Temperature and salinity controls on methanogenesis in an artificial freshwater lake (Cardiff Bay, Wales)

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Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

September 2015

Cardiff University
School of Earth and Ocean Sciences
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SUMMARY

Cardiff Bay is an artificial freshwater lake created by the impoundment of 100 ha of estuarine intertidal mudflats in the early 2000s. This dramatic environmental change is reflected in the sedimentary record as a sharp transition between lacustrine gyttja and estuarine clay sediments. This study utilises a combination of geochemical, molecular genetic and novel cultivation based approaches to explore how the methanogen community of Cardiff Bay sediments has responded to the transition from brackish to freshwater conditions caused by impoundment, and how they might respond to future climate change.

Microbial methanogenesis is active in newly deposited Cardiff Bay lacustrine gyttja sediments. Porewater methane concentrations regularly exceed saturation limits, suggesting that ebullition may be a direct pathway for atmospheric methane emissions. Sediment slurry incubations suggest that methane production in Cardiff Bay sediments would be further enhanced by both increased substrate availability and climate relevant temperature increases. Hydrogenotrophic methanogenesis mediated by the genus Methanobacterium is the major pathway for methane production in Cardiff Bay, which is atypical for either estuarine or freshwater environments, and reflects the importance of recalcitrant terrestrial carbon inputs. In fact, cultivation based experiments provide tentative evidence that syntrophic acetate and methylamine utilizing consortia involving hydrogenotrophic methanogens were able to outcompete acetotrophic and methylotrophic methanogens under certain environmental conditions.

The temperature and salinity ranges and characteristics, and substrate types, of Cardiff Bay methanogenic communities varied little between sediments deposited pre- and post-impoundment. Differences between the methanogen community composition of lacustrine gyttja and estuarine clay sediments were driven predominantly by a reduction in the quantity and availability of organic matter with increasing sediment depth. A phylogenetically and physiologically diverse range of methanogens were enriched from Cardiff Bay sediments, including thermophilic methanogens and selected marine methanogens. Methanogen strains isolated from Cardiff Bay were closely related to cultivated strains, yet displayed novel physiological characteristics. These included a strain of the thermophilic genus Methanothermobacter able to produce methane at 25 °C, and a strain of the marine genus Methanolobus which grew under freshwater conditions (0.01 M NaCl).
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1. INTRODUCTION

1.1. Prokaryotes and biogeochemical cycling

On sub-tectonic timescales the biogeochemical cycles of the elements fundamental to life: hydrogen, carbon, nitrogen, oxygen and sulphur, are driven predominantly by microbiologically mediated redox reactions (Falkowski et al. 2008). These redox reactions couple the transformation of inorganic elements to release of energy which is utilised for biosynthesis, and are under thermodynamic control. The biogeochemical cycles of these elements are coupled, both directly and indirectly, due to assimilatory (accumulation of elements during biosynthesis) and dissimilatory (microbial oxidation or reduction of inorganic compounds) processes (Burgin et al. 2011). Prokaryotes also play a fundamental role in biogeochemical cycling of other critical elements such as iron, manganese, phosphorus and sulphur (Ehrlich and Newman 2008).

1.2. Energy conservation in prokaryotes

Reactions which transfer electrons from one reactant to another, known as redox reactions, are the basis for energy utilization in living organisms. Electrons are transferred from the energy source with most negative electrode potential (the primary electron donor or PED) to the energy source with most positive electrode potential (the terminal electron acceptor or TEA). The electron donors necessary for redox reactions are generated either via light energy (phototrophy) or from oxidation of chemical compounds (chemotrophy). Chemotrophs are divided into those that utilise inorganic compounds (chemolithotrophs) or organic compounds (chemoorganotrophs) as an electron donor. The metabolism of prokaryotes can be further divided into those which utilise inorganic carbon molecules (autotrophs) or organic carbon molecules (heterotrophs) as a carbon source for biosynthesis.

Energy released during redox reactions is transferred by creation of molecules such as adenine tri-phosphate (ATP), which are later hydrolysed to provide energy for biosynthetic reactions. Electrons are transferred along a redox gradient between the PED and TEA via a series of intermediate carrier enzymes associated with the plasma membrane. The specific carrier enzymes utilised vary depending on the redox pair utilised. The carrier membranes are ordered according to their electrode potential,
from negative to positive, resulting in stepwise release of energy as electrons are passed down the redox chain. Electrons are transferred between electron acceptors within the redox chain by intermediate electron carriers such as nicotinamide adenine dinucleotide (NAD+). NAD+ is reduced to NADH by acceptance of 2 electrons and 1 proton. Protons are released into the cell cytoplasm by NADH. Simultaneously, protons are translocated from one side of the cell membrane to the other, creating an electrochemical gradient across the cell membrane called the proton motive force (PMF). Protons diffuse back into the cytoplasm, along the proton motive force, via membrane bound enzymes such as adenosine triphosphatase (ATPase). The energy generated by diffusion of the protons into the cell drives phosphorylation of adenosine diphosphate (ADP) to form ATP. This process is known as oxidative phosphorylation, and is utilised by all organisms which conserve energy via chemiosmosis.

$$\Delta G^0 = -nF(E^{\circ}\text{PED} - E^{\circ}\text{TEA})$$

\(n\) = number of transferred electrons  
\(F\) = Faraday constant (96500 kJ V\(^{-1}\))  
\(E^{\circ}\) = electrode potential

*Equation 1.1.1. Energy yield of a redox reaction under standard conditions with respect to electrode potential of electron donor and electron acceptor*

The net free energy change of the reaction (Equation 1.1.1.), referred to as \(\Delta G^0\), is determined by the difference in standard electrode potential \(E^{\circ}\) between the electron donor and the terminal electron acceptor under standard conditions (pH 7, 25°C, concentrations of products and reactants 1 M, partial pressure of gases at 1 bar). If \(\Delta G\) is negative then the reaction is exergonic, and can proceed without additional energy input. Estimates of the minimum \(\Delta G\) required in order for prokaryotes to utilise the energy released from redox reactions range from around 10-20 kJ mol\(^{-1}\) (Schink 1997; Hoehler et al. 1998). There are two main modes of energy metabolism: respiration and fermentation. In respiration NADH is recycled to NAD+ within the cell by transfer of electrons to the TEA. No external TEA or electron transport chain is required for fermentation of organic compounds. Instead pyruvate is reduced by NADH to form various different compounds (depending on the metabolism of the bacteria) via internally balanced redox reactions during which oxidation of an organic molecule is coupled to reduction of another organic molecule. In at least one of these reactions ATP is generated by substrate level phosphorylation. The energy yield from substrate
level phosphorylation during fermentation is far lower than the energy yield from oxidative phosphorylation during respiration, as the electron donor compound is not fully oxidized. Recently a third mode of energy conservation, flavin based electron bifurcation (FBEF) has been recognised, which is used by strictly anaerobic prokaryotes including methanogens and acetogens (reviewed by Buckel and Thauer 2013). Flavin based enzymes bifurcate their two electrons; one towards an electron acceptor with higher redox potential than the flavin enzyme, and one with lower redox potential. The highly exergonic oxidation of the electron acceptor with more positive redox potential enables endergonic reduction of the electron acceptor with lower redox potential (Nitschke and Russell 2012).

1.3. Organic matter diagenesis in sedimentary environments

1.3.1. The Redox Cascade

Eukaryotes are only able to utilise oxygen as an electron acceptor for respiration, however, prokaryotes are able to use a range of inorganic and organic compounds as electron acceptors. As stated above, the energy yield of redox reactions depends on the difference in electrode potential of the PED and the TEA. The energy yield of a given redox reaction ($\Delta G$) is directly linked to the minimum substrate and TEA concentration at which the redox reaction becomes thermodynamically feasible (according to a rearrangement of equation 1.1.2.). The greater the energy yield of a redox reaction, the lower the concentration of substrate and terminal electron acceptor at which $\Delta G$ becomes negative, and the greater the energy available for biosynthesis. Availability of fermentation products (e.g. $H_2$, acetate) is normally limiting in sediments, and microorganisms which utilise a redox pair with greater energy yield ($\Delta G$) maintain concentrations of common substrates (e.g. organic compounds, $H_2$) below the minimum threshold of microorganisms which utilise a less favourable electron acceptor (Lovley and Goodwin 1988). In sedimentary environments this results in a vertical zonation of respiration processes (see Figure 1.1.1.) according to the availability of the TEA ($O_2 \rightarrow NO_3^- \rightarrow Mn$ (IV) $\rightarrow Fe$ (III) $\rightarrow SO_4^{2-} \rightarrow CO_2$) and their respective energy yields (Froelich et al. 1979).

Aerobic respiration (oxygen used as TEA) has the greatest $\Delta G^o$, and therefore occurs first within the sequence. Aerobic respiration has a high energy yield, and hence the
biomass and activity of aerobes creates rapid oxygen removal and limitation in sediments. At the sediment depth where oxygen diffusion becomes the limiting factor on the rate of aerobic respiration, then anaerobic respiration processes occur. Nitrate, nitrite, manganese (IV) and iron (III) are utilised as terminal electron acceptors by facultatively anaerobic bacteria in the suboxic zone and anoxic zones. Sulphate is utilised by anaerobic sulphate reducing bacteria (SRB) in the anoxic zone. The final terminal organic matter oxidation process is methanogenesis. Methanogenesis also occurs exclusively in anoxic environments, and is mediated by a group of anaerobic archaea belonging to the Euryarchaeota (known as the methanogens).
Reduced nitrogen, manganese, iron and sulphur compounds produced in the anoxic zone diffuse upwards to be reoxidized via biogenic (chemolithotrophic respiration) and abiogenic reactions in the sub-oxic and oxic zones, maintaining availability of TEA compounds. Methane oxidation under sub-oxic and anoxic conditions is coupled with nitrate and nitrite (Raghoebarsing et al. 2006; Ettwig et al. 2008), manganese (Beal et al. 2009) or iron (Beal et al. 2009) reduction and performed by anaerobic methanotrophic archaea (ANME). Methane is also oxidized in anoxic sediments, at the interface between the sulphate reduction and methanogenic zones, by a consortia of ANME archaea and SRB (Boetius et al. 2000). In reality the idealized zonation shown in Figure 1.1.1. is rarely observed as disturbance from burrowing organisms introduces oxidized compounds into anoxic sediment layers, anoxic microniches develop within oxic sediment layers, and oxidized recalcitrant minerals become more deeply buried.

\[ \Delta G = \Delta G^\circ + RT \ln K \]

\( \Delta G \) = energy yield under given conditions
\( \Delta G^\circ \) = energy yield under standard conditions (25 °C, gases at 1 bar partial pressure, concentration of products and reactants at 1 M, pH 7)
\( R \) = ideal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\))
\( T \) = temperature (K)
\( K \) = ratio of product to reactant concentrations

_Equation 1.1.2. Energy yield of a redox reaction_

1.3.2. Energetics of sediment diagenesis in aqueous environments

Biodegradation of high molecular weight organic matter in anoxic environments involves a consortium of functional groups of prokaryotes including hydrolytic bacteria, fermentative bacteria (primary and secondary fermenters), and anaerobic terminal oxidizers such as sulphate reducing bacteria (SRB) and methanogens. Figure 1.1.2. shows the process of anaerobic organic matter degradation and interactions of the different functional groups involved. Hydrolytic bacteria hydrolyse high molecular weight polymeric organic matter (e.g. polysaccharides, proteins, nucleic acids, and lipids) to oligomers and soluble monomers (e.g. sugars, amino acids, purines, pyrimidines, fatty acids, and glycerol), typically through the use of extracellular hydrolytic enzymes. The resulting oligomers and monomers are then further broken down by primary fermenters to highly reduced compounds such as fatty acids (primarily
propionate and butyrate), succinate, lactate, alcohols, CO\(_2\) and H\(_2\). H\(_2\) consumption by anaerobic respirers (including hydrogenotrophic methanogens) benefits primary fermenters by maintaining the low H\(_2\) partial pressures required for oxidation of NADH to be thermodynamically favourable. This allows primary fermenters to reoxidize NADH to produce H\(_2\) rather than utilising an internal electron acceptor, enabling them to conserve more ATP by synthesizing more acetate and less highly reduced products. This relationship is synergistic, as acetate, H\(_2\), and CO\(_2\) can be utilised directly by anaerobic respirers.

![Figure 1.1.2. Schematic diagram showing carbon and electron flow during anaerobic degradation of polymeric organic matter (adapted from Meganigal et al. 2005)](image_url)
The remaining organic matter (i.e. fatty acids of two or more carbon atoms, alcohols of more than 2 carbon atoms, and branched chain or aromatic fatty acids) are oxidized by secondary fermenters, generating acetate and formate, \( \text{CO}_2 \) and \( \text{H}_2 \). Interspecies transfer of \( \text{H}_2 \) and formate is considered essential for oxidation of highly reduced compounds by the secondary fermenters, as the oxidation process is thermodynamically unfavourable unless \( \text{H}_2 \) partial pressures are kept very low (<\(10^{-3}\) bar, Shrestha and Rotaru 2014) by \( \text{H}_2 \) scavengers such as sulphate reducers and methanogens (Stams et al. 2006). Secondary fermenters also generate electrons for interspecies transfer mediated by shuttle molecules (such as humic substances, flavins and sulphur compounds) or direct interspecies electron transfer (DIET) via cell-to-cell pili connections or minerals (Shrestha and Rotaru 2014). \( \text{H}_2 \) and \( \text{CO}_2 \) are converted to methane via hydrogenotrophic methanogenesis, or utilised by acetotrophic bacteria to form acetate. Acetate can be converted to methane via either acetotrophic methanogenesis, or syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (Zinder and Koch 1984; Lee and Zinder 1988). A number of syntrophic fatty acid oxidizing bacteria have been isolated and characterized, including syntrophic butyrate, propionate, and acetate oxidizers (Hattori 2008).

Methanogenesis is often the major pathway for anaerobic organic matter mineralization in freshwater environments (e.g. Capone and Kiene 1988) but is replaced by sulphate reduction in marine environments such as saltmarsh soils and marine sediments (Capone and Kiene 1988; Weston et al. 2006). SRB outcompete methanogens for common substrates in sulphate-rich environments due to their higher affinity for \( \text{H}_2 \) (Oremland and Taylor 1978; Kristjansson et al. 1982; Oremland and Polcin 1982; Lovley and Klug 1983) and acetate (Oremland and Polcin 1982; Schonheit et al. 1982; Lovley and Klug 1983) which enables them to maintain sediment \( \text{H}_2 \) and acetate concentrations at too low a concentration for methanogens to function effectively. SRB are more metabolically diverse than methanogens and are able to use almost all products of primary fermentation, so by the same principle syntrophs (secondary fermenters) are also unable to compete with SRB for the products of primary fermentation.
1.4. Environmental controls on microbially mediated redox reactions

Physical and chemical environmental controls include temperature, pressure, pH, salinity, ionising radiation, exposure to toxic compounds (e.g. oxygen for anaerobes), and substrate and electron acceptor concentrations. Temperature and salinity are probably the two factors most likely to affect microbial communities and processes in surface sediment environments.

1.4.1. Temperature

The temperature range for prokaryotic growth ranges from below -40 °C (Price and Sowers 2004) to 122 °C under elevated pressures (Takai et al. 2008). There is no apparent temperature minima for prokaryotic growth (Price and Sowers 2004), but stability of nucleic acids is thought to limit the likely maximum temperature to ~150 °C (Wiegel and Canganella 2001). Most prokaryotes can grow over a temperature range of approximately 45°C (Cavicchioli 2006), but have a narrow window of optimal growth. The temperature optima of an organism is usually based on growth rate, but may also be based on growth yield, as these are not necessarily concurrent. The temperature maxima of an organism is very close, often 3-5 °C above, the temperature optimum. Conversely, the growth yield optima of pure culture strains grown under laboratory conditions often appears to be several degrees centigrade lower than the growth rate optima (Overmann et al. 2006). Prokaryotes are grouped broadly into psychrophiles, mesophiles, thermophiles and hyperthermophiles according to their cardinal temperatures (Table 1.1.1). Other temperature groupings are also sometime utilised to describe organisms which do not fit into the traditional classifications (Table 1.1.1.), but are not as widely recognised.

<table>
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<tr>
<td></td>
<td>Minimum</td>
</tr>
<tr>
<td>Psychrophile*</td>
<td>&lt;5</td>
</tr>
<tr>
<td><em>Psychrotolerant</em>*</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Mesophile*</td>
<td>&gt;0</td>
</tr>
<tr>
<td><em>Facultative thermophile</em>*</td>
<td>-</td>
</tr>
<tr>
<td><em>Moderate thermophile</em>*</td>
<td>-</td>
</tr>
<tr>
<td>Thermophile†</td>
<td>-</td>
</tr>
<tr>
<td>Hyperthermophile†</td>
<td>-</td>
</tr>
</tbody>
</table>

* defined according to Morita (1975)
** defined according to Bertrand et al. (2015)
† defined according to Stetter (1996)
Temperature affects the fluidity of cell membranes, reducing the efficiency of chemiosmosis and affecting other cross membrane processes such as nutrient uptake (Madigan et al. 2003). Bacteria and archaea both vary the composition their lipid side chains in response to temperature alteration. Short chain fatty acids, unsaturated fatty acids and branched fatty acids help maintain bacterial cell membrane fluidity at low temperatures, whereas saturated long chain fatty acids help increase stability at high temperatures (Booth 1999). In general, thermophiles have a higher proportion of long chain and branched fatty acids than mesophiles, and vice versa for psychrophiles (Russell and Fukunaga 1990). Archaea decrease the degree of saturation in their hydrocarbon side chains at low temperatures (Nichols and Franzmann 1992), and at high temperatures they increase cyclization of their hydrocarbon side chains and replace diether membrane lipids with tetraether membrane lipids (de Rosa and Gambacorta 1988; de Rosa et al. 1991). Tetraether lipids span across the width of the cell membrane, further enhancing stability at high temperatures, and are often found in thermophilic archaea. High temperatures also affect the permeability of the cell wall. Some thermophiles, particularly hyperthermophiles, utilise Na\(^+\) for energy coupling during chemiosmosis as temperature affects cell membrane permeability to Na\(^+\) less than cell membrane permeability to protons.

Both high and low temperatures may cause protein denaturation, reducing the efficiency of an enzyme system. Prokaryotes synthesise cold shock or heat shock proteins in response to transient shifts in temperature. Heat shock proteins help maintain cellular processes such as transcription, translation and protein folding. Production of chaperone proteins also helps maintain protein structure and folding (Jaenicke and Sterner 2006). Such chaperone proteins are synthesized in all organisms, but hyperthermophiles have a specific set of heat shock proteins (Holden et al. 2000). The proteins of thermophiles have a relatively high number of hydrogen bonds and salt bridges, and multimeric proteins also have increased numbers of ionic bonds, all of which contribute to structural stability at elevated temperatures (Jaenicke and Sterner 2006). Thermophiles also have reduced numbers of thermolabile amino acids (Singer and Hickey 2003). Hyperthermophiles are also unique in that they encode reverse DNA gyrase, which may help maintain the stability of DNA at high temperatures (Forterre 2002). At transcriptomic level double stranded structural RNAs (tRNA and rRNA) have high G+C content, and mRNA is enriched in purines, which is
thought to increase the thermal stability of these molecules. Adaptations to low temperature seen in psychrophilic prokaryotes include RNA helicases which prevent stabilization of DNA and RNA structure at low temperatures. Other psychrophilic adaptations include enzymes with high specific activity at low temperatures, proteins with reduced numbers of ion pairs, hydrogen bonds, and hydrophobic interactions (to reduce stabilization and increases flexibility), and synthesis of proteins and organic molecules which act as antifreeze molecules and cryoprotectants (D’Amico et al. 2006).

Temperature is an important controlling factor on most biochemical processes, as almost all redox reactions (even if exergonic) have an activation energy (Lengeler et al. 1999). Similar to chemical reactions, the temperature dependence of microbial metabolism and growth rates is described by the Arrhenius equation (Equation 1.1.3., Arrhenius 1889). When applied to growth rates of physiological processes, the activation energy ($E_a$) is a measure of the temperature response of the organism or process. The Q10 coefficient describes the factor by which a rate increases for a 10°C temperature increase, and is often used as a measure of the temperature sensitivity of biochemical processes and biological systems (e.g. Roussel et al. 2015). The Q10 coefficient for most chemical and biological processes, including the microbial growth rates, is 2 (ie a doubling of rate with 10 °C increase in temperature) (Lengeler et al. 1999).

$$k = Ae^{-Ea/(RT)}$$

$k = \text{rate constant of reaction at } 0 ^\circ$  
$A = \text{collision or frequency factor (h}^{-1})$  
$E_a = \text{activation energy of reaction}$  
$T = \text{temperature (K)}$  
$R = \text{ideal gas constant (8.314 J K}^{-1} \text{ mol}^{-1})$  

*Equation 1.1.3. Arrhenius equation*  

1.4.2. Salinity  
Prokaryotic life can exist at salt concentrations up to 5.2. M, although the phylogenetic and metabolic diversity of prokaryotes decreases significantly at salinities greater than approximately 1.7 M (Oren 2001). Prokaryotes are loosely defined according to the minimum, optimum and maximum salt concentration for growth as shown in Table
1.1.2., however these values can vary substantially depending on temperature. Cell membranes are permeable to water, but impermeable to most solutes. Therefore there is a risk of plasmolysis in high salinity environments, and it is necessary to raise the osmotic potential within the cell in order to maintain cell turgor. In addition, high salt concentrations within the cell disrupt protein stability (Oren 2006). Non-halophiles and halotolerant prokaryotes synthesize compatible solutes such as dimethylsulfonopropionate, glycerol, mannitol, sucrose, trehalose, betaine, and ectoin in response to salinity stress. Compatible solutes are substances which raise the osmolarity of the cytoplasm in order to balance external osmotic pressure without interfering with cell metabolism (Roberts 2005). In contrast, halophilic archaea and halophilic bacteria maintain intracellular salt (primarily K+ and Cl−) concentrations isotonic with the external environment, and possess enzymes that are salt adapted or even salt dependent (Oren 2006).

Table 1.1.2. Salinity classification of prokaryotes (adapted from Oren 2006).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Salt content optima and range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-halophilic</td>
<td>&lt;0.2 M</td>
</tr>
<tr>
<td>Halotolerant</td>
<td>Optima &lt; 0.2 M, range up to 2.5 M</td>
</tr>
<tr>
<td>Slight halophile</td>
<td>0.2-0.5 M</td>
</tr>
<tr>
<td>Moderate halophile</td>
<td>0.5-2.5 M</td>
</tr>
<tr>
<td>Extreme halophile</td>
<td>2.5-5.2 M</td>
</tr>
</tbody>
</table>

1.5. Methane

1.5.1. Methane production

Methane (CH₄) is a highly reduced hydrocarbon molecule produced via both abiogenic (thermogenic and pyrogenic) and biogenic (largely microbial) processes. Natural abiogenic methane production is largely generated by thermocatalytic processes occurring under high temperature and pressure. These include thermal decomposition of organic matter, and inorganic reactions between water and reduced minerals in ultramafic rocks (Horita and Berndt 1999). Pyrogenic methane production predominantly occurs due to incomplete combustion of organic matter during wildfires or of fossil fuels (Kirschke et al. 2013). Althoff et al. (2014) recently discovered that methane may be formed from organosulphur compounds via sulphoxidation of methylsulphides under highly oxidative conditions at ambient atmospheric pressure and temperature, however the environmental significance of this novel pathway for methane production is currently unknown.
Anaerobic biogenic methane production is predominantly carried out by a group of microorganisms known as the methanogens which belong to the domain Archaea and kingdom Euryarchaeota (discussed in section 1.6.). There are several other types of prokaryotes able to produce trace amounts of methane under anaerobic conditions as a by-product of their metabolism including strains of Bacteroides (McKay et al. 1982), Clostridia (Rimbault et al. 1988), and many strains of sulphate-reducing bacteria (e.g. Postgate 1969; Schauder et al. 1986). These groups are known as the ‘mini-methane’ producers (Whitman et al. 2006). The methanogens are unique in that they are only able to grow through methane formation (Whitman et al. 2006). Stable carbon (\(\delta^{12}\text{C}/\delta^{13}\text{C}\)) isotope composition can be used to distinguish between methane produced by abiogenic and biogenic processes, as the 3 main types of emissions have different isotopic \(\delta^{13}\text{C}\) signatures; −55 to −110 ‰ for biogenic emissions, −25 to −55‰ for thermogenic emissions, and −13 to −25‰ for pyrogenic emissions (Whiticar 1999; Neef et al. 2010; Monteil et al. 2011). Stable carbon isotopes can also be used to distinguish the biochemical pathway by which biogenic methane was generated. \(\delta^{13}\text{C}\) of methane generated by hydrogenotrophic methanogenesis ranges from -110 to -60 ‰, compared to -50 to -20 ‰ for acetotrophic methanogenesis (Whiticar et al. 1986). It may also be possible to identify methane generated by methylotrophic methanogenesis from the carbon isotope composition (Penger et al. 2012), however this method is not widely utilised.

Traditionally it has been assumed that biogenic methane production occurs only under anoxic conditions. However, the observation that oxic ocean waters are often supersaturated with methane with respect to atmospheric concentrations (termed the ‘oceanic methane paradox’, Kiene 1991) prompted investigation into the possibility of aerobic biogenic methane production mechanisms. Work by Karl et al. (2008), Metcalf et al. (2012) and Kamat et al. (2013) showed that the Thaumarchaeal group Nitrosopumilus maritimus (widely detected in marine waters), is able to produce methane from methylphosphonate, and that this aerobic methane production may be generated through oxidation of the C-P bond in methylphosphonate. Methane supersaturation has also been recorded in the oxic, surface waters of freshwater lakes (Grossart et al. 2011; Bogard et al. 2014). However, experimental evidence described in both Grossart et al. (2011) and Bogard et al. (2014) suggests that, in contrast to marine environments, aerobic methane production in oxic freshwater environments is
predominantly mediated by methanogenic archaea associated with photoautotrophic phytoplankton.

1.5.2. Trends in atmospheric methane concentrations

Methane has a global warming potential twenty-five times higher than carbon dioxide over a 100 year period on a per molecule basis (Shindell et al. 2009), and is thought to account for between 20-30% of total greenhouse gas radiative forcing since the industrial revolution (Solomon 2007). Global atmospheric methane concentrations have risen from ~ 715 ppb during the pre-industrial period to 1799 ppb in 2010 (Kirschke et al. 2013), an increase which far surpasses the range of natural variation (320-790 ppb) recorded over the past 6.5 kyr (Solomon 2007). The global methane budget is not well constrained, partly due to large discrepancies between estimates of global atmospheric emissions derived from ‘bottom-up’ (process studies of sources) and those derived from ‘top-down’ (direct observation of atmospheric concentrations) (Nisbet et al. 2014).

Atmospheric methane emissions are generated from both biogenic and abiogenic processes, and derived from both natural and anthropogenic means. Figure 1.1.3. shows the relative contribution of each of the major sources of atmospheric methane emissions. Over 70 % of total annual atmospheric methane emissions are derived from biogenic sources (Denman et al. 2007), hence the global methane cycle is strongly under microbial control. In order to fully anticipate how methane emissions may vary in response to future climate change, knowledge of the physical (e.g. temperature, salinity) controls on the structure and functioning of the methanogenic communities in these ecosystems is required (Schimel and Gulledge 1998; Schimel 2004; Singh et al. 2010; Liu et al. 2012). Schimel and Gulledge (1998) and Singh et al. (2010) argue that in order to predict atmospheric emissions from key methanogenic environments it is necessary to understand physical controls on methanogen community compositions and structure, including physiological response of component members at a species level. The major sink for methane is oxidation with hydroxyl (OH-) in the troposphere, forming formaldehyde (CH2O), carbon monoxide (CO), and where nitrous oxides (NOx) are available, ozone (O3). Methane oxidation in the troposphere accounts for ~90 % of the global sink (Kirschke et al. 2013). Methane is also oxidized by chlorine radicals and
atomic oxygen radicals in the stratosphere (Cicerone and Oremland 1988), and by chlorine in the marine boundary layer (Allan et al. 2007), each of which account for ~3% of the global sink (Kirschke et al. 2013). Methane produced in anoxic sediments and soils is also oxidized aerobically and anaerobically by methanotrophic bacteria before it reaches the atmosphere. It is estimated that approximately 60% of methane produced in anoxic environments such as soils and sediments is oxidized microbially before it reaches the atmosphere (Reeburgh 2007), equivalent to approximately ~4% of the global methane sink (Kirschke et al. 2013).

The rate of increase in atmospheric methane concentrations decreased significantly in the latter half of the 20th century from ~12 ppbv year⁻¹ during the 1980s to ~3 ppbv year⁻¹ during the late 1990s (Dlugokencky et al. 2011). The mechanisms behind this decrease are unclear. The reduction in the rate of increase in atmospheric methane emissions was initially attributed to an increase in sink strength, which is oxidation with hydroxyl in the stratosphere (Khalil 2000). However, a synthesis of more recent studies suggests that a combination of stable to decreasing microbial methane production, and decreasing methane production from fossil fuel combustion, is the most likely

Figure 1.1.3. Sources of atmospheric methane emissions as percentage of global budget of 500-600 Tg year⁻¹ (adapted from Conrad 2009).
explanation for the reduction in growth rate of atmospheric methane concentrations observed between the mid-21\textsuperscript{st} century and 2006 (Kirschke et al. 2013). Small variations in the strength of the tropospheric hydroxyl sink (Montzka et al. 2011) may also play a role in modulating atmospheric emission trends (Kirschke et al. 2013). Despite a brief hiatus in the post-industrial trend of rising atmospheric methane concentrations between 1999 and 2006, levels have once again begun to increase (Rigby et al. 2008). Currently, atmospheric methane concentrations are rising by \~6 ppbv year (Nisbet et al. 2014), but the reason for this variability is uncertain due to incomplete knowledge of the factors controlling the global atmospheric methane flux. It is not known whether the post-2006 trend is a short term fluctuation, or represents the beginning of a new period of rising atmospheric methane concentrations (Dlugokencky et al. 2009).

1.5.3. Methane as an energy source

In an age of declining traditional petroleum resources, biogenic methane production could also be harnessed to increase the efficiency of fossil fuel extraction and provide a wealth of alternative energy sources. Recently it has been suggested that the microbial community of ‘spent’ petroleum reserves could be engineered to enable enhanced recovery of the residual products as natural gas (Parkes 1999; Vazquez-Duhalt and Quintero-Ramirez 2004; Gieg et al. 2008; Jones et al. 2008; Gray et al. 2011). Microbial enhanced oil recovery (MEOR) techniques typically involve inoculation with nutrients and substrates to stimulate methanogenic communities indigenous to reservoirs through injection of nutrients, or alternatively introduction of cultivated exogenous microbial communities (Sen 2008; Kobayashi et al. 2012). Methane production increases the pore pressure of reservoir formations and reduces oil viscosity as methane dissolves in oil (Sen 2008). Increased biomass production also alters the physical properties of the oil, and aids oil transport towards the production wells (Kobayashi et al. 2012). Methane produced can also be recovered directly, and this is termed ‘residual oil gasification’ (Maurice et al. 2010; Hu et al. 2015). The environmental conditions in petroleum fields are extreme, with high temperatures and salinities, and with only refractory organic matter available for microbial degradation. Biodegradation of oil is an important source of methane in such reservoirs, despite the extreme conditions (Jones et al. 2008). As such, the success of these approaches is
dependent on knowledge of the metabolic and physiological characteristics of indigenous microorganisms to meet the challenge of maintaining growth and increasing yield in these harsh conditions (Lin et al. 2014). Methanogenic degradation of waste organic matter in anaerobic digesters, such as sewage, animal manure and waste food, produces biogas which is composed mainly of methane and which can be burnt directly in a boiler to produce heat or burnt in CHP (Combined Heat and Power) unit to produce heat and electricity. Biogas can also be cleaned to remove carbon dioxide and other non-methane gases, and then fed directly into the national grid or used as a vehicle fuel. It is hoped that better understanding of the environmental controls on methanogenic communities, and specifically methanogens, will enable the energy generating processes described above to be exploited to their full potential (e.g. Blake et al. 2015).

1.6. The Archaea

All methanogens fall within the Euryarchaeal branch of the domain Archaea, traditionally considered one of the 3 domains of life (Woese and Fox 1977). The Archaea were first recognised as a separate evolutionary domain from the Bacteria and Eukarya in the late 1970’s following the advent of molecular microbiology. Initially classification was based on protein and small subunit ribosomal RNA analyses (Woese and Fox 1977; Woese et al. 1990), which have been subsequently confirmed by comparative genomics (Gribaldo and Brochier-Armanet 2006). Until relatively recently the Archaea were thought to be predominantly extremophiles (with the exception of the methanogens), constrained to a small number of habitats, and with a minor role in biogeochemical cycling. However, utilisation of next generation genomic techniques has shown that they are abundant in habitats such as marine waters, and are thought to be the dominant group of organisms in deep sea sediments (Kozubal et al. 2012) and geothermal environments (Offre et al. 2013). Members of the domains Bacteria and Archaea are both unicellular prokaryotic organisms, characterised by lack of a nucleus and small genome size, however there are substantial phenotypic differences between the Bacteria and Archaea relating to cell wall and membrane structure and
cellular functions such as gene transcription (key differences summarised in Table 1.1.3.).

For a considerable period of time the Crenarchaeota and Euryarchaeota (Woese et al. 1990) were the only widely recognised archaeal phyla. The Crenarchaeota consists of one class, Thermoprotei, and 5 orders - Acidilobales, Desulfurococcales, Fervidicoccales, Sulfolobales, and Thermoproteales. All of these orders are thermophilic or hyperthermophilic, and depend on sulphur for their metabolism. The phylogeny of the Euryarchaeota is less well defined than the Crenarchaeota, and consists of 8 classes (Methanobacteria, Methanomicrobia, Methanopyri, Methanococci, Thermoplasmatales, Archaeoglobi, Halococci, Thermococci and Halobacteria). These orders include organisms from psychrophiles to hyperthermophiles, and with a diversity of metabolisms and habitats. Three new archaeal phyla, the Thaumarchaeota (Könneke et al. 2005; Brochier-Armanet et al. 2008), Korarchaeota (Barns et al. 1996; Elkins et al. 2008), and Nanoarchaeota (Huber et al. 2002) have recently been described. There are very few cultivated species within the Thaumarchaeota, and all are nitrogen oxidizers belonging to the order Crenarchaeales (Brochier-Armanet et al. 2012). The candidate species ‘Nanoarchaeum equitans’ is an obligate syntroph which grows in coculture with the sulphur reducing Ignicoccus hospitalis, and is the only cultivated member of the Nanoarchaeota, (Huber et al. 2002). The candidate species ‘Korarchaeum

<table>
<thead>
<tr>
<th>Trait</th>
<th>Archaea</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall</td>
<td>Absence of peptidoglycan. Cells walls may contain pseudomurein, complex polysaccharides and glycoproteins.</td>
<td>Peptidoglycan present</td>
</tr>
<tr>
<td>Membrane lipids</td>
<td>Isoprenoid ethers built on glycerol-1-phosphate</td>
<td>Straight chain fatty acids bound to C1 and C2 atoms of glycerol by ester linkages (similar to Eukarya)</td>
</tr>
<tr>
<td>Histone-like proteins</td>
<td>Yes (similar to Eukarya)</td>
<td>No</td>
</tr>
<tr>
<td>Initiator tRNA in protein synthesis</td>
<td>Methionine (similar to Eukarya)</td>
<td>N-formylmethionine</td>
</tr>
<tr>
<td>RNA polymerase structure</td>
<td>Many (8-12 subunits)</td>
<td>1 (4 subunits)</td>
</tr>
<tr>
<td>Spore formation</td>
<td>No</td>
<td>Yes (some)</td>
</tr>
<tr>
<td>Transcription factors utilised</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1.1.3. Some key differences between the archaea and bacteria.
cryptophilum’, obtained in enrichment culture, is the only cultivated member of the Korarchaeota (Elkins et al. 2008).

A wealth of new, uncultured candidate archaeal phyla have been proposed during the last 10 years, based on metagenomic analyses. Figure 1.1.4. shows a schematic tree of the archaea including the recently identified ‘Lokiarchaeota’. Spang et al. (2015) recently identified the candidate phyla ‘Lokiarchaeota’, whose genomes encode eukaryote protein homologs involved in the manipulation of membranes or in the formation of a cytoskeleton which have not previously been observed in Archaea. Also included are the ‘Aigarchaeota’ (Nunoura et al. 2011) and ‘Bathyarchaeaota’ (Meng et al. 2014) which are more often placed within the Thaumarchaota (e.g. Forterre 2015; Spang et al. 2015), and the ‘Geoarchaeota’ (Kozubal et al. 2012) which were subsequently identified as a deeply branching lineage within the Crenarchaeota (Guy et al. 2014). The Crenarchaeota, Thaumarchaeota and Korarchaeota form an archaeal superphylum known as either the Protoarchaeota or the TACK superphylum (Guy and Ettema 2011). Rinke et al. (2013) proposed grouping 5 extremophilic candidate phyla, the ‘Nanoarchaeota’ (Huber et al. 2002), ‘Nanohaloarchaeota’ (Narasingarao et al. 2012), ‘Diapherotrites’ (Takai and Horikoshi 1999), ‘Aenigmarchaeota’ (Takai et al. 2001) and ‘Pavarchaeota’ (Baker et al. 2006; Baker et al. 2010), into a superphylum named DPANN. However, subsequent analyses have grouped these candidate phyla within the phylum Euryarchaeota (Williams and Embley 2014; Forterre 2015). The Thermoplasmatales, Methanomasiliicoccales and a number of uncultivated lineages form the sub-phylum Diaforarchaea (Petitjean et al. 2015).

1.7. Phylogeny and diversity of the methanogens

The methanogens are a physiologically diverse group of prokaryotes within the Euryarchaeota, united by the ability to synthesise methane stoichiometrically from a narrow range of substrates in anoxic conditions. To date (January 2016) there are seven recognised orders of methanogens; Methanococcales, Methanomicrobiales, Methanobacteriales, Methanocellales, Methanosarcinales, Methanopyrales, and the Methanomassiliicoccales (see Table 1.1.4.). The methanogen orders are sometimes referred to as either “Class 1” (Methanobacteriales, Methanococcales, and Methanopyrales) or “Class 2” (Methanomicrobiales and Methanosarcinales) based on
16S rRNA gene sequences (Bapteste et al. 2005). It is unclear why the methanogens do not appear as a monophyletic group within the Euryarchaeota, but are instead interspersed between the Archaeoglobales and Halobacteriales (see Figure 1.1.4.).

Hypotheses include that: (i) non-methanogenic classes within the Euryarchaeota lost the ability to synthesise methane, (ii) methanogenic orders acquired the ability to

Figure 1.1.4. Schematic phylogenetic tree of the archaea (adapted from Forterre 2015).

Schematic phylogenetic tree of the archaea adapted from Forterre (2015), which was based on the ribosomal protein tree produced by Brochier-Armanet et al. (2011) and protein tree of Petitjean et al. (2015).
synthesize methane through horizontal gene transfer, or (iii) that the Archaeoglobi and methanogen orders share a different common ancestor than the remaining orders within the Euryarchaeota (Liu 2010a).

1.8. Physiology and metabolic properties of the methanogens

The majority of cultivated methanogen species are mesophilic, although a number of psychrophilic and thermophilic, and hyperthermophilic methanogen species have also been described (Garcia et al. 2000). The temperature range of cultivated methanogen species ranges from -2 °C (Reid et al. 2006) to 122 °C (Takai et al. 2008).

1.8.1. Morphology

Methanogens have a diverse range of morphologies including rods, chains of rods, regular and irregular cocci, sarcina, filaments, spirilla, and the disc-like morphology of *Methanoplanus* spp. Some species are motile, some have sheathes, and several members of the *Methanosarcina* and *Methanothrix* contain gas vacuoles (Whitman et al. 2006). Methanogen cell walls consist of pseudomurein or protein sub-units, hence they are resistant to antibiotics that inhibit murein peptidoglycan cell wall synthesis in the Bacteria (Hilpert et al. 1981). There are 4 main types of cell wall chemistries; pseudopeptidoglycan, methanochondroitin, protein/glycoprotein, or S-layer (Albers and Meyer 2011). The cell membrane lipids of methanogens consist of isoprenoid ethers built on glycerol-1-phosphate (Sprott 2001).

1.8.2. Nutritional and biochemical properties

Growth of most methanogens is stimulated by addition of acetate, which is utilised as carbon source (Balch et al. 1979). Some methanogens also require amino acids, vitamins (e.g. riboflavin, pantothenic acid, thiamine, biotin; Garcia et al. 2000), or cofactors necessary for methanogenesis which they are unable to synthesize themselves (Balch and Wolfe 1976; Tanner and Wolfe 1988). Methanogens indigenous to rumen environments often need mixed branched fatty acids (Garcia et al. 2000). All methanogens require a supply of trace metals (including nickel, cobalt, molybdenum and iron; Jarrell and Kalmokoff 1988) as metalloenzymes play an important role in all methanogenic biochemical pathways (Lessner 2001). A few
<table>
<thead>
<tr>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanobacteria</td>
<td>Methanobacteriales</td>
<td>Methanobacteriaceae</td>
<td>Methanobacterium</td>
<td>22</td>
<td>H₂ + CO₂, formate, alcohols</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanobrevibacter</td>
<td>15</td>
<td>H₂ + CO₂, formate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanothermabacter</td>
<td>8</td>
<td>H₂ + CO₂, formate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanosphaera</td>
<td>2</td>
<td>Ethanol + H₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanothermus</td>
<td>2</td>
<td>H₂ + CO₂</td>
</tr>
<tr>
<td>Methanococci</td>
<td>Methanococcales</td>
<td>Methanocaldococcaee</td>
<td>Methanocaldococcus</td>
<td>7</td>
<td>H₂ + CO₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanotiss</td>
<td>2</td>
<td>H₂ + CO₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanococcus</td>
<td>4</td>
<td>H₂ + CO₂, formate, pyruvate + CO₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanococcaceae</td>
<td>Methanococcus</td>
<td>2</td>
<td>H₂ + CO₂, formate</td>
</tr>
<tr>
<td>‘Methanomicrobia’</td>
<td>Methanomicrobiales</td>
<td>Methanomicrobiaceae</td>
<td>Methanomicrobiurn</td>
<td>4</td>
<td>H₂ + CO₂, formate, alcohols</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanogenium</td>
<td>7</td>
<td>H₂ + CO₂, formate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanocorpusculaceae</td>
<td>Methanocorpusculum</td>
<td>4</td>
<td>H₂ + CO₂, formate, alcohols</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanocalculaceae</td>
<td>Methanocalculus</td>
<td>5</td>
<td>H₂ + CO₂, formate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanoregulaceae</td>
<td>Methanolinena</td>
<td>2</td>
<td>H₂ + CO₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanosphaera</td>
<td>1</td>
<td>H₂ + CO₂, formate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanoregula</td>
<td>Methanoregula</td>
<td>2</td>
<td>H₂ + CO₂, formate + CO₂</td>
</tr>
<tr>
<td>Methanosarcinales</td>
<td>Methanosarcinaceae</td>
<td>Methanosarcina</td>
<td>Methanosarcina</td>
<td>13</td>
<td>H₂ + CO₂, methanol, methylamines, acetate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanolobus</td>
<td>7</td>
<td>Ethanol, methylamines</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanohalobium</td>
<td>Methanohalobium</td>
<td>1</td>
<td>Ethanol, methylamines</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanococccoides</td>
<td>Methanococccoides</td>
<td>4</td>
<td>Ethanol, methylamines</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanohalophilus</td>
<td>Methanohalophilus</td>
<td>4</td>
<td>Ethanol, methylamines, methyl sulphides</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanosalum</td>
<td>Methanosalum</td>
<td>1</td>
<td>Ethanol, methylamines, dimethylsulphide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanimicrococcus</td>
<td>Methanimicrococcus</td>
<td>1</td>
<td>Ethanol, methylamines</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanomethylivorans</td>
<td>Methanomethylivorans</td>
<td>3</td>
<td>Methanol, methylamines, dimethylsulphide,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>methanethiol</td>
</tr>
<tr>
<td>Methanocellales</td>
<td>Methanocellaceae</td>
<td>Methanocellaceae</td>
<td>Methanococcus</td>
<td>1</td>
<td>Methylamines</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanothrix</td>
<td>3</td>
<td>Acetate</td>
</tr>
<tr>
<td>Methanomassiliicoccales</td>
<td>Methanomassiliicoccaee</td>
<td>Methanomassiliicoccus</td>
<td>Methanomassiliicoccus</td>
<td>1</td>
<td>Methanol, methylamines + H₂</td>
</tr>
<tr>
<td>Methanopyri</td>
<td>Methanopyrales</td>
<td>Methanopyraceae</td>
<td>Methanopyrus</td>
<td>1</td>
<td>H₂ + CO₂</td>
</tr>
</tbody>
</table>

* Proposed by Oren (2014) to replace the name Methanosaetaceae.
thermophilic methanogens also require tungsten (e.g. Winter et al. 1984; Zellner et al. 1987).

1.8.3. Substrates for methanogenesis

Pure cultures of methanogens are known to be able to convert around 20 substrates to methane. Many more substrates can be indirectly utilised by methanogens via syntrophic reactions involving methanogens and anaerobic bacteria (see section 1.3.2.). There are 3 main pathways of methane production: (i) the reduction of carbon dioxide, (ii) disproportionation of methyl substrates, and (iii) fermentation of acetate-type substrates. Until recently it was thought that all methanogens utilised the same biochemical pathway to metabolise a given substrate, however it seems likely the Methanomassiliicoccales utilise a biochemical pathway substantially different from any other methylotrophic methanogens (Lang et al. 2015). All pathways involve the demethylation of methyl-coenzyme M to methane catalysed by methyl-coenzyme M, and the reduction of the heterodisulfide of coenzyme M and coenzyme B catalysed by heterodisulfide reductases, but are differentiated by the origin of the methyl group and the method by which the methyl group is acquired (Lessner et al. 2001).

1.8.3.1. Methanogenesis from CO₂-type compounds

Isolated species from 5 out of the 7 orders of methanogens (the Methanobacteriales, Methanomicrobiales, Methanococcales, Methanopyrales, and Methanocellales) are only capable of reducing carbon dioxide to methane (Table 1.1.4.). Carbon dioxide is reduced to a methyl group using electrons derived primarily either from hydrogen or formate (Table 1.1.5.), although carbon monoxide and short chain alcohols (ethanol, propanol, isopropanol, 2-butanol and cyclopentanol) can also be used as electron donors by a limited number of species. Primary alcohols are oxidized to acetate and secondary alcohols are oxidized to ketones (Table 1.1.5.). Despite the diversity of methanogen species able to reduce carbon dioxide to produce methane, it is estimated that only 30 % of biogenic methane is generated using this pathway in freshwater environments (Whiticar et al. 1986).
Table 1.1.5. Stoichiometric equations and energy yields for methanogenesis (adapted from Whitman et al. 2006)

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Substrate</th>
<th>Stoichiometric equation</th>
<th>Energy yield ($\Delta G^0$, kJ mol$^{-1}$ CH$_4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrogenotrophic</strong></td>
<td>Carbon dioxide</td>
<td>$\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O}$</td>
<td>-135.6</td>
</tr>
<tr>
<td></td>
<td>Formate</td>
<td>$4\text{HCOOH} \rightarrow \text{CH}_4 + 3\text{CO}_2 + 2\text{H}_2\text{O}$</td>
<td>-130.1</td>
</tr>
<tr>
<td></td>
<td>Carbon monoxide</td>
<td>$4\text{CO} + 5\text{H}_2\text{O} \rightarrow \text{CH}_4 + 3\text{HCO}_3^- + 3\text{H}^+$</td>
<td>-196.0*</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>$2\text{CH}_3\text{CH}_2\text{OH} + \text{HCO}_3^- \rightarrow \text{CH}_4 + 2\text{CH}_3\text{COO}^- + \text{H}_2\text{O} + \text{H}^+$</td>
<td>-116.3</td>
</tr>
<tr>
<td></td>
<td>Propanol</td>
<td>$\text{CO}_2 + 4(2\text{propanol}) \rightarrow \text{CH}_4 + 4(\text{acetone}) + 2\text{H}_2\text{O}$</td>
<td>-36.5</td>
</tr>
<tr>
<td><strong>Acetotrophic</strong></td>
<td>Acetate</td>
<td>$\text{CH}_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_4 + \text{HCO}_3^-$</td>
<td>-31.0</td>
</tr>
<tr>
<td><strong>Methylotrophic (disproportionation)</strong></td>
<td>Methanol</td>
<td>$4\text{CH}_3\text{OH} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 2\text{H}_2\text{O}$</td>
<td>-104.9</td>
</tr>
<tr>
<td></td>
<td>Methylationm</td>
<td>$4\text{CH}_3\text{NH}_3\text{Cl} + 2\text{H}_2\text{O} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 4\text{NH}_4\text{Cl}$</td>
<td>-75.0</td>
</tr>
<tr>
<td></td>
<td>Dimethylamine</td>
<td>$2(3\text{CH}_3)\text{NH}_2\text{Cl} + 2\text{H}_2\text{O} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 2\text{NH}_2\text{Cl}$</td>
<td>-73.2</td>
</tr>
<tr>
<td></td>
<td>Trimethylamine</td>
<td>$4(3\text{CH}_3)\text{NHCl} + 6\text{H}_2\text{O} \rightarrow 9\text{CH}_4 + 3\text{CO}_2 + 4\text{NH}_4\text{Cl}$</td>
<td>-74.3</td>
</tr>
<tr>
<td></td>
<td>Dimethylsulphide</td>
<td>$2(3\text{CH}_3)\text{S} + 3\text{H}_2\text{O} \rightarrow 3\text{CH}_4 + \text{HCO}_3^- 2\text{H}_2\text{S} + \text{H}^+$</td>
<td>-73.8</td>
</tr>
<tr>
<td><strong>Methylotrophic (reduction with H$_2$)</strong></td>
<td>Methanol</td>
<td>$\text{CH}_3\text{OH} + \text{H}_2 \rightarrow \text{CH}_4 + \text{H}_2\text{O}$</td>
<td>-112.5</td>
</tr>
</tbody>
</table>

*as calculated by (Sowers and Ferry 2002)
1.8.3.2. Methanogenesis from acetotrophic compounds

Acetate fermentation is only carried out by members of 2 genera, the Methanosarcina and Methanothrix (both within the order Methanosarcinales, Table 1.1.4.). During acetotrophic methanogenesis the C-1 carboxyl group of acetate is oxidized to bicarbonate, providing electrons for the reduction of the C-2 methyl group to methane (Whitman et al. 2006). The free energy yield of this pathway is considerably lower than for most hydrogenotrophic or methylotrophic substrates (Table 1.1.5.).

1.8.3.3. Methanogenesis from methyl compounds

The Methanomassiliicoccales, many genera within the order Methanosarcinales, and the genus Methanosphaera (order Methanobacterales) utilise methylated compounds as substrates for methanogenesis. Compounds commonly used by methylotrophic methanogens include methanol, methylated amines, tetramethylammonium, dimethylethylamine (Whitman et al. 2006). Methylated sulphides, such as dimethylsulphide and methylmercaptopropionate may also be used (Sowers and Ferry 2002). It was recently discovered that some strains of Methanococcoides (Watkins et al. 2012; Watkins et al. 2014) and Methanolobus (Ticak et al. 2014) can also utilise glycine-betaine, choline, or N,N – dimethylethanolamine directly for methanogenesis. This ability may be present in other methylotrophic methanogens, but is as yet untested. In the presence of an external electron acceptor (e.g. H₂) the methyl groups of methylated compounds are reduced completely to CH₄ (Table 1.1.5.). In the absence of an external electron donor some methyl groups are oxidized to CO₂ via reversal of the reductive C-1 pathway, which provides electrons for the reduction of other methyl groups to CH₄ (Table 1.1.5.). Members of the Methanomassiliicoccales, the genus Methanosphaera and the species Methanomicarococcus are unable to oxidize methyl groups independent of an external electron donor, and therefore require the presence of H₂ for methanogenesis (Whitman et al. 2006; Lang et al. 2015).

1.9. Ecology of the methanogens

Methanogens inhabit a diverse range of anoxic ecosystem types ranging from psychrophilic to thermophilic, and freshwater to hypersaline. Methanogenesis is typically the final step in degradation of organic matter to CO₂ and CH₄ in anoxic environments with high organic matter loading and/or a limited supply of alternative
electron acceptors (such as NO$_3^-$, Mn or Fe oxides, and SO$_4^{2-}$). Such environments include freshwater sediments, wetlands and swamps, estuaries, continental shelf environments, and water-logged soils such as rice paddies (Ferry and Lessner 2008). Methanogenesis has also been detected in a wide variety of ‘extreme’ environments, such as deep marine and terrestrial sediments, coal and shale deposits, petroleum reservoirs, granitic aquifers, mafic and ultra-mafic hydrothermal systems, hypersaline brines, solar salterns, permafrost soils, glacial lakes, and icesheets (Whitman et al. 2006). They are partially responsible for generating the methane in gas hydrates, even at great depth (Marchesi et al. 2001). The microbiology of methane production in natural sedimentary environments such as marine sediments (Sowers and Ferry 2002; Reeburgh 2007; Ferry and Lessner 2008), wetlands (Bridgham et al. 2013), soils (Nazaries et al. 2013), and, in recent years, permafrost soils (Jansson and Tas 2014) has been increasingly well studied and reviewed. This study will focus on methanogenesis in an artificial freshwater lake (Cardiff Bay, Wales) which was created by impoundment of estuarine, intertidal mudflats in the early 2000s. To put the following chapters into context, methanogenesis in estuarine sediments, freshwater lake sediments, and artificial lakes will be discussed in more detail below.

1.9.1. Methanogenesis in estuarine sediments

Estuaries are characterised by longitudinal salinity gradients from head (freshwater endpoint) to mouth (marine endpoint), caused by mixing of marine and riverine waterbodies. The areal extent of estuarine environments is approximately 0.4 % of the marine environments, yet they contribute 7.4% of marine atmospheric methane emissions (Bange et al. 1994). Sulphate reduction accounts for 50 % of organic matter degradation in marine environments (Jørgensen, 1982). Sulphate reduction is the dominant anaerobic terminal oxidation process in estuarine sediments (Wellsbury et al. 1996), with increasing importance of methanogenesis towards the freshwater end point (e.g. Takii and Fukiu, 1991; Wellsbury et al. 1996; Purdy et al. 2002; O’Sullivan et al. 2013). Atmospheric methane emissions from estuarine environments are also influenced by organic matter availability and lability, with highest emissions from tidal flats and marsh environments (Bianchi et al. 2006).
The methanogen community of estuarine tidal and intertidal sediments are typically dominated by the orders Methanosarcinales and Methanomicrobiales (Banning et al. 2005; Wilms et al. 2006; Li et al. 2012; Zeleke et al. 2012; Chen et al. 2013a,b; O’Sullivan et al. 2013; Webster et al. 2014; Xie et al. 2014). The orders Methanocellales (Li et al. 2012; Zeleke et al. 2013; Chen et al. 2013a,b; Xie et al. 2014) and Methanococcales (Li et al. 2012; Zeleke et al. 2013) have also been detected at low abundance in estuarine sediments. The order Methanosarcinales (Wilms et al. 2006; Li et al. 2012; Chen et al. 2013a,b; Webster et al. 2014) are commonly present in low abundance, but were the dominant methanogen order in sediments of a tropical estuaries on the western coast of India (Singh et al. 2011). Several studies investigating variation in sediment methanogen community composition along the spatial salinity gradient in estuarine environments (Purdy et al. 2002; O’Sullivan et al. 2013; Webster et al. 2014; Xie et al. 2014) have observed niche partitioning in the distribution of different methanogen genera based on substrate utilisation capability. Webster et al. (2014) found that the methylotrophic methanogen genera Methanococcoides (family Methanosarcinaceae, order Methanosarcinales) and Methanolobus family Methanosarcinaceae, order Methanosarcinales) were only detected at the marine endpoint of the Colne Estuary, UK. Purdy et al. (2002) also found that the genus Methanolobus was restricted to the marine endpoint of the Colne Estuary, UK. Similarly, Xie et al. (2014) reported a decrease in the abundance of the genus Methanococcoides with decreasing salinity between the marine and freshwater endpoints of the Pearl River estuary in China.

The methylotrophic genera Methanococcoides and Methanolobus, and hydrogenotrophic Methanococcus are the genera most commonly detected in marine sediments by cultivation (Ferry 1993). Watkins et al. (2012) used a combination of molecular genetic and cultivation approaches to show that the genus Methanococcoides is ubiquitous in marine sediments. Sulphate reducing bacteria (SRB) outcompete methanogens for common substrates (such as acetate and hydrogen) in sulphate-rich environments by virtue of their higher substrate affinity (Lovley et al. 1982; Oremland and Polcin 1982). Therefore, non-competitive substrates such as methylated amines are particularly important precursors for methanogenesis in saline environments if sulphate is present. When sulphate is depleted, hydrogenotrophic methanogenesis predominates (Liu and Whitman 2008).
1.9.2. Methanogenesis in freshwater lake sediments

Freshwater lakes are one of the major sources of atmospheric methane emissions, contributing between 6 and 16 % of natural atmospheric methane emissions (Bastviken et al. 2004). Methanogenesis is the dominant anaerobic terminal oxidation process in freshwater environments, accounting for approximately 50% of organic matter degradation (Lovley et al. 1982). Rates of methane production in freshwater lake sediments are strongly dependent on organic matter availability (Bastviken et al. 2009). Acetotrophic methanogenesis is usually the main pathway for methane production in freshwater environments (70 % of total methane production; Whiticar et al. 1999), however there are some exceptions to this rule (Smith et al. 1993; Lovley et al. 1982; Wand et al. 2006; Mandic-Mulec et al. 2012; Conrad et al. 2011). Methylotrophic methanogenesis is only a minor pathway for methane in freshwater lake sediments (Lovley and Klug 1983b; Conrad and Claus 2005), as the precursors for methylated compounds (e.g. osmoregulants such as glycine-betaine) are not abundant in freshwater environments (Zinder and Brock 1978; Lovley and Klug 1983b; Lomans et al. 1997).

Despite the importance of freshwater lakes in global atmospheric emissions relatively little is known about the methanogen ecology of freshwater lake sediments. Similar to estuarine tidal and intertidal sediments, the Methanomicrobiales and Methanosarcinales are the dominant methanogen orders in freshwater lake sediments (Falz et al. 1999; Chan et al. 2005; Karr et al 2006; Schwarz et al. 2007; Lehours et al. 2007; Mandic-Mulex et al. 2012). The hydrogenotrophic genus Methanoregula (family Methanomicrobiaceae, order Methanomicrobiales) and acetotrophic genus Methanothrix (family Methanotrichaceae, order Methanosarcinales) are detected in almost all freshwater lake sediments (Borrel et al. 2011). 16s rRNA sequences affiliated with the orders Methanocellales and Methanobacteriales are also occasionally detected, but usually at lower abundance than the Methanomicrobiales and Methanosarcinales (Borrel et al. 2011). Methylotrophic methanogens are rarely detected in freshwater lake sediments (Borrel et al. 2011), although the type strain for the methylotrophic *Methanomethylovorans hollandica* was isolated from sediments of a freshwater lake (Lomans et al. 1999).
1.9.3. Methanogenesis in impounded freshwater environments

Impounded freshwater reservoirs are also an important source of atmospheric methane emissions which have received relatively little attention. The global number of impoundments which are built for hydropower, agricultural and recreational purposes, has increased dramatically between the mid-21st century and early 2000s (Downing et al. 2006). The growth rate in annual construction of large scale reservoirs has decreased significantly over the past 3 decades due to environmental concerns, however the rate of increase of small scale reservoirs continues to rise (Chao et al. 2008). Several studies have recorded exceptionally high rates of atmospheric methane emissions from temperate impounded reservoirs (e.g. DelSontro et al. 2010; Sobek et al. 2012; Maeck et al. 2013; Maeck et al. 2014). Riverine impoundments typically have high sedimentation rates which lead to a build-up of sediments beneath a shallow water column unless the reservoir is dredged (DelSontro et al. 2010). High sedimentation rates mean that organic matter is quickly shuttled into the anoxic zone of sediments, and hence is available to sustain methanogenesis (Sobek et al. 2012).

Increased atmospheric temperatures may also increase the efficiency of methane export from anoxic lake sediments to the atmosphere. There are four main pathways by which methane produced in sediments reaches the atmosphere - diffusion at the water/air interface (either from surface waters or when methane saturated deep waters are exposed through seasonal convective overturning), ebullition, and advection through plants (Bastviken et al. 2004). Diffusion is the main pathway of methane transport in deep waterbodies (>50 m, McGinnis et al. 2006), but ebullition becomes important when the water column is <10 m depth as limited dissolution of methane bubbles occurs during transit (Ostrovsky et al. 2008). Methane diffusion is a slow process, and a significant proportion of methane that enters the watercolumn through diffusive flux is oxidized by methanotrophic bacteria (Segers 1998).

It is estimated that between 30-99% of methane produced in anoxic lake sediments is oxidized by methanotrophic bacteria (Bastviken et al. 2008), and the relative contribution of the diffusion and ebullition as pathways of methane is an important factor in determining the proportion of methane generated in anoxic sediments that is oxidized before reaching the atmosphere (Maeck et al. 2014). In contrast, ebullition
represents a direct pathway between sediments and the atmosphere. Ebullition is an important pathway for methane transport in impounded reservoirs, and such environments often have high methane production rates (DelSontro et al. 2010; Sobek et al. 2012; Martinez and Anderson 2013). Ebullition is temperature dependent, with increased rates recorded during the summer season (Casper et al. 2000; Martinez and Anderson 2013; Wik et al. 2013; Wik et al. 2014). Hence the relative importance of ebullition as a pathway for atmospheric methane emissions from freshwater lakes is expected to increase as a result of future climate change (Thornton et al. 2015).

1.10. Aims and objectives

Cardiff Bay is an unusual aquatic habitat, having recently undergone rapid and dramatic environmental change. The impoundment of Cardiff Bay in 2001 transformed a 100 ha of estuarine intertidal mudflats into an artificial freshwater lake. The overarching aim of this study was to determine how the sedimentary methanogenic community in Cardiff Bay has developed as a result of impoundment, and also to investigate the impact of future climate change. More specifically, the objectives were to determine how the transition from estuarine to freshwater salinities has impacted on the types of methanogens present, their substrates, and their temperature and salinity characteristics.

This was achieved through:

- comparison of the salinity and temperature functional operating range and optima of methanogens and methanogenic (methanogens plus syntrophs) communities in sediments deposited pre- and post-impoundment (estuarine clay and freshwater gyttja respectively).
- enrichment and isolation of methylotrophic, acetotrophic and hydrogenotrophic methanogens representative of the salinity and temperature ranges of Cardiff Bay sedimentary methanogenic communities.
- physiological and metabolic characterisation of methanogens isolated from Cardiff Bay sediments.
2. GENERAL MATERIALS AND METHODS
Note: This chapter contains a description of materials and methods common to all of the following results (Chapters 4 to 7). Materials and methods specific to a particular results chapter are described within the relevant chapter.

2.1. Sediment coring
Sediment cores were obtained from Cardiff Bay, Wales using a specially designed multicorer (Duncan and Associates, Cumbria, UK) mounted on the boat R.V. Guiding Light and operated via a hydraulic A-frame (Wigmore Wright, Penarth, Wales) with electrohydraulic winch system (Spencer Carter, Falmouth, England) (Figure 2.0.1.). The multicorer consists of a central section which holds four core sleeves, four spring-loaded ‘arms’ which hold the rubber bungs which plug the bottom of the core sleeves, and an outer frame. The multicorer is able to retrieve sediment cores of 120 mm diameter and up to 450 mm in length, which are held within clear plastic core sleeves sealed with rubber bungs. The multicorer is lowered down to the sediment surface using the electrohydraulic winch, and when the corer frame reaches the sediment surface then the central section of the corer is automatically released and sinks into the sediment by gravity. After a period of time (dependant on the sediment composition, and determined by trial and error) the corer is slowly lifted from the sediment bed, and once suitable clearance is obtained the ‘arms’ swing round to plug the bottom of the core sleeves. The speed at which the central core section sinks into the sediment is controlled by a valve connected to the central section of the corer. This allows the core sleeves to be lowered slowly in fine grained or loosely consolidated sediments in order to minimise sediment disturbance. Cores were stored at in darkness at 4 °C overnight before further processing to minimise changes to geochemical conditions or microbial community composition.

2.2. Sediment sampling for slurry preparation, geochemical analysis and molecular analysis
Sediment cores were sectioned using a hydraulic extrusion system (Duncan and Associates, Cumbria, UK). Core sectioning was carried out on the laboratory bench top, and performed as rapidly as practicable. Briefly, the bottom bung in a core sleeve was removed and replaced with a piston, and the core placed on the hydraulic extrusion assembly. The top bung of the core sleeve was also then removed. Water
was pumped slowly into the base of the hydraulic extrusion assembly, pushing the piston and overlying sediment upwards. Core collars were used to ensure that cores were sectioned at either 2 or 5 cm intervals as required. A sterile aluminium plate was used to slice the whole round core section once the correct depth of sediment had been extruded from the core sleeve. Replicate cores sectioned to provide inoculum for

Figure 2.0.1. Sediment sampling using multicorer aboard the R.V. Guiding Light in Cardiff Bay, Wales
A: Corer being lowered down to sediment surface using winch. Core sleeves are empty, and corer arms are in upright position.
B: Two out of four sediment cores have been successfully retrieved. Corer arms have been released, and are now holding rubber bungs in position to cap core sleeves.
sediment slurries were sectioned at 0-5 (January and June 2012 cores), 10-15 (January 2012 core only) and 25-30 (January 2012 and June 2012 cores) depth, and whole round core sections of 5 cm depth were transferred into a wine bag and homogenised under an N₂ atmosphere as described by Cragg et al. (1992). The homogenised sediment preparation was then dispensed into slurry media bottles as appropriate (described in sections 4.2.2. and 5.2.2.).

Sediment cores sectioned for geochemical analysis or molecular genetic analysis were sectioned at either 2 cm (January 2012 core) or 5 cm (June 2012 and April 2014 core) depth. Sediment minicores for geochemical and molecular genetic analysis were then taken from whole round cores of 5 cm or 2 cm depth using sterile 5 ml syringes with luer end removed. The syringes were inserted vertically into the surface of a sediment slice, avoiding the perimeter of the cores in order to minimise the likelihood of contamination, and then carefully removed with the plunger still fully withdrawn. To obtain porewaters for ion and cation analysis 5 ml of sediment (from one of more sediment minicores) was extruded into a 15 ml centrifuge tube and centrifuged (Rotanta 460 R, Hettich, Tutlingen, Germany) at 1270 x g (RCF) for 5 minutes. Of the resulting supernatant, 1 ml was filtered into 1.5 ml Chromacol glass vials (Thermo Fisher Scientific, MA, USA) using a 0.22 µm Millex® syringe filter (Merck Millipore, Darmstadt, Germany) and stored at -20 °C until analysis. Samples were then analysed according to section 2.6.2. For gas analysis, 3 ml of sediment was extruded into 20 ml of 10 % (w/v) KCl in a 50 ml Chromacol glass vial (Thermo Fisher Scientific, MA, USA), and stoppered with a rubber bung and aluminium crimp. Samples were homogenised using a mechanical shaker (Stuart Scientific Flask Shaker SF1, Keison, Chelmsford, UK) for one hour, and stored upside down overnight at 20 °C in the dark to allow equilibration between slurry and headspace. 2 ml of headspace gas was analysed according to section 2.6.1. Sediment minicores (5 ml) for molecular analysis were stored at -80 °C until further analysis.

2.3. Cultivation techniques

The methanogens are strictly anaerobic prokaryotes that thrive only under reducing conditions. While many fermenters and sulphate reducers can cope with short time oxygen exposure, methanogens are the most oxygen-sensitive microorganisms
known. This sensitivity requires a range of precautions to prevent oxygen exposure and also the use of specifically designed anoxic, reducing media.

### Table 2.0.1. Mineral salts composition of FeS reduced methanogen cultivation media

<table>
<thead>
<tr>
<th>Mineral salt</th>
<th>Solution concentration</th>
<th>Media salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freshwater</td>
<td>Brackish</td>
</tr>
<tr>
<td>NaCl</td>
<td>-</td>
<td>0.2 g</td>
</tr>
<tr>
<td>MgCl₂ * 6 H₂O</td>
<td>-</td>
<td>0.25 g</td>
</tr>
<tr>
<td>CaCl₂ * 2 H₂O</td>
<td>-</td>
<td>0.1 g</td>
</tr>
<tr>
<td>KCl</td>
<td>-</td>
<td>0.1 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.4 M</td>
<td>1 ml</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.04 M</td>
<td>1 ml</td>
</tr>
<tr>
<td>NaBr</td>
<td>0.84 M</td>
<td>-</td>
</tr>
<tr>
<td>NaF</td>
<td>0.07 M</td>
<td>-</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>0.15 M</td>
<td>-</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.4 M</td>
<td>-</td>
</tr>
<tr>
<td>SL10*</td>
<td>-</td>
<td>1 ml</td>
</tr>
<tr>
<td>SeWo†</td>
<td>-</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

* Unchelated trace element solution (Widdel et al. 1983).
† Selenite-tungstate solution (Widdel and Bak 1992).
N.B. Addition mass/volumes are per litre

#### 2.3.1. Preparation of methanogen cultivation media

Methanogen media was prepared under anoxic conditions using a glass vessel (Figure 2.0.2.) based on the design of Widdel (1980). Basal mineral salts (Table 2.0.1.), SL10 trace element solution (Table 2.0.2.), and selenite-tungstate solution (Table 2.0.3.) were dissolved in reverse osmosis water and autoclaved within the media vessel for 1 hour at 121 °C. During autoclaving the tube leading to the glass bell was closed using a steel clip to prevent media loss. At least one of the screw caps of the side ports on top of the vessel was loosened during autoclaving to allow pressure exchange between the vessel interior and exterior. After autoclaving the media was removed from the autoclave at temperatures between 75 and 80°C and connected to a gas line. This was done to take advantage of the fact that the freshly boiled media is almost gas free, and therefore oxygen free. The incoming gas was sterilised by passing through a glass wool or cotton filter. After the vessel was connected the gas was kept flowing for about 5 minutes with one or both side ports partly opened to allow replacement of the headspace air in the vessel with oxygen-free gas, N₂/CO₂ or H₂/CO₂ (for carbonate buffered media; 80:20 v/v, 5 kPa) or N₂ (for phosphate buffered media; 5 kPa) headspace. The media becomes saturated with the gas present in the headspace whilst cooling. Temperature-sensitive (e.g. vitamins, iron (II) chloride) or volatile (e.g.
bicarbonate, sulphide) media components were added to the cold media from sterile stock solutions via the side ports of the media vessel. Contamination of the media with airborne microbes is extremely unlikely due to the counter-flow of sterile headspace gas. The pH of the media was adjusted to 7.2-7.4 using autoclaved 1M NaOH or HCl if necessary. Media was dispensed under an autoclaved glass bell (to maintain sterility) into autoclaved screw top bottles for storage.

Figure 2.0.2. Schematic diagram of media vessel used to prepare and dispense FeS-reduced mineral salts media.

2.3.1.1. Buffering of methanogen media

The standard buffer used for methanogen media was a mixture of carbon dioxide and bicarbonate, which is also the natural buffer system in most aquatic and sedimentary systems. While carbon dioxide is added via the gas headspace, bicarbonate stock solutions are prepared alongside the media. For one litre of media 30 ml of a 1 M NaHCO₃ solution was prepared and filled into a screw-cap bottle, ensuring that the bottle was only filled to a maximum of 60%. The screw cap with a rubber septum was tightly closed to prevent a loss of carbon dioxide during autoclaving. For safety reasons, the bottle with the bicarbonate solution is autoclaved within a plastic container, and is removed once the solution has reached room temperature.
2.3.1.2. Reducing agents

Reducing agents are added to scavenge any oxygen that may still be present in the media after autoclaving or that diffuses in during incubation, and also to lower the redox potential of the media. The most common reducing agents used in microbiology are sodium dithionite (which is very selective for sulphate reducers) or and sodium sulphide. Iron monosulphide (FeS) was used in this study rather than sodium sulphide. Iron monosulphide offers a number of advantages compared with sodium sulphide: (i) the added iron represent additional oxygen scavenging potential and iron reacts faster with oxygen than sulphide, (ii) as most of the sulphide is present in FeS particles, the free sulphide concentration is lower than in standard sulphide-reduced media, (iii) because during oxidation the colour changes from black to orange (iron oxohydroxide), there is no need for the addition of a redox indicator (resazurin) which may be inhibitory for some microorganisms, (iv) FeS particles offer surfaces for attachment. FeS is particulate, therefore accurate addition is very difficult. For this reason, iron (II) and sulphide were added separately from sterile stock solutions (see 2.3.1.3.). As iron (II) chloride solution is very acidic, and the sodium sulphide solution is very alkaline, it is necessary to let the media pH stabilise for 15 minutes after addition of these compounds before testing the media pH.

2.3.1.3. Media additions

The composition of the different media supplements are given in Tables 2.0.2. - 2.0.5. The vitamin solution is sterile-filtered and the glass bottles wrapped with aluminium foil to prevent photodegradation. The vitamin solution was stored at 4°C. The trace metal solution is prepared using HCl as most metals would precipitate as metal oxides at neutral pH. The use of complexation agents, such as EDTA, was avoided as they may be inhibitory to methanogens. Selenite and tungstate are only soluble under alkaline conditions, and therefore cannot be added to the trace element solution. The trace element and selenite-tungstate solution are autoclaved, and can be stored at room temperature.
Table 2.0.2. Composition of SL10 trace element solution (Widdel et al. 1983)

<table>
<thead>
<tr>
<th>Compound</th>
<th>mg l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl₂.6H₂O</td>
<td>190</td>
</tr>
<tr>
<td>MnCl₂.2H₂O</td>
<td>100</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>70</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>36</td>
</tr>
<tr>
<td>NiCl₂.6H₂O</td>
<td>24</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>6</td>
</tr>
<tr>
<td>CuCl₂.2H₂O</td>
<td>2</td>
</tr>
</tbody>
</table>

To prepare SL10 trace element solution add 1.5 g FeCl₂.4H₂O to 10 ml of HCl (25%) and mix well. Add the above compounds and distilled water to make up to 1000 ml total volume, and mix well. Decant into screw top bottles and autoclave at 120 °C for 30 minutes.

Table 2.0.3. Composition of selenite and tungstate (SeWo) solution (Widdel and Bak 1992)

<table>
<thead>
<tr>
<th>Compound</th>
<th>mg l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>400</td>
</tr>
<tr>
<td>Na₂SeO₃.5H₂O</td>
<td>6</td>
</tr>
<tr>
<td>Na₂WO₃.2H₂O</td>
<td>8</td>
</tr>
</tbody>
</table>

To prepare solution add the above components to 1000 ml of distilled water, and mix well. Decant into screw top bottles and autoclave at 120 °C for 30 minutes.

Table 2.0.4. Temperature sensitive additions to methanogen cultivation media

<table>
<thead>
<tr>
<th>Media additions</th>
<th>Solution concentration</th>
<th>ml l⁻¹ of media</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃ solution</td>
<td>1 M</td>
<td>30</td>
</tr>
<tr>
<td>10 vitamin solution*</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Na₂S³</td>
<td>1 M</td>
<td>1.2</td>
</tr>
<tr>
<td>FeCl₂⁵</td>
<td>1 M in 0.1 M HCl</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Composition of 10 vitamin solution (Balch et al. 1979) shown in Table 2.0.5.

a To prepare Na₂S solution add distilled water to serum vial and sparge with N₂ for 15 minutes. Add Na₂S.9H₂O using plastic tweezers, and flush vial headspace with N₂. Seal vial with butyl rubber stopper and crimp. The resulting solution has a pH of around 14, and is therefore considered sterile.
b To prepare to FeCl₂ solution add 0.1 M HCl into a glass vial with magnetic follower and gas with N₂ for 15 minutes. Add FeCl₂.4H₂O and mix, whilst flushing vial with N₂. Seal vial with butyl rubber stopper and crimp. Autoclave for 30 minutes at 120 °C.

Table 2.0.5. Composition of 10 vitamin solution (Balch et al. 1979)

<table>
<thead>
<tr>
<th>Compound</th>
<th>mg l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Aminobenzoic acid</td>
<td>25</td>
</tr>
<tr>
<td>D(+) biotin</td>
<td>10</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>25</td>
</tr>
<tr>
<td>Ca-D(+) pantothenate</td>
<td>25</td>
</tr>
<tr>
<td>Pyridoxine-dihydrochloride (vitamin B6)</td>
<td>50</td>
</tr>
<tr>
<td>Folic acid</td>
<td>10</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>25</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>25</td>
</tr>
<tr>
<td>Thiamine-hydrochloride</td>
<td>25</td>
</tr>
<tr>
<td>Cyanocobalamin (vitamin B12)</td>
<td>5</td>
</tr>
</tbody>
</table>

To prepare the 10 vitamin solution add the above compounds to 1000 ml of distilled water and mix well. Filter sterilize the resulting solution into autoclaved screw cap bottles covered in foil. Store at 4°C.
2.3.2. Preparation of methanogen cultivation tubes

10 ml of media was transferred from screw cap bottles into 20 ml anaerobic tubes (Bellco, New Jersey, USA) under an N₂/CO₂ or N₂ gas stream using a sterile glass syringe. Methanogen cultivation tubes were flushed with either N₂/CO₂ (80:20 v/v, 150 kPa) or N₂ (150 kPa) using a gassing cannula (Figure 2.0.4.) for 1 minute prior to the transfer of media, and for 30 seconds following the transfer of media. Once media transfer and headspace flushing was complete the tubes were sealed with a butyl rubber bung and aluminium crimp.

![Figure 2.0.3. Schematic diagram showing gassing cannula used to flush the headspace of anaerobic cultivation tubes during addition of FeS-reduced mineral salts media.](image)

2.4. Temperature gradient incubator

The temperature gradient incubator (previously described by Barnes et al. 1998) consists of an aluminium block encased in a foam insulating later, and within a wooden housing (Figure 2.0.5.). The aluminium block is heated at one end by a heating rod and cooled at the opposite end using a chiller. A constant temperature gradient can be maintained across the aluminium block by adjusting the heater and chiller settings as desired. There are 12 fixed temperature sensors, and 47 incubation cells evenly spaced across the width of the aluminium block. The temperature of a given incubation cell can be calculated from a linear regression of the temperatures measured by the sensors (and can be checked manually with aid of thermometer).

2.5. Microscopy

Microscopy was undertaken using a phase-contrast microscope (Axioskop, Zeiss, Germany) with x100 magnification, oil immersion objective and x10 magnification
eyepiece. F420 autofluorescence, indicative of methanogens (Doddema and Vogels 1978), was visualized using a UV light source in conjunction with a blue violet excitation band pass filter (390-440 nm) and dichromatic FT beam splitter (460 nm). Photographic images were taken using Nikon Coolpix 4.0 megapixel digital camera attached to the eyepiece of the microscope.

2.6. Geochemical analyses

2.6.1. Natural gas analysis

Headspace gas samples were analysed using a modified Perkin Elmer Arnel Clarus 500 Natural Gas Analyzer (Perkin Elmer, MA, USA) with Flame Ionisation Detector (FID) and Thermal Conductivity Detector (TCD). The use of a packed column 2 channel system allows simultaneous determination of hydrocarbons, carbon dioxide, and hydrogen. Gas samples with a minimum volume of 2 ml were introduced to the valve injection system via a gas tight syringe. The oven was operated under isothermal conditions (110 °C), the FID at 250 °C with helium carrier gas, and TCD at 150°C with argon carrier gas. Peaks were identified against a gas standard, and quantification was carried out using calibration curves derived from three gas standards (Scott Speciality Gases, Pennsylvania, USA) of differing concentrations. Detection limits were 0.5 ppm for methane, 70 ppm for hydrogen, and 50 ppm for carbon dioxide.
2.6.2. Ion chromatography

Anion chromatography was carried out using a Dionex ICS-2000 Ion Chromatography System (Dionex, Camberley, UK) with a KOH eluent (flow rate 0.9 ml min\(^{-1}\)), fitted with Dionex AS50 autosampler (Dionex, Camberley, UK). Ion separation was conducted using 2 Ionopac AS15 columns in series, with an Anion Self-Regenerating Suppressor (ASRS-ULTRA II 4-mm) unit in combination with a DS6 Heated Conductivity Cell (Dionex, Camberley, UK). The eluent gradient was as follows: (i) 6 mM KOH for 38 minutes, (ii) increase of 16 mM min\(^{-1}\) for 4 minutes to final concentration 70 mM KOH, (iii) 70 mM KOH for 17 minutes, (iv) decrease of 64 mM KOH min\(^{-1}\) to 6 mM KOH final concentration, (v) 6 mM KOH for 12 minutes. Cation chromatography was carried out using a Dionex ICS-2000 Ion Chromatography System (Dionex, Camberley, UK) with methanesulfonic acid eluent (32 mM, flow rate 0.9 ml min\(^{-1}\)), fitted with Dionex AS50 autosampler (Dionex, Camberley, UK). Ion separation was conducted on an Ionopac CS16 column (50 °C), with a CSRS 300 4 mm suppressor in combination with a DS6 Heated Conductivity Cell (50 °C). Calibration was achieved using 5 (for anions) or 7 (for cations) standards containing the compounds of interest in sequentially increasing concentrations. All calibrations were linear, and calibrations with a coefficient of <0.95 % were rejected.

2.7. Porosity

3-4 ml of sediment was placed in pre-weighed screw cap glass scintillation vials and weighed (wet weight, WW). Samples were dried at 60 °C for 36 hours, and reweighed (dry weight, DW). Sediment porosity (Φ) was calculated according to Equation 2.0.1. after Schulz and Zabel (2006).

\[
\Phi = \left[\frac{(WW-DW)}{WW}\right] \times 100
\]

*Equation 2.0.1. Calculation of sediment porosity*

2.8. Sediment organic carbon and carbonate content

Loss on ignition (LOI) was used to determine sediment organic matter content and carbonate content using the protocol described by Heiri et al. (2001). Approximately 1 g of dried sediment from porosity analyses was weighed (DW\(_{60}\)), and heated to 550 °C in a muffle furnace for 4 hours in a ceramic crucible, then reweighed once cool (DW\(_{550}\)).
The sediment was then heated for a further 2 hours at 950 °C, then reweighed once cool (DW \text{950}). Sediment organic matter content was calculated according to Equation 2.0.2., and sediment CaCO\textsubscript{3} content calculated according to Equation 2.0.3.

LOI at 550°C (LOI\textsubscript{550}) and LOI at 950 °C (LOI\textsubscript{950}) values normally correlate well with sediment organic matter and calcium carbonate content (Dean Jr 1974; Heiri et al. 2001; Santisteban et al. 2004), however results cannot be used quantitatively to compare facies unless absolute carbon content has been determined using an alternative method (Santisteban et al. 2004).

\[ \text{Organic matter (weight \%, LOI}_{550} = \frac{(\text{DW}_{60} - \text{DW}_{550})}{\text{DW}_{60}} \times 100 \]

*Equation 2.0.2. Sediment organic matter content*

\[ \text{Calcium carbonate (weight \%, LOI}_{950} = \frac{(\text{DW}_{950} - \text{DW}_{550})}{\text{DW}_{60}} \times 1.36 \times 100 \]

*Equation 2.0.3. Sediment calcium carbonate content*

### 2.9. Molecular genetic analyses

#### 2.9.1. DNA extraction from sediment and sediment slurries

Whole community genomic DNA was extracted from sediment and slurry samples using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) using a modified method detailed in Table 2.0.6. DNA extractions were visualized via 1.2% (w/v) agarose gel electrophoresis and stained with 0.05 µl ml\textsuperscript{-1} SYBR® Safe DNA gel stain (Life Technologies Ltd, Paisley, UK) and quantified against the Hyperladder \textsuperscript{TM} 100bp (Bioline Reagents Ltd, London, UK) DNA quantification marker. DNA was visualised using either the Gene Genius Bio Imaging System with Gene Snap software (Syngene, Cambridge, UK), or Versa Doc 4000 with Quantity One version 1.6.3. software (Biorad, California, USA).

#### 2.9.2. DNA extraction from methanogen cultures

Genomic DNA was extracted from methanogen cultures using the nexttec 1-step DNA Isolation from Bacteria kit (MP Sciences, Wellingborough, UK) according manufacturer’s instructions. DNA extractions were visualized and quantified as detailed in section 2.9.1.
2.9.3. DNA amplification
Polymerase Chain Reaction (PCR) mixtures were prepared under aseptic conditions, and amplifications were carried out using a DNA Engine Dyad thermal cycler (MJ Research, Boston, USA). Primers were synthesised by Eurofins MWG Operon (Ebersburg, Germany) and all primer compositions and cycling conditions are shown in Table 2.0.6. PCR reactions were prepared in 0.2 ml thin wall tubes (Alpha Laboratories, Hampshire, UK) and contained 1 µl template DNA, 1.25 units Taq DNA polymerase (PCR Biosystems, London, UK), 0.5 µl primer (20 pmol µl⁻¹), 10 µl reaction buffer (PCR Biosystems, London, UK) and 33.75 µl sterile molecular biology grade water (Severn Biotech Ltd, Kidderminster, UK). 5 µl of bovine serum albumin (10 mg µl⁻¹) was also added to PCR master mix for the first round of nested PCR if amplifying from sediment or slurry DNA extractions, and the volume of water reduced correspondingly. Sediment and sediment slurry DNA extractions were diluted 1:10 for use as a template for PCR reactions. For nested PCR reactions 1 µl of a 1:10 dilution of first round PCR product was used as a template for 2nd round PCR product.

<table>
<thead>
<tr>
<th>Step</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add 120 µL of MT lysis buffer and 800 µL of sodium phosphate buffer to either 1 g of sediment or 3 ml of slurry.</td>
</tr>
<tr>
<td>2</td>
<td>Twice homogenize sample for 30 seconds using the FastPrep instrument on speed setting 5.5. Centrifuge for 8 minutes at 16000 g.</td>
</tr>
<tr>
<td>3</td>
<td>Transfer supernatant from Lysing Matrix tube to a 1.5 ml high recovery APEX NoStick Microcentrifuge Tube (Alpha Laboratories Ltd, Hampshire, UK) containing 250 µl of Protein Precipitation Solution (PPS). Invert 10 times, and centrifuge for 5 minutes at 16000 g.</td>
</tr>
<tr>
<td>4</td>
<td>Transfer supernatant from non-stick eppendorf to a 15 ml universal tube containing 1 ml of Binding Matrix. Shake by hand for 3 minutes, and then incubate for 30 minutes at room temperature.</td>
</tr>
<tr>
<td>5</td>
<td>Resuspend solution in universal tube and transfer to a Spin Filter tube. Centrifuge for 1 minute at 14000 g. Discard solution in catch tube after centrifugation. Repeat step until all of the sample had passed through the Spin Filter.</td>
</tr>
<tr>
<td>6</td>
<td>Add 50 µl of SEWS-M to Spin Filter tube, and centrifuge for 1 minute at 14000g. Place filter was in new catch tube, and add 100 µl of sterile molecular biology grade water (Severn Biotech Ltd, Kidderminster, UK) to the sample pellet. Incubate for 30 minutes at room temperature.</td>
</tr>
<tr>
<td>7</td>
<td>Centrifuge at 14000 g for 2 minutes to elute the DNA into catch tube. Discard filter.</td>
</tr>
</tbody>
</table>

2.9.4. DGGE protocol
PCR-DGGE was used to investigate the methanogen community structure of sediment, sediment slurries, and enrichment cultures. A nested PCR approach was utilised as initial testing demonstrated that for the majority of enrichment samples,
direct amplification using archaeal DGGE primers did not produce sufficient PCR product for DGGE analysis. Archaeal primer pairs 109f/958r (first round) and SAF-GC/598r (second round) were chosen as in silico testing suggests good potential coverage of archaeal sequences, particularly for methanogens (Nicol et al. 2003; Webster et al. 2006). Although there is concern that primer bias from nested PCR higher than from single step PCR (e.g. Muylaert et al. 2002; Zwart et al. 2005; Park et al. 2010), several studies have shown that the use of nested PCR has minimal effect on the bacterial community composition as observed using PCR-DGGE (Boon et al. 2002; Dar et al. 2005; Nikoluasz et al. 2005).

Denaturing Gradient Electrophoresis (DGGE) was carried out using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, California, USA) with 16 x 16 cm glass plates of 1 mm thickness. 8 % (w/v) polyacrylamide gels with a 30 % (126 g l⁻¹ urea, 12 % v/v formamide) to 60 % (252 g l⁻¹ urea, 24 % v/v formamide) denaturing gradient were prepared using 1 x TAE buffer, and poured using a 50 ml Gradient Mixer (Fisher Scientific, Loughborough, UK). DGGE marker standards were constructed by mixing bacterial 16S rRNA gene amplicons from a range of bacterial species according to Webster et al. (2003). 8 µl of DGGE marker was run on the first and last lane of every gel to allow normalization of gels during banding pattern analysis. 10 µl of PCR product was loaded per lane, and gels electrophoresed for 15 minutes at 80 V, then a further 285 minutes at 200 V (total electrophoresis time of 5 hours). Gels were stained with SYBR gold nucleic acid gel stain (Invitrogen, California, USA) for 30 minutes, and imaged under UV light using the VersaDoc MP 4000 Imaging System with Quantity 1 Software (Biorad, California, USA). Individual bands were excised from DGGE gels using a sterile scalpel and stored in sterile 0.2 ml PCR tubes (Alpha Laboratories, Hampshire, UK) at -20 °C until reamplification. If a band was present at a particular position in more than one fingerprint on the DGGE gel then the band of interest was excised from at least two differing samples. All unique bands were excised and sequenced.

2.9.5. Reamplification of DGGE bands

Excised DNA bands were washed with sterile molecular biology grade water (Severn Biotech Ltd, Kidderminster, UK) for 10 minutes, air dried, crushed into 10 µl sterile molecular biology grade water, then frozen for a minimum of 6 hours. 1 µl of the thawed
supernatant was used as the template for reamplification using primers SAF-GC and Parch 519r-AT-M13F (archaeal 16S rRNA gene DGGE bands; Table 2.0.7.).

2.9.6. DNA sequencing
DNA sequencing was carried out by the Molecular Biology Support Unit at School of Biosciences, Cardiff University using a ABI 3130xl Prism Genetic Analyzer automated capillary sequencer (Applied Biosystems, California, USA) with BigDye terminator chemistry (Version 3.1.).

2.9.7. Phylogenetic analysis
Sequence chromatograms were viewed and edited using either MEGA version 6.0 (Tamura et al. 2013) or BioEdit version 7.2.5 (available from http://www.mbio.ncsu.edu/bioedit/bioedit.html). Nucleotide assignment was checked manually against sequence chromatograms. The portion of sequences relating to forward or reverse primers were removed, and ambiguous peaks labelled with N. Poor quality regions were excluded from further analyses. Sequence similarity searches were performed against the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) sequence database using the BLASTN program.

2.9.8. DGGE fingerprint analysis
2.9.8.1. Band class identification and quantification of band intensity
DGGE gel images were scanned into Gel Compar version 5.5 (Applied Maths, Belgium) as an inverted 16-bit TIFF file. Tone curve settings were adjusted for individual gels using a linear transformation. The spectral analysis program included within the software was used to determine the optimum disk size rolling-ball background subtraction and least-squares filtering cut-off for each gel. A median filter was applied to all images to produce smooth densitometric curves. Intragel normalization was achieved using molecular markers located in the first and last lane of each gel. Band searching (minimum profile relative to maximum lane profile = 1 %, grey zone = 5 %, shoulder sense = 6) and band matching (position tolerance = 1 %, optimization = 5 %) were performed automatically, and checked by visual inspection.
<table>
<thead>
<tr>
<th>Primer Description</th>
<th>Primer Sequence (5’ – 3’)</th>
<th>Amplicon length</th>
<th>PCR cycling parameters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YCCGGCGTTGAMTCCAATT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Archaeal 16S rRNA gene DGGE</td>
<td>CCTAYGGGGGCAGCAGCGG</td>
<td>154 (plus 40 bp clamp)</td>
<td>35 cycles 94°C, 30 s; 53.5°C, 30 s; 72°C, 60 s.</td>
<td>Nicol et al. (2003)</td>
</tr>
<tr>
<td>SAF-GC (i)</td>
<td>AGCCTACGGGGCGCAGAGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAF-GC (ii)</td>
<td>TTACC CGGGCGCKGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parch 519r</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reamplification of archaeal 16S rRNA gene DGGE bands</td>
<td>CCTATGGGGGCAGCGGG</td>
<td>154 (plus 80 bp linkers)</td>
<td>35 cycles 94°C, 30 s; 53.5°C, 30 s; 72°C, 60 s.</td>
<td>O'Sullivan et al. (2008)</td>
</tr>
<tr>
<td>SAF-GC</td>
<td>AGCCTACGGGGCGCAGAGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAF-GC (i)</td>
<td>TTACC CGGGCGCKGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAF-GC (ii)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Parch519r-AT-M13</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sequencing of reamplified DGGE bands</td>
<td>CAGGAAACAGCTATGAC</td>
<td>-</td>
<td>-</td>
<td>TOPO TA cloning® (Invitrogen)</td>
</tr>
<tr>
<td>M13r</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanogen mcrA gene</td>
<td>GCCMATGCGARATHGGWATGTC</td>
<td>760</td>
<td>30 cycles 94 °C, 40 s; 50 °C, 90 s; 72 °C, 180 s.</td>
<td>Hales et al. (1996)</td>
</tr>
<tr>
<td>ME1F</td>
<td>TCATKGCRTAGTDDGRTAGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME2R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial 16S rRNA gene DGGE</td>
<td>CCTACGGGGAGGCAGCAGCAG</td>
<td>194 (plus 40 bp clamp)</td>
<td>10 cycles 94°C, 30 s; 55°C, 30 s; 72°C, 60 s.</td>
<td>Muyzer et al. (1993)</td>
</tr>
<tr>
<td>357F-GC</td>
<td>ATTACCGCGGCTCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>518R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a All PCR programmes started with 5 min at 94°C and finished with 5 min at 72°C
b GC clamp: CGCCCGCGCGCGCGCCGGCGGGCAGGGCGAGCGG (attached at 5’ end of primer)
c M13R-GC linker: CAGGAAACAGCTATGACGGGGCGGCGGCGGGCGGACGGGG (attached at 5’ end of primer)
d Primer SAF-GC is a mixture of primers SAF-GC (i) and SAF-GC (ii) at a 2:1 molar ratio.
Band classes were labelled according to phylogenetic information obtained from the bands excised and sequenced from the DGGE gel. Band intensity was quantified according to band relative surface area (integrated peak area as a percentage of the total band area of the sample pattern). DGGE finger print processing using Gel Compar therefore generated a square matrix containing the presence and relative abundance of DGGE band classes for each sample, which was exported to Microsoft Excel for further analysis.

2.9.8.2. Calculation of genus relative abundance from band class intensity values

Methanogen sequences retrieved from DGGE gels cannot be identified reliably at species level due to the short sequence length (typically 100-250 bp), and therefore they were identified at genus level. The relative abundance of a given genus in a sample pattern was determined by summing the relative band surface area values of all band classes affiliated to the genus of interest, and calculating this value as a percentage of the total relative band surface area of identified band classes in the sample pattern of interest. DGGE band excision and sequencing combined with fingerprint band class analysis (as described above) showed that in many cases the DNA sequence associated with a particular band class present on a DGGE gel was not unique to that band class. In fact it was frequently observed that multiple band classes (up to 8 or 9) on a DGGE fingerprint were attributable to identical DNA sequences (shown in Appendices 2 and 3). In a small number of cases multiple banding was an artefact of degenerate primers (incidences indicated in Appendices 2 and 3), but in general there was no clear explanation for this phenomena.

Many organisms have 2 or more heterogeneous 16s rDNA operons which generate multiband profiles during PCR-DGGE analysis as a result of their differing sequences. However, in this study multiband profiles with identical sequences were observed. Multiple banding can also be linked to heteroduplex bands which are formed by reannealing of non-identical single stranded template molecules during PCR, however heteroduplex bands are typically observed only in the uppermost few centimetres of DGGE gels (Neilson et al. 2013) and this region of DGGE gels was excluded from band class analyses during this study. Shadow bands, described as low intensity
bands which closely follow high intensity bands with the same oligonucleotide sequence, are frequently reported in (Janse et al. 2004). Shadow bands are visible in some of the DGGE profiles presented in this study, and are particularly common in samples with low genus diversity (e.g. Figures A3.1. and 3.2., Appendix 3). Neilson et al. (2013) suggest that multiband profiles may occur when amplicons with identical oligonucleotide sequences but differing stable structural conformations are formed during PCR, which likely explains the remaining multiband profiles seen in this study. As DGGE gels produced during this study exhibited multiband profiles it was not possible to use band presence-absence data to measure community diversity. The number of band classes associated with a given sequence was not always consistent between lanes on a DGGE gel, and for this reason it would also not be appropriate to use band presence-absence data for intergel or intragel statistical comparison of sample community composition. Instead analysis has been limited to calculation of genus relative abundance as described above. The metabolic and physiological characteristics of methanogens are well conserved at genus level, with perhaps the exception of the genus *Methanosarcina*, therefore this approach is also well suited to the aims of the study. Often not all band classes on a DGGE gel could be identified from bands excised and sequenced. This mainly occurred because the imaging software used for fingerprint analysis can identify bands which were too faint to be identified by eye, but also on occasion when excised bands did not generate usable sequences.
3. METHANOGENESIS IN CARDIFF BAY SEDIMENTS

This chapter describes the environmental history of Cardiff Bay and uses sediment geochemistry, Methane Production Rates (MPR), and methanogen community structure to characterise methanogenesis in Cardiff Bay sediments. The aim of this chapter is to provide a basis for interpretation of the experimental studies investigating environmental controls on methanogenesis presented in subsequent chapters.

3.1. Location and environmental history of Cardiff Bay, Wales

3.1.1. Pre-barrage environment

The Severn Estuary has a tidal range of approximately 14.5 m in the Cardiff region, hence a large portion of tidal mudflats were exposed for 12-18 hours per day prior to barrage impoundment (Crompton 2002). The influence of the incoming tide from the Severn Estuary was evident as far upstream as Blackweir on the River Taff and the Arjo Wiggins weir on the River Ely (Cardiff Harbour Authority 2012).

3.1.2. Construction of Cardiff Bay barrage

Construction of Cardiff Bay barrage began in 1994, and was completed in late 2000. The barrage consists primarily of an embankment of 800 m length and 8.0 m height constructed from armoured rockfill overlying a sand base. Five sluice gates control water flow between the impoundment and the estuary, and a silt sump is positioned on the landward side of the sluices to trap saline ingress. Three navigable locks, a fish pass, and harbour breakwater caissons are also incorporated into the barrage structure. Substantial dredging works were undertaken at the site of the barrage before construction, and throughout the bay after impoundment for navigation and environmental purposes (map shown in Appendix 1). Dredging works to remove silt from the lake bed were undertaken during the winter of 2000/2001, during which time the Bay was regularly flushed with saline estuarine water. Final impoundment under freshwater conditions occurred in spring 2001.
3.1.3. Post-barrage environment

Cardiff Bay has a mean depth of 4.03 m, and maximum depth of 13.39 m. At the coring location for this study (shown in Figure 3.0.1.) the mean water depth is approximately 8 m. Cardiff Bay is a heavily managed environment; Cardiff Harbour Authority (CHA) is responsible for maintaining water quality in Cardiff Bay and the lower reaches of the Taff and Ely rivers. Dissolved oxygen (DO) concentrations are maintained at 5 mg/L by bed mounted diffuse aerators under the Cardiff Bay Barrage Act 1993. Continuous monitoring stations measure water quality parameters (dissolved oxygen, pH, turbidity, temperature, salinity and conductivity) at 6 locations and 2 water column depths within Cardiff Bay and the Taff and Ely rivers. More in depth chemical analysis (biochemical oxygen demand, nutrients, metals suspended solids, dissolved CaCO₃) is carried out on a monthly basis. CHA also monitor levels of pathogenic microbes (Enterococci, faecal coliforms, Streptococci, Enterovirus, Cryptosporidium, and Salmonella) and algae community composition on a weekly to biweekly basis, and removes large floating organic debris on the water surface as required.

3.1.4. Pollution inputs to Cardiff Bay

The Taff and Ely rivers and tributaries drain agricultural land in their upper reaches (75 % of catchment area), industrialised areas in their mid reaches (15 % of catchment area), and urbanised areas in their lower reaches (10 % of catchment area).
area), and densely populated urban land in their lower reaches (Environment Agency 2010). Both the Taff and Ely river valleys are steep sided and prone to flash flooding events (Natural Resources Wales 2009). In the 19th and 20th centuries, the Taff and Ely rivers were heavily polluted by discharge from coal mining and associated industries (Davies et al. 1997), sewage, and sewage treatment effluence (Williams and Simmons 1999). Although water quality has improved significantly during the past 20 years as a result of more stringent regulation (Jüttner et al. 2010), both rivers are still affected by a variety of pollutant sources including wastewater and solid waste from Combined Sewer Overflows (CSOs), illegal tipping, treated and untreated discharge from disused coal mines and nutrient loading from agricultural activity (Natural Resources Wales 2009). Cardiff Bay also received direct pollutant inputs, alongside contributions from the Taff and Ely. Sixteen sewage and pollutant outfalls were diverted away from Cardiff Bay prior to impoundment, but CSOs still discharge directly into Cardiff Bay waters under periods of high rainfall (Hill et al. 1996; Harris et al. 2002). In addition the dense residential, commercial and industrial development in the urban area immediately surrounding Cardiff Bay is also likely to be a direct source of nutrient and pollutant run-off (Jüttner et al. 2010). Cardiff Bay was identified as a Sensitive Area under the Urban Waste Water Treatment Directive (UWWTD, 91/271/EEC) in 2002, and Dwr Cymru Welsh Water (DCWW) was required to reduce the nutrient content of treated effluent from the 5 wastewater treatment plants which discharge into the Taff and Ely. Studies carried out between 2001 and 2010 classified Cardiff Bay as eutrophic to hypertrophic based on water column ammonia, orthophosphate, and nitrate levels (Vaughan et al. 2008) and diatom assemblage data (Jüttner et al. 2010). However, the bacteriological water quality is routinely assessed as good (Cardiff Harbour Authority 2012) and there have been no algal blooms within the main body of the Cardiff Bay in recent years (Lee In prep.)

3.2. Description of Cardiff Bay sampling regime

Core samples were taken from a site in the southwest of the bay (Figure 3.0.1.; 51° 26’ 56.3”, 03° 10’ 17.8” W) in January 2012, June 2012, and April 2014. Cores were analysed for one or more of the following; porewater geochemistry, sediment porosity profiles, sediment organic matter and carbonate content, molecular genetic analysis of sediment microbial community, or to provide inoculum for the
Table 3.0.1. Geochemical, molecular genetic and cultivation based analyses undertaken for each sampling date.

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Porewater geochemistry</th>
<th>Organic matter content</th>
<th>Porosity</th>
<th>Molecular genetic analysis</th>
<th>Inoculum for cultivation based experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>January 2012</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>June 2012</td>
<td></td>
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</tr>
<tr>
<td>April 2014</td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
cultivation based experiments of this thesis (Chapters 4, 5 and 6). Table 3.0.1. shows how cores from each sampling date were utilised. Cores were obtained as described in section 2.1. and sub-sampled for geochemical and molecular genetic analysis as described in section 2.2.

![Sediment core taken from Cardiff Bay in April 2014](image)

**Figure 3.0.2. Sediment core taken from Cardiff Bay in April 2014**

### 3.3. Core description

The upper ~20 cm of sediment cores consisted of a brown-black sediment with high abundance of partially degraded leaf debris and other plant matter, consistent with a lacustrine gyttja (Hansen 1959; Wetzel 1975). The upper 2-3 cm of each core was loosely consolidated and consisted primarily of flocculent matter, with significant evidence of bioturbation. The lacustrine gyttja graded sharply (over approximately 1-1.5 cm depth) into well consolidated, dense grey clay at ~20 cm depth in the June 2012 and April 2014 cores, and ~23 cm depth in the January 2012 cores. The clay lithology
showed no evidence of bioturbation. Intact shells belonging to marine bivalve taxa, for example *Cerastoderma* spp., are sometimes found in the clay lithology at this location (Thomas 2014). Numerous voids were observed in the clay sediment, and also less commonly in the gyttja. Bubbles were frequently emitted from sediment cores disturbed by movement, or during subcoring, suggesting that the voids observed in sediment cores occur *in situ* and were not produced by coring.

3.4. Sediment porosity

The method for determining sediment porosity is described in section 2.7. Sediment porosity decreased slightly with increasing sediment depth within the gyttja lithology in the January 2012 and April 2014 cores. In January 2012 the core porosity decreased from 80 % at 1 cm depth to 64 % at 23 cm depth. In the April 2014 core, sediment porosity was stable at approximately 83 % until 12.5 cm depth then decreased slightly to 73 % at 17.5 cm depth (see Figure 3.0.3.). In contrast porosity increased from 78 % at the sediment surface to a maximum of 82 % at 12.5 cm depth in the June 2012 core, then decreased slightly to 68 and 72 % at 17.5 and 22.5 cm respectively. In the April 2014 core, there was a marked decrease in porosity in the clay sediments (48 and 66 % at 22.5 and 27.5 cm depth respectively compared to the gyttja lithology, however only a slight decrease occurred >23 cm depth in the January 2012 core (64-69 %).
3.5. Sediment geochemistry

Geochemical analysis methods are described in section 2.6.

3.5.1. Sediment organic and carbonate carbon profiles

The sediment organic matter content (weight %, LOI$_{550}$) in the April 2014 core decreased slightly from 17.7 % at 2.5 cm depth to approximately 16.8 % between 7.5 and 17.5 cm depth (Figure 3.0.4.). Sediment calcium carbonate content (weight %, LOI$_{950}$) increased from 0.07 % at 2.5 cm depth to 0.10 % at 7.5 cm depth, then decreased to 0.06% again at 17.5 cm depth. There was a large decrease in organic matter and increase in calcium carbonate over the lacustrine and estuarine sediment interface. Organic carbon decreased to approximately 7 % between 22.5 and 26.5 cm depth, whereas calcium carbonate increased to ~0.4%.

![Figure 3.0.4. Sediment organic matter and CaCO$_3$ depth profile](image)

3.5.2. Chloride

Chloride concentrations increased with sediment depth in all cores (Figure 3.0.5.), with a lower rate of increase over 0 to 10 cm depth than between 10 and 25-30 cm depth. The January 2012 profile increased slightly from 0.8 mM at 1 cm depth to 3.8 mM at 9 cm depth, then more rapidly to 40.5 mM at 27 cm depth. The April 2014 profile was almost identical to the January 2012 profile over the top 25 cm, with concentrations...
increasing from 1.1 to 3.8 mM between 2.5 and 7.5 cm depth, then again to 24.7 mM at 17.5 cm depth. However, chloride concentrations at 27.5 cm depth were approximately 6 mM lower than in the January 2012 profile. Chloride concentrations in the June 2012 profile were lower throughout the core depth compared with the January or April profiles, particularly below 10 cm depth, although the characteristic depth profile shape present in January and April was maintained. The June 2012 profile increased from 0.6 mM at 2.5 cm depth to 1.4 mM at 7.5 cm depth, to a maximum of 33 mM at 27.5 cm depth.

3.5.3. Sulphate

As typical for freshwater environments, sediment sulphate concentrations were very low (<0.07 mM) in all 3 cores (Figure 3.0.5.). Highest concentrations occurred in the January 2012 profile. Concentrations increased from 0.05 mM between 1 and 3 cm depth to a peak of 0.06 mM at 5 cm depth, then decreased to 0 between 13 and 15 cm depth. Concentrations remained at 0.01 mM at 17 cm depth and below. The June 2012 profile was very similar to the January 2012 profile over the top 15 cm, with concentrations decreasing from 0.26 mM at 2.5 cm to 0.1 mM between 12.5 and 22.5 cm depth. However, the June 2012 showed a small subsurface peak of 0.05 mM at 27.5 cm depth. Sulphate concentrations were below minimum detection limits (<0.01 µM) between 2.5 and 17.5 cm in the April 2014 core, and was measurable at 0.04 mM at 22.5 cm depth.

3.5.4. Methane

Sediment porewater methane concentrations differed significantly in depth profile and concentration range between the 3 sampling times (Figure 3.0.5.). The January 2012 core had a small peak in methane (0.14 mM/L wet sediment) at 1 cm depth. There was a gradual increase in methane with sediment depth from 4 µmol/L wet sediment at 3 cm to 164 µmol/L wet sediment at 23 cm depth, then a sharp rise in concentrations over the 2 deepest data points peaking at 2.4 mmol/L wet sediment at 27.5 cm depth. The June 2012 and April 2014 cores had shallower peaks in methane production than the January 2012 core, with maximum methane concentrations occurring at 17.5 and 12.5 or 22.5 cm depth respectively. The June 2012 profile had a gentle increase in methane concentration between 2.5 and 7.5 cm, then a more rapid increase in
concentrations plateauing at 1.2-1.4 mM/L wet sediment between 17.5 and 22.5 cm depth. Including duplicate core measurements, the methane concentration at 2.5 cm depth ranged from 0.02-0.6 mM/L wet sediment, and at 22.5 cm depth it ranges from ~1.4 mM/L to 2.6 mM/L wet sediment. This significant variation reflects the highly heterogeneous nature of Cardiff Bay sediments. The June 2012 core had large gas bubbles throughout the upper 20 cm, hence multiple minicores were taken in an attempt to measure for the heterogeneous nature of the sediment. Methane concentrations in the April 2014 core were significantly higher between 2.5 and 12.5 cm depth than in the January 2012 or June 2012 cores. Methane concentrations were ~1 mM/L wet sediment between 2.5 and 7.5 cm depth, rising to a peak of 1.8 mM/L wet sediment at 12.5 cm depth. Methane concentrations at 17.5 cm depth (1.5 mM) were comparable with the June 2012 core, then decreased to ~0.5 mM/L wet sediment at 22.5 cm depth.

3.5.5. Porewater geochemical profiles of methanogen substrates

3.5.5.1. Acetate

Sediment acetate profiles differed considerably between the three sampling times (Figure 3.0.5.). Acetate concentrations in the January 2012 profile were relatively high at 1 cm depth (16 µM) but decreased rapidly to 5 µM at 3 cm depth, then more slowly to a minimum of 1.7 µM at 9 cm depth. Increasing concentrations in the bottom half of the core reach a peak of 15 µM at 17 cm depth, then decrease to ~11 mM at 27 cm depth. The June 2012 profile had high acetate concentration in the bottom half of the core, with concentrations rising steadily from 1.4 µM at 2.5 cm depth to ~10.5 µM at 12.5-17.5 cm depth, then dropping slightly to 9.4 µM at 22.5 cm depth. In contrast, highest acetate concentrations in the April 2014 core occurred at 2.5 cm depth, and were more than four times higher than maximum acetate concentrations in the other cores. The acetate profile was similar to the other cores below this depth, with concentrations decreasing rapidly to below minimum detection limits (<1 µM) between 12.5 and 17.5 cm depth, then increasing to a second peak of 13.8 mM at 22.5 cm depth.
3.5.5.2. Hydrogen

Hydrogen concentrations in the January 2012 varied little with sediment depth. Concentrations were ~1.4-1.6 µM between 1 and 15 cm depth. There was a small peak at 17 cm depth (2 µM), then a decrease to 0 at 21 cm depth. Concentrations then increased again to a maximum of 2.6 µM at 25 cm depth, before decreasing to 1.3 µM at 27 cm. In the June 2012 core, concentrations decreased slowly and consistently from 2.4 µM at 2.5 cm depth to 1.6 µM at 7.5 cm depth, and remained at that level until 22.5 cm depth. In the April 2014 core, hydrogen concentrations decreased from ~5 µM at 2.5 cm depth to ~0.8 µM at 7.5 and 12.5 cm depth. Hydrogen concentrations were below minimum detection limits at 17.5 cm depth, then increased again at 22.5 cm depth to between 3-7 µM.

3.5.5.3. Methylated amines

Trimethylamine (TMA), dimethylamine (DMA) and monomethylamine (MMA) were not detected at any depth in sediment cores from Cardiff Bay. The minimum detection limit for TMA, DMA and MMA are not certain, however concentrations of 1 µM can be reliably measured in freshwater samples.

3.5.6. Porewater profiles of general organic matter degradation indicators

3.5.6.1. Carbon dioxide

Sediment porewater carbon dioxide concentrations were slightly higher in the January 2012 core than in either the June 2012 or April 2014 core (Figure 3.0.5.). Concentrations ranged from 1.7-5.5 mM/L in the January 2012 core compared to 0-3 mM/L in the June 2012 or April 2014 cores. Carbon dioxide concentrations in the January 2012 core were highest at 1 cm depth (5.5 mM) then decreased consistently to 1.7 mM at 7 cm depth. There was then a very gradual increase in concentrations with depth, reaching a smaller second peak at 19 cm (2.9 mM), then decreased to 1.8 mM at 27 cm depth. Carbon dioxide concentrations in the June 2012 and April 2014 core showed a general decrease with increasing sediment depth. In the June 2012 core concentrations decreased from 2.8 mM at 2.5 cm depth to 0.01 mM at 22.5 cm depth. In the April 2014 core concentrations decreased from 2 mM at 2.5 cm to 0.5 mM at 17.5 cm, and were below minimum detection limits (50 ppm, equivalent to approximately 8 µM/L wet sediment) at 22.5 cm depth.
Figure 3.0.5. Sediment porewater geochemical profiles
NB. Methane concentrations shown on log scale.
3.5.6.2. Ammonium

N.B. There is was no ammonium profile for the June 2012 core as insufficient porewater was obtained during core processing.

Ammonium concentrations in the January 2012 core increased slightly from 0.2 to 0.28 mM between 1 and 3 cm depth, then decreased consistently to 0.1 mM at 11 cm depth (see Figure 3.0.5.). Between 11 and 19 cm depth there was a substantial increase in ammonium concentrations, reaching a peak of 1.4 mM at 19 cm depth. There was a small decrease to 1 mM at 21 cm depth, but concentrations returned to 1.4 mM by 25 cm depth. Ammonium concentrations in the April 2014 (average of 375 µM) core were slightly higher than the January 2012 core (average of 244 µM) over the top 15 cm depth. There was a slight decrease between 2.5 cm (0.35 mM) and 7.5 cm depth (0.30 mM), then an increase to a maximum of ~0.47 mM between 12.5 and 17.5 cm depth. Ammonium concentrations at 22.5 cm (0.28 mM) depth were substantially lower than at comparable depths in the January 2012 core.

3.5.7. Depth profile for rates of acetotrophic and hydrogenotrophic methanogenesis

This data is from S. Thomas, PhD thesis, Cardiff University (2014). $^{14}$C labelled acetate and bicarbonate were used to determine rates of acetotrophic and hydrogenotrophic methanogenesis in Cardiff Bay sediments in January 2012. Methods are described in Thomas (2014). Down core profiles for rates of acetotrophic and hydrogenotrophic methanogenesis are shown in Figure 3.0.6.

3.5.7.1. Hydrogenotrophic methanogenesis

Rates of hydrogenotrophic methanogenesis were relatively low (~1 nmol/cm$^3$/d) over the top 3 cm of sediment. There were large peaks in hydrogenotrophic methanogenesis, with rates reaching a maximum of 11 nmol/cm$^3$/day between 5 and 7 cm depth, and again between 11 and 17 cm depth. Rates then decreased rapidly with depth between 19 and 27 cm depth (within the clay layer), reaching a minimum of 0.03 nmol/cm$^3$/day at 27 cm depth.
3.5.7.2. Acetotrophic methanogenesis

Rates of acetotrophic methanogenesis were significantly (mainly between 100-1000 times) lower than rates of hydrogenotrophic methanogenesis at a given depth, and ranged from 0 to 170 pmol/cm$^3$/day. Surprisingly, there was a small peak (60 pmol/cm$^3$/day) in acetotrophic methanogenesis over the top 3 cm depth. Like the hydrogenotrophic methanogenesis depth profile there was a larger subsurface peak (170 pmol/cm$^3$/day) between 15 and 17 cm depth, followed by rapid decrease in rates to 10 pmol/cm$^3$/day within the clay layer at 27 cm depth. Both the turnover and rate depth profiles for acetotrophic methanogenesis closely followed the acetate porewater depth profile, indicating that acetate availability was likely limiting rates of acetotrophic methanogenesis in Cardiff Bay sediments (Thomas 2014).

3.5.8. Sediment methanogen population structure

PCR-DGGE was used to characterise the archaeal community composition of Cardiff Bay sediments. PCR, DGGE, band re-amplification, sequencing, and fingerprint analysis were carried out as described in sections 2.9.2.-2.9.8. The two methanogen OTUs (labelled I and II in Figure 3.0.7., and represented by 4 different band classes) obtained during PCR-DGGE profiling of Cardiff Bay 0-5 and 10-15 cm sediment
archaeal community structure were most closely related (99-100 % 16S rRNA gene sequence similarity) to members of the hydrogenotrophic genus *Methanobacterium* (family Methanobacteriaceae, order Methanobacterales). One *Methanobacterium* band class was also detected in the 25-30 cm sediment, however 25 out of 26 band classes detected on the 30-35 cm fingerprint either did not provide usable sequences or were too faint to excise (Figure 3.0.7.).
Poor quality sequences are sometimes caused by co-migration of similar nucleotide sequences, but the sequences from 25-30 cm estuarine clay sediments had almost no identifiable peaks and did not resemble the ‘noisy’ profiles indicative of mixed template DNA. They may also result from incomplete amplification of partially degraded DNA from dead cells present in older marine clay sediments. The proportion of high molecular weight DNA is thought to decrease with sediment depth in marine environments, reflecting the increasing age of sediments (Coolen and Overmann 1998; Schippers and Neretin 2006). The unidentified bands present in the estuarine clay lithology at 25-30 cm depth may represent archaeal lineages previously active under estuarine conditions but which were not able to adapt to the rapid decrease in salinity caused by impoundment. Alternatively these DGGE bands which did not provide high quality chromatograms could represent sequences from uncultivated archaeal lineages, perhaps very rare or unique, which did not anneal well with the primers used in this study.

3.6. Discussion

3.6.1. Core lithology and environmental change

The sharp boundary between lacustrine gyttja (Hansen 1959; Wetzel 1975) and brown-grey clay in the sediment cores (described in 3.3.) delineates the transition from estuarine intertidal mudflat to freshwater lake following impoundment. The sharp transition between lithologies reflects the rapidity of environmental change caused by impoundment, and was caused by removal of surficial silty estuarine tidal flat sediment by the dredging programme carried out between barrage construction and final impoundment. Hence the grey clay observed at the bottom of the sediment cores is likely not representative of the surface sediments of the pre-impoundment tidal mudflats. The volume of estuarine sediments removed by dredging is unknown, and therefore, it is difficult to estimate the age of the clay formation without using direct dating techniques, for example radionuclide dating (e.g. Arnaud et al. 2006). The significant decrease in organic matter content between the gyttja and clay lithologies further supports the clay layer having a different source from the overlying gyttja sediments. The grey clay layer could even represent a much older Holocene (late Flandrian) estuarine facies such as the Awre and Northwick (<200 BP), Rumney,
(<1500 BP), or Wentlooge (>5000 BP) formations (Allen 1987; Edwards 1997; Allen 2001).

The Loss On Ignition (LOI) method which was used to produce depth profiles of sediment organic matter and carbonate content (Figure 3.0.4.) is a qualitative rather than quantitative method of determination. \( \text{LOI}_{550} \) values are influenced by the organic carbon content of the sediment (Santisteban et al. 2004), and by the wide range of dehydration temperatures of clay minerals (Dean Jr 1974; Ran et al. 2000; Heiri et al. 2001), but broadly reproduce trends in downcore organic carbon content determined using volumetric combustion techniques (Santisteban et al. 2004). For this reason the Cardiff Bay \( \text{LOI}_{550} \) values cannot be quantitatively compared to similar lacustrine or estuarine habitats.

3.6.2. Salinity depth profile

The consistent increase in porewater chloride concentrations with sediment depth (Figure 3.0.5.) is replicated in sediments across the areal extent of Cardiff Bay (Thomas 2014). The chloride depth profile is typical of a diffusion controlled profile, and is probably also a consequence of the transition from a brackish, estuarine environment to the current lacustrine freshwater environment. Downcore salinity gradients have previously been observed in sedimentary environments exposed to salinity alteration as a result of environmental change (e.g. The Black Sea, Jørgensen et al. 2001; Knab et al. 2009). However, maximum porewater chloride concentrations (~40 mM Cl\(^-\), equivalent to salinity of 2.1‰) in Cardiff Bay sediments are at least 10-fold lower than in mudflats located immediately adjacent to the seawards side of the barrage (23.7 ‰; Thomas 2014). There are several possible explanations for this difference. The porewater salinity of the intertidal mudflats which were present prior to impoundment is not known, but they would have been continuously influenced by both by freshwater exiting the Ely and Taff rivers, and incoming brackish water from the Severn Estuary during high tide. As the mudflats were exposed for a large portion of the day any rainfall would have also influenced porewater salinity of the uppermost sediments.
The vertical salinity gradient in estuarine sediment porewaters is determined by the balance between the salinity of the porewater and salinity of overlying waters during high tide, and is dependent on sediment porosity and the duration of exposure to incoming saline waters (Chapman and Wang 2001). Porewater salinity in surficial estuarine sediments responds rapidly (within the order of days) to exposure to high or low salinity overlying water (Chapman and Wang 2001), although this may not be the case for the compacted clayey sediments such as those which underlie Cardiff Bay lacustrine gyttja layer. River flows were exceptionally high during the period immediately prior to final impoundment, and Cardiff Bay waters were effectively freshwater before impoundment (Crompton 2002). This may explain the relatively low salinities in the estuarine clay sediments relative to salinity of open estuarine waters, particularly as dredging immediately preceding closure of the barrage would have churned up sediments and overlying water. An alternative explanation for increased chloride concentrations at depth is intrusion of saline water either through the barrage or saline groundwater below the barrage (e.g. Wilms et al. 2006). However intrusion through the barrage is minimised by the use of locks and a saline sump (Crompton 2002). Salinity is one of the water quality parameters routinely monitored by Cardiff Harbour Authority under the Cardiff Bay Barrage Bill (1993), and operation of the Barrage is carefully controlled to minimise saline intrusion.

3.6.3. Concurrent sulphate reduction and methanogenesis in surface sediments

Sulphate reduction is usually considered to be a minor terminal oxidation process in freshwater sediments as electron acceptor (sulphate) availability is limited (Lovley and Klug 1986; Capone and Kiene 1988). Porewater sulphate concentrations decreased rapidly over the upper ~10 cm (Figure 3.0.5.), indicating that sulphate reduction occurred concurrently with methane production within this depth range. Sulphate Reducing Bacteria (SRB) outcompete methanogens for common substrates due to their higher affinity for hydrogen and acetate (Oremland and Polcin 1982; Lovley and Klug 1983a) when sulphate concentrations exceed approximately 0.03 mM (Lovley and Klug 1986). In Cardiff Bay sediments, sulphate reduction did not completely inhibit methane production in upper sediment layers. However, porewater methane concentrations in the June 2012 and April 2014 cores, and rates of both acetotrophic and hydrogenotrophic methanogenesis for the January 2012 core, were notably higher.
below 5 cm depth (Figure 3.0.6.), where sulphate porewater concentrations are <0.03 mM.

3.6.4. Seasonal variation in porewater methane concentrations, and implications for atmospheric methane emissions

The depth profiles and range of porewater methane concentration in Cardiff Bay sediments varied between sampling dates. Maximum methane porewater concentrations in the gyttja sediments (Figure 3.0.5.) increased significantly between January (0.18 mM/L wet sediment at 17 cm depth), April (1.9 mM/L wet sediment at 12.5 cm depth) and June (5.0 mM/L wet sediment at 22.5 cm depth). Seasonal variation in porewater methane concentrations and methane production rates are commonly reported in lake sediments and other freshwater environments, with increased porewater methane concentrations and methane production rates observed in summer months (e.g. Kelly and Chynoweth 1981; Schulz and Conrad 1995; Nusslein and Conrad 2000; Earl et al. 2003; Chan et al. 2005; Lofton et al. 2014).

Similar to several previous studies (Kuivila et al. 1989; Sobek et al. 2012; Zhu et al. 2012; Zhang et al. 2014) porewater methane concentrations and rates of methanogenesis in Cardiff Bay lacustrine gyttja sediments increased with depth, but were significantly lower in the estuarine clay sediments than in the lacustrine gyttja sediments in the April and June cores. In April and June methane concentrations were highest in the gyttja sediment, concurrent with the depth at which rates of acetotrophic and hydrogenotrophic methanogenesis peaked in the January core. A small peak also occurred within the depth range in the January core, however, maximum methane occurred within the estuarine clay sediments.

The depth at which highest methane concentrations occurred within the lacustrine gyttja sediments also varied between seasons. Rates of hydrogenotrophic and acetotrophic methanogenesis were relatively low at this depth (total methane production <25 µmol/cm³/day), and therefore, pockets of methane physically entrapped within sediments (Kiene and Capone 1985) or methane adsorbed to clay sediment particles (Sugimoto et al. 2003; Dan et al. 2004) are the most likely explanations for high concentrations at this depth. It is not clear why such high porewater methane
concentrations are not seen at this depth in the April and June cores, but is perhaps linked in heterogeneous spatial distribution of methane bubbles in the sediment. Continuously low rates of methane production in the estuarine clay layer could produce significant quantities of methane over a long period of time. Porewater carbon dioxide and ammonium concentrations were also elevated in clay sediments in the January core compared to the other sampling dates. This subsurface peak could also be linked to fermentative degradation of organic matter, coupled with low rates of porewater exchange between the low porosity clay layer and overlying gyttja sediments, leading to a localised ‘hotspot’ of high methane, carbon dioxide and ammonium concentrations. Alternatively, these high concentrations might reflect advective groundwater flow (e.g. Burnett et al. 2003) rather than in situ microbial activity.

Maximum porewater methane concentrations in January and April were within the range of values commonly observed in natural eutrophic lake sediments (Table 3.0.2.)

<table>
<thead>
<tr>
<th>Site</th>
<th>Trophic status</th>
<th>Typical maximum porewater methane concentrations (mM/L sediment)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural lakes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lake Biwa (Dan et al. 2004)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Lake Washington, Washington, USA (Kuivila et al. 1989)</td>
<td>Mesotrophic</td>
<td>0.3</td>
</tr>
<tr>
<td>Lake Kinneret (Bar Or et al. 2014)</td>
<td>Meso-eutrophic</td>
<td>1.3</td>
</tr>
<tr>
<td>Lake Constance, Germany (Schulz and Conrad 1995)</td>
<td>Meso-eutrophic</td>
<td>0.75</td>
</tr>
<tr>
<td>Xuanwu Lake, Nanjing, China (Zhu et al. 2012)</td>
<td>Eutrophic</td>
<td>0.97</td>
</tr>
<tr>
<td>Wulongtan Lake, Nanjing, China (Zhang et al. 2014)</td>
<td>Eutrophic</td>
<td>0.2</td>
</tr>
<tr>
<td>Priest Pot, Cumbria, UK (Earl et al. 2003)</td>
<td>Hypereutrophic</td>
<td>1</td>
</tr>
<tr>
<td>Lake Orn, Denmark (Thamdrup and Schubert 2013)</td>
<td>Eutrophic</td>
<td>1.5</td>
</tr>
<tr>
<td><strong>Artificial lakes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiff Bay (this study)</td>
<td>Eutrophic</td>
<td>1.8-2.5 (5 in one sample from June 2012 core)</td>
</tr>
<tr>
<td>Lake Wohlen, Switzerland (Sobek et al. 2009)</td>
<td>Meso-eutrophic</td>
<td>5</td>
</tr>
<tr>
<td>Rzesrow Reservoir, Poland (Gruca-Rokosz and Tomaszek 2015)</td>
<td>Eutrophic</td>
<td>0.3</td>
</tr>
</tbody>
</table>

such as Lake Orn, Denmark (Thamdrup and Schubert 2013), Lake Kinneret, Israel (Bar Or et al. 2014), Priests Pot, Cumbria, UK (Earl et al. 2003) and in newly settled sediments of a shallow eutrophic lake which is regularly dredged (Xuanwu Lake, Nanjing, China; Zhu et al. 2012). Similarly high porewater methane concentrations may
also occur in oligotrophic lakes, for example Lake Biwa, Japan (Dan et al. 2004). Thomas (2014) also concluded that methane production rates in Cardiff Bay sediments are also broadly comparable with mesotrophic and eutrophic lakes and tidal river systems from around the world. However, methane concentrations in Cardiff Bay sediments were notably lower than the maximum methane concentrations (ranging from 2-5 mM/L, seen Table 3.0.2.) in a comparable small, shallow impounded artificial freshwater lake (Lake Wohlen, Switzerland; Sobek et al. 2012), with the exception of the high concentrations (> 5 mM) in deeper sediments of the June 2012 replicate cores.

Despite this, Cardiff Bay porewater methane concentrations still exceeded theoretical saturation limits at some depths (Figure 3.0.8.), even at the lower end of the in situ temperature range (5 °C). Methane super saturation would explain the gas voids in intact sediment cores, and also the large variability in methane concentrations between replicate samples from the same sediment core (Figure 3.0.5.). Gas bubbles form when the maximum solubility of the porewater is exceeded, and rise up through the sediment and water column (ebullition) by buoyancy (Boudreau et al. 2005). Ebullication provides a direct route for methane transport from sediment to atmosphere in shallow lakes (<10m) as bubbles are not dissolved (McGinnis et al. 2006) or oxidized (Bastviken et al. 2008) during transport through the water column. Porewater methane concentrations were measured at close to or above theoretical saturation limits at
several depths within Cardiff Bay sediments during each sampling campaign (Figure 3.0.8.), indicating that ebullition may be a pathway for atmospheric methane emissions from Cardiff Bay. It is difficult to quantify the importance of ebullition as a pathway for methane emissions due to the stochastic nature of bubble formation (Bastviken et al. 2008; DelSontro et al. 2010; Bussmann et al. 2013). Previous studies have found that ebullition may account for a higher proportion of methane emissions in shallow freshwater reservoirs (Huttunen et al. 2002; DelSontro et al. 2010), and is attributed to a relatively high rate of supply of reactive organic matter to anoxic sediments (Sobek et al. 2012).

3.6.5. Hydrogenotrophic methanogenesis is the dominant pathway for methane production in Cardiff Bay sediments

Rates of hydrogenotrophic methanogenesis exceeded rates of acetotrophic methanogenesis by a factor of 10-100 in Cardiff Bay gyttja sediments, and by a factor of 2 in clay sediments (Figure 3.0.6.). Acetotrophic methanogenesis is the dominant pathway for methane production in the majority of freshwater lake sediments (Borrel et al. 2011). There are some exceptions; for example hydrogenotrophic methanogenesis is the major pathway for methane production in sediments of permanently ice covered Arctic and Antarctic lakes (Smith et al. 1993; Karr et al. 2006) temperate eutrophic lakes (Lovley et al. 1982; Eller et al. 2005; Mandic-Mulec et al. 2012), and tropical lakes located in the Amazon floodplain in Brazil (Conrad et al. 2011). The factors which determine the relative importance of hydrogenotrophic and acetotrophic methanogenesis in lake sediments remain unclear (Conrad et al. 2011).

PCR-DGGE analysis suggest that Cardiff Bay lacustrine gyttja archaeal communities are heavily dominated by the hydrogenotrophic *Methanobacterium* spp. (family Methanobacteriaceae, order Methanobacterales) at both 0-5 and 10-15 cm depth (Figure 3.0.7.). The only archaeal sequences successfully retrieved from the clay sediments using PCR-DGGE were also closely related to *Methanobacterium*. 16S rRNA gene pyrosequencing analysis of April 2014 sediments from 0-5 and 25-30 cm depth also showed that approximately 46% of all methanogen operational taxomic units (OTUs; equivalent to ~6 % of total archaeal OTUs) present in Cardiff Bay sediments at 0-5 cm depth and 95 % of methanogen OTUs (equivalent to total ~1.7 %
of archaeal OTUs) at 25-30 cm depth were affiliated with the order Methanobacterales (Gordon Webster, unpublished data). Interestingly, OTUs affiliated with the methylotrophic, H₂ dependent order Methanomassiliicoccales (Dridi et al. 2012) were also detected in the 0-5 cm sediments at equal proportion (~6 % total archaeal OTUs) to the Methanobacterales using pyrosequencing (Gordon Webster, unpublished data), but were not detected using PCR-DGGE. Primer mismatch may be the reason that Methanomassiliicoccales OTUs were not detected in Cardiff Bay lacustrine sediment using PCR-DGGE in this study, although in silico comparison of primers used in this study (109f/958r and SAf, Parch 519r) using the Ribosomal Database Project (RDP) probe check tool (available at https://rdp.cme.msu.edu/probematch) suggests that for all primers coverage is comparable to that of the Methanobacterales.

OTUs belonging to the orders Methanosarcinales, Methanocellales, Methanomicrobiales, Methanococcales and Methanomicrobiales were also detected at very low relative abundance (<1 % of total archaeal OTUs) in 0-5 cm sediments by pyrosequencing analysis (Gordon Webster, unpublished data), whereas Methanosarcinales (0.02% of total archaeal OTUs) was the only additional order detected in 25-30 cm sediments. The low relative abundance of OTUs belonging to methanogen orders other than the Methanobacterales (at 0-5 and 25-30 cm depth) and Methanomassiliicoccales (at 0-5 cm depth only), may explain why they were not detected using PCR-DGGE in this study as threshold for detection is thought to be around 1-2% of template rDNA (1-2% of the rDNA template, Muyzer et al. 1993; Murray et al. 1996; Stephen et al. 1999).

It is also notable that although general archaeal primers (109f/958r and SAf-GC/Parch519r; Table 2.0.7., Chapter 2) were used for PCR-DGGE analysis of the archaeal community in Cardiff Bay sediments, only one of the operational taxonomic units (OTUs) detected in Cardiff Bay sediments was not affiliated with the Methanobacterium. In contrast, pyrosequencing analysis (Gordon Webster, unpublished data) showed that 71 % of archaeal OTUs in 0-5 cm sediments and 90 % of OTUs detected in 25-30 cm sediments were affiliated with the ‘Bathyarchaeota’ (MCG, Meng et al. 2014). The general archaeal primer set SAf/Parch519r (used as the second round primers for nested PCR during PCR-DGGE analysis) are a poor match
to the ‘Bathyarchaeota’ (Teske and Sørensen 2007), suggesting that primer mismatch prevented detection of ‘Bathyarchaeota’ sequences by PCR-DGGE.

All molecular genetic techniques based on PCR have inherent bias due to primer specificity (Von Wintzingerode et al. 1997). The difference in primers pairs used for PCR-DGGE analysis (109f/958r and SAf/Parch519r) and for pyrosequencing analysis (515f/806r, Caporaso et al. 2010) may have played a role in the differing results obtained. Studies that have used PCR-DGGE and pyrosequencing in parallel to investigate the microbial community composition of natural environments have concluded that results obtained using the two different methods are largely congruent (e.g. Roh et al. 2009; Cleary et al. 2012; Pires et al. 2012), but have differing biases. The major bias associated with pyrosequencing is overestimation of community diversity and richness (Kunin et al. 2010; Pinto and Raskin 2012). In contrast, the main problems with PCR-DGGE is the inability to detect minor community members (Casamayor et al. 2013), and the difficulty with accurate characterisation of highly complex communities (Muyzer et al. 1993; Muyzer and Smalla 1998).

The *Methanobacterium* sequences obtained using PCR-DGGE from Cardiff Bay sediments were closely related to the type strain sequences of cultivated *Methanobacterium* species, which as for all members of the Methanobacteriaceae, are obligate hydrogenotrophs. This is surprising as although the functional characteristics associated with the dominant methanogen genera are consistent with the major pathway for methane production in Cardiff Bay sediments, *Methanobacterium* spp. are only minor constituents of methanogen communities in many freshwater lake sediments (Borrel et al. 2011). Instead, genera belonging to the order Methanomicrobiales and family Methanotrichaceae usually predominate in freshwater lake sediments (Borrel et al. 2011, Table 1). The lakes in which OTUs belonging to the genus *Methanobacterium* or order Methanobacteriales have been detected are eutrophic (Chan et al. 2002; Earl et al. 2003; Liu et al. 2009; Ye et al. 2009) and/or sub-tropical or tropical (Schwarz et al. 2007; Conrad et al. 2010; Conrad et al. 2011; Zhu et al. 2012). However, to date no studies describe a freshwater lake sediment methanogen community where the sediment methanogen community is dominated by *Methanobacterium* spp. or the Methanobacteriales. In fact, the only lake sediment in
which *Methanobacterium* spp. constitute a significant proportion of the methanogen community acidic bog lake in Germany (Lake Fuchskuhle, Chan et al. 2002).

The Methanomicrobiales and Methanotrichaceae (in addition to the Methanosarcinaceae) are also the most abundant methanogen groups in many estuarine sediments (Banning et al. 2005; Li et al. 2012; Chen et al. 2013a,b; O'Sullivan et al. 2013; Zeleke et al. 2013; Webster et al. 2014; Xie et al. 2014). *Methanobacterium* are often detected in low relative abundance in estuarine sediments (e.g. Chen et al. 2013b; Zeleke et al. 2013; Webster et al. 2014; Xie et al. 2014), but often only at tens of centimetres below the sediment surface. Interestingly, the only putative methanogen sequences detected in estuarine sediments in Portugal (Abreu et al. 2001) and tropical estuarine sediments from the west coast of India (Singh et al. 2010b) belonged to the Methanobacteriales, and Saia et al. (2010) enriched *Methanobacterium* like strains from highly polluted tropical estuarine sediments from Brazil.

Radioisotope tracer experiments show low rates of acetotrophic methanogenesis at most depths in Cardiff Bay sediments, yet no OTUs related to the acetotrophic *Methanosarcinaceae* (order Methanosarcinales) or *Metanosaetaceae* (order Methanosarcinales) were detected using PCR-DGGE (Figure 3.0.7.). Pyrosequencing analysis suggests that the Methanosarcinales were present at low relative abundance in both lacustrine gyttja and estuarine clay sediments (6 % and 1 % of methanogen OTUs at 0-5 cm and 25-30 cm depth respectively; Gordon Webster, unpublished data). Rates of acetotrophic methanogenesis are <6 % of rates of hydrogenotrophic methanogenesis in lacustrine gyttja sediments, and therefore, it is reasonable to assume that the size of the acetotrophic methanogen population is far smaller than the hydrogenotrophic methanogen population. It is also plausible that acetate utilisation in Cardiff Bay sediments proceeds via syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis (Zinder and Koch 1984; Lee and Zinder 1988). If so, this would be consistent with acetate being the major precursor to methane production in freshwater environments, and also the observed dominance of hydrogenotrophic methanogens in Cardiff Bay sediments. Syntrophic acetate oxidation is usually associated with high temperature environments (Hattori 2008), but has also been identified in sediments of subtropical Lake Kinneret, Israel where $^{14}$C acetate was
converted to $^{14}$C methane in the absence of acetotrophic methanogens (Nüsslein et al. 2001; Nüsslein et al. 2003). Conrad et al. (2010) speculate that syntrophic acetate oxidation occurs alongside acetotrophic methanogenesis in sediments of Lake Mussura and Lake Battata, Amazonia, Brazil. It is not possible to conclusively determine whether syntrophic acetate oxidation occurs in Cardiff Bay sediments without further investigation.

In contrast to previous studies investigating depth related changes in methane production, there is no clear depth dependent variation in the relative contribution of hydrogenotrophic and acetotrophic methanogenesis (Figure 3.0.6.), or relative abundance of acetotrophic and hydrogenotrophic methanogen guilds (Figure 3.0.7.) in Cardiff Bay lacustrine gyttja sediments. Several studies report an increase in the relative contribution of hydrogenotrophic methanogenesis with depth in lake sediments (Koizumi et al. 2004; Chan et al. 2005; Lofton et al. 2015) and/or increase in abundance of hydrogenotrophic methanogens (Chan et al. 2005; Borrel et al. 2012c), which is attributed to a decrease in availability of labile organic matter with depth (Chan et al. 2005). Lack of depth variation in Cardiff Bay sediments could be because hydrogenotrophic methanogenesis is dominant at all sediment depths, and could be also due to the young age of the lacustrine gyttja sediments.

3.6.6. Methanogenesis in Cardiff Bay vs intertidal sediments of the Severn Estuary

There are also clear differences between the methanogen community composition in Cardiff Bay sediments and intertidal sediments of the open Severn Estuary, which suggests that impoundment (and the subsequent salinity reduction) has had a significant effect on methane production in Cardiff Bay. The Methanomicrobia (including the orders Methanomicrobiales, Methanosarcinales, and Methanocellales) were the dominant methanogen class in intertidal sediments of the Wentlodge levels, Severn Estuary (Williams 2015). In contrast, members of the Methanomicrobia were not detected in Cardiff Bay sediments using PCR-DGGE (Figure 3.0.7.), and pyrosequencing analysis (Gordon Webster, unpublished data) suggests that the Methanomicrobiales, Methanosarcinales and Methanocellales represent only 9.8 % of methanogen OTUs at 0-5 cm depth and 1.4 % of OTUs at 25-30 depth (Gordon Webster, unpublished data). The Methanobacteria (order Methanobacteriales) were
the dominant methanogen type in Cardiff Bay lacustrine gyttja and estuarine clay sediments, but were present in Severn Estuary sediments at significantly lower relative abundance than the Methanomicrobia (7 % and 11 % of archaeal 16S rRNA gene OTUs respectively; Williams, 2015).

The ratio of hydrogenotrophic and acetotrophic methanogenesis in intertidal Severn Estuary sediments also varies significantly from Cardiff Bay lacustrine gyttja sediments. Thomas (2014) found that maximum rates of acetotrophic and hydrogenotrophic methanogenesis in intertidal sediments of the Severn Estuary (Woodhill Bay, Portishead) were very similar (~0.07 pmol/cm³/day, site ST4) between 0 cm and 20 cm depth, although the depth distribution of peak acetotrophic and hydrogenotrophic methanogenesis rates differed. Although hydrogenotrophic methanogenesis is the dominant pathway for methane production in Cardiff Bay sediments at all depths (Figure 3.0.6.), the relative contribution of acetotrophic methanogenesis is far greater in the estuarine clay sediments (~33 %) than the lacustrine gyttja sediments (max. 6 %). The increased importance of acetotrophic methanogenesis in the estuarine clay sediments could be a relic of the estuarine origin of these sediments, although it was not accompanied by an increase in the abundance of acetotrophic methanogens in estuarine clay sediments (section 3.5.3.) and nor does the methanogen community composition of estuarine clay sediments resemble that of intertidal sediments of the Severn Estuary (as discussed above). However, when comparing the methanogen community of the estuarine clay lithology in Cardiff Bay sediments and intertidal mudflats of the Severn Estuary it is important to consider that the estuarine clay lithology in Cardiff Bay is likely representative of the bottom of estuarine intertidal mudflats (less compacted overlying silts were removed by dredging), whereas the sediments investigated by Williams (2015) and Thomas (2014) were surficial (<20 cm depth) and consisted of fine sand and mud. The relative abundance of different methanogen classes and families, and the relative contribution of hydrogenotrophic and acetotrophic methanogenesis, altered over 20 cm depth in the Severn Estuary intertidal mudflats and probably continues to vary with increased sediment depth.
3.7. Conclusions

The impoundment of Cardiff Bay and resulting transition from intertidal estuarine sediments to artificial freshwater lake is reflected in sediment cores as a transition in core lithology between lacustrine gyttja and estuarine clay, and a low but gradually increasing downcore salinity gradient. The lacustrine gyttja sediments have markedly higher organic matter content and porosity, and lower salinity, than the underlying estuarine clay sediments. Downcore porewater methane concentrations are mainly within the typical range for natural freshwater lake sediments, however, maximum methane concentrations in some cores reach the elevated levels recorded in similar artificial impounded freshwater reservoirs. Cardiff Bay porewater methane concentrations were either close to or exceeded theoretical saturation limits at several sediment depths in cores taken during the winter, spring and summer seasons, indicating sediments may be a source of atmospheric methane emissions via ebullition. The methanogen community structure and function of Cardiff Bay sediments is atypical of either estuarine or lacustrine sediments, and does not resemble the methanogen community composition of Severn Estuary intertidal mudflat sediments. Methanogenic activity was far higher in the lacustrine gyttja layer than the underlying estuarine clay layer. Hydrogenotrophic methanogenesis was the major pathway for methane production in Cardiff Bay sediments at all sediment depths, and the sediment methanogen community was unusually and consistently dominated by the hydrogenotrophic methanogen genus *Methanobacterium.*
4. SALINITY GRADIENT EXPERIMENT

4.1. Rationale

Salinity is one of the most important environmental controls on microbial community composition, structure and function. Global studies of microbial distribution identify salinity as a key environmental determinant in structuring bacterial (Lozupone and Knight 2007) and archaean (Auguet et al. 2009; Logares et al. 2009) communities. Saline and non-saline environments harbour phylogenetically distinct microbial communities, suggesting divergent evolutionary pathways for marine and freshwater microbial taxa (Logares et al. 2009). The mechanism behind this split is uncertain, however, adaptation to physicochemical differences between saline and non-saline environments such as ionic composition, ionic strength and osmolarity are likely important (Lozupone and Knight 2007; Logares et al. 2009). Ionic composition, particularly sulphate concentration, is a key control on methanogen distribution.

Increased abundance of methylotrophic methanogens is seen in sulphate rich environments (discussed in section 1.9.1.). Osmolarity also affects methanogen community composition, and dominant pathway of methanogenesis independently of sulphate availability. NaCl has been shown to have an inhibitory effect on methane production in pure cultures (Patel and Roth 1977) and in variety of natural and experimental settings (Liu and Boone 1991; Borzenkov et al. 1997; Mishra et al. 2003). Mishra et al. (2003) suggest that increased chloride concentrations cause disruption to the cation-anion equilibrium of cells, leading to a reduction in metabolic activity. Methanogens are able to grow across an extremely broad range of salinities, from 0 to >5 M NaCl (Hoehler et al. 2010). The maximum salinity at which a metabolic process can occur is largely determined by bioenergetics constraints as osmoregulation has an energetic cost. Hence the maximum salinity at which the three major pathways for methanogenesis occur varies according to the typical energetic yield of the dissimilation reaction (Oren 2001). Methylotrophic methanogens are detected at salinities of up to 270 g/L (energy yield under standard conditions -79 to -191 kJ/mol substrate), compared 120 g/L (-34 kJ/mol hydrogen) for hydrogenotrophic methanogenesis and 40 g/L (-31.1 kJ/mol acetate) for acetotrophic methanogenesis (Oren 1999, 2001).

To date, research into salinity control on methane production consists of studies investigating methane production along natural estuarine salinity gradients (Purdy et
al. 2002; O’Sullivan et al. 2013; Webster et al. 2014; Xie et al. 2014) and studies using mesocosm experiments to model the effects of marine encroachment or saline groundwater intrusion on low salinity coastal freshwater sediments (e.g. Baldwin et al. 2006; Weston et al. 2006; Edmonds et al. 2009; Weston et al. 2011). Studies consistently find that the methanogen community structure and function varies between the freshwater and marine endpoints of estuarine gradients (Purdy et al. 2002; Webster et al. 2014; Xie et al. 2014). Increasing sodium chloride concentration is linked to an alteration in the rate of methane production in freshwater sediment mesocosm and bioreactor experiments (Baldwin et al. 2006; Edmonds et al. 2009; Weston et al. 2011), however, this it is not always accompanied by an alteration in methanogen community composition (Baldwin et al. 2006; Edmonds et al. 2009). This disparity suggests that sulphate availability (and hence competitive interactions with sulphate reducing bacteria), rather than ionic strength and osmolarity, determines the spatial variability in methanogen community composition observed in longitudinal studies of estuarine sediments.

The aims of this study are to investigate (i) whether the salinity and substrate characteristics of methanogen populations in Cardiff Bay lacustrine gyttja and estuarine clay methanogen populations differ, (ii) how the lacustrine gyttja and estuarine clay methanogenic (methanogens plus substrates) respond to long term alteration in salinity conditions, and (iii) whether the methane production potential of Cardiff Bay sediments is greater under low salinity conditions. Salinity manipulation of sediment slurry microcosms is used to investigate salinity control on total methane production, methanogen community structure, and methane production pathways in methanogenic communities from post-impoundment lacustrine gyttja and pre-impoundment estuarine clay sediment layers. Enrichment culture experiments are used to determine whether substrate utilization capability, and identity or abundance of methanogen populations mediating methylotrophic, acetotrophic and hydrogenotrophic methane production pathways, differs according to sediment depth or salinity.
4.2. Methods for salinity gradient experiment

4.2.1. Sediment coring and geochemical analyses

Sediment cores were taken from Cardiff Bay in January 2012 and minicores for geochemical analysis (porewater anion and cation constituents, and gas measurements) were taken at 2 cm intervals between 0 and 35 cm depth. Detailed methods for sediment coring, geochemical analysis are shown in Chapter 2. Results of sediment geochemical analysis described in Chapter 3.

4.2.2. Preparation of slurries for salinity gradient experiment

Sediment from 0-5 (upper lacustrine gyttja), 10-15 (mid lacustrine gyttja) and 25-30 cm (estuarine clay) depth was used to prepare 1:10 v/v slurries in 200 ml crimp vials with butyl rubber stoppers and aluminium crimps. 10 ml of homogenised sediment was added to 90 ml of marine, brackish, and freshwater media under an N₂/CO₂ atmosphere, and slurry vials shaken for 3 hours at room temperature using a mechanical shaker (Stuart Scientific Flask Shaker SF1, Keison, Chelmsford, UK) to standardise slurry composition. Duplicate slurries were prepared for each sediment depth (i.e. 0-5, 10-15 or 25-30 cm) and media salinity combination (i.e. marine, brackish or freshwater) to form a total of 18 slurries. Figure 4.0.1. shows the process of slurry preparation.

4.2.3. Preparation and incubation of salinity gradient microcosms

One of the duplicate slurries for each sediment depth and media salinity combination was incubated at 10 °C (average water temperature 12.2 °C between 2003-2011, Lee In prep.) in the dark for 2 years. Samples for molecular analysis were taken from these microcosms prior to incubation, and samples for headspace gas and molecular analysis taken after 2 years incubation. Headspace gas samples were taken at 3 monthly intervals between 2 and 2.5 years incubation to determine whether methanogenesis was still active.

4.2.4. Preparation and incubation of salinity gradient enrichment cultures

The other duplicate slurry for each sediment depth and media composition combination were used to inoculate salinity gradient enrichment cultures. Salinity gradient enrichment cultures were prepared using three different inoculum volumes: 5 ml (1:30 v/v sediment), 1 ml (1:150 v/v sediment) and 0.1 ml (1:1500 v/v sediment).
Salinity gradient microcosms
Incubation at 10°C for 2 years

1:30 and 1:150 v/v salinity gradient enrichments
Incubation at 25 °C until headspace CH₄ concentration >50 ppt in at least one replicate

1:1500 v/v salinity gradient enrichments
Incubation at 25 °C for 12 weeks

For each sediment sampling depth
- Duplicate 1:10 v/v primary slurries for each salinity condition
- Triplicate secondary slurries for each slurry dilution/substrate combination

For each sediment depth/salinity combination
- 1:30 and 1:150 v/v salinity gradient enrichments
- 1:1500 v/v salinity gradient enrichments

0-5
10-15
25-30

H₂/CO₂
Methylamine
Acetate

Figure 4.0.1. Diagram showing work flow for preparation of salinity gradient microcosms and enrichments
Enrichment cultures were prepared in Bellco tubes containing either 10 or 15 ml of the appropriate media. The media volume was varied to ensure the headspace of cultures were equal regardless of the volume of sediment added. Enrichment cultures were amended with either methylealmine hydrochloride, sodium acetate, or sodium formate to a final concentration of 10 mM, with a N₂/CÖ₂ headspace (80:20 v/v, 150 kPa). Enrichment cultures were prepared in triplicate for each sediment depth, media salinity, substrate, and inoculum volume combination (Figure 4.0.1).

Salinity gradient enrichment cultures were incubated at 25 °C for the duration of the experiment. Headspace gas samples were taken from 1:30 and 1:150 v/v enrichments on a bi-weekly basis until one of the three replicates had a headspace methane concentration of ≥50 parts per thousand (ppt). 1:30 v/v enrichments with a headspace methane concentrations of ≥40 ppt were then subcultured (detailed further in section 6.2.1.). In addition, 3 ml of culture media was sampled for molecular analysis and stored in a sterile APEX NoStick Microcentrifuge Tube (Alpha Laboratories Ltd, Hampshire, UK) at -80 °C until further use. All 1:1500 v/v enrichments were sampled for headspace gas concentrations after 12 weeks incubation.

4.2.5. Radiotracer activity measurements

Radiotracer experiments were conducted on slurry from salinity gradient microcosms after 2 years incubation to determine rates of dimethylamine, acetate, and bicarbonate conversion to methane for each depth/salinity condition. 3 ml aliquots of microcosm slurry were dispensed into 10 ml crimp vials stoppered with butyl rubber bungs and aluminium crimps under an N₂/CÖ₂ (80:20, 0.5 kPa) headspace. Slurries were sampled for ion chromatography analysis to provide dissolved acetate and dimethylamine concentrations. Slurries were injected with 1 µl of either bilabelled dimethylamine [¹⁴C] hydrochloride (3.80 kBq, American Radiolabelled Chemicals), sodium [1, 2 -¹⁴C] acetate (1.26 kBq, American Radiolabelled Chemicals), or sodium [¹⁴C] bicarbonate (5.35 kBq, American Radiolabelled Chemicals) and incubated at 10 °C in the dark. Time zero controls (incubations stopped immediately after injection) were also carried out for each radiolabelled substrate. Three different incubation periods were used for each substrate; dimethylamine and acetate amended slurries were incubated for 3, 7, and 14 hours, and bicarbonate amended slurries for 7, 14, and 28 hours. A variety of incubation periods were used in order to account for potential differences in cold pool
dimethylamine, acetate and bicarbonate concentrations between different slurry aliquots. Incubations were stopped using 1 ml of 1 M NaOH and stored upside down until analysis.

$^{14}$C labelled methane production was separated and analysed using a method modified from Parkes et al. (2010). Briefly, headspace gas was purged from vials by flushing with N$_2$/O$_2$ (95:5 v/v) at a flow rate of 35 ml min$^{-1}$. The gas was then passed through multiple dehydrated silica gel traps in order to remove the water vapour component, through a KOH trap to remove the CO$_2$ component, then finally through a copper oxide furnace (Carbolite, UK) heated to 800 °C to convert the remaining CH$_4$ into CO$_2$ (Cragg et al. 1990). The resulting CO$_2$ was bubbled through three 20 ml scintillation vials in series (Lab Logic, Sheffield, UK) which contained scintillation fluid (Scintisafe 3, Thermo Fisher Scientific, UK) with 0.7 % β-phenethylamine (Sigma Aldrich, UK) to fix $^{14}$CO$_2$ as a carbonate salt. Radioactivity was then measured using a Packard Tri-Carb 2900TR (Packard, UK) scintillation counter. Each scintillation vial was counted for three times for 20 minutes, with a counting window of 4-156 keV. Results from the three counts were then averaged to give the final count.

$$TM = \frac{(DPM_{in})}{[(DPM_{out} \times IDF)/(t/24)]}$$

*Equation 4.1.1. Turnover to methane*

$$MPR \text{ (pmol ml}^{-1} \text{ day}^{-1}) = \frac{[(DPM_{out} \times IDF)/(IV \times DPM_{in})]/(t/24) \times C}{t/24}$$

*Equation 4.1.2. Methane production rate*

TM = Turnover to methane

DPM$_{in}$ = DPM injected into samples (determined from pre-made standards), corrected for blanks and divided by 2 for sodium [1, 2-$^{14}$C] acetate and bilabelled dimethylamine [$^{14}$C] hydrochloride

DPM$_{out}$ = total of the average DPM values for the three scintillation vials counted for each sample (minus total average DPM value for blanks)

IDF = Isotope discrimination factor (1.06 for acetate and methylamine, 1.12 for bicarbonate)

IV = volume of radiolabelled substrate injected to sample (µl)

t = incubation time (hours)

C = substrate cold pool (pmol ml$^{-1}$)

Acetate and dimethylamine cold pools were measured using ion chromatography, and the bicarbonate cold pool was calculated from slurry headspace CO$_2$ concentrations according to Henry’s Law. Turnover and rates were calculated as described in Parkes
et al. (2012) except that total methane disintegration per minute (DPM) values (after correction for time zero values) for samples amended with dimethylamine \([^{14}\text{C}]\) hydrochloride and dual labelled sodium \([1, 2\ ^{14}\text{C}]\) acetate doubled to account for the differential between turnover to methane in DPM relative to total turnover, and the stoichiometry of acetate \((\text{CH}_3\text{COOH}:\text{CH}_4 = 1:1)\) and dimethylamine \([\text{[CH}_3]_2\text{CH}_4\text{CO}_2 = 1:1.5:1]\) conversion to methane. Turnover to methane (TM) was then calculated using equation 4.1.1. Methane production rates (MPR) were calculated using equation 4.1.2.

4.2.6. Molecular analysis of slurries and methanogen enrichments

PCR-DGGE was used to monitor archaeal populations in salinity gradient microcosms and enrichments. DNA extraction, PCR, DGGE, band re-amplification, sequencing, and fingerprint analysis were carried out as described in sections 2.9.1.-2.9.8. PCR products from salinity gradient enrichments were run on DGGE gels alongside PCR products from the salinity gradient 5\textsuperscript{th} subcultures (data presented in Chapter 6), with the 0-5 cm enrichments and 5\textsuperscript{th} subcultures on one DGGE gel and the 25-30 cm enrichments and 5\textsuperscript{th} subcultures on another. A total of 46 bands were excised and sequenced from the DGGE gel for the 0-5 cm enrichments and 5\textsuperscript{th} subcultures, and a total of 50 bands were excised and sequenced from the DGGE gel for the 25-30 cm enrichments and 5\textsuperscript{th} subcultures. The relative abundance of methanogen genera present in each sample was calculated from the summed band intensity of all DGGE bands identified in the fingerprint affiliated with a given genera as detailed in section 2.9.8.2.

4.3. Results

4.3.1. Substrate utilisation in salinity gradient enrichments

Note: Headspace methane concentrations in 1:30 and 1:150 v/v enrichments were measured until at least one out of three replicates had a headspace methane concentration of 50 ppt. Headspace methane concentrations in 1:1500 v/v enrichments were measured once after 12 weeks of incubation, and the methane concentrations used for comparison.

4.3.1.1. Methylamine utilisation in salinity gradient enrichments

Methane production results for salinity gradient enrichments amended with methylamine are shown in Table 4.0.1. Headspace methane concentrations in most
1:30 v/v enrichments exceeded 50 ppt at the first sampling point, except for the marine 10-15 cm and freshwater 25-30 cm enrichments (which reached 50 ppt at the second sampling point). Overall, the rate of methane production was generally slower in the 1:150 v/v enrichments than 1:30 v/v enrichments, and showed more evidence of depth and salinity variation. The 1:150 v/v 0-5 cm freshwater enrichment, brackish 0-5cm enrichment and brackish 10-15 cm enrichment had produced 50 ppt of methane by the first sampling point (same as the corresponding 1:3 v/v enrichments), and all other freshwater and brackish 1:150 v/v enrichments were successful by the second sampling point. There is clear depth dependent variation in the rate of methane production.

Table 4.0.1. Methane production in salinity gradient enrichments amended with methylamine (10 mM final concentration)

<table>
<thead>
<tr>
<th>Salinity</th>
<th>Sediment depth</th>
<th>Enrichment dilution</th>
<th>1:30 v/v</th>
<th>1:150 v/v</th>
<th>1:1500 v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R1</td>
<td>R2</td>
<td>R3</td>
</tr>
<tr>
<td>Freshwater</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>4</td>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>6</td>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Brackish</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>4</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Marine</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>4</td>
<td>13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>4</td>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>4</td>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Key for 1:30 and 1:150 v/v enrichments

- **Number of weeks until headspace methane concentration >50 ppt in one of three replicates**
  - 3-4 weeks
  - 5-6 weeks
  - 7-8 weeks
  - 9-10 weeks
  - >10 weeks

Key for 1:1500 v/v enrichments

- **Headspace methane concentration (ppt)**
  - + >100 ppt
  - >10 ppt
  - >1 ppt
  - <1 ppt
production in the marine 1:150 v/v enrichments amended with methylamine. The marine 25-30 cm enrichment had produced 50 ppt of methane by the second sampling point (comparable with freshwater and brackish methylamine 1:150 v/v enrichments from 25-30 cm depth), but the 10-15 cm enrichment took until the third sampling point, and the 0-5 cm enrichment took >12 weeks incubation.

Headspace methane concentrations in the 1:1500 v/v methylamine enrichments partially support the results of the 1:30 and 1:150 v/v enrichments as methane production was consistently high in freshwater enrichment replicates, but was very erratic in the marine 0-5 cm enrichment replicates. Headspace methane concentrations in 1:1500 v/v marine enrichments do not show the depth dependent trend evident in 1:150 v/v enrichments. A minimum of two out of three replicates from all freshwater enrichments, and the marine 10-15 cm depth enrichment had produced <100 ppt methane after 3 months incubation. Headspace methane concentrations in the brackish 0-5 cm and 10-15 cm enrichments and the marine 25-30 cm enrichment were less consistent between replicates than seen for the freshwater enrichments, ranging from <1-10 ppt to >100 ppt. The marine 0-5 cm enrichments showed greatest variation in methane production; one replicate had a headspace concentration >100 ppt, whereas the other two had headspace concentrations <1 ppt. No methane production occurred in the 1:1500 v/v brackish 25-30 cm methylamine enrichments.

4.3.1.2. Acetate utilisation in salinity gradient enrichments

Methane production results for salinity gradient enrichments amended with acetate are shown in Table 4.0.2. Methane production in the 1:30 v/v brackish 0-5 cm, marine 10-15 cm, and freshwater 25-30 cm was as rapid as their respective corresponding methylamine enrichments, with headspace methane concentrations reaching 50 ppt by the first sampling point for the brackish 0-5 cm enrichment and second sampling for the marine 10-15 cm and freshwater 25-30 cm enrichments. Methane production was slower in acetate amended enrichments than their respective methylamine amended enrichments under all other depth/salinity combinations. The freshwater and marine 0-5 cm and freshwater and brackish 10-15 cm enrichments took until the second sampling point to produce 50 ppt of methane, and brackish 25-30 cm and marine 25-30 cm enrichments took until the third sampling point to produce 50 ppt of
methane. The difference in time taken for headspace methane concentrations to reach 40 ppt in 1:30 v/v enrichments compared to 1:150 v/v enrichments was also greater for the acetate amended enrichments than for the methylamine amended enrichments. The majority of depth/salinity combinations had produced 50 ppt of methane by the third sampling point, but the brackish and marine 25-30 cm enrichments took significantly longer again (12-13 weeks). Headspace methane concentrations in the 1:1500 v/v acetate amended enrichments showed a similar depth/salinity trend to the 1:30 and 1:150 v/v acetate enrichments. Headspace methane concentrations exceeded 100 ppt in all freshwater 0-5 cm replicates, and two out of three replicates

<table>
<thead>
<tr>
<th>Salinity</th>
<th>Sediment depth</th>
<th>Enrichment dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:30 v/v</td>
</tr>
<tr>
<td>Freshwater</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>6</td>
</tr>
<tr>
<td>Brackish</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>8</td>
</tr>
<tr>
<td>Marine</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>8</td>
</tr>
</tbody>
</table>

**Key for 1:30 and 1:150 v/v enrichments**

*Number of weeks until headspace methane concentration >50 ppt in one of three replicates*

- 3-4 weeks
- 5-6 weeks
- 7-8 weeks
- 9-10 weeks
- >10 weeks

**Key for 1:1500 v/v enrichments**

*Headspace methane concentration (ppt)*

- + >100 ppt
- + >10 ppt
- + >1 ppt
- - <1 ppt
for the brackish 0-5 cm and freshwater and marine 10-15 cm enrichments. One out of three replicates for the marine 0-5 cm and freshwater 25-30 cm enrichments had a headspace methane concentration of >100 ppt, but the remaining replicates showed little evidence of substantial methane production (headspace methane concentrations <10 ppt). Similarly only one out three of the brackish 10-15 cm enrichments produced methane (>10 but <100 ppt in this case). Marine and brackish 25-30 cm enrichments did not produce methane.

Table 4.0.3. Methane production in salinity gradient enrichments amended with formate (10 mM final concentration)

<table>
<thead>
<tr>
<th>Salinity</th>
<th>Sediment depth</th>
<th>Enrichment dilution</th>
<th>1:30 v/v</th>
<th>1:150 v/v</th>
<th>1:1500 v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshwater</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>R1</td>
<td>6</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>R2</td>
<td>6</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>R3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brackish</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td></td>
<td>10</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td></td>
<td>6</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td></td>
<td>9</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Marine</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td></td>
<td>8</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td></td>
<td>8</td>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td></td>
<td>6</td>
<td>13</td>
<td>-</td>
</tr>
</tbody>
</table>

Key for 1:30 and 1:150 v/v enrichments

Number of weeks until headspace methane concentration >50 ppt in one of three replicates

- 3-4 weeks
- 5-6 weeks
- 7-8 weeks
- 9-10 weeks
- >10 weeks

Key for 1:1500 v/v enrichments

Headspace methane concentration (ppt)

- >100 ppt
- >10 ppt
- >1 ppt
- <1 ppt
4.3.1.3. Formate utilisation in salinity gradient enrichments

Methane production results for salinity gradient enrichments amended with formate are shown in Table 4.0.3. Formate amended enrichments were mostly slower to produce methane than those amended with methylamine or acetate, particularly the 1:150 v/v inoculum enrichments. However the 1:30 v/v freshwater 0-5 cm, freshwater and brackish 10-15 cm, and marine 25-30 cm enrichments, had produced 50 ppt of methane by the second sampling point (5-6 weeks), similar to the corresponding acetate enrichments. The marine 0-5 cm and 10-15 cm 1:30 v/v enrichments were successful by the third sampling point (compared to the second sampling point for corresponding acetate enrichments), but the brackish 0-5 cm and freshwater and brackish 25-30 cm enrichments took between 9 and 10 weeks.

The time taken for freshwater 0-5 cm and 10-15 cm 1:150 v/v enrichments to produce 50 ppt of methane was very similar to their 1:30 v/v equivalents, and the marine 10-15 cm 1:150 v/v enrichment was also successful by the fourth sampling point (9 weeks). The brackish and marine 0-5 cm enrichments, and all 25-30 cm enrichments, took >10 weeks to produce sufficient methane (>50 ppt) for subculturing. In general, headspace methane concentrations in formate amended 1:1500 v/v slurries was lower than in methylamine or acetate amended 1:1500 v/v slurries, and headspace methane concentrations only exceeded 100 ppt under in 2 replicates (belonging to the freshwater 0-5 and 25-30 cm enrichments). Methane production is clearly fastest in freshwater 0-5 cm and 10-15 cm 1:30 and 1:150 v/v enrichments, however there is little variation between different substrate/salinity conditions in the 1:1500 v/v enrichments after 12 weeks incubation. Headspace methane concentrations were consistently between 10-100 ppt for brackish and marine 0-5 cm enrichments, and also for the brackish 10-15 cm enrichment. All freshwater enrichments and the marine 10-15 cm enrichments showed more variability in headspace methane concentrations between replicates; two out of three replicates fell within the 10-100 ppt bracket and one in the >100 ppt bracket. Brackish 25-30 cm enrichment replicates did not produce methane, and only one in three replicates from the marine 25-30 cm and freshwater 10-15 cm enrichments produced methane.
4.3.2. Methanogen community structure in salinity gradient enrichments

Depth and salinity substrate utilisation patterns differed little between 1:30 and 1:150 v/v enrichments for a given sediment depth/salinity/substrate combination, or in fact between the 0-5 and 10-15 cm (upper and lower lacustrine gyttja) enrichments for each salinity/substrate combination. Therefore, only 0-5 cm and 25-30 cm 1:30 v/v enrichments were analysed for molecular diversity in order to explore the differences between these two sediment layers with distinctly different origins (lacustrine gyttja and estuarine clay). The 1:1500 v/v enrichments were not sampled for molecular analysis as several sediment depth/salinity/substrate combinations did not produce methane. All 16s rRNA sequences retrieved from DGGE bands had >97% sequence similarity to methanogen type strains.

### Table 4.0.4. Methanogen community structure in 1:30 v/v salinity gradient enrichments amended with methylamine (10 mM final concentration)

<table>
<thead>
<tr>
<th>Genus</th>
<th>Mb</th>
<th>Msc</th>
<th>Mi</th>
<th>Mmv</th>
<th>Mbv</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upper lacustrine (0-5 cm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Brackish</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Freshwater</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Estuarine (25-30 cm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brackish</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Freshwater</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Key**

<table>
<thead>
<tr>
<th>Genus relative abundance (%)</th>
<th>Mb</th>
<th>Msc</th>
<th>Mi</th>
<th>Mmv</th>
<th>Mbv</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++</td>
<td>&gt;70</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>++</td>
<td>&gt;40</td>
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<td></td>
</tr>
<tr>
<td>+</td>
<td>&gt;3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>&lt;3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

 Mb = Methanobacterium spp.
 Msc = Methanosarcina spp.
 Mi = Methanolobus spp.
 Mmv = Methanomethylovorans spp.
 Mbv = Methanobrevibacter spp.

4.3.2.1. Methanogen population structure in methylamine amended 1:30 v/v salinity gradient enrichments

Table 4.0.4. shows the relative abundance of methanogen genera detected in salinity gradient enrichments amended with methylamine. The hydrogen utilising genus *Methanobacterium* was the dominant genus in 25-30 cm (estuarine clay) and 0-5 cm (upper lacustrine gyttja) enrichments amended with methylamine under all salinity conditions. Specialist methylotrophic genera were also present in most methylamine enrichments, but only in relatively low relative abundance (<40 %). *Methanolobus* spp.
(family Methanosarcinaceae, order Methanosarcinales) were detected in the marine enrichments, whereas the 0-5 cm brackish enrichment and both the 0-5 and 25-30 cm freshwater enrichments contained Methanomethylovorans spp. (family Methanosarcinaceae, order Methanosarcinales). The freshwater 0-5 cm and brackish 25-30 cm enrichments also contain a third genus, the facultatively methylotrophic genus Methanosarcina (family Methanosarcinaceae, order Methanosarcinales).

4.3.2.2. Methanogen population structure in acetate amended 1:30 v/v salinity gradient enrichments

Table 4.0.5. Methanogen community structure in 1:30 v/v salinity gradient enrichments amended with acetate (10 mM final concentration)

<table>
<thead>
<tr>
<th>Genus relative abundance (%)</th>
<th>Mb</th>
<th>Msc</th>
<th>Ml</th>
<th>Mmv</th>
<th>Mbv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper lacustrine (0-5 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brackish</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Freshwater</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Estuarine (25-30 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brackish</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Freshwater</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: Mb = Methanobacterium spp.  
Msc = Methanosarcina spp.  
Ml = Methanolobus spp.  
Mmv = Methanomethylovorans spp.  
Mbv = Methanobrevibacter spp.

4.3.2.2. Methanogen population structure in acetate amended 1:30 v/v salinity gradient enrichments

Table 4.0.5. shows the relative abundance of different methanogen genera detected in salinity gradient enrichments amended with acetate. The substrate versatile and facultatively acetotrophic genus Methanosarcina was the dominant genus in the freshwater 25-30 cm (>70 %) enrichment, and both the marine (>70 %) and freshwater (40-70 %) 0-5 cm enrichments. Methanobacterium spp. were also detected in low relative abundance in the freshwater 0-5 cm and 25-30 cm enrichments, but not the marine 0-5 cm enrichment. Methanobacterium was the dominant genus in the marine 25-30 cm enrichment, and in both 25-30 cm and 0-5 cm brackish enrichments. Methanosarcina spp. were also present at low relative abundance in the 25-30 cm and 0-5 cm brackish enrichments, and members of the hydrogenotrophic genus Methanobrevibacter (family Methanobacteriaceae, order Methanobacteriales) and
methylotrophic genus *Methanomethylovorans* were identified in low relative abundance in the freshwater and brackish 0-5cm enrichments.

### 4.3.2.3. Methanogen population structure in formate amended 1:30 v/v salinity gradient enrichments

Table 4.0.6. shows the relative abundance of different methanogen genera detected in salinity gradient enrichments amended with formate. *Methanosarcina* was the dominant genus in the marine 25-30 cm enrichment amended with formate, with *Methanobacterium* spp. also present in low relative abundance. In contrast, *Methanobacterium* was the dominant genus in the low salinity 25-30 cm enrichments and all 0-5 cm enrichments. *Methanosarcina* spp. were also detected in low relative abundance in the freshwater 25-30 cm enrichments, and the marine and freshwater 0-5 cm enrichments, but not in the brackish 0-5 cm enrichment. In addition, *Methanobrevibacter* spp. were detected in low relative abundance in the marine and brackish 0-5 cm enrichments. Surprisingly the obligately methylotrophic *Methanomethylovorans* was identified at low relative abundance in both the freshwater 25-30 cm enrichment and brackish and marine 0-5 cm enrichments.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Mb</th>
<th>Msc</th>
<th>Mi</th>
<th>Mmv</th>
<th>Mbv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper lacustrine (0-5 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Brackish</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Freshwater</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Estuarine (25-30 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marine</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brackish</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Freshwater</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Key**

- **+++** >70
- **++** >40
- **+** >3
- **-** <3

*Mb* = *Methanobacterium* spp.
*Msc* = *Methanosarcina* spp.
*Ml* = *Methanolobus* spp.
*Mmv* = *Methanomethylovorans* spp.
*Mbv* = *Methanobrevibacter* spp.
4.3.3. Methane production in salinity gradient microcosms

Headspace methane concentrations in salinity gradient microcosms after 2 years incubation at 10 °C were influenced by both salinity and sediment depth (Table 4.0.7.). Methane headspace concentrations in the marine microcosms increased slightly with sediment depth from <30 ppm in the 0-5 cm and 10-15 cm microcosm to ~700 ppm in the 25-30 cm microcosm. In contrast, headspace methane concentrations in the freshwater and brackish 0-5 cm and 10-15 cm microcosms were ~2 orders of magnitude higher than in the corresponding marine microcosms, and decreased with sediment depth. Greatest methane production occurred in the brackish 0-5 cm microcosm (~17,000 ppm), with headspace methane concentrations in the brackish 10-15 cm microcosm and freshwater 0-5 cm and 10-15 cm microcosms being approximately half as high. There was a substantial decrease in headspace methane concentrations between freshwater and brackish 10-15 cm microcosms (~7,400 and 6,100 ppm respectively), and the freshwater and brackish 25-30 cm microcosms (~500 and 200 ppm respectively). Headspace methane concentrations in the freshwater and brackish 25-30 cm microcosms were similar in magnitude to the marine 25-30 cm microcosm (<700 ppm).

4.3.4. Methanogenic substrate utilisation in salinity gradient microcosms

Headspace methane concentrations in the freshwater salinity gradient microcosms continued to rise between 2 and 2.5 years incubation (data not shown) demonstrating that methane production was ongoing at all depths, however no corresponding increase occurred in the marine or brackish microcosms. Therefore, radiotracer measurements were used to determine methane production rates in freshwater microcosms only. Microcosm acetate concentrations were very low (<5 µM) in the 0-5 and 10-15 cm microcosms, and below the minimum detection limit (<1 µM) in all other microcosms (Table 4.0.7.). Headspace hydrogen concentrations were also very low (between 10 and 20 ppm), although slightly higher in the brackish 0-5 cm microcosms (36 ppm, Table 4.0.7.). As cold pool acetate and dimethylamine concentrations were below minimum detection limits, results from acetate and dimethylamine radiotracer experiments are presented as turnover time rather than rates of methylotrophic and acetotrophic methanogenesis (except for the 0-5 cm freshwater microcosm amended with 14C-acetate). Substrate turnover and rates of acetotrophic and hydrogenotrophic methanogenesis are shown in Table 4.0.8.
4.3.4.1. Acetate turnover in freshwater gradient microcosms

Acetate turnover was 10-fold faster in the 10-15 cm (34 days) freshwater microcosm than either the 0-5 cm (172 days) or 25-30 cm (541 days) microcosms. Acetate turnover rates also peaked in situ between 12 and 15 cm depth in Cardiff Bay sediments (Figure 3.0.6., Chapter 3). The rate of acetotrophic methanogenesis in the 0-5 cm freshwater salinity gradient microcosm (35 pmol/ml/day) was comparable to in situ rates of acetotrophic methanogenesis in Cardiff Bay sediments in the top 5 cm depth (maximum of 60 pmol/cm$^3$/day at 3 cm depth; Figure 3.0.6., Chapter 3).

4.3.4.2. Dimethylamine turnover in salinity gradient microcosms

Dimethylamine turnover was similar to acetate turnover in the 0-5 cm (40 days) and 25-30 cm (354 days) freshwater microcosms (Table 4.0.8.). However, in direct contrast to acetate turnover results, average dimethylamine turnover in the 10-15 freshwater microcosm (1,235 days) was considerably slower than in the 0-5 and 25-30 cm freshwater microcosms. Dimethylamine turnover was not measured in Cardiff Bay sediments as dimethylamine could not be detected in sediment porewaters.

4.3.4.3. Bicarbonate turnover in salinity gradient microcosms

Bicarbonate turnover was an order of magnitude slower than acetate or dimethylamine turnover for every microcosm depth (Table 4.0.8.), however turnover does not reflect substrate concentrations and cannot be directly compared between different substrates, as reflected by the difference in rates of acetotrophic (35 pmol/ml/day) and hydrogenotrophic (9.2 nmol/ml/day) methanogenesis in the 0-5 cm freshwater microcosm. Rates of bicarbonate methanogenesis decreased consistently with increasing sediment depth from 9.2 nmol/ml/day in the 0-5 cm freshwater microcosm to 1.5 nmol/ml/day in 25-30 cm freshwater microcosm. Rates of hydrogenotrophic methanogenesis in salinity gradient microcosms had a rather different depth distribution to in situ sediment hydrogenotrophic methanogenesis rates, although overall rates were very similar. In Cardiff Bay sediments hydrogenotrophic methanogenesis rates (Figure 3.0.6., Chapter 3) were consistently higher between 7 and 17 cm sediment depth (~ 8-10 nmol/cm$^3$/day) than over the upper 5 cm depth (1-1.5 nmol/cm$^3$/day between 1 and 4 cm, 9 nmol/cm$^3$/day at 5 cm depth). However rates of hydrogenotrophic methanogenesis were lower at 25-30 cm depth than at shallower depths in both salinity gradient microcosms and sediments. It is surprising that
### Table 4.0.7. Methane production, substrate concentration and methanogen population structure in salinity gradient microcosms

<table>
<thead>
<tr>
<th>Media salinity</th>
<th>Sediment depth</th>
<th>Methane (ppm)</th>
<th>Mb as % of total relative band surface area</th>
<th>Substrate concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Slurry acetate (µM)</td>
</tr>
<tr>
<td>Freshwater</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>7,280</td>
<td>83</td>
<td>4.16</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>7,429</td>
<td>84</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>537</td>
<td>77</td>
<td>ND</td>
</tr>
<tr>
<td>Brackish</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>17,460</td>
<td>93</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>6,124</td>
<td>86</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>232</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>Marine</td>
<td>Upper lacustrine gyttja (0-5 cm)</td>
<td>28.4</td>
<td>92</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Lower lacustrine gyttja (10-15 cm)</td>
<td>7.4</td>
<td>81</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Estuarine clay (25-30 cm)</td>
<td>673</td>
<td>76</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mb = Methanobacterium spp.
ND = not detected.

### Table 4.0.8. ¹⁴C labelled substrate turnover, and rates of acetotrophic and hydrogenotrophic methanogenesis, in freshwater salinity gradient microcosms

<table>
<thead>
<tr>
<th>Sediment depth (cm)</th>
<th>Dimethylamine</th>
<th>Acetate</th>
<th>Bicarbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative turnover (days)</td>
<td>Relative turnover (days)</td>
<td>Rate (pmol/ml/day)</td>
</tr>
<tr>
<td>0-5</td>
<td>40</td>
<td>172</td>
<td>35.0</td>
</tr>
<tr>
<td>10-15</td>
<td>1,235</td>
<td>34</td>
<td>-</td>
</tr>
<tr>
<td>25-30</td>
<td>354</td>
<td>541</td>
<td>-</td>
</tr>
</tbody>
</table>
maximum rates of both acetotrophic and bicarbonate methanogenesis in salinity gradient microcosms were similar to in situ sediment values at the corresponding depth ranges (Figure 3.0.6., Chapter 3) despite the salinity gradient microcosms having been incubated for 2 years under a closed system.

4.3.1. Methanogen population structure in salinity gradient microcosms

*Methanobacterium* was the only genus identified in the salinity gradient microcosms. Table 4.0.7. shows the relative band surface area of *Methanobacterium* operational taxonomic units (OTUs) in salinity gradient microcosms as a percentage of the total relative band surface for each sediment depth/salinity condition. The relative band surface area of *Methanobacterium* OTUs was high (varying between 76 and 93%) except for the brackish 25-30 cm microcosm where it was only 4%. The remainder of DGGE bands excised from the brackish 25-30 cm salinity gradient microcosm were of poor quality and not identifiable.

4.4. Discussion

4.4.1. Salinity and substrate range of the lacustrine gyttja and estuarine clay methanogen communities

Enrichment culture experiment results show that the salinity range and substrate utilization capability of Cardiff Bay methanogen communities did not differ with either sediment depth. Significant methane production was stimulated in both lacustrine gyttja (0-5 and 10-15 cm depth) and estuarine clay (25-30 cm depth) enrichments by addition of methylamine, acetate, or formate when incubated under freshwater, brackish or marine conditions (Tables 4.0.1 – 4.0.3.). The majority of the methanogen genera enriched from lacustrine gyttja and estuarine clay sediments under marine conditions (*Methanolobus*, *Methanomethylovorans*, *Methanosarcina*, *Methanobrevibacter*, Tables 4.0.4. to 4.0.6.) were not detected in Cardiff Bay sediments using PCR-DGGE (Figure 3.0.7., Chapter 3). The increase in relative abundance of these genera in the marine salinity gradient enrichments relative to under in situ conditions strongly suggests that members of these genera were active under marine conditions.
4.4.2. Salinity control on methanogen community composition

It is difficult to conclusively identify the methanogen populations responsible for methane production in salinity gradient enrichments as *Methanobacterium* spp. were detected in all enrichments (Tables 4.0.4. - 4.0.6.), including those amended with methylamine or acetate (sometimes as the dominant genus). The obligately hydrogenotrophic genus *Methanobacterium* is heavily dominant in the methanogen community under *in situ* conditions (Figure 3.0.7., Chapter 3). For this reason it seems likely that *Methanobacterium* spp. in enrichments amended with methylamine or acetate are a relic of sediment inoculum methanogen community, although theoretically *Methanobacterium* could have been participating in syntrophic oxidation of acetate (or even methylamine, discussed further in sections 6.5.4. and 6.5.5.).

The identity of the dominant methylotrophic and hydrogenotrophic methanogen genera in salinity gradient enrichments differed according to salinity (Tables 4.0.4. and 4.0.6. respectively), and were distributed in close accordance with the known salinity characteristics of cultivated methanogen genera. *Methanolobus* spp. were enriched on methylamine under marine conditions (0.55 M NaCl), which is close to the salinity optimum of the majority of *Methanolobus* type strains (~ 0.5 M NaCl; Table 7.0.4., Chapter 7). Most *Methanolobus* type strains were isolated from saline environments (the majority from marine sediments; Table 7.0.4., Chapter 7), and *Methanolobus* spp. are frequently detected in marine sediment environments (Ferry and Lessner 2008). In contrast, *Methanomethylovorans* spp. were detected predominantly in freshwater and brackish salinity gradient enrichments amended with methylamine (Table 4.0.4.). *Methanomethylovorans* type strains have a salinity optima of <0.1 mM NaCl and salinity maxima of ≤ 0.4 mM (Liu, 2010; Cha et al. 2013), which is consistent with a preference for lower salinity conditions. *Methanomethylovorans* type strains have previously been isolated from freshwater lake and wetland habitats (Lomans et al. 1999; Simankova et al. 2003; Cha et al. 2013), however *Methanomethylovorans* spp. are rarely detected in freshwater lakes sediment methanogen communities (Borrel et al. 2011).

The population structure of the hydrogenotrophic methanogen communities (targeted in formate amended enrichments) also differs according to salinity (Table 4.0.6.), but it is difficult to determine the extent of salinity control without conclusively identifying
which genera are active in enrichments. Methanobacterium was the dominant methanogen genera in the 0-5 cm marine enrichments amended with formate (Table 4.0.6.), yet results of sediment microcosm incubations suggest that the Methanobacterium strains present in 0-5 cm sediments were not active under marine conditions (Table 4.0.7., discussed further in section 4.4.3.). Very few Methanobacterium type strain species are able to tolerate marine salinities (equivalent to approximately ~0.55 M NaCl), and only three type strains (Methanobacter petrolearium and Methanobacterium subterraneum, Kotelnikova et al. 1998; Mori and Harayama 2011) have salinity optima >0.3 M NaCl (Table 7.0.2., Chapter 7). Just three Methanobacterium type strains have been isolated from marine (Shlimon et al. 2004) or saline (Mori and Harayama 2011) environments out of a total of 18 validly described species. However, perhaps the increased substrate concentrations in enrichments (initially amended with 10 mM methylamine, acetate or formate) enabled growth of halotolerant Methanobacterium not active under marine conditions in sediment microcosms.

The distribution of Methanosarcina spp. and Methanobrevibacter spp. in formate amended enrichments also shows evidence of salinity control (Table 4.0.6.). However, members of the Methanosarcina are not known to use formate as an electron donor (Whitman et al. 2006), and it is therefore surprising that they have been enriched in cultures amended with formate. Maeder et al. (2006) found that a strain of Methanosarcina barkeri (strain Fusaro) isolated from a temperate freshwater coastal lagoon possessed a complete formate dehydrogenase operon with high similarity to sequenced catabolic formate dehydrogenase of several formate-utilizing methanogens. However, it did not utilize formate for growth, nor did formate addition enhance growth in conjunction with catabolic substrates (Maeder et al. 2006). Furthermore, genome sequencing of other strains of Methanosarcina barkeri has not revealed a formate dehydrogenase operon (Boone and Mah, 2001). Therefore, it seems unlikely that Methanosarcina strains native to Cardiff Bay sediments possess a unique ability to use formate as an electron donor. More likely Methanosarcina spp. utilising formate indirectly for methane production. Perhaps Methanosarcina spp. were utilising acetate produced by formate-utilizing acetogenic bacteria. Alternatively, there is experimental evidence for syntrophic formate oxidation in anaerobic environments carried out by formate oxidizing bacteria in partnership with hydrogenotrophic
methanogens (Dolfing et al. 2008). Formate utilising syntrophs in the environment are in direct competition with methanogens able to use formate as an electron donor (e.g. *Methanobacterium, Methanobrevibacter*), but have less energy available to them. Dolfing et al. (2008) argue that, in this respect, competition between syntrophic formate utilisation can be considered analogous to competition between acetotrophic methanogens and syntrophic acetate oxidizing consortia.

*Methanosarcina* spp. were detected in both freshwater and marine estuarine clay (25-30 cm) enrichments amended with formate, however the relative abundance of Methanosarcina was significantly higher under marine conditions (>70 % compared to >3 % in freshwater 25-30 cm enrichment). Similarly, *Methanosarcina* spp. were present in the marine lacustrine gyttja (0-5 cm) enrichment at >3% relative abundance, but were not detected in the lower salinity (freshwater and brackish) 0-5 cm enrichments. *Methanosarcina* strains have previously been isolated from environments of wide ranging salinities, including both freshwater and marine sediments (Liu, 2010). All species of *Methanosarcina* are able to tolerate a very wide range of salinities (with the exception of *Methanosarcina horonobensis*, Shimizu et al. 2011), reaching up to >1M NaCl in some cases (Sowers et al. 1984; Lyimo et al. 2000). The salinity optima of *Methanosarcina* type strains is also broad, ranging from 0.2 - 0.6 M NaCl (Table 7.0.5., Chapter 7). The increased relative abundance of *Methanosarcina* spp. in marine enrichment relative to low salinity enrichments suggests that they are outcompeted by *Methanobacterium* and *Methanobrevibacter* spp., which have lower and narrower salinity optima than *Methanosarcina* spp. but are able to utilise formate directly.

The *Methanobrevibacter* also play a role in hydrogenotrophic methanogenesis in the lacustrine gyttja (0-5 cm) brackish and marine enrichments, but *Methanobrevibacter* spp. were not detected in lacustrine gyttja enrichments (0-5 cm) under freshwater conditions. *Methanobrevibacter* spp. are infrequently detected in sedimentary environments of any salinity. In fact, *Methanobrevibacter* species have repeatedly been suggested as a ‘microbial source tracker’ for ruminant (Ufnar et al. 2007) and human fecal (Johnston et al. 2010; Ahmed et al. 2011) contamination of surface and recreational waters as they are so rarely detected in pristine lake and coastal sediments. There is very little published information available with regards to the salinity characteristics of *Methanobrevibacter* type strains, although it is known that some species (*Methanobrevibacter millerae* and *ollayae*) can tolerate salinities in
excess of 2.6 M NaCl (Rea et al. 2007). *Methanobrevibacter* OTUs detected in salinity gradient enrichments were closely related to *Methanobrevibacter aboriphilus* (Zeikus and Henning, 1975). *Methanobrevibacter* *aboriphilus* grows optimally under freshwater conditions (0-0.1 M optimum) but can also tolerate salinities of up to 0.6 M (Asakawa et al. 1993). The salinity distribution of *Methanobrevibacter* spp. in 0-5 cm salinity gradient enrichments (highest relative abundance under marine conditions) does not correspond with the known salinity characteristics of *Methanobrevibacter aboriphilus*. This suggests that *Methanobrevibacter* spp. were not able to compete with *Methanobacterium* spp. under low salinity conditions, but become competitive at higher salinities by virtue of a wider salinity range.

In contrast to methylotrophic and hydrogenotrophic methanogenesis, acetotrophic methanogenesis was mediated by a single genus (*Methanosarcina*) under both freshwater and marine conditions (Table 4.0.5.). As previously discussed, the *Methanosarcina* are common in both freshwater and estuarine sediments, and many species type strains are able to grow under a wide ranges of salinities. In addition, the *Methanosarcina* are one of only two methanogen genera (the other being *Methanothrix*) which utilise acetate for methane production. Hence it is not surprising that *Methanosarcina* were dominant in enrichments under all salinity conditions.

4.4.3. Growth of ‘marine methanogens’ from lacustrine gyttja sediments enabled by substrate addition

The ecological strategy of the methanogen genera active under marine conditions may explain why ‘marine’ methanogens could be enriched from upper lacustrine gyttja (0-5 cm) sediments (Tables 4.0.4.-4.0.6.) but were not active in the lacustrine gyttja microcosms (Table 4.0.7.). The relative abundance of the methylotrophic genus *Methanolobus* is likely limited by substrate availability under *in situ* conditions. Almost all *Methanobrevibacter* species type strains originate from environments with high volatile fatty acid concentrations such as human and animal gastrointestinal tracts, faeces and anaerobic digesters, which is indicative of a strategic adaptation to high substrate availability. *Methanobrevibacter* spp. are usually the dominant methanogen genera in these environments (Liu and Whitman 2008), but have also been detected in less organic-rich environments, such as rice field soils (e.g. Asakawa et al. 1993; Großkopf et al. 1998) and subsurface marine sediments (Marchesi et al. 2001;
Newberry et al. 2004; Parkes et al. 2005). *Methanosarcina* spp. have low affinity for acetate (Jetten et al. 1992), and acetate concentrations in Cardiff Bay sediment porewaters and marine sediment microcosms were 10-fold lower than the minimum threshold (0.2 mM, Jetten et al. 1992) for this genus (Table 4.0.7.). There is very limited information available about hydrogen threshold values of facultatively hydrogenotrophic *Methanosarcina* spp. Lu et al. (2005) and Erkel et al. (2006) speculate that *Methanosarcina* strains native to rice field soils have low affinity for hydrogen.

Overall, it seems likely that increased substrate concentrations in enrichments (amended to 10 mM final concentrations of methylamine, acetate or formate) enabled an increase in the activity of halotolerant *Methanosarcina* and *Methanobrevibacter* strains indigenous to Cardiff Bay sediments. The only other difference in incubation conditions between the microcosm and enrichment salinity gradient experiments was that the microcosm experiments were incubated at 10 °C, whereas the enrichments were incubated at 25 °C in order to increase growth rates. The increased incubation temperature of the enrichment experiment could have enabled growth of more mesophilic strains of *Methanolobus, Methanosarcina, Methanobrevibacter*, or halotolerant strains of *Methanobacterium*.

4.4.4. Does the estuarine clay methanogen community have increased relative abundance of ‘marine’ methanogens?

Given the differing origins of Cardiff Bay lacustrine gyttja (0-5 and 10-15 cm depth) and estuarine clay (25-30 cm depth) sediments, it might be expected that the relative abundance of methanogens active under marine conditions would be higher in the estuarine clay sediment than in the lacustrine gyttja sediments. Unfortunately it was not possible to determine the relative abundance of methylotrophic, acetotrophic and hydrogenotrophic methanogens active under different salinity conditions in the upper (0-5 cm) and lower lacustrine (10-15 cm) gyttja sediments, as at least one replicate for each salinity/substrate conditions was successful even at the highest dilution (1:1500 v/v, Figures 4.0.1 – 4.0.3.). However, the 1:1500 v/v estuarine clay enrichments amended with acetate were not successful under marine conditions (Figure 4.0.2.), which demonstrates that the absolute abundance of marine acetotrophic methanogens (*Methanosarcina* spp.) in estuarine clay (25-30 cm) sediments is lower than the relative
abundance of marine acetotrophic methanogen in the lacustrine gyttja (0-5 and 10-15 cm) sediments. This is surprising given that the estuarine clay sediments are of marine origin.

The fact that the 1:1500 v/v estuarine clay enrichments amended with acetate were not successful under marine conditions also demonstrates that the absolute abundance of acetotrophic methanogens active under marine conditions is lower than the absolute abundance of acetotrophic methanogens active under freshwater conditions in the estuarine clay sediments. Acetotrophic methanogenesis is typically only a minor pathway for methane production in marine sediments as methanogens are outcompeted for acetate by SRB (Lovley et al. 1982; Oremland and Polcin 1982). In addition, there are only 2 acetotrophic methanogen genera, *Methanosarcina mazei* (Sowers et al. 1984) and *Methanothrix pelagica* (Mori et al. 2012), which grow optimally under marine salinities. Therefore, it is perhaps not surprising that acetotrophic methanogens active under freshwater conditions are more abundant in the estuarine clay sediment than acetotrophic methanogens active under marine conditions, despite the marine origin of the estuarine clay sediments. The 1:1500 v/v 25-30 cm brackish enrichments amended with acetate also did not produce methane, however the validity of these results is uncertain. The brackish 1:1500 v/v enrichments were unsuccessful under all salinity/substrate conditions, and the low relative abundance of methanogen genera in the brackish 25-30 cm microcosm (Table 4.0.7.) suggests that some other factor was inhibiting methanogenesis in these slurries. Perhaps there the sediment slurry inoculum (duplicate slurry to the brackish 25-30 cm microcosm) had become partially oxidized prior to inoculation of salinity gradient enrichments.

As for the lacustrine gyttja (0-5 and 10-15 cm) enrichments, estuarine clay (25-30 cm) enrichments targeting methylotrophic methanogens were successful at the highest dilution under all salinity conditions (Table 4.0.1.). However, the dramatic decrease in the time taken for 1:150 v/v marine enrichments amended with methylamine to produce 50 ppt of methane with increasing sediment depth (Table 4.0.1.) suggests that the absolute abundance of methylotrophic methanogens active under marine conditions (*Methanolobus* spp.) is higher in the estuarine clay (25-30 cm) sediments than in the upper and lower lacustrine gyttja sediments (0-5 and 10-15 cm depth respectively). This depth trend is not seen in the 1:30 v/v enrichments, but may be obscured by the
coarse sampling resolution. In addition, methane production in the lacustrine gyttja (0-5 and 10-15 cm) enrichments amended with methylamine was clearly slowest under marine conditions (Table 4.0.1), whereas the rapidity of methane production in estuarine clay (25-30 cm) enrichments amended with methylamine did not differ with salinity. As discussed above, most *Methanolobus* spp. are adapted to marine environments, and higher abundance of *Methanolobus* spp. in estuarine clay sediments may be a relic of their marine origin.

4.4.5. Do fermentative bacteria limit the salinity range for methane production from lacustrine gyttja sediments?

Lacustrine gyttja (0-5 cm and 10-15 cm) and estuarine clay (25-30 cm) sediment methanogenic communities (methanogen plus syntrophs) exhibited differing salinity requirements, with highest methane production occurring under marine conditions in the 25-30 cm sediment microcosm and lowest methane production occurring under marine conditions for the 0-5 and 10-15 cm sediment microcosms (Table 4.0.7.). The intertidal mudflats present in Cardiff Bay prior to impoundment would have been exposed to both freshwater (from the Taff and Ely rivers, and rainfall during low tide) and marine (incoming tide from the Severn Estuary) waters on a daily basis, and therefore it is not surprising that the methanogenic community is active under both freshwater and marine conditions. In addition, the incoming tide would provide a source of fermentative bacteria and methanogens. In contrast, Cardiff Bay lacustrine gyttja sediments were laid down post-impoundment, hence the methanogenic communities will be derived predominantly from freshwater and terrestrial sources. Headspace methane concentrations in the estuarine clay microcosms were very low even after 2 years incubation (< 1000 ppm in 25-30 cm microcosms compared to >6000 ppm in 0-5 cm microcosms, Table 4.0.7.).

Low methane production in the estuarine clay microcosms reflects the low organic matter content of these sediments compared to the overlying lacustrine gyttja sediments (Figure 3.0.4, Chapter 3), and corresponds with relatively low *in situ* rates of methanogenesis in Cardiff Bay sediments (Figure 3.0.6., Chapter 3). Although methane production was very low in the estuarine clay microcosms, it is likely autochthonous in origin as the physical sediment homogenization process for preparation of Cardiff Bay primary slurries (see section 2.2.) was designed to maximize
porewater methane degassing prior to preparation of the secondary slurries (such as the salinity gradient microcosms), and to prevent allochthonous methane outgassing or desorption from sediment particles affecting interpretation of methane production results.

It is surprising that methane production was more rapid in the upper lacustrine gyttja (0-5 cm) brackish microcosm than upper lacustrine gyttja (0-5 cm) freshwater microcosm (Table 4.0.7.), given that in situ porewater salinities at 0-5 cm depth (< 1 mM Cl\(^-\); Figure 3.0.6., Chapter 3) are more similar to the conditions of the freshwater microcosm (0.01 M NaCl). This indicates that the salinity optima of the upper lacustrine gyttja (0-5 cm) methanogenic community is slightly higher than the in situ porewater salinity. In contrast, methane production in the mid lacustrine gyttja (10-15 cm) microcosms was approximately equal under freshwater and brackish conditions (Table 4.0.7.). However, total methane production is more likely to have been limited by substrate availability in these microcosms as the quantity of degraded plant matter in lacustrine gyttja sediments decreased with increasing sediment depth (section 3.3.1.), and the lability of organic matter also typically decreases with increasing sediment depth (Schulz and Zabel 2006). Methane production from estuarine clay (25-30 cm) microcosms was approximately 50 % lower under brackish conditions than under marine and freshwater conditions (Table 4.0.7.), however the low proportion of methanogen OTUs detected in this sediment microcosm suggest that perhaps the microcosms became slightly oxidized either prior to or during incubation.

Previous studies investigating the salinity control on methane production in freshwater sediments have also found that methane production decreased with increasing salinity (Baldwin et al. 2006; Weston et al. 2006). Baldwin et al. (2006) investigated salinity control on methane production, in sediments of a freshwater wetland adjacent to the Murray River, Australia using sediment microcosms incubated at 0 to 0.1 M NaCl. Maximum rates of methane production in sediment microcosms decreased significantly with increasing salinity, particularly between microcosms incubated between 0 and 0.01 M NaCl, despite the absence of sulphate (and hence presumably competition with SRB). Weston et al. (2006) incubated sediment cores from the freshwater estuarine sediments in a flow through bioreactor, during which time the salinity of cores was increased from 0 to 10 ‰ (approximately 0.15 M NaCl) using artificial seawater (containing sulphate). Methane production rates decreased with
increasing salinity, as sulphate reduction became the dominant terminal oxidation process.

Despite the differing salinity ranges of the lacustrine gyttja and estuarine clay methanogenic communities, molecular genetic results indicate that hydrogenotrophic methanogenesis mediated by *Methanobacterium* was the dominant pathway for methane production in active methanogenic sediment microcosms under all depth/salinity conditions (Table 4.0.7). This is supported by radiotracer measurements for the freshwater microcosms (Table 4.0.8) which show that hydrogenotrophic methanogenesis was the dominant pathway for methane production in both lacustrine gyttja and estuarine clay microcosms. Edmonds et al. (2009) investigated salinity control on archaeal community composition in the salinity ramp experiment described by Weston et al. (2006) (detailed above), and also found that the archaeal community of freshwater estuarine sediment did not differ when incubated under salinities ranging from 0-0.15 M NaCl. In contrast, Baldwin et al. (2006) found that the archaeal community composition of microcosms incubated at $<0.06$ M NaCl differed from those incubated at higher salinities, and were characterised by reduced abundance of the facultatively acetotrophic genus *Methanosarcina*. Acetotrophic methanogenesis is only a minor pathway for methane production in Cardiff Bay sediments (Figure 3.0.6, Chapter 3) and in salinity gradient microcosms (Table 4.0.8), which could explain why there is no apparent variation in archaeal community composition in Cardiff Bay salinity gradient microcosms incubated under salinities ranging from freshwater (0.01M NaCl) to marine (0.55 M NaCl).

There are several potential explanations as to why the methanogen community composition of the estuarine clay (25-30 cm) marine microcosm does not differ from the methanogen community composition of the lacustrine gyttja (0-5 and 10-15 cm) microcosms or the low salinity (freshwater and brackish) estuarine clay microcosms. Firstly, perhaps the *Methanobacterium* community of lacustrine gyttja (0-5 and 10-15 cm) and estuarine clay (25-30 cm) sediments differed at species or strain level, and therefore *Methanobacterium* species or strains present in microcosms from each depth had differing salinity characteristics. PCR-DGGE does not allow retrieved sequences to be accurately identified at species level, so it is not possible to determine whether this was the case. In addition, the limit of detection for PCR-DGGE is approximately 1-2% of the rDNA template (Muyzer et al. 1993; Murray et al. 1996; Stephen et al. 1999),
which is perhaps not sufficiently sensitive to identify very small scale changes in population structure between microcosms. Methane production in the estuarine clay (25-30 cm) salinity gradient microcosms under marine conditions may have been mediated by genera other than *Methanobacterium*. This hypothesis is supported by enrichment of *Methanosarcina* spp. under marine conditions in salinity gradient enrichments targeting hydrogenotrophic methanogens (Tables 4.0.4. – 4.0.6.). Low substrate availability in estuarine clay microcosms may have limited the increase in biomass of methanogens active under marine conditions, hence the lack of observable difference in community composition as determined using PCR-DGGE.

Finally, the salinity characteristics of the hydrolytic and fermentative bacteria guilds present in Cardiff Bay sediments might differ with sediment depth. Salinity is thought to be the main control on global distribution of bacterial taxa (Lozupone and Knight 2007), however, it is not known whether methanogen (plus syntrophs) distribution is similarly affected. The estuarine clay sediments at 25-30 cm depth may still harbour hydrolytic and fermentative guilds active under marine conditions, whereas the lacustrine gyttja (0-5 and 10-15 cm) sediments do not. If this is the case, then lack of primary or secondary fermentative bacteria active under marine conditions could have prevented methanogenic activity in the marine lacustrine gyttja (0-5 cm and 10-15 cm) microcosms (as methanogens are reliant on the activity of hydrolytic and fermentative bacteria for substrate supply) without leading to alteration of the archaeal community structure. Again, substrate additions to salinity gradient enrichments would have enabled methanogens active under marine conditions to grow as they would not be reliant on substrate supply from syntrophs (discussed in section 4.4.2.). Neither Baldwin et al. (2006) or Edmonds et al. (2009) detected any variation in bacterial community composition with increasing salinity. Instead, Baldwin et al. (2006) suggest that the reduction in methane production with increased salinity was linked to inhibition of acetotrophic methanogens rather than inhibition of fermentative bacteria. However, the maximum salinities tested by (0.06 M NaCl and 0.15 M NaCl respectively) are far lower than the media salinity in the marine microcosms of this study (0.55 M NaCl).

4.4.6. Enhanced methane production potential under freshwater conditions

Results from salinity gradient microcosms and salinity gradient enrichments show that the methane production potential of Cardiff Bay sediments is enhanced under low
salinity conditions. Headspace methane concentrations in the low salinity (freshwater and brackish) lacustrine gyttja (0-5 and 10-15 cm) salinity gradient microcosms was 10-fold higher than methane production in the estuarine clay (25-30 cm) microcosms after 2 years incubation (Table 4.0.7.) Also, in contrast to the brackish and marine microcosms, methane production was ongoing in the freshwater lacustrine gyttja (0-5 and 10-15 cm) and estuarine clay (25-30 cm) microcosms even after two years incubation. This suggests that organic matter in the lacustrine gyttja and estuarine clay sediment may be more available under low salinity conditions than under marine conditions. Lower rates of methane production in estuarine clay microcosms is at least partly linked to reduced substrate availability (as discussed above), however results from salinity gradient enrichments (which were amended with relatively high quantities of known methanogen substrates) support the trend seen in the lacustrine gyttja and estuarine clay microcosms. Methane production was more rapid in lacustrine gyttja enrichments (0-5 and 10-15 cm) than estuarine clay (25-30 cm) enrichments for four out of 9 of the 1:30 v/v salinity/substrate combinations (Tables 4.0.1. - 4.0.3), and only slower than under one salinity/substrate condition. Similarly, methane production was more rapid in lacustrine gyttja (0-5 and 10-15 cm) enrichments than estuarine clay (25-30 cm) enrichments for seven of the nine 1:150 v/v salinity/substrate combinations (Tables 4.0.1. - 4.0.3), and only slower under one salinity/substrate condition (discussed in section 4.4.3.) This indicates that even when substrate availability is non-limiting, methane production is enhanced under low salinity conditions.

4.5. Conclusions

The salinity range and substrate characteristics of Cardiff Bay lacustrine gyttja and estuarine clay methanogen populations are very similar, despite the differing origins of the pre- and post-impoundment sediments. The identity of Cardiff Bay methylotrophic, acetotrophic and hydrogenotrophic methanogen populations enriched under substrate amended (methylamine, acetate of formate) conditions did not vary with sediment depth or salinity, with both ‘marine’ and ‘freshwater’ methanogens enriched from both lacustrine gyttja (0-5 and 10-15 cm) and estuarine clay (25-30 cm) sediments. This included a methylotrophic methanogen genus rarely detected outside of marine environments (*Methanolobus*). The methanogen community composition in salinity gradient enrichments targeting methylotrophic and hydrogenotrophic methanogens altered consistently with salinity. The identity of the dominant methylotrophic and
hydrogenotrophic genera enriched under marine and low salinity conditions corresponds well with their environmental distribution and known salinity characteristics of their respective cultured species type strains. Interestingly, the *Methanobacterium* were present in almost all salinity gradient enrichments, even those amended with substrates that they cannot utilise. Hence, syntrophs and their salinity requirements may be important in Cardiff Bay sediments.

There was minimal evidence for increased abundance of marine methanogens in the estuarine clay sediments deposited pre-impoundment relative to the lacustrine gyttja sediments deposited post-impoundment. In fact, the abundance of hydrogenotrophic and acetotrophic methanogens active under marine conditions decreased with sediment depth, and the abundance of acetotrophic and hydrogenotrophic methanogens active under marine conditions was lower than the abundance of hydrogenotrophic methanogens active under low salinity conditions in the estuarine clay sediments. The only indication of the more saline origin of the estuarine clay sediments is that methylotrophic methane production under marine conditions was more rapid in estuarine clay enrichments than lacustrine gyttja enrichments.

In contrast to the methanogen communities, the salinity range of lacustrine gyttja (0-5 and 10-15 cm) methanogenic communities (methanogen plus syntrophs) was more limited than the estuarine clay methanogenic communities, even though the methanogenic community structure was apparently still dominated by *Methanobacterium* under all salinity (marine, brackish and freshwater) conditions even after two years incubation. This suggests that the fermentative bacterial population of Cardiff Bay lacustrine gyttja sediments might be more sensitive to salinity than the methanogen population (as substrate amendment removes the need for syntrophs).

Overall, results from the salinity gradient enrichments and microcosms suggest that the methane production potential of Cardiff Bay sediments is enhanced under the present day low salinity conditions. Methane production was more rapid and sustained in low salinity lacustrine gyttja microcosms than in the estuarine clay microcosms, with total methane production a minimum of 10-fold higher in the low salinity lacustrine gyttja microcosms than the estuarine clay microcosms. Methane production was also more rapid under low salinity conditions than marine conditions in the majority of lacustrine gyttja and estuarine clay methanogen enrichments.
5. TEMPERATURE GRADIENT EXPERIMENT

5.1. Rationale

Cold and temperate soils and sediments often contain microbial populations with mesophilic or thermophilic temperature adaptations (Isaksen et al. 1994; Nozhevnikova et al. 1997; Fey et al. 2001; Wu et al. 2006; Hubert et al. 2009; Sawicka et al. 2012). These populations may not be active under *in situ* conditions, but the temperature adaptation ‘fingerprint’ of a specific process can be compared between environments to investigate the effect of allochthonous sources of microorganisms with different temperature characteristics on microbial community composition and their carbon mineralization temperatures (Sawicka et al. 2012). Many previous studies of methanogenesis in freshwater lake sediments have shown that the temperature range for methane production far exceeds the habitat temperature range, with methane production rates being positively correlated with temperature (Zeikus and Winfrey 1976; Kelly and Chynoweth 1981; Thebrath et al. 1993; Nozhevnikova et al. 1997; Nozhevnikova et al. 2003; Glissmann et al. 2004; Nozhevnikova et al. 2007).

Methanogenic decomposition of organic matter involves a consortia of anaerobic microorganisms including hydrolytic bacteria, fermentative (primary fermenters and secondary syntrophs) bacteria and methanogens. However little is known about how the temperature characteristics of each functional group interact to produce the overall methane production temperature response observed in field and experimental studies (Yvon-Durocher et al. 2014). There is evidence to suggest that methanogens are more responsive to temperature increase than other functional groups involved in anaerobic organic matter degradation. Q10 values for anaerobic carbon mineralization (fermentation processes) typically range from 1 to 4, whereas Q10 values of methanogen pure cultures can reach up to 12 (Segers 1998). The different functional groups involved in methanogenesis also demonstrate different temperature optima and ranges. Finke and Jørgensen (2008) and Weston and Joye (2005) found that the microbial functional groups mediating organic matter degradation in marine sediments dominated by sulphate reduction exhibit variable temperature responses, leading an imbalance in production and consumption of metabolic intermediates (volatile fatty acids and hydrogen).
The relative contribution of different methanogenic pathways (methylo trophic, acetotrophic and hydrogenotrophic) also varies with temperature in a consistent manner in freshwater ecosystems. Acetotrophic methanogenesis is dominant at low temperatures, with increasing contribution of hydrogenotrophic methanogenesis with increasing temperature (Schulz et al. 1997; Chin et al. 1999a; Fey and Conrad 2000; Glissmann et al. 2004; Nozhevnikova et al. 2007). However, there is no consensus as to what drives the observed variation in function, and whether there is a corresponding alteration in methanogen community structure (Glissmann et al. 2004; Noll et al. 2010). Characterising the effects of dual temperature and substrate addition on Cardiff Bay methanogenic communities is also of applied interest as freshwater lakes are a significant source of atmospheric methane emissions (section 1.9.2.) and are particularly vulnerable to climate change enhanced eutrophication Jeppesen et al. 2010; West et al. 2012).

The aims of this study are to determine whether (i) the temperature range and characteristics of Cardiff Bay ‘artificial’ lake sediment methanogen populations are similar to that of previously tested terrestrial and fluvial environments, (ii) methane production rates, or the temperature range for methanogenesis, is enhanced by substrate addition, (iii) the temperature range for methane production is constrained by the temperature characteristics of the fermentative bacteria on which methanogens rely for substrate supply, and (iv) methanogen community structure and function alters in response to temperature.

5.2. Methods

5.2.1. Coring and geochemical analyses

Sediment cores were taken from Cardiff Bay in June 2012 and minicores for geochemical analysis (porewater anion and cation constituents, and gas measurements) were taken at 5 cm intervals between 0 and 30 cm depth. Results of sediment geochemical analysis are shown in Chapter 3.

5.2.2. Preparation of master sediment slurries

Sediment cores were sectioned at 0-5 (lacustrine gyttja) and 25-30 (estuarine clay) depth, and an amended and an unamended master slurry produced from each depth using a 25% inoculum of homogenised sediment. The substrate amended master
slurries were prepared using bicarbonate buffered, brackish slurry media amended with 2 mM Na-acetate and HCl-methylamine, and cooled under a H2/CO2 (20:80, 0.5 kPa) headspace. Unamended slurries were prepared using bicarbonate buffered, brackish slurry media cooled under an N2/CO2 (80:20, 0.5 kPa) headspace. Slurry media was prepared in 2 L modified Duran® bottles (Figure 5.0.1.) using the method described in section 2.3.1., but without the addition of vitamin, trace element, or selenite-tungstate solutions. Slurry bottles were autoclaved with a screw cap lid to enable addition of temperature sensitive media additions and pH adjustment through the main port. Once the media had cooled to room temperature the screw cap lid was replaced with an air tight rubber bung and open top cap to enable gas sampling through the top port. A constant gas flow (N2/CO2 or H2/CO2, 5 kPa) was maintained throughout cooling, media additions, pH adjustment and cap replacement in order avoid oxygen ingress. The homogenised sediment was dispensed into the master slurry bottles under an N2/CO2 (80:20 v/v, 100 kPa) or H2/CO2 (80:20 v/v, 100 kPa) gas to form the master slurry. The master slurries were placed on an orbital shaker (model 3019, GFL, Burgwedel, Germany) for between 12-24 hours at 10 °C in the dark to ensure full mixing and standardize geochemical conditions between slurries. 5 ml of homogenized sediment from each depth was sampled for molecular and geochemical analysis.

5.2.3. Preparation of temperature gradient slurries and microcosms

The master slurries were dispensed into 20 ml thermal gradient vials (containing 10 ml slurry) and 100 ml microcosm vials (containing 50 ml slurry) sealed with a butyl rubber bung and aluminium crimp. Slurry transfer was carried out in an anaerobic cabinet under N2 atmosphere, and vials were immediately flushed with either H2/CO2 (amended slurries; 20:80 v/v, 0.5 kPa) or N2/CO2 (unamended slurries; 80:20 v/v, 0.5 kPa).
Rubber bung to allow headspace gas sampling from top port during incubation

Gas inlet for flushing headspace and deoxygenating media

Gas inlet tube fitted with in line filter (Pall Acro® 0.2 µM vent filter)

Gas inlet tube extends to bottom of bottle to ensure media is deoxygenated

Sampling outlet connected to cut-off syringe with luer end and stopcock via neoprene rubber tubing

*Figure 5.0.1. Modified Duran bottles used for preparation of sediment master slurries*
5.2.4. Incubation and analysis of temperature gradient slurries and microcosms

Individual temperature gradient slurry vials were incubated in the temperature gradient apparatus over a range of 0-65 °C at intervals of approximately 1.5 °C. Triplicate slurries from each sediment depth were incubated at each temperature point, alternating between amended and unamended slurries (Figure 5.0.2.). An amended and unamended 100 ml microcosm vial for each sediment depth was incubated at 4, 10, 25, 38, 55 and 66 °C for 2 years before being analysed. Amended temperature gradient slurries were sacrificed at 2, 4, and 10 days (referred to as T=2, T=4 and T=10) and unamended slurries at 6, 12, and 30 days (referred to as T=6, T=12, and T=30). Samples for headspace gas concentration were also taken from a representative amended and unamended temperature gradient slurry from each depth prior to incubation (referred to as T=0). Vials were shaken well by hand, and 2 ml of headspace gas sampled for natural gas analysis. 2 ml of slurry was processed for anion and cation chromatography, and the remaining slurry was transferred aseptically into a sterile falcon tube (VWR International, Pennsylvania, USA) and stored at -80 °C for molecular genetic analysis.

![Image](image_url)

**Figure 5.0.2.** Schematic diagram showing slurry vial incubation in temperature gradient apparatus

Each circle represents a slurry vial (colour coded according to slurry condition) and each column represents a temperature point.

5.2.5. Preparation and incubation of single substrate methanogen enrichments

After 10 days incubation (final sacrifice for amended slurries) amended slurries at selected temperature points (Tables 5.0.1. and 5.0.2.) were subcultured into methanogen cultivation tubes containing brackish methanogen cultivation media and either 10 mM sodium acetate, methylamine hydrochloride, or with a H₂/CO₂ headspace
Chapter 5 – Temperature Gradient Experiment

(20:80 v/v, 0.5 kPa). The headspace methane concentration of single substrate enrichments was measured on a weekly basis for 3 weeks after the initial inoculation, then on a biweekly basis for the following 19 weeks.

Methane production was negligible at temperatures below 20 °C during the 10 day incubation period for amended slurries and could not be used to inoculate further methanogen enrichments. Instead 4 and 10 °C single substrate methanogen enrichments were inoculated from the 4 and 10 °C indicator vials once significant methane production had occurred after approximately 6 months of incubation. Headspace methane concentrations in 4 and 10 °C enrichments were measured on a monthly or bimonthly basis for 18 months following the initial inoculation.

Table 5.0.1. Methane production in single substrate enrichments inoculated with 0-5 cm amended temperature gradient slurries

<table>
<thead>
<tr>
<th>Thermal gradient temperature (°C)</th>
<th>Incubation temperature (°C)</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Methylamine</td>
</tr>
<tr>
<td>4.0</td>
<td>4.0</td>
<td>+</td>
</tr>
<tr>
<td>10.0</td>
<td>10.0</td>
<td>+</td>
</tr>
<tr>
<td>19.8</td>
<td>25.0</td>
<td>+</td>
</tr>
<tr>
<td>36.8</td>
<td>38.0</td>
<td>+</td>
</tr>
<tr>
<td>45.3</td>
<td>46.0</td>
<td>+</td>
</tr>
<tr>
<td>53.8</td>
<td>55.0</td>
<td>+</td>
</tr>
<tr>
<td>59.4</td>
<td>55.0</td>
<td>+</td>
</tr>
<tr>
<td>59.4</td>
<td>66.0</td>
<td>-</td>
</tr>
<tr>
<td>65.1</td>
<td>66.0</td>
<td>-</td>
</tr>
<tr>
<td>65.1</td>
<td>77.0</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: Number of weeks taken for enrichment headspace methane concentration to exceed 40 ppt was not measured for 4 and 10 °C enrichments.

Key

Number of weeks until headspace methane concentration >50 ppt

1
2
3
6
8
>8
N.D.
5.2.6. Molecular analysis of temperature gradient slurries

PCR-DGGE was used to monitor archaeal populations in temperature gradient slurries and microcosms. DNA extraction, PCR, DGGE, band re-amplification, sequencing, and fingerprint analysis were carried out as described in sections 2.9.1 - 2.9.8. PCR products from the temperature gradient slurries were run on two DGGE gels; one containing samples from the 0-5 cm amended and unamended slurries, and the other containing samples from the 25-30 cm amended and unamended slurries. A total of 50 bands were excised and sequenced from the 0-5 cm amended and unamended slurries, and 48 bands were excised and sequenced from the 25-30 cm amended and unamended slurries. PCR products from the temperature gradient microcosms were run on two different DGGE gels. Products from the 10, 25, 55 and 66 °C 0-5 cm amended microcosms and 4-66 °C 0-5 and 25-30 cm unamended microcosms were run on one DGGE gel, and a total of 45 bands were excised and sequenced from this gel. Products from the 4 and 38 °C 0-5 cm amended microcosms and the 55 and 66°C...
25-30 cm amended microcosms were run on a second gel, from which 10 bands were excised and sequenced. The relative abundance of methanogen genera present in each sample was calculated from the summed band intensity of all DGGE bands identified in the fingerprint affiliated with a given genus as detailed in section 2.9.8.2.

5.3. Results

5.3.1. Methane production

Headspace methane temperature profiles for each sampling time point are shown in Figures 5.0.3. (0-5 cm amended), 5.0.4. (25-30 cm amended), 5.0.5. (0-5 cm unamended), and 5.0.6. (25-30 cm unamended). Methane production occurred between ~4-65 °C in amended and unamended slurries from both 0-5 cm and 25-30 cm sediment depths. Putative psychrophilic peaks and clear mesophilic and thermophilic peaks in methane production can been seen for all four slurry conditions. The psychrophilic, mesophilic, and thermophilic temperature ranges for prokaryotic growth (Table 5.0.3.) are indicated in Figures 5.0.3.-5.0.6. by coloured shading, and are color coded according to Table 5.0.3.

<table>
<thead>
<tr>
<th>Temperature range in this study</th>
<th>Classification</th>
<th>( T_{\text{min}} ) (°C)</th>
<th>( T_{\text{opt}} ) (°C)</th>
<th>( T_{\text{max}} ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psychrophilic (0-20°C)</td>
<td>Psychrophile*</td>
<td>&lt;5</td>
<td>( \leq 15 )</td>
<td>( \leq 20 )</td>
</tr>
<tr>
<td></td>
<td>'Psychrotolerant'*</td>
<td>&lt;5</td>
<td>( \leq 25 )</td>
<td>( \leq 35 )</td>
</tr>
<tr>
<td>Mesophilic (25-45 °C)</td>
<td>Mesophile*</td>
<td>&gt;0</td>
<td>25-42</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>'Facultative thermophile”**</td>
<td>-</td>
<td>25-45</td>
<td>50-60</td>
</tr>
<tr>
<td>Thermophilic (45-65°C)</td>
<td>'Moderate thermophile†</td>
<td>-</td>
<td>45-65</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Thermophile†</td>
<td>-</td>
<td>65-80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hyperthermophile†</td>
<td>-</td>
<td>&gt;80</td>
<td>-</td>
</tr>
</tbody>
</table>

* defined according to Morita (1975)
** defined according to Bertrand et al. (2015)
† defined according to Stetter (1996)

5.3.1.1. Methane production in the psychrophilic temperature range

Methane production at psychrophilic temperatures (below ~20 °C) was an order of magnitude slower than in the mesophilic or thermophilic temperature ranges. Headspace methane concentrations exceeded \( T=0 \) values in amended slurries at temperatures above 2.8 °C (but not at 0) and in unamended slurries at temperatures above 1.4 °C (lowest incubation temperature; 25-30 cm slurries). However, the
heterogeneous nature of sediment slurries and low concentrations of methane present at these temperatures make it difficult to distinguish whether this is due to microbial methane production or sediment outgassing. Clear and consistent increases in headspace methane concentration with increasing incubation occurred above 5.6 °C in amended slurries and 4.2 °C in unamended slurries. It is not possible to accurately state whether substrate addition has had an effect on the minimum temperature at which methane production occurs. The incubation temperatures of amended and unamended slurries are not identical, and therefore cannot be directly compared.

Headspace methane concentrations increased with temperature throughout the psychrophilic temperature range. There was a defined psychrophilic methane peak at 19.8°C in the 0-5 cm amended slurries after 10 days incubation (Figure 5.0.3., point 1), and also a 'shoulder' in the mesophilic methane peak at 19.8 °C in the 25-30 cm amended slurries (Figure 5.0.4., point 1) after 10 days incubation. In the 0-5 cm unamended slurries there was a defined methane peak at 15.6 °C after 12 days incubation (Figure 5.0.3., point 1), and a shoulder in the mesophilic methane peak at 15.6 °C after 30 days incubation (Figure 5.0.3., point 2). However there was no evidence of a psychrophilic methane peak at 15.6 or 19.8 °C in the 25-30 cm unamended slurries (Figure 5.0.5.). Methane concentrations in the 25-30 cm unamended slurry increased consistently with temperature to a maximum at 21.2 °C (Figure 5.0.6., point 1), but it is not clear whether this low temperature maxima is a true psychrophilic peak or whether it forms the lower end of the mesophilic methane peak.

Headspace methane concentrations in the 0-5 cm amended slurry were at least two times higher than headspace methane concentrations in the 25-30 cm amended slurry at temperatures below 19.8 °C after 2 and 4 days incubation and 11.3 °C after 10 days incubation. The difference in methane concentrations between the 0-5 cm and 25-30 cm amended slurries increased significantly relative at the upper end of the psychrophilic temperature range (>11.4 °C). At the final time point (10 days incubation) headspace concentrations in the 0-5 cm amended slurries at 14.1 and 17.0 °C were 6.5 and 3.1 times higher respectively than in the corresponding 25-30 cm slurries.
Figure 5.0.2. Temperature gradient geochemical and methanogen community structure profiles for 0-5 cm Cardiff Bay amended slurries

Figure 5.0.3. Geochemical and methanogen community structure profiles in lacustrine gyttja (0-5 cm) amended temperature gradient slurries
Figure 5.0.4. Geochemical and methanogen community structure profiles in estuarine clay (25-30 cm) amended temperature gradient slurries.
Figure 5.0.5. Geochemical and methanogen community structure profiles in lacustrine gyttja (0-5 cm) unamended temperature gradient slurries
Figure 5.0.6. Geochemical and methanogen community structure profiles in estuarine clay (25-30 cm) unamended temperature gradient slurries.
In contrast, headspace methane concentrations in 0-5 cm and 25-30 cm unamended slurries are very similar below 21.2 °C at all temperature points, but after 6 days incubation the headspace methane concentration in the 0-5 cm unamended slurries was 2.6 times higher than in the 25-30 cm unamended slurries at 24.0 °C. The difference in headspace methane concentrations in the 0-5 and 25-30 cm unamended slurries at 24.0 °C decreased with increasing incubation time; after 12 and 30 days incubation headspace methane concentrations were only 1.3 times higher in the 0-5 cm unamended slurries than 25-30 cm unamended slurries.

Methane production was clearly far slower in unamended slurries than amended slurries at psychrophilic temperatures, as headspace methane concentrations in the 0-5 cm and 25-30 cm amended slurries after 10 days incubation exceeded headspace methane concentrations in the corresponding 0-5 and 25-30 cm unamended slurries after 12 days incubation.

5.3.1.2. Methane production in the mesophilic temperature range

The mesophilic peaks in methane production were generally broader and less defined than either the psychrophilic or thermophilic peaks, particularly at the final sampling time point. The temperature at which the mesophilic methane maxima occurred also shifted between sampling points. In the 0-5 cm amended slurries mesophilic methane peaks could be seen at 36.8 and 42.5 °C after 2 days incubation (points 2 and 3, Figure 5.0.3.), but at 36.9 °C after 4 and 10 days incubation (points 4 and 5, Figure 5.0.3.). Strangely there is a second, smaller mesophilic methane peak at 45.3 °C (point 6, Figure 5.0.3.) in the 0-5 cm amended slurry after 10 days incubation. This second peak consists of only one data point, and may be an aberration. The mesophilic methane peak in the 25-30 cm amended slurries occurred at 39.6 °C after 2 days incubation (point 2, Figure 5.0.4.), but shifted upwards with increasing incubation time to 42.5 °C after 4 days incubation (point 3, Figure 5.0.4.), before moving down to 36.8 °C after 10 days incubation (point 4, Figure 5.0.4.).

There was a mesophilic temperature peak at 32.5 °C in both the 0-5 (point 3, Figure 5.0.5.) and 25-30 cm (point 3, Figure 5.0.6.) unamended slurries after 6 days incubation. In the 0-5 cm unamended slurry it remained at 32.5 °C (point 4, Figure 5.0.5.) at 12 days incubation, with no defined mesophilic peak after 30 days incubation. In the 25-30 cm unamended slurries there were 2 mesophilic peaks at 26.9 and 38.2
°C (points 3, and 4, Figure 5.0.6.). There was no defined mesophilic methane peak in either the 0-5 or 25-30 cm unamended slurries after 30 days incubation (Figures 5.0.5. and 5.0.6.), although headspace methane concentrations at 38.2 and 41 °C in the 25-30 cm slurry were slightly elevated relative to the remainder of the mesophilic temperature range (points 5 and 6, Figure 5.0.6.).

Headspace methane concentrations at the mesophilic temperature maxima were higher in the 0-5 cm amended slurries than the 25-30 cm amended slurries after 2 (120 and 50 ppt respectively) and 10 (447 and 322 ppt respectively) days incubation, but were roughly equivalent after 4 days incubation (~150 ppt). The magnitude of the mesophilic methane maxima did not differ between 0-5 and 25-30 cm unamended slurries after 6 and 12 days incubation, however methane concentrations at the mesophilic maxima were approximately a third higher in the 0-5 cm unamended slurry (124 ppt) than the 25-30 cm unamended slurry (91 ppt) after 30 days incubation. Methane production in the mesophilic temperature range was also substantially more rapid in the amended slurries than unamended slurries. Maximum methane concentrations in amended slurries were ~460 (0-5 cm, 36.8 °C) and 320 ppt (25-30 cm, 36.8 °C) after 10 days incubation, compared to ~125 ppt (0-5 cm, 41.0 °C) and 90 ppt (25-30 cm, 38.2 – 41.0 °C) in the unamended slurries after 30 days incubation.

5.3.1.3. Methane production in the thermophilic temperature range

The thermophilic peak in methane concentrations was present in 0-5 cm slurries from the first sampling time point (T=2 for amended slurry, T=6 for unamended slurry) onwards, signifying that a viable population of thermophilic methanogens were present in Cardiff Bay sediments throughout the core depth. The thermophilic peak in methane concentrations was significantly higher than the mesophilic peak at every time point in 0-5 cm slurries, particularly in the amended slurry. This suggests that the thermophilic methanogen population responded more rapidly to the change in temperature, and particularly to increased substrate availability, than the psychrophilic or mesophilic methanogen population. There was no defined thermophilic methane peak in the amended and unamended 0-5 cm slurries at their respective first sampling points (T=2 for the amended slurries, T=6 for the unamended slurries), but was not visible in the 25-30 cm slurries until the second sampling points (T=4 for the amended slurry, T=12 for the unamended slurry). The thermophilic methane maxima exceeded the
mesophilic maxima at the first sampling point in both 0-5 cm amended slurry, however it remains smaller than the mesophilic methane maxima until the second sampling point for the 25-30 cm amended slurries and the third sampling point (T=30) for the 25-30 cm unamended slurries.

The temperature optima of the thermophilic methane peak also varied between slurry conditions. If the optima was determined according to the temperature at which maximum methane concentrations occurred in the first 2 sampling points, then the thermophilic methane temperature optima for the 0-5 cm unamended slurry (point 5, Figure 5.0.5.), and both the amended (point 5, Figure 5.0.4.) and unamended (point 7, Figure 5.0.6.) 25-30 cm slurries, is approximately 58-60 °C. In contrast, headspace methane concentrations were 3-fold higher at 65 °C (point 7, Figure 5.0.3.) than at 63 °C in the 0-5 cm amended slurry at the first sampling time point, suggesting that the temperature optima of the thermophilic peak might even have been above 65°C for this slurry condition.

As seen in the psychrophilic and mesophilic temperature ranges, maximum headspace methane concentrations in the thermophilic temperature range were higher in the 0-5 cm amended slurries (maximum of ~480 ppt at 65.0 °C) than the 25-30 cm amended slurries after 2 days incubation (maximum of 20 ppt at 56.6 °C) but were roughly equivalent after 10 days incubation (~600 and 560 ppt respectively at >51 °C). Headspace methane concentrations in the amended slurries increased very rapidly in the thermophilic temperature range between the 2 and 4 day profiles, but there was negligible increase between the 4 and 10 day profiles. This suggests that either production inhibition or lack of substrate may have inhibited thermophilic methane production in amended slurries between 4 and 10 days incubation. The magnitude of the thermophilic methane maxima was very similar in 0-5 and 25-30 cm unamended slurries at 6 and 12 days incubation, however concentrations were approximately a third higher in the 0-5 cm unamended slurries after 30 days incubation.

The difference between maximum methane concentrations in the amended and unamended slurries is slightly reduced in the thermophilic temperature range compared to the mesophilic temperature range. Maximum headspace methane concentrations in the 0-5 and 25-30 cm amended slurries (~600 ppt and 560 ppt respectively) are approximately three-fold higher than maximum headspace methane
concentrations in the 0-5 and 25-30 cm unamended slurries (190 ppt and 180 ppt respectively). However, the disparity between mesophilic and thermophilic headspace methane concentrations in 0-5 and 25-30 cm slurries after the maximum incubation period (10 days for the amended slurries, 30 days for the unamended slurries) is notably higher at mesophilic and thermophilic temperatures (headspace methane concentrations 3-4 times higher in the 0-5 cm slurries) than at psychrophilic temperatures (headspace methane concentrations in 0-5 cm slurries ≤ 1.3 times higher than in 25-30 cm slurries).

5.3.2. Temperature profiles of methanogen substrates

Headspace hydrogen concentration temperature profiles for each sampling time point and slurry acetate and methylamine concentration temperature profiles the final sampling time points are shown in Figures 5.0.3. (0-5 cm amended), 5.0.4. (25-30 cm amended), 5.0.5. (0-5 cm unamended), and 5.0.6. (25-30 cm unamended).

5.3.2.1. Hydrogen production and utilisation

Hydrogen depletion was evident at temperatures above 25.5°C in the 0-5 cm amended slurries after 2 days incubation (Figure 5.0.3.), including clear peaks in hydrogen utilisation at 33.9, 42.5 - 45.2, and 65 °C where concentrations have been reduced from ~160 ppt to ~0.1 ppt. These hydrogen utilisation peaks correspond directly with methane production maxima (points 2, 3 and 7, Figure 5.0.3.). There was also an additional fourth, smaller utilisation peak at 54.8 °C where concentrations have been reduced to 115 ppt (point 8, Figure 5.0.3.) which does not correspond to a methane peak.

There was limited hydrogen depletion between 25.5 and 34.0 °C in the 25-30 cm amended slurry after 2 days incubation, with significant depletion at temperatures >34.0 °C only (Figure 5.0.4.). The 25-30 cm amended slurries also showed smaller peaks in hydrogen utilisation at 42.5 °C and 56.6 °C (points 6 and 7, Figure 5.0.4.) where concentrations were reduced to 53 ppt and 130 ppt respectively.

The 4 day incubation profile for the 0-5 cm amended slurry showed a shallow reduction in hydrogen concentration with increasing temperature between 11.3 and 19.8 °C, then a marked reduction between 19.8 (148 ppt) and 28.3 °C (<0.01 ppt). Hydrogen concentrations remained <0.01 ppt at temperatures >28.3 °C. A similar pattern
occurred in the 25-30 cm slurry with limited depletion and temperatures below 25.5 °C, then rapid removal at 31.1 °C and above (headspace hydrogen concentrations <0.01 ppt). After 10 days rapid significant hydrogen removal occurred at temperatures >11.3 °C in the 0-5 amended slurries, and >14.1 °C in the 25-30 cm amended slurry. Hence the minimum temperature for rapid hydrogen removal shifted to slightly lower temperatures over the 10 day period of incubation.

Hydrogen concentrations in unamended slurries were far lower than in amended slurries; typically less than 30 ppm, and never more than 120 ppm. There was a notable peak in hydrogen concentrations (~120 ppm) in the 6 day incubation profiles of both slurries at 15.6 °C in the 0-5 cm unamended slurry (point 6, Figure 5.0.5.) and 9.9-12.7 °C in the 25-30 cm slurry (point 8, Figure 5.0.6.), which is removed by day 12. This early peak in hydrogen concentrations is likely a slurring effect (Arnosti et al. 2005; Finke et al. 2007a; Finke et al. 2007b; Finke and Jorgensen 2008), caused by mechanical disruption of syntrophic communities combined with low temperatures where terminal oxidizers may respond more slowly than fermentative organisms (Weston and Joye 2005).

The 12 day profiles for both slurry depths had a shallow increase in hydrogen concentration with temperature, and there was even a large peak at 65 °C in the 25-30 cm slurry. However, in general, the average and range of hydrogen concentrations decreased with increasing incubation time. The hydrogen concentration range in 0-5 cm slurries was 0-122 ppm (average of 21.8 ppm) after 6 days incubation, which was reduced to 0-77 ppm (average of 32.2 ppm) after 12 days incubation, and 0-36.5 ppm (average of 16.5 ppm) after 30 days incubation. For 25-30 cm slurries, the range at 6 days incubation was 0-119 ppm (average of 17.8) and at 12 days was 0-162 ppm (average of 21.2 ppm), which reduced significantly to 0-32 ppm (average of 15.4 ppm) at 30 days incubation. It is important to note that the low concentrations of hydrogen seen in the unamended slurries are close to the minimum detection limit for hydrogen (~3 ppm), meaning confident interpretation of hydrogen temperature profiles in the unamended slurry is not feasible.

### 5.3.2.2. Acetate production and utilisation

There was evidence of both significant acetate production in 0-5 cm and 25-30 cm amended and unamended slurries. At T=0 the acetate concentration was 2.4 mM in
both 0-5 cm and 25-30 cm amended slurries, and concentrations remained between 2-2.4 mM between 0 and 5.6 °C for both slurry depths at the final sampling time point. Acetate concentrations then increased rapidly with increasing temperature, starting slightly below the temperature at which methane concentrations also first increased (11.3 °C in the 0-5 cm amended slurries [Figure 5.0.3.], 14.1 °C in the 25-30 cm amended slurries [Figure 5.0.4.]). In the 0-5 cm amended slurries acetate concentrations increased rapidly between 8.4 and 19.8 °C, reaching >10 mM at 19.4 °C. In the 25-30 cm amended slurries concentrations began to increase at approximately 11.3 °C, reaching 9.0 mM at 19.8 °C. Acetate concentrations remained very high (>7.5 mM) throughout the mesophilic temperature range in both 0-5 cm and 25-30 cm slurries, but showed a clear inverse relationship with maximum headspace methane concentrations. This was particularly evident in the 0-5 cm slurry where there was an acetate minima and methane peak at 36.8 °C (point 5, Figure 5.0.3.), whereas in the 25-30 cm amended slurry acetate concentrations remain stable at ~8 mM throughout the temperature range of the mesophilic methane peak (19.1-45.3 °C). Interestingly the acetate maxima at 45.3 °C in the 0-5 cm amended slurries corresponds directly with a small sub-thermophilic methane peak (point 6, Figure 5.0.3.). Acetate concentrations then decreased substantially with increasing temperature to <4 mM between 45.3 °C and 51 °C in both the 0-5 cm and 25-30 cm amended slurries, coincident with the onset of the thermophilic methane peak (point 9 in Figure 5.0.3. and point 8 in Figure 5.0.4.). In the 25-30 cm amended slurries acetate concentrations rose again significantly at temperatures >51 °C, reaching >10 mM at 65 °C (whilst methane production was very limited, point 10 in Figure 5.0.4.).

Acetate concentrations in 0-5 cm and 25-30 cm unamended slurries exceeded the T=0 concentrations by 4-5 fold at 1.6 °C, and increased to a maximum of 5.8 and 3.6 mM respectively at 7.1 °C. Acetate concentrations then gradually decreased with increasing temperature and headspace methane concentrations above 7.1 °C (point 7 in Figure 5.0.5., point 9 in Figure 5.0.6.), down to <30 µM at 29.7 °C in the 0-5 cm slurries and at 24.0 °C in the 25-30 cm slurry (coincident with the respective mesophilic peaks in methane concentration). Acetate concentrations remained <30 µM until 41 °C in the 0-5 cm amended slurry, then increased gradually with temperature to a maximum of 370 µM at 63.7 °C. Acetate concentrations were also generally low (<20 µM) in the mesophilic and low thermophilic temperature range in the 25-30 cm unamended
slurries, but increased significantly above 49.5 °C to a maximum of 2.2 mM at 63.7 °C. In both the 0-5 cm and 25-30 cm unamended slurries there was a small peak in acetate concentrations coincident with the interface between the mesophilic and thermophilic methane concentration peaks (90 µM at 43.9 °C and 696 µM at 46. 7°C respectively, point 10 in Figure 5.0.6.).

5.3.2.3. Methyamine utilisation

Methyamine concentrations showed an inverse relationship with methane concentrations in the amended slurries, particularly in the mesophilic and thermophilic temperature ranges. Methyamine concentrations in the T=0 slurry were 1.7 mM for the 0-5 cm amended slurry and 1.2 mM for the 25-30 cm amended slurry. Methyamine concentrations in the 0-5 cm amended slurry remained at 1.7 mM between 0 and 8.5 °C., then decreased gradually between 8.5 °C and 22.6 °C and more rapidly between 22.6 and 33.9 °C. There was no methyamine depletion in 25-30 cm slurries at temperatures below 25.5 °C, but methyamine concentrations also decreased rapidly between 25.5 and 33.9 °C. Methyamine was completely depleted (minimum detection limit <1 µM) in both 0-5 cm and 25-30 cm slurries between 33.9 and 39.6 °C, broadly coincident with the mesophilic methane maxima (point 5 in Figure 5.0.3. and point 4 in Figure 5.0.4.). Methyamine concentrations were also reduced to 0.14 mM in the 0-5 cm slurry and completely depleted in the 25-30 cm slurry at 52.8 °C, coincident with part of the thermophilic methane maxima (point 9 in Figure 5.0.3. and point 8 in Figure 5.0.4.). Similar to the trend in acetate concentrations in amended slurries, significantly less methyamine depletion occurred at the methane minima between the mesophilic and thermophilic methane peaks (point 6, Figure 5.0.2. and point 11, Figure 5.0.4.).

As typical of freshwater environments, methyamine concentrations were below minimum detection levels (5 µM) in unamended time 0 slurries, and remained below minimum detection levels at all temperatures in both 0-5 cm and 25-30 cm unamended slurries.

5.3.3. Carbon dioxide production and consumption

Carbon dioxide concentrations in the 0-5 cm amended slurries at T=2 ranged between 35.0 - 40.0 ppt between 0-39.6 °C. There was a slight rise in carbon dioxide concentrations with increasing temperature, to a maximum 51.0 ppt at 45.2 °C,
followed by a decrease to between 36.0 - 42.0 ppt between 45.2 and 62.3 °C. At the highest temperature point (65.0 °C) concentrations rose again almost 5-fold to 188 ppt (point 7, Figure 5.0.3). Carbon dioxide concentrations remained between 30.0-42.0 ppt between 1.4 and 28.3 °C after 4 days incubation, but were substantially higher (generally >150 ppt) at temperatures above 28.3 °C. After 10 days incubation concentrations were 30.0-38.0 at temperatures ≤11.3 °C, rising sharply to 158 ppt at 14.1 °C, then more gradually to 266 ppt at 48.1 °C. Concentrations then decreased to 182-210 ppt at temperatures >48.1 °C.

In 25-30 cm amended slurries carbon dioxide concentrations ranged from 31.0 - 40.0 ppt at temperatures >39.6 °C after 2 days incubation. Concentrations rose to 56.0 ppt at 45.3 °C, then decreased to 40.0-46.0 ppt at temperatures >45.3 °C. After 4 days incubation carbon dioxide concentrations remained at 31.5 - 41.4 ppt below 25.5 °C, rose sharply to 207 ppt at 28.3°C (point 3, Figure 5.0.4.), then decreased to 52.2-53.5 ppt between 31.1 and 34.0 °C. There was a second large peak in carbon dioxide concentrations at 36.8 °C (224 ppt), and concentrations continued to increase with temperature to a maximum of 280 ppt at 45.3 °C, then decreased rapidly to 46.2 ppt at 48.1 °C. A third period of elevated carbon dioxide concentrations occurred in the thermophilic temperature range, with concentrations increasing with temperature from 179 ppt at 51.0 °C to 211 ppt at 62.3 °C, followed by a decrease to 45.9 ppt at 65.0 °C. After 10 days incubation carbon dioxide concentration remain at approximately 35 ppt until 17 °C, and then increase sharply with temperature alongside methane and acetate concentrations (and inversely to hydrogen concentrations, point 1 in Figure 5.0.4.). Maximum carbon dioxide concentrations occurred at 45.3 °C, coincident with the methane minima between the mesophilic and thermophilic methane peaks, and also methylamine and acetate maxima (point 11, Figure 5.0.4.). Carbon dioxide concentrations in the thermophilic temperature range are relatively low compare to the mesophilic temperature range, with the exception of a large peak (348 ppt) at 65.0 °C (point 10, Figure 5.0.4.) coincident with the temperature at which thermophilic methanogenesis ceases in the 25-30 cm amended slurries. Headspace carbon dioxide concentrations in the thermophilic temperature range show a similar temperature distribution to acetate, and inverse temperature distribution to methane.

In unamended slurries carbon dioxide concentrations increased gradually with increasing temperature, and varied little with incubation time. In the 0-5 cm unamended
slurries concentrations increased from 118 to 171 ppt between 1.4 and 63.7 °C after 6 days incubation, 103 to 180 ppt after 12 days incubation, and 118 to 163 ppt after 30 days incubation. In the 25-30 cm unamended slurries concentrations increased from 139 to 170 ppt between 1.4 and 63.7 °C after 6 days incubation, 155 to 182 ppt after 12 days incubation, and 149 to 167 ppt after 30 days incubation.

5.3.4. Methanogen community structure in temperature gradient slurries

All 16s rRNA sequences retrieved from DGGE bands had >97% sequence similarity to methanogen type strains. 16s rRNA sequences affiliated with the hydrogenotrophic genera *Methanobacterium* and *Methanothermobacter* (family Methanobacteriaceae, order Methanobacteriales) were detected in both amended and unamended slurries from 0-5 cm and 25-30 cm depth. The genus *Methanobacterium* contains predominantly mesophilic species and a few psychrophilic species, whereas the genus *Methanothermobacter* consists only of thermophilic species (Liu 2010a). A third hydrogenotrophic genus, *Methanoculleus* (family Methanomicrobiaceae, order Methanomicrobiales) was also identified in the 0-5 cm amended and unamended slurries only. The genus *Methanosarcina* (family Methanosarcinaceae, order Methanosarcinales) was detected in the 25-30 cm amended and unamended slurries only. The genus *Methanosarcina* has exceptionally wide ranging physiology in terms of both temperature optima and substrate utilisation. Members of the genus *Methanosarcina* include psychrophiles, mesophiles, and thermophiles, and are typically able to utilise at least two of the three main classes (hydrogenotrophic, acetotrophic, and methylotrophic) of methanogen substrates (Liu 2010b). The temperature distribution of the genera *Methanobacterium*, *Methanothermobacter*, *Methanoculleus* and *Methanosarcina* in temperature gradient slurries at the final sampling time point is shown in Figures 5.0.3. (0-5 cm amended), 5.0.4. (25-30 cm amended), 5.0.5. (0-5 cm unamended), and 5.0.6. (25-30 cm unamended).

The genus *Methanobacterium* was dominant between 5.6 and 59.4 °C in both 0-5 cm and 25-30 cm amended slurries. The relative abundance of *Methanobacterium* spp. was slightly lower (85-90 %) between 5.6 and 22.6 °C than between 36.8 and 59.4 °C (90-95 %) in the 0-5 cm amended slurries. The genus *Methanothermobacter* was dominant at 62.3 and 65.1 °C. The relative abundance of *Methanothermobacter* spp. at 62.3°C (96.2 %) was significantly higher than at 65.1 °C (76.5 %), with a
corresponding increase in the relative abundance of Methanobacterium spp. from 3.8 % at 62.3 °C to 23.5 % at 65.0 °C.

The relative abundance of Methanobacterium spp. in 25-30 cm amended slurries rose from 81.9 % at 5.6 °C to 99 % between 14.1 and 22.6 °C. However, Methanosarcina were present at comparatively high relative abundance at 5.6 °C (12.8 %), which decreased to <1 % at 14.1 and 22.6 °C. The relative abundance of Methanosarcina spp. increased substantially in the mesophilic and lower thermophilic temperature ranges, showing a positive relationship with methane concentrations, and an inverse relationship with acetate and methylamine concentrations. There was a large peak in the relative abundance of Methanosarcina spp. at 36.8°C (42.1 %) and a smaller peak at 53.8 °C (30.9 %), coincident with the methane maxima and acetate and methylamine minima at 10 days incubation (points 4 and 8, Figure 5.0.4.). At the methane minima between the mesophilic and thermophilic methane peaks (53.8 °C, point 11 in Figure 5.0.4.) the relative abundance of Methanobacterium spp. increased to 86.3 °C, and the relative abundance of Methanosarcina spp. decreased to 13.7 %. At 59.4 °C, above the maximum temperature of methylamine depletion, the relative abundance of Methanobacterium spp. increased once again to 74.6 %. The relative abundance of Methanosarcina spp. decreased from 16.6 % at 59.4 °C to 0 % at 65.0 °C, with a parallel increase in the relative abundance of Methanothermobacter spp. from 8.7 % to 96.7 % over the same temperature range.

The genus Methanobacterium was also the dominant in unamended slurries at psychrophilic and mesophilic temperatures, with the genus Methanothermobacter dominant at thermophilic temperatures. The relative abundance of Methanobacterium spp. in the 0-5 cm unamended slurries was >95 % between 7.1 and 35.4 °C, decreasing to 84 % at 52.4 °C and <20 % at 60.9 °C and above. The relative abundance of Methanothermobacter spp. was 84.4 % at 60.9 °C, but also decreased at the highest temperature point (63.7 °C) to 80.5 %. There was a shallow peak (relative abundance 8.3 %) in Methanothelus spp. at 52. 4 %, however the relative abundance of Methanothelus spp. was <1 % at all other temperatures.

The relative abundance of Methanobacterium spp. was between 95-100 % between 7.1 and 21.2°C in the 25-30 cm unamended slurries, which decreased gradually over the mesophilic temperature range to 77.0 % at 52.4 °C. The relative abundance of
Methanosarcina spp. increased over the mesophilic temperature range from 0 % at \( \leq 21.2 \, ^\circ C \) to 19 % between 43.9-52.4 °C and 28.4 % at 52.4 °C. There was then a sharp decrease in relative abundance of Methanosarcina spp. at 63.7 °C (6.2 %) coincident with the marked increase in acetate concentrations to 2.2 mM (point 9, Figure 5.0.6.). The relative abundance of Methanothermobacter spp. was less than 6 % in the psychrophilic and mesophilic temperature ranges, but increased consistently with temperature at \( \geq 59.4 \, ^\circ C \), reaching a maximum 96.7 % at 63.7 °C. Interestingly, the switch in dominance between Methanobacterium and Methanothermobacter occurred slightly after the interface between the mesophilic and thermophilic methane peaks in the amended slurry, but before the intersection in the unamended slurry.

5.3.5. Geochemical profiles and methanogen community structure in temperature gradient microcosms

Headspace methane and hydrogen profiles, slurry methylamine and acetate profiles, and relative abundances of different methanogen genera are shown in Figure 5.0.7. (0-5 cm, amended), Figure 5.0.8. (25-30 cm, amended), Figure 5.0.9. (0-5 cm, unamended) and Figure 5.1.0. (25-30 cm, unamended). The psychrophilic, mesophilic, and thermophilic temperature ranges for prokaryotic growth (Table 5.0.3.) are indicated in Figures 5.0.7.-5.1.0. by coloured shading, and are colour coded according to Table 5.0.3. All 16s rRNA sequences retrieved from DGGE bands had \( >97\% \) sequence similarity to methanogen type strains.

5.3.5.1. 0-5 cm amended microcosms

The headspace methane profile in the 0-5 cm amended microcosm did not have the clearly defined psychrophilic, mesophilic, and thermophilic peaks visible in the parallel 0-5 cm amended temperature gradient slurries. Headspace methane concentrations increased sharply from \( \sim 93 \) ppt at 4°C to 649 ppt at 10 °C, followed by a slight increase to \( \sim 750 \) ppt between 25 and 66°C. Methane concentrations were only approximately 150 ppt higher between 25 and 66 °C in the 2 year slurry than the highest methane concentrations produced in the 10 day temperature gradient slurries (\( \sim 550 \) ppt between 51 and 65 °C). Methane concentrations also did not vary in this temperature range, suggesting substrate limitation prevents further methane production.
The community composition, however, still showed a clear separation between *Methanobacterium* and *Methanothermobacter* dominated temperature ranges. The
relative abundance of *Methanobacterium* spp. decreased gradually over the psychrophilic and mesophilic temperature range from 100 % at 5.6 °C to 89 % at 38°C. The hydrogenotrophic genus *Methanocella* (family Methanocellaceae, order Methanocellales) was also present at low relative abundance (6%) at 25 °C. The genus *Methanocella* contains both mesophilic and thermophilic species. The relative abundance of *Methanothermobacter* spp. was below 1 % between 4 and 25 °C, increased slightly to 10 % at 38 °C, and then increased dramatically to 97 and 89 % at 55 and 66 °C respectively.

The temperature profile of hydrogen and acetate concentrations in the 0-5 cm amended microcosm show an inverse relationship with the headspace methane concentrations. Highest concentrations of hydrogen and acetate occurred at 4°C, coincident with relatively low methane concentrations. Hydrogen concentrations were depleted significantly below the concentration to which they were initially set (160 ppt), but were markedly higher at 4 °C (~4.2 ppt) than at 10 °C and above. Headspace hydrogen concentrations were reduced to ~0.2 ppt between 10 and 38 °C, decreasing ~0.1 ppt at 55 and 66 °C. Acetate concentrations at 4°C (25 mM) were significantly higher than the level to which they were originally amended (2 mM), but concentrations were depleted to >20 µM between 10 and 38 °C. Acetate concentrations at 55 and 66 °C were 485 and 250 µM respectively, which although lower than the initial concentrations (2 mM), is more than ten-fold higher than acetate concentrations in the mesophilic microcosms. Methylamine concentrations were also depleted below the level to which they were initially amended (2 mM) at all temperatures. Methylamine concentrations were highest (485 µM) at 4 °C, as also seen in the hydrogen and acetate profiles, but were depleted below minimum detection levels at higher temperatures in 0-5 cm amended microcosms.
5.3.5.2. 25-30 cm amended microcosms

Headspace methane concentrations increased slightly between 4 and 10 °C from 81 ppt to 108 ppt (compared to 649 ppt for the 0-5 cm amended slurry), then more rapidly to ~850 ppt between 25 and 38 °C. In contrast to the methane temperature profile of the temperature gradient slurries, headspace methane concentrations in the 25-30 cm amended microcosms decreased significantly between the mesophilic (~850 ppt) and thermophilic temperature ranges (95 ppt at 55 °C, 2 ppt at 66 °C). In addition, maximum headspace methane concentrations were higher in the 25-30 cm amended microcosm (876 ppt at 25 °C) than the 0-5 cm amended microcosm (797 ppt at 25°C) after 2 years incubation.

The methanogen community structure of the 25-30 cm amended microcosms also differed from the 25-30 cm amended temperature gradient slurries as the genus *Methanosarcina* was not detected in the 25-30 cm amended microcosms. However the temperature distribution of the genera *Methanobacterium* and *Methanothermobacter* was very similar to the 25-30 cm amended temperature gradient slurries. The relative abundance of *Methanobacterium* spp. ranged from 94 to 100 % between 4 and 25 °C. At 38 °C the relative abundance of *Methanobacterium* spp. and *Methanothermobacter* spp. was approximately equal, and at 55 and 66 °C the relative abundance of *Methanothermobacter* spp. rose above 90 %.

Highest hydrogen and acetate concentrations occurred in the 4 and 10°C 25-30 cm amended microcosms, coincident with relatively low methane concentrations. Headspace hydrogen concentrations decreased rapidly from 13.6 ppt at 4°C to 0.02 ppt between 25 and 38 °C. Slurry acetate concentrations were greatly elevated above the level to which microcosms were initially amended level (2 mM) at 4°C (24.6 mM), but decreased rapidly to 20 µM at 10 °C. Acetate concentrations remained extremely low (below 10 µM) between 25 and 46 °C. There was a significant increase in acetate concentrations between the mesophilic and thermophilic temperatures ranges, coincident with the decrease in headspace methane concentrations, with acetate concentrations rising to >30 mM at 55 and 65°C. Conversely hydrogen concentrations increased only slightly between the mesophilic and thermophilic temperature ranges, reaching 0.7 ppt at 55°C and 3.6 ppt at 66°C. Methylamine concentrations were below
minimum detection levels between 4 and 55 °C, but showed no evidence of depletion from the initial amended level at 66 °C.

Figure 5.0.8. Geochemical and methanogen community structure profiles in estuarine clay (25-30 cm) amended microcosms
5.3.5.3. 0-5 cm unamended microcosms

Methane concentrations increased consistently with temperature from 34 ppt at 4 °C to c. 75 ppt between 25 and 38 °C (compared to ~110 ppt in 30 day temperature gradient profile), then increased again more sharply to 116 ppt at 55°C (~168 ppt in 30 day temperature gradient profile) and 122 ppt at 66°C. Maximum headspace methane concentrations in the mesophilic and thermophilic 0-5 cm unamended microcosms were lower than detected in the corresponding 0-5 cm unamended temperature gradient slurries after 30 days incubation. The community composition profile reflect the transition between the mesophilic and thermophilic peaks, with *Methanobacterium* dominant between 4 and 38 °C (99-100 %) and *Methanothermobacter* dominant at 55 and 66 °C (90-98 %). The genus *Methanocella* was also detected at low relative abundance (0.4 %) at 55 °C. Similar to the parallel 0-5 cm unamended temperature gradient slurries, hydrogen concentrations in the 0-5 cm unamended microcosms were extremely low throughout the temperature range of incubation. Hydrogen concentrations decreased from 26 ppm at 4 °C to a minimum of 9 ppm at 38 °C, before increasing gradually to 40 ppm at 66°C. Acetate concentrations were substantially lower than in the parallel 0-5 cm unamended temperature gradient slurries at temperatures below 25 °C, decreasing from 33 µM at 4 °C to 3 µM at 25°C. Acetate concentrations increased gradually with temperature over the mesophilic and thermophilic temperature ranges, as also seen in the 0-5 temperature gradient slurries, to reach a maximum of 307 µM at 66 °C. Methylamine was detected at low concentrations (15 µM) in the 66 °C 0-5 cm unamended microcosm, but was below minimum detection limits (~2 µM) at lower temperatures.

5.3.5.4. 25-30 cm unamended microcosm

gradient slurries, hydrogen concentrations in the 0-5 cm unamended microcosms were extremely low throughout the temperature range of incubation. Hydrogen concentrations decreased from 26 ppm at 4 °C to a minimum of 9 ppm at 38 °C, before increasing gradually to 40 ppm at 66°C. Acetate concentrations were substantially lower than in the parallel 0-5 cm unamended temperature gradient slurries at temperatures below 25 °C, decreasing from 33 µM at 4 °C to 3 µM at 25°C. Acetate concentrations increased gradually with temperature over the mesophilic and thermophilic temperature ranges, as also seen in the 0-5 temperature gradient slurries, to reach a maximum of 307 µM at 66 °C. Methylamine was detected at low
concentrations (15 µM) in the 66 °C 0-5 cm unamended microcosm, but was below minimum detection limits (~2 µM) at lower temperatures. The headspace methane temperature profile of the 25-30 cm unamended microcosms was very similar to the 0-5 cm unamended microcosms, however methane concentrations in the 25-30 cm unamended microcosms were approximately 50 % lower than in the 0-5 cm unamended microcosms at each temperature point. Headspace methane

\[ \text{Figure 5.0.9. Geochemical and methanogen community structure profiles in lacustrine gyttja (0-5 cm) unamended microcosms} \]
concentrations in the 25-30 cm unamended microcosms after 2 years incubation were also lower than in the corresponding 25-30 cm unamended temperature gradient slurries after 30 days incubation. Methane concentrations in the 25-30 cm unamended microcosms increased gradually with temperature between the psychrophilic and mesophilic temperature ranges from 15 ppt at 4 °C to ~33 ppt at 25 and 38 °C. Methane concentrations then increased more rapidly between the mesophilic and thermophilic temperature ranges to ~60 ppt at 55 and 66 °C. The relative abundance of
*Methanobacterium* spp. was between 89-99 % in the 4, 10, 25 and 38 °C 25-30 cm unamended microcosms, with *Methanothermobacter* spp. also detected at low relative abundance (6-10 %) in the 4 and 10 °C microcosms. The relative abundances of *Methanobacterium* spp. and *Methanocella* spp. were approximately equal in the 55°C 25-30 cm unamended microcosm, but *Methanothermobacter* was the dominant genus at 66 °C (90.6 %).

Highest concentrations of hydrogen and acetate occurred in the psychrophilic and thermophilic temperature ranges. Hydrogen concentrations increased from 32 ppm in the 4 °C microcosm to a peak of 152 ppm at 10°C, before decreasing to minimum of 11 ppm at 38 °C. Hydrogen concentrations then increased significantly again between the mesophilic and thermophilic temperature ranges, reaching a second peak of 207 ppm at 66 °C. The acetate concentration in the 4°C microcosm was 27 mM, which decreased rapidly to below 40 µM between 10 and 66 °C.

### 5.3.6. Temperature range and methane production rates of single substrate enrichments

Single substrate enrichments were inoculated using slurry from the 0-5 cm and 25-30 cm amended temperature gradient slurries from a number of incubation temperatures. The temperatures selected were chosen to coincide with peaks and troughs in methane production or potential substrate utilisation. Single substrate enrichments with either methylamine, acetate, or H₂/CO₂ were inoculated for each of the temperatures chosen. Tables 5.0.1. and 5.0.2. show the temperature gradient temperature, subsequent incubation temperature, and rate of methane production in single substrate enrichments measured as number of weeks elapsed to obtain 40 ppt of methane in headspace for enrichments inoculated with 0-5 cm amended slurries and 25-30 cm amended slurries respectively. Hydrogen, acetate, and methylamine single substrate enrichments were successful at both 4 and 10 °C when inoculated with 0-5 cm and 25-30 cm amended temperature gradient slurry. The time taken for enrichments to produce 50 ppt of methane was not recorded due to the very slow rate of methane production at these low temperatures.

Enrichments amended with hydrogen grew over the greatest temperature range and had the highest temperature maximum of the three methanogenic substrates tested. Hydrogen enrichments were successful at incubation temperatures between 4 and 77
°C for the 0-5 cm amended slurry. The temperature range of successful enrichments from the 25-30 cm amended temperature gradient slurries was significantly lower than for the 0-5 cm amended temperature gradient slurries, ranging from 4 to 55 °C. Rates of methane production in hydrogen enrichments were similar for both sediment depths. Both 0-5 cm and 25-30 cm hydrogen enrichments produced 50 ppt of methane within 1 week of incubation at incubation temperatures between 25 and 55 °C. The 0-5 cm hydrogen enrichments incubated at 66 and 77 °C did not produce 50 ppt of methane until between 1 and 2 weeks.

Acetate enrichments were successful between 4 and 65 °C when inoculated with 0-5 cm temperature gradient slurry, and between 4 and 55 °C when inoculated with 25-30 cm temperature gradient slurry. The rate of methane production in 0-5 cm acetate enrichments was substantially faster than in 25-30 cm acetate enrichments. 0-5 cm acetate enrichments produced 55 ppt of methane within 1 week at temperatures between 38 and 55 °C, whereas 25-30 cm acetate enrichments took 8 weeks at 38 °C and more than 8 weeks at 46 and 55 °C. 0-5 cm acetate enrichments were slowest at 25 °C (2 weeks) and 66 °C (temperature gradient incubation temperature 59.4 °C; 8 weeks), however 25-30 cm acetate enrichments took more than 8 weeks at both of these temperatures.

Methylamine enrichments grew between 4 and 55 °C when inoculated with either the 0-5 cm or 25-30 cm temperature gradient slurry, which was the lowest temperature maximum or range of the three methanogen substrates tested. 0-5 cm methylamine enrichments took less than 1 week to produce 50 ppt of methane between 25 and 46 °C, and 1-2 weeks at 55 °C. Similar to acetate enrichments, 25-30 cm methylamine enrichments were considerably slower to produce methane than 0-5 cm acetate enrichments, taking more than 8 weeks to reach a headspace methane concentration of 50 ppt at all temperatures.

5.4. Discussion

5.4.1. Temperature range and optima for methanogenesis exceed Cardiff Bay environmental temperature range

The seasonal average water temperature for Cardiff Bay varies from 6.5 °C in winter (December to January) to 18.1 °C in summer (June to August), and the minimum and maximum average monthly water temperature during the period 2003-2012 were 2.9
C and 21.7 °C respectively (Lee In prep.). Cardiff Bay is a shallow, polymictic lake (Thomas 2014) and water temperatures at the water-sediment interface will be the same (or at maximum of a few degrees lower) than in the overlying water column. It is, therefore, surprising that the temperature optima and range for methane production from Cardiff Bay sediments far exceed the in situ temperature range. Previous studies investigating the temperature characteristics of freshwater sediments and soils have also found that the temperature optima for methanogenesis is significantly higher than the environmental temperature range (Zeikus and Winfrey 1976; Thebrath et al. 1993; Westermann 1994; Schulz and Conrad 1996; Nozhevnikova et al. 1997; Schulz et al. 1997; Yao and Conrad 2000; Fey et al. 2001; Nozhevnikova et al. 2003; Glissmann et al. 2004; Metje and Frenzel 2005; Metje and Frenzel 2007; Nozhevnikova et al. 2007; Jerman et al. 2009). Interestingly, the temperature optima for methane production broadly increases with the average in situ temperature of a range of environments (Table 5.0.4.). The in situ temperature range of the northern peatlands, characterised by Metje and Frenzel (2005) and Metje and Frenzel (2007), is -1.1°C, yet the temperature optimum for methane production is 25-28°C. Similarly, Blake et al. (2015) found that the temperature optima for methane production from Arctic wetland sediments was ~30 °C; far higher that the environmental temperature range of -1.4 – 14.1 °C. There is also a significant disparity between the in situ temperature range (4-5 °C) and methanogenic temperature optima (30-40°C) of boreal and temperate lakes such as Lakes Baldegg and Soppen, Switzerland (Nozhevnikova et al. 1997), Lake Constance, Germany (Thebrath et al. 1993; Schulz and Conrad 1996) and Lake Mendota, Wisconsin, USA (Zeikus and Winfrey 1976). Rice field soils have significantly higher in situ temperature range (15-30 °C; Schutz et al. 1990) than boreal and temperate wetland or lake sediments, yet the temperature optima for methane production in rice field sediments, 37-41 °C (Yang and Chang 1998; Yao and Conrad 2000; Fey et al. 2001), is only slightly higher than lake sediments (maximum of 40 °C).

5.4.1. Psychrotolerant and mesophilic methanogen populations active in Cardiff Bay sediments under in situ conditions

It is not clear whether the methanogen populations responsible for the mesophilic peak in methane production in lake sediments are those which are active in situ. On the one hand, methane production rates are uniformly highest at mesophilic temperatures (Zeikus and Winfrey 1976; Thebrath et al. 1993; Schulz and Conrad 1996;
Table 5.0.4. Temperature range and optima for methanogenesis in peatland soils, rice field soils and lake sediments

<table>
<thead>
<tr>
<th>Study environment</th>
<th>In situ temperature range</th>
<th>Temperature optima for methane production</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peatland soils</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidic peat mire, northern Scandinavia (Metje and Frenzel, 2005)</td>
<td>Average -1.1 (max. 13.4°C)</td>
<td>25°C</td>
</tr>
<tr>
<td>Subarctic permafrost, northwestern Siberia (Metje and Frenzel, 2007)</td>
<td>Average -7.4°C (max. 18.5°C)</td>
<td>26-28°C</td>
</tr>
<tr>
<td>Fen, Slovenia (Jerman et al. 2009)</td>
<td>Range 1-20°C</td>
<td>38°C</td>
</tr>
<tr>
<td><strong>Lake sediments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Littoral, Lake Constance (Thebrath et al. 1993)</td>
<td>4°C</td>
<td>30-40°C</td>
</tr>
<tr>
<td>Profundal, Lake Constance (Schulz et al. 1997)</td>
<td>4°C</td>
<td>34°C</td>
</tr>
<tr>
<td>Lake Mendota (Zeikus and Winfrey, 1976)</td>
<td>4-23°C</td>
<td>35-42°C</td>
</tr>
<tr>
<td>Lakes Baldegg and Soppen (Nozhevnikova et al. 1997; 2003)</td>
<td>5°C</td>
<td>30-35°C</td>
</tr>
<tr>
<td>Cardiff Bay (this study)</td>
<td>Range 6-19°C</td>
<td>32-38°C</td>
</tr>
<tr>
<td><strong>Rice field soils</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taiwan (Yang and Chang, 1998)</td>
<td></td>
<td>37°C</td>
</tr>
<tr>
<td>Italy and the Philippines (Yao and Conrad 2000)</td>
<td>15-30°C (Schutz et al. 1990)</td>
<td>32-41°C</td>
</tr>
<tr>
<td>Italy (Fey et al. 2001)</td>
<td></td>
<td>41°C</td>
</tr>
</tbody>
</table>

N.B. Only studies where the temperature range under investigation exceeded the temperature optima for methanogenesis were included. Temperature optima for methanogenesis is temperature at which methane production most rapid.
Nozhevnikova et al. 1997; Nozhevnikova et al. 2003; Glissmann et al. 2004; Nozhevnikova et al. 2007), and (to date) all methanogen strains isolated from lake sediments have either been mesophilic (Zhu et al. 2011; Ganzert et al. 2014; Schirmack et al. 2014) or psychrotolerant (e.g. Simankova et al. 2001; Borrel et al. 2012a). In contrast, psychrophilic peaks in methane production are evident at 10, 20 °C, and 25 °C in boreal and temperate wetland soils (Svensson 1984; Wagner and Pfeiffer 1997; Avery et al. 2003), and both psychophilic (e.g. Zhang et al. 2008; Zhou et al. 2014) and psychrotolerant (Kotsyurbenko et al. 2007; Krivushin et al. 2010) methanogen strains have been isolated from these environments. However, Nozhevnikova et al. (2003) note that there was a ‘shoulder’ at 6 and 15 °C in the mesophilic peak in the methane temperature profile for Lake Baldegg sediments (Nozhevnikova et al. 2003) and at approximately 25 °C in the temperature profile of Lake Constance (Schulz et al. 1997). They suggest that these ‘shoulders’ are psychrophilic peaks in methane production obscured by the more rapid response of mesophilic methanogen populations incubated at their optimum temperature. Subsequent cultivation experiments by Nozhevnikova et al. (2003) provide further evidence that methanogen populations adapted to temperatures <25°C were present in sediments of Lake Baldegg, however they did not determine whether the sediment methanogen community structure more closely resembled that of the psychrophilic methanogenic enrichments or mesophilic methanogenic enrichments.

Similar psychrophilic ‘shoulders’ were present in headspace methane temperature profiles of both the amended and unamended Cardiff Bay lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) temperature gradient slurries. As *Methanobacterium* spp. are the dominant community members in Cardiff Bay sediments under *in situ* conditions (Figure 3.0.7., Chapter 3), and were also dominant at psychrophilic and mesophilic temperatures in temperature gradient slurries (Figures 5.0.3. - 5.0.5.) it seems likely that methane production in Cardiff Bay sediments is mediated by psychrotolerant or mesophilic *Methanobacterium* spp. However, *Methanobacterium* strains active at *in situ* temperatures and in psychrophilic slurries and microcosms may differ at species or strain level from those active in mesophilic slurries and microcosms.
5.4.2. Methane production is stimulated by substrate addition and by climate relevant increases in temperature

The temperature characteristics of the *in situ* methanogen populations are an important factor in determining how methane production from lake sediments might respond to future climate change. The most recent IPCC report predicts an increase in global mean surface temperature relative to period 1986-2005 of 1-2°C for the period 2046-2065 and 1-3.7 °C for 2081-2100 (depending on emissions scenario; Stocker et al. 2014). If the predicted increase in surface temperature is applied directly to water temperatures then the seasonal temperature average for Cardiff Bay waters would increase from 6-18 °C (current) to between 7-20°C during 2046-2065 and between 7-22 °C during 2081-2100. Temperature gradient slurries results show that methane production rates are extremely sensitive to temperature increase within the environmentally relevant temperature range for climate change. Figure 5.1.1. shows that methane headspace concentrations increase exponentially with temperature between ~0 and 25 °C in both 0-5 cm and 25-30 cm amended (R² = 0.93 and R² = 0.94 respectively) and unamended (R² = 0.95 and R² = 0.94 respectively) slurries. Avery et al. (2003) also recorded an exponential increase in methane production rates for freshwater estuarine sediments incubated over a temperature range of 0-25 °C.

Climate change may render freshwater lakes increasingly vulnerable to eutrophication, induced by a combination of rising temperatures and increased nutrient and terrestrially derived dissolved organic carbon (DOC) loading from their catchment areas. It is in this context relevant that substrate addition stimulates methane production throughout the temperature range under investigation including the environmentally relevant temperature range for climate change (see Figure 5.1.1.). This finding supports results of previous studies (Schulz and Conrad 1995; Schwarz et al. 2008; West et al. 2012; Lofton et al. 2015) which found that methane production rates in lake sediments increased when amended with algal biomass, terrestrially derived organic matter, or direct methanogen substrates (acetate and hydrogen). It is not clear whether increased substrate availability leads to an alteration in methanogen community structure or abundance in lake sediments. Schwarz et al. (2008) detected a rapid increase in abundance of the obligately acetotrophic family ‘Methanotrichaceae’ (previously referred to as Methanosetaceae; Oren 2014) in amended sediment cores from Lake Constance, Germany in response to
Figure 5.1.1. Methane production rates in lacustrine gyttja and estuarine clay sediments increase exponentially with temperature between 0 and 25 °C. A shows the headspace methane concentration for 0-5 and 25-30 cm amended and unamended temperature gradient slurries plotted over a temperature range of 0-25 °C. Note that headspace methane concentrations in A are plotted on a log_{10} scale. A linear regression was fitted to each of the headspace methane concentration plots shown in A. B shows the gradient of the linear regression fitted to each of the headspace methane concentration plots shown in A. The R^2 value for each of the linear regressions is shown in the legend of B.
supplementation with labile organic matter (algal biomass). However, there was no alteration in archaeal community structure in amended (supplemented with algal biomass or terrestrially derived organic matter) and unamended cores from Lake Diamond, Michigan, USA (West et al. 2012), or between methanogen community structure in amended (supplemented with methanogen substrates) and unamended temperature gradient slurries or microcosms from Cardiff Bay.

5.4.3. Thermophilic methanogen communities in Cardiff Bay sediments

Most existing studies of temperature control on methanogenesis in natural environments confined the temperature range under investigation to within 10 °C of the maximum temperature experienced in situ (typically 30-40 °C), however many of those of that extended the temperature range under investigation to 50 °C or above also identified a second thermophilic peak in methane production which was clearly distinct from the lower temperature mesophilic peak. Yao and Conrad (2000) and Fey et al. (2001) detected a thermophilic methane production optima at 50 °C in rice field soils from Italy, and Yang and Chang (1998) detected a thermophilic methane peak in Taiwanese rice field soil at their maximum incubation temperature of 60 °C. The presence of moderately thermophilic methanogen communities is widespread in rice field soils from Italy, China and the Philippines, and was also detected in flooded riparian soils from the Netherlands (Wu et al. 2006). The capability for high temperature methane production is less consistent in temperate lake sediments than rice paddy fields. This lack of consistency could be linked to the lower in situ temperatures of temperate lake sediments (<25 °C), which are consistently below the minimum growth temperature of all known thermophilic methanogen species. Thermophilic methane peaks were identified at 50 °C in sediments from Lake Mendota (Zeikus and Winfrey 1976) and Lake Baldegg (Nozhevnikova et al. 1997), but not in sediments from Lake Constance (Thebrath et al. 1993; Schulz et al. 1997) which had very limited methane production above 45 °C.

The maximum temperature for methane production in Cardiff Bay sediments (>65 °C for 0-5 cm sediments) is also higher than in many previous studies. However, methane production is recorded up to 60 °C in rice field sediments by (Yang and Chang 1998; Fey et al. 2001) and is ongoing at 70 °C in lake sediments (Nozhevnikova et al. 1997). Most studies also report a lag time of between 10 and 20 days incubation before the
onset of thermophilic methanogenesis (Nozhevnikova et al. 1997; Yang and Chang 1998; Yao and Conrad 2000; Fey et al. 2001), which is attributed to a small population of thermophilic methanogens which are activated by temperature increase. In contrast, substantial thermophilic methane production occurred within 48 hours in Cardiff Bay amended slurries and within 6 days in unamended slurries. *Methanothermobacter* spp. were not detected in the original sediments (Figure 3.0.7., Chapter 3), and therefore must constitute a small proportion of the total archaeal population. The maximum temperature for methane production from Cardiff Bay sediments was slightly lower for the estuarine clay (25-30 cm) sediments than the lacustrine gyttja (0-5 cm) sediments, although the dominant methanogen genus at thermophilic temperatures (*Methanothermobacter*) is the same for both sediment depths. The temperature optima for *Methanothermobacter* type strains ranges from 55 to 70°C, and the upper temperature limit from 65 to 80°C. It is possible that different species or strains of *Methanothermobacter* inhabit the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) sediments. Interestingly, thermophilic methanogenesis has not been detected in temperate (Jerman et al. 2009), boreal (Metje and Frenzel 2005), subarctic (Metje and Frenzel 2007) or arctic (Blake et al. 2015) wetland soils. Thermophilic Sulphate Reducing Bacteria (SRB) have previously been identified in temperate wetlands (Rahman et al. 2004) and permanently cold marine sediments (Isaksen et al. 1994; Hubert et al. 2009; Hubert et al. 2010), but perhaps the mechanism which provides thermophilic SRB to sediments does not seed methanogens.

### 5.4.4. Potential sources and survival mechanisms of thermophilic methanogens in Cardiff Bay sediments

Both of the thermophilic methanogen genera (*Methanobacterium* and *Methanoculleus*) enriched in temperature gradient slurries or microcosms have previously been enriched or isolated from temperate environments. *Methanothermobacter*-like strains were enriched from temperate freshwater lake sediments by Nozhevnikova et al. (1997), and a strains of *Methanoculleus thermophilus* have been isolated from temperate, brackish estuarine sediments (Rivard and Smith 1982) and freshwater lake sediments (Harris et al. 1984). The origins and survival mechanisms of thermophilic methanogens in cold and temperate environments are not well understood. Endosporulation enables certain bacterial groups to survive hostile environments as dormant cells (Hubert et al. 2009; Portillo et al. 2012; de Rezende et al. 2013),
however, methanogens do not form spores. It is often stated that methanogens do not have resting stages of any form, yet there is a growing body of evidence to suggest *Methanosarcina* spp. alter their cell morphology in response to oxygen exposure and other environmental stressors, possibly to a resting form (Robinson 1986; Fetzer et al. 1993; Liu et al. 2008; Angel et al. 2011). Assuming methanogens do not actually form true survival stages, low temperature environments containing thermophilic methanogens must receive continuous sources of fresh inocula. Hubert et al. (2009) hypothesise that geothermal fluids seed surface ocean sediments with thermophilic anaerobic bacterial spores native to the deep, hot biosphere via cold seeps and sea floor pockmarks associated with deep petroleum deposits, mid-ocean ridge spreading centres, and fractures in the ocean crust. Bonjour et al. (1988), Fey et al. (2001) and Marchant et al. (2008) suggest aerial transfer of microorganisms from volcanic, geothermal, or hydrothermal habitats as a source and dispersal mechanism for thermophilic aerobes in terrestrial environments. Neither of these mechanisms are likely sources of thermophilic prokaryotes for Cardiff Bay.

Other potential sources of thermophilic prokaryotes in temperate soils are bioreactors (Van Lier et al. 1996; Sekiguchi et al. 1998) and compost (Derikx et al. 1989; Thummes et al. 2007a; Thummes et al. 2007b). Thummes et al. (2007b) suggest that commercial composting plants supply thermophilic microorganisms to temperate soils via bioaerosols generated during the composting process and through compost application to agricultural land. Thermophilic methanogens detected in compost include *Methanothermobacter* spp., *Methanosarcina thermophila*, *Methanoculleus thermophilus* and *Methanocellales* spp. (Thummes et al. 2007a; Thummes et al. 2007b), and members of each of these genera (in addition to the genus *Methanoculleus*) were detected in Cardiff Bay temperature gradient slurries or microcosms (Figures 5.0.3.-5.1.0.). Methane production occurs in commercial composting plants, regardless of the source material for composting, and despite the use of aeration methods to maintain aerobic degradation conditions (Thummes et al. 2007a).

Anaerobic bioreactors are increasingly used to treat organic matter and wastes from the food and agricultural industries, in addition to their more traditional usage during sewage treatment processes. The genera *Methanothermobacter* (Sekiguchi et al. 1998; Hori et al. 2006; Chen et al. 2008; Franke-Whittle et al. 2014). *Methanoculleus*
Chapter 5 – Temperature Gradient Experiment

(Hori et al. 2006; Kobayashi et al. 2008; Sasaki et al. 2011; Wagner et al. 2011) and *Methanosarcina* (Hori et al. 2006; Kobayashi et al. 2008; Sasaki et al. 2011) are also genera often abundant in thermophilic anaerobic bioreactors. The end products of the anaerobic digestion process are biogas (predominantly methane and carbon dioxide) and digestate. The fluid and liquid components of digestate, after further processing, are commonly used as fertilisers and soil conditioners for agricultural soils. Digestate usage is subject to compliance with microbiological pathogen and nutrient limits set by the European Commission (Saveyn and Eder 2014) and the British Standards Institute (PAS 2013). In order to achieve the necessary criteria a pasteurization step must be incorporated into the anaerobic digestion process either before feedstock is added to the digestion vessel, or of the resulting digestate. This typically involves heat treatment to 70°C for one hour, or use of an aerobic composting step. Some plants are exempt from these procedures depending on the operating temperature and hydraulic retention time of the digester, the feedstock source, and intended end use of the digestate. Anaerobic digestors are not generally considered to be a source of thermophilic methanogens to the environment due to the strict criteria governing usage or disposal of digestate (Thummes et al. 2007b). However it is important to note that ‘pasteurization does not aim to achieve sterilisation’ (PAS 2013), and high temperature thermophiles may be able to survive this treatment. Thermophilic methanogens enriched during this study were active at temperatures considerably higher than 70°C, suggesting that they may be able to survive pasteurization processes.

If thermophilic methanogens are able to survive pasteurisation, then digestate from anaerobic bioreactors would be a source of thermophilic methanogens to agricultural soils as per compost. The catchment areas of the Taff and Ely rivers and their respective tributaries are composed of 75 % agricultural land and 15 % industrialised areas (Environment Agency Wales, Environment Agency 2010), and therefore it is probable that the Taff and Ely rivers receive soil and leachate input from agricultural land treated with sludge from anaerobic digesters. Thermophilic methanogens transferred into Cardiff Bay via agricultural soils would have to survive oxygen exposure during storage, fertiliser application, residence in aerated soils, and transport downstream within oxic river waters. Whether this is feasible is not known. Several recent studies have demonstrated that methanogens are not as oxygen sensitive as previously believed (e.g. Liu et al. 2008; Angel et al. 2011; Angel et al. 2012; Tang et
Experimental evidence demonstrates methanogens are able to survive the desiccation stress experienced during airborne transport, and can be cultured from soil treated with compost for several months following the last application of compost (Thummes et al. 2007b).

Landfill leachate is another potential source of the thermophilic methanogens in Cardiff Bay sediments. The deeper layers of older, stabilized landfills can reach temperatures conducive to growth of thermophilic methanogens. 16s rRNA gene sequences affiliated with the thermophilic genus *Methanothermobacter* (Chen et al. 2003; Song et al. 2015) and thermophilic species *Methanoculleus thermophilus* (Uz et al. 2003) have been detected in partially degraded refuse and leachate from municipal solid waste landfills. Leachate from several tips and landfill sites situated around the perimeter of Cardiff Bay was discharged into the waters prior to construction of Cardiff Bay Barrage (Environmental Advisory Unit 1991). Discrete leachate outfalls were diverted into the Severn Estuary prior to impoundment, and preventative measures put in place to reduce the risk of diffusive input, however limited input still occurs via the foul drainage system (Environmental Advisory Unit 1991).

Thermophilic prokaryotes detected in cold and temperate environments are often thought to be inactive under *in situ* conditions, however this is not necessarily a valid assumption. Alternatively, in some environments thermophilic prokaryotes may survive through temporary bursts of activity when environmental conditions become favourable (Portillo et al. 2012). For example, Cockell et al. (2015) speculate that thermophilic *Geobacillus* are able to survive in Icelandic basalt and obsidian outcrops because the high thermal conductivity and low albedo of the outcrops causes temperatures to exceed their minimum growth temperature under certain environmental conditions. Hence thermophilic prokaryotes with a minimum temperature of 36 °C are able to survive in a habitat where the mean air temperature is <10 °C. Some thermophilic organisms are active over exceptionally large ranges, which could enable their survival in temperate environments (Wiegel 1990). Strains of *Methanothermobacter thermautotrophicus* grow at temperatures as low as 22 °C, and continue to produce methane down to 15 °C (Wiegel 1990). A *Methanothermobacter* strain isolated from Cardiff Bay sediments produced methane when incubated at 15°C, but not at 10 °C (see section 7.6.2). *Methanothermobacter*-like strains could also be enriched from the estuarine and lacustrine 4 and 10°C sediment slurry microcosms at 66°C under a
H₂/CO₂ atmosphere, demonstrating that viable *Methanothermobacter* cells remain after 2 years incubation at temperatures significantly below their minimum growth temperature (data not shown). Similarly, Rivard and Smith (1982) enriched a strain of *Methanoculleus thermophilus* from estuarine sediments in the effluent channel for cooling waters from a coastal nuclear power plant which utilizes seawater as a coolant. They hypothesize that warm waters released from the coolant towers may have provided a temporary microhabitat for thermophilic methanogens in this otherwise inhospitable environment.

5.4.5. Acetogenesis vs hydrogenotrophic methanogenesis in low temperature freshwater environments

In common with previous studies investigating low temperature methanogenesis in freshwater lake sediments (e.g. Nozhevnikova et al. 2003) there was substantial acetate accumulation at psychrophilic (<25 °C) temperatures amended and unamended temperature gradient slurries and microcosms from both sediment depths of Cardiff Bay (Figures 5.0.3. - 5.1.0.). This is surprising given that radiotracer studies show high rates of hydrogenotrophic methanogenesis in cores incubated at 10 °C (Figure 3.0.6., Chapter 3), and porewater acetate concentrations are very low (<20 mM) in sediment core profiles (Figure 3.0.5., Chapter 3). Homoacetogens are active in almost all anoxic environments, and produce acetate through degradation of sugars and carbon dioxide reduction. Kotsyurbenko et al. (2001) suggest that homoacetogens can outcompete some types of hydrogenotrophic methanogens at low temperatures <15 °C when hydrogen availability is non-limiting by virtue of their higher growth rate $V_{\text{max}}$ (Kotsyurbenko et al. 2001, discussed further in section 6.5.3.).

Physical homogenization of sediments is necessary for slurry based experiments to reduce the effects of sediment heterogeneity and produce robust results, but physical disruption of sediment causes transient increases in concentrations of acetate and hydrogen, presumably due to disruption of syntrophic relationships (Arnosti et al. 2005; Finke et al. 2007a; Finke et al. 2007b; Finke and Jorgensen 2008). Hoehler et al. (1999) found that acetogenic populations in marine sediments dominated by sulphate reduction and methanogenesis were able to respond quickly to a transient increase in hydrogen availability. The temperature gradient slurry and microcosm methane and methanogen community structure profiles indicate that that the dominant methanogen
groups in Cardiff Bay sediments are mesophilic. Therefore perhaps sediment acetogen populations were able to make use of the hydrogen transitorily formed as a result of slurrying (Figure 5.0.5. and 5.0.6. at 6 days incubation) more rapidly than the mesophilic hydrogenotrophic methanogens, and continued to dominate after the initial hydrogen addition by virtue of increased population size.

The minimum temperature at which acetogenesis occurred at the final temperature gradient sampling point (10 days for amended slurries, 30 days for unamended slurries) was significantly higher in amended temperature gradient slurries (8.5 °C., Figure 5.0.5.) than unamended temperature gradient slurries (1.4 °C. Figure 5.0.6.), despite high substrate availability and lack of competition from methanogens. The concurrent sharp increase in acetate and methane production and decrease in hydrogen concentrations at ~11-15 °C indicates that high hydrogen concentration probably inhibited acetogenesis at low temperatures in amended temperature gradient slurries. In contrast, there was substantial methane and acetate production in the 4 °C amended microcosms (93 ppt and 81 ppt respectively in the 0-5 cm and 25-30 cm amended microcosms, Figures 5.0.7. and 5.0.9. respectively). Presumably acetogenesis became thermodynamically viable during the long incubation period (2 years) of the 4 °C amended microcosms once hydrogen concentrations had been sufficiently reduced by hydrogenotrophic methanogenesis. In contrast to previous lake sediment studies (Glissmann et al. 2004), acetotrophic methanogens were not detected in psychrophilic temperature gradient slurries or microcosms. Perhaps acetate accumulation did not prompt an increase in the abundance of acetotrophic methanogens in the temperature gradient slurries or 4°C microcosms, and acetotrophic methanogens were not responsible for acetate consumption in the 10 and 25°C microcosms. Acetate consumption may have proceeded via syntrophic acetate oxidation at low temperatures. Alternatively perhaps the abundance of acetotrophic methanogens remained below the detection threshold for PCR-DGGE (1-2% of the rDNA template, Muyzer et al. 1993; Murray et al. 1996; Stephen et al. 1999).

Acetogenesis and methanogenesis occurred concurrently at temperatures >8.5 °C in the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) amended temperature gradient slurries after 10 days incubation (Figures 5.0.3. and 5.0.4.), presumably enabled by non-limiting substrate (H2/CO2) availability. Over longer time periods the balance between acetate production and consumption in the mesophilic 0-5 cm and
25-30 cm microcosms and thermophilic 0-5 cm microcosms altered, and acetate concentrations were reduced to relatively low levels (<0.01 mM in mesophilic microcosms and <0.5 mM in thermophilic microcosms, Figures 5.0.7. - 5.1.1.). This reduction in acetate concentration was not seen in the estuarine clay (25-30 cm) amended microcosms. Instead acetate concentrations in the estuarine clay (25-30 cm) amended microcosms increased dramatically from <0.01 mM in the mesophilic temperature range to 30 and 37 mM respectively in the 55 and 66 °C amended microcosms. This was accompanied by a decrease in methane concentrations from >800 ppt in the mesophilic temperature range to 95 ppt at 55 °C and 2.7 ppt at 66 °C.

The estuarine clay (25-30 cm) temperature gradient slurries did not produce methane at 65 °C, suggesting that the numbers of viable thermophilic methanogens present in 25-30 cm sediments was lower in the lakestrine gyttja (0-5 cm) sediments. Relatively low abundances of thermophilic methanogens in estuarine clay (25-30 cm) sediments could explain why rates of acetate utilization were lower in estuarine clay (25-30 cm) microcosms. Strangely, acetate concentrations in the estuarine clay (25-30 cm) unamended microcosms were lower than in the lacustrine gyttja (0-5 cm) unamended microcosms at thermophilic temperatures (Figures 5.0.9. and 5.1.0.), however perhaps acetate production in unamended temperature gradient slurries and microcosms was limited by substrate availability.

5.4.6. Temperature dependent variation in methanogen community structure and function

Methylamine and hydrogen depletion were more rapid in the mesophilic temperature range than psychrophilic temperature range in both the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) amended temperature gradient slurries. Hydrogen depletion was most rapid at 34 and 42.5-45.3 °C in the lacustrine gyttja (0-5 cm) amended slurry (Figure 5.0.3., 2 day incubation profile), and at 42.5-45.3 °C in the estuarine clay (25-30 cm) amended slurry (Figure 5.0.4., 2 day incubation profile). Methylamine depletion was most rapid at 33.9-39.6 °C in the lacustrine gyttja (0-5 cm) amended slurries, and at 36.8-39.6 °C in the estuarine clay (25-30 cm) amended slurries (Figures 5.0.3. and 5.0.4. respectively, 10 day incubation profile). There was a clear mesophilic acetate minima in the lacustrine gyttja (0-5 cm) amended slurries, which coincided directly with the mesophilic methane peak slurry (Figure 5.0.3., 10 day incubation profile). However, it is not certain whether this acetate minima was due to relatively low levels of
acetogenesis (perhaps due to competition with hydrogenotrophic methanogens for substrate) or acetate utilisation by acetotrophic methanogens. There was no clear mesophilic acetate minima in the estuarine clay (25-30 cm) amended slurries, instead acetate concentrations remained stable at ~8 mM throughout the temperature range of the mesophilic methane peak (Figure 5.0.4., 10 day incubation profile). Successful acetate amended single substrate enrichments were obtained at 38 and 46 °C for both 0-5 and 25-30 cm slurries (Tables 5.0.1. and 5.0.2.), which strongly suggests that acetotrophic methanogens active at mesophilic temperatures are present in both lacustrine gyttja and estuarine sediments.

Previous studies also have also found that rates of acetotrophic and hydrogenotrophic methanogenesis increase with temperature between the psychrophilic and mesophilic ranges in lake sediments (Schulz et al. 1997; Glissmann et al. 2004; Nozhevnikova et al. 2007), rice field sediments (Chin et al. 1999a; Fey and Conrad 2000) and cold wetland sediments (Blake et al. 2015; Fu et al. 2015). In each of these studies acetotrophic methanogenesis predominates at low temperatures, and the contribution of hydrogenotrophic methanogenesis increases with temperature. This alteration in the rates and relative contributions of the acetotrophic and hydrogenotrophic methanogenesis was not accompanied by a change in methanogen community structure in sediments of Lake Dagow (Glissmann et al. 2004). In rice field sediments methanogen community structure differs with temperature, but temperature controls methanogen community structure both directly and indirectly depending on the duration of incubation. Fey and Conrad (2000) recorded a decrease in the relative abundance of the hydrogenotrophic Methanocellaceae and Methanosarcinaceae between 10 and 37 °C, and a corresponding increase in the relative abundance of acetotrophic Methanotrichaceae. Results of Fey and Conrad (2000) directly contradict other studies investigating temperature control on methanogen community structure in rice field sediments (Chin et al. 1999a; Chin et al. 1999b; Wu et al. 2002) who found that sediments incubated at 30 °C had a higher relative abundance of Methanosarcinaceae and Methanocellaceae than sediments incubated at 15 °C, and vice versa for Methanotrichaceae, Methanomicrobiaceae and Methanobacteriaceae. In long term incubation experiments (e.g. Fey and Conrad 2000) methanogen community structure is affected both directly (e.g. temperature characteristics of methanogen strains) and indirectly (e.g. substrate availability) by temperature. Under
steady state conditions acetate concentrations decreased with increasing temperature, selecting for Methanotrichaceae at mesophilic temperatures as a function of their lower substrate threshold (Fey and Conrad 2000). However, in short term incubations (e.g. Chin et al. 1999a; Chin et al. 1999b; Wu et al. 2002) high acetate concentrations sustain Methanosarcinaceae at mesophilic temperatures.

There was clear correspondence between temperature dependent variation in methanogen community structure and function in the estuarine clay (25-30 cm) slurries. Mesophilic and thermophilic peaks in relative abundance of the mixotrophic genus *Methanosarcina* corresponded well with the mesophilic and thermophilic methylamine minima and a plateau in acetate concentrations in the estuarine clay (25-30 cm) amended slurries (Figure 5.0.4.). The temperature distribution of *Methanosarcina* was very similar in the estuarine clay (25-30 cm) unamended slurries to the estuarine clay (25-30 cm) amended slurries. In the estuarine clay (25-30 cm) unamended slurries the relative abundance of *Methanosarcina* began to increase at >21.1 °C, coincident a sharp reduction in acetate concentrations and increase in methane concentrations. (Figure 5.0.6., 10 day temperature profile). Acetate concentrations in the estuarine clay (25-30 cm) unamended slurries were below minimum detection levels at mesophilic and thermophilic temperatures, and methylamine was presumably not available for consumption (as concentrations of methylated amines were below minimum detection limits in Cardiff Bay sediments, section 3.5.5.3.), therefore it is not clear what methanogenic pathway was being utilised by *Methanosarcina* present in the estuarine clay (25-30 cm) unamended slurries.

The genus *Methanosarcina* (nor any other acetotrophic or methylotrophic methanogen genera) was not detected in the lacustrine gyttja (0-5 cm) temperature gradient slurries (Figures 5.0.3. and 5.0.5.), nor the lacustrine gyttja (0-5 cm) microcosms (Figures 5.0.7. and 5.0.9.) or estuarine clay (25-30 cm) microcosms (Figures 5.0.8. and 5.1.0.). It is possible that acetic consumption in these slurries and microcosms could have been due to syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis. However, syntrophic acetate oxidation is favoured by low hydrogen concentrations (Hattori 2008) and hydrogen was added in large excess (headspace hydrogen concentration ~160 ppt) to amended slurries prior to incubation. In addition, syntrophic acetate oxidation is usually associated with high temperature environments.
Methylamine is a non-competitive substrate for methanogenesis (Oremland and Polcin 1982) which is only used by methanogens under anaerobic conditions. Given the close similarity between the temperature distribution of methylamine and acetate in the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) amended slurries, and acetate in the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) unamended slurries, it is probable than *Methanosarcina* are also present in the lacustrine gyttja (0-5 cm) temperature gradient slurries and lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) microcosms at abundances below the minimum detection limit of PCR-DGGE. The sensitivity of PCR-DGGE has been estimated to be approximately 1-2% of the rDNA template (Muyzer et al. 1993; Murray et al. 1996; Stephen et al. 1999), although the use of nested PCR increases the sensitivity of PCR based analyses (Vissers et al. 2009).

5.4.7. Evidence for methylotrophic and acetotrophic methanogenesis at thermophilic temperatures

Hydrogenotrophic methanogenesis is the major pathway for methane production at temperatures above ~45 °C in both lake sediments (Zeikus and Winfrey 1976; Nozhevnikova et al. 2007) and rice field sediments (Fey et al. 2001; Wu et al. 2006; Conrad et al. 2009; Noll et al. 2010; Rui et al. 2011). Rates of acetotrophic methanogenesis were typically very low (<5 % of total methane production) in lake sediment and rice field sediments at thermophilic temperatures, and the cessation of acetotrophic methanogenesis is accompanied by an abrupt switch in methanogen composition in rice field sediments. Fey et al. (2001), Noll et al. (2010), and Conrad et al. (2009) detected a sharp increase in the relative abundance of the hydrogenotrophic Methanocellaceae, and decrease in the hydrogenotrophic *Methanobacteriaceae* and acetotrophic Methanosarcinaceae and Methanotrichaceae, in the interval between approximately 42 and 46°C. Methanocellaceae was heavily dominant at >46°C. Methanobacteriaceae, Methanomicrobiaceae and very rarely Methanosarcinaceae, are occasionally detected in rice field sediments incubated at 45 °C (Wu et al. 2006). In general, methanogen diversity at thermophilic temperatures is severely reduced relative to mesophilic temperatures (Conrad et al. 2009).
In contrast to previous studies, there was a thermophilic peak in methylamine utilisation in Cardiff Bay amended slurries at 53.8-56.6 °C, with a corresponding peak in relative abundance of *Methanosarcina* spp. in the estuarine clay (25-30 cm) amended slurries only. Significant methylamine depletion was evident at temperatures <62.3 °C in amended slurries (Figures 5.0.3. and 5.0.4.), and successful methanogenic enrichments were obtained on methylamine when subcultured from the 59.4 °C lacustrine gyttja (0-5 cm) amended slurry and 62.3 °C estuarine clay (25-30 cm) amended slurry (although subcultures were incubated at 55 °C, Table 5.0.1 and 5.0.2.). Methylation concentrations were depleted significantly below the level to which they were originally amended (2 mM) in 66°C amended microcosms (Figures 5.0.7. and 5.0.9.), suggesting that the temperature maxima for methylamine utilisation may be higher than indicated by methylamine profiles in the short term (T=10) amended temperature gradient profiles. The genus *Methanosarcina* was not detected in the estuarine clay (25-30 cm) amended or unamended temperature gradient slurries at the highest temperature point (Figures 5.0.4.-5.0.6.), which concurs with the known temperature maxima for the methylamine and acetate utilising *Methanosarcina thermophila* (55 °C; Zinder et al. 1985).

There was also a thermophilic peak in acetate utilisation in Cardiff Bay slurries, demonstrated by the acetate minima (<2 mM) at 53.8-56.6. °C in the lacustrine gyttja (0-5 cm) amended slurries (Figure 5.0.3.) and 51.0-62.3 °C (2.5-3.0 mM) in the estuarine clay (25-30 cm) amended slurries (Figure 5.0.4.) after 10 days incubation. Again, this thermophilic acetate minima broadly coincides with a peak in the relative abundance of the mixotrophic genus *Methanosarcina* in the estuarine clay (25-30 cm) slurries. As discussed for the mesophilic acetate minima, it is not clear from the geochemical profiles of the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) whether this thermophilic acetate minima is due to reduced levels of acetate production, or high rates of acetotrophic methanogenesis. High acetate concentrations (10.9 mM) in the estuarine clay (25-30 cm) amended slurries (Figure 5.0.4.), and elevated (> 3mM) acetate concentrations in the lacustrine gyttja (0-5 cm) amended slurries (Figure 5.0.3.) at 65 °C, demonstrate that acetogens in Cardiff Bay sediments were still active at this temperature. However, successful single substrate enrichments were obtained using acetate at temperatures up to 66°C from the lacustrine gyttja (0-5 cm) amended slurries (Table 5.0.1.) and up to 55 °C from the estuarine clay (25-30 cm amended slurry).
cm) amended slurries (Table 5.0.2.), indicating that Cardiff Bay lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) methanogenic communities are able to convert acetate to methane at thermophilic temperatures.

The methanogen genera detected in lacustrine gyttja (0-5 cm) temperature gradient slurries and microcosms at thermophilic temperatures (*Methanobacterium* and *Methanothermobacter*) are not known to utilise acetate for methanogenesis, suggesting that acetate consumption at thermophilic temperatures may occur via syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis. Syntrophic acetate oxidation is the major pathway for acetate utilisation at high temperatures in rice field sediments (Conrad et al. 2009; Liu and Conrad 2011; Rui et al. 2011), and is also an important methanogenic pathway in permanently high temperature environments such as high temperature oil fields and anaerobic digesters (Hao et al. 2011; Mayumi et al. 2011; Fotidis et al. 2012). *Methanothermobacter* (dominant at thermophilic temperatures in all slurries and microcosms) and *Methanoculleus* (detected at 52.4 °C in 0-5 cm unamended slurries) are known to participate in syntrophic acetate oxidation in anaerobic digesters (e.g. Lee and Zinder 1988; Schnürer et al. 1999; Hori et al. 2006).

An alternative explanation is that acetate was utilised by an acetotrophic methanogen genera present at too low abundance for detection using PCR-DGGE. This could be *Methanosarcina thermophila* (as discussed above), but the maximum temperature for *Methanosarcina thermophila* is lower than the maximum temperature at acetate utilisation occurred in lacustrine gyttja (0-5 cm) slurries and single substrate enrichments (65 and 66 °C respectively). The acetotrophic methanogen species *Methanothrix thermophila*, has an upper temperature limit of ~70 °C (Liu 2010b). *Methanothrix thermophila* was enriched from Cardiff Bay sediments (section 6.5.2) but was not detected in Cardiff Bay temperature gradient slurries or microcosms. It is not clear which process is responsible for methylamine depletion in lacustrine gyttja (0-5 cm) temperature gradient slurries at thermophilic temperatures, unless methylotrophic methanogens (possibly *Methanosarcina thermophila*) are also present in abundances too low for detection using PCR-DGGE.
5.5. Conclusions

In common with previous studies of lake, wetland, and rice field sediments the temperature range for methane production in Cardiff Bay sediments was far greater than the environmental temperature range. Cardiff Bay sediments contained mesophilic and thermophilic methanogen populations, which responded rapidly to incubation at temperatures higher than the environmental temperature range. The temperature range for methanogenesis were not constrained by fermentative syntrophs, and nor did it differ between lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) sediments. Agricultural soils seeded with thermophiles during fertilisation with industrially produced compost or anaerobic digester sludge may provide a source of thermophilic methanogens to Cardiff Bay sediments. The dominant methanogen genera altered between psychrophilic and mesophilic temperatures (Methanobacterium) and thermophilic temperatures (Methanothermobacter) in both short term incubations (temperature gradient slurries) and long term incubations (microcosms), but did not differ between the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) sediments. Methanogen community structure and function varied with temperature in estuarine clay (25-30 cm) temperature gradient slurries, with elevated relative abundance of the mixotrophic genus Methanosarcina coincident with mesophilic and thermophilic peaks in methylamine (and possibly also acetate) utilisation. However, this temperature distribution of Methanosarcina was not replicated in the lacustrine gyttja (0-5 cm) slurries or lacustrine gyttja and estuarine clay (25-30 cm) microcosms, which may reflect the limited sensitivity of PCR-DGGE as a technique for characterising community composition and structure.

There was some evidence for the existence of a psychrophilic methanogen population in Cardiff Bay sediments, with a temperature optima (~20 °C) close to in situ temperatures. However, the overall temperature optima for methane production of the methanogen populations active in Cardiff Bay sediments was significantly higher than the average in situ temperature, supporting results of previous studies which have shown that methanogenesis in low temperature and temperate freshwater ecosystems is temperature limited (e.g. Zeikus and Winfrey 1976). There was an exponential increase in methane production rates with temperature over the environmentally relevant temperature range for climate change, and methane production in this temperature range was further enhanced by substrate addition. These results further
confirm that methane production in temperate freshwater lake ecosystems is highly sensitive to climate change (Duc et al. 2010; West et al. 2012).
6. CULTIVATION EXPERIMENT

6.1. Rationale

For the past three decades, cultivation-independent methods such as PCR-based molecular genetic techniques (DGGE, T-RFLP, cloning and next generation sequencing), non-PCR-based molecular genetic techniques (e.g., Fluorescent In Situ Hybridization (FISH) and microarrays), and Stable Isotope Probing (SIP), have predominantly been used to study archaeal and bacterial biodiversity and ecology. These techniques have substantially increased our knowledge of prokaryotic diversity. However, cultivation-based studies are invaluable in yielding information about microbial community, population physiology and metabolism (Fry 2000; Ellis et al. 2003) that provide indispensable context to genomic data (Nichols 2007). The temperature and salinity gradient experiments presented in Chapters 4 and 5 demonstrate that methanogen populations from Cardiff Bay sediments are active at salinities and temperatures which far exceed the in situ range, and that methanogen community structure and function alters in response to temperature and salinity control.

To further understand the temperature and salinity response of Cardiff Bay methanogen communities, it is necessary to investigate the temperature and salinity characteristics of the individual methanogen populations which collectively determine the community level response.

A key advantage of cultivation-based studies is that they enable isolation of some indigenous strains in pure culture for physiological characterisation. The conventional process of isolate purification from enrichment cultures involves subculturing to increase the relative abundance of target organisms, followed by repeated series of dilutions in liquid media, or single colony isolation, to generate an axenic culture (Alain and Querellou 2009). However organisms obtained in pure culture during cultivation-based studies are often not those which are numerically abundant in the environment of interest (Amann et al. 1995; Liesack et al. 1997; Großkopf et al. 1998; Eilers et al. 2000; Kopke et al. 2005). Instead isolated strains are those most suited to the defined conditions of cultivation, and are often fast-growing organisms able to quickly adapt to increased substrate availability (termed r-strategists) (Eilers et al. 2000; Kopke et al. 2005). Depending on the purpose and aims of a study this effect can be a benefit, as selectivity can assess functionally important but low biomass prokaryotes not detected
using general primers and/or target prokaryotes with specific features such as anaerobes, psychrophiles and piezophiles. Hence, cultivation can effectively be utilised to explore the ‘rare biosphere’ (Shade et al. 2012).

The aim of this study was to isolate the methanogen strains which contributed significantly to the methanogen community salinity and temperature response presented in Chapters 4 and 5. This was achieved by using conventional isolation techniques (subculturing and dilution-to-extinction) to purify methanogen strains active across the range of temperatures and salinities tested. This approach was used with the understanding that isolation techniques select for methanogen strains best adapted to the defined cultivation conditions. In this way, the ecological (temperature or salinity) niche of the different culturable methanogen populations in Cardiff Bay sediments was explored. Methanogen community structure dynamics was monitored at 3 stages throughout the isolation process (enrichment, 5th subculture and final methanogen monoculture or isolate) in order to obtain maximum information about ecological interactions between competing methanogen populations, and providing an indication of how representative the methanogen monoculture or pure cultures were of the total culturable population, and also how the cultivation conditions might be modified to select for other methanogen populations present in sediments and enrichments.

6.2. Methods

6.2.1. Enrichment of methanogens

Single substrate enrichments from the salinity gradient experiment and temperature gradient experiments (Tables 5.0.1. and 5.0.2., Chapter 5) were incubated until the headspace methane concentration was in excess of 50 parts per thousand (ppt), then subcultured using a 1:20 v/v inoculum. The incubation temperature, media composition, and substrate additions of the original enrichments were maintained in all cases. This process was repeated five times for each enrichment condition, with the aim of promoting an increase in abundance of methanogen strains best adapted to the enrichment conditions (i.e. media salinity, incubation temperature, and substrate addition). A 5 ml sample of culture media was taken from the 5th subculture for molecular analysis.
6.2.2. Purification of isolated methanogen strains

A selection of single substrate enrichments from the salinity gradient enrichments (Table 6.0.1) and temperature (Table 6.0.2) gradient experiments were chosen for further study. Methanogen strains were purified using dilution-to-extinction enrichment (Overmann 2006) in liquid media. Each enrichment underwent a minimum of 5 rounds dilution-to-extinction over a dilution range of $10^{-1}$ to $10^{-10}$. The incubation temperature, media composition, and substrate additions of the original enrichments were maintained in all cases. The methanogen cultivation media was supplemented with 1 mg ml$^{-1}$ penicillin sodium salt (Apollo Scientific, Manchester, UK), 0.1 mg ml$^{-1}$ vancomycin sodium salt (Apollo Scientific, Manchester, UK), and 0.55 mg ml$^{-1}$ ampicillin sodium salt (Apollo Scientific, Manchester, UK) during at least 2 rounds of dilution-to-extinction to suppress the growth of bacterial contaminants. Where possible, cultures were purified until only a single methanogen strain and no viable bacteria were present (referred to as ‘pure cultures’). In some instances it was not possible to eradicate contaminating bacterial strains during the time period available, and instead, cultures were purified until a single methanogen strain was present (referred to as ‘methanogen monocultures’). Methanogen monocultures and pure cultures were maintained in media without antibiotics after the dilution-to-extinction process had ceased.

Table 6.0.1. Salinity gradient experiment cultivation series

<table>
<thead>
<tr>
<th>Cultivation series/media salinity</th>
<th>Enrichment</th>
<th>5th subculture</th>
<th>Methanogen monoculture or pure culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lacustrine gyttja (0-5 cm)</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Marine</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Brackish</td>
<td>Y</td>
<td>Y</td>
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</tr>
<tr>
<td>Freshwater</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Estuarine clay (25-30 cm)</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
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<td>Y</td>
<td>Y</td>
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</tr>
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<td>Y</td>
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</tr>
<tr>
<td>Freshwater</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
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</tbody>
</table>

N.B. 1:30 v/v salinity gradient enrichments from salinity gradient experiment were subcultured to generate the salinity gradient cultivation series listed in this table.

6.2.3. Purity tests for methanogen monocultures isolated methanogen strains

Isolate culture purity was verified using a combination of tests. 5 ml of isolate culture was sampled for Polymerase Chain Reaction (PCR) using the bacterial domain specific primers 357f-GC and 518r. DNA extraction and PCR, were carried out as described in sections 2.9.1-2.9.3. Isolate cultures were tested for heterotrophic growth
in basal salt media of the appropriate salinity (section 2.3.1.) amended with yeast extract (0.03 g l⁻¹) and peptone (0.06 g l⁻¹), and inspected visually for contaminants using a combination of phase contrast and epifluorescence microscopy.

### Table 6.0.2. Temperature gradient experiment cultivation series

<table>
<thead>
<tr>
<th>Cultivation series/temperature (°C)</th>
<th>Substrate</th>
<th>Enrichment</th>
<th>5(^{th}) subculture</th>
<th>Methanogen monoculture or pure culture</th>
</tr>
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<tr>
<td>Lacustrine gyttja (0-5 cm)</td>
<td>Methylamine</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>H₂/CO₂</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>4</td>
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<td>N</td>
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<td>Acetate</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>H₂/CO₂</td>
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<td>Y</td>
<td>N</td>
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<td>Acetate</td>
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<td>Y</td>
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<td>H₂/CO₂</td>
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<tr>
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<td>H₂/CO₂</td>
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<tr>
<td>Estuarine clay (25-30 cm)</td>
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<td>H₂/CO₂</td>
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<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
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<td>H₂/CO₂</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
</tbody>
</table>

#### 6.2.4. Molecular analysis of salinity and temperature gradient 5\(^{th}\) subcultures

PCR-DGGE was used to monitor archaeal populations in salinity gradient microcosms and enrichments. DNA extraction, PCR, DGGE, band re-amplification, sequencing,
and fingerprint analysis were carried out as described in sections 2.9.1.-2.9.8. PCR products from salinity gradient 5th subcultures were run on DGGE gels alongside PCR products from the salinity gradient enrichments as described in section 4.2.6. PCR products from the 0-5 cm and 25-30 cm temperature gradient 5th subcultures were run on one DGGE gel, and PCR products from the 0-5 cm and 25-30 cm 4 – 25 °C 5th subcultures were run on another. A total of 16 bands were excised and sequenced from the DGGE gel for the 4-25°C 5th subcultures, and 33 bands were excised and sequenced from the DGGE gel for the 38-66 °C 5th subcultures. The purpose of this study was to track changes in the dominant methanogen genus throughout the cultivation process, and therefore only bands positions which produced a strong signal in one or more samples were excised, re-amplified and sequenced from the 5th subculture samples. The relative abundance of methanogen genera present in each sample was calculated from the summed band intensity of all DGGE bands affiliated with a given genera as detailed in section 2.9.8.2.

6.2.5. Molecular analysis of methanogen monocultures and pure cultures
DNA extraction, PCR and sequencing were carried out as described in sections 2.8.1.-2.8.3. and 2.8.6. using 16s rRNA gene primers 109f/958r.

6.3. Results
6.3.1. Methanogen community structure dynamics in salinity gradient cultivation series

The relative abundance of methanogen genera detected at different stages of cultivation (slurry, enrichment, 5th subculture and methanogen monoculture/pure culture) are shown in Table 6.0.3. (lacustrine gyttja [0-5 cm] salinity gradient cultivation series) and Table 6.0.4. (estuarine clay [25-30 cm] salinity gradient cultivation series).

6.3.1.1. Methylamine amended salinity gradient cultivation series

The hydrogenotrophic genus *Methanobacterium* (family Methanobacteriaceae, order Methanobacterales) was the dominant genus in enrichment cultures amended with methylamine under all depth/salinity conditions, although methylotrophic genera (*Methanomethylovorans*, *Methanolobus*, or *Methanosarcina*, family Methanosarcinaceae, order Methanosarcinales) were also present at low relative abundance. The dominant genus switched to *Methanolobus* in the 25-30 cm and 0-5
cm marine 5\textsuperscript{th} subcultures and the 25-30 cm brackish 5\textsuperscript{th} subculture, although *Methanobacterium* spp. were still detected at low relative abundance in both the marine 5\textsuperscript{th} subcultures. The facultatively methylotrophic genus *Methanosarcina* was also detected at low relative abundance in the marine 0-5 cm 5\textsuperscript{th} subculture only. *Methanolobus* spp. were eventually obtained in pure culture from 25-30 cm sediments.
under marine conditions, and in monoculture from 0-5 cm sediments under marine conditions. Hydrogenotrophic genera remained dominant in the freshwater and brackish 0-5 cm 5th subcultures, with *Methanomethylovorans* spp. (freshwater 5th

<table>
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<th>Salinity</th>
<th>Substrate</th>
<th>Genus</th>
<th>Mb</th>
<th>Msc</th>
<th>Ml</th>
<th>Mmv</th>
<th>Mbv</th>
<th>Mf</th>
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<td>5th subculture</td>
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<td>++</td>
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</tr>
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<td>++</td>
<td>-</td>
<td>-</td>
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</tr>
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<td>+</td>
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<td>+</td>
<td>-</td>
<td>+++</td>
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<tr>
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<tr>
<td>H₂ + CO₂</td>
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<table>
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<th>Key</th>
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<td>&gt;3</td>
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<td>-</td>
<td>&lt;3</td>
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</tbody>
</table>
subcultures), *Methanolobus* spp. (freshwater 0-5 cm 5\textsuperscript{th} subculture) and *Methanosarcina* spp. (freshwater and brackish 0-5 cm 5\textsuperscript{th} subcultures) still only present at low relative abundance. The genus *Methanomethylovorans* was obtained in monoculture from 25-30 cm sediments under freshwater conditions, but surprisingly the hydrogenotrophic genus *Methanobrevibacter* (family Methanobacteriaceae, order Methanobacterales) was obtained in monoculture from 0-5 cm sediments under freshwater conditions. Members of the genus *Methanobrevibacter* are not known to utilise methylamine as a substrate, and were not detected in the 0-5 cm freshwater cultivation series at enrichment or 5\textsuperscript{th} subculture stage. The genus *Methanobrevibacter* also became the dominant methanogen genera in 0-5 cm brackish enrichments at 5\textsuperscript{th} subculture stage, even though methylotrophic methanogens were also present in this cultivation series at both enrichment and 5\textsuperscript{th} subculture stage.

\section*{6.3.1.2. Acetate amended salinity gradient cultivation series}

*Methanosarcina* was the dominant genus at all stages of cultivation for both the 0-5 cm and 25-30 cm freshwater cultivation series and the 0-5 cm marine cultivation series, although *Methanobacterium* spp. were also detected at low relative abundance in the freshwater enrichments, freshwater 5\textsuperscript{th} subcultures, and the marine 0-5 cm 5\textsuperscript{th} subculture. *Methanosarcina* spp. were only present at low relative abundance in the 25-30 cm brackish and marine enrichments, but *Methanosarcina* became the dominant genus in the corresponding 5\textsuperscript{th} subcultures and was obtained in monoculture from the 25-30 cm marine cultivation series. Members of the obligately hydrogenotrophic genus *Methanobacterium* were still present at low relative abundance in marine and brackish 5\textsuperscript{th} subcultures. *Methanobrevibacter* spp. and *Methanomethylovorans* spp. (neither of which are genera known to use acetate as a substrate) were also present in the marine and brackish 5\textsuperscript{th} subcultures, although not identified at the enrichment stage.

\section*{6.3.1.3. Hydrogen amended salinity gradient cultivation series}

*Methanobacterium* was the dominant genus in the freshwater and brackish 25-30 cm enrichments and 5\textsuperscript{th} subcultures amended with hydrogen. *Methanobacterium* strains were obtained in monoculture or pure culture from freshwater cultures under these salinity/substrate conditions. *Methanobrevibacter* spp. and *Methanosarcina* spp. were also identified in the 0-5 cm freshwater 5\textsuperscript{th} subculture, but were not seen in the 25-30 cm freshwater and brackish cultivation series at any stage of cultivation. Both
Methanobacterium spp. and Methanobrevibacter spp. were detected in the 0-5 cm brackish cultivation series. Methanobacterium was the dominant methanogen genus at the enrichment stage, and Methanobrevibacter was the dominant genus at 5th subculture stage. There was also a switch in dominant genus between enrichment and 5th subculture stage in the marine enrichments amended with hydrogen. Methanobacterium was the dominant genus in the 0-5 and 25-30 cm marine cultivation series at enrichment stage, however Methanosarcina was the dominant genus at 5th subculture stage and Methanosarcina strains were obtained in monoculture or pure culture from these cultivation series. The 25-30 cm marine enrichments contained a relatively high number of different hydrogenotrophic genera. Methanobacterium spp. were still present at low relative abundance in both the 0-5 cm and 25-30 cm marine 5th subcultures. The hydrogenotrophic genera Methanobrevibacter and Methanofollis (family Methanomicrobiaceae, order Methanomicrobiales) were also present in the 25-30 cm marine 5th subculture.

6.3.2. Methanogen community structure dynamics in temperature gradient cultivation series

The relative abundances of methanogen genera detected at different stages of cultivation (slurry, enrichment, 5th subculture and methanogen monoculture/pure culture) are shown in Table 6.0.5. (lacustrine gyttja [0-5 cm] temperature gradient cultivation series) and Table 6.0.6. (estuarine clay [25-30 cm] temperature gradient cultivation series).

6.3.2.1. Methylamine amended temperature gradient cultivation series

Cultivation results suggest that the facultatively methylotrophic genus Methanosarcina was responsible for methylamine utilisation in the 0-5 cm temperature gradient cultivation series at temperatures between 4 and 46 °C. Methanosarcina was the dominant genus in the 0-5 cm 4 and 25°C 5th subcultures, with Methanoregula spp. (family Methanoregulaceae, order Methanomicrobiales) also present at low relative abundance at 4 °C. Hydrogenotrophic genera dominated the 10, 38, and 46 °C 0-5 cm 5th subcultures; Methanobacterium at 10 °C, and both Methanobacterium and Methanocella (family Methanocellaceae, order Methanocellales) at 38 °C, and Methanocella at 46 °C. The genus hydrogenotrophic Methanothermobacter (family Methanobacteriaceae, order Methanobacteriales) was also detected at low abundance.
in the 46°C 5th subculture. Members of the genera *Methanosarcina* and *Methanomethylovorans* were identified in the 10 and 38 °C 5th subcultures at low relative abundance, and a strain of *Methanosarcina* was obtained in monoculture from the 38 °C cultivation series. Similarly, methylotrophic methanogen genera were not detected in the 46 °C 5th subculture, yet a strain of *Methanosarcina* was obtained in methanogen monoculture.

*Methanomethylovorans* was the dominant methanogen genus present in 25-30 cm 5th subcultures at 4 and 10°C, yet at 25°C the genus *Methanococccoides* was dominant. The genus *Methanosarcina* was also present in these 5th subcultures, but only at low relative abundance. The hydrogenotrophic genera *Methanocellla* and *Methanobacterium* were the dominant genera in the 38 and 46 °C 5th subcultures respectively. The genus *Methanosarcina* was also present at low relative abundance in the 46 °C 5th subculture. The genus *Methanomethylovorans* was only detected at low abundance in the 38 °C 5th subculture, yet was obtained in monoculture in this cultivation series. The hydrogenotrophic *Methanothermобacter* (family Methanobacteriaeeae, order Methanobacteriales) was the only genus detected in the 0-5 cm methylamine amended cultures at 55 °C, and was the dominant genus in 25-30 cm methylamine amended cultures. A strain of *Methanothermобacter* was also obtained as a methanogen monoculture from the 55 °C 0-5 cm methylamine cultivation series.

6.3.2.2. Acetate amended temperature gradient cultivation series

The facultatively acetotrophic genus *Methanosarcina* was the dominant genus in both the 0-5 cm and 25-30 cm 4, 10 and 25 °C 5th subcultures; however a strain belonging to the acetotrophic genus *Methanothrix* (family Methanotrichaceae, order Methanosarcineae) was obtained in monoculture from the 25 °C cultivation series. Interestingly several hydrogenotrophic genera (*Methanoregula* and *Methanocorpusculum* [family Methanocorpusculaceae, order Methanomicrobiales]) were present at low relative abundance in these low temperature cultivation series. The genera *Methanoregula* and *Methanocorpusculum* were not detected in mesophilic or thermophilic enrichments.

Similar to the mesophilic and thermophilic methylamine cultivation series, hydrogenotrophic methanogen genera such as *Methanobacterium*, *Methanocellla*, and
Methanothermobacter were dominant in the 0-5 and 25-30 cm acetate amended 5\textsuperscript{th} subcultures at 38 and 46 °C. Methanosarcina spp. were only present at low relative abundance in the 38 °C 0-5 cm acetate 5\textsuperscript{th} subculture, yet a strain of Methanosarcina was obtained in monoculture from this cultivation series. There were no acetotrophic methanogens detected in the 38 °C 25-30 cm acetate 5\textsuperscript{th} subculture, yet a strain belonging to the genus Methanotrich was obtained in monoculture.

Methanotrich was the only genus present in the 55 °C 0-5 cm acetate 5\textsuperscript{th} subculture, and a strain of Methanotrich was obtained in monoculture from this cultivation series. Methane production in the 0-5 cm 55 °C acetate amended subcultures was too slow to permit 5 subcultures during the time available, thus there is no data with regards to methanogen community composition under these cultivation conditions. Hydrogenotrophic methanogen genera dominated the 0-5 cm 5\textsuperscript{th} subculture at 66 °C, and a strain belonging to the genus Methanothermobacter was obtained in pure culture from this cultivation series.

6.3.2.3. Hydrogen amended temperature gradient cultivation series

There was a defined temperature dependent succession in the relative abundance of different hydrogenotrophic genera in the 0-5 cm H\textsubscript{2} + CO\textsubscript{2} 5\textsuperscript{th} subcultures. Methanobacterium was the only genus detected in Cardiff Bay sediments (Figure 3.0.7., Chapter 3), and also in psychrophilic temperature gradient slurries and microcosms (Figures 5.0.3. – 5.1.0., Chapter 5). However, Methanobacterium spp. were only present at low relative abundance in the 4 and 10 °C hydrogen cultivation series at 5\textsuperscript{th} subculture. The genera Methanosarcina and Methanoregula were dominant in the 4 and 10 °C 0-5 cm 5\textsuperscript{th} subcultures respectively, and Methanocorpusculum was dominant in the 4 and 10 °C 25-30 cm 5\textsuperscript{th} subcultures.

The genus Methanobacterium was dominant in mesophilic (25, 38 and 46 °C) 0-5 cm cultivation series at enrichment stage, and Methanobacterium strains were obtained in monoculture from the 25 °C cultivation series and in pure culture from the 38 °C cultivation series. The genera Methanocella and Methanosarcina were also present at low relative abundance in the 38 °C cultivation series at 5\textsuperscript{th} subculture stage. Methanocella succeeded Methanobacterium as the dominant genus in the 46 °C
Table 6.0.5. Relative abundance of different methanogen genera detected at varying stages of cultivation in the lacustrine gyttja (0-5 cm) temperature gradient cultivation series

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<th>Mcl</th>
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N.B. Mb = Methanobacterium, Msc = Methanosarcina, Mcl = Methanocella, Mtb = Methanothermobacter, Mmv = Methanomethylovorans, Mt = Methanothrix, Mg = Methanogenium, Mcp = Methanocorpusculum, Mr = Methanoregula

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Table 6.0.5. continued. Relative abundance of different methanogen genera detected at varying stages of cultivation in the lacustrine gyttja (0-5 cm) temperature gradient cultivation series

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Key
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N.B. Mb = Methanobacterium, Msc = Methanosarcina, Mcl = Methanocella, Mt = Methanothermobacter, Mmv = Methanomethylovorans, Mt = Methanothrix, Mg = Methanogenium, Mcp = Methanocorpusculum, Mr = Methanoregula

* cultivation series inoculated with temperature gradient slurry from 59.4°C, subcultures incubated at 66 °C.
** cultivation series inoculated with temperature gradient slurry from 65.1 °C, subcultures incubated at 66 °C.
*** cultivation series inoculated with temperature gradient slurry from 65.1 °C, subcultures incubated at 77 °C.
Table 6.0.6. Relative abundance of different methanogen genera detected at varying stages of cultivation in the estuarine clay (25-30 cm) temperature gradient cultivation series

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<td>38</td>
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<td>Methanamine</td>
<td>5th subculture</td>
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<tr>
<td>55</td>
<td>Enrichment</td>
<td>+</td>
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<td>H₂ + CO₂</td>
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</tr>
</tbody>
</table>

Key: Genus relative abundance (%)

- +++ >60
- ++ >30
- + >3
- - <3

N.B. Mb = Methanobacterium, Msc = Methanosarcina, Mcc = Methanococcoides, Mcl = Methanocella, Mtb = Methanothermobacter, Mmv = Methanomethylovorans, Mt = Methanothrix, Mg = Methanogenium, Mcp = Methanocorpusculum, Mr = Methanoregula
cultivation series at 5\textsuperscript{th} subculture. The genera \textit{Methanocella} and \textit{Methanothermobacter} were both present at high relative abundance in the 55 °C cultivation series at 5\textsuperscript{th} subculture stage, \textit{Methanothermobacter} became the dominant genus at 5\textsuperscript{th} subculture stage in the 66 °C cultivation series (and was obtained in pure culture).

The temperature succession of hydrogenotrophic genera shown in the 0-5 cm cultivation series was also evident in the 25-30 cm temperature gradient cultures, but the temperature separation between the genera \textit{Methanocella} and \textit{Methanothermobacter} was not as defined. In addition, the genus \textit{Methanosarcina} (rather than \textit{Methanobacterium}) was the dominant genus in the 25 °C 0-5 cm cultivation series at 5\textsuperscript{th} subculture stage. The genus \textit{Methanobacterium} was dominant in the 38 °C cultivation series at 5\textsuperscript{th} subculture, the genera \textit{Methanocella} and \textit{Methanothermobacter} were present at high relative abundance in the 46 °C cultivation series 5\textsuperscript{th} subculture, and \textit{Methanothermobacter} was the dominant genus in the 55 °C cultivation series 5\textsuperscript{th} subculture.

6.4. Discussion

6.4.1. Methanogen diversity in Cardiff Bay sediments

Cultivation has proven to be an extremely powerful tool for investigating the microbial diversity of an environment. Molecular genetic analysis (PCR-DGGE using archaeal specific primers) of Cardiff Bay sediment methanogen community composition presented a picture of a methanogen community of extremely limited diversity and dominated heavily by \textit{Methanobacterium} phylotypes (Figure 3.0.7., Chapter 3). Subsequent cultivation experiments exploiting a wide range of temperatures and salinities have demonstrated that the Cardiff Bay sediments contain viable strains belonging 13 genera, representing 6 different families and 4 (out of a possible total of 7) methanogen orders. The Methanopyrales, Methanomasiliicoccales and Methanococcales were the only methanogen orders not enriched in salinity or temperature gradient cultivation series during this study. Members of the Methanopyrales were also not detected by pyrosequencing analysis of the archaeal community composition (Gordon Webster, unpublished data). All cultivated members of the Methanomasiliicoccales reduce methylamines or methanol using hydrogen for methane production (Lang et al. 2015). Cultivation series set up in this study were not
amended with both methanol and hydrogen, which may explain why Methanomasilliicoccales were not enriched despite their relatively high abundance in lacustrine gyttja sediments (section 3.4.5.). Neither the in situ sediment conditions nor the range of cultivation conditions used in this study were capable of supporting growth of Methanococcales or Methanopyrales. Members of the Methanococcales require minimum NaCl concentrations of 0.6 M for growth (Whitmam et al. 2006) and the maximum NaCl concentration of media used in this study was 0.55 M. The family Methanococcales has been detected in Cardiff Bay sediments by pyrosequencing analysis of the archaeological community composition (Gordon Webster, unpublished data), but presumably Methanococcales strains present in Cardiff Bay sediments are also not active under in situ conditions. Members of the Methanopyrales are hyperthermophiles with a minimum growth temperature of 84 °C (Whitmam et al. 2006), whereas the maximum incubation temperature used in this study was 77 °C. Furthermore, both orders are marine in origin (Whitmam et al. 2006).

A greater number of discrete genera were detected in temperature gradient enrichments and 5th subcultures than in salinity gradient enrichments and 5th subcultures, which was due to the large temperature range over which enrichments were incubated. All salinity gradient cultures were incubated at 25 °C, whereas temperature gradient enrichments were incubated over a temperature range of 4 to 77 °C. A number of genera that were not identified in salinity gradient cultures were detected in 4 and 10 °C temperature gradient cultures, including Methanocorpusculum, Methanoregula, Methanogenium (family Methanomicrobiaceae, order Methanomicrobiales), and Methanothrix. In addition, putatively thermophilic members of the genera Methanothermobacter, Methanocella, and Methanothrix were detected in temperature gradient enrichments incubated at 38 °C and above.

6.4.2. Methanogen monocultures and isolates obtained at mesophilic and thermophilic temperatures have defined temperature or salinity niches

Subculturing and dilution-to-extinction generated methanogen monocultures or pure cultures of methanogen genera with the targeted metabolism for the majority of depth/salinity or temperature/substrate combinations. Hydrogenotrophic methanogen genera were dominant at all stages of cultivation in cultivation series targeting hydrogen utilisers, however the relative abundance of different hydrogenotrophic
genera shifted away from the dominant sediment or enrichment genera and towards genera best adapted to the given temperature or salinity conditions. Hydrogenotrophic methanogenesis is the major pathway for methane production in Cardiff Bay sediments (Figure 3.0.6., Chapter 3), therefore, hydrogenotrophic methanogen genera likely constitute a large proportion of the sediment methanogen community, and hence are easily cultivated.

Rates of acetotrophic (and presumably also methylotrophic) methanogenesis were far lower than hydrogenotrophic methanogenesis in Cardiff Bay sediments (Figure 3.0.6., Chapter 3), and the size of the acetotrophic and methylotrophic sediment methanogen population was likely correspondingly smaller. Lower abundance of methylotrophic and acetotrophic methanogens in the starting sediment slurry inoculum may explain why the dominant methanogen genera in cultivation series amended with methylamine or acetate were putative hydrogenotrophs (typically *Methanobacterium* spp.) until the 5th subculture or monoculture/pure culture stage of cultivation in psychrophilic (4 and 10 °C °C) and lower mesophilic (25 °C) cultivation series (including salinity gradient cultivation series). PCR does not distinguish between living and dead cells. Therefore, if the relative increase in biomass of methylotrophic and acetotrophic methanogenesis as a proportion of total archaeal biomass was very small for each successive subculture, then perhaps the hydrogenotrophic genus *Methanobacterium* appeared to be dominant in cultures amended with methylamine and acetate even if not active. At higher temperatures (46 °C and above) the dominance of hydrogenotrophic methanogens in acetate amended cultivation series was more likely due to syntrophic acetate oxidizing consortia successfully competing with acetotrophic methanogens for substrate. It is not clear what process supplies hydrogen or formate to support hydrogenotrophic methanogenesis in methylamine amended cultivation series. Potential explanations are discussed further in section 6.5.5.

The rapidity with which acetotrophic or methylotrophic methanogen genera became dominant within the cultivation series amended with acetate and methylamine varied according to both salinity and sediment depth. In general, methylotrophic methanogens became established more rapidly in brackish or marine cultivation series than freshwater cultivation series, and more rapidly in the estuarine clay (25-30 cm) than lacustrine gyttja (0-5 cm) cultivation series. Acetotrophic methanogen genera demonstrated the opposite pattern. Acetotrophic methanogenesis is the main pathway
for methane production in freshwater environments (Whiticar et al. 1986), whilst the methylotrophic methanogenesis is a very minor pathway (Lovley and Klug 1983b; Conrad and Claus 2005). This contrasting pattern in the rapidity establishment of methylotrophic and acetotrophic methanogens in freshwater and marine cultivation series reflects the relative importance of the methylotrophic and acetotrophic methane production pathways in freshwater and marine environments.

The identity of the methanogen strains obtained in monoculture at mesophilic and thermophilic temperatures consistently changed according to the temperature, and the temperature at which each monoculture strain was obtained was similar to the temperature optima of the closest cultivated relative (Table 6.0.7). One of the major criticisms levelled at cultivation as a tool for investigating microbial ecology is that traditional cultivation techniques select for organisms with fast growth rates, high yields, and able to tolerate high nutrient concentrations (e.g. Großkopf et al. 1998). However, a number of the strains obtained in monoculture or pure culture in this study do not fit this description. In particular, the genera Methanocella and Methanothrix have been identified as difficult to cultivate using conventional cultivation methods. Methanocella spp. were outcompeted by Methanobacterium spp. in high H2 enrichments derived from environmental samples (Sakai et al. 2007; Sakai et al. 2009). Likewise, Methanosarcina spp. outcompeted Methanothrix spp. in enrichments amended with high acetate concentrations (Janssen 2003).

The salinity optima and range of the closest cultured relative of methanogen strains obtained in marine cultivation series was consistently higher than the salinity optima and range of the closest cultured relative of methanogen strains obtained in freshwater cultivation series (Tables 6.0.8 and 6.0.9), with the exception of the estuarine clay (25-30 cm) acetate cultivation series which generated strains closely related to Methanosarcina lacustris under both freshwater and marine conditions. The type strain species for Methanosarcina lacustris was isolated from freshwater lake sediment (Simankova et al. 2001), but unfortunately there is no published data with regards to the salinity range or optima of Methanosarcina lacustris strains. Methanosarcina lacustris is not known to use acetate as a growth substrate, although the ability to use acetate as a substrate may differ between strains of a Methanosarcina species (discussed further in section 6.4.6). In this study, strains closely related to Methanosarcina lacustris were cultivated on marine (25-30 cm acetate cultivation
series and 0-5 cm hydrogen cultivation series) and freshwater (25-30 cm acetate cultivation series), demonstrating that *Methanosarcina lacustris* strains native to Cardiff Bay sediments have a wide salinity range. Overall, this suggests that the cultivation process selected the strains present in Cardiff Bay sediments best adapted to the temperature or salinity of incubation, rather than strains most abundant in the enrichment inoculum or those with highest growth rate and yield.

Interestingly almost all methanogen strains obtained as methanogen monocultures or pure cultures from Cardiff Bay sediments were closely related to type strain species enriched from low salinity environments, such as freshwater lakes, anaerobic digesters, rice field sediments and peat bogs (Tables 6.0.7., 6.0.8., 6.0.9.). Even methanogen strains enriched on marine media (*Methanolobus profundi* and *Methanosarcina mazei*) had salinity optima (approx. 0.5 M) slightly below marine conditions (approx. 0.55 M NaCl). 16s rRNA gene sequences belonging to the genus *Methanofollis* were detected at 5th subculture stage in marine cultivation series only, yet again the NaCl tolerance range of most type strain species demonstrated a preference for low salinity environments, although *Methanofollis liminatans* and *Methanofollis tationis* are able to tolerate NaCl concentrations up to 0.4 M (Zellner et al. 1999).

It is surprising that methanogen strains with 16r rRNA gene sequences most similar to *Methanobacterium subterraneum* and *Methanolobus profundi* were present in Cardiff Bay sediments, as both of these species originate from deep biosphere habitats. In addition, *Methanobacterium subterraneum* is an alcaliphilic strain with pH optima (7.8-8.8, range 6.5-9.2) far higher than the pH of the growth media used during cultivation (7.2-7.4). However, phylotypes closely related to *Methanobacterium subterraneum* are often detected in anaerobic digester sludge (e.g. Leclerc et al. 2004; Hwang et al. 2008; Kovacik et al. 2010; Kobayashi et al. 2011), and have been identified in sediments of small, low order freshwater streams (Porat et al. 2010). *Methanolobus profundi* has been detected in activated sludge of a seawater-processing wastewater treatment plant (Sánchez et al. 2011), and also in in sediments of low order freshwater streams (Porat et al. 2010).

Low temperature cultivation series were not progressed to cultivation stage, and, therefore, it is not possible to identify whether methanogen strains present at this
temperature were competitive due to psychrophilic or psychrotolerant temperature characteristics. One of the Methanosarcina strains isolated at 25 °C is closely related to Methanosarcina lacustris (see Table 6.0.7.), and the type strain for Methanosarcina lacustris grows at temperatures as low as 1°C. In addition psychrotolerant strains of Methanosarcina mazei, Methanosarcina lacustris and Methanomethylovorans hollandica were isolated from cold terrestrial habitats by Simankova et al. (2003), and strains closely related to each of these species were obtained in monoculture or pure culture at 25 °C in this study. It is interesting to note that only four of the methanogen monoculture strains obtained in this study (Methanomethylovorans hollandica [Table 6.0.9.] Methanothrix concilii, Methanosarcina lacustris, and Methanobacterium subterraneum [see Table 6.0.7.]) belong to species which have type strains known to grow at the average summer water temperature (18.6 °C) in Cardiff Bay (Lee In prep.). All other strains obtained in monoculture or pure culture from Cardiff Bay sediments have a temperature minima >20 °C, although it is important to take into account that it is difficult to accurately determine the minimum temperature limit of methanogen strains due to exceptionally slow rate of growth at low temperatures.

6.4.3. Cultivation bias controls the dominant methanogen genera in low temperature cultivation series

The identity of dominant methanogen genera obtained in psychrophilic cultivation series amended with hydrogen was not directly influenced by the temperature adaptation. The average water temperature of Cardiff Bay waters ranges from 6.5 and 18 °C (average summer and winter temperature, Lee et al. In Prep.), and therefore it would be reasonable to assume that methanogen genera enriched in 4 and 10 °C cultivation series were closely representative of the active in situ methanogen population (Methanobacterium spp.). Sediment radiotracer and PCR-DGGE results indicated that methane production in Cardiff Bay sediments is predominantly hydrogenotrophic and mediated by the genus Methanobacterium, yet Methanobacterium strains were not enriched in low temperature cultivation series targeting hydrogenotrophic methanogens. However, Methanobacterium strains were enriched and obtained in monoculture or pure culture from higher temperature (25 and 38 °C) Cardiff Bay cultivation series, and have previously been isolated from freshwater lake sediments using similar cultivation techniques (Zeikus and Winfrey 1976; Kendall and Boone 2006; Borrel et al. 2011; Borrel et al. 2012a). One hypothesis
<table>
<thead>
<tr>
<th>Cultivation substrate/temperature</th>
<th>Closest cultured relative, accession number (similarity)</th>
<th>Temperature optimum (range) of type strain for closest cultured relative</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methylamine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Methanosarcina lacustris DSM 13486, AB973355.1 (100%)</td>
<td>Anoxic lake sediments, 25°C (1-35°C)(^a)</td>
</tr>
<tr>
<td></td>
<td>(i) Methanosarcina spelaei DSM 26047, LC006853.1 (99%)</td>
<td>(i) Floating biofilm in sulphurous lake waters, 33°C (0-54°C)(^b)</td>
</tr>
<tr>
<td></td>
<td>(ii) Methanosarcina sicilae T4M, NR104757.1 (99%)</td>
<td>(ii) Marine sediments, 40°C (25-45°C)(^c)</td>
</tr>
<tr>
<td></td>
<td>(iii) Methanosarcina vacuolata Z761, NR_104728.1 (99%)</td>
<td>(iii) Anaerobic digester, 37-40°C (18-42°C)(^d)</td>
</tr>
<tr>
<td></td>
<td>(iv) Methanosarcina barkeri DSM 800, AB973359.1 (99%)</td>
<td>(iv) Sheep rumen, 45°C (25-50°C)(^e)</td>
</tr>
<tr>
<td>38</td>
<td>Methanomethylovorans hollandica DSM 15978, NR102454.1</td>
<td>Eutrophic pond, 34-37°C (12-4b °C)(^f)</td>
</tr>
<tr>
<td>46</td>
<td>Methanosarcina barkeri DSM 800, AB973359.1 (99%)</td>
<td>Sewage sludge, 45°C (25-50°C)(^g)</td>
</tr>
<tr>
<td><strong>Acetate</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Methanothrix concilii GP6, NR102903.1 (96%)</td>
<td>Anaerobic digester, 35-40 (10-45°C)(^h)</td>
</tr>
<tr>
<td>38</td>
<td>Methanosarcina barkeri DSM 80, AB973360.1 (99%)</td>
<td>Sheep rumen, 45°C (25-50°C)(^i)</td>
</tr>
<tr>
<td></td>
<td>Methanothrix harundicea Bac, NR043203.1 (98%)</td>
<td>Anaerobic digester, 34-37°C (25-45°C)(^j)</td>
</tr>
<tr>
<td>46</td>
<td>Methanothrix thermophila PT, NR074214.1 (99%)</td>
<td>Anaerobic digester, 55-60°C (&gt;30&lt;70°C)(^k)</td>
</tr>
<tr>
<td><strong>Hydrogen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Methanobacterium subterraneum A8p, NR028247.1 (98%)</td>
<td>Deep granitic groundwater, 20-40°C (3.6-45°C)(^l)</td>
</tr>
<tr>
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<td>Methanobacterium formicum FCam*, AF028689.1 (98%)</td>
<td>Rice field soils, 37-45°C (range ND)(^m)</td>
</tr>
<tr>
<td></td>
<td>Methanobacterium palustre Z2*, DQ649332.1 (98%)</td>
<td>Anaerobic digester, 30-45°C, (range ND)(^n)</td>
</tr>
<tr>
<td>38</td>
<td>Methanobacterium bryantii M.O.H, NR042781.1 (99%),</td>
<td>Anaerobic digester, 37-39°C (range ND)(^o)</td>
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<tr>
<td></td>
<td>Methanobacterium vetereum MK4, NR115935.1 (99%)</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Methanobacterium formicicum MF, NR115168.1 (99%)</td>
<td>Unknown, 37-45°C (range ND)(^p)</td>
</tr>
<tr>
<td>55</td>
<td>Methanothrix concilii** HZ2254 (100%)</td>
<td>Rice field soil, 55°C (37-60°C)(^q)</td>
</tr>
<tr>
<td></td>
<td>Methanothrix concilii** HZ2254 (100%)</td>
<td>Rice field soil, 55°C (37-60°C)(^r)</td>
</tr>
<tr>
<td>66</td>
<td>Methanothermobacter thermoautotrophicus Delta H, NR074260.1 (100%)</td>
<td>Sewage sludge, 65-70 (40-75°C)(^s)</td>
</tr>
</tbody>
</table>

* This strain is not the type strain for the species, but was more closely related to the strain of interest than the type strain of the species. ** The 46 and 55 °C hydrogen 5th subculture were not progressed to methanogen monoculture/pure culture stage, however Methanocella was the dominant genus in the 46 and 55 °C hydrogen 5th subcultures and Methanocella sequences obtained from PCR-DGGE analysis of the 46 and 55 °C hydrogen 5th subculture showed <95 % similarity to any other methanogen species.

\(^a\)Simankova et al. (2001), \(^b\)Ganzert et al. (2014), \(^c\)Ni et al. (2014), \(^d\)Zhilina and Zarvazin (1987), \(^e\)Bryant and Boone (1987b), \(^f\)Lomans et al. (1999), \(^g\)Patel and Spott (1990), \(^h\)Ma et al. (2006), \(^i\)Kamagata et al. (1992), \(^j\)Kotelnikova et al. (1998), \(^k\)Bryant and Boone (1987a), \(^l\)Zellner et al. (1998), \(^m\)Krivushin et al. (2010), \(^n\)Lu and Lu (2012), \(^o\)Zeikus and Wolfe (1972)
for the absence of *Methanobacterium* spp. in low temperature cultivation series targeting hydrogenotrophic methanogens is that either the slurrying process used during preparation of slurries, or high substrate concentration in enrichments and subcultures amended with a H$_2$/CO$_2$ headspace indirectly discriminates against *Methanobacterium* spp. by promoting growth of acetogenic bacteria hence reducing substrate availability for hydrogenotrophic methanogens. Acetate is the major precursor for methane production in sediments of many freshwater lakes (Kuivila et al. 1989; Kotsyurbenko et al. 1993; Thebrath et al. 1993; Schulz and Conrad 1996; Nozhevnikova et al. 1997; Schulz et al. 1997; Falz et al. 1999; Nüsslein et al. 2001; Nozhevnikova et al. 2003; Glissmann et al. 2004; Nozhevnikova et al. 2007). Kotsyurbenko (2005) suggests that homoacetogens are able to outcompete methanogens in cold environments if one or more of the following applies; (i) the hydrogenotrophic methanogen populations are not psychroactive, (ii) hydrogen concentrations are high (homoacetogens are competitive with psychroactive hydrogenotrophic methanogens at hydrogen concentrations >0.1 kPa, and dominant at >1 kPa). Under hydrogen-limited conditions hydrogenotrophic methanogens outcompete acetogenic bacteria for substrate due to a higher affinity for hydrogen. However acetogens can outcompete hydrogenotrophic methanogens at temperatures <15 °C when hydrogen availability is non-limiting by virtue of their higher growth rate $V_{\text{max}}$ (Kotsyurbenko et al. 2001).

If psychoactive hydrogenotrophic methanogen populations are present and hydrogen concentrations are low (as typical in natural environments) then hydrogen utilization proceeds directly via methanogenesis. Psychoactive hydrogenotrophic methanogen populations are presumably present in Cardiff Bay sediments, however hydrogen concentrations were significantly higher in enrichments and subcultures than in Cardiff Bay sediment porewaters (~0-5 μM/L wet sediment). Significant acetate production occurred at <20 °C in unamended temperature gradient slurries (Chapter 5), indicating that amendment with high substrate levels may not be the sole factor in promoting acetogenesis over methanogenesis. The culture media composition will also had a selective effect on the methanogen types which developed in salinity and temperature gradient cultivation series, and may perhaps have disadvantaged psychoactive hydrogenotrophic *Methanobacterium* strains which compete successfully with acetogens under in situ sediment conditions.
Table 6.0.8. Identity and temperature characteristics of closest cultured relative for methanogen monocultures and pure cultures obtained from lacustrine gyttja (0-5 cm) salinity gradient cultivation series

<table>
<thead>
<tr>
<th>Cultivation substrate/salinity</th>
<th>Closest cultured relative, accession number (similarity)</th>
<th>Salinity optimum (range) of species type strain for closest cultured relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine Methylamine</td>
<td>Methanobrevibacter aboriphilus DM-1, NR_042783.1 (99%)</td>
<td>Decaying plant matter, 0-0.1M (0-0.6)</td>
</tr>
<tr>
<td>Freshwater Methylamine</td>
<td>Methanobrevibacter aboriphilus DM-1, NR_042783.1 (99%)</td>
<td>Decaying plant matter, 0-0.1M (0-0.6)</td>
</tr>
<tr>
<td>Marine Acetate</td>
<td>Methanosarcina mazei DSM 2053, AB97338.1 (99%)</td>
<td>Anaerobic digester, 0.1-0.3M (0.1-1M)</td>
</tr>
<tr>
<td>Freshwater Acetate</td>
<td>Methanosarcina spelaei DSM 26047, LC006853.1 (99%)</td>
<td>Floating biofilm in sulphurous lake waters, 0.05M (&lt;0.02-&gt;0.6)</td>
</tr>
<tr>
<td>Marine Hydrogen</td>
<td>Methanosarcina lacustris DSM 13486, AB973355.1 (99%)</td>
<td>Anoxic lake sediments, ND</td>
</tr>
<tr>
<td>Freshwater Hydrogen</td>
<td>Methanobrevibacter aboriphilus DM-1, NR_042783.1 (99%)</td>
<td>Decaying plant matter, 0-0.1M (0-0.6)</td>
</tr>
</tbody>
</table>

*Mochimaru et al. (2009), Zeikus and Henning (1975), Mah et al. (1980), Ganzert et al. (2014), Simankova et al. (2001),

Table 6.0.9. Identity and salinity characteristics of closest cultured relative for methanogen monocultures and pure cultures obtained from estuarine clay (25-30 cm) salinity gradient cultivation series

<table>
<thead>
<tr>
<th>Cultivation substrate/salinity</th>
<th>Closest cultured relative, accession number (similarity)</th>
<th>Salinity optimum (range) of species type strain for closest cultured relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine Methylamine</td>
<td>Methanobrevibacter aboriphilus DM-1, NR_042783.1 (99%)</td>
<td>Decaying plant matter, 0-0.1M (0-0.6)</td>
</tr>
<tr>
<td>Freshwater Methylamine</td>
<td>Methanomethylovorans hollandica DSM 15978, NR_102454.1 (99%)</td>
<td>Freshwater lake sediments, 0-0.04M (0-0.3M)</td>
</tr>
<tr>
<td>Marine Acetate</td>
<td>Methanosarcina mazei DSM 2053, AB97338.1 (99%)</td>
<td>Anaerobic digester, 0.1-0.3M (0.1-1M)</td>
</tr>
<tr>
<td>Freshwater Acetate</td>
<td>Methanosarcina spelaei DSM 26047, LC006853.1 (99%)</td>
<td>Floating biofilm in sulphurous lake waters, 0.05M (&lt;0.02-&gt;0.6)</td>
</tr>
<tr>
<td>Marine Hydrogen</td>
<td>Methanosarcina lacustris DSM 13486, AB973355.1 (99%)</td>
<td>Anoxic lake sediments, ND</td>
</tr>
<tr>
<td>Freshwater Hydrogen</td>
<td>Methanobrevibacter aboriphilus DM-1, NR_042783.1 (99%)</td>
<td>Decaying plant matter, 0-0.1M (0-0.6)</td>
</tr>
</tbody>
</table>

*Mochimaru et al. (2009), Lomans et al. (1999), Simankova et al. (2001), Mah et al. (1980), Wagner et al. (2013), Ni et al. (2014), Zellner et al. (1988)*
The media acetate concentration of 5th subcultures was not measured. However, significant acetate production occurred at temperatures <25 °C in 0-5 cm and 25-30 cm temperature gradient slurries and microcosms, and the 4 °C 25-30 cm hydrogen 5th subculture and 10°C 0-5 cm hydrogen 5th subculture contained 16s rRNA gene sequences affiliated with the acetogenic bacterial genus *Acetobacterium* (data not shown). The presence of *Acetobacterium* in the 0-5 cm 4°C hydrogen 5th subculture in conjunction with the facultatively acetotrophic genus *Methanosarcina*, suggests that methane production proceeded via acetate formation and acetotrophic methanogenesis under these cultivation conditions. It is surprising that acetotrophic methanogens were not detected in the 10 °C 0-5 cm hydrogen 5th subculture given that *Acetobacterium* spp. were present. Perhaps acetate concentrations in the 10 °C 0-5 cm hydrogen 5th subculture were sufficiently high to inhibit acetotrophic methanogenesis. Nozhevnikova et al. (2007) found that psychrophilic methanogenesis was inhibited for a period of up to 3 months in sediment slurries from Lake Baldegg, Switzerland amended with 20 mM acetate. Although psychrophilic acetotrophic methanogen populations eventually adapted to the high acetate concentrations. Inhibition of methanogenesis by high substrate concentrations has also be noted for a pure culture of *Methanosarcina* (Westermann et al. 1989) and in a psychrophilic wastewater treatment plant (Rebac et al. 1995)

Interestingly the hydrogenotrophic methanogens dominant in the 10 °C 0-5 cm hydrogen 5th subculture and 4 and 10 °C 25-30 cm 5th subculture was dominated by genera belonging to the order Methanomicrobiales. The Methanomicrobiales (and Methanosarcinales) are frequently detected in freshwater lake sediments (Borrel et al. 2011), but were not detected in the Cardiff Bay sediments used to inoculate these cultivation series using PCR-DGGE (Figure 3.0.7., Chapter 3). *Methanoregula* (dominant in the 0-5 cm 10 °C hydrogen 5th subculture) is the most commonly detected, and often most abundant, methanogen genus in freshwater lake sediments (Borrel et al. 2011). *Methanoregula* spp. are also prevalent in temperate oligotrophic fen and peatland soils (Sun et al. 2012). *Methanoregula* type strains have an extremely narrow salinity range. The salinity maxima of *Methanoregula boonei* and *Methanoregula formicica* are 50 and 171 mM NaCl respectively (Bräuer et al. 2011; Yashiro et al. 2011), which may explain the absence of *Methanoregula* spp. in 25-30 cm low temperature cultivation series. The current porewater salinity in Cardiff Bay sediments
at 25-30 cm depth (estuarine clay lithology; Figure 3.0.5., Chapter 3) is ~0.04 M Cl⁻, which is within the salinity range of *Methanoregula* type strains. However, assuming the porewater salinity of the estuarine intertidal mudflats present in Cardiff Bay prior to impoundment (now represented by clay lithology in sediment cores at >20 cm depth) was similar to the current salinity of intertidal mudflats located immediately adjacent to the seawards side of Cardiff Bay Barrage (23.7 ‰; Thomas 2014) then *Methanoregula* spp. would probably not have been active in these sediments. It is also of interest that the genus *Methanoregula* is believed to be adapted to low substrate environments (Borrel et al. 2011; Sun et al. 2012). The first isolated strain of *Methanoregula* (*Methanoregula boonei*), was isolated on low substrate, nutrient poor media (Bräuer et al. 2011), and *Methanoregula* strains are often cultivated in co-culture with H₂ syntrophs (Sakai et al. 2009; Yashiro et al. 2011). In addition, *Methanoregula* strains have low growth rates compared to hydrogenotrophic methanogen genera common in high substrate environments such as *Methanobacterium* (Bräuer et al. 2011). Perhaps adaptation to low substrate environments enables *Methanoregula* spp. to thrive alongside acetogens in the low temperature cultivation series amended with H₂/CO₂.

The genus *Methanocorpusculum* (dominant in the 4 and 10 °C 25-30 cm hydrogen 5th subcultures, Table 6.0.6.) are commonly detected in high substrate environments. In contrast to the genus *Methanoregula, Methanocorpusculum* are important members of the methanogen community in high substrate environments such as waste treatment plants (McKeown et al. 2009). Most *Methanocorpusculum* type strains have been isolated from anaerobic digester sludges (Zellner et al. 1987; Xun et al. 1989; Zellner et al. 1989). Also, unlike the genus *Methanoregula, Methanocorpusculum* spp. were detected in both the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) cultivation series, and *Methanocorpusculum* type strains are able to grow under salinities ranging from freshwater to marine (Zellner et al. 1987; Zellner et al. 1989; Zhao et al. 1989). However the environmental distribution of the genus *Methanocorpusculum* indicates a preference for low salinity environments. For example, Waldron et al. (2007) recorded a significant decrease in the abundance of *Methanocorpusculum* across a salinity gradient from 0.7 to 26.6 g/L NaCl in well formation waters of organic rich shales in the Michigan Basin, and Purdy et al. (2002) identified *Methanocorpusculum* in the freshwater and brackish sediments of the Colne Estuary, UK but not in the marine sediments. In addition, *Methanocorpusculum* have more complex nutritional
requirements than other genera within the Methanomicrobiales, requiring a combination of peptone, yeast extract or rumen fluid for cultivation (Liu 2010d), which is not indicative of organisms adapted to oligotrophic environments. However *Methanocorpusculum* spp. have also been identified in environments characterised by recalcitrant organic matter such as well production waters of coal and shale beds (Waldron et al. 2007; Strapoc et al. 2008). The type strain for *Methanocorpusculum labreanum* was isolated from freshwater sediments (Zhao et al. 1989), but they are not common constituent of methanogen communities in freshwater lake sediments (Borrel et al. 2011). The genera *Methanobacterium* and *Methanocorpusculum* both contain psychrotolerant (but not psychrophilic) species originally isolated from temperate environments, although Simankova et al. (2003) isolated a psychrotolerant strain of *Methanocorpusculum* (thought to be a new ecotype of existing species) from cold sediments of a freshwater lake in Russia. Presumably a combination of temperature characteristics and substrate adaptation of indigenous sediment strains favours growth of *Methanocorpusculum* spp. over *Methanobacterium* spp. in the 4 and 10 °C 25-30 cm cultivation series (Table 6.0.6.).

6.4.4. Role of syntrophic methanogenic consortia in acetate and methylamine oxidation at mesophilic and thermophilic temperatures (38-66°C)

Interestingly, the freshwater 0-5 cm methylamine cultivation series (*Methanobrevibacter*), 55 °C 0-5 cm methylamine cultivation series (*Methanothermobacter*), and 66 °C 0-5 cm acetate cultivation series (*Methanothermobacter*) ultimately generated monocultures of obligately hydrogenotrophic methanogen genera. In addition, only hydrogenotrophic methanogen genera were detected in the 5th subculture of the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) 46 °C methylamine and acetate cultivation series (*Methanobacterium, Methanocella* and *Methanothermobacter*). Hydrogenotrophic methanogens were also dominant at 5th subculture in the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) 38 °C acetate and methylamine cultivation series (*Methanocella*), although methylotrophic and acetotrophic methanogen genera were eventually obtained in monoculture or pure culture at 38 °C.

In cultivation series where methylotrophic or acetotrophic methanogen genera were obtained in monoculture or pure culture (38, 46, and 55 °C methylamine and acetate
cultivation series) then it seems likely that hydrogenotrophic methanogen genera were able to outcompete methylotrophic or acetotrophic methanogen genera until at least the 5th subculture stage. Under some cultivation conditions, including the 66 °C lacustrine gyttja (0-5 cm) acetate cultivation series, methanogen genera with the targeted metabolism were not detected at any stage of cultivation. Therefore it seems likely that there are no methylamine or acetate utilising methanogen strains able to survive cultivation conditions present in Cardiff Bay sediments. The genus *Methanosarcina* was detected at 46 °C and 55 °C in the mixed substrate estuarine clay (25-30 cm) enrichments, and temperature-substrate utilization patterns appeared very similar between estuarine clay (25-30 cm) and lacustrine gyttja (0-5 cm) amended temperature gradient slurries (Figures 5.0.3. and 5.0.4, Chapter 5.), leading to the assumption that *Methanosarcina* was responsible for methylamine and acetate utilisation in both 0-5 cm and 25-30 cm temperature gradient enrichments (discussed in sections 5.4.6. and 5.4.7.). Most species of *Methanosarcina* are able to utilise hydrogen, and *Methanosarcina* spp. may be able to compete successfully with *Methanobacterium* spp. and *Methanothermobacter* spp. in the range between the typical temperature optima of *Methanobacterium* species (~38 °C) and *Methanothermobacter* species (55-60 °C) species (Liu 2010b). However in cultivation series such as the freshwater lacustrine gyttja (0-5 cm) methylamine cultivation series, where the methylotrophic genus *Methanomethylovorans* was present at both enrichment and 5th subculture stage (Table 6.0.3.), it appears that *Methanobrevibacter* spp. were able to outcompete methanogen genera directly able to utilise methylamine.

The hydrogenotrophic methanogen genera dominant in the 38, 46 and 66 °C acetate 5th subcultures were likely participating in syntrophic acetate oxidation. Syntrophic acetate oxidation has previously been detected in temperate anoxic environments (Nüsslein et al. 2001; Chauhan and Ogram 2006; Conrad et al. 2010), albeit rarely. Several studies have demonstrated that syntrophic acetate oxidation can be induced in rice paddy field soils incubated at thermophilic temperatures (Conrad et al. 2009; Noll et al. 2010; Liu and Conrad 2011; Rui et al. 2011). The genera *Methanobacterium*, *Methanocella* and *Methanothermobacter* have been implicated in syntrophic acetate oxidation in various different methanogenic environments (Zinder and Koch 1984; Sekiguchi et al. 1998; McHugh et al. 2003; Pham et al. 2009; Hao et al. 2011; Liu and Conrad 2011; Mayumi et al. 2011; Rui et al. 2011). Oxidation of acetate to hydrogen
and carbon dioxide becomes more thermodynamically favourable with increasing temperature (Hattori et al. 2008), and syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis is the major pathway for methane production in high temperature petroleum fields (Mayumi et al. 2011; Mayumi et al. 2013) and thermophilic anaerobic bioreactors (Hattori 2008). The sediment content of 5th subcultures is negligible (2.5 µL for thermal gradient cultures, 0.3 µL for salinity gradient cultures), meaning bacterial degradation of sediment organic matter is not a significant source of substrates for hydrogenotrophic methanogens in 5th subcultures amended with acetate. Furthermore, the presence of hydrogenotrophic genera which were not detected in sediment samples using PCR-DGGE (ie Methanocella and Methanothermobacter demonstrates that the cultivation conditions favoured an active increase in the population size of hydrogenotrophic methanogens.

Syntrophic acetate oxidizing consortia are less competitive for acetate than acetotrophic methanogens (Hattori et al. 2008). Methane production occurred primarily via syntrophic acetate oxidation in the profundal sediments of Lake Kinneret in the absence of acetotrophic methanogens (Nüsslein et al. 2001), but switched to acetotrophic methanogenesis when acetotrophic methanogens were present (Schwarz et al. 2007). Therefore it is surprising that there is a decrease in the relative abundance of facultatively acetotrophic genus Methanosarcina between enrichment and 5th subculture stage in mesophilic estuarine clay (25-30 cm) acetate cultivation series, and that acetotrophic methanogens were present in 38 °C 5th subcultures at lower abundance than the hydrogenotrophic methanogen genera potentially involved in syntrophic reactions. Most species of Methanosarcina have the capability either to perform acetate oxidation directly or act as hydrogen scavengers in partnership with syntrophic acetate oxidizing bacteria (Karakašhev et al. 2006). Methanosarcina spp. in the 38 °C 0-5 cm acetate 5th subculture could have been utilising either pathway (although it is not possible to determine which from the data available. Methanothrix (obtained in monoculture from the 38 °C 25-30 cm acetate cultivation series) are obligate acetotrophs (Liu 2010c), yet were still outcompeted by hydrogenotrophic methanogens at 5th subculture stage.

Studies of methane production in anaerobic bioreactors show that syntrophic acetate oxidation occurs when the activity of acetotrophic methanogens is suppressed by unfavourable environmental factors such as high ammonia concentrations (Schnürer
et al. 1999; Karakashev et al. 2006; Schnürer and Nordberg 2008), low (<0.5 mM, Petersen and Ahring 1991) or high acetate concentrations (>50 mM, Hao et al. 2011; Hao et al. 2013) or high pH conditions (>7.5, Hao et al. 2013). Conversely, high hydrogen concentrations (Hattori et al. 2008) and high CO₂ concentrations (Mayumi et al. 2013; Kato et al 2014) favour growth of acetotrophic methanogens over syntrophic acetate oxidizing consortia. The culture media ammonia, hydrogen, and carbon dioxide concentrations were not measured in salinity and temperature gradient cultivation series cultures. However, cultures were incubated with a N₂/CO₂ (80:20, 0.5 kPa) headspace and in bicarbonate buffered media, hence media carbon dioxide concentrations would have been relatively high. Culture media acetate concentration (max. 20 mM) and pH (7.2-7.4) were below the level at which acetotrophic methanogenesis is thought to be inhibited. Batch cultures represent a closed environment and growth conditions (acetate and carbon dioxide concentrations, pH) will probably have varied throughout the duration of incubation as a result of metabolic processes occurring within. For this reason the relative contribution of syntrophic acetate oxidation and acetotrophic methanogenesis also may differ throughout the period of incubation of a culture, explaining the presence of both acetotrophic and hydrogenotrophic methanogens in 5th subcultures. Another explanation is that perhaps build-up of ammonium under closed conditions in temperature gradient slurries promoted the growth of syntrophic acetate oxidizing consortia at this stage of cultivation, and syntrophic acetate oxidizing consortia may have continued to dominate through successive subcultures due to numerical dominance. Although cultivation conditions appear to favour the syntrophic acetate oxidation pathway, the process of dilution-to-extinction favours acetotrophic methanogens as it disrupts syntrophic relationships which rely on close proximity of syntrophic partners (e.g. DIET; Summers et al. 2010). Hence acetotrophic methanogens were obtained in monoculture from 38 °C acetate cultivation series despite having low relative abundance at the 5th subculture stage.

6.4.5. Methylamine oxidation at mesophilic and thermophilic temperatures

Hydrogenotrophic methanogen genera in methylamine 5th subcultures may potentially have survived through scavenging hydrogen produced by methylotrophic methanogenesis. Finke et al. (2007a) suggested that interspecies hydrogen transfer between methylotrophic and hydrogenotrophic methanogens occurs in sulphate
depleted methanogenic enrichments. Methanogenesis from methylated compounds generates methane via a reductive pathway, and carbon dioxide via an oxidative pathway. Electrons from the oxidation of methyl groups of methylated compounds are transferred from the oxidative pathway to the reductive pathway to form methane. When extracellular hydrogen concentrations are maintained at low levels, for example by SRB (Finke et al. 2007a), these electrons are transferred to protons forming hydrogen rather than methane (Phelps et al. 1985).

However, there are several problems with the hypothesis above. Firstly, this process gives an increase in free energy yield of 15 kJ/mol under sulphate-reducing conditions, but when extracellular hydrogen concentrations are determined by hydrogenotrophic methanogens, then this pathway represents a loss in reducing power (unless the same methanogen utilises both methylamine and hydrogen) (Finke et al. 2007a). Secondly it cannot explain how hydrogenotrophic methanogen genera were obtained in monoculture from 0-5 cm sediments from enrichments amended with methylamine under freshwater conditions (Methanobrevibacter) and under brackish conditions at 55 °C (Methanothermobacter) with no methylotrophic methanogens present. Also the methylotrophic genera Methanobacterium, Methanomethylovorans and Methanosarcina were detected using PCR-DGGE in many other cultures, which indicates that their absence from certain cultures was not the result of methodological (e.g. PCR) limitations.

A second hypothesis is that consortia of bacteria and methanogens mediate methane production in these cultures, potentially via a mutualistic or even syntrophic partnership with methylamine oxidizing bacteria. This hypothesis is supported by the fact that hydrogenotrophic methanogen genera were obtained in monoculture in the 0-5 cm freshwater methylamine cultivation series and the 0-5 cm 55 °C methylamine cultivation series. Syntrophic acetate and formate oxidation involving a consortia of fermentative bacteria and hydrogenotrophic methanogens have been described in a number of different environments (Dolfing et al. 2008), although apparently operating at the limits of thermodynamic feasibility (Schink 2006). Under standard thermodynamic conditions methylamine disproportionation to methane is more energetically favourable than acetate fermentation to methane or hydrogenotrophic methanogenesis utilising formate as an electron donor, however syntrophic methylamine oxidation has not yet been described. Methylotrophic bacteria able to
degrade methylated amines under oxic conditions are phylogenetically diverse and ubiquitous in the environment (Chistoserdova et al. 2009; Chistoserdova 2011b), including sediments of freshwater lakes (e.g. Miller et al. 2005; Nercessian et al. 2005; Chistoserdova 2011a). Kim et al. (2001) and Kim et al. (2003) isolated strains of denitrifying bacteria able to degrade trimethylamine under anoxic conditions using nitrate as an electron acceptor, and more recently Yang et al. (2013) found that mixed bacterial cultures obtained from activated sludge and rice paddy soil could effectively reduce methylamine concentrations in industrial wastewaters. However, apart from direct methanogen degradation of methylamines (King 1984), very little is known about prevalence and pathways for anaerobic oxidation of methylated compounds in the natural environment, or identity of organisms responsible.

6.4.6. Consistency of methanogen population structure in cultivation series incubated under identical incubation conditions.

In common with other cultivation based studies (e.g. Kopke et al. 2005) there was a lack of consistency between cultivation series. In theory, both the lacustrine gyttja (0-5 cm) 25 °C methylamine, acetate and hydrogen temperature gradient cultivation series and the lacustrine gyttja (0-5 cm) brackish methylamine, acetate and hydrogen salinity gradient cultivation series should produce identical results as their conditions of cultivation (incubation temperature of 25 °C, brackish media composition, and substrate addition) were exactly same, however this was not the case. Two of the genera which were dominant in salinity gradient cultivation series, *Methanobrevibacter* (dominant genus in the salinity gradient brackish 0-5 cm 5th subcultures for all substrates) and *Methanolobus* (dominant genus in salinity gradient brackish 25-30 cm methylamine 5th subculture), were not detected in at any stage in the temperature gradient cultivation series. The dominance of *Methanobrevibacter* spp. in salinity gradient cultivation series (rather than *Methanobacterium* spp. as in temperature gradient cultivation series) might be linked to differences in the substrate with which the initial salinity and temperature gradient enrichments were amended. The salinity gradient cultivation series targeting hydrogenotrophic methanogens were amended with formate rather than hydrogen at enrichment stage. Only approximately half of *Methanobacterium* and *Methanobrevibacter* type strains are able to utilise formate (Liu 2010b). If *Methanobacterium* strains present in Cardiff Bay sediments are not able to utilise formate, whilst *Methanobrevibacter* strains are, then that could have led to
preferential enrichment of *Methanobrevibacter* spp. in salinity gradient cultivation series.

*Methanobacterium* spp. and *Methanosarcina* spp. were both frequently detected in temperature and salinity gradient cultivation series, however the genus *Methanobacterium* was the dominant in the 25 °C estuarine clay (25-30 cm) 5th subculture amended with hydrogen (and was obtained in monoculture) whilst *Methanosarcina* was the dominant genus in the brackish estuarine clay (25-30 cm) 5th subculture amended with hydrogen. Temperature gradient enrichments were prepared using mixed substrates (methylamine, acetate and hydrogen) whilst salinity gradient enrichments were prepared using single substrates. The use of mixed substrates in temperature gradient enrichments may perhaps have given *Methanosarcina* spp. a competitive advantage over other methanogen genera due to their unique capability to utilise all 3 substrates. The sediment cores used in temperature and salinity gradient experiments were taken at different time points (June and January respectively) and, therefore, differences in the methanogen population composition of the starting inoculum may also have contributed to differing results in salinity and temperature gradient cultivation series.

Results of this study contrast to a previous cultivation based study of bacterial diversity in tidal flat sediments (Kopke et al. 2005) in that several strains were obtained in monoculture from more than one cultivation series and on a range of substrates. Strains closely related to *Methanosarcina lacustris* were obtained on acetate under both marine and freshwater conditions from 25-30 cm sediments, on hydrogen under freshwater conditions at 25 °C, and on methylamine under brackish conditions at 25°C from 0-5 cm sediments. The type strain for *Methanosarcina lacustris* does not use acetate (Simankova et al. 2001), however it is not unusual for the substrate characteristics of *Methanosarcina* species to vary at strain level. For example, Maestrojuan and Boone (1991) and Harris (1987) found that the *Methanosarcina mazei* type strain S-6 grew on H\(_2\)/CO\(_2\), whereas Mah (1980) and Mah and Kuhn (1984) found that it did not. *Methanosarcina mazei* strain MC3 also does not grow on H\(_2\)/CO\(_2\) (Touzel and Albagnac 1983).

It is noteworthy that methanogen strains closely related to the species *Methanosarcina mazei* were always obtained in monoculture from marine cultivation series, but were
obtained on hydrogen from lacustrine gyttja (0-5 cm) sediments and on acetate from estuarine clay (25-30 cm) sediments. Also, strains closely related to *Methanosarcina spelaei* were obtained from lacustrine gyttja (0-5 cm) sediments on acetate under freshwater conditions, but on methylamine under brackish conditions at 38 °C. As all 3 *Methanosarcina* strains were detected in both lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) cultivation series, it is not clear why the cultivation conditions under which they were obtained in monoculture differs (assuming the salinity characteristics does not differ between strains of the same species). The presence of other methanogens and bacterial syntrophs in cultivation series, which may well differ according to salinity, sediment depth within core, and between sediment cores taken at differing sampling times, will also impact on the dominant methanogen species in a cultivation series. Further work would be needed to ascertain whether the interplay of substrate and salinity alters the competitive interaction between these species to produce the observed pattern of results, or whether it is a demonstration of the difficulty in obtaining consistent results in cultivation based experiment.

6.4.7. Comparison between lacustrine gyttja and estuarine clay cultivation series

The methanogen population structure in mesophilic and thermophilic lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) cultivation series were very similar at enrichment stage and 5th subculture, therefore the decision was made not to progress the estuarine clay (25-30 cm) cultivation series further except for at one temperature point (38°C) for comparison. However, the strain obtained as a monoculture or pure culture differed at genus level for the methylamine and acetate 38 °C cultivation series (Table 6.0.9.), and species level for the hydrogen 38 °C cultivation series. Again, it is uncertain whether this disparity reflects differences in the composition or competitive interactions between methanogen populations native to the lacustrine gyttja (0-5 cm) and estuarine clay (25-30 cm) sediment layers, or if differences during the cultivation process (i.e. first enrichment amended with mixed substrates or single substrates, enrichments targeting hydrogenotrophic methanogens amended with H₂/CO₂ or formate) led to one genus being obtained in monoculture over the other. The diversity of methanogen genera in temperature and salinity gradient cultivation series is higher in cultures inoculated with lacustrine gyttja (0-5 cm) sediments than estuarine clay (25-30 cm) sediments, as *Methanobrevibacter*, *Methanocorpusculum*, and *Methanogenium* were only detected in 0-5 cm sediment cultures. Conversely, the
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genus *Methanococcoides*, which is widely considered to inhabit only marine environments (*Watkins* 2012) was enriched from the estuarine clay (25-30 cm) sediments only (Table 5.0.6.). It is not surprising that *Methanococcoides* was enriched from the estuarine sediments given their marine origin.

6.5. Conclusions

The phylogenetic diversity of organisms present in the mid and late stages of Cardiff Bay cultivation series (5th subculture and monocultures or pure culture) far exceeded the diversity of organisms present in the original sediments (Figure 3.0.7., Chapter 3), or initial temperature (Figures 5.0.3. and 5.0.4., Chapter 5) and salinity gradient (Tables 4.0.4. – 4.0.6., Chapter 4) enrichments. This finding reinforces the importance of cultivation as a tool for exploring the diversity of environmental prokaryotic communities. Tracing methanogen population dynamics throughout the isolation process has provided important information about the relevance of the monocultures and pure cultures generated in shaping the community temperature or salinity response of the Cardiff Bay methanogen communities. This included competitive interactions between acetate-oxidizing syntrophic methanogenic consortia and acetotrophic methanogens, and also between methylotrophic methanogens and potentially methylotrophic bacteria and syntrophs.

Temperature adaptation was fundamental in determining the types of methanogen strains obtained as methanogen monocultures or pure cultures. Dominant methanogen genera in temperature and salinity gradient enrichments were presumably those either most abundant in the original sediments and/or fast-growing, whereas, methanogens strains obtained as methanogen monocultures or pure cultures were those whose temperature optima most closely matched the temperature of incubation and media composition used. However, there is also evidence to suggest that syntrophic acetate (and possibly methylamine) oxidation coupled with hydrogenotrophic methanogenesis is a dominant pathway for acetate and methylamine oxidation at mesophilic and thermophilic temperatures. The tentative evidence for a syntrophic association between methylotrophic bacteria and hydrogenotrophic methanogens is novel.

The dominant genera in low temperature cultivation series (Tables 6.0.5. and 6.0.6., Chapter 6), where conditions (temperature, media salinity, substrate type) were as close as possible to *in situ* conditions, were not the methanogen genera most abundant
in the original sediment (Figure 3.0.7., Chapter 3). This is attributed to the high substrate conditions of cultivation favouring growth of acetogenic bacteria over hydrogenotrophic methanogens (Conrad 1999), and demonstrates the difficulties involved in culturing organisms that are most abundant in the environment. In general this study disputes conclusions of previous cultivation based studies that cultivation conditions are highly selective (Kopke et al. 2005), although it is clear that small variations in cultivation conditions can have a substantial impact on microbial community dynamics.
7. PHYSIOLOGICAL CHARACTERISATION OF CARDIFF BAY ISOLATES

7.1. Rationale

A number of methanogen strains with diverse phylogenetic affiliation were isolated from Cardiff Bay sediments following enrichment in sediment slurries subjected to temperature or salinity manipulation. This included strains affiliated with the thermophilic, hydrogenotrophic methanogen genus *Methanothermobacter* and the marine, methylotrophic genus *Methanolobus*. The temperature minima of isolated *Methanothermobacter* type strains (see Table 7.0.1.) is significantly higher than the *in situ* temperature range of Cardiff Bay (maximum average summer water temperature 18 °C, Lee In prep.). Type strains of the methylotrophic genus *Methanolobus* have mainly been isolated from moderate to high salinity environments (Liu et al. 2010c), yet *Methanolobus* spp. were enriched from Cardiff Bay lacustrine gyttja and estuarine clay sediments and a strain of *Methanolobus* was isolated from Cardiff Bay estuarine clay sediments (maximum porewater salinity of 0.04 M NaCl). Assuming that the physiological characteristics of the *Methanothermobacter* and *Methanolobus* strains isolated from Cardiff Bay sediments closely reflect the physiological characteristics of previously cultured representatives of these genera, it is difficult to understand how these strains are surviving *in situ*.

Methanogen strains belonging to the genera *Methanobacterium* and *Methanosarcina* were also isolated from Cardiff Bay sediments. *Methanobacterium* is the dominant methanogen genus in both Cardiff Bay lacustrine gyttja and estuarine clay sediments (Figure 3.0.7., Chapter 3) although both genera are relatively rare in freshwater lake sediments (Borrel et al. 2011). Members of the genus *Methanosarcina* are typically able to tolerate a wide range of salinities (Table 7.0.5.), however members of the genus *Methanobacterium* are sensitive to salinity (Whitman et al. 2006; Teske et al. 2014). Salinity characterisation data is currently available for 14 out of the 22 validly described *Methanobacterium* type strain species; 8 have a salinity maxima <0.55 M, and only 2 out of the remaining 6 species have salinity optima of >0.55 M (Table 7.0.2.). Furthermore, results of the temperature gradient study presented in Chapter 4 indicated that *Methanobacterium* in Cardiff Bay sediments are predominantly mesophilic (section 5.4.2.). Therefore it is interesting to determine whether isolated *Methanobacterium* strains isolated from Cardiff Bay sediments are tolerant of the pre-
and post-impoundment salinity conditions in Cardiff Bay or active at \textit{in situ} temperatures.

This study investigates the physiological and metabolic characteristics of the methanogen strains isolated from Cardiff Bay sediments in order to (i) determine whether isolates share the physiological and metabolic characteristics of the most closely related isolated and characterised methanogen strains, and (ii) develop better understanding of the ecological niche of these isolates.

7.2. Methods

6 methanogen strains were obtained in pure culture from temperature and salinity gradient cultivation series as described in section 6.2. The cultivation conditions under which each isolate was obtained are shown in Table 7.0.3.

The temperature and salinity characteristics of \textit{Methanobacterium} isolates (CB01, CB02 and CB03) were investigated in detail as \textit{Methanobacterium} is an important methanogen genus in Cardiff Bay sediments (Figure 3.0.7., Chapter 3). This included temperature and salinity range, but also temperature and salinity specific growth rate and biomass production characteristics. Temperature and salinity range were the only parameters determined for the \textit{Methanolobus} (CB04), \textit{Methanosarcina}, (CB05) and \textit{Methanothermobacter} (CB06), as these are minor community members under \textit{in situ} conditions.

7.2.1. Specific growth rate and biomass production temperature profiles for strains CB01, CB02 and CB03

Strains CB01, CB02, and CB03 were incubated in duplicate at 2-3 °C intervals over a temperature range of ~4 to 56 °C using a temperature gradient block (see section 2.4.). Cultures were prepared in 10 ml tubes containing 4.5 ml media and a 5% inoculum. Headspace gas samples were taken for natural gas analysis at regular intervals (from daily to weekly) until the culture had been growing in stationary phase for a minimum period of 48 hours. Once cultures reached the stationary phase of growth 1-1.5 ml of culture media was transferred into a sterile 2 ml siliconized microcentrifuge tube (Alpha Laboratories, Hampshire, UK) and stored at -20 °C until further use.
Table 7.0.1. Physiological characteristics of strain CB06 and Methanothermobacter type strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Salinity range (NaCl M)†</th>
<th>Temperature range (°C)‡</th>
<th>Substrates</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB06</td>
<td>&lt;0.01-&gt;0.55</td>
<td>15/25-77</td>
<td>H₂/CO₂ (some strains use formate)</td>
<td>Freshwater lake sediment</td>
</tr>
<tr>
<td>Methanothermobacter thermautotrophicus²</td>
<td>0.0017-0.6</td>
<td>40-75 (65-70)</td>
<td>H₂/CO₂</td>
<td>Sewage sludge</td>
</tr>
<tr>
<td>Methanothermobacter wollei²</td>
<td>0-0.34</td>
<td>34-74 (55-65)</td>
<td>H₂/CO₂, formate</td>
<td>Sewage sludge/river sediment</td>
</tr>
<tr>
<td>Methanothermobacter thermophilus³</td>
<td>ND</td>
<td>45-65 (57)</td>
<td>H₂/CO₂</td>
<td>Anaerobic digester sludge</td>
</tr>
<tr>
<td>Methanothermobacter marburgensis⁵</td>
<td>0.0017-0.6</td>
<td>45-&gt;70 (65)⁵</td>
<td>H₂/CO₂ (formate, methanol/ethanol)</td>
<td>Oil sands from hot oil field</td>
</tr>
<tr>
<td>Methanothermobacter tenebrarum⁶</td>
<td>0.0001-0.04</td>
<td>45-80 (70)</td>
<td>H₂/CO₂</td>
<td>Well formation waters in gas field</td>
</tr>
<tr>
<td>Methanothermobacter defluvi¢</td>
<td>0.014-0.34</td>
<td>45-65 (60-65)</td>
<td>H₂/CO₂, formate</td>
<td>Anaerobic digester sludge</td>
</tr>
</tbody>
</table>


† Optima shown in brackets.

N.B. Two temperatures separated by a forward slash indicates that a very small amount of methane production was observed at the first temperature, and significant methane production was observed at the second temperature.

Table 7.0.2. Physiological characteristics of strains CB01, CB02, CB03 and Methanobacterium type strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature range (°C)‡</th>
<th>Salinity range (NaCl M)†</th>
<th>Substrates</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB01</td>
<td>13/22-54/51 (34-40)</td>
<td>&lt;0.01-0.46 (0.06)</td>
<td>H₂/CO₂ (methanol/ ethanol and 2-propanol/2-butanol/cyclopentanol)</td>
<td>Freshwater lake sediment</td>
</tr>
<tr>
<td>CB02</td>
<td>13-51/54 (38-40)</td>
<td>&lt;0.01-&gt;0.55</td>
<td>H₂/CO₂, formate</td>
<td>Freshwater lake sediment</td>
</tr>
<tr>
<td>CB03</td>
<td>10/28-48/54 (44)</td>
<td>&lt;0.01-&gt;0.55</td>
<td>H₂/CO₂ (formate, methanol/ethanol)</td>
<td>Freshwater lake sediment</td>
</tr>
<tr>
<td>Methanobacterium ivanoviiᵃ</td>
<td>15-55 (45)</td>
<td>ND</td>
<td>H₂/CO₂</td>
<td>Oil field well formation waters</td>
</tr>
<tr>
<td>Methanobacterium lacusᵇ</td>
<td>14-41 (30)</td>
<td>0-0.4 (0.1)</td>
<td>H₂/CO₂, methanol/H₂</td>
<td>Freshwater lake sediments</td>
</tr>
<tr>
<td>Methanobacterium beijingenseᶜ</td>
<td>25-50 (37)</td>
<td>0-0.5</td>
<td>H₂/CO₂, formate</td>
<td>Anaerobic digester</td>
</tr>
<tr>
<td>Methanobacterium oryzaeᵈ</td>
<td>20-42 (40)</td>
<td>0-0.4</td>
<td>H₂/CO₂, formate</td>
<td>Rice field sediments</td>
</tr>
<tr>
<td>Methanobacterium conglobense&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25-50 (37-42)</td>
<td>ND</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt; (2-propanol, 2-butanol and cyclopentanol)</td>
<td>Anaerobic digester</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>----------------</td>
<td>-----</td>
<td>-------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Methanobacterium palustre&lt;sup&gt;†&lt;/sup&gt;</td>
<td>20-45 (33-37)</td>
<td>0-0.3 (0.2)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;, formate, 2-propanol (2-butanol)</td>
<td>Anaerobic digester</td>
</tr>
<tr>
<td>Methanobacterium subterraneum&lt;sup&gt;§&lt;/sup&gt;</td>
<td>3.6-45 (20-40)</td>
<td>0.2-1.4 (0.2-1.25)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;, formate</td>
<td>Deep, granitic groundwaters</td>
</tr>
<tr>
<td>Methanobacterium alcaliphilum&lt;sup&gt;§&lt;/sup&gt;</td>
<td>ND (37)</td>
<td>ND</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Sediments of alkaline lake</td>
</tr>
<tr>
<td>Methanobacterium bryantii&lt;sup&gt;†&lt;/sup&gt;</td>
<td>ND (37-39)</td>
<td>ND</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt; (2-propanol, 2-butanol and cyclopentanol)</td>
<td>Anaerobic digester</td>
</tr>
<tr>
<td>Methanobacterium veterum&lt;sup&gt;i&lt;/sup&gt;</td>
<td>10-46 (28)</td>
<td>0.3</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;, methanol/H&lt;sub&gt;2&lt;/sub&gt;, methylamine/H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Permafrost soil</td>
</tr>
<tr>
<td>Methanobacterium uliginosum&lt;sup&lt;k&lt;/sup&gt;</td>
<td>15-45 (40)</td>
<td>ND</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Marshy sediment</td>
</tr>
<tr>
<td>Methanobacterium aarhusense&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5-48 (45)</td>
<td>ND</td>
<td>ND</td>
<td>Marine sediment</td>
</tr>
<tr>
<td>Methanobacterium movens&lt;sup&gt;m&lt;/sup&gt;</td>
<td>10-50 (35-38)</td>
<td>0-1.7 (0-0.3)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt; only</td>
<td>Freshwater lake sediments</td>
</tr>
<tr>
<td>Methanobacterium kanagiense&lt;sup&gt;n&lt;/sup&gt;</td>
<td>15-45 (40)</td>
<td>0-1.2 (0.086)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt; only</td>
<td>Rice field soil</td>
</tr>
<tr>
<td>Methanobacterium petrolearium&lt;sup&gt;p&lt;/sup&gt;</td>
<td>20-40 (35)</td>
<td>0-1.2 (0-0.68)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Sludge from crude oil storage tank</td>
</tr>
<tr>
<td>Methanobacterium arcticum&lt;sup&gt;r&lt;/sup&gt;</td>
<td>15-45 (37)</td>
<td>0-0.3 (0.1)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;, formate</td>
<td>Arctic permafrost soil</td>
</tr>
<tr>
<td>Methanobacterium paludis&lt;sup&gt;q&lt;/sup&gt;</td>
<td>16-40 (32-37)</td>
<td>0-0.25 (0.025-0.075)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Peat bog</td>
</tr>
<tr>
<td>Methanobacterium espanolae&lt;sup&gt;r&lt;/sup&gt;</td>
<td>&gt;15-&lt;50 (35)</td>
<td>ND</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt; (2-propanol, 2-butanol)</td>
<td>Sludge from bleach craft mill</td>
</tr>
<tr>
<td>Methanobacterium formicicum&lt;sup&gt;s&lt;/sup&gt;</td>
<td>ND (37-45)</td>
<td>Isolated at 0.25, range ND</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt; and formate</td>
<td>Sewage sludge</td>
</tr>
<tr>
<td>Methanobacterium flexile&lt;sup&gt;m&lt;/sup&gt;</td>
<td>10-50 (35-38)</td>
<td>0-1.0 (0.1)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt; and formate</td>
<td>Freshwater lake sediments</td>
</tr>
<tr>
<td>Methanobacterium movilense&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0-44 (33)</td>
<td>0.02-0.6 (0.08)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;, 2-propanol, 2-butanol, formate</td>
<td>Sediment of subsurface lake</td>
</tr>
<tr>
<td>Methanobacterium ferruginis&lt;sup&gt;r&lt;/sup&gt;</td>
<td>20-45 (40)</td>
<td>0-1.2 (0.34)</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Pipe transporting gas field brine</td>
</tr>
</tbody>
</table>

<sup>a</sup>Belyaev et al. (1983), <sup>b</sup>Borrel et al. (2012a), <sup>c</sup>Ma et al. (2005), <sup>d</sup>Joulian et al. (2000), <sup>e</sup>Cuzin et al. (2001), <sup>f</sup>Zellner et al. (1988), <sup>g</sup>Kotelnikova et al. (1998), <sup>h</sup>Worakit et al. (1986), <sup>i</sup>Balach et al. (1979b), <sup>j</sup>Krivushin et al. (2010), <sup>k</sup>König (1984), <sup>l</sup>Shlimon et al. (2004), <sup>m</sup>Zhu et al. (2011), <sup>n</sup>Kitamura et al. (1988), <sup>o</sup>Mori and Harayama (2011), <sup>p</sup>Shcherbakova et al. (2011), <sup>q</sup>Cadillo-Quiroz et al. (2014), <sup>r</sup>Patel et al. (1993), <sup>s</sup>Bryant and Boone (1987a), <sup>t</sup>Schirmack et al. (2014).

* Not tested with ethanol.
† Optima shown in brackets.
N.B. Substrates listed in brackets were oxidized but not used for growth, or only produced very small quantities of methane.
Two temperatures separated by a forward slash indicates that a very small amount of methane production was observed at the first temperature, and significant methane production was observed at the second temperature.
Table 7.0.3. Cultivation conditions under which methanogen isolates obtained from temperature and salinity gradient cultivation series and genus identification

<table>
<thead>
<tr>
<th>Sediment depth (cm)</th>
<th>Incubation temperature (°C)</th>
<th>Media salinity (NaCl M)</th>
<th>Substrate addition (headspace or media concentration)</th>
<th>Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB01</td>
<td>0-5</td>
<td>38</td>
<td>H2/CO2 (80:20, 0.1 MPa)</td>
<td>Methanobacterium</td>
</tr>
<tr>
<td>CB02</td>
<td>0-5</td>
<td>38</td>
<td>H2/CO (80:20, 0.1 MPa)</td>
<td>Methanobacterium</td>
</tr>
<tr>
<td>CB03</td>
<td>25-30</td>
<td>25</td>
<td>H2/CO (80:20, 0.1 MPa)</td>
<td>Methanobacterium</td>
</tr>
<tr>
<td>CB04</td>
<td>25-30</td>
<td>25</td>
<td>HCl-methylamine (20 mM)</td>
<td>Methanobacterium</td>
</tr>
<tr>
<td>CB05</td>
<td>0-5</td>
<td>25</td>
<td>H2/CO (80:20, 0.1 MPa)</td>
<td>Methanothermobacter</td>
</tr>
<tr>
<td>CB06</td>
<td>0-5</td>
<td>66</td>
<td>H2/CO (80:20, 0.1 MPa)</td>
<td>Methanosarcina</td>
</tr>
</tbody>
</table>

Table 7.0.4. Physiological characteristics of strain CB04 and Methanolobus type strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature range (°C)†</th>
<th>Salinity range (NaCl M)†</th>
<th>Min NaCl tested</th>
<th>Substrates</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB04 (this study)</td>
<td>10-30</td>
<td>&lt;0.01-0.55</td>
<td>0.01</td>
<td>Methanol, MMA</td>
<td>Freshwater lake sediments</td>
</tr>
<tr>
<td>Methanolobus profundia</td>
<td>9-37 (30)</td>
<td>0.1-1.2 (0.5)</td>
<td>0</td>
<td>Methanol, MMA, DMA, TMA</td>
<td>Deep, subsurface gas field sediments</td>
</tr>
<tr>
<td>Methanolobus oregonensis</td>
<td>20-40 (35-37)</td>
<td>0.1-0.6 (0.48)</td>
<td>0</td>
<td>Methanol, MMA, DMA, TMA</td>
<td>Saline, alkaline aquifer</td>
</tr>
<tr>
<td>Methanolobus vulcanii</td>
<td>13-45 (37)</td>
<td>0.1-1.2 (0.5)</td>
<td>0</td>
<td>Methanol, MMA, DMA, TMA</td>
<td>Marine sediments</td>
</tr>
<tr>
<td>Methanolobus tindarius</td>
<td>10-45 (25)</td>
<td>0.06-1.27 (0.47)</td>
<td>ND</td>
<td>Methanol, MMA, DMA, TMA</td>
<td>Marine sediments</td>
</tr>
<tr>
<td>Methanolobus bombayensis</td>
<td>22-40 (37)</td>
<td>0.3-2.0 (0.5)</td>
<td>~ 0.1</td>
<td>Methanol, MMA, DMA, TMA</td>
<td>Marine sediments</td>
</tr>
<tr>
<td>Methanolobus taylorii</td>
<td>11-40 (37)</td>
<td>0.2-1.2 (0.5)</td>
<td>ND</td>
<td>Methanol, MMA, DMA, TMA</td>
<td>Estuarine sediments</td>
</tr>
<tr>
<td>Methanolobus zinderi</td>
<td>25-50 (40-50)</td>
<td>0.05-1.8 (0.2-0.6)</td>
<td>0</td>
<td>Methanol, MMA, DMA, TMA</td>
<td>Subsurface, saline coal seam</td>
</tr>
<tr>
<td><em>Methanolobus psychrophilus</em>*</td>
<td>18 (0-25)</td>
<td>0.005-0.8 (0.2-0.25)</td>
<td>0</td>
<td>Methanol, TMA*, DMS</td>
<td>Permanently cold wetland soil</td>
</tr>
<tr>
<td>Methanolobus chelungpuianus</td>
<td>24-45 (37)</td>
<td>0-0.68 (0-0.08)</td>
<td>0</td>
<td>Methanol, TMA</td>
<td>Deep, subsurface sandstone formation</td>
</tr>
</tbody>
</table>

a Mochimaru et al. (2009), b Liu et al. (1990), c Kadam and Boone (1995), d König and Stetter (1982), e Kadam et al. (1994), f Doerfert et al. (2009), g Zhang et al. (2008), h Wu and Lai (2011).

* Growth with MMA and DMA not tested
** Not yet validly described
† Optima shown in brackets.
7.2.2. Temperature range profiles for strains CB04, CB05 and CB06

Strains CB04, CB05 and CB06 were tested for growth at 4, 10, 15, 25, 38, 46, 55, 66 and 77°C in duplicate. Cultures were prepared in methanogen cultivation tubes containing 10 ml media and a 0.25% inoculum, with a H₂/CO₂ (80:20, 0.1 mPa) headspace. Temperature testing was carried out using the same media composition and substrate additions as used during the isolation process (Table 7.0.3.). Positive growth was recorded if methane production was observed in at least one of the duplicate cultures after a 6 month incubation period.

7.2.3. Specific growth rate and biomass production salinity profiles for strains CB01, CB02 and CB03

Strains CB01, CB02 and CB03 were incubated in duplicate between 0.01 and 0.55 M NaCl at intervals of 0.05 M. Samples were taken for headspace gas analysis at regular intervals, and the volume of headspace gas removed was replaced with an equal volume of substrate gas mixture (H₂/CO₂).

7.2.4. Salinity range profiles for strains CB04, CB05 and CB06

Strains CB04, CB05 and CB06 were tested for growth in duplicate at 0.01, 0.5, and 0.55 M NaCl. Cultures were prepared in methanogen cultivation tubes containing 10 ml media and a 0.25% inoculum, and the headspace was flushed with N₂/CO₂ (80:20, 0.1 mPa) for strains CB04 and CB05 or H₂/CO₂ (80:20, 0.1 mPa) for strain CB06. Salinity testing was carried out using the incubation temperature and substrate additions used during the isolation process (Table 7.0.3.). Positive growth was recorded if methane production was observed in at least one of the duplicate cultures after a 6 month incubation period.

7.2.5. Substrate usage

All isolated strains were tested for growth on choline, betaine, glycolate, trimethylamine, dimethylamine, methylamine, dimethylsulphide, methanethiol, acetate, formate, ethanol/methanol, cyclopentanol/ isopropanol/ isobutanol (10 mM), and H₂/CO₂ (80:20, 0.1 mPa).
Cultures were prepared in methanogen cultivation tubes containing 10 ml media and a 0.25% inoculum. Substrate testing was carried out using the same media composition and incubation temperature as during the isolation process (Table 7.0.3.)

7.2.6. Protein extraction

Protein was extracted from culture media using a method modified from Bradford (1976) and Hippe et al. (1979). 1 - 1.5 ml of cell suspension was centrifuged at 1400 rpm for 15 minutes, and the resulting supernatant removed. The cell pellet was then washed twice with 0.5 ml sterile 0.9 % NaCl, and resuspended in 0.5 ml 1M NaOH for 10 minutes at room temperature. Samples were then heated for 15 s at 100 °C and immediately cooled on ice. Once cool, samples were diluted 1:10 with deionized water.

7.2.7. Protein concentration

Protein concentration was determined using a modification of the Bradford method (Bradford 1976) described in Ernst and Zor (2010), whereby absorbance is measured at both 590 and 450 nm. The traditional Bradford protocol only generates a linear calibration for protein concentrations between 2-10 mg/ml, whereas the method of Ernst and Zor (2010) produces a linear calibration at concentrations <0.05 µg/ml. Standards were prepared using bovine serum albumin (Sigma-Aldrich, Missouri, USA) diluted in deionized water to give final concentrations of 0 (reagent blank), 1, 2.5, 5, 7.5 and 10 µg/ml of protein. 0.5 ml of sample protein extraction or standard protein solution (Bovine Serum Albumin [BSA]; Sigma-Aldrich, Missouri, USA) was added to 0.5 ml Bradford reagent (Sigma-Aldrich, Missouri, USA) and incubated at room temperature for 5 minutes in 1 ml disposable Fisherbrand™ semi-micro plastic cuvettes (ThermoFisher Scientific, California, USA). A cuvette containing only 1 ml of deionized water was used as a blank.

Sample and standard absorbance was measured at 595 and 450 nm using a Jenway J6300 spectrophotometer (Bibby Scientific, Staffordshire, UK). A calibration curve was obtained by plotting the ratio of absorbance values at 595 and 450 nm (see Equation 7.1.) against BSA concentration. Sample protein concentration was calculated from the equation describing linear regression of the calibration curve, taking into account the protein concentration of the culture inoculum.
**Equation 7.0.1. Ratio of absorbance at 595 and 450 nm**

\[
\text{Absorbance ratio} = \frac{A_{595}^{\text{sample}} - A_{595}^{\text{water blank}}}{A_{450}^{\text{sample}} - A_{450}^{\text{water blank}}}
\]

7.2.8. Conversion from protein concentration to biomass production

Protein concentration was converted into biomass concentration assuming that protein represents 50% of the weight of dry biomass (Archer 1984). The inoculum biomass concentration was subtracted from sample biomass concentration to give ‘biomass production’ values.

7.2.9. Calculation of specific growth rate

The specific growth rate (\(\mu\)) of a culture was calculated by linear regression of plots showing the logarithm of headspace methane accumulation over time during the exponential phase of growth (Powell 1983; Lyimo et al. 2009; Watkins et al. 2012; Watkins et al. 2014).

7.2.10. Molecular genetic identification of methanogen isolates

DNA extraction and PCR, were carried out as described in sections 2.9.1.-2.9.3. using 16s rRNA gene primers 109f/958r and mcrA functional gene primers ME1/ME2. Both the 16s rRNA and mcrA genes were targeted in order to try and obtain a species level classification for the isolates obtained during this study.

7.2.11. Phylogenetic classification of methanogen isolates

Type strain sequences were obtained from the National Centre for Biotechnology Information (NCBI, [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) GenBank® sequence database. 16s rRNA nucleotide sequences were aligned using ClustalW (Larkin et al. 2007) within MEGA version 6.0 (Tamura et al. 2013). Neighbour joining trees were constructed in MEGA version 6.0 using the Jukes-Cantor algorithm with 1000 bootstrap replicates.

7.3. Physiological characteristics of *Methanobacterium* strains CB01, CB02, and CB03

Table 7.0.2. lists the temperature, salinity and metabolic characteristics of strains CB01, CB02 and CB03 and type strains of the genus *Methanobacterium*. 
7.3.1. Phylogenetic affiliation of *Methanobacterium* strains

Figure 7.0.1 shows the phylogenetic placement of strains CB01, CB02 and CB03 in the genus *Methanobacterium*. Strain CB01 had 99 % 16s rRNA gene similarity to four type strains; *Methanobacterium arcticum* type strain M2 (NR_115811) isolated from Arctic permafrost soil (Rivkina et al. 2007), *Methanobacterium bryantii* M.o.H type strain (NR042781, submitted by Wright and Pimm 2003) isolated from an anaerobic digester (Barker and Buswell 1956), *Methanobacterium uliginosum* type strain P2St (NR_104694), and *Methanobacterium veterum* type strain MK4 (NR_115935) isolated from...
from Arctic permafrost soil (Rivkina et al. 2007). These 4 species form a distinct clade (Figure 7.0.1.) alongside Methanobacterium espanolae type strain GP9 and Methanobacterium ivanovii type strain OCM 140. However, the mcrA gene sequence for CB01 was most similar (99%) to Methanobacterium ivanovii type strain DSM 2611 (EF465107; Ma and Dong, unpublished data) and Methanobacterium bryantii type strain DSM 863 (AF313806) isolated from Italian rice field soils (Lueders et al. 2001).

Strains CB02 and CB03 clustered tightly with Methanobacterium formicicum and Methanobacterium palustre (Figure 7.0.1.). Strain CB02 had 100% 16s rRNA gene similarity to Methanobacterium formicicum strains BEG1 (JN243320), HWS1 (JN243318) and TAF1 (JN243315) which were isolated from anaerobic digester slurry (Stantscheff et al. unpublished data), and 99 % similarity to Methanobacterium formicicum type strain MF (NR_115168) which was isolated from sewage sludge (Balch et al. 1979a). Strain CB02 could not be amplified using mcrA primers ME1/ME2.

Strain CB03 had 98% 16s rRNA gene similarity to Methanobacterium palustre type strain F (NR041713, submitted by Joulian et al. 2000) originally isolated from a peat bog (Zellner et al. 1988) and Methanobacterium formicicum type strain MF (NR115168, submitted by Wright and Pimm 2003) isolated from sewage sludge (Balch et al. 1979a). The mcrA gene sequence fragment for strain CB03 had greatest (99 %) similarity to Methanobacterium strain EO9F.3 (KP006500) isolated from an anaerobic digester (Kern, T. and Rother, M. unpublished), and the validly described species type strain with most similar mcrA sequence was Methanobacterium congolense type strain NBRC 105227 (96 %, AB542748, Mori and Harayama 2011) which was isolated from an anaerobic digester containing cassava peel (Cuzin et al. 2001).

7.3.2. Temperature characteristics of Methanobacterium strains

Strain CB01 produced methane at temperatures ≥12.9°C, although significant methane production (headspace concentration >0.5 ppt) and a clear exponential growth phase only occurred at temperatures ≥22°C (see Figure 7.0.2.). The specific growth rate increased from 0.35 day⁻¹ at 24.6°C to a maximum of ~1.8 day⁻¹ between 33.7-40.2 °C. Specific growth rate values were still relatively high (1.4 day⁻¹) at 46.7°C, but by 53.2°C methane production was limited to <2 ppt and there was no exponential growth phase. Biomass production was evident at all temperatures where methane production occurred (12.9-55.8 °C), but production was highest ~9-11 µg/ml between
24.6 and 46.7°C with the temperature range over which methane production occurred in an exponential pattern. There is no defined temperature optima for average biomass production values as seen for average specific growth rate values. In fact biomass production values at 33.7 and 37.6 °C are relatively low compared to the preceding and subsequent temperature points. Also biomass production is highly variable (5-6 µg/ml difference between replicates) in cultures incubated at 40.2 and 46.7 °C. Interestingly although methane production was limited at temperatures >46.7°C, biomass production values at 55.8 °C (~6 µg/ml) are a third higher than maximum biomass production at temperature <24.6 °C (4 µg/ml).

Strain CB02 had uniformly low specific growth rates (0.05-0.065) between 12.9 and 22.0 °C, which increased rapidly to a maximum of ~1.15 at 37.6-40.2 °C (Figure 7.0.3.). Specific growth rates decreased rapidly with temperature at >40.2 °C, and no
exponential methane production phase was seen at temperatures \(>50.6\, ^\circ\text{C}\). Similar to strains CB01 biomass production occurred at all temperatures where methane production occurred \(\geq12.9\, ^\circ\text{C}\) irrespective of whether an exponential growth phase occurred. However average biomass production was relatively low \(<8\, \mu\text{g/ml}\) at temperatures \(<31.1\, ^\circ\text{C}\) and \(>50.6\, ^\circ\text{C}\). Surprisingly biomass production was no higher at 22.0 and 28.5 \(^\circ\text{C}\) than in cultures incubated at \(<22.0\, ^\circ\text{C}\) (where there was no exponential growth phase). Average biomass production increased from 15.8 \(\mu\text{g/ml}\) at 31.1 \(^\circ\text{C}\) to a maximum of 32.0 \(\mu\text{g/ml}\) 46.7 \(^\circ\text{C}\). As seen for strain CB01 average biomass production was relatively low \(14.0\, \mu\text{g/ml}\) at the temperature of maximum specific growth rate \(33.7\, ^\circ\text{C}\) relative to the preceding and following temperature points.

\[\text{Figure 7.0.3. Strain CB02 temperature profile for specific growth rate and biomass production at 0.05 M NaCl}\]

N.B. Replicates which did not produce methane were excluded from the average specific growth rate or average biomass calculations.
CB03 produced >0.5 ppt methane between 18.7 and 44.2°C, however only cultures incubated between 28.1 and 44.2 °C had an exponential growth phase during the 40 day period of incubation (Figure 7.0.4.). Average specific growth rate increased sharply from 0.6 at 28.1 °C to 1.4 at 37.5°C. There was a slight decrease in specific growth rate between 37.5 and 40.1 °C to 1.2 day⁻¹, however specific growth rates then increased again to their maximum value of 1.55 day⁻¹ at 44.2 °C. Biomass production only occurred within the temperature range of exponential growth apart from a small peak (2.9 µg/ml) at 9.3°C. Average biomass production increased from 11.1 µg/ml at 28.1 °C to a maximum of 16.2 µg/ml at 37.5 °C, and then decreased steadily to 0 at 48.2 °C.

![Figure 7.0.4. Strain CB03 temperature profile for specific growth rate and biomass production at 0.01 M NaCl](image)

N.B. Replicates which did not produce methane were excluded from the average specific growth rate or average biomass calculations.
7.3.1. Salinity characteristics of *Methanobacterium* strains

Strain CB01 produced methane between 0.01 and 0.46 M, although methane production was not accompanied by biomass production at 0.46 M (Figure 7.0.5.). Average specific growth rate increased rapidly with NaCl concentration from 0.9 day$^{-1}$ at 0.01 M, to a maximum of ~1.4 day$^{-1}$ at 0.06 M. Average specific growth rate then decreased to ~0.6 at 0.26 M, remaining between 0.6-0.8 day$^{-1}$ until 0.46 M, before decreasing to 0 and 0.51 and 0.55 M. The average specific biomass salinity profile was broadly similar to the average specific growth rate salinity profile. Specific biomass increased sharply from 4.3 µg/ml at 0.01 M to a maximum of 10.3 µg/ml 0.06 M, coinciding with peak specific growth rate. There was a second peak in average specific biomass production (10.1 µg/ml), set within a general decrease in biomass production with increasing salinity at NaCl concentrations greater than the biomass production optima (0.06 M NaCl). No biomass production occurred at >0.46 M NaCl.

Figure 7.0.5. Strain CB01 salinity profile for specific growth rate and biomass production at 38°C

N.B. Replicates which did not produce methane were excluded from the average specific growth rate or average biomass calculations.
Strain CBO2 showed methane production and growth throughout the salinity range of investigation (Figure 7.0.6.). The specific growth rate of strain CBO2 varied little throughout the salinity range of investigation, ranging between 0.4 and 0.75 µg/ml, with the exception of a slight peak (0.88 µg/ml) at 0.26 M. The average biomass production salinity profile bore little resemblance to the specific growth rate salinity profile, and was highly unusual in that there were 2 regions of high biomass production at opposing ends of the salinity spectrum. Average biomass production increased from 14.5 µg/ml at 0.01 M to a maximum of 30.4 µg/ml at 0.16 M, before decreasing to a minimum of 6.7 µg/ml at 0.26 M. Average specific biomass was highly variable at salinities 0.26 M. Values were between 6.9 µg/ml at 0.31, 0.36 and 0.46 M, but increased to 19-22 µg/ml at 0.41, 0.51 and 0.55 M.

Strain CBO3 produced methane throughout the salinity range of investigation, with specific growth rate values ranging between 0.16 and 0.44 day\(^{-1}\) (Figure 7.0.7.).

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**Figure 7.0.6. Strain CB02 salinity profile for specific growth rate and biomass production at 38°C**

N.B. Replicates which did not produce methane were excluded from the average specific growth rate or average biomass calculations.
Highest values (>0.34 day\(^{-1}\)) occurred between 0.01-0.06 M, at 0.21 M, and between 0.41-0.51 M. Specific biomass values were relatively low between 0.01-0.26 M (8.5 to 17.3 µg/ml), but increased to ~18.0-28.0 µg/ml between 0.31 and 0.51 M. At 0.55 M the specific biomass decreased again to 14.3 µg/ml.

7.3.2. Substrate characteristics of Methanobacterium strains

Strains CB01, CB02 and CB03 used H\(_2\)/CO\(_2\). Strain CB02 also used formate. Strain CB03 produced small quantities of methane (increase in culture headspace methane concentration <1 ppt) from formate, and strains CB01 and CB03 produced very low quantities of methane (increase in culture headspace methane concentration <0.1 ppt) when incubated with a combination of methanol and ethanol. Strain CB01 also produced <0.1 ppt methane when incubated with a combination of isobutanol, isopropanol, and cyclopentanol. There was no methanogenesis from any of the other substrates tested.
7.4. Physiological characteristics of *Methanolobus* strain CB04

Table 7.0.4. lists the temperature, salinity and metabolic characteristics of strain CB04 and type strains belonging to the genus *Methanolobus*.

7.4.1. Phylogenetic affiliation of *Methanolobus* strain CB04

Strain CB04 had 99% 16s rRNA gene similarity to uncultured clone NR9_09_007 (HQ654836) detected in mud volcano fluids from the terrestrial Nirano mud volcano field (Wrede et al. 2012). The closest cultured relatives to strain CB04 were *Methanolobus profundi* type strain MobM (NR_041665) isolated from deep petroleum reservoir sediment (Mochimaru et al. 2009) and *Methanolobus taylorii* type strain GS-16 (NR_028238; submitted by Woese, unpublished data) isolated from estuarine sediments (Kiene et al. 1986), which both had 98% 16s rRNA similarity. Strain CB04 has 96% mcrA gene similarity to *Methanolobus profundi* type strain MobM (AB703629; submitted by Mori and Suzuki, unpublished data). Figure 7.0.8. shows the phylogenetic placement of strain CB04 within the genus *Methanolobus*.

![Figure 7.0.8. 16s rRNA phylogenetic tree (neighbour-joining) showing placement of strain CB04 within the genus Methanolobus. Numbers represent % of bootstrap values based on 1000 replicates. Bootstrap values <50 % are excluded. Accession numbers are given in brackets. Scale bar represents 0.01 base changes.](image)

7.4.2. Temperature characteristics of *Methanolobus* strain CB04

Strain CB04 produced methane at 10 and 30°C, but not at 4 or 38 °C (Table 7.0.4.). The minimum temperature for strain CB04 was similar to closely related cultivated species such as *Methanolobus profundi*, *Methanolobus tindarius*, *Methanolobus*...
vulcanii and Methanolobus taylorii. But it is noteworthy that the maximum temperature for CB04 was approximately 10°C lower than any other Methanolobus type strain species. Zhang et al. (2008) isolated a psychrophilic strain of Methanolobus (“Methanolobus psychrophilus”) from permanently cold wetland soil of the Tibetan plateau which had a temperature maxima of 25 °C, however “Methanolobus psychrophilus” was active at temperatures down to 0°C whereas strain CB04 did not produce methane at 4 °C.

7.4.3. Salinity characteristics of Methanolobus strain CB04

Strain CB04 produced methane at 0.01, 0.05 and 0.55 M NaCl. Strain CB04 was able to tolerate a far lower salinity, at 0.01 M, than has previously been tested for Methanolobus type strain species, with the exception of Methanolobus chelungpuianas (Wu and Lai 2011).

7.4.4. Substrate characteristics of Methanolobus strain CB04

Strain CB04 was able to utilise MMA and methanol but, unlike previously isolated strains of Methanolobus, it did not utilise TMA or DMA. Almost all methanogen species able to utilise DMA and TMA are also able to utilise MMA, with the exception of Methanosarcina horonobensis (Shimizu et al. 2011). Recent studies have shown members of the methylotrophic methanogen genera Methanococcoides (Watkins et al. 2012; Ticak et al. 2014; Watkins et al. 2014) and Methanolobus (Ticak et al. 2014) are able to utilise quaternary amines for methanogenesis, however, strain CB04 did not utilise choline or glycine betaine for growth. The ability to utilise quaternary amines varies between closely related strains (>99 % 16s rRNA gene sequence similarity) of a given species (Ticak et al. 2014).

7.5. Physiological characteristics of Methanosarcina strain CB05

Table 7.0.5. lists the temperature, salinity and metabolic characteristics of strain CB05 and type strains belonging to the genus Methanosarcina.
<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature range (°C)</th>
<th>Salinity range (NaCl M)</th>
<th>Substrates</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB05 (this study)</td>
<td>4-38</td>
<td>&lt;0.01-&gt;0.55</td>
<td>Methanol, MMA, DMA, TMA, acetate, H₂/CO₂</td>
<td>Freshwater lake sediments</td>
</tr>
<tr>
<td>Methanosarcina siciliaeᵃ</td>
<td>25-45 (40)</td>
<td>0-1.7 M (0.4-0.6)</td>
<td>TMA, methanol, DMS</td>
<td>Marine sediments</td>
</tr>
<tr>
<td>Methanosarcina acetivoransᵇ</td>
<td>15-45 (35-40)</td>
<td>0.1 -1 (0.1-0.6)</td>
<td>Methanol, MMA, TMA, acetate</td>
<td>Marine sediments</td>
</tr>
<tr>
<td>Methanosarcina thermophilaᶜ</td>
<td>35-55 (50)</td>
<td>ND</td>
<td>Methanol, MMA, TMA, acetate, H₂/CO₂</td>
<td>Anaerobic digester sludge</td>
</tr>
<tr>
<td>Methanosarcina semesiaeᵈ</td>
<td>15-39 (30-35)</td>
<td>0.1-1 (0.2-0.6)</td>
<td>Methanol, MMA, TMA, DMS</td>
<td>Mangrove sediments</td>
</tr>
<tr>
<td>Methanosarcina balticaᵉ</td>
<td>4-27 (25)</td>
<td>0.2-1.2 (0.3-0.4)</td>
<td>Methanol, MMA, TMA, acetate</td>
<td>Marine sediments</td>
</tr>
<tr>
<td>Methanosarcina vacuolataᶠ</td>
<td>18-42 (37-40)</td>
<td>ND</td>
<td>Methanol, MMA, DMA, TMA, acetate, H₂/CO₂</td>
<td>Anaerobic digester sludge</td>
</tr>
<tr>
<td>Methanosarcina spelaehᵍ</td>
<td>0-54 (33)</td>
<td>0.02-&gt;0.6 (0.05)</td>
<td>Methanol, MMA, DMA, TMA, acetate, H₂/CO₂</td>
<td>Biofilm on subsurface</td>
</tr>
<tr>
<td>Methanosarcina barkerʰ</td>
<td>25-50 (45)</td>
<td>0.1-0.6 (0.1-0.2)</td>
<td>Methanol, MMA, DMA, TMA, acetate, H₂/CO₂</td>
<td>Sewage sludge</td>
</tr>
<tr>
<td>Methanosarcina horonobensisᵢ</td>
<td>20-42 (37)</td>
<td>0-0.35 (0.1)</td>
<td>Methanol, DMA, TMA, acetate, DMS</td>
<td>Deep, subsurface aquifer</td>
</tr>
<tr>
<td>Methanosarcina lacustrisiᵣ</td>
<td>1-35 (25)</td>
<td>ND</td>
<td>Methanol, MMA, DMA, TMA, methanol, H₂/CO₂</td>
<td>Freshwater lake sediments</td>
</tr>
<tr>
<td>Methanosarcina mazeʰ</td>
<td>25-48 (40-42)</td>
<td>0.1-1 (0.1-0.3)</td>
<td>Methanol, MMA, DMA, TMA, acetate, H₂/CO₂</td>
<td>Sewage sludge</td>
</tr>
<tr>
<td>Methanosarcina soligelidiᵣ</td>
<td>0-54 (28)</td>
<td>0.02-0.6 (0.2)</td>
<td>Methanol, acetate, H₂/CO₂</td>
<td>Permafrost soil</td>
</tr>
</tbody>
</table>


† Optima shown in brackets.
7.5.1. Phylogenetic affiliation of *Methanosarcina* strain CB05

Strain CB05 clustered alongside *Methanosarcina soligelidi* and *Methanosarcina mazei* within the genus *Methanosarcina* (Figure 7.0.9.). Strain CB05 had 100 % 16s rRNA similarity to *Methanosarcina* strain TMA3RMK (KC989921) which was detected in an anaerobic digester in India and *Methanosarcina* strain BEG3 (KC989921) which was detected in a biogas plant in Germany. The type strain with most similar 16s RNA gene sequence was *Methanosarcina mazei* type strain DSM 2053 (99%; AB973358) isolated from sewage sludge (Mah and Kuhn 1984). Strain CB04 had 100 % mcrA gene sequence similarity to *Methanosarcina mazei* strain MT (AY260440) isolated from permanently cold tundra wetland soil (Simankova et al. 2003) and *Methanosarcina mazei* strain NBRC 101201 (AB703645; Mori and Suzuki, unpublished data), but no mcrA sequence was available for the *Methanosarcina mazei* species type strain.

![Phylogenetic tree](image)

*Figure 7.0.9. 16s rRNA phylogenetic tree (neighbour-joining) showing placement of strain CB05 within the genus Methanosarcina.*

Numbers represent % of bootstrap values based on 1000 replicates. Bootstrap values <50 % are excluded. Accession numbers are given in brackets. Scale bar represents 0.01 base changes.
7.5.2. Temperature characteristics of *Methanosarcina* strain CB05

Strain CB05 produced methane at 4 and 38°C, but not at 46°C. The temperature range of strain CB05 indicated that, in common with most species of *Methanosarcina* (exceptions are *Methanosarcina baltica*, *Methanosarcina soligelidi*, and *Methanosarcina lacustris*) strain CB05 is a mesophile. However the temperature minima for strain CB05 (4°C) was significantly lower than for other mesophilic species of *Methanosarcina* (usually 15-20°C). Several novel species of *Methanosarcina* have recently been isolated from low temperature freshwater environments which have mesophilic temperature optima (>25 °C) but which are able to withstand temperatures as low as 0°C. *Methanosarcina soligelidi* was isolated from permafrost affected soil in north eastern Siberia, and had a temperature range of >54 °C (Wagner et al. 2013). *Methanosarcina spelaei*, isolated from a floating biofilm in a subsurface lake in Romania (water temperature 22-24 °C), also has temperature range >54°C (Ganzert et al. 2014). In addition, Simankova et al. (2003) isolated a novel ecotype of *Methanosarcina mazei* (strain MT) able to tolerate low temperatures (optima 35 °C, range 5-40 °C) from permanently cold permafrost soil in Russia.

7.5.3. Salinity characteristics of *Methanosarcina* strain CB05

Strain CB05 produced methane at 0.01, 0.05 and 0.55 M NaCl. *Methanosarcina* species are usually able to tolerate a wide range of salinities (often <0.1 - >1 M NaCl, Table 7.0.5.), with the exception of *Methanosarcina horonobensis* which has a low salinity maxima (0.35 M NaCl). However the minimum NaCl concentration tested in studies describing *Methanosarcina* type strain species is 0.1 M NaCl, with the exception of *Methanosarcina soligelidi* (Wagner et al. 2013) and *Methanosarcina spelaei* (Ganzert et al. 2014) which were tested to a minimum of 0.02 M. It is therefore of interest that strain CB04 tolerated salinities as low as 0.01 M NaCl.

7.5.4. Substrate characteristics of *Methanosarcina* strain CB05

Strain CB05 used methylated amines (MMA, DMA, TMA), methanol, acetate and H₂CO₂ for growth. *Methanosarcina mazei* strain MT is also able to utilise all of these substrates. *Methanosarcina* are nutritional generalists, and often capable of utilising methylated compounds, acetate and H₂/CO₂ for growth.
7.6. Physiological characteristics of *Methanothermobacter* strain CB06

Table 7.0.1 lists the temperature, salinity and metabolic characteristics of strain CB06 and species type strains belonging to the genus *Methanothermobacter*.

7.6.1. Phylogenetic affiliation of *Methanothermobacter* strain CB06

Strain CB06 has 99% 16s rRNA gene similarity to *Methanothermobacter thermautotrophicus* type strain Delta H (AE000666), *Methanothermobacter defluvii* type strain ADZ (NR_028248), *Methanothermobacter marburgensis* type strain Marburg (X15364), *Methanothermobacter wolfeii* type strain DSM 2970 (NR_040964), *Methanothermobacter thermophilus* type strain M (NR_028250) and *Methanothermobacter thermoflexus* type strain IDZ (NR_028249). Strain CB05 had 96% mcrA gene similarity to *Methanothermobacter marburgensis* type strain Marburg (AB842181; Mori and Suzuki, unpublished data). Figure 7.1.0 shows the phylogenetic placement of strain CB06 within the genus *Methanothermobacter*.

![Figure 7.1.0. 16s rRNA phylogenetic tree (neighbour-joining) showing placement of strain CB06 within the genus Methanothermobacter](image)

*Figure 7.1.0. 16s rRNA phylogenetic tree (neighbour-joining) showing placement of strain CB06 within the genus Methanothermobacter*

Numbers represent % of bootstrap values based on 1000 replicates. Bootstrap values <50% are excluded. Accession numbers are given in brackets. Scale bar represents 0.02 base changes.

7.6.2. Temperature characteristics of *Methanothermobacter* strain CB06

Strain CB06 produced significant methane at 25°C and 77°C. Only a small amount of methane (<300 ppm) was produced at 15°C, and no methane production occurred at 88°C. Strain CB06 had a significantly lower temperature minima than most
**Methanothermobacter** species type strains. The lowest temperature minima of a *Methanothermobacter* type strain species is 34°C (*Methanothermobacter wolfeii*), and all other species type strains have a temperature minima of 45°C. The temperature maxima of strain CB06 is within the typical range for *Methanothermobacter* type strain species (65-80°C). Overall strain CB06 has a very wide temperature range (>52°C).

7.6.3. Salinity characteristics of *Methanothermobacter* strain CB06

Strain CB06 produced methane at 0.01, 0.05 and 0.55 M NaCl, in common with most *Methanothermobacter* type strain species (with the exception of (*Methanothermobacter defluvii, Methanothermobacter tenebrarum, and Methanothermobacter wolfeii)*).

7.6.4. Substrate characteristics of *Methanothermobacter* strain CB06

Strain CB06 was only able to utilise H₂/CO₂, and did not require acetate or yeast extract for growth. Most *Methanothermobacter* type strain species are only able to utilise H₂/CO₂, and the ability to utilise formate as a substrate varies between strains of the same species (Wasserfallen et al. 2000).

7.7. Discussion

7.7.1. Identity of isolates

All of the methanogen strains isolated from Cardiff Bay sediments were closely related (98-99% 16s rRNA gene sequence fragment) to existing cultivated methanogen species. It is not possible to identify the Cardiff Bay strains at species level from 16s rRNA (~900 bp) or mcrA (~700 bp) gene fragment sequence analysis alone. The range of within-genus sequence similarity is 88.2-99.9 % for the 16s rRNA gene and 69.2-100% for the mcrA gene (Springer et al. 1995; Steinberg and Regan 2008). The 16s rRNA gene sequence identity threshold widely used to identify prokaryotes at species level is 97 % (Stackebrandt and Goebel 1994), however 16s rRNA gene fragments obtained from Cardiff Bay isolates often showed >97 % similarity to 3-5 different species. In addition, most strains showed maximum 16s rRNA sequence similarity to more than one species type strain. For this reason DNA-DNA hybridisation is often used to determine new species, as the genomic relatedness between strains can be below the species threshold (70%; Tindall et al. 2010) even when the 16s rRNA sequence similarity >97 %. For example the type strain for *Methanosarcina soligelidi* (SMA-21ᵀ)
has 99.9% 16s rRNA sequence similarity to the type strain for *Methanosarcina mazei* (DSM 2053\textsuperscript{T}), but only 22 % genomic relatedness (Wagner et al. 2013).

The mcrA sequence cut-off for methanogen species is less well defined. Springer et al. (1995) found that the average sequence distance between species of *Methanosarcina* is approximately 3 times higher for the mcrA gene than the 16s rRNA gene, and Hunger et al. (2011) suggest that 85.5 % mcrA sequence identity is equivalent to 97 % 16s rRNA gene sequence identity. Both Steinberg and Regan (2008) and Yang et al. (2014) suggest that an mcrA sequence similarity cut-off value of 84 % provides maximum agreement with previously defined 16s rRNA gene thresholds. As for the 16s rRNA gene sequence analysis, all Cardiff Bay methanogen strains showed >84 % sequence similarity to more than one species type strain. All except one of the Cardiff Bay strains showed highest mcrA gene sequence similarity to one particular species type strain, with the exception of strain CB01 which had 98 % similarity to 2 different closely related species of *Methanobacterium*.

Strains CB01, CB02, CB04 and CB05 could be identified to species level using a combination of phylogenetic and physiological characterisation data, although each of the strains isolated from Cardiff Bay had slightly different physiological or metabolic characteristics than the type strain for their species. Phylogenetic (16s rRNA and mcrA gene fragments) and physiological (temperature, salinity, substrates) characterisation suggest that CB01 was a strain of *Methanobacterium bryantii*, CB02 was a strain of *Methanobacterium formicicum*, and CB04 was a strain of *Methanolobus profundi*. However there is no clear evidence that strain CB01 utilised primary or secondary alcohols for growth, whereas the type strain for *Methanobacterium bryantii* is able to oxidize 2-propanol, 2-butanol and cyclopentanol, but does not oxidize methanol. It is not known whether type strain for *Methanobacterium bryantii* can oxidize ethanol. The phylogenetic and physiological characteristics of strain CB05 are almost identical to *Methanosarcina mazei* strain MT, a psychrotolerant ecotype of *Methanosarcina mazei* isolated from permafrost soil (Simankova et al. 2003).

The species affiliation of strains CB03 and CB06 remains uncertain. For strain CB03 there was little similarity between the *Methanobacterium* type strains with most similar 16s rRNA sequence (*Methanobacterium palustre* and *Methanobacterium formicicum*) and the type strain species with most similar mcrA sequence (*Methanobacterium*
Intriguingly the 16s rRNA nucleotide sequence obtained from strain CB03 had <95 % similarity to any strain of *Methanobacterium congolense* recorded in the NCBI database. Strain CB03 had a wider salinity range and higher temperature optima than *Methanobacterium palustre*, which supports placement within the species *Methanobacterium congolense* as indicated by mcrA analysis. However strain CB03 produced small quantities of methane from formate and from a methanol/ethanol mixture, which are not reported as substrates for *Methanobacterium congolense* (Cuzin et al. 2001). Conversely strain CB03 did not produce methane when amended with an isopropanol/isobutanol/cyclopentanol mixture, whereas *Methanobacterium congolense* is able to oxidize each of these alcohols (Cuzin et al. 2001). There is no published information about the salinity range or optima of *Methanobacterium congolense* available for comparison with the salinity characteristics of strain CB03. *Methanothermobacter* species differ little in their physiological traits or 16s rRNA gene sequences (with the exception of *Methanothermobacter crinale* and *Methanothermobacter tenebrarum*), and species definitions are predominantly based on cell morphology and protein or antigen fingerprinting (Wasserfallen et al. 2000; Ding et al. 2010). The 16s rRNA gene sequence identity and physiological characteristics (excluding growth temperature minima) of strain CB06 were consistent with both *Methanothermobacter thermautotrophicus* and *Methanothermobacter marburgensis*, however strain CB06 showed greater mcrA gene sequence similarity to *Methanothermobacter marburgensis* than *Methanothermobacter thermautotrophicus*.

### 7.7.2. Environmental relevance of isolates

In general the phenotypic traits (temperature and salinity range, substrate utilisation) of the methanogen strains isolated from Cardiff Bay were strongly representative of the phenotypic traits characteristic of the genus to which they belong. For example, the genus *Methanobacterium* consists of mesophilic hydrogenotrophic species, and all three *Methanobacterium* isolates (CB01, CB02, and CB03) were mesophiles which utilised H₂/CO₂ (and sometimes formate) for growth. The genus *Methanolobus* contains obligate methylotrophs and strain CB04 was only able to utilise methylated substrates for growth; whereas the genus *Methanosarcina* contains nutritional generalists, and strain CB05 was able to utilise methylated compounds, acetate and H₂/CO₂ for growth. The genus *Methanothermobacter* contains hydrogenotrophic
thermophiles, and strain CB06 has temperature and substrate characteristics consistent with this description.

Four out of five Cardiff Bay isolates (all except strain CB01) are able to tolerate both freshwater (0.01M NaCl) and marine (0.55M NaCl) salinities, suggesting that they would be able to tolerate the salinity transition caused by impoundment of Cardiff Bay (~27.3 ‰ to <2.1 ‰, approximately equivalent to 0.4 M to 0.01 M NaCl) and could be of estuarine or marine origin. The specific growth rate of Methanobacterium strains CB02 and CB03 varied little throughout the salinity range 0.01-0.55 M NaCl (Figures 7.0.6. and 7.0.7.), suggesting that these strains are halotolerant. However, biomass production was notably enhanced at salinities >0.26 M NaCl for strain CB03 (Figure 7.0.7.), which could suggest that strain CB03 is slightly halophilic. Surprisingly, Methanobacterium strain CB01 is the only non-halophile strain isolated from Cardiff Bay sediments (Figure 7.0.5.).

Physiological characterisation of Methanolobus strain CB04 (Table 7.0.4.) confirms that it is able to tolerate the low freshwater salinities now present in Cardiff Bay sediments (0.01-0.04 mM Cl–). Methanolobus strain CB04 differs from Methanolobus profundus in that MMA was the only methylamine utilised for methane production. Methanosarcina horonobensis and Methanolobus chelungpuianas are both methylotrophic methanogen species which can utilise DMA or TMA but not MMA, but to date (01/04/2015) there are no recorded incidences of methylotrophic methanogen strains able to utilise MMA but not DMA or TMA. The ability to utilise DMA or TMA but not MMA for growth may be linked to higher energetic yield of DMA and TMA. Methanogenic disproportionation of trimethylamine gives a free energy yield of -191 kJ per mol of substrate, compared to -92 kJ per mol substrate for methylamine. The biochemical pathway of methylamine disproportionation has been studied extensively in the genera Methanosarcina and Methanococcoides. Methanosarcina and Methanococcoides have substrate specific methyltransferases to initiate methanogenesis from methylamines; MtmB for MMA, MtbB for DMA, and MttB for TMA (Galagan et al. 2002; Williams et al. 2010). These methyltransferases catalyse activation and transfer of a methyl group from methylated amines to a Co (I) cognate corrinoid protein, generating a methylated Co (III) cognate corrinoid protein (Lessner 2001). Each methyltransferase has a separate cognate corrinoid protein; MtmC, MtbC and MttC for MMA, DMA and TMA respectively. The corrinoid proteins are
demethylated by a single enzyme (MtbA; CoM methyltransferase) to produce methyl-CoM. From this point onwards disproportionation of the different methylamines follows a common biochemical pathway. *Methanolobus psychrophilus* strain R15 has the mtmB, mtbB and mttB genes (Jiang 2013; Chen et al. 2014), however methylamine disproportionation has been little studied for the genus *Methanolobus*. *Methanococcoides burtonii* strain DSM6242 and *Methanosarcina acetivorans* strain C2A have 2 or 3 paralogs of the genes which encode each different methyltransferase (Deppenmeier et al. 2002; Galagan et al. 2002; Li et al. 2005a, b; Williams et al. 2010). It is thought that multiple gene copies result from past duplication events, and do not provide a selective advantage to the organisms which carry them (Li et al. 2005a). Williams et al. (2010) found that only 2 out of 3 mttB paralogs and 1 out of 2 mtbB paralogs are utilized during TMA demethylation by *Methanococcoides burtonii* strain DSM62.

Strain CB05 presumably either does not have the mtbB and mttB genes encoding DMA and TMA methyltransferases, or is unable to express them. Lyimo et al. (2000) found that cells of *Methanosarcina semesiae* MD1T grown on dimethylsulphide and transferred to methanol or TMA (or vice versa) exhibited a lag phase of up to 15 days before methane production began. This was presumably the time required for synthesis of the relevant methyltransferases. However no lag phase occurred when *Methanosarcina semesiae* MD1T cells grown on TMA were transferred to DMA or MMA (Lyimo et al. 2000). Strain CB04 cultures were incubated for 3 months in the presence of DMA and TMA, which is ample time for synthesis of the appropriate methyltransferases, with no evidence of methane production. One hypothesis could be that strain CB05 has lost the ability to utilise DMA and TMA as a result of competition with *Methanomethylovorans* spp. under low salinity conditions in situ. Members of the genus *Methanomethylovorans* also utilise methylamines and methanol for methanogenesis, but have lower salinity optima and maxima (<0.1 M and <0.4 M NaCl respectively, Lomans et al. 1999; Jiang et al. 2005; Cha et al. 2013) than *Methanolobus profundii* (0.5 and 1.2 M NaCl respectively, Mochimaru et al. 2009), and originate from freshwater environments. *Methanomethylovorans* outcompeted *Methanolobus* for methylamine at low salinities in salinity gradient enrichments (section 4.4.1.), suggesting *Methanolobus* strain CB05 may also be outcompeted for higher energy yield methylamines under environmental conditions.
Interestingly, the temperature optima of *Methanobacterium* strains CB01, CB02 and CB03 (either 38-40 or 44 °C; Table 7.0.2) was higher than environmental temperature range for Cardiff Bay (maximum 21.7 °C). This is of particular interest for these strains, as *Methanobacterium* is the dominant genus in Cardiff Bay sediments under *in situ* conditions, and may help explain the strong increase in methane production rate with increasing temperature between 0-25°C (Figure 5.1.1., Chapter 5). The phenomenon of temperature optima of a strain being higher than the *in situ* temperature range has previously been recorded for methanogen strains isolated from cold and temperate environments (e.g. Harris et al. 1984; Franzmann et al. 1977). *Methanothermobacter* strain CB06 produced methane at temperatures as low as 15 °C (Table 7.0.1.), providing an explanation for how this thermophilic strain could survive in Cardiff Bay sediments. Wiegel (1990) also isolated a strain of *Methanothermobacter thermautotrophicus* (JW501) able to produce methane at 15 °C, although methane production was not accompanied by growth at temperatures below 18 °C. If this were also the case for strain CB06, it would indicate an allochthonous rather than autochthonous origin for strain CB06 in Cardiff Bay sediments.

### 7.7.3. Comparison of temperature and salinity characteristics of specific growth rate and biomass production in isolated strains

The specific growth rate of *Methanobacterium* species type strains generally ranges between ~0.43 day⁻¹ (*Methanobacterium petrolearium*; Mori and Harayama 2011) to ~1.2 day⁻¹ (*Methanobacterium beijingense*; Ma et al. 2005), although Kotelnikova et al. (1998) report a maximum value of 9.6 day⁻¹ for *Methanobacterium subterraneum*. The maximum specific growth rate for Cardiff Bay strains CB01, CB02, and CB03 (1.8, 1.2 and 1.6 day⁻¹ respectively, Figures 7.0.2.-7.0.4.) are relatively high for *Methanobacterium*, although direct comparison of rates is not possible unless cultivation conditions are carefully standardised (Leigh 2011). Biomass production data was not converted into specific growth yield for comparison with previous studies, as it is well documented that the growth yield of hydrogenotrophic methanogens varies depending on H₂ availability (De Poorter et al. 2007). When growth rate and biomass production experiments are carried out in batch culture, as for this study, hydrogen partial pressures alter throughout the duration of incubation.

The temperature maxima for average biomass production is lower than the average specific growth rate temperature maxima for strains CB01 and CB03. In addition, strain
CB01 did not show a defined temperature peak as seen for specific growth rate values (Figure 7.0.2.). Instead average biomass production remained relatively stable throughout the temperature range of exponential growth (31-37°C). The average biomass production maxima (46.7 °C) for strain CB02 occurred at a slightly higher temperature than the average specific growth rate temperature maxima (40.2 °C), however biomass production values for strain CB02 (Figure 7.0.3.) varied even more between replicates than for strains CB01 or CB03 (Figures 7.0.2 and 7.0.4. respectively). Maximum biomass production for strains CB01, CB02 and CB03 occurred at a lower temperature than either maximum average biomass production or maximum average specific growth rate. All cultures were sampled for biomass after a set period of incubation, usually 3-5 days after exponential phase had ceased in one or more cultures. Therefore, perhaps cell death outweighed cell production during this period in those cultures which first exited the exponential phase of growth, leading to the appearance of a decrease or plateau in biomass production at the optimum temperature for specific growth rate.

It is also well documented that methanogens without cytochromes (which includes the Methanobacteriales; Jussofie and Gottschalk 1986) are able to decouple anabolism and catabolism. This occurs when rates of catabolism are too low to support biosynthesis (Schill et al. 1996; Schill et al. 1999), where biosynthesis is limited by availability of nutrients other than hydrogen (e.g. iron; Liu et al. 1999), and at high hydrogen concentrations where “specific maintenance” requirements and degree of proton leakage and slippage processes are relatively higher than under low hydrogen conditions (De Poorter et al. 2007). However, catabolic rates are relatively high throughout these temperature ranges, and hydrogen availability did not vary between cultures. A second possibility is that nutrient availability is the limiting factor on biomass production throughout these temperature ranges, causing the plateau in biomass production seen for strain CB01. If this were the case, then the temperature maxima for specific growth rate presumably demonstrates where maximum biomass production would occur if all necessary nutrients were available in unlimited supply. Strains CB02 and CB03 had biomass production maxima closer to the specific growth rate maxima than strain CB01, suggesting that whichever nutrient is limiting for CB01 biomass production had less impact on strains CB02 and CB03. The Methanobacterium are predominantly autotrophs, but some species benefit from addition of acetate as a
carbon source. For example *Methanobacterium espanolae* is stimulated by acetate (Patel et al. 1993). In addition, *Methanobacterium alcalophilum* and *Methanobacterium beijingense* require yeast extract for growth (Worakit et al. 1986; Ma et al. 2005). Perhaps lack of acetate or yeast extract limited the rate of biomass production in strain CB01 cultures, particularly around the optimum temperatures.

It is also notable that relatively high biomass values occurred at low and high temperatures (below and above the maximum temperature where methane production occurred in an exponential manner) in the CB01 and CB02 temperature profiles (Figures 7.0.2. and 7.0.3.). Limited methane production also occurred outside the temperature range of exponential methane production, therefore it is feasible that some biomass production could have occurred at these temperatures. Also sample protein concentrations were very low (<1.5 µg/ml) at temperatures <25 °C and >51°C for strains CB01 and CB02 (Figures 7.0.2. and 7.0.3.). Protein standards (generated at 0, 1, 2.5, 5, 7.5 and 10 µg/ml) produced a linear calibration between 0 and 10 µg/ml, however the variation between standard replicates is noticeably greater at 2.5 µg/ml and below (see Appendices 4, 5, and 10). Perhaps the assay method or spectrophotometer is not suitable for precise quantification of very low protein concentrations.

Average specific growth rate and average biomass production profiles were rather different in the CB02 salinity profile (Figure 7.0.6.). The specific growth rate of strain CB02 varied little with salinity between 0.01 and 0.55 M NaCl, yet there was a distinct minima in biomass production between 0.26 and 0.36 M NaCl which was not present in the specific growth rate profile. The fact that exponential methane production occurred within this salinity range strongly suggests that biomass production must have occurred in these cultures, hence the apparent absence of biomass production is probably a measurement error. Biomass concentrations in the CB02 salinity gradient range from 2.5-7.5 µg/ml, comfortably within the mid-range of concentrations tested using protein standards, suggesting an error may lie in protein extraction rather than detection.

Average specific growth rate and average biomass production values were not consistent between temperature and salinity gradient profiles for a given strain (Figures 7.0.1. – 7.0.7., summarised in Table 7.0.6.). Specific growth rate was substantially
higher in temperature gradient profiles than salinity gradient profiles for all 3 strains when incubated under equivalent temperature and salinity conditions, and vice versa for biomass production (with the exception of strain CB03 where biomass production values were very similar). The most likely explanation for increased specific growth rate values in temperature gradient cultures is that vials used for temperature characterisation experiments have a slightly wider circumference than the vials used for salinity gradient characterisation (diameter of 23 mm compared to 18 mm). The increased area of interface between media and headspace gas atmosphere might have enabled a faster rate of gas drawdown, potentially sustaining faster rates of methanogenesis. In addition, cells tend to cluster around FeS particles in media, which settle to the bottom of culture tubes during incubation unless vigorously agitated. Therefore there is approximately 5.2 cm depth of media between the gas headspace and majority of cells in salinity gradient culture tubes, and 3.2 cm depth of media in temperature gradient culture tubes. As hydrogen has low solubility (0.8 mM at 1 bar, 20°C), very small differences in cultivation conditions may have a significant effect on hydrogen dissolution kinetics and thus on hydrogen availability to cells.

Reduced biomass production in temperature gradient cultures compared to salinity gradient cultures also was probably a consequence of the different culture vials used for temperature and salinity characterisation. CB01 and CB02 salinity gradient cultures had a relatively short exponential phase (3-4 days) followed by a period of linear growth which continued for >10 days (data shown in Appendices 5 and 7). In contrast, CB01 and CB02 temperature gradient profiles and CB03 salinity and temperature gradient profiles had an exponential phase of approximately 5-6 days and then entered the

| Table 7.0.6. Comparison of specific growth rate and biomass production in temperature and salinity characterisation experiments for strains CB01, CB02 and CB03 |
|---------------------|------|------|------|
|                     | CB01 | CB02 | CB03 |
| **Temperature profile** |      |      |      |
| Specific growth rate (day⁻¹) | 1.8  | 1.1  | 0.6  |
| Biomass production (µg/ml)  | 8.8  | 14.0 | 11.2 |
| **Salinity profile** |      |      |      |
| Specific growth rate (day⁻¹) | 1.4  | 0.7  | 0.4  |
| Biomass production (µg/ml)  | 10.3 | 20.8 | 12.1 |

N.B. Specific growth rate and biomass production values shown for strains CB01 and CB02 were measured at at 0.06 M NaCl/38 °C during salinity testing, and 0.05 M NaCl/37.6 °C during temperature testing. Specific growth rate and biomass production values shown for strain CB03 were measured at 0.01 M NaCl/25 °C during salinity testing and 0.01 M NaCl/28.1 °C during temperature testing.
stationary phase (data shown in Appendices 6, 9, 11 and 12). A linear growth pattern usually indicates that either substrate, nutrient, or space availability is limiting growth (De Poorter et al. 2007). Media of identical composition was used for salinity and temperature characteristics cultures, and the vial headspaces of salinity and temperature gradient cultures were amended with H₂/CO₂ to an equal partial pressure. However, CB01 and CB02 temperature gradient cultures did not have a linear methane production phase. Biomass production still occurs in the linear methane production phase, therefore the overall period during which biomass production occurred in the CB01 and CB02 temperature gradient cultures (5-6 days) is much shorter than in the CB03 temperature gradient cultures and all salinity gradient cultures (13 days plus). This may explain why overall biomass production was lower in the CB01 and CB02 temperature gradient cultures than in the corresponding salinity gradient cultures despite extra 2 days of exponential growth. Alternatively perhaps the lower area of interface between media and headspace gas atmosphere in salinity gradient cultures induced more direct coupling between anabolism and catabolism, hence increasing biomass production rates. Many studies have shown that the growth yield of hydrogenotrophic methanogens grown under laboratory conditions is higher under hydrogen limited conditions than when hydrogen is present under excess (Schönheit et al. 1980; Tsao et al. 1994; Schill et al. 1996; Liu et al. 1999; De Poorter et al. 2007).

There was significant variability in specific growth rate and biomass production between replicates for all three isolates. This was partly due to low biomass production which could not be accurately measured, and may also be linked to variation in the biomass with which culture tubes were originally inoculated. As stated previously, cells tend to cluster around FeS particles present in media. Tubes of growing cultures used to inoculate temperature and salinity characterisation cultures were shaken thoroughly before extraction of media, but it is still very difficult to ensure even distribution of cells between each inoculum. Batch cultivation using anaerobic tubes has been demonstrated to be an accurate and effective method of investigating biomass production for methanogens which utilise dissolved substrates (e.g. Watkins et al. 2012; Watkins et al. 2014), however Leigh (2011) observe that it is difficult to prevent variation in methane and biomass production in batch cultures supplied with gaseous substrates even when gassing supply is constant and cultures are agitated during incubation. Cultures were not agitated during incubation in this study, nor the
headspace gas (\(\text{H}_2:\text{CO}_2\)) continuously exchanged, hence the observed variability in specific growth rate and biomass production between replicates.

7.8. Conclusions

16s and mcrA gene sequence analysis in combination with physiological and metabolic characterisation strongly suggests that all strains isolated from Cardiff Bay sediments belong to previously isolated methanogen species. However several isolates, particularly those not commonly detected in temperate freshwater sediments (\textit{Methanolobus} and \textit{Methanothermobacter} strains), have atypical physiological characteristics which may enable their survival in Cardiff Bay sediments. \textit{Methanothermobacter} strain CB06 and \textit{Methanosarcina} strain CB05 have lower temperature minima than their respective species type strains. \textit{Methanothermobacter} strain CB06 produced methane at 25°C (but not 15°C), whereas the minimum temperature of the most closely related \textit{Methanothermobacter} type strain species is 34°C. This adaptation could enable viable cells to survive in temperate sediments for long periods of time. \textit{Methanosarcina} strain CB05 is psychrotolerant and active even at 4°C, whereas the species type strain for \textit{Methanosarcina mazei} is mesophilic. \textit{Methanolobus} strain CB04 has a minimum salinity of 0.01 M, lower than most previously isolated \textit{Methanolobus} strains, and is only able to utilise methylamine as a substrate. It is not clear how the loss of ability to utilise more energetically favourable substrates such as DMA and TMA would be advantageous, and perhaps \textit{Methanolobus} strain CB04 is outcompeted for TMA and DMA by other methylotrophic methanogens present in Cardiff Bay sediments which have freshwater salinity optima (such as \textit{Methanomethylovorans} spp. and \textit{Methanosarcina mazei}). All isolates could tolerate NaCl concentrations corresponding to Cardiff Bay water salinity under both current and pre-impoundment conditions (0.01-0.46 M NaCl). \textit{Methanobacterium} strains CB02 and CB03 were classified as halotolerant, and were also able to tolerate full marine conditions. In contrast \textit{Methanobacterium} strain CB01 was non-halophilic and the only isolate not able to tolerate full marine salinities. Interestingly, whilst \textit{Methanobacterium} was the dominant genus in Cardiff Bay sediments, all three \textit{Methanobacterium} isolates were mesophilic and showed limited only very slow growth at temperatures within the environmental range for Cardiff Bay (<19 °C). Specific growth rate and biomass production showed comparable temperature or salinity distributions for strains CB01 and CB03, however the salinity distribution of biomass
production for strain CB02 did not correspond with specific growth rate values. The reason for this discrepancy is unknown. In general there was significant variability in biomass production (and to a lesser extent specific growth rate) between replicates for hydrogenotrophic strains. This demonstrates the complications inherent in assessing growth kinetics of methanogen strains which utilise gaseous substrates, and the difficulties in obtaining accurate measurements of biomass when biomass production rates are very low.
8. GENERAL DISCUSSION AND CONCLUSIONS

8.1. Introduction and aims of study
Cardiff Bay is an unusual aquatic habitat, having recently undergone rapid and
dramatic environmental change. The impoundment of Cardiff Bay in 2001 transformed
100 ha of estuarine intertidal mudflats into an artificial freshwater lake. The overarching
aim of this study was to determine how the sedimentary methanogenic community in
Cardiff Bay has developed as a result of impoundment, and also to investigate the
impact of future climate change. More specifically, the objectives were to determine
how transition from estuarine to freshwater salinities has impacted on the types of
methanogens present, their substrates, and their temperature and salinity
characteristics.

This was achieved through:

- comparison of the salinity and temperature functional operating range and
  optima of methanogens and methanogenic (methanogens plus syntrophs)
  communities in sediments deposited pre- and post-impoundment (marine clay
  and freshwater gyttja respectively).
- enrichment and isolation of methylotrophic, acetotrophic and hydrogenotrophic
  methanogens representative of the salinity and temperature ranges of Cardiff
  Bay sedimentary methanogenic communities.
- physiological and metabolic characterisation of methanogens isolated from
  Cardiff Bay sediments.

8.2. Cardiff Bay as a potential source of atmospheric methane emissions
Sediment geochemical and cultivation results demonstrate that an active and
culturable methanogen population has been established in Cardiff Bay sediments
despite the significant environmental changes wrought by impoundment and the
relative newness of the lacustrine gyttja sediments. The total (hydrogenotrophic plus
acetotrophic) rate of methanogenesis in sediment cores from Cardiff Bay taken during
January 2012 is comparable with maximum rates in similar meso- and eutrophic lake
sediments (Thomas 2014). This is surprising given that prior to impoundment the
dominant anaerobic terminal oxidation process in Cardiff Bay sediments would likely
have been sulphate reduction, as it is in intertidal sediments of the Severn Estuary
(Wellsbury et al. 1996; Thomas 2014). In addition, the silty surface layer of the
estuarine mudflats present in Cardiff Bay prior to impoundment was removed by dredging during construction of the barrage. Therefore, the lacustrine gyttja sediments deposited post-impoundment may be lying directly above an ancient estuarine clay layer laid down 200-500 years BP (section 3.6.1.). Rates of methanogenesis in the estuarine clay layer are currently very low (Figure 3.0.6., Chapter 3), and hence, the potential it has as an inoculum and source of methanogens for the overlying lacustrine gyttja sediments is presumably limited.

Porewater methane concentrations in the lacustrine gyttja layer are substantially higher in cores taken during June 2012 and April 2014 than in cores taken in January 2011 (Figure 3.0.8., Chapter 3), suggesting that in common with most natural methanogenic environments (Yvon-Durocher et al. 2014) rates of methanogenesis increase seasonally with temperature. Porewater methane concentrations exceeded theoretical saturation limits at environmentally relevant temperatures (5-15 °C) in the estuarine clay layer in January 2012 and in the lacustrine gyttja layer in April 2014 and June 2012 (Figure 3.0.8., Chapter 3), indicating that ebullition may be an important pathway for atmospheric methane emissions from Cardiff Bay. A study by Zhu et al. (2012) demonstrated that methanogens can rapidly colonize and become active in newly settled sediments, and it seems that this is also the case in Cardiff Bay.

Experimental evidence from sediment microcosms shows that the methane production potential of the newly deposited lacustrine gyttja sediments is ten-fold higher than the methane production potential of the estuarine clay sediments (Table 4.0.7., Chapter 4). However, whether the increased methanogenic potential of the lacustrine gyttja sediments deposited post-impoundment translates into increased atmospheric methane emissions is uncertain without further work (discussed further in section 8.9.5). There is a growing body of evidence that shallow, freshwater environments are much more important contributors of atmospheric methane emissions than previously thought (Bastviken et al. 2008; Downing 2010). In particular, recent investigation into methane production in artificial impounded freshwater reservoirs have shown that such environments can be methane production and emission ‘hotspots’ (DelSontro et al. 2010; Sobek et al. 2012; Maeck et al. 2013). This is important as the number of freshwater lake impoundments continues to grow globally (Downing et al. 2006; Chao et al. 2008). The role of freshwater lakes, both impounded and natural, to global atmospheric methane emissions is expected to increase in the future as a result of
Chapter 8 – General Discussion and Conclusions

rising atmospheric temperatures (Tranvik et al. 2009). Experimental results from this study support this assumption as methane production in Cardiff Bay sediments showed a very strong positive relationship with temperature (Figure 5.1.1, Chapter 5) over the environmentally relevant temperature for climate change (increase of 1-3.7 °C for 2081-2100, Stocker et al. 2014). Experimental results also indicated that this positive relationship would be enhanced by increased supply of labile, autochthonous organic matter (Figure 5.1.1, Chapter 5), which is pertinent to similar, non-managed environments as freshwater lakes are susceptible to global-warming induced eutrophication (Jeppesen et al. 2010).

8.3. Hydrogenotrophic methanogenesis is the dominant pathway for methane production in Cardiff Bay sediments

8.3.1. Factors determining the relative contribution of hydrogenotrophic and acetotrophic methanogenesis in freshwater environments

The factors which determine the relative importance of hydrogenotrophic and acetotrophic methanogenesis in lake sediments remain unclear (Conrad et al. 2011). Whiticar et al. (1986) hypothesized that the extent of alteration of sedimentary organic matter by sulphate reducing bacteria (SRB) determines whether acetotrophic or hydrogenotrophic methanogenesis is the dominant pathway for methane production in sedimentary environments. They suggested that SRB outcompete methanogens for acetate in marine environments, and typically exhaust the available pool of acetate-producing organic matter within the sediment sulphate reduction zone. For this reason, hydrogenotrophic methanogenesis is the dominant pathway for methane production in marine sediments (Liu and Whitman 2008). In freshwater environments, where sulphate availability is typically very limited, acetate is normally the major precursor for methane production. However this study, and several other studies of methanogenesis in freshwater lakes (Smith et al. 1993; Eller et al. 2005; Karr et al. 2006; Conrad et al. 2011; Mandic-Mulec et al. 2012), have shown that hydrogenotrophic methanogenesis may be the dominant pathway for methane production in some environments with very limited sulphate availability.

The main factors thought to control methanogenesis are temperature and the concentration and quality of organic matter (Schulz and Conrad 1996; Conrad 2005). Several authors have suggested that hydrogenotrophic methanogenesis predominates
in freshwater environments which receive limited supply of labile organic matter (Hornibrook et al. 2000; Conrad 2005) or where substrate availability is limited by the activity of fermentative and syntrophic bacteria (Conrad et al. 2011). Thomas (2014) argued that limited availability of labile organic matter explains why hydrogenotrophic methanogenesis is the dominant pathway for methane production in Cardiff Bay sediments. Cardiff Bay is a managed environment, and potential sources of labile organic matter such as algal blooms are rapidly removed from the water surface if present. In addition, Dwyr Cymru Welsh Water (DCWW) significantly reduced the nutrient content of treated effluent discharged into the rivers Taff and Ely following designation of Cardiff Bay as ‘sensitive water’ under the Urban Waste Water Treatment Directive (UWWTD) in 2002 (Cardiff Harbour Authority 2012). This was expected to reduce the incidence of algal blooms in Cardiff Bay, and none has been observed since the early 2000’s (Lee In prep.) In addition, Cardiff Bay is flushed regularly during winter to maintain optimum water levels (average residence time of 3.3 days in 2008; Alix 2010), meaning there is little time for algal blooms to develop.

Studies of methane production in lake sediments have shown that labile, autochthonous sources of organic matter stimulate methanogenesis to a greater extent that allochthonous, terrestrially derived organic matter (Schwarz et al. 2008; West et al. 2012). Cardiff Bay is designated eutrophic according to both water chemistry conditions (period 2001-2005; Vaughan et al. 2008) and diatom assemblage data (Jüttner et al. 2010), and surface sediments contain a high content of leaf litter and other plant debris. However, plant debris is largely composed of lignin and cellulose and is relatively recalcitrant to bacterial degradation (e.g. Ruiz-Dueñas and Martínez 2009). The predominance of recalcitrant organic matter in Cardiff Bay sediments may explain why rates of hydrogenotrophic methanogenesis exceed rates of acetotrophic methanogenesis in Cardiff Bay sediments.

PCR-DGGE analysis of the methanogen community composition in Cardiff Bay sediments shows that hydrogenotrophic Methanobacterium spp. represent a significant proportion of the methanogen community at all sediments depths (Figure 3.0.7., Chapter 3), yet Methanobacterium spp. are thought to be adapted to high substrate conditions (Chan et al. 2005; Lu et al. 2005; Karadagli and Rittmann 2007; Ye et al. 2009; Borrel et al. 2011). Perhaps the Methanobacterium population is supported by the high quantities of recalcitrant organic matter present in lacustrine
gyttja sediments. It is notable that *Methanobacterium* was also the only methanogen genus detected in unamended temperature gradient microcosms incubated at 4-38 °C (Figures 5.0.7, -5.1.0., Chapter 5) and salinity gradient microcosms (incubated at 25 °C) after a 2 year incubation period (Table 4.0.7, Chapter 4). Substrate availability in unamended microcosms was presumably extremely limited after such a lengthy incubation period. This could suggest that in contrast to previous studies, the *Methanobacterium* populations native to Cardiff Bay sediments are well adapted to very low substrate conditions. Alternatively, it is possible that *Methanobacterium* spp. were dominant numerically in microcosms but were not representative of the active methanogen populations.

The increase in relative contribution of acetoclastic methanogenesis with increasing sediment depth is more difficult to explain. Rates of both hydrogenotrophic and acetotrophic methanogenesis decrease significantly between the lacustrine gyttja and estuarine clay layers (Figure 3.0.6., Chapter 3), but the decrease in hydrogenotrophic methanogenesis is more marked. There is a 20-fold decrease in the average rate of hydrogenotrophic methanogenesis between lacustrine gyttja (taken as 0-25 cm depth) and estuarine clay sediments (>25 cm depth), compared to only a 6 fold decrease in the average rate of acetotrophic methanogenesis. This corresponds to a significant increase in the relative contribution of acetoclastic methanogenesis from <6 % in the lacustrine gyttja sediments to ~33 % in the estuarine clay sediments. The increase in the relative contribution of acetotrophic methanogenesis with sediment depth contradict results of previous studies, which generally show that the relative contribution of acetotrophic methanogenesis decreases with increasing sediment depth in freshwater lakes (Chan et al. 2005; Lofton et al. 2014). Assuming that the hydrogenotrophic *Methanobacterium* populations native to Cardiff Bay sediments are supported by the high availability of degraded plant material present in the lacustrine gyttja sediments, then it makes sense that the relative contribution of hydrogenotrophic methanogenesis is lower in the estuarine clay sediments as degraded plant matter was not observed in the estuarine clay layer during coring.

The link between labile organic matter availability and relative contribution of hydrogenotrophic and acetotrophic methanogenesis in freshwater sediments has been disputed by a number of studies. For example, Mandic-Mulec et al. (2012) found that hydrogenotrophic methanogenesis was the dominant pathway for methane production
in temperate sediments of Lake Bled, Slovenia despite the availability of labile organic matter. A combination of lipid biomarker and geochemical evidence suggests that methanogens are in competition with SRB for acetate in Lake Bled sediments (Mandic-Mulec et al. 2012), which may partially explain why hydrogenotrophic methanogenesis is dominant in Lake Bled sediments. Porewater sulphate depth profiles in Cardiff Bay sediments are indicative of sulphate consumption (Figure 3.0.5., Chapter 3), yet porewater sulphate concentrations are low (<0.08 mM) compared to other freshwater systems (typically 0.1-0.2 mM, Borrel et al. 2011). Therefore, it seems unlikely that sulphate reduction is the major pathway for acetate consumption in Cardiff Bay sediments. Peatlands are also low salinity methanogenic environments characterised by recalcitrant organic matter. Whilst hydrogenotrophic methanogenesis is typically dominant in acidic ombrotrophic bogs, acetotrophic methanogenesis is dominant in minerotrophic fens (Galand et al. 2005; Bridgham et al. 2013). It is sometimes argued that that pH is major controlling factor on the relative contribution of acetotrophic and hydrogenotrophic methanogenesis in ombrotrophic bogs and minerotrophic fens (Kotsyurbenko et al. 2007), but experimental evidence suggests that this is not the case (Ye et al. 2012). Unfortunately porewater pH in Cardiff Bay sediments was not measured, however it seems unlikely that the Cardiff Bay sediments are particularly acidic.

Temperature is another factor thought to play a role in determining the relative contribution of acetotrophic and hydrogenotrophic methanogenesis in low salinity environments. The activity of \( \text{H}_2 \)-supplying fermentative and syntrophic bacteria in lake sediments increases with temperature (Falz et al. 1999), indirectly stimulating hydrogenotrophic methanogenesis (Lofton et al. 2015). Conversely, heterotrophic acetogens are more active at temperatures <20 °C, which likely stimulates the activity of acetotrophic methanogens (Fey and Conrad 2000). It is notable that many of the lake and estuarine sediments where hydrogenotrophic methanogenesis is the major pathway for methane production (Nüsslein et al. 2001; Conrad et al. 2011) or the sediment methanogen community is dominated by hydrogenotrophic methanogen genera (Abreu et al. 2001; Singh et al. 2010b) are located in sub-tropical or tropical geographic zones. However, temperature is clearly not the defining factor in determining the relative contribution of hydrogenotrophic and acetotrophic methanogenesis in freshwater lake sediments as hydrogenotrophic methanogenesis
also prevails over acetotrophic methanogenesis in lake sediments in polar (Smith et al. 1993) and temperate (Lovley et al. 1982; Eller et al. 2005; Mandic-Mulec et al. 2012) freshwater lakes. Hydrogenotrophic methanogen genera (Methanobacterium or Methanothermobacter) were dominant in Cardiff Bay sediment slurries and microcosms at temperatures ranges from 0-65 °C (Figures 5.0.3.-5.1.0., Chapter 5), affirming that temperature is not the major control on the relative contribution of acetotrophic and hydrogenotrophic in Cardiff Bay sediments.

8.3.2. Importance of syntrophic processes involving hydrogenotrophic methanogens

The importance of hydrogenotrophic methanogenesis is reinforced by cultivation based experiments which indicated that a methanogenic consortia consisting of methylamine or acetate oxidizing bacteria and hydrogenotrophic methanogens were able to outcompete specialist methylotrophic or acetotrophic methanogens respectively under certain conditions (discussed in sections 6.5.4. and 6.5.5.). Preferential enrichment of methylamine and acetate utilising methanogenic consortia occurred at mesophilic and thermophilic temperatures (38-66 °C) in both estuarine clay and lacustrine gyttja temperature gradient cultivation series (Tables 6.0.5. and 6.0.6., Chapter 6). Preferential enrichment of methylamine utilising methanogenic consortia over methylotrophic methanogens was also seen at 25 °C, but only in the lacustrine gyttja cultivation series incubated under freshwater conditions (Table 6.0.3, Chapter 6). The factors promoting hydrogenotrophic methanogenesis over acetotrophic methanogenesis in select low salinity environments are not well understood. Recent studies have indicated that geochemical factors such as ammonium, acetate and carbon dioxide concentration, pH, and temperature are important in determining the pathway of acetate consumption in anaerobic methanogenic environments. Environmental factors known to favour syntrophic acetate oxidation over direct oxidation include; high temperatures (Hattori 2008), high ammonia concentrations (Schnurer et al. 1999; Karakashev et al. 2006; Schnurer and Nordberg 2008), low (Petersen and Ahring, 1991) or high (Hao et al. 2011) acetate concentrations, and also high pH conditions (Hao et al. 2013). One potential explanation is that high ammonium, carbon dioxide and acetate concentrations caused by cultivation conditions may have contributed to the dominance of syntrophic oxidation processes in Cardiff Bay mesophilic and thermophilic cultivation series amended with acetate (discussed in
Another hypothesis is that the apparent prevalence of syntrophic processes involving acetate, and also possibly methylamine, in Cardiff Bay mesophilic and thermophilic methanogenic communities may somehow be linked to dominance of hydrogenotrophic methanogenesis in sediments. Those factors which result in the dominance of hydrogenotrophic methanogenesis in Cardiff Bay sediments (and perhaps other low salinity environments) might also support the dominance of syntrophic processes over direct methanogenic utilisation at mesophilic and thermophilic temperatures. Perhaps enhanced sensitivity of Cardiff Bay bacterial communities to salinity (relative to methanogens, discussed in section 4.4.5.) has temporarily disrupted the structure and function of the anaerobic prokaryotic community, altering normal carbon and electron flow pathways. Finally, perhaps low hydrogen availability in Cardiff Bay sediments promotes syntrophic acetate oxidation over direct acetate utilisation. The $\Delta G'$ for syntrophic acetate oxidation increases at low hydrogen partial pressures (Hattori et al. 2008), whereas low concentrations of hydrogen have been shown to inhibit acetotrophic methanogens in pure culture experiments (Ferry et al. 1993). Various lines of evidence suggest that a high proportion of the organic matter in Cardiff Bay sediments is of low ‘quality’ (discussed in section 8.3.1.) suggesting that hydrogen availability may be limited.

8.3.3. Role of methylotrophic methanogenesis in Cardiff Bay sediments
Interestingly, pyrosequencing analysis of the methanogen community composition of Cardiff Bay sediments (Gordon Webster, unpublished data) showed that members of the methylotrophic Methanomassiliicoccales are abundant (46 % of methanogen OTUs) in Cardiff Bay lacustrine gyttja sediments. However, Methanomassiliicoccales spp. were not cultivated from Cardiff Bay sediments, perhaps due to the range of cultivation conditions utilised (section 6.5.1.). It is also unclear whether the Methanomassiliicoccales are active in Cardiff Bay sediments under in situ conditions, or whether they are simply representative of faecal contamination from agricultural land and water treatment effluent inputs to the Taff and Ely rivers. To date, cultivated strains belonging to the order Methanomassiliicoccales have been enriched or isolated from a very narrow range of gastrointestinal-type habitats, including human faeces, anaerobic digester sludge, and the intestinal tract of termites (Borrel et al. 2012b; Dridi et al. 2012; Borrel et al. 2013a; lino et al. 2013). The Methanomassiliicoccales are one of the three methanogen types dominant in rumen environments (Janssen and Kirs
However, several studies have pointed out that the environmental distribution of phylotypes affiliated with the Methanomassiliicoccales also includes rice paddy fields, natural wetlands, subseafloor and freshwater sediments (Paul et al. 2012; Borrel et al. 2013b; Iino et al. 2013; Poulsen et al. 2013).

All isolated or enriched strains of Methanomassiliicoccales reduce methylated amines or methanol using hydrogen as an electron donor (Lang et al. 2015). Bacterial degradation of plant matter (Wang and Lee 1994), specifically lignin (Donnelly and Dagley 1980) and pectin (Schink and Zeikus 1980), is a potential source of methylated amines and methanol in freshwater environments. Hence the high quantities of degraded plant matter observed in Cardiff Bay lacustrine gyttja sediments (section 3.3.) are a potential source of substrate for Methanomassiliicoccales in this freshwater environment. However, it seems likely that in common with most freshwater environments (Lovley and Klug 1983b; Conrad and Claus 2005), methylotrophic methanogenesis is only a very minor pathway for methane production in Cardiff Bay sediments. Porewater concentrations of methylamines were below minimum detection levels in Cardiff Bay sediments (section 3.5.5.), indicating that their availability under in situ conditions is low. Dimethylamine turnover was broadly comparable with acetate turnover in freshwater salinity gradient microcosms, however trimethylamine (TMA), dimethylamine (DMA), and monomethylamine (MMA) concentrations were below minimum detection limits in salinity gradient slurries. This indicates that rates of methylotrophic methanogenesis were significantly lower than hydrogenotrophic methanogenesis in freshwater salinity gradient microcosms. Overall it seems that, although numerically significant, methylotrophic Methanomassiliicoccales are not major contributors to methane production in Cardiff Bay sediments.

### 8.4. Phylogenetic and physiological diversity of methanogens in Cardiff Bay sediments

Cultivation based studies of methanogenesis in natural environments are restricted, partly due to the perceived difficulty and laborious nature of culturing under strict anaerobic conditions. In addition, the diversity of methanogen strains enriched and isolated is often very low (e.g. Großkopf et al. 1998; Orphan et al. 2000; Kendall and Boone 2006; Kendall et al. 2007). This study used a wider range of incubation conditions (temperature, salinity, and substrate) than typically used in cultivation studies, which enabled a far wider range of methanogen genera to be cultivated from
Cardiff Bay sediments. The phylogenetic diversity of methanogen taxa detected included 4 out of a total of 7 known methanogen orders. The physiological diversity includes taxa active at temperatures from 4 °C to 77 °C and salinities from freshwater (0.01 M NaCl) to marine (0.55 M NaCl). Studies investigating the microbial community of an environment often describe the phylogenetic diversity as ‘high’ (Fierer and Lennon 2011), yet many of the taxa detected are only present in very low abundance. Bacterial communities typically consist of a relatively low number of abundant taxa and a relatively high number of rare taxa which are often termed the “rare biosphere” (Sogin et al. 2006; Lynch and Neufeld 2015). The ‘everything is everywhere, but the environment selects’ hypothesis (Baas-Becking, 1934; referenced in De Wit and Bouvier 2006) supports the existence of a microbial seed bank of dormant organisms which is often invoked to explain cultivation of thermophilic bacteria from cold and temperate soils and sediments (Hubert et al. 2010; Portillo et al. 2012) and marine bacterial taxa from water and soils of freshwater environments and air (Comte et al. 2014; Zhang et al. 2014).

The ecological function of the rare biosphere is uncertain. In some environments, such as soil, a large proportion of taxa are dormant under the current environmental conditions, but can be revived if and when conditions become favourable (Lennon and Jones 2011; Aanderud et al. 2015). However, other studies have found that at least some members of the rare biosphere are active under in situ conditions (Pedrós-Alió 2012; Hugoni et al. 2013). The existence of dormant taxa in a microbial community is often referred to as the ‘microbial seed bank’, and is thought to be important for maintaining ecosystem diversity (Jones and Lennon 2010) and resilience to environmental change (Lennon and Jones 2011). This study shows that rare taxa (not identified in sediment using PCR-DGGE, and therefore presumably <1-2 % abundance in the template DNA extraction; Muyzer et al. 1993; Murray et al. 1996; Stephen et al. 1999) reflect the survival of methanogens capable of growth at thermophilic temperatures (≥ 65°C) and under marine salinities in temperate, freshwater Cardiff Bay sediments.

Comte et al. (2014) argue that the ‘marine’ bacteria enriched from freshwater lake waters and sediments were generalist taxa which are most commonly found in marine environments but are also able to survive under low salinity conditions. Comte et al. (2014) also observed that the average niche breadth of taxa present in cultures
increased at higher salinities. This description of marine ‘generalist’ taxa could also be applied to the *Methanolobus* isolate (methanogen strain CB04) obtained from Cardiff Bay sediments, as CB04 was active under both freshwater (0.01 M NaCl) and marine (0.55 M NaCl) conditions. *Methanolobus* are one of the most commonly detected genera in marine sediments (Ferry 1993), but evidently extremely rare in freshwater lake sediments (Borrel et al. 2011). Enrichment of *Methanolobus* strains from Cardiff Bay lacustrine gyttja sediment suggests *Methanolobus* spp. may be part of the ‘rare biosphere’ in freshwater environments. As *Methanolobus* are rarely detected in freshwater lake sediments (Borrel et al. 2011, Table 1) their presence may reflect the marine history of Cardiff Bay sediments, and/or continuous inoculation of Cardiff Bay sediments with marine methanogen communities.

Thermophilic bacteria may survive in soil and surface sediment environments either by (i) very slow growth at low temperatures, or (ii) short periods of rapid growth when environmental conditions are favourable, perhaps for a short or seasonal period annually (Portillo et al. 2012) or within temporary microniches created by thermal energy release during intense microbial degradation of organic matter (Wiegel and Kevbrin 2004). In contrast, thermophilic endospores in permanently cold sediments have been described as ‘misplaced organisms’ which are supposedly introduced to the seafloor by geofluids originating from deep, hot petroleum bearing sediments or crustal sediments (Hubert et al. 2010). Thermophilic methanogens can also be readily cultivated from temperate environments (Nozhevnikova et al. 1997; Fey et al. 2001; Wu et al. 2006). It is difficult to explain the presence of thermophilic methanogens in temperate environments as methanogens are not known to form spores or resting stages, although apparently capable of dormancy for extended periods of time (Zinder 1993; Rothfuss et al. 1997). A thermophilic methanogen strain belonging to the genus *Methanothermobacter* isolated from Cardiff Bay sediments (strain CB06) had an exceptionally large temperature range (≥15 - ≤77 °C; Table 7.0.5., Chapter 7), and hence could well be active *in situ*. Such a large temperature range is not unique for *Methanothermobacter* strains (also reported by Wiegel 1990), but it is not known whether this temperature flexibility also applies to the thermophilic methanogen strains belonging to the genera *Methanocella*, *Methanogenium*, *Methanosarcina* and *Methanothrix* which were also enriched from Cardiff Bay sediments at 55 and 66 °C (Tables 6.0.5. and 6.0.6., Chapter 6).
Since impoundment the rivers Taff and Ely have been the major sources of water and sediment to Cardiff Bay. There is a growing body of evidence to suggest that the microbial community of lake waters is structured by the microbial community of upstream soils and waterbodies. Crump et al. (2012) characterised the archaeal and bacterial microbial community along a hydrological continuum spanning from soil waters and headwaters to lake waters in arctic tundra, and found that microbial diversity in downstream waters was structured by inoculation from soils and species sorting processes. Besemer et al. (2013) and de Oliveira and Margis (2015), who also conducted longitudinal studies of bacterial diversity in fluvial networks, reiterate the importance of source headwaters in maintaining bacterial diversity in aquatic waters. Whether the same structuring processes apply to soil and sediment microbial populations, including obligate anaerobes such as methanogens, is not known. However large scale metagenomics studies have shown that there is substantial overlap between the prokaryotic communities of soil and freshwater aquatic environments, despite their differing habitat characteristics (Lozupone and Knight 2007; Tamames et al. 2010). This is consistent with their conclusions that salinity is the dominant environmental structuring influence on prokaryotic community.

8.5. Physiological flexibility of Cardiff Bay methanogens

All of the methanogen strains obtained in monoculture or pure culture in this study are closely related to previously isolated and characterised methanogen species type strains (Tables 6.0.7.-6.0.9., Chapter 6). However in many cases, the environmental conditions of Cardiff Bay sediments do not correspond with the known temperature or salinity ranges of the closely related methanogen type strains. The unexpectedly low temperature minimum of *Methanothermobacter* strain CB06, and unexpectedly low salinity minimum of *Methanolobus* strain CB04, are described above. The *Methanobacterium* strains (methanogen strains CB01, CB02, CB03) isolated from Cardiff Bay sediments had temperature optima significantly higher than the *in situ* temperature range for Cardiff Bay (6.5-18.1 °C; Lee et al. In Prep). However, all three isolated also had a temperature minima which was lower than typical of closely related *Methanobacterium* species, and fell within the *in situ* temperature range for Cardiff Bay (Table 7.0.2., Chapter 7). Similarly, 2 out of the 3 Methanobacterium strains isolated from Cardiff Bay sediments (CB02 and CB03) were able to tolerant marine salinities (0.55 M), which is also relatively unusual for members of the *Methanobacterium* (Table
7.0.2., Chapter 7). Again, this demonstrates the ability of these methanogen strains to survive under apparently unfavourable conditions and adapt to environmental change.

8.6. Factors influencing the depth dependent variation of the methanogen community in Cardiff Bay sediments

Overall, results indicate that the in situ and culturable methanogen community of lacustrine gyttja and estuarine clay sediments are very similar in composition, and in their salinity and temperature range and characteristics. However, cultivation based experiments revealed that there are some subtle differences in between the methanogen community composition and activity of lacustrine gyttja and estuarine clay sediments. Many of the differences in composition and function between lacustrine gyttja and estuarine clay methanogen communities appear to be predominantly driven directly or indirectly by contaminant loading from agricultural activities upstream and/or the difference in organic matter content of the lacustrine gyttja and estuarine clay sediments. Cardiff Bay receives both direct inputs of faecal contamination from combined sewer overflows (CSOs), and also likely indirect inputs from agricultural land and water treatment effluent discharges to the Taff and Ely rivers. Another important factor is depth variation in substrate availability. There is a significant decrease in organic matter content with increasing sediment depth from ~17% in the lacustrine gyttja sediments to ~7% in the estuarine clay sediments (Figure 3.0.4., Chapter 3). The organic matter content and lability of sediments usually decreases with sediment depth (Schulz and Zabel 2006), but in this case the reduction is exaggerated by the difference in ages and sources (increased proportion of terrestrial organic matter) of the lacustrine gyttja sediments and estuarine clay sediments. The lacustrine gyttja sediments were all deposited <15 years ago, whilst the estuarine clay sediments do not even represent the surface sediments of the original intertidal mudflats present in Cardiff Bay prior to impoundment, and may in fact be much older (<200 to >5,000 years BP, section 3.4.1.) subsurface sediments deposited under estuarine conditions.

The significant decrease in organic matter content between lacustrine gyttja and estuarine clay sediments is reflected in reduced methane production rates, both in in situ and in temperature and salinity gradient sediment slurries. Hydrogenotrophic and acetotrophic methane production rates in Cardiff Bay sediments are dramatically lower in the estuarine clay lithology than lacustrine gyttja lithology (Figure 3.0.6., Chapter 3). This decrease in methane production rate with increasing sediment depth is also
consistently observed in amended and unamended slurries and enrichments in the salinity and temperature gradient experiments. Methane production is consistently more rapid in both the amended and unamended lacustrine gyttja (0-5 cm) temperature gradient slurries (Figures 5.0.3. and 5.0.5., Chapter 5) than estuarine clay (25-30 cm) temperature gradient slurries (Figures 5.0.4. and 5.0.6., Chapter 5). Final methane concentrations in amended and unamended lacustrine gyttja temperature gradient microcosms were also higher than in the corresponding estuarine clay microcosms at all temperatures (Figures 5.0.7. - 5.1.0., Chapter 5). Also, in general, methane production was more rapid in the lacustrine gyttja (0-5 and 10-15 cm) salinity gradient enrichments than the corresponding estuarine clay enrichments (Tables 4.0.1. - 4.0.3., Chapter 4). Final methane concentrations in the freshwater and brackish lacustrine gyttja salinity gradient microcosms (which were not amended with methanogen substrates) were an order of magnitude higher in the estuarine clay microcosms, even after 2 years incubation (Table 5.0.7., Chapter 5). More rapid methane production in lacustrine gyttja substrate amended salinity and temperature gradient short-term incubation enrichments suggests that the size of the in situ methanogen community is greater in lacustrine gyttja sediments than estuarine clay sediments, again this probably reflects depth dependent differences in substrate concentrations. The relative contribution of these two factors (contaminant loading and organic matter quantity and lability) to observed differences in the methanogen community composition and structure of Cardiff Bay lacustrine gyttja and estuarine clay sediments are discussed below.

Impoundment of Cardiff Bay will have increased the residence of time of river waters within the area, and removed the diluting effect of the tidal Severn Estuary waters. The main difference between the in situ sediment methanogen community composition of lacustrine gyttja and estuarine clay sediments is the decrease in the relative abundance of Methanomassiliicoccales with increasing sediment depth (from 46 % of the methanogen phylotypes at 0-5 cm depth to 3 % at 25 cm depth; Gordon Webster, unpublished data). Methanomassiliicoccales spp. are probably not active in Cardiff Bay sediments (section 8.3.2.), and the reduction in relative abundance of Methanomassiliicoccales with depth in Cardiff Bay sediments may be linked to increased retention time and reduced dilution following impoundment. The main difference between the methanogen community of lacustrine gyttja and estuarine clay
salinity gradient enrichments was that *Methanobrevibacter* spp. were detected in lacustrine gyttja enrichments of all salinities, but were not present in clay enrichments under any salinity/substrate combination. As discussed in section 4.4.3. *Methanobrevibacter* are typically found in gastrointestinal tracts of animals and humans and are characterised by high concentrations of volatile fatty acids (Liu and Whitman 2008). In fact, *Methanobrevibacter* species have repeatedly been suggested as a ‘microbial source tracker’ for ruminant (Ufnar et al. 2007) and human fecal (Johnston et al. 2010; Ahmed et al. 2011) contamination of surface and recreational waters as they are so rarely detected in pristine lake and coastal sediments.

The estuarine clay layer (25-30 cm sediments) of Cardiff Bay sediments have slightly higher salinity than 0-5 cm sediments (Figure 3.0.5., Chapter 3), and also lower organic matter content (Figure 3.0.4., Chapter 3). It is unlikely that salinity is the discriminating factor for the depth distribution of *Methanobrevibacter* in Cardiff Bay sediments, as *Methanobrevibacter* strains were detected in marine salinity gradient enrichments (Tables 4.0.4.-4.0.6.). As previously mentioned *Methanobrevibacter* spp. are typically found in high substrate environments, therefore perhaps substrate availability controls the depth distribution of *Methanobrevibacter* in Cardiff Bay sediments. *Methanobrevibacter* phylotypes detected in salinity gradient enrichments were closely related to *Methanobrevibacter aboriphilus*, as was a *Methanobrevibacter* strain obtained in monoculture from Cardiff Bay (section 6.5.2). *Methanobrevibacter aboriphilus* was originally isolated from decaying plant material (Asakawa et al. 1993), and Cardiff Bay sediments contain a significant amount of degraded plant material in the top 5 cm sediment depth (section 3.3.1) which could explain the unusual presence of *Methanobrevibacter* in freshwater sediments. Therefore the depth distribution of *Methanobrevibacter* also likely reflects both impoundment conditions (as for Methanomassiliicoccales), but also depth variation in organic matter content.

The temperatures profiles of dominant members in lacustrine gyttja and estuarine clay methanogen communities were very similar, however there were significant differences in the temperature distribution of more minor community members both in short term (temperature gradient slurries) and long term (temperature gradient microcosms) incubation experiments. The relative abundance of *Methanosarcina* and *Methanoculleus* differed between lacustrine gyttja and estuarine clay temperature gradient enrichments. The mixotrophic genus *Methanosarcina* was identified in high
relative abundance (>30 %) at mesophilic and thermophilic temperatures in clay microcosms, coincident with rapid methylamine depletion (Figures 5.0.3. and 5.0.5., Chapter 5). However, *Methanosarcina* were not detected in the lacustrine gyttja slurries, although methylamine and acetate (potential substrates for *Methanosarcina*) temperature profiles were very similar in clay and gyttja sediment slurries (Figures 5.0.2. - 5.0.5., Chapter 5). In addition, the relative abundance of *Methanosarcina* in lacustrine gyttja sediments was higher than in estuarine clay sediments (6 % and 1% of methanogen OTUs respectively; Gordon Webster, unpublished data). *Methanosarcina* spp. are adapted to high substrate conditions (Jetten et al. 1992), and therefore it makes sense that the *in situ* relative abundance of *Methanosarcina* was higher in lacustrine gyttja sediments than estuarine clay sediments. This suggests that perhaps *Methanosarcina* were not detected in lacustrine gyttja sediments by PCR-DGGE because the total abundance of archaeal DNA was higher in lacustrine gyttja sediments than estuarine clay sediments. Rates of both hydrogenotrophic and acetotrophic methanogenesis decrease substantially between lacustrine gyttja and estuarine clay sediments (Figure 3.0.6., Chapter 3), and presumably the size of the total methanogen population decreases concordantly.

The presence of *Methanoculleus* spp. in the 0-5 cm unamended temperature gradient slurries only is less clear cut. The genus *Methanoculleus* was detected in low relative abundance (8% of genus relative abundance according to PCR-DGGE analysis) at 52.4 °C in the unamended gyttja temperature gradient slurries (Figure 5.0.4., Chapter 5) and also at <1 % relative abundance at psychrophilic temperatures in the gyttja amended and unamended temperature gradient slurries (Figures 5.0.3 and 5.0.5., Chapter 5). *Methanoculleus* spp. were not detected in the estuarine clay temperature gradient slurries (Figures 5.0.4. and 5.0.6., Chapter 5), nor microcosms from either sediment type (Figures 5.0.7. – 5.1.0., Chapter 5). The genera *Methanoculleus* and *Methanothermobacter* (dominant in thermophilic Cardiff Bay slurries and microcosms; Figures 5.0.3.-5.1.1., Chapter 5) are frequently a major constituent of the methanogen community in thermophilic anaerobic digesters (Hori et al. 2006; Kobayashi et al. 2008; Sasaki et al. 2011; Wagner et al. 2011). Their presence in these environments is indicative of an adaptation to relatively high substrate, high temperature environments. Adaptation to high substrate availability might explain why *Methanoculleus* spp. were not enriched from estuarine clay sediments, as *in situ* substrate availability in estuarine
clay sediments is likely lower than in lacustrine gyttja sediments. Similarly, substrate availability in both lacustrine gyttja and estuarine clay temperature gradient microcosms was likely very low after 2 years incubation, hence *Methanoculleus* spp. may have been outcompeted by methanogens better adapted to low substrate concentrations during the incubation period.

Fluctuating VFA (Volatile Fatty Acid) and hydrogen concentrations affect methanogen community dynamics within high substrate environments. Hori et al. (2006) investigated microbial community succession in a thermophilic anaerobic digester, and found that the methanogen community composition was linked to VFA (specifically propionate and acetate) concentration. The genera *Methanoculleus*, *Methanothermobacter* and *Methanosarcina* were all present during the initial period of incubation, during which time VFA concentrations were relatively low for anaerobic digester sludge, and *Methanoculleus* was most abundant out of these three genera. During the period of acidification (decreasing pH and sharp increase in acetate and propionate concentrations) there was a dramatic increase in the relative abundance of *Methanothermobacter* spp. and decrease in the relative abundance of *Methanoculleus* spp. The opposite pattern was observed in relative abundance of both *Methanoculleus* spp. and *Methanothermobacter* spp. once the slurry pH had been stabilised, and VFA concentrations began to decrease once again. The authors suggest that relatively low, for anaerobic digester sludge, VFA (acetate concentration <1 mM) and hydrogen concentrations promote growth of *Methanoculleus* spp. over *Methanothermobacter* spp. Acetate concentrations were significantly higher in the lacustrine gyttja and estuarine clay amended temperature gradient slurries at the final sampling time point (1.8 and 3.4 M respectively at 53.8 °C; Figures 5.0.3. and 5.0.4., Chapter 5) than in unamended temperature gradient slurries (0.06 M and 0.08 M respectively at 52.4 °C; Figures 5.0.4. and 5.0.5., Chapter 5) due to significant thermophilic acetogenesis in slurries amended with H₂/CO₂. Perhaps growth of *Methanoculleus* spp. was selected for by relatively low (< 1 mM) acetate concentrations in unamended temperature gradient slurries. Hence, enrichment of *Methanoculleus* spp. from Cardiff Bay lacustrine gyttja sediments (0-5 cm depth) was controlled by both in situ substrate availability (higher in lacustrine gyttja sediments, hence higher in situ relative abundance of *Methanoculleus* spp. in lacustrine gyttja sediments) and cultivation
conditions (lower VFA and acetate concentrations in unamended temperature gradient slurries than amended temperature gradient slurries).

The methanogen community composition of temperature gradient microcosms incubated for a 2 year period also differed with sediment depth. The genus *Methanocella* was detected in high relative abundance (48 % of relative band surface area; Figure 5.0.9., Chapter 5) at 55 °C unamended clay microcosm, but were only present at <5 % relative band surface area in the amended and unamended gyttja microcosms or the amended clay microcosms. There is significant experimental evidence to suggest that the *Methanocellales* are adapted to low substrate concentrations (Lu et al. 2005; Conrad et al. 2006; Sakai et al. 2007; Sakai et al. 2009; Lu and Lu 2012). Methanogen communities in the unamended estuarine clay microcosms would have experienced more severe substrate limitation than microcosms which were amended with methanogen substrates prior to incubation (amended microcosms), or the 0-5 cm microcosms as lacustrine gyttja sediments contained high organic matter concentrations (~17 % wt ; Figure 3.0.4., Chapter 3) and degraded plant matter. Low substrate availability may have selected for *Methanocella* spp. in the unamended clay microcosms more strongly than in the amended microcosms or the unamended lacustrine gyttja microcosm, although *Methanocella* spp. are also clearly present in the lacustrine gyttja sediments as they appeared in 0-5 cm cultivation series at 5th subculture and monoculture stage (Table 6.0.5.).

Differences between the lacustrine gyttja and estuarine clay methanogen communities are more evident from parallel lacustrine gyttja and estuarine clay cultivation series at the later stages (i.e. 5th subculture and monoculture/pure culture) of cultivation than at the initial stages of cultivation (i.e. temperature gradient slurries/salinity gradient enrichments). The increasing selection pressure from cultivation conditions throughout the cultivation process, from enrichment to monoculture or pure culture, reinforces differences in the physiology of methanogen communities in the lacustrine gyttja and estuarine clay sediments and in competitive interactions with bacterial syntrophs and other methanogens. *Methanomethylovorans* spp. and *Methanobacterium* spp. were obtained in monoculture from the freshwater estuarine clay cultivation series amended with methylamine and hydrogen respectively, however *Methanobrevibacter* were obtained from the corresponding lacustrine gyttja cultivation series. As discussed above the depth distribution of *Methanobrevibacter* is linked to both impoundment
conditions and sediment organic matter. Where present, *Methanobrevibacter* are apparently able to outcompete *Methanomethyllovorans* for methylamine (presumably aided by a methylamine degrading bacterial population) and *Methanobacterium* for \( \text{H}_2/\text{CO}_2 \) under laboratory conditions (Tables 5.0.5. and 5.0.6., Chapter 5). Whether this is also the case under in Cardiff Bay sediments under *in situ* conditions is not known as *Methanobacterium* was the only genus detected in Cardiff Bay sediments using PCR-DGGE (Figure 3.0.7., Chapter 3). *Methanosarcina* spp. were obtained in monoculture from the lacustrine gyttja methylamine and acetate cultivation series incubated at 38 °C (Table 6.0.5., Chapter 6), whereas *Methanomethyllovorans* spp. and *Methanothrix* spp. were obtained from the corresponding estuarine clay methylamine and acetate cultivation series respectively (Table 6.0.6., Chapter 6). Cultivation of *Methanosarcina* from the lacustrine gyttja lithology and *Methanothrix* spp. from the estuarine clay lithology makes sense considering their respective growth strategies (discussed above).

8.7. Marine legacy evident in methanogen community of sediments deposited pre-impoundment

There is also some limited evidence for salinity control on the methanogen community of lacustrine gyttja and estuarine clay sediments. There were consistent differences in the dominant methanogen genera at 5th subculture in 4 °C and 10 °C cultivation series. *Methanosarcina* and *Methanoregula* were the dominant genera in the 4 °C and 10 °C lacustrine gyttja 5th subcultures amended with \( \text{H}_2/\text{CO}_2 \) respectively (Table 6.0.5., Chapter 6), whereas *Methanocorpusculum* was the dominant genus in the corresponding estuarine clay 5th subcultures amended with \( \text{H}_2/\text{CO}_2 \) (Table 6.0.6., Chapter 6). *Methanoregula* was only enriched from the lacustrine gyttja sediments, possibly reflecting the highly limited salinity range tolerated by members of this genus (discussed in section 6.5.3.). The maximum salinity tolerated by *Methanoregula* species type strains are 0.05 M (*Methanoregula boonei*; Bräuer et al. 2011) and 0.17 M NaCl (*Methanoregula formicica*; Yashiro et al. 2011), which although greater than the current porewater salinity of the estuarine clay lithology (max. 0.04 M, equivalent to ~2.1 ‰; Figure 3.0.5., Chapter 3), is significantly lower than the porewater salinity of intertidal mudflat sediments located immediately adjacent to the seawards side of Cardiff Bay barrage (23.7 ‰; Thomas 2014). In contrast, members of *Methanocorpusculum* often have brackish salinity optima of ~0.2-0.25 M, and are
active at marine salinities (Zellner et al. 1987; Zellner et al. 1989; Zhao et al. 1989), with the exception of *Methanocorpusculum aggregans* (Ollivier et al. 1985). Methylamine utilising methanogen populations active under marine conditions (dominated by *Methanolobus*) are present in higher abundance in the estuarine clay sediment (Table 4.0.4., Chapter 4), which may also be a legacy of marine influence on the methanogen community of the estuarine clay sediments.

8.8. Did the pre-impoundment methanogen community adapt to post-impoundment freshwater conditions?

The similarity between the methanogen community composition, and also their temperature and salinity range and characteristics, of the Cardiff Bay lacustrine gyttja and estuarine clay lithologies suggests that either; (i) the methanogen community of the estuarine intertidal mudflats which existed prior to construction of the Cardiff Bay barrage in 2001 were able to adjust to a reduction in porewater salinity of ~400 mM Cl− following impoundment, or (ii) the porewater salinity of Cardiff Bay sediments has always been lower than the outside Severn Estuary. There are several lines of evidence to suggest that the archaeal community of the pre-2001 estuarine intertidal mudflats differs from the archaeal community of intertidal sediments in Severn Estuary, and that the methanogen community of Cardiff Bay sediments may have always been adapted to low salinity conditions even prior to impoundment.

The methanogen community composition of both the lacustrine gyttja and estuarine clay sediments differs from the methanogen community composition of intertidal sediments in the open Severn Estuary, suggesting that the environmental factors controlling methanogenesis in these two environments are not the same. The major difference between the methanogen community composition of Severn Estuary intertidal sediments (Williams 2015) and Cardiff Bay lacustrine gyttja and estuarine clay sediments (this study and unpublished data provided by Gordon Webster, Cardiff University) is that the Methanomicrobia is the dominant methanogen class in Severn Estuary intertidal sediments, as typical of both estuarine and freshwater lake environment (Banning et al. 2005; Li et al. 2012; Chen et al. 2013a; Chen et al. 2013b; O'Sullivan et al. 2013; Zeleke et al. 2013; Webster et al. 2014; Xie et al. 2014). However, Methanobacteria is the dominant methanogen class in Cardiff Bay sediments, with the Methanomicrobia only 9.8 and 1.4 % of sequences in Cardiff Bay
lacustrine gyttja and estuarine clay sediments respectively unpublished (Gordon Webster, unpublished data).

Differences between the methanogen community composition of Cardiff Bay lacustrine gyttja sediments and Severn Estuary intertidal sediments can be explained by differences in the sediment and organic matter sources to Cardiff Bay, however the methanogen community composition of Cardiff Bay estuarine clay sediments might be expected to have similar composition to Severn Estuary intertidal sediments given that they share the same sediment source (and same salinity at the time of deposition). One potential explanation for the relatively low abundance of Methanomicrobia in Cardiff Bay estuarine sediments relative to Severn Estuary sediments is that members of the Methanomicrobia present in the original Severn Estuary tidal flat sediment were unable to adapt to post-impoundment reduction in porewater impoundment. Current porewater salinity in the estuarine clay lithology (max. ~40 mM Cl\(^-\); Figure 3.0.5., Chapter 3), although higher than the porewater salinity of Cardiff Bay lacustrine gyttja sediments, is still 10-fold lower than the porewater salinities of intertidal mudflats located immediately adjacent to the seawards side of the barrage (23.7 ‰, Thomas 2014).

The Methanomicrobia are common in both freshwater, lacustrine and brackish estuarine environments (discussed in section 3.4.5.), but conceivably the Methanomicrobia populations of these two different environments may differ at genus or species level. The genera within the Methanomicrobia most commonly detected in lake sediments are the *Methanoregula*, *Methanolinea* and *Methanospirillum* (Borrel et al. 2011). Unfortunately most studies of methanogen community composition and structure in estuarine sediments, including the Williams (2015) study of methanogen community composition in Severn Estuary sediments, do not investigate the methanogen community composition at genus level. However, Purdy et al. (2002), O’Sullivan et al. (2013) and Webster et al. (2014) all investigated methanogen community composition of intertidal sediments between the freshwater and marine endpoints of the Colne Estuary, UK. The diversity of methanogen genera detected was slightly different in each study, which may be partly due to methodological biases. Purdy et al. (2002) and O’Sullivan et al. (2013) both used the 16s rRNA gene as a genetic marker, although Purdy et al. (2002) constructed a clone library whilst O’Sullivan et al. (2013) used PCR-DGGE for community profiling. In contrast Webster
et al. (2014) used pyrosequencing of mcrA amplicons. The differing methodological biases associated with DGGE and pyrosequencing are discussed in full in section 3.6.6.

Purdy et al. (2002) compared the methanogen community composition in brackish sediments East Hill Bridge (salinity <1 %$_0$) to that in marine sediments at the mouth of the Colne Estuary (Colne Point, salinity 39.3-53.3 %$_0$; Munson et al. 1997). Phylotypes belonging to the genera *Methanosarcina*, *Methanococcoides*, *Methanolobus*, and *Methanothrix* (Methanosarcinales), and *Methanogenium*, *Methanoculleus*, *Methanocorpusculum* (Methanomicrobiales) were identified. *Methanococcoides* spp. and *Methanoculleus* spp. were only present at the marine end, whilst *Methanocorpusculum* spp. and *Methanosarcina* spp. were only detected at the freshwater end. In contrast O'Sullivan et al. (2013), who also investigated the sediment methanogen community in Colne Estuary sediments spanning the full salinity range from freshwater to marine, only detected one genus belonging to the Methanosarcinales (*Methanococcoides*) and one belonging to the Methanomicrobiales (*Methanospirillum*). *Methanococcoides* spp. were not detected at the freshwater endpoint whereas *Methanospirillum* spp. were detected at all salinities. Webster et al. (2014) analysed the same samples as O'Sullivan et al. (2013), but detected a far greater diversity of genera than either O'Sullivan et al. (2013) or Purdy et al. (2002).

Webster et al. (2014) detected the genera *Methanosarcina*, *Methanolobus*, *Methanomethylovorans* and *Methanococcoides* (Methanosarcinales), *Methanogenium* (Methanomicrobiales) and *Methanobrevibacter* (Methanobacterium). *Methanolobus* spp. were only detected at the marine end, whilst *Methanococcoides* spp. were detected at the marine and brackish sites. In contrast, *Methanomethylovorans* spp. were only detected at the freshwater end. Webster et al. (2014) also detected the genera *Methanothrix* and *Methanoculleus* at the freshwater endpoint only using primers targeting the 16s rRNA gene, but not with mcrA primers.

Xie et al. (2014) investigated relative abundance of methanogen genera along estuarine salinity gradient of Pearl River, China and detected the genera *Methanosarcina*, *Methanothrix*, *Methanococcoides* (Methanosarcinales), *Methanoregula*, *Methanolinea* (Methanomicrobiales), *Methanocella* (Methanocellales) and *Methanobrevibacter*, *Methanobacterium* (Methanobacteriales). There was a decrease in abundance of all genera with increasing porewater salinity, with the
exception of the genus *Methanococoides* which present at the most marine site. In addition the genus *Methanocella* was present at highest abundance in sites of intermediate salinity between freshwater and marine. The genera *Methanolinea* and *Methanoregula*, both of which are genera known to be sensitive to salt content (Imachi et al. 2008; Bräuer et al. 2011; Cadillo-Quiroz et al. 2014) and abundant in freshwater lake sediments (Table 1; Borrel et al. 2011) were not present at the marine site.

Overall, the identity of genera belonging to the Methanosarcinales detected in estuarine sediments is fairly consistent between studies. *Methanococoides* spp. were detected in all studies, and *Methanosarcina* spp. in all except O'Sullivan et al. (2013). There is a clear and consistent trend in the salinity distribution of the Methanosarcinales; particularly the *Methanococoides* (which was never detected at the freshwater site in the Colne or Pearl River estuaries) and to a lesser extend *Methanolobus* (only detected at the marine site of Colne Estuary by Webster et al. 2014). In contrast, there was little consistency between the identities of genera belonging to the Methanomicrobiales, even between the three studies which all focussed on the Colne Estuary, UK. There is also no clear trend in the salinity distributions of the genera which were consistently detected between studies. For example the genus *Methanoculleus* was only detected at the marine site of the Colne Estuary by Purdy et al. (2002), yet only detected at the freshwater site by Webster et al. (2014). These studies generally suggest that high salinity is more discriminative than low salinity for genera belonging to the Methanomicrobiales. For example, *Methanocorpusculum* was only detected at the freshwater site of the Colne Estuary by Purdy et al. (2002) and *Methanoregula* and *Methanolinea* were not detected at the marine site of Pearl River Estuary by Xie et al. (2014). Therefore, the reduction in porewater salinity that occurred in Cardiff Bay sediments following impoundment would presumably not have discriminated strongly against members of the Methanomicrobiales present in Cardiff Bay estuarine intertidal sediments prior to impoundment.


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Methanothrix, and Methanosarcina), Methanobacteria (Methanobacterium) and also the Methanococci (genus Methanococcus) from an intertidal mudflat on the southern bank of the Severn Estuary (Woodhill Bay, Portishead). Strains belonging to each of these genera were also enriched from Cardiff Bay sediments, with the exception of the genus Methanococcus. The genera Methanococcoides, Methanococcus and Methanolobus are the three methanogen strains most commonly cultivated from marine sediments (Ferry 1993), and the low salinity of Cardiff Bay lacustrine gyttja sediments likely explains why Methanococcus spp. were not enriched in this study. The salinity range of the Methanococcus strains isolated from Woodhill Bay, Portishead by Watkins (2012) was not determined, however, the minimum salinity tolerated by type strain species is 0.3-0.5 M Na⁺ (Oren 2014b). Hence, the relatively low (max. 0.04 M Cl⁻) salinity in Cardiff Bay sediments may explain the absence of Methanococcus in the estuarine clay lithology of Cardiff Bay sediments following impoundment (assuming that they were present in Cardiff Bay intertidal mudflats prior to impoundment). In contrast, Watkins (2012) found that Methanococcoides strains isolated from Woodhill Bay, Portishead had a minimum salinity for growth of 0.03 M Cl⁻. Similarly, the Methanolobus strain isolated from Cardiff Bay sediments (CB03) was able to tolerate both freshwater (0.01 M NaCl) and marine salinities (0.55 M NaCl), and all the Methanolobus species type strains isolated from saline environments are able to tolerate salinities at least as low as 0.1 M NaCl (Table 7.0.4., Chapter 7).

Furthermore, both the lacustrine gyttja and estuarine clay methanogen populations demonstrate a clear preference for low salinity conditions over marine conditions (section 4.4.6.). The methanogen community of the estuarine clay sediments may have developed a preference for low salinity conditions during the time period between impoundment (2001) and present day (2015) in response to lowered porewater salinities. Alternatively, the porewater salinity of the intertidal estuarine intertidal mudflats which preceded impoundment may have always been lower than the porewater salinity of other intertidal and tidal sediments in the open estuary. Prior to impoundment most of sediment and organic matter in Cardiff Bay came from the Severn Estuary (Environmental Advisory Unit 1991), thus the microbial community composition of Cardiff Bay sediments would probably have been influenced by the Severn Estuary. However, if locally the sediment porewater salinity of the intertidal mudflats which preceded impoundment was also strongly influenced by freshwater
discharged from the Taff and Ely rivers, then the salinity characteristics of the methanogen community of Cardiff Bay sediments has likely always differed from the intertidal mudflat sediments of the open Severn Estuary. It may also be important that Cardiff Bay has a relatively sheltered position compared to the rest of the estuary (Environmental Advisory Unit 1991), and was not impacted by wave and current action to the same extent as open estuary sediments prior to impoundment. Cardiff Bay’s position next to the mouth of these two relatively large rivers mean that would also have received a high loading of terrestrially derived plant debris, which is now represented by the surficial lacustrine gyttja sediments (section 3.3.1.). Probably the relatively undisturbed nature of the sediment (excluding the dredging carried out prior to impoundment, section 3.1.2.) and river flows with heavy loading of terrestrially derived organic debris was a highly influential factor controlling the methanogen sediment diversity both pre- and post-impoundment.

8.9. Conclusions
In conclusion it is clear that microbial methanogenesis is active in Cardiff Bay sediments despite the dramatic environmental change caused by impoundment, dredging, and the subsequent transition from brackish, estuarine intertidal mudflats to artificial freshwater lake. Rates of methanogenesis are now typical of eutrophic freshwater lakes, and porewater methane concentrations regularly exceeded theoretical saturation limits suggesting that (as typical of impounded environments) ebullition may be a direct pathway for atmospheric methane emissions in Cardiff Bay sediments. Long term sediment microcosm incubations (> 2 years) show that the post-impoundment lacustrine gyttja sediments have the potential to produce 10-fold more methane than pre-impoundment estuarine clay sediments. Short term sediment slurry incubations (maximum of 30 days) indicate that this methane production is greatly enhanced by both increased substrate availability and small scale, climate relevant, temperature increases.

Methane production in Cardiff Bay sediments is unusual for a freshwater environment in that hydrogenotrophic methanogenesis is a more important pathway for methane production than acetotrophic methanogenesis. The sediment methanogen community is dominated by hydrogenotrophic methanogens (\textit{Methanobacterium}), although culturable acetotrophic and methylotrophic methanogens are also present. The novel methanogen order Methanomassiliicoccales is present in lacustrine gyttja sediments
in high abundance, but it is likely not active under in situ conditions. Intriguingly, putatively syntrophic acetate and methylamine utilizing methanogenic consortia involving hydrogenotrophic methanogens appeared to outcompete direct methylotrophic and acetotrophic methanogens at mesophilic and thermophilic temperatures. It is not clear why hydrogenotrophic methanogenesis is prevalent in Cardiff Bay sediments and a limited number of other freshwater environments, however, for Cardiff Bay it seems likely that the high abundance of plant-derived recalcitrant organic matter in lacustrine gyttja sediments is the driving factor behind the pathway for methane production and methanogen community composition.

Cultivation based experiments demonstrated that Cardiff Bay sediments has a diverse physiologically and phylogenetically variable methanogen community which is active under salinities ranging from freshwater (0.01 M NaCl) to marine (0.55 M NaCl), and temperatures from <4 to >65 °C. A wide variety of methanogen strains were enriched under differing temperature and salinity condition, including: *Methanobacterium, Methanobrevibacter* and *Methanothermobacter* (order Methanobacteriales, class Methanobacteria), *Methanosarcina, Methanothrix, Methanolobus, Methanomethylovorans, Methanococcaoides* (order Methanosarcinales, class Methanomicrobia), *Methanocella* (order Methanocellales, class Methanomicrobia), *Methanogenium, Methanoregula, Methanofollis* and *Methanocorpusculum* (order Methanomicrobiales, class Methanomicrobia). Whether methanogens active at the extremes of temperature and salinity are allochthonous or autochthonous in source is not clear. Cardiff Bay, and the rivers discharging into it, receive input from potential sources of thermophilic methanogens, such as anaerobic digesters, silage, agricultural soils and landfill. Each of these possible sources could provide a continuous supply of thermophilic methanogens to Cardiff Bay sediments, explaining the presence of viable thermophilic methanogens in an environment where average monthly water temperature does not exceed 21.7 °C (Lee, In Prep). Marine ingression and aerosols of marine water are a consistent potential source of marine methanogens.

Physiological characterisation of pure culture isolates from Cardiff Bay has extended the growth range of known type strains. A strain of *Methanothermobacter* isolated from Cardiff Bay sediments is produces methane at 15 °C, which is 20 °C lower than type strains of the thermophilic genus *Methanothermobacter*. In addition, a strain of *Methanolobus profundii* isolated from Cardiff Bay grew at freshwater salinities (0.01 M),
which has not previously been recorded for *Methanolobus* type strains isolated from aquatic environments. Hence it appears that methanogen genera dominant in enrichments incubated under marine or thermophilic conditions are also active in Cardiff Bay sediments under *in situ* salinities and temperatures, and are likely active participants in organic matter mineralization in Cardiff Bay sediments. The majority (2 out of 3) *Methanobacterium* strains isolated from Cardiff Bay sediments were able to tolerate marine salinities (0.55 M NaCl; Table 7.0.2., Chapter 7), which is relatively uncommon in *Methanobacterium* species (Table 7.0.2., Chapter 7). This unusually large salinity tolerance may perhaps have enabled them to survive the transition from the brackish, estuarine salinities present prior to impoundment to the freshwater conditions present today.

The salinity and temperature optima and range of methanogen communities in sediments laid down pre-impoundment (estuarine clay) and post-impoundment (lacustrine gyttja) are very similar, as are salinity and temperature dependent variations in dominant methanogen genera. Depth dependent variation in the identity of minor methanogen community members (*Methanobrevibacter*, *Methanosarcina*, *Methanoculleus*, *Methanocella*) observed in temperature and salinity gradient enrichments and cultivation series was largely attributable to differences in the organic matter content and age of the lacustrine gyttja and estuarine clay sediments, and hence, availability of labile organic matter. Both lacustrine gyttja and estuarine clay methanogen communities demonstrated a preference for low salinity conditions. Given the strong similarity in temperature and salinity characteristics of lacustrine gyttja and estuarine clay methanogen communities, this suggests that either (i) the methanogen community composition of Cardiff Bay sediments has always been strongly influenced by the freshwater input from the Taff and Ely rivers, or (ii) the viable methanogen community of the pre-impoundment sediments has been replaced by the lacustrine methanogen community.

**8.10. Future work**

8.10.1. Utilisation of different molecular genetic techniques to further explore the archaeal diversity of Cardiff Bay sediments

It would be interesting to investigate use NGS (Next Generation Sequencing) technologies to investigate microbial diversity in Cardiff Bay cultivation series. NGS
technologies such as pyrosequencing by 454 Life Sciences and Roche Diagnostics and sequencing platforms provided by Ion Torrent and Illumina have been widely used to investigate microbial community diversity in the natural environment since the early to mid-2000s. Several studies investigating microbial community diversity of the natural environment have found that NGS technologies uncover greater microbial diversity than more traditional methods such as DGGE or clone libraries used in conjunction with Sanger sequencing (Biddle et al. 2008; Kim et al. 2008; Quince et al. 2008; Hamdan et al. 2012). PCR-DGGE was used in this study as it would detect dominant methanogens in Cardiff Bay sediments for contrast with results of enrichment and cultivation under different environmental conditions. However, more in depth analysis of certain samples would help to determine the fuller methanogen diversity and whether there is a difference between lacustrine gyttja and estuarine clay methanogen communities. In particular, it would be interesting to more fully characterise changes in methanogen diversity with depth in Cardiff Bay sediments. PCR-DGGE analysis suggests that there are limited differences in methanogen community with sediment depth; yet differences in the identity and abundance of minor community members would not be picked up by PCR-DGGE, and numerically minor community members may be functionally important (Campbell et al. 2011; Hugoni et al. 2013; Wilhelm et al. 2014). However, the dominance of hydrogenotrophic methanogens detected using PCR-DGGE does agree with the dominance of hydrogenotrophic methanogens in intact sediments, and in temperature and salinity gradient microcosms. In particular it would be interesting to conduct detailed characterisation of the methanogen community in lacustrine gyttja (0-5 cm) amended temperature gradient slurries within the temperature ranges corresponding with the mesophilic thermophilic acetate and methylamine minima (Figure 5.0.2., Chapter 5). It is not clear which community members are responsible for acetate and methylamine consumption in the 0-5 cm temperature gradient slurries. Full community composition characterisation might help pinpoint if, and how, methylamine and acetate are converted to methane at these temperatures discussed further in 8.10.4.).

8.10.2. Source and distribution pathway of thermophilic methanogens in Cardiff Bay sediments
Several potential sources of thermophilic methanogens in Cardiff Bay sediments have been suggested in section 5.4.5., for example landfill leachate, agricultural soils which
have been treated with fertiliser derived from commercial composting, or digestate from thermophilic anaerobic digesters. However, there are substantial areas of uncertainty within these potential pathways, not least with regards to how thermophilic methanogens survive low temperatures (thought to be below their minimum growth temperatures) and exposure to oxygen. A combination of molecular genetic characterisation and cultivation could be used to investigate presence of thermophilic archaea and bacteria in leachate obtained from landfill sites in close proximity to Cardiff Bay, agricultural soils which drain into the rivers Taff and Ely (the rivers which feed into Cardiff Bay) and in the sediments and waters of the rivers Taff and Ely themselves. This would help to constrain a source and a potential transfer pathway for thermophilic methanogens in Cardiff Bay sediments.

One potential explanation is that methanogens could survive transfer downstream in oxic river water within anoxic microniches in particulate organic matter. Several studies have suggested methanogens are able to survive within anoxic microniches in particulate organic matter (Burke et al. 1983; Marty 1993; Sieburth 1993; Karl and Tilbrook 1994; Schulz et al. 2001) or the digestive tracts of zooplankton (Oremland 1979; de Angelis and Lee 1994) in oxic ocean and freshwater lake waters. More recently a study by Grossart et al. (2011) provided convincing evidence that methanogens attached to photoautotrophic cyanobacteria and algae may be responsible for methane production in the oxic watercolumn of Lake Stechlin, Germany. Physiological characterisation of a strain belonging to the genus *Methanothermobacter* isolated from Cardiff Bay sediments indicates that the temperature minima of thermophilic methanogens present in Cardiff Bay may be within the environmental temperature range of Cardiff Bay sediments, and far lower than previously recorded for closely related cultivated strains. In addition to *Methanothermobacter*, thermophilic methanogen strains belonging to the genera *Methanothrix*, *Methanosarcina*, *Methanocella* and *Methanoculleus* were also enriched from Cardiff Bay sediments. It would be informative to obtain pure cultures belonging to these genera to test whether they are also able to survive at environmental temperatures.

However, even the suggested high temperature sources upstream act as an enrichment culture for thermophilic methanogens and seeds Cardiff Bay with thermophilic methanogens, it is still difficult to explain how these sources themselves
were initially inoculated. The waste feedstock being degraded in these sources (landfill, compost or anaerobic digestion) must contain small populations of thermophilic methanogens which then increase in abundance when the temperature becomes favourable, yet there is no clear explanation for the existence of these thermophilic populations in the waste feedstock.

8.10.3. Utilisation of different cultivation techniques in order to isolate environmentally relevant methanogen strains of *Methanobacterium*

It was disappointing that temperature gradient cultivation series incubated at 4 °C and 10 °C did not enrich strains belonging to the genus *Methanobacterium* or the family Methanomassiliicoccales, as these were potentially important members of the methanogen community in Cardiff Bay sediments. The *Methanobacterium* strains which were isolated grew extremely slowly at temperatures <25 °C, meaning that they are unlikely to be abundant in sediments under *in situ* conditions. It seems likely that relatively high substrate concentrations in the laboratory cultivation series may have promoted growth of acetogens coupled with acetotrophic methanogens in low temperature enrichment series targeting hydrogenotrophic methanogens (e.g. Conrad et al. 1989; Kotsyurbenko et al. 2001; Kotsyurbenko 2005), and hence, indirectly promoted growth of hydrogenotrophic methanogen genera not detected in the original sediment (*Methanoregula* and *Methanocorpusculum*, discussed in section 6.5.3.). Sakai et al. (2009) describe a low substrate method of methanogen cultivation which was utilised to isolate the first pure culture strain of *Methanocella* from rice field sediments (Sakai et al. 2007). This involves supplying enrichments with indirect substrates such as propionate and ethanol which are degraded to H₂ by heterotrophic fermentative bacteria which have a syntrophic relationship with hydrogenotrophic methanogens. Previous attempts to enrich and isolate *Methanocella* using traditional cultivation methods failed as *Methanocella* were outcompeted by faster growing *Methanobacterium* and *Methanospirillum* (Sakai et al. 2009). *Methanobacterium* strains adapted to *in situ* temperatures might have been enriched in this study if a co-culture method, similar to that described by Sakai et al. (2009), had been used.

High substrate concentrations, however, may not be the explanation for preferential enrichment of *Methanosarcina*, *Methanocorpusculum* and *Methanoregula* over *Methanobacterium* in low temperature cultivation series. Borrel et al. (2012a) and Cadillo-Quiroz et al. (2014) isolated psychrotolerant strains of *Methanobacterium* from
freshwater lake sediments using conventional cultivation methods (Hungate tubes with a 80:20 H₂/CO₂ headspace). It is possible that other aspects of cultivation, such as media composition, are inhibitory to the strains of *Methanobacterium* which are active *in situ*. For example, it was relatively recently discovered that some methanogens are very sensitive to sodium sulphide (Bräuer et al. 2004). Sodium sulphide is commonly used as a reducing agent for methanogen cultivation media, often added at a concentration of several millimolar (Whitman et al. 2006). The type strains for the species *Methanosphaerula palustris* (Cadillo-Quiroz et al. 2009) and *Methanoregula boonei* (Bräuer et al. 2011), both isolated from peat bog environments, are very sensitive to sodium sulphide. Sulphate and sulphide concentrations in peat bogs environments are typically very low (e.g. Pester et al. 2010). *Methanosphaerula palustris* is inhibited by 0.1 mM sodium sulphide (Cadillo-Quiroz et al. 2009), whilst concentrations greater than 2 mM may inhibit *Methanoregula boonei* (Bräuer et al. 2004). In this study sodium sulphide was added to methanogen cultivation media to a concentration of 1.2 mM (see section 2.3.1.), however the final concentration of sodium sulphide in media will have been significantly reduced by reaction between sodium sulphide and iron chloride to form iron sulphide.

There are currently no published studies to suggest that *Methanobacterium* is sensitive to sodium sulphide, however strains indigenous to Cardiff Bay may be inhibited by this or another (as yet unknown) compound within the media used for cultivation. Sulphate concentrations, and likely also sulphide concentrations, are very low (<0.07 M) in Cardiff Bay sediments. In addition, it is often stated that only a small proportion (<1 %) of prokaryotic taxa are culturable under conventional laboratory conditions (Amann et al. 1995). All enriched or isolated strains affiliated with the Methanomassiliicoccales reduce methylated amines or methanol with H₂, which was a substrate combination not utilised for enrichments prepared in this study. It would be very interesting to attempt to culture Methanomassiliicoccales strains from Cardiff Bay sediments, particularly given very low number of strains (1) currently isolated. Better knowledge about the metabolic and physiological traits of this newly discovered methanogen family would also perhaps provide information about the importance of methylotrophic methanogenesis and methyl substrates in methane production in freshwater environments.
8.10.4. Further investigation into syntrophic relationships between acetate and methylamine utilising bacteria and hydrogenotrophic methanogens

One particularly intriguing aspect of this study was enrichment of hydrogenotrophic methanogens in cultivation series amended with acetate and methylamine. In fact, a strain of the strictly hydrogenotrophic genus *Methanobrevibacter* was obtained in monoculture from a cultivation series amended with methylamine. Amendment with acetate could conceivably result in enrichment of hydrogenotrophic methanogens under conditions where syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis dominates due to substrate inhibition of acetotrophic methanogens. Temperature and pH, in addition to ammonium, acetate, and carbon dioxide concentrations, are known to influence the relative importance of direct and syntrophic oxidation of acetate in anaerobic digesters (Peterson and Ahring, 1991; Schnurer et al. 1999; Karakashev et al. 2006; Schnurer and Nordberg 2008; Hao et al. 2011; Zhao et al. 2013; Kato et al. 2014).

There is no known pathway for microbial hydrogen production from methylamine. In section 6.5.5. it is tentatively suggested that hydrogenotrophic methanogens are supported through a syntrophic association with anaerobic methylamine oxidizing bacteria, akin to syntrophic acetate or formate oxidation, involving transfer of either dissolved H₂ or electrons via DIET. A variety of cultivation and molecular genetic techniques could be used to investigate the existence and functioning of this pathway further. DNA-based SIP (Stable Isotope Probing) using isopycnic separation of ¹³C and ¹²C labelled methylamine and acetate in combination with molecular genetic screening such as PCR-DGGE could be used to identify bacteria and methanogens which are directly utilising methylamine as a substrate. In order to identify any syntrophic relationship between methylamine oxidizing bacteria and hydrogenotrophic methanogens it would be necessary to use deuterated methylamine as a substrate. Radajewski et al. (2000) used deuterium-labelled substrates, but found that isopycnic separation of the ²H labelled DNA using centrifugation is only half as efficient as with the more commonly used ¹³C-labelled substrates. GC-IRMS (gas chromatography-isotope ratio mass spectrometry) could be used to measure incorporation of ²H into bacterial and archaeal lipids. Deuterated substrates are rarely used in SIP based studies as it is thought that there is significant exchange with ¹H during cell metabolism (e.g. Uphaus et al. 1967; deGraaf et al. 1996), and the relative impact of an additional
neutron on the total mass is substantial compared to more commonly used carbon isotopes (Radajewski et al. 2003).

Microscopy techniques such as Fluorescence In Situ Hybridization (FISH) would reveal if aggregates of methylamine oxidizing bacteria and hydrogenotrophic methanogens exist within cultures, which would imply a syntrophic relationship between the different functional groups. Again, this process would not be straightforward as it would be necessary to first identify and obtain gDNA sequences for the methylamine oxidizing bacteria in order to design suitable hybridization probes. Microautoradiography-Fluorescence In Situ Hybridisation (MAR-FISH) with $^{14}$C-labelled methylamine could be used to try and identify which organisms are utilising methylamine. Ultimately the aim would be to isolate potential bacterial syntrophs, and conduct co-culture experiments with hydrogenotrophic methanogens.

8.10.5. Relationship between temperature and atmospheric methane flux from Cardiff Bay sediments

Section 5.4.3. shows that the rate of methane production from lacustrine and estuarine Cardiff Bay sediments increases exponentially over the environmental temperature range relevant to current IPCC climate change predictions (Figure 5.0.6., Chapter 5). Whether this effect is sustainable over longer incubation periods (>30 days) is unknown. Freshwater lakes are a significant source of atmospheric methane emissions (6-16% of global non-anthropogenic emissions; Bastviken et al. 2004). In particular, impounded artificial freshwater lakes often emit methane at high rates, which is thought be linked to high sedimentation rates rapidly shifting labile organic carbon into deeper anoxic sediment layers (Sobek et al. 2012). Lake atmospheric methane emissions depend on many different factors including activity rate of aerobic and anaerobic methane oxidizing bacteria in the sediment and water column and the relative contribution of diffusion and ebullition as routes of methane escape to the lake surface. Extensive characterisation of temperature control on methane production and consumption and diffusion/ebullition was beyond the scope of this study. However, given the shallow water depth and strong temperature dependence of methane production in Cardiff Bay sediments, it would be interesting to explore further whether the high rates of methane production in Cardiff Bay sediments translate into significant atmospheric emissions.
The presence of numerous gas voids in Cardiff Bay sediments indicates that ebullition could be an important pathway of methane from Cardiff Bay. Measuring methane ebullition is difficult because of the large temporal and spatial variability in outgassing events. Lake atmospheric methane emissions are traditionally measured using floating surface gas traps, but there is concern that results do not accurately reflect atmospheric emissions in environments where ebullition is important. It has recently been suggested that ‘microbubble’ transport may also be an important, and hitherto unmeasured, pathway of transport from sediment to atmosphere in freshwater lakes (McGinnis et al. 2006; Prairie and del Giorgio 2013). Hydroacoustic technologies, such as echosounding, have been used successfully to quantify water column bubble volume in shallow water environments (e.g. Ostrovsky et al. 2008). A disadvantage of such using hydroacoustic technologies is that they obviously do not distinguish between carbon dioxide and methane bubbles. Furthermore, Del Sontro (2011) measured atmospheric methane emissions from Lake Kariba, Zimababwe using both an echosounder and traditional gas traps and found that estimates of atmospheric methane emissions varied little between methods. It seems likely that gas traps are appropriate for use in environments with small areal extent.
References


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