibited F_{420} autofluorescence, were non-motile and were irregular cocci approximately 1-2 μm in diameter. They could not be identified as *Methanogenium* by morphology; they also resembled members of the genera *Methanoculleus and Methanococcus*. All four previously described strains of the genus *Methanogenium* can utilise H_2/CO_2 as well as formate for methanogenesis and require acetate as a carbon source, other growth factors may also be necessary (Table 3.13). Whilst *M. cariaci* strain JR1^T does not growth below 15 ºC (enrichment culture P10H was kept at 10 ºC) the type strains *M. frigidum* Ace-2^T and *M. marinum* AK-1^T can (Table 3.13). Both *M. marinum* AK-1^T and *M. organophilum* CV^T require vitamins; a vitamin solution was added to all media used in this study (Section 2.2.1.1).

In summary, the *Methanogenium* strains cultured in this study are typical of the genus.

Table 3.13 Characteristics of the type strains of the genus *Methanogenium*.

Characteristic	*M. cariaci* JR1^T (DSM 1497)	*M. frigidum* Ace-2^T (OCM 469)	*M. marinum* AK-1^T (DSM 15558)	*M. organophilum* CV^T (DSM 3596)
Habitat	Sediment in Cariaco Trench	Anoxic hypolimnion of Ace Lake, Antarctica	Skan Bay, Alaska, 38-45 cm below sediment surface	Marine mud
Shape	Irregular cocci	Irregular cocci	Irregular cocci	Irregular cocci
Size (μm)	2.6	1.2-2.5	1-1.2	0.5-1.5
Motility	-[1]	-	-	-
Substrates				
H_2/CO_2	+	+	+	+
Formate	+	+	+	+
Ethanol	-	-	-	+
1-propanol	-	-	-	+
2-propanol	-	-	-	+
2-butanol	-	-	-	+
Required growth factors	Acetate or yeast extract	Acetate[2]	Acetate, thiamine, Riboflavin, B12 & peptone[3]	Acetate, biotin, B12 and 4-aminobenzoate[4]
Temperature range (°C)	15-25	0-17	5-25	Optimum 30-35 Maximum 39 Minimum not stated
pH range	6.0-7.6	6.3-8.0	5.5-7.5	Optimum 6.4-7.3
Na^+ M range	0.2-0.8	0.10-0.85	0.25-1.25	Optimum 0.34
Reference	Romesser *et al.* (1979)	Franzmann *et al.* (1997)	Chong *et al.* (2002)	Widdel *et al.* (1988)

[1] Not seen to be motile but has peritrichous flagella
[2] Peptone and yeast extract stimulatory
[3] Yeast extract stimulatory
[4] Trypticase and yeast extract stimulatory

3.3.2.3 *Methanoplanus*

Tamar enrichment culture T3AFr contained what was thought of at first to be a mixed population of F_{420} auto-fluorescent rods and irregular cocci (all were non-motile). However, it became apparent that what appeared to be rods were discs seen edge on. Disc-shaped cells are a characteristic of the genus *Methanoplanus* (Wildgruber *et al.*, 1982). Archaeal 16S rRNA gene sequencing demonstrated a 99% sequence identity to *Methanoplanus limicola* strain DSM 2279^T (Table 3.1). The strain *Methanoplanus endosymbiosus* MC1^T is an endosymbiont of the marine cillate *Metopus contorus* (van Bruggen *et al.*, 1986), the methanogens in the Tamar enrichment culture were free living not endosymbiots. All three previously described strains grow on H_2/CO_2 or formate (Table 3.14) and acetate is required as a carbon source by *Methanoplanus limicola* strain DSM 2279^T and M*ethanoplanus petrolearius* strain SEBR 4847^T (Ollivier *et al.*, 1997; Wildgruber *et al.*, 1982). The type strains are capable of growing under freshwater or marine conditions.

As the type strains of *Methanoplanus* do not utilize acetate as a catabolic substrate then the members of this genus in the Tamar acetate enrichment culture T3AFr probably utilized hydrogen produced from acetate in a syntrophic relationship and bacteria.

Table 3.14 Characteristics of type strains of the genus *Methanoplanus*.

	M.endosymbiosus strain DSM 3599^T	*M. limicola* strain DSM 2279^T	*M.petrolearius* strain SEBR 4847^T
Habitat	Endosymbiont of the saprophilic marine cillate *Metopus contorus*	Drilling waste swamp, Naples, Italy	Offshore oilfield, Gulf of Guinea
Shape	Discs occurring singly	Discs occurring singly	Irregular discs Occurs singly/pairs
Size (μm)	1.6-3.3	1.6-2.8	1-3
Motility	ND	Weakly motile	Non-motile
Substrates	H_2/CO_2, formate	H_2/CO_2, formate	H_2/CO_2, formate
Required growth factors	None[1]	Acetate[2]	Acetate[1]
Temperature range (°C)	16-36	17-41	24-44
pH range	6.1-8.0	Optimum 6.5-7.5	5.3-8.4
Na^+ M range	0.0-0.8	0.1-0.9	0.0-0.9
Reference	Van Bruggen *et al*, (1986)	Wildgrubber *et al.* (1982)	Olivier *et al*. (1997)

[1]Yeast extract is stimulatory
[2]Yeast extract or peptone is stimulatory

3.3.2.4 *Methanospirillum*

Five Tamar estuary enrichment cultures, three with marine medium T3H, T3E(38) and T20H, and two with freshwater medium T20HFr and T20AFr (Table 3.1) contained autofluorescent rods that were curved and approximately 10 μm in length (Figure 3.15). Less common, but seen in all enrichment cultures, were long wavy filaments that could exceed 100 μm in length (Figure 3.15). When viewed with F_{420} autofluorescence, gaps could be seen at intervals along the filament (Figure 3.15). Morphologically, the F_{420} autofluorescent cells were similar to forms of the genus *Methanospirillum* (Ferry *et al.*, 1974) and to *Methanobacterium oryzae* strain Fpi^T (rod 3–10 μm long to filamentous 40 μm long, Joulian *et al.* (2000)). 16S rRNA gene PCR-DGGE band sequencing of all the cultures identified methanogens with 97% 16S rRNA gene identity to *Methanospirillum hungatii* strain JF1^T (Tables 3.1) All previously described strains grow on H_2/CO_2 and formate (Table 3.15) with *Methanospirillum hungatii* strain SK and *Methanospirillum* strain TM20-1 also growing on 2-propanol (Widdel, 1986).

Growth of *Methanospirillum hungatii* strain GP1 in brackish concentrations of Na^+ (0.23 M) have been noted to result in the formation of predominantly the filamentous form (70 to 300 μm long) with the growth rate being one-third of the optimum growth rate. In the Tamar enrichment cultures with marine or freshwater medium the predominant type was the single cell/ short wavy filament. Of the described strains, *Methanospirillum* strain TM20-1 is the most halotolerant showing growth up to 0.35 M Na^+ (Tonouchi, 2002), Table 3.16. A salt-tolerant strain from marine sediment has been isolated but a full description of this strain has not been published (Ferry & Boone, 2001).

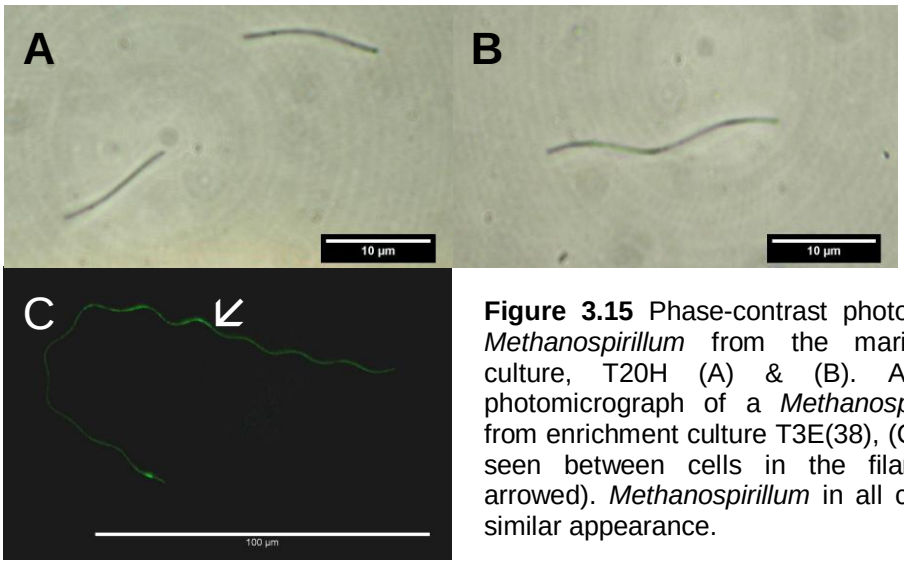


Figure 3.15 Phase-contrast photomicrographs of *Methanospirillum* from the marine enrichment culture, T20H (A) & (B). Autofluorescence photomicrograph of a *Methanospirillum* filament from enrichment culture T3E(38), (C). Gaps can be seen between cells in the filament (example arrowed). *Methanospirillum* in all cultures were of similar appearance.

Table 3.15 Characteristics of the type strains of *Methanospirillum*.

	M. hungatii strain JF1^T	*M.lacunae* strain Ki8-1^T	*Methanospirillum* strain TM20-1
Habitat	Sewage sludge	Puddly soil, Japan	Paddy field soil, Japan
Shape	Curved rods	Curved rods	Curved rods
Length (μm)	7.4-20 also filaments 15 to several hundred μm	11-25	6-13 occasional wavy filaments > 100 μm
Substrates	H_2/CO_2, formate[1]	H_2/CO_2, formate	H_2/CO_2, formate, 2-propanol
Required growth factors	None[2]	Acetate or yeast extract	Acetate
Temperature range (°C)	20-50	15-37	15-40
pH range	6.5-10.0	6.0-9.5	6.2-8.6
Na^+ M range	ND	<0.2	0-0.35
Reference	Ferry *et al.* (1974)	Iino *et al.* (2010)	Tonouchi (2002)

[1] Strain SK can utilize 2-propanol (Widdel, 1986)
[2] Yeast extract and tryptase are stimulatory

3.3.3 *Methanosarcinales*

Strains from three genera of the order *Methanosarcinales* were enriched in this study: *Methanosaeta* (Section 3.3.3.1), *Methanosarcina* (Section 3.3.3.2), and *Methanococcoides* (Section 3.3.3.3). Figure 3.16 is a 16S rRNA gene phylogenetic tree including *Methanosaeta* and *Methanosarcina* strains enriched in this study. *Methanococcoides* strains enriched in this study are included in Figure 3.19.

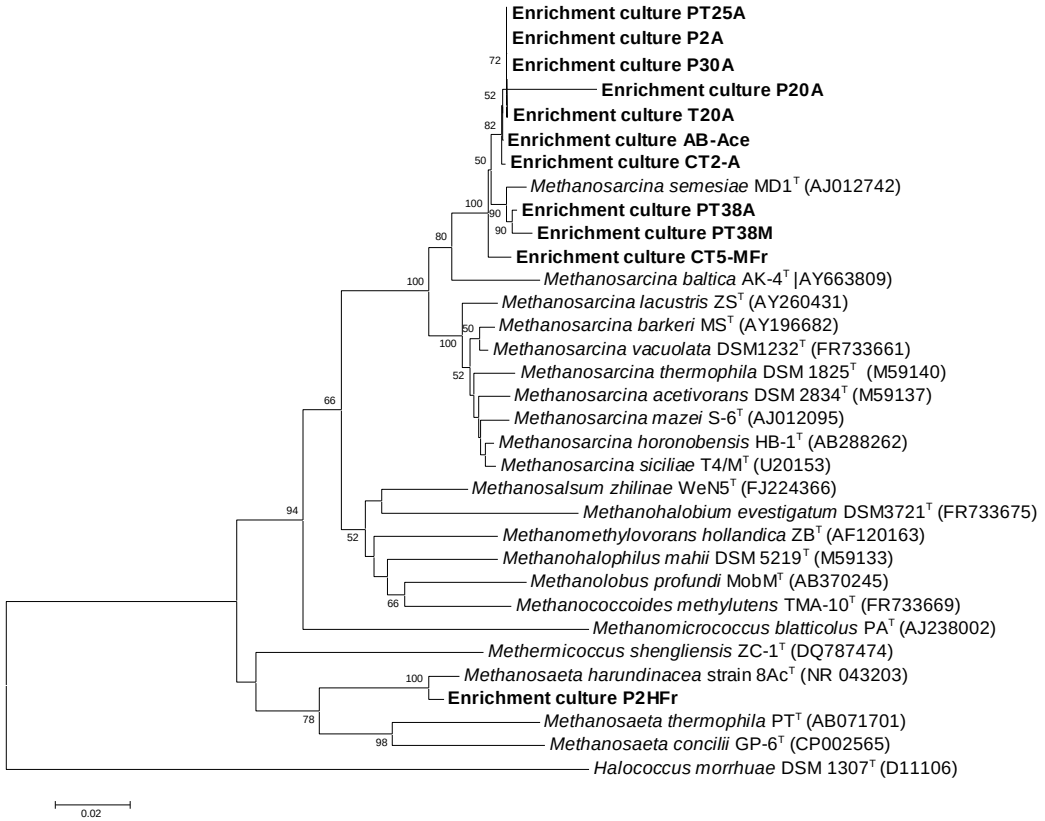


Figure 3.16 16S rRNA gene phylogenetic tree (neighbour-joining) of the order *Methanosarcinales*. Accession numbers in brackets. Numbers at nodes are percentage bootstrap values based on 1000 replicates (only bootstrap values >50 are shown). Bar, 0.02 substitutions per site. *Halococcus morrhuae* sequence used as an outgroup.

3.3.3.1 *Methanosaeta*

Two enrichment cultures, Portishead enrichment culture P2HFr and Tamar enrichment culture T3A contained distinctive flat-ended non-motile rods approximately 5 μm long (straight rods can be as long as 15 μm and filaments as long as 50 μm could occasionally be seen) that appeared to contain vesicles (Figure 3.16).

These disappeared when cells were centrifuged indicating that they were gas vesicles. The F_{420} autofluorescence of cells from both cultures was very weak or not observed. Morphologically, the flat-ended rods with gas vesicles resembled *Methanosaeta thermophila* strain P_T^T (Table 3.16). Gas vesicles as well as being a characteristic of *Methanosaeta thermophila* strain P^T are occasionally seen in *Methanosaeta* strains in mesophilic anaerobic digestors (Kamagata & Mikami, 1991). However, they have not been described as a characteristic of the other type strains, *M. harundinacea* strain $8Ac^T$ or *M. concilii* strain $GP6^T$ (Ma *et al.*, 2006; Patel & Sprott, 1990). All three *Methanosaeta* species can grow as a chain of cells within a common sheath (Kamagata & Mikami, 1991; Ma *et al.*, 2006; Patel & Sprott, 1990). No other cells in these cultures resemble described strains of *Methanosaeta*. The presence of *Methanosaeta* in these enrichment cultures was confirmed by 16S rRNA gene sequencing (Table 3.1).

As with the cells in the enrichment cultures, *Methanosaeta concilii* strain $GP6^T$ exhibits very weak or no observable autofluorescence whereas *Methanosaeta thermophila* strain P_T^T has strong autofluorescence (Kamagata & Mikami, 1991). The strength of F_{420} autofluorescence has not been described for *Methanosaeta harundinacea* strain $8Ac^T$ (Ma *et al*, 2006). Kamagata & Mikami (1991) measured the coenzyme F_{420} content of *Methanosaeta thermophila* strain P^T that was determined to be ten times higher than that of *Methanosaeta concilii* strain $GP6^T$; 63.5 nmol g^{-1} (dry wt) and 6.1 nmol g^{-1} (dry wt) respectively. The F_{420} content of *Methanosaeta harundinacea* strain $8Ac^T$ has not been determined.

All previously described species of *Methanosaeta* utilize only acetate as a substrate for methanogenesis (Kamagata & Mikami, 1991; Ma *et al.*, 2006; Patel & Sprott, 1990). *Methanosaeta harundinacea* requires yeast extract for growth whereas *Methanosaeta concilii* does not (Ma *et al.*, 2006; Patel & Sprott, 1990). The culture grew without the addition of yeast extract.

All previously described *Methanosaeta* species are non-marine and their salinity ranges have not been determined (Kamagata & Mikami, 1991; Ma *et al.*, 2006; Patel & Sprott, 1990). However, *Methanosaeta* have been identified in marine environments such as Jade Bay (Dangast, Germany) (Kittelmann & Friedrich, 2008).

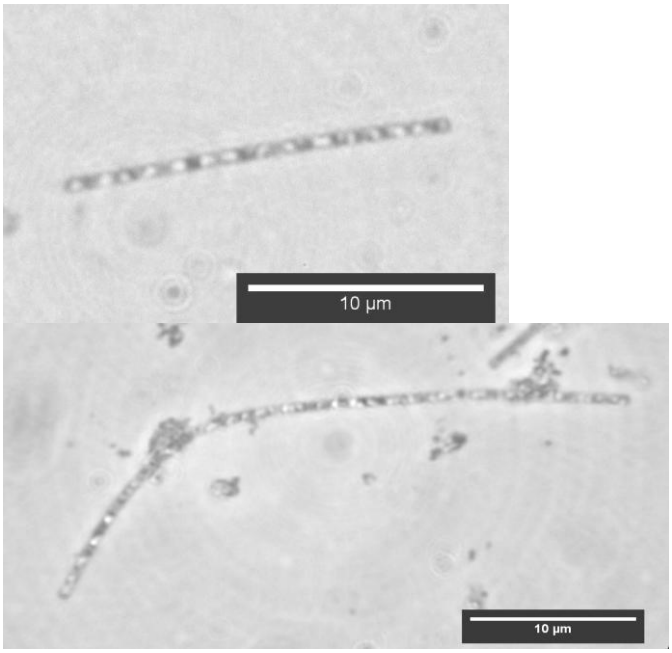


Figure 3.17 Phase-contrast photomicrographs of cells resembling *Methanosaeta thermophila* strain P^T. F_{420} autofluorescence of such cells is, at best, very weak. Top: Enrichment culture T3A. Bottom: Enrichment culture P2HFr.

Table 3.16 Characteristics of the type strains of the genus *Methanosaeta*.

	M. concilii strain GP6^T (DSM 3671T)[1]	*M. harundinacea* strain 8Ac^T (JCM 13211)	*M. thermophila* strain P_T^T (DSM 6174T)
Habitat	Pear waste fermentor inoculated with anaerobic sludge	Anaerobic digestor, beer-manufacture wastewater	Thermophilic anaerobic digester
Shape	Non-motile rods with flat ends Can occur as long filaments	Non-motile rods with flat ends Occurrs singly, in pairs or as long filaments	Non-motile rods with flat ends Can form filaments up to 100 µm long Gas vesicles present
Size (µm)	2.5 to 6.0	3-5	3.0
Motility	-	-	-
Substrates	Acetate	Acetate	Acetate
Required growth factors	None[2]	Yeast extract	None[3]
Temperature range (°C)	>10-<45	24-45	35-65
pH range	6.6-7.8	6.5-9.0	6.1-7.5
Na^+ M range	ND	ND	ND
Reference	Patel (1984) Patel & Sprott (1990)	Ma *et al*. (2006)	Kamagata & Mikami (1991)

[1] Previously *Methanothrix concilii* (Patel & Sprott, 1990)
[2] Yeast extract (0.05% w/v) is inhibitory
[3] Yeast extract (0.1% w/v) is inhibitory

3.3.3.2 *Methanosarcina*

All Portishead enrichment cultures with acetate as a substrate (P2A, P20A, P30A) as well as Portishead temperature gradient enrichment with acetate (PT25A, PT38A and PT46A) contained methanogens identified by 16S rRNA gene sequencing as members of the genus *Methanosarcina* (Tables 3.1 and 3.7) The cells were similar, they were non-motile, approximately 1-3 μm in diameter and occurred as single cells or, more commonly, as aggregates and exhibited F_{420} autofluorescence (Figure 3.18). A Portishead enrichment culture with methylamine as a substrate (PT38M) contained F_{420} autofluorescent, non-motile, irregular cocci approximately 1-2 μm in diameter, which occurred singly or occasionally as loose aggregates of cells (Figure 3.18). Tamar enrichment cultures T3H, T20A, T20AFr and T3E(38) contained F_{420} autofluorescent non-motile cocci, with diameters of approximately 1 μm in diameter occurring singly and in loose aggregates (Figure 3.18). The cocci in enrichment cultures T3H occurred as aggregates and single cocci were not seen (Figure 3.18). All of these cultures contained members of the genus *Methanosarcina* (Table 3.1). The Aarhus Bay enrichment culture AB-Ace and, Guaymas Basin enrichment cultures CT2-A and CT5-MFr also contained members of this genus (Table 3.1). All of the enrichment cultures contained F_{420} autofluorescent non-motile cocci, with diameters of approximately 1 μm in diameter occurring singly and attached to FeS particles.

There are ten species of *Methanosarcina* (Euzéby, 2011) including four species, in addition to *M. semesiae* strain MD1^T, that were isolated from marine environments; *M. acetivorans* strain C2A^T from Scripps Canyon, California (Sowers *et al.*, 1984), *Methanosarcina baltica* strain GS1-A^T from the Gotland Deep, Baltic Sea (von Klein *et al.*, 2002b), *M. frisia* strain C16^T from a southern North Sea mud shoal (Blotevogel & Fischer, 1989) and *M. siciliae* strain T4/M^T (originally known as *Methanolobus siciliae*) from marine canyon sediments (Elberson & Sowers, 1997b; Ni & Boone, 1991). *M. frisia* is regarded as a heterotypic synonym of *M. mazei* (Maestrojuán *et al.*, 1992). Characteristics of these species are given in Table 3.17.

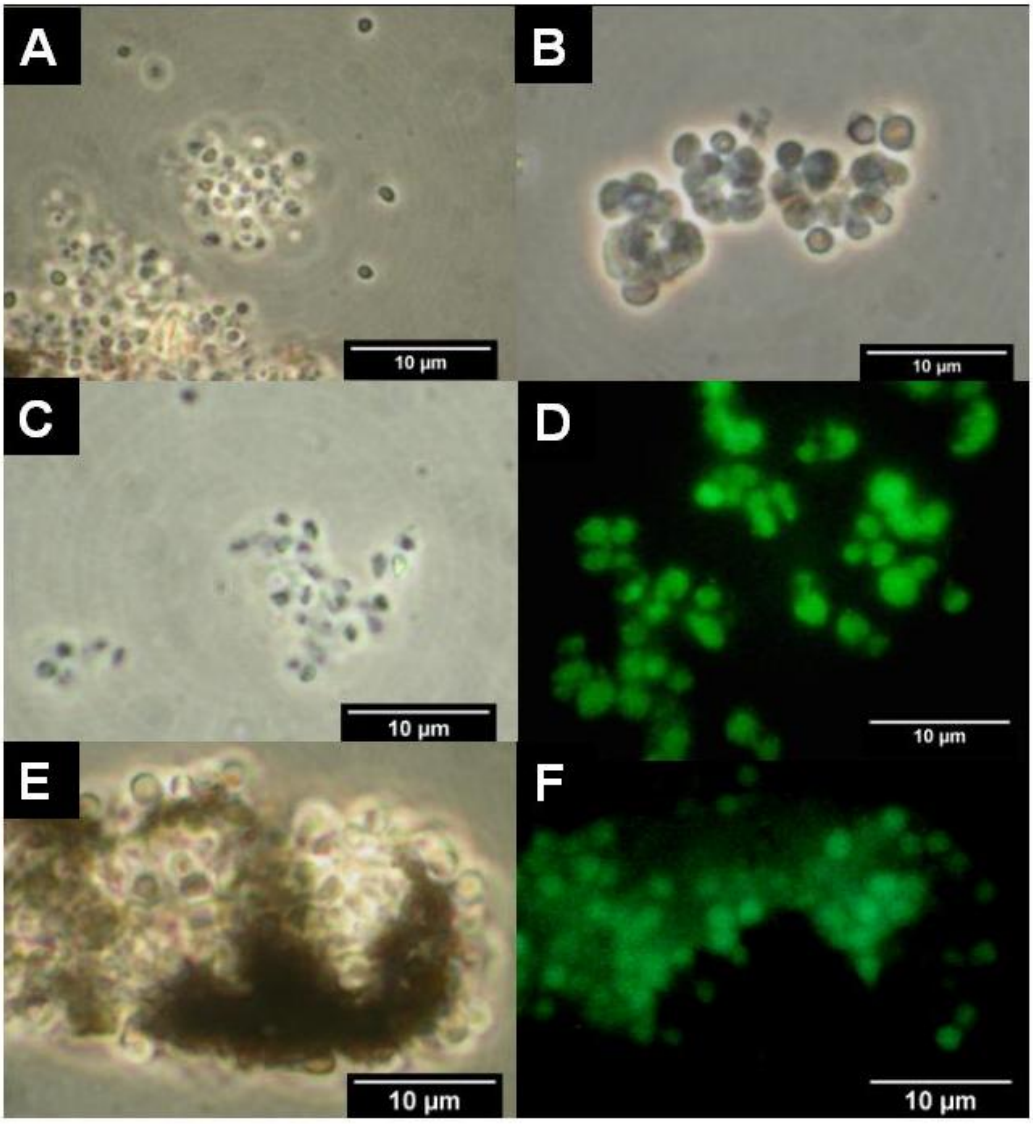


Figure 3.18 Photomicrographs of *Methanosarcina* enrichment cultures. Phase contrast photomicrographs of enrichment cultures T20A (A), T3H (B) and PT38M (C). F_{420} autofluorescent photomicrograph of enrichment culture PT38A (D). Phase contrast and autofluorescent photomicrographs of the same group of cells from enrichment culture P2A (E and F).

Table 3.17 Characteristics of the type strains of *Methanosarcina mazei* and the type strains of the marine *Methanosarcina*. Where ranges are not available the optimum values are given.

	Type Strains				
Characteristic	*M. acetivorans* Strain C2A (DSM 2834)	*M. baltica* Strain GS1-A^T (DSM 14042)	*M. mazei* Strain S-6^T (DSM 2053)	*M. semesiae* Strain MD1^T (DSM 112914)	*M. siciliae* Strain T4/M^T (DSM 3028)
Habitat	Scripps Canyon, La Jolla, USA	Gotland Deep, Baltic Sea	Anaerobic sewage digester	Mangrove forest sediment, Tanzania	Marine sediment, Sicily, Italy
Shape	Irregular cocci Singly/pairs/ aggregates	Irregular cocci Singly/pairs/ tetrads	Irregular cocci Irregular clumps	Irregular cocci Aggregates not seen	Irregular cocci Singly or aggregates
Size (μm)	Cocci 1.9	Cocci 1.5-3.0	Cocci 1.0-3.0 Clumps 20-100	0.8-2.1	1.5-3.0
Motility	-	ND	-	-	-
Substrates					
Acetate	+	+	+	-	-[4]
Formate	-	-	+	-	-
H_2/CO_2	-	-	+	-	-
DMS	ND	-	ND	+	+
Methanol	+	+	+	+	+
Methylamine	+	+	+	+	+
Dimethylamine	+	+	ND	+	+
Trimethylamine	+	+	+	+	+
Required growth factors	None	None	Yeast extract, trypticase	None	None
Stimulatory factors	Yeast extract	ND	Sludge supernatent	None stated	Yeast extract
Temp. range (°C)	10-50	4-27	20-45[1]	18-39 [3]	20-50
pH range	5.5-8.0	4.0-8.5	6.1-8.0	6.2-8.3	5.8-7.2
Na^+ range (M)	0.1-0.6	0.2-1.2	0.1-1.0[1]	0.0-1.0	0.4-0.6 (optimum)
Reference	Sowers *et al* (1984)	Von Klein *et al.* (2002b)	Mah & Kuhn (1984)	Lyimo *et al* (2000)	Ni & Boone (1991)

[1] Maestrojuán and Boone (1991)
[2] May grow slowly on H_2/CO_2 (Zinder *et al.*, 1985)
[3] Lower temperatures not tested Lyimo *et al* (2000)
[4] *M. siciliae* strain C25 is able to utilise acetate (Elberson & Sowers, 1997a)
ND, not determined

Six enrichment cultures contained methanogens with a 98-99% 16S rRNA gene sequence similarity to *M. semesiae* strain MD1^T (AB-Ace, CT2-A, CT5-MFr, P2A, P10A and P30A). All of these enrichment cultures except CT5-MFr only acetate as a growth substrate (Table 3.1). The methanogen may have been utilizing acetate directly or alternatively, syntrophic acetate oxidation may have occurred and the methanogens were using hydrogen as a substrate. One of the characteristics of strain *M. semesiae* strain MD1^T, is the inability to utilise acetate or H_2/CO_2 as growth substrates (Lyimo *et al.*, 2000), Table 3.17. The results of this study suggest that the substrate range of the species *M. semesiae* maybe wider than that of *M. semesiae* strain MD1^T. *Methanosarcina* were identified only once in an enrichment culture with H_2/CO_2 as a growth substrate, enrichment culture T3H, and the 16S rRNA gene sequence had a higher 16S rRNA gene sequence identity to *M. mazei* S-6^T than to any other strain (Table 3.1).

The morphology of the cells with high 16S rRNA gene sequence identity to *M. semesiae* strain MD1^T also differed from the description of this strain, which describes their shape as irregular cocci (Lyimo *et al.*, 2000). Only the cells in enrichment culture PT38M (methylamine as a growth substrate) match this description although they can form aggregates, which is not a characteristic of *M. semesiae* strain MD1^T. Members of the genus *Methanosarcina* are known to have a variety of morphologies. Four *Methanosarcina* morphologies have been described; single cells, communal cysts (multiple cocci within a common envelope), small aggregates and large aggregates (Maestrojuán & Boone, 1991). Growth substrates can be a factor affecting the morphology of *Methanosarcina* cells. For example, the morphology of *M. acetivorans* strain C2A^T depends on whether it is grown on acetate or methylamines (Sowers *et al.*, 1984). With acetate it forms aggregates of two to twelve cells and communal cysts, when grown on methylamines aggregates and communal cysts did not occur. *M. mazei* strain S-6^T has even been observed to have different morphologies when subcultures have been made under apparently identical conditions (Maestrojuán & Boone, 1991).

In summary, thirteen enrichment cultures contained members of the genus *Methanosarcina*, six of which were closely related to the species *M. semesiae* by 16S rRNA gene sequencing (Table 3.1). Unlike the type strain *M. semesiae* MD1^T they were able to utilise acetate and exhibited a wider range of morphologies.

3.3.3.3 *Methanococcoides*

With one exception (enrichment culture PT38M) all marine medium enrichment cultures with methylamine or methanol as a catabolic substrate contained methanogens of the genus *Methanococcoides* (Table 3.1 and Figure 3.19 for phylogenetic tree of the genus *Methanococcoides*). All cells exhibited F_{420} autofluorescent (autofluorescence faded rapidly in some cultures) and were irregular cocci 1-2 μm in diameter. They were non-motile and had a tendency to attach to FeS particles in the medium (Figure 3.20). In enrichment cultures AB-Bet and AB-MeOH, clumps of cells were seen (Figure 3.20). The genus and sixteen strains isolated from the enrichment culture are discussed in Chapters 6 and 7.

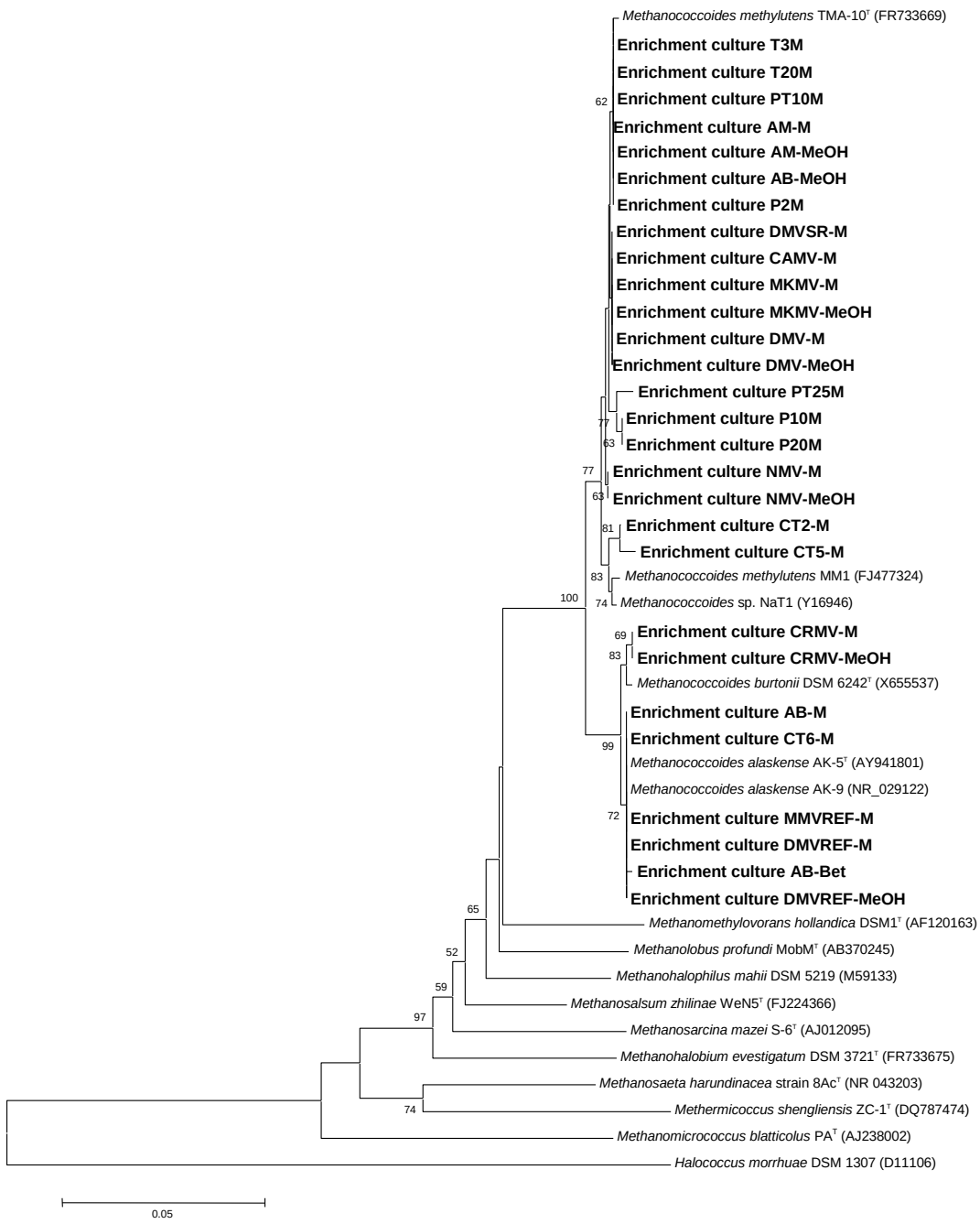


Figure 3.19 16S rRNA gene sequence phylogenetic tree (neighbour-joining), showing enrichment culture sequences of the genus *Methanococcoides*. Accession numbers in brackets. Numbers at nodes are percentage bootstrap values based on 1000 replicates (only bootstrap values >50 are shown). Bar, 0.01 substitutions per site.

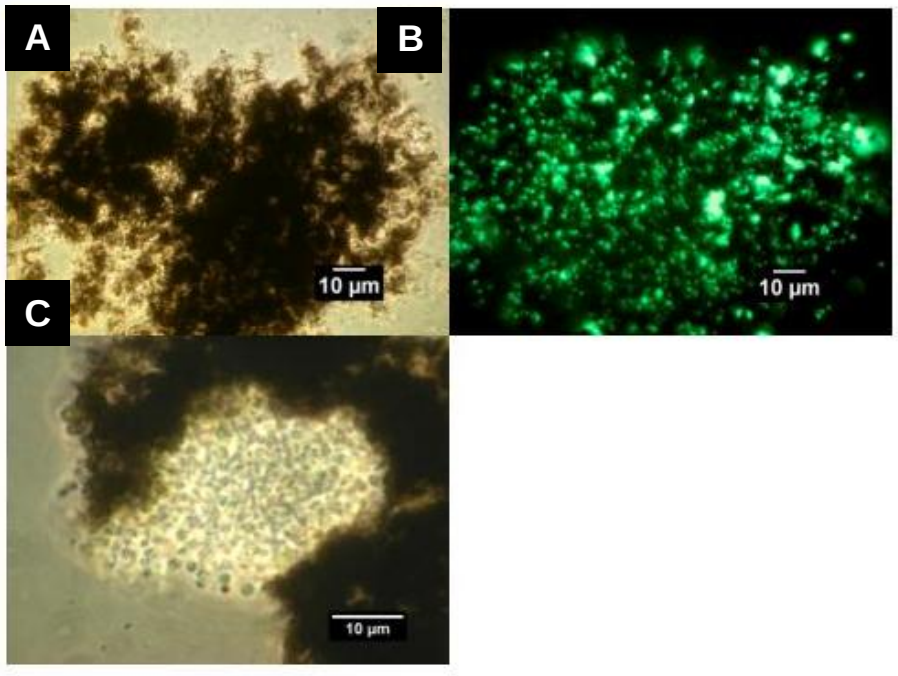


Figure 3.20 Phase-contrast (A) and autofluorescent (B) photomicrographs of the same FeS particle in a Meknes MV enrichment culture (cells attached to particles is seen in all *Methanococcoides* enrichment cultures). Aarhus Bay enrichment culture AB-MeOH (C), the dark area surrounding the clump of cells is a FeS particle.

3.3.4 *Methanococcales*

This order was represented by only one genus in this study and was cultured from only one location. Three Portishead enrichment cultures (P2H, P10H and P30H) contained members of the genus *Methanococcus* (Table 3.1). They all had 100% 16S rRNA gene sequence identities to *Methanococcus maripaludis* KA1. Two strains were isolated from Portishead, strain PM4 from enrichment culture P10H and strain PM5 from enrichment culture P30H. See Section 6.3 for a description of these strains.

3.4 Discussion

3.4.1 Shallow Water Sites

3.4.1.1 Aarhus Bay

This study was undertaken in two parts, the first part made use of slurry (Section 2.1.1.2) that had been prepared for the study by Webster *et al*. (2011) and the second part used slurry prepared specifically for this study.

Methanogens belonging to the genera *Methanococcoides* (AM-M) and *Methanogenium* (AM-H) were identified in the first set of Aarhus Bay enrichment cultures (Table 3.1). Prior to this study, samples of the same sediment slurry (Chapter 2.1.1.2) were used to investigate the prokaryotic community of the Aarhus Bay SMTZ (Webster *et al.*, 2011). As part of that study, methanogenesis rates were determined using radioisotopes. Methanogenesis from [^{14}C] acetate or [^{14}C] bicarbonate was not detected within the SMTZ (methanogenesis from non-competitive substrates was not investigated) (Webster *et al.*, 2011).

16S rRNA gene PCR-DGGE (Webster *et al.*, 2011) demonstrated the presence of *Archaea* in the SMTZ; they were represented by members of the Miscellaneous *Crenarchaeota* Group (MCG), MBG-D/ *Thermoplasmatales* groups and ANME-1 as well as by novel *Euryarchaeota*. No methanogen sequences were detected (Webster *et al.*, 2011). The Archaeal community structure was further assessed in sediment slurries to which acetate and glucose were added (Webster *et al.*, 2011). Archaeal 16S rRNA gene PCR-DGGE did not show any difference to the initial gene clone library results; however, *mcrA* PCR-DGGE detected the presence of *Methanosarcina* sp. Additional enrichment cultures contained methanogens belonging to the orders *Methanosarcinales* and *Methanomicrobiales* (Webster *et al.*, 2011). The order *Methanomicrobiales* includes the genus *Methanogenium.*

Despite using the same sediment slurry as Webster *et al.* (2011), *Methanosarcina* were not detected in enrichment from this study. The unamended sediment slurry had been stored at 10 ºC for approximately two years prior to the start of this study and

this may, to some extent, explain why *Methanosarcina* was not cultured although it had previously been detected by molecular means. *Methanosarcina* were subsequently cultured from the Aarhus Bay SMTZ in this study (enrichment culture AB-Ace, Table 3.1) using sediment slurry prepared from a more recently collected sediment core from the same site and using acetate as a growth substrate (Section 2.1.1.2).

16S rRNA gene sequences obtained in this study from the second series of enrichment cultures (core May 2010) had high sequence identities to two *Methanococcoides* species. The 16S rRNA gene sequences obtained from enrichment cultures with methanol as a substrate (AB-MeOH) had 99% sequence identity to *M. alaskense* AK-5^T whilst those from the methylamine enrichment culture (AB-M) had a 99% sequence identity to *M. methylutens* TMA-10^T (Table 3.1).

3.4.1.2 Portishead

This study also consisted of two parts, sediment slurries prepared for this study and subcultures of sediment slurries that were part of a temperature gradient experiment.

Three genera of methanogens were enriched in cultures from Portishead tidal flat sediment using marine medium, *Methanococcoides* (methylamine as a substrate), *Methanococcus* (H_2/CO_2 as a substrate), *Methanogenium* (H_2/CO_2 as a substrate) and *Methanosarcina* (acetate or methylamine as substrates) and two additional genera using freshwater medium, *Methanoculleus* (H_2/CO_2 as a substrate) and *Methanosaeta* (H_2/CO_2 as a substrate), Table 3.1. With marine medium the same substrate gave rise to the same methanogen at all depths (0-2, 18-21 and 30-35 cm). *Methanosaeta* were cultured from the uppermost layer (0-2 cm depth) using freshwater medium and *Methanoculleus* from the other two layers also using freshwater medium (Table 3.1).

Four genera of methanogens were identified by 16S rRNA gene PCR-DGGE in the Portishead temperature gradient experiment slurries: *Methanobacterium*, *Methanococcoides*, *Methanoculleus* and *Methanosarcina* (Figure 3.21 and Table 3.18, Dr Gordon Webster, unpublished data). Sequences identified as *Methanococcoides* fell into two groups, those with a 98-100% sequence identity to *M. alaskense* (bands 7.1 and 13.2), and those with a 97-99% sequence identity to *M. methylutens* (bands

13.1, 20.1, 30.1, 37.1, 40.1 and 40.2). The DGGE gel showed bands corresponding to *M. alaskense* and *M. methylutens* occurred in the temperature range 13-20 ºC with bands corresponding to *M. alaskense* at lower temperatures and *M. methylutens* at higher temperatures (Figure 3.21).

16S rRNA gene PCR-DGGE indicated the presence of four species of the genus *Methanosarcina*: *M. lacustris*, *M. baltica*, *M. semesiae* and *M. thermophila* (Figure 3.21 and Table 3.18). DGGE band sequencing identified a *Methanoculleus* strain (99% 16S rRNA gene sequence identity to *Methanoculleus thermophilus* strain NG-1) at 44 and 50 ºC. Only one DGGE band sequence was identified as possibly representing a methanogen capable of utilising a substrate other than H_2/CO_2 for methanogenesis above 44 ºC, *Methanosarcina thermophila* DSM 1825, which was identified at 54 ºC.

All four of the genera identified by 16S rRNA gene PCR-DGGE in the temperature gradient experiment were also identified in subcultures of the temperature gradient slurries undertaken in this study (Table 3.7). In addition, methanogens of the hydrogen-utilizing genus *Methanogenium* were enriched in this study at 10 and 25 ºC (Table 3.7). 16S rRNA gene PCR-DGGE of the temperature gradient slurries did not identify a specific hydrogen-utilizing methanogen below 44 ºC (Figure 3.20 and Table 3.18); the only methanogens identified that may have been able to utilize hydrogen below this temperature were *Methanosarcina* sp.

The difference between the temperature gradient slurries and the subcultures may be explained by the medium and substrates used by the two. The temperature gradient slurries were given a combination of three substrates, acetate, H_2/CO_2, and methylamine in a medium without added vitamins (Dr Gordon Webster) whilst the subcultures prepared by this study used medium containing vitamins (Section 2.2.1.1) and only one substrate (the slurry was subcultured into tubes with either acetate, H_2/CO_2 or methylamine). Some *Methanogenium* strains require vitamins for growth *e*.g. *M. marinum* AK-1^T and *M. organophilum* CV^T (Table 3.13).

Using enrichment cultures prepared by this study, *i.e.* not subcultures of the temperature gradient enrichment cultures, the hydrogen-utilizing genus

Methanococcus was enriched at 25 ºC and subsequent two strains were isolated (strains PM4 and PM5). This genus was not grown in subcultures of the temperature gradient enrichment cultures nor was it identified in the temperature gradient slurries using 16S rRNA gene sequencing (Figure 3.21 and Table 3.18),

Methanoculleus was also enriched in this study from the same sediment slurry as *Methanococcus* using freshwater medium and H_2/CO_2 as a substrate (enrichment cultures P20HFr and P30HFr, Table 3.1). Freshwater medium also resulted in the culture of another genus not seen in the temperature gradient experiment, *Methanosaeta* (enrichment culture P2HFr, Table 3.1).

A methanogen closely related to *Methanosarcina semesiae* MD1^T was identified in the temperature gradient slurries at 44 ºC but was not detected at lower or higher temperatures. This study enriched *Methanosarcina semesiae* related methanogens with acetate as a substrate (identified by 16S rRNA gene sequencing, Table 3.1); enrichment cultures with methylamine as a growth substrate, incubated below 38 ºC, resulted in the growth of *Methanococcoides* sp. not *Methanosarcina* sp. As all species of *Methanosarcina* can utilize methylamine it appears that this strain of *Methanosarcina* was out-competed by *Methanococcoides* at lower temperatures.

Methanosarcina strains other than *Methanosarcina semesiae* were identified using 16S rRNA gene PCR-DGGE in the temperature gradient enrichment cultures below 44 ºC (Figure 3.21 and Table 3.18). These were presumably using acetate and/or hydrogen as substrates. The *Methanosarcina semesiae* related methanogens either cannot use or are out-competed for these substrates below 44 ºC. *Methanosarcina semesiae* MD1^T has been described as not being able to use acetate or hydrogen as growth substrates (Lyimo *et al.*, 2000). Alternatively, their absence may have been due to their inability to grow at these lower temperatures; this is unlikely as *Methanosarcina* species are mesophilic or psychrotolerant (Whitman *et al.*, 2006). *Methanosarcina semesiae* was presumably identified at 44 ºC due to the inability of *Methanococcoides* to grow at this temperature (*Methanococcoides* strain PM1 isolated from this location has an upper growth temperature of 40 ºC, Table 6.4). The absence of *Methanosarcina semesiae* above 44 ºC, where no other methylamine consumers

were identified, is presumably due to this strain reaching its upper temperature limit for growth.

Methanosaeta related strains were present in the 0-2 cm depth freshwater culture with H_2/CO_2 (culture P2HFr, Table 3.1) rather than hydrogen utilising *Methanoculleus* enriched at the other two depths, 10-15 cm and 30-35 cm depth (enrichment cultures P10HFr and P30HFR, Table 3.1). *Methanosaeta* can only utilize acetate as a growth substrate (Whitman *et al.*, 2006) and acetate was not added to this enrichment culture. The only source of acetate would have been as a product of hydrogen utilization by acetogenic bacteria. Normally, acetogenic bacteria are out-competed by hydrogenotrophic methanogens except under low temperature conditions (Kotsyurbenko *et al.*, 2001). That acetogenic bacteria were consuming hydrogen in enrichment culture P2HFr may have been due to the lack of a freshwater tolerant hydrogenotrophic methanogen at this particular depth.

As *Methanosaeta* were not enriched in marine medium it raises the question whether they are active at seawater salinity. *Methanosarcina* were enriched in seawater medium with acetate rather than *Methanosaeta,* and *Methanosaeta* were not observed when the acetate levels declined (*Methanosaeta* have a lower threshold for acetate than *Methanosarcina*, Table 1.2). *Methanosaeta* are usually found in freshwater were they may be ubiquitous (Purdy *et al.*, 2002) but have also been identified in the marine environment, *e.g.* tidal-flat sediments (Kittelmann & Friedrich, 2008).

Figure 3.21 16S rRNA gene DGGE profile of day 100 of the Portishead temperature gradient experiment (Dr Gordon Webster, unpublished data). All numbered bands were excised; the BLASTN results for the excised bands are given in Table 3.18. M, marker.

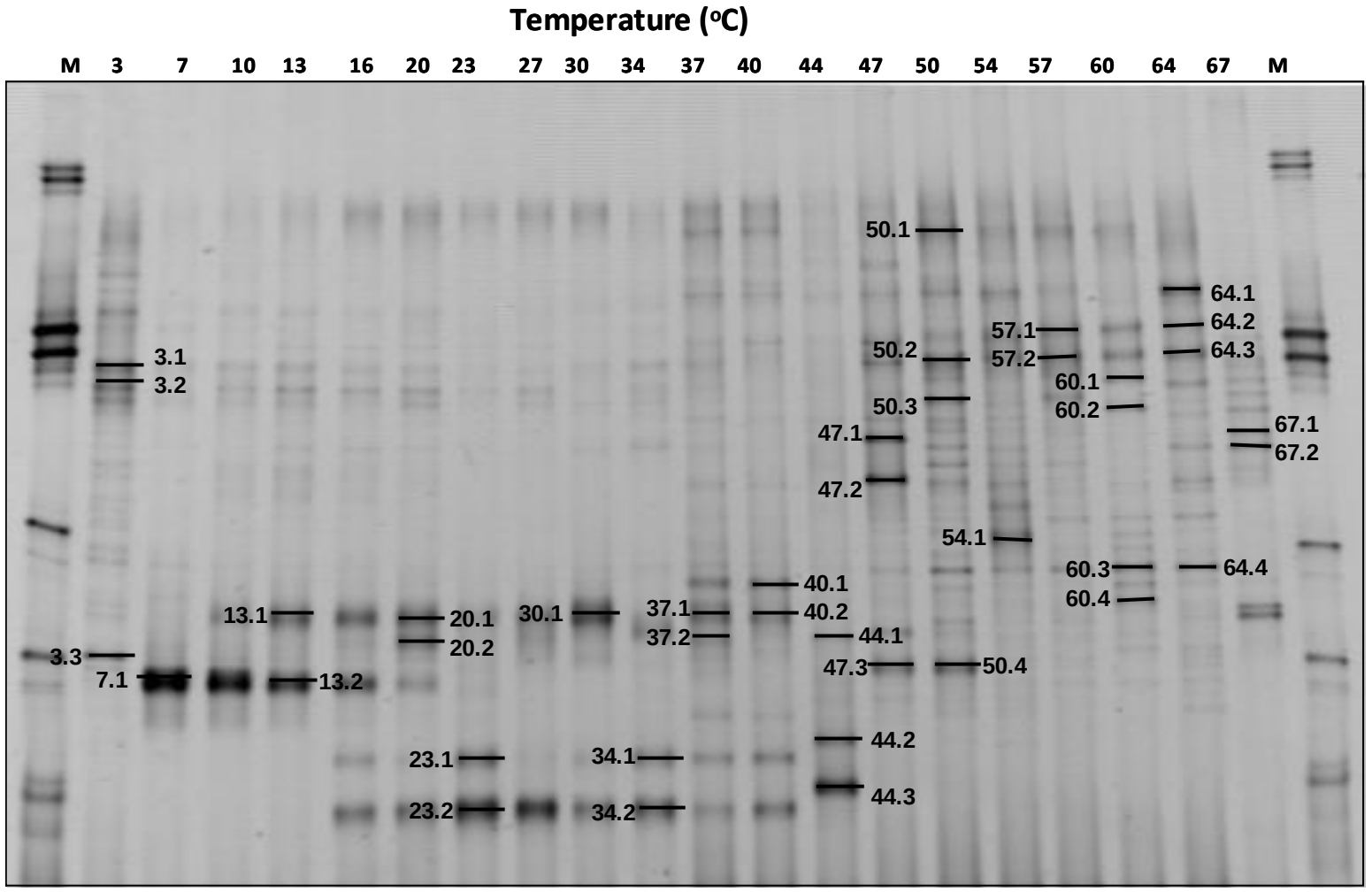


Table 3.18 Archaeal 16S rRNA gene DGGE band identities for the Portishead temperature gradient experiment, day 100 (Dr Gordon Webster, unpublished data). The digit(s) before the decimal place of the DGGE band number indicates the incubation temperature.

DGGE Band number	Closest match by BLASTN search (accession number)	% Sequence identity	Phylogenetic affiliation
3.1	Uncultured euryarchaeote clone AMIX-1C (FJ609955)	100	MBG-D
3.2	Uncultured archaeon clone HWA1012-2-82 (HM244355)	97	MBG-D
3.3	Uncultured archaeon clone HNDA53 (HM171880)	100	MCG
7.1	*Methanococcoides alaskense* strain AK-5^{T} (NR_029122)	100	*Methanosarcinales*
13.1	*Methanococcoides methylutens* strain MM1 (FJ477324)	98	*Methanosarcinales*
13.2	*Methanococcoides alaskense* strain AK-5^{T} (NR_029122)	99	*Methanosarcinales*
20.1	*Methanococcoides methylutens* strain MM1 (FJ477324)	99	*Methanosarcinales*
20.2	*Methanosarcina lacustris* strain MS (AY260431)	100	*Methanosarcinales*
23.1	*Methanosarcina baltica* strain AK-4 (AY663809)	98	*Methanosarcinales*
23.2	*Methanosarcina baltica* strain AK-4 (AY663809)	96	*Methanosarcinales*
30.1	*Methanococcoides methylutens* strain MM1 (FJ477324)	99	*Methanosarcinales*
34.1	*Methanosarcina baltica* strain AK-4 (AY663809)	97	*Methanosarcinales*
34.2	*Methanosarcina baltica* strain AK-4 (AY663809)	97	*Methanosarcinales*
37.1	*Methanococcoides methylutens* strain MM1 (FJ477324)	97	*Methanosarcinales*
37.2	*Methanosarcina lacustris strain* MS (AY260431)	100	*Methanosarcinales*
40.1	*Methanococcoides methylutens* strain MM1 (FJ477324)	99	*Methanosarcinales*
40.2	*Methanococcoides methylutens* strain MM1 (FJ477324)	98	*Methanosarcinales*
44.1	*Methanoculleus thermophilus* strain NG-1 (EF118906)	99	*Methanomicrobiales*
44.2	*Methanosarcina semesiae* strain MD1^{T} (NR_028182)	98	*Methanosarcinales*
44.3	*Methanosarcina semesiae* strain MD1^{T} (NR_028182)	97	*Methanosarcinales*
47.1	*Methanobacterium formicicum* strain MG-134 (HQ591420)	100	*Methanobacteriales*
47.2	*Methanobacterium formicicum* strain M6 (EU544028)	100	*Methanobacteriales*
50.1	Uncultured Crenarchaeon clone Zeebrugge_A78 (HM598528)	99	RC-VI
50.2	Uncultured Crenarchaeon clone Zeebrugge_A78 (HM598528)	98	RC-VI
50.3	Uncultured Crenarchaeon clone Kazan-2A-24 (AY592001)	82	*Crenarchaeota*
50.4	*Methanoculleus thermophilus* strain NG-1 (EF118906)	99	*Methanomicrobiales*
54.1	*Methanosarcina thermophila* DSM 1825 (M59140)	98	*Methanosarcinales*
57.1	Uncultured Crenarchaeon clone Zeebrugge_A78 (HM598528)	98	RC-VI
57.2	Uncultured Crenarchaeon clone Zeebrugge_A78 (HM598528)	98	RC-VI
60.1	Uncultured Crenarchaeon clone Kazan-2A-47 (AY592023)	100	*Crenarchaeota*
60.2	Uncultured Crenarchaeon clone Kazan-2A-47 (AY592023)	100	*Crenarchaeota*
60.3	Uncultured Crenarchaeon clone LPROCKA51 (FJ902278)	93	MCG
60.4	Uncultured Crenarchaeon clone HWA5257-3-53 (HM244193)	97	MCG
64.1	Uncultured Crenarchaeon clone Zeebrugge_A103 (HM598552)	97	MG1
64.2	Uncultured Crenarchaeon clone Zeebrugge_A78 (HM598528)	98	RC-VI
64.3	Uncultured Crenarchaeon clone Zeebrugge_A78 (HM598528)	98	RC-VI
64.4	Uncultured Crenarchaeon clone Kazan-2A-33/BC1 (AY592009)	100	MCG
67.1	Uncultured archaeon clone MgvArc02 (GU993946)	98	*Crenarchaeota*
67.2	Uncultured archaeon clone MgvArc02 (GU993946)	98	*Crenarchaeota*

3.4.1.3 Tamar

As with Portishead sediment, *Methanococcoides* (methylamine as a substrate), *Methanobacterium* (formate as a substrate), *Methanoculleus* (formate as a substrate) *Methanosarcina* (acetate and H_2/CO_2 as substrates) and *Methanosaeta.* Additionally *Methanoplanus* (acetate, formate and H_2/CO_2 as substrates) and *Methanospirillum* (H_2/CO_2 and formate as substrates) were enriched from Tamar sediments. Unlike the Portishead H_2/CO_2 enrichment cultures, members of the genus *Methanococcus* were not cultured (Table 3.1).

The 16S rRNA gene PCR-DGGE (Figure 3.8 and Table 3.8) and enrichment culture results (Table 3.1) together suggested that members of the genus *Methanococcoides* are important components of the *in situ* population of the Tamar Estuary sediment. The presence of *Methanococcoides* at 3 cm depth corresponds to a peak in methane concentration (28 μM l^{-1} wet sediment, Figure 3.8). As *Methanococcoides* are only able to use methylated compounds as growth substrates this implies that these substrates are also important at this site.

Unlike Portishead were the same methanogens were cultured at all three depths (except when freshwater medium was used), the methanogens enriched from Tamar sediments differed between the two depths studied (2-4 and 18-21 cm depth), Table 3.1. Both depths had representatives of the genera *Methanococcoides* and *Methanosarcina* whereas members of the genera *Methanoplanus* and *Methanoculleus* were only enriched from sediment at 2-4 cm depth (Table 3.1). Whilst PCR-DGGE detected a *Methanobacterium* related sequence at 20 cm depth (Figure 3.9 and Table 3.8), *Methanobacterium* were enriched from sediment taken from both 3 and 20 cm depth (Table 3.1).

Possible syntrophic relationship was noted in enrichment cultures T3AFr and T20AFr to which acetate was added as a growth substrate. Enrichment cultures T3AFr and T20AFr contained methanogens related to *Methanoplanus limicola* and *Methanospirillum hungatii* respectively (16S rRNA gene sequencing results, Table 3.1). None of these methanogens are known to utilize acetate as a growth substrate; acetate utilization is limited to the genera *Methanosaeta* and *Methanosarcina*.

Members of the genera *Methanoplanus* and *Methanospirillum* can utilize hydrogen as a substrate. *Methanoplanus* and *Methanospirillum* in enrichment cultures T3AFr and T20AFr are likely to be utilizing hydrogen produced from acetate as part of a syntrophic relationship as the oxidation of acetate to hydrogen is not energetically favourable unless the hydrogen concentration is kept at a low level (Schink, 1997). A syntrophic relationship is also likely with enrichment cultures T3E and T3E(38) which had ethanol added as a growth substrate. The methanogens identified in these two enrichment cultures were members of the genus *Methanobacterium* (Table 3.1), a genus for which ethanol has not been described as a growth substrate (Whitman *et al.*, 2006). Methanogens of the genus *Methanobacterium* can utilize hydrogen as a substrate, hydrogen that can be produced by the degradation of ethanol in a syntrophic relationship (see Section 1.3.2 for the classic example of syntrophy between strain S and strain M.o.H with ethanol as a substrate).

3.4.2 Mud Volcano Sites

3.4.2.1 Dvurechenski MV (Sorokin Trough, Black Sea)

This is the first study to culture methanogens from Dvurechenski MV or any mud volcano in the Sorokin Trough. The three strains isolated from Dvurechenski MV were all members of the methylotrophic genus *Methanococcoides* (Table 3.1).

Prior to this study, methanogenesis rate measurements were measured using [^{14}C]acetate and [^{14}C]bicarbonate with samples taken from the same cores as the samples used for enrichment cultures (Table 3.19; Dr Barry Cragg, unpublished data). A methylamine methanogenesis rate measurement was not undertaken.

Table 3.19 Methanogenesis rates for the Dvurechenski MV Geographic Centre and Reference Site determined using [^{14}C] acetate and [^{14}C] bicarbonate (data, Dr Barry Cragg).

	Methanogenesis rate/ pmol cm^{-3} d^{-1}	
	Geographic Centre	Reference Site
[^{14}C] acetate	Not detected	0.06
[^{14}C] bicarbonate	190	250

No enrichment cultures were prepared from shallow sediment from the Geographic Centre. The Reference Site sediment slurry with hydrogen was prepared (by Dr Barry Cragg) using sediment from a depth corresponding to the hydrogen methanogenesis peak (28 cm depth) but no methane production was noted and it was not subcultured in this study. Bicarbonate methanogenesis was high compared with deep-sea sediment from the Cascadia Margin (up to 50 pmol cm^{-3} d^{-1}), Woodlark Basin (up to 20 pmol cm^{-3} d^{-1}) and Napoli MV (up to 14 pmol cm^{-3} day^{-1}) (Cragg *et al.*, 1996; Lazar *et al.*, 2011b; Wellsbury *et al.*, 2002).

No published study has reported the presence of methanogens at Dvurechenski MV. The isotopic composition of methane at Dvurechenski MV $\delta^{13}C$ varied between –62 and –66 indicated a mainly biogenic origin for the methane (Blinova *et al.*, 2003). Only one study has looked at Archaea in the Sorokin Trough and this investigated carbonate crusts covered with microbial mats at NIOZ, Odessa, and Kazakov mud volcanoes (Stadnitskaia *et al.*, 2005). 16S rRNA gene sequences were not closely related to known methanogens, two sequences (AY847616 and AY847617) had 85% identity to *Methanolobus* and one sequence (AY847618) had 85% identity to *Methanosaeta*. Other sequences were related to ANME-1, MBG-B and miscellaneous *Crenarchaeota*.

A study using fluorescence in situ hybridization (FISH) has detected methanogens in sediment in the deeper parts of the Black Sea (Ince *et al.*, 2006) . Sediment from a water depth of 2235 m (approximately 200 m deeper than the Sorokin Trough) were taken from a location in the central area of the Black Sea lying to the south of the Sorokin Trough (other areas of the Black Sea were also sampled). A range of FISH probes used with sediment from 0 to 27 cm deep detected *Methanosarcina* spp., *Methanosarcina* related methanogens, *Methanosarcinaceae*, *Methanosaeta* sp., *Methanococcales*, *Methanobacteriales*, and *Methanogenium* related methanogens.

3.4.2.2 Gulf of Cadiz Mud Volcanoes

Only two genera of methanogen were enriched from Gulf of Cadiz mud volcanoes, the widely distributed methylated compound utilizing *Methanococcoides* and the hydrogen utilizing *Methanogenium* that was only cultured from Darwin MV (Table 3.1). Of the six mud volcanoes studied, methanogens were successfully cultured from

five; the exception was Bonjardim mud volcano. All successful cultures grew with either methanol or methylamine as a growth substrate. The Mercator MV enrichment cultures that originally produced methane from benzoate or hexadecane (Table 3.5) were not successfully subcultured. As methanogens are not known to degrade benzoate or hexadecane, the initial methane production would have been the result of a syntrophic relationship (Section 1.3.2). The production of methane from hexadecane can involve acetogenic (syntrophic) bacteria that breakdown hexadecane to acetate and hydrogen methanogens producing methane from acetate and hydrogen. Hexadecane enrichment cultures investigated by Zengler *et al*. (1999) identified bacteria affiliated with the *Deltaproteobacteria* and methanogens related to the genera *Methanosaeta*, *Methanospirillum* and *Methanoculleus*. Subculturing of enrichment cultures in this study may have disrupted syntrophic relationships; the conditions may not have suited the syntrophic partners resulting in unsuccessful subcultures. Interestingly, no methanogenesis was noted for the acetate and H_2/CO_2 enrichment cultures using sediment from the same depth as the hexadecane enrichment culture.

Sediment from Bonjardim MV was analyzed using 16S rRNA PCR-DGGE by Sas (2009). Archaea were detected but could not be identified as the DGGE bands were so faint that they could not be excised for sequencing. Sas (2009) also investigated Captain Arutynov and Meknes mud volcanoes. Only four archaeal phylotypes were identified in sediment from Captain Arutynov MV using a 16S rRNA gene library. The most common taxa were represented by one phylotype of ANME-2. There were also two phylotypes of the Miscellaneous *Crenarchaeota* Group and a single phylotype of the genus *Methanococcoides* (99% sequence identity to *Methanococcoides burtonii* DSM 6242^T). *McrA* DGGE-PCR failed to identify methanogens at Meknes MV but did identify sequence belonging to *mcrA* group a (equivalent to the ANME-1 16S rRNA group), however, a *mcrA* clone library did identify *Methanococcoides* (95% sequence identity to *Methanococcoides burtonii* DSM 6242^T) (Sas, 2009).

16S rRNA sequences obtained from enrichment cultures (sediment from 10 cm depth) had a 99% identity to *Methanococcoides methylutens* strain NaT1 and sequences with a 98% identity to *Methanogenium cariaci* DSMi1497^T (Sas, 2009). DNA extracted

from enrichment cultures by Sas (2009) had *mcrA* sequences that had a 97% identity to M*ethanogenium organophilum mcrA*. Whilst members of this genus can use hydrogen as a substrate, a radiotracer study of hydrogenotrophic methanogenesis did not detect methanogenesis at 10 cm depth at Meknes MV (Sas, 2009). This suggested that the *mcrA* sequence did not represent an active hydrogen utilizing methanogen at this location and this is consistent with the findings of this study.

A study of the prokaryotic diversity below the Captain Arutyunov MV SMTZ (30-40 cm depth) by Niemann *et al.* (2006) did not detect known methanogens (16S rRNA gene clone library). The majority of archaeal sequences were related to ANME-and AMNE-2 groups. The remaining sequences were affiliated with the Marine Benthic Groups B and D and the rest being unclassified.

3.4.2.3 Eastern Mediterranean Mud Volcanoes

Two cores were taken from Napoli MV, cores MEDKUL-3 and MEDKUL-4 (Section 2.1.3.2). This study enriched methanogens from core MEDKUL-3 and Lazar *et al*. (2011b) enriched methanogens from core MEDKUL-4. Core MEDKUL-3 was also used for radiotracer experiments for the study by Lazar *et al*. (2011b)(work undertaken at Cardiff University).

This study isolated two *Methanococcoides* strains from core MEDKUL-3 using methylamine as a substrate and normal marine salinity medium (strains NM1 and NM2). No methane was produced in the acetate and H_2/CO_2 cultures with any medium.

Lazar *et al*. (2011b) enriched *Methanococcoides* from core MEDKUL-4; enrichment cultures of sediment from 0-20 cm depth contained methanogens with16S rRNA gene sequences with 98% identity to *M. methylutens* TMA-10^T (Lazar *et al.,* 2011b). Representatives of two genera of methanogen where present in enrichment cultures from 80-100 cm depth (this was deeper than the sediment used in this study); an enrichment culture with trimethylamine as a substrate cultured methanogens with a 99% 16S rRNA gene sequence identity to *Methanococcoides methylutens* TMA-10^T and an enrichment culture with H_2/CO_2 as a substrate contained methanogens with a 95% sequence identity to *Methanogenium marinum* (Lazar *et al.*, 2011b).

An archaeal 16S rRNA gene profile of core MEDKUL-4 showed low diversity (Lazar *et al.*, 2011b). No methanogens were identified; the excised DGGE band sequences had phylogenetic affiliations with ANME-1, ANME-2, ANME-3 and *Archaea* from marine benthic group D (MBG-D).

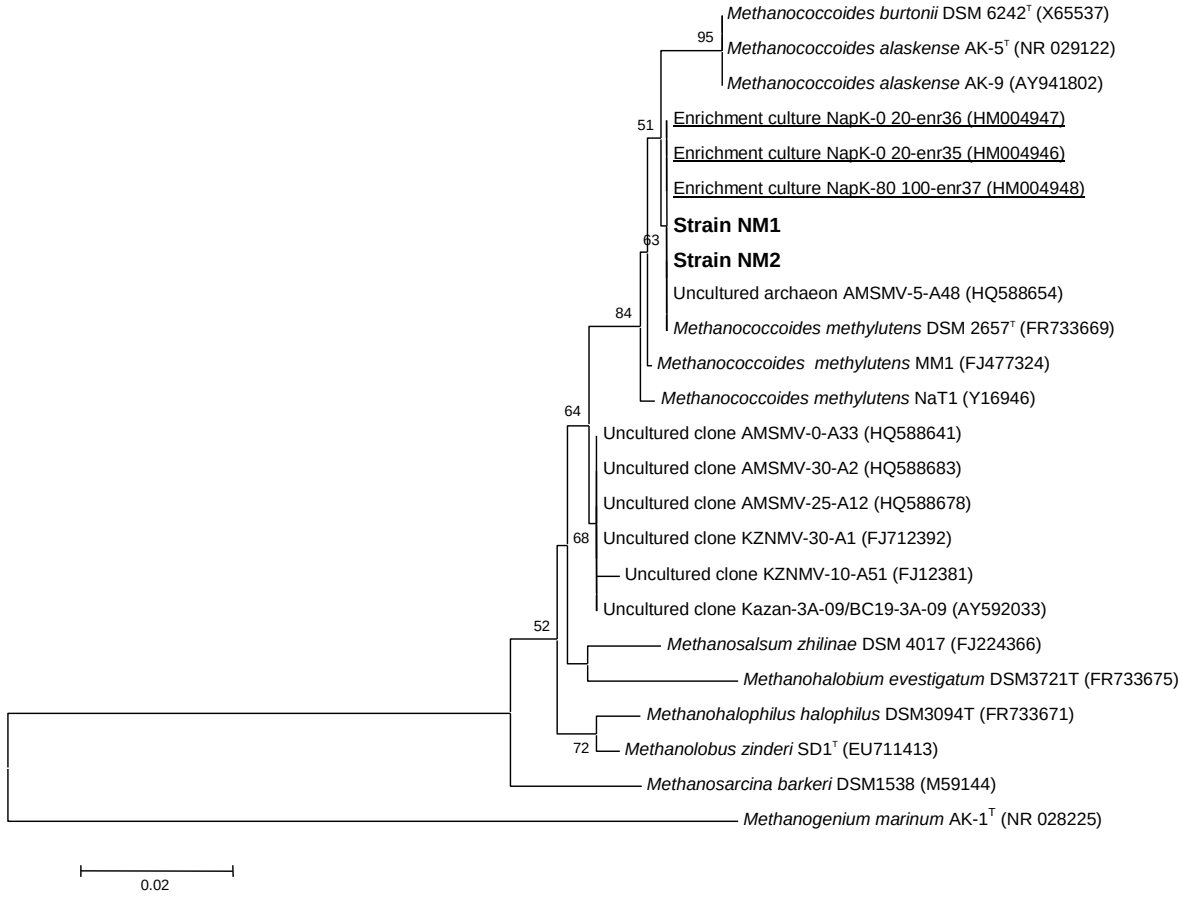


Figure 3.22 16S rRNA phylogenetic tree (neighbour joining) of Napoli MV methanogens. Enrichment cultures and isolated strains sequences from this study (core KUL-3) are in bold. Sequences from core KUL-4 are underlined (Lazar *et al*, 2011). Numbers at nodes are percentage bootstrap values based on 1000 replicates. Bar, 0.02 substitutions per site. Sequences NapK-0-enr35/6/7 Lazar *et al*. (2011a), sequence names starting AMSMV Pachiadaki *et al*. (2011), sequences starting KZNMV Pachiadaki *et al*. (2010) and sequence Kazan-3A-09 Heijs *et al*. (2007).

Lazar et al. (2011a) also cultured *Methanococcoides* from sediment taken from below a microbial mat (consisting of filamentous bacteria possibly of the genus *Beggiatoa* or *Thioploca*) at Napoli MV. Methanogens in enrichment cultures using sediment from

0-2 cm depth and from 2-4 cm depth had 98% 16S rRNA gene sequence identity to *Methanococcoides methylutens* TMA-10^T and 98% identity to *Methanococcoides burtonii* DSM 6242^T respectively (Lazar *et al.*, 2011a).

This study and the other two Napoli MV studies (Lazar *et al.*, 2011a; Lazar *et al.*, 2011b) cultured *Methanococcoides* using normal marine salinity media (0.5 M Na^+). The media used did not represent *in situ* conditions more than a few cm below the sediment surface, the Na^+ concentration for the depth interval 0-20 cm was approximately 1.0 M and increased to 4.5 M at 100 cm depth (Lazar *et al.*, 2011b). *Methanococcoides* strains NM1 and NM2 (Table 3.1) were isolated by this study from sediment taken from 0-5 cm depth. *Methanococcoides* strain NM1 can grow with Na^+ concentrations up to 0.8 M (Table 6.4) so it is possible that conditions allow growth of *Methanococcoides* for a few cm below the sediment surface at Napoli MV. Radio-labelled methylamine turnover rates peaked at 5 cm depth (Lazar *et al.*, 2011b).

At the microbial mat site, near the centre of the mud volcano, the porewater Na^+ concentration was found to be below 0.8 M until approximately 3 cm depth (Lazar *et al.*, 2011a). There was a higher archaeal diversity at this site compared with the MEDKUL-3/4 site, the 16S rRNA gene library had sequences representing MBG- D, Rice Cluster V (RC-V), Deep-Sea Hydrothermal Vent Euryarchaeotal group 4 (DHVE-4), terrestrial Miscellaneous Euryarchaeotal Group (TMEG), marine group II (MG-II), Miscellaneous Crenarchaeotic Group (MCG), and marine benthic group B (MBG-B) but not methanogens (Lazar *et al.*, 2011a).

The salinity conditions at depth at Napoli MV are too high to allow the growth of any the previously described strains of *Methanococcoides* or *Methanogenium* (*Methanococcoides* Na^+ concentration range 0.03-1.3 M (Table 6.4) and *Methanogenium* Na^+ concentration range 0.01-1.3 M (Table 3.13)). The upper salinity limit for the growth of described *Methanococcoides* strains is higher than that indicated by the previously described strains as *Methanococcoides* phylotypes have been detected below a hypersaline microbial mat at Baja California (7.5% NaCl) and in hypersaline sediments from Guerrero Negro, Baja California Sur (8.5% NaCl)(Orphan *et al.*, 2008b).

Methanogenesis from H_2/CO_2 has not been demonstrated *in situ* above 1.5 M Na^+ (Oremland & King, 1989) and the highest concentration tolerated by an isolated strain (*Methanocalculus halotolerans*) is 2.1 M Na^+ (Ollivier *et al.*, 1998). The upper limited for methanogens utilising acetate is possibly lower but little data is available (Oren, 1999; Oren, 2011). Methanogens utilising methylated compounds can grow at high Na^+ concentrations; *Methanohalobium evestigatum* can grow up to 5.1 M Na^+ (Zhilina & Zavarzin, 1987) and *Methanohalophilus* strain Z7302 can grow at Na^+ concentrations as high as 4.4 M (Lai & Gunsalus, 1992).

Known halophilic methanogens have not been identified in the sediment at Napoli MV (Lazar *et al.*, 2011a; Lazar *et al.*, 2011b). The Na^+ concentration in the Napoli MV sediments increased from 1.1 M in the depth interval 0-20 cm to 4.6 M at 100-120 cm depth which was the lowest depth measured (Lazar *et al.*, 2011b). Below this depth it may be too saline for methanogenesis to occur by known methanogens using acetate, H_2/CO_2 or methylated compounds. The sulphate concentration exceeded 10 mM throughout the section (>25 mM near the surface, Figure 3.4) and this would also appear to exclude methanogenesis using acetate or H_2/CO_2. However, radiotracer measurements (Lazar *et al.*, 2011b) showed that bicarbonate methanogenesis occurred at about 70 cm depth (approximately 14 pmol cm^{-3} day^{-1} methane) as did methanogenesis from acetate but at a much lower rate (approximately 0.6 pmol cm^{-3} day^{-1} methane). The rate measurements were carried out using subcores of core MEDKUL-3 and the only addition was the radioactive substrate (Dr Barry Cragg, personnel communication) so the sediment was as close to its *in situ* conditions as possible. Methanogenesis from hexadecane was also seen at approximately 65 cm depth (Lazar *et al.*, 2011b). In this study, none of the hexadecane-amended slurries (three salinities) for the depth interval 60-65 cm in this study produced methane. As methanogens cannot directly degrade hexadecane the subculturing probably disturbed a syntrophic partnership between methanogens and bacteria.

An attempt was made by this study to culture methanogens from 0-5 and 60-65 cm depths using two hypersaline media (salinities of 126 and 252‰, Section 2.2) but as previously mentioned it was not successful. The composition of the hypersaline media was based on those used to simulate the hypersaline conditions at Bannock Basin (Eastern Mediterranean) (Sass *et al.*, 2008).

Whilst this study was unsuccessful in culturing methanogens from Amsterdam mud volcano, *Methanococcoides* has been detected at Amsterdam MV by 16S rRNA gene sequencing (Pachiadaki *et al.*, 2010; Pachiadaki *et al.*, 2011). It was the only genus of methanogen detected.

3.4.3 Guaymas Basin - Deep Water, Non-Mud Volcano Site

This study investigated sediment from three closely spaced sites (all within 50 m of each other) approximately 65 km to the north of the sites investigate in other studies (see below). Whereas *Methanococcoides* was readily cultured from all three sites (CT2, CT5 and CT6) acetoclastic methanogens were only cultured at site CT2 (enrichment culture CT2-A) and hydrogen-utilizing methanogens were only enriched from site CT5 (enrichment culture CT5-H). There are no published culture studies of Guaymas Basin sediments for comparison; however, methanogens have been detected by molecular means by three studies, Table 3.20. The sites known as Everest Mound (Figure 2.8), Orpheus and, Rebecca's Roost (all within 18 km of each other) in the southern Guaymas vent field were studied by Teske *et al*. (2002). Everest Mound was also investigated by Dhillon *et al*. (2005) and a site approximately 2 km SE of Everest Mound was investigated by Holler *et al*. (2011).

Table 3.20 Methanogens enriched from Guaymas Basin (northern vent field, this study) and methanogens identified in the southern trough by three studies using 16S rRNA gene sequencing.

Genus	This study	Dhillon *et al*. (2005)	Teske *et al*. (2002)	Holler *et al*. (2011)
Methanococcoides	+	+	+	+
Methanosarcina	+	-	-	-
Methanocorpusculum	-	+	+	-
Methanogenium	+	-	-	-
Methanocaldococcus	-	+	-	-

All studies detected, or cultured, the methylated substrate utilizing methanogen *Methanococcoides*. Dhillon *et al*. (2005) identified *Methanocaldococcus* in the sediment of the southern vent field. *Methanocaldococcus* is a genus of hyperthermophilic methanogens; its identification is not unexpected as the Guaymas Basin is underlain by a hydrothermal system. This study did not attempt to culture

hyperthermophiles, all culture work in this study was carried out at 25 ºC, which was representative of the upper 7 cm of the sediment from which the samples used in this study were taken. Neither Dhillon *et al*. (2005), Holler *et al.* (2011) or Teske *et al*. (2002) detected an acetate utilizing methanogen. Whilst this study enriched a *Methanosarcina* strain with acetate as a substrate, *Methanosarcina* can also utilize methylated compounds. Although Guaymas Basin is rich in organic matter, acetate may not by an important methanogenic substrate.

3.4.4 Substrates

3.4.4.1 Possible Additional Substrates

This study has enriched methanogens that can utilise acetate, hydrogen and methylamine from all of the shallow water sites and Guaymas Basin. In addition, Tamar methanogens were cultured with formate and a wide range of potential substrates was tested with Aarhus Bay enrichment cultures. The mud volcano sites were generally limited to methanogenesis from methanol and methylamine. The identity of methanogens cultured in this study allows us to infer other possible growth substrates at these sites.

All previously described strains of the genera *Methanococcus, Methanoplanus* and *Methanospirillum* can utilize formate as well as H_2/CO_2 as substrates (Whitman *et al.*, 2006). The previously described strains of *Methanoculleus, Methanogenium and Methanosarcina* can all utilize H_2/CO_2 and some previously described strains of *Methanobacterium* (Section 3.3.1) and *Methanogenium* (Chapter 3.3.2.2) can also utilize formate. Formate was only tested as a substrate with Aarhus Bay and Tamar enrichment cultures. As previously noted, methane production from formate was poor from the Aarhus Bay enrichment culture and formate was consumed by acetogens. Some previously described strains of *Methanobacterium, Methanoculleus, Methanogenium* and *Methanospirillum* can also utilize alcohols including 1-propanol, 2-propanol, 2-butanol and cyclopentanol (Sections 3.3.1, 3.3.2.1, 3.3.3.2 and 3.3.2.4). One previously described strain of *Methanococcus* can also use pyruvate for methanogenesis although a strain isolated from Portishead in this study (strain PM4) did not (Section 6.3.6). The genus *Methanosarcina* has the widest substrate range of

all the methanogens (Whitman *et al.*, 2006). All described strains can utilize methylamines and methanol for methanogenesis and some strains can utilize acetate, H_2/CO_2, formate, DMS and methanethiol.

3.4.4.2 Competition for Methylamine

With two exceptions, the addition of methylamine to enrichment cultures in this study resulted in the growth of *Methanococcoides* even if *Methanosarcina* have been cultured from the same sediment using acetate as a substrate (Table 3.1). The exceptions being the 38 ºC enrichment culture PT38M (Section 3.3.3.2.) and the Guaymas Basin freshwater culture CT5-MFr (Section 3.3.2.2). 38 ºC is close to the upper temperature range of *Methanococcoides* and *Methanococcoides* appear to be found only in the marine environment (for a discussion of the environments in which *Methanococcoides* are found see Chapter 8). The presence of both *Methanococcoides* and *Methanosarcina* in the temperature gradient experiment between 16 and 40 ºC (Figure 3.21 and Table 3.18) may be due to *Methanosarcina* using acetate and/or H_2/CO_2 as a metabolic substrate rather than methylamine (all three substrate were added to the temperature gradient enrichment cultures).

3.4.5 Oxygen Sensitivity

Four genera of methanogens (*Methanococcus*, *Methanococcoides*, *Methanosaeta*, *Methanosarcina*, identified by 16S rRNA gene sequencing) were cultured from the 0-2 cm depth sediments from Portishead. Oxygen may not diffuse to this depth but the sediments are bioturbated and reworked by the tides. 0-2 cm straddles the aerobic and dysaerobic zones (Raiswell & Canfield, 1998); aerobic zone 0-0.5 cm (Webster *et al.*, 2010). The mudflat bed level varies on a tide by tide basis, the bed level being approximately 0.1 cm higher after a spring tide (Whitehouse & Mitchener, 1998) and the annual change in bed level is of the order of 10 cm with seasonal storms (Whitehouse & Mitchener, 1998). Methanogens can survive in generally aerobic conditions by making use of anaerobic microenvironments created by the action of bacteria (Kato *et al.*, 2004). A member of the genus *Methanosaeta* was cultured in this study only from this upper level. Oxygen is reported to completely inhibit growth and methane formation by *Methanothrix soehngenii* (*Methanothrix* and *Methanosaeta* refer to the same taxon [Garrity *et al.*, 2011]), however, they do not loose their

viability when exposed to high concentrations of oxygen (Huser *et al.*, 1982). The genome of *Methanococcoides burtonii* encodes genes for oxidative stress enzymes that target oxygen radicals, including catalase and superoxide reductase (Williams *et al.*, 2010b).

3.5 Summary

This extensive culture-dependent survey of methanogens in marine sediments, enriched methanogens from all except one of the locations investigated (only Amsterdam MV was unsuccessful). In total nine genera of methanogen were enriched from twelve sites (Tables 3.1 and 3.21) ranging from mudflats to mud volcanoes at ~ 2 km water depth. These nine genera represent four of the six orders of methanogens; the orders not cultured were *Methanocellales* (previously Rice Cluster 1) and *Methanopyrales*, an order that contains only hyperthermophilic methanogens. The two tidal-flat sites (Portishead and Tamar) had the highest diversity with the mud volcano sites having the lowest.

Members of the genus *Methanococcoides* were cultured from all sites; sixteen strains from seven locations were subsequently isolated (see Chapter 6 for further details). As this genus can only use methylated compounds as growth substrates this implies that these substrates are important in marine sediments. This study indicated that *Methanococcoides* could out-compete *Methanosarcina* under certain circumstances for methylamine. In addition, two strains of *Methanococcus* were isolated from Portishead sediments.

This is believed to be the first study to culture methanogens taken from the Sorokin Trough of the Black Sea, Darwin MV located in the Gulf of Cadiz, and sediments from the Tamar estuary and Portishead. Despite molecular studies indicating the presence of methanogens at Amsterdam MV, none were enriched by this study. Although the Napoli MV sediments were hypersaline, no halophilic methanogens were enriched from this location.

Hydrogen and acetate utilizing methanogens have been cultured from sediments with high levels of sulphate, the sulphate concentration in Portishead and Tamar sediments ranged from 10-30 mM (Figure 2.12). Portishead and Tamar sediments also contained methanogens that could be enriched using freshwater medium (Table 3.1).

Figure 3.21 Summary of methanogen genera identified at the locations invested in this study.

			Gulf of Cadiz mud volcanoes									
	Aarhus Bay	Guaymas Basin	Bonjardim	Captain Arutynov	Carlos Ribeiro	Darwin	Mercator	Meknes	Dvurechenskii MV	Napoli MV	Portishead	Tamar
Methanobacterium											+	+
Methanococcus											+	
Methanococcoides	+	+	+	+	+	+	+	+	+	+	+	+
Methanoculleus											+	+
Methanogenium	+	+				+						+
Methanoplanus												+
Methanosaeta											+	+
Methanosarcina	+	+									+	+
Methanospirillum												+

Chapter 4
Choline Enrichment Cultures

4.1 Introduction

Choline is widely distributed in membrane lipids (King, 1984). Whilst, choline has not been shown to be a growth substrate for methanogens it can be degraded to compounds that can utilized by methanogens. For example, *Desulfovibrio* can degrade choline to trimethylamine, acetate and ethanol (Hayward & Stadtman, 1959), Equation 4.1.

$$2(CH_3)_3N^+CH_2CH_2OH + H_2O \rightarrow 2(CH_3)_3N^+H + CH_3CH_2OH + CH_3COO^- + H^+ \quad \text{(Equ. 4.1)}$$

The same products have been noted for the degradation of choline by *Clostridium* sp. (Bradbeer, 1965). Some genera of methanogens can utilize trimethylamine as a growth substrate (Equation 4.2) producing ammonium as well as methane as end products with dimethylamine and methylamine as intermediate products (Hippe *et al.*, 1979).

$$4(CH_3)_3NH^+ + 9H_2O \rightarrow 9CH_4 + 3HCO_3^- + 3H^+ + 4NH_4^+ \quad \text{(Equ. 4.2)}$$

In a coculture experiment with *Desulfovibrio* strain G1 and *Methanosarcina barkeri* strain Fusaro (DSM 804), choline was degraded to methane, ammonia, hydrogen sulfide, and carbon dioxide in the presence of sulphate (Fiebig & Gottschalk, 1983). The overall reaction is given by Equation 4.3.

$$4\,(CH_3)_3N^+CH_2CH_2OH + SO_4^{2-} + 13\,H_2O \rightarrow 13\,CH_4 + 7\,HCO_3^- + 4\,NH_4^+ + HS^- + 6\,H^+ \quad \text{(Equ. 4.3)}$$

In the absence of sulphate, equal amounts of acetate and ethanol were produced from choline agreement with Equation 4.1 and ethanol was not converted to methane by this coculture, indicating the absence of hydrogen transfer between *Desulfovibrio* strain G1 and *Methanosarcina barkeri* strain Fusaro. *Methanosarcina barkeri* strain Fusaro was able to utilize acetate as a substrate (Fiebig & Gottschalk, 1983).

The bacterium *Eubacterium limosum* has been determined to degrade choline to *N*, *N*-dimethylethanolamine, acetate and butyrate (Equation 4.4) rather than trimethylamine, acetate and ethanol (Muller *et al.*, 1981). Neither *N*, *N*-dimethylethanolamine nor butyrate has been described as substrates for methanogens.

$$7(CH_3)_3N^+CH_2CH_2OH + 2HCO_3^- \rightarrow 7(CH_3)_2NCH_2CH_2OH + 1.5\ CH_3COO^- + 1.5\ CH_3CH_2CH_2COO^- + 8H^+ \quad \text{(Equ. 4.4)}$$

Although choline (*N*,*N*,*N*-trimethylethanolamine) has not been described as a substrate for methanogenesis it could, in principal, be degraded to methane and ethanolamine (Equation 4.5) with the production of the intermediate products *N*,*N*-dimethylethanolamine and *N*-monomethylethanolamine. This degradation would be analogues to the degradation of trimethylamine.

$$4\ (CH_3)_3N^+CH_2\ CH_2OH + 9\ H_2O \rightarrow 9\ CH_4 + 4\ NH_2CH_2CH_2OH + 3\ HCO_3^- + 7\ H^+ \quad \text{(Equ. 4.5)}$$

The objective of this enrichment culture study was to determine the route of choline degradation in a number of methanogen enrichment cultures and to identify the microorganisms involved. The sediment used for these enrichment cultures came from Guaymas Basin (cores CT2, CT5 and CT6, see Section 2.1.6 for further details) and Aarhus Bay (core May 2010, see Section 2.1.1.2 for core details) and were chosen as they had become available at the same time and no other sediment was available from other sites. The enrichment cultures used in this study were the fourth subculture of the original enrichment culture; choline was added as the sole substrate to the original enrichment culture and all subcultures.

4.2 Method

Ion chromatography was used to detect acetate, formate, propionate, methylamines, ammonium, choline, *N*-monomethylethanolamine and ethanolamine. Whilst ion chromatography could detect ammonium or ethanolamine, it could not distinguish between the two in the same sample (Dr Erwan Roussel, personal communication), hence, these are reported as a combined measurement. Nor was it possible, again for technical reasons, to detect *N,N*-dimethylethanolamine. The ethanol concentration of the samples was not measured, as the analytical facilities were not available. Methane concentrations are given for total methane, both headspace and dissolved methane.

4.3 Results

4.3.1 Guaymas Basin, Enrichment Culture CT2

After 48 days incubation at 25 °C, 5.0 mM choline was consumed and, 5.4 mM ammonium/ethanolamine, 12.1 mM methane and 4.6 mM acetate were produced (Figure 4.1). As the concentration of choline declined, the concentration of methylamine increased reaching a maximum concentration of 3.0 mM on day 24 and declined to below detection limits by day 36. Choline was consumed by day 15. Two days after the detection of methylamine, dimethylamine was detected and the concentration increased to 0.8 mM on day 21 and was consumed by day 27. Propionate was detected on the same day as dimethylamine and it concentration increased to 0.6 mM and remained at this level until the end of the experiment. Formate was also detected; it peaked at 0.5 mM on day 24 and declined thereafter. Trimethylamine and *N*-monomethylethanolamine were not detected. 5.0 mM of choline would be expected to produce 11.3 mM methane (Equation 4.2); 12.1 mM methane was measured.

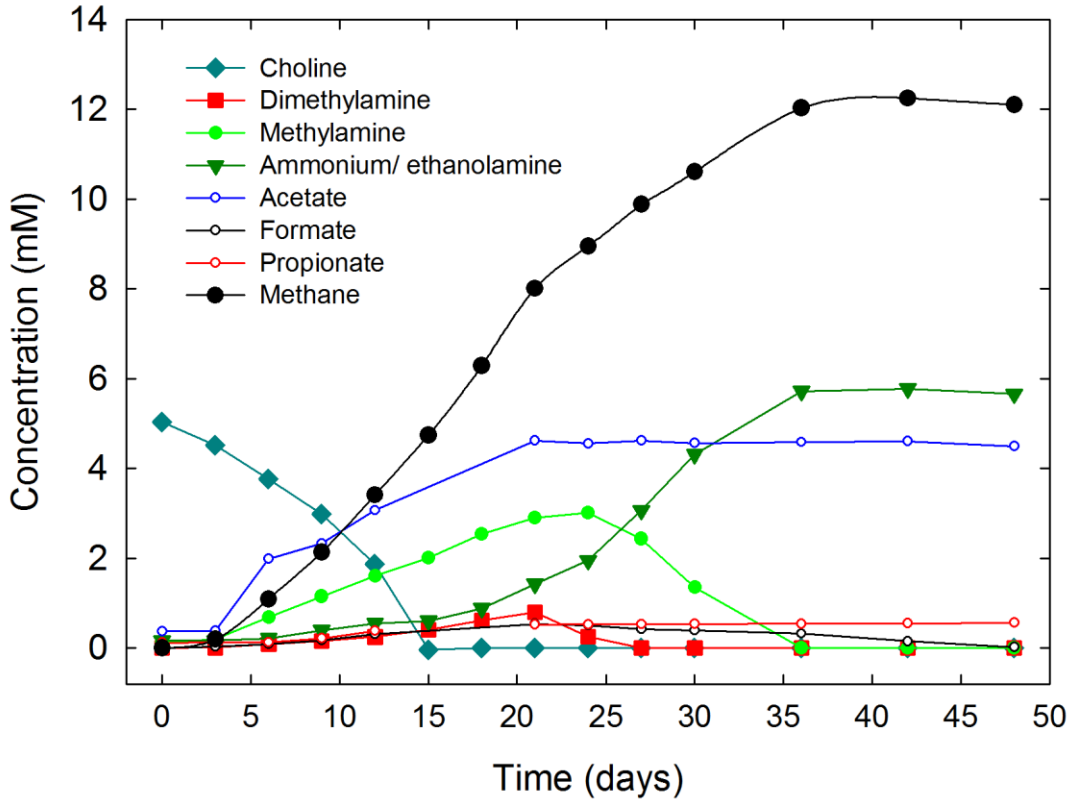


Figure 4.1 Guaymas Basin enrichment culture CT2, degradation of choline to methane with the production of dimethylamine and methylamine. Acetate and small amounts of formate and propionate were also produced. Incubated at 25 ºC for 48 days.

4.3.2 Guaymas Basin, Enrichment Culture CT5

As the concentration of choline declined (starting concentration 5.4 mM) the concentration of trimethylamine increased and reached a maximum concentration of 4.8 on day 6 (Figure 4.2). Also as the trimethylamine was consumed, methylamine was produced reaching a maximum concentration of 3.3 mM on day 24 and then declined to below detection levels by day 48. Dimethylamine was also transitorily produced reaching maximum concentration of 1.1 mM on day 24 and then declined. Ammonium/ethanolamine increased from day 18 reaching a maximum of 5.8 mM by day 48. Also, as trimethylamine decreased methane increased reaching a maximum concentration of 12.0 mM on day 48. Acetate was produced as choline decreased reaching a maximum concentration of 5.4 mM on day 15. Low concentrations of

formate and propionate, both < 0.1 mM, were also detected (not shown in Figure 4.2) and remained close to this concentration after 48 days.

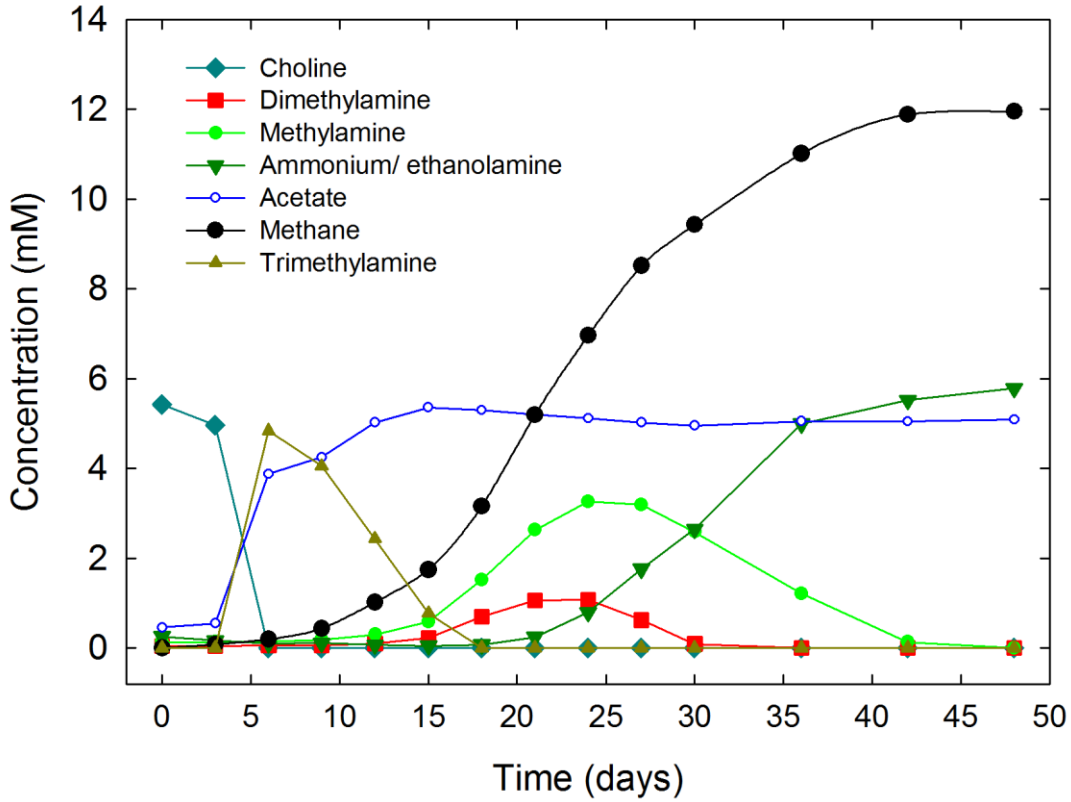


Figure 4.2 Guaymas Basin enrichment culture CT5, degradation of choline to methane with the production of trimethylamine and the intermediate products dimethylamine and methylamine. Acetate and was also produced. Incubated at 25 ºC for 48 days.

4.3.3 Guaymas Basin, Enrichment Culture CT6

The degradation of choline proceeded at a much lower rate than with cultures CT2 and CT6 and was incomplete even after 62 days incubation with only 1 mm choline consumed (Figure 4.3). At day 0, 0.09 mM methylamine and 0.05 mM dimethylamine were present (presumably carried over with the inoculum), both declined to below detection limits by day 12. By day 62, 3.2 mM methane, 1.3 mM acetate and 2.0 mM ammonium/ ethanolamine had been produced. The rate was methane and ammonium/ ethanolamine was higher between days 0 and 12 than during the rest of the incubation (Figure 4.3).

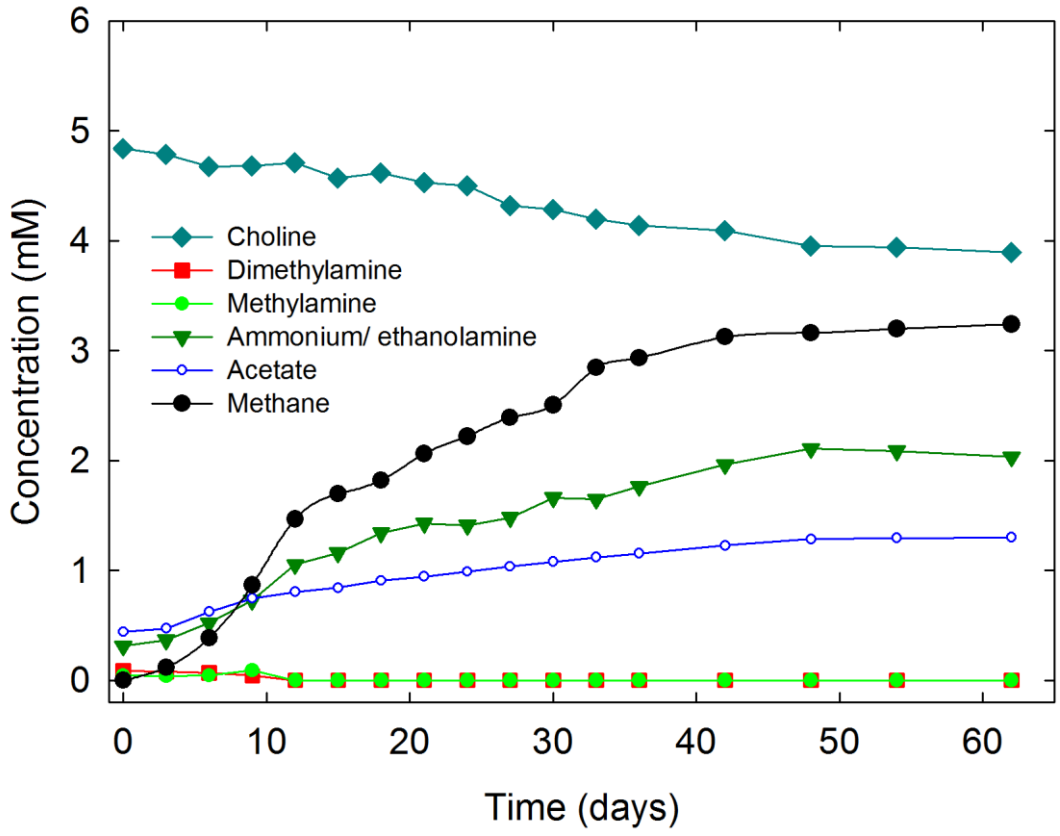


Figure 4.3 Guaymas Basin culture CT6, degradation of choline to methane and acetate. Incubated at 25 ºC for 62 days.

4.3.4 Aarhus Bay, Enrichment Culture AB

The starting concentration of choline (5.5 mM) steadily decreased to 2.4 mM on day 54 and then rapidly decreased to below detection limits by day 62 (Figure 4.4). Acetate increased from 0.4 mM on day 0 to 3.7 mm on day 88. During the time period covering days 54-68, both the rate of acetate and methane production increased. They then returned to their pre-day 54 rates. By day 88, the methane concentration had increased to 9.5 mM. The 3.1 mM choline consumed by day 54 would theoretically, if trimethylamine had been an intermediate, have produced 7.0 mM methane. By day 54 only 4.8 mM methane had been produced. The remaining 2.4 mM choline was consumed between days 54 and 62 (Figure 4.4).

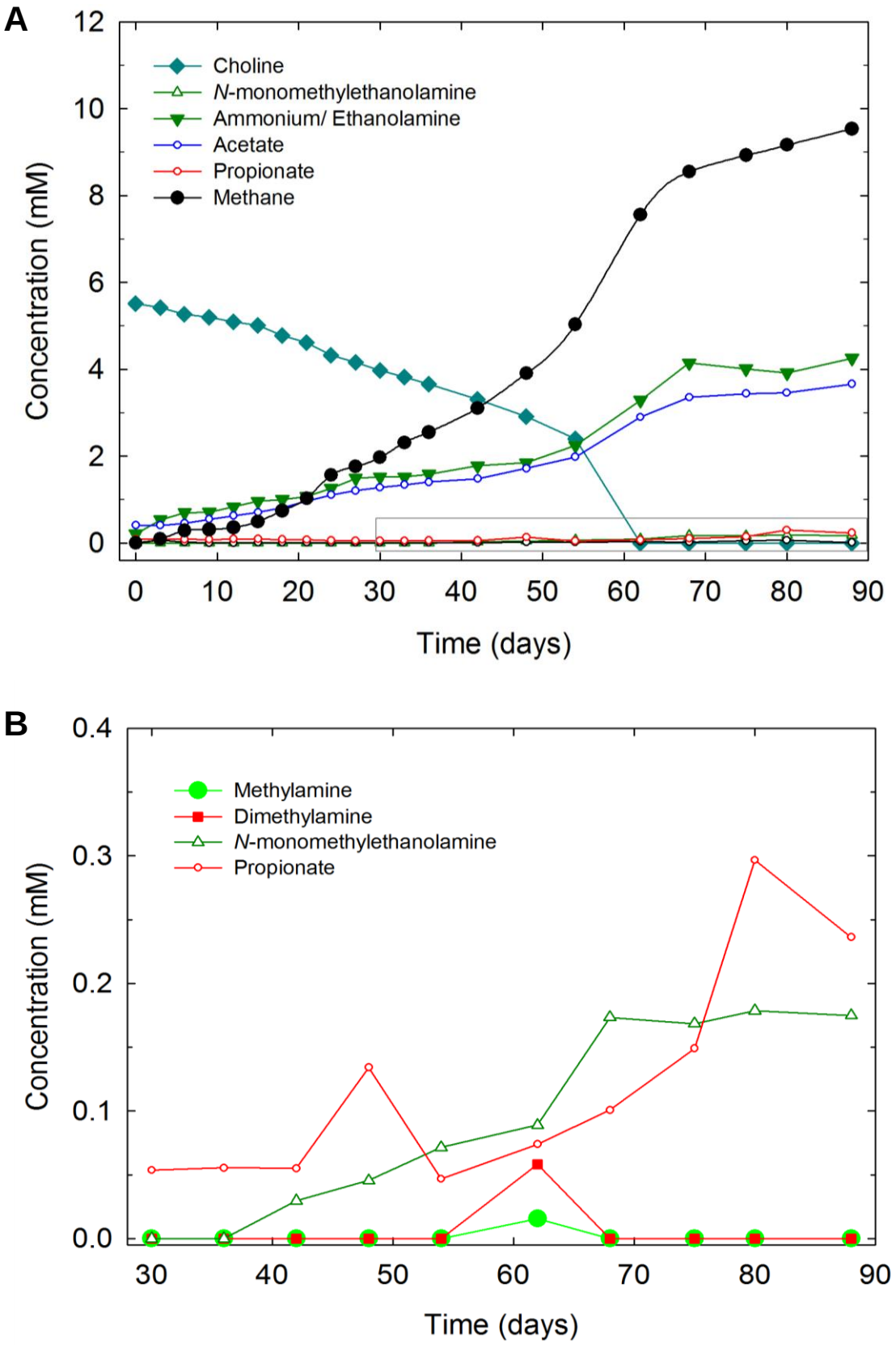


Figure 4.4 (A) Aarhus Bay enrichment culture AB, degradation of choline to methane and acetate. (B) Enlarged view of days 30-60 to show methylamine, dimethylamine, *N*-monomethylethanolamine and propionate. Incubated at 25 ºC for 88 days.

4.3.5 DGGE Results

4.3.5.1 Archaea

No methanogens other than *Methanococcoides* (94-100% 16S rRNA gene sequence identity) were identified by 16S PCR-DGGE band sequencing in cultures CT5, CT6 and Aarhus Bay (Figure 4.5 and Table 4.1). Sequences with a 93-97% 16S rRNA gene sequence identity to *Methanosarcina* were identified in culture CT2 as well as fainter DGGE bands corresponding to *Methanococcoides* (Figure 4.5 and Table 4.1).

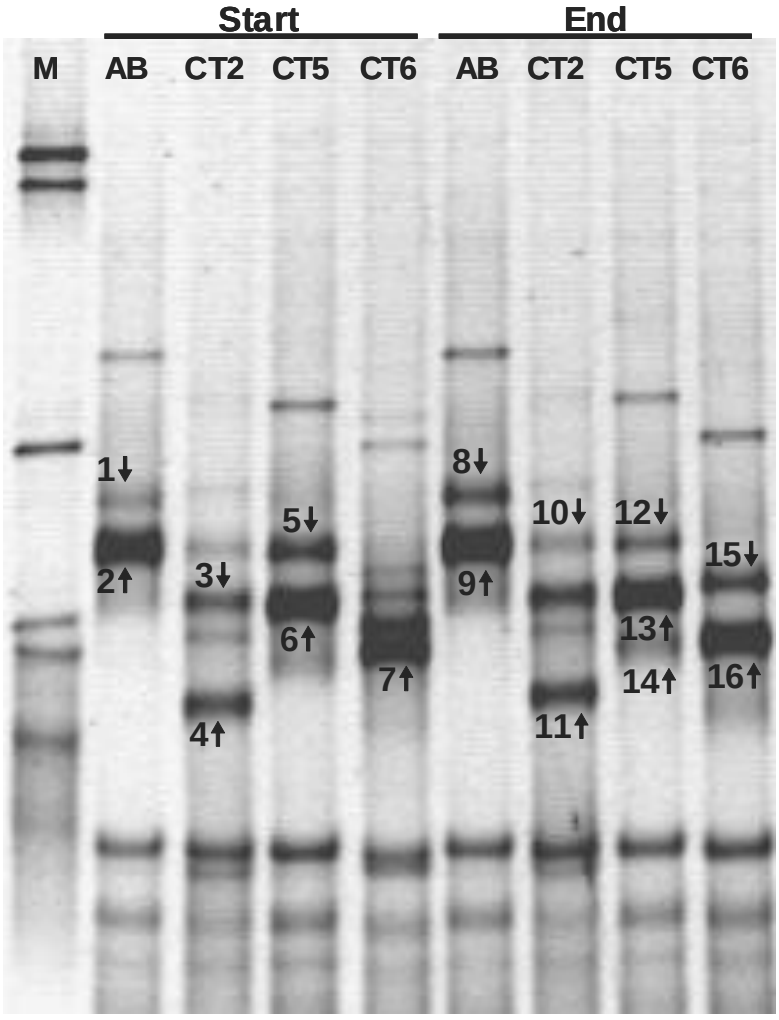


Figure 4.5 Archaeal DGGE profile (16S rRNA gene) of Aarhus Bay (AB) and Guaymas (CT2, CT5 and CT6) choline enrichment cultures. M, marker.

Table 4.1 Choline enrichment culture Archaeal 16S DGGE band identities. All cultures contained methanogens belonging to the genus *Methanococcoides*. The only other genus of methanogen detected was *Methanosarcina* and this was present in culture CT2 only.

Culture	DGGE Band	Nearest match by BLASTN search (accession number)	% similarity	Nearest cultivated match by BLASTN search	% similarity
AB	1	*Methanococcoides methylutens* TMA-10^{I} (FR733669)	100	*Methanococcoides methylutens* TMA-10^{I} (FR733669)	100
AB	2	*Methanococcoides methylutens* TMA-10^{I} (FR733669)	99	*Methanococcoides methylutens* TMA-10^{I} (FR733669)	99
CT2	3	Uncultured archaeon clone Zeebrugge_A57 (HM598511)	95	*Methanosarcina baltica* AK-5^{I} (AY663809)	93
CT2	4	Uncultured archaeon clone Zeebrugge_A57 (HM598511)	97	*Methanosarcina baltica* AK-5^{I} (AY663809)	94
CT5	5	*Methanococcoides methylutens* MM1 (FJ477324)	100	*Methanococcoides methylutens* MM1 (FJ477324)	100
CT5	6	*Methanococcoides methylutens* MM1 (FJ477324)	99	*Methanococcoides methylutens* MM1 (FJ477324)	99
CT6	7	*Methanococcoides alaskense* AK-5^{I} (NR_029122)	96	*Methanococcoides alaskense* AK-5^{I} (NR_029122)	96
AB	8	*Methanococcoides methylutens* TMA-10^{I} (FR733669)	98	*Methanococcoides methylutens* TMA-10^{I} (FR733669)	98
AB	9	*Methanococcoides methylutens* TMA-10^{I} (FR733669)	99	*Methanococcoides methylutens* TMA-10^{I} (FR733669)	99
CT2	10	Uncultured archaeon clone BCMS-19 (AJ579734)	97	*Methanococcoides methylutens* MM1 (FJ477324)	96
CT2	11	Uncultured archaeon clone Zeebrugge_A57 (HM598511)	99	*Methanosarcina baltica* AK-5^{I} (AY663809)	97
CT5	12	*Methanococcoides methylutens* NaT1 (Y16946)	99	*Methanococcoides methylutens* NaT1 (Y16946)	99
CT5	13	*Methanococcoides methylutens* MM1 (FJ477324)	98	*Methanococcoides methylutens* MM1 (FJ477324)	98
CT5	14	*Methanococcoides methylutens* MM1 (FJ477324)	94	*Methanococcoides methylutens* MM1 (FJ477324)	94
CT6	15	*Methanococcoides alaskense* AK-5^{I} (NR_029122)	100	*Methanococcoides alaskense* AK-5^{I} (NR_029122)	100
CT6	16	*Methanococcoides alaskense* AK-5^{I} (NR_029122)	100	*Methanococcoides alaskense* AK-5^{I} (NR_029122)	100

4.3.5.2. Bacteria

Bacterial 16S PCR-DGGE showed the presence of a number of bacterial types in each enrichment culture (Figure 4.6 and Table 4.2). All cultures contained bacteria related to *Clostridium* and *Desulfovibrio*. The two cultures (CT2 and CT5) that rapidly degraded choline to trimethylamine had prominent DGGE bands at the start of the incubation with 95-100% 16S rRNA gene sequence identity to *Desulfovibrio* sp. NA81 isolated from a Wadden Sea tidal flat (Figure 4.6 and Table 4.2, bands 8, 9 and 12). This strain has been determined to degrade choline (Dr Henrik Sass, personal communication). Less intense bands also related to *Desulfovibrio* sp. NA81 were also present in the other two cultures. At the end of the incubation, bands corresponding to *Desulfovibrio* sp. NA81 were not identified in culture CT2.

Culture AB also contained bacteria related to *Desulfovibrio* (band 1) at the start of the incubation. Although excised, band 5 was not successfully sequenced; however, it did correspond to bands 16, 19 and 22, which had 95-96% 16S rRNA gene sequence identities to *Clostridium* sp. (Figure 4.6 and Table 4.2). The remaining sequences were not closely related to cultivated strains. At the end of the incubation, sequences related to *Desulfovibrio portus* (band 21), *Clostridium litorale* (band 24) and *Sulforimonas autotrophica* (band 20). Neither *Desulfovibrio portus* or *Clostridium litorale* have been reported to degrade choline and *Sulforimonas autotrophica* is a sulphur- and thiosulphate-oxidizing bacterium (Inagaki *et al.*, 2003). Whilst not having been described as degrading choline, *Clostridium halophilium* and *C. litorale* have both been described as degrading betaine in the Stickland reaction to trimethylamine (Fendrich *et al.*, 1990).

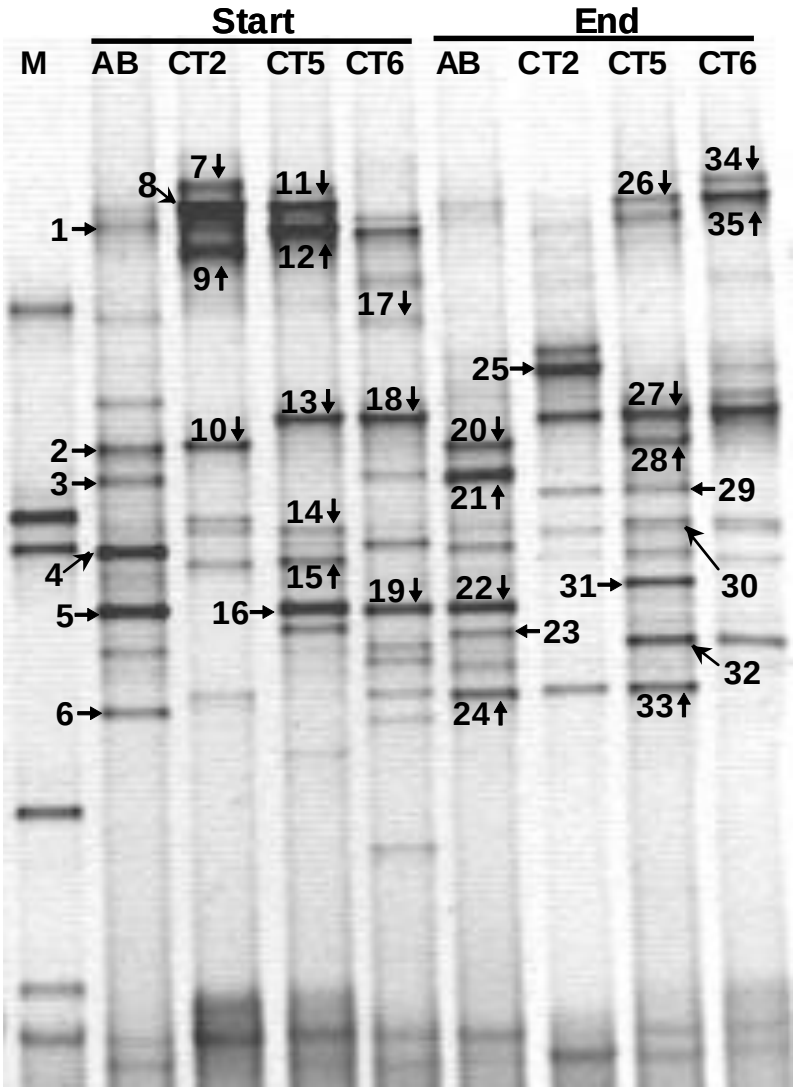


Figure 4.6 Bacterial DGGE profiles (16S rRNA gene) of Aarhus Bay (AB) and Guaymas (CT2, CT5 and CT6) choline enrichment cultures. M, marker.

Table 4.2 Bacterial 16S rRNA gene DGGE band identities for Guaymas choline enrichment cultures CT2, CT5, CT6 and Aarhus Bay culture AB with choline a catabolic substrate. Bands 3, 5, 14 and 30 were not successfully sequenced.

Culture	Band	Nearest match BLASTN search (accession number)	% identity	Nearest cultivated match BLASTN search (accession number)	% identity	Phylogenetic affiliation
AB	1	*Desulfovibrio* sp. NA302 (AJ866944) Tidal flat sediment	96	*Desulfovibrio oceani* subsp. galateae I9 (FJ655908) Coastal waters	95	*Deltaproteobacteria*
AB	2	Uncultured bacterium clone 154G2.13 (JF827488)	97	*Capnocytophaga gingivalis* ACTC_H303_86 (GU410391) oral cavity	89	*Bacteroidetes*
AB	4	Uncultured bacterium clone P2_CO9038_HCB (FJ810789) River sediment	90	Uncultured bacterium clone P2_CO9038_HCB (FJ810789) River sediment	90	*Firmicutes*
AB	6	*Clostridium aminobutyricum* DSM 2634^T (X76161) Swamp mud	90	*Clostridium aminobutyricum* DSM 2634^T (X76161) Swamp mud	90	*Firmicutes*
CT2	7	Uncultured bacterium clone GL63_B012 (HQ287202) Hot spring	96	*Alkaliphilus peptidifermentans* Z-7036^T (EF382660) Soda lake	95	*Firmicutes*
CT2	8	*Desulfovibrio* sp. NA81 (AJ866930) Tidal flat sediment	100	*Desulfovibrio* sp. NA81 (AJ866930) Tidal flat sediment	100	*Deltaproteobacteria*
CT2	9	*Desulfovibrio* sp. NA81 (AJ866930) Tidal flat sediment	96	*Desulfovibrio* sp. NA81 (AJ866930) Tidal flat sediment	96	*Deltaproteobacteria*
CT2	10	Delta proteobacterium clone S130(3)-2 (GU136555) Marine sediment	98	*Desulfovibrio oceani* subsp. galateae I9 (FJ655908) Coastal waters	97	*Deltaproteobacteria*
CT5	11	Uncultured bacterium clone C04_PCE (FJ810692) River sediment	97	*Clostridium propionicum* JCM 1430 (AB649276)	96	*Firmicutes*
CT5	12	*Desulfovibrio* sp. NA81 (AJ866930) Tidal flat sediment	96	*Desulfovibrio* sp. NA81 (AJ866930) Tidal flat sediment	96	*Deltaproteobacteria*
CT5	13	Uncultured bacterium clone LXE62 (JF514278) Seawater	96	Candidatus "*Cloacamonas acidaminovorans*" (CU466930) Anaerobic reactor	88	
CT5	15	Uncultured bacterium clone C8_10.1_2 (F1717183)Marine sediment	99	Bacterial isolate G200VII (AJ786050) Tidal flat sediment	99	
CT5	16	Firmicutes enrichment culture clone 4.176 Bac Band3 (FN548086) Marine sediment, Meknes MV	98	*Clostridium halophilum* M1^T (X77837) Hypersaline mud	95	*Firmicutes*
CT6	17	Uncultured bacterium clone DUNE-t8C2-97 (HM117456) Marine sediment	94	*Winogradskyella exilis* 022-2-26^T (FJ595484) Isolated from the starfish *Stellaster equestris*	93	

Table 4.2 (continued) Bacterial 16S DGGE band identities for Guaymas cultures CT2, CT5, CT6 and Aarhus Bay culture AB

Culture	Band	Nearest match BLASTN search (accession number)	% identity	Nearest cultivated match BLASTN search (accession number)	% identity	Phylogenetic affiliation
CT6	18	Uncultured bacterium clone C8_10.1_2 (F1717183) Marine sediment	99	Bacterial isolate G200VII (AJ786050) Tidal flat, Wadden Sea	98	
CT6	19	Uncultured bacterium clone MS048 (FR691508) River sediment	98	*Clostridium aminobutyricum* DSM 2634^T (X76161) Swamp mud, N. Carolina	96	*Firmicutes*
AB	20	Uncultured bacterium clone Pohang_WWTP_December.2006_095 (HQ510090) Activated sludge wastewater	100	*Sulfurimonas autotrophica* DSM 16294 (CP002205) Mid Okinawa Trench hydrothermal field	99	*ε-Proteobacteria*
AB	21	*Desulfovibrio portus* MSL^T (AB110541) Niida River estuary	99	*Desulfovibrio portus* MSL^T (AB110541) Niida River estuary	99	*Deltaproteobacteria*
AB	22	Uncultured Clostridium sp. clone C10 (EF221807) Anaerobic reactor	98	*Clostridium aminobutyricum* DSM 2634^T (X76161) Swamp mud, N. Carolina	96	*Firmicutes*
AB	23	Firmicutes clone SSHA-74 (AJ306755) Environmental sample	96	*Fusibacter paucivorans* strain TERI GP8 (EU664984) Oilfield formation water	95	*Firmicutes*
AB	24	Uncultured bacterium clone s5_8_I_8 (AJ306755) Marine sediment	99	*Clostridium litorale* W6^T (NR_029270) Marine sediment	98	*Firmicutes*
CT2	25	Uncultured bacterium clone AN05aug-164 (AB429720) Marine sediment	95	"*Croceimarina litoralis*" strain IMCC1993 (EF108214)	93	*Flavobacteria*
CT5	26	*Desulfovibrio* sp. NA81 (AJ866930) Tidal flat sediment	98	*Desulfovibrio* sp. NA81 (AJ866930) Tidal flat sediment	98	*Deltaproteobacteria*
CT5	27	Uncultured bacterium clone H5i (JF971573) Seawater	99	*Arcobacter cryaerophilus* strain NW94 (JF915357) Salmon intestine	98	*ε-Proteobacteria*
CT5	28	Uncultured bacterium clone T6-Ph07-962 (AJ575998) Deep sea hydrothermal vent	100	*Sulurimonas autotrophica* DSM 16294^T (CP002205) Mid Okinawa Trench hydrothermal field	99	*ε-Proteobacteria*

Table 4.2 (continued) Bacterial 16S DGGE band identities for Guaymas cultures CT2, CT5, CT6 and Aarhus Bay culture AB.

Culture	Band	Nearest match BLASTN search (accession number)	% identity	Nearest cultivated match BLASTN search (accession number)	% identity	Phylogenetic affiliation
CT5	29	Sphingobacteria clone JAM-BA0302 (AB362263) Methane-rich sediments	92	*Meniscus glaucopis* strain ATCC 29398 (GU269545) Anaerobic digestor	87	*Bacteroidetes*
CT5	31	Uncultured bacterium clone A5053_B39 (GU3900850) Sulfidic cave stream	96	*Flavobacterium anhuiense* D3^T (NR_044388) Soil	94	*Bacteroidetes*
CT5	32	Firmicutes clone GoMGC019S6 (AY211742) Marine sediment	99	*Clostridiisalibacter paucivorans* 37HS60^T (NR_044043) Olive mill wastewater	98	*Firmicutes*
CT5	33	"*Clostridium sediminis*" DY192 (HQ696463)	99	*Clostridium caminithermale* Dvird3^T (NR_041887) Deep-sea hydrothermal chimney	98	*Firmicutes*
CT6	34	Desulfovibrio sp. NA202 (AJ866943) Tidal flat sediment	94	*Desulfovibrio oceani* subsp. galateae I9 (FJ655908) Coastal waters	94	*Deltaproteobacteria*
CT6	35	*Desulfovibrio* sp. NA202 (AJ866943) Tidal flat sediment	98	*Desulfovibrio oceani* subsp. galateae I9 (FJ655908) Coastal waters	98	*Deltaproteobacteria*

4.4 Discussion

For Guaymas Basin enrichment culture CT5 (Figure 4.2), degradation of choline clearly followed the trimethylamine pathway, the degradation of choline to trimethylamine, acetate and presumably ethanol (the presence of ethanol was not determined) with the production of dimethylamine and methylamine as intermediate products. By reference to Equation 4.1, the consumption of choline would produce an equal amount of trimethylamine; the 5.4 mM choline added to the enrichment culture resulted in the production of at least 5.0 mM trimethylamine (Figure 4.2) as some of the trimethylamine would have been consumed.

Enrichment culture CT5 contained bacteria related to those known to degrade choline to trimethylamine (*Clostridium* and *Desulfovibrio*) although this did not necessarily mean that the strains identified in the enrichment culture did so. The ability to degrade choline is common in *Clostridia* sp. Möller *et al* (1986) tested 92 strains of the genus *Clostridia* (representing 60 species) for the ability to degrade choline. 32 strains (representing 16 species) were determined to degraded choline and all of these strains degraded choline to trimethylamine and acetate (ethanol not measured) (Möller *et al.*, 1986). None of the strains produced *N*,*N*-dimethylethanolamine from choline and no strain degraded *N*,*N*-dimethylethanolamine (Möller *et al.*, 1986).

Acetate was seen to accumulate in all enrichment cultures (Figures 4.1-4.4). Methanogens with the ability to use acetate as a growth substrate are found in only two genera: *Methanosaeta* and *Methanosarcina*. *Methanosarcina* were identified only in enrichment culture CT2 (Figure 4.5 and Table 4.1). Whilst not all strains of *Methanosarcina* are able to use acetate as a growth substrate, *Methanosarcina* were enriched from core CT2 with acetate (CT2-A, Table 3.1) where the acetate was either utilized directly or it was consumed by way of a syntrophic partnership (Section 1.3.2). The acetate in choline enrichment culture CT2 accumulated rather than being consumed despite the presence of *Methanosarcina* indicating that neither of these two processes were important in this enrichment culture.

The excess methane produced by enrichment culture CT2 (11.3 mM expected, 12.1 mM measured) may be accounted for by the presence of *Methanosarcina* and

syntrophic bacteria. In the absence of sulphate, *Desulfovibrio desulfuricans* has been shown to produce acetate and hydrogen from ethanol if the hydrogen is kept at a low concentration by a hydrogen-utilizing organism (Bryant *et al.*, 1977b), the metabolism of *Methanosarcina* is varied and some strains are able to utilize hydrogen as a substrate (Whitman *et al.*, 2006).

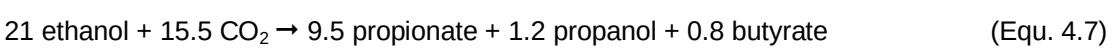
Figures 4.1 and 4.2 showing the consumption of choline and the production of end and intermediate products are similar except for the rate of choline consumption and the appearance of trimethylamine. Enrichment culture CT5 took 6 days to consume 5.4 mM choline whereas enrichment culture CT2 took 15 days to consume 5 mM. As with enrichment culture CT5, the degradation of choline in enrichment culture CT2 may have followed the trimethylamine pathway with dimethylamine and methylamine as possible intermediate products of the degradation of choline. The absence of trimethylamine in this enrichment culture may have been the result of trimethylamine being consumed as fast as it was being produced from choline. Alternatively, choline may have been degraded to *N*,*N*-dimethylethanolamine (which could not be detected due to technical reasons) which itself was degraded to dimethylamine and acetate. The degradation of choline to *N*,*N*-dimethylethanolamine would also result in the production of acetate and butyrate (Equation 4.4); butyrate was not detected in the enrichment culture. Butyrate can be degraded to acetate as described by Dwyer *et al*. (1988) for a syntrophic partnership between *Methanospirillum hungatei* strain PM-1 and anaerobic bacterium NSF-2 (Equation 4.6).

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \qquad \text{(Equ. 4.6)}$$

The higher than expected concentration of methane in culture CT6 for the amount of choline consumed is difficult to account for (3.2 mM measured rather than the theoretically expected 2.3 mM). As *Methanococcoides* (the only methanogen genus identified in this culture, Figure 4.5 and Table 4.1) can only utilize methylated compounds as growth substrates (Chapter 7) there is nothing identified in the culture that can result in the amount of methane detected. The discrepancy could have been due to choline and methane concentration measurement errors, however, the stoichiometrically of other enrichment cultures balanced.

Whilst having a similar bacterial composition to enrichment culture CT5, enrichment culture CT6 degraded choline comparatively slowly (there is the possibility that choline slowly degraded to trimethylamine without biological action) Enrichment culture CT6 developed differences on the DGGE profile between day 0 and day 64 (Figure 4.6). Band sequences corresponding to *Clostridibacter paucivorans* (band 32, 98% 16S gene sequence identity) and *Desulfovibrio* sp. (band 34, 94% 16S gene sequence identity) were present at the end of the incubation and not at the start (at least not detectable).

The presence of propionate in enrichment culture AB and CT2 (Figures 4.4 and 4.1) may have been due to the fermentation of ethanol (if produced by the degradation of choline) to propionate (Equation 4.7). For example, *Clostridium neopropionicum* has been determined to produce propionate, propanol and butyrate from the fermentation of ethanol, Equation 4.7 (Tholozan *et al.*, 1992).



(Equ. 4.7)

The production of a lower concentration of methane by culture AB than that expected from the degradation of choline via trimethylamine suggested that some of the choline was degrade to *N*,*N*-dimethylethanolamine (Equation 4.4). PCR-DGGE (Figure 3.8 and Table 3.7) did not detect bacteria related to *Eubacterium limosum* (known to degrade choline to *N*,*N*-dimethylethanolamine, Equation 4.4) although some of the identified bacteria may have the ability to degrade choline to *N*,*N*-dimethylethanolamine. The detection of *N*-monomethylethanolamine in culture AB (Figure 4.4 B) may have indicated that some of the choline was being degraded directly by methanogens by way of the demethylation of choline to *N*,*N*-dimethylethanolamine and then to *N*-monomethylethanolamine. This process has been identified in pure cultures of methanogen of the genus *Methanococcoides* isolated by this study from sediment taken from Aarhus Bay (Section 7.3.2.2.). The production and slow utilization of *N*,*N*-dimethylethanolamine could have allowed it to accumulated in the medium (*N*,*N*-dimethylethanolamine could not be detected for technical reasons), and unconsumed *N*,*N*-dimethylethanolamine could account for the lower than expected methane production by enrichment culture AB.

The degradation of choline was slow in enrichment culture AB until day 54 (Figure 4.4). The slow degradation of choline, as with culture CT6, possibly reflected a low population of suitable bacteria. The rapid choline consumption between days 54 and 62 may have been due to an increase in bacterial numbers thus increasing the degradation rate of choline. There may also have been an increase in the consumption of choline by methanogens. The detection of methylamine and dimethylamine between days 54 and 68 (Figure 4.4 B) is consistent with the formation of trimethylamine from choline with the trimethylamine being rapidly consumed; however, the amount of methane produced during this period (3.6 mM) was less than would be produced from the degradation of choline via trimethylamine (5.4 mM). On the basis of methane production, at least some of the choline was degraded to a product that was not detected, presumably *N*,*N*-dimethylethanolamine. After the consumption of methylamines on day 68, the methane increase may have been due to the consumption of *N*,*N*-dimethylethanolamine that had accumulated in the medium.

Bacterial 16S rRNA gene PCR-DGGE (Figure 4.6 and Table 4.2) showed the presence of two sequences in the AB enrichment culture at the end of the incubation that were not apparent at the start (bands 23 and 24). These sequences were related to *Fusibacter paucivorans* strain TERI GP8 (16S rRNA gene sequence 95% identity) and *Clostridium litorale* W6^T ((16S rRNA gene sequence 98% identity) respectively. It is possible that these two strains were, at least in part, responsible the rapid degradation of choline between days 54 and 62.

4.5 Summary

In Guaymas Basin enrichment culture CT5, choline was degraded to methane and ethanol via trimethylamine, dimethylamine and methylamine. The choline in enrichment cultures CT2 and CT6 may also have been degraded via trimethylamine and/or possibly *N*,*N*-dimethylethanolamine. Aarhus bay enrichment culture AB, may also have degraded choline, via separate pathways, to trimethylamine and *N*,*N*-dimethylethanolamine. The presence of *N*-monomethylethanolamine in enrichment culture AB may have indicated that *N*,*N*-dimethylethanolamine was demethylated by the action of a methanogen (experiments with pure methanogen

cultures have shown that choline can be degraded to methane and ethanolamine via the intermediates *N*,*N*-dimethylethanolamine and *N*-monomethylethanolamine, Section 7.3.2.2). Methane and acetate were present in all choline enrichment cultures with a small quantity of propionate (< 0.5 mM) present in enrichment cultures CT2, CT5 and AB. Interpreting data from mixed methanogen and bacterial enrichment cultures is not straightforward and other explanations for the degradation of choline are possible.

All enrichment cultures contained methanogen closely related to *Methanococcoides* (identified by 16S rRNA gene PCR-DGGE) with enrichment culture CT2 also containing *Methanosarcina* (also identified by 16S rRNA gene PCR-DGGE).

Chapter 5
The Effect of Pressure on Methanogenesis in Enrichment Cultures

5.1 Introduction

To determine whether methanogens could be enriched at elevated hydrostatic pressure and, if they could be enriched whether there would be any difference in the types of methanogens enriched, a series of enrichment cultures were incubated at a hydrostatic pressure of 40 MPa (400 bar). Portishead sediment was used for this study; conventional enrichment cultures (atmospheric pressure, 0.1 MPa) had already been undertaken using acetate, H_2/CO_2 and methylamine as growth substrates (Section 3.2.5.1).

5.2 Method

The enrichment cultures were prepared as described in Section 2.4. In summary, 30 ml serum bottles (completely filled, no headspace) contained sediment slurry (25% v/v) made with sediment taken from Portishead (Figure 2.6) with either acetate, formate or methylamine as a substrate. Three replicate enrichment cultures with the same substrate were incubated at 0.1 and 40 MPa at 25 ºC for 90 days in a custom-made pressure vessel. At the end of the incubation period, samples were taken to determine acetate, ammonium, formate, methane and methylamine, sulphate concentrations (Section 2.5) and for archaeal 16S rRNA gene PCR-DGGE (Section 2.8.3). As the vials used for enrichment cultures under elevated pressure could not have a headspace, H_2/CO_2 was replaced with formate. Many but not all, H_2/CO_2 utilizing methanogens are able to use formate as a growth substrate, *e.g*. *Methanobacterium*, *Methanoculleus* and *Methanogenium* (Whitman, 2006).

5.3 Results

The amount of methane produced over the 90 day incubation was not significantly different between 0.1 and 40 MPa for methylamine and formate (Table 5.1). However, significantly less methane ($p < 0.05$, *t*-test) was produced by the acetate-amended slurries kept at 40 MPa than those kept at 0.1 MPa (Table 5.1). There was a marked difference in the amount of sulphate reduced in the acetate-amended cultures at 0.1 MPa and 40 MPa, 3.24 and 1.39 mM respectively (Table 5.1). Pressure appeared to have no affect on the formate-amended enrichment cultures, in both cases the formate was completely consumed and there was no significant difference between the concentrations of sulphate remaining in the 0.1 and 40 MPa cultures at the end of the incubation (Table 5.1). The amount of sulphate consumed was significantly higher ($p < 0.05$) in the methylamine-amended cultures at 40 MPa than at 0.1 MPa.

The production of methane from methylamine results in the production of an equal amount of ammonium (Equation 5.1). The ammonium concentrations at the end of the incubation for both pressures were consistent with this; at 0.1 MPa, 3.84 mM methylamine was consumed and 3.90 ± 0.08 mM ammonium was produced, and at 40 MPa, 3.84 mM methylamine was consumed and 3.93 ± 0.03 mM ammonium was produced

Five of the sequenced DGGE bands had high 16S rRNA gene sequence identities (98-100%) to *Methanococcoides methylutens* and unsequenced bands corresponding to the position of these bands are seen in all amended slurries (Figure 5.1 and Table 5.2). Other bands present in all amended slurries, although not prominent in the 0.1 MPa methylamine amended slurry, are related to the uncultured Marine Benthic Group D (MBG-D). They had been enriched during the 90 day incubation, as bands corresponding to MBG-D were not seen in the unamended slurry sampled on Day 0. Other DGGE band sequences were related to the Marine Crenarchaeota Group (MCG) and were seen in the enrichment culture and the unamended sediment slurry on day 0 (Figure 5.1).

Table 5.1 Ion and gas chromatography results for Portishead sediment slurries incubated for 90 days at pressures of 0.1 and 40 MPa and a temperature of 25 ºC. The results for Day 30 are the mean of three replicates plus or minus one standard deviation. For Day 0, the values are for a single measurement (the triplicate cultures were produced from one of three slurries, acetate, formate and methylamine amended). Cultures to which methylamine was added also had their ammonium concentrations measured. Significant differences existed between pressures only where indicated.

Pressure (MPa)		Day 0	Day 90
0.1	Acetate (mM)	5.54	0.03 ± 0.00
	Sulphate (mM)	3.25	0.01 ± 0.00
	Methane (mM)	-	1.02 ± 0.07*
40	Acetate (mM)	5.54	5.03 ± 0.11
	Sulphate (mM)	3.25	1.86 ± 0.58
	Methane (mM)	-	0.39 ± 0.02*
0.1	Formate (mM)	4.97	0.01 ± 0.00
	Sulphate (mM)	3.24	1.45 ± 0.05
	Methane (mM)	-	0.07 ± 0.00
40	Formate (mM)	4.97	0.01 ± 0.00
	Sulphate (mM)	3.24	1.29 ± 0.21
	Methane (mM)	-	0.25 ± 0.12
0.1	Methylamine (mM)	3.84	0.00 ± 0.00
	Ammonium (mM)	0.35	4.25 ± 0.08
	Sulphate (mM)	3.24	2.38 ± 0.22**
	Methane (mM)	-	1.80 ± 0.42
40	Methylamine (mM)	3.84	0.00 ± 0.00
	Ammonium (mM)	0.35	4.28 ± 0.03
	Sulphate (mM)	3.24	1.64 ± 0.34**
	Methane (mM)	-	2.40 ± 0.16

*Significantly less methane ($p < 0.05$, *t*-test) present in the acetate-amended slurries kept at 40 MPa than those incubated at 0.1 MPa.
**Significantly more sulphate was reduced at 40 MPa than at 0.1 MPa (*t*-test, $p < 0.05$).

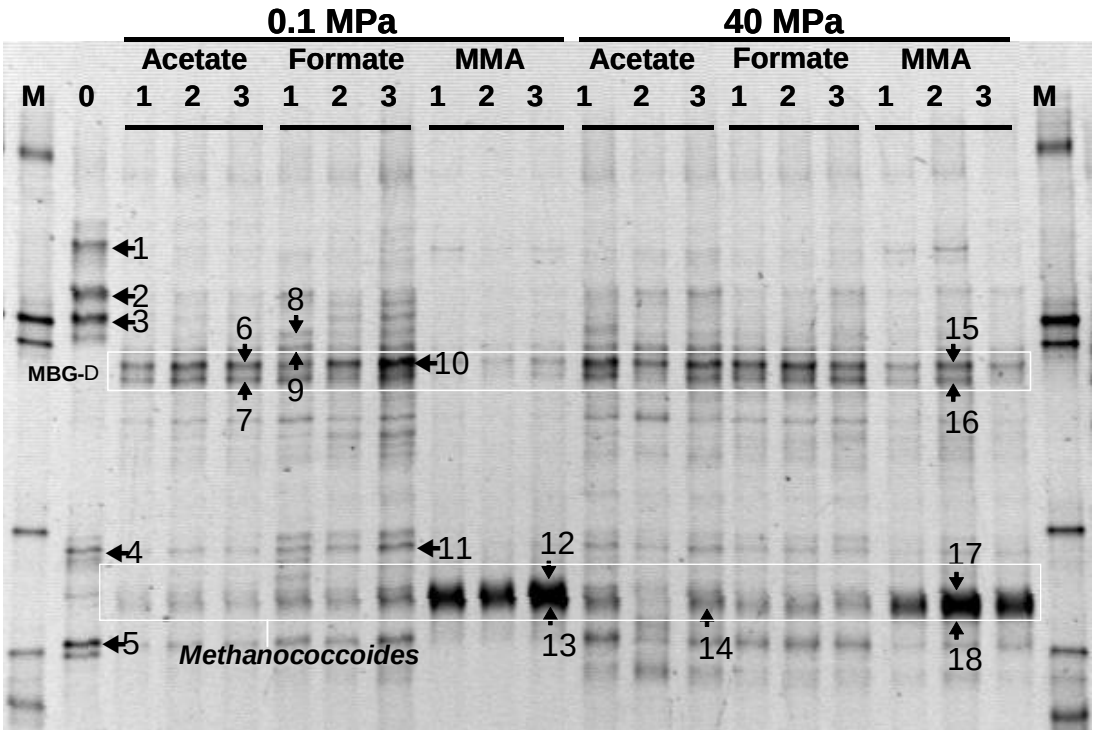


Figure 5.1 DGGE profile (Archaeal 16S rRNA gene sequencing) of Portishead slurries incubated at 0.1 MPa and 40 MPa with acetate, formate or methylamine (MMA) as substrates (all in triplicate). All incubations were for 90 days at 25 ºC. Position of sequenced MBG-D related bands and corresponding unsequenced bands are indicated. Likewise for *Methanococcoides*. All numbered bands were excised; the BLASTN results for the excised bands are given in Table 5.2. Columns marked 'M' are markers and the column marked '0' is unamended Portishead slurry on day 0.

Table 5.2 Phylogenetic affiliations of sequenced archaeal 16S rRNA gene DGGE bands of Portishead pressure slurries.

DGGE Band	Pressure (MPa)	Nearest match by BLASTN search (accession number)	% identity	Nearest cultivated match by BLASTN search (accession number)	% identity	Affiliation
1	0.1	Uncultured clone pIta-vmat-64 (AB301896) Hot spring microbial mat, Okinawa	96	Candidatus *Nitrosopumilus* sp. NM25 (AB546961) Ammonia-oxidzing archaeon, Tanoura Bay, Japan	93	MG1
2	0.1	Uncultured clone 3H3M_ARC113 (JN229822) Marine sediment, Porcupine Seabight	98	No significant similarity		Saltmarsh Group
3	0.1	Uncultured clone 3H3M_ARC113 (JN229822) Marine sediment, Porcupine Seabight	98	No significant similarity		Saltmarsh Group
4	0.1	Uncultured clone HSZ-S54 (HQ267310) Sediment, Yellow River Delta	95	No significant similarity		MCG
5	0.1	Uncultured clone HNDA53 (HM171880) Marine sediment, Hainan Island, China	98	No significant similarity		MCG
6	0.1	Uncultured clone ACSAS2P1C3 (FJ685731) Mangrove sediment, Western Ghats, India	98	No significant similarity		MBG-D
7	0.1	Uncultured clone ACSAS2P1C3 (FJ685731) Mangrove sediment, Western Ghats, India	98	No significant similarity		MBG-D
8	0.1	Uncultured clone A1001R001_G15 (AB654223) Rice paddy soil, Japan	86	"*Nitrososphaera gargensis"* Ga9.2 (GU797786) Hot spring, Russia	85	*Crenarchaeota*
9	0.1	Uncultured clone MKCSM-5 (DQ363839) Mangrove soil, China	92	No significant similarity		*Thermoplasmatales*
10	0.1	Uncultured clone 48H-0S-16 (GUU270204) Cold seep, Okhotsk Sea	98	No significant similarity		*Euryarchaeota*
11	0.1	Uncultured clone SH3B_1H3b_A074 (HQ606180) Sediment, South China Sea	94	No significant similarity		MCG
12	0.1	*Methanococcoides methylutens* TMA-10^T (FR733669) Scripps Canyon, California, USA	100	*Methanococcoides methylutens* TMA-10^T (FR733669) Scripps Canyon, California, USA	100	*Methanosarcinales*
13	0.1	*Methanococcoides methylutens* TMA-10^T (FR733669) Scripps Canyon, California, USA	100	*Methanococcoides methylutens* TMA-10^T (FR733669) Scripps Canyon, California, USA	100	*Methanosarcinales*

Table 5.2 (Continued) Phylogenetic affiliations of sequenced archaeal 16S rRNA gene DGGE bands of Portishead pressure slurries.

DGGE Band	Pressure (MPa)	Nearest match by BLASTN search (accession number)	% identity	Nearest cultivated match by BLASTN search (accession number)	% identity	Affiliation
14	40	*Methanococcoides methylutens* TMA-10^{I} (FR733669) Scripps Canyon, California, USA	98	*Methanococcoides methylutens* TMA-10^{I} (FR733669) Scripps Canyon, California, USA	98	*Methanosarcinales*
15	40	Uncultured clone AM1X-1C (FJ609955) Estuarine sediment, California, USA	100	No significant similarity		MBG-D
16	40	Uncultured clone ACSAS2P1C3 (FJ685731) Mangrove sediment, Western Ghats, India	97	No significant similarity		MBG-D
17	40	*Methanococcoides methylutens* MM1 (FJ477324) Mangrove sediment, Tanzania	99	*Methanococcoides methylutens* MM1 (FJ477324) Mangrove sediment, Tanzania	99	*Methanosarcinales*
18	40	*Methanococcoides methylutens* TMA-10^{T} (FR733669) Scripps Canyon, California, USA	100	*Methanococcoides methylutens* TMA-10^{T} (FR733669) Scripps Canyon, California, USA	100	*Methanosarcinales*

5.4 Discussion

The results of this study were unexpected, the methanogen identified in all slurries using 16S RRNA gene PCR-DGGE was *Methanococcoides methylutens* (Figure 5.1 and Table 5.2). Members of the genus *Methanococcoides* not able to utilize acetate or formate as a substrate (Whitman *et al.*, 2006). Previous Portishead enrichment cultures with acetate as a growth substrate had cultured methanogens closely related to *Methanosarcina* (Table 3.1) and H_2/CO_2 enrichment cultures contained methanogens of the genera *Methanobacterium*, *Methanococcus, Methanoculleus* and *Methanogenium*, some or all strains of these genera can also utilize formate (Tables 3.1 and 3.7).

The enrichment of *Methanococcoides methylutens* without the addition of methylamines or methanol may be explained by the breakdown of organic matter supplying methanol and/or methylamines that can be utilized by *Methanococcoides methylutens* (the production of methylamines from betaine, choline and TMAO is described in Section 1.3.6.1). One quarter of the slurry used in this experiment was, by volume, sediment. The production of methane from methylamines will result in the formation of ammonium (Equation 5.1) and as noted above the amount of ammonium produced in the methylamine-amended cultures was consistent with the amount of methylamine consumed (Table 5.1). If significant amounts of methylamines had been produced and consumed in the sediment slurries than this would have been reflected in additional amounts of ammonium being produced in the methylamine-amended slurries; however, ammonium did not significantly accumulate above the expected concentration of 3.8 mM ammonium. Methanogenesis using methanol as a substrate would not produce ammonium (Equation 5.2). Hence, this or other compounds (see Chapter 7 for other methylated compounds that do not result in ammonium production) from the sediment could still explain the presence of *Methanococcoides.*

$$4\ \text{Methylamine} + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + H^+ + 4\ NH_4^+ \qquad \text{(Equ. 5.1)}$$

$$4\ \text{Methanol} \rightarrow 3\ CH_4 + HCO_3^- + H^+ + H_2O \qquad \text{(Equ. 5.2)}$$

Whilst the concentrations of methylamine consumed and ammonium produced were in agreement, the concentrations of methylamine consumed and methane produced were not. One mole of methylamine was expected to give rise to three-quarters of a mole of methane (Equation 5.1); at 0.1 MPa and 40 MPa the expected concentrations of methane were 2.88 mM. The 0.1 MPa enrichment culture contained 1.80 ± 0.42 mM methane and the 40 MPa enrichment culture contained 2.40 ± 0.0.16 mM methane. This shortfall may have been due to methane consumption although no known methane consuming *Archaea* were identified in these or any of the other enrichment cultures (Figure 5.1 and Table 5.2). Another possibility is methane loss from the vials by diffusing through the butyl rubber stopper and in the case of the 40 MPa enrichment cultures escape of methane during depressurization. If methane loss occurred in the methylamine enrichment cultures then it most likely occurred with the other enrichment cultures and the results in Table 5.1 are an underestimate.

Acetate and hydrogen utilising methanogens had previously been enrichment in Portishead sediment taken from the same depth range and incubated at the same temperature (Table 3.1). The absence of these methanogens was not due to elevated hydrostatic pressure, as they were not enriched in the atmospheric pressure (0.1 MPa) enrichment cultures. However, the conditions differed from previous enrichment cultures in one aspect, the lack of a headspace. The use of high pressure required that the serum bottles had to be completely filled with sediment slurry; the atmospheric pressure control enrichment cultures were also completely filled with sediment slurry. This factor did not affect the enrichment of *Methanococcoides* (Figure 5.1 and Table 5.2). Acetate and formate utilizing methanogens may have been out-competed by sulphate-reducing bacteria.

One mole of acetate is consumed for every mole of sulphate reduced (Equation 5.3). The acetate-amended cultures at both pressures contained more acetate than sulphate (Table 5.1). Whilst all of the acetate was consumed in the 0.1 MPa enrichment cultures, only 10% of the acetate had been consumed in the 40 MPa cultures leaving 5.0 mM available for methanogenesis. It may have been left unconsumed due to the absence of acetoclastic methanogens or acetoclastic methanogenesis was inhibited at 40 MPa. In contrast, four moles of formate are required to reduce one mole of

sulphate (Equation 5.4) and there was insufficient formate in the formate-amended cultures to account for the concentration of sulphate reduced (Table 5.1).

$$CH_3COO^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^- \quad \text{(Equ. 5.3)}$$

$$4\ CHOO^- + SO_4^{2-} + H^+ \rightarrow 4\ HCO_3^- + HS^- \quad \text{(Equ. 5.4)}$$

The absence of formate utilizing methanogens may then be due to competition from sulphate-reducing bacteria, the reduction of sulphate was not adversely affected by elevated hydrostatic pressure as the remaining sulphate was similar at both 0.1 and 40 MPa (Table 5.1).

As *Methanosarcina* had previously been identified at Portishead (Table 3.1) and all members of the genus are able to utilize methylamine as a growth substrate (Whitman, 2006) they would be in competition with *Methanococcoides* for this substrate. In this study, no methylamine-amended enrichment culture with marine medium incubated below 38 ºC enriched *Methanosarcina* (Table 3.1) and this enrichment study was no exception.

The surprising finding was the presence of the archaeal MBG-D in acetate and formate amended slurries with active sulphate reduction and methanogenesis. In addition, MBG-D was also clearly present in methylamine-amended slurries at 40 MPa. MBG-D have been identified in marine sediments from many sites including Aarhus Bay (Webster *et al.*, 2011), Portishead (Webster *et al.*, 2010) and Eel River Basin (Beal *et al.*, 2009). They have also been detected at non-marine sites such as Qinghai Lake (NW China) (Jiang *et al.*, 2008). Members of MBG-D are *Euryarchaeota* and the closest cultured relatives of many MBG-D members are methanogens (80% 16S rRNA gene squence identity) (Beal *et al.*, 2009).

The metabolism of members of MBG-D is not currently known (Beal *et al.*, 2009). They were enriched in this study regardless of the substrate added to the sediment slurry (acetate, formate or methylamine). Substrate(s) was possibly supplied by the breakdown of organic matter that would have occurred at a higher rate at the

incubation temperature of 25 °C compared with the *in situ* temperature of 3 °C at Portishead mudflat at the time of sediment collection in December 2010. As there was a shortfall in the amount of methane in the methylamine enrichment cultures (and possibly in the other enrichment cultures) there is the possibility that at least some members of MBG-D are methanotrophs.

5.5 Summary

Enrichment cultures with sediment collected from a Portishead mudflat and acetate, formate and methylamine as substrates were incubated for 90 days at atmospheric pressure (0.1 MPa), and elevated hydrostatic pressure (40 MPa). In all cases the methanogens enriched were members of the methylotrophic genus *Methanococcoides*; acetate or formate utilizing methanogens were not identified in the enrichment cultures. Along with *Methanococcoides*, members of the uncultured *Euryarchaeota* group MBG-D were enriched in all enrichment cultures.

Chapter 6
Characterisation of Methanogen Strains

6.1 Introduction

Sixteen strains of the genus *Methanococcoides* and two strains of the genus *Methanococcus* were isolated from enrichment cultures; the strains and the locations from which they were isolated are summarized in Table 6.1. The *Methanococcoides* strains were characterised; the phylogeny and morphology of all sixteen strains are detailed in Section 6.2 and the results of the substrate utilization tests are presented in Chapter 7. One *Methanococcoides* strain from each location (strains AM1, BSM1, DM1, MKM1, NM1, PM1 and TM1) was selected for further characterisation including pH, Na^+ concentration and temperature ranges. The opportunity was also taken to determine the upper and lower pH range of *M. burtonii* DSM 6242^T which had not been previously determined (strain obtained from DMSZ). Three strains from this study (BSM1, NM1 and TM1) as well as the type species of the genus, *M. methylutens* TMA-10^T, were tested to determine growth with a range of hydrostatic pressures. The characterisation of the two *Methanococcus* strains (PM4 and PM5) isolated from a mudflat at Portishead (UK) is detailed in Section 6.3 and is limited to phylogeny, morphology and substrate utilisation.

Table 6.1 Summary of strains isolated by this study.

Strain	Location	Genus
AM1, AM2 & AM3	Aarhus Bay	*Methanococcoides*
BSM1, BSSM2 & BSM3	Dvurechenskii MV	*Methanococcoides*
DM1	Darwin MV	*Methanococcoides*
MKM1 & MKM2	Meknes MV	*Methanococcoides*
NM1 & NM2	Napoli MV	*Methanococcoides*
PM1, PM2 & PM3	Portishead	*Methanococcoides*
PM4 & PM5	Portishead	*Methanococcus*
TM1 & TM2	Tamar	*Methanococcoides*

6.2 Description of *Methanococcoides* Strains

6.2.1 Introduction to the Genus *Methanococcoides*

The genus *Methanococcoides* is a member of the family *Methanosarcinaceae* of the order *Methanosarcinales* (Whitman *et al.*, 2006). This order has three validly described species: *Methanococcoides methylutens* TMA-10^T (Sowers & Ferry, 1983), *Methanococcoides burtonii* DSM 6262^T (Franzmann *et al.*, 1992) and *Methanococcoides alaskense* AK-5^T (Singh *et al.*, 2005). *Methanococcoides methylutens* TMA-10^T is the type species of the genus (Sowers & Ferry, 1983). A second strain of *Methanococcoides alaskense*, strain AK-9, was described along with the type strain AK-5^T (Singh *et al.*, 2005). Descriptions of two strains of *Methanococcoides methylutens*, in addition to the type strain, have been published; strain NaT1 (Tanaka, 1994) and strain MM1 (Lyimo *et al.*, 2009b). A fourth species, *Methanococcoides euhalobius* (Obraztsova *et al.*, 1987), has been transferred to the genus *Methanohalophilus* on the basis of 16S rRNA sequence data (Davidova *et al.*, 1997). Morphologically and physiologically, *Methanococcoides euhalobius* was similar to *Methanococcoides methylutens* except that it could grow in the presence of NaCl up to a concentration of 2.3 M (Obraztsova *et al.*, 1987). A possible additional strain of *Methanococcoides methylutens* has been described which was enriched from mangrove sediment using trimethylamine as a substrate. Its DNA base content was found to be consistent with the type description of

Methanococcoides methylutens (Mohanraju *et al.*, 1997). However, no 16S rRNA or *mcrA* sequence data are available for this strain.

Methanococcoides methylutens TMA-10^T was isolated from sediment, consisting of sand and sea grass debris, taken from the Sumner branch of Scripps Canyon, La Jolla, California, USA (Sowers & Ferry, 1983). *Methanococcoides burtonii* DSM 6262^T was isolated from water taken from 26 m depth in Ace Lake, Antarctica (Franzmann *et al.*, 1992). The salinity of Ace Lake ranged from 0.6% at the surface to 6.3% at 26 m depth where the temperature was always less than 2 ˚C. Both strains of *Methanococcoides alaskense* (AK-5^T and AK-9) were isolated from Skan Bay, Unalaska Island, Alaska (Singh *et al.*, 2005) . The sediment was rich in organic matter (diatoms and kelp) and annual temperatures ranged from 1 to 6 ˚C. *Methanococcoides* strain NaT1 was isolated from a sand sample collected in Tokyo Bay (Asakawa *et al.*, 1998; Tanaka, 1994) and *Methanococcoides* strain MM1 was isolated from the Mtoni creek mangrove forest (Dar es Salaam, Tanzania) which is dominated by the mangrove species *Sonneratia alba*, *Avicennia marina*, *Rhizophora mucronata*, and *Ceriops tagal* (Lyimo *et al.*, 2002; Lyimo *et al.*, 2009b).

6.2.2 Phylogenetic Analysis of *Methanococcoides* Strains

The 16S rRNA gene sequences of all sixteen isolated strains were compared with those of all previously described strains. The strains isolated in this study fall into two broad groups on the basis of 16S rRNA gene sequences; strains BSM1, BSM2 and BSM3 have 99% sequence identity to *M. alaskense* AK-5^T whilst the other strains have 99% sequence identity to *M. methylutens* TMA-10^T (Table 6.2, Figure 6.1). Phylogenetic analysis using *mcrA* gene sequences was also undertaken with comparison with sequences for *M. alaskense* AK-5^T, *M. burtonii* and *M. methylutens* TMA-10^T (*mcrA* sequences for *M. alaskense* AK-9 and *Methanococcoides* strains NaT1 and MM1 were not available). On the basis of *mcrA* gene sequences all sixteen strains are most closely related to *Methanococcoides methylutens* TMA-10^T, $\geq 95\%$ sequence identity (Table 6.2, Figure 6.2). On both the 16S rRNA and *mcrA* phylogenetic trees the Portishead strains PM1 and PM2 form a clade of their own and, strain PM3 forms a group with Tamar strains TM1 and TM2. The Cadiz MV strains DM1, MKM1, MKM2 and the Napoli MV strains NM1 and NM2 also form a distinct

group on both 16S rRNA and mcrA trees. The 16S rRNA gene phylogenetic tree shows a grouping of *M. methylutens* TMA-10^T, AM1, AM2, AM3, TM1, TM2 and PM3. The *mcrA* gene phylogenetic tree shows that AM1, AM2, AM3 form a group with *M. methylutens* TMA-10^T and TM1, TM2 and PM3 form another group; however, this has very low bootstrap support.

Table 6.2 Percentage sequence identity (16S rRNA and *mcrA*) between the sixteen strains isolated in this study and the three type strains of the genus *Methanococcoides*.

Strain	Percentage Blastn Match					
	16S			*mcrA*		
	M.methylutens Strain TMA-10^T	*M.alaskense* Strain AK-5^T	*M.burtonii* Strain DSM 6262^T	*M.methylutens* Strain TMA-10^T	*M.alaskense* Strain AK-5^T	*M.burtonii* Strain DSM 6262^T
AM1	99	98	98	99	95	95
AM2	99	98	98	99	95	95
AM3	99	98	98	99	95	95
BSM1	98	99	99	95	96	93
BSM2	98	99	99	95	96	93
BSM3	97	99	98	95	96	93
DSM1	99	98	98	97	95	95
MKM1	99	98	98	97	95	95
MKM2	99	97	98	97	95	95
NM1	99	98	98	97	95	95
NM2	99	98	98	97	95	95
PM1	99	98	97	97	95	95
PM2	99	98	97	97	96	95
PM3	99	98	98	98	96	96
TM1	99	98	97	96	96	96
TM2	99	98	98	98	96	96

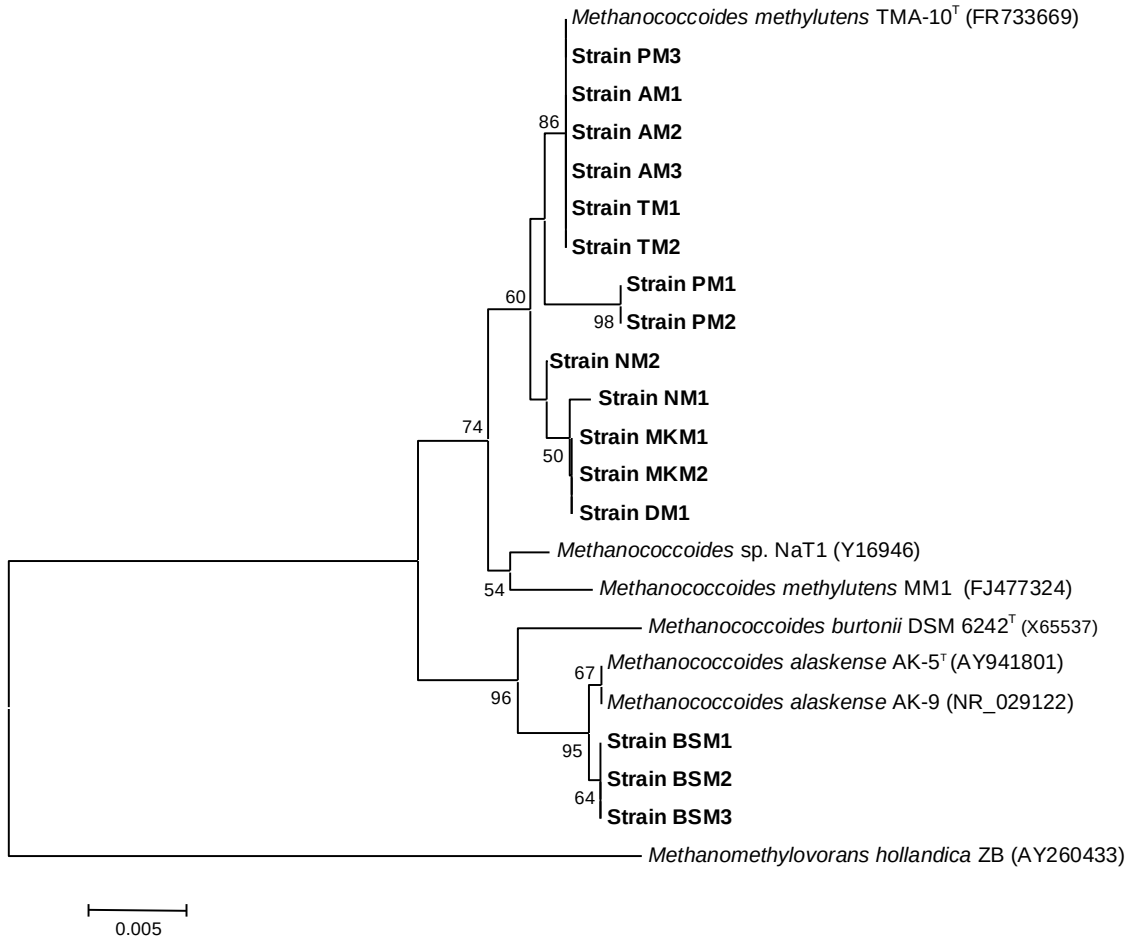


Figure 6.1 Neighbour-joining 16S rRNA phylogenetic tree showing all sixteen *Methanococcoides* strains isolated in this study and sequences of all published strains. Numbers at nodes represent percentage bootstrap values (1000 replicates and only bootstrap values > 50% shown). Bar, 0.005 substitutions per site.

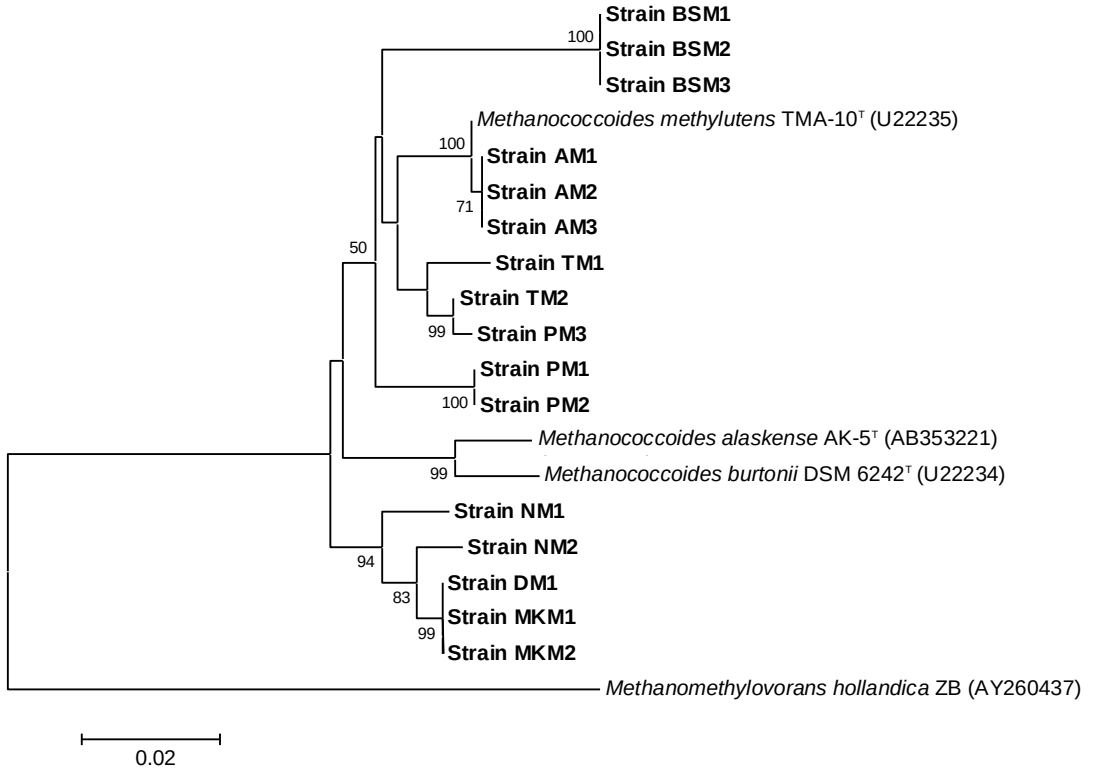


Figure 6.2 Neighbour-joining *mcrA* phylogenetic tree showing all *Methanococcoides* strains isolated in this study. Numbers at nodes represent percentage bootstrap values (1000 replicates and only bootstrap values > 50% shown). Bar, 0.02 substitutions per site. Sequences are not available for *Methanococcoides* sp NaT1 and MM1.

6.2.3 Morphology of the *Methanococcoides* Strains

Cells of all seven *Methanococcoides* strains investigated in this study were irregular cocci and ranged from 0.9 to 2.1 μm in diameter (Figure 6.3). All occurred singly or in pairs with a number of strains occurring as clumps of cells (Table 6.3). All strains were autofluorescent and non-motile. Cells of all strains, with the exception of BSM1, stained Gram negative; strain BSM1 lysed during the staining process. The presence or absence of flagella has not been determined for strains isolated in this study. Although non-motile this does not exclude the presence of flagella, *M. alaskense* strain AK-9 was observed to be non-motile but electron microscopy revealed the presence of two appendages resembling flagella (Singh *et al.*, 2005). Cell of all strains were susceptible to lysis by 0.01% SDS indicating a proteinaceous cell wall (Boone & Whitman, 1988). With the exception of strain TM1, cells from the isolated strains lysed in distilled water. After 30 minutes in distilled water not all cells of strain TM1 had lysed and the remaining cells were seen to be spherical or near spherical rather than irregularly shaped indicating their ability to resist the osmotic pressure.

Cells of the previously described strains are also irregular cocci occurring either singly or, with two exceptions, as pairs (Table 6.3). No clumps, other than those of up to four cells in *M. burtonii* (Franzmann *et al.*, 1992), have been reported.

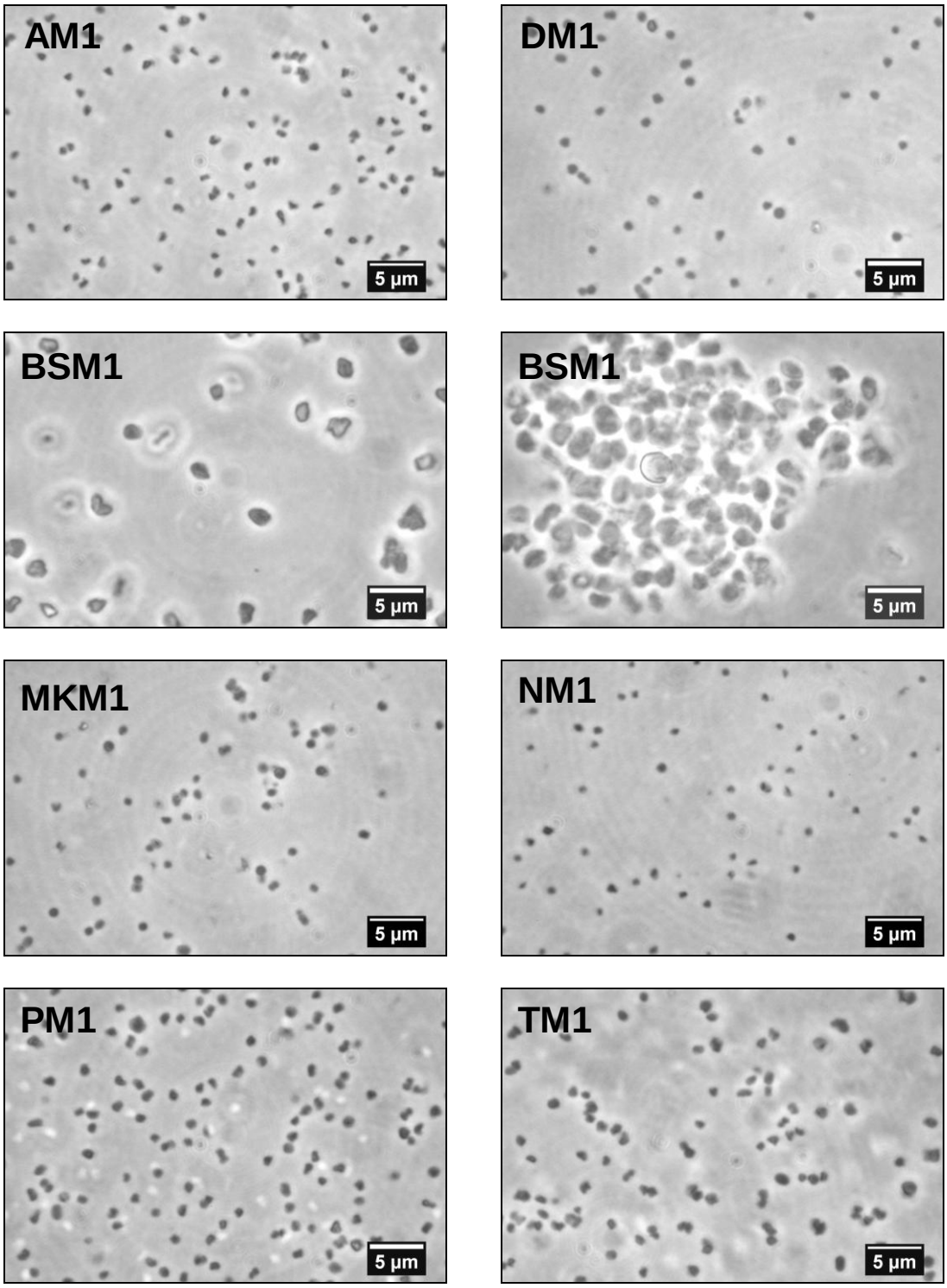


Figure 6.3 Phase contrast photomicrographs of selected *Methanococcoides* strains. Strain BSM1 is shown as single cells and as an aggregation of cells.

Table 6.3 General characteristics of *Methanococcoides* strains isolated in this study and comparison with published strains, *M.alaskense* AK-5^T (Singh *et al.*, 2005), *M.burtonii* DSM 6262^T (Franzmann *et al.*, 1992), *M.methylutens* TMA-10^T (Sowers & Ferry, 1983), MM1 (Lyimo *et al.*, 2009b) and NaT1 (Tanaka, 1994). Cell size +/- one standard deviation.

Strain	Shape	Size (μm)	*Occurrence*	Motility	Gram stain	Lysis	
						SDS (0.01%)	dH_2O
AM1	Irregular cocci	1.1 ±0.3	Singly/pairs	-	-ve	+	+
AM2	Irregular cocci	0.9 ±0.1	Singly/pairs	-	-ve	+	+
AM3	Irregular cocci	1.0 ±0.1	Singly/pairs/aggregates	-	-ve	+	+
BSM1	Irregular cocci	1.5 ±0.5	Singly/pairs/aggregates	-	lysed	+	+
BSM2	Irregular cocci	2.1 ±0.6	Singly/pairs/aggregates	-	-ve	+	+
BSM3	Irregular cocci	1.9 ±0.3	Singly/pairs/aggregates	-	-ve	+	+
DM1	Irregular cocci	1.0 ±0.1	Singly/pairs	-	-ve	+	+
MKM1	Irregular cocci	1.1 ±0.3	Singly/pairs	-	-ve	+	+
MKM2	Irregular cocci	1.0 ±0.2	Singly/pairs	-	-ve	+	+
NM1	Irregular cocci	0.9 ±0.1	Singly/pairs/aggregates	-	-ve	+	+
NM2	Irregular cocci	1.0 ±0.1	Singly/pairs	-	-ve	+	+
PM1	Irregular cocci	1.0 ±0.1	Singly/pairs	-	-ve	+	+
PM2	Irregular cocci	1.0 ±0.3	Singly/pairs	-	-ve	+	+
PM3	Irregular cocci	0.9 ±0.1	Singly/pairs	-	-ve	+	+
TM1	Irregular cocci	1.2 ±0.6	Singly/pairs	-	-ve	+	+[1]
TM2	Irregular cocci	1.0 ±0.1	Singly/pairs	-	-ve	+	+
M.methylutens TMA-10^T	Irregular cocci	1-3	Singly/pairs	-	-ve	+[2]	+[3]
M. burtonii DSM6262^T	Irregular cocci	0.8-1.8	Singly/clumps up to 4 cells. No pseudosarcina	+[6]	lysed	+[3]	+
M. alaskense AK-5^T	Irregular cocci	1.5-2.0	Singly	-	-ve	+	+[3]
M. alaskense AK-9	Irregular cocci	1.0-1.5	Singly	-[5]	-ve	+	ND
M.methylutens MM1	Irregular cocci	1.3 (0.2)	Singly/pairs	-	ND	+	ND
M.methylutens NaT1	Irregular cocci	0.5-1.2	Singly/pairs	+[6]	+ve	+	ND

[1] incomplete lysis, remaining cells spherical
[2] also lysed by 0.001% Triton X-100 (Sowers & Ferry, 1983)
[3] determined by this study, not included in the species description
[6] single flagellum (Franzmann *et al.*, 1992)
[5] flagella-like appendages (Singh *et al.*, 2005)
[6] type of flagella not determined (Tanaka, 1996)
-ve, stained Gram negative +ve, stained Gram positive ND, not determined
-, property of the strain +, not a property of the strain

6.2.4 pH Range

The pH range for seven representative strains of *Methanococcoides* isolated in this study has been determined (Figure 6.4). Overall, the pH ranges for growth for each strain was similar. Growth occurred over the pH range 6.0 (strains AM1, PM1 and TM1) to pH 9.5 (strain BSM1). The optimum for each strain was around pH 7.0 except for strain DM1 whose optimum was around pH 8.0. The published growth range for *Methanococcoides burtonii* is from pH 6.8 to 8.2 with pH values outside of this range not determined in the original study (Franzmann *et al.*, 1992). This study determined that the minimum and maximum pH values for growth of *M. burtonii*

were pH 6.6 and pH 8.9 respectively (Table 6.4). Results are summarized and compared to pH ranges for the previously described strains, Table 6.4.

6.2.5 Na^+ Concentration Range

Figure 6.5 shows the specific growth rates for seven representative strains with concentrations of Na^+ ranging from 0.03 to 1.5 M. Strains, except strain MKM1, have an optimum Na^+ concentration less than that of seawater (0.65 M Na^+). The optimum Na^+ concentration of strain MKM1 is 0.65 M. Strains AM1, BSM1, DM1, PM1 and TM1 were able to grow at the lowest concentration tested (0.03 M Na^+). Strain TM1 also grew at the highest concentration (1.3 M Na^+) and had the widest Na^+ concentration range for growth. The growth rates are summarized in Table 6.4 where comparison is made with data for the previously described strains.

6.2.6 Temperature Range

Figure 6.6 shows the growth rates (average of two samples) for the seven representative strains. The data is presented as specific growth rate (μ) at a range of temperatures and as Arrhenius plots (log of the specific growth rate constant against the reciprocal of absolute temperature) the slope of which was used to calculate activation energy (E_a) and Q_{10} values (Table 6.5). With the exception of strains AM1 and PM1, strains were able to grow at 6 ºC (typical bottom seafloor temperature). No strain grew at 0 ºC (strains were monitored for six months) and strain TM1 grew at the lowest temperature (3 ºC). The highest recorded temperature for growth was 40 ºC (strains PM1 and TM1). The doubling times at T_{opt} and 25 ºC (the general incubation temperature) for seven representative strains are given in Table 6.6. Strain TM1 had the shortest doubling times at both T_{opt} and 25 ºC, 0.3 and 0.6 days respectively. Strain MKM1 had the longest doubling time at T_{opt} (1.2 days) and both strain MKM1 and strain BSM1 had the longest doubling times at 25 ºC (1.3 days). The Arrhenius plots (Figure 6.6) show deviations from linearity at upper temperature ranging from 25 to 34 ºC and at lower temperatures ranging from 8 to 16 ºC. The Q_{10} value for the strains tested in this study are in the range 2.6 to 2.8 with E_a values in the range 63 to 73 kJ mol^{-1} (Table 6.5).

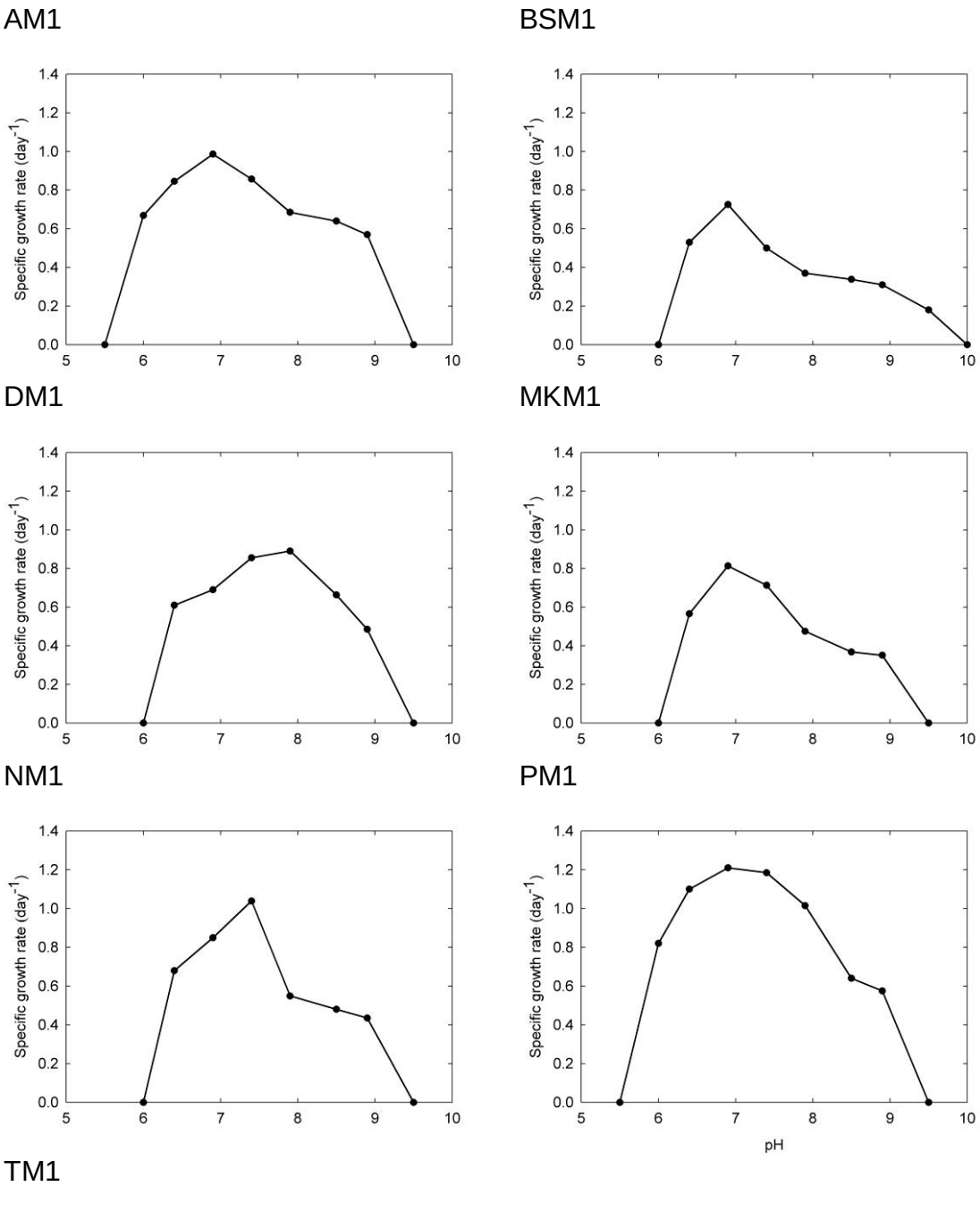


Figure 6.4 Specific growth rates, determined over a pH range 5-10, of representative strains from each of the seven sites investigated in this study. Strains grown in marine medium with methylamine (10 mM) as a substrate and incubated at 25 ºC. Specific growth rates are the average of two samples.

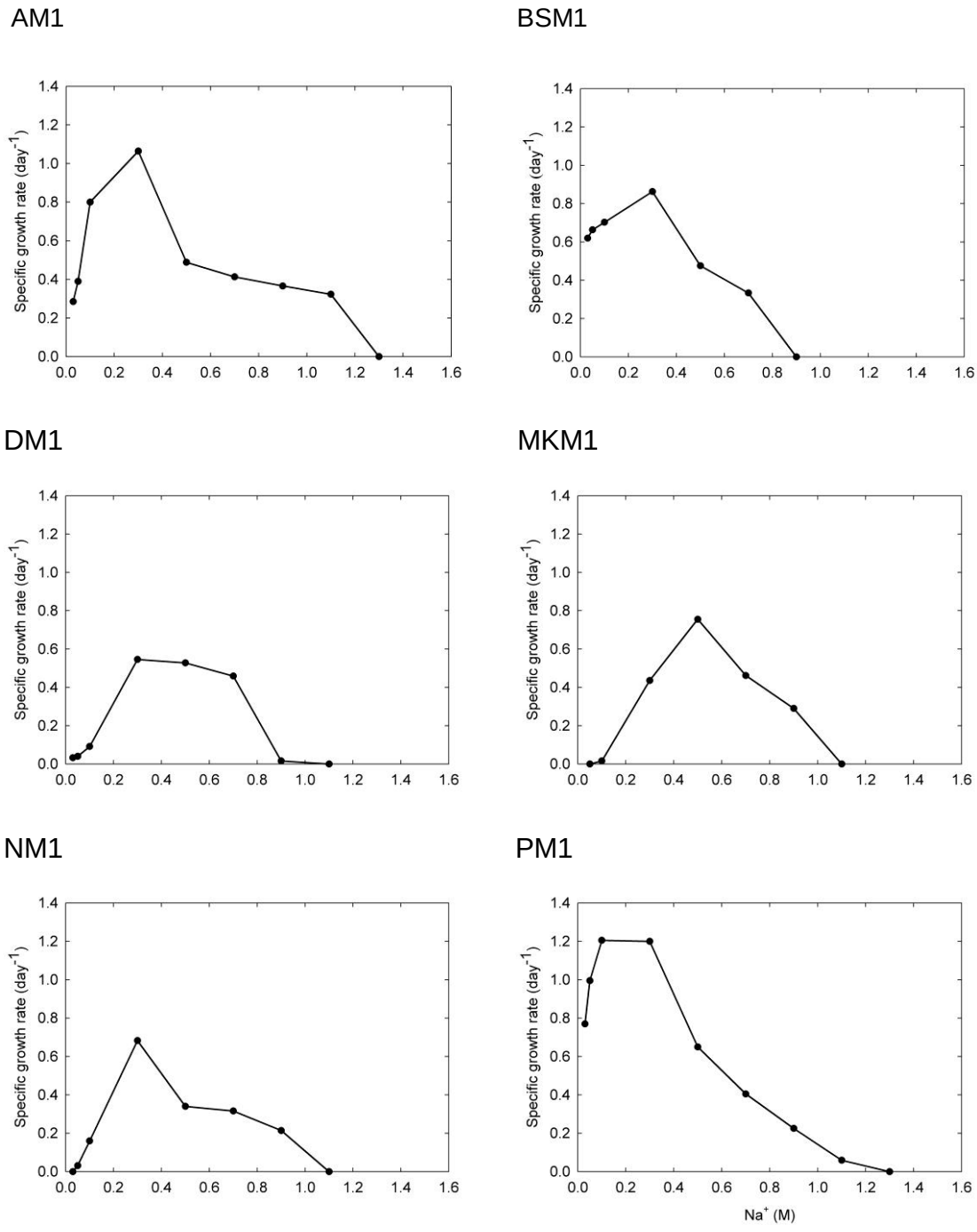
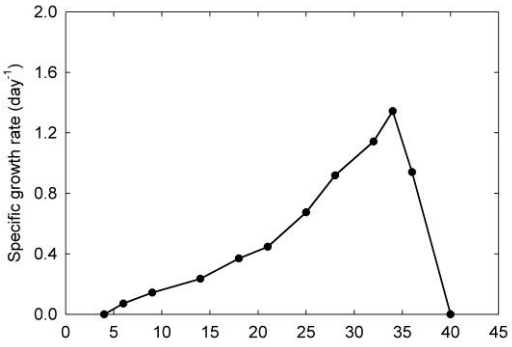
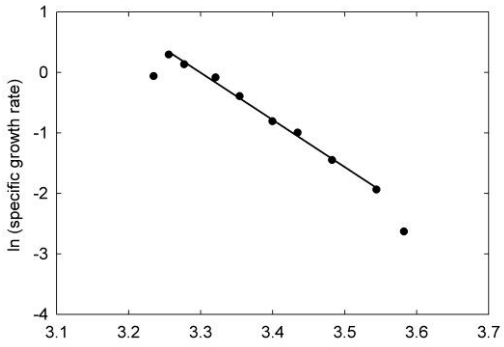


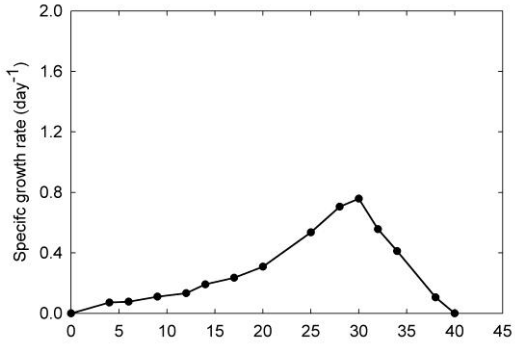
Figure 6.5. Specific growth rates, determined over a Na^+ concentration range of 0.03 to 1.5 M, of representative strains from each of the seven sites investigated in this study. Strains grown in marine medium with methylamine (10 mM) as a substrate and incubated at 25 ºC. Specific growth rates are the average of two samples.

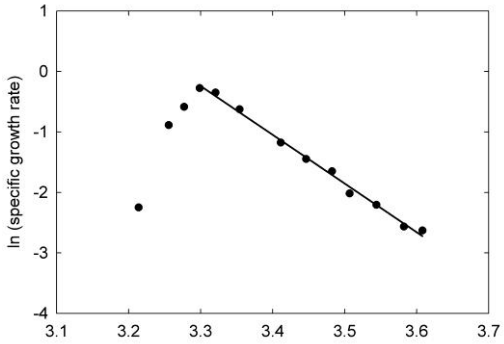
AM1



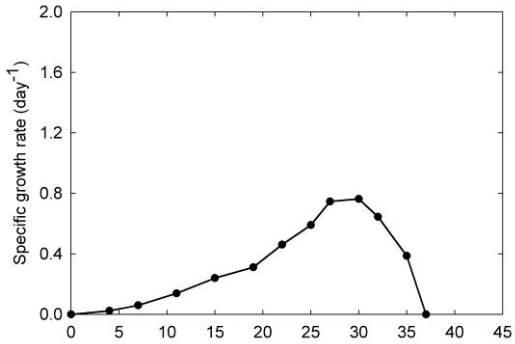


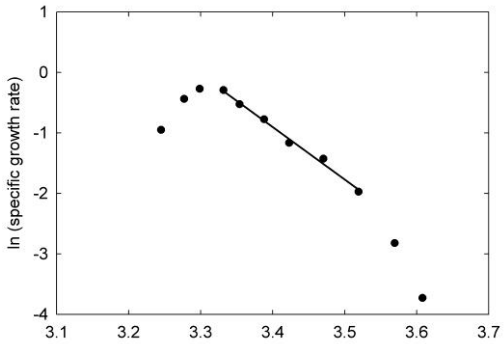
BSM1



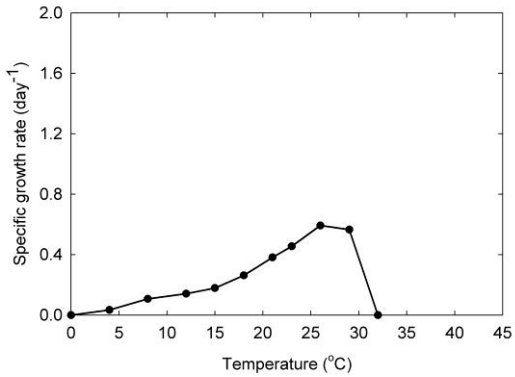


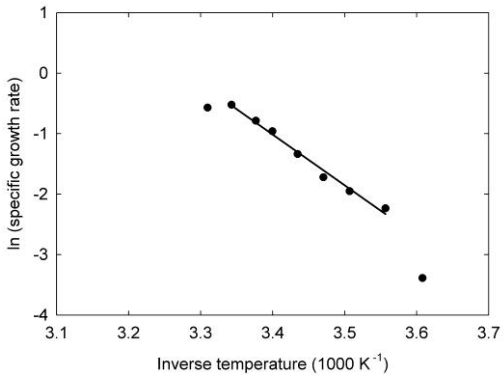
DM1





MKM1





NM1

PM1

TM1

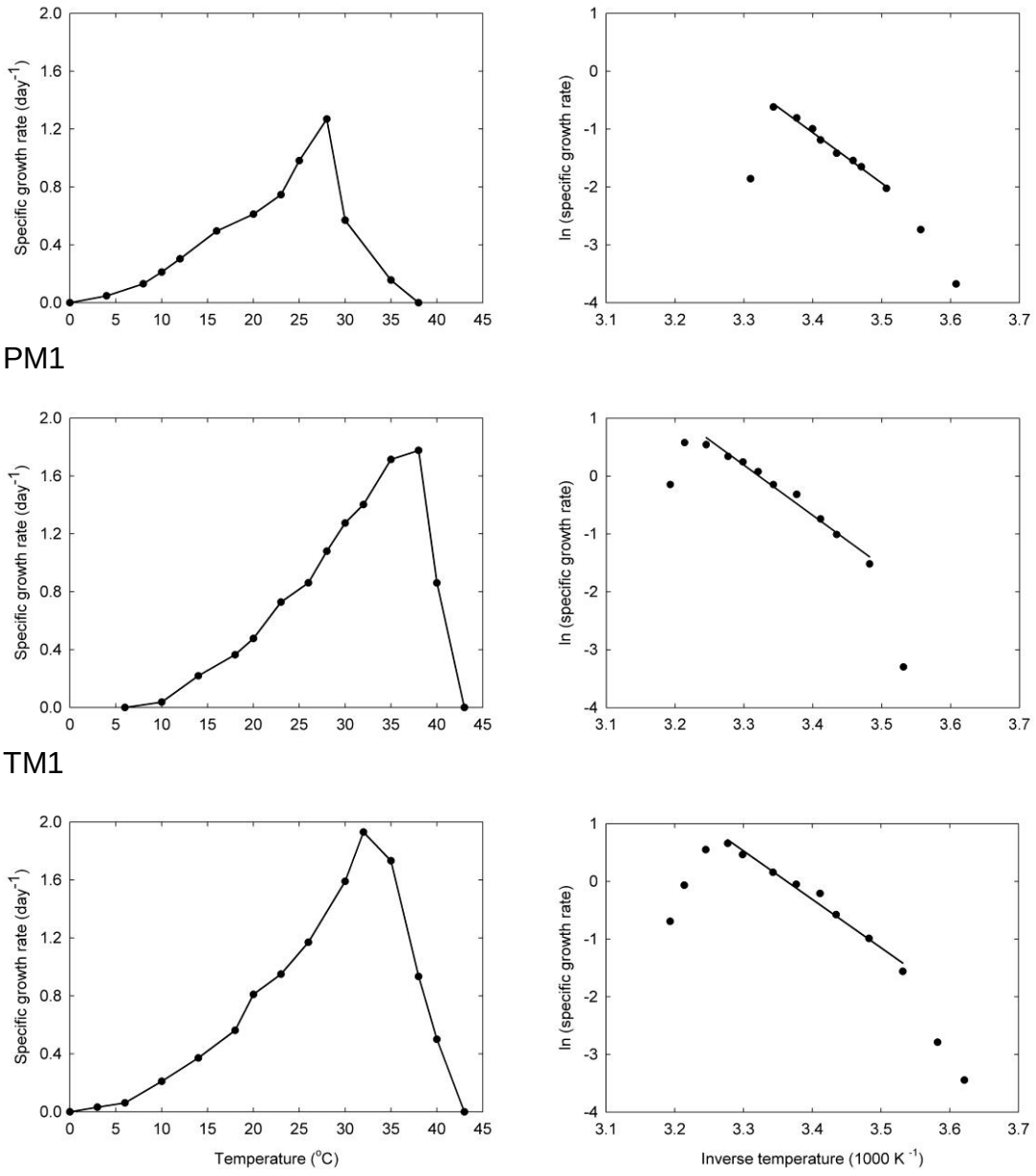


Figure 6.6 Growth with increasing temperature for representative strains from each of the seven sites investigated in this study. Left hand column, specific growths rates, right hand column, Arrhenius plots (log specific growth rate against inverse temperature in K). Strains grown in marine medium with methylamine (10 mM) as a substrate and incubated at 25 ºC. Growth rates are the average of two samples.

Table 6.4 Growth ranges for pH, Na^+ concentration and temperature of the sixteen strains isolated in this study and five published strains of *Methanococcoides*. This study also determined the minimum and maximum pH values for *M. burtonii* as these were not described by Franzmann *et al* (1992).

	pH			Na^+ (M)			Temperature (ºC)			*In situ*
Strain	minimum	optimum	maximum	minimum	optimum	maximum	minimum	optimum	maximum	temp. (ºC)
AM1	6.0	6.9	8.9	0.03	0.1-0.3	1.1	6	36	36	10
BSM1	6.6	6.9	9.5	0.03	0.3	0.7	6	30	38	9
DM1	6.6	7.6-7.9	8.9	0.03	0.3-0.5	0.7	6	27-30	35	5
MKM1	6.6	6.9-7.6	8.9	0.1	0.5	0.9	6	26-29	29	7
NM1	6.6	7.6	8.9	0.05	0.3	0.9	6	28	35	16
PM1	6.0	6.9-7.6	8.9	0.03	0.1-0.3	1.1	10	35-38	60	22[16]
TM1	6.0	6.6-6.9	8.9	0.03	0.1-0.3	1.3	3	32-35	60	12
M.alaskense AK-5^{T} [1]	6.3	7.3	7.3	0.1	0.3-0.6	0.7	-2.3 [9]	23.6	30.6	1-6
M.burtonii DSM 6262^{T} [2]	6.8 [6]	7.7	8.2 [7]	0.2	0.2	0.5	1.7 [10]	23-26	29	<2
M.methylutens TMA-10^{T} [3]	6.0	7.0-7.5	8.0	0.15	0.26-0.66	1.1	ND[11]	35	35	Not given
M.methylutens MM1 [6]	6.3	7.0-7.8	8.3	0.05	0.3	0.6	ND[12]	32	35	Not given
M.methylutens NaT1 [5]	6.2	6.6-7.5	8.5 [8]	ND[13]	0.3-0.6	ND[13]	25	35	60	Not given

[1] Singh *et al* (2005)
[2] Franzmann et al (1992)
[3] Sowers & Ferry (1983)
[6] Lyimo et al (2009)
[5] Tanaka (1996)
[6] Not determined below 6.8 (Franzmann et al, 1992), this study 6.6
[7] Not determined above 8.2 (Franzmann et al, 1992), this study 8.9
[8] Not determined above 8.5 (Tanaka, 1996)
[9] T_{min} using square root equation (Singh et al, 2005)
[10] T_{min} if growth initiated at 20 ºC otherwise it is 5.6 ºC. T_{min} using square root equation is –2.5 ºC
[11] Growth not determined below 15 ºC (Sowers & Ferry, 1983)
[12] Growth not determined below 23 ºC (Lyimo *et al.*, 2009b)
[13] Not determined below 0.1 M Na^+ or above 0.5 M Na^+ (Tanaka, 1994)
[16] December 2009, 20 cm depth. In December 2010 it was 3 ºC
ND, not determined

Table 6.5 Q_{10} values and activation energies (E_a) for seven representative strains isolated in this study and *Methanococcoides methylutens* TMA-10^T. Activation energies (E_a) for the strains isolated in this study are for the temperature range 15 to 25 °C. Values for *M. methylutens* calculated from published data (Sowers & Ferry, 1983), also for the temperature range 15 to 25 °C. Methylamine was used as a substrate.

Strain	Q_{10}	E_a (kJ mol^{-1})
AM1	2.5	65
BSM1	2.6	67
DM1	2.7	72
MKM1	2.7	70
NM1	2.8	73
PM1	2.7	72
TM1	2.7	70
Methanococcoides methylutens TMA-10^I	2.6	63

Table 6.6 Doubling time for seven representative *Methanococcoides* strains at T_{opt} and 25 ºC.

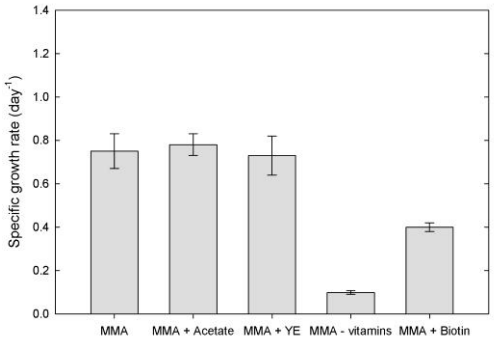
		Doubling time	
Strain	T_{opt} (ºC)	At T_{opt} (days)	At 25 ºC (days)
AM1	36	0.5	1.0
BSM1	30	0.9	1.3
DM1	30	0.9	1.2
MKM1	26	1.2	1.3
NM1	28	0.6	0.7
PM1	38	0.6	0.9
TM1	32	0.3	0.6

6.2.7 Growth Factors

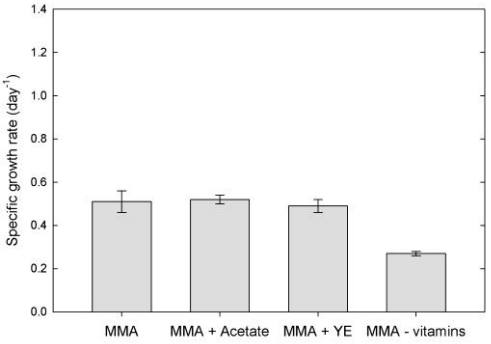
None of the *Methanococcoides* strains tested in this study required yeast extract (2g l^{-1}) or acetate (3 mM) for growth nor did these additions stimulate growth (Figure 6.7). Six of the seven strains tested in this study were found to have lower growth rates when grown in vitamin free medium vitamins (four transfers into vitamin free media with 3% inoculum) compared to media with vitamins (Figure 6.7). One strain, strain NM1, did not grow without vitamins. Of those strains that grew without vitamins, the specific growth rate of strain BSM1 was the least affected (specific growth rate without vitamins was 56% of that with vitamins) and the specific growth rate of strain PM1 was the most affected (specific growth rate without vitamins was

only 16% of that with vitamins). The addition of biotin to strain AM1 did not increase the growth rate to that of the full vitamin solution indicating that an additional vitamin or vitamins was required.

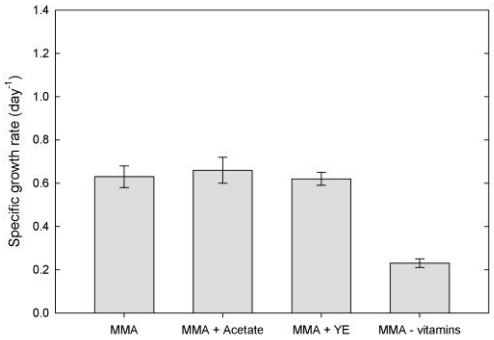
AM1



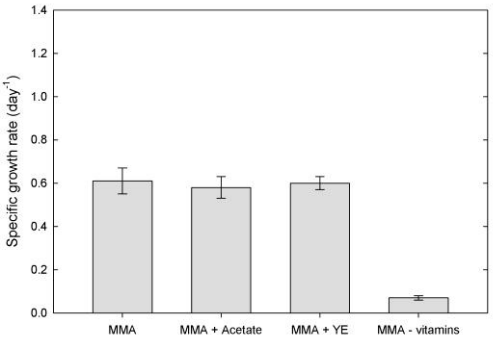
BSM1



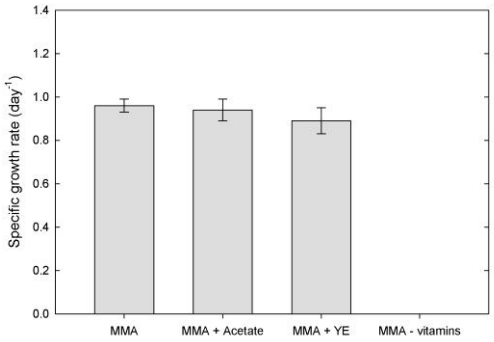
DM1



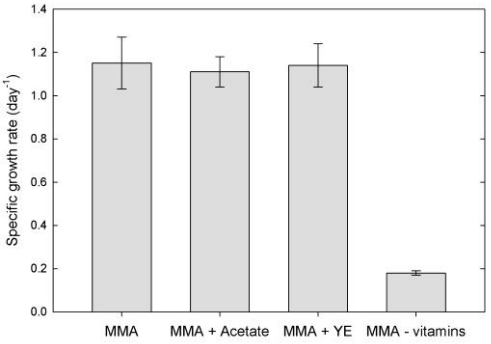
MKM1



NM1



PM1



TM1

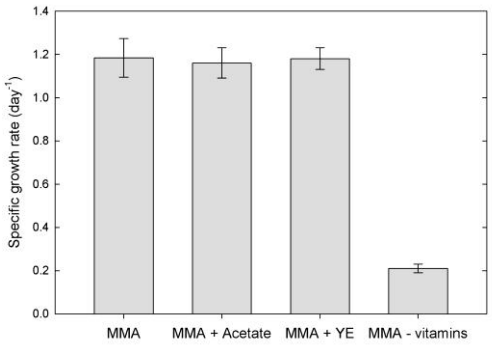


Figure 6.7 Specific growth rates (average of three samples, error bars represent one standard deviation) for strains grown at 25 °C on marine medium with 20 mM methylamine and vitamins (MMA), 20 mM methylamine with vitamin medium and 3 mM acetate (MMA + Acetate), 20 mM methylamine with vitamin medium and 0.2% yeast extract (MMA + YE) and 20 mM methylamine and medium without vitamins (MMA – vitamins). MMA + biotin, biotin added as the only vitamin. Four 3% transfers were made into vitamin free medium.

6.2.8 Pressure

Strains NM1, TM1 and *M. methylutens* TMA-10^T consumed all of their growth substrate (5 mM methylamine) during four weeks incubation at hydrostatic pressures in the range 0.1 to 50 MPa (Figure 6.8). Strain TMA-10^T consumed only 0.6 mM methylamine at 60 MPa and no methylamine was consumed at 70 MPa. Strain TM1 did not consume any methylamine at 60 MPa and Strain NM1 consumed all of its substrate at 60 MPa, 0.2 mM at 70 MPa and no methylamine was consumed at 80 MPa. Strain BSM1 consumed all of its substrate at 0.1 and 20 MPa. At 60 MPa it consumed 6.6 mM methylamine and only 0.3 mM methylamine was consumed at 50. Substrate consumption increased at 60 MPa and consumption declined to 0 mM at 80 MPa. For strains NM1 and TM1 the protein concentration declined above 20 MPa. The protein concentrations of strains BSM1 and *M. methylutens* TMA-10^T declined above 0.1 MPA but the decline at 20 MPA was not significant (p>0.05) for both strains. As strains BSM1, NM1 and *M. methylutens* TMA-10^T were able to grow above 50 MPa they can be classified as piezotolerant; only those strains whose optimal growth occurs above a pressure of 10 MPa can be regarded as peizophiles (Fang *et al.*, 2010). As strain TM1 did not grow above 50 MPa it can be classified as piezosensitive (Fang *et al.*, 2010).

All strains were successfully cultured after depressurization from 20, 60 and 50 MPa. After depressurization from 60 MPa strains BSM1, NM1 and *M. methylutens* TMA-10^T cultured successfully but strain TM1 did not grow. Strains BSM1, NM1 and *M. methylutens* TMA-10^T were successfully cultured after depressurization from 70 MPa and strains BSM1 and NM1 were successfully cultured after depressurization from 80 MPa (no other strains were pressurized to 80 MPa).

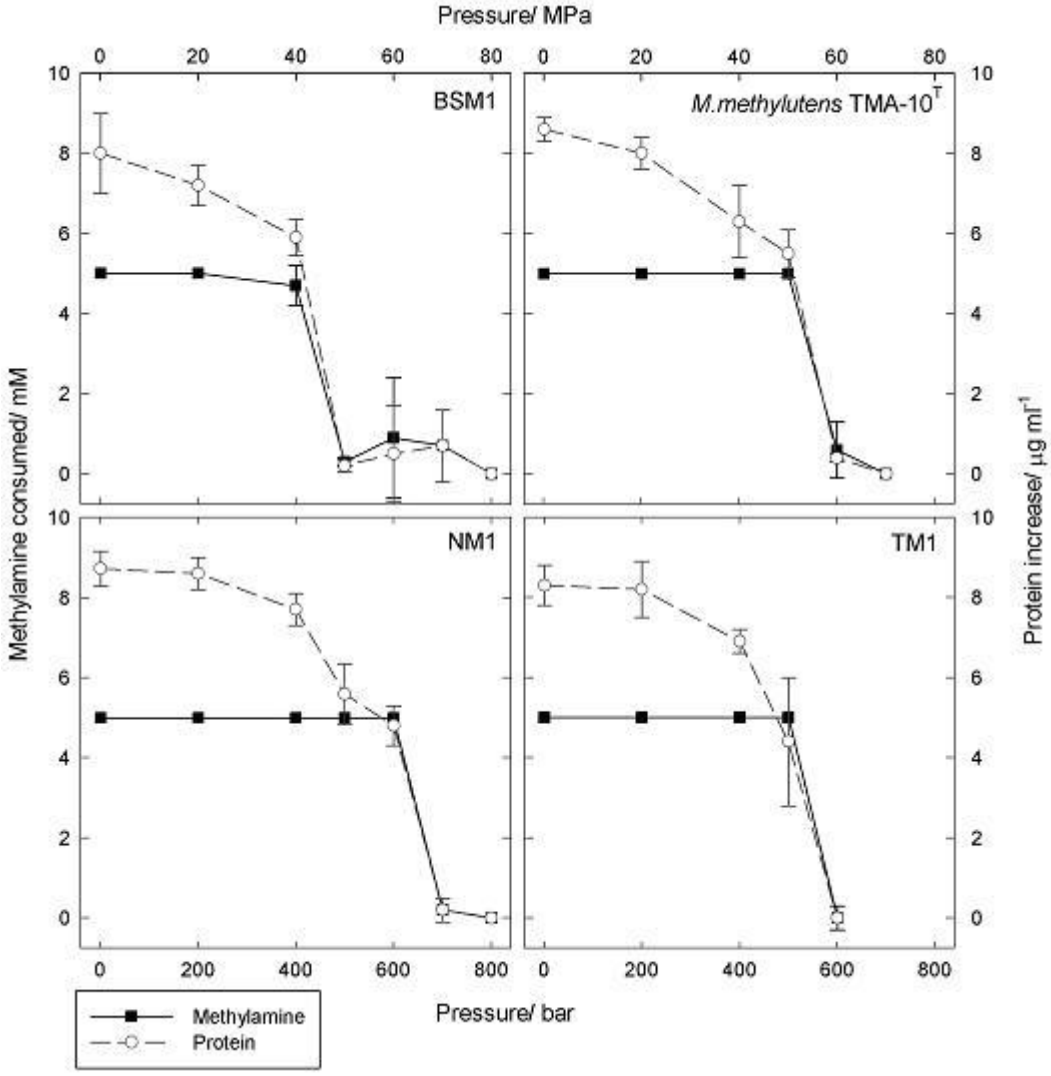


Figure 6.8 Response of strains BSM1, NM1, TM1 and type strain *M. methylutens* TMA-10^T to increasing hydrostatic pressure measured by substrate consumption (all cultures were given 5 mM methylamine) and increase in protein concentration. All strains incubated in triplicate, all values are the mean of three samples and error bars represent one standard deviation. Strain TM1 was isolated from a tidal flat, *M. methylutens* TMA-10^T was isolated from a water depth of 65 m and strains BSM1 and NM1 were isolated from water depths of 2000 m (equivalent to a pressure of 20 MPa).

6.2.9 Discussion

6.2.9.1 Morphology

Morphologically, the sixteen strains were similar to the descriptions of previously described strains (Table 6.3). However, strains AM3, BSM1, BSM2, BM3 and NM1 were observed to form aggregates of cells a feature not previously reported for the genus *Methanococcoides* (large clusters of cells were also seen in an Aarhus Bay *Methanococcoides* enrichment culture, Figure 3.18). Bacteria can be divided into one of two groups, Gram-positive and Gram-negative. The difference being a result of the structural and the chemical composition of the bacterial cell envelope. As *Archaea* lack typical Gram-negative or Gram-positive cell wall structures, *Archaea* are described as staining Gram-negative rather than being Gram-negative (Boone & Whitman, 1988). Members of the genus *Methanococcoides* have a cell wall with a protein S-layer (Section 1.4.1, Figure 1.4) and as a consequence should stain Gram-negative. Methanogens with proteinaceous cell walls may lyse during the drying stage of Gram staining (Boone & Whitman, 1988), this occurred with *Methanococcoides burtonii* DSM 6262^{T} (Franzmann *et al.*, 1992) and in this study with strain BSM1. With the exception of *Methanococcoides* strain NaT1 (which stained Gram positive), strains that were successfully stained, stained Gram-negative (Table 6.3).

6.2.9.2 pH Range

Prokaryotes whose optimal growth is below pH 5.5 can be classified as acidophiles, those with growth optima above pH 8.5 can be classified as alkaliphiles and those that have an optimum pH between these two values are neutrophiles (Overmann, 2006). As all *Methanococcoides* strains isolated in this study and the published strains have a pH optima between pH 5.5 – 8.5 (Figure 6.4 and Table 6.4) they can be classified as neutrophiles. Whilst strain BSM1 has an optimal pH <9 it can grow between pH 9 – 10 allowing it to be classified as alkalitolerant (Yumato, 2002). The order *Methanosarcinales* does not contain any examples of acidophiles although it does include two alkaliphiles, *Methanolobus oregonensis* with an optimum pH 8.6 (Liu *et al.*, 1990) and *Methanosalsum zhilinae* with an optimum pH 9.2 (Mathrani *et al.*, 1988a).

6.2.9.3 Na^+ Concentration Range

Prokaryotes with optimal growth < 0.2 M Na^+ and highest growth > 0.2 M Na^+ can be classified as being halotolerant, those whose optimal growth lies between 0.2 and 0.5 M Na^+ are slight halophiles and those with optimum growth between 0.5 and 2.5 M Na^+ are moderate halophiles (Oren, 2006). On the basis of this classification then all seven strains isolated in this study were slight halophiles as were the previously described strains except *M. methylutens* TMA-10^T that is a moderate halophile (Figure 6.5 and Table 6.4). Whilst none of the strains isolated in this study nor any of the published strains can tolerate hypersaline conditions, *Methanococcoides* 16S rRNA and *mcrA* gene sequences have been detected in hypersaline environment (Orphan *et al.*, 2008b).

6.2.9.4 Temperature Range

Prokaryotes can be divided into four categories on the basis of their minimum (T_{min}), optimum (T_{opt}), and maximum (T_{max}) temperatures: psychrophiles (T_{opt} ≤15 ºC), mesophiles (T_{opt} between 20 and 62 ºC), thermophiles (T_{opt} 62–70 ºC) and hyperthermophiles (T_{opt} >70 ºC) (Overmann, 2006). On the basis of this classification scheme all seven of the *Methanococcoides* strains isolated in this study were mesophiles as were the type strains and other previously described strains (Figure 6.6 and Table 6.4). Prokaryotes capable of growing at temperatures between 0-5 ºC and possessing a T_{max} >25 ºC can also be classified as psychrotolerant (Morita, 1975). On this basis *M. alaskense*, *M. burtonii* and, strains BSM1, DM1, MKM1, NM1 and TM1 can be classified as psychrotolerant. For comparison, other members of the order *Methanosarcinales* can grow at temperatures as low as 1 ºC (*Methanosarcina lacustris* [Simankovaa, 2001]) and as high as 60 ºC (*Methanohalobium evestigatum* (Zhilina & Zavarzin, 1987)). For all of the previously described strains and the strains isolated in this study the value of T_{opt} and T_{max} are higher than their *in situ* temperatures (Table 6.4).

Low temperatures result in a number of problems for the microorganism, as the temperature decrease the rates of chemical reactions, cold denaturation of proteins occurs and the fluidity of the cell membrane decreases and will ultimately lose its function (D'Amico *et al.*, 2006). The loss of membrane fluidity will result in a

decreased affinity of the cell for substrates and the organism will be nutrient-limited (Nedwell, 1999). Increasing temperatures will increase reaction rates and the microorganism will continue to grow with increasing temperature until some aspect of cell metabolism is thermally compromised such as the denaturation of key proteins (Cavicchioli, 2006).

For all of the published strains and the strains isolated in this study, the values of T_{opt} are higher than the *in situ* temperatures with the difference ranging from 13 to 26 ºC (Table 6.4). Strain PM1 (T_{opt} 35-38 ºC) was isolated from sediment taken from Portishead (Severn Estuary, UK) during June 2009 when the *in situ* temperature was 22 ºC, the difference between the T_{opt} and the *in situ* temperature was much greater in December 2010 when the *in situ* temperature was only 3ºC at 20 cm depth (a temperature below the T_{min} of strain PM1, Table 6.3). The phenomenon of T_{opt} being higher than the *in situ* temperature has also been seen with other mesophilic methanogens and with the psychrophilic methanogen *Methanogenium frigidum* Ace-2 (T_{opt} 15 ºC) that was isolated from Ace Lake (Antarctica), which had an *in situ* temperature of 1.9 ºC (Franzmann *et al.*, 1997). This phenomenon is not restricted to methanogens, it is also seen in bacteria *e.g.* sulphate-reducing bacteria isolated from Arctic sediments were determined to have an optimum temperature 15 ºC higher than the *in situ* temperature (Knoblauch & Jørgensen, 1999).

It would seem that the methanogens isolated in this study are not well adapted to their *in situ* temperatures and would have an ecological advantage if the optimal growth temperature were closer to the *in situ* temperature. Whilst they are not functioning near their optimum temperature they are able to grow at the *in situ* temperatures (using the *in situ* temperature measurements taken at the time the samples were taken), Table 6.4.

The T_{min} of the seven methanogen strains investigated in this study were higher than those of *Methanococcoides alaskense* strains AK-5^T and AK-9 (Singh *et al.*, 2005) and, *M. burtonii* DSM 6262^T (Franzmann *et al.*, 1992), Table 6.4. *M. alaskense* strains AK-5^T and AK-9 have T_{min} values based on the Ratkowsky model where the regression line derived from a plot of $\sqrt{\mu}$ against temperature is extrapolated to the

temperature axis to give T_{min} (Ratkowsky *et al.*, 1983). However, the temperature so derived is not a minimum growth temperature (T_{min}), it is a notional or conceptual temperature usually 2-3 °C lower than the observed minimum growth temperature (McMeekin *et al.*, 1993). The observed minimum growth rate for *M. burtonii* was 5.6 °C, which was 8.1 °C higher than the minimum temperature based on the Ratkowsky model (Franzmann *et al.*, 1992). *M. burtonii* could grow slowly at 1.7 °C, the *in situ* temperature of Ace Lake, if growth was first initiated in fresh medium at a higher temperature (Franzmann *et al.*, 1992). The predicted growth temperature of *M. alaskense* strains AK-5^T and AK-9 were -2.3 and -10.7 °C respectively but the actual growth temperature of strain AK-5^T was not determined below 15 °C and strain AK-9 did not grow at 5 °C (Singh *et al.*, 2005). With a T_{min} of 25 °C *Methanococcoides* sp. NaT1 has the highest T_{min} of all the strains (Tanaka, 1996). Cultures of *Methanococcoides* sp. NaT1 were not available for further testing (K. Tanaka, personal communication).

The temperature dependence of the growth rate can be described by the Arrhenius equation (Arrhenius, 1889), Equation 6.1.

$$k = A\, e^{-Ea/RT} \qquad \text{(Equ. 6.1)}$$

Where k is the specific growth rate, A is the collision or frequency factor, E_a is the activation energy, R is the universal gas constant (8.3 kJ mol^{-1}), and T is the absolute temperature in Kelvin. Any change in temperature will lead to an exponential change in the growth rate the extent of which depends on the activation energy (E_a). The linear portion of the Arrhenius plot corresponds to the physiologically 'normal' temperature range for growth, above and below this temperature range there is a deviation from linearity (Hébraud & Potier, 1999). Temperatures outside the linear range of the Arrhenius plot are stress inducing. The linear range for strains in this study range from 8-16 ºC to 25-36 ºC (Figure 6.6). The activation energy (E_a), also known as the 'temperature characteristic' (Hanus & Morita, 1968), can be obtained from the Arrhenius plot and is a measure of the temperature response (Overmann, 2006). Q_{10} is the factor by which the reaction rate increases with a 10 °C temperature increase (Isaksen & Jørgensen, 1996) and can be calculated from the E_a (Section

2.3.3). A Q_{10} value of one indicates that a reaction is independent of temperature; values higher than one indicate a dependence of temperature, for example, a value of two would indicate a doubling of the reaction rate with an increase in temperature of 10 ºC. Published values of E_a and Q_{10} for methanogens are rare and there are no published values for any member of the genus *Methanococcoides*. To allow for comparison with the type strain *M. methylutens* TMA-10^T the temperature data provided by Sowers (1983) was replotted and Q_{10} and E_a determined, the values were similar to those obtained for strains isolated in this study (Table 6.5). Possibly the only previously described strain that has had its Q_{10} value determined is "*Methanothrix soehngenii*" which when grown on acetate as the growth substrate in the temperature range 10-20 ºC had a Q_{10} value of 2.1 (Huser *et al.*, 1982). Studies that have determined E_a and Q_{10} values for methanogenesis are generally environmental studies of wetlands such as swamps or peat bogs that, presumably, have mixed populations of methanogens. Slurries of swamp sediment have been found to Q_{10} values ranging from 1.9 to 2.5 and E_a values ranging from 67 to 67 kJ mol^{-1} (Westermann, 1993) and a study of a blanket bog found Q_{10} values ranging from 3.0 to 6.8 and E_a values ranging from 76 to 106 kJ mol^{-1} (Macdonald *et al.*, 1998).

6.2.9.5 Growth Factors

Organic compounds such as acetate, yeast extract or vitamins, when added to the medium can stimulate growth of methanogens (Overmann, 2006). The growth of *M. methylutens* TMA-10^T was stimulated by B vitamin solution (1% v/v), trypticase (0.1% wt/v) and rumen fluid (10% v/v) (Sowers & Ferry, 1983). As with strain NM1 (Figure 6.7), no significant growth was found for *M. methylutens* TMA-10^T in vitamin free medium (Sowers & Ferry, 1985). The addition of biotin to the growth medium of *M. methylutens* TMA-10^T resulted in growth comparable to that with the addition of the full vitamin solution (Sowers & Ferry, 1985). The results for strain AM1 (Figure 6.7) indicated that the addition of biotin to vitamin free medium was not sufficient to restore the growth rate and that another vitamin or vitamins was required. As with most of the strains tested in this study, *M. burtonii* DSM 6262^T did not require vitamins for growth although it was not stated whether the addition of vitamins stimulated growth (Franzmann *et al.*, 1992).

M. methylutens TMA-10^T, *M. burtonii* DSM 6262^T, *M. alaskense* AK-5^T and *Methanococcoides* strain NaT1 did not require yeast extract for growth although it did stimulate growth for *M. methylutens* TMA-10^T, *M. burtonii* DSM 6262^T and *M. alaskense* AK-5^T (Franzmann *et al.*, 1992; Singh *et al.*, 2005; Sowers & Ferry, 1983; Tanaka, 1994). 0.5g l^{-1} yeast extract suppressed growth in strain NaT1 (Tanaka, 1994) and the effect of the addition of yeast extract was not determined for strain MM1 (Lyimo *et al.*, 2009b). A proteomic study found that there was only a marginal response by *M. burtonii* DSM 6262^T to the supply of exogenous amino acids (amino acids stated to be added in the form of yeast extract although the composition of yeast extract can vary) indicating that *M. burtonii* DSM 6262^T was predisposed to the endogenous synthesis of amino acids (Williams *et al.*, 2010a). The effects of the addition of peptone (0.05-0.2% final concentration) has only been tested with *M. alaskense* AK-5^T and was found to increase the specific growth rate (Singh *et al.*, 2005). The strains tested in this study and the *Methanococcoides* type strains are able to grow without the addition of acetate to the medium as a carbon source (Franzmann *et al.*, 1992; Singh *et al.*, 2005; Sowers & Ferry, 1985).

6.2.9.6 Hydrostatic Pressure

The deep sea has been defined as water depth of 1000 m or greater (Jannasch & Taylor, 1984). Hydrostatic pressure increases by approximately 0.1 MPa (1 bar) per 10 m water depth. Strains BSM1 and NM1 were isolated from Dvurechenskii and Napoli mud volcanoes respectively, which are located at approximately 2000 m water depth and would experience an *in situ* pressure of 20 MPa (200 bar). Elevated hydrostatic pressure affects a number of cellular processes including cell division, DNA replication, enzyme function, flagella function, membrane structure and protein synthesis (Bartlett, 2002). The effects of pressure on *Escherichia coli* are given in Table 6.7.

Table 6.7 Processes inhibited by elevated pressure in *E.coli*. Data from (Yayanos & Pollard, 1969) except motility, (Meganathan & Marquis, 1973).

Process	Inhibitory pressure (MPa)
Motility	10
Cell division	20-50
Growth	50
DNA replication	50
Translation	60
Transcription	77

Elevated pressures act to decrease the system volume changes associated with the equilibrium and rates of biochemical processes. The relationships of pressure (P) to equilibrium and rate processes are given by Equations 6.2 and 6.3 were R is the gas constant, T is the absolute temperature, and $K1$ and $k1$ are the equilibrium and rate constants, respectively, at atmospheric pressure; K_p and k_p are the constants at a higher pressure. ΔV is the difference between the initial and final volumes (reaction volume) and $\Delta V‡$ is the apparent volume change (activation volume).

$$Kp = K1\, e^{-P\Delta V/RT} \qquad \text{(Equ. 6.2)}$$

$$kp = k1\, e^{-P\Delta V‡/RT} \qquad \text{(Equ. 6.3)}$$

Small changes in volume can lead to large changes in Kp or kp; reactions that result in an increased volume will be inhibited. Methane production from methylamine will result in an increase in reaction volume. However, methane can dissolve in solution. Whilst the solubility of methane at atmospheric pressure is low it increases with increasing pressure; the solubility of methane at 30 ºC and 0.1 MPa is 1 mM increasing to 232 mM at 80 MPa (Duan *et al.*, 1992).

Proteins are some of the most pressure sensitive macromolecules in the cell, they adapt to restricted volume by conformation changes thereby affecting their function. Protein synthesis is also susceptible to high pressure, uncharged ribosomes (those without mRNA and tRNA) have been found to dissociate at pressures greater than 60 MPa (Gross *et al.*, 1993). Dissociation of ribosomes results in a large negative volume change and is therefore favoured at high pressure (Gross *et al.*, 1993). Lipid membranes are more sensitive to the effects of elevated pressures than are proteins.

Under elevated pressure, lipids pack more closely to adapt to the restricted volume and the membrane fluidity decreases and becomes impermeable. Proton-translocating ATPase is also sensitive to pressure (Marquis & Bender, 1987). The ability of ATPase to move protons across the membrane is affected more by elevated pressure than its ability to catalyze ATP hydrolysis, which results in cells expending more energy pumping protons across the membrane (Marquis & Bender, 1987).

Few studies have been carried out on the effects of pressure on methanogens. Those that have been undertaken have been with thermophiles. *Methanothermococcus thermolithotrophicus* had its growth enhanced up to 50 MPa when kept at optimum temperature (Bernhardt *et al.*, 1987) and *Methanopyrus kandleri* grown at 60 MPa was found to have a higher maximum growth temperature than at atmospheric pressure (0.1 MPa) (Takai *et al.*, 2008). This study is the first to investigate the growth of mesophilic methanogens under elevated hydrostatic pressure. The decline in protein concentration with increasing pressure, even when the substrate has been fully consumed, may be due to the inhibition of protein synthesis with increasing pressure, the increased use of energy for maintaining cell function rather than growth or a combination of these and other factors such as the loss of membrane fluidity. Strains BSM1 and NM1 grew as well at 20 MPa, the *in situ* pressure of the sediment from which they were isolated, as they did at 0.1 MPa. The ability of the strains, except strain TM1, to grow following exposure to the highest hydrostatic pressure at which they were tested indicated that they were inhibited by high hydrostatic pressure rather than being killed by high hydrostatic pressure or by the process of depressurization.

6.3 Description of *Methanococcus* Strains

6.3.1 Introduction to the Genus *Methanococcus*

The genus *Methanococcus* is a member of the family *Methanococcaceae* of the order *Methanococcales* with five validly published species (Table 6.8). The type strain of the genus is *Methanococcus vannielii* DSM 1126^T (Stadtman & Barker, 1951). All previously described strains of the genus *Methanococcus* have been isolated from marine environments (Table 6.8). A number of species previously assigned to the genus *Methanococcus* have been transferred to other genera *e.g*. the original type strain of the genus, *Methanococcus mazei*, was transferred to the genus *Methanosarcina* after re-characterization of the strain (Mah & Kuhn, 1984) and thermophilic *Methanococcus* strains were transferred to the genus *Methanothermococcus* (Whitman, 2001b). The morphology and growth substrate range of the genus *Methanococcus* is similar to that of the genera *Methanogenium* and *Methanomicrobium* (Whitman, 2001b).

Table 6.8 Published strains of the genus *Methanococcus* including type strains.

Species	Strain	Habitat	Reference
M. aeolicus	Nankai-2 Nankai-3^T (DSM 17508)	Nankai Trough sediment, Japan	Kendall *et al* (2006)
	PL-15/h^P	Marine sediment, Lipari Islands near Sicily, Italy	Kendall *et al* (2006)
M. maripaludis	JJ^T (DSM 2067)	Top 10 cm of sediment, Pawley's Island salt marsh, South Carolina, USA	Jones *et al* (1983b)
	C5, C6 and C7	Salt-marsh sediments, Georgia, USA	Whitman *et al*. (1986)
M. vannielii	SB^T (DSM 1226)	San Francisco Bay sediment	Stadtman & Barker (1951)
M. voltae	PS^T (DSM 1537)	Sediment, Waccasassa estuary, Florida, USA	Ward *et al* (1989)
	P2F9701a	Estuarine environment, Erlin Shi, Taiwan	Lai & Shih (2001)
*M. deltae**	ΔRC^T (DSM 2771)	Mississippi River delta sediment, East Bay, Gulf of Mexico	Corder *et al* (1983)

**M. deltae* was validly published but is considered to be a heterotypic synonym of *M. maripaludis* (Keswani *et al.*, 1996).

6.3.2 Phylogeny

On the basis of 16S rRNA and *mcrA* gene sequences, strains PM4 and PM5 are identical. Both strains have 16S rRNA sequences identical to *Methanococcus maripaludis* strains KA1, Mic1c10, Mic6co8, S2 and X1, Figure 6.9. There is a 97% sequence identity to the *M. maripaludis* type strain JJ^T. Also, the *mcrA* sequences of strains PM4 and PM5 are identical to *M. maripaludis* strains S2 and X1, Figure 6.10.

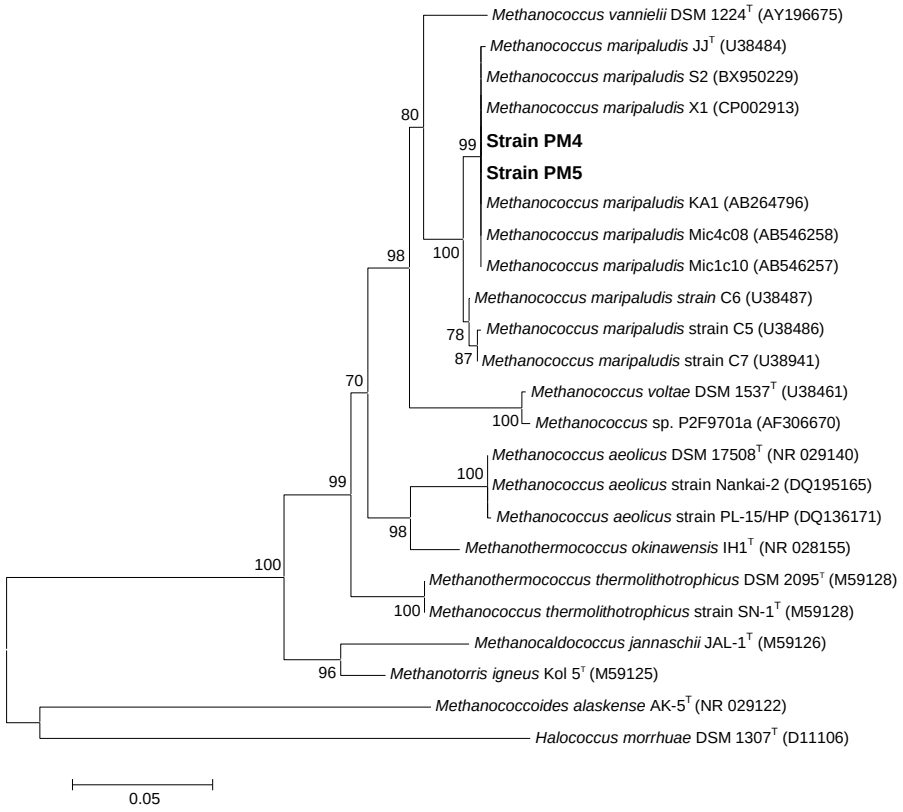


Figure 6.9 Phylogenetic tree (neighbour-joining) showing the positions of strains PM4 and PM5 relative to *Methanococcus* species as well as representatives of the families *Methanococcaceae* and *Methanocaldococcaceae* (*Methanotorris igneus* and *Methanocaldococcus jannaschii* originally classified as *Methanococcaceae*) based on 16S rRNA gene. Accession numbers in brackets. Numbers at nodes are percentage bootstrap values based on 1000 replicates and only bootstrap values > 50% are shown. Bar, 0.05 substitutions per site. *Halococcus morrhuae* sequence used as an outgroup.

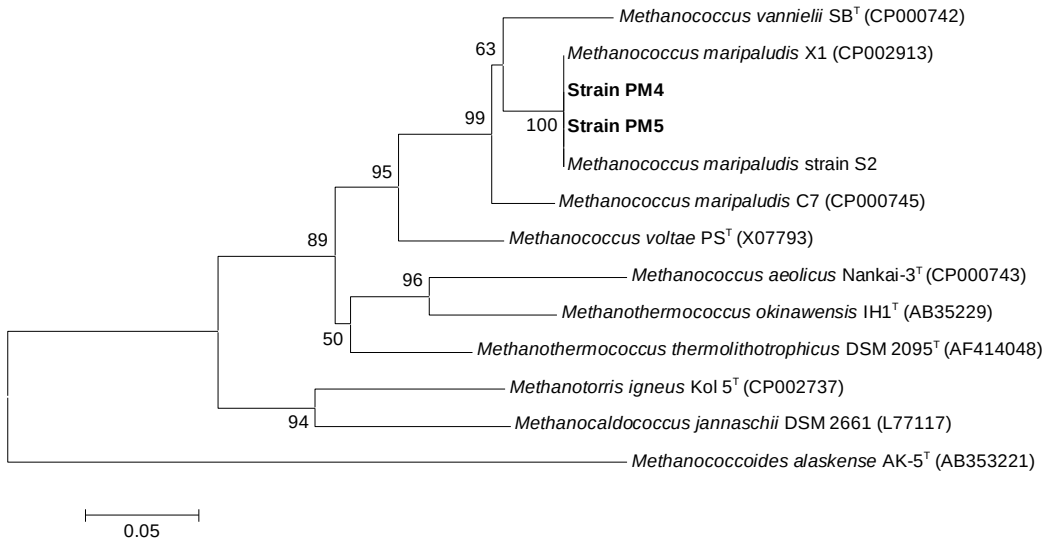


Figure 6.10 Phylogenetic tree (neighbour-joining) showing the positions of strains PM4 and PM5 relative to *Methanococcus* species as well as representatives of the families *Methanococcaceae* and *Methanocaldococcaceae* (*Methanotorris igneus* and *Methanocaldococcus jannaschii* originally classified as *Methanococcaceae*) based on *mcrA* sequences. Numbers at nodes are percentage bootstrap values based on 1000 replicates and only bootstrap values > 50% are shown. Bar, 0.05 substitutions per site. *Methanococcoides alaskense* AK-5 sequence used as an outgroup. Accession numbers in brackets.

6.3.3 Morphology

Strains PM4 and PM5 are irregular cocci with a diameter of approximately 1 μm in diameter (Figure 6.11) and stained Gram negative. Cells occur singly or as pairs and lyse in distilled water or SDS (0.01%). Morphology and other characteristics are compared in Table 6.9.

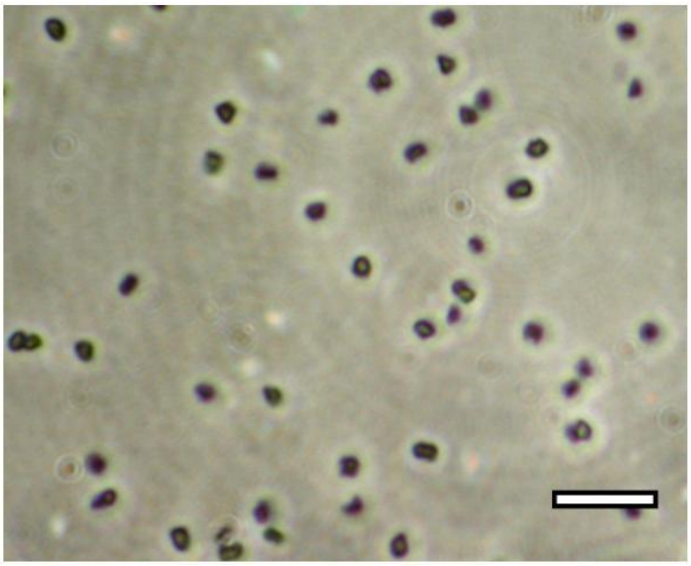


Figure 6.11 Phase-contrast photomicrograph of strain PM4 (strain PM5 has an identical appearance). Scale bar, 5 μm.

6.3.4 Substrates and Growth Factors

Strains PM4 and PM5 were able to utilize H_2/CO_2 and formate for methanogenesis. Additional catabolic substrate tests were carried out on strain PM4 only. Methane was not produced from acetate, propionate, pyruvate, ethanol, methanol, 1-propanol, 2-propanol, cyclopentanol, betaine, dimethylglycine, choline, or trimethylamine (see Table 6.9 for comparison to other strains).

Strain PM4 did not require acetate, yeast extract or vitamins for growth (Figure 6.12). The addition of yeast extract increased the growth rate of strain PM4 by 23% when grown on formate. After four transfers (3% inoculum) into vitamin free medium the growth rate was reduced to 59% of the growth rate determined with vitamins (Figure 6.12). Whilst acetate was not required for growth it did reduce the lag time of strain PM4 grown on formate by 36 hours.

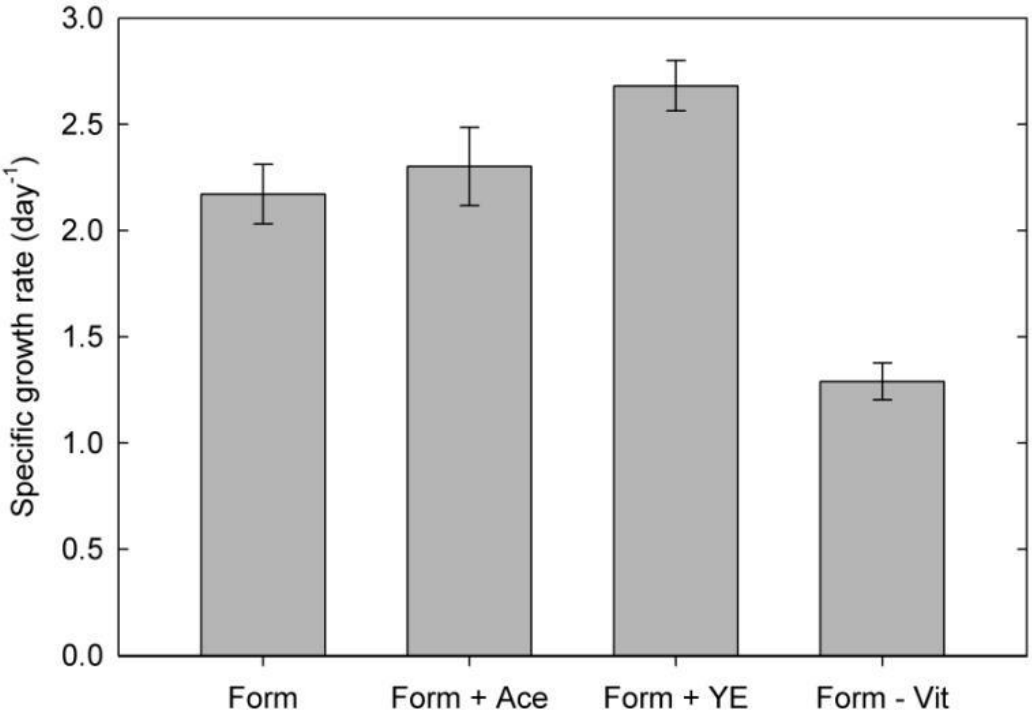


Figure 6.12 Comparison of specific growth rates for *Methanococcus* strain PM4 growing on mineral medium with vitamins and the following additions; formate only (Form), formate and acetate (Form + Ace), formate and yeast extract (Form + YE). The specific growth rate was also determined in the absence of vitamins, four transfers (3% inoculum) into vitamin free medium (Form – Vit). All cultures incubated at 25 ºC.

6.3.5 Other Phenotypic Characteristics

The grow of strains PM4 and PM5 with increasing pH, Na^+ concentration and temperature were not determined. All incubations were carried out at 25ºC, a pH of 7.2 and a Na^+ concentration of 0.6 M all of which are within the ranges of previously described strains of *Methanococcus* (Table 6.9).

Table 6.9 Characteristics of members of the genus *Methanococcus* and strain PM4. Strain PM5 was not tested for substrate utilisation or for the requirement for growth factors. Other characteristics of PM5 are the same as PM4. In addition to the substrates listed below, PM4 did not utilise 1-propanol, 2-propanol, cyclopentanol, betaine, choline or dimethylglycine as substrates for methanogenesis. Also, Nankai-3^T did not utilize ethanol, 1-propanol or 2-propanol (Kendal et al, 2006).

	Strain								
Characteristic	PM4	*M. aeolicus* Strain Nankai-3^T	*M. aeolicus* Strain Nankai-2	*M. aeolicus* Strain PL-15/H^P	*M. deltae* Strain ΔRC^T	*M. maripaludis* Strain JJ^T	*M. vannielii* Strain SB^T	*M. voltae* Strain PS^T	*M. voltae* P2F9701a
Size/ µ	1.0 (0.2)[1]	1.5-2.0	1.5-2.0	1.5-2.0	1.0-1.5	1.2 x 1.6	1.3	1-2	0.7-1.1
Shape	Irregular cocci	Cocci	Cocci	Cocci	Irregular cocci	Pleomorphic cocci	Irregular cocci	Irregular cocci	Cocci
Occurrence	Singly/pairs	Singly[2]	Singly[2]	Singly[2]	Singly/pairs	Singly/pairs	Singly/pairs	Singly/pairs	Singly/pairs
Motility		+	+	+	-	+[3]	+	+[6]	+[5]
Gram stain	-	-	-	-	ND	-	-	Cells lyse	-
Lysis SDS	+	+	+	+	+	+	+[6]	+	+
Lysis dH_2O	+	+[6]	+[6]	+[6]	+	+	+[6]	+	+
Temp. range/ ºC	ND	<20-55	<10-50	<20-65	30-65	18-67	<20-65	<20-65	30-62
pH range	ND	7.5-5.5	6.3-5.5	6.5-8.0	ND	6.6-8.2	6.5-8.0	6.5-8.0	6.3-8.2
Na^+ range/ M	ND	0.05-1.0	0.05-1.0	0.05-0.8	0.03-1.1	0.05-0.8	0.05-0.8	0.08-0.8	0.02-1.03
Substrates									
H_2/CO_2	+	+	+	+	+	+	+	+	+
Formate	+	+	+	+	+	+	+	+	+
Pyruvate	-	ND	ND	ND	ND	-	+[7]	ND	ND
Acetate	-	-	-	-	-	-	-	-	-
Propionate	-	ND	ND	ND	ND	-	-	-	-
Ethanol	-	-	-	-	ND	-	-	-	-
Methanol	-	-	-	-	-	-	-	-	-
Trimethylamine	-	-	-	-	-	ND	ND	-[8]	-
Growth factors									
Acetate	-	-	-	-	-	-[9]	-	+[10]	-[11]
Yeast extract	-	-	-	-	-	-	-[6]	-[6]	+
Vitamins	-	-	-	-	-	-	-[6]	+	ND
Reference	This study	(Kendall *et al.*, 2006)	(Kendall *et al.*, 2006)	(Kendall *et al.*, 2006)	(Corder *et al.*, 1983)	(Jones *et al.*, 1983b)	(Stadtman & Barker, 1951)	(Ward *et al.*, 1989)	(Lai & Shih, 2001)

[1] Mean with standard deviation in brackets (n = 30)
[2] Occurs singly or as pairs (Whitman & Jeanthon, 2006)
[3] Weakly motile
[5] Heavily flagellated
[6] Whitman & Jeanthon (2006) - not in original publication
[7] Yang *et al* (1992)
[8] Whitman *et al* (1982)
[9] Growth stimulated (Whitman & Jeanthon, 2006)
[10] Optimal growth with 2.5 mm acetate (Whitman *et al.*, 1982)
[11] Did not enhance cell growth but greatly reduced lag time
[6] Peritrichous flagella
-, property of the strain +, not a property of the strain ND, not determined

6.3.6 Discussion

Strains PM4 and PM5 have 100% 16S rRNA and *mcrA* gene sequence identity to *M. maripaludis* strain S2. Strain S2 is also known as strain LL which is a strain derived from *M. maripaludis* JJ^T and used in genetic research (Hendrickson *et al.*, 2004 ; Kessler *et al.*, 1998) . Strain S2 has had its genome sequenced (Hendrickson *et al.*, 2004). *M. maripaludis* JJ^T was isolated from sediment taken from the top 10 cm of a *Spartina alterniflora* dominated region of Pawley's Island salt marsh, South Carolina, USA (Jones *et al.*, 1983b). Strains PM4 and PM5 are also closely related to *M. maripaludis* strains C5, C6 and C7 that were isolated from a salt marsh at Sapelo Island, Georgia, USA (Whitman *et al.*, 1986). This is an area of extensive intertidal systems subjected to twice daily tidal flooding and like Pawley's Island salt marsh is dominated by the plant *Spartina alterniflora* (Jones & Paynter, 1980).

Strains PM4 and PM5 also have high sequences identities to *M. maripaludis* strain X1. Although this strain has not been isolated, its genome has been sequenced and it is believe to be the first genome from a noncultured microorganism which has been reconstructed directly from *de novo* sequencing of a metagenomic data pool (Wang *et al.*, 2011). Found in a subsurface thermophilic saline oil reservoir strain X1 has not yet been successfully cultured in the laboratory under reservoir conditions (Wang *et al.*, 2011).

Other close relatives of strains PM4 and PM5 are two crude-oil related strains of *M. maripaludis*. *M. maripaludis* strains Mic6c08 and Mic1c10 were isolate from a crude-oil storage tank (Mori *et al.*, 2010). Strain Mic1c10 is an iron-corroding organism. *M. maripaludis* strain KA1, isolated from sludge collected from the bottom of crude-oil storage tank, also oxidized iron (Uchiyama *et al.*, 2010).

All previously described members of the genus *Methanococcus* are able to utilize H_2/CO_2 and formate for methanogenesis (Table 6.9). It has been reported that *Methanococcus voltae* strain A3, *Methanococcus maripaludis* strain JJ1 (not JJ^T) and *Methanococcus vannieli* strain SB^T produced methane from pyruvate in the absence of H_2 (Yang *et al.*, 1992). Acetate was produced in addition to methane, Equation 6.6. After three months incubation, strain PM4 failed to produce methane from pyruvate.

$$6 \text{ pyruvate} \rightarrow 6 \text{ acetate} + 3\ CO_2 + CH_6 \qquad \text{(Equ. 6.6)}$$

Of the previously described strains only *Methanococcus voltae* strain PS^T required acetate as a carbon source for growth on H_2/CO_2 or formate, Table 6.8 (Whitman *et al.*, 1982). It is also the only member of the genus that has a requirement for isoleucine and leucine (2-methlbutyrate and isovalerate can be substituted respectively). As with strain PM4, the addition of acetate to cultures of *Methanococcus voltae* strain P2F9701a had the effect of reducing the lag time (Lai & Shih, 2001). Jones *et al* (1983) reported that acetate did not stimulate the growth of *Methanococcus maripaludis* strain JJ^T, however, Whitman & Jeanthon (2006) reported that acetate did stimulate growth in this strain. No previously described strain has a requirement for yeast extract although growth of *Methanococcus voltae* strain PS^T was greatly stimulated by its addition (Whitman *et al.*, 1982). The requirement for vitamins has not been reported for any strain although the growth of *Methanococcus voltae* strain PS^T was stimulated by pantothenate (Whitman *et al.*, 1982).

6.4 Summary

Sixteen methanogen strains isolated in this study from seven locations (Aarhus Bay, Darwin MV, Dvurechenskii MV, Meknes MV, Napoli MV, Portishead and Tamar) were closely related to members of the genus *Methanococcoides* as determined by 16S rRNA and *mcrA* genes sequences. The sixteen strains were morphologically very similar to previously described strains expect that a number of strains form aggregates of cells, a feature not previously described for this genus. Growth ranges with temperature, Na^+ concentration or pH are broadly similar but this study has extended the lower Na^+ concentration for growth from 0.15 to 0.03 M and a number of strains have an optimum Na^+ concentration lower than that previously described. Previous reports that members of this genus do not require acetate or yeast extract as growth factors are confirmed. This is the first study to investigate the affects of elevated hydrostatic pressure on members of the genus *Methanococcoides* and strains can grow under pressure of at least 50 MPa. The results of the substrate tests are to be found in Chapter 7.

Two strains isolated from a Portishead mudflat (strains PM4 and PM5) are closely related to *Methanococcus* strains isolated from salt-marsh sediments and crude-oil storage tanks (Figure 6.9 and Table 6.9). Morphologically both strains are similar to previously described strains (Table 6.9). As with all published strains of *Methanococcus*, strains PM4 and PM5 use H_2/CO_2 and formate as catabolic substrates; unlike *Methanococcus vannielii* strain SB^T, strain PM4 did not use pyruvate as a catabolic substrate. Strain PM4 did not require acetate, yeast extract or vitamins for growth (Figure 6.12), only *Methanococcus voltae* strains have a requirement for growth factors (Lai & Shih, 2001; Ward *et al.*, 1989).

Chapter 7
Methanococcoides Substrates

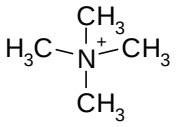
7.1 Introduction

Members of the genera *Methanococcoides* utilize a limited range of substrates. All previously described strains are able to utilise methylamine, dimethylamine and trimethylamine as substrates (Franzmann *et al.*, 1992; Lyimo *et al.*, 2009b; Singh *et al.*, 2005; Sowers & Ferry, 1983; Tanaka, 1994) and, with the exception of *M. alaskense* (Singh *et al.*, 2005), are also able to utilise methanol as a substrate. The only other substance that has been reported to support methanogenesis in this genus is tetramethylammonium, which was utilised by *Methanococcoides* strain NaT1 (Tanaka, 1994).

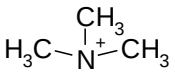
All sixteen *Methanococcoides* strains isolated in this study were tested with a range of potential methanogenic substrates (Section 7.3.1). Whilst not having been reported as growth substrates for *Methanococcoides*, acetate, formate and H_2/CO_2 were tested as they are used by other methanogen genera. Strains were also tested with a range of methylated compounds in addition to the methylated compounds known to be growth substrates for other strains of *Methanococcoides* (structural formulae given in Figure 7.1). Methylphosphonic acid, methyliodide and the methylated sulphur compounds methionine, methanethiol and dimethylsulfide were tested as potential growth substrates. Of these compounds only methanethiol and DMS have been previously shown to be substrates for methanogenesis, both are used by some *Methanosarcina* species and, a number of *Methanolobus* species are able to use DMS (Whitman, 2006). Methylphosphonic acid has not been shown to be a substrate for methanogens although some species of the bacterium *Escherichia coli* can produce methane from it (Section 1.1). Other substrates tested were ethanol, isopropanol, cyclopentanol and pyruvate. These are all known to be substrates for some methanogens (Whitman *et al.*, 2006).*M. methylutens* TMA-10^T (Sowers & Ferry, 1983), *M. burtonii* DSM 6242^T

(Franzmann *et al.*, 1992) and *M. alaskense* strain AK-5^T (Singh *et al.*, 2005) were also tested with a range of potential substrates that had not been previously tested.

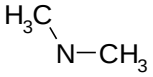
Utilisation of methylamine, dimethylamine, and trimethylamine by strain AM1 were further investigated using a combination of ion and gas chromatograph (Section 7.3.2). The utilisation of the novel substrates choline and betaine were also investigated further (Section 7.3.3). The methylated compounds choline (*N*,*N*,*N*-trimethylamine), *N*,*N*-dimethylethanolamine and *N*-monomethylethanolamine like trimethylamine, dimethylamine and methylamine have three, two and one methyl groups respectively and in addition have a hydroxyl group (Figure 7.1). Betaine (*N*,*N*,*N*-trimethylglycine), dimethylglycine (*N*,*N*-dimethylglycine) and sarcosine (*N*-methylgylcine) are similar to choline, *N*,*N*-dimethylethanolamine and *N*-monomethylethanolamine but posses a carboxyl group rather than a hydroxyl group (Figure 7.1).



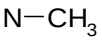
Tetramethylammonium*



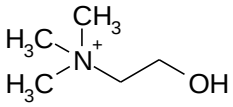
Trimethylamine*



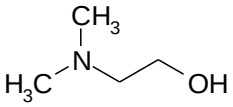
Dimethylamine *



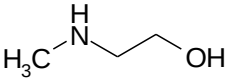
Methylamine*



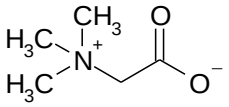
Choline



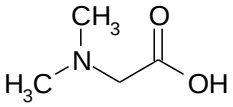
N,*N*-dimethylethanolamine



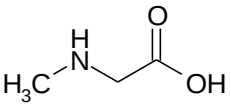
N-monomethylethanolamine



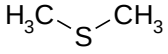
Betaine



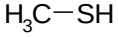
Dimethylglycine



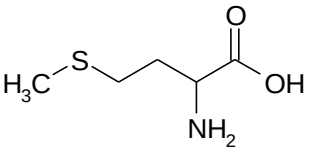
Sarcosine



DMS



Methanethiol



Methionine

Figure 7.1 Structural formulas of methylated compounds tested as potential catabolic substrates in this study. An asterisk indicates a methylated compound known to be a growth substrate for one or more strains of *Methanococcoides*.

7.2. Methods

A general survey of substrate usage was undertaken on all sixteen *Methanococcoides* strains with the following: acetate (10 mM), betaine (10 mM), choline (10 mM), cyclopentanol (10 mM), DMS (10 mM), ethanol (10 mM), formate (10 mM), H_2/CO_2 (80:20, 0.1 MPa), isopropanol (10 mM), methanethiol (1 mM), methanol (10 mM), methyliodide (1 mM), methylamine (10 mM), propionate (10 mM), pyruvate (10 mM) and tetramethylammonium (10 mM). Tube headspaces were sampled at approximately 30 day intervals to test for the presence of methane and samples were compared to controls without methanogenic substrates.

Additional studies with methylamines, betaine and choline cultures were performed in triplicate with 150 ml serum bottles containing 30 ml of medium and incubated at 25 °C. The methylamines, choline, *N*,*N*-dimethylethanolamine and *N*-monomethylethanolamine were detected using ion chromatography. Unfortunately, betaine could not be detected using the available analytical equipment (ion chromatography and HPLC). The serum bottles were sampled at one to two day intervals for both gas and liquid. The concentrations of these compounds, when used as the sole substrate, were 5 mM. When betaine and choline were used in conjunction with trimethylamine the concentrations were 2.5 mM except for the delayed addition of trimethylamine to cultures growing on choline when 5 mM of trimethylamine was used. *Methanococcoides methylutens* TMA-10^T with methyliodide as a substrate were monitored for methane production only as analytical equipment was not available to measure the substrate concentrations.

7.3 Results

7.3.1 General Survey

All strains isolated in this study were able to utilise methylamine, dimethylamine, trimethylamine and methanol for methanogenesis (Table 7.1). *M. alaskense* AK-5^T was able to utilise methanol at a concentration of 10 mM. In addition a number of strains, AM1, DM1, MKM1, MKM2, PM1, PM2 and TM1 also utilised tetramethylammonium for methanogenesis (Table 7.1).

None of the strains isolated in this study or any previously described strain of *Methanococcoides* was capable of utilizing acetate, cyclopentanol, dimethylglycine, ethanol, formate, H_2/CO_2, isopropanol, methanethiol, methionine, methylphosphonic acid, propionate, pyruvate, or sarcosine for methanogenesis (Table 7.1).

Betaine was utilised by three strains, MKM1, NM1 and PM2. Strains AM1, BSM1, DM1, NM1, PM1 and PM2 were able to utilise choline for methanogenesis and all strains that utilised choline also utilized *N,N*-dimethylethanolamine. In addition, strains AM2, AM3, BSM3, TM1, TM2 and type strains *M.methylutens* TMA-10^T and *M.alaskense* AK-5^T utilised *N,N*-dimethylethanolamine but not choline. No strain was able to utilise *N*-monomethylethanolamine for methanogenesis. There was a marked difference in the speed of utilization of choline by the various strains (see Section 7.4.2 for growth rates). The addition of sodium 2-bromoethanesulfonate (BES) inhibited growth of strain AM1 on choline and the growth of strain NM1 on betaine. BES is a specific inhibitor of methanogenesis: it is a structural analogue of the final enzyme of methane formation, methyl-CoM reductase (Section 1.5). This demonstrates that methylation of CoM was necessary for the metabolism of betaine and choline.

Methyliodide at a concentration of 1 mM did not result in methanogenesis with any of the strains. 1 mM methyliodide was found to inhibit methanogenesis when added to cultures of *Methanococcoides methylutens* TMA-10^T growing on methylamine (Figure 7.2).

Table 7.1 Substrate utilization of all sixteen *Methanococcoides* strains isolated in this study, the three type strains and strains MM1 and NaT1. In addition to the substrates listed below, strain NaT1 did not utilize fructose, glucose, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxycinnamate, acetone, *N*,*N*-dimethylformamide, dimethylsulfoxide, methoxyacetate and 2-methoxyethanol for methanogenesis (Tanaka, 1994). All concentrations 10 mM except methyliodide (1 mM), methanethiol (1 mM) and H_2/CO_2 (80:20, 0.1 MPa overpressure).

	Strains and Type Strains																				
Substrates	AM1	AM2	AM3	BSM1	BSM2	BSM3	DM1	MKM1	MKM2	NM1	NM2	PM1	PM2	PM3	TM1	TM2	*M. alaskense* AK-9^T	*M. burtonii* DSM 6242^T	*M. methylutens* TMA-10^T	MM1	NaT1
Acetate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-[1]	-[2]	-[3]	-[4]	-[5]
Pyruvate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-
Formate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-[1]	-[2]	-[3]	-[4]	-[5]
H_2/CO_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-[1]	-[2]	-[3]	-[4]	-
Cyclopentanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-
Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-[2]	-	ND	-[5]
Isopropanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-[2]	-	ND	-
Methanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[4]	+[2]	+[3]	+	+
Methylamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[2]	+[3]	+	+
Dimethylamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[2]	+[3]	+	+
Trimethylamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[1]	+[2]	+[3]	+	+
Tetramethylammonium	+	-	-	-	-	-	+	+	+	+	-	+	+	-	+	-	-	-	-	ND	+
Choline	+	-	-	-	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	ND	-[5]
N,N-dimethylethanolamine	+	+	+	+	+	-	+	-	-	+	-	+	+	-	+	+	-	+	+	ND	-[5]
N-monomethylethanolamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-[5]
Ethanolamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND
Betaine	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	ND	-[5]
N,N-dimethylglycine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-[5]
Sarcosine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-[5]
DMS	+	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-[1]	-[2]	-	-[4]	ND
Methanethiol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-[4]	-
Methylphosphonic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND
Methionine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND
Methyliodide	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+[6]	ND	ND

[1] Singh et al (2005)
[2] Franzmann et al (1992)
[3] Sowers & Ferry (1983)
[4] Lyimo et al (2009)
[5] Tanaka (1994)
[6] <200 µM methyliodide

+, is a substrate
-, is not a substrate
ND, not determined

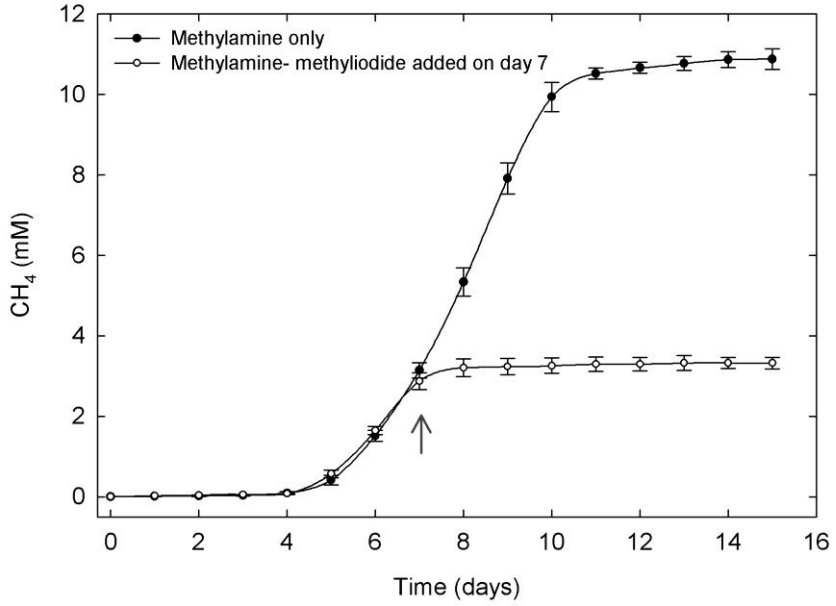


Figure 7.2 Inhibition of methanogenesis by *Methanococcoides methylutens* TMA-10^T with methyliodide. Two triplicate sets of cultures, both initially with only methylamine added. On day 7 (arrow), 1 mM of methyliodide was added to one set of cultures (open circles).

Six strains, strains AM1, AM2, AM3, PM2, TM1 and TM2, produced methane from DMS. The methane yield was low and further testing was undertaken (Section 7.3.2.5).

7.3.2 Additional Substrate Studies

7.3.2.1 Methylamines

The consumption of methylamine, dimethylamine and trimethylamine and the production of end products and intermediate products were monitored for Aarhus Bay strain AM1 only.

A. Methylamine

Figure 7.3 shows that as methylamine was consumed, methane and ammonium were produced. On average 5.3 mM methylamine was consumed and 5.4 mM ammonium and 3.8 mM methane were produced.

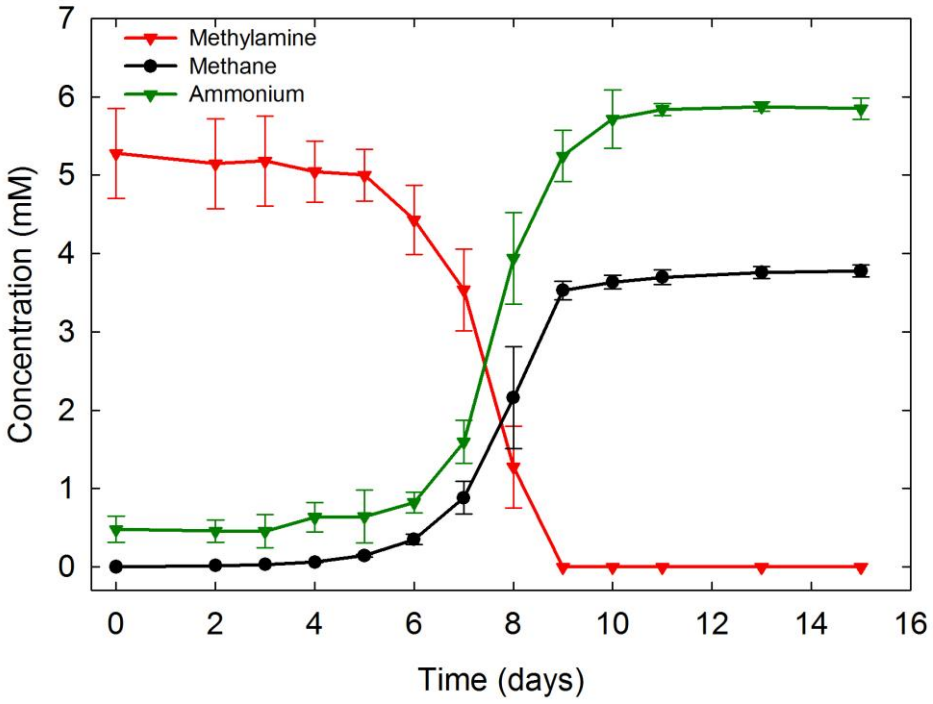


Figure 7.3 Strain AM1 with methylamine as a catabolic substrate; the end products are methane and ammonium. All values are the average of three replicates with the error bars indicating one standard deviation. All incubations carried out at 25 ˚C.

B. Dimethylamine

Figure 7.4 shows that on average 4.7 mM dimethylamine was consumed and 4.9 mM ammonium and 6.8 mM methane was produced. Dimethylamine consumption, methane production and ammonium production started on the same day, day 5. Methylamine appeared as an intermediate, which peaked at 1.1 mM before starting to decline on day 7 when the dimethylamine concentration had dropped to 0.4 mM. The presence of an intermediate showed that at least some and possibly all of the dimethylamine was not demethylated in one step. As methane was produced at each demethylation step but ammonium was only produced when methylamine was demethylated, then the demethylation of methylamine and dimethylamine must have occurred together as both ammonium and methane were produced at the same time. As methylamine accumulated transiently in the medium this indicated that it was metabolised at a slower rate then dimethylamine. Hence, the rate of ammonium production should be slightly slower than methane production (this is seen in Figure 7.4).

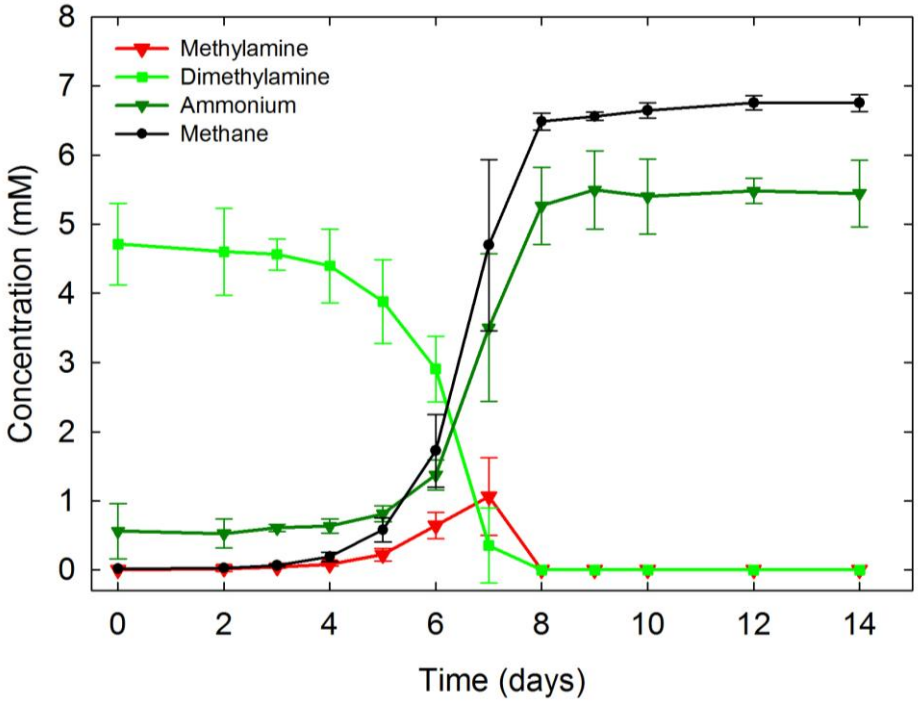


Figure 7.4 Strain AM1 with dimethylamine as a catabolic substrate; the end products are methane and ammonium with methylamine as an intermediate product. All values are the average of three replicates with the error bars indicating one standard deviation. All incubations carried out at 25 ˚C.

C. Trimethylamine

The AM1 cultures consumed on average 4.7 mM trimethylamine and produced 4.7 mM ammonium and 10.8 mM methane (Figure 7.5). Both dimethylamine and methylamine were produced as intermediates, reaching concentrations of 1.0 mM and 3.7 mM respectively by day 8 and both declined thereafter. As dimethylamine did not accumulate to the starting concentration of trimethylamine, dimethylamine and trimethylamine demethylation must have occurred concurrently. Trimethylamine concentration decreased but an increase in ammonium concentration was not detected until day 8 indicating that the demethylation of methylamine had begun on that day. As 0.4 mM ammonium had been produced, and hence 0.4 mM methylamine consumed, by day 8, and dimethylamine and trimethylamine concentrations did not reach zero until day 9, methylamine must have been demethylated concurrently with the other methylamines.

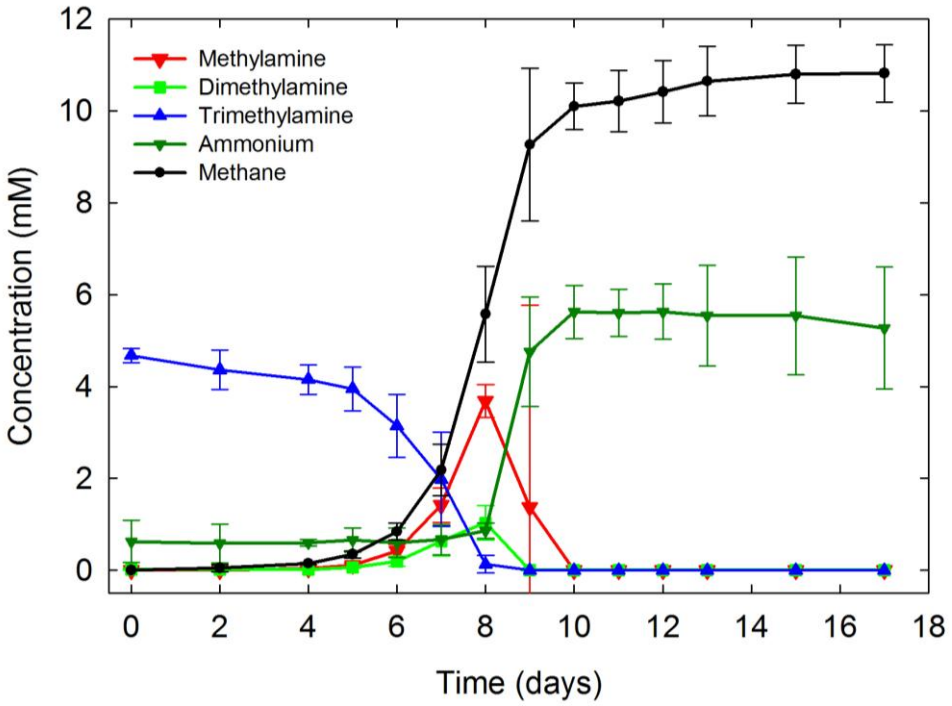


Figure 7.5 Strain AM1 with trimethylamine as a catabolic substrate; the end products are methane and ammonium with methylamine and dimethylamine as intermediate products. All values are the average of three replicates with the error bars indicating one standard deviation. All incubations carried out at 25 ˚C.

7.3.2.2 Choline

Strains AM1 and DM1 was chosen for further study of choline metabolism: strain AM1 was the fastest growing strain, strain DM1 was slower growing but the second strain to be identified to grow on choline. Choline utilisation by strain AM1 was studied with choline on it own and with choline in combination with trimethylamine to investigate preferential and/or co-metabolism.

A. Choline Only

As choline was consumed, methane and ethanolamine were produced, Figure 7.6. On average, 4.8 mM choline was consumed and 4.7 mM ethanolamine and 11.3 mM methane were produced. Two intermediate products were detected, *N*,*N*-dimethylethanolamine and *N*-monomethylethanolamine. The presence of intermediate products indicated that choline was not completely demethylated in one step. On day 4, the choline concentration had started to decrease, the methane

concentration had started to increase and ethanolamine (the end product of the complete demethylation of choline) was first seen. This indicated that the processes of demethylation of choline and the intermediate products must have operated at the same time. *N*,*N*-dimethylethanolamine had accumulated to 0.3 mM by day 10 and declined thereafter. *N*-monomethylethanolamine had accumulated to 1.2 mM by day 11 and as it accumulated to such a relatively high concentration the production rate must have exceeded the consumption rate.

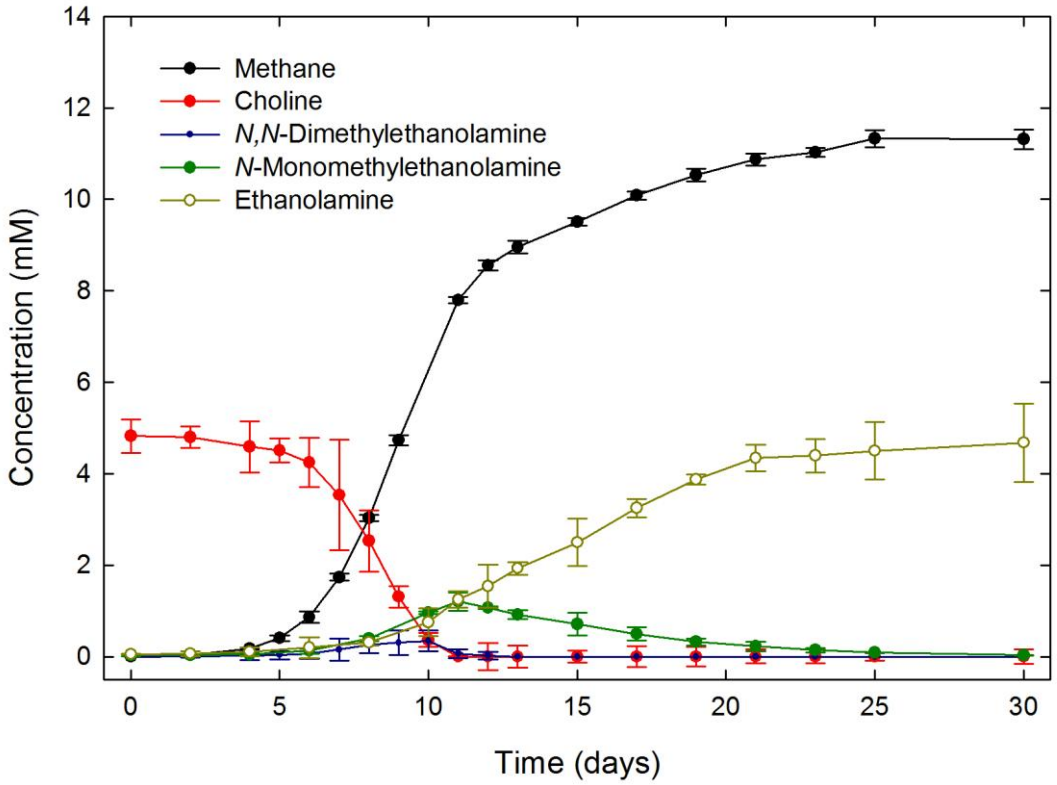


Figure 7.6 Metabolism of choline by strain AM1. Methane was measured by gas chromatography and, choline, *N*,*N*-dimethylethanolamine, *N*-methylethanolamine and ethanolamine were measured by ion chromatography. All values are the average of three replicates with the error bars indicating one standard deviation. All incubations carried out at 25 ˚C.

B. Choline and Trimethylamine Added at the Same Time

When 3 mM each of choline and trimethylamine were added to cultures on day 0, the trimethylamine was consumed first producing methane and ammonium, Figure 7.7. Methylamine was totally consumed by day 9 (methylamine, dimethylamine and the choline intermediates are not shown for clarity). Except for 0.2 mM choline consumed during growth on trimethylamine (days 6-8) choline was not utilised until day 14, but was totally consumed by day 31 with production of ethanolamine (see also Figure

7.6). The methane curve of Figure 7.7 is a typical diauxic growth curve: two exponential growth phases separated by a lag period.

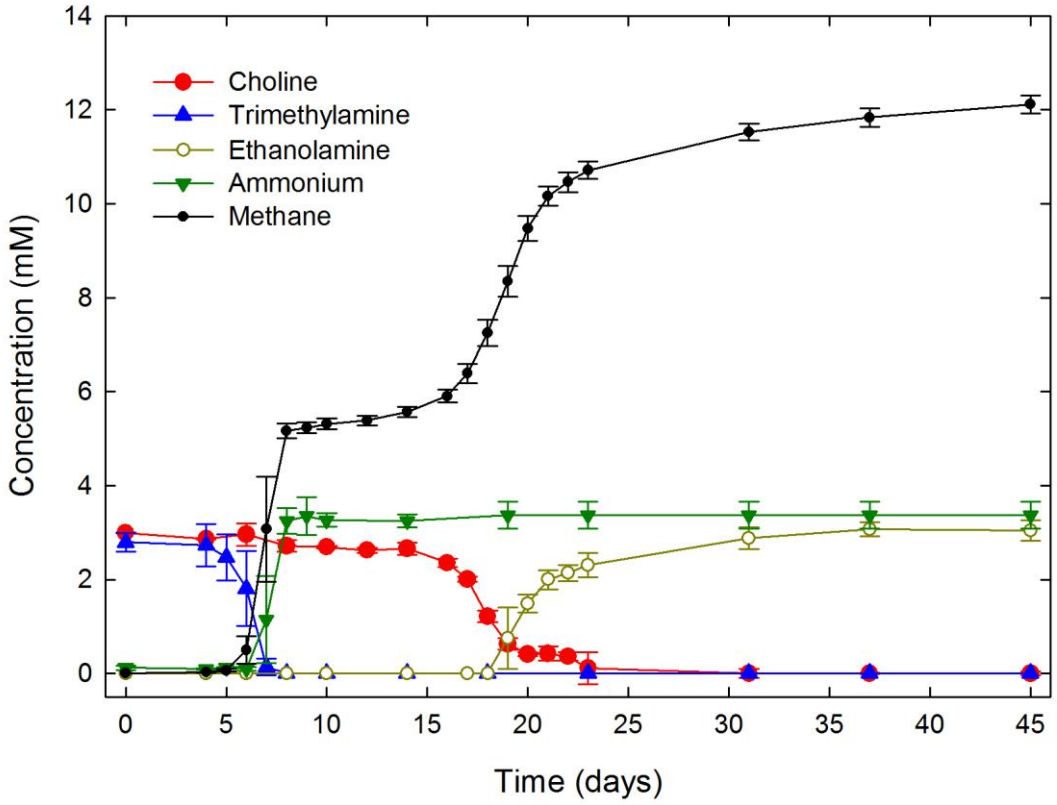


Figure 7.7 Metabolism of choline and trimethylamine, present in the medium from day 0, by strain AM1. All values are the average of three replicates with the error bars indicating one standard deviation. All incubations carried out at 25 ˚C.

C. Choline with the Delayed Addition of Trimethylamine

The result differed when 3 mM choline was added on day 0 and the addition of trimethylamine was delayed until after methanogenesis had started, Figure 7.8. The trimethylamine was added on day 7 when 0.6 mM CH_4 had been produced and trimethylamine was consumed concurrently with choline. Both choline and trimethylamine had been consumed by day 15.

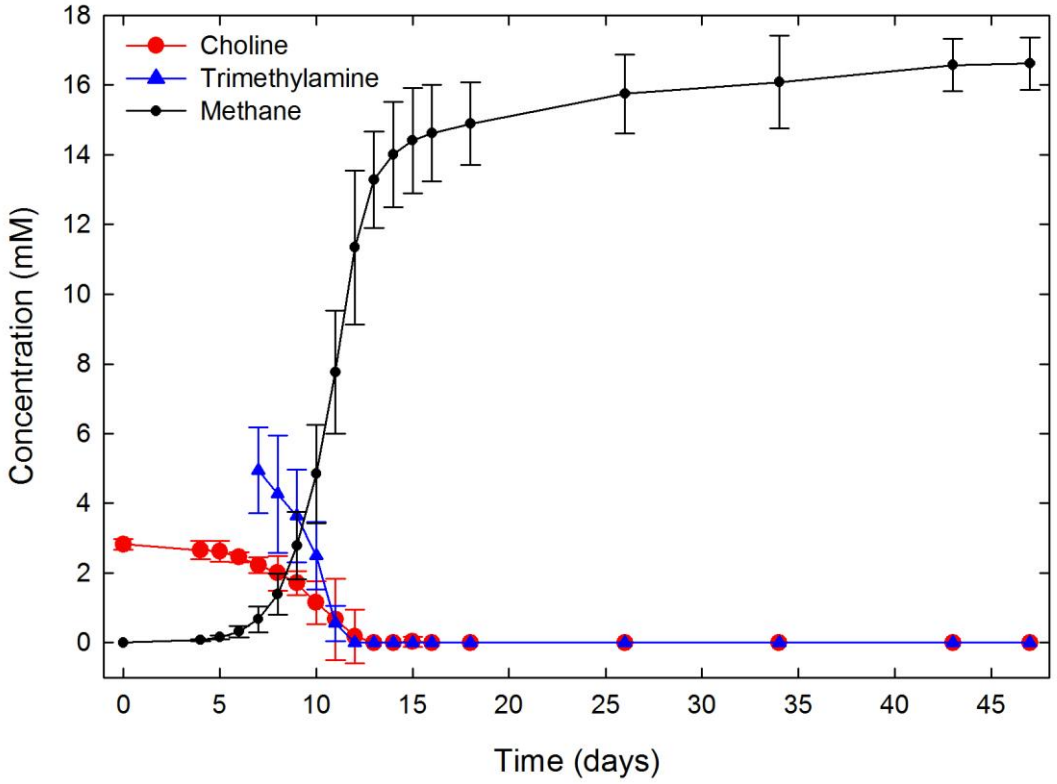


Figure 7.8 Metabolism of choline and trimethylamine by strain AM1 when choline (2.5 mM) was added on day 0 and the addition of trimethylamine (5 mM) was delayed until day 6. All values are the average of three replicates with the error bars indicating one standard deviation. All incubations carried out at 25 ˚C.

D. Other Strains

As identified in the initial substrate utilization survey (Section 7.3.1), strain DM1 utilised choline much more slowly than strain AM1. Of the 5.0 mM choline added on day 0, 4.7 mM had been consumed after 256 days incubation (Table 7.2). *Methanococcoides burtonii* DSM 6242^{T}, a strain that utilized *N*,*N*-dimethylethanolamine but not choline, consumed only half of the 10 mM substrate after 180 days incubation (Table 7.2). The other strains that produced methane from choline or *N*,*N*-dimethylethanolamine (both 10 mM) were part of the initial substrate utilization survey and incubations were undertaken in duplicate (Table 7.2). Despite lengthy incubation times (220-500 days, Table 7.2), none of these strains consumed all of their substrate

Table 7.2 Incubation time and methane produced from choline (10 mM) or *N*,*N*-dimethylethanolamine (10 mM) by strains isolated in this study and by the type strain of the genus *M. methylutens* TMA-10^T. The results are the average of two samples. 10 mM choline and 10 mM *N*,*N*-dimethylethanolamine may be expected to produce 14 and 22.5 mM methane respectively. The result for strain AM1 is the mean of three samples and the starting choline concentration was 4.8 mM.

Substrate	Strain	Incubation time (days)	Methane produced (mM)
Choline	AM1	25	11.3
	BSM1	320	4.7
	NM1	220	5.5
	PM1	500	3.6
	PM2	500	4.3
N,*N*-dimethylethanolamine	AM2	230	2.5
	AM3	230	3.7
	BSM3	400	4.0
	M. methylutens TMA-10^T	220	6.0

7.3.2.3 Betaine

Betaine metabolism was studied using strain NM1 as it was the first strain in which betaine was identified as a substrate, the two other strains that utilised betaine did so over a similar time period. Betaine was used as a growth substrate on its own and in combination with trimethylamine.

A. Betaine Only

5 mM betaine was added to cultures of NM1 on day 0 and methane was detected by day 2 and was produced exponentially between days 4 and 10 (Figure 7.9). Methane production continued subsequently at a lower rate, until a maximum value of 3.57 mM was reached on day 18. At the end of the experiment the protein content of the cultures had, on average, increased by 9.6 ±0.3 μg ml^{-1}.

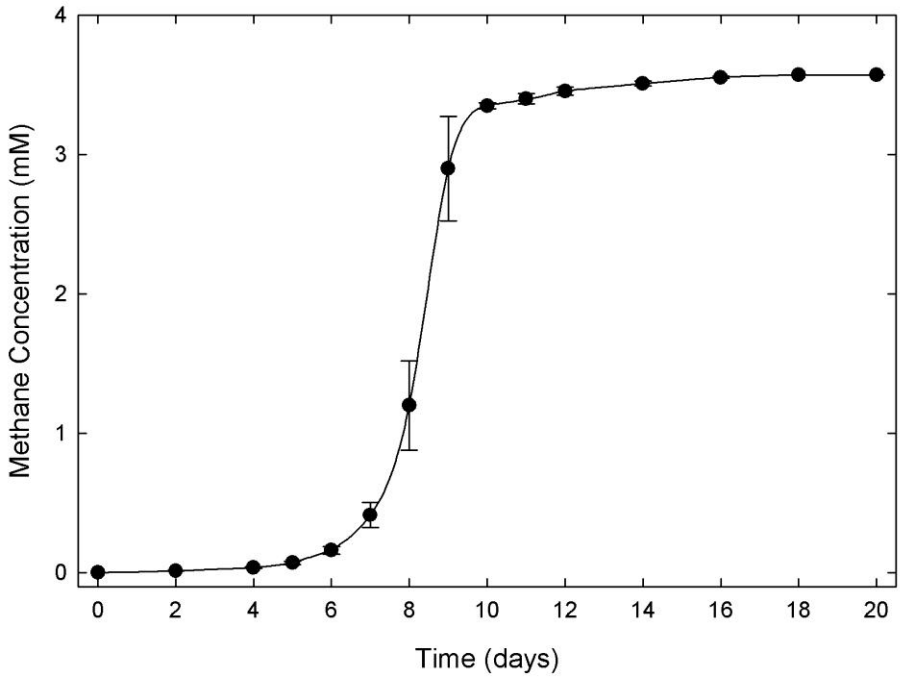


Figure 7.9 Methane production by strain NM1 with betaine as a growth substrate. All values are the average of three replicates with the error bars indicating one standard deviation. All incubations carried out at 25 ˚C. Analytical facilities for the measurement of betaine were not available; hence methane results only.

The other strains that utilized betaine, strains MKM1 and PM2, could produce methane at a rate comparable to strain NM1 and all produced a concentration of methane consistent with the demethylation of betaine to dimethylglycine. In separate substrate tests, both of these strains and strain NM1 were unable to produce methane from dimethylglycine.

B. Betaine and Trimethylamine Added at the Same Time

2.5 mM of betaine and 2.5 mM trimethylamine were added on day 0 to cultures of NM1 (Figure 7.10). Methane was produced exponentially until day 6 then increased slowly until day 26 when the maximum methane concentration of 7.6 mM was reached. As with strain AM1 (Figure 7.5), dimethylamine and methylamine were intermediates in the degradation of trimethylamine. As 5.6 mM of methane will have been produced from 2.5 mM trimethylamine then the remaining 2 mM methane would have come from the consumption of betaine. 2 mM methane is consistent with the expected amount of methane production from the consumption of 2.5 mM betaine and

the production of 2.5 mM dimethylglycine. However, methylamine was consumed by day 7 and after day 7 a further 0.8 mM methane had been produced. This is consistent with the consumption of 1.1 mM betaine. Hence, 1.4 mM betaine must have been consumed before the end of methane production from methylamine so betaine was consumed along with the methylamines. There is no indication that methane production from betaine started at the same time as the consumption of trimethylamine.

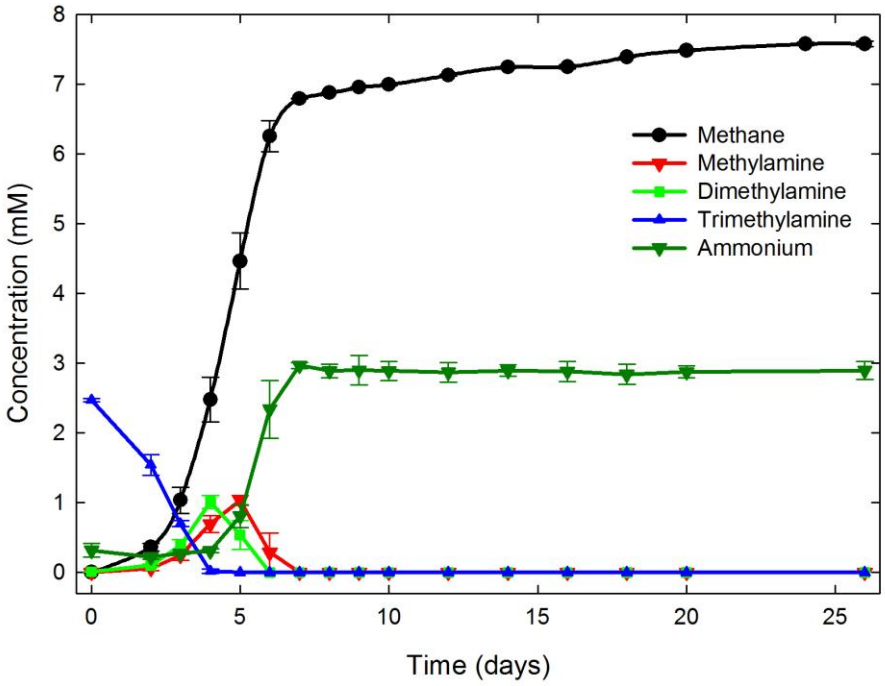


Figure 7.10 Growth of strain NM1 with betaine and trimethylamine added to the culture on day 0. As with strain AM1, dimethylamine and methylamine were seen as intermediates in the degradation of trimethylamine. All values are the average of three replicates with the error bars indicating one standard deviation. All incubations carried out at 25 ˚C.

7.3.2.4 Methyliodide

Methyliodide concentrations less than 1 mM were tested with *Methanococcoides methylutens* TMA-10^T to determine if low concentrations could be a methanogenic substrate rather than an inhibitor of methanogenesis. No methane was produced by *M. methanococcoides* TMA-10^T at methyliodide concentrations of 500, 400, 320, and 240 μM. The addition of 5 mM methylamine to these cultures did not result in methanogenesis. One of triplicate samples to which 160 μM methyliodide was added

produced methane (Figure 7.11). The other two samples failed to produce methane when 5 mM methylamine was added. All samples to which methyliodide was added at concentrations of 40 and 80 μM result in methanogenesis, Figure 7.11.

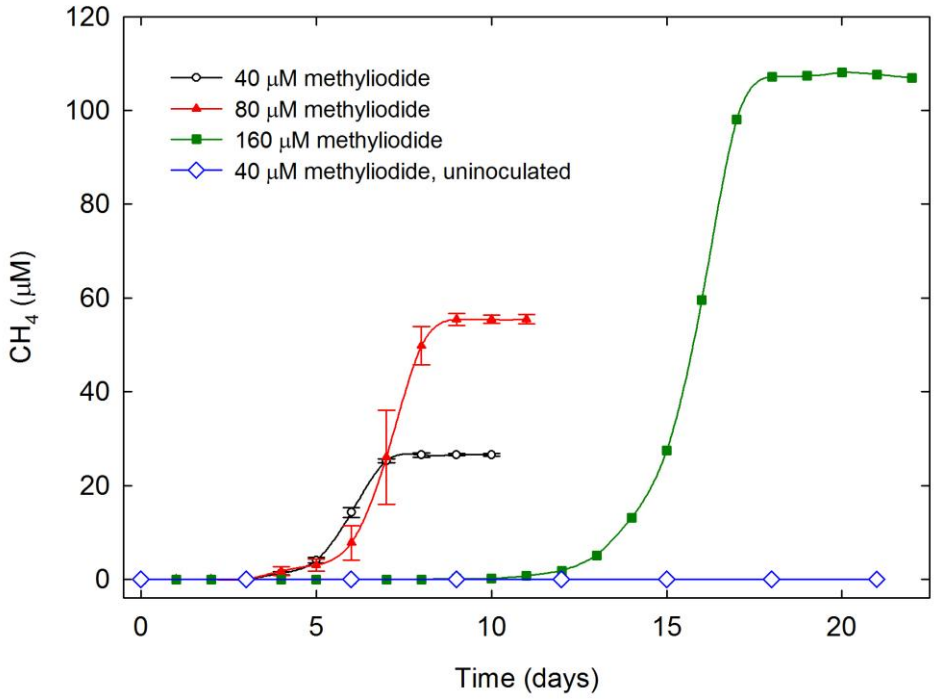


Figure 7.11 Growth of *Methanococcoides methylutens* TMA-10^T with methyliodide. Values for 40 and 80 μM methyliodide are the average of three replicates with error bars representing one standard deviation. The results for 160 μM methyliodide are for a single culture (only one of three cultures produced methane). Uninnocculated bottles (triplicate) with the addition of 40 μM methyliodide did not produce methane.

7.3.2.5 DMS

A lower concentration of DMS (2.5 mM) was used with strains previously identified as producing methane in case the original concentration was inhibitory. With the exception of strain AM1, all cultures produced methane within four to six days of the addition of DMS (Table 7.3). Only one of three replicates of strain AM2 produced methane and did so after a lag period of 40 days. No methane was detected in uninoculated serum bottles with 1.0 mM DMS added. Theoretically, 1 mM of DMS should yield 1.5 mM methane. Strains AM2 and PM2 came close to this (Table 7.3). The worst performer was strain AM1 which had a methane yield of only 0.6 mM per mM DMS after 66 days incubation.

Table 7.3 Methane produced by *Methanococcoides* strains from DMS (1.0 mM) at the end of a 66 day incubation. Results are the mean of three replicates, plus or minus one standard deviation except for strain AM2 where only one replicate produced methane. Complete demethylation of 1.0 mM DMS would theoretically yield 1.5 mM methane.

Strain	Methane produced (mM)	DMS consumed (mM)	Methane per mM DMS
AM1	0.24 ± 0.09	0.39 ± 0.11	0.6
AM2	1.21	0.92	1.3
AM3	0.31 ± 0.07	0.33 ± 0.10	0.9
PM2	0.85 ± 0.18	0.64 ± 0.15	1.3
TM1	0.34 ± 0.23	0.37 ± 0.16	0.9
TM2	0.94 ± 0.24	0.96 ± 0.05	1.0

7.3.2.6 Growth Rates and Methane Yields

For strains AM1 and NM1, the specific growth rates for methylamine, dimethylamine, trimethylamine and methanol in addition the specific growth rates for the novel substrates choline and betaine were measured (Figure 7.12). The methane yields for these catabolic substrates for strains AM1 and NM1 plus the methane yield for DM1 growing on choline are given in Table 7.4. For both strains the specific growth rate with trimethylamine was higher than that of methylamine and the growth rates with the methylamines are broadly similar for both strains. Methanol resulted in a much higher growth rate with strain AM1 than with strain NM1, as did tetramethylammonium. The specific growth rate with choline was similar to that of methylamine with strain AM1, and the specific growth rate for strain NM1 with betaine was similar to that with methylamine/dimethylamine.

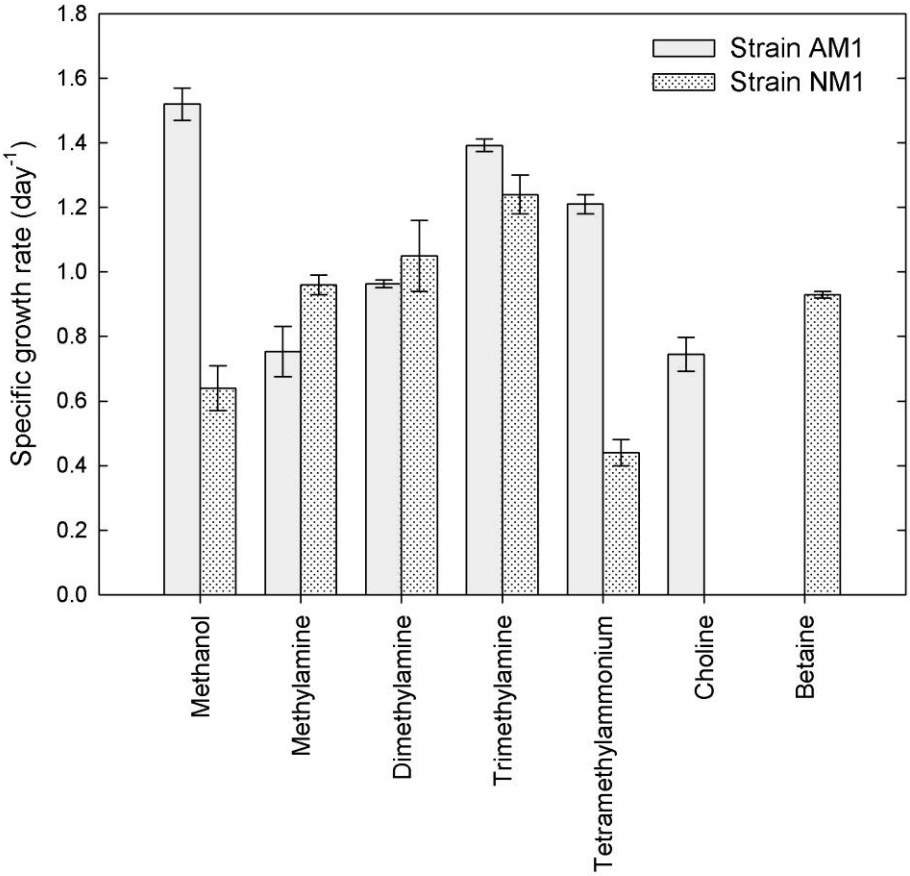


Figure 7.12 Comparative specific growth rates for strains AM1 and NM1 grown on a range of substrates (incubated at 25 ºC, 10 mM of each substrate). Results are the mean of three replicates; error bars represent one standard deviation.

Table 7.4 Starting substrate concentrations, end methane concentrations, mM methane per mM substrate and mM methane per *N*-methyl group for strains AM1, DM1, NM1 isolated in this study, *Methanococcoides burtonii* DM 6242^T test in this study and *Methanococcoides* strain NaT1 for comparison. Results for strains AM1, DM1 and NM1 are averages of three samples plus or minus one standard deviation.

Strain	Substrate	Substrate/ mM	Methane/ mM	Methane/ mM		Ammonium / mM	Ethanolamine / mM
				Per mM of substrate	Per mM of N-methyl group		
AM1	Methylamine	5.28 ±0.23	3.78 ±0.04 (3.96)	0.72 ±0.03 (0.75)	0.72 ±0.03 (0.75)	5.35 ±0.07	
	Dimethylamine	4.71 ±0.24	6.75 ±0.05 (7.07)	1.43 ±0.07 (1.50)	0.72 ±0.03 (0.75)	4.88 ±0.31 (4.71)	
	Trimethylamine	4.67 ±0.06	9.83 ±0.26 (10.51)	2.10 ±0.08 (2.25)	0.70 ±0.03 (0.75)	4.65 ±0.57 (4.67)	
	Choline	4.82 ±0.15	11.32±0.21 (10.87)	2.36 ±0.06 (2.25)	0.79 ±0.02 (0.75)		4.78 ±0.35 (4.82)
DM1	Choline[1]	4.96 ±0.07	11.18±0.04 (11.16)	2.19 ±0.13 (2.25)	0.73 ±0.04 (0.75)		4.70 ±0.01[2] (4.96)
NM1	Methylamine	5.35 ±0.02	3.10 ±0.03 (4.01)	0.63 ±0.01 (0.75)	0.63 ±0.01 (0.75)	ND	
	Dimethylamine	5.08 ±0.18	6.59 ±0.11 (7.62)	1.32 ±0.02 (1.50)	0.66 ±0.01 (0.75)	ND	
	Trimethylamine	4.89 ±0.05	10.27±0.16 (11.00)	2.10 ±0.04 (2.25)	0.70 ±0.01 (0.75)	ND	
	Betaine	5	3.57 ±0.01 (3.75)	0.7 (0.75)	0.7 (0.75)		
M. burtonii[3]	*N*,*N*-dimethylethanolamine	5.0 ±0.02	3.35 ±0.16 (7.5)	1.23 ±0.05 (1.50)	0.62 ±0.03 (0.75)		0.02 ±0.01 (5.0)
NaT1[4]	Methanol	10	6.00 (7.5)	0.60 (0.75)	0.60 (0.75)		
	Methylamine	10	7.20 (7.5)	0.72 (0.75)	0.72 (0.75)	ND	
	Dimethylamine	10	13.3 (15.0)	1.33 (1.50)	0.67 (0.75)	ND	
	Trimethylamine	10	26.2 (22.5)	2.12 (2.25)	0.71 (0.75)	ND	

[1] After 256 days incubation on average 0.2 mM *N*-methylethanolamine remained, utilization of this could result in an additional 0.15 mM methane being produced.
[2] If the ethanolamine produced from the remaining 0.2 mM *N*-methylethanolamine is taken into consideration there would be 4.9 mM ethanolamine.
[3] After 180 days incubation on average 2.48 mM *N,N*-dimethylethanolamine was unconsumed, when consumed this could produce an additional 3.48 mM methane.
[4] Tanaka (1994)

7.4 Discussion

7.4.1 Methylated Compounds as Substrates

All *Methanococcoides* strains isolated in this study plus all of the published strains are able to use methylamine, dimethylamine, trimethylamine and methanol as growth substrates. *M. alaskense* AK-5^T was originally described as not utilising methanol as a substrate (Singh *et al.*, 2005). This study showed that it did utilize methanol although the concentration tested in this study (10 mM) was lower than that originally used (40 mM).

7.4.1.1 Methylamines

The concentrations of methane and ammonium produced by strain AM1 growing with methylamine, dimethylamine and trimethylamine agreed with the expected stoichiometry. A ratio of methylamine, dimethylamine or trimethylamine to ammonium of 1:1 is to be expected from the total degradation of methylamine, dimethylamine or trimethylamine (Equations 7.1 to 7.3). Theoretically the ratio of methylamine consumed to methane produced is 1:0.75 (Equation 7.1); the experimental result was in broad agreement giving a ratio of 1:0.72 (Figure 7.3 and Table 7.4). The theoretical ratio of dimethylamine consumed to methane produced is 1:1.5 (Equation 7.2), the actual ratio was found to be 1:1.43 (Figure 7.4 and Table 7.4). With trimethylamine as a substrate the theoretical ratio of substrate utilized to methane produced is 1:2.25 (Equation 7.3); the ratio found in this study was 1:2.31 (Figure 7.5 and Table 7.4). The agreement between measured and expected values indicates that the products and reactants have been measured accurately. Hence, data presented for these and other substrates can be considered robust.

$$4CH_3NH_2^+ + 3H_2O \rightarrow 3CH_4 + HCO_3^- + H^+ + 4NH_4^+ \quad \text{(Equ. 7.1)}$$

$$2(CH_3)_2NH_2^+ + 3H_2O \rightarrow 3CH_4 + HCO_3^- + H^+ + 2NH_4^+ \quad \text{(Equ. 7.2)}$$

$$4(CH_3)_3NH^+ + 9H_2O \rightarrow 9CH_4 + 3HCO_3^- + 3H^+ + 4NH_4^+ \quad \text{(Equ. 7.3)}$$

The degradation of trimethylamine to ammonium and methane has been documented for *Methanosarcina barkeri* strain Fusaro, which belongs to the same taxonomic

order as the genus *Methanococcoides*. Hippe *et al* (1979) showed that dimethylamine and methylamine were intermediate products in its degradation to methane and ammonium as was the situation with trimethylamine usage by strain AM1 in this study (Figure 7.5). However, during methanogenesis from dimethylamine as a substrate, methylamine accumulation was not seen with *Methanosarcina barkeri* strain Fusaro (Hippe *et al* 1979). This was presumably due to methylamine being degraded to methane and ammonium as quickly as it was being produced from dimethylamine, unlike the situation with strain AM1 which clearly shows methylamine as a metabolic intermediate (Figure 7.4).

The methylotrophic metabolism of *Methanococcoides burtonii* DSM 6242^T has been studied using proteomic data and complete genome sequence data (Williams *et al.*, 2010a). Methyltransferases initiate the processing of methanogenic substrates and there is a methyltransferase specific to each substrate. The specific methyltransferase are MttBC, MtbBC and MtmBC for trimethylamine, dimethylamine and methylamine respectively and each methyltransferase has a corresponding corrinoid protein (a protein containing a cyclic system of four pyrrole rings), which is methylated by its methyltransferase (Williams *et al.*, 2010a). The methylated corrinoids are then demethylated by the same enzyme (MtbA, methylamine:CoM MT) to generate methyl-CoM. There is also a specific methyltransferase (MtaBC) and associated corrinoid protein for methanol demethylation and a unique enzyme (MtaA, methanol:CoM MT) to generate methyl-CoM. Methyl-CoM is the key intermediate in the methylamine and methanol methyl transfer pathways (Williams *et al.*, 2010a). A specific methyltransferase for tetramethylammonium has been identified in *Methanococcoides methylutens* strain NaT1 (Asakawa *et al.*, 1998); when grown on trimethylamine rather than tetramethylammonium, strain NaT1 did not exhibit tetramethylammonium methyltransferase activity.

Multiple copies of each of the methyltransferases are present in *Methanococcoides burtonii* DSM 6242^T, although two methylamine methyltransferases appear to be non-functional, and one set of trimethylamine methyltransferases is membrane associated. The membrane associated methyltransferase may be a permease (a membrane transport protein that facilitates diffusion of a specific molecule into the cell) that may allow the direct coupling of trimethylamine uptake to demethylation (Williams *et al.*,

2010a). No mechanism for methanol uptake was identified. Multiple copies of each of the methyltransferases is a feature also seen in the genomes of *Methanosarcina* species (Allen *et al.*, 2009; Paul *et al.*, 2000).

A transcriptional profiling study of methyltransferase genes in *Methanosarcina mazei* strain Gö1 (DSM 7222) during growth on trimethylamine has identified different gene expression during early and late exponential growth phases (Krätzer *et al.*, 2009). During the first stage, trimethylamine was taken up from the medium by means of a trimethylamine permease (MttP1), demethylated by the methyltransferase MttBC1 and a quantity of the dimethylamine so formed was excreted into the medium. Whilst trimethylamine was being utilized, the expression of the methylamine transferase (mtmBCt) was low. In the second stage, when the trimethylamine had been consumed, the cells switched their metabolism and the gene thought to encode for the dimethylamine permease (mtbP) was highly expressed, expression of the gene thought to encode the methylamine permease (MtmP) was increased and, the genes responsible for trimethylamine metabolism were downregulated. This pattern of gene regulation may also occur in *Methanococcoides* strains and would explain why the intermediate products of trimethylamine degradation accumulate in the medium and why only methylamine is detected towards the end of methanogenesis (Figure 7.5). The presence of intermediate products and the existence of three specific methyltransferases indicate that trimethylamine is demethylated one methyl group at a time to methane and finally ammonium. However, there does appear to be overlap in the process as during trimethylamine degradation by strain AM1 (Figure 7.5) some of the methylamine was degraded concurrently with other methylamines.

7.4.1.2 Choline

The degradation of choline to methane and ethanolamine is analogous to the degradation of trimethylamine to methane and ammonium. Both choline and trimethylamine are totally demethylated and intermediate products with two and one methyl groups are formed (Figures 7.5 and 7.6). In the case of trimethylamine the intermediates are dimethylamine and methylamine whereas the intermediates of choline degradation are *N*,*N*-dimethylethanolamine and *N*-monomethylethanolamine.

The amount of choline consumed and the amount of ethanolamine produced was consistent with a 1:1 ratio as would be expected from the total demethylation of choline (Equation 7.4). The ratio of choline to methane (1:2.56) was consistent with the expected ratio of choline to methane of 1:2.25 if choline was totally demethylated to ethanolamine (Equation 7.4).

$$4\ (CH_3)_3N^+CH_2\ CH_2OH + 9\ H_2O \rightarrow 9\ CH_4 + 4\ NH_2CH_2CH_2OH + 3\ HCO_3^- + 7\ H^+ \qquad \text{(Equ. 7.4)}$$

The ratio of methane produced per methyl group of choline consumed was 0.79 ±0.02 (0.73 ±0.04 for strain DM1) (Table 7.4). This compares to 0.70-0.73 for the methylamines (Table 7.3). The value for choline is slightly higher than the value expected considering that some of the choline would have been used for biosynthesis and theoretically only 75% of methyl groups can be converted to methane (Section 1.4) but this is probably within experimental error. Ethanolamine, the end product of the demethylation of choline, was not metabolized either during the metabolism of choline (Figure 7.7) or as a sole substrate (Table 7.1).

As in the case of trimethylamine, the degradation of choline to methane and ethanolamine may require the action of three methyltransferases and associated corrinoid proteins, one each for choline, *N*,*N*-dimethylethanolamine and *N*-monomethylethanolamine. The inability of some strains tested in this study to utilize choline whilst being able to utilize *N*,*N*-dimethylethanolamine may be explained by the lack of a choline methyltransferase and/or its associated corrinoid protein. The next step in the utilization of choline, the demethylation of the methylated corrinoid proteins, may by analogy with the utilization of trimethylamine, be carried out by a single enzyme. Given the substrate specificity of methyltransferases for methylamines and methanol it may be expected that specific methyltransferases exist for choline, *N*,*N*-dimethylethanolamine, *N*-methylethanolamine and betaine. However, a less specific methyltransferase has been identified in *Methanosarcina barkeri* strain MS that can utilize both DMS and methylmercaptopropionate (MMPA). The ability to utilise choline and betaine are not necessarily found in the same *Methanococcoides* strain (Table 7.1) indicating the presence of a specific betaine methyltransferase.

None of the strains that utilised choline or *N*,*N*-dimethylethanolamine were able to utilise *N*-monomethylethanolamine (Table 7.1) except as a breakdown product of *N*,*N*-dimethylethanolamine (Figure 7.6). The inability to utilise *N*-monomethylethanolamine also occurred when added to cultures of AM1 grown on choline (concentrations of 1 and 10 mM tested). An inability of the strains to take up *N*-monomethylethanolamine from the medium can be rejected as strain AM1 excretes *N*-monomethylethanolamine into the medium and subsequently utilises it for methanogenesis (Figure 7.6). It may be that a considerable length of time is required to induce the pathways required for the utilization of *N*-monomethylethanolamine but the strains tested for *N*-monomethylethanolamine utilization have been monitored for methanogenesis over a period exceeding 200 days. It is possible that the presence of *N*-monomethylethanolamine cannot initiate the pathway required for its own utilization, it may be utilized non-specifically by the choline and/or *N*,*N*-dimethylethanolamine pathways rather than have a dedicated pathway. This would require the methyltransferases for choline and/or *N*, *N*-dimethylethanolamine to be less specific than those for trimethylamine and dimethylamine. Also the utilization of *N*-monomethylethanolamine is much slower than that of choline and *N*,*N*-dimethylethanolamine (Figure 7.6) then the choline and/or *N*,*N*-dimethylethanolamine pathways would have to remain active for a corresponding length of time.

Cultures of strain AM1 with choline and trimethylamine added at the same time exhibited diauxic growth (Figure 7.7). The classic example of diauxic growth is *Escherichia coli* grown on a glucose-lactose mixture (Magasanik, 1970). *E. coli* requires the enzyme β-galactosidase in order to utilize lactose and β-galactosidase is subject to catabolite repression; if glucose is present then β-galactosidase is not synthesized. Catabolite repression is removed once the glucose is consumed and after a lag period β-galactosidase is synthesized and the lactose is consumed. Diauxic growth is also exhibited by *Streptococcus salivarius* grown on a glucose-lactose mixture (Plamondon *et al.*, 1999). When *S. salivarius* cells are grown on lactose only, the addition of glucose causes a switch from lactose to glucose metabolism within a few minutes (Plamondon *et al.*, 1999).

It appears that the pathway for choline degradation in strain AM1 needs to be induced and that it is not induced in the presence of trimethylamine, which leads to diauxic growth (Figure 7.7). However, once induced the pathway remains active in the presence of trimethylamine (Figure 7.8) unlike *S. salivarius* grown on lactose. When present together, trimethylamine may be consumed in preference to choline due to the higher free energy available from trimethylamine ($\Delta G°'$ –167 kJ per mol trimethylamine and –108 kJ per mol choline, Table 7.5).

The catabolic repression of one substrate by another has not been described for any *Methanococcoides* strain. However, it has been noted with *Methanolobus taylorii* GS-16; cells grown on DMS were able to utilize both DMS and trimethylamine simultaneously, however, cells grown on trimethylamine did not metabolize [^{14}C]DMS to $^{14}CH_4$ (Oremland *et al.*, 1989).

7.4.1.3 Betaine

Like choline and trimethylamine, betaine has three methyl groups (Figure 7.1). The amount of methane produced from betaine by strain NM1 (Figure 7.9 and Table 7.4) was consistent with the removal of only one methyl group to produce dimethylglycine as 5 mM betaine would be expected to produce 3.75 mM methane if a single methyl group was removed to produce dimethylglycine (Equation 7.5), 3.57 ±0.01 mM methane was actually produced. Neither dimethylglycine nor sarcosine was utilized as a growth substrate by any strain including those that utilized betaine (Table 7.1).

$$4\ (CH_3)_3N^+CH_2COOH.Cl^- + 3\ H_2O \rightarrow\ 3\ CH_4 + 4\ (CH_3)_2NCH_2COOH + HCO_3^- + 5\ H^+ + 4\ Cl^- \qquad \text{(Equ. 7.5)}$$

If betaine were utilised for methanogenesis in a manner analogous to that of trimethylamine then betaine would be degraded to methane and glycine with dimethylglycine (two methyl groups) and sarcosine (one methyl group) as intermediates. This would yield three times the amount of methane actually detected (Equation 7.6).

$$4\ (CH_3)_3N^+CH_2COOH.Cl^- + 9\ H_2O \rightarrow 9\ CH_4 + 4\ NH_2CH_2COOH + 3\ HCO_3^- + 7\ H^+ + 4\ Cl^- \qquad \text{(Equ. 7.6)}$$

Energetically the degradation of betaine to methane and glycine is favourable (Table 7.5). The simultaneous addition of betaine and trimethylamine to NM1 cultures did not result in diauxic growth (Figure 7.9). Due to the inability to detect betaine and dimethylglycine it is not clear if betaine is consumed alongside trimethylamine. It may be the case that both pathways are always active and do not need to be induced. However, betaine may be utilized after trimethylamine on the basis of the free energy available from the substrates ($\Delta G^{\circ\prime}$ -167 kJ per mol trimethylamine and –80 kJ per mol betaine, see Section 7.4.2).

7.4.1.4 Tetramethylammonium

Several strains isolated in this study utilized tetramethylammonium as a growth substrate at a concentration of 10 mM (Table 7.1). The significance of tetramethylammonium as a catabolic substrate *in situ* is uncertain, as there are no reports of tetramethylammonium occurring in marine sediments. It does, however, occur in nature, tetramethylammonium (tetramine) has been detected in the salivary glands of marine gastropods *e.g*. *Neptunea antiqua* (Anthoni *et al.*, 1989) and *Neptunea arthritica* (Asano & Itoh, 1960).

7.4.1.5 DMS

This is the first time a strain of *Methanococcoides* has been reported to produce methane from DMS. Other genera of the *Methanosarcinales* (*Methanosarcina*, *Methanolobus* and *Methanomethylovorans*) contain DMS utilizing methanogens. As with members of the genera *Methanosarcina* and *Methanolobus* (Whitman *et al.*, 2006), the ability of *Methanococcoides* to utilize DMS is not present in all strains. Of the three *Methanococcoides* strains isolated from Portishead, for instance, only one strain (PM2) has been noted to produce methane from DMS. Aarhus Bay strains AM1. AM2 and AM3 all utilized methane but only strain AM2 exhibited a prolonged lag time.

The strains that produced methane from DMS were strains isolated from tidal flats (TM1, TM2 and PM2) and a shallow bay (AM1-AM3) not from deep water. With the exception strain AM2, strains previously grown on trimethylamine produced methane from DMS with a lag time of two to six days. Only one of three replicates of strain AM2 produced methane and this was after a lag period of 40 days. A long lag

period, ~3-4 weeks, was noted before growth of *Methanolobus taylorii* GS-16^T on DMS after growth on trimethylamine for over one year (Oremland *et al.*, 1989).

The poor methane yield of strain AM1 grown on DMS (Table 7.4) may have been due to inhibition, however, the concentrations of DMS used (1 mM) is below reported inhibitory levels. Oremland et al. (1989) found DMS concentrations >10 mM were inhibitory to *Methanolobus taylorii* GS-16^T and no growth occurred with > 20 mM DMS. Lyimo *et al*. (2000) did not test for toxic levels of DMS for *Methanosarcina semesiae* MD1^T but did find that there was no methane production with DMS concentrations ≥30 mM. Alternatively, there may be a threshold level for uptake of DMS.

The genome of *Methanococcoides burtonii* DSM 6242^T has been found to lack the genes encoding the specific methylthiol methyltransferases required for the metabolism of DMS that are found in some *Methanosarcina* species (Tallant & Krzycki, 1997).

7.4.1.6 Methyl Iodide

Methyliodide, at concentrations < 200 μM was a substrate for methanogenesis (Figure 7.1) and methane was produced in agreement with Equation 7.7, the ratio of methyliodide to methane produced was 1:0.75.

$$4\ CH_3I + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 5\ H^+ + 4\ I^- \qquad \text{(Equ. 7.7)}$$

At concentrations > 200 μM, methyliodide did not result in methanogenesis and 1 mM has been shown to inhibit methanogenesis from methylamine (Figure 7.2). Methyl bromide, methyl chloride and methyl fluoride are known inhibitors of methanogenesis (Chan & Parkin, 2000; Janssen & Frenzel, 1997; Oremland *et al.*, 1994). The only report in the literature of methyl iodide inhibiting methanogenesis was the inhibition of methane production from acetate by an unidentified strain of *Methanosarcina barkeri* at a concentration of 50 μM (Fischer & Thauer, 1990). Sediment slurries have been incubated with [^{14}C]-methyl iodide and they did produce $^{14}CH_4$ and $^{14}CO_2$ (Oremland *et al.*, 1994). This was considered to be due to

methanogens utilizing the methylated sulfur intermediates of methyl iodide degradation rather than methanogens directly utilizing methyl iodide (Oremland *et al.*, 1994).

7.4.2 Growth Rates

Tetramethylammonium, trimethylamine, dimethylamine, methanol, betaine and choline did not give rise to the same specific growth rate (Figure 7.12). For strain AM1 the specific growth rate was lower with methylamine then it was with trimethylamine and growth on methanol was faster than that on trimethylamine. Strain NM1 has broadly similar specific growth rates to strain AM1 for the methylamines but the specific growth rate with methanol was lower than that with methylamine. In the case of *Methanococcoides burtonii* DSM 6242^T (the only previously described strain for which growth rates have been given for the methylamines and methanol) the fastest growth occurred with trimethylamine followed by dimethylamine and then methylamine, the slowest growth rate was with methanol (Franzmann *et al.*, 1992). Given that the first stage of methanogenesis has methyltransferases that are unique for each substrate (at least for the methylamines, methanol and tetramethylammonium) then variations in the specific growths rate may, in part, by due to the action of the methyltransferases. With methanol there is an additional unique step before a common pathway is joined (see Section 7.4.1) and this may also be the case with betaine, choline and tetramethylammonium. The presence of permeases may allow the direct coupling of substrate uptake to demethylation, a potential trimethylamine permease was identified in *M. burtonii* DSM 6242^T (Williams *et al.*, 2010a).

The marked difference in the specific growth rates with methanol may indicate an inhibitory effect of methanol on strain NM1. If this was the case then strain NM1 was more sensitive to methanol than strain AM1 as the same concentration was used with both strains (10 mM). A concentration of 20 mM was used with *M. methylutens* MM1 and this did not appear to be inhibitory as the specific growth rate with methanol was the same as with trimethylamine (Lyimo *et al.*, 2000; Lyimo *et al.*, 2009b). These concentrations were low compared to the 94 mM given to *M. burtonii* DSM 6242^T (Franzmann *et al.*, 1992). The only described strain of *Methanococcoides* reported not

to grow on methanol was *M. alaskense* AK-5^T which failed to grow with a concentration of 40 mM (Singh *et al.*, 2005). This study found that *M. alaskense* AK-5^T grew with 10 mM methanol. There was a marked difference in specific grow rates with tetramethylammonium (Figure 7.12), 10 mM was used with both strains and the same concentration was used with the only described strain to utilize tetramethylammonium as a substrate (strain NaT1), unfortunately the growth rates of the substrates used were not given (Tanaka, 1994).

7.4.3 Energetics of Methylated Substrates

A study by Bose *et al*. (2006) found that *Methanosarcina acetivorans* C2A grown on acetate would initially consume only trimethylamine when transferred into medium containing both trimethylamine and methanol. Only when trimethylamine had been consumed and both dimethylamine and methylamine had accumulated in the medium did rapid methanol consumption begin (a relatively small amount of methanol had been utilised before the concentration of dimethylamine had peaked). The order in which the substrates were consumed was explained by the free energy available from the substrates when expressed per mole substrate. The free energy of a methanogenic reaction is usually reported in terms of free energy per mole CH_4; when expressed this way the highest value is for methanol ($\Delta G°'$ -105 kJ per mol CH_4) and the free energy available from methylamine, dimethylamine and trimethylamine are very similar, $\Delta G°' \sim$ -74 kJ per mole CH_4 (Table 7.5). However, trimethylamine has the highest free energy if it is expressed as per mole of substrate and it was suggested that the substrates used by *Methanosarcina acetivorans* C2A were consumed in order of free energy so expressed (Bose *et al.*, 2006).

That trimethylamine is consumed by strain AM1 in preference to choline (Figure 7.7) may be due to choline having a lower free energy per mole of substrate than trimethylamine ($\Delta G°'$ -108 and –167 kJ per mol respectively). An extensive literature review failed to find the free energies of formation ($\Delta G°_f$) for choline and ethanolamine that were required to calculate free energy. However, the free energy was calculated using predicted $\Delta G°_f$ values (Table 7.5). The predicted $\Delta G°_f$ values were obtained from Leibovic & d'Anterroches (2011) and were calculated using the Joback method, a method uses molecular structure to predict thermodynamic

properties (Joback & Reid, 1987). As predicted rather than actual values were used for the calculation the result must be treated with caution. The result shows that the free energy available from choline when expressed per mole of substrate is lower than that of trimethylamine and slightly lower than that of dimethylamine. Unlike the situation with *Methanosarcina acetivorans* C2A, no significant amount of choline was consumed by strain AM1 until several days after the complete consumption of the methylamines (Figure 7.7). Strain NM1 consumed at least some of the betaine at the same time as the consumption of the methylamines (Figure 7.10) although it is not known if it was consumed alongside trimethylamine or alongside dimethylamine or methylamine. The predicted free energy (again, the actual free energy of formation is not available) would suggest the consumption of betaine before methylamine (Table 7.5).

Table 7.5 Free energy ($\Delta G°'$) of methanogenic reactions. Most of the data used for the calculations was obtained from Thauer *et al*. (1977). The $\Delta G°_f$ values for choline, ethanolamine, betaine and dimethylglycine where not available, instead predicted values were used. These were obtained from Leibovic & d'Anterroches (2011) and were calculated using the Joback method (Joback & Reid, 1987). As a predicted value for betaine was not available, the predicted value of betaine chloride was used. The free energy results calculated using predicted values should be treated with caution.

Substrate	Reaction	$\Delta G°'$ (kJ)		
		Per reaction	Per mol CH_4	Per mol substrate
Trimethylamine	$4(CH_3)_3NH^+ + 9H_2O \rightarrow 9CH_4 + 3HCO_3^- + 3H^+ + 4NH_4^+$	−669	−74	−167
Dimethylamine	$2(CH_3)_2NH_2^+ + 3H_2O \rightarrow 3CH_4 + HCO_3^- + H^+ + 2NH_4^+$	−220	−73	−110
Methylamine	$4CH_3NH_2^+ + 3H_2O \rightarrow 3CH_4 + HCO_3^- + H^+ + 4NH_4^+$	−225	−75	−56
Methanol	$4\ CH_3OH \rightarrow 3\ CH_4 + HCO_3^- + H^+ + H_2O$	-315	-105	-79
Choline	$4\ (CH_3)_3N^+CH_2\ CH_2OH + 9\ H_2O \rightarrow 9\ CH_4 + 4\ NH_2CH_2CH_2OH + 3\ HCO_3^- + 7\ H^+$	-430	-48	-108
N,*N*-Dimethylethanolamine	$2\ (CH_3)_2NCH_2CH_2OH + 3\ H_2O \rightarrow 3\ CH_4 + 2\ NH_2CH_2CH_2OH + HCO_3^- + H^+$	-190	-63	-95
N-Monomethylethanolamine	$4\ CH_3NHCH_2CH_2OH + 3H_2O \rightarrow 3\ CH_4 + 4\ NH2CH_2CH_2OH + HCO_3^- + H^+$	-192	-64	-48
Betaine chloride (degraded to dimethylglycine)	$4\ (CH_3)_3N^+CH_2COOH.Cl^- + 3\ H_2O \rightarrow 3\ CH_4 + 4\ (CH_3)_2NCH_2COOH + HCO_3^- + 5\ H^+ + 4\ Cl^-$	-319	-106	-80
Betaine chloride (degraded to glycine)	$4\ (CH_3)_3N^+CH_2COOH.Cl^- + 9\ H_2O \rightarrow 9\ CH_4 + 4\ NH_2CH_2COOH + 3\ HCO_3^- + 7\ H^+ + 4\ Cl^-$	-698	-78	-175
Methyliodide	$4\ CH_3I + 3\ H_2O \rightarrow 3\ CH_4 + HCO_3^- + 5\ H^+ + 4\ I^-$	-495	-165	-124

7.4.4 *Methanococcoides* Phylogeny and Substrate Usage

The utilization of catabolic substrates other than methylamines and methanol by *Methanococcoides* strains is indicated on a 16S rRNA phylogenetic tree (Figure 7.13). Tetramethylammonium, betaine and DMS are seen to be utilized by strains more closely related to *M. methylutens* TMA-10^T than to *M. alaskense* AK-5^T/ *M.burtonii* DSM 6242^T. Tetramethylammonium was utilized by all strains in the PM1/ PM2 and NM1/ MKM1/ MKM2/ DM1 groups. Strain PM3 is the only strain in that group not to make use of either choline or *N*,*N*-dimethylethanolamine as a substrate.

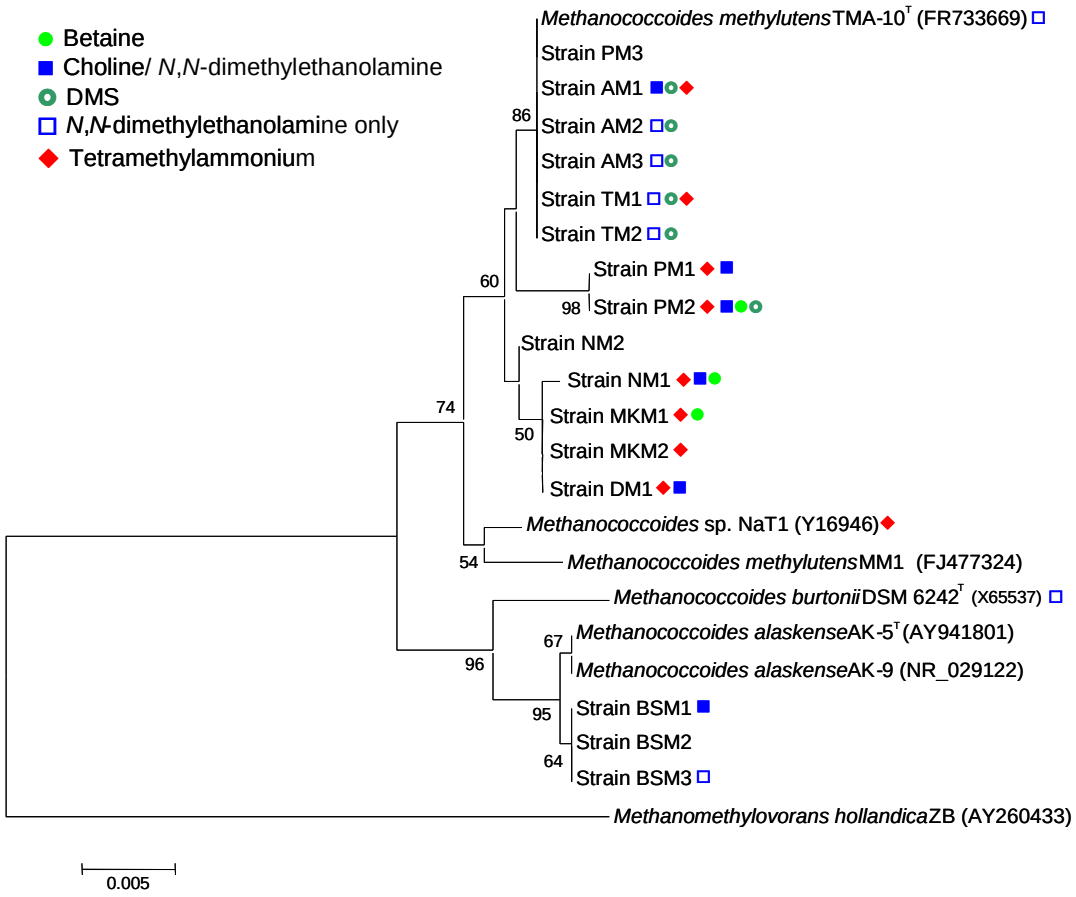


Figure 7.13 *Methanococcoides* phylogenetic tree based on 16S rRNA gene sequences with utilization of betaine, choline, DMS and *N*,*N*-dimethylethanolamine indicated.

DMS utilization is found only in strains that also utilize choline/ *N*,*N*-dimethylethanolamine or *N*,*N*-dimethylethanolamine. However, utilization of choline or *N*,*N*-dimethylethanolamine is not limited to strains that can utilize DMS (Figure 7.13).

7.4.5 Other Substrates

The common methanogen substrates acetate and H_2/CO_2 were not utilized by any *Methanococcoides* strain in this study (Table 7.1) or by any of the previously described strains (Franzmann *et al.*, 1992; Lyimo *et al.*, 2009b; Singh *et al.*, 2005; Sowers & Ferry, 1983; Tanaka, 1994).

The genus *Methanosarcina* utilizes a relatively wide range of catabolic substrates and some strains can utilise acetate and/or hydrogen (Whitman *et al.*, 2006). Hydrogen methanogenesis in *Methanosarcina* requires the action of three hydrogenases (Ech, Frh/Fre and Vho) (Meuer *et al.*, 2002), these have not been identified in the genome of *Methanococcoides burtonii* DSM 6242^T (Allen *et al.*, 2009). For methanogenesis from acetate, *Methanosarcina* require carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) to catalyze the methylation of methanopterin (MPT) by dismutation of acetyl CoA and acetate kinase (AckA) and phophotransacetylase (Pta), which are required for generating acetyl CoA from acetate (Allen *et al.*, 2009). Whilst the *M. burtonii* DSM 6242^T genome has a copy of the CODH/ACS gene, it lacks the genes for acetate kinase (AckA) and phophotransacetylase (Pta) (Allen *et al.*, 2009). Phylogenetic analysis of *Methanosarcina* has determined that the ancestor of *Methanosarcina* acquired the genes for acetate kinase (AckA) and phophotransacetylase (Pta) by horizontally transfer from an organism of the bacterial class *Clostridia* (Fournier & Gogarten, 2008).

7.5 Summary

Sixteen *Methanococcoides* strains isolated in this study from seven locations (Aarhus Bay, Darwin MV, Dvurechenskii MV, Meknes MV, Napoli MV, Portishead and Tamar) have been tested with a range of potential growth substrates. For all strains methylamine, dimethylamine, trimethylamine and methanol were growth substrates and seven strains also utilized tetramethylammonium. Some strains also utilized the

novel catabolic substrates betaine and choline, which have only previously been reported as methanogenic compounds in mixed culture (Hippe *et al.*, 1979). Choline was degraded in a manner analogous to that of trimethylamine. DMS, a substrate of closely related genera (some strains of *Methanolobus* and *Methanosarcina*), was used by a small number of strains. Methyliodide inhibited methanogenesis in *Methanococcoides methylutens* TMA-10^T at a concentration of 1 mM, and lower concentrations resulted in methanogenesis. Acetate and H_2/CO_2 were not determined to be substrates for any of the *Methanococcoides* strains isolated in this study.

Chapter 8
General Discussion

8.1 Introduction

A considerable number of molecular studies have been undertaken to assess the diversity of methanogens in marine sediments; however, few culture-dependent surveys have been undertaken. The main objectives of this project were to explore the culturable diversity of marine methanogens by:

- enriching methanogens from a variety of marine environments using a range of substrates and conditions
- obtaining pure methanogen cultures from these environment
- characterizing and identifying isolated methanogens and comparing them to known isolates
- testing for possible new methanogenic substrates

8.2 Summary of Main Findings

This study was possibly the widest culture-dependent survey of marine methanogens that has been undertaken. During the course of this project knowledge of marine methanogens was expanded by culturing methanogens from five Gulf of Cadiz mud volcanoes (Captain Arutynov, Carlos Ribeiro, Darwin, Meknes and Mercator), Napoli MV, Dvurechenskii MV, Aarhus Bay, Guaymas Basin, and Portishead and Tamar tidal flats (Chapter 3).

Of the sixteen genera that have been isolated from marine sediments (Chapter 1), nine were detected in enrichment cultures in this study. The genera detected were *Methanobacterium*, *Methanococcoides*, *Methanococcus*, *Methanoculleus*, *Methanogenium*, *Methanoplanus*, *Methanosaeta*, *Methanosarcina* and *Methanospirillum*. Of these nine genera *Methanococcoides*, *Methanogenium* and

Methanosarcina were the most commonly cultured and the least common were *Methanococcus*, *Methanoplanus* and *Methanospirillum* (Table 3.1). The two tidal flat locations had the highest methanogen diversity of the all the sites studied (Chapter 3). Eighteen methanogen strains were isolated during the course of this study. Sixteen of the strains were members of the genus *Methanococcoides* and two were members of the genus *Methanococcus* (Chapter 3). Prior to this study, the description of only six strains (three of them type strains) of *Methanococcoides* had been published. All published strains were from shallow marine environments, *M. methylutens* TMA-10^T from the Sumner branch of Scripps Canyon (California, USA), *M. burtonii* DSM 6242^T from Ace Lake (Antarctica), *M. alaskense* AK-5^T and AK-9 from Skan Bay (Alaska, USA), *M. methylutens* MM1 from Mtoni Creek mangrove swamp (Tanzania) and *Methanococcoides* strain NaT1 from Tokyo Bay (Japan). This study isolated *Methanococcoides* strains from Aarhus Bay (three strains), Dvurechenskii MV (three strains), Darwin MV (one strain), Meknes MV (two strains), Napoli MV (two strains), Portishead intertidal sediments (three strains) and Tamar intertidal sediments (two strains). This is the first time that a methanogen had been isolated and characterized from the Gulf of Cadiz mud volcanoes Darwin and Meknes, Napoli MV and Dvurechenskii MV. *Methanococcoides* has previously been identified through molecular methods and culture studies of Meknes and Napoli mud volcanoes (Lazar *et al.*, 2011a; Lazar *et al.*, 2011b; Sas, 2009) but there is no report in the literature of methanogens at Dvurechenskii MV or other localities in the Sorokin Trough (Crimea, Black Sea). Two *Methanococcus* strains were isolated from Portishead intertidal sediments, which was the only site from which *Methanococcus* was cultured.

All *Methanococcoides* strains isolated in this study and the three type strains (obtained from the DSMZ collection) were tested with a wide range of potential growth substrates. This was the most comprehensive substrate test undertaken on this genus. This study identified four compounds not previously identified as direct methanogenic substrates; betaine, choline, *N*,*N*-dimethylethanolamine and methyliodide (Chapter 7). Choline was degraded to ethanolamine in a way analogous to the degradation of trimethylamine, in both cases methyl groups were removed one by one leading the production of two and one methyl group intermediates (for further details see Chapter 7). *M. methylutens* TMA-10^T was found to produce methane from low concentrations (< 200 μM) of methyliodide (Chapter 7). Half of the strains were

able to utilize tetramethylammonium, a compound previously only reported as a growth substrate of *Methanococcoides* strain NaT1 (Tanaka, 1994). Additionally, dimethylsulphide (DMS) previously identified as a substrate in two other genera of methanogens (*Methanosarcina* and *Methanolobus*) was identified as a substrate for six *Methanococcoides* strains. All *Methanococcoides* strains (previously published strains and strains isolated in this study) have been shown to utilize methanol, methylamine, dimethylamine and trimethylamine as growth substrates.

One representative *Methanococcoides* strain from each of the seven locations investigated by this study had its growth range with Na^+ concentration, temperature and pH determined (Chapter 6). The ranges where not markedly different from those of published strains. *Methanococcoides* strains are mesophilic or psychrotolerant, halotolerant and neutrophilic (Chapter 6). Four strains, including one type strain, were also tested under elevated hydrostatic pressure; this is the first study to do this (Chapter 6).

Using the data from this study (Chapters 6 and 7) and for the previously described strains of *Methanococcoides* the genus can now be summarized as having the following characteristics:

- irregular cocci, 0.5-2 μm diameter, occurring singly, in pairs and occasionally forming clusters, may be motile, does not form spores
- methylamine, dimethylamine, trimethylamine and methanol are growth substrates for all strains tested
- betaine, choline, DMS, methyliodide, tetramethylammonium are additional growth substrates for some strains
- acetate is not required as a carbon source, yeast extract is not required but vitamins may be required
- mesophilic or psychrotolerant, halotolerant and neutrophilic
- growth occurs under hydrostatic pressure as high as 70 MPa (four strains tested)

Few non-thermophilic methanogens have been isolated from the deep sea. Methanogen strains isolated and characterized in this study are not only the deepest characterized strains of *Methanococcoides* they are the deepest characterized non-thermophilic methanogens (Table 8.1). Consistent with these results, *Methanococcoides* have been detected using molecular techniques at water depths of 3850 m at Porto MV, Gulf of Cadiz (Sas, 2009) and at 6400 m water depth in the Japan Trench (Li *et al.*, 1999). This is supported by this study showing that *Methanococcoides* strains can grow at hydrostatic pressure of 70 MPa, equivalent to 7000 m water depth (Chapter 6).

Table 8.1 Methanogens isolated from the deep marine environment.

Strain	Water depth (m)	Site	Reference
BSM1, BSM2 & BSM3	2060	Dvurechenskii MV	This study
NM1&NM2	1938	Napoli MV	This study
DM1	1100	Darwin MV	This study
MKM1 and MKM2	650	Meknes MV	This study
Mesophiles			
Methanococcus aeolicus strain Nankai-3^T	950	Nankai trough	Kendall *et al* (2006b)
Methanoculleus submarinus strain Nankai-1^T	950	Nankai trough	Mikucki *et al* (2003)
Methanosarcina baltica strain GS1-A^T	241	Gotland Deep, Baltic Sea	Von Klein *et al* (2002)
Thermophiles			
Methanocaldococcus indicus strain *SL43^T*	2420	Hydrothermal chimney Central Indian Ridge	L'Haridon (2003)
Methanocaldococcus infernos strain ME^T*	3000	Hydrothermal field, Mid-Atlantic Ridge	Whitman (2001b)
Methanocaldococcus jannaschii strain *JAL-1^T**	2600	White smoker East Pacific Rise	Jones *et al* (1983a)
Methanocaldococcus vulcanius strain M7^T*	2600	Hydrothermal field East Pacific Rise	Jeanthon *et al* (1999)
Methanopyrus kandleri strain AV19^T	2000	Guaymas hot vent area	Kurr *et al* (1991)
Methanotorris formicicus strain Mc-S-70^T	2421	Black smoker Central Indian Ridge	Takei *et al* (2004)

* Transferred from the genus *Methanococcus*

8.2 Comparisons With Other Marine Culture-based Studies

The number of published cultivation-based studies investigating the diversity of marine methanogens is limited to three studies, Skan Bay (Kendall *et al.*, 2007), Hydrate Ridge (Kendall & Boone, 2006) and Shimokita Peninsula (Imachi *et al.*, 2011). Another study, whose primary purpose was to assess the effect of the pollutant perchloroethene (PCE) on tidal flat prokaryotes, cultured methanogens from Jade Bay (Wadden Sea, Germany). The methanogens cultured from these sites are summarized in Table 8.2.

Skan Bay (Unalaska Island, Alaska, USA) is 65 m deep, the sediment temperature is in the range 1-6 °C and contains a large amount of organic matter (2 -3% of sediment dry weight) (Kendall *et al.*, 2007). The sulphate concentration at the sediment surface was ~21 mM and below the SMTZ (30-45 cm depth) methane accumulated to 3.2 mM and its biogenic origin was indicated by a δ^{13} value of -80‰ (Kendall *et al.*, 2007). Enrichment cultures covering approximately 5 cm intervals from 0-60 cm sediment depth were set up with acetate, H_2/CO_2 or methylamine as substrates. At all depths investigated, the methanogen enriched on methylamine was *Methanococcoides* (Kendall *et al.*, 2007). *Methanococcoides alaskense* AK-5^T and AK-9 had been previously isolated from a sediment depth of 31-42 cm at Skan Bay using trimethylamine as a substrate (Singh *et al.*, 2005). H_2/CO_2 utilizing methanogens were also cultured at all depths and were phylogenetically related to described species of the genus *Methanogenium* (Kendall *et al.*, 2007). In contrast, an acetoclastic methanogen was only enriched from one depth, (34-39 cm, <0.1 mM sulphate) and was identified as a member of the genus *Methanosarcina* (98% 16S rRNA sequence identity to *M. lacustris*). Whilst three genera of methanogens were cultured by Kendall *et al*. (2007), only two genera were identified in a sediment 16S rRNA gene clone library and one of these was of a genus not cultured, *Methanogenium marinum* AK-1 (99% sequence identity) detected in shallow and mid depth sediments (3-45 cm) and *Methanosaeta* sp. (95% sequence identity) which was only detected in mid depth sediment (33-45 cm) (Kendall *et al.*, 2007). The type strain of *Methanogenium boonei* AK-7^T was isolated from 39-41 cm sediment depth (Kendall *et al.*, 2007). In addition there were sequences related to ANME-1,

AMNE-2a/b/c and MBG-D. Less than 2% of clones in the 16S rRNA gene clone library were identified as methanogens.

The cultivation study of Hydrate Ridge (offshore Oregon, USA) methanogens used sediment taken by pushcores from 800 m water depth (Kendall & Boone, 2006). Sediment from 31-41 cm sediment depth was incubated at 4-15 ºC with acetate, formate or trimethylamine in bicarbonate buffered medium with peptone and yeast extract. Sulphate data for the sediment cores used in this study was not available beyond 25 cm depth, at this depth the sulphate concentration was < 5 mM, although the profile shows likely sulphate depletion by 31-41 cm depth (Valentine *et al.*, 2005). Methanogens identified in enrichment cultures with trimethylamine were *Methanococcoides* (98% 16 rRNA gene identity to *Methanococcoides methylutens* TMA-10^T). Enrichment cultures with formate cultured *Methanogenium* (97% 16S rRNA gene identity to *Methanogenium cariaci*) and a *Methanosarcinales* related cluster, not closely related to any cultured methanogen, was enriched using trimethylamine (Kendall & Boone, 2006). Also enriched were ANME-1/2a/2c, MBG-B and MBG-D. No acetate utilizing methanogens were cultured even after a two-year incubation period. Unlike Skan Bay, 16S rRNA gene clone library sequences did not detect any methanogens and MPN estimated only 10^1-10^2 methanogen cells ml^{-1}.

A different cultivation technique was used by Imachi *et al.* (2011) to culture methanogens from sediment taken from a water depth of 1180 m at Shimokita Peninsula (Japan). A continuous-flow bioreactor system was used to enrich methanogens before using conventional enrichment techniques. The reactor medium contained glucose, acetate, propionate and yeast extract. After enrichment in the reactor, methanogens were isolated using batch cultures with butyrate, H_2/CO_2, formate, trimethylamine, methanol, 2-propanol or propionate as growth substrates. H_2/CO_2 and formate cultures also contained yeast extract and 1 mM acetate as a carbon source. Both 16 rRNA and *mcrA* genes were analysed. All of the archaeal 16S rRNA gene phylotypes detected in the sediment prior to enrichment were closely related to MBG-B, MBG-D and MCG; no methanogen-related sequences were detected. However, after 357 days incubation in the bioreactor, *Methanobacterium*, *Methanococcoides* and *Methanosarcina* were identified. The *mcrA* gene phylotypes

were affiliated with the genera *Methanobacterium*, *Methanococcoides*, *Methanosarcina* and *Methanobrevibacter* (all ≥ 97% sequence identity). Representatives of all four genera identified by *mcrA* sequencing were subsequently isolated. Batch cultures from the original sediment, *i.e.* cultures not involving the use of a continuous-flow bioreactor system, did not enrich methanogens.

Sediment taken from Jade Bay (Wadden Sea, Lower Saxony, Germany) was incubated with a hydrogen headspace at 20 ºC with or without PCE for 51 days (Kittelmann & Friedrich, 2008). The sediment depth and sulphate concentrations were not stated in this study but referred to as upper sediment, Llobet-Brossa *et al*. (2002) report sulphate concentrations 20-17 mM in the upper 25 cm of the sediment at Jade Bay. Using 16S rRNA gene sequencing, methanogens closely related to *Methanocorpusculum*, *Methanococcoides*, *Methanogenium*, *Methanoplanus*, *Methanosarcina* and *Methanosaeta* were cultured with or without the addition of PCE. *Methanoculleus* was only identified in cultures without PCE. *Methanococcoides* was cultured although no methylated substrate was added, as with the elevated hydrostatic pressure enrichment cultures in this study (Chapter 5), the sediment must have contained sufficient precursors to methylated compounds to facilitate the enrichment of *Methanococcoides*.

In addition, Goffredi *et al* (2008) described what could be regarded as a natural enrichment culture study, the effect of a whale fall on the methanogen population in Monterey Canyon (California, USA). The sulphate concentration beneath a whale carcass located at 2893 m water depth was found to be reduced from 28 mM to ~ 9 mM. Three genera of methanogen were identified using DNA techniques, *Methanogenium* (99% 16S rRNA gene sequence identity to *Methanogenium marinum*), *Methanococcoides* (99% 16S rRNA gene sequence identity to *Methanococcoides alaskense*) and *Methanosarcina* (98% 16S rRNA gene sequence identity to *Methanosarcina baltica*). No methanogens were detected at a reference site 10 m away from the whale carcass. At another Monterey Canyon whale fall site (1820 m water depth) *Methanolobus* in addition to *Methanococcoides* and *Methanosarcina* were identified using 16 rRNA gene sequences (Goffredi *et al.*, 2008).

The culture-dependent studies described above (Table 8.2) showed that *Methanococcoides* is ubiquitous in marine sediment with *Methanosarcina* and *Methanogenium* also having a wide distribution. *Methanococcoides* was enriched in this study rather than *Methanosarcina*, when using marine medium and methylamine as a substrate (Table 3.1) despite both genera being able to metabolise methylamine (Whitman *et al.*, 2006). The Skan Bay study enriched *Methanosarcina* with acetate as a substrate yet from the same sediment depth an enrichment culture with methylamine selected for *Methanococcoides* (Kendall *et al.*, 2007). Also, in the Shimokita Peninsula study *Methanococcoides* was cultured with trimethylamine whilst *Methanosarcina* was cultured with acetate/yeast extract or methanol (Imachi *et al.*, 2011). All three studies, therefore, suggest that *Methanococcoides* can out-compete *Methanosarcina* for methylamines.

Table 8.2 Comparison of the results of this study and previously described marine methanogen culture studies. Members of the genera *Methanococcoides*, *Methanogenium* and *Methanosarcina* were the most commonly cultured methanogens. In addition, this study isolated *Methanococcoides* from Napoli and Gulf of Cadiz mud volcanoes and *Methanogenium* from Darwin MV.

	Culture-dependent studies							
	This Study				Previous studies			
Genus	Aarhus Bay	Portishead	Guaymas Basin	Tamar	Hydrate Ridge[1]	Jade Bay[2]	Skan Bay[3]	Shimokita[4]
Methanobacterium		+		+				+
Methanobrevibacter								+
Methanococcoides	+	+	+	+	+	+	+	+
Methanococcus		+						
Methanocorpusculum						+		
Methanoculleus		+		+		+		
Methanogenium	+		+	+	+	+	+	
Methanolobus								
Methanoplanus				+		+		
Methanosaeta		+	+	+		+		
Methanosarcina	+	+		+	+	+	+	+
Methanospirillum				+				

[1] Kendal & Boone (2006)
[2] Kittelmann & Friedrich (2008)
[3] Kendall *et al*. (2007)
[4] Imachi *et al*. (2011)

8.3 Substrates

8.3.1 Non-Competitive Substrates

In marine sediments with high sulphate concentrations, methanogens that can make use of non-competitive substrates (such as methylamine) would be expected. All methanogens that disproportionate methylamines, methanol and other methyl containing compounds are members of the order *Methanosarcinales* (Whitman *et al.*, 2006). *Methanosarcinales* contains seven genera of obligatory methylotrophic methanogens, *Methanococcoides*, *Methanolobus*, *Methanosalsum*, *Methanohalobium Methanohalophilus*, *Methanomethylovorans* and *Methanosphaera*. In addition, members of the genus *Methanosarcina* can utilize methylated substrates and may also utilize acetate and/or H_2/CO_2 (Whitman *et al.*, 2006). Of all these genera only *Methanococcoides*, *Methanolobus* and *Methanosarcina* have type species isolated from marine environments. *Methanomethylovorans* have been isolated from freshwater environments (Jiang *et al.*, 2005 ; Lomans *et al.*, 1999), type species of the genus *Methanosphaera* are intestinal methanogens and use methanol only in conjunction with hydrogen (Biavati *et al.*, 1988; Miller & Wolin, 1985). The type strains of the genus *Methanohalophilus* are moderately halophilic with optimum growth rates in the range 0.5 to 2.5 M NaCl (Boone *et al.*, 1993; Liu *et al.*, 1990; Mathrani *et al.*, 1988b; Paterek & Smith, 1988; Wilharm *et al.*, 1991). *Methanohalobium evestigatum*, the only member of that genus, is extremely halophilic with an optimal salinity of 4.3 M NaCl (Zhilina & Zavarzin, 1987).

Whilst *Methanococcoides* and *Methanosarcina* were cultured in this study, *Methanolobus* was notable by its absence. The genus *Methanolobus* is morphologically similar to *Methanococcoides* and also utilizes methylamines and methanol as substrates (some strains can also utilize DMS). The main physiological difference between the described strains of the two genera is the temperature range. The T_{min} of the marine *Methanolobus* species range from 9-45 ºC compared with 1.7-40 ºC for the genus *Methanococcoides*. However, the wetland *Methanolobus* strain "*Methanolobus psychrophilus*" can grow in the range 0-25 ºC (Zhang *et al.*, 2008) and *Methanolobus* have been identified at Shallow Bay (Signy Island, South Orkney Islands) which is frozen during winter (Purdy *et al.*, 2003).

Methanolobus and *Methanococcoides* can occur in the same sediment *e.g.* Colne Point (Purdy *et al.*, 2002), Shallow Bay (Purdy *et al.*, 2003) and Monterey Canyon (Goffredi *et al.*, 2008) so the absence of *Methanolobus* does not appear to be due to competitive exclusion. Interestingly in a hypersaline mat at Baja California, the two genera were vertically separated. *Methanolobus* were identified in superficial layers below the microbial mat, whilst *Methanococcoides* were identified below this in coarse-grained unconsolidated sediment (Orphan *et al.*, 2008a). It is not clear why *Methanolobus* has not been enriched at the locations investigated by this study, but it may indicate a more restricted geographical distribution for *Methanolobus* than *Methanosarcina* and *Methanococcoides*. Media bias was considered in this study but the same medium allowed the culture of nine other genera of methanogens. The non-detection by this study of *Methanolobus* in Tamar estuary sediment using 16S rRNA gene PCR-DGGE (Section 3.2.5.2.) does not seem to be the result of primer specificity as both sets of primers used for the nested PCR (109F & 958R and SAf & PARCH519R, Table 2.9) match *Methanolobus* sequences in the NCBI database.

8.3.2 Competitive substrates

This study enriched both genera of acetoclastic methanogens, *Methanosaeta* and *Methanosarcina*. *Methanosaeta* were only enriched from Portishead and Tamar tidal flats (Table 3.1). *Methanosaeta* have been detected along the Colne Estuary (Carbonero, 2010), and it was argued that they originated from the freshwater end of the estuary, as they would be out-competed for acetate by sulphate-reducing bacteria at the marine end of the estuary A freshwater origin would have explained the decreasing number of *Methanosaeta* with increasing salinity that had been observed and the lack of change in genetic diversity along the estuary (Carbonero, 2010). In this study *Methanosaeta* were cultured from Portishead only with freshwater medium (strain P3HFr), which would be consistent with a freshwater origin; however, *Methanosaeta* were only enriched from Tamar sediment with marine medium. Whilst this study also enriched *Methanosarcina* with acetate, the high sulphate concentrations *in situ* (except for Aarhus Bay where sediment was taken from the SMTZ) might have resulted in *Methanosarcina* metabolism switching to methylotrophy. As *Methanococcoides* appears to outcompete *Methanosarcina* for methylamine, *Methanosarcina* may be utilizing other methylated substrates such a DMS and methanol.

Both tidal flat sites investigated in this study had a wide diversity of hydrogen utilizing methanogens, whilst other sites were limited to *Methanogenium* and possibly *Methanosarcina*. Other culture-dependent studies also cultured hydrogen-utilizing methanogens including *Methanogenium* (Table 3.1). This study and the Skan Bay study cultured hydrogen-utilizing methanogens from sediment with high sulphate levels.

A study of saltmarsh sediment from Colne Point (UK) found that low levels of hydrogenotrophic methanogenesis (measured using [^{14}C] bicarbonate) occurred in sediment simultaneously with sulphate reduction, although hydrogenotrophic methanogenesis was three orders of magnitude lower than the rate of sulphate-reduction (Senior *et al.*, 1982). Other studies, for example, the [^{14}C] bicarbonate study of sediment from Aust Wrath (Severn estuary, UK) have also found low rates of hydrogenotrophic methanogenesis in the presence of 18 mM sulphate (Wellsbury *et al.*, 1996).

The hypothesis of hydrogenotrophic methanogenesis taking place in sulphate-depleted microenvironments (environments where reduction is so active that it exceeds the rate of sulphate diffusion) was dismissed as improbable when the minimum radius for a spherical microenvironment was calculated using the method of Jørgensen (1977) as it required a 5.9 cm radius microenvironment (Senior *et al.*, 1982). It was concluded that hydrogenotrophic methanogens persisted within the sediment with extremely low levels of activity in the presence of sulphate-reducing bacteria.

The hydrogen utilizing methanogens, and indeed the acetoclastic methanogens, cultured from sediment just beneath the oxidized surface sediments could be responding to occasionally occurring inputs of high-levels of organic matter, which would over the short term, reduce sulphate levels sufficiently to reduce the competition from sulphate-reducing bacteria. An extreme example of this being the Monterrey Bay whale-fall study previously described. A radiotracer study of methanogenesis at Cape Lookout Bight (North Carolina, USA) demonstrated hydrogenotrophic methanogenesis (using [^{14}C] bicarbonate) in zones of high sulphate concentration and suggested that the input of organic substrates may have been high

enough to allow the simultaneous hydrogenotrophic methanogenesis and sulphate reduction (Grill & Martens, 1983).

At Cape Lookout Bight the increase in temperature from 5 ºC in winter to 28 ºC in summer and the resulting increase in the activity of sulphate-reducing bacteria causes the depth of sulphate penetration into the sediment column to decrease from below 20 cm in winter to 8-10 cm in summer and there is a shift from sulphate reduction to methanogenesis (Klump, 1989). The response of sulphate-reducers at Portishead and Tamar to temperature increases may also decrease sulphate penetration and allow a switch from sulphate-reduction to methanogenesis. However, there are no seasonal data for sulphate penetration for either of these sites. At Dangast tidalflat (Wadden Sea, Germany) the sediment contains sulphate to at least 1 m depth all year and is dominated by sulphate-reduction (Finke *et al.*, 2007).

The heterogeneous distribution of organic matter in marine sediments creates a mosaic of microenvironments, some of which will favour methanogens over sulphate-reducing bacteria. It has been suggested (Boone, 1984) that within sedimentary particle microenvironments the partial pressure of hydrogen is higher than in the surrounding porewater. This would favour the growth within the particles of methanogens with lower affinity and threshold values for hydrogen. A study of an anaerobic digester (Tomei *et al.*, 1985) found that methanogens were attached to butyrate degrading bacteria (*Clostridium*/ *Desulfotomaculum*), it was suggested that interspecies attachment may be a method to overcome lower affinities for hydrogen, resulting in methanogenesis despite a lower affinity for hydrogen than sulphate-reducing bacteria with a higher affinity for hydrogen. For example, attachment might allow the methanogen to scavenge hydrogen before it diffuses into the porewater (Tomei *et al.*, 1985). Interspecies attachment may be species specific; the butyrate degrading bacteria attached to *Methanobacterium* sp., but not to *Methanospirillum* sp. or the sulphate-reducing *Desulfovibrio* sp. (Tomei *et al.*, 1985).

Rather than a symbiotic relationship between methanogens and bacteria, methanogenesis may result from the symbiotic relationship between methanogens and ciliates (large unicellular eukaryotes). Anaerobic ciliates such as *Metopus*, *Plagiopyla*

and *Sonderia* possess hydrogenosomes, which are organelles that couple substrate level phosphorylation to pyruvate fermentation with hydrogen formation and methanogens act as a sink for the hydrogen (Finlay & Fenchel, 1992). *Methanoplanus endosymbiosus* MC1^T was isolated from the marine ciliate *Metopus contortus* taken from decomposing sea-grass and mud from the shallow shore of the Wadden Sea (van Bruggen *et al.*, 1986). A *Methanobacterium* related methanogen was detected in the marine ciliate *Parduzcia orbis* taken from the upper 2 cm of sediment of Santa Barbara Basin (California, USA) (Edgcomb *et al.*, 2011). No ciliates were detected in this study; this was not surprising, as the culture conditions were not tailored for anaerobic ciliates.

8.3.3 Tetramethylammonium

After the methylamines and methanol, tetramethylammonium was the most common substrate, used by 9 out of 19 strains of *Methanococcoides* tested in this study (Table 7.1). Tetramethylammonium is not known to be a substrate in any other genus of methanogen. Whilst tetramethylammonium occurs naturally, *e.g.* in marine gastropods (Section 7.4.1.4), its distribution in marine sediments is unknown. Outside of the genus *Methanococcoides*, tetramethylammonium has been identified as a substrate for the aerobic methylotroph *Paracoccus kocurii* B^T(Ohara *et al.*, 1990). The source of tetramethylammonium was wastewater from a semiconductor factory. Bacteria related to *Pseudomonas aminovorans,* and *Methylobacterium* sp. have also been identified as utilizing tetramethylammonium (Urakami *et al.*, 1990). Again, the source of tetramethylammium was not natural; the soil the bacteria were cultured from was contaminated with tetramethylammonium that was manufactured in a nearby factory. The use of tetramethylammonium as a methanogenic substrate was first identified in *Methanococcoides* strain NaT1 isolated from Tokyo Bay (Tanaka, 1994); tetramethylammonium was one of a number of potential substrates tested with strain NaT1, it is not known if this site was contaminated with tetramethylammonium.

The strains isolated by this study that utilized tetramethylammonium were from tidal-flats, a shallow bay and mud volcanoes (Table 7.1). It seems unlikely that all of these sites were contaminated with a compound used in the electronics industry so either tetramethylammonium occurs naturally in marine sediments or

tetramethylammonium is not the natural target of the enzymes that degrades it. It may be a case of substrate ambiguity (the activity of an enzyme with substrates whose structure resembles the native substrate). We then have a mystery as to the identity of the native substrate. Also, did the ability of *Methanococcoides* to utilize tetramethylammonium (or a similar substrate) evolve independently in a range of marine environments or was it a general property, which has been lost by some strains.

8.3.4 Betaine and Choline

Choline has previously been tested as a possible growth substrate with *Methanosphaera stadtmanae* MCB-3^T(Miller & Wolin, 1985), *Methanohalophilus mahii* SLP^T(Paterek & Smith, 1988), *Methanosarcina bakeri* strains MS^T, Fusaro, 3 and *Methanosarcina vacuolata* DSM 1232^T (previously *M. barkeri* strain "Zhilina") (Hippe *et al.*, 1979). In all cases choline was not identified as a substrate. Betaine is not a growth substrate for *Methanohalophilus mahii* SLP^T (Paterek & Smith, 1988) and *Methanosarcina barkeri* strains MS^T, Fusaro and 3. In addition, *Methanosarcina vacuolata* DSM 1232^T does not utilize betaine, dimethylglycine, sarcosine or tetramethylammonium (Hippe *et al.*, 1979).

Rather than being a growth substrate, betaine has been identified as a compatible solute for *Methanosarcina barkeri* strains MS^T, 227, Fusaro and LBS, *M. Mazei* strains S-6^T and LYC, *M. thermophila* strain TM-1^T, *M. acetivorans* strains C2A^T and C2E, and *M. vacuolata* Z-761^T (Sowers & Gunsalus, 1995). The same study determined that the addition of glycine betaine to culture medium resulted in partial repression of *de novo* synthesis of the compatible solutes α -glutamate and *N*,-acetyl-β-lysine synthesis. Betaine has also been determined to be a compatible solute in *Methanohalophilum mahii* SLP^T (Robertson *et al.*, 1990). Members of the genus *Methanococcoides* have not been shown to utilize betaine as a compatible solute but *Methanococcoides burtonii* DSM 6242^T does possess an ATP-binding cassette (ABC) transport system for glycine betaine uptake (Williams *et al.*, 2010a), the betaine is possibly used as a cryoprotectant (Campanaro *et al.*, 2011).

Despite previous studies, which showed that betaine and choline could only be used by methanogens in mixed culture (Hippe *et al.*, 1979), this study has shown that some

Methanococcoides strains can directly utilize betaine and/or choline as growth substrates (Table 7.1). Some bacteria can degrade these compounds to trimethylamine and the free energy available to methanogens from trimethylamine ($\Delta G°'$ -167 kJ mol^{-1} substrate) is possibly higher than that available from the direct utilization of betaine ($\Delta G°'$ -80 kJ mol^{-1} substrate) or choline ($\Delta G°'$ -108 kJ mol^{-1} substrate) (Table 7.5). Indeed, this study has demonstrated that strain AM1 will preferentially utilize trimethylamine when simultaneously presented with choline and trimethylamine (Figure 7.7). An advantage of the direct utilization of these substrates is that methanogens are not reliant on the presence of bacteria to degrade choline to trimethylamine (Section 1.3.6.1). With the exception of strain AM1, all the strains in this study that utilized choline utilized it slowly, much more slowly than trimethylamine (Chapter 7). In contrast, those methanogens that utilized betaine did so reasonably quickly. The direct utilization of betaine would be particularly advantageous because some bacteria such as *Desulfobacterium autotrophicum*, degrade betaine to dimethylglycine (Heijthuijsen and Hansen, 1989), which is a compound that has not been identified as a substrate for methanogenesis by this or other studies.

If direct utilization of betaine and choline is advantageous for *Methanococcoides*, it is puzzling that strains isolated from the same sediment do not all have this ability. Only one of the two strains isolated from Napoli MV degraded betaine and only one of three strains from Aarhus Bay was able to degrade choline (Table 7.1). The isolated strains were checked regularly by microscopy for signs of bacterial contamination, none was seen. If bacteria were present as contaminants in the methanogen cultures and they were degrading betaine or choline to methylamines they would also be producing acetate and/or butyrate (see Section 1.3.6.1), these products were never identified by ion chromatography. The accumulation of acetate was seen in the choline enrichment cultures CT2 and CT5 containing both methanogens and bacteria (Section 4.3).

These newly discovered methanogenic degradation pathways for betaine and choline are illustrated in Figure 8.1 along with bacterial degradation pathways of betaine, choline and TMAO outlined in Section 1.3.6 to illustrate possible indirect metabolite supply to methanogens. Depending on the bacterial pathways taken for the

degradation of betaine, one or two different types of bacteria may be involved in breaking it down to a substrate suitable for methanogenesis if the methanogen cannot use betaine directly. In the case of choline, up to three types of bacteria may be involved if degradation proceeds via betaine and *N*,*N*-dimethylglycine.

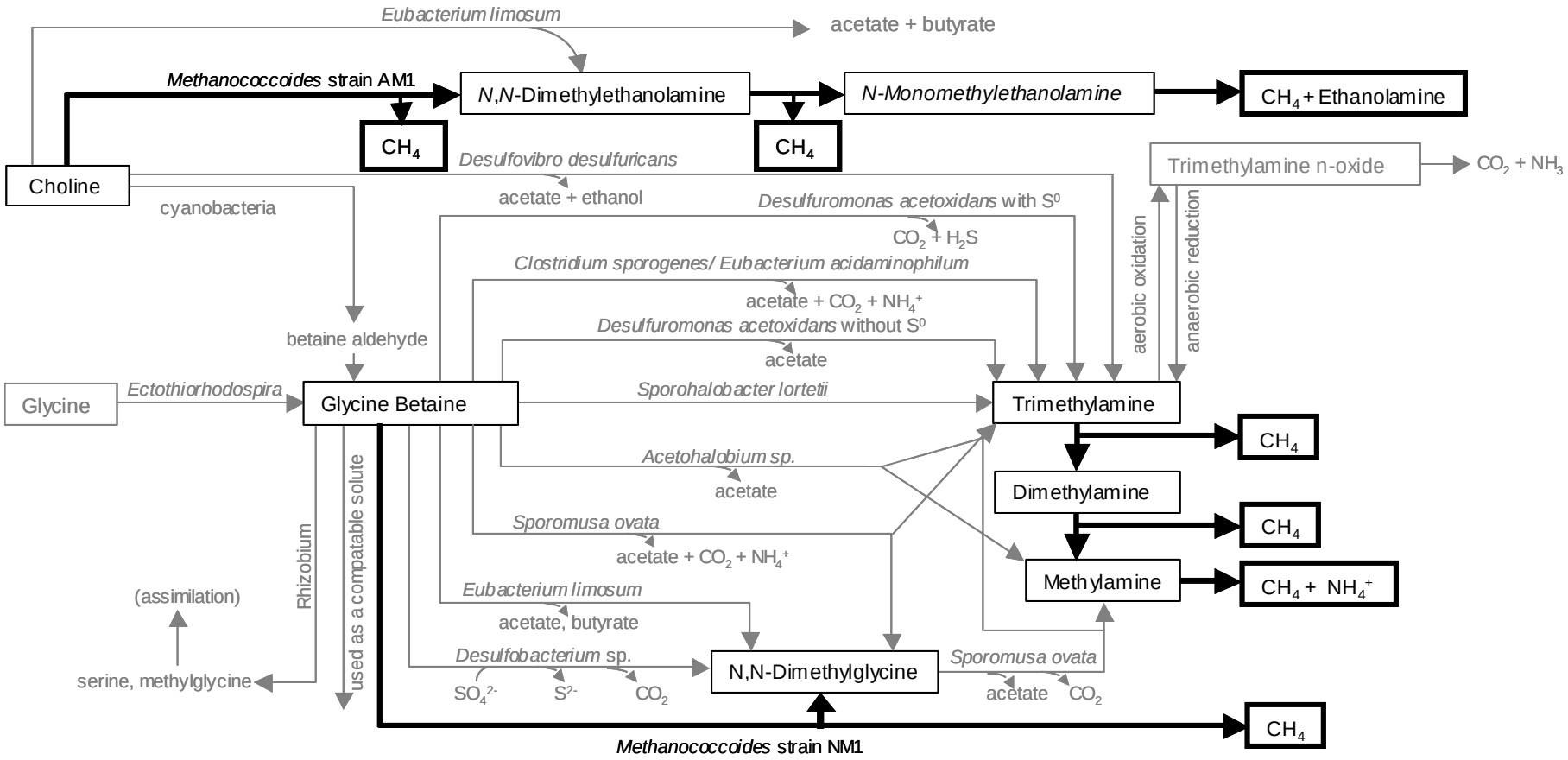


Figure 8.1 Degradation pathways of betaine and choline to trimethylamine by bacteria (gray) and the direct degradation of betaine and choline by methanogens isolated in this study (black). For details of the bacterial degradation of betaine and choline see Chapter 1. The methanogenic degradation of choline proceeds in an analogous manner to that of trimethylamine, the methyl groups are removed one at a time resulting in the production of two intermediate products. The end products of trimethylamine degradation by methanogens are methane and ammonium; the direct methanogenic degradation of choline produces methane and ethanolamine as end products. The difference in end products may be used to determine which pathway is being used to degrade choline. The direct methanogenic degradation of betaine appears to produce methane and dimethylglycine. Dimethylglycine may be degraded to methylamine and trimethylamine by bacteria such as *Sporomusa ovata.*

8.3.5 Origin of the Ability to Utilize Substrates

Novel genes can be acquired by means of gene duplication and the divergence in function of the duplicated gene (Ohno, 1970). The genome of *Methanococcoides burtonii* has been found to contain several copies of each of the methyltransferases for methanol, methylamine, dimethylamine and trimethylamine utilization and their associated corrinoid proteins (Allen *et al.*, 2009). Multiple copies of methyl transferases have also been identified in the genome of *Methanosarcina acetivorans* (Galagan *et al.*, 2002). The corrinoid proteins and methylcobamide:CoM methyltransferase of *Methanosarcina* have been studied by Burke *et al*. (1998), the corrinoid proteins for methylated thiols, methanol and methylamines are homologous to the B_{12} binding domain of methionine synthase. The three methylcobamide:CoM methyltransferases (one for the methylamines, one for methanol and one for methylated thiols) have a 50% sequence similarity to one another at the amino acid level and share an ancestor with uroporphyrinogen decarboxylase. In contrast, the methanol, methylamine, dimethylamine, and trimethylamine methyltransferases have no significant homology with one another (Paul *et al.*, 2000; Rother & Krzycki, 2010) and are therefore not a result of gene duplication. It was suggested by Burke *et al*. (1998) that the methylotrophic ability of *Methanosarcina barkeri* was due to the recruitment of existing protein families to perform new functions, their subsequent duplication and then dedication to a specific methylotrophic pathway and this would appear to be the case with the corrinoid proteins and methylcobamide:CoM methyltransferases.

The genomes of *Methanococcoides burtonii* DSM 6424^T, *Methanosarcina acetivorans* C2A, *Methanosarcina barkeri* Fusaro, and *Methanosarcina mazei* Gö1 have shown that, with one exception, all methylamine, dimethylamine and trimethylamine methyltransferase genes have an amber codon (the amber codon codes for pyrrolysine, the twenty-second amino acid) (Paul *et al.*, 2000). Among the seven trimethylamine, dimethylamine, or methylamine methyltransferase genes identified in *Methanococcoides burtonii* DSM 6424^T, only *mttB3* lacks an internal amber codon (Williams *et al.*, 2010a). The absence of MttB-3 in the expressed proteome is consistent with MttB-3 not being synthesized with pyrrolysine and not being able to function in the metabolism of trimethylamine (Williams *et al.*, 2010a). A number of reasons have been put forward to explain the need for an amber codon; pausing at the

amber codon during translation may allow proper folding of MttB-3, or it could be involved in residue modification that is necessary for catalytic activity (James *et al.*, 2001). The presence of an amber codon is not required in the genes that encode for methanol and methylthiol methyltransferases (Paul *et al.*, 2000). Rother (2010) has suggested that rather than the *mttB3* gene being non-functional it may be specific for tetramethylammonium methyltransferase, as the encoding gene for this has not yet identified. However, this study has determined that *M. burtonii* does not utilize tetramethylammonium as a substrate (Chapter 7). As *Methanococcoides burtonii* utilized *N*,*N*-dimethylethanolamine (Chapter 7) then MttB-3 could be a candidate for *N*,*N*-dimethylethanolamine methyltransferase.

Prokaryotes can also acquire new genes by way of horizontal gene transfer (HGT). HGT involves transfer of genes between different species, in contrast to the usual vertical inheritance. The genus *Methanosarcina* acquired its ability to use acetate as a growth substrate by HGT from an organism of the bacterial class *Clostridia* (Fournier & Gogarten, 2008). If utilization of the novel substrates identified in *Methanococcoides* is a result of HGT then where did the genes come from? The three strains that utilized DMS came from shallow water environments were *Methanosarcina* were also present (Chapter 3) so they are an obvious source. Alternatively, DMS utilization may have been a feature of the genera *Methanococcoides*, *Methanolobus* and *Methanosarcina* that has been lost by may strains. No functional or non-functional DMS methyltransferase was identified in the genome of *Methanococcoides burtonii* (Allen *et al.*, 2009). Several strains of bacteria that can utilize betaine and/or choline were described in Chapter 1 (see also Figure 8.1) and *Desulfovibrio* and *Clostridium* are possible source for the enzymes required for betaine and choline utilization. A difficulty with HGT is that an additional enzyme is not sufficient for the utilization of a methylated substrate, an associated corrinoid protein is also required and possibly a methylcobamide:CoM methyltransferase (Chapter 7). For the complete degradation of choline to methane and ethanolamine, three methyltransferase, three corrinoid proteins and at least methylcobamide:CoM methyltransferase protein are required.

8.3.6 Other Substrates

This study identified methyl iodide as both an inhibitor and as a substrate for *Methanococcoides methylutens* TMA-10^T depending on concentration (Chapter 7). Use of methyl iodide as a substrate has not been reported for any other methanogen and as only one strain was tested it is unclear whether this a common substrate for *Methanococcoides* sp. Utilization of methyl iodide, if this occurs under *in situ* conditions, would involve methanogens in the marine iodide cycle, a cycle in which methyl iodide plays an important role transporting iodine to the atmosphere where it participates in ozone destruction (Amachi *et al.*, 2001).

It has been mentioned (Chapter 1) that methane could be produced aerobically in seawater by the decomposition of methylphosphonic acid, a process involving nitrogen-fixing bacteria(Karl *et al.*, 2008). This study (Chapter 7) has found that methylphosphonic acid is not, however, a substrate for anaerobic methanogenesis.

8.3.7 Hydrogen Leakage

Methylotrophic methanogens have been observed to produce hydrogen at the expense of methane (Phelps *et al.*, 1985); (Finke *et al.*, 2007). For example, a co-culture of the methanogen *Methanosarcina barkeri* strain MS and the sulphate-reducing *Desulfovibrio vulgaris* strain Madison resulted in the methanogen diverting 58% of the reducing power from methane to hydrogen production and increased carbon dioxide production (Finke *et al.*, 2007). Methanogenesis using methylated substrates produces carbon dioxide and methane through an oxidative and a reductive pathway; electrons from the substrates methyl group are transferred from the oxidative to the reductive pathway to produce carbon dioxide (Figure 8.2). Depending on the ambient hydrogen concentration, electrons can be transferred to protons via hydrogenases to produce hydrogen rather than methane (Phelps *et al.*, 1985). The loss of hydrogen from the cell is controlled by the extracellular hydrogen concentration; hydrogenotrophic methanogens or sulphate-reducing bacteria determine the magnitude of hydrogen loss and sulphate-reducing bacteria reduce the environmental hydrogen concentration to a lower level than hydrogenotrophic methanogens (Section 1.3.3).

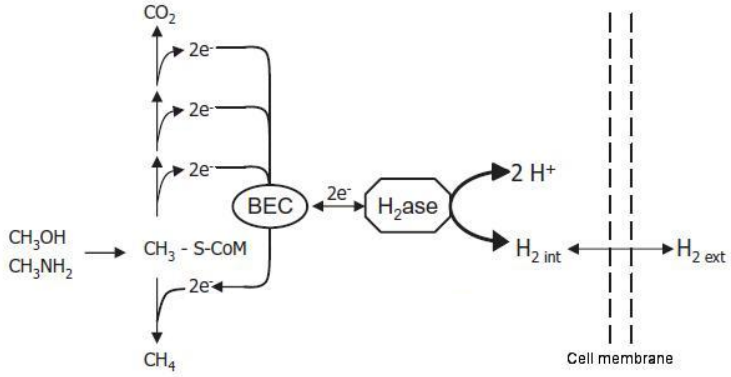


Figure 8.2 Methanogenesis pathways from methanol and methylamine. Biological electron carriers (BEC) transfer electrons from the oxidative pathway to the reductive pathway. BEC can also transfer electrons to protons via hydrogenases (H_2ase) to form H_2 if the ambient H_2 levels are low. Diagram from Finke *et al.* (2007).

Hydrogen leakage may be a benefit or a detriment to the methanogen depending on the external hydrogen concentration. In the presence of sulphate -reducing bacteria the leakage of hydrogen results in a favourable free energy yield: up to an extra 15 kJ mol^{-1} substrate if the energy can be conserved (Finke *et al.*, 2007). Under sulphate free conditions were the external hydrogen concentration is controlled by hydrogenotrophic methanogens the loss of reducing power as hydrogen represents a loss of free energy to the methanogen unless the methanogen can simultaneously utilize both methylated substrates and hydrogen, a situation that can occur in some strains of *Methanosarcina* (Finke *et al.*, 2007).

Hydrogen leakage would occur in methanogens with hydrogenases, the only genus of marine methylotrophic methanogen known to have hydrogenases is the genus *Methanosarcina*. It is not known if the required energy conservation mechanism exists in *Methanosarcina*, however, membrane-bound hydrogenases may be involved in energy conservation (Finke *et al.*, 2007) and such hydrogenases are present in *Methanosarcina* sp. (Deppenmeier 1996).

The marine *Methanosarcina* species *M. acetivorans* and *M. baltica* do not utilize hydrogen as a substrate (Sowers *et al.*, 1984; von Klein *et al.*, 2002a). The genome of *M. acetivorans* has been sequenced and compared to the genome sequences of *M. barkeri* and *M. mazei* (Galagan *et al.*, 2002). *M. acetivorans* lacks the F_{420}-

reducing hydrogenase encoded by the *fre* operon and the energy-conserving ferredoxin-dependent hydrogenase encoded by the *ech* operon. *M. acetivorans* does posses homologs of the *M. barkeri* F_{420}-reducing hydrogenase encoded by the *frh* operon and the homologs of two *M. mazei* operons encoding F_{420}-nonreducing hydrogenases. It is not known if *Methanolobus* sp. possess hydrogenases but *Methanolobus* sp. are not known to utilize hydrogen as a substrate (Whitman *et al.*, 2006). No hydrogenases were identified in the genome of *Methanococcoides burtonii* DSM 6242^T (Allen *et al.*, 2009) .

As noted in Section 3.4.4.2, this study cultured *Methanococcoides* with methylamine or methanol as a substrate except for one enrichment culture kept at 38 ºC and using freshwater medium (conditions not favourable to *Methanococcoides* strains cultured in this study, Chapter 6). Further work needs to be carried out to determine if hydrogen leakage gives an advantage to one genera rather than the other under sulphate and sulphate-free conditions.

8.4 *Methanococcoides* Environments

This study cultured *Methanococcoides* from a range of marine environments, tidal-flats, a shallow bay and deep-water sediments. Published strains have been isolated from a marine lake, a mangrove swamp and shallow water sediments. The environmental distribution of *Methanococcoides* was determined by a literature and NCBI database search. The distribution of *Methanococcoides* is illustrated in Figure 8.3 and details are given in Table 8.3.

Methanococcoides is predominantly found in marine environments (Figure 8.3 and Table 8.3). Exceptions are *Methanococcoides mcrA* gene sequences that were detected at Lake Wallendorf (Germany) but this is a saline lignite mine lake (NCBI database, accession number JF973601), the Greenland ice-sheet where *Methanococcoides* and other prokaryotes are believed to have been deposited by marine aerosols (Miteva *et al.*, 2009) and a terrestrial saline mud volcano in Taiwan (NCBI database, accession number GU553549). The genus has not been reported to be present in freshwater environments. A study of methanogens along the course of

the Colne estuary from freshwater to marine conditions identified *Methanococcoides* only at the marine end of the estuary (Purdy *et al.*, 2002).

Temperature ranges determined by this study and for published strain of *Methanococcoides* show that *Methanococcoides* are psychrotolerant or mesophilic (Chapter 4). *Methanococcoides* gene sequences have been detailed in a number of environments with low temperatures such as submarine permafrost sediments (Koch *et al*., 2009), cold anoxic brine (Perreault *et al*., 2007) and sediment at a seasonally frozen bay (Purdy *et al*., 2003). The upper temperature limit of the genus may be higher than that of the cultured strains (40 ^{o}C) as a *mcrA* clone from 9-11 cm sediment depth at the Guaymas Basin (temperature range of ~40-60 ^{o}C) has been identified as a member of the genus *Methanococcoides* (Dhillon *et al.*, 2005). However, this may not represent an active methanogen. Also in agreement with this study and properties of the described strains there are no reports in the literature of *Methanococcoides* under extreme pH conditions (this study has determined the highest pH for growth to be pH 8.9 and the lowest is pH 6.0, Chapter 6).

In addition to the water depths already mentioned, a number of molecular genetic studies have identified *Methanococcoides* in water depth 500-3850 m, Arakawa *et al*. (2006); Fang *et al*. (2006); Goffredi *et al.* (2008); Heijs *et al.* (2007); Hongchen *et al.* (2007); Lanoil *et al.* (205); Omoregie *et al.* (2009); Orphan *et al.* (2001); Sas (2009). At depths below about 1000 m seawater has a constant temperature of around 4–5 °C (Feller & Gerday, 2003) requiring *Methanococcoides* to be both piezophilic and psychrophilic.

Methanococcoides have also been identified in seawater. Seawater particles from the North Sea (north-west of Terschelling, Netherlands) were found to contain methanogens identified as *Methanococcoides methylutens*, >97% 16S rRNA sequence identity (van der Maarel *et al.*, 1999). The same study also found *Methanococcoides methylutens* in the digestive tract of a flounder (*Platichthys flesus*). Unlike *Methanobrevibacter* and *Methanosphaera* species, *Methanococcoides* are not known to be gut methanogens. It is possible that the presence of methanogens in the flounder was due to the ingestion of sediment particles rather than being permanent gut residents (van der Maarel *et al.*, 1999). This may be an area for further study.

Methanococcoides as well as members of the genera *Methanosarcina*, *Methanoculleus*, *Methanothermobacter*, *Methanosaeta*, *Methanopyrus* and *Methanocaldococcus* have been detected in the permanently oxic water of the Gulf of Aqaba (northern end of the Red Sea) (Ionescu *et al.*, 2009). An enrichment culture containing marine plankton from oxygenated seawater off the Californian coast was found to contain methanogens whose morphology, physiology and DNA base content was consistent with *Methanococcoides methylutens* (Cynar & Yayanos, 1991). These methanogens, and those in the Gulf of Aqaba study, may have been in the guts of herbivorous plankton, in faecal pellets or in aggregations of particles (Cynar & Yayanos, 1991).

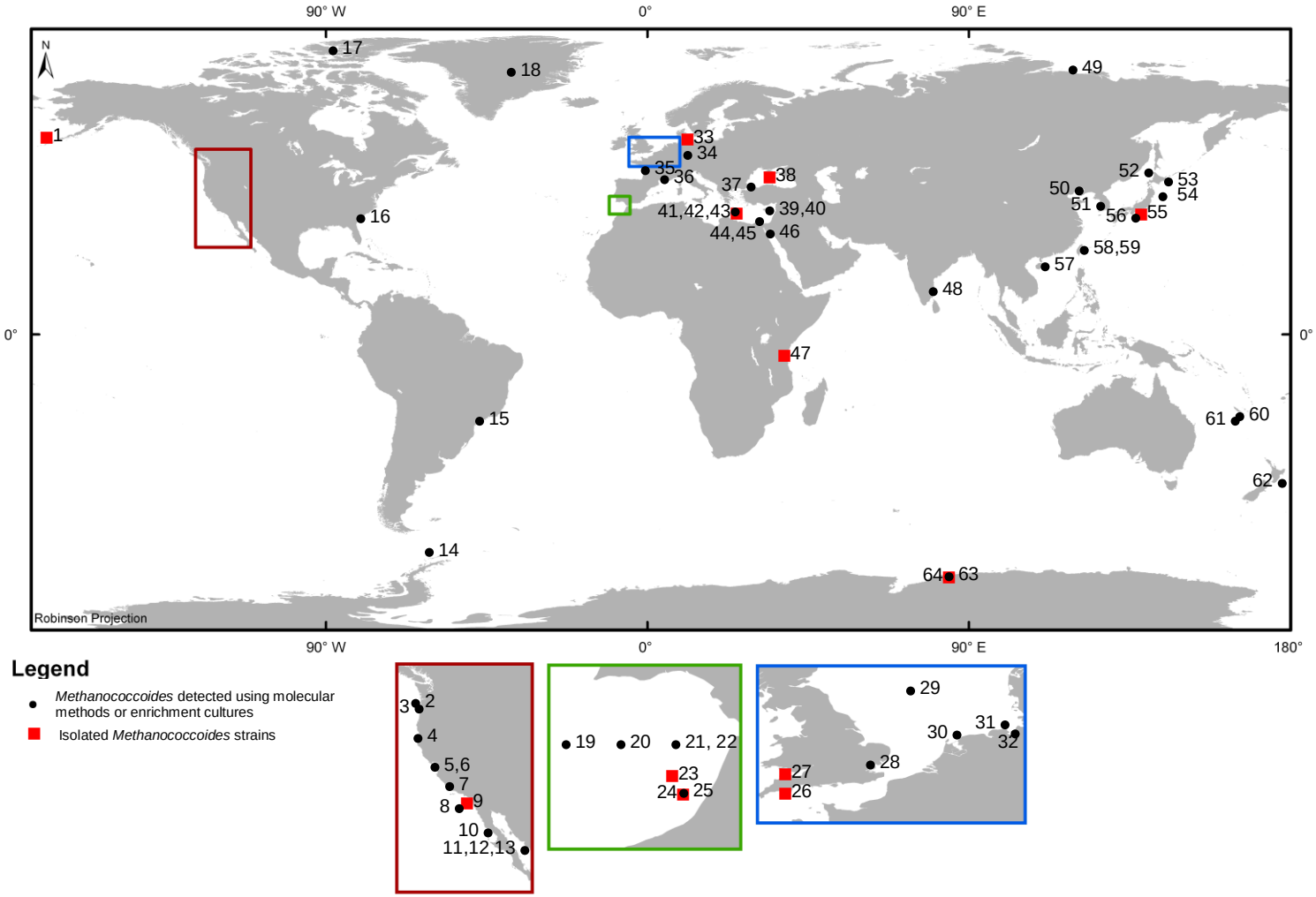


Figure 8.3 World map showing locations from which *Methanococcoides* strains have been isolated, cultured or detected by molecular means. See Table 8.2 for details of locations.

Table 8.3 Locations from which *Methanococcoides* strains have been isolated, cultured or identified by 16S rRNA or *mcrA* gene surveys. Location numbers refer to locations in Figure 6.2.

Location	Location	Comments	Reference
1	Skan Bay, Alaska	Type strain *Methanococcoides alaskense* AK-5^T and strain AK-9 from anoxic sediment (2-7 cm depth) 65 m below sea level. Sediment temperature range 1 to 6 ^oC.	Singh *et al.* (2005)
2	Cascadia Margin	16S rRNA clone sequence V.8ArD4 from core ODP892b 99% identity to *M. burtonii* This sediment from 675 m water depth contained methane hydrates.	Lanoil *et al.* (2005)
3	Hydrate Ridge, Oregon	Enrichment cultures identified by 16S rRNA sequencing, 98% identity to *M. methylutens*. Sediment taken from water depth of 800 m.	Kendall & Boone (2006)
4	Eel River Basin	16S rRNA clone sequence Eel-36a2HII from sediment at 4-7 cm depth, 99% identity to *M. burtonii*. Water depth ~500 m.	Orphan *et al.* (2001)
5	Monterey Canyon, California	16S rRNA clone sequence R8_0s_B2 (EU084526) from a whale-fall sediment, 2893 m water depth. 99% identity to *M. burtonii.*	Goffredi *et al*. (2008)
6	Monterey Canyon, California	16S rRNA clone sequence MC (F17.1_C05) from marine sediment.	Hallam *et al.* (2003)
7	Santa Barbara Basin	16S rRNA clone sequence SB-24a1C2 (AF354132), 99% identity to *M. alaskense*, from sediment at water depth ~500 m.	Orphan *et al.* (2001
8	Californian coast	*Methanococcoides* strain (based on morphology, physiology and DNA base content) from a marine plankton sample from the upper 125 m of the water column 140 km off the Californian coast.	Cynar & Yayanos (1991)
9	Scripps Canyon, La Jolla, California	Type strain *Methanococcoides methylutens* TMA-10^T from sediment (0 to 60 cm depth) 19 m below sea level.	Sowers & Ferry (1983)
10	Salton Sea , California, USA	Uncultured clone SS_WC_05 (FJ656257), 97% identity to *M. methylutens*, from water column (6 m depth) of hypersaline lake.	Swan *et al.* (2010
11	Guerrero Negro, Baja California Sur, Mexico	mcrA clone sequences ET3 D2B (ACD02064), AT1A4 (ACD02054), ET4 E11F (ACD02071), AH 1D6 (ACD02055), ET4 H3Cp (ACD02072) and ET2 H: ET3 B5C (ACD02061) from a hypersaline cyanobacterial mat under 1m of water.	Orphan *et al.* (2008)
11	Guaymas Basin (southern trough), Gulf of California, Mexico	mcrA clone sequence B09 (AY837774) from hydrothermal sediments, 95% identity to *M. alakense*. Sample from 9 to 11 cm depth, temperature ~40 to 60 ^oC.	Dhillon *et al.* (2005)
12	Guaymas Basin (southern trough), Gulf of California, Mexico	*mcrA* clone sequence Guaymas_37enr_mcrA62 (FR682809)	Holler *et al.* (2011)
13	Guaymas Basin, Gulf of California, Mexico	Enrichment culture, sediment taken from the northern trough of Guaymas Basin.	This study

Table 8.3 (continued) Locations from which *Methanococcoides* strains have been isolated, cultured or identified by 16S rRNA or *mcrA* surveys. Location numbers refer to locations in Figure 6.2.

Number	Location	Comments	Reference
14	Shallow Bay, Signy Island, South Shetland Islands	16S rRNA clones from sediment (0 to 1 0 cm deep), 99% identity to *M. burtonii*. Bay frozen during the winter and ice free during the summer.	Purdy *et al*. (2003)
15	Santos-Sao Vicente estuary, Brazil.	16S rRNA clone sequence E_H05 (AY454744) from the marine end of the estuary. 98% identity to *M. methylutens.*	Piza *et al*. (2005)
16	Sapelo Island, Georgia, USA	Sediment from a salt marsh. *Methanococcoides* identification based on morphology and substrate utilisation only.	Franklin *et al.* (1988)
17	Gypsum Hill, Axel Heiberg Island, Canada	16S rRNA clone sequence GH-A115(6) (DQ521136), 99% identity to *M. alaskense*, top 10 cm of sediment at a cold (6.9 °C) anoxic brine spring	Perreault *et al*. (2007
18	Greenland Icesheet	16S rRNA clone sequence K2 from the Greenland Ice Sheet Project Two (GISP2) ice core. Believed to have been deposited from marine aerosols.	Miteva *et al.* (2009)
19	Porto Mud Volcano, Gulf of Cadiz	Sediment from 3850 m below sea level.	Sas (2009)
20	Carlos Ribeiro mud volcano, Gulf of Cadiz	*Methanococcoides* enrichment culture from Carlos Ribeiro mud volcano sediment.	This study
21	Captain Arutyunov mud volcano, Gulf of Cadiz	*Methanococcoides* enrichment cultures from Captain Arutyunov mud volcano sediment.	This study
22	Captain Arutyunov mud volcano, Gulf of Cadiz	Enrichment culture clone sequence CpA ArcA33 (FN547401) from 750 m below sea level.	Sas (2009)
23	Darwin mud volcano, Gulf of Cadiz	*Methanococcoides* strain DM1 isolated from Darwin MV sediment.	This study
24	Meknes mud volcano, Gulf of Cadiz	*Methanococcoides* strains MKM1 & MKM2 isolated from Meknes MV sediment.	This study
25	Meknes Mud Volcano, Gulf of Cadiz	Enrichment culture clone (FN547401) from 750 m below sea level.	Sas (2009)
26	Tamar estuary, UK	*Methanococcoides* strains TM1 & TM2 isolated from Tamar estuary.	This study
27	Portishead, UK	*Methanococcoides* strains PM1, PM2 & PM3 isolated from Portishead intertidal sediment	This study
28	Colne Point, Colne estuary, Colchester, UK	16S rRNA clone sequence 2C30(CP) (AF015972) from marine sediment, 98% identity to *M. methylutens*.	Purdy *et al*. (2002)
29	Tommeliten Seep, EKOFISK, North Sea	16S rRNA clone sequence Tomm05_1274_3_Arch90 (FM179838), 99% identity to *M. methylutens*, from marine sediment 75 m below sea level.	Wegener *et al*. (2008

Table 8.3 (continued) Locations from which *Methanococcoides* strains have been isolated, cultured or identified by 16S rRNA or *mcrA* surveys. Location numbers refer to locations in Figure 6.2.

Number	Location	Comments	Reference
30	North Sea near Terschelling, Netherlands	16S rRNA clone sequences from suspended particulate matter in the North Sea and 16S rRNA clones from the intestine of a flounder (*Platichthys flesus*). 97% sequence identity to *M. methylutens*.	Van der Maarel *et al*. (1999)
31	Island of	16S rRNA PCR-DGGE of sediment from a back-barrier tidal-flat, 95% sequence identity to *M. methylutens*.	Wilms *et al*. (2006)
32	Jade Bay, Germany	16S rRNA clone sequences TfC20L41Ar, TfC20L51Ar, TfC20L24Ar and TfP20L25/41Ar from tidal flat sediment at a meso- to macrotidal embayment	Kittelmann & Friedrich (2008)
33	Aarhus Bay, Denmark	*Methanococcoides* strains AM1, AM2 and AM3 isolated from Aarhus Bay sediment.	This study
34	Lake Wallendorf, Germany	McrA sequence (JF973601) from a saline lignite mine lake. 94% sequence identity to *M. alaskense*.	NCBI database
35	Marennes-Oleron Bay, French Atlantic coast	mcrA clone sequence M43 DNA 0 cm bsf MOBOcr43040 (AM942090) and M43 DNA 90 cm bsf MOBOcr43977 (AM942096) from a macro-tidal bay sediment. 95% identity to *M. alaskense*.	Roussel *et al*. (2009a)
36	Gulf of Fos, French Mediterranean coast	16S rRNA clones, 99% identity to *M. burtonii*, from sediments at 20 m water depth and temperature 9 to 19 ^{o}C.	Miralles *et al.* (2010)
37	Sea of Marmara, Turkey	16S rRNA clone sequence Ma29_4a_82 (HM109892), 99% identity to *M. methylutens*.	Quaiser *et al.* (2011)
38	Sorokin Trough, Black Sea	*Methanococcoides* strains BM1, BM2 & BSM3 isolated from Black Sea sediment.	This study
39	Amsterdam MV	16S rRNA clone sequence AMSMV-25-A12 (HQ588678), 96% identity to *M. methylutens*.	Pachiadaki *et al*. (2011)
40	Kazan mud volcano, Eastern Mediterranean Sea	16S rRNA clone sequence Kazan-3A-09/BC19-3A-09 (AY592033), 95% identity to *M. methylutens* from 1673m water depth.	Heijs *et al*. (2007)
41	Napoli Mud Volcano, Eastern Mediterranean Sea	Enrichment culture clone NapK-0_20-enr35 (HM004946) 99% 16S rRNA sequence identity to *M. methylutens.*	Lazar *et al*. (2011)
42	Napoli Mud Volcano, Eastern Mediterranean Sea	*Methanococcoides* strains NM1 & NM2 isolated from Napoli MV sediment.	This study
43	Napoli Mud Volcano, Eastern Mediterranean Sea	Enrichment culture clone NapMat-0_2-enr30, 98% 16S rRNA sequence identity to *M. methylutens.*	Lazar *et al*. (2011a)

Table 8.3 (continued) Locations from which *Methanococcoides* strains have been isolated, cultured or identified by 16S rRNA or *mcrA* surveys. Location numbers refer to locations in Figure 6.2.

Number	Location	Comments	Reference
44	Nile Deep Sea Fan, Amon Mud volcano	16 rRNA similar to *Methanococcoides*. Cultured with methanol. Marine sediment 1120 m below sea level.	Omoregie *et al.* (2009)
45	Nile Deep Sea Fan, Isis Mud volcano	16 rRNA similar to *Methanococcoides*. Cultured with methanol. Marine sediment 992 m below sea level.	Omoregie *et al*. (2009)
46	Gulf of Aqaba, northernmost part of the Red Sea	16S rRNA clone sequences related to *M. burtonii* from oxic seawater.	Ionescu *et al.* (2009)
47	Tanzania	*M. methylutens* strain MM1 isolated from a mangrove forest sediment.	Lyimo *et al*. (2009)
48	Pichavaram, SE India	Mangrove swamp isolate. Morphology, physiology and DNA base content consistent with the type description of *M. methylutens*.	Mohanraju *et al.* (1997)
49	Cape Mamontovy Klyk, Siberia	16S rRNA clone sequences subM_C2_1246c (EU489462) and subM_C2_1246d (EU489463) from submarine permafrost sediment samples near Cape Mamontovy Klyk.99% identity to *M. burtonii.*	Koch *et al.* (2009)
50	Bohai Bay, China	16S rRNA clone sequence SCA81, 99% sequence identity to *M. alaskense*, from contaminated soil in the Jidong Oilfield.	Liu *et al.* (2009)
51	Ganghwa Island, Korea	16S rRNA clone sequence BS1-1-84, 99% identity to *M. methylutens*, from tidal-flat sediments.	Kim *et al.* (2005)
52	Sagami Bay, Japan	16S rRNA clone (AB188805), 99% identity to *M. alaskense*, from cold seep sediment at a depth of 1174 m.	Fang *et al.* (2006)
53	Shiribeshi seamount, north-eastern Japan Sea	16S rRNA clone sequence AJS72-22 (AB239074) and AJS72-003 (AB239072), both with a 95% identity to *M. methylutens*, from a deep-sea (~ 3000 m) cold seep sediment.	Arakawa et al. (2006)
54	Shimokita Peninsula, Japan	Sediment from 1180m water depth. *Methanococcoides* cultured using a continuous flow bioreactor rather than batch culture.	Imachi *et al.* (2011)
55	Tokyo Bay, Japan	*Methanococcoides* strain NaT1 isolated from sandy sediment, first strain shown to grow of tetramethylammonium.	Tanaka (1994)
56	Japan Trench	16S rRNA clone sequence JTA175 (AB015279), 99% identity to *M. alaskense*, from sediment at a depth 6400 m below sea level.	Li *et al.* (1999)
57	Qiongdongnan Basin, China	16S rRNA clone sequence SCS-QBS-A36 (EF104090), 99% identity to M. alaskense, from the continental slope of the Qiongdongnan Basin at a water depth of 1508 m.	Hongchen *et al*. (2007)

Table 8.3 (continued) Locations from which *Methanococcoides* strains have been isolated, cultured or identified by 16S rRNA or *mcrA* surveys. Location numbers refer to locations in Figure 6.2.

Number	Location	Comments	Reference
58	Yung-An Ridge, SW Taiwan	16S rRNA clone sequence ORI-860-26-P_S008-010_261A05 (GU553549), 98% sequence identity to *M. alaskense*, from methane seep sediment	NCBI database
59	Lei-Gong-Huo mud volcano, Taiwan	16S rRNA clone sequence LGH02-A-02 (HQ916478), 97% identity to *M. methylutens*, saline terrestrial mud volcano	NCBI database
60	Fairway Basin, New Caledonia	16S rRNA clone sequence 3022T75G71 (AM989382) from subsurface marine sediment. 99% sequence identity to *M. methylutens.*	Roussel *et al.* (2009b)
61	New Caledonian Basin, New Caledonia	16S rRNA clone sequences 3018T15E52 (AM989361) and 3018T240E87(AM989369) from subsurface marine sediment. 99% sequence identity to *M. methylutens*.	Roussel *et al.* (2009b
62	Hikurangi Margin, New Zealand	16S clone sequence NZ_309_Arch75 (JF268337), 96% sequence identity to *M. methylutens*, marine methane seep at 1056 m water deep.	NCBI database
63	Ace Lake, Vestfold Hills, Antarctica	Type strain *Methanococcoides burtonii* DSM 6242^T isolated from water at a depth of 24 m and temperature < 2^{o}C. Salinity similar to seawater.	Franzmann *et al*. (1992)
64	Ace Lake, Vestfold Hills, Antarctica	16S rRNA clone sequence ACE3_A (AF142978) from anoxic lake at a depth of 25 m at a temperature of 2.5 ^{o}C. 99% sequence identity to *M. burtonii.*	Bowman *et al.* (2000)

8.5 Methylated Substrates in Marine Sediments

Given the wide distribution of *Methanococcoides* (Table 8.3) and its ability to utilize only methylated compounds as growth substrates (Table 7.1) this implies a wide distribution of methylated compounds in marine sediments. The ^{13}C fractionation study by Whiticar (1986) indicates that marine methanogenesis occurs primarily by the reduction of carbon dioxide. *Methanosarcina barkeri* grown by Londry (2008) on acetate, H_2/CO_2, methanol and trimethylamine under substrate limited and substrate replete conditions resulted in considerable variation in the ^{13}C content of the methane produced. The discrimination against ^{13}C during methanogenesis was similar for methylated substrates and H_2/CO_2; acetate did result in a lower fractionation (Londry *et al.*, 2008). Conrad (2005) also found that cultures producing methane from methanol and trimethylamine (*Methanosarcina* and *Methanococcoides*) exhibited large fractionation factors that could be mistaken for H_2/CO_2 methanogenesis. In addition, Summons (1998) determined that the fractionation of trimethylamine methanogenesis was in the range of H_2/CO_2 fractionation.

Methylamines are present in the porewater of marine sediments and bound in an exchangeable form to sedimentary particles (Wang & Lee, 1990). The affinity of methylamines for particles depends on the clay and organic matter content and competition with cations in seawater as methylamines occur predominantly as protonated compounds at the pH of seawater or lower (Wang & Lee, 1990). A number of studies have determined the environmental concentrations of methylamines (Table 8.4). Methylamines were found to occur throughout the year in sediment porewaters and in the solid phase in anoxic sediments of a salt marsh at Flax Point (New York, USA) (Wang & Lee, 1994). Calculations by Wang & Lee (1994) indicated that 30-130 mmol methylated amines m^2 y^{-1} could be produced solely by *S. alterniflora* decomposition at Flax Point. Measurement of dissolved methylamines in the Thames estuary over a tidal cycle showed that increases in concentration were coincident with the remobilisation of seabed sediments (Fitzsimons *et al.*, 2006). Model calculations showed that desorption of methylamines from the remobilised sediments accounted for > 90% of the concentration increase.

At Norsminde Fjord (Denmark) both the seasonal efflux of trimethylamine and its distribution pattern were found to be influenced by burrowing infauna (Sørensen &

Glob, 1987). Significant accumulations of trimethylamine were limited to the upper few centimetres of sediment with dense populations of benthic invertebrates (Sørensen & Glob, 1987). The highest trimethylamine concentrations (10 μM) occurred together with the highest faunal density in spring and summer. The distribution of trimethylamine in saltmarsh sediments was also found to related to the abundance of benthic invertebrates (Wang & Lee, 1994). *Nereis succinea* (common clam worm) released methylamine at a rate > 1 mmol day^{-1} when immersed in seawater. *Corophium volutator* (mud shrimp) in estuarine sediments were found to release trimethylamine when placed in low-salinity water (Sørensen & Glob, 1987). However, a study of a mudflat at Burnham Overy Staithe (Norfolk, UK) did not find any correlation between salinity and dissolved methylamines (cores were taken at different stages of the tidal cycle) (Fitzsimons *et al.*, 2001). The salinity effects may have been masked by adsorption, diffusion or microbial uptake of methylamines (Fitzsimons *et al.*, 2001). Methylamines have not only been detected in coastal sediments, low levels have also been reported in continental shelf sediments (Table 8.4). Pore water and sediment concentrations of methylamines from a variety of marine locations including salt marshes, inter-tidal zones and continental shelves are given in Table 8.4.

Reactions of proteins and amino acids have been proposed as an alternative source for methylamines by Mitterer *et al* (2001). Cores (500 m long) taken from the Great Australian Bight (off the southern coast of Australia) contained sulphate concentrations 15-50 mM throughout their length as well as methane. $\delta^{13}C$ values of - 76 to -80 ‰ are in the usual range for microbially produced methane. In addition, the maximum down-hole temperatures ~25-30 ºC were too low to have generated any significant amounts of thermogenic methane. Proteins and amines are usually mineralised or otherwise incorporated into humic substances before burial; however, biogenic carbonates (unlike siliciclastic sediments) usually have a protein matrix in the mineral structure. The proteins are thereby protected from rapid mineralisation or incorporation into humic substances. After burial the proteins can be hydrolyzed to free amino acids that in turn can be converted to amines. The cores contained 0.1 –1.0 nM mg^{-1} dry sediment glycine and 12-50 nM g^{-1} dry sediment methylamine. The decarboxylation of glycine yields methylamine.

Table 8.4 Pore water and sediment concentrations of methylamines from a variety of marine locations.

Pore water (μM)			Sediment (μmol/g dry weight)			Envionment	Reference
MMA	DMA	TMA	MMA	DMA	TMA		
bdl	48	29	bdl	007	0.02	Buzzard Bay (Massachusetts, USA)	Lee & Olson (1984)
1.9	2.1	4.3	nd	nd	nd	Continental shelf	Ferdelman *et al* (1997)
bdl	0.5	0.1	bdl	0.02	0.002	Continental margin of Mexico	Lee & Olson (1984)
1.0	0.3	0.04	nd	nd	nd	Continental slope, Louisiana, USA	Whelan *et al* (1985)
bdl	nd	15	nd	nd	nd	Estuarine sediment	Glob & Sorensen (1987)
1.4	1.9	2.2	8.4	8.0	10.0	Estuarine sediment	Fitzsimons *et al* (2006)
nd	nd	2.2-2.4	nd	nd	nd	Intertidal zone	King *et al* (1984)
2	3.6	0.6	0.6	0.02	0.1	Salt marsh, Flax Pond, New York, USA	Wang & Lee (1994)
0-319	0-9	1-43	0.05-1.91	0.01-0.34	0.01-0.98	Salt marsh, Oglet Bay, Mersey, UK	Fitzsimons *et al* (1997)
nd	nd	4.7	nd	nd	4.6	Salt marsh, Burnham Overy Staithe, UK	Fitzsimons *et al* (2001)

bdl, below detection limit
nd, not determined

8.6 Future Work

This study extended the range of growth substrates for the genus *Methanococcoides*. The novel *Methanococcoides* catabolic substrates betaine, choline and *N*,*N*-dimethylethanolamine and other methylated substrates should also be tested with the type strains of *Methanolobus* and *Methanosarcina* as direct betaine/ choline utilization may be present in other methylamine utilizing marine methanogens. Whilst dimethylglycine and sarcosine were not found to be substrates in this study, they may be substrates for other genera of methanogens. It will also be interesting to test betaine as a catabolic substrate with *Methanohalophilus* strains. As this is a halophilic genus then there would be a ready supply of betaine in high salt environments. Some *Methanohalophilus* strains make use of betaine as a compatible solute, however, there are strains including *Methanohalophilus mahii* SLP^T where betaine has not been identified as a compatible solute (Lai *et al.*, 1991), they may use betaine as a growth substrate rather than as a compatible solute. These strains contain the compatible solutes L-α-glutamic acid, β-glutamine and *N*,-acetyl-β-lysine (Lai *et al.*, 1991). Betaine should also be tested with the *Methanosarcina* strains that have been identified as using betaine for osmoregulation to see if betaine would be used as a growth substrate if no other substrate were available.

The isolation and characterization of other methanogens enriched in this study requires further work. It may prove difficult to isolate methanogens enriched from the Tamar estuary as several genera of methanogens are present in many of the enrichment cultures (Chapter 3). As methanogens that can grow under normal marine salinity and freshwater conditions are present in Tamar and Portishead tidal flat sediments, the use of a salinity gradient to culture methanogens would provide information on the distribution of methanogens along the estuary and may aid the in the isolation of methanogens.

The two *Methanococcus* strains (PM4 and PM5) isolated by this study need to be fully characterized, no tests had yet been undertaken to determine salinity pH and temperature ranges.

The two genera of methanogens cultured from mud volcanoes in this study may reflect the low diversity of methanogens at these locations or that a batch enrichment process was not suitable to culture additional genera from these locations. A cultivation approach using a continuous-flow bioreactor that was successfully used by Imachi *et al.* (2001) may yield further genera of methanogens from these locations.

Co-culture studies of *Methanococcoides* and *Methanosarcina* with methylamines and methanol should be undertaken to determine the conditions under which the two genera can co-exist or out-compete each other. In particular, the culturing of *Methanococcoides* and *Methanosarcina* with sulphate-reducing medium and sulphate-reducing bacteria to determined whether hydrogen leakage by *Methanosarcina* would give it an advantage over *Methanococcoides*. This study could also be extended to include the other marine methylotrophic methanogen, *Methanolobus*.

As methylated compounds appear to be important substrates for methanogenesis in marine sediments then studies need to be undertaken the concentrations of such compounds in a wide range of marine environments and attempts made to determine the degradation routes of substrates such as betaine and choline. Further investigation is also required to determine the significance of the ability of a number of *Methanococcoides* strains to grow on tetramethylammonium.

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Appendix

Bacteria Isolated from Methanogen Enrichment Cultures

During the course of this study, three strains of bacteria were isolated from methanogen enrichment cultures using shake tubes/ dilution to extinction and YPG marine medium. Strain AB1 was isolated from Aarhus Bay enrichment culture AM-M (Chapter 3), strain NB1 was isolated from Napoli MV enrichment culture NMV-M, and strain TB1 was isolated from Tamar enrichment culture T20M.

Strain AB1 had a 98% 16S rRNA sequence identity to *Lutibacter litoralis* strain CL-TF09 (NR_043301). Figure A.1 is a 16S rRNA phylogenetic tree including strain AB1. Strain NB1 had a 93% 16S rRNA sequence identity to *Fusibacter paucivorans* strain SEBR 4211 (NR_024886) and strain TB1 had a 89% 16S rRNA sequence identity to *Coprococcus catus* strain GD/7 (EU266552). Figure A.2 is a 16S rRNA phylogenetic tree including strains NB1 and TB1.

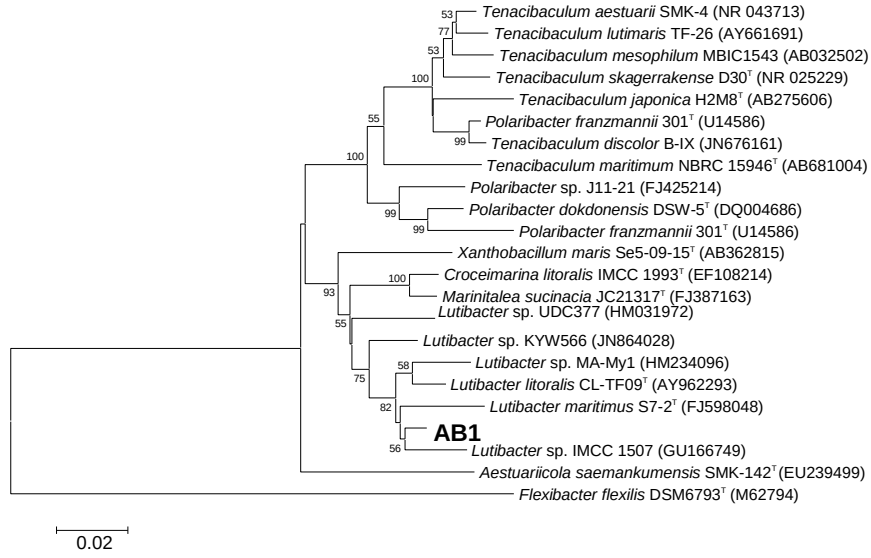

Figure A.1 16S rRNA gene sequence phylogenetic tree (neighbour-joining) including bacterial strain AB1 isolated from an Aarhus Bay methanogen enrichment culture. Accession numbers in brackets. Numbers at nodes are percentage bootstrap values based on 1000 replicates (only bootstrap values >50 are shown). Bar, 0.01 substitutions per site.

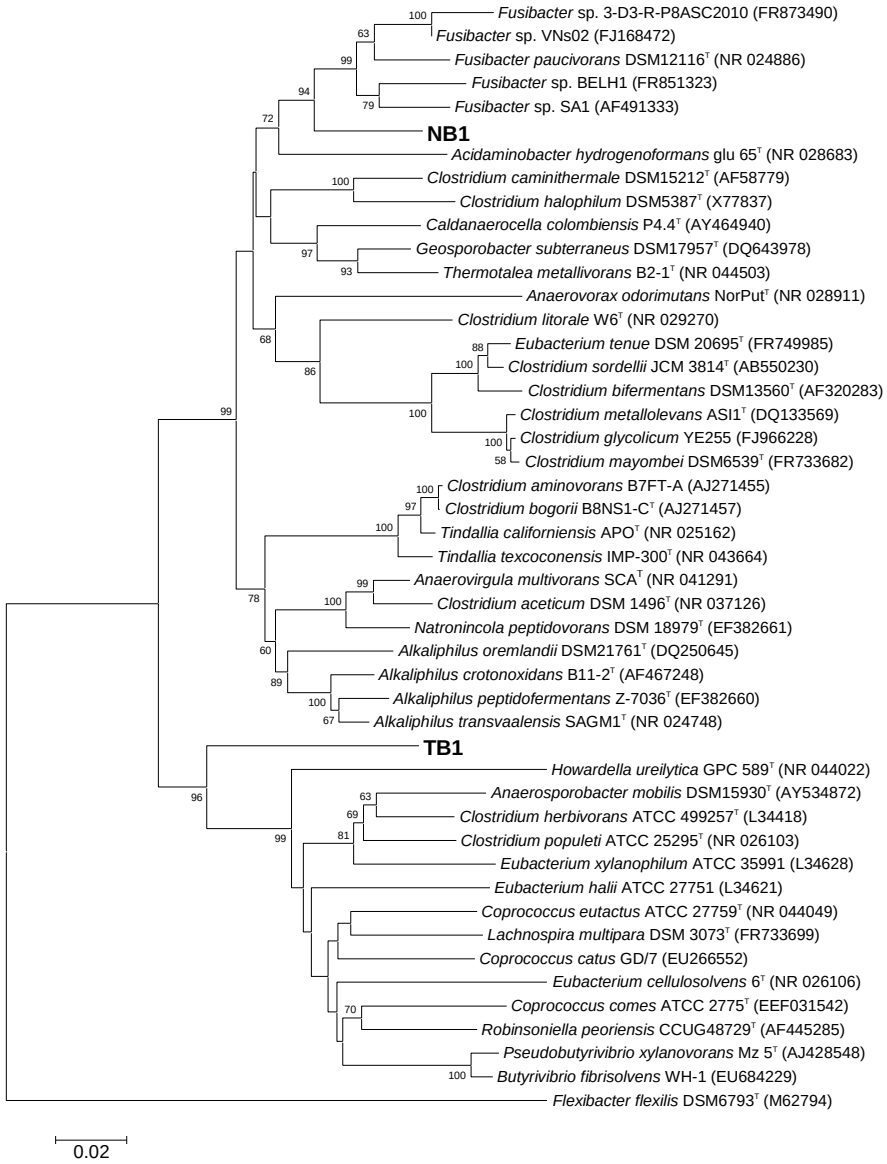

Figure A.2 16S rRNA gene sequence phylogenetic tree (neighbour-joining) including bacterial strains NB1 and TB1 isolated from a Napoli MV methanogen enrichment culture and a Tamar estuary methanogen enrichment culture, respectively. Numbers at nodes are percentage bootstrap values based on 1000 replicates (only bootstrap values >50 are shown). Bar, 0.01 substitutions per site.

Strains AB1 and NB1 have not been characterised; strain TB1 has been partially characterised (characterisation undertaken by Dr Henrik Sass). Strain TB1 was rod-shaped with one to three subterminal to lateral flagella (Figure A.3). The strain did not form spores. The pH range was 5.5-8.9. Tested substrates are listed in Table A.1.

Table A.1 Bacterial strain TB1: substrate utilization. The concentration of the potential substrate in mM is in parenthesis (except YE and peptone were a percentage is given).

Potential Substrate	Utilization
YE (0.5%)	-/+
Peptone (0.4%)	-/-
Sucrose (10)	++/++
Cellobiose (4)	-/-
Lactose (8)	-/-
Maltose (10)	-/-
Trehalose (3)	+
Fructose (20)	+
Fucose (20)	-/-
Galactose (20)	-/-
Glucose (20)	+
Mannose (20)	+
Arabinose (20)	-/-/+
Ribose (20)	+
Xylose (20)	-/-
Gluconate (20)	(+)/+
Glucosamine (20)	+/-
Mannitol (20)	+
Sorbitol (20)	-/-/-/-
Erythritol (10)	(+)/-/-
Rhamnose	-/-
Formate (20)	-/-/+/+
Acetate (20)	-/-
Propionate (10)	-/-
Butyrate (15)	-/-
Methanol (10)	-/-/-/-
Propanol (10)	-/-
Butanol (10)	-/-
Ethylene Glycol	-/-/-
Aspartate (20)	+/+
Cysteine (20)	-/-
Glutamate (20)	-/-
Histidine (12.5)	-/-
Lysine (20)	-/-
Methionine (12.5)	+
Phenylalanine (10)	-
Proline (20)	(+)/-/+
Serine (20)	-/-
Threonine (20)	+/(+)
Tryptophan (5)	-/-
Tyrosin	+/+
Betaine	-/-/-/+

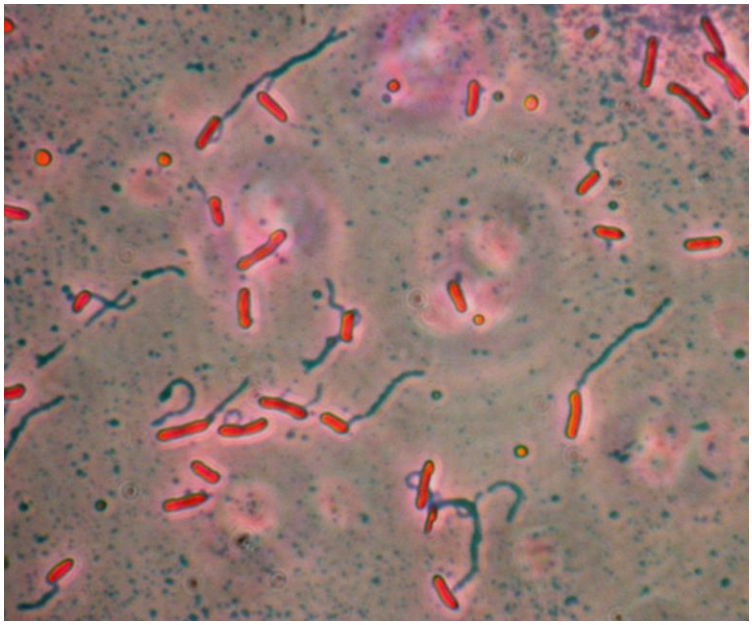

Figure A.3 Phase contrast photomicrograph of strain TB1 stained with flagella stain.